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Salmonella

A Diversified Superbug

Edited by Yashwant Kumar

SALMONELLA – A DIVERSIFIED SUPERBUG

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Meet the editor

Mr. Yashwant Kumar is currently working as a Technical Supervisor at the National Salmonella and Escherichia Centre in Kasauli, India. He is experienced in surveillance activities related to Salmonella and Escherichia coli, as well as the production of diagnostic reagents. Mr. Kumar has experience in different bacteriological, virological, molecular techniques, and lyophilization. In addition, his expertise extends to the maintenance of different bacterial strains, such as production, quality control, and quality assurance of immunobiologicals in facilities regulated by cGMP and cGLP guidelines to name just a few. He is actively involved in the field of Microbiology, and he has published papers in different Indian and international journals. Mr. Kumar trained for Instrumental Methods in Drug Analysis, LAMP and Real Time PCR, Yeast Molecular Biology, Biochemical Analysis, and Microbial Technology. He has been a reviewer of four international journals and has a vast knowledge of computer applications.

Contents

Preface

The genus *Salmonella* comprises a diverse range of bacteria which contain a collective of some 2579 serovars in *Salmonella enterica* and *Salmonella bongori*. Being the etiological agent across a spectrum of diseases, ranging from reptiles to humans, *Salmonella* presents an array of continuously emerging challenges in front of the researchers. *Salmonella* is ubiquitous and possess the capability to respond and survive in a wide variety of environments.

Salmonella is a genus of bacteria that is a major cause of food-borne illness throughout the world, posing a high economic burden. The bacteria is generally transmitted to humans through consumption of contaminated food that originates from animals - primarily meat, poultry, eggs and milk and other dairy products.

Because of the increasing resistance to a wide range of antimicrobial agents, including third generation cephalosporins, the antimicrobial chemotherapy using available antibiotics has been hindered. The scenario has been further aggravated by the emergence of plasmid-borne fluoroquinolone resistance in both typhoidal and non-typhoidal *Salmonella*e and horizontal transfer of gene coding for antimicrobial resistance. Resistance to cephalosporins, due to the production of extended – spectrum β-lactamases (ESBLs) is an ever increasing problem, and it is a cause of serious concern worldwide. These enzymes have been detected in many species of the family enterobacteriaceae, including different serovars of *Salmonella enterica.* The ability of *Salmonella* to exist in biofilm further complicates its removal and ensures its prolonged existence.

Although several reports have been published highlighting the re-emergence of susceptibility to the conventionally used drugs, antimicrobial resistance continues to be the major problem in front of health authorities. This also points towards the ever changing fluidic nature of antibiogram patterns, which necessitates strong and effective surveillance systems able to spot changes in the antibiogram patterns to enable health authorities to respond to these changes immediately.

There have been plenty of available research publications focusing on different aspects of *Salmonella* in the last decade. So, what is the need for a book on *Salmonella*? The answer is simple. Today, the number of infections due to *Salmonella* in humans and animals resulting in high morbidity, mortality, and ultimately leading to huge economic losses

has increased. It becomes essential to study different aspects of *Salmonella*, in terms of its pathogenicity, resistance, and immunoprophylaxis to provide better understanding of this bacterium which may help in combating this microorganism efficiently. In this book, an effort has been made to compile different studies on *Salmonella* by internationally acclaimed experts, providing a timely snapshot of the current state of research on *Salmonella.* The book is not only informative, but also enjoyable to read. The book contains six sections - each with a different focus on *Salmonella* in terms of the its impact on the environment, antimicrobial resistance, genetics, immunology, pathogenesis, and application of novel techniques.

Section I comprises of the studies on environmental interactions of *Salmonella,* including biofilm production and its existence in different ecological niches. Section II presents different studies on emerging problem of antimicrobial resistance in *Salmonella* group. The section also discusses the possibility of employing different plant metabolites for treatment of *Salmonella* infections. Section III gives insight into different genetic aspects of *Salmonella,* including genetic diversity and evolutionary aspects. Section IV covers different studies revealing several immunological responses to *Salmonella.* It also discusses the development of vaccines using *Salmonella* as a live carrier of the antigens. Additionally, the section also presents the studies on the evaluation of the *Salmonella* vaccines for control of the disease. Section V deals with different aspects of *Salmonella,* covering mechanisms of pathogenesis, insect-bacteria association and its role in nosocomial infections, and use of animal models for studies on pathogenesis. Section VI covers the application of rapid and advanced techniques for the diagnosis of *Salmonella* infections in outbreak investigations. Moreover, the section also provides insight into immunoimmobilization of *Salmonella* for biosensor applications.

I put together this book and it is my hope that it will provide information for all researchers dealing with Salmonella. The book will assist in better understanding of various facets of *Salmonella* and provides an up-to-date insight into the research on this disease.

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Part 1

Environmental Interactions

1

Invasion and Survival of Salmonella in the Environment: The Role of Biofilms

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1. Introduction

Bacteria compose the majority of living biomass on Earth and play a vital role in the recycling of elements critical to sustaining life. We are discovering that they often exist as interlinked, multispecies colonies termed biofilms. They are all around us, on us, and in us. In fact, over 99% of microorganisms on Earth live as biofilms. They play a critical role in the ecology of the earth and the sustainability of life. For many years, studies of bacterial physiology focused primarily on the planktonic state neglecting the bacteria within the biofilm. The biofilm state is now recognized as the predominant form in which bacteria endure the stresses of the environment (An and Parsek, 2007; Hall-Stoodley et al., 2004; Hoffman et al., 2005; Karatan and Watnick, 2009; Stoodley et al., 2001)

Bacterial biofilms have long been recognized as participants in tooth decay, slippery rock surfaces, and contaminated water. Now these colonies are being investigated as perpetrators of persistent low-level food contamination which threaten animal and human health. Bacteria existing as biofilms are capable of surviving for extended periods in various environments, such as water, animal manure, and a range of agricultural soil types. For example, human pathogens can attach to and colonize the surfaces of plants and form biofilms on plant tissues (Annous et al., 2006). These biofilms are problematic because they are extremely hearty and difficult to remove by simple washing techniques. Causing, foodborne illnesses associated with human consumption of contaminated fresh fruits and vegetables (Fett and Cooke, 2003; Sivapalasingam et al., 2004). Living in biofims is advantageous for bacteria as it increases survival chances when confronted with unpredictable environmental stresses such as: temperature changes, desiccation, ultraviolet rays, etc.

In recent years, bacterial biofilms have been increasingly linked to food safety issues worldwide. The culprits of three recent foodborne illness outbreaks in cantaloupe melons, apples, and leafy greens have been identified as pathogenic bacteria existing in biofilms (Annous et al., 2009). They have also been implicated as the cause of many chronic infections in humans and are frequently associated with implanted devices, such as catheters, prosthetics, and contact lenses (Prouty et al., 2002). There is increasing interest in biofilms found on mucosal surfaces, such as the colon, particularly with respect to their role in disease processes (Macfarlane and Macfarlane, 2006).

There are numerous definitions of biofilms but all share the common threads of a concept involving an assemblage of microorganisms in which some of the bacteria adhere to the surface and exude an extracellular polymeric substance (EPS) that forms a matrix for further cellular attachment. The matrix is comprised of proteins, polysaccharides, extra-cellular DNA, and the various organisms involved. Biofilms can range from simple single species monolayer matrices, to complex multi-organism communities and sometimes even involve higher level organisms such as nematodes and larvae (Cloete et al., 2009).

Initially, the term "biofilm" was used informally among scientists for many years. It first appeared in a scientific journal in 1977 (Montana State University, http://www.biofilm.montana.edu/node/2930). Early researchers examining the phenomenon of microbes attaching to surfaces include Windogradsky, Cholodny, and Conn in the 1930's (Lappin-Scott, 1999). An important observation made by these scientists, was that bacteria which grew attached to a surface (in this case glass slides immersed in soil slurry) were phenotypically different from those cultured from the water phase of soil slurry (Lappin-Scott, 1999). Henrici studying freshwater bacteria observed that "for the most part water bacteria are not free floating but grow attached to the surfaces" (Lappin-Scott, 1999). These early researchers described how bacteria that were attached to surfaces exhibited diverse populations and developed into "microbial films". ZoBell's research from the early 1930's, focused on the role of bacteria in biofouling (the unwanted accumulation of microorganisms on surfaces) (Lappin-Scott, 1999). In fact ZoBell & Allen (1935), report the first apparatus specifically designed to examine bacterial attachment to surfaces. It was a carrier that held 16 glass slides and was designed to be lowered into the ocean where marine microbes could attach to the glass. Using this apparatus, ZoBell & Allen found a greater diversity of bacteria in the biofilm "lawn" on the slide than that which could be cultured from the sea water.

The bacteria found in biofilms are phenotypically distinct from their planktonic form. These changes include alterations in the regulation of large suites of genes (Hall-Stoodley et al., 2004; Karatan and Watnick, 2009). The transformation from planktonic existence to biofilm formation is a complex process, often triggered by various alterations in the surrounding environment. Bacteria in biofilms exhibited: protein profiles that more closely resemble those of exponentially growing planktonic cells (Mikkelsen et al., 2007); significant differences in the genes that are expressed (Teplitski et al., 2006; Trevors, 2011); and significant differences in the degree of resistance to antibiotics and disinfectants (Brooun et al., 2000; Ryu and Beuchat, 2005).

Bacteria living within biofilms can exhibit 1000 times more resistance to antimicrobials than their planktonic peers. The close proximity of fellow bacteria within this community allows for the increased incidence of gene transfer; resulting in increased genetic diversity, including augmented antimicrobial resistance. Biofilms impart increased levels of protection against environmental stresses, such as depleted nutrient, moisture and oxygen levels; inhospitable surrounding pH and salinity; excessive shear forces and UV exposure, and even metal toxicity. Additionally, life in a biofilm protects against attacks by a host immune system's protective proteins and signaling molecules, phagocytes, antibiotics and disinfectants (Jefferson, 2004; Mara and Horan, 2002).

Even after more than 80 years of research, there are still many unanswered question about the formation, function, maturation and eventual death of biofilms. Biofilms are typically attached and sessile. However, they have become ubiquitous in the environment because,

portions can detach and relocate to other hospitable surroundings. There is widespread scientific interest in investigating the molecular mechanisms underlying life in these intriguing bacterial communities that are able to inhabit such diverse environments.

2. Biofilm development

Despite the years of research into the mechanism of bacterial attachment, there remain many basic facets of the process that are still a mystery. The nuances of the attachment are difficult to elucidate. What is known is that the multifaceted process involves a complete alteration in life style of the bacteria involved. A generalized model for bacterial transformation from a planktonic to biofilm existence can be made (Lemon et al., 2008). This model contains five major phases: attachment; formation; micro-colony development; maturation; and finally detachment/dispersal of the biofilm. Each phase can be described by key features and triggers unique to that phase of development and will be discussed in the remainder of this section.

Cell attachment occurs in five stages. The first stage is a reversible stage where cells lightly attach to the surfaces. It is followed by a second, more permanent stage, where the cells affix themselves securely by forming an adhesive exopolymeric compound. Then in stage three, the biofilm begins to expand by the recruitment of cells into micro-colonies. In stage four, the mature biofilm is characterized by the development of a three-dimensional structure containing cells packed in clusters with channels forming to aid in the movement of nutrients and molecules to cells beneath the colony surface. In the fifth and final stage, the cells detach which facilitates dispersal and the initiation of new similar biofilms at more favorable locations. It is important to note that cell division is uncommon in mature biofilms, and energy is used predominantly to produce exopolysaccharides (Watnick and Kolter, 2000).

Bacteria within biofilms exhibit a range of phenotypes; some of these do not exist in the planktonic phase. These phenotypes include: freely suspended naked cells (resuming their planktonic state); cells reversibly attached to a surface; cells irreversibly attached to a surface and not encapsulated by EPS; embedded attached cells surrounded by EPS matrix or deeply embedded attached cells within a the three dimensional microbial stack; embedded cells sloughed into suspension; and planktonic daughter cells (Parry, 2004).

Quorum sensing allows bacterial cells to communicate resulting in a cohesiveness of function that benefits an entire population and allow the community to operate as a living system (Smith and Chapman, 2010). The channels between cell clusters deliver water and nutrients to each cell and facilitate waste removal. These structures combined with strong adhesive properties and sophisticated cell-cell communication make biofilms highly resistant to conventional cleansing agents such as biocides and disinfectants. Not surprisingly, once biofilms form, they are difficult to eliminate.

2.1 Attachment

Surface attachment offers distinct advantages for bacteria which depend on the diffusion of nutrients and wastes for their well-being. Most natural aqueous environments contain only dilute substances which can be used for metabolism and growth. On the other hand, natural surfaces tend to collect and concentrate nutrients by charge-charge or hydrophobic interactions; which provide bacteria exposure to more concentrated foodstuffs. Biofilms are

initiated when individual motile bacteria localize onto a surface and begin major physiological alterations. This initial attachment is reversible but encourages aggregation and attachment of more planktonic bacteria and other organisms. During this phase the attraction is mediated by weak forces, such as van der Waals, acid-base and simple electrostatics processes.

2.2 Formation

Permanent formation and expansion of the biofilm occurs when the initial transient attachment is reinforced by the production of cell surface adhesive compounds, pilli and fimbriae (Kaplan, 2010). The complex transition from transient to permanent attachment is associated with the formation of a monolayer via the up-regulation of genes responsible for the production of an extracellular matrix composed of exopolysaccharides, and extracellular DNA. Bacterial motility is lost by removal of cell flagellum by protease and replacement with a holdfast protrusion composed of oligomers of *N*-acetylglucosamine. The holdfast is composed of a strong adhesive polysaccharide that ensures a tight bond to the surface (Karatan and Watnick, 2009). In some strains of bacteria cell wall bound surface proteins called biofilm-associated protein (BAP) begin to be expressed and promote cell to cell interactions and the development of the extracellular matrix (Lasa and Penades, 2006).

Further development of the biofilm is promoted by the production of molecules which cause potassium leakage and trigger the activation of a membrane kinase (Lopez et al., 2009). In addition, the transcription of flagellar genes is repressed when the monolayer stage is achieved. Transcription of a large number of methyl-accepting chemotaxis genes are activated in the monolayer stage. Studies suggest that chemotaxis proteins influence monolayer formation. One possibility is that flagellar rotation pausing, which plays a role in the response to chemoattractants, also enhances the transition to permanent attachment (Karatan and Watnick, 2009). Some of the different components involved in the formation of the matrix include pilli and extracellular DNA (Banas and Vickerman, 2003; Kachlany et al., 2001; Petersen et al., 2005).

2.3 Micro-colony development

Now that the bacteria are sessile and biofilm formation is initiated, the bacteria actively multiply and communicate via quorum sensing signals. Once the quorum sensing threshold is achieved, exopolysaccharide production begins and micro-colonies develop through a variety of mechanisms. *Pseudomonas aeruginosa* use flagella and pili-mediated twitching motility to redistribute across the surface. *Escherichia coli* utilize fimbriae, flagella and pili for the same purpose. Others spread and generate micro-colonies through cell division, where the daughter cells spread outward and upward (Cloete et al., 2009).

2.4 Maturation

Maturation results in the formation of pillars and masses of tightly packed cells intermixed with fluid filled channels allowing for the exchange of nutrients, oxygen, and waste products between the biofilm and the surrounding liquid (Cloete et al., 2009). EPS is a key component of the biofilm matrix and may be composed of a number of sugar monomers such as glucose, galactose, mannose and xylose and some non-carbohydrate substitutes

(such as acetate, pyruvate, succinate, and phosphate). Most EPS molecules are neutral or polyanionic in nature, which aids in immune evasion and tolerance toward antibacterial agents. Enzymatic alteration of EPS is thought to significantly change its physicochemical properties and consequently the entire structure. Some examples of polymeric biofilm matrix constituents include the glucan polysaccharides produced by *Streptococcus mutans* (Banas and Vickerman, 2003), proteinaceous fimbriae produced by *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* (Kachlany et al., 2001; Lamont et al., 2002); extracellular, double-stranded DNA in biofilms produced by *A. actinomycetemcomitans*, *S. mutans,* and *Streptococcus intermedius* (Inoue et al., 2003; Petersen et al., 2004; Petersen et al., 2005) and a wide variety of proteins, glycoproteins, glycolipids, and enzymes.

Mature biofilms are intricate structures containing sectors with distinctive microenvironments that differ in cell densities, oxygen and nutrient levels, and pH ranges. As a result, the metabolic and reproductive functionality of the bacteria located in these distinct sectors are quite divergent (Kaplan, 2010). Metabolically dormant cells located in the interior of the colony are often more resistant to the actions of antimicrobial agents that target actively growing cells near the exterior (Fux et al., 2005).

2.5 Detachment and dispersal

The fifth and final phase of a biofilm lifecycle is detachment and dispersal. Growth and detachment are interdependent. Under robust conditions, the detachment rate has been shown to increase with increasing growth rates (Gjaltema et al., 1997). This phase leads to colonization of new areas offering fresh resources, which is critical for long-term survival. This phase is also important in the dissemination of infection and therefore, disease transmission in clinical and public health settings. As with all aspects of the biofilm lifecycle, the processes surrounding detachment and dispersal are very intricate; involving a wide variety of environmental and physiological triggers and signal transduction pathways (Karatan and Watnick, 2009). Individual bacteria employ somewhat different methods of dispersal, which can be divided into three discrete stages: (a) detachment of cells from the colony; (b) relocation of cells to an alternative site; and (c) re-attachment of the cells to a new substrate site (Kaplan, 2010). Rochex et al. (2009) found that one dominant species often comprises most of the weakly cohesive, thick top layer of the biofilm; while a more diverse population comprises the strongly cohesive, thin basal layer. These findings suggest that determining species diversity may be an important parameter in understanding detachment and dispersal.

2.5.1 Key factors for detachment

Both biochemical and physical factors participate in the major processes facilitating biofilm detachment; those being erosion; sloughing; abrasion, grazing, and human intervention Numerous biochemical factors involved in detachment are: the production of EPS-degrading enzymes; lytic bacteriophage activation; expression of phosphodiesterases; and quorum-sensing signaling. Physical detachment factors are : microbiologically generated gas bubbles; the presence of cross-linking cations; nutrient limitations; metabolite accumulations; changes in osmolarity; high cell density growth; and fluidic shear factors (Thormann et al., 2006).

2.5.2 Erosion, sloughing and abrasion

Erosion and sloughing are two mechanisms of spontaneous biofilm cellular detachment. The distinction between erosion and sloughing has a considerable effect on bacterial species competition within biofilms and thus morphology (Telgmann et al., 2004). Erosion is the continual detachment of single cells or small fragments from the biofilm at low levels over the course of formation. Researchers have noted that the rate of erosion from the biofilm increases with increased matrix thickness and fluidic shear forces at the cell-liquid interface. An increase in the flow velocity causes the hydrodynamic boundary layer to decrease, resulting in amplified turbulence at the biofilm surface. Sloughing is the swift, massive loss of large chunks of biofilm greater than or equal to the overall thickness. Sloughing is a more random occurrence than erosion and is thought to result from nutrient or oxygen depletion within the structure and is more commonly observed in thicker systems (Donlan, 2002).

Erosion and sloughing occur when local shear forces overwhelm the cohesiveness of the biofilm. Overall cohesiveness is strongly influenced by the composition and the structure of the polymeric matrix, which is dependent on the formation history, the environmental growth conditions and the developmental stage of the biofilm. The resulting strength of biofilm attachment is contingent on cell density, composition of extracellular polymeric substances, and levels of specific compounds, such as the calcium. Fast growing organisms with high initial cell growth rates favor the development of protrusions and the formation of a heterogeneous biofilm structure. Shear forces more easily erode these protrusions (Telgmann et al., 2004).

Abrasion is the loss of biofilm due to collision of particles from the surrounding fluid with the exposed surface. Biofilms in fluidized beds, filters, and particle-laden environments such as surface waters are often subjected to abrasion (Donlan, 2002). Work by Rochex et al. (2009) demonstrated that abrasion characteristics, such as particle collision frequency and pressure strongly affect biofilm detachment rates. Experiments by Gjaltema et al. (1997) have shown that inter-particle collisions cause an on-going abrasion of the biofilm

2.5.3 Grazing and human intervention

A key mortality factor in the control of bacteria within biofilms is grazing. Grazing is the uptake and killing of bacteria by phagocytic protozoa and metazoa in close association with biofilms. These biofilm-associated protozoa exhibit three modes of predation: 1) planktonic, but swimming close to the biofilm surface; 2) surface attachment on biofilm, but feeding on suspended prey; and 3) feeding directly on biofilm as prey. Protozoans benefit from this association as demonstrated by their increased numbers and taxa diversity when associated with a biofilm community compared to the surrounding plankton environment (Boenigk and Arndt, 2000).

Protozoa exhibit a sizeable diversification of morphologies consequent to developing a variety of means to capture and engulf their bacterial prey. However, they are commonly grouped into flagellates, ciliates, and amoebae. All three free-living groups efficiently graze on bacteria exposed on the biofilm surface. Flagellates and ciliates contain feeding types primarily focused on suspended bacteria with only a few that preferably feed on surface-bound bacterial prey (Parry, 2004). For instance, the flagellate *Rhynchomonas nasuta* feed on attached *Pseudomonas* spp. at rates between 13 and 120 bacteria per

flagellate per hour (Boenigk and Arndt, 2000). Sibille et al. (1998) found that a mixed population of flagellates could consume on average 12 suspended bacteria per flagellate per hour. The ciliate *Euplotes* spp. grazes on adherent *Vibrio natriegens* and *Pseudomonas fluorescens* at rates of 120 and 882 bacteria per ciliate per hour, respectively (Lawrence and Snyder, 1998), while Ayo et al. (2001) found that in general ciliates showed a grazing rate of ≤20 free swimming bacteria per ciliate per hour.

Amoebae protozoans feed almost exclusively on surface-bound bacteria (Parry, 2004). Amoebae species such as *Hartmanella cantabrigiensis*, *Platyamoeba placida*, *Saccamoeba limax*, *Vahlkampfia avara* eat attached *Escherichia coli* at rates of 15 to 440 bacteria per amoeba per hour (Heaton et al., 2001).

Many predators are selective and remove only a subset of the microbial community thus altering the biofilm community structure (Parry, 2004). Morphological differences in biofilm structure correlate with predation. Without the pressure of predation a flat, compact structure results. Conversely in the presence of predators, an open and heterogeneous structure results.

Metazoa (rotatoria, nematoda, and oligochaeta) are the main group of higher level predators responsible for grazing. Their grazing on biofilms initially decreases microbial biomass, and unless grazing pressure is severe, the secondary microbial community that develops will have increased rates of metabolic activity and growth. Total microbial biomass will be greater and the turnover rates of both the substrates and microorganisms will increase. The diversity of the community structure will decrease as the biofilm community shifts towards faster growing organisms.

Bacterial predators, such as *Bdellovibrio bacteriovorus; Micavibrio* spp.*;* and *Hyphomicrobium* spp. also play a vital role in the life and death of biofilms. *Bdellovibrio bacteriovorus* is a gram-negative, aerobic bacterium that preys upon a wide variety of other gram negative bacteria, including *E. coli*; which, in simple biofilms, can devastate a community altogether (Dashiff et al., 2010). Additionally, *Micavibrio s*pp. is also a gram-negative, aerobic bacterium that also preys on bacteria and biofilm structures. Unlike *Bdellovibrio* which penetrate their prey, *Micavibrio* attach to the outside surface and eventually lyse their host bacteria. *Bdellovibrio* and *Micavibrio* spp. have been shown to be extremely host specific; for example, *Micavibrio aeruginosavorus* strain ARL-13 preys only on *Pseudomonas aeruginosa*. In static and flow cell experiments, *M. aeruginosavorus* not only modified *P. aeruginosa* biofilm structure, but also decreased bacterial viability. The alterations were likely caused by increased cell-cell interactions brought about by the presence of the predator (Donlan, 2002).

Human intervention involves both mechanical action and the use of disinfectants. Any type of brush or scouring pad provides the agitation required to disrupt the biofilm structure. Once the community has been physically disrupted the addition of a surfactant and disinfectant is required to complete the destruction process. In the case of contact lens , Wu et al. (2011) found that *Staphylococcus aureus* or *Pseudomonas aeruginosa* biofilms required rubbing and rinsing with multipurpose disinfecting solutions followed by tissue-wiping and air-drying to remove them from the surface. *Listeria monocytogenes,* an important foodborne pathogen, has the ability to form persistent biofilm matrices in food processing environments. Soni & Nannapaneni, (2010) determined that a cocktail of different bacteriophages may be essential for their removal. Lequette et al. (2010) found that

solubilization of polysaccharidases and proteases in a buffer containing surfactants, along with dispersing and chelating agents, enhanced their efficiency of removing biofilms by targeting several components of EPS of *Bacillus* spp. and *Pseudomonas* spp..

Biofilms have been extensively studied in the dental industry. Periodontitis is a chronic bacterial infectious disease whose hallmark is the presence of a bacterial biofilm at the gum line. The condition necessitates thorough removal of the biofilm for therapy. However, debridement using hand instruments or oscillating scalers is both technically demanding and time consuming, and may lead to severe root damage over time (Petersilka, 2011). Air-polishing with glycine powder proved to be an easy, safe and effective means of biofilm removal from teeth (Petersilka, 2011).

3. Quorum sensing

For many years, bacteria were believed to exist as individual cells that existed to find nutrients and multiply. The discovery of intercellular communication among bacteria led to the realization that bacteria are capable of coordinated activity that was once thought to be restricted to higher organisms (reviewed in (Waters and Bassler, 2005). The ability to behave collectively has obvious advantages, for example, the ability to migrate to a more suitable environment or better nutrient supply and to adopt new modes of growth, such as biofilm formation, which may afford protection from harmful environments. This intercellular communication is called quorum sensing. The mechanism used for quorum sensing is the process of recognition of and response to small molecules, called autoinducers, secreted by the bacteria themselves. The process of biofilm creation in a variety of bacteria has been shown to specifically involve quorum sensing. These autoinducers are used by bacteria to regulate their behavior according to population density. The phenomenon relies on the principle that when a single bacterium releases autoinducers into the environment, the concentration is too low to be detected. However, when sufficient bacteria are present, autoinducer concentrations reach a threshold level that allows the bacteria to sense a critical mass and respond by the activation or repression of target genes (de Kievit and Iglewski, 2000). Quorum sensing manifests itself as a synchronization of individual behavior into cooperative group activity, often resulting in a change of phenotype within a population once bacterial densities have reached a threshold level. The specific threshold level can be different for each population. Examples of density-dependent changes include the turning on of bioluminescence within *Vibrio fischeri*, conjugal transfer in *Agrobacterium tumefaciens*, swarming in *Serratia liquefacians*, production of virulence factors in *Burkholderia cepacia* and *Pseudomonas aeruginosa*, and biofilm formation in numerous species including *Pseudomonas aeruginosa*, *Pantoea stewartii* and *Vibrio cholera* (Bottomley et al., 2007; Davies et al., 1997; Nadell et al., 2008; Ward et al., 2004).

4. Biofilm and virulence

Many bacterial pathogens including *Listeria monocytogenes, Salmonella* spp., *Shigella* spp., *Staphylococcus aureus, Escherichia coli,* and *Enterobacter* spp., utilize a biofilm strategy to survive inhospitable conditions and to cause disease. Tamayo et al. (2010) found that pathogenic *Vibrio cholera* in both dispersed and intact biofilms vastly out-competed planktonic populations. Huang et al. (2008) found that that *Streptococcus mutans* utilizes the

general secretory pathway to secrete virulence factor proteins and the level of SecA, the key factor in the general secretory pathway, was influenced significantly by biofilm formation. PrfA is the critical virulence transcription factor that regulates the switch from extracellular, flagellum-propelled bacterium to intracellular pathogen in *L. monocytogenes*. Lemon et al. (2010) reported the first evidence that PrfA has a significant positive impact on extracellular biofilm development. Mutants lacking *prfA* were defective in surface-adhered biofilm indicating that PrfA positively regulates biofilm establishment and has a role in modulating the life-style of *L. monocytogenes*. This could provide selective pressure to maintain this critical virulence regulator when *L. monocytogenes* is outside host cells. The human-enteropathogenic species *Yersinia enterocolitica* and *Y. pseudotuberculosis* and the highly virulent plague bacillus *Y. pestis*, represent ideal species to study how bacteria adapt from different environments and evolve to be highly virulent. The work of Hinchliffe et al. (2008) found that several alleged virulence determinants of the *Yersinia* species, regulated by a phosphorelay, also regulated proteins involved in biofilm formation, motility, mammalian cell adhesion and stress survival. *Escherichia coli* are one of the first colonizers of the gastrointestinal tract of newborns and a normal component of the gastrointestinal flora of almost every human being. Found in concentrations up to 10^8 cells ml^{-1} it is a major source for the spread of potentially pathogenic *E. coli* to susceptible sites via the fecal route. Adherence and invasion of intestinal epithelial cells mediated by type 1 fimbriae is a feature of *E. coli* strains isolated from lesions of Crohn's disease.

Salmonella enterica serovar Enteritidis has emerged as one of the most important foodborne pathogens for humans. It is often associated with consumption of contaminated produce, poultry meat and eggs. The spiA gene within S. enterica serovar Typhimurium encodes an outer-membrane component of the SPI-2 type III secretion system that is essential for virulence in host cells. Dong et al. (2011) found that that the spiA gene is also critical to biofilm formation. Biofilm cells, from *Listeria monocytogenes* and *S. enterica* serovar Typhimurium , which survived disinfection, seem to develop a stress response and become more virulent, which may compromise food safety and increase public health risk (Rodrigues et al., 2011a). Legendre et al. (2011) showed that adhered *S. enterica* serovar Enteritidis bacteria were more resistant to antibacterial agents than their planktonic counterparts. Xu et al. (2010) found that the enterotoxin production and invasion ability of biofilm *S. enterica* serovar Typhimurium cells is enhanced under acidic stress conditions. Further, cells of *S. enterica* serovar Typhimurium, collected from a biofilm, showed increased adhesive ability within the spleens of mice. The invasion of *S. enterica* serovar Typhimurium into the intestinal epithelial cells is the crucial step in pathogenesis. Wilson et al. (2007) reported that *S. enterica* serovar Typhimurium samples grown during the weightlessness of space flight exhibited enhanced virulence in a mouse infection model, along with extracellular matrix accumulation consistent with a biofilm.

5. Biofilm development in Salmonella

Scientific understanding of the formation process of biofilms by Salmonella is insufficient and replete with opportunities for further exploration. While some generalities can be made, each species has its own idiosyncrasies relating to the influence of local environmental conditions, gene expression and protein production and secretion. Some of these differences will be discussed in the following section.

In recent years, outbreaks Salmonellosis have often been traced back to contaminated plant sources (CDC, 2011). Lately it has been determined that contamination of plants with Salmonella is not superficial, but due to specific attachment of the bacteria to plant tissues by surface molecules (Barak et al., 2005; 2002). Salmonella uses extracellular matrix components, such as thin aggregative fimbriae and polymers (cellulose and O-antigen capsules) to colonize the plants, forming a biofilm, which is ultimately consumed by and causes illness in humans (Barak et al., 2007). The determination that Salmonella specifically attaches with biofilm formation, challenges the public concept that cleaning vegetables by simply rinsing with water is adequate for bacterial removal. These surface molecules appear to aid this pathogen in the utilization of plants as vectors for spreading and increase the risk of contamination of fresh produce.

Iturriaga, et al. (2007) found that during growth of tomatoes in greenhouses or during postharvest handling, higher humidity promotes biofilm development on the surface of the fruit. These biofilms provide a protective environment for pathogens and reduce the effectiveness of sanitizers and other inhibitory agents used to clean the fruit prior to consumption. *S. enterica* serovar Montevideo was shown to grow on tomato surfaces under a wide range of temperature and relative humidity combinations even when external nutrients were scarce. These findings reinforce the importance of maintaining fruits and vegetables under proper storage conditions to reduce the incidence of Salmonella biofilm development.

Fifteen *S. enterica* serotypes, Anatum; Baidon; Caracase; Cubana; Give; I 13,23,d-; Isangi; Montevideo; Muenchen; Newport; Onderstepoort; Senftenberg; Teko; Wandsbek and Weltevideo, found to form biofilms, were identified from various foods, spices and water sample (Xia et al., 2009). Pulse Field Gel Electrophoresis showed that eight out of the 15 serotypes had patterns indistinguishable from patterns of strains from human clinical samples or foods (US PulseNet National database); indicating that the isolates could potentially infect humans and cause salmonellosis.

Patel & Sharma, (2010) investigated the ability of five *S. enterica* serovars to attach to and colonize intact and cut lettuce (Iceberg, Romaine) and cabbage surfaces. They found that biofilm formation was significantly affected by the serovars used. Generally, *S. enterica* serovars Tennessee and Thompson showed significantly more biofilm formation than serovars Braenderup, Negev, and Newport; and were thus classified as strong biofilm producers according to the criteria suggested by Stepanovic et al. (2004). The criteria states, that strong biofilm producer had four times the optical density (OD) cutoff, which is three standard deviations above the mean OD of the negative control. Understanding the attachment mechanisms of Salmonella to vegetables may be useful in developing new intervention strategies to prevent contamination.

Kim and Wei, (2009) demonstrated that the knockout of the *yjc*C gene, encoding putative diguanylate cyclase/phosphodiesterase, in *S. enterica* serovar Typhimurium DT104 enhanced biofilm formation by the mutant in meat and poultry broths and on contact surfaces. This work also showed that biofilm formation by *S. enterica* serovar Typhimurium DT104 could be affected by the type of food products, since the *yjc*C mutant produced greater biofilms in meat and poultry broths than in vegetable broths. Therefore, the prevention of bacterial biofilm formation on food contact surfaces is critical for controlling cross-contamination of *S. enterica* serovar Typhimurium DT104 in food processing.

Bhowmick, et al. (2011)found the existence of an alternative biofilm regulatory pathway in *S. enterica* serovar Weltevreden from seafood isolates. This is the most prevalent serovar associated with seafood. While human illness caused by this serovar is rare in Europe and United States, it has been reported in Asia. In *S. enterica* serovar Typhimurium the gcpA gene plays a critical role in biofilm formation under low nutrient conditions. In *S. enterica* serovar Weltevreden deletion of the gcpA gene resulted in its inability to produce cellulose and failure to produce biofilm on polystyrene substrate. This indicated that in the case of *S. enterica* serovar Weltevreden, gcpA is critical for activating cellulose synthesis and biofilm formation. The characterization of genes involved in biofilm formation will help in defining critical control points within the process that may be manipulated to control for or possible eliminate the development of biofilms in certain environments.

Small RNAs (sRNA) are non-coding RNA molecules, 50-250 nucleotides in length, produced by bacteria. Kint et al. (2010) showed that biofilm formation is influenced by the sRNA molecule in various *S. enterica* serovar Typhimurium mutants. The sRNA was encoded in the same region as the quorum sensing synthase luxS. Quorum sensing represents a coordinated gene expression response in bacteria, stimulated by local population density. Autoinducer-2 (AI-2) is considered a universal signaling molecule in quorum sensing that is widespread in bacteria, and the LuxS enzyme is required for AI-2 synthesis. Quorum sensing plays an important role in biofilm formation and survival (see section 3). MicA is a family of small RNA molecules highly conserved in several Enterobacteriaceae. These sRNA's are reported to be a regulatory mechanism necessary for biofilm formation in many bacterial species and whose balanced expression level is essential for mature Salmonella biofilm formation.

The *ydcI* gene is differentially regulated in response to conditions of low fluid shear force that increase bacterial virulence and alter other phenotypes in *S. enterica* serovar Typhimurium (Jennings et al., 2011). They found that the *S. enterica* serovar Typhimurium strain in which *ydcI* expression is induced; invaded cells at a level 2.8 times higher than that of the wild type strain. Further, induction of *ydcI* resulted in the formation of a biofilm in stationary cultures, indicating that the *ydcI* gene encodes a conserved DNA binding protein involved with aspects of prokaryotic biology related to stress related biofilm production and possibly virulence. Further, these studies indicate that the *S. enterica* serovar Typhimurium *ydcI* gene is conserved across genera and has auto-regulated expression. When induced, it alters the interactions of *S. enterica* serovar Typhimurium host cells and expedites biofilm formation.

Human-to-human transmission of *S. enterica* serovar Typhimurium makes this a pathogen of global concern. Random transposon mutants of this serovar were screened for impaired adherence and biofilm formation on cholesterol-coated surfaces; 49 mutants with this phenotype were found (Crawford et al., 2010). It was determined that genes involved in flagellum biosynthesis and structure primarily mediated the attachment to cholesterol. In addition, the presence of the flagellar filament enhanced binding and biofilm formation in the presence of bile. This improved understanding of the early events during biofilm development, specifically how Salmonella bind to cholesterol, provides potential therapeutic targets for alleviating asymptomatic gallbladder carriage of *S. enterica* serovar Typhimurium.

6. Biofilm and Salmonella survival

Salmonella enterica serovar Enteritidis is a significant biofilm-forming pathogen. The survival of Salmonella on equipment and instruments in the food industry might be one of the most important contributing factors to food contamination and the subsequent foodborne infection. Further, the biofilm formation ability of foodborne pathogens has attracted much attention in the medical field and food industry due to its potential risks, including transfer of antimicrobial resistance and virulence factors (Xu et al., 2010).

Hasegawa et al. (2011) found that the ability of Salmonella strains to survive in the presence of acetic acid and rice vinegar paralleled their ability to form biofilms. Thus, Salmonella with a high biofilm-formation capability might be more difficult to kill in a food production setting. Salmonella cells embedded in these matrices show reduced susceptibility to trisodium phosphate, desiccation, and chlorination. Further, the connection between biofilm-forming ability and risk of foodborne outbreaks has been suggested in Salmonella. The work of Vestby et al. (2009) showed a correlation between persistence and biofilm establishment of Salmonella thus this may be an important factor for its longevity in the factory environment. These Salmonella strains appear to be a greater risk to human health via food contamination by surviving for longer periods (Iibuchi et al., 2010).

Mangalappalli-Illathu et al. (2008) found significant differences in the pattern and degree of resistance between planktonic and biofilm *S. enterica* serovar Enteritidis cells to benzalkonium chloride (BC). They established that the biofilm phenotype resulted in an early, more efficient adaptive response, and produced a higher proportion of adapted individuals than the planktonic phenotype. Once adapted, these cells were better able to survive BC than the planktonic cells. It is worth mentioning that disrupted BC adapted biofilm cells seem to have a better likelihood to attach, multiply, and form biofilms in BC-containing environments if the concentration is sublethal. The presence of these BC adapted *S. enterica* serovar Enteritidis biofilm cells presents a potential problem in environments such as health care facilities, the food industry, and households.

The presence of *S. enterica* serovars in animal feed ingredients is a well-known problem, resulting in contamination that vectors Salmonella infections in livestock farms. Dual-species biofilms favored Salmonella growth compared to Salmonella in mono-species biofilms, where biomass increased 2.8-fold and 3.2-fold in the presence of Staphylococcus and Pseudomonas, respectively (Habimana et al., 2010). Thus contamination with Salmonella in the presence of other bacteria will only exacerbate the problem of dissemination of Salmonella.

Fresh fruits and vegetables have been increasingly associated with outbreaks of foodborne illness. Salmonella contamination was higher on members of the Brassicaceae family (radish, turnip, and broccoli) than on lettuce, tomatoes, and carrots when sown and grown in contaminated soil. Vegetables that had soft rot exhibited twice the Salmonella contamination as did healthy produce. This could be stress related or possibly because the vegetables are already immunocompromised (Barak and Liang, 2008). Biofilm formation on plant tissue enabled foodborne pathogens to survive in the harsh phyllosphere and decreased the efficacy of commonly used sanitizers (Critzer and Doyle, 2010). Lapidot and Yaron, (2009) demonstrated that *S. enterica* serovar Typhimurium could be transferred from irrigation water to the edible parts of parsley plants. This work also revealed that *S. enterica* serovar

Typhimurium formed aggregates at a depth of 8 to 32 µm beneath the leaf surface. Penetration was most likely achieved through the roots or the phyllosphere. They further determined that, curli and cellulose, both components involved in the formation of biofilms, play a major role in the transfer or survival of *S. enterica* serovar Typhimurium in the plant. Incidences of salmonellosis caused by eating fresh produce continue to increase. This appears to be the result of *S. enterica* serovar Typhimurium attaching to and colonizing plants, rather than incidental contamination. *S. enterica* serovar Typhimurium that preferentially colonize roots use a hydrolase for swarming or biofilm production on plants; this multicellular behavior of *S. enterica* serovar Typhimurium has emerged as central to plant colonization (Barak et al., 2009).

A series of studies from our lab provided a molecular-based characterization of both the biofilm and planktonic populations from continuous-flow culture community. These studies examined the ability of *S. enterica* serovar Typhimurium to colonize a defined microfloral community established to model chicken ceca at day-of-hatch, 7 and 14 days old. The bacterial communities were allowed to equilibrate biofilm and planktonic populations for 3 weeks prior to introduction of *S. enterica* serovar Typhimurium. The one common factor relating to successful invasion of the community was the presence of *S. enterica* serovar Typhimurium within the biofilm. If the introduced *S. enterica* serovar Typhimurium could invade and sequester within the biofilm, then colonization appeared long-term. However if it only invaded the planktonic portion, then it was unable to gain a foothold and did not persist within the community (Crippen et al., 2008; Sheffield et al., 2009a, b).

7. Salmonella biofilms in the environment

Salmonella causes an estimated 93.8 million human infections and 155,000 deaths annually worldwide (Majowicz et al., 2010). The U.S. Centers for Disease Control and Prevention (CDC) have estimated that over 1.4 million cases of infection and 600 deaths related to salmonellosis may occur every year, accounting for about 31% of all food-related deaths in the USA (Wang et al., 2010). Poultry, poultry products, red meat, pork, wild game, and vegetables are all possible vehicles of transmission to humans.

7.1 Poultry

There are many avenues for Salmonella persistence in large scale poultry houses; one is to develop biofilms. Poultry feed has been demonstrated to be a leading source of Salmonella introduction into a poultry production facility (Park et al., 2011). Further, containers used in transporting live poultry between production and processing units have also been incriminated as primary sources of contamination for processed poultry products (Ramesh et al., 2002).

7.2 Non-poultry food animals

In developed countries, the production of food animals (i.e. cattle and hogs) is often limited to highly concentrated rearing facilities, also known as concentrated animal feeding operation (CAFO). This provides a conduit for the spread of Salmonella serovars to a large number of individuals within the herd, as demonstrated by the *S. enteritidis* pandemic in the 1990s, which affected both developed and developing countries (Hendriksen et al., 2011).

Additionally, where wild game is still a key food source, the incidence of Salmonella in feces is upwards of 22% of the wild boar and 48% of the wild rabbit populations in some areas (Vieira-Pinto et al., 2011). This demonstrates the potential for the exchange of bacterial pathogens between wild and domestic animals, which is cause of concern for the welfare of both the wild and the domestic populations, as well as for the humans in contact with them.

Antimicrobial resistance gene-bearing organisms that move from nutritionally rich to more dilute environments, such as when inadvertently washed from CAFO's into the surrounding watershed, survive longer in biofilms (Engemann et al., 2008). Additionally, antimicrobial resistance genes readily transfer into biofilms, which can then be transferred into the surrounding environment, in particular aquatic systems. These organisms are then accessible to wild fauna also utilizing the environment. Many studies have been performed investigating wild animals acting as reservoirs of disease for domestic animals. However, the influence of domestic animals serving as a reservoir of diseases transferable to wildlife is rarely considered. Domestic stock, particularly ungulates, have introduced many diseases into wildlife populations, sometimes with catastrophic results for that population and wildlife conservation on the whole (Mathews, 2010).

7.3 Processed foods

Food processing or handling equipment may provide a niche in which pathogenic bacteria such as *S. enterica* can grow rapidly into highly hydrated biofilms resulting in cross-contamination from food processing surfaces to food products. This cross-contamination can potentially lead to foodborne illnesses. Such cross-contamination of food products has been observed from the use of inadequately cleaned/sanitized processing equipment. Some examples include pumps, containers, or tanks first used for handling raw food materials and subsequently used for processed food products without first undergoing proper sanitation procedures (Jun et al., 2010). Predictably, the food industry has increased interest in chemical, physical, and biological interventions that mitigate food-borne pathogens on these products (Ha and Ha, 2011). *Salmonella* spp. is one of the most commonly isolated pathogens associated with fresh produce (Wong et al., 2011). Penteado & Leitao, (2004) demonstrated that low acid fruits are good substrates for the survival and growth of *S. enterica* serovar Enteritidis, a known biofilm forming pathogen.

8. Salmonella biofilm control measures

Salmonella enterica is a major cause of bacterial food-borne diseases worldwide, and serovars, such as Typhimurium, can cause a localized self-limiting gastroenteritis in humans. In immunocompromised people, Salmonella infections are often fatal if they are not treated promptly with antibiotics (Janssens et al., 2008). While Salmonella infections are most commonly treated using fluoroquinolones (e.g., ciprofloxacin) and extended spectrum cephalosporins (e.g., cefotaxime), there are disturbing reports regarding the development of resistance against these antimicrobials. Further, Salmonella is able to form biofilms on a variety of biotic and abiotic surfaces, where they are a double threat in that they allow the Salmonella to survive and spread in the environment outside the host (Janssens et al., 2008). The Salmonella found in these biofilms show an even higher tolerance to antibiotics than most Salmonella and according to the National Institutes of Health; approximately 80% of persistent bacterial infections in the United States are caused by biofilms (NIH, 1997).

Therefore, the need for alternative strategies to combat the spread of bacterial biofilm related infections is emerging (Janssens et al., 2008).

8.1 Chemical control

Salmonella in biofilms is less susceptible to disinfectants than planktonic Salmonella (Wong et al., 2010); therefore the eradication of biofilm sequestered pathogens is more challenging. *S. enterica* can itself form biofilms that are relatively resistant to chemical sanitizing treatments. The use of glutaraldehyde, formaldehyde, and peroxygen at a concentration of 1.0% in field conditions is insufficient to eradicate Salmonella biofilms (Marin et al., 2009). However, Rodrigues et al. (2011a) and Wong et al. (2011) showed sodium hypochlorite to be one of the most effective disinfectants against biofilms; with the ability to eradicate biofilms at concentrations as low as 3.125 mg per ml. Rodrigues et al. (2011a) also found that bacterial cells from biofilms, which survived disinfection, appeared to develop a stress response and/or become more virulent. The main finding of this work is the worrying fact that, even at concentrations that lead to significant reduction in biofilm biomass, disinfectants may actually enhance virulence within the surviving cells. Adding to this is the fact that the biofilm forms of Salmonella have significantly increased antibiotic resistance properties compared to their planktonic forms (Papavasileiou et al., 2010). These studies confirm that the biofilm form of Salmonella is not only more difficult to remove during sanitation procedures, but has an increased potential to compromise food safety and potentiate public health risk.

In further work, Rodrigues et al. (2011b) examined the adhesion, formation and viability within biofilms of *S. enterica* serovar Enteritidis on regular (granite, marble, stainless steel) and triclosan-impregnated kitchen bench stones (Silestones). Triclosan is a polychlorophenoxy phenol compound with broad spectrum antimicrobial activity that works by targeting lipid biosynthesis and inhibiting cell growth. Salmonella cells adhered equally well (4 to 5 log CFU per cm^2) to all surfaces, with the exception of silestone, which exhibited a potential for bacteriostatic activity. Less *S. enterica* serovar Enteritidis biofilms formed on impregnated silestones and cell viability was one to two logs lower than on other materials (Rodrigues et al., 2011b).

Hasegawa et al. (2011) observed a positive relationship between acid tolerance and biofilm-formation capability in Salmonella by examining the ability of strains to survive and form biofilms in the presence of acetic acid and rice vinegar. It has been suggested that a positive relationship exists between biofilm formation and increased risk of foodborne outbreaks. Therefore, when developing strategies for the prevention of Salmonella contamination of foods it is important to consider the biofilm-formation capability of each particular strain (Hasegawa et al., 2011).

Rosenberg et al. (2008) demonstrated that biofilm formation can be prevented through controlled release of nature-derived antimicrobials, such as salicylate-based poly (anhydride esters). The inhibition of the biofilm appeared to be caused by the irreversible interaction of salicylic acid molecules with the cells. The inhibition was not caused by interference with attachment but rather, via another mechanism essential for biofilm development that remains to be elucidated.

Another promising area of biofilm control is the use of essential oils from a variety of plants. The efficacy of essential oils from the leaves of *Myrcia ovata* Cambess for antimicrobial

activity and prevention of the formation of microbial biofilms by *Enterococcus faecalis* was examined (Candido et al., 2010). The essential oil from this plant is commonly used in Brazil for the treatment of gastric illnesses. This oil showed antimicrobial activity against *E. faecalis, E. coli, P. aeruginosa, S. choleraesuis, Staphylococcus aureus, Streptococcus pneumoniae* and *Candida parapsilosis*. Further, at a concentration as low as 0.5 % it appreciably reduced the formation of biofilm by *E. faecalis* (Candido et al., 2010).

8.2 Predation

Protozoa are important participants within microbial food webs; however protozoan feeding preferences and their effects with respect to bacterial biofilms are not very clear. Work by Chabaud et al. (2006) demonstrated that protozoan grazing had a substantial effect on the removal of pathogenic coliforms in septic effluent and in the presence of a biofilm. Coliform survival was 10 times lower in a septic effluent with protozoa than without them. Further, removal of the bacteria within the biofilm was 60% higher in the presence of protozoa.

A landmark study examined the predatory range of *Myxococcus virescens* and *Myxococcus fulvus*, on a variety of human pathogens, including *Staphylococcus aureus*, *Mycobacterium phlei*, *Shigella dysenteriae*, *Vibrio cholerae*, *Proteus* X, and several Salmonella isolates (Mathew and Dudani, 1955). With the exception of M. phlei, all of the examined pathogenic species were completely or partially lysed, indicating that deciphering the predatory mechanism utilized by *Myxobacteria* species is of practical importance to improve our understanding of how to treat bacterial infectious diseases.

In 1983 Lambina and colleagues (Lambina et al., 1983) isolated a new species (*Micavibrio* spp.) of exoparasitic bacteria with an obligatory parasitic life cycle. They are gram negative, small curved rod shaped (0.5 x 1.5 mm), bacteria with a single polar flagellum. A titer as low as 10 plaque forming units per well of *M. aeruginosavorus* was sufficient to produce a 78% reduction in a *P. aeruginosa* biofilm after 30 min exposure in a static assay (Kadouri et al., 2007).

Dopheide et al. (2011) examined the grazing interactions of two ciliates, the free-swimming filter feeder *Tetrahymena* spp. and the surface-associated predator *Chilodonella* spp., on biofilm-forming bacteria. They found that both ciliates readily consumed cells from both *Pseudomonas costantinii* and *Serratia plymuthica* biofilms. They also found that both ciliates used chemical cues to locate biofilms. Further, using confocal microscopy they discovered that *Tetrahymena* spp. had a major impact on biofilm morphology, forming holes and channels throughout *S. plymuthica* biofilms and reducing *P. costantinii* biofilms to isolated, grazing-resistant microcolonies. Grazing by *Chilodonella* spp. resulted in the development of less-defined trails through *S. plymuthica* biofilms and caused *P. costantinii* biofilms to become homogeneous scatterings of cells (Dopheide et al., 2011).

Bdellovibrio spp. are small, predatory bacteria that invade and devour other gram-negative bacteria. Under dilute nutrient conditions, bdellovibrio prevented the formation of simple bacterial biofilms and destroyed established biofilms (Nunez et al., 2005). During the active prey-seeking period of its life cycle, it moved through water or soil searching for prey. Once it encountered a prey cell, bdellovibrio attached to the prey bacterium's surface, broke the outer membrane, and killed the prey cell by halting its respiration and growth. During the growth period, this predator utilized the prey's macromolecules for fuel and the carcass

provided a protected, nutrient-rich habitat for development. Once the prey resource was exhausted, bdellovibrio divided into multiple progeny that lyse the remains of the prey and swim away to pursue new prey. Depending on the prey and the environmental conditions, its life cycle takes roughly 3–4 h (Berleman and Kirby, 2009; Nunez et al., 2005). While many predatory bacteria have been identified, most have been studied only superficially. Predation behavior has evolved a number of times. Examples of predatory bacteria are found in diverse genera, within the *Proteobacteria*, *Chloroflexi*, and *Cytophagaceae* (Berleman and Kirby, 2009). Dashiff et al. (2010) has demonstrated that predatory bacteria, *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus*, are able to attack bacteria from a variety of genus, including *Acinetobacter, Aeromonas, Bordetella, Burkholderia, Citrobacter, Enterobacter, Escherichia, Klebsiella, Listonella, Morganella, Proteus, Pseudomonas, Salmonella, Serratia, Shigella, Vibrio* and *Yersinia*. Further, predation occurred on single and multispecies planktonic cultures, as well as on monolayer and multilayer biofilms. Finally, *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* have the ability to reduce many of the multidrug-resistant pathogens associated with human infection (Dashiff et al., 2010).

8.3 Radiation

Niemira & Solomon, (2005) found that while the radiation sensitivity of Salmonella is isolate specific, the biofilm associated cells of *S. enterica* serovar Stanley were significantly more sensitive to ionizing radiation than the respective planktonic cells. The dose of radiation value required to reduce the population of *E. coli* O157:H7 by 90% (D10) was highly dependent on the isolate. One isolate exhibited significantly ($P < 0.05$) higher D10 values for planktonic cells than those observed for biofilm cells indicating a significantly increased sensitivity to irradiation for cells in the biofilm habitat. However, for another isolate of *E. coli* O157:H7 exhibited exactly the opposite results. It appears that culture maturity had a more significant influence on the irradiation efficacy of planktonic cells than on biofilm-associated cells of *E. coli* O157:H7 (Niemira, 2007).

9. Future outlook

Current research investigating Salmonella biofilms covers efforts to fully understand the multifaceted process of biofilm development and the intricate relationships between biofilms and virulence, and to develop more effective and environmentally friendly control methods. In the following section we will discuss some of the most recent work reported in these areas.

Shah et al. (2011) have found an association between the pathogenicity of *S. enterica* serovar Enteritidis strains and the differential production of type III secretion system proteins during the production of biofims. In addition several factors including motility, fimbriae, biofilm production, and the presence of large molecular mass plasmids can augment pathogenicity. Such research will provide more insights into molecular basis of *S.* Enteritidis virulence and thus delineate a new direction for the reduction of virulence in *S.* Enteritidis. Based on recent finding, solid murine tumors might represent a unique model to study biofilm formation *in vivo*. Crull et al. (2011) found that systemic administration of *S. enterica* serovar Typhimurium to tumor bearing mice resulted in preferential colonization of the tumors by Salmonella and retardation of tumor growth. Ultrastructural analysis of these tumors did not detect the Salmonella intracellularly, but revealed that the bacteria had

formed biofilms. This model could provide the means for further clarification of the biofilm development process. Research by Sha et al. (2011) utilized the high resolution tool, Rep-PCR, to differentiate closely related microbial strains among Salmonella. This methodology could provide more discriminatory information essential to pin pointing bacterial sources, which is critical to maintaining food safety and public health in the future.

Perez-Conesa et al. (2011) tested eugenol and carvacrol delivered within surfactant micelles at concentrations of 0.9 and 0.7%, respectively. Eugenol is a component of essential oils primarily from clove, nutmeg, cinnamon, and bay leaf; and carvacrol is a predominant phenol found in wild oregano oil. These oils decreased viable counts of 48 hr biofilms of pure *E. coli* O157:H7 or *L. monocytogenes* on stainless steel surfaces by 3.5 to 4.8 logs of CFU per cm2, respectively, within 20 minutes of exposure. Thus, micelle-encapsulated eugenol and carvacrol appear to be good vehicles to deliver hydrophobic antimicrobials through the exopolymeric structure to cells embedded within biofilms. Potentially, these oils could be used in combination with other treatments to diminish biofilm formation on food and food contact surfaces.

The pathogenicity of several significant human pathogens has been linked to the activity of AI-2 quorum sensing signaling, which is also involved with the development of biofilms (Roy et al., 2011). The ubiquitous nature of AI-2 makes it an excellent target as a potential antimicrobial therapy against a broad spectrum of pathogens. Additionally, as AI-2 is not essential for cell growth or survival, interference with its synthesis and processing will probably not stimulate development of resistance. However, as with any single piece of the biofilm pathogenicity puzzle, it is unlikely that quorum sensing quenching drugs will be the "magic bullet" for the treatment of bacterial infections. Therefore, according to Roy et al. (2011) a mixed therapy of quorum sensing quenchers and traditional antibiotics appears to be a promising approach for the future. Finally, it is important that our understanding of signaling molecules be increased, thereby allowing the identification of potential new antimicrobial therapies.

Many questions remain to be answered on the path to understanding the complicated processes involved in the development and expansion of biofilms in human, animal and environmental settings. What specific factors, both biotic and abiotic, govern the initiation and continuation of the biofilm process? What impact does quorum sensing have on the initiation and differential development of the unique biofilm characteristics? What influences the ability of Salmonella to form biofilms and the development of virulence and antibiotic resistance? The final question is how to use this knowledge to manage the environment, and components involved in the biofilm development process to reduce their negative impact on human and animal health.

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2

Motility and Energy Taxis of *Salmonella* spp.

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1. Introduction

1.1 Flagellar motility

The essential morphological prerequisite for active bacterial motility is the flagellum. Besides that, only flotation with the help of self-produced gas vesicles in some cyanobacteria and gliding of filamentous rod-shaped bacteria are known mechanisms of flagella-independent active motion (Madigan & Martinko, 2006). The flagellum is a tail-like protrusion that can be found in many bacterial species. Besides its main function – locomotion – it is also involved in passing the mucosa barrier (Erdem et al., 2007), regulation of auto-aggregation (Ulett et al., 2006), aggregation on solid surfaces, which precedes biofilm formation (O'Toole & Kolter, 1998), and in the export of virulence factors and other proteins (Samudrala et al., 2009). Some bacteria own even more than one flagellum. According to number and arrangement of flagella, different schemes are distinguished (Hahne et al. 2004). Monotrichous bacteria, like *Vibrio cholerae* have only a single flagellum at one cell pole. The amphitrichous flagella arrangement scheme is characterized by single flagella on each of both cell poles, as observable for most *Campylobacter* spp. Lophotrichous flagellated bacteria, e.g. *Pseudomonas aeruginosa*, have multiple flagella on one cell pole and peritrichous bacteria, like *Salmonella* spp. have multiple flagella randomly distributed over the whole cellular surface.

The flagellum acts in principle like a marine screw propeller. Its rotational direction is by definition described by an external observer looking down the flagellar filament toward the bacterial cell (Adler, 1975). The flagellar mechanics is the only known real-rotating joint in the biological world. Its rotation frequency is around 100 Hz (Lauga et al., 2006). The direction of the flagellar motor and in consequence of the flagellar filament determines whether there is a thrust or drag impulse acting on the bacterium. The rotational direction can be reversed in a very short time, thus thrust and drag impulse momentum can switch suddenly. In general, the flagellum pushes the bacterium by providing a pressure gradient, which is relatively high near the filament and acts as a centrifugal force (Gebremichael et al., 2006). According to the physical law of the conservation of the angular momentum, the bacterial body rotates slowly in the counter direction at a rotation frequency of about 10 Hz (Lauga et al., 2006). A counter-clockwise rotation of the flagella causes a bacterial cell to move straight forwards, whereas a clockwise rotation causes the bacterium to tumble. The bacterial movement is controlled by conformational transitions in the flagellar filament between left- and right-handed supercoils (Kitao et al. 2006). These transitions are realized

by a high flexible structure of the flagellar filament, due to "sliding"-interactions and "switch"-interactions", which stabilize inter- and intrasubunit interactions (Kitao et al. 2006). In case of a counter-clockwise flagellar rotation, several filaments of a left-handed helical structure form a bundle and act as propeller. If the flagellar motor rotates clockwise a transition into a right-handed helix of the filament structure is induced and the bundle is feazed (Larsen et al. 1974). The flagella of peritrichous bacteria are synchronized some way that they all rotate in the same orientation. They unite to form a rear-facing bundle that pushes the bacterium forward (Adler, 1975). In amphitrichous bacteria, the flagella of both poles rotate in opposite directions. Thus, the flagellum of the rear-end rotates comparable to monotrichous bacteria in order to provoke a thrust impulse, whereas the flagellum of the bow-end is bent backwards and turns around the front end of the bacterium. Thereby, the thrust impulse is increased. If the direction of the flagellar rotation is reversed, the filaments are fold over. The rear-end of the bacterium becomes the bow-end and the bow-end becomes the rear-end. In consequence, the bacterium swims in the opposite direction. In case of Gram-negative bacteria like *Salmonella* sp., the process of active bacterial movement is divided into continuously alternating phases of slow, non-directed movement called "tumbles" and phases of fast, straight-lined movement called "runs" (Adler, 1975). During a "tumble", the bacterium stops and turns in a more or less randomly chosen direction. It is a passive phase of re-orientation due to a rotational motion, where the non-spherical shape of the bacterial cell affects the way that it is rotated by the shear flow of the surrounding medium. Then the bacterium starts a fast, rectilinear "run", driven by the rotation of the flagella until it stops again and the next motion cycle begins. When the rotational direction of the flagella of peritrichous-flagellated bacteria is to be inverted, the individual flagellum is directed radially from the bacterial cell body in a way that it is sticking out. The dragging effects on the bacterial body outweigh each other to the mean positions in which the bacterium tumbles in a random motion in one place. The reversal of the flagellar rotation and the associated change in the direction of motion plays an important role in (chemo)-tactic movements (Adler, 1975).

1.2 Chemotaxis

Chemotaxis is the process in which bacteria direct their locomotion dependent on the concentration of certain substances in their environment. Compounds affecting chemotaxis are called chemotaxins or chemoeffectors. Chemotaxis in the direction of a higher concentration of the chemoeffector is defined as positive and these kind of compounds are called chemoattractors. On the contrary, chemotaxis away from the higher concentration is defined as negative and these chemotaxins are called chemorepellents. Energy sources usually attract motile bacteria whereas bacteriotoxic agents act as repellents (Fig. 1). The finding, that bacteria move actively towards or away from certain substances, was already made at the end of the 19th century by Engelmann (Engelmann, 1881) and Pfeffer (Pfeffer, 1884 & 1888). Thus, with the help of chemotaxis bacteria direct their movement to find favourable niches with high chemoattracor and low chemorepellent concentrations. This decision-making is based on temporal sensing. As indicated above the overall motion of a bacterium is composed of alternating phases of straight swimming and thumbling. In the presence of a chemical gradient the straight swimming phases last longer, and if the bacterium is moving nat along this gradient, it starts sooner to tumble and tries to reorientate depending on the chemotaxins

concentration (Adler, 1975). The essential prerequisites for chemotaxis are, as already mentioned, a flagella mediated motility, a variety of individual chemoreceptors and a highly conserved chemosensory signal-transduction system.

2. Flagellar motility and chemotaxis

2.1 Experimental approaches

Before the mechanisms of flagellar motility and chemotaxis will be discussed, the most common tools or experimental approaches to study and record bacterial motility and taxis will be presented: microscopy and chemotaxis assay.

2.1.1 Microscopy

Conventional light microscopy is not sufficient to visualize flagellar filaments because of their thinness and the swiftness. One very early approach to visualize flagella of living bacterial cells is dark field microscopy (Macnab, 1976). Since light is scattered by dirt particles reducing the contrast, it has to be considered that the medium and the specimen slides must be remarkably clean. A great advance in this field is video-enhanced differential interference-contrast microscopy (Block et al., 1991). Video microscopy combined with computer based image processing made it possible to detect very small objects like particular microtubules of ≈ 25 nm in diameter. Computerized image analysis offers the option to estimate values like mean cell run speed and average tumbling frequency and their variation in the presence or absence of attractants or repellents (Staropoli & Alon, 2000). Phase-contrast video microscopy combined with the analysis of superimposed image series is a very useful tool, especially for the study of the taxis to and the motion near solid surfaces (Lauga et al., 2006). A further helpful method, although not specifically associated with flagellar motility and chemotaxis, is fluorescence microscopy, which can be used to visualize protein-protein-interactions in the chemoreceptor signaltransduction pathway and the fagellar motor, in combination with green fluorescence fusion proteins (Pierce et al., 1999; Khan et al., 2000).

2.1.2 Chemotaxis assays

Another easy to handle experimental set of tools is composed from different kinds of chemotaxis assays (Miller et al. 2009). One semiquantitative variant is based on changes of the opalescence of a semi-solid agar due to the concentration of bacterial cells (Hugdahl et al., 1988). In a first step, a phosphate buffered saline-agar solution is mixed with a bacterial suspension of a specifiy optical density and poured into a petri dish. After solidification, paper discs with the chemotaxins are placed onto the agar surface following incubation of three to four hours. A more opaque zone can be seen in the surrounding of chemoattractants (see Fig. 1A), whereas chemorepellents are girdled by a more transparent halo (see Fig. 1 B). Other versions of the agar based chemotaxis assay deal with pure – bacteria free - agar plates. After solidification of the agar small recessions are cut into the agar and are filled with either a bacterial suspension or the test solution (Köhidai, 1995). A variation of this assay uses parallel channels (PP-technique) cut from each of both recesses connected by a third perpendicular channel between these two to facilitate diffusion of bacterial suspension and test solution (Köhidai, 1995).

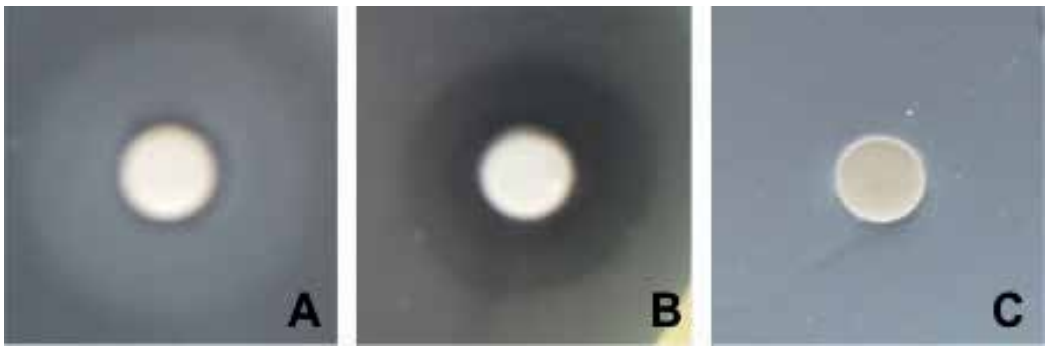

Fig. 1. Examples for chemotaxis: A: Attraction towards towards L-asparagine; B: Repulsion from deoxycholic acid; C: Control (PBS)

A second method is a capillary assay. In this assay, heparinized glass capillaries bridge between a bacterial suspension and a test solution (Koppelhus et al., 1994; Leick & Helle, 1983). Two-phase assays in spectrophotometer plastic cuvettes are suitable to monitor the chemotaxis-mediated migration between the two fluid phases (Koppelhus et al., 1994). The T-maze assay allows the quantification of the chemoresponse of two substances in direct comparison, using a T-shaped experimental arrangement of three containers (Van Houten et al., 1982).

The use of a so-called Boyden chamber is a third variant to study chemotaxis. Chambers divided by filters are a third variant of chemotaxis assays. The suspension of motile cells is placed into the upper vessel of the so-called *Boyden chamber* (Boyden, 1962). The test solution with the chemoeffector is filled into the lower vessel. A filter membrane separates both parts of the Boyden chamber. The pore diameter must be chosen according to the size of the organism allowing its transmigration. To simulate *in vivo* conditions, the filter membranes can be optionally covered with extracellular matrix proteins like collagen, elastin or fibrin. Modifications of this technique connect the vessels either horizontally (Zigmond, 1988) or as concentric rings (Zicha et al., 1991). Multiwell chambers make the parallel testing of different substances in one occasion feasible.

2.2 Molecular structure of the flagella motor and chemoreceptors

2.2.1 Molecular structure and synthesis of the flagellar apparatus

Non-flagellar Type III secretory systems and the flagellar apparatus share a common basic architecture. Thus, it seems apparently that both go back to a common evolutionary origin (Toft & Fares, 2008).

Basically, the flagellum is comprised of three parts: a helical filament, the hook and the basal body (see Fig. 2). The filament of bacterial flagella is built up of multiple subunits of the flagellin protein FliC (Samatey et al., 2004), which form so called protofilaments. Eleven circular arranged protofilaments stacked into a left- or right-handed helix, according to the direction of rotation (see above) comprise the filament (O'Brian & Bennet, 1972). It has a length of about 10 µm and a diameter of about 20 nm. If a flagellum is virtually flattened, it shows a constant interspace between adjacent turns, corresponding to the wavelength of a

"sine wave" (Madigan & Martinko, 2006). This wavelength is specific for any bacterial species and is determined by the structure of the flagellin protein and the rotational direction of the filament (Madigan & Martinko, 2006).

The hook tethers the filament to the basal body. Electron microscopic studies demonstrated that the hook of *S. typhimurium* has length of 55 ± 6 nm (Hirano et al., 1994). The interdigitated hook-subunits make up a bended tube with a 2 to 3 nm wide central channel, which continues in the rod as well as in the filament (Shaik et al., 2005). The hook connects the filament to the motor portion of the flagellar apparatus. The hooks flexibility permits the transmission of torque from the motor to the helical propeller when both are not in a coaxial orientation to each other (Berg & Anderson, 1973). A so-called gap compression/extension mechanism and mutual sliding of the hook-subunits allows continuous structural change of the hook during flagellar rotation at low energy cost (Furuta et al., 2006). The hook facilitates the synchronization of several filaments bundled together at one cell pole (Macnab, 1977; Berg & Anderson, 1973). The two hook-associated proteins (HAP 1 and 3) form a small hook-filament junction, which acts as an adaptor for transition between the hook that is flexible in bending but rigid against twisting and the much more stiff filament (Samatey et al., 2004).

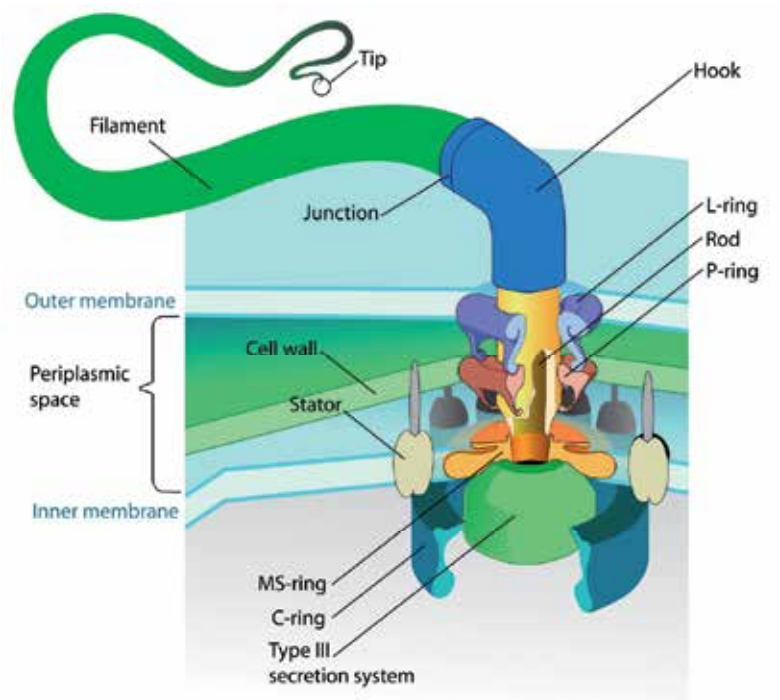

Fig. 2. The flagellum of Gram-negative bacteria, like *Salmonella* sp., is a complex structure consisting of the propeller like acting filament, the flexiple hook and the flagellar ATP-driven motor, which is comprised of four ring-structures and the static motor complexes. An integrated flagellar type III secretion system exports actively the proteins for flagellar assembly but also some virulence associated factors. (Copyright: Wikimedia Commons, public domain aviable from http://upload.wikimedia.org/wikipedia/commons/1/15/ Flagellum _base_diagram_en.svg)

The third part of the flagella – the basal body is comprised of a rod sticking in four ring-like structures: the L-ring (associated with lipopolysaccharides) and the P-ring (associated with peptidoglycanes) forming an outer cylinder embedded in the plasma membrane, the MS-ring, building up a motor mounting plate; and the versatile C-ring (Macnab, 1999). Gram-positive bacteria lacking an outer membrane lack consequentely the outer ring structures.

Overall more than 40 genes are involved in flagellar synthesis and subsequent motility in *S. typhimurium* (Shaik et al. 2005). The MS- and the C-ring, the export apparatus as well as the motor and switch are the first synthesized compounds of the flagella (Katayama et al., 1996; Macnab, 2003). The following assembly steps utilize the type III export apparatus, while the export substrates are supplied via an delivery apparatus located in a patch of membrane near the center of the MS-ring to the channel (Suzuki et al., 1998; Macnab, 2003). It follows the formation of the rod and the other two rings. The proteins of which the rod is comprised are FlgB, FlgC, FlgF and FlgG (Homma et al., 1990). The bifunctional FlgJ protein, which has a muraminidase activity to open the peptidoglycan layer for penetration of the sprouting rod, is also able to bind other rod constituents (Hirano et al., 2001), and thus may act as a rod capping protein promoting the assembly of the rod out of its four components (Nambu et al.; 1999, Macnab, 2003). The assembly of the basal body is finished by the synthesis of the periplasmic P-ring made out of FlgI (Homma et al., 1987) and the outer membrane L-ring consisting of FlgH-subunits (Jones et al., 1990). In the following step, the rod cap is dislodged, while the hook-cap, consisting of about 5 FlgD-subunits, is mounted (Macnab, 2003) and the hook is attached on the basal body. It consists, comparable to the flagellar filament, of about 120 copies of a single kind of protein, FlgE – the so-called hook protein (Samatey et al., 2004). Parallel, the L-ring is assembled (Kubori et al., 1992; Ohnishi et al., 1987). After this, two junction protein zones, made either of FlgK or FlgL, are attached, and a so-called filament-cap out of FliD-proteins is mounted on the hook (Homma et al., 1985; Ikeda et al., 1987 & 1989). Cap proteins assist the organization of the flagellin proteins to form a new filament (Ikeda et al., 1985). Between hook and cap, a junction zone is synthesized before the protofilaments are assembled (Macnab, 2003). The flagellin molecules pass the channel inside the hook and the filament and add on successively at the lower end. The flagellar assembly starts beneath the cap and grows from its tip to its base. A mature flagellum is composed of approximately 20 000 copies of flagellin protein. A broken flagella can be repaired with newly synthesized flagellin units from the cytoplasm passing through the filament channel (Homma & Iino, 1985). The proteins, which built up the flagellum, are translocated to the distal part of the growing flagellum through the central channel by a flagellar type III homologous protein secretion system (Ibuki et al., 2011). This secretion system is comprised of two classes of proteins: soluble and membrane associated ones. The essential soluble compounds of this ATP-consuming process are the soluble FliI-ATPase, its regulator FliH and the FliJ-protein, that promotes the hexamerization of FliI-ATPases (Ibuki et al., 2011). The remaining soluble components are specific chaperones: FlgN for the hook-filament junction proteins, FliT for the filament cap protein, and FliS for flagellin (Macnab, 2003). The six membrane associated components FlhA, FlhB, FliO, FliP, FliQ, and FliR form a complex within the MS-ring (Macnab, 2003).

A rotary motor is in principle built up of two functional components: the rotor and the stator. The flagellar motor consists of the static Mot-complexes, which were affixed in the inner cytoplasmatic membrane and the rotating C-ring. The Mot-complexes are

transmembrane structures made from two proteins MotA and MotB (Macnab, 2003). The cytoplasmic C-ring contains the motor/switch proteins – the Fli-proteins. The FliG-proteins generate the torsional moment, while working against the Mot-complexes. The switch-proteins, in *Salmonella* sp.: FliG, FliM and FliN can reverse the flagellar rotational direction in response to intracellular signals (Francis et al., 1994; Yamaguchi et al. 1986). The FliM-protein is the final effector of a sensory transduction chain (Bren et al., 1998; Sockett et al., 1992). Thus, the stator is formed by the Mot-proteins, which mantle the C- and the MS-rings. C- and MS-rings, as well as the rod, collectively form the rotor.

Driven by a transmembrane proton gradient, the flagellar motor is able to perform the clockwise and a counterclockwise rotation of the filament, which is reversed stochastically in the absence of any stimulus. The protons are pumped from outside across the cytoplasmic membrane through the Mot-complexes. Calculations showed that about 1000 protons must be translocated to perform a single rotation of the flagellar filament. In the proposed proton pump model, the protons flowing through the stator channels exert electrostatic forces on helically arranged charge clusters on the rotor rings. Most probably, the protons bind temporary to a specific aspartate residue of MotB, causing a change of the stators conformation that drives the rotor through an elementary rotational step (Kojima & Blair, 2001; Macnab, 2003). In the next step, the aspartate residue gets deprotonated and the stator returns to its original conformation. These resulting series of interactions between positive and negative charges generate a torsional moment as protons flow through the Mot-complexes.

2.3 Chemoreceptor structure and signal transduction

Presence and concentration of chemotaxins are detected by a family of chemoreceptors sharing a common two-component system architecture (Miller et al. 2009). Such two-component systems are generally comprised of a membrane associated histidine autokinase (CheA) and a cytoplasmic response regulator (CheY; Lux et al. 2004). Methyl-accepting chemotaxis proteins (MCPs), embedded in the cytoplasmic membrane, sense the environmental signals via their *N*-terminal periplasmic sensory domain to their *C*-terminal cytoplasmic signaling domain. The MCP-monomers have a molecular mass of about 60 kDa and form constitutively very stable homodimers, which are arranged in groups of three (Lux et al. 2004). CheW linker proteins tether the CheA histidine kinase to the MCPs (Miller et al. 2009). This inhibits autophosphorylation of CheA, which in turn reduces the phosphorylation of CheY response regulator at a conserved histidine residue. Hypophosphorylated CheY can diffuse freely in the cytoplasm and can interact with FliM, the switch protein of the flagellar motor (Mot), which is the final effector of sensory transduction chain (Bren et al., 1998; Sockett et al., 1992). Thus, it triggers counterclockwise rotation of the flagella, which leads to bacterial "running". If a bacterium moves along a gradient of a chemoattractant, the intracellular concentration of phosphorylated CheY decreases. Consequently, the frequency of flagella switching decreases, and the number of site directed "runs" along the gradient increases. Thus, addition of an attractant triggers a counterclockwise rotation of the filament (Bren & Eisenbach, 2000).

In the opposite case, decreasing ligand occupancy of the MCPs leads to increased autophoshorylation of CheA and in consequence to an amplified phosphorylation of CheY

and CheB. Phosphorylated CheY binds as well to the motor switch but triggers a clockwise flagellar rotation resulting in bacterial "tumbling".

CheB is a receptor-demethylating enzyme, which is also activated by phosphorylation. The phosphatase CheZ is responsible for the dephosphorylation of phosphate-activated CheY (Bourret & Stock, 2002).

The result of this chemosensing in three-dimensional spatial gradients of different chemoattractors and chemorepellents is a stereoscopic "zigzag" path of motion (Berg & Brown, 1972), until the bacterium reaches a niche with an equilibrium between the varying chemoeffectors (Miller et al., 2009).

2.4 Sensory adaptation

Sensory adaption means reestablishment of the prestimulus state in the perpetual presence of the stimulus. Adaptation to chemotactical stimuli is mostly due to modulation of the methylation of certain sites of the MCP receptors. The central players in the process of sensory adaption are the methyltransferase CheR, the methylesterase CheB, and the cytoplasmic domains of the MCP-receptors that have adjacent to the CheA and CheW binding sites, sites for methylation and demethylation of glutamyl side chains (Macnab, 2003).

CheR catalyzes in a S-adenosylmethionine consuming reaction the methylation of the specific glutamate residues on the cytoplasmic domains of the MCPs (Bren & Eisenbach, 2000). This reaction enhances the CheA autophosphorylation favouring clockwise flagallar rotation and is triggered by attracting stimuli (Borkovich et al., 1992; Ninfa et al., 1991). The cytoplasmatic domains of the MCPs have a specific domain, which is methylated by CheR, and a distinct CheR-binding site consisting of a pentapepetide that is only present in high-abundance receptors. It was shown that CheR bound to the binding sites onto the high-abundance receptors methylates the designated sites of the low-abundance receptors (Le Moual et al., 1997; Li et al., 1997).

Its antagonist is the methylesterase CheB, which demethylates the MCPs during adaptation to repelling stimuli. Additionally CheB has an amidase activity catalyzing the conversion of glutamamine residues into glutamate on the MCPs (Djordjevic et al., 1998). The liberation of glutamate residues inhibits the autophosphorylation of CheA favouring a counterclockwise rotation of the flagellum. In addition CheB itself is regulated by CheA-mediated phosphorylation (Hess et al., 1988; Lupas & Stock, 1989). Phosphorylation inhibits the methylesterase activity, while the unphosphorylated enzyme has less methylesterase activity. The binding sites on CheA for CheY and CheB are identical. Thus CheB competes with CheY (Li et al., 1995).

Furthermore a high methylation rate decreases the receptors affinity to chemoattractants (Bornhorst et al., 2000; Li et al., 2000). It was also suggested that a deferred activation of CheZ, which is responsible for an enhanced CheY dephosphorylation, is involved in the process of sensory adaptation (Blat et al., 1998).

These regulatory effects occur only after the initial chemotactic response and the steady state of all these parallel-acting adaptational processes determines the extent of reaction to a certain chemoeffector (Alon et al., 1999).

2.5 Specific Salmonella chemoreceptors

Altogether, it is difficult to identify chemoreceptors specific for a certain taxin, because the different MCPs can compensate each other in many cases, Thus, knockout mutants of chemoreceptor genes show often no defects in their phenotype (Vegge et al., 2009; Tareen et al. 2010). Up to now, four chemoreceptor specificities are identified for *Salmonella* spp.

The Tar chemoreceptor is specific for aspartate and initiates attractant signalling (Foster et al. 1985; Milburn et al., 1991). The same receptor molecule interacts also with the periplasmic maltose-binding protein, and senses in this way chemoattraction towards maltose (Mowbray & Koshland 1987; Gardina et al., 1992). It was also demonstrated that this receptor mediates attractant responses to phenol and repellent responses to glycerol and nickel or cobalt ions in *Escherichia coli* as well as thermoresponses (Lee & Imae, 1990).

The ligand serine mediates positive taxis via the Tsr receptor, whereas Tsr sensing due to leucine and glycerol results in a repulsion of the bacteria. (Lee & Imae, 1990; Jeffrey & Koshland, 1993; Oosawa & Imae, 1984; Springer et al., 1977). Tsr functions also as thermoreceptor. Temperature increase leads to smooth swimming of bacterial cells, whereas temperature decrease induces tumbling (Lee et al., 1988).

S. typhimurium demonstrates attraction towards citrate and metal-citrate complexes, but repulsion from phenol. This behavior gives the name to the third chemoreceptor in this schedule – Tcp, that stands for taxis to citrate and away from phenol (Yamamoto & Imae, 1993).

The *trg* gene encodes a fourth chemoreceptor of the MCP family specific for ribose/galactose (Blat & Eisenberg, 1995, Kasinkas et al. 2007).

2.6 Virulence factors secreted via the flagellar type III secretion system

As mentioned above, the flagellar apparatus is a homologue of a type III secretion system that is able to secrete specific peptides and proteins in an ATP dependent mechanism into the environment (Collazo & Galán, 1996; Eichelberg et al., 1994). Among these secreted proteins are mostly structural components of the flagella, for example flagellin monomers, and the hook protein, but also several virulence factors. It functions as a molecular syringe – the so-called injectisome – that is used by bacteria to inject effector proteins directly into the interior of host cells (Mota et al., 2005a+b; Arnold et al. 2009). Thus, these proteins play an important role for host cell invasion and the pathogenesis of salmonellosis. It was shown, that the *N*-terminal 30 residues of these effector proteins form a taxonomically universal, type III specific secretion signal (Arnold et al. 2009; Samurdrala et al. 2009). About 65 type III secretion system substrates are known for *S. typhimurium* (Samurdrala et al. 2009). Five well described proteins involved in host cell invasion, typically the M-cells of the ileal Peyers' Patches, are InvE, Sipa, Sipb, SipC, and SipD. InvE plays a pivotal role for triggering cellular mechanisms, which lead to bacterial entry. It is required for translocation of other effector proteins into the cytosol of host cells and forms complexes with SipA, SipB, and SipC. (Kubori & Galán, 2002). Comparable to InvE the effector protein SipD, which has been shown to be important for liver and ileum colonization, is suggested to modulate the secretion of SipA, SipB, and SipC (Gong et al., 2010). Cell invasion occurs via a ruffle-mediated mechanism, which is initiated by the activation of specific signal transduction cascades and rearrangement of the actin cytoskeleton. The actin rearrangements are realized

by SipA interworking with SopE, a guanine-nucleotide exchange factor for Rho GTPases, and SptP, a protein tyrosine phosphatase, (Brumell et al., 1999) as well as SipC that binds and bundles F-actin (Myeni & Zhou, 2010).

SipB interacts after entering the cytosol of macrophages with cell signalling pathways to induce apoptosis (Hersh et al. 1999). It associates with caspase-1 and promotes the proteolytic activation of this protease.

Two further proteins entering macrophages are SrfN and PagK2, which were shown to be essential for full virulence and are suggested to interact with host cellular components (Yoon et al. 2011). These two effector proteins are translocated independently of the injectisome. Thus, the flagellar type III secretion system is the only protein export mechanism in *Salmonella* sp.

2.7 Role of chemotaxis and flagellar motility for the pathoegenesis of salmonelosis

The ability for directed movement and taxis towards and away from chemoeffectors plays a crucial role for the pathogenesis of salmonellosis. Amongst others, *Salmonella* bacteria are able to persist inside the inner leaf tissue of plants (Kroupitski et al., 2009; Goldberg et al., 2011). It was shown that flagellar motility and chemotaxis towards nutrients produced by photosynthetically active cells are crucial for entry into iceberg lettuce leaves via open stomata and invasion into the plant tissue (Kroupitski et al., 2009). Enteropathogens have the ability to adapt to the phyllosphere environment. They obviously interact with epiphytic bacteria (Beuchat, 2002; Brandl, 2006; Heaton & Jones 2008) and become part of phylloplane biofilms, where they gain protection from environmental stressors (Fett, 2000). Plants that might become contaminated by the use of germ-containing water for irrigation or *Salmonella*-containing liquid manure for fertilization might function as source of infection (Beuchat, & Ryu, 1997; Brandl, 2006; Horby et al., 2003). Internal persistence after entering the plant tissue explains the failure of lavation and sanitizers to eradicate *Salmonella* in leafy greens.

Furthermore, flagellar movement and chemotaxis are also pivotal for the intestinal colonization of the different *Salmonella* hosts, especially for the competition for nutrients with other bacteria of human microbiome (Stecher et al., 2008). Even the induction of colitis depends on a functioning flagellar movement and chemotaxis (Stecher et al., 2004).

3. Conclusion

The flagellar apparatus is an evolutionary ancient multifunctional tool involved in motility, bacterial cell aggregation, biofilm formation, protein export, and a virulence factor injection via the injectisome. It is also the prototype of a sensing system, coupling energy taxis and motility. The research on chemotaxis and flagellar motility is almost as old as bacteriology itself, starting at the end of the 19th century. The research on *Salmonella* sp. plays here a special role, as most of the knowledge about thermo- and chemotaxis, MCP-receptor signal transduction, MCP-receptor sensory adaptation, structure, synthesis, and function of the flagellar apparatus as well as effector protein secretion via a flagellar type III homologue secretion system was made using *Salmonella* sp. and *E. coli* as model organism.

Thus, the flagellar apparatus regulated by energy taxis may be the most important structure for intestinal colonization and pathogenesis of salmonellosis.

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3

Influence of a Salt Water Regulator on the Survival Response of *Salmonella Paratyphi* in Vembanadu Lake: India

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1. Introduction

Contamination of environmental water by pathogenic microorganisms and subsequent infections originated from such sources during different contact and non- contact recreational activities are a major public health problem worldwide particularly in developing countries. The main pathogen frequently associated with enteric infection in developing countries are *Salmonella* enterica serovar typhi and paratyphi. Although the natural habitat of *Salmonella* is the gastrointestinal tract of animals, it find its way into natural water through faecal contamination and are frequently identified from various aquatic environments (Baudart *et al*., 2000; Dionisio *et al*., 2000; Martinez -Urtaza *et al*., 2004., Abhirosh *et al*., 2008). Typhoid fever caused by *S. enterica* serotype typhi and paratyphi are a common infectious disease occurring in all the parts of the world with its highest endemicity in certain parts of Asia, Africa, Latin America and in the Indian subcontinent with an estimated incidence of 33 million cases each year with significant morbidity and mortality (Threlfall, 2002). In most cases the disease is transmitted by polluted water (Girard *et al*., 2006) because of the poor hygienic conditions, inadequate clean water supplies and sewage treatment facilities. However in developed countries the disease is mainly associated with food (Bell *et al*., 2002) especially shellfish (Heinitz *et al*., 2000).

Salmonella, since being allochthonous to aquatic environments, the potential health hazard is dependent on their period of survival outside the host and retention of critical density levels in the receiving water in a given time frame during transmission via the water route. In general, the major environmental factors influencing the enteric bacterial survival following their exposure to aquatic environments are water temperature (Anderson *et al*., 1983), adsorption and sedimentation processes (Auer & Niehaus, 1993), sunlight action (Sinton *et al*., 1999), lack of nutrients (Sinclair & Alexander, 1984),

predation by bacteria or protozoa (Hahn & Hofle, 2001), bacteriophage lysis (Ricca & Cooney, 1999), competition with autochthonous microbiota (McCambridge & McKeekin, 1981) and antibiosis (Colwell, 1978).

Although *Salmonella* spp. has been isolated from fresh, estuarine and marine waters, they showed differential survival response to those aquatic environments and the results were sometimes contradictory in relation to salinity. For instance, it has been reported that *Salmonella* showed very low survival in sea water (Lee *et al*., 2010) on the contrary Sugumar & Mariappan (2003) found that they exhibited very long survival up to 16 to 48 week in sea water. But it is also documented that it survived for 54 days (Moore *et al*., 2003) and 58 days in freshwater Sugumar & Mariappan (2003). However, when *Salmonella* suspended in stabilization ponds effluent and rapidly mixed with brackish water, survival time was particularly short, whereas it was prolonged when the bacteria was submitted to a gradual increase in salinity (Mezrioui *et al*., 1995). Therefore the survival of pathogenic bacteria in estuarine environments in response to varying saline concentration due to the mixing of salt water with freshwater has of particular health significance especially in locations where contact and non recreation takes place.

Hence the present study has been carried out in Vembanadu Lake that lies 0.6-2.2 m below mean sea level (MSL) along the west coast of India (9°35'N 76°25'E) and has a permanent connection with the Arabian Sea (Fig.1). As the north-east monsoon recedes, the area is exposed to tidal incursion of saline water from the Arabian Sea. In order to prevent the saline incursion during certain periods of the year, a salt water regulator is constructed in the lake. It divides the lake into a freshwater region on the southern part and a saline lagoon on the northern part. As a result, during the closure and opening of the regulator the water quality on both regions of the regulator may change in terms of its salinity and a progressive saline gradient may occur throughout the lake when the regulator is open. On the other hand over 1.6 million people directly or indirectly depend on it for various purposes such as agriculture, fishing, transportation and recreation. As a result water related diseases are very common in this region particularly in young children but none of them were reported officially. Enteric fever caused by *Salmonella* enterica serovars paratyphi *A*, B and C and Newport have been reported in India (Misra *et al*., 2005; Gupta *et al*., 2009).

Since die-off of enteric bacteria in aquatic environment could be attributed to a variety of interacting physical, chemical and biological factors and processes (Rhoder & Kator, 1988), in our previous studies in the Vembanadu lake we have evaluated the effect of sunlight, chemical composition of the estuarine water (Abhirosh & Hatha, 2005) effect of biological factors such as protozoan predation, predation by bacteriophages, autochthonous bacterial competition (Abhirosh *et al*., 2009) on the survival of *Salmonella* and other organisms. However, the effect of salinity, since being important on the survival of enteric bacteria has not been evaluated in Vembanadu lake. As we already reported the presence of different *Salmonella* serotypes such as *Salmonella paratyphi* A, B, C and *Salmonella Newport* in Vembanadu lake (Abhirosh *et al*., 2008), in this study our aim was to evaluate the health risk associated with *S. paratyphi* when released into the water by studying the survival responses to the salinity changes (saline gradient) caused by the saltwater regulator in Vembanadu lake using microcosm experiments at 20°C and 30°C.

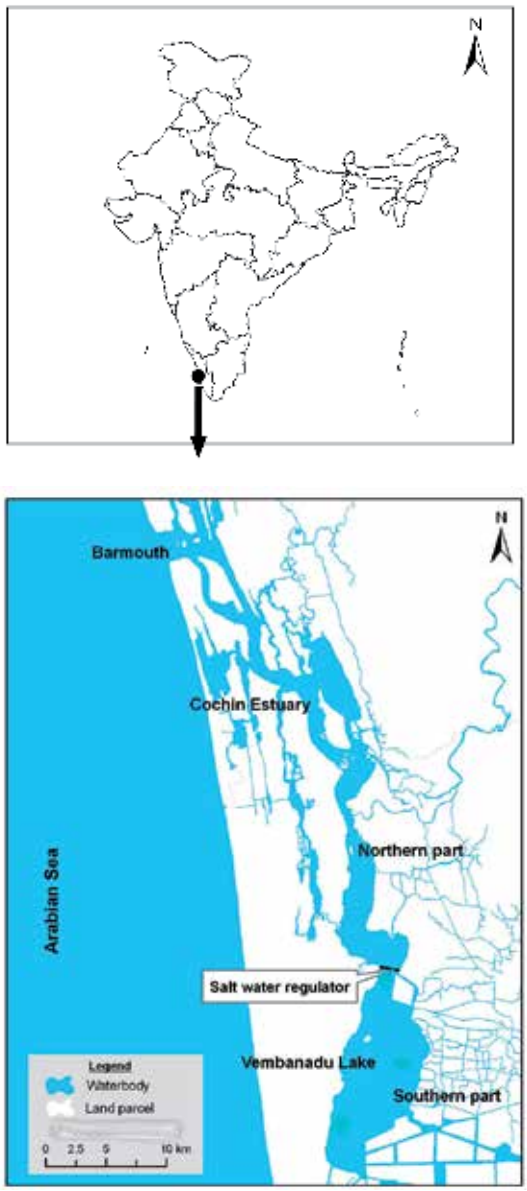


Fig. 1. Map showing Vembanadu Lake

2. Materials and methods

2.1 Test organism and water sample

A pure culture of *S. paratyphi* isolated from the Vembanadu lake was used for the survival experiments. All experiments were conducted in filter sterilized lake water in order to avoid the effect of predation. When saltwater regulator is closed the saline intrusion from northern part is prevented and the water on the southern part becomes freshwater. Therefore, to

imitate the actual condition on the southern part of the lake, experiments were conducted in water collected from the lake when the salinity was 0 ppt (freshwater microcosm). To study the survival of the test organisms during mixing of water from northern and southern part of the Vembanadu lake, experiments were conducted in mixing water samples collected when the regulator was open (mixing water microcosm). Besides, in order to study the survival in all possible saline gradient throughout the year, survival experiments were conducted in lake water with salinity concentration ranged from 0-25 ppt. The test solutions of desired saline concentrations were prepared using fresh lake water with NaCl.

2.2 Preparation of inocula

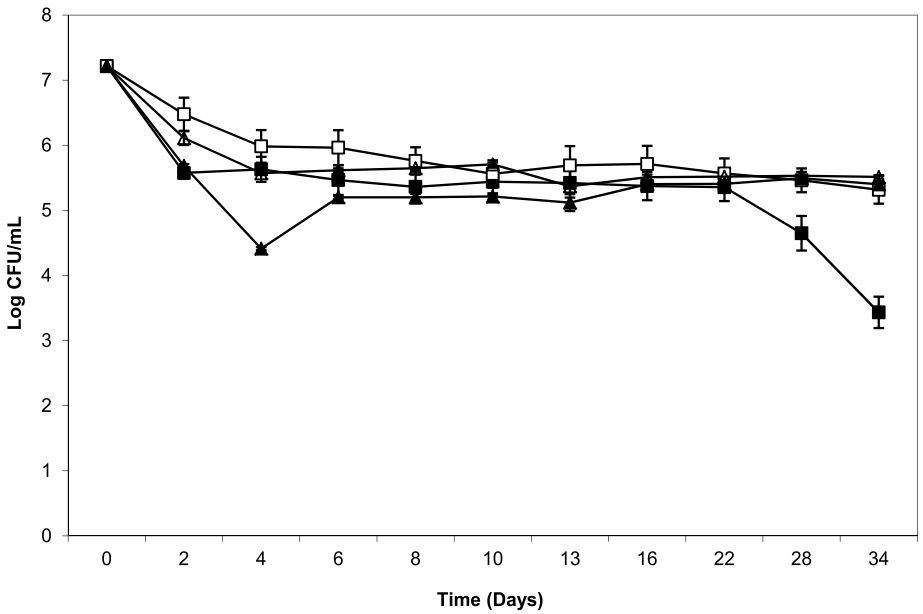
The inocula were prepared as previously described by Abhirosh & Hatha (2005). *S. paratyphi* was grown in Tryptone Soy Broth (TSB) and incubated at 37°C for 24 h. After incubation, the cells were concentrated by centrifugation at 1400 × g for 15 min and washed twice with sterile isotonic saline. After the final wash, the cells were re-suspended in sterile isotonic saline for inoculation into the microcosms. Then 1 ml washed cell suspension of *S. paratyphi* was inoculated into each microcosm containing different test solution (250 ml Erlenmeyer flask with 100 ml) at a concentration of 10^{6-7} CFU/ mL. All inoculated microcosm were incubated at 20°C and 30°C. The microcosms were incubated at 20°C in order to find out the survival at low temperature as the temperature goes down to 20°C in winter as well as at a certain depth. The enumeration of culturable bacteria were done after 2, 4, 6, 8, 10, 13, 16, 22, 28 and 34 days using spread plate technique on TSA agar plates and the colony forming units were counted.

2.3 Decay rate and statistical calculation

The decay rates of culturable *S. paratyphi* cells were calculated as per first order decay model using the following equation $\text{Log } N_t/N_0 = -kt$, where N_t is the number of bacteria at time t, N_0 is number of bacteria at time 0, and t is expressed in days; k is the first-order constant calculated by linear regression technique. T_{99} (time required for 2 log reduction) values were calculated using the decay constant (k) in the following equation, $T_{99} = -2/k$.The difference in the survival at different salinities and temperature was analysed using two way analysis of variance (ANOVA).

3. Results and discussion

The survival curves of *S. paratyphi* in freshwater and mixing water at 20°C and 30°C are given in Fig. 2 and the inactivation rates and T_{99} values are given in Table 1. The results revealed that *S. paratyphi* showed significantly ($p<0.01$) higher survival at 20°C (T_{99}= 25.99) compared to 30°C (T_{99}= 17.68) in freshwater water indicating their better survival capacity at low temperature. However *S. paratyphi* did not show much difference in the survival response in mixing water at both temperature and the T_{99} respectively was 16.37 days at 20°C and 15.12 days at 30°C. The results revealed that *S. paratyphi* cells remained viable until 34 days at a high density of 10^5 CFU/mL. The salinity of the mixing water when it was collected was 12.77 ppt and the average saline concentration of the lake water was 12.5ppt when it was monitored over 2hr interval in a day.



Freshwater 30°C■ and 20°C □; mixing water 30°C ▲and 20°C Δ

Fig. 2. The survival curves of *S. paratyphi* in freshwater and mixing water at 20°C and 30°C

Days	Freshwater 30°C	Freshwater 20°C	Mixing water 30°C	Mixing water 20°C
0	0.00	0.00	0.00	0.00
2	-1.64	-0.74	-2.48	-2.05
4	-1.59	-1.23	-3.75	-2.59
6	-1.75	-1.25	-2.96	-2.54
8	-1.86	-1.46	-2.96	-2.51
10	-1.78	-1.66	-2.95	-2.45
13	-1.80	-1.53	-3.04	-2.79
16	-1.84	-1.50	-2.76	-2.65
22	-1.86	-1.65	-2.75	-2.64
28	-2.57	-1.75	-2.67	-2.63
34	-3.78	-1.90	-2.76	-2.65
K value	-0.11	-0.07	-0.13	-0.12
T99	17.68	25.99	15.20	16.37

Table 1. Inactivation rates of *S. paratyphi* in freshwater and mixing water at 30°C and 20°C

Even though the survival time was longer, in agreement with our results Sugumar & Mariyappan (2003) reported that *Salmonella* survived up to 24 weeks in sterile freshwater microcosm at 30°C but at low temperature it survived for 58 weeks. It is also documented that it survived for 54 days (Moore *et al*., 2003) in freshwater. Since *S. paratyphi* did not show much difference in survival response in mixing water at both temperatures, similar to our results Rhodes and Kator (1988) reported that *Salmonella* populations exhibited significantly less die-off in filtered estuarine water at temperatures of <10°C. In sterile estuarine water virtually unaltered bacterial densities over a 10-day period have also been reported by McCambridge & McMeekin (1980a,b). It has been documented in other studies that low temperature is favorable for the survival of *Salmonella* in (Vasconcelos & Swartz, 1976; Hernroth *et al*., 2010) and other enteric bacteria in aquatic environments (Craig *et al*., 2004; Sampson *et al*., 2006; Silhan *et al*., 2006).

The aim of conducting this survival experiments in freshwater and mixing water was to evaluate the public health risk associated with *S. paratyphi* in Vembanadul lake during the closure and subsequent opening of the regulator. While addressing this issue it has been noticed that similar to other studies *S. paratyphi* could survive very long time in freshwater and mixing water until the end of the experimental period. Therefore the log term survival potential *S. paratyphi* in freshwater may pose health risk since people use this region for their freshwater needs and we have already recorded high abundance of indicator bacteria and enteric pathogens (*Salmonella* serotypes such as *S. paratyphi* A, B, C and S. Newport) on the southern part during the closure of the saltwater regulator (Abhirosh *et al*., 2008). During the closure of the saltwater regulator the water on southern part of the lake become fresh and the natural flow is prevented which results in the accumulation of organic load in the southern part of the lake, giving proper environmental conditions for the multiplication of bacteria. Besides, the high survival capacity noticed at low temperature further increases the health risk during monsoon season because of the drop down of the water temperature to nearly 20°C and we already reported high prevalence of indicator and pathogenic bacteria in southern part of the lake during monsoon season (Abhirosh *et al*., 2008) and every year waterborne disease outbreaks occur during monsoon season. Prolonged survival of *S. paratyphi* in mixing water suggests that it can remain viable in water at high concentration (10^5CFU/ml) when the saltwater is open. It was almost similar to the results we obtained for *S. typhimurium* in Cochin estuary where we found it remained viable at even higher density (10^6 CFU/mL) until the end of experiment (Abhirosh & Hatha . 2005) at 20°C and 30°C. Our results are also in agreement with other studies that better survival of enteric bacteria in estuarine and other aquatic environments (Rhodes and Kator, 1988; Placha *et al*., 2001).

It has been reported that *Salmonella* may be of prolonged public health significance once it is introduced into tropical surface waters than *E. coli* (Jimenez *et al.,* 1989). Sporadic outbreaks of enteric fever due to *S. enterica* serovars paratyphi A, have been reported in India with an annual incidence of 3 million cases (Threlfall,2002; Misra *et al*., 2005). *S. enterica* serovar paratyphi A has emerged as an important cause of enteric fever in India Gupta *et al*. (2009). These reports suggest that the high survival of *S. paratyphi* in Vembanadu lake could be a public health concern.

In order to assess the survival in all possible saline concentrations on both sides of the salt water regulator, survival experiment were conducted in lake water at 5, 10, 15, 20, and

25ppt at 20°C and 30°C and the results are represented in Fig 3-8 and the inactivation rates are given in Table 2 and 3. When the saltwater is closed the saline concentration on Northern part was reported to a maximum of 20ppt. Even though no significant variation in the survival response of *S. paratyphi* was noticed at 0, 5, 10, 15 and 20 ppt ($p>0.05$), they exhibited an extended survival for 34 days at 20°C and 30°C. They showed enhanced survival in water at 0 ppt at both temperatures as evident from T_{99} values and it was 25.99 days at 20°C and 17.68 days at 30°C (Table 2 and 3). However as time goes depending on the increasing saline concentration from 5to 25 ppt it showed gradual decrease in the T_{99} values at both temperatures. The lowest T_{99} was observed at 25 ppt (8.61 and 7.25) and showed a significant ($p<0.0001$) decline of cultural cells at both temperature indicating the deleterious effect of high saline concentration. However the most suitable condition for their growth was found to be at 0 and 5 ppt and suggests that they can survive well at low salinity levels in Vembanadu lake. The results indicate that *Salmonella* can survive well in water weakly diluted or with gradually increasing saline concentrations. In agreement with our results Mezrioui *et al*. (1995) reported that when *Salmonella* suspended in stabilization ponds effluent and rapidly mixed with brackish water survival time was particularly short as we found at 25 ppt where it showed a sudden decline at both temperature, whereas it was prolonged when the bacteria was submitted to a gradual increase in salinity.

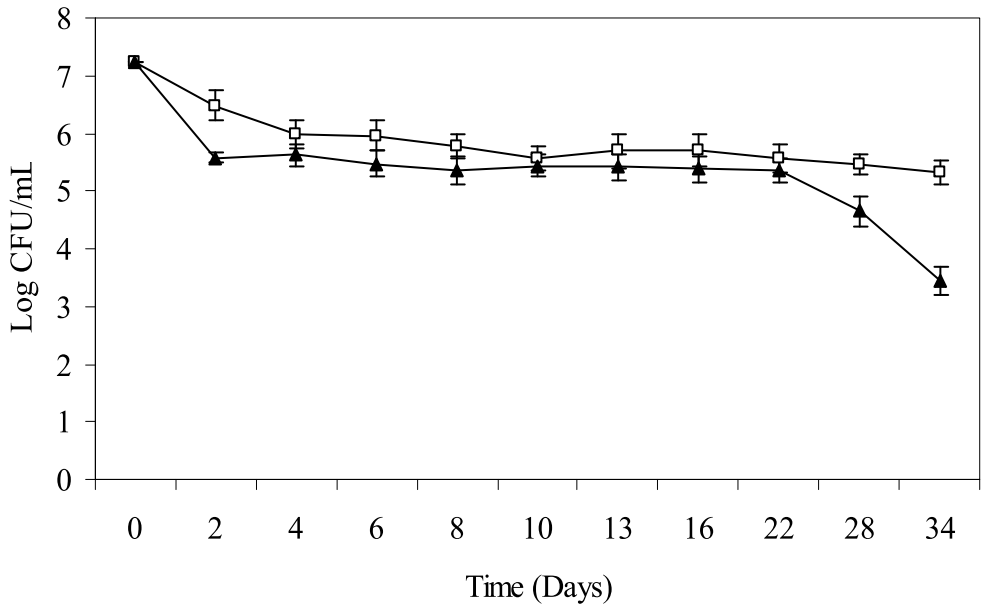


Fig. 3. Survival curves of *S. paratyphi* in fresh sterile water at 0 ppt at 20°C (□)and 30°C (▲) (Mean ±SD, n = 4).

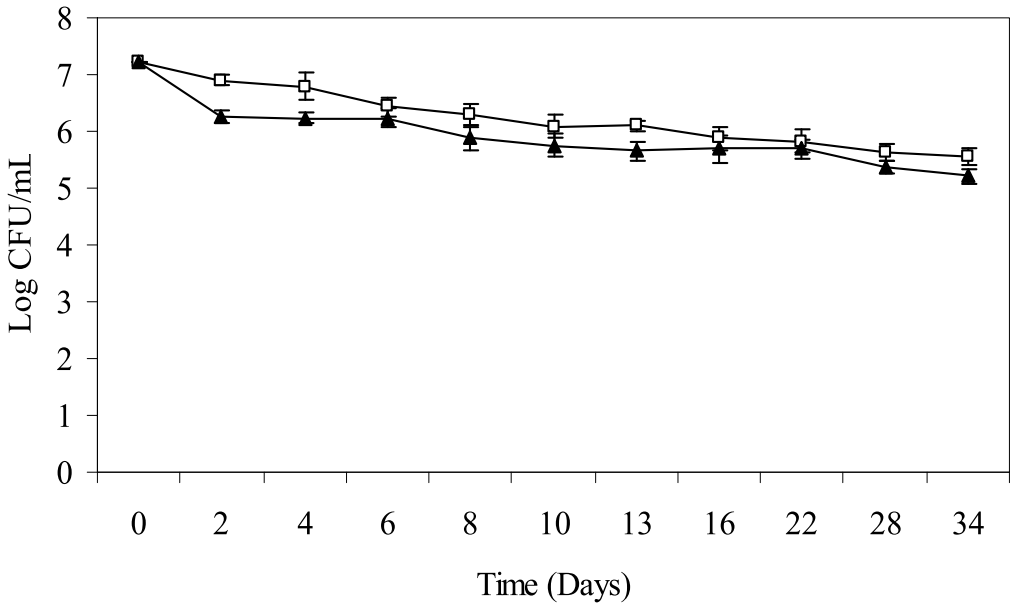


Fig. 4. Survival curves of *S. paratyphi* in sterile water at 5 ppt at 20°C (□)and 30°C (▲) (Mean ±SD, n = 4).

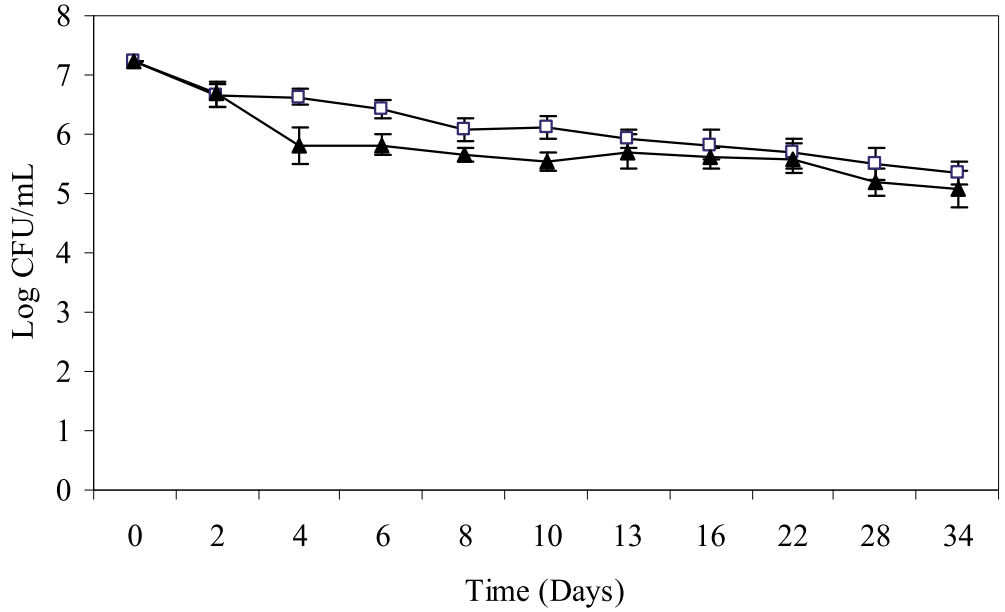


Fig. 5. Survival curves of *S. paratyphi* in sterile water at 10 ppt at 20°C (□)and 30°C (▲) (Mean ±SD, n = 4).

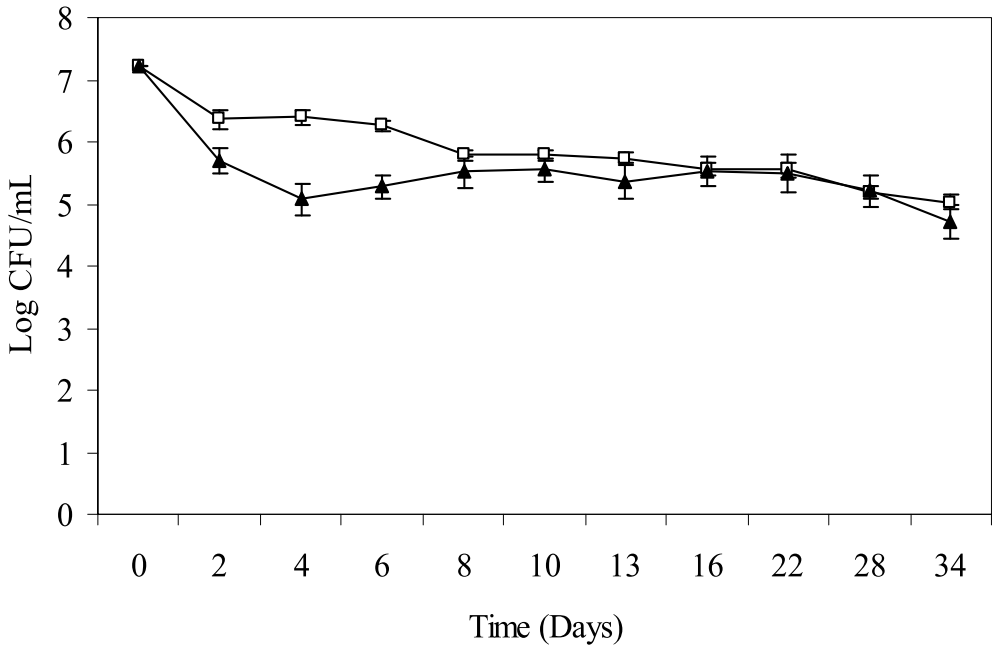


Fig. 6. Survival curves of *S. paratyphi* in sterile water at 15 ppt at 20°C (□)and 30°C (▲) (Mean ±SD, n = 4).

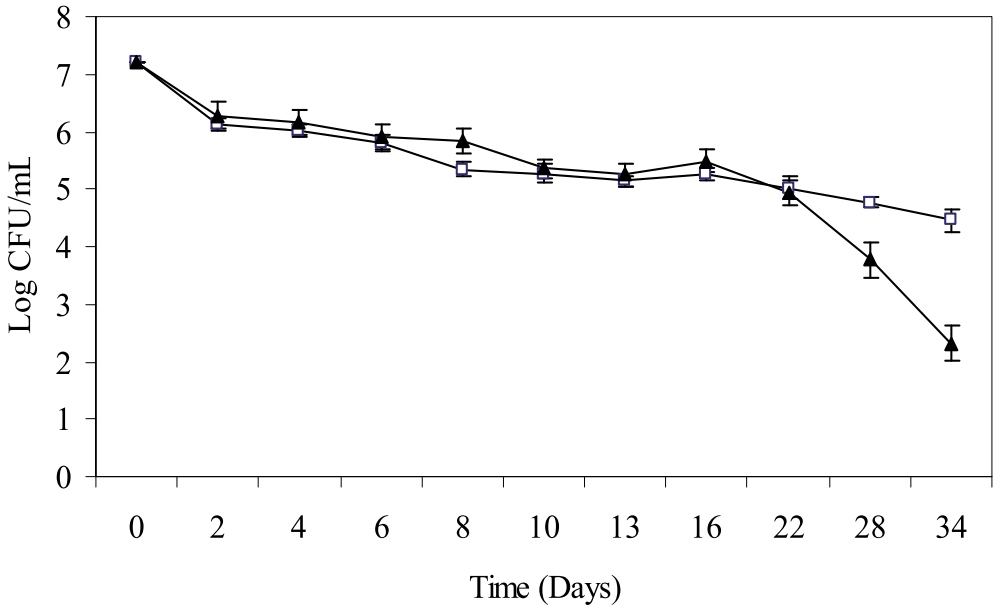


Fig. 7. Survival curves of *S. paratyphi* in sterile water at 20 ppt at 20°C (□)and 30°C (▲) (Mean ±SD, n = 4).

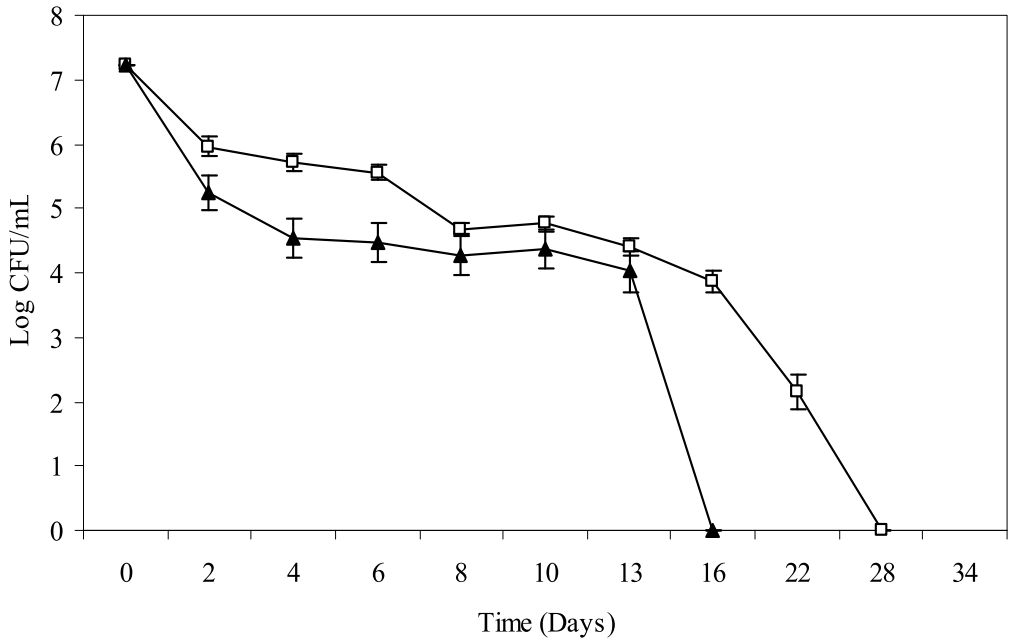


Fig. 8. Survival curves of *S. paratyphi* in sterile water at 25 ppt at at 20°C (□)and 30°C (▲) (Mean ±SD, n = 4).

Days	Saline concentration					
	0 ppt	5 ppt	10ppt	15ppt	20ppt	25 ppt
0	0.00	0.00	0.00	0.00	0.00	0.00
2	-0.74	-0.31	-0.58	-0.85	-1.10	-1.26
4	-1.23	-0.43	-0.59	-0.82	-1.19	-1.49
6	-1.25	-0.78	-0.79	-0.96	-1.40	-1.67
8	-1.46	-0.92	-1.14	-1.44	-1.87	-2.54
10	-1.66	-1.13	-1.10	-1.44	-1.95	-2.46
13	-1.53	-1.12	-1.28	-1.47	-2.07	-2.80
16	-1.50	-1.34	-1.39	-1.65	-1.96	-3.35
22	-1.65	-1.38	-1.53	-1.67	-2.20	-5.07
28	-1.75	-1.59	-1.72	-2.03	-2.44	-7.22
34	-1.90	-1.66	-1.87	-2.19	-2.76	-
k	-0.07	-0.06	-0.06	-0.08	-0.10	-0.23
T99	25.99	31.74	28.85	24.54	19.23	8.61

Table 2. Inactivation rates of *S. paratyphi* in water at different saline concentration at 20°C

	Saline concentration					
Days	0 ppt	5 ppt	10ppt	15ppt	20ppt	25 ppt
0	0.00	0.00	0.00	0.00	0.00	0.00
2	-1.64	-0.97	-0.53	-1.52	-0.94	-1.98
4	-1.59	-0.98	-1.40	-2.14	-1.07	-2.68
6	-1.75	-0.98	-1.39	-1.95	-1.32	-2.74
8	-1.86	-1.35	-1.56	-1.70	-1.37	-2.94
10	-1.78	-1.47	-1.68	-1.67	-1.85	-2.85
13	-1.80	-1.56	-1.51	-1.85	-1.97	-3.20
16	-1.84	-1.53	-1.61	-1.69	-1.74	-7.22
22	-1.86	-1.52	-1.63	-1.72	-2.28	-
28	-2.57	-1.83	-2.02	-2.01	-3.45	-
34	-3.78	-2.00	-2.15	-2.52	-4.90	-
K	-0.11	-0.07	-0.08	-0.09	-0.13	-0.27
T99	17.68	26.14	24.03	21.56	14.95	7.25

Table 3. Inactivation rates of *S. paratyphi* in water at different saline concentration at 30°C

We clearly observed that the decline of cell density with increasing saline concentration. Similar results were reported previously when a freshwater bacteria was exposed to brakish water, Painchaud *et al*. (1987, 1995; Painchaud and Therriault 1989). Similar gradients were reported in other estuaries (Albright, 1983) Rivers (Prieur, 1987). Painchaud *et al*.(1995) reported that no mortality resulted from exposure to water with a salinity of >10ppt and high bacterial count at saline concentration between 0-5. He also reported drastic decline of bacteria at higher salinity (20ppt). This is in agreement with our results that we observed high survival rate at 0 and 5 ppt which was found to be the most suitable condition for the growth whereas at 25 ppt a drastic decline was noticed indicates the deleterious effect of high saline concentration.

At higher saline concentration, for example in sea water, enteric bacteria are subjected to an immediate osmotic upshock, and their ability to overcome this by means of several osmoregulatory systems could largely influence their subsequent survival in the marine environment (Gauthier *et al*., 1987; Davies *et al*., 1995). This osmotic shock might be the reason for the sudden decline of cells at 25ppt. However there are contradicting reportes related to enteric bacterial survival in sea water. Lee *et al* (2010) Gerba and McLeod, (1976) reported that noh halophilic bacterial like *Salmonella* and *E. coli* do not survive well in seas water whereas Sugumar & Mariappan (2003) reported very long survival up to 16 to 48 week in sea water. Upon an osmotic upshift, bacterial cells accumulate or synthesize specific osmoprotectant molecules, in order to equalize osmotic pressure and avoid drastic loss of water from the cytoplasm (Csonka & Epstein, 1996). Although the accumulation or synthesis

of such molecules (trehalose, glycine betaine, glutamic acid) has been reported in *Salmonella* spp. in estuarine waters, in the present study *S. paratyphi* might not overcome the stress caused by the high saline concentration at 25ppt whereas all other saline concentration tested were not found to be lethal.

The maximum saline concentration during the closure of the regulator on the Northern part of the Vembanadu lake is 20 ppt and minimum is 0ppt. Therefore in a year the possible seasonal salinity changes in Vembanadu lake could be between 0-20ppt. It has been generally assumed that when the regulator is closed the bacterial density on the Northern part would be very low because of the increasing saline concentration compared to Southern part. But it has been clearly observed that *S. paratyphi* exhibited high survival capacity in all possible saline gradients from 0 to 20ppt. The result indicates that *S. paratyphi* could survive very long time throughout Vembanadu lake irrespective of the saline concentration. Since the opening and closing of the regulator related to water quality and recreational activities has always been a topic of endless debate, the results indicates that the opening and closing of the salt water regulator does not have any significant impact on the survival (reduction in survival) of the enteric pathogens in relation to saline concentration in Vembanadu lake. However, if the saline concentration reaches 25 ppt it will negatively affect their survival ($p<0.0001$) but the maximum salinity so far reported is 20ppt. Since the lake is being used for various recreational activities the long term survival of *S. paratyphi* in all season regardless of saline concentration in Vembanadu lake could be a public health concern.

4. Conclusions

The results of the microcosm experiment revealed that *S. paratyphi* has a better survival capacity over a wide range of saline concentration from 0 to 20 ppt in Vembanadu lake. It exhibited significantly higher survival at 20^{o}C compared to 30^{o}C. It also showed prolonged survival in all other saline concentration at a higher density at both the temperature and the most suitable saline concentration was found to be 5 ppt. The result indicates that *S. paratyphi* could survive very long time throughout Vembanadu lake irrespective of the saline concentration. The opening and closing of the salt water regulator does not have any significant impact on the survival (reduction in survival) of the enteric pathogens in relation to saline concentration in Vembanadu lake. However, if the saline concentration reaches 25 ppt it will negatively affect their survival but the maximum salinity so far reported is 20ppt. Since the lake is being used for various recreational activities the long term survival of *S. paratyphi* in all season regardless of saline concentration in Vembanadu lake could be a public health concern.

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Part 2

Antimicrobial Chemotherapy

4

Antibiotic Resistance and the Prospects of Medicinal Plants in the Treatment of Salmonellosis

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1. Introduction

Salmonella enterica serotype typhi is the aetiological agent of typhoid fever, a multisystemic disease with protean manifestations and initial lesions in the bowel. Typhoid fever still remains a major public health problem in developing countries even in the twenty first century (Lin et al., 2000; Otegbayo et al., 2003). This was also the case in America and Europe three centuries ago, until measures for sanitary disposal and supply of potable water were put in place. Unacceptable morbidity and mortality are still recorded in developing countries in spite of availability of several drugs over the years for the treatment of typhoid fever. There is enough evidence to show that the prevalence of typhoid fever in any community is an index of communal hygiene and effectiveness of sanitary disposal. In Nigeria, as in other developing countries of the world, studies have estimated over 33 million cases and 500,000 deaths due to typhoid fever per year (Institute of Medicine, 1986). Otegbayo (2005) enumerated several factors responsible for the failure of public health measures to tame the tide of the continuing rise in the incidence, prevalence, morbidity and mortality of typhoid fever.

Salmonellosis is an infection with Salmonella bacteria, often restricted to the gastro-intestinal tract and is often a self limiting disease. Most individuals infected with *Salmonella typhimurium* experience mild gastrointestinal illness involving diarrhoea, chills, abdominal cramps, fever, head and body aches, nausea, and vomiting (Honish, 1999). Infections are usually self-limiting, and antimicrobial treatment is not recommended for uncomplicated illnesses (Aserkoff and Bennet, 1969; Gill and Hammer, 2001). However, extraintestinal infection can occur, particularly in very young, elderly, and immunocompromised patients (Angulo and Swerdlow, 1995; Thuluvath and McKendrick, 1998). In these cases, effective antimicrobial treatment is essential (Cruchaga et al., 2001). Every year, approximately 40,000 cases of salmonellosis are reported in the United States. The actual number of infections may be thirty or more times greater (CDC, 2006). In many parts of the world, such cases are either not documented or because many milder cases are not diagnosed or reported. Cases, however, of systemic disease due to *Salmonella typhimurium* and other salmonellae have been reported (Panhot and Agarwal, 1982; Varma et al., 2005). Salmonelloses have been

reported to be season dependent and occur more in the winter than summer and often referred to as gastroenteritis or diarrheoa. Likewise more cases of diarrhoea caused by enterobacteriacea especially *E. coli*, occurring more during wet season than dry season (Olowe et al., 2003). Children are the most likely to get salmonellosis, however the elderly, and the immunocompromised are the most likely to have severe infections. It is estimated that approximately 600 persons die each year with acute salmonellosis as reported by Centre for disease control (CDC, 2006).

2. Multidrug-resistant (MDR) strains of *Salmonella*

Multidrug-resistant (MDR) strains of *Salmonella* are now encountered frequently and the rates of multidrug-resistance have increased considerably in recent years (CDC, 2006). Even worse, some variants of *Salmonella* have developed multidrug-resistance as an integral part of the genetic material of the organism, and are therefore likely to retain their drug-resistant genes even when antimicrobial drugs are no longer used, a situation where other resistant strains would typically lose their resistance (CDC, 2006). Most of the strains of *Salmonella typhimurium* isolated in a study in western part on Nigeria were resistant to drugs like streptomycin, amoxicilin, tetracycline, ampicillin, kanamycin and chloramphenicol (Olowe et al., 2007). This data is alarming since the isolates were already showing high resistance to drugs that are meant as alternate therapy to salmonellosis treatment; especially isolates from blood were resistant to the commonly used antibiotics. Drug-resistant *Salmonella* emerged in response to antimicrobial usage in food animals, which has also contributed or resulted in major outbreaks of salmonellosis (Olowe et al., 2007). Selective pressure from the use of antimicrobials is a major driving force behind the emergence of resistance, but other factors also need to be taken into consideration. Four types of species namely *S. typhi* (55.5%) *S. paratyphi* A (48.1%), *S. paratyphi* B (25.9%) and *S. typhimurium* (22.2%) were isolated from food samples in Namakkal and the isolates showed multiple drug resistance, and 100 % resistant to Vancomycin, Novobiocin, Nitrofurantoin, Ciproflaxacin, and Methicillin (Jegadeeshkumar et al., 2010). Jegadeeshkumar et al. (2010) also observed increasing resistance for Amoxiclave (92.55 %) and Bacitracin (78.57 %). Akinyemi et al. (2000) reported that out of the total blood samples cultured, 101 (15.9%) isolates of *Salmonella* species were isolated of which 68 (67.3%) were *S. typhi*, 17 (16.8%) and 16 (15.8%) were *S. paratyphi A* and *S. arizonae* respectively. All the *S. typhi* and *S. paratyphi* isolates showed resistance to two or more of the 10 of 12 antibiotics tested particularly the 3-first-line antibiotics commonly used (chloramphenicol, ampicillin and cotrimoxazole) in the treatment of typhoid fever in Nigeria.

Since the discovery of antibiotics and their uses as chemotherapeutic agents, there was a belief in the medical fraternity that this would lead to the eradication of infectious diseases. However, diseases and disease agents that were once thought to have been controlled by antibiotics are returning in new forms resistant to antibiotic therapies (Levy and Marshall, 2004). Incidents of epidemics due to such drug resistant microorganisms are now a common global problem posing enormous public health concerns (Iwu et al., 1999). The global emergence of multi-drug resistant bacterial strains is increasingly limiting the effectiveness of current drugs and significantly causing treatment failure of infections (Hancock, 2005). Examples include methicillin-resistant staphylococci, pneumococci resistant to penicillin and macrolides, vancomycin-resistant enterococci as well as multidrug resistant gram-

negative organisms (Norrby et al., 2005). As resistance to old antibiotics spreads, the development of new antimicrobial agents has to be expedited if the problem is to be contained. However, the past record of rapid, widespread and emergence of resistance to newly introduced antimicrobial agents indicates that even new families of antimicrobial agents will have a short life expectancy (Coates et al., 2002). Confronted with a possible shortage of new antimicrobials, there is need to ensure a careful use of our available drugs. This has led to calls for controlled use of antibiotics through the reduction of dosage used per regime of treatment or by regulating prescriptions in areas such as animal husbandry and aquaculture (Hernandez, 2005). While, reduced use could lead to delayed resistance development, the emergence of resistant strains is from an evolutionary viewpoint inevitable. It becomes imperative therefore that alternative approaches are explored. Targeting and blocking resistance processes could be an attractive approach. The presence of efflux pumps and multidrug resistance (MDR) proteins in antibiotic resistant organisms contribute significantly to the intrinsic and acquired resistance in these pathogens. The discovery and development of new compounds that either block or circumvent resistance mechanisms could improve the containment, treatment, and eradication of these strains (Oluwatuyi et al., 2004).

3. Problems of antibiotic resistance

The origin of antibiotic resistance extends much further back in evolutionary terms and reflects the attack and counter-attack of complex microbial flora in order to establish ecological niches and survive. Early treatment failures with antibiotics did represent a significant clinical problem because other classes of agents, with different cellular targets, were available. The emergence of multiple resistances is causing major problems in the treatment options today. Several factors drove this situation in the 1970s and 1980s, including the introduction of extended-spectrum agents and advances in medical techniques such as organ transplantation and cancer chemotherapy. The net result has been a huge selective pressure in favour of multiply resistant species. Coupled with this, there has been a sharp decline in the introduction of agents acting on new cellular targets over the last 30 years compared with the 20-year period following World War II. Smith (2004) reported that the resistant organisms causing concern among Gram-positive organisms at present are methicillin resistant *Staphylococcus aureus* (MRSA) and coagulase-negative staphylococci, glycopeptides intermediate sensitivity *S. aureus* (GISA), vancomycin-resistant *Enterococcus* (VRE) species and penicillin-resistant *Streptococcus pneumoniae*. Concerns among the Gram-negative organisms include multidrug-resistant *Pseudomonas aeruginosa, Stenotrophomonas maltophilia* and *Acinetobacter baumannii* and members of the Enterobacteriaceae with extended-spectrum beta- lactamases.

When infections become resistant to first choice or first line antimicrobials, treatment has to be switched to second-or third-line drugs, which are nearly always expensive (Sibanda and Okoh, 2007). In many poor countries in developing nations, the high cost of such replacement drugs is not easy to come-by, with the result that some diseases can no longer be treated in areas where resistance to first-line drugs is widespread (WHO, 2002). The alarming challenges facing physicians and pharmacist now, is the need to develop alternative approaches in addition to the search for new antimicrobial compounds (Sibanda and Okoh, 2007). Plants might hold a promise for combating the problem of antibiotic resistance.

4. Mechanisms of antibiotic resistance in pathogenic bacteria

Resistance to antimicrobial agents typically occurs as a result of four main mechanisms namely enzymatic inactivation of the drug (Davies, 1994), alteration of target sites (Spratt, 1994), reduced cellular uptake (Smith, 2004) and extrusion by efflux (Nakaido, 1994). It has been reported that chemical modifications could be significant in antibiotic resistance, though exclusion from the cell of unaltered antibiotic represents the primary means in denying the antibiotic access to its targets and this is believed to enhance resistance even in cases where modification is the main mechanism (Li et al., 1994b).

4.1 Alteration of target site

Chemical modifications in the antibiotic target may result in reduced affinity of the antibiotic to its binding site (Lambert, 2005). These mechanisms have been reported to be employed by a number of pathogenic bacteria in over-powering the effect of antibiotics and are usually mediated by constitutive and inducible enzymes. Resistance to macrolides, lincosamide and streptogramin B antibiotics (MLSB resistance) in pathogenic *Streptococcus* species is a result of methylation of the N6 amino group of an adenine residue in 23S rRNA. This is presumed to cause conformational changes in the ribosome leading to reduced binding affinity of these antibiotics to their binding sites in the 50S ribosomal subunit (Seppala et al., 1998; Kataja et al., 1998). Beta-lactams antibiotics function by binding to and inhibiting the biosynthetic activity of Penicillin Binding Proteins (PBPs), thereby blocking cell wall synthesis.

4.2 Enzymatic inactivation

The production of hydrolytic enzymes and group transferases is a strategy employed by a number of pathogens in evading the effect of antibiotics (Wright, 2005). Genes that code for antibiotic degrading enzymes are often carried on plasmids and other mobile genetic elements. The resistance to-lactam antibiotics by both gram negative and gram positive bacteria has long been attributed to -lactamases (Frere, 1995). These enzymes confer significant antibiotic resistance to their bacterial hosts by hydrolysis of the amide bond of the four membered-lactam ring (Wilke et al., 2005). Resistance to aminoglycosides in gram-negative bacteria is most often mediated by a variety of enzymes that modify the antibiotic molecule by acetylation, adenylation or phosphorylation (Over et al., 2001).

4.3 Antibiotic efflux pump

It is now widely recognized that constitutive expression of efflux pump proteins encoded by house-keeping genes that are widespread in bacterial genomes are largely responsible for the phenomenon of intrinsic antibiotic resistance (Lomovskaya and Bostian, 2006). Several studies have shown that active efflux can be a mechanism of resistance for almost all antibiotics (Li et al., 1994a; Gill et al., 1999; Lin et al., 2002). The majority of the efflux systems in bacteria are non-drug-specific proteins that can recognize and pump out a broad range of chemically and structurally unrelated compounds from bacteria in an energy-dependent manner, without drug alteration or degradation (Kumar and Schweizer, 2005).

The consequence of this drug extrusion is that, it leads to a reduced intracellular concentration of the antimicrobial such that the bacterium can survive under conditions of elevated antimicrobial concentration (Marquez, 2005). The MIC of the drug against such organisms will be higher than predicted. Multi-drug resistance efflux pumps are ubiquitous proteins present in both gram-positive and gram-negative bacteria as either chromosomally encoded or plasmid encoded (Akama et al., 2005). Although, such proteins are present constitutively in bacteria, the continued presence of the substrate induces over-expression (Teran et al., 2003). This increased transcription is responsible for the acquired resistance. In gram negatives bacteria, the effect of the efflux pumps in combination with the reduced drug uptake due to the double membrane barrier is responsible for the high inherent and acquired antibiotic resistance often associated with this group of organisms (Lomovskaya and Bostian, 2006).

The NorA protein which is the best studied chromosomally encoded pump in pathogenic gram positive bacteria (Hooper, 2005) has been reported to be present in *S. epidermidis* but appears to be absent in *Enterococcus faecalis* or in gram-negative organisms, such as *E. coli* and *K. pneumoniae* (Kaatz et al., 1993). Over expression of the NorA gene in *S. aureus* confers resistance to chloramphenicol and hydrophilic fluoroquinolone antimicrobials (Hooper, 2005; Kaatz and Seo, 1995; Hooper, 2005 instead of Hooper, 2005; Kaatz and Seo, 1995). QacA is a member of the major facilitator super-family of transport proteins, which are involved in the uniport, symport, and antiport of a wide range of substances across the cell membrane (Mitchell et al., 1998). The QacA multidrug exporter from *S. aureus* mediates resistance to a wide array of monovalent or divalent cationic, lipophilic, antimicrobial compounds. QacA provides resistance to these various compounds via a proton motive force-dependent antiport mechanism (Brown and Skurray, 2001).

Baucheron et al. (2004) reported the resistance of *Salmonella enteric* serovar Typhimurium to fluororoquinolones, chloramphenicol-florfenicol and tetracycline is highly dependent on the presence of AcrAB-TolC efflux pump. *S. enterica* includes nontyphoidal Salmonella belonging to different serotypes on the basis of the flagellar and somatic antigens, and represents one of the most important food-borne pathogens causing gastroenteritis in humans (Neidhardt, 1996). Nontyphoidal *S. enterica* strains are easily passed from animals to humans and are thus classified as zoonotic pathogens. They can colonize or infect humans as well as a variety of domesticated and wild animals ranging from mammals to birds and reptiles. Most infections are related to ingestion of contaminated food products rather than person-to-person transmission or direct fecal-oral transmission (Mead et al., 1999). Several *S. enterica* isolates are characterized by the presence of host-adapted virulence plasmids encoding genes contributing to colonization and resistance to complement killing, such as the spvA, spvB and spvC (salmonella plasmid virulence) and the rck (resistance to complement killing) genes (Guiney et al., 1994).

In the last few years, Salmonella shows increasing antimicrobial resistance rates in isolates obtained from both food animals and humans. *S. enterica* strains belonging to different serotypes and showing multiple antibiotic resistance (to four or more antimicrobials) are now widespread in both developed and developing countries, most of these strains are zoonotic in origin, acquire their resistance in the food-animal host and cause human infections through the food chain (Threlfall, 2002). Salmonella infections have been associated with the ingestion of poultry, meat, milk and dairy products (Bean et al., 1996).

Most infections result in self-limiting diarrhea and do not require antimicrobial treatment. However, severe life-threatening bacteremias and other deep-seated infections do occur, particularly in children and immunocompromised hosts and in these cases an antimicrobial therapy is recommended (Blaser and Feldman, 1981). Good drugs for Salmonella infections include fluoroquinolones, ampicillin, trimethoprimsulfamethoxazole or third-generation cephalosporins. Rising rates of resistance to ampicillin and trimethoprimsulfamethoxazole have significantly reduced their efficacy and fluoroquinolones are not approved for the use in children. Consequently, extended-spectrum cephalosporins have become the current drugs of choice for the treatment for invasive infections in children (Hohmann, 2001). The emergence of Salmonella species that are resistant to extended-spectrum cephalosporins (Herikstad et al., 1997; Threlfall et al., 1997) is cause of worldwide concern.

4.4 Importance of plasmids in the dissemination of resistance in *S. enterica*

Since the aminoglycoside antibiotic apramycin was licensed for veterinary use in the 1980s, resistance to apramycin and the related antibiotic gentamicin, one the most frequently used aminoglycoside in human therapy (Sibanda and Okoh, 2007). In the United Kindom, during the period 1982-84, the incidence of resistance to apramycin in salmonellas increased from 0.1% in 1982 to 1.4% in 1984 (Wray et al., 1986). Resistance to both apramycin and gentamicin was detected in different Salmonella serotypes, as well in *Escherichia coli*. In particular, the incidence of *S. enterica* (S.) *typhimurium* definitive type 204c (DT204c) from calves showed a dramatically increase (Wray et al., 1986). In *S. typhimurium* DT204c the gentamicin resistance was specified by three types of plasmids of the I1 incompatibility group, which also conferred resistance to apramycin (Threlfall et al., 1986). Most of these plasmids produced the enzyme aminoglycoside 3-N-acetyltransferase IV and the resistance was transferable by conjugation in most of the strains examined. The increasing incidence of the gentamicin-resistant *S. typhimurium* DT204c was also observed in humans, providing the first evidence that the use of apramycin in animal husbandry gave rise to resistance to gentamicin, an antimicrobial used for human therapy (Threlfall et al., 1986).

In the 1990s, the increasing frequency of *E. coli* and Salmonella with plasmids conferring resistance to gentamicin and apramycin was reported in other European countries (Chaslus-Dancla et al., 1991; Pohl et al., 1993). Gentamicin- and apramycin- resistant strains were isolated from both humans and cattle in France and Belgium and six different types of replicons were identified (Pohl et al., 1993). During the 1990-1997 alarming reports pointed out the rapid development in several countries of resistance to β-lactam antibiotics in Salmonella (Threlfall et al., 1997). A survey conducted between 1987 and 1994 in France, demonstrated a dramatic increase (from 0 to 42.5%) in the prevalence of β-lactam resistance among Salmonella isolates. Several types of β-lactamases were found on plasmids belonging to different incompatibility groups Q, P, F and HI (Llanes et al., 1999).

Resistance to β-lactams in Gram-negative bacteria is mediated predominantly by two major types of β- lactamases: the chromosomally-encoded enzymes of the Amber class C (e.g. AmpC β-lactamase in *Citrobacter, Enterobacter, Serratia spp, Morganella morganii* and *Pseudomonas aeruginosa*) or by plasmid-encoded enzymes of the Amber class A, in species that do not produce AmpC β-lactamases, such as *E. coli, Salmonella spp*., and *Shigella spp*. (Bauernfeind et al., 1998a).

Extended-spectrum β-lactamase (ESBL), evolved from the blaTEM-1, blaTEM-2, and blaSHV-1 genes, extending resistance to new third-generation cephalosporins. During the last decade, infections caused by *S. enterica* carrying ESBLs have been reported, and most of the ESBL-producing strains were found to carry plasmids encoding the blaTEM- 1, and blaSHV-1 gene derivatives (Hammani et al., 1991; Morosini et al., 1995; Tassios et al., 1997; Villa et al., 2000; Mulvey et al., 2003).

In Southern Italy, during the period 1990 to 1998, several epidemiologically unrelated *S. enteriditis* isolates showing resistance to expanded-spectrum cephalosporins were recurrently isolated from ill patients. Most of these strains carried the blaSHV-12 gene located on conjugative plasmids (Villa et al., 2002a). Notably, this was the first case of acquisition of the blaSHV-12 gene by Salmonella in Italy and worldwide. However, SHV-12-encoding plasmids were previously encountered in *K. pneumoniae* isolated from hospitals throughout Italy (Laksai et al., 2000; Pagani et al., 2000). Therefore, it is plausible that SHV-12-encoding plasmids originated from nosocomial bacterial pathogens and were horizontally transmitted to *S. enteriditis* strains.

In 1999, the spread of a *S. typhimurium* clone resistant to third generation cephalosporins has been reported in Russia, Hungary and Greece. In this case non distinguishable institutional and community outbreak of *S. typhimurium* isolates harboured a transferable plasmid containing the blaCTX-M gene (Tassios et al., 1999). The relatedness of resistance plasmids harboured by strains of various origins can be demonstrated by incompatibility grouping, restriction fragmentation pattern analysis and identification of specific resistance determinants located within the plasmids. These analyses may allow a better understanding of how resistance plasmids propagate, helping to trace their evolution.

The *S. typhimurium* pSEM plasmid has a very similar restriction pattern to that of the *K. oxytoca* plasmid, pACM1. The pSEM plasmid was identified in 1997 in *S. typhimurium* strains isolated from children in Albania (Villa et al., 2000), while pACM1 was isolated from *K. oxytoca* strains responsible of a nosocomial outbreak in the USA (Preston et al., 1997; Preston et al., 1999). Both plasmids belonged to the same IncL/M group and conferred resistance to expanded-spectrum cephalosporins by the blaSHV-5 gene. Both plasmids carried a class 1 integron conferring aminoglycoside resistance by the aacA4, aacA1 and aadA1 resistance gene cassettes (Villa et al., 2000; Preston et al., 1997). Thus, these plasmids could be members of a family of broad-host-range replicons widely spreading among Gram-negative pathogens. Other plasmids of the IncL/M group showing similar restriction profiles, and carrying the blaSHV-5 gene and a class 1 integron, were previously described in several countries in Europe from clinical isolates of *P. aeruginosa* and *K. pneumoniae* (de Champs et al., 1991; Petit et al., 1990; de Champs et al., 1991; Prodinger et al., 1996; Preston et al., 1997 instaed of de Champs et al., 1991; Petit et al., 1990; Preston et al., 1997, Prodinger et al., 1996). The identification of a family of related plasmids has serious public health implications, since it demonstrates that broad-host-range plasmids carrying resistance to clinically relevant antibiotics can spread worldwide among bacteria responsible of both nosocomial and community-acquired infections. The fact that conserved plasmids can be identified in a wide variety of pathogens isolated in different countries, illustrates the important role of plasmids in the dissemination of antimicrobial resistance among Gram-negative bacteria.

Recently, a case of treatment failure due to ceftriaxone resistant *S. anatum* has been reported in Taiwan (Su et al., 2003). In this study, ceftriaxone-susceptible *S. anatum* was

initially isolated from the urine of a 70-year-old diabetic patient hospitalized for the treatment of a large pressure sore in the sacral area and urinary tract infection. The unexpected emergence of the resistance during the treatment with ceftriaxone led to systemic bacteraemia by *S. anatum* and to the fatal outcome in the patient. The emergence of the resistance has been linked to the *in vivo* acquisition of a resistance plasmid carrying the CTX-M3 β-lactamase by the susceptible *S. anatum* strain. In the same hospital this β-lactamase has been previously identified in clinical isolates of *E. coli, K. pneumoniae* and *Enterobacter cloacae*, suggesting that such bacteria may have acted as reservoirs of the resistance plasmid (Su et al., 2003).

4.5 Plasmid-mediated resistance to expanded-spectrum cephalosporins encoded by the CMY-2 AmpC β- lactamase

The ampC genes were regarded as exclusively chromosomal until 1989, when an AmpC-type β-lactamase was found for the first time on transmissible plasmids (Bauernfeind et al., 1998a). Plasmid-mediated AmpC β-lactamases belong to the homogeneous group of genes related to the chromosomal ampC gene of *Citrobacter freundii* (cmy-2, bil-1 and lat genes), the cmy-1, fox and mox family, or originate from the *Morganella morganii* AmpC β-lactamase (DHA-1) (Bauernfeind et al., 1998a). The latter was identified on a plasmid in *S. enteriditis* (Barnaud et al., 1998; Verdet et al., 2000). The first case of plasmid-mediated CMY-2 in Salmonella was reported on a conjugative plasmid of *S. senftenberg* recovered in 1994 from stool of an Algerian child (Koeck et al., 1997). Review of 1996 data from the National Antimicrobial Resistance Monitoring System (NARMS) in the United States identified only 1 (0.1%) Salmonella isolate among 1272 human Salmonella isolates showing expanded spectrum cephalosporin resistance (Dunne et al., 2000). However, in 1999, NARMS reported the emergence of domestically acquired broad-spectrum cephalosporin resistant Salmonella, most of them producing the CMY-2 AmpC β-lactamase (Dunne et al., 2000).

S. typhimurium strains carrying indistinguishable CMY-2-encoding plasmids were isolated in Nebraska from a patient and cattle during a local outbreak of salmonellosis, demonstrating that the Salmonella-resistant strain evolved primarily in livestock (Fey et al., 2000). Since 1998, *Salmonella* of human and animal origin were reported to show resistance to expanded-spectrum cephalosporins, in Iowa (Winokur et al., 2000; Winokur et al., 2001). Molecular studies demonstrate the emergence of plasmid encoded CMY-2 β-lactamase in most cephalosporin resistant isolates. During the 1998-1999 periods, nearly 16% of *E. coli* isolates and 5.1% of *Salmonella* isolates from clinically ill animals in Iowa produced CMY-2 (Winokur et al., 2000; Winokur et al., 2001).

Similar plasmids, carrying the cmy-2 gene were also reported in Salmonella isolated from animals in Illinois (Odeh et al., 2002). The spreading of the CMY-2-carrying plasmids in the USA was confirmed by molecular analysis of domestically acquired Salmonella strains of human origin isolated in nine different States, representing the 87% of the total expanded-spectrum cephalosporin resistant Salmonella collected by the Center for Disease Control and Prevention (CDC) during the 1996-1998 surveillance periods. The isolates were distinguishable by their chromosomal DNA patterns, thus demonstrating that they did not represent the epidemic spread of a clonal strain (Dunne et al., 2000).

4.6 The use of resistance modifying agents in combination with antibiotics to overcome resistance

The selection pressure exerted by the continued presence of bactericidal or bacteriostatic agents facilitates the emergence and dissemination of antibiotic resistance genes. Over generations, the genotypic makeup of bacterial populations is altered (Taylor et al., 2002). The clinical implications of this are that many infections become untreatable resulting in serious morbidity and mortality. Although the introduction of new compounds into clinical use has helped to curtail the spread of resistant pathogens, resistance to such new drugs, has developed in some cases. It has been observed by several studies that antibiotic combinations can have synergistic benefits and interactions between existing antibiotics (Bayer et al., 1980; Hallander et al., 1982; Hooton et al., 1984; Cottagnoud et al., 2000; instaed of Bayer et al., 1980; Hooton et al., 1984; Cottagnoud et al., 2000; Hallander et al., 1982.). Several current therapeutic regimes are based on synergistic interactions between antibiotics with different target sites. As new antimicrobial compounds are discovered, there is need to assess their potentials in combination therapies with old antibiotics that have been rendered ineffective by the development of resistant strains, even when such compounds are not directly evidently inhibitory. Taylor et al. (2002) suggested that the use of agents that do not kill pathogenic bacteria but modify them to produce a phenotype that is susceptible to the antibiotic could be an alternative approach to the treatment of infectious disease. Such agents could render the pathogen susceptible to a previously ineffective antibiotic, and because the modifying agent applies little or no direct selective pressure, this concept could slow down or prevent the emergence of resistant genotypes. The inhibition of resistance expression approach was successfully used in the production of Augmentin, a combination of amoxycillin and clavulanic acid (Reading and Cole, 1977). In this case, clavulanic acid is an inhibitor of class-A-lactamases which is coadministered with amoxicillin. The combination has been used clinically since the late 1970s (Neu et al., 1993). A similar approach can be used for target-modifying enzymes and for efflux systems. A number of *in vitro* studies have reported the use of plant extracts in combination with antibiotics, with significant reduction in the MICs of the antibiotics against some resistant strains (Al-hebshi et al., 2006; Darwish et al., 2002; Al-hebshi et al., 2006; Betoni et al., 2006 instead of Al-hebshi et al., 2006; Darwish et al., 2002; Betoni et al., 2006). The curative effect of plant extracts in this combination study has been variably referred to as resistance modifying/modulating activity (Gibbons, 2004). This ability of plant extracts to potentiate antibiotics has not been well explained. It is speculated that inhibition of drug efflux, and alternative mechanisms of action could be responsible for the synergistic interactions between plant extracts and antibiotics (Lewis and Ausubel, 2006; Zhao et al., 2001; Lewis and Ausubel, 2006; instead of Lewis and Ausubel, 2006; Zhao et al., 2001).

4.7 Efflux pump inhibition in combination with antibiotics as a strategy for overcoming resistance

The discovery and development of clinically useful Efflux Pump Inhibitors (EPIs) that decrease the effectiveness of efflux pumps represents a significant advance in the development of therapeutic regimes for the treatment of MDR-related conditions. This approach termed the EPI strategy (Lomovskaya and Bostian, 2006), is based on blocking the activity of the pumps, resulting in the accumulation of the antibiotic inside the bacterial cell, consequently increasing access to its target sites. In addition, this will lead to increased susceptibility of the bacterium, thus implying that the therapeutic effect of the drug is

achieved with low concentrations. Combining broad spectrum efflux pump inhibitors with current drugs that are pump substrates can recover clinically relevant activity of those compounds and thus may provide new dimensions to the ever increasing need for development of new antimicrobial agents. This approach will in addition lead to the preservation and improvement of the usefulness of old and cheap antibacterial agents. Ultimately this could reduce the appearance and spread of resistant mutants (Kaatz, 2002).

4.8 Role of Ethnopharmacology in the treatment of Salmonellosis

Herbal medicine is used globally and has a rapidly growing economic importance. In developing countries, traditional medicine is often the only accessible and affordable treatment available. In Africa, 80% of the population uses traditional medicine as the primary health care system (Fisher and Ward, 1994). Traditional medicine is also gaining more respect by national governments and health providers. Peru's national program in complementary medicine and the Pan American health organization recently compared complementary medicine in clinics and hospitals within the Peruvian social security system (Lima, 2000). Plants have been used in traditional medicine for several thousand years (Abu – Rabia, 2005). The knowledge of medicinal plants has been accumulated in the course of many centuries based on different medicinal system. During the last few decades there is an increasing interest in the study of medicinal plants and their traditional use in different parts of the world (Rossato et al., 1999). There are considerable economic benefits in the use of medicinal plants for the treatments of various diseases (Azaizeh, 2003). Due to less communication means, poverty, ignorance and unavailability of modern health facilities, most of the rural people are forced to practice traditional medicines for their common day ailments. Most of these people form the poorest link in the trade of medicinal plants (Khan, 2002). A vast knowledge of how to use the plants against different illness may be expected to have accumulated in areas were the use of plants is still of great importance (Diallo et al., 1999). In the developed countries, 25% of the medical drugs are based on plants and their derivatives (Principe, 1991). A group of World Health Organization experts, who met in Congo, Brazzaville in 1976, sought to define traditional African medicine as the sum total of practices, measures, ingredients and procedures of all kinds whether material or not, which from time immemorial has enabled the African to guard against diseases to alleviate his / her suffering and cure him / herself (Busia, 2005). Traditional medical knowledge of medicinal plants and their use by indigenous cultures are not only useful for conservation of community health care and biodiversity but also for community health care and drug development in the present and future (Pei, 2001).

A number of workers have evaluated the anti typhoidal activities of medicinal plants and some of them proved to be promising. For example: Aliero and Wara (2009) evaluated the efficacy of *Leptadenia hastata* (Pers.) Decne extracts against five selected bacterial species and two fungal species. Aqueous extract markedly inhibited the growth of *Salmonella paratyphi* and *Escherichia coli* and *Pseudomonas aeruginosa*. The result obtained in this study has provided a scientific support for the claimed ethnomedical uses of aqueous extracts of *L. hastata* in the treatment of bacterial diseases and suggest the potential of methanol extract as a source of antifungal agent. Evans et al. (2002) evaluated the efficacy of *Euphobia hirta*; *Citrus aurantifolia*, *Cassia occidentalis*, and *Cassia eucalyptus* claimed by the Nupes tribe of Nigeria to be effective in the treatment of typhoid fever. The result of *invitro* antimicrobial

analysis showed that, only *Cassia eucalyptus* showed inhibition of *Salmonella typhi* growth and concluded that the plant is efficacious and contains natural compounds that could be used in the treatment of typoid fever. Similarly, In Camerron, Nkuo-Akenji et al. (2001) evaluated the effects of herbal extracts derived from plants commonly prescribed by traditional practitioners for the treatment of typhoid fever against *Salmonella typhi*, *S. paratyphi* and *S. typhimurium* using formulations often prescribed by the traditional healers which includes; 1) Formulation A comprising *Cymbogogon citratus* leaves, *Carica papaya* leaves, and *Zea mays* silk. 2) Formulation B comprising *C. papaya* roots, *Mangifera indica* leaves, *Citrus limon* fruit and *C. citratus* leaves. 3) *C. papaya* leaves. 4) *Emilia coccinea* whole plant. 5) *Comelina bengalensis* leaves. 6) *Telfaria occidentalis* leaves. 7) *Gossypium arboreum* whole plant. The result obtained in this study, showed that Formulation A elicited inhibitory activity at a lower range of 0.02 to 0.06 mg/ml. Similarly, Formulation B elicited bacterial activity at the lowest range of 0.06 to 0.25mg/ml. *C. bengalensis* leaves on the other hand, showed the lowest activity with a concentration range of 0.132 to 2.0 mg/ml and 1 to 4 mg/ml in MIC and MBC assays respectively. The result demonstrated that *S. paratyphi* was most sensitive to the formulations (concentration range of 0.02 to 1 mg/ml in both MIC and MBC assays) while *S. typhimurium* was the least sensitive and concentrations of up to 4 mg/ml were required to be bactericidal.

Iroha et al. (2010) evaluated the anti *Salmonella typhi* activity of ethanol, hot and cold crude water extracts of *Vitex doniana* (root), *Cassia tora* (Leaf), *Alstonia boonei* (bark), *Stachytarpheta jamaicensis* (leaf), and *Carica papaya* (leaf) used as traditionally medicine in Ebonyi state, Nigeria. Ethanol extracts of *Vitex doniana* exhibited anti-typhoid activity against 9(90%) of the test organisms, *A. boonei* exhibited activity against 8(80%) of the test organisms, *C. papaya* against 2(20%), *C. tora* against 6(60%), and *S. jamaicensis* against 6(60%). Hot water extract of *Vitex doniana* showed anti-typhoid activity against 7(70%) of the test organisms, *A. boonei* against 9(90%), *C. papaya* against 1(10%), *C. tora* against 8(80%) and *S. jamaicensis* against 7(70%). Cold water extract of *V. doniana,* had anti-typhoid activity against 6(60%) of the test organisms, *A. boonei*, against 6(60%), *C. papaya* against 0(0%), *C. tora* against 6(60%) and *S. jamaicensis* against 4(40%). MIC of ethanol, hot and cold water extracts of *V. doniana*, *A. boonei, C. papaya, C. tora and S. jamaicensis,* fall within 0.4 -128, 0.8 -128, 64 -128, 32 – 128 and 32 – 128. MIC of hot water extracts were within 16 -128, 0.8 – 128, 128 -512, 0.8 – 512 and 0.8 – 128 while MIC of cold water extract are within 64 – 128, 64 – 512, 64 – 512, 64 – 512 and 128-512 respectively. The results of these findings showed that ethanol and hot water extracts of *V. doniana* and *A. boonei* had the best antityphoid activity.

The work of Oluduro and Omoboye (2010) investigated the antibacterial potency and synergistic effect of crude aqueous and methanolic extracts of nine plant parts against multi-drug resistant *S. typhi* tested against nine plant parts: unripe C*arica papaya* fruit, *Citrus aurantifoliia*, *Anana sativus*, *Citrus paradisi*, *Cymbopogon citratus*, *Cocos nucifera* leaves of *Carica papaya,* leaves of *Euphorbia heterophylla* and *Gossypium* spp. Both the aqueous and methanol extracts of each plant material and mixture showed appreciable antimicrobial activities on *S. typhi.* Antimicrobial activity increased with increasing concentration of the extracts. Synergistic activity of crude aqueous and crude methanolic extracts of the plant parts, in various combinations of two to nine against the test organism ranged from 10-33 mm zone of growth inhibition. The antibacterial efficacy of the mixture of extracts from plant parts increased considerably compared to the low activities recorded with the extract of

individual plant parts (P>0.05). Methanolic extracts of each plant material and mixture produced greater antimicrobial activity than the aqueous extracts at all concentrations. The minimum inhibitory concentration (MIC) of the individual plant parts ranged between 0.1 and 1.0 mg/ml in aqueous extracts and 0.01 and 0.1 mg/ml in methanol extracts while the MICs of the combined extracts ranged between 0.1 and 0.01 mg/ml in aqueous extracts and 0.01 and 0.0001 mg/ml in methanolic extracts. The combined or synergistic activity of the plant parts compared favourably with the standard antibiotics of choice for salmonella-infections therapy, and contained two or more phytochemicals responsible for their antimicrobial activities. There is the need therefore to develop effective combination of antimicrobial agents in purified form from higher plants and their parts for clinical trials. Frey and Meyers (2010) reported that *Achillea millefolium, Ipomoea pandurata, Hieracium pilosella*, and *Solidago canadensis* exhibited antimicrobial properties as expected, with particularly strong effectiveness against *S. typhimurium*. In addition, extracts from *Hesperis matronalis* and *Rosa multiflora* also exhibited effectiveness against this pathogen.

4.9 Plants as sources of new antimicrobials and resistance modifying agents

Plants have traditionally provided a source of hope for novel drug compounds, as plant herbal mixtures have made large contributions to human health and well-being (Iwu et al., 1999). Owing to their popular use as remedies for many infectious diseases, searches for substances with antimicrobial activity in plants are frequent (Shibata et al., 2005; Betoni et al., 2006). Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties (Cowan, 1999; Lewis and Ausubel, 2006). Examples of some of these compounds are shown in Table 1. Literature is awash with compounds that have been isolated from a variety of medicinal plants. Despite this abundant literature on the antimicrobial properties of plant extracts, none of the plant derived chemicals have successfully been exploited for clinical use as antibiotics (Gibbons, 2004). A significant part of the chemical diversity produced by plants is thought to protect plants against microbial pathogens. Gibbons (2004) reported that a number of plant compounds often classified as antimicrobial produce MIC greater than 1,000 µg/ml, which are of no relevance from a clinical perspective. Tegos et al. (2002) suggested that a vast majority of plant compounds showing little *in vitro* antibacterial activity are not antimicrobial but are regulatory compounds playing an indirect role in the plant defence against microbial infections. The observation that plant derived compounds are generally weak compared to bacterial or fungal produced antibiotics and that these compounds often show considerable activity against gram-positive bacteria than gram-negative species has been made by many (Nostro et al., 2000; Gibbons, 2004). This observations led Tegos et al. (2002) hypothesizing that; Plants produce compounds that can be effective antimicrobials if they find their way into the cell of the pathogen especially across the double membrane barrier of Gram negative bacteria. Production of efflux pump inhibitors by the plant would be one way to ensure delivery of the antimicrobial compound. This hypothesis has been supported by the findings of Stermitz et al. (2000 a,b), who observed that *Berberis* plants which produce the antimicrobial compound, berberine, also make the MDR inhibitors 5-methoxyhydnocarpin D (5-MHC-D) and pheophorbide A. The MDR inhibitors facilitated the penetration of berberine into a model gram-positive bacterium, *S. aureus*. In testing their hypothesis, Tegos et al. (2002), showed that two MDR inhibitors (INF271 and MC207110) dramatically increased the effectiveness of thirteen

putative plant antimicrobial compounds against gram-negative and gram positive bacteria including isolates known to express efflux pumps.

Class of compound	Examples	Plant sources	Reference
Coumarins and their derivatives	Asphodelin A 4'-*O*-D-glucoside Asphodelin A	*Asphodelus microcarpus*	El-Seedi (2007)
Simple phenols	Epicatechin Epigallocatechin Epigallocatechin gallate Epicatechin gallate	*Calophyllum brasiliense* *Camellia sinensis*	Pretto et al. (2004) Mabe et al. (1999) Hamilton-Miller (1995)
Flavonoids	Isocytisoside Eucalyptin	*Aquilegia vulgaris* L. *Eucalyptus maculate*	Bylka et al. (2004) Takahashi et al. (2004)
Flavones	Luteolin GB1(hydroxybiflavanonol)	*Senna petersiana* *Garcinia kola*	Tshikalange et al. (2005) Madubunyi (1995) Han et al. (2005)
Tannins	Ellagitannin	*Punica granatum*	Machado et al. (2002)
Alkaloids	Berberine	*Mahonia aquifolium*	Cernakova and Kostalova (2002)
Terpenes	Ferruginol, (Diterpene) Epipisiferol (Diterpene) 1-Oxoferruginol	*Chamaecyparis lawsoniana* *Salvia viridis*	Smith et al. (2007) Ulubelen et al. (2000)

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Table 1. Examples of some plants with active antimicrobial derived compounds.

These studies have provided the bases for understanding the action of plant antimicrobials, namely that vast majority of such compounds are agents with weak or narrow-spectrum activities that act in synergy with intrinsically produced efflux pump inhibitors. There is

reason therefore to believe that, plants could be a source of compounds that can increase the sensitivity of bacterial cells to antibiotics. Such compounds could be useful particularly against antibiotic resistant strains of pathogenic bacteria. The rich chemical diversity in plants promises to be a potential source of antibiotic resistance modifying compounds and has yet to be adequately explored.

4.10 Resistance modifying activities of plants crude extracts: The basis for isolation of potentially useful compounds

If the isolation of resistance modifying compounds from plants is to be realistic, screening for such activities in crude extracts is the first step in identifying leads for isolation of such compounds, and some plants have provided good indications of these potentials for use in combination with antimicrobial therapy. Typical examples are as follows: Aqueous extracts of tea (*Camellia sinensis*) have been shown to reverse methicillin resistance in MRSA and also, to some extent, penicillin resistance in beta lactamase-producing *Staphylococcus aureus* (Stapleton et al., 2004). Forty to one hundred fold dilutions of tea extracts was able to reduce the MICs of high- level resistant MRSA (256 µg/ml) to less than 0.12 µg/ml for methicillin and penicillin (Yam et al., 1998; Stapleton et al., 2004). Aqueous crude khat (*Catha edulis*) extracts of Yemen showed varying antibacterial activities with a range of 5-20 mg/ml-1 against periodontal bacteria when tested in isolation. Addition of the extracts at a sub- MIC (5 mg/ml) resulted in a 2 to 4-folds potentiation of tetracycline against resistant strains *Streptococcus sanguis* TH-13, *Streptococcus oralis* SH-2, and *Fusobacterium nucleatum* (Al-hebshi et al., 2006). Betoni et al. (2006), observed synergistic interactions between extracts of guaco (*Mikania glomerata*), guava (*Psidium guajava*), clove (*Syzygium aromaticum*), garlic (*Allium sativum*), lemongrass (*Cymbopogon citratus*), ginger (*Zingiber officinale*), carqueja (*Baccharis trimera*), and mint (*Mentha pieria*) from Brazil and some antibiotics which represented inhibitors of protein synthesis, cell wall synthesis, nucleic acid synthesis and folic acid synthesis against *Staphylococcus aureus*. Darwish et al. (2002) reported that sub-inhibitory levels (200 µgml−1) of methanolic extracts of some Jordanian plants showed synergistic interactions in combination with chloramphenicol, gentamicin, erythromycin and penicillin G against resistant and sensitive *S. aureus*. The methanolic extract of *Punica granatum* (PGME) showed synergistic interactions with chloramphenicol, gentamicin, ampicillin, tetracycline, and oxacillin. The bactericidal activity of the combination of PGME (0.1×MIC) with ampicillin (0.5×MIC) by time-kill assays, reduced cell viability b 99.9 and 72.5% in MSSA and MRSA populations, respectively (Braga et al., 2005). The ethanol extracts of the Chinese plants, *Isatis tinctoria* and *Scutellaria baicalensis* in combination with ciprofloxacin had synergistic activities against antibiotic resistant *S. aureus* (Yang et al., 2005). The combinations of pencillin with ethanolic extracts of *Paederia scandens* and *Taraxacun monlicum* showed a strong bactericidal activity on two strains of *S. aureus* (Yang et al., 2005). When Ciprofloxacin was incorporated at sub-inhibitory concentrations (1/8MIC) to the crude chloroform extracts of *Jatropha elliptica* and the mixture assayed against NorA expressing *S. aureus*, the activity of the extract was enhanced. This suggests the presence of an inhibitor of the pump which could restore the activity of Ciprofloxacin (Marquez et al., 2005). In another study, Ahmad and Aqil (2006) observed that crude extracts of Indian medicinal plants, *Acorus calamus, Hemidesmus indicus, Holarrhena antidysenterica* and *Plumbago zeylanica* showed synergistic interactions with tetracycline and ciprofloxacin

against extended Spectrum beta-lactamase (ESBL), producing multidrug-resistant enteric bacteria with ciprofloxacin showing more synergy with the extracts than tetracycline.

4.11 Plant compounds as resistance modifying agents

Some isolated pure compounds of plant origin have been reported to have resistance modifying activities *in vitro*. Examples of some of the compounds are given in Table 2. This has prompted the search for such compounds from a variety of medicinal plants. Some of the compounds which have been observed to have direct antimicrobial activity have been

Compound	Plant source	Antibiotics potentiated	Reference
Ferruginol 5-Epipisiferol	*Chamaecyparis lawsoniana*	Oxacillin, Tetracycline, Norfloxacin Tetracycline	Smith et al. (2007)
2,6-dimethyl-4-phenylpyridine-3,5-dicarboxylic acid diethyl ester	*Jatropha elliptica*	Ciprofloxacin, Norfloxacin, Pefloxacin, Acriflavine and Ethidium bromide	Marquez et al. (2005)
Carnosic acid carnosol	*Rosmarinus officinalis*	Erythromycin	Oluwatuyi et al. (2004)
Ethyl gallate	*Caesalpinia spinosa*	Bate-lactams	Shibata et al. (2005)
Methyl-1-_-acetoxy-7-_-14 _-dihydroxy-8,15-isopimaradien-18-oate Methyl-1-_-14-_-diacetoxy-7-_-hydroxy-8,15-isopimaradien-18-oate	*Lycopus europaeus*	Tetracycline and Erythromycin	Gibbons et al. (2003)
Epicatechin gallate Epigallocatechin gallate	*Camellia sinensis*	Norfloxacin Imipenem Panipenem beta-Lactams	Gibbons et al. (2004) Hu et al. (2002) Zhao et al. (2001)

Sibanda and Okoh, 2007

Table 2. Some antibiotic resistance modifying compounds from plants.

shown to be potentiating against the activity of antibiotics when used at low MIC levels. The antimicrobial properties of tea (*Camellia sinensis*) have been found to be a result of the presence of polyphenols (Yam et al., 1998; Stapleton et al., 2004; Si et al., 2006). Bioassay directed fractionation of the extracts revealed that epicatechin gallate (ECG), epigallocatechin gallate (EGCG), epicatechin (EC), and caffeine (CN) are the bioactive components. ECG and CG reduced MIC values for oxacillin from 256 and 512 to 1 and 4 mgl-1 against MRSA (Shibata et al., 2005). Ethyl gallate, a conginer of alkyl gallates purified from a dried pod of tara (*Caesalpinia spinosa*) native to South America, intensified lactam susceptibility in MRSA an MSSA strains (Shibata et al., 2005). The abietane diterpenes, (carnosic acid carnosol) isolated from the aerial parts of *Rosmarinus officinalis* by fractionation of its chloroform extract at 10 µgml-1, potentiated the activity of erythromycin (16 - 32 fold) against strains of *S. aureus* that express the two efflux proteins MsrA and TetK. Additionally, carnosic acid was shown to inhibit ethidium bromide efflux in a NorA expressing *S. aureus* strain (Oluwatuyi et al., 2004). A penta-substituted pyridine, 2, 6-dimethyl-4-phenylpyridine-3, 5-dicarboxylic acid diethyl ester and proparcine have been isolated from an ethanol extract of rhizome of *Jatropha elliptica* by bioassay guided fractionation. The pyridine at a concentration of 75 µgml-1 was shown to increase by 4-fold, the activity of ciprofloxacin and norfloxacin against NorA expressing *S. aureus* when tested at sub-inhibitory concentrations (Marquez et al., 2005). Smith et al. (2007) screened active compounds from the cones of *Chamaecyparis Lawsoniana* for resistance modifying activities and observed that Ferruginol and 5-Epipisiferol were effective in increasing the efficacy of tetracycline, norfloxacin, erythromycin and Oxacillin against resistant *S. aureus*. The majority of researches on the combinations between plant extracts and antibiotics have been focused on the identification and isolation of potential resistance modifiers from such natural sources which are considered to be positive results. However, it is likely that such combinations could produce antagonistic interactions that most studies have considered irrelevant and therefore ignored (Sibanda and Okoh, 2007).

5. Suggested solutions to challenges in management

There are still loopfuls of challenges in many developing countries for the management of typhoid fever. Otegbayo (2005) gave the following suggestions as solution for typhoid fever management. This include among others, the improvement in personal and communal hygiene, effective waste disposal system and provision of potable water. Effective treatment of index cases, health education both for the populace and physicians are other important measures. Determination of drug sensitivity patterns and aggressive policy will be quite helpful. The difficulty in diagnosis could also be overcome by making laboratory facilities such as culture media available. Parry et al. (2002) recently suggested the use of conjugate Vi vaccine as part of the Expanded Programme of Immunization. The cost-effectiveness of this latter measure may however be negative for resource – poor countries, where preventive measures by way of improved sanitation and provision of potable water would be more beneficial. Above all, resources should be made available, accessible and affordable to the common man; National Health Insurance appears to be the answer to this as well as economic empowerment of the people in emerging economies.

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5

Antibiotic Susceptibility Patterns of *Salmonella* Typhi in Jakarta and Surrounding Areas

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1. Introduction

Typhoid fever, also known as enteric fever, is a potentially fatal multi systemic illness caused primarily by *Salmonella* enterica serotype Typhi (*S*. Typhi). The classic presentation of the disease includes fever, malaise, diffuse abdominal pain, and constipation. Untreated, typhoid fever may progress to severe condition like delirium, intestinal hemorrhage, bowel perforation, and death. The disease remains a critical public health problem in developing countries. In 2000, it was estimated that over 2,16 million of typhoid occurrences worldwide, resulting in 216,000 deaths, and that more than 90% of this morbidity and mortality occurred in Asia [1]. A report from World Health Organization in 2008 on typhoid fever in five Asian countries showed the annual typhoid incidence (per 100,000 person years) among 5-15 years age group varied from 24.2 and 29.3 in Vietnam and China, to 180.3 in Indonesia; and to 412.9 and 493.5 in Pakistan and India, respectively; multidrug resistant *S*. Typhi were 23% (96/413) [2]. Further, unlike *S*. Typhi originated from Pakistan, Vietnam and India, those from Indonesia collected in North Jakarta, were all susceptible to antibiotic tested, i.e. Chloramphenicol, Ampicillin, Trimethoprim-Sulfamethoxazole; none of multidrug resistance were found. Nalidixic acid resistance was rather high in Pakistan, India and Vietnam, but none was found in Indonesia [2].

In Indonesia the prevalence of typhoid fever was 358-810 per 100,000 populations in 2007, with 64% of the disease was found in people aged 3-19 years. Mortality rate varies from 3.1-10.4% among hospitalized patients. Hatta and Ratnawati, 2008 reported a rise of resistance of *S*. Typhi to 6.8% to all three of first line drugs (Chloramphenicol, Ampicillin, Co-trimoxazole) in South Sulawesi (East of Indonesia) [3]. Antibiotics Fluoroquinolone and 3rd generation of Cephalosporin are frequently used for therapy of patients suspected typhoid fever in the past decade in many places especially in endemic countries including Indonesia due to resistance issues against conventional antibiotics [4, 5, 6, 7]. This study aimed to overview antibiotic susceptibility of *S*. Typhi originated from Jakarta and surrounding areas in particular to those

recommended by Performance Standard for Antimicrobial Susceptibility Testing for Clinical and Laboratory Standard Institute within 9 years period up to 2010.

2. Materials and methods

2.1 Specimens

Specimens used in the study were blood received in our laboratory i.e. Laboratory of Clinical Microbiology Faculty of Medicine University of Indonesia (CML-FMUI) Jakarta between 2002-2007. Our laboratory accepted specimens from hospitals, mainly the National Hospital Cipto Mangunkusumo (a tertiary general public hospital), primary health cares, private practices, and individuals. As in CML-FMUI, blood specimens from in and outpatients as well as other sources examined in Siloam Hospital Kebon Jeruk and St. Carolus Private Hospital in 2008-2010 were also included.

2.2 Culture and antibiotic susceptibility tests

Culture and antibiotic susceptibility tests were established in each of the above-mentioned institutions. Microbiology tests were performed according to microbiology standard practices and Performance Standards for Antimicrobial Susceptibility Testing for the Clinical and Laboratory Standards Institute (CLSI) [8]. Cultures were performed using Bac-T Alert™ (Enseval)/Bactec™ 9050 (Becton Dickinson), and sub cultured were on Salmonella-Shigella and MacConkey agar. Microorganism identification was determined using conventional biochemical reactions i.e. acid production from glucose, lactose, maltose, mannitol and saccharose, IMViC tests (Indole, Methyl Red, Voges Proskauer and Citrate) and H2S production in TSI agar. In recent years, API20E biochemical identification system (BioMerieux, Paris, France) was used instead. Susceptibility of microorganisms to antibiotics was assessed using the disc diffusion method. Antimicrobial susceptibility results were categorized in to three groups: Sensitive (S), Intermediate (I) and Resistant (R) according to CLSI guidelines. The antibiotics susceptibility data was then entered into the WHO-Net 5.4 program.

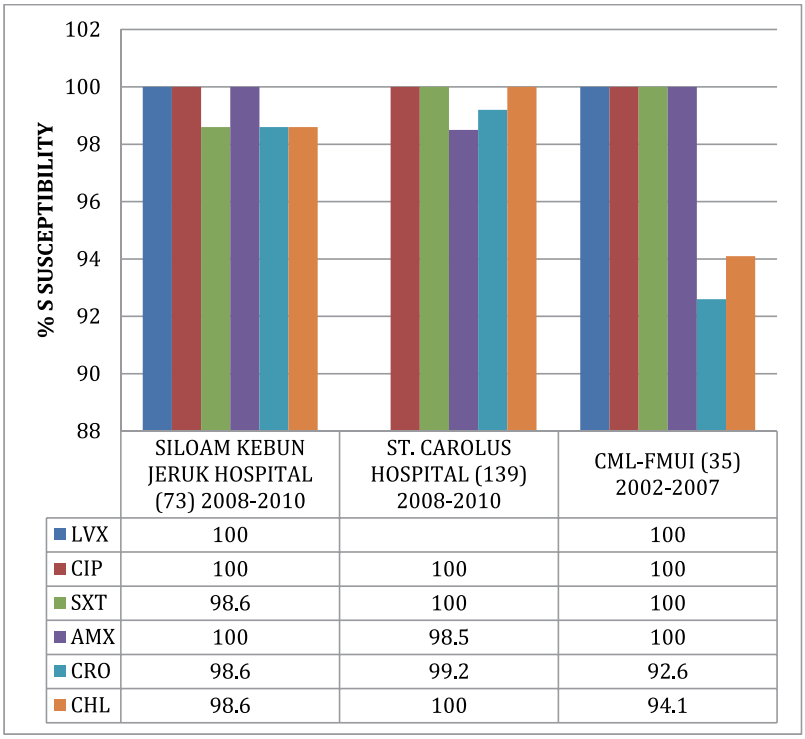
2.3 Antibiotics

Standard disc diffusion method was employed. The following antibiotic discs Chloramphenicol (CHL) 30µg, Amoxycillin (AMX) 25µg, Trimethoprim-Sulfamethoxazole (SXT) 1.25/23.75µg, Ceftriaxone (CRO) 30µg, Ciprofloxacin (CIP) 5µg, and Levofloxacin (LVX) 5µg were included in the study. These antibiotics are frequently used to treat typhoid fever in Jakarta, Indonesia. Susceptibility of *S.* Typhi to antibiotics was tabulated, and good activity in-vitro was defined by antimicrobial susceptibility of 80% or greater. Minimal inhibitory concentration was not examined.

3. Results

During nine years period from 2002-2010, 247 isolates of *S.* Typhi were collected, in which 35 isolates were from CML-FMUI, 73 and 139 isolates came from Siloam Kebon Jeruk and St. Carolus Hopitals respectively. In 2002-2007, all *S.* Typhi isolated in CML-FMUI was susceptible to antibiotics Levofloxacin, Ciprofloxacin, Trimethoprim-Sulfamethoxazole and

Amoxycillin (Figure 1). In 2008-2010, all of *S*. Typhi isolated in Siloam Hospital Kebon Jeruk was susceptible to Levofloxacin and Ciprofloxacin as also found in St. Carolus Hospital except antibiotic Levofloxacin was not tested on isolates from St. Carolus Hospital (see Figure 1). Susceptibility of the microorganism to Trimethoprim-Sulfamethoxazole showed almost similar pattern to those of CML-FMUI in the earlier years ranging from 98.6% to 100%, and so Amoxycillin that was 98.5% to 100%. Susceptibility of these microorganisms to Ceftriaxone seemed to increase from 92.6% in 2002-2007 to 98.6% or greater in 2008-2010. Lastly, although antibiotic Chloramphenicol was scarcely used in the treatment of typhoid fever compared to Fluoroquinolones, this antibiotic was still effective. The susceptibility of *S*. Typhi isolates to Chloramphenicol was 94.1% in 2002-2007, and was apparently increase to 98.6% or greater in 2008-2010 (Figure 1). Overall, during 9 years period up to 2010, antibiotic Chloramphenicol, Amoxycillin, Trimethoprim-Sulfamethoxazole, Ceftriaxone, Ciprofloxacin and Levofloxacin showed good activity in-vitro against *S*. Typhi originated from Jakarta and adjacent areas.



	SILOAM KEBUN JERUK HOSPITAL (73) 2008-2010	ST. CAROLUS HOSPITAL (139) 2008-2010	CML-FMUI (35) 2002-2007
LVX	100		100
CIP	100	100	100
SXT	98.6	100	100
AMX	100	98.5	100
CRO	98.6	99.2	92.6
CHL	98.6	100	94.1

Fig. 1. Susceptibility of *S*.Typhi to Antibiotics in Jakarta and Surrounding Areas

4. Discussions

Jakarta, as a capital city, is the biggest urban area in Indonesia. The city connects to five other satellites cities i.e. Bogor, Depok, Tangerang, Bekasi, Karawang, and it is densely populated where some districts are still impoverished. Health care system in the country does not support extensive program that covers laboratory examinations. This reflected in

the limited samples received in the laboratories. Clinical Microbiology Laboratory of Faculty of Medicine University of Indonesia is located in the center of Jakarta and known as the referral Microbiology Laboratory as it is part of the Department of Microbiology FMUI. The laboratory receives specimens from other laboratories, hospitals, primary health cares and also individuals. Furthermore, Siloam Kebon Jeruk in West Jakarta and St. Carolus in Central Jakarta are private hospitals; their laboratories serve for the hospitalized, outpatients and also other sources. In this study, 247 isolates of *S.* Typhi examined during 2002-2010 were susceptible to antibiotics Chloramphenicol, Amoxycillin, Trimethoprim-Sulfamethoxazole, Ceftriaxone, Ciprofloxacin, and Levofloxacin. In addition, *S.* Typhi was in fact still susceptible to antibiotic Tetracycline (Data not shown).

Some reports on antibiogram of *S.* Typhi from other institutions within the country were similar i.e. Central Laboratory of Cipto Mangunkusumo Hospital reported all S. Typhi isolated from hospitalized typhoid fever cases in 2009 were all susceptible to antibiotics (Chloramphenicol, Cotrimoxazole, Ceftriaxone, Cefuroxime, Ampicillin-Sulbactam, Amoxycillin-Clavulanic acid, Ciprofloxacin, Levofloxacin) [9]. In 2003-2005, ninety isolates of *S.* Typhi were collected from some districts in East Jakarta. All of these isolates were susceptible to first line drugs (Chloramphenicol, Trimethoprim-Sulfamethoxazole, Ampicillin) and Tetracycline except for only 1 isolate which was resistant to Chloramphenicol [10].

Data obtained from outside Jakarta such from Pakanbaru in Sumatra Island in 2009-2010 also showed very similar susceptibility patterns. Those *S.* Typhi isolates were all susceptible to Chloramphenicol, Tetracycline, Trimetoprim-Sulfamethoxazole, Amoxycillin-Clavulanic Acid, Cefotaxim, Cefepime, Ceftazidime, Cefazolin, Ceftriaxone and Ciprofloxacin [11]. *S.* Typhi isolated from South Sulawesi, however, showed an increase resistant against Chloramphenicol and Ciprofloxacin between 2001-2007, which was 1.04% to 7.84% and 0.11% to 6.83% respectively [3]. In 2003, a collaborated study on enteric bacteria in patients with diarrhea had been carried out in United States Naval Medical Research Unit, Jakarta that involved many health institutions from many cities in Indonesia including Medan, Padang, Batam, Jakarta, Pontianak, Denpasar and Makassar. A total of 111 *S.* Typhi had been isolated from feces, and all were susceptible to antibiotic tested i.e. Ampicillin, Trimethoprim-Sulfamethoxazole, Chloramphenicol, Tetracyclin, Cephalotin, Ceftriaxone, Norfloxacin and Ciprofloxacin. Nalidixic acid resistance was not found [12].

Resistance to Chloramphenicol was reported to emerge in only two years after its introduction in 1948, and was not until 1972 that typhoid fever caused by Chloramphenicol-resistant *S.* Typhi became a major problem; outbreaks occurred in Mexico, India, Vietnam, Thailand, Korea, and Peru (cited from Parry et al, 2002 [13]). Toward the end of the 1980s and the 1990s, *S.* Typhi developed resistance simultaneously to all the drugs that were then used as first line treatment (Chloramphenicol, Trimethoprim, Sulfamethoxazole, and Ampicillin). Despite multidrug-resistant *S.* Typhi are still common in many areas of Asia, strains that are fully susceptible to all first line antibiotics have reemerged in some areas [13]. Chau et al, 2007 reported that of eight endemic countries from Indian continent to China, multidrug resistance (MDR) *S.* Typhi varied from 16 to 37% and Nalidixic acid resistance were 5 to 51% [7]. In some places Nalidixic Resistant *S.* Typhi (NRST) was reported to cause more complication and poorer outcome of the disease; the presence of NRST is critical and influenced the successful rate of therapy with Fluoroquinolone [14].

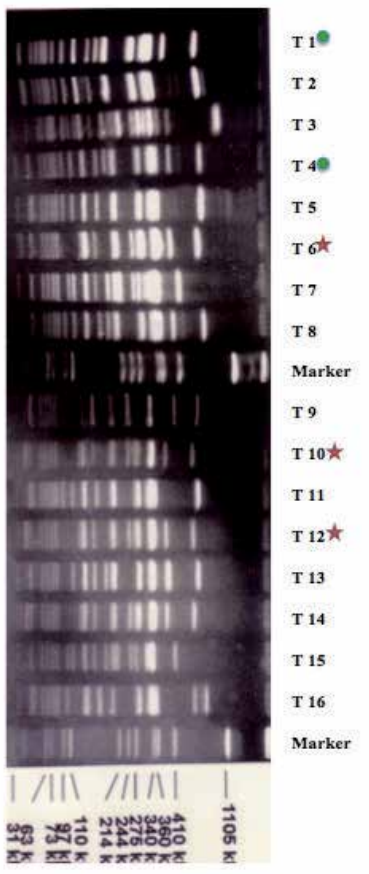
Despite an increase resistance elsewhere, certain areas especially in Northern India, reported that Chloramphenicol resistance has reduced from a high of 18% to only 2% [15].

Differences in the antibiotic susceptibility profiles as well as clinical appearance of typhoid fever cause by *S*. Typhi rely on many factors. Conditions such as disease control programs, inadequate policy of using antibiotics, local conditions that include personal hygiene, availability of clean drinking water, food handling and sanitation contribute to the complexity and outcome of the disease. Nevertheless, one important element of the diverse clinical manifestation of typhoid fever is the presence of genome plasticity of *S*. Typhi. Many studies had been conducted in the late nineties on genome profile of *S*. Typhi and showed the diversity of the genome. Some of strains originated from a certain region or country share some degree of similarity to other strains from different places. Our earlier study on genetic relationship using pulsed-field gel electrophoresis (PFGE) found that *S*. Typhi originated from five cities i.e. Medan, Jakarta, Pontianak, Makassar and Jayapura in Indonesia expanding from west to east part of the country, had clusters of endemic strains in certain geographic areas [16]. The presence of specific strains in localized area might have been the reason of varied symptoms of the disease and, possibly their susceptibility to antibiotics. Despite heterogeneity and different clonality of the 33 isolates of *S*. Typhi used in the study, these endemic strains were in fact all susceptible to Chloramphenicol, Ampicillin and Cotrimoxazole [16].

Some investigators reported a correlation of certain genome profiles of *S*. Typhi strains and their ability to cause a fatal typhoid fever [17]. Others reported specific flagellar types were associated with severe outcome of the disease [18]. A study carried out in our laboratory by Tjita in 2000 showed that 3 *S*. Typhi strains had identical genome profiles deduced from PFGE [19]. Each of the strains showed different susceptibility against several antibiotics i.e. one strain was resistant to Tetracycline; another two were multi resistant to Chloramphenicol/Tetracycline, and Ampicillin/Chloramphenicol/ Tetracycline respectively (see Figure 2). In addition to the findings, two other *S*. Typhi strains which resistant to Ampicillin and Tetracycline were found to be an identical strains [19]. The mentioned conditions could have been the reasons that conventional drugs such as Chloramphenicol, Amoxycillin, Trimethoprim- Sulfamethoxazole and Tetracyclin still have good activity in-vitro against *S*. Typhi strains in Jakarta and surrounding areas. In recent years, antibiotic Fluoroquinolone has been widely used in the treatment of typhoid fever in Indonesia, and it showed superiority in term of efficacy and safety [20]. Previous reports by Ochiai et al, 2008 [2] and Tjaniadi et al, 2003 [12] showed Nalidixic acid resistant *S*. Typhi was not found thus far in Indonesia. Cautious is adviced, however, since quinolone resistant *S*. Typhi strains have been an important issue in regional and global [6, 21, 22, 23].

In conclusion, despite resistance issue of *S*. Typhi from other countries, this study showed that most of all *S*. Typhi isolated in certain places in Jakarta and neighboring areas were susceptible to antibiotic tested (Chloramphenicol, Amoxycillin, Trimethoprim-Sulfamethoxazole, Ceftriaxone, Ciprofloxacin, Levofloxacin). This information is important since antimicrobial therapy plays a key role in management of typhoid fever disease. The susceptibility profiles, however, were only derived from certain strains, which may not represent all strains, which present in Indonesia. Therefore it is necessary to perform cultures and antibiotic sensitivity tests on patients with suspected typhoid fever, and so the

patients can be treated with definitive antibiotic therapy. Needless to say, adequate antibiotic therapy will prevent the spread of antibiotic-resistant *S*. Typhi strains. Lastly, promotion of public health such as personal hygiene, sanitations, clean drinking water, food handlings and also vaccination are equally important as prevention of the disease.



Sixteen *S*. Typhi isolates were originated from patients with typhoid fever in Jakarta. The genome was digested *Xba*I restriction enzyme. T6, T10 and T12 [★] were identical strains, which showed different susceptibility patterns i.e. T6 resistant to Tetracycline, T10 resistant to Chloramphenicol/Tetracycline, T12 resistant to Ampicillin/Chloramphenicol/Tetracycline. T1 and T4 [●] had identical genome profiles but each showed different susceptibility patterns i.e. T1 resistant to Ampicilin, and T4 resistant to Tetracycline. Modified from Tjita, 2000 [19].

Fig. 2. Genome profile of *S*. Typhi isolates from Jakarta using Pulsed-Field Gel Electrophoresis

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6. References

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6

Salmonellas Antibiotic Sensibility and Resistance; The Sensitivity to Herb Extracts and New Synthesize Compounds

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1. Introduction

Although the use of antibiotics in the developed countries is done under certain principles, there is an opposite situation at the use of antibiotics in the underdeveloped countries which is irregular. The prevalence of antibiotic use in the fight against infectious diseases additionally raises the problem of increasing resistance of the micro-organisms against antibiotics. There is also an increase in the resistance of antibiotics which is used against *Salmonella*. The newly synthesized chemical compounds and extracts derived from plants which are alternative to existing antibiotics determining the sensitivity to *Salmonella*, plays an important role for increasing the options of alternative antibiotics.

1.1 Appearance and staining characteristics

Salmonella bacteria are asporogenic, capsule-free, motile via peritrichous cilium (*Salmonella gallinarium* or *Salmonella pulorum* are immotile), rod-shaped bacteria with an approximate length of 2,0-5,0 μm, width of 0,7-1,5 μm. They are stained well with bacteriologic stains and they are gram-negative (Picture 1). Most of them have type 1 (mannose sensitive (ms), hemagglutinating); S. Gallinarium and some origins have type 2 fimbriae. S. paratyphi As do not have fimbriae.

1.2 Reproduction and biochemical characteristics

Salmonella bacteria reproduce in many ordinary mediums. They are aerobe and facultative anaerobe. Their reproduction temperature limit is very wide even they reproduce at 37° C best. (20°C- 42°C). This is extremely important for reproduction of *Salmonellas* which cause food intoxication at room temperature. They like to produce at average pH of 7,2. They make homogenous turbidity in bouillon and similar liquid medium. They make round, slab sided, mostly tumescent colonies with a diameter of 2-3 mm, regular surface. In colonies of various *Salmonellas*, some differences may exist in terms of size, protuberance, surface and side. *Salmonella typhi* may also make gnome colonies which may reach to 0,2-0,3 mm diameter within the first 24 hours. Biochemical characteristics of bacteria which are obtained

from these colonies are same as normal colonies; and they are agglutinated with O serums only antigenically and they differ from bacteria in S colonies in terms of not reacting with anti H, anti Vi serums. If they are reproduced in mediums including sulfurous compounds, sulfates and tiosulfates which may be assimilated, normal colonies occur from bacteria that make gnome colonies.

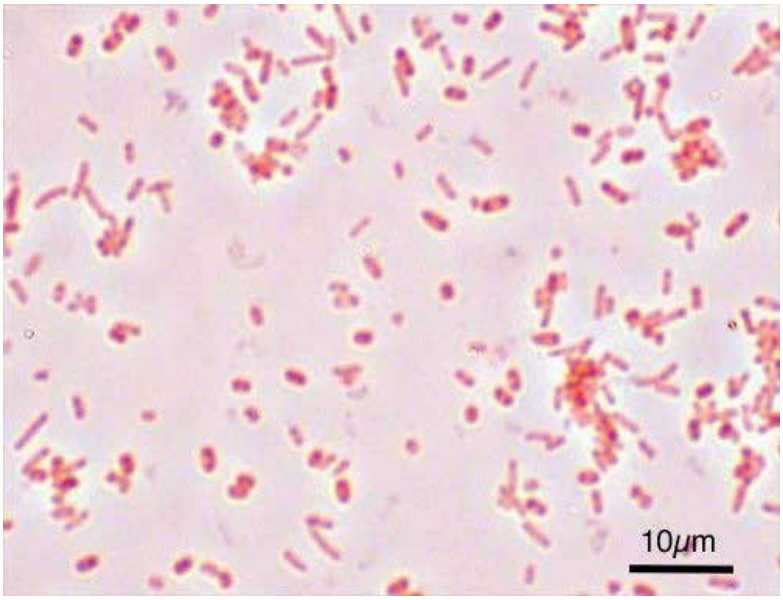

Fig. 1. Microscopic View of *Salmonella*

Some of *Salmonellas*, S. Schottemuelleri (s. paratyphi) in particular and some others form M colonies in appropriate mediums. It is detected that these bacteria have M antigens and agglutination is prevented by anti O and anti H serums. Furthermore, R colonies are formed by *Salmonella* which reproduce in inappropriate mediums (Picture 2).

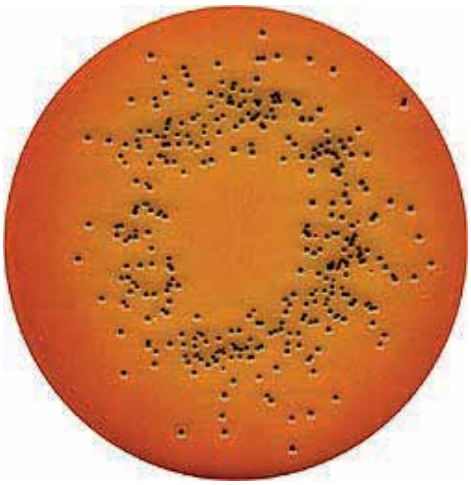

Fig. 2. *Salmonella* colonies

Salmonellas are not effective on lactose. This characteristics is important in first differentiation from Escherichias. As these bacteria which are planted in a separator plaque medium (endo, EMB) including lactose and an appropriate reagent are not effective on lactose, they make colorless colonies; however those effective on lactose make dark red, black, greenish bright colonies (Picture 3).

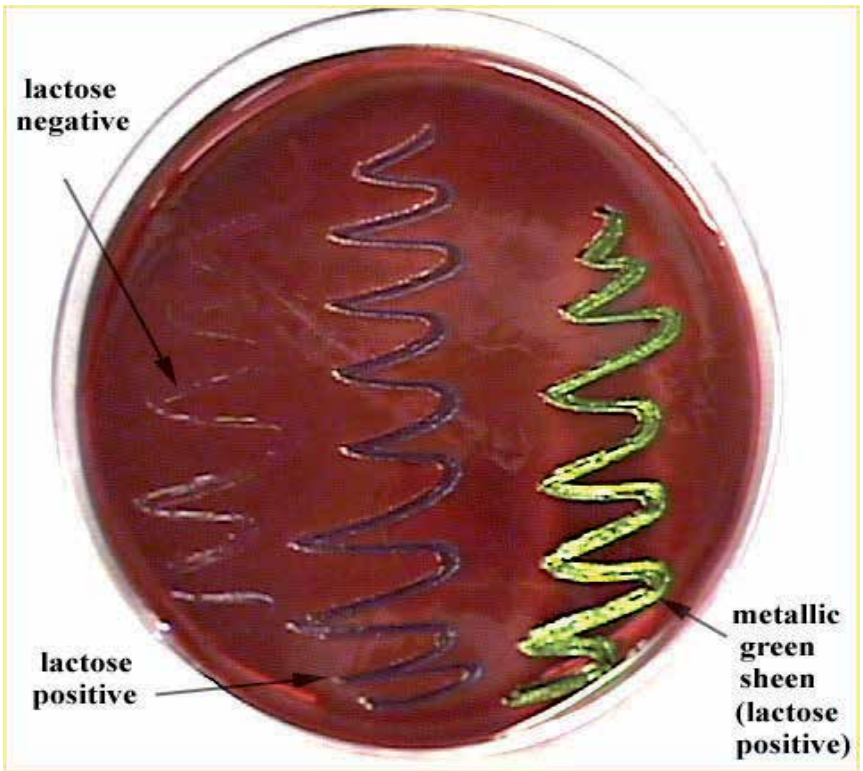

Fig. 3. View of *Salmonella* and Lactose Positive Colonies

Salmonellas do not effect on sucrose, adonitole and salicin in usual other than lactose. They digest glucose, mannite and maltose by producing acid and gas except *Salmonella typhi* and S. gallinarum; and *Salmonella typhi* and gallinarum digest them by producind acid only. They produce H2S in general (except S. paratyphi A); they are indole negative, methyl red positive, Vogesproskauter negative and they reproduce in citrated mediums (Simmon), they do not digest urea. They could not be produced in KCN (potassium cyanide 0,5%) mediums. ONPG (orthonitro phenyl galactopyranoside) assay is negative. (They do not have beta galactosidase enzymes that may digest lactose). This assay is positive in Arizona. Biochemical characteristics of *Salmonellas* were shown in Table 1.

Motility	+
Indole	-
H2S	+
Oxidase	-
Urease	-
Nitrate reduction	+
Citrate Utilization	+
MR	+
VP	-
Lysine decarboxilation	+
Ornithine decarboxilation	+
Phenylalanine deamination	-
Malonate Utilization	-
Lactose	-
Sucrose	-
Salicine	-
Inositol	-
Amygdalin	-
Gas Production from glucose	+
β-galaktosidase (ONPG Test)	-
Reproduction in KCN	-
	(-) Negative (+) Positive

Table 1. Biochemical characteristics of *Salmonellas*

2. Resistence in *Salmonellas*

Salmonella bacteria are resistless to heat. They die at 55°C in 20 minutes. They do not have resistance to dryness. But they may stay alive in humid environments away from daylight, sewages, well water and soil for a long time. They are very resistant to cold. Staying alive in cold food and drinks has an epidemiological importance. They may stay alive in liyophilized situations for years.

Antiseptics effect rapidly providing the direct contact. Chlorine within normal concentrations kill *Salmonellas* in the water. However, effect of these agents to *Salmonellas* in stool particles and other organic substances lower.

To differ them from other bacteria in terms of accompanying coliform and intestinal settlement habits, their status against various chemical agents and stains were examined and consequently special cases of *Salmonellas* appeared against some of them.

Malachite green, a stain kills E.coli or slower their reproduction within suitable densities, however it does not effect S. typhi. Similarly, while paratyphoid bacillus are very resistant and typhoid bacillus is quite resistant to Brillant green stain, dysentery bacillus and coliforms are very sensitive. Although lithium chloride inhibits E.coli similarly, it s inefficient to *Salmonella typhi*. Differently, sodium tetrathionate increases Slamonella reproduction although it is noneffective to coliforms.

To preserve *Salmonellas*, after making immersing culture to a vertical agar including 1% agar and 2% Na H PO_4 $12H_2O$ and reproduction is provided, they may stay alive in tightly closed agar in the dark for months. Furthermore, *Salmonellas* may be lyophilized.

Although sodium deoxycholate reduces reproduction of coliforms, they do not effect reproduction of *Salmonellas* and Shigellas.

Resistance against chemotherapeutics in *Salmonellas* has appeared quite lately. Although an important resistance has not been observed in *Salmonellas* against antimicrobic agents such as chloramphenicol, tetracyclines, kanamycin, ampicilin, streptomycin, sulfonamids until 1960, increasingly resistant strains against tetracyclins, ampicilin, streptomycin, sulphonamids have been detected from that day to this. Resistance to chloramphenicol develops slower than others. Resistance depends on plasmids.

3. Antibiotic resistance in microorganisms

Microorganisms may become resistant to antibiotics that they are in effect spectrum in time due to any reason. Antibiotic resistance in bacteria is called as inability to treat the infection disease against treatment doses of antibiotics that are in effect spectrum of microorganisms naturally and used routinely.

There are various predictions recently about where antibiotic resistance genes which are thought to be appeared after wide usage of antibiotics in medicine come from. According to an opinion, resistance genes has developed as a protection mechanism in bacteria species that produce antibiotic first. Resistance genes in these species are in the same group with antibiotic production genes. Another possibility is that antibiotic resistance genes has developed from normal genes present in the microorganism. However, possibility of development of antibiotic resistance due to spontaneous mutation is very low. (10^{-5}-10^{-10}).

3.1 Resistance problem against antibiotics

Antibiotics which has been started to be used within last 50-60 years provided the most important contribution in human life and enabled treatment of many infectious diseases successfully. Antibiotics which are one of the most important inventions of humanity lost their effects significantly because of resistance developed after inappropriate and unnecessary usage. Microorganisms gain a sustaining power, namely resistance eventually against antimicrobic agents which are used to destroy themselves. The resistance developed against antimicrobic agents is a very important problem which will threat all humanity today. Hospital infections which develop by resistant origins against many drugs mainly in hospitals increase hospitalization and death rates and cause more additional cost. Today, resistance not only in hospital sources, but also in sources acquired from the society increases significantly and this case augments the problem and carries it to serious levels.

Resistance may also develop to other antimicrobis which are close in terms of structure or effect form to a chemotherapeutic agent in a microorganism species which has become resistant to such antimicrobic agent; and this condition is called as cross resistance. The condition that a microorganism becomes resistant to many antimicrobic agents with different structure and effect is called as multiple-drug resistance.

3.2 Natural (intrinsic) resistance

It is a resistance type without hereditary characteristic. This means becoming resistant of a microorganism due to its structure. Absence of the target molecule that the antimicrobic agent is effective by binding is responsible from the natural resistance in general. Any origin of the resistant species to an antimicrobic agent is not effected from such antibiotic.

Many gram negative bacteria show resistance to vancomycine and methicillin and enterococcus show resistance to cephalosporin because of their cell wall structure. As passage of aminoglycosides into cell membrane is an oxygen dependent, energy requiring case, aminoglycosides are not effective on anaerobes since sufficient drug can not enter into the cell in obligate anaerobe bacteria that oxidative phosphorylation is not present.

3.3 Acquired (hereditary) resistance

It is the resistance type acquired. Here, the drug is effective when bacteria population contacts the antimicrobic agents first; however, resistance develops against the antimicrobic agent in the microorganism population during the contact period or repeated treatments. The resistance developed against antimicrobics occurs by this manner and resistant origins appear and diffuse by selection after genetic change. Genetic resistance is under control of chromosome, plasmide, transposon. Microorganisms become resistant against antimicrobics by using one or more resistance mechanisms.

4. Mutation

Changes which has been occurred in alignment or structure of nucleotide pairs forming gene structure in DNA and modified protein structure coded and function as well are called as mutation. As a result of mutations, mutants which show resistance to various drugs, disinfecting agents, chemotherapeutics, inhibitors, ultraviolet beams, phages and such agents may appear.

5. Genetic material transfer between bacteria

In bacteria, genetic material may be transferred into another bacteria partially and three basic mechanisms play role in genetic material transfer after these transfers.

5.1 Transformation

It is recombination of free DNA fragment which was left into the environment by the donor bacteria without any mediator (another bacteria or bacteriophage) in the environment with the receiver's own genetic elements. In another words; if a microorganism is produced in an environment including genetic material (DNa fragments) of another microorganism which is very close in terms of DNA composition and planting from this liquid medium into a solid medium after a certain period, it is seen that some colonies have different morphologies and genetic material of them are similar to original colonies of dead microorganisms which give the genetic material.

Some specific characters has been able to be transferred to receiver bacteria by transformation. These include lactose and galactose positive genes, resistance against

antibacterial substances, virulence etc. These factors are transferred into mRNA by transcription after combination with donor DNA and it is transloced from here and causes appearance of new characters in the cell. Although transformation is not successful among enterobacters, it is reported recently that transformation was detected in high calcium ion concentrations in Escherichia coli.

5.2 Transduction

Transfer of genetic material from a donor bacteria into a receiver bacteria via bacteriophages is called as transduction. Gen transfer via transduction is detected in Gram negative (*Salmonella*, E.coli, Shigella, Proteus, Vibrio, P. Aeruginosa etc.) and Gram positive (staphylococcus and bacillus) microorganisms.

5.3 Conjugation

Conjugation is a method of gene transfer that genetic material transfer realized as a result of physical relation of donor cell with the receiver cell. For realization of conjugation between two cells, cells should contact with each other. Generally, contact occurs via sex pilus which are synthesized by special genes in sex factors in the cell. These are longer and thicker tan other normal pilus (fimbria). They serve as a pipe or passage bridge as they are hollow. Genetic material passes from here and is transmitted into the receiver. Normal pilus do not have roles on gene transmission.

Another way in conjugation is direct contact. In this way, membranes of two bacteria combine and form a pore on the combination point. And DNA transmission occurs via this pore. Receiver population is very wide in conjugation and it may occur between different species and genus.

There are two types of conjugative structures consisting of chromosomal elements known as conjugative transposones and plasmides.

5.3.1 Conjugative transposons

Conjugative tronsposons are genetic elements of a bacteria which may replace from chromosome of a bacteria to its plasmid or to a chromosome or plasmids. They show similar characteristics with plasmids and bacteriophages. The difference from plasmids is inability to replicate by themselves. These structures are in Gram positive bacteria and Bacteroides species and cause diffusion of antibiotic resistance genes among various bacteria groups. Conjugative transposons form a circular intermediate form by binding covalently after leaving from bacterial DNA. This intermediate form may be transmitted to another region in the same cell or to another cell and be bounded to genomes or plasmids of the receiver cell.

5.3.2 Plasmids

Genes that make bacteria resistant against antimicrobial agents may be present on chromosomes of bacteria as well as they are carried on small DNA fragments called as plasmid. The term plasmid was first used by Lederberg to define all extrachromosomal hereditary elements. Today, this phrase was limited by extrachromosomal DNA which replicates independent from chromosome. Plasmids are circular extrachromosomal DNA

molecules with fibril pairs and they code various activities which are not required for aliveness of the bacteria in natural environments and conditions.

The most common antibiotic resistance in bacteria caused by plasmid. Genes that control antibiotic resistance of bacteria exist on R (resistance) plasmids and these plasmids cause spreading of the resistance by being transmitted to other bacteria. Existence of resistance genes on plasmids and their ability to be transmitted showed that plasmids are basic vectors for spresding of resistance genes among bacteria populations. Plasmids on bacteria of enteobacteriacea family are related with transmission of various genetic characteristics such as drug resistance, hemolysin, enterotoxin and co-lysin production, tolerance to heavy metals, resistance to ultraviolet beams, carbohydrate fermentation and H2S production.

Plasmids acquire their multiple drug genes via 2 paths.

1. To form plasmids that show multiple drug resistance via subsequent transposon insertion (This path is not used by many plasmids).
2. To acquire multiple drug resistance genes by receiving linear DNA fragments that may be inserted into DNA like transposons called as integrons.

Integrons are integrated only to a single point differently from transposons and they do not code transposase. Integrase enzymes are coded by plasmids that they are integrated. Many integrons carry promoter-free antibiotic resistance genes and are integrated to plasmids specific to direction. Many resistance gene may be present consecutively on a plasmid.

6. Resistance mechanisms of bacteria against antibiotics

Various resistance mechanisms has developed in bacteria which are coded by any of abovementioned paths against antibiotics which are compounds with low molecular weight and suppress bacteria reproduction or kill them.

6.1 Resistance dependent on external membrane in gram negative bacteria

Target regions of beta lactam antibiotics are on the outer surface of the cytoplasmic membrane and targets of many other antibiotics are in the cell. Therefore, all antibiotics should pass the external membrane barrier to reach to target regions in gram negative bacteria. Passage from the external membrane is via pores. Requirement of antibiotics diffusing from pores gives a minor resistance to all Gram negative bacteria (5 to 10 times) and mutations that may occur in pores may increase this resistance ratio. Some of external membrane pores are specific and some of them are non-specific pores. Mutations on non-specific pores may provide resistance to more than one antibiotic type.

6.2 Enzymatic inactivation of antibiotics

An important resistance mechanism especially in gram negative bacteria against beta-lactam antibiotics is beta-lactamase. The beta-lactamase enzyme which is present on some microorganisms (Staphylococcus aureus, E. coli, *Salmonella* spp., Shigella spp., etc.) and is coded by R-plasmids hydrolyzes C-N bound in beta-lactam bond in the structure of antibiotics such as penicillin, cephalosporin, ampicillin, cloxacilin etc. and inactivates antibiotics (Arda, 2000). Beta lactamases are released into periplasmic space in Gran

negative bacteria and into extracellular environment in Gram positive bacteria. Since external membrane pores limits antibiotic passage and beta lactamases are released into periplasmic space, resistance of gram negative bacteria can be obtained with a lower enzyme level than gram positive bacteria.

The most important mechanism of the resistance against aminoglycosides is enzymatic inactivation of the antibiotic. Aminoglycoside modifying enzymes inactivates antibiotic by addition of a group such as phosphoryl, adenyl or acetyl. These enzymes are on the outer side of the cytoplasmic membrane in gram negative bacteria. Therefore, a decrease occurs both in passage of antibiotics from cytoplasmic membrane and in their power to inhibit protein synthesis. Although an enzyme which inactivates tetracycline in aerobe conditions recently, it was understood that this enzyme is not effective clinically and in terms of total resistance of tetracycline.

6.3 Active ejaculation of antibiotics

Ejaculation type resistance is first observed in tetracycline. A cytoplasmic membrane protein catalyzes energy dependent ejaculation of bacteria of tetracycline. Genes that code this protein are both in Gram negative (tet A- tet G) and in Gram positive bacteria (tet K - Tet L). This type of resistance was detected in staphylococcus species for macrolide antibiotics. Another ejaculation system with a low efficiency was also found for fluoroquinolones.

6.4 Modification of target areas of antibiotics

The resistance mechanism which plays role in the resistance against beta lactam antibiotics is changing specific binding regions of penicillin binding proteins (PBP) which are the target region of the antibiotic. This type of resistance is common among Gram positive bacteria. The "mec" gene which gives methicillin resistance is the best defined gene among these resistance genes. Furthermore, this type of resistance was also detected against glycopeptides, tetracyclines, macrolides, lincosamide, quinolon, rifampicin, trimethoprim and sulphonamides.

7. Antibiotic resistance conditions of *Salmonella* origins

Resistance against chemotherapeutics in *Salmonellas* has appeared quite lately. While no resistance has been observed in *Salmonellas* against chloramphenicol, kanamycine, ampicillin, streptomycin and sulphonamides until 1960, an increasing resistance has been developed against tetracyclins in particular, ampicillin, streptomycin and sulphınamides since 1960s. However, the most effective chemotherapeutic agents on *Salmonella* species are chloramphenicol, tetracyclines, ampicillin and gentamicin.

S. typhi origins were sensitive to all antibiotics including chloramphenicol in particular until 1970s. After a wide epidemia created by chloramphenicol resistant S. typhi origins in 1972, these resistant sources were found in many countries mostly in India, Mexico, Thailand and Vietnam.

First trimethoprim resistance for S. typhimurium has been detected in 1973 and then it is reported in 7% of human sources. No trimethoprim resistance was detected in S. typhi until 1980. During past 20 years, antibiotic resistance and multiple drug resistance has increased

in *Salmonella* species. Resistance cases were reported in non-typhoidal *Salmonellas* in many countries in South America, Middle-east and South Asia. *Salmonella* sources that show aggressive multiple drug resistance is a big problem in many countries. Antibiotic resistance is commonly under control of plasmids in *Salmonella* sources. Plasmids are gained as a result of antibiotic pressure which is used common in feeds of livestock and in medicine and in veterinary. Resistance plasmids gains resistance genes from other plasmids in the same source or plasmids that is carried by other bacteria origins involved in chromosome or host organism. Resistance may also develop spontaneous mutation of a chromosomal gene as a response to selective antibiotic pressure.

8. Determination of antimicrobial characteristics of essential oils and methods used

Herbal extracts and essential oils have been used for long years for different purposes. However, their use for wider purposes by utilizing from their different features and studies related with them are continued rapidly. The most emphasized subject is antimicrobial characteristics. By using these characteristics, essential oils are started to be used in protection of raw and processed food, as additives in modern drugs and in natural treatments. There are many articles published associated with research of antimicrobial characteristics of essential oils and herbal extracts. In these studies, a kind of essential oil is studies by targeting against a pathogen microorganism.

This information is usually useful, however every study has procedural differences. Antimicrobial test methods used differ from each other. Furthermore, there are differences between selected oils or plants that they were extracted in terms of place where they are picked up and extraction methods. It is more likely that study results may differ due to these factors.

Until 1960s, many methods for drug, especially antibiotic sensitivity tests of microorganisms and many different modifications of these methods were reported. Superiority and area of use of every methods are limited. To interpret results with a highest level, all characteristics of the method should be understood well. There are two basic methods that are used to detect antibiotic sensitivity of bacteria. These methods are "Titration (Dilution) Methods" that antibiotics interact with microorganisms after serial dilution and "Diffusion Methods" which are performed by placing a test substance impregnated paper disc to medium surface after planting the culture to be tested.

Essential oils have some characteristics such as volatility, hydrophobicity and having special odors activating in respiratory system. Essential oils are heterogenous mixtures that organic substances are present as a mixture. These final characteristics reveal that especially odorous oils may be biologically active. In fact, essential oils have various pharmacological activities. The most reported characteristic is antimicrobial effect. Tests that reveal such characteristics does not depend on a certain standardization and they may be performed in any laboratory randomly. Techniques used are generally agar diffusion and dilution methods.

Dilution techniques were developed to detect sensitivity of a microorganism to antibiotics. However, they are also used to determine antimicrobial characteristics in plant extracts or essential oils. It depends on serial dilution of antimicrobial agent and inoculating bacteria culture. After incubation, the effect of antimicrobial agent tested with which concentration

against the microorganisms is determined according to presence and absence of reproduction. Presence and absence of reproduction is performed via turbidity detection and low final concentration value that no reproduction occurs is defined as Minimum Inhibiting Concentration(MIC) value. This technique is macro-both dilution technique which has been performed in standard assay tubes for a long time. Recently, a method that acts with the principle of this method but requires very less medium and test material was started to be used in testing synthetic and natural antimicrobial agents excepts antibiotics. The method which is more advantageous mostly than other diffusion techniques and reveal MIC value accurately is micro tube dilution or microbroth dilution methods. In this methods, plates including 80, 96 or more pits which were developed commercially are used. Material dilutions are prepared in these pit series and agent and the microorganism are activated by adding less culture. After the incubation, absence and presence of reproduction by turbidity determination. Turbidity detection procedure may be performed by simply observation or by using special turbidity readers. Although this method is mostly used for antibiotics, they are also used for plant extracts and essential oils. The most important advantage is to perform the assay with 10 to 25 µl. Because it is very hard to obtain essential oils plenty. Another advantage is to allow testing many agents at the same time.

Another method used in antimicrobial tests is agar diffusion method. This technique is commonly preferred due to easy testing of essential oils. Agar diffusion technique has been used to determine antimicrobial characteristics of various agents since early 1940s. Qualitative and quantitative information may be revealed by this method. In agar diffusion technique, an appropriate medium including test organism is used by a hole system including the substance to be tested. The essential oil which was dissolved homogenously is put on medium with certain volumes. Holes contact with the medium. By this method, sometimes essential oil impregnated paper discs are used instead of making a hole on the medium. Consequently, essential oil is diffused to previously microorganism vaccinated medium from holes or paper discs. Structural characteristics of the agent used may be effective on diffusing percentage or period and may effect test results. If the agent used is effective at the end of incubation period, significant, inhibition zones occur around holes indicating no reproduction. Oil quantity applied and diameter of the disc or hole used are important parameters in this method. Because, diameters of inhibition zones occurred at the end of incubation are under the control of these parameters. Thickness of the medium that the hole is opened effects the diameter of inhibition zone. A certain period should pass for formation of inhibition zone. This period is called as "critical period" (T erit). Before this period, inhibition zones may not become significant or when incubation is performed over this period, occurred zones start to disappear. Furthermore, density of inoculum used should be certain and fixed. Because, an agent which be effective normally will be seemed as ineffective due to microorganism concentration and will not form an inhibition zone or will not be within real sizes. Therefore, inoculum concentration should be hold in critical level. If density of microorganism is at the required level, duration of the incubation period is not very important. Diameters of inhibition zones formed is measured via a scale and recorded. Increasing or decreasing concentrations of the agent is put into the pits and increase or decrease in diameters of zones proportionally are expected. However, it is reported that there is a definite parallelism between zone diameter values obtained via agar diffusion method and corresponding agent concentrations and real MIC values, but zone diameters obtained are not compatible with MIC values.

Another method which has been started to be used frequently to determine microbiological activity of essential oils recently is bioautography. Bioautography method is very easy and correct results giving method to try plant extracts or pure substances against botanic and human pathogens. In this method, it is revealed that which of organic compounds forming the essential oil is responsible from the activity as well as antibacterial characteristics of the essential oil. This method bases on principles of agar diffusion technique. However, it may differ in terms of application of the substance to be tested and evaluation of results. The biggest difference is that thin layer chromatography (TLC) is used in the method and the essential oil is activated by test microorganisms after application to TLC plates. By the help of TLC technique, compounds in the essential oil are removed roughly and the compound which is responsible from the activity is revealed. Test substance is applied to both TLC plates in the method and one of plates are accepted as reference plate. The other is the plate that microorganisms are applied. Fractions are marked by making the reference plate colored with reagents or examining under 254 or 336 nm UV light. After incubation of the plate used in the assay, the inhibition zone presence is determined by such substance and Rf value of that substance is calculated. Rf value (Retention Factor) is found by calculation the ratio of the distance that such substance has moved on the plate to the distance that dissolving agent has moved. Referans olarak saklanan İTK plağındaki maddeler ile inhibisyon zonlarının oluşturduğu maddelerin R_f değerleri karşılaştırılarak zonu oluşturan madde işaretlenmekte ve bu aşamadan sonra zonu oluşturan madde çeşitli yöntemlerle referans plaktan izole edilerek tayin yoluna gidilmektedir. In fact, bioautography method is suitable to reveal compounds with high antimicrobial activity such as antibiotics. It reveals the most active components among plant extracts or similar organic compounds. Three bioautography was reported since today. These are;

a. Direct bioautography method that microorganism is directly placed on TLC plate,
b. Contact biouatography method that the substance moved on TLC plate is isolated and transferred into inoculated medium by the microorganism.
c. Immersion bioautography or "Agar-overlay biouautography" that is performed by pouring the medium which was inoculated by a certain microorganism on TLC plate.

The last method is formed by combining direct bioautography and contact bioautography. Direct biouatography is especially used for bacteria and spore producing fungus. This method is quite sensitive and very net inhibition zones may be observed at the end of the assay. However, disadvantage of this method is difficulty of development of microorganisms on TLC plate. This problem is not present in contact biouatography method, but isolation and transfer of the substance from TLC plate cause some problems. Bigger than required inhibition zones appear and this causes difficulty in discrimination between active components. It is reported that immersion bioautography which is the combination of both methods are generally used for yeasts and bacteria. By pouring certain medium on TLC plate, active substances are tested in place and no reproduction problem occurs because sufficient medium is used. The problem of this technique is different diffusing coefficients of active substances. As a solution, a softer medium is obtained by decreasing the agar amount on the medium which is poured on TLC plate and diffusions of components on the plate into inoculated agar is facilitated. Whether which of these three methods is used, tetrazolium salts are used in general to provide determination of inhibition zones that are expected to form after incubation or to visualize. These reactive substances provide microorganisms to be colored purple and formation of colorless inhibition zones are provided at backstage.

There are various methods for determination of antimicrobial effects of volatile substances, especially essential oils in steam phase. The most applicable method is micro-atmosphere method. It is reported that this method is more appropriate for filament fungus in general. Petri plates with a size of 120 mm including medium is used in the method. Fungal spores which are prepared in sterile distilled water are inoculated on the centre of the medium as final concentration to be 10^4 spore/spot. The oil to be tested is impregnated purely with different amounts to the filter paper which has the same size with the petri plate. Sole microorganism is inoculated to one of the plates to use as control, no test substance is applied. Separate petri plates are used to try different quantities of the essential oil. Prepared filter paper is placed into the cap of the petri and petri plate is closed and incubated in a reverse position for 2 to 12 days. Plates are controlled during this 12 days and development status of inoculated microorganisms are controlled. Usability of essential oils in elimination or disarming of fungal or bacterial load in the air has been searched. So, it has been stated that there would be chance to protect the atmosphere of library, museum, hospital, cinema etc places without damaging to people against to microbial flora through essential oil. Some of researchers observed the inhibition instead of emission the pure essential oil into filter paper to put directly into cover section of petri cup. To prevent lost of essential oil, around of petri cup is covered with parafilm and is let to incubation in converse type. At the end of 3 days incubation period, petri cup is opened and steam of essential oil is released and it is left 3 days more to incubation. This second time applied incubation provides to determine whether antimicrobial affect of essential oil is at bactericidal feature or not. If essential oil steam only inhibited development of microorganism and it has been informed that the microorganisms at the plate will be developed at the end of this period. Some of the researchers searched whether steam pressure has any affect or not through emission the essential oil in various diameter filter papers and applying different quantities. For this purpose, volatile pure materials have been tested and consequently it has been set forth that steam pressure doesn't support steam activity of essential oil.

9. The methods that are used to determine antimicrobial features

9.1 Resuscitation of microorganisms

The bacteria occurring in lyophilize cultures, are extracted from their tubes under aseptic terms and they are transmitted into Nutrient Broth tubes to resuscitate. At the end of incubation period at 37°C for 24 hours, the cultures are planted in sole colony into Mueller Hinton Agar (MHA) plates and then they are left into incubation again. At the end of this period the purity of microorganisms are checked and they are transmitted to 2 ml micro-reaction tubes (eppendorf) including 1,5 ml 15% sterile glycerol solution which was prepared previously. Those tubes are kept in -85°C as to be used later.

9.2 Agar diffusion method

For the Agar diffusion method, first of all 25 ml Mueller Hinton Agar (MHA) has been poured into sterile glass petris in 120 mm diameter and the petris have been left for frozen on a smooth surface. The bacteria that will be used are developed in 5 pursuant to McFarland cloudy gauge at Müller Hinton Broth (MHB). Then 1 ml is taken from bacteria

solution and poured into petri cups in 120 mm diameter that were prepared previously and the bacteria is provided to spread over medium through sterile drigalski spatula. Then those petris are left to dry in sterile cabinet with semi opened cover. Approximately six reservoirs at per petri is opened on drying surface medium through sterile corc-borer with 6 mm diameters. Essential oil are weighted in 1 mg and solved in 1 ml DMSO and double layer serial dilutions are prepared. Essential oil concentration from 1 mg/ml to 15.6 µl is obtained. Those dilutions are pipette in 50 µl into opened holes on mediums. After completing pipette process, the materials are stored in fridge for 30 minutes for diffusion of materials into medium and then they are left into incubation at 37°C for 24-48 hours.

9.3 Microbroth dilution method

The cultures are cultivated in petries which include MHA to regenerate them and incubated for a period of 24 hours after they are taken out from -85°C. After incubation, some colonies are developed in medium and these colonies are transferred to 10ml of tubes that contain Mueller Hinton Broth (MHB) and again incubated at 37°C for a period of 24 hours. After a period of 18-24 hours of incubation, the cultures developed in liquid medium are again transferred to double power of MHB tubes in definite amounts after they are tuned up in haze as per Mc Farland No: 0.5 (approximately 10^8 cfu/ml).

The essential oils to be tested are transferred to 4mg of sterilized flakons and these are dissolved by adding 2ml of sterilized Dimethyl-Sulfoxide(DMSO) in 25% ratio. The essential oils should be fully dissolved in DMSO and a homogenous mixture should be obtained. Beginning from stocked solutions obtained, essential oils in micro-reaction tubes (eppendorf) are diluted with sterile distilled water and a series of combinations (1, 1/2, 1/4, 1/8, ...) are prepared from 2mg/ml to 1.95 µg/ml.

Micro titration petries (Brand) having 96"U" type bores are used for experiment. 100 µl of diluted mixtures are transferred to each bore column series via micropipettes. In addition to the essential oils to be tested, to control the solvents the DMSO and Standard antibiotic chloramphenicol (Sigma) are tested as positive control. After all concentrations are transferred to the bores, the microorganisms are added to them. To do this, the microorganism cultures whose haze is tuned up as per McFarland No:05 before are transferred to the reservoirs that are produced proper to multichannel automatic pipetors and 100 µl of mixture are pipeted to each line of bores as one microorganism is in the same eline. After these procedures, the cover of micro titration petries are closed and they are incubated at 37°C for period of 24 hours. At the end of incubation period, to be able detect the regeneration some TTC salt is sprayed over petries. Later it is left to incubation and coloring process at 37°C for a period of 3 hours. At the end of incubation, the areas that are not colored are the ones that no regeneration is obtained.

9.4 Detection of antimicrobial compounds in essential oils by bio-autography method

9.4.1 Thin layer chromatography (TLC) system

Thin layer chromatography plaques (Aldrich) that are coated with silica gel 60 GF_{254} adsorbent and in 0.2mm of height are used in appropriate sizes over aluminum supports. These plaques are kept in their special protected boxes in room temperature, droughty, and dark places. Pure

essential oils are applied over plaques as 1 µl by using capillary tubes. 9:1 (v/v) proportion Hexane-Ethyl Acetate dissolvent as 20 ml is prepared and filled in a covered rectangular glass container. The TLC plaque where essential oil applied parts are marked is immersed vertically into dissolvent, but one should be careful as the dissolvent not to reach the oily parts. The TLC plaques are developed in this system as twins. While one of the plaques is taken out and kept aside for experiment, the other one is cleaned out from dissolvent by evaporating it and analyzed under 254/364nm wave lengths and UV active spots are marked. To be able to detect the compounds that do not absorb UV, anisaldehyde / H_2SO_4 color reactive is sprayed over the plaque and it is heated at 110°C for a period of 1-3 minutes.

9.4.2 Preparation of microorganisms

A cell suspension of approximately 1.5 x10^9 cfu/ml in MHB is prepared for bio-autography method which contains bacterial culture that is prepared the day before in MHB medium and tuned up in haze as per McFarland No:5.

9.4.3 Assessment of activity

Pre-prepared molten agar that is delivered in 20ml of Erlenmeyer is kept in water-bath and then 2ml of bacterial suspension culture is added into agar and a final concentration of 1.5x10^8 cfu/ml is obtained. TLC plaques which are pre-prepared and not processed with reactive are placed in a petri that is filled with Nutrient Agar. Then molten agar that is kept in hot water-bath and inoculated with microorganisms in a well stirred situation is added into the petri as a thin layer not much than 1mm. These plaques are incubated for a period of 24-48 hours at 37°C. At the end of incubation period, formed inhibition zones and their R_f values of corresponding reference plaque fractions are measured and registered.

9.4.4 Gas chromatography (GC)

The compounds in essential oil are isolated and evaluated under following conditions by taking into account their attachment periods in gas chromatography (R_t) and their relative rates.

GC Analysis Conditions	
System	Shimadzu GC-17A
Column	CPSil 5CB
Carrying Gas	Nitrogen (1ml/min.)
Splitting Rate	50:1
Detector	FID
Temperatures	
Injection	250°C
Column	60°C//5°C/min//260°C-20 min
Detector	250°C,

Table 2.

9.4.5 Gas chromatography / mass spectrometer(GC/MS)

After isolating compounds in essential oil within a gas chromatography column, spectrums of each compound is determined by using a mass spectrometer. All evaluations are made primarily by using "TBAM Essential Oil Compounds Library". Furthermore, Wiley and Adams-LIBR library scanning software" and ''The Wiley/NBS Registry of Mass Spectral Data'' systems are used.

GC/MS Analysis Conditions	
System	Shimadzu GCMS-QP5050A
Column	CPSil 5CB (25mx0.25mm i.d.)
Carrying Gas	Helium (1ml/min)
Temperatures	
Injection	250^{o}C
Column	60^{o}C//5^{o}C/min.//260^{o}C-20 min.
Splitting Rate	50:1
Power of Electrons	70 eV
Mass Spectrum	35-400 m/z

Table 3.

9.4.6 Dispense of preparative TLC plaque

Readymade TLC plaque coated in size of 20x20 is used for preparative purposes to isolate active compounds in essential oil. Neutral essential oil is applied over plaque as a thin layer and eliminated in hexane: ethylacetat (9:1 v/v) dissolvent system within a TLC vessel. At the end of this process, a narrow fraction of TLC plaque is cut out vertically and this sample is analyzed under 254-364nm UV light and R_f value of this to be isolated fraction is determined by the application of anisaldehyde/ H_2SO_4 reactant. Subsequent to this process, sample plaque fraction is laid alongside of TLC plaque that is not processed with reactant yet and by taking into account the R_f value, the area on the plaque where the target substance is present is determined and silica gel over the aluminum support is rubbed out. Silica gel and substance compound is transferred to a glass funnel where glass wool is present and acetone is poured over them as a solvent. The substance is dissolved with acetone and resolving silica gel in the funnel gathers in the Erlenmeyer downside. Later on by using a vacuumed rotavapor, the solvent is removed in 40^{o}C.

10. References

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7

Xylariaceae Endophytic Fungi Metabolites Against *Salmonella*

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1. Introduction

For several years, natural products have been used directly as drugs or have provided the basic chemical architecture for deriving such drugs. Natural products are naturally derived metabolites and/or byproducts from plants, animals or microorganisms. These products have been exploited for human use for thousands of years and plants have been chief source of compounds used for medicine. Besides plants, microorganisms constitute a major source of natural products with desirable bioactive properties. The ultimate purpose of the researchers is looking for new sources of metabolites. The marine organisms, for example, have been studied with more attention in the recent decades. Endophytic fungi appear to be another interesting source of research. Because of what appears to be their contribution to the host plant, the endophytes may produce a plethora of substances that may have potential use to modern medicine, agriculture and industry.

Infections caused by pathogenic microorganisms are responsible for high rates of morbidity and mortality in world (Coelho et al., 2007; Souza et al., 2007). These infections can occur in invasive form, and are an increasing problem due to the increase of their incidence in hospitals, especially in patients who are undergoing cancer treatment, transplantation or are immunosuppressed for other reasons (Oliveira et al., 2001).

The genus *Salmonella* is extremely heterogeneous, comprising almost 2000 serotypes, of which only a few are major human pathogens (Kaufmann et al, 2001). However, despite this apparent complexity, *Salmonella* species are actually quite similar genetically, with the serotype differences based on surface antigen differences such as LPS and flagella (Kaufmann et al, 2001). The two main symptoms of salmonellosis are typhoid or typhoid-like fever and gastroenteritis (Tahergorabi et al, 2011). Salmonella spp. are a leading cause of acute gastroenteritis in several countries, and salmonellosis remains an important public health problem worldwide, particularly in the developing countries (Mead et al., 1999). Food is a transmission vehicle for *Salmonella* that causes about 96% of all salmonellosis cases (Tahergorabi et al, 2011). Gastroenteritis-causing pathogens are the second leading cause of morbidity and mortality worldwide, with children under the age of 5 years at greatest risk

(Guerrant et al, 2001). Such serious infections are most common in children and the elderly (Rotimi et al, 2008).

S. typhi remains an important health threat for humankind, with more than 16 million cases and 600000 deaths annually, worldwide (Kaufmann et al, 2001). Ironically, typhoid fever is declining worldwide, but non-typhoidal *Salmonella* infections are increasing rapidly, due to increased automation in food processing and other factors (Kaufmann et al, 2001). Non-typhoidal species also cause serious disease in immunocompromised individuals. (Rotimi et al., 2008). Up to a decade ago, in many countries, conventional 1st-line antimicrobial agents, such as ampicillin, chloramphenicol, and trimethoprim–sulfamethoxazole, were the drugs of choice for the treatment of lifethreatening salmonella infections (Rotimi et al 2008). However in the past two decades, these species are also becoming increasingly resistant to most antibiotics, which has significantly increased the concern about these food and water-borne pathogens (Threlfall et al., 1997; Therefall, 2002; Wedel et al., 2005).

In recent years, several powerful new technologies have been developed that have significantly enhanced our knowledge of *Salmonella* pathogenesis (Beuzon & Holden, 2001; Kaufmann et al., 2001). Novel approaches for development of new antibiotics have been pursued, such as combinatory chemistry tools but only a few new antibiotics are produced by the pharmaceutical industry nowadays (Coates & Hu, 2007) Despite of the huge expectative on synthetic molecules with effective antimicrobial properties, natural products are still a worth promise.

Thus, the search for new compounds with antimicrobial activity from plants and fungi has been the subject of intense research in recent years (Harvey, 2007; Lee et al., 2007; Hostettmann et al., 2003). This is due mainly to the fact that the plants are widely used in folk medicine to combat various diseases in humans caused by bacteria and fungi (Stefanello et al., 2006; Duarte et al., 2004; Cruz et al., 2007). In this sense, many researchers are aiming to scientifically prove the use of plant extracts as an effective control of infections of the skin (Weckesser et al., 2007), the mouth (More et al., 2008) and other infections caused by a range of Gram-positive and Gram-negative bacteria (Vuuren, 2008; Lee et al., 2007; Chauhan et al.,2007).

2. Endophytic fungi

Endophyte is one which resides in the tissues beneath the epidermal cell layers without causing any immediate, overt negative effects (Stone et al., 2000). It is worthy to note that studies have shown that, nearly 300000 plant species that exist on earth, each individual plant is the host to one or more endophytes, the population of a given endophytic species varies from several to a few hundreds strains (Strobel & Daisy, 2003; Huang et al., 2007; Yu et al., 2010). The endophytes may live in plants air parts, especially in leaves, but can also be found living in intracellular gaps of roots, that is one of the main entrance door for these microorganisms (Azevedo et al., 2001). The endophytic colonization can be positive to host plants. Both fungi and bacteria are the most common microbes existing as endophytes, but the most frequently isolated are fungi.

The endophytes transmission from one generation to another may occur vertically, among seeds, during plants reproduction, or horizontally, where fungi spores are transmitted by air way, water or insects (Carroll, 1988). The microorganism penetration may occur from natural gaps or insects. The roots are the entrance main door (Kobayashi & Palumbo, 2000).

Of the myriad of ecosystems on earth, those having the greatest biodiversity seem to be the ones also having endophytes with the greatest number and most diversity (Strobel et al., 2005). The observation of Moricca & Ragazzi (2008) indicates that the type of interaction between an endophyte and a plant is controlled by the genes of both organisms and modulated by the environment. The fungi produce secondary metabolites compounds that have various biological activities, and have great bioactive potential (Petrini, 1991). The symbiosis among plants and fungi, mainly endophytic fungi, might be an important source of active pharmacologic compounds.

Beyond production of substances that come from secondary metabolism as the majority of antimicrobial, the symbiosis among plants and endophytic fungi can lead to other benefits to host plants as substances that improve growth and host competitively in nature (Hallman et al., 1997; Azevedo et al., 2000). Besides pharmacological potential, these microorganisms can also be highlighted for their capacity to produce interesting substances for farming, as growth plants regulators or insecticides, acting an important role from the ecological point of view (Souza, 2004). Only a handful of plants have ever been completely studied relative to their endophytic biology, consequently is an opportunity to find novel endophytic microorganisms and antimicrobial metabolites produced by them.

2.1 Biological activity from medicinal plants endophytes

The search for substances with pharmaceutical utility was one of the reasons that boosted the endophytic fungi researches. Beyond the studies about endophytic colonization, the characterization of new metabolites produced from a symbiotic association between fungus and host plant lead to isolation of various compounds with commercial importance. A diversity of biosynthetic classes metabolites were isolated from endophytic fungi and most of them showed interested pharmacological effects (Tan et al., 2001; Gunatilaka, 2006)

The development of new agents with pharmacological proprieties still is a great challenge for science and represents and endless research area. In the last 40 years, plenty of metabolites with different carbon skeleton were isolated from fungi. The isolation of cyclosporine in 1970, as a metabolite from *Cylindrocarpon lucidum* and *Tolypocladium inflation* fungi, represented and important step in the immunosuppressive treatment. (Hanson, 2008). Thought the past of the years, another studies were published describing interesting metabolites isolation. Borges et al. (2006) described the presence of derivatives anthraquinones produced by *Phoma sorghina*, and endophytic fungus associated to a medicinal plant *Tithonia diversifolia* (Asteracea). This plant extracts are used for the treatment of malaria, diarrhea, fever, hepatitis and wounds (Gu et al., 2002, Cos et al., 2002). There are also attributed anti-inflammatory, amebic, antispasmodic, antifungal, antibacterial and antiviral activities (Goffin et al., 2002, Cos et al., 2002).

Lu & co-workers (2000) in a research at Nanjing, China, observed the presence of 11 bioactive metabolites produced by *Colletotrichum* sp. and endophytic fungus isolated from *Artemisia annu*a (Asteracea), traditional plant from Chinese medicine. When tested against bacteria, some of there metabolites showed inhibitory activity against gram-negative and positive bacteria, as *Pseudomonas sp*. and *Bacillus subtilis.* Other metabolites were active against pathogenic fungi, as *Candida albicans* and *Aspergillus niger* in the concentration of 200 µg/mL.

2.2 Xylariaceae metabolites biological activity

The Xylariaceae family is considered a great source of a variety of bioactive compounds, showing plenty of chemical structures and biological activity. As an example, can be highlighted the taxol, a diterpene derived that have been used as an effective anti-cancer agent (Stierle et al., 1993). Among fungi that belong to Xylariaceae, the genus *Xylaria* is an important source of new secondary metabolites, with a variety of chemical structures and distinct biological activities.

The chemical investigation of fungi of Xylariaceae family showed as a potential source of biotechnological products, mainly with pharmacological proprieties. The study leadered by Healy et al. (2004) resulted on the isolation of xanthones, compounds isolated from endophytic fungi identified as *Xylaria sp.* The fungus isolated from *Glochidion ferdinandi* plant, and the metabolites extracted showed important pharmacological activities (Peres & Nagem, 1996; Peres et al., 2000) as for example, anti-inflammatory (Lin et al., 1996), antimicrobial (Malet-Cascon et al., 2003), antioxidants (Minami et al., 1994), antifungical (Rocha et al., 1994) e anticancer properties (Ho et al., 2002).

Krohn et al. (2004), developed an similar research, describing the syntesis of xyloketal D, a natural product that belongs to a group of secondary metabolites isolated from *Xylaria* sp. The biotechnological interested for this group is based on inhibitory activity over acethylcholinesterase enzyme.

Based on the abundance of secondary metabolites found in Xylariaceae family, Liu et al. (2008), identified and described the biological activity of 7-amino-4-methylcoumarin, a compound extracted from *Xylaria* sp YX-28 endophytic fungus. The chemical investigation of *Xylaria* (Xylariacea) genus fungus leads to potentials sources of natural products, as Xylarenal A, a terpenoid isolated form *Xylaria persicaria* fermentation (Smith et al., 2002) e xylactam, a nitrogened compound obtained from *Xylaria euglossa* ascomycete (Wang et al, 2005).

The *Hypoxylon, Nodulisporium* and *Daldinia* constitute one of the largest and most important genus of Xylariaceae, and they show a great diversity and production of secondary metabolites (Laessoe et al., 2010; Stadler et al., 2001; Kamisuki et al., 2007). The figure 1 shows some chemical structures isolated from genus *Hypoxylon, Nodulisporium* and *Daldinia.*

2.3 Antimicrobial activity of endophytic fungi metabolites

There is a general call for new antibiotics that are highly effective, possess low toxicity and will have minor environmental impact. This search is driven by the development of resistance infectious microorganisms (e.g. *Staphylococcus, Mycobacterium, Streptococcus*) to existing compounds and by the menacing presence of naturally resistant organisms (Strobel et al., 2005). In support of this idea, metabolites of endophytes have been reported to inhibit a number of microorganisms (Petrini, 1991; Gurney & Mantle, 1993). Many important antifungal and antibacterial chemotherapeutics are either microbial metabolites or their semi-synthetic derivates.

Between the years of 1981 to 2006, the Food and Drug Administration (FDA) had approved 1,184 new drugs among about 609 (51.4%) were natural products related: 55 were natural products, 270 natural product derived by chemical modification (semi-synthetic), 52 were done by synthesis where the active core came from a natural product, and 232 were synthesized by imitating a natural product (Newman & cragg, 2007).

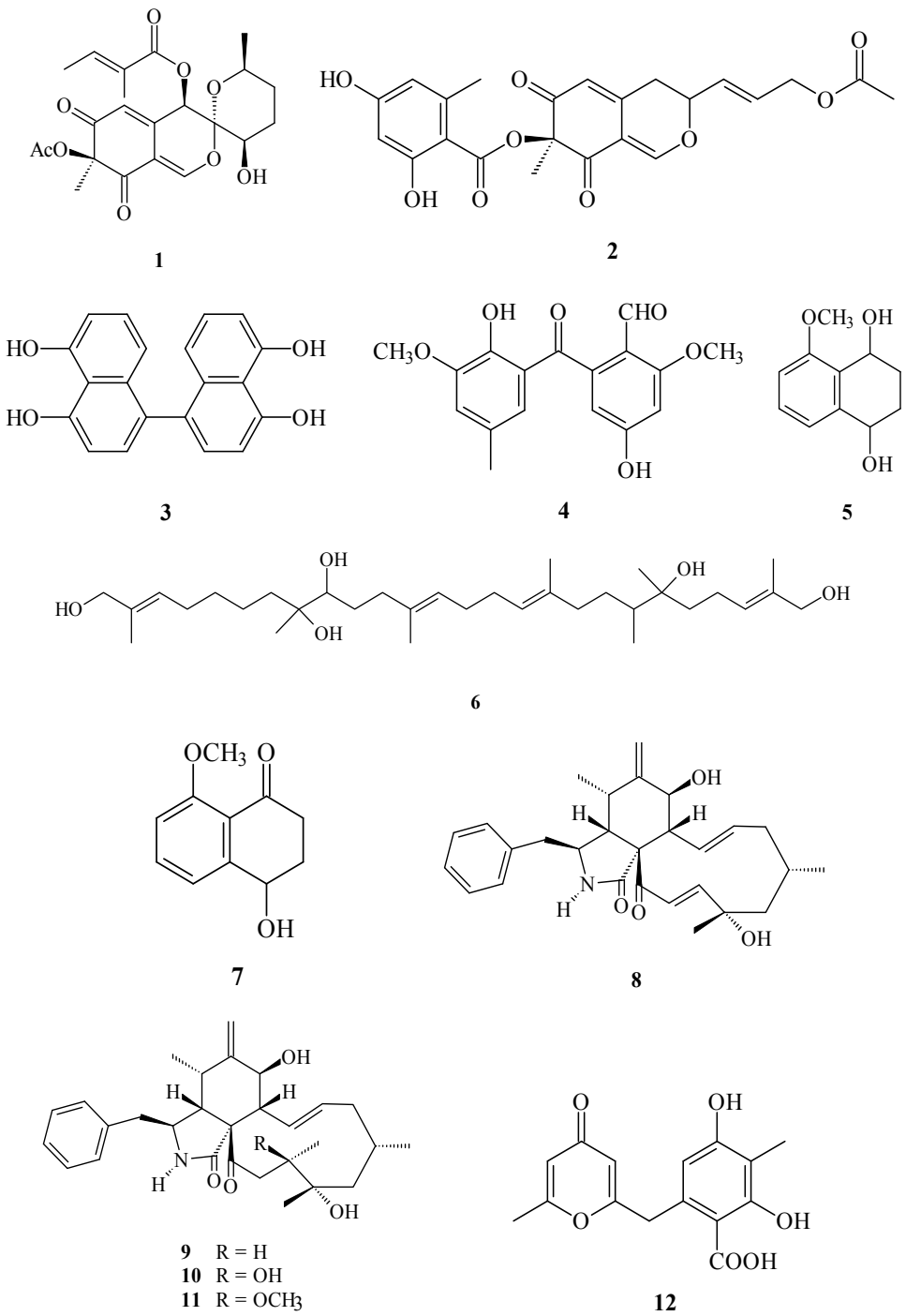


Fig. 1. Chemical structures isolated from species of the genus *Nodosporium, Hypoxylon* and 7 *Daldinia*.1= daldinin C; 2= hypomiltin; 3= BNT; 4= dalninal A; 8= cytochalasin 8 II; 9= cytochalasin I; 10= cytochalasin III; 11= cytochalasin IV; 12= macrocarpon A

Natural products from endophytic microbes have been observed to inhibit or kill a wide variety of harmful microorganisms including, but not limited to phytopathogens, as well as bacteria, fungi, viruses and protozoan that affect humans and animals (Strobel et al., 2005). Taechowisan et al. (2008), described the biological activity of chemical compounds of *Streptomyces* sp., an endophytic fungus isolated from Alpinia galanga (Zingiberaceae) roots, medicinal plant collected in Nakom Pathom, Tailândia, surrounds. According to the authors, the isolated substances showed antimicrobial activity against the following microorganism: Staphylococcus aureus ATCC25932, *Bacillus subtilis* ATCC6633, *Escherichia coli* ATCC10536, *Pseudomonas aeruginosa* ATCC27853, *Candida albicans* ATCC90028 e *Colletrotrichum musae*.Taechowisan et al. (2008), described the biological activity of chemical compounds of *Streptomyces* sp., an endophytic fungus isolated from Alpinia galanga (Zingiberaceae) roots, medicinal plant collected in Nakom Pathom, Tailândia, surrounds. According to the authors, the isolated substances showed antimicrobial activity against the following microorganism: Staphylococcus aureus ATCC25932, *Bacillus subtilis* ATCC6633, *Escherichia coli* ATCC10536, *Pseudomonas aeruginosa* ATCC27853, *Candida albicans* ATCC90028 e *Colletrotrichum musae*. Rocha et al. (2010) observed antagonistic activity of endophytic fungi against to conidia of *Microcyclus ulei*, the agent of South American Leaf Blight responsible for the weak development of rubber plantations in Latin America. Endophytic fungi were isolated from *Hevea brasiliensis* (the rubber tree) leaves, cultivars FX3864, CDC312, MDF180, exhibiting distinct resistance levels to the attack by *M. ulei*. Lyophilized culture filtrates obtained from fungal isolates, grown in liquid malt extract medium, were tested *in vitro* and showed activity against germination of *M. ulei* and exhibited marked inhibitory activity on *M. ulei* conidia germination *in vitro*. The lyophilized culture filtrate of eleven foliar endophytic isolates achieved high inhibitory activity on *Microcyclus ulei* conidia germination and belong to seven genera: *Fusarium* sp., *Gibberella* sp., *Glomerella cingulata*, *Microsphaeropsis* sp., *Myrothecium* sp., *Pestalotiopsis* sp. and *Phomopsis* sp.

Davis et al. (2005) also tested antimicrobial activity of endophytic fungi. After chemical analysis of endophytic fungi cultures, *Eupenicillium* sp. Isolated from an endemic plant in Australia, *Glochidium ferdinandi* (Euphorbiaceae), the authors verified the presence of the following compounds: phomoxin B e C, eupenoxide e phomoxin. The isolated compounds were tested against plenty of microorganism associated to nosocomial infections, including *Staphylococcus aureus* drug multi-resistant (MRSA), *Staphylococcus aureus* (NCCLS 29523), *Escherichia coli* (ATCC 25922), *Enterococcus faecalis* (NCCLS 29212), *Pseudomonnas aeruginosa* (ATCC 27853), *Streptococcus pyogenes* (ATCC 19615), *Acinetobacter anitratus* e *Candida albicans* (ATCC 60193).

The fungus was isolated from a traditional medicinal Chinese plant Ginkgo biloba L. (Ginkogoaceae). The metabolite was identified by NMR and mass spectra. The isolated compound showed activity against Staphylococcus aureus (MIC, 16 µg.mL-1), Escherichia coli (MIC, 10 µg.mL-1), Salmonnela typhia (MIC, 20 µg.mL-1), Salmonnela typhimurium (MIC, 15µg.mL-1), Salmonella enteriditis (MIC, 8,5 µg.mL-1), Aeromonas hydrophila (MIC, 4µg.mL-1), Yerisinia sp. (MIC, 12,5 µg.mL-1), Vibrio anguillarum (MIC, 25 µg.mL-1) Shigella sp. (MIC, 6,3 µg.mL-1), Vibrio parahaemolyticus (MIC, 12,5 µg.mL-1) Candida albicans (MIC, 15 µg.mL-1), Penicilium expansum (MIC, 40 µg.mL-1) e Aspergillus niger (MIC, 25 µg.mL-1).According many researchers (Arnold and Lutzoni 2007; Huang et al., 2008; Tejesvi et al., 2009), endophytes of tropical plants constitute a species-rich ecological

assemblage of fungi and should be included in screening programs for novel metabolites (Suryanarayanan et al., 2009).

Metabolites biologically active have been isolated and characterized (Shulz et al., 2002; Tejesvi et al., 2009; Aly et al., 2010), and could be new molecules for many applications. Shulz and Boyle (2005) and Aly et al. (2010) point that these active metabolites belong to the chemical groups, such as phenols, steroids, flavonoids, quinines, terpenoids, xantones, peptides, cytocatalasins, alkaloids, aliphatic compounds, and phenylpropanoids.

3. Xylariaceae metabolites and *Salmonella* strains

The Xylariaceae endophytics fungus were previously isolated by our research group from a cultivate specimem of *Mikania laevigata*, Asteraceae family (Ribeiro, 2011). This plant constituted in a Brazilian medicinal plant more used for respiratory infections. The endophytics fungus species were submitted to sequencing the rDNA ITS region which resulted in the identification of three strains of *Nodulisporium* sp., three strain of *Hypoxylon* sp., one strain of *Daldinia* sp and four strains unknown. The crude extracts above fungi were obtained by its cultivation on potato dextrose agar at 28 °C in Erlenmeyer flasks (3 × 100 m L), for 7 days. The cultures were filtered to separate broth and mycelia. The mycelia were extracted by reflux with methanol (100 mL) to furnish the respective crude extract (Ribeiro et al, unpublished data)

For antimicrobial test four isolates of *Salmonella* were tested: *S. enteritidis* CCMB 522; *S. carrau* CCMB 523 and two isolated from from food (*Salmonella* sp. CCMB 270 and CCMB 281).

3.1 Determination of minimun inhibitory concentration (MIC)

The broth microdilution susceptibility test was used to determinate the minimum inhibitory concentration (MIC) for bacteria as recommended by CLSI (2003). The extract was dissolved in dimethyl sulfoxide (DMSO) and water (50:50) to reduce the inhibitory potential of DMSO and then the extract were sterilized by filtration through cellulose acetate membrane (0.22 mm). Geometric dilutions were prepared using 96-well, flat-bottom microdilution plate which received 90 mL of DMSO extracts diluted in water in lines A1 to A9 containing 90 mL of previously Mueller-Hinton broth two times concentrated. So, the first wells (A1 to A9) contained crude extracts diluted in a concentration from 1 $mg.mL^{-1}$ until 0.008 $mg.mL^{-1}$ (H1 to H9). The suspension of the micro-organism test was adjusted to 1.5 x 10^8 cells mL^{-1} for bacteria in 0.45% sterile saline. After the dilutions were carried out in all wells, each well received 10 µL of the microbial suspension performing a total volume of 100 mL by well (90 µL of the extract and HCM + 10 µL micro-organism). The plates were incubated at 37 ° C for 24 hours. After the incubation period, were added 30 mL of rezasurin (7-hydroxy-3H-fenoxazina-3-one-10-oxide) at final concentration of 0, 01% for quantitative analysis of microbial growth and determining the relative antimicrobial activity of each sample dilution. All tests were performed in triplicate. Dilutions of the antibiotic chloramphenicol (10 $mg.mL^{-1}$) were used as positive control for comparison of data between independent experiments and as indicators for assessing the relative level of inhibition of the samples tested. Controls of the microbial viability, sterility of the medium, sterility the extract and the potential for inhibition of DMSO on the micro-organisms tested were also carried out. In this work a representative results of MIC values was regarded as equal to or less than 0.5 $mg.mL^{-1}$ of the extracts tested.

3.2 Determination of minimum microbicide concentration (MMC)

After determining the MIC, the minimal microbicidal concentration (MMC) was done. Aliquots of 5 µL of the wells were plated on Mueller Hinton Agar (MHA) and incubated at 37 ° C for 24 hours. The MMC was considered the lowest concentration of the extract which showed no cell growth on the surface of (MHA).

3.3 Xylariaceae extracts against *Salmonella*

Twelve extracts were tested against four strains of *Salmonella.* The results are shown in Table 1.

Extracts		*Salmonella* sp. CCMB 270	*Salmonella* sp. CCMB 281	*Salmonella Enteritidis* CCMB 522	*Salmonella carrau* CCMB 523
Nodulisporium sp. (specie 1)	MIC	0,25	0,5	0,5	0,25
	MMC	0,5	1	-	1
Nodulisporium sp. (specie 2)	MIC	0,5	0,5	0,25	0,5
	MMC	1	1	-	-
Nodulisporium sp. (specie 3)	MIC	0,5	0,5	0,25	0,5
	MMC	1	1	-	-
Hypoxylon sp. (specie 1)	MIC	1	0,5	0,5	0,5
	MMC	1	1	-	-
Hypoxylon sp. (specie 2)	MIC	0,5	0,5	0,5	0,25
	MMC	1	1	-	1
Hypoxylon sp. (specie 3)	MIC	0,5	0,5	0,25	0,5
	MMC	1	1	-	-
Daldinia sp.	MIC	0,5	0,5	N/A	N/A
	MMC	1	1	N/A	N/A
Specie 3 (Unknown)	MIC	0,5	0,5	0,25	0,25
	MMC	1	1	-	-
Specie 8 (Unknown)	MIC	0,5	0,5	0,5	0,25
	MMC	1	1	-	1
Specie 10 (Unknown)	MIC	0,5	0,5	N/A	N/A
	MMC	1	1	N/A	N/A
Specie 12 (Unknown)	MIC	0,25	0,5	0,25	0,5
	MMC	0,5	1	-	-
Specie 14 (Unknown)	MIC	0,5	0,5	0,25	0,5
	MMC	1	1	-	-
CONTROL	CHLORAN	0,31	0,31	0,16	0,31
	DMSO	1	1	1	1

N/A: not applied; CHLORAN: Chloramphenicol; DMSO: dimethylsulfoxide

Table 1. Minimum inhibitory concentration (MIC) and minimal microbicidal concentration (MMC) (mg.mL^{-1}) of Xylariaceae endophytic fungal extracts on *Salmonella* strains

The microdilution technique for determination of minimum inhibitory concentrations (MIC) is often considered as the best methodology for assessing susceptibility or resistance of bacteria to antibiotics (Rivers et al., 1988; Reis, 2006; Alves et al., 2008). According to Ostrosky et al. (2008), MIC has several advantages and one is that this method can be 30 times more sensitive than other methods used in the literature. The DMSO control showed growth inhibition in a dilution corresponding to 1 $mg.mL^{-1}$ extract through MIC determination. Therefore, results were considered representative for MIC values at or below the next lower dilution of the extracts tested (0.5 $mg.mL^{-1}$). DMSO is a substance that facilitates the diffusion (Vieira, 2005), but it was necessary to control the solvent, since this can enhance the activity of the antimicrobial agent (Herschler, 1970; Ribeiro et al., 2001). All the extracts tested (three strain of *Nodulisporium* sp., three strain of *Hypoxylon* sp., one strain of Daldinia sp. and five strain unknown) samples shown antimicrobial activity less than the representative value for DMSO for MIC (Table 1). Most of samples studied demonstrated MMC= 1.0 $mg.mL^{-1}$ for *Salmonella* sp. CCMB 270 and *Salmonella* sp. CCMB 281. These results show that crude extracts had the same antimicrobial effect on salmonella strains studied**.** None of the tested extracts showed activity against all microorganisms, because some MIC values obtained were 1 $mg.mL^{-1}$ and were not considered as representative (Table 1). However, the fact of not showing detectable antimicrobial activity does not mean that the fungal extracts evaluated did not possess bioactive compounds against microorganisms tested.

The strain *Salmonella* enteritidis CCMB 523 seems more sensitive compared to the extracts tested, since it was inhibited at 0.25 $mg.mL^{-1}$ by 6 (60%) of the extracts evaluated (Figure 2). Under the same conditions, *Salmonella* carrau CCMB 523 was inhibited by 4 (40%), *Salmonella* sp. CCMB 270 was inhibited by 2 (20%) of the extracts evaluated, while *Salmonella* sp. CCMB 281 was not inhibited at this concentration by none of the tested extracts.

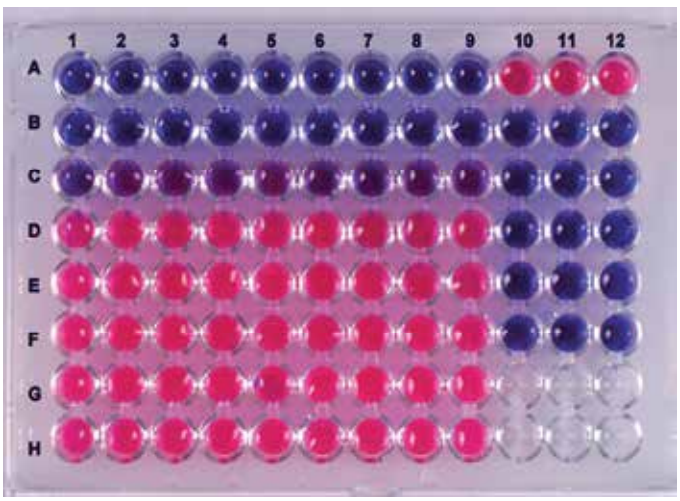


Fig. 2. Determination of minimum inhibitory concentration. Representation of serial dilution of methanolic extracts against *Salmonella enteritidis*. Columns 1-3: **Specie 14 (Unknown)** extract, MIC = 0.25 $mg.mL^{-1}$, Columns 4-6: ***Hypoxylon* sp. (specie 3)** extract, MIC = 0.25 $mg.mL^{-1}$ and Column 7-9: **Specie 12 (Unknown)** extract, MIC = 0.25 $mg.mL^{-1}$. Line A 10-11-12: microbial growing control of microorganism tested. Lines B 10-11-12, C 10-11-12 and D 10-11-12: control of extracts and lines F 10-11-12 and G 10-11-12: Control of the sterility of the culture medium (HCM).

The Gram-negative bacteria are reported to possess resistance to several antibiotics. The complexity of Gram-negative bacteria makes them less susceptible to antimicrobial agents (Tadeg et al., 2005).Variations related to determining the MIC of natural extracts can be attributed to several factors. Thus, there is no standardized method for expressing the results of antimicrobial testing of natural products (Fennel et al., 2004; Ostrosky et al., 2008). So the results can be influenced by microorganisms used for testing, the selected method and the solubility characteristics of each substance (Vanden et al., 1991; Valgus et al., 2007).

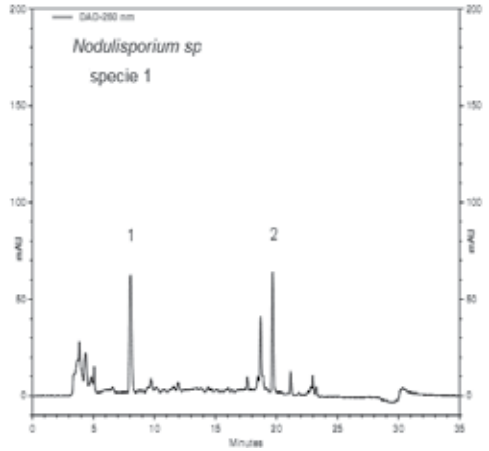
Through the use of the MIC was possible to demonstrate quantitatively the concentration of extracts that inhibited each microorganism, but this test only indicates the concentration able to cause growth inhibition and does not identify whether the inhibition was bactericidal or bacteriostatic. For this, we used the test Microbicide Minimum Concentration (MMC). Observing the results, the *Nodulisporium* sp. (specie 1) and Specie 3 (Unknown) extracts showed microbicidal activity up to 0.5 $mg.mL^{-1}$ against *Salmonella* sp. CCMB 270.

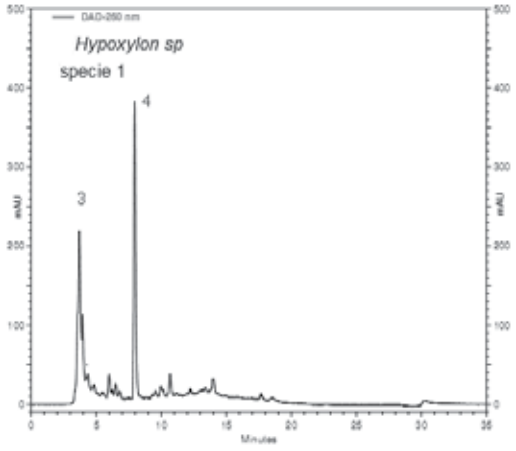
3.4 HPLC-DAD extracts analysis

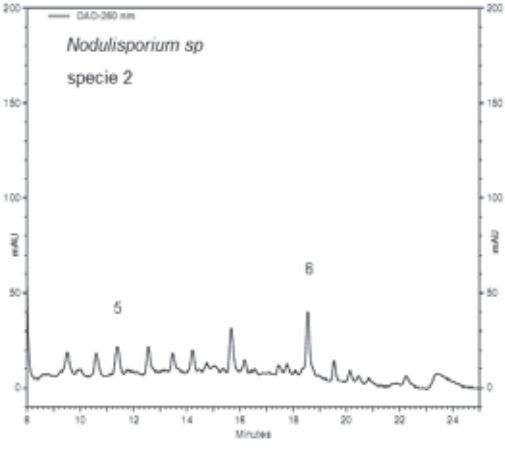
Chromatographic techniques are used to separate the constituents from a mixture of substances aiming isolation and identification, being used to chemical investigation of crude extracts and identification of secondary metabolites of interested (Strege, 1999). High Performance Liquid Chromatography (HPLC) is one of methods of choice for determination of secondary metabolites profile to fungi isolated from Xylariaceae family. Ascomycets as *Daldinia, Hypoxylon* and *Xylaria* have been extensively studied using this method for chemical profile determination of majority and minority sample components. The analyses are based on retention time and UV absorption spectra. This technique also facilitates uncolored metabolites detection (Stadler et al., 2004).

The crude extract obtained from methanol extraction and tested to biological activity were analysed and monitored by High Performance Liquid Chromatography with diode array detector (HPLC-DAD), Hitachi, Laechrom Elite model, LiCospher 100 RP18 (5 µm) column, with 150 mm x 04 mm dimensions, Merck, equipped with diodo array detector (DAD). The mobile phase was composed of solvent (A) H_2O/H_3PO_4 0.1% and solvent (B) MeOH. The solvent gradient was composed of A (75-0%) and B (25-100%) for 25 minutes. A flow rate of 1.0mL/min was used, and 20µL of each sample was injected. Chromatographic peaks were monitored at 260 nm and characterized by retention time and UV-vis spectrum (200-600 nm). The HPLC-DAD chromatograms of the methanolic crude extracts from mycelium of *Nodulisporium* sp. (three strains), hypoxylon sp. (three strains) and *Daldinia* sp. were showed in Figure 2. The Figure 3 showed the HPLC-DAD chromatograms of the methanolic crude extracts from mycelium of five unknown species of Xylariaceae.

The *Nodulosporium* sp (specie 2) and *Daldinia* sp demonstrated to contain lack compounds in this analysis conditions when *Nodulosporium* (species 1 and 3) and all species de *Hypoxylon* showed two majoritary compound in the respective chromatograms. In the specific case of unknown species was to verify the presence de several compounds in the respective HPLC-DAD chromatograms (Figure 3). In the crude extract of unknown species 3, 10 and 12 the peaks eluted between 15 to 20 minutes, while in species 6 and 10 the peaks were eluted before 10 minutes, showing two majoritary peaks as the same profile for *Hypoxylon* chromatograms.


DAD-280 nm
Nodulisporium sp
specie 1
1
2
mAU
Minutes


DAD-280 nm
Hypoxylon sp
specie 1
3
4
mAU
Minutes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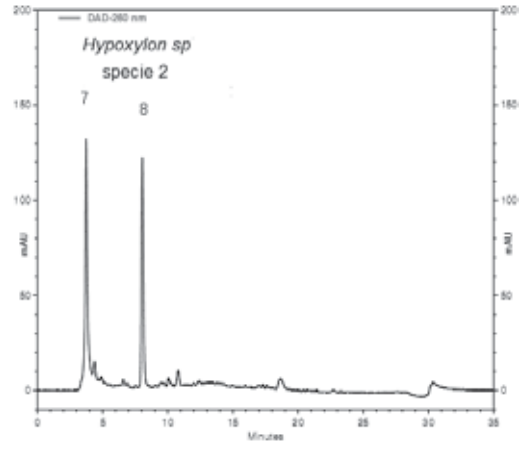
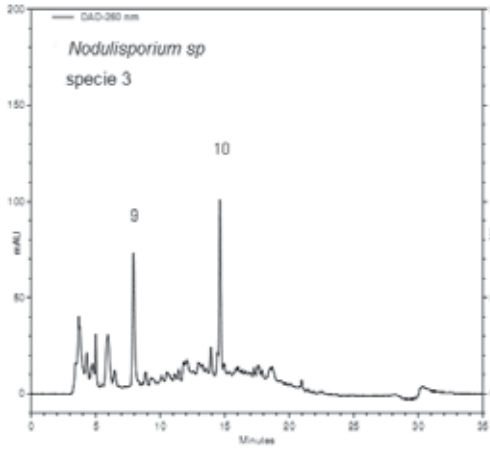
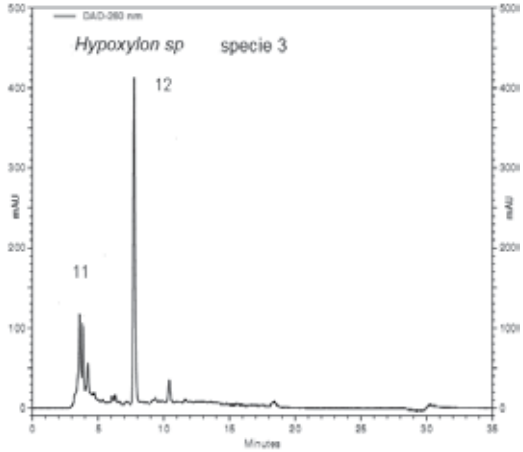
Nodulisporium sp
specie 2
5
6
mAU
Minutes


DAD-280 nm
Hypoxylon sp
specie 2
7
8
mAU
Minutes


DAD-280 nm
Nodulisporium sp
specie 3
9
10
mAU
Minutes


DAD-280 nm
Hypoxylon sp
specie 3
11
12
mAU
Minutes

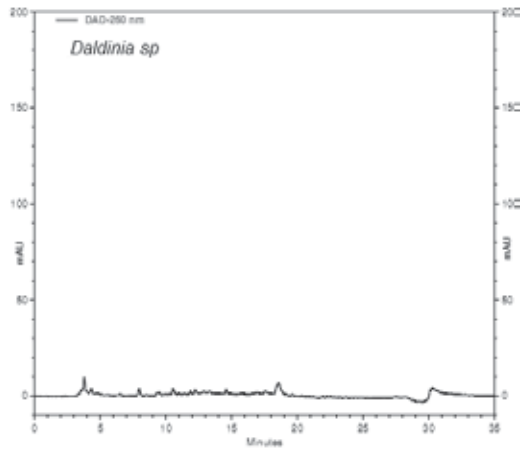
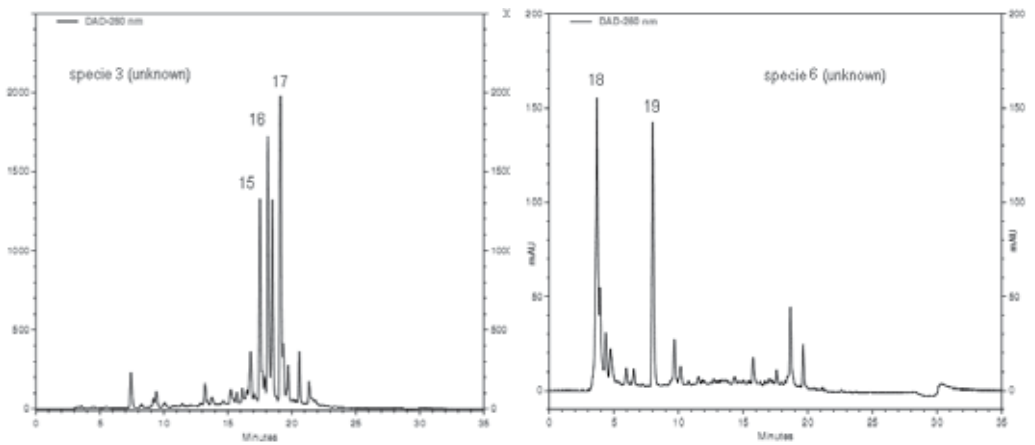


Fig. 3. HPLC-DAD chromatograms of methanolic extracts obtained from *Nodulisporium* sp., *Hypoxylon* sp. and *Daldinia* sp

The *Nodulisporium* chromatograms showed two majoritary compounds eluted in slightly different times but showed similar UV characteristics, suggesting to have the same chromophores (236 and 292 nm). The *Hypoxylon* chromatograms also showed two majoritary compounds eluted in about 3.7 and 7,8 minutes with the same UV profile (260 and 280 nm). On the basis of spectral identification it can be suggest that these compounds might be identified as phenolics.

A possible explanation for the antimicrobial activity of the methanolic extract against salmonella may be the fact that one or some of its constituents caused a significant inhibition of bacterial mobility besides ion permeability alteration on the into bacteria membrane. Antimicrobial activity of phenolic compound toward microorganism, as *salmonella*, is well documented and support this chemical investigation (Orsi et al, 2005**;** Nohynek et al, 2006). Addition studies are being performed for compounds isolation and identification.



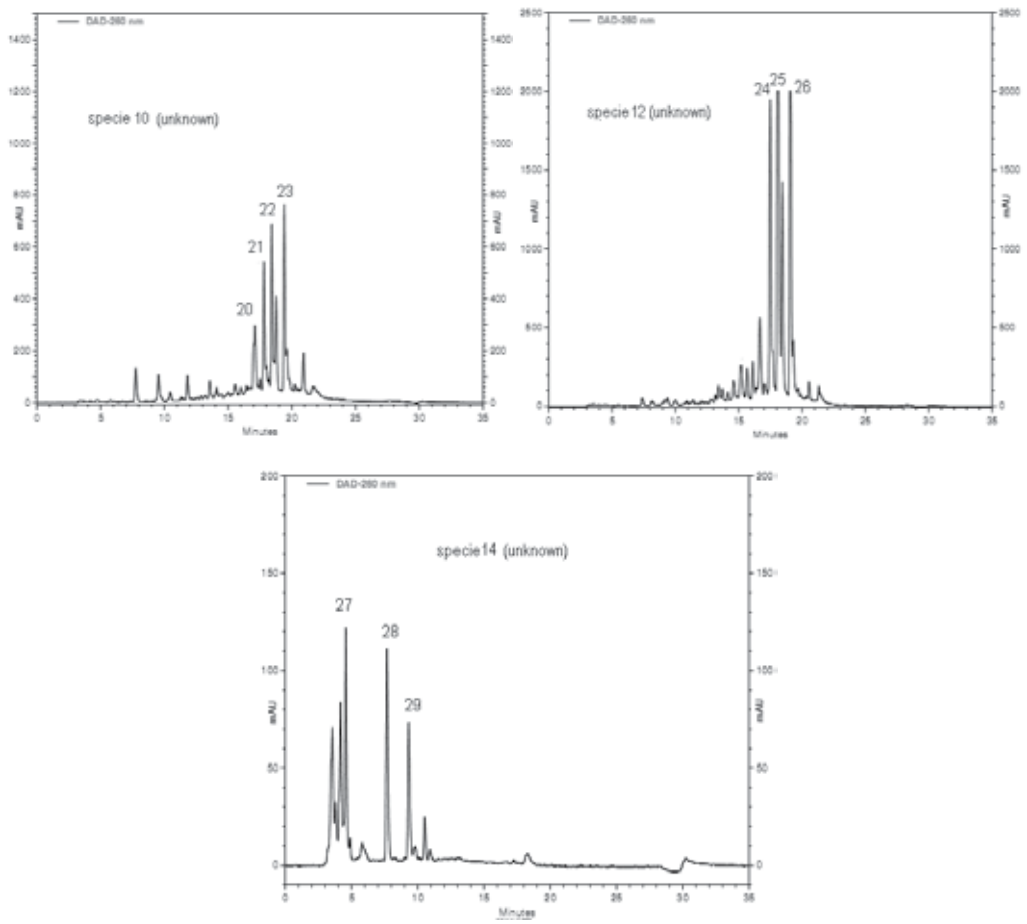


Fig. 4. HPLC-DAD chromatograms of methanolic extracts obtained from five unknown species

4. Conclusion

This work evaluated the antimicrobial activity against four *Salmonella* species of the crude extracts of endophytic fungi. Eleven fungi were isolated from *Mikania laevigata* (Asteraceae), a Brazilian medicinal plant and identified by our group and identified as being from Xylariaceae family. All the extracts of *Nodulisporium* sp., *Hypoxylon* sp., *Daldinia* sp. and unknown species showed similar antimicrobial activity. The HPLC-DAD analysis showed that extracts may contain phenolics compounds comum in others genus.

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8

Nanotechnology Tools for Efficient Antibacterial Delivery to *Salmonella*

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1. Introduction

In recent years, an increasing number of salmonellosis outbreaks have been recorded around the world, and probably there should be more cases that were not detected or reported (1). Many different types of *Salmonella* exist, some of which cause illness in both animals and people, and some types cause illness in animals but not in people. The various forms of *Salmonella* that can infect people are referred to as serotypes, which are very closely related microorganisms that share certain structural features. Some serotypes are only present in certain parts of the world (1). *Salmonella* spp are gram negative anaerobic and intracellular bacteria. Salmonellosis, mainly due to *Salmonella typhimurium*, occurs more frequently in HIV-infected patients than in healthy individuals and the frequency of bacteraemia is much higher in such patients (2).

Despite the discovery of new antibiotics, treatment of intracellular infections often fails to eradicate the pathogens completely. One major reason is that many antimicrobials are difficult to transport through cell membranes and have low activity inside the cells, thereby imposing negligible inhibitory or bactericidal effects on the intracellular bacteria (3). In addition, antimicrobial toxicity to healthy tissues poses a significant limitation to their use (3). Therefore, the delivery of the drug to the bacterial cells is currently a big challenge to the clinicians. This is on top of the problems posed by the emerging Multi-Drug Resistant species. Moreover, the reduced membrane permeability of microorganisms has been cited as a key mechanism of resistance to antibiotics (4).

Indeed, the challenge is to design the means of carrying an antibiotic into bacterial cells. The pioneer concept of targeted drugs was developed by Ehrlich in 1906 and defined as the 'magic bullet'. Since then targeted drug delivery has involved design and development of small molecule drugs that can specifically interact with the intended receptors in intended tissues. For example prodrugs can be designed for brain delivery of the active drug (5). Another common example is colon delivery of prodrugs designed to release the drug by taking advantage of the bacterial reductase enzymes in colon (6).

However, the drug development process is inevitably lengthy and breakthroughs are quite scarce which has led to the ever increasing cost of discovery and development of new drugs (7). On the other hand, nanotechnology offers a more convenient method for targeted therapy.

Logistic targeting strategies can be employed to enable the drug to be endocytosed by phagocytic cells and then released into the bacteria. To reach the above goal, a drug carrier is generally needed for a drug to arrive at the target site (8). The first study employing a drug carrier for targeted drug delivery was published approximately 40 years ago, using antibodies as carriers of radioactivity for the specific recognition of tumor cells (9). The ideal drug carrier ensures the timely release of the drug within the therapeutic window at the appropriate site, is neither toxic nor immunogenic, is biodegradable or easily excreted after action, and is preferably cheap and stable upon storage (10). Out of different types of drug carriers that have been investigated, many are soluble macromolecular carriers or liposomes (11-15).

By searching all published work on drug carriers it can be concluded that "the ideal drug carrier" does not exist. The suitability of a drug carrier is determined by the disease that will be targeted, its access to the pathological site, and the carriers' ability to achieve appropriate drug retention and timely drug release (16). When these types of formulations are administered by the intravenous route, phospholipidic, polymeric or metal particles are localized preferentially in organs with high phagocytic activity and in circulating monocytes, ensuring their clearance (8). The ability of circulating carriers to target these cells is highly dependent on tissue characteristics and on the carrier's properties. The liver rather than the spleen or bone marrow captures the submicronic particles (8). Immediately after injection, the foreign particles are subjected to opsonization by plasma proteins. This is the process by which bacteria are altered by opsonins so as to become more readily and more efficiently engulfed by phagocytes. In this way, 'classical' or 'conventional' carriers are recognized by the mononuclear phagocytic system (8).

The approaches for drug carrier to improve the drug's antibacterial efficacy are shown in Figure 1. In most cases, i.v. administration of the formulation is needed particularly for passive and active targeting.

The local administration of drug/carriers will increase the residence time of antibiotics at the site of infection (17-19). These carriers are generally investigated with the intention to treat local infections in body parts with limited blood flow as in bone, joint, skin, and cornea.

In passive targeting after i.v. administration of carriers which tend to be taken by phagocytic cells, drug-carrier complex will target intracellular infections. These infections are often difficult to treat as a result of limited ability of the antimicrobial agent to penetrate into cells. This approach makes use of the recognition of drug carriers (nanoparticles) as foreign material in the bloodstream by the phagocytic cells of the mononuclear phagocyte system, the cell type often infected with microorganisms (20, 21).

Regarding the other two approaches (passive targeting with long-circulation time, and active targeting) the targeting of infectious foci is not restricted to mononuclear phagocyte system tissues. In passive targeting a drug carrier with long duration of circulation is used and this is an area which has extensively been investigated, whereas in active targeting carriers specifically bind to the infectious organism or host cells involved in the inflammatory response.

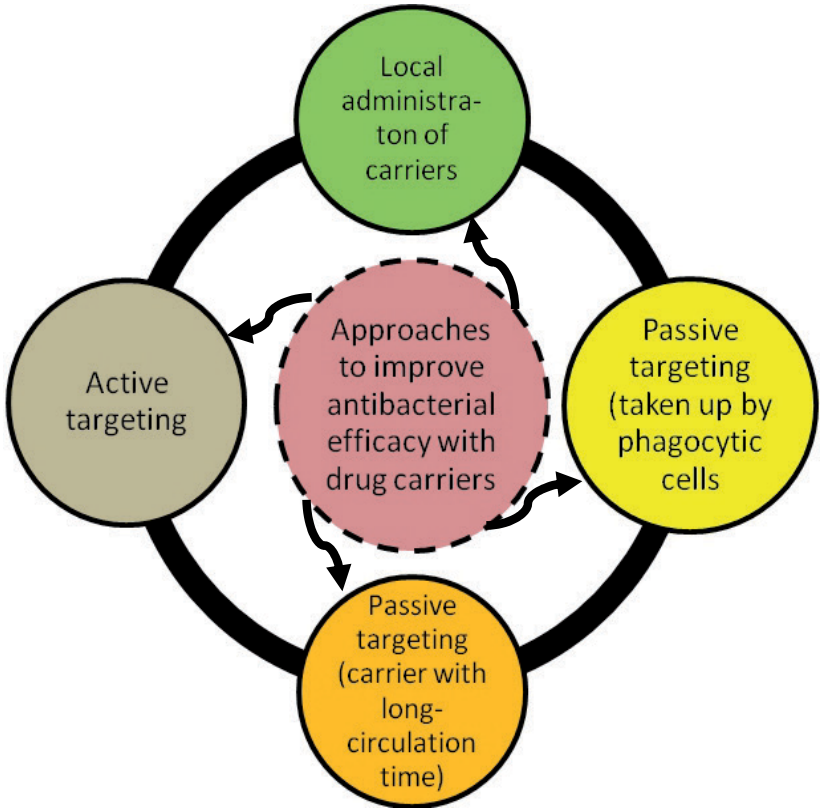


Fig. 1. Drug carrier approaches targeting bacterial infections to improve antibacterial efficacy of drugs.

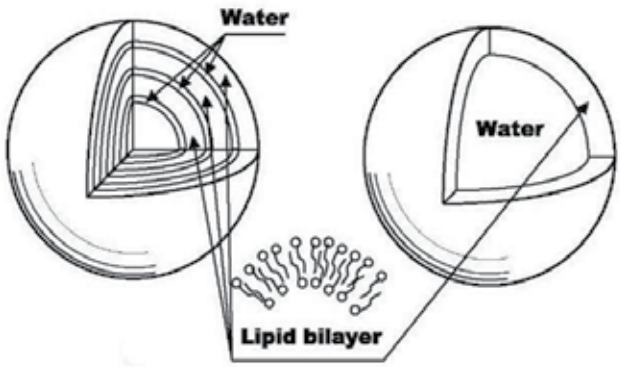
This chapter focuses mainly on the current research for increasing anti-*salmonella* performance of antibiotics by means of liposomes and nanoparticle systems. Structure, properties, advantages and disadvantages of these drug delivery systems have been discussed. It is clear that such systems may improve the antibiotic efficacy by increasing the drug concentration at the surrounding of the bacteria.

2. Liposomes for antisalmonellosis drug delivery

2.1 Introduction

Liposomes are composed of small vesicles of a bilayer of phospholipid, encapsulating an aqueous space ranging from about 30 to 10000 nm in diameter (Figure 2). They are composed of one or several lipid membranes enclosing discrete aqueous compartments. The enclosed vesicles can encapsulate water-soluble drugs in the aqueous spaces, and lipid soluble drugs can be incorporated into the membranes. They are used as drug carriers in the cosmetic and pharmaceutical industry. The main routes of liposome administration are parenteral, topical and inhalation, and, in a few occasions, possibly other routes of administration can be used. Majority of current products are administered parenterally (22).

Liposome structure was first described in 1965, and they were proposed as a drug delivery nanoparticle platform in 1970s. In 1995, Doxil (doxorubicin liposomes) became the first liposomal delivery system approved by the Food and Drug Administration (FDA) to treat AIDS associated Kaposi's sarcoma (23). Liposomal drug delivery systems can be made of either natural or synthetic lipids. The main building blocks of some liposomal formulations are phospholipids (22). These are natural biomacromolecules that play a central role in human physiology as they are structural components of biological membranes and support organisms with the energy (24). They are amphiphilic molecules, poorly soluble in water, consisting of a hydrophilic part containing hydroxyl groups (the polar head), a glycerol backbone and two fatty acid chains, which form the hydrophobic part. One of the most commonly used lipids in liposome preparation is phosphotidylcholine, which is an electrically neutral phospholipid that contains fatty acyl chains of varying degrees of saturation and length. Cholesterol is normally incorporated into the formulation to adjust membrane rigidity and stability (8). Liposomes can be characterized in terms of size and lamellarity as small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) and multi lamellar vesicles (MLV). MLVs are usually considered large vesicles and aqueous regions exist in the core and in the spaces between their bilayers. The structure of these liposomes is shown in Figure 2.



(a) (b)

Fig. 2. Schematic structures of (a) multilamellar and (b) unilamellar liposomes (the picture was taken from http://what-when-how.com/nanoscience-and-nanotechnology/nanoencapsulation-of-bioactive-substances-part-1-nanotechnology).

The main advantages of liposomes as drug delivery systems can be in their versatile structure that can be easily modified according to experimental needs; they can also encapsulate hydrophilic drugs in their aqueous compartments and hydrophobic drugs in their bilayers, while amphiphilic drugs will be partitioned between the two. Moreover, being mainly made of phospholipid, they are non-toxic, non-immunogenic and fully biodegradable. Methods for preparing liposomes can take into consideration parameters such as the physicochemical characteristics of the liposomal ingredients, materials to be contained within the liposomes, particle size, polydispersity, surface zeta potential, shelf time, batch-to-batch reproducibility, and the possibility for large-scale production of safe and efficient products (23).

2.2 Preparation of liposoms

Liposome formation happens spontaneously when phospholipids are dispersed in water. However, in order to obtain the desired formulation with particular size and structure, various methods such as thin film method (24), sonication (25), extrusion (26), injection methods (27), dehydrated-rehydrated vesicles (28), reverse phase evaporation (29) and one step method (30) have to be used.

Each technique is briefly described below, but for more details, it is recommended to refer to the cited references. In brief, in thin film method liquids are dissolved in organic solvents and the solvent is removed under vacuum or nitrogen stream to form a thin film on the wall of a flask or test tube. In order to complete the formation of liposomes aqueous phase is added to the lipid film at a temperature above the phase transition of the lipid (24).

The sonication method is usually used to reduce the particle size and lamellarity of MLVs. In case of using the probe sonicator, the reduction in size of the liposomes can be guaranteed (25).

In order to get very homogeneous vesicles with a predetermined size, the extrusion technique is used. MLVs are extruded under pressure through particular filter with well-defined pore sizes from 30 nm to several micrometers. If the extrusion is repeated several times unilamellar liposomes can be formed (26).

Very small unilamellar vesicles with a particle size of 30 nm can be prepared using the ethanol injection method. Generally, lipids are dissolved in ethanol and injected rapidly into the aqueous solution, under stirring. At the end, the injected ethanol has to be removed from the system (27).

As dehydrated-rehydrated vesicles are able to hold high amounts of hydrophilic drugs under mild conditions, therefore this method is suitable for the drugs that are losing their activity under harsh conditions (28). Empty liposomes, usually unilamellar vesicles, are disrupted during a freeze drying step in the presence of the drug meant to be encapsulated. A controlled rehydration is obtained in the presence of concentrated solution of the drug. This technique can produce large oligolamellar liposomes of a size around 400 nm to several micrometers. It has been shown that in case of producing smaller liposomes (100-200 nm) sucrose can be added (31).

In the reverse phase evaporation technique which is similar to thin film technique, lipids are dissolved in organic solvent and the solvent is removed by evaporation (29). The thin film is resuspended in diethyl ether followed by the addition of third of water and the suspension is sonicated in a bath sonicator. The emulsion is evaporated until a gel is formed and finally the gel is broken by the addition of water under agitation. The traces of organic solvent should be removed by evaporation (29).

Finally, in the one-step method, lipid dispersion should be hydrated at high temperatures under nitrogen gas stream. This method has the capability to produce liposomes in the range of 200-500 nm (30).

2.3 Targeted delivery by liposomes

The main methods of delivery from liposome to cytoplasm include the exchange of membrane and lipids, contact release, adsorption, fusion and endocytosis. Through these

processes, drugs can be released into the bacterial or eukaryotic cells. Liposomal formulations have been used for the delivery of antitumor anthracyclines such as doxorubicin (23) and antifungal agent amphotericin B. Targeted delivery of liposomes to tumor cells has been explored through arsenoliposomes (32). Liposomes for antibacterial chemotherapy are under intensive research to enhance the antibacterial activity and improve pharmacokinetic properties. Advantages of liposomal antibiotics include improved pharmacokinetics, decreased toxicity, enhanced activity against intracellular pathogens, target selectivity and as a tool to overcome bacterial drug resistance (3).

Some liposomes are unique because they can be selectively absorbed by tissues rich in reticuloendothelial cells, such as the liver, spleen and bone marrow. This can serve as a targeting mechanism, but it also removes liposomes from the circulation rather rapidly. Although the poor stability of liposomes, particularly the rapid uptake from the body is not desirable, it could be useful for eradicating the infection by 'passive targeting' through macrophage activation and killing or elimination of parasitic infections.

On the other hand, surface charge and phospholipid composition can affect the interactions of liposomes with bacterial cell surface. For example it has been shown that cationic liposome formulations are more efficient in binding to skin bacterial cells (33).

Moreover, by attaching targeting ligands such as immunoglobulines (34), antibody segments, aptamer (35), peptides and small molecule ligands, and oligosaccharide chains (36), to the surface of the liposomes, they can selectively bind to microorganisms or infected cells and then release the drug payloads to kill or inhibit the growth of the microorganisms (23). The highly specific liposomes are those containing antibodies or immunoglobulin fragments which have affinity to specific receptors on the surface of the infected tissue cells or pathogens (3).

Biofilm surface characteristics have also been used for targeted delivery. Biofilms are microbial aggregations that are covered in an extracellular matrix of polymeric substances. The matrix is usually composed of complex mixture of oligomeric and polymeric molecules such as proteins, lipids and polysaccharides which, as Microbial Associated Molecular Patterns (MAMPs), elicit host defenses (37). Pathogens are much more difficult to control when living in biofilms. This is partly due to the matrix preventing drug transport to the microbial cells. Moreover, bacteria in biofilms grow slower and have reduced metabolic activity, and therefore they are expected to be less susceptible to the antibiotics (38). Currently a great deal of research is focused on exploring new chemotherapeutic targets in biofilms (37). On the other hand liposomes have proven efficient in targeting and eradication of various types of biofilms. Examples are immunoliposomes with high affinity to various oral bacteria including Streptococcus oralis (34) and polysaccharide-coated liposomes for the efficient delivery of metronidazol to periodontal pocket biofilm (39).

pH-sensitive liposomes offer another method for targeting and efficiently delivering the liposomal content into cytoplasm. Such liposomes are stable at physiological pH but undergo destabilization under acidic conditions. Therefore, they are able to promote fusion of target plasma or endosomal membranes, the so called 'fusogenic' properties, at acidic pH (40). Several mechanisms can trigger pH-sensitivity in liposomes. One of the most widely used methods is the use of a combination of phosphatidylethanolamine (PE) or its derivatives with compounds containing an acidic group that act as a stabilizer at neutral pH (41). Other more recent methods include the use of novel pH-sensitive lipids, synthetic

fusogenic peptides/proteins (42) and association of pH-sensitive polymers with liposomes (43). pH-sensitive liposomes have found applications in many therapeutic area including the antibiotic delivery to intracellular infections (44).

2.4 Pharmacokinetics consideration of liposomal drug delivery

Liposomal carriers can lead to sustained release of antibiotics during drug circulation in the body. Thus, appropriate levels of drug will be available for a longer duration in comparison with the conventional antibiotic formulations where the outcome is a quick and short effect (45). However, conventional liposomes are quickly opsonized after intravenous administration and therefore they are taken up by the mononuclear phagocyte as foreign antigens. As a consequence blood circulation time is lowered. By controlling the physicochemical properties of the vesicles (size and charge distribution, membrane permeability, tendency for aggregation or fusion, drug encapsulation efficiency, membrane rigidity) and therefore their interaction with the biological environment, many different types of liposomes with the aim of obtaining longer circulation half-lives can be developed (8).

The plasma circulation time of antibiotics can be improved by encapsulation in polyethylene glycol-coated (pegylated) (STEALTH) liposomes. The PEG coating forms a hydration layer that retards the reticuloendothelial system recognitions of liposomes through sterically inhibiting hydrophobic and electrostatic interactions with plasma proteins (46). Other methods that can confer hydrophilicity or steric repulsion are by the use of compounds having sialic residues, or through MLVs containing phospholipids with long saturated chains and negative surface charge (47). The increased half lives of stealth liposomes increase their ability to leave the vascular system into some extravascular regions.

2.5 Antibiotic loaded liposomes against *Salmonella* spp

One of the distinguishing features of liposomes is their lipid bilayer structure, which mimics cell membranes and can readily fuse with the cell membrane and deliver the antibiotic contents into the cellular cytoplasm. As a result, drug delivery may be improved to bacterial and eukaryotic cells alike. By directly fusing with bacterial membranes, the drug payloads of liposomes can be released into the cell membranes or to the interior of the bacteria. In terms of extracellular pathogens, improved antibiotic delivery into the bacterial cells is of particular importance especially since it can interfere with some of the bacterial drug-resistance mechanisms which involve low permeability of the outer membrane or efflux systems (48).

Liposomes are particularly successful in eradicating intracellular pathogens. Examples of these include liposomal formulations of antituberculosis agents isoniazid and rifampin (49), and ampicillin loaded liposomes for eradication of Listeria monocytogenes (50). This is partly due to improved drug retention in the infected tissue and the decreased toxicity as a result of sustained release of drug from liposomes. Moreover, liposomal formulations often have improved antibiotic pharmacokinetics with extended circulation time and prolonged tissue retention.

Liposomal chemotherapeutics for the treatment of salmonellosis may employ some of the conventional antibiotics with proven inhibitory or cidal activity *in vitro*. Bacterial gastro-intestinal infections with *Salmonella typhi* may be treated with chloramphenicol. Alternatives to

chloramphenicol include amoxicillin, co-trimoxazole and trimethoprim (51). Recently treatment with cephalosporins and fluoroquinolones has become popular, as several members of these antibiotic families have been shown to be effective. The treatment of paratyphoid fever is the same as that for typhoid (51). *Salmonella* food-poisoning is self-limiting and does not require antibiotic therapy, unless the patient is severly ill or blood cultures indicate systemic infection. In this case, third generation cephalosporins or fluoroquinolones are the most reliable agents (51). Ceftriaxone or a first generation fluoroquinolone such as ciprofloxacin, ofloxacin or pefloxacin but not norfloxacin have been recommended as the first choice in typhoid and paratyphoid by The Sanford Guide to Antimicrobial Therapy (52). The improved efficiency of liposome formulations of antibiotics has been shown *in vitro* and *in vivo*. The *in vitro* infection models utilize macrophages infected with *salmonella*.

2.5.1 Penicillin loaded liposomes

The tissue distribution of ampicillin loaded liposomes was studied in normal noninfected mice and showed that ampicillin concentrated mostly in the liver and spleen (53). The Liposome formulation of ampicillin was significantly more effective than free ampicillin in reducing mortality in acutely infected mice with *Salmonella typhimurium* C5. These liposomes were quite efficient in targeting ampicillin to the spleen but were less effective in targeting ampicillin to the liver and reducing mortality in acute salmonellosis (53).

2.5.2 Cephalosporine loaded liposomes

Third generation cephalosporines have been indicated as suitable candidates for the treatment of *Salmonella* infections (52). Liposome formulations of these antibiotics may improve pharmacokinetics and also the targeted delivery to the intracellular infections. In a study with cephalotin, treatment of infected macrophages with multilamellar liposome-encapsulated cephalothin enhanced the intraphagocytic killing of *Salmonella typhimurium* over that by macrophages treated with free cephalothin (54). Resident murine peritoneal macrophages were shown to be capable of interiorizing the liposome-antibiotic complex leading to a relatively high intracellular concentration of cephalothin. The intracellular killing of the bacteria was maximal at 60 min of incubation; at this time, 60% of the interiorized organisms had been killed (54).

Desiderio & Campbell infected mice with *Salmonella typhimurium* to investigate the effectiveness of liposome-encapsulated cephalothin treatment (55). In the study they also compared the results with formulations containing free cephalothin. They showed that following intravenous administration, liposome-encapsulated cephalothin was cleared from the circulation more rapidly and concentrated in the liver and spleen. Treatment of infected mice with the liposome antibiotic complex was more efficacious in terms of reducing the number of *Salmonella typhimurium* in these organs compared to the injection of free antibiotic, although treatment did not completely eliminate the bacteria from this site (55).

Another study showed that egg phosphatidylcholine liposomes containing cephapirin were relatively stable in serum, and provided acceptable serum levels of cephapirin for 24 hr after i.v. administration while free drug at a similar dosage was undetectable in 3-5 hr. Moreover, the liposome formulation, as opposed to the free drug, could be used successfully for prophylaxis. Cephapirin activity in the spleen and liver was greatly increased and persisted

for at least 24 hr when iv injections of the liposome formulation was used. This formulation of liposome, in contrast with the other liposome formulation containing tris salt of cholesterol hemisuccinate, could prolong survival in mice infected with *Salmonella typhimurium* (56).

Ceftiofur sodium is a third generation broad spectrum cephalosporin widely used clinically to treat respiratory diseases and mastitis. Its spectrum also covers *Salmonella* spp. The liposome formulations of ceftiofur were prepared in order to increase drug half life *in vivo* for veterinary purposes (57). The pharmacokinetic study in healthy cows showed that liposome preparations provided therapeutically effective plasma concentrations for a longer duration (elimination half life of more than double) than with the drug alone. These liposomes were stable and the minimum inhibitory concentrations against *Salmonella enteritidis* were 1⁄4th that of free ceftiofur sodium (57).

2.5.3 Aminoglycoside loaded liposomes

Despite the susceptibility of *Salmonella* spp to aminoglycosides, their use against many important intracellular bacterial infections has been limited due to the cell membrane permeability problems. Lutwyche et al. prepared several liposomal encapsulation formulations including pH-sensitive DOPE-based carrier systems containing gentamicin in order to achieve intracellular antibiotic delivery and therefore increase the drug's therapeutic activity against intracellular pathogens (58). They reported the superiority of some of the pH-sensitive liposomes over conventional liposome formulations, which was associated with the intracellular delivery of the antibiotic and was dependent on endosomal acidification. This liposomal carrier demonstrated pH-sensitive fusion that was dependent on the presence of unsaturated phosphatidylethanolamine (PE) and the pH-sensitive lipid N-succinyldioleoyl-PE. These formulations also efficiently eliminated intracellular infections caused by a recombinant hemolysin-expressing *Salmonella typhimurium* strain which escape the vacuole and reside in the cytoplasm. Moreover, *in vivo* pharmacokinetics and biodistribution tests confirmed that encapsulation of gentamicin in pH-sensitive liposomes significantly increased the concentrations of the drug in plasma compared to those of free gentamicin. Furthermore, liposomal encapsulation increased the levels of accumulation of drug in the infected liver and spleen by 153- and 437-folds, respectively (59).

Other investigations have indicated that even with conventional liposomes, liposome encapsulated gentamicin is less toxic in mice than is free gentamicin and is extremely effective-therapy for disseminated *Salmonella* infections in mice. For example when gentamicin sulfate was encapsulated in liposomes composed solely of egg phosphatidylcholine, the mean half-lives of the encapsulated drug in serum were around four times that of free (nonencapsulated) gentamicin in mice and rats following i.v. administration. Moreover, liposome encapsulation led to higher and more prolonged activity in organs rich in reticuloendothelial cells especially in spleen and liver. In acute septicemia infections in mice, the liposomal formulation showed enhanced prophylactic activity when compared with the free drug. In a model of murine salmonellosis, liposomal gentamicin greatly enhanced the survival rate (60). Similarly, a single iv injection of low dose gentamicin loaded multilamellar liposomes (composed of egg phosphatidylcholine, egg phosphatidylglycerol, cholesterol and alpha-tocopherol) resulted in 80% survival of mice infected with *Salmonella Dublin,* while zero survival was observed when treated with the same amount of free gentamicin. Higher concentrations of free gentamicin led to

neuromuscular paralysis, while the slow release of this dose from liposomes increased the survival rate to 100%. After the single dose treatments with liposomes, high concentrations of the drug were detectable for 10 days (61). The liposome-encapsulated gentamicin has also been proven successful in the treatment of Mycobacterium Avium-M intracellular complex (MAC) bacteremia in AIDS patients. In this case, MAC colony counts in blood fell by 75% or more when given intravenously twice weekly for 4 weeks (62).

Another effective antibiotic for liposomal formulation which attracted the interest of researchers is streptomycin. Conventional liposomal formulation of streptomycin made with egg yolk phosphatidylcholine was investigated using *in vivo* model of *Salmonella* infection in mice. Liposome-entrapped streptomycin prolonged the survival to more than 15 days for all mice infected with the virulent strain of *Salmonella enteritidis,* while treatment with the same dose of free streptomycin resulted in all of the mice dying between days 5 and 7. The prolongation of survival was due to suppression of the multiplication of *S. enteritidis.* Furthermore, the liposome-entrapped drug was less toxic than the free drug when applied at high doses. A tissue distribution study in various organs demonstrated that liposomal streptomycin was selectively accumulated in the spleen and liver with concentrations in these organs about 100 times higher than those in mice receiving the free drug (63).

In contrast to this, another investigation on *S. enteritidis* indicated a less concentration of streptomycin administered using some of the liposome formulations in the liver and spleen in comparison with the free drug (9). In this study, several formulations of streptomycin sulfate liposomes, prepared from a mixture of L-a-dipalmitoy phosphatidyl choline (DPPC) and cholesterol with or without a charge inducing agent, were used in drug targeting experiments using Swiss mice. The biodistribution results indicated that although, in comparison with the free drug, some of the liposome formulations exhibited 2-3 times higher concentration of streptomycin in the liver and spleen, this effect decreased over time from one to seven days. Despite this, the survival rate experiments indicated a definite protection against *Salmonella enteritidis* exhibited by the liposome-encapsulated streptomycin compared to the free drug (64). Therefore, it seems that the liposome formulation plays the major role in the targeting effect and the delivery efficiency of the liposomes for intracellular infections.

2.5.4 Fluoroquinolone loaded liposomes

Ciprofloxacin is a synthetic bactericidal fluoroquinolone which inhibits the activity of bacterial DNA gyrase, resulting in the degradation of bacterial DNA by exonuclease activity. Consequently, ciprofloxacin has broad-spectrum efficacy against a wide variety of bacteria, including the family *Enterobacteriaceae* of which *Salmonella* spp is a member of (65). It has been used in the treatment of individuals with *Salmonella* infections, including those with typhoid fever and chronic typhoid carriers (52). Despite the enormous success with ciprofloxacin, there are some factors which limit the drug's clinical utility, such as its poor solubility at physiological pH and rapid renal clearance. Several investigations have focused on the formulation of this drug as liposomes, in order to improve the drug delivery.

Ciprofloxacin loaded liposomes, consisting of dipalmitoyl-phosphatidylcholine, dipalmitoyl-phosphatidylglycerol and cholesterol, were used to treat *Salmonella Dublin* infected mice (66). It has been reported that a single injection of liposome formulation was 10 times more effective than a single injection of free drug at preventing mortality. Treatment with liposomal ciprofloxacin produced dose-dependent decreases in bacterial

counts in spleen, stool, and Peyer's patches, indicating that the drug had distributed to all areas of inflammation, not just to the major reticuloendothelial system organs. Although liposome formulation was cleared rapidly from the blood, drug persisted in the liver and spleen for at least 48 h after administration of a dose (66).

In a similar study, Webb et al. encapsulated ciprofloxacin into large unilamellar liposomes. The LUVs composed of dipalmitoylphosphatidylcholine-cholesterol, distearoylphosphatidylcholine-cholesterol, or sphingomyelin-cholesterol. In comparison with the free drug, the liposomal formulations increased the circulation lifetime of the drug by >15 fold and resulted in 10^3 to 10^4 fold fewer viable *Salmonella typhimurium* in the livers and spleens after intravenous administration (67). These results show the utility of liposomal encapsulation in improving the pharmacokinetics, biodistribution, and antibacterial efficacy of ciprofloxacin.

3. Polymeric nanoparticles for antisalmonellosis drug delivery

3.1 Introduction

Nanoparticles (NP) are solid colloidal particles with particle sizes smaller than 1000 nm. However, most nanoparticles utilized in drug delivery are in the size range of 100–200 nm. Nanoparticles can be classified into two main subgroups: nanospheres and nanocapsules. Nanospheres have a matrix-type structure, and drug molecules can be adsorbed on their surface or entrapped inside their matrix. Nanocapsules have a capsule-like structure and possess the capability of encapsulating the drug molecules inside the capsule or adsorbed to them externally. Because these systems have unique characteristics, such as very small particle size, high surface area, and possibility of surface modification, they have been attracting much interest for drug-delivery purposes during recent years. Nanoparticles are able to adsorb and/or encapsulate a drug, thus protecting it against chemical and enzymatic degradation. Generally, the drug is dissolved, entrapped, encapsulated or attached to a NP matrix and depending upon the method of preparation, nanoparticles, nanospheres or nanocapsules can be obtained. Owing to their polymeric nature, nanoparticles (Figure 3) may be more stable than liposomes in biological fluids and during storage.

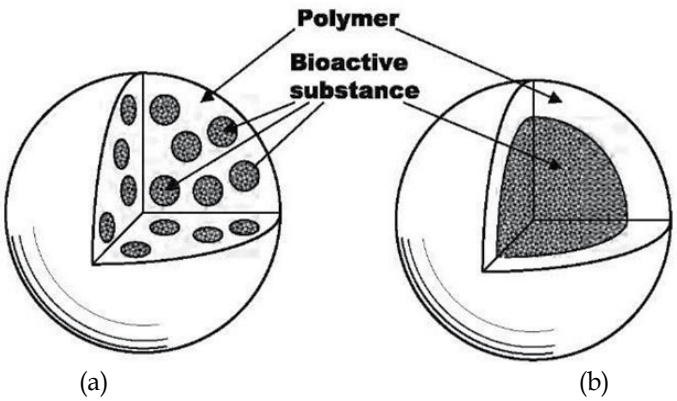


Fig. 3. Schematic structures of (a) nanosphere and (b) nanocapsule type nanoparticles (the picture was taken from http://what-when-how.com/nanoscience-and-nanotechnology/nanoencapsulation-of-bioactive-substances-part-1-nanotechnology).

Nanocapsules are vesicular systems in which the drug is confined to a cavity surrounded by a unique polymer membrane, while nanospheres are matrix systems in which the drug is physically and uniformly dispersed. In order for nanoparticles to minimize the side effects, the polymers associated with nanoparticles must be degraded *in vivo* due to intracellular polymeric overloading. Thus in recent years, biodegradable polymeric nanoparticles have attracted considerable attention as potential drug delivery devices in view of their applications in the controlled release of drugs, their ability to target particular organs, as carriers for DNA in gene therapy, and their ability to deliver proteins, peptides and genes through a peroral route of administration (68). Most polymers PLGA, chitosan, gelatin, alginate, and poly cyanoacrylate can be used in the formulation of nanoparticles.

It is believed that nanoparticles could be effective in increasing drug accumulation at the site of infection with reduced toxicity and side effects after parenteral or oral administration (69, 70). Polymeric nanoparticles have been explored to deliver a variety of antimicrobial agents to treat various infectious diseases and have shown great therapeutic efficacy (71).

3.2 Antibiotic loaded cyanoacrylate nanoparticles

The polymers involved in nanoparticle structure should be degraded in order to release the drug, therefore, there should be a direct correlation between the rate of degradation and the drug release rate. If degradation happens in the presence of esterase, it was shown that the degradation of the polymer in esterase-free medium is low, therefore, the drug release rate is low accordingly. The drug release was increased when the medium contained carboxyesterase (72).

The *in vitro* interaction between [^{3}H]ampicillin-loaded polyisohexylcyanoacrylate nanoparticles and murine macrophages infected with *Salmonella typhimurium* was investigated and the results showed that the uptake of nanoparticle-bound [^{3}H]ampicillin by non-infected macrophages was six- and 24-fold greater respectively compared to free [^{3}H]ampicillin. However, there was no difference between nanoparticle-bound ampicillin and free ampicillin in terms of bactericidal activity against intracellular *Salmonella typhimurium*. This unexpected observation might be accounted for by bacterium-induced inhibition of phagosome-lyosome fusion within the macrophages, thereby preventing contact between the bacteria in the phagosomes and the nanoparticles in the secondary lysosomes (73).

In another study the intracellular distribution of (^{3}H)ampicillin-loaded polyisohexylcyanoacrylate nanoparticles in the same cells using ultrastructural autoradiography was investigated by the same authors (74). Ampicillin penetration and retention into the cells obviously increased by means of nanoparticles. After 2-4 h treatment with the nanoparticle formulation, numerous intracellular bacteria were seen to be in the process of destruction. After 12 h treatment, numerous spherical bodies and larger forms were seen in the vacuoles and it was an indication of marked damaging action of the ampicillin on the bacterial walls. The targeting of ampicillin therefore allowed its penetration into the macrophages and vacuoles infected with *Salmonella typhimurium* (74).

Pinto-Alphandary et al. used transmission electron microscopy to prove that ampicillin which usually penetrates into cells at a low level is directly carried in when loaded on nanoparticles, and brought into contact with intracellular bacteria (75). They concluded that ampicillin loaded polyisohexylcyanoacrylate nanoparticles is an ideal formulation when an intracellular targeting for ampicillin is needed.

Page-Clisson et al. (76) investigated the antibacterial efficiency of polyalkylcyanoacrylate nanoparticles loaded with ciprofloxacin and ampicillin against *Salmonella typhimurium.* It was shown that i*n vivo* treatment with ciprofloxacin led to a significant decrease of bacterial counts in the liver whatever the stage of infection and the form used. However, none of the treatments were able to sterilize the spleen or the liver (76).

Ampicillin was also attached to nanoparticles of polyisohexylcyanoacrylate (PIHCA) for the treatment of C57BL/6 mice experimentally infected with *Salmonella typhimurium* C5. The injection of the nanoparticles containing ampicillin treated all mice, whereas by the injection of non-loaded nanoparticles all mice died within 10 days (77).

3.3 Antibiotic loaded PLGA nanoparticles

Some polymeric nanoparticles may be more effective than liposomes in acute salmonellosis model due to better stability of nanoparticles in serum compared to liposomes. Therefore it is believed that antibiotic loaded nanoparticles can improve the targeting, particularly in the case of intracellular bacteria. For example, gentammicin (78), azithromicin and clarithromicin loaded nanoparticles using poly(lactide-co-glycolide) [PLGA] (79, 80) were more effective than corresponding intact drug against *Salmonella typhimurium.*

As mentioned before, nanoparticles should be degraded *in vivo* to avoid side effects and it has been shown that PLGA nanoparticles fulfill such requirements. Therefore, in most cases for antibiotics such as rifampcin (81), amphotericin (82), azithromycin (79) and clarithromycin (80) PLGA nanoparticle preparations have been recomended.

Mohammadi *et al*., showed that azithromycin and clarithromycin-loaded (PLGA) nanoparticles (NPs) prepared with three different ratios of drug to polymer have better antibacterial activity against *Salmonella typhi* (79). In other words, the nanoparticles were more effective than pure azithromycin and clarithromycin against *Salmonella typhi* and *S. aureus,* respectively, with the nanoparticles showing equal antibacterial effect at 1/8 concentration of the intact drug. Both studies on azithromycin and clarithromycin proved that the antibacterial activity of nanoparticles were about 8-fold more than the free azithromycine and clarithromycin (Figure 4). The higher antibacterial effect of clarithromycin and azithromycin may have resulted from higher bacterial adhesion of the nanoparticles. For example, an adhesion of Eudragit nanoparticles containing PLGA to the *S. aureus* bacteria was reported (83). Although, Figure 4 shows that the ratio of drug:PLGA has no significant effect on antibacterial activity of azithromycin and clarithromycin, Table 1 shows that the particle size of nanoparticles, their zeta potential and the encapsulation efficiency are remarkably dependent on the ratio of drug:polymer used in the formulations. This indicates that by controlling the ratio of drug:carrier the desirable particle size and zeta potential could be achieved. As it is shown in Figure 5 all nanoparticles were spherical in appearance.

Several investigations have shown that nanoparticles could not be very effective on all different types of bacteria and that the antibacterial effect depends on bacterial type (84). For example, recently Martins et al. evaluated the antibacterial activity of PLGA nanoparticles containing violacein against different bacteria (84). Although, they showed that the MIC with nanoparticles is 2-5 times lower than free violacein against *Staphylococcus aureus*, the results failed to show any significant activity against *Escherichia coli* and *Salmonella enterica*.

Formulations	Encapsulation efficiency (%)	Mean particle size (nm)	Zeta potential (mV)
AZI:PLGA (1:1)	50.5 ± 3.4	252 ± 5	-5.6 ± 2.15
AZI:PLGA (1:2)	66.8 ± 2.8	230 ± 7	-11.10 ± 1.87
AZI:PLGA (1:3)	78.5 ± 4.2	212 ± 4	-15.56 ± 2.53
CLR:PLGA (1:1)	57.4 ± 4.3	280 ± 15	-6.3 ± 1.70
CLR:PLGA (1:2)	72.9 ± 3.2	223 ± 12	-10.08 ± 1.63
CLR:PLGA (1:3)	80.2 ± 4.0	189 ± 10	-14.26 ± 1.92

Table 1. Encapsulation efficiency, mean particle size and zeta potential of various formulations containing Azithromycine and clarithromycin (data taken from references 79, 80)

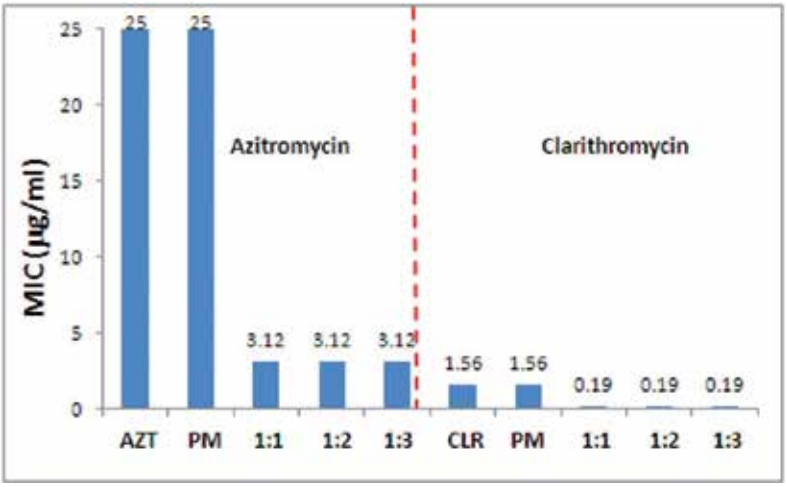
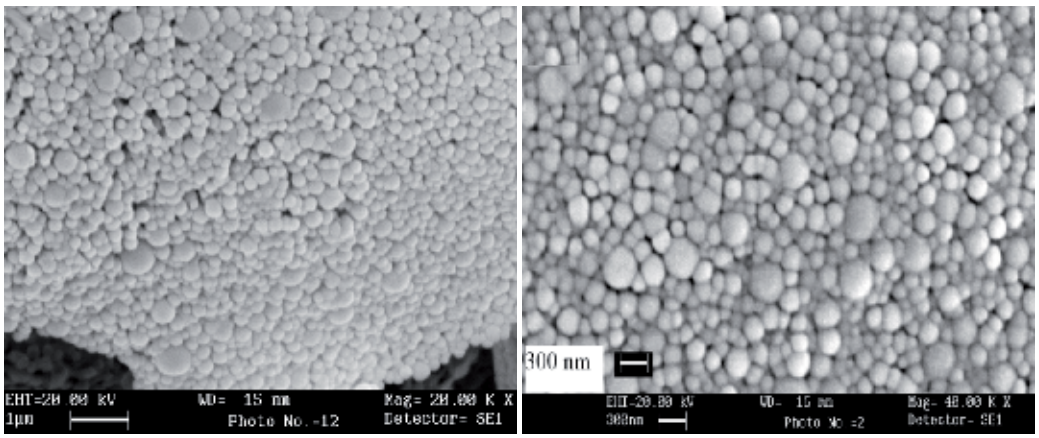


Fig. 4. Minimum inhibitory concentrations (MICs) of the intact AZT, CLR, physical mixtures (PM) and drug-loaded nanoparticles suspensions with different drug:PLGA ratios (data are reproduced from references 79, 80).



(a) (b)

Fig. 5. SEM images of clarithromycin and azithromycin-loaded nanoparticles with the ratio of drug:PLGA 1:2 (SEM taken from ref. 80).

3.4 High loading antibiotic nanoparticles

One of the problems with antibiotic loaded nanoparticles is that in some cases the capacity of a polymeric drug carrier should be engineered to incorporate high concentrations of antibiotics to achieve the required dosage, yet avoid side effects that may be associated with higher amounts of carriers. This seems a difficult task, however, Ranjan *et al* introduced two novel technologies by which high concentrations of gentamicin could be incorporated into the formulations (85).

In the first technology, Ranjan *et al*., made an attempt to enhance antibacterial efficacy of gentamicin using a new technology called core-shell nanostructures (78). In this research pluronic based core-shell nanostructures encapsulating gentamicin were prepared. The maximum antibiotic loading was 20% in their formulation with a zeta potential of -0.7. It was shown that when using core-shell nanostructures containing gentamicin, not only that significant reduction in toxicity and side effects was evident, but also the percentage of viable bacteria in the liver and spleen was significantly reduced (78).

In the second technology, Ranjan et al (85) incorporated gentamicin into macromolecular complexes with anionic homo- and block-copolymers via cooperative electrostatic interactions between cationic drugs and anionic polymers (Figure 6). They showed the possibility of incorporating 26% by weight of gentamicin in the nanoplexes with average diameter of 120 nm and zeta potential of -17 (85). This was 6% more drug loading compared to their previous study. Their study showed that in addition to the high loading of drug carried by these polymeric nanoplexes, the nanoplexes can potentially improve targeting of interacellular pathogens such as *salmonella*.

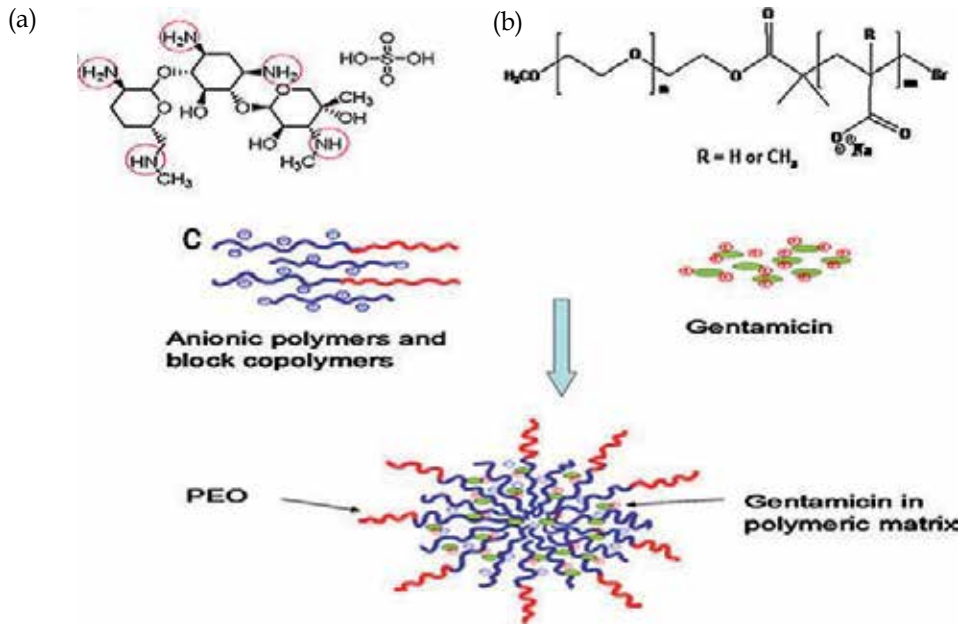


Fig. 6. (a) Gentamicin is cationic aminoglycoside antibiotic with five amino groups, (b) anionic block copolymers for electrostatic complexation to gentamicin, (c) strategy to incorporate gentamicin within polymeric nanoplex (Figure was taken from ref 85).

3.5 Xerogel systems containing antibiotic

During the last fifteen years, a special attention has been dedicated on silica xerogel system to treat diseases due to intracellular pathogens (86-92). The properties of silica xerogel systems such as size, zeta potential, pore structure, and the surface characteristics make them suitable carriers for therapeutics to target the replicative niche of intracellular pathogen. These are ideal systems for the delivery of gentamicin as this antibiotic does not kill intracellular *Salmonella* due to the polar nature of the drug which is associated with low level of intracellular penetration. A study showed that when gentamicin was incorporated into silica xerogel formulations, 31% of the drug entrapped in the matrix system remained biologically active and the bactericidal effect was retained after drug release. The results showed that by incorporation of PEG the drug release can be modulated. Administration of two doses of the xerogel formulations showed a remarkable reduction in the load of *Salmonella entrica* in the spleen and liver of the infected mice (86). A similar study was performed by another group on gentamicin silica xerogel systems showing that the silica xerogel was more effective in clearing the infection in the liver compared to the same dose of the free drugs (87).

3.6 Vaccine delivery by polymeric nanoparticles

Ochoa et al (93) made an attempt to use nanoparticle for the delivery of vaccines. An immunogenic subcellular extract obtained from whole *Salmonella Enteritidi*s cells (HE) was encapsulated in nanoparticles made with the polymer Gantrez (HE-NP). When they studied the immunogenicity and protection of HE-loaded nanoparticles against lethal *Salmonella Enteritidis* in mice, an increase in survival was observed compared to a control group (80% of the mice immunized with the HE-loaded nanoparticle formulation survived even when administered 49 days before the lethal challenge). They noticed that the cytokines released from *in vitro*-stimulated spleens showed a strong gamma interferon response in all immunized groups at day 10 post-immunization. However, the immunity induced by HE-loaded nanoparticles at day 49 post-immunization suggests the involvement of a TH2 subclass in the protective effect. It can be concluded from their study that, HE-nanoparticles may represent an important alternative to the conventional attenuated vaccines against *Salmonella Enteritidis* (93).

4. Metal nanoparticles as antisalmonellosis agents

In the fast-developing field of nanotechnology, metal nanoparticles are of great interest due to their multiple applications as chemical catalysts, adsorbents, biological stains, and building blocks of novel nanometer scale optical, electronic, and magnetic devices. Metal nanoparticles are pure metal nano sized material (Figure 7) with the size of usually up to 200 nm. They have been suggested to be suitable for biological applications. It was shown that if the size of these nanoparticles is less than 50 nm they are the most suitable particles as therapeutic agents as the biosystem fails to detect them (94).

Different types of nanometals including copper, magnesium, zinc, titanium, gold, and silver have been investigated but silver nanoparticles have been employed and investigated most extensively compared to the other metals since ancient times to fight infections and control spoilage (95-97). A large number of successful *in vitro* studies were performed for the

evaluation of the antisalmonella effect of metal nanoparticles. These nanoparticles are usually nonspecific and are broad spectrum antibacterial. It is also reported that silver can cause argyrosis and argyria and is toxic to mammalian cells (98). As silver attacks a broad range of targets in the microbes, therefore it is difficult for microbes to develop resistance against silver (99). This property of silver makes it an excellent candidate for antimicrobial effect.

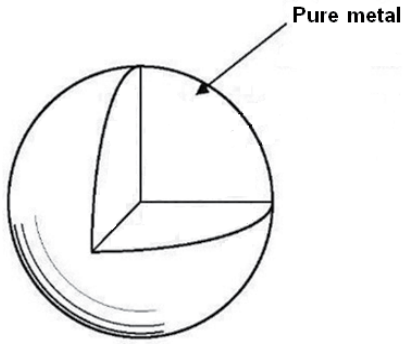


Fig. 7. Schematic structure of a metal nanoparticle

In terms of production, it is suggested that monodispersed particles (very narrow particle size distribution) rather than polydispersed nanoparticles (broad particle size distribution) are preferred. This is because the former distribution is believed to be more effective against microbes due to the high surface/volume fraction so that a large proportion of silver atoms can be in direct contact with their environment (100).

Recently, the potential use of silver nanoparticles on pathogenic bacteria was reviewed (101). There are various physical, chemical or biological methods which can be used to produce metallic nanoparticles. Among these, it seems, the biological method is popular due to the reliability and being eco-friendly. This method has attracted the attention of researchers in the field (102-108). In fact, a number of different species of bacteria and fungi are able to reduce metal ions producing metallic nanoparticles with antimicrobial properties. Recently, it has been shown that silver nanoparticles produced by the fungus *F. acuminatum* have efficient antibacterial activity against multidrug resistant and highly pathogenic, *Salmonella typhi* (109). Additionally, plant extracts can also be used to obtain metallic nanoparticles (110). Metal nanoparticles were also modified to be used in the prevention of biofilm formation on the implanted devices (111-114), however, care must be taken when this type of metal nanoparticles are used due to potential risk on patient's health (115-117).

Researchers suggested that to achieve a better utilization of the antimicrobial activity, metal nanoparticles may be combined with nontoxic and biocompatible polymers. For example, in an attempt NaPGA- (poly (g-glutamic acid)) and CaPGA-coated magnetite nanoparticles were synthesized (118) and their antibacterial activity against *Salmonella enteritidis, Staphylococcus aureous* and *Eschercia coli* were tested. The results showed that both produced nanoparticles were more effective against *Salmonella enteridis* compared to commercial antibiotics, linezolid and cefaclor. In addition, these nanoparticles showed no toxicity toward human skin fibroblast cells.

In few cases polymers such as PVP have been used as steric stabilizers to obtain monodispersed silver nanoparticles (119, 120). Although silver nanoparticles have the

capability to remain dispersed in liquids without major signs of agglomeration, in case of the appearance of aggregation hydrophilic surfactants, proteins, amino acids and PVA (poly vinyl alcohol) can be used (121-125). Metal nanoparticles have also found application in various other fields, i.e. catalysis and sensors as mentioned before (126-128). However, their undesirable and unforeseen effects on the environment and in the ecosystem should not be ignored (129, 130). The antibacterial effect of silver and copper nanoparticles was also investigated on *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* (131). The results showed that the efficiency of silver and copper nanoparticles were different on different bacteria. Among the bacteria used, *B. subtilis* showed the highest sensitivity to copper nanoparticles compared to silver, whereas silver nanoparticles were more effective on the other two bacteria compared to copper nanoparticels (133).

Interesting results were reported by Patil *et al* when they synthesized and tested chloramphenicol loaded nano-silver particles against *Salmonella typhi* (97). For the first time they used PVP in their formulations containing silver as a carrier for chloramphenicol. In the formulation, PVP played a dual role. It acts as a stabilizer and linker for binding chloramphenicol to the silver nanoparticles (Figure 8). The nanoparticles showed considerably enhanced activity against clinically isolated *Salmonella typhi*.

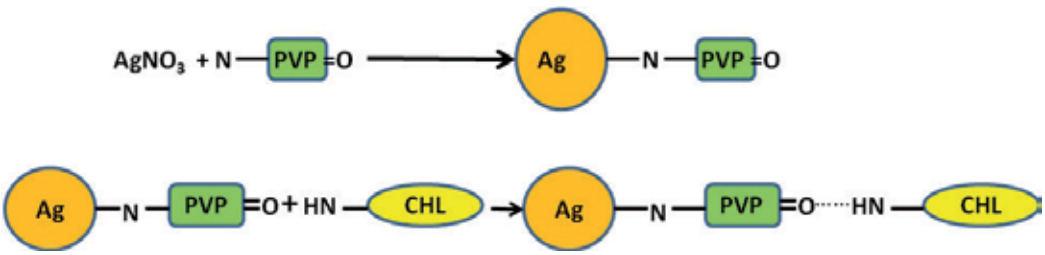


Fig. 8. Top: schematic representation of the synthesis of silver nanoparticles (PVP as a stabilizer); bottom: schematic representation of the synthesis of chloramphenicol loaded silver nanoparticles (PVP as a linker) (figure was reproduced from ref 97).

The summary of some of metal nanoformulations are listed in Table 2.

Gold and platinum nanoparticles have also attracted the attention of researchers due to their antibacterial activity (132, 133). Several research groups studied the cytotoxicity of gold nanoparticles in different cell types (134, 135). It was shown that citrate-capped gold nanoparticles were not cytotoxic to baby hamster kidney cells and human hepatocellular liver carcinoma cells, but cytotoxic to human carcinoma cells at certain concentrations (135).

Despite all research data about the toxicity of gold nanoparticles, still more research for better understanding of gold nanoparticles toxicity is reqired. Recently, Wang *et al* prepared 16 nm gold nanospheres stabilized with citrate ions and their antimicrobial activity was tested against *Salmonella typhi* bacteria strain TA 102 (133). The results showed that gold nanoparticles are not mutagenic or toxic in *Salmonella*, but is photomutagenic to the bacteria. The photomutogenicity was due to the presence of citrate and Au^{3+} ions used during the preparation of gold nanoparticles. Their final results showed that although there was a good surface interaction between gold nanoparticles and the bacteria, the gold nanoparticles were not able to penetrate into the bacteria.

Type of nanoparticle	Type of *Salmonella*	Reference
ASAP Nano-silver Solution	*Salmonella typhi*	(136)
silver colloid nanoparticles	*Salmonella enteric*	(137)
silver–silicon dioxide hybrid	*Salmonella enteric*	(137)
ZnO nanoparticles	*Salmonella typhimurium*	(138)
Spherical silver nanoparticles	*Salmonella typhimurium*	(139)
Zinc oxide QuantumDots	*SalmonellaEnteritidis*	(140)
Silver nanoparticles	*Salmonella typhi*	(141)
Silver nanoparticles	*Salmonella typhimurium*	(142)
Silver bionanoparticles	*Salmonella typhi*	(143)
Silver bionanoparticles	*Salmonella paratyphi*	(144)
TiO2 nanoparticles	*Salmonella typhimurium*	(145)
ZnO nanoparticles	*Salmonella typhimurium*	(145)
Silver nanoparticles	*Salmonella typhus*	(146)
Iron nanoparticles	*Salmonella paratyphi*	(147)
silver nanoparticles	Not specified	(148)
Silver Nanoparticles	*Salmonella typhimurium*	(149)
Silver bionanoparticles	*Salmonella typhi*	(150)
Ag–SiO2 anoparticles	*Salmonella typhimurium*	(151)
$Zn_{1-x}Ti_xO$ (x = 0, 0.01, 0.03 and 0.05) nanoparticles	*Salmonella typhi*	(152)
platinum nanoparticles	*Salmonella Enteritidis*	(153)
CuO nanoparticles	*Salmonella paratyphi*	(154)

Table 2. Various metal naoparticles used against different microbes

Similar study was carried out on gold and platinum nanoparticles (132) and the results showed that gold nanoparticles can interact with *Salmonella Enteritidis* but did not penetrate the bacterial cell, whereas platinum nanoparticles were observed inside bacterial cells due to binding to DNA. They concluded that gold nanoparticles can be used alongside with bacteria to deliver the nanoparticles to specific points in the body for targeted delivery.

A major controversy with metal nanoparticles is that whether they are toxic to bacteria or bacteria develops resistance mechanism against these nanoparticles. If the former is true, there might be a devastating effect to the ecosystem which will lead to a global destabilization. Nanoparticles have a greater potential to travel through an organism and could be more toxic due to their larger surface area and specific structural/chemical properties.

Although the evolution of nanotechnology is about to bring various advantages to our lives over conventional formulations but the lung toxicity of metal nanoparticles (155)

should be carefully considered as these nanoparticles are very small and light, and they have larger surface area with a greater potential to travel through an organism or tissues (156). These small particles can travel via nasal nerves to the brain (156, 157). It has been shown that most of metallic nanoparticles such as TiO_2, Ag, Al, Zn, Ni exhibit cellular toxicity on human alveolar epithelial cells (158). The results reported by Park et al (158) showed that these metal nanoparticles could damage the cell directly or indirectly. The cell damage is probably dependent on the size, structure, and composition of the nanoparticles, yet more studies are needed for better understanding of the toxicity mechanism of the metal nanoparticles.

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9

Mexican Plants Used in the Salmonellosis Treatment

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1. Introduction

Diarrhoeal diseases constitute a major public health problem, particularly in developing countries, where the rate of mortality and morbidity is very high (Bern et al., 1992). The World Health Organization (WHO) has estimated that 1.5 billion episodes of diarrhea occur every year in these countries, resulting in 3 million deaths (Alper 2003). In Mexico, intestinal infectious diseases are the second cause of morbidity, for which children younger than 5 years of age and adults over 65 years are the most affected (Secretaría de Salud, 2005). These diseases are the 18th leading cause of mortality in the general population and, more importantly, the primary cause of death in children (1 to 4 years) and the fourth for infants (1 to 12 months) (Secretaría de Salud, 2005). In addition, certain social groups are more prone to suffer intestinal infections. These diseases are the second cause of mortality among the Mexican rural population (Tapia-Conyer, 1994).

One study suggests that at least 37% of the Mexican population uses medicinal plants. From the 3034 plants used as medicine in Mexico, 1024 (34%) are used to treat diseases in the digestive tract (Argueta et al., 1994). The high percentage of species used to treat gastrointestinal diseases may reflect the high level of incidence of these illnesses among rural and native communities where the plants are their principal, if not only, available health resource (Tapia-Conyer, 1994; Waller, 1993).

The etiological agents of diarrhea described in epidemiological studies are transmitted as waterborne and foodborne. Some foodborne pathogens have been recently considered as emerging diseases (WHO Media Centre, 2007), despite the fact they have been known since a long time ago. For example, outbreaks of salmonellosis have been described for many decades, and yet their incidence has increased over the last 25 years. Diarrhoeal infections can be caused by many etiological agents, but mainly by enterobacteria such as *Escherichia coli*, *Salmonella spp*., *Shigella spp*., *Campylobacter jejuni* and *Vibrio cholerae*; as well as parasites such as *Entamoeba histolytica* and *Giardia intestinalis*, and some rotaviruses are also important agents (Guerrant et al., 1990). *Salmonella spp*. is a facultative, gram negative, flagellated member of the *Enterobacteriaceae* family. The most extensive accepted classification of

Salmonella strains is based on the diversity of two differentially expressed H flagellar antigens: flagellin phase I and phase II antigens (codified by fliC and fljB genes), and the O antigens of the bacterial lipopolysaccharide, both determined by serotyping. Until now, 2501 serotypes have been described; which turns Salmonella classification into a complex and laborious process in the clinical laboratory; therefore, several PCR based methods have recently been developed, and were reported to be a simple, highly sensitive, fast and reliable alternative when compared to traditional clinical laboratory methods.

As a part of our contribution in this area, we have studied an important plant used in this public health problem. *Piqueria trinervia* Cav. is a perennial herb of the family *Asteraceae* that grows commonly in open areas of the pine-oak forests of the mountains throughout Mexico and Central America. It is employed to treat diarrhea, dysentery, ''empachos'' (a cultural disease with manifestations of various gastrointestinal disorders), intestinal infections, stomach pain, and typhoid fever (Argueta et al., 1994). To cure diarrhea or intestinal infections, an infusion or decoction is prepared with 10 g of the aerial parts in 0.5L of water; the tea is consumed throughout the day until the sickness disappears (Linares, 1991). The biological and chemical study of this plant would be described in the present chapter.

2. Traditional medicine in the treatment of Salmonellosis

The use of medicinal plants has occurred in Mexico since pre-Hispanic times. Aztecs had traditionally viewed the disease and health as dynamic changes in the body. According with this, health is maintained when the body is balanced, and the disease comes when this balance is lost (Ortiz de Montellano 1987; Viesca Trevino 1986). The most common causes of illness were the "bad" spirits called "ehecatl". This concept still remains until our days in the Nahua region. These spirits can get into the body of a person and bring bad luck, illness or even death. This concept can be easily associated etiologic with the popular cause of "mal aire" (bad air: offensive spirits in the air) found in different areas of Mesoamerica. Treatment for the disease among the Nahua is a series of techniques to get the offensive spirit of the patient's body, including complex rituals that use a combination of prayers, invocations, and medicinal plants. An important part of the ritual is the "limpia" or the ritual of the cleanliness, through which the body is cleaned of the spirits that cause disease. The specialist in healing restores the balance by removing the spirit from the body of the person, and then he use different formulations based on medicinal plants to complete the treatment of the patient. Aztec/Nahua healing specialists are very knowledgeable about plants that can be used medicinally and they have passed this knowledge from generation to generation.

The Yucatán peninsula is a region particularly recognized for its culture, endemic elements and ancient Mayan buildings. Furthermore, although less known, there is an important ancestral knowledge about the medicinal properties of plants, which is widely used by the general population (Ankli et al., 1999). The Mayan traditional healers keep the knowledge of the different properties of the plants, and how to make medicinal mixtures. These formulations (also known as potions) vary according to the disease to be treated and are used since ancestral times (Leonti et al., 2003) to cure some of the most common health problems in the population, such as diarrheal, cutaneous diseases and those from the respiratory tract as documented by Roys (1931). Among the infectious bowel diseases in the Mayan traditional medicine, there is a group known as dysenteries classified as white and red. The main symptom associated with the red dysentery is bloody diarrhea, and the main

symptom associated with the white dysentery is foamy diarrhea (Roys, 1931); other symptoms include abdominal cramps, fever or severe pain during defecation (Waldman et al., 1994). Since the traditional diseases are not easy to correlate with the causal agents of infectious bowel diseases, it has taken into account the most characteristic symptoms: bloody and/or mucous diarrhea, foamy diarrhea, and abdominal pain to select the microorganisms to be tested

With the presence of nearly 10,000,000 indigenous people speaking nearly 85 different languages, who still depend upon plants for primary therapy from the diverse flora (almost 5,000 medicinal plants), Mexico represent a good area to perform ethnopharmacological studies. Studying the biological diversity of plants related to their traditional use as medicines can lead us to understand how they act and to assure the rational exploitation of the resources and their further development as phytomedicines. Because medicinal plants continue to be culturally suitable as treatments for several illnesses, it is important to document their uses and perform studies about their pharmacological activities to assure their efficacy and safety. Despite the vast literature that exists in Mexico (in Spanish) about ethnobotanical studies, only a few efforts to publish these data in international journals have been done. However, Heinrich and his group published more than 18 works (i.e. Weimann and Heinrich, 1997; Heinrich et al., 1998) with a combination of adequate field work and appropriate interpretation of the data.

The people in Mexico still depend upon the use of medicinal plants to treat simple health problems, including those who live in regions where it is still possible to find people who speak the pre-Hispanic Nahua language. The cultural knowledge about the use of medicinal plants converges with the richness in the surrounding flora making possible to select different regions in Mexico to study selected traditionally used medicinal plants.

3. Mexican medicinal plants used in Intestinal diseases

Today in Mexico there is a strong attachment to the use of plants to cure various diseases. For large sectors of Mexican society, particularly for the indigenous, traditional medicine is the main or only source to address the health problems (Tapia-Conyer, 1994).

The presence of traditional medicine is clearly observed in the southern areas of Mexico such as Chiapas and Oaxaca, where native culture prevails (Lozoya, 1990). Although there are medical services in some rural communities, people prefer traditional medicine. In addition to the cultural, traditional medicine is more accessible to people living in rural communities, either because health facilities are far from the locality or because it is more expensive medical treatment with allopathic (Frei et al., 1998).

In a study from 1983 to 1985 in rural areas of Mexico, through surveys of the program IMSS-COPLAMAR clinics to traditional doctors, they enlisted the 1.950 most used medicinal species in the country. 140 plant species were highlighted for the frequency of use, of which 38.0% were used to cure digestive disorders (deworming, antispasmodics, laxatives, anti-diarrhea and cholera), 13.6% were used to cure diseases of the respiratory system and 13.5% for the treatment of skin lesions. (Lozoya et al., 1987).

Between 1994 and 1995, the most common diseases treated by traditional healers in rural areas of the country were digestive (44.0%), respiratory (11.0%) and injuries (9.0%) (Lozoya,

1990). Most of the medicinal plants used in three indigenous communities of Oaxaca (Maya, Nahua and Zapotec) were used to treat gastrointestinal, skin and respiratory diseases (Heinrich et al., 1992). Zapotec Healers from the Tehuantepec Isthmus used 205 plant species, of which 46.1% were used to treat skin problems and 39.6% to treat gastrointestinal diseases (Frei et al., 1998).

According to traditional Mexican medicine, there are diverse healing strategies, as well as different preferences for the plant parts used for various diseases. It is generally accepted that the beneficial effects of medicinal plants can be obtained from active constituents present in the whole plants, parts of plant (as flowers, fruits, roots or leaves), or plant materials or combinations thereof, whether in crude or processed state.

According with our etnobotanical research, the next plant are the most used in treatment of intestinal diseases: Buddleia scordioides, Byrsonima crassifolia, Geranium seemannii, Guazuma ulmifolia, Larrea tridentate, Persea americana, Piqueria trinervia, Psidium guajava.

3.1 Escobilla (*Buddleia scordioides* H. B. K)

Buddleja scordioides HBK (Buddlejaceae) is a shrub which grows in the Chihuahuan desert and in the state of Coahuila, Nuevo León, Tamaulipas, Durango, Zacatecas, Aguas Calientes, San Luis Potosí, Guanajuato, Queretaro, Hidalgo, Jalisco, México and Distrito Federal (Avila et al., 2005). *Buddleja scordioides* HBK (KUNTH), *Loganiaceae*, is commonly known as escobilla, butterfly-bush, mato, salvia real and salvilla (Martínez, 1979).

Its medicinal use includes the treatment of eating disorders, especially stomach aches and diarrhea. Also used as eupeptic, it is recommended to prepare branch or root infusions, these parts are boiled in milk and given to children when they have colic (Argueta, 1994).This plant is widely used for the treatment of diarrhea, stomacha che (colic) and gastrointestinal disorders.

It was reported that the amebicide activity of *B. cordata* is caused by linarin. This plant can be a source for this compound since it constitutes 24% of the methanol extract of aerial parts.

It was found that extracts of the aerial parts of *B. scordioides* and *B. perfoliata* showed antispasmodic activity and had a relaxing effect on rabbit jejunum and ileum of guinea pigs. Such activities may be the cause of its use in traditional medicine in the treatment of gastrointestinal pain, spasms and cramps (Cortés et al., 2006). Decoctions of this plant are commonly used orally or topically for treating several illnesses such as diarrhea, headache, and hurts (Avila et al., 2005).

In addition, a verbascoside with antibacterial activity, triterpenoid saponins and other glycosides, have been extracted from this species. The presence of some flavonoids such as rutine, quercetin and quercitrin has also been reported (Cortés 2006)

3.2 Nanche (*Byrsonima crassifolia* (L.) Kunth)

Byrsonima crassifolia (Malpighiaceae) is a tropical tree widely distributed in Mexico, Central and South America. The pharmacological activities of *B. crassifolia* extracts as a bactericide, fungicide, leishmanicide, and as a topical anti-inflammatory (Maldini et al. 2009) have been described. *B. crassifolia* is popularly known as "nanche" and it has been used medicinally

since prehispanic times, mainly to treat gastrointestinal afflictions and gynecological inflammation (Heinrich et al. 1998). The most often medicinal use of *B. Crassifolia* has been as an antidiarrheal, but has been also indicated to treat other disorders of the digestive system such as dysentery, stomach pain, indigestion and poor digestion. A decoction of the bark is usually used to treat these conditions (Martinez, 1959; Argueta, 1994). Some other reports indicate that *B. crassifolia* has been employed in the treatment of nervous excitement and to induce a pleasant dizziness (Maldonado 2008).

Different phytochemical studies have been carried out to isolate the main active compounds. The presence of terpenes, saponins, flavonoids and glycosides has been reported from the root of *B. crassifolia* and saponins, flavonoids, glycosides and tannins have been isolated from the stem as well.

3.3 Pata de León (*Geranium seemannii* Peyr)

Geranium seemanni Peyr is a perennial herbaceous plant, with flowers with different colours varying from purple to white (Rzedowski Rzedowski, 1995). It has been located in the states of Sinaloa, Chihuahua, Coahuila, Nuevo Leon, Tamaulipas, Durango, Zacatecas, San Luis Potosi, Aguascalientes, Guanajuato, Mexico City, Queretaro, Hidalgo, Guerrero, Jalisco, Michoacan, Morelos, Oaxaca , Puebla, Tlaxcala, Veracruz and Chiapas (Sanchez, 1979; Rzedowski and Rzedowski, 1995).

The Codigo Florentino, one of the most important books written in ethnobotany in the century XVI, mention that the ground plant applied in the face can remove stains on the skin face. Francisco Hernandez, in the same century says: "is astringent, cures dysentery and other flows, eye inflammation, hemorrhoids and indigestion, and cools down some fevers, among other diseases."

Currently the most common use of this plant is for digestive disorders such as vomiting and diarrhea. The decoction of the plant can be used as antigastralgic and the infusion of the leaves as a purgative. The decoction of the stem, leaves and flowers is usually used to relieve the itch (Argueta, 1994).

This specie has not been studied to analyze its bioactive compounds. However, different species of the *Geranium sp*, used with therapeutic purposes in Mexico, are reported in the literature (Calzada et al., 1999; Serkedjieva e Ivancheva, 1999; Akdemir et al., 2001). The geranin is the most abundant tannin founded in the *Geranium* genre. According with Okuda et al., in 1980, a 9.8 to 12% of this compound is present in dry leaves of the plant. (-)-Epicatechin, (+)-catechin, β-sitosterol-3-O-β-glucoside, tiramine y saccharose were isolated from the roots of the *G. mexicanum* (Calzada et al., 2005).

The aqueous and methanolic extracts prepared from the aerial part and root of *G. mexicanum* showed antimicrobial activity against strains of *E. coli*, *Shigella sonnei*, *S. flexneri* and *Salmonella sp*. (Alanis et al., 2005).

3.4 Guacimo (*Guazuma ulmifolia* Lam.)

Trees of *Guazuma ulmifolia* Lam. (*Sterculiaceae*), commonly known as guácimo, caulote, tapaculo, or aquiche, occupy dry lowlands from Peru, north and east to Venezuela and to

northern México. The species is common in pastures and fencerows and its foliage and fruits are valuable cattle and horse fodder (Seigler et al., 2005).

This plant is used in the Mexican traditional medicine to treat various diseases. In Guerrero, Puebla and Veracruz, is used to treat gastrointestinal disorders. The decoction of the bark, leaves or buds, are used to treat diarrhea. A tea prepared with guacimo shoots, shoots of guayaba (*Psidium guajava*), the stem of nanche (*Byrsonimia crassifolia*) and oak (Quercus sp.) is used in children suffering intestinal infection with diarhea (Argueta, 1994). *G. ulmifolia* is used in some Mixe communities of Oaxaca and Veracruz to treat diarrhea (Leonti et al., 2003). The species is widely used by the Zapotec of Oaxaca to treat gastrointestinal diseases. Indigenous healers recognize plants with astringent properties (high levels of tannins) as useful in the treatment of gastrointestinal disorders (Frei, 1998).

Tannins are the main components of *Guazuma sp*. The variation of these compounds depends on the part of the plant, thus the leaves has 0.145 mg/g, in the leaves with stems 0.115 mg/g, in the stems 0.087 mg/g, and were not detected in fruit (Ortega et al., 1998).

The following compounds were isolated from the ethanolic extract of the stem bark, tannin acid, (-)-epicatechin-[4β→8]-(-)-epicatechin-4β-benzilthioether, (-)-epicatechin-[4β→6]-(-)-epicatechin-4β-benzilthioether; (-)-epicatechin; the dimers procyanidin B2 and B5; the trimers procyanidin C1, (-)-epicatechin-[4β→6]-(-)-epicatechin-[4β→8]-(-)-epicatechin and (-)-epicatechin-[4β→8]-(-)-epicatechin-[4β→6]-(-)-epicatechin and the tetramer (-)-epicatechin-[4β→8]-(-)-epicatechin-[4β→8]-(-)-epicatechin-[4β→8]-(-)-epicatechin (Hörr et al., 1996).

3.5 Gobernadora (*Larrea tridentata* (DC) Cav.)

Larrea tridentata (Sesse and Moc. Ex DC, Zygophyllaceae) also known as gobernadora, coville, larrea, chaparral, or creosote bush, is a shrubby plant belonging to the family Zygophyllaceae. *L. tridentada* is a common shrub of North American warm deserts. Its dominance has increased within 19 million ha of lands previously considered desert grasslands in response to disturbances such as grazing (Arteaga et al., 2005). While often viewed as an indicator of desertified conditions and the focus of extensive control efforts it is also an important plant with a long history of medicinal use (Arteaga et al., 2005).

Tea brewed from the leaves of *L. tridentata* has been used in traditional medicine to treat digestive disorders, rheumatism, venereal disease, sores, bronchitis, chicken pox, and the common cold (Sinnott et al., 1998). This plant is often used to treat gynecological problems. In cases of infertility, the decoction of the leaves is used in vaginal washings or taken as a tea for nine days after the period (for three consecutive months)(Argueta, 1994). Among the proposed medicinal properties of creosote bush, the most prominent is its antioxidant effects (Sheikh et al., 1997).

Phytochemical studies carried out on *L. tridentate* showed that it contains a series of lignans, flavonoids, condensed tannins, triterpene saponins, and naphthoquinones (Abou-Gazar et al., 2004). The extracts or constituents of *L. tridentata* have been reported to possess antioxidant, anti-HIV, antimicrobial, enzyme inhibitory, anti-tumor, and anti-hyperglycemic (Abou-Gazar et al., 2004) activities. The plant contains the powerful antioxidant, nordihydroguaiaretic acid (NDGA) which is suspected to contribute to the toxic effects associated with the consumption of chaparral products (Sinnott et al., 1998).

3.6 Aguacate (*Persea americana* Miller)

Persea americana mill (lauraceae) is a tree plant also called avocado or alligator pear. It is chiefly grown in temperate regions and sparsely grown in tropical regions of the world. Since ancient times this plant has been valued for its nutritional and medicinal properties. The fruit is highly prized for its aroma and exquisite taste (Lozoya and Lozoya, 1982). Avocado is used in 25 states in Mexico to treat various digestive disorders (Lozoya et al., 1987) as dysentery, stomach pain, constipation, stomach gas, vomiting, among others (Argueta, 1994). Peel of the avocado is used in infusion for treating intestinal parasites (Martinez 1959; Lozoya and Lozoya, 1982; Argueta, 1994). It is recommended for anemia, exhaustion, hypercholesterolemia, hypertension, gastritis, and gastroduodenal ulcer. The infusion prepared from the leaves is used in the treatment of diarrhea and some cases of indigestion. The leaves have also been reported as an effective antitussive, antidiabetic, and relief for arthritis pain by traditional medicine practitioners. Analgesic and antiinflammatory properties of the leaves have been reported (Adeyemi et al., 2002).

The result of the phytochemical screening of the aqueous leaf extract of *Persea americana* revealed that the extract contained various pharmacologically active compounds such as saponins, tannins, phlobatannins, flavonoids, alkaloids, and polysaccharides.

From the aqueous extract of the avocado leaves, two new monoglycosyl flavonols were isolated, 3-O-α-D-of kaempferol and quercetin arabinopyranoside, along with the 3-O-α-L-ramnopiranosido-kaempferol (Afzeliana), 3-O-α-L-ramnopiranosido-quercetin (quercitrin), 3-O-β-gluco-pyranoside-quercetin and quercetin 3-O-β-galactopyranoside, quercetin (Almeida et al., 1998).

3.7 Guayaba (*Psidium guajava* L.)

Psidium guajava, a tropical fruit guava of the family *Myrtaceae*, is widely recognized as a plant of many herbal medicines. The leaf, root, and bark of *P. guajava* are used in indigenous herbal medicine for the treatment of various ailments including those that are bowel related. In 23 states of Mexico is used to treat gastrointestinal diseases (Lozoya et al., 1987), most notably diarrhea. Generally, a decoction or infusion prepared with the leaves of guava tree is taken against gastrointestinal diseases (Argueta, 1994). The Tzotzil prepare an infusion to relieve severe diarrhea, weakness, vomiting, stomach pain, when present watery or bloody stools that can last days (Argueta, 1994).

The decoction of the leaves has showed *in vitro* antimicrobial activity against *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, *Proteus mirabilis*, and *Shigella dysenteria*. Another paper showed the effectiveness of the leaf extract against *Staphylococcus aureus* (Gnan and Demello, 1999). It was shown to antibacterial in another study and in addition to *Staphylococcus aureus* was also useful against *Streptococcus spp*. The leaves are rich in tannin, and have antiseptic properties. Modern proof of the traditional use can be found in modern studies. The methanolic extract of *P. guajava* (leaves) showed significant inhibitory activities against the growths of 2 strains of *Salmonella*, *Shigella spp*. (*Shigella flexneri*, *Shigella virchow* and *Shigella dysenteriae*) and 2 strains of the enteropathogenic *Escherichia coli*. The results have confirmed the effectiveness of this medicinal plant as an antidiarrheal agent. Guava sprout extracts (P. guajava) by 50% diluted ethanol showed the most effective inhibition of *E. coli*, while those in 50% acetone were less effective. It is concluded that guava sprout extracts constitute a

treatment option for diarrhoea caused by *E. coli* or by *S. aureus* produced toxins, due to their quick therapeutic action, easy availability in tropical countries and low cost.

The leaves contains essential oil with the main components being α-pinene, β-pinene, limonene, menthol, terpenyl acetate, isopropyl alcohol, longicyclene, caryophyllene, β-bisabolene, caryophyllene oxide, β-copanene, farnesene, humulene, selinene, cardinene and curcumene. The essential oil from the leaves has been shown to contain, nerolidiol, β-sitosterol, ursolic, crategolic, and guayavolic acids have also been identified. In addition, the leaves contain an essential oil rich in cineoland four triterpenic acids as well as three flavonoids; quercetin, its 3-L-4-4-arabinofuranoside (avicularin) and its 3-L-4-pyranoside with strong antibacterial action.

4. The *Asteraceae* (*Compositae*) family

The family *Asteraceae* (*Compositae*) is one of the largest families of flowering plants with about 1100 currently accepted genera and 25000 species (Heywood, 1977). It is of worldwide distribution particularly in semiarid region of the tropics and subtropics. The most members are evergreen shrubs or subshrubs or perennial rhizomatous herbs; biennial and annual herbs are also frequent. It is generally accepted that *Compositae* are a "natural" family with well established limits and a basic uniformity of floral structure imposed on all members by the common possession of charaters such as the aggregation of the flowers into capitula and the special features of the stamensand corolla.

Plants in *Asteraceae* are medically important in areas that don't have access to Western medicine. They are also commonly featured in medical and phytochemical journals because the sesquiterpene lactone compounds contained within them are an important cause of allergic contact dermatitis. *Asteraceae* (*Compositae*) are useful for therapeutic application due to their antihepatoxic, choleretic, spasmolytic, antihelminthic, antiphlogistic, antibiotic or antimicrobial activities. Some of them possess remarkable bacteriostatic and fungistatic properties and they probably participate in the pharmaceutical activity of some drugs and hence the elucidation of the structure of some members of the family. Some preliminary studies of *Piqueria trinervia* have demonstrated that active substances are present in these plants.

4.1 Piqueria trinervia Cav from the work "antibacterial activity of *Piqueria trinervia*, a Mexican medicinal plant used to treat diarrhea", Pharmaceutical Biology, 2007, vol. 45, No. 6, pp. 446–452

Piqueria trinervia Cav. is a perennial herb of the family *Asteraceae* that grows commonly in open areas of the pine-oak forests of the mountains throughout Mexico and Central America. It is usually called ''hierba de San Nicolás'' or ''hierba de tabardillo.'' During the 16th century, it was known by its Nahuatl name as ''cuapopolchi'' and was used as a febrifuge as well as in the treatment of various gastrointestinal ailments including diarrhea. Today, it is employed to treat diarrhea, dysentery, ''empachos'' (a cultural disease with manifestations of various gastrointestinal disorders), intestinal infections, stomach pain, and typhoid fever (Argueta et al., 1994). To cure diarrhea or intestinal infections, an infusion or decoction is prepared with 10 g of the aerial parts in 0.5L of water; the tea is consumed throughout the day until the sickness disappears. This preparation is used as an enema for 2 days, once in the morning and once at night (Torres, 1984). To cure ''empacho,'' people drink a half glass of the root decoction of the tea on an empty stomach for 2 days or until the sickness disappears.

4.2 Biological activity in salmonellosis

Because of its long history in traditional medicine in Mexico, the antimicrobial activity of *Piqueria trinervia* was investigated. Previous studies reported antibacterial activity only from the aerial parts. In our study, antimicrobial activity of extracts derived from both above-ground and root portions of ''hierba de tabardillo'' was evaluated using bacteria that are common to intestinal infections.

4.3 Plant material

The plant material used in this study was collected in the Ajusco zone, Distrito Federal, Mexico, during May and June. The voucher specimen is deposited in the National Herbarium (MEXU) located at the Instituto de Biología, UNAM. The plant was divided in three parts, thick (primary) roots, thin (secondary, 5mm or less) roots, and aerial parts (stem, leaf, flower, and fruit), and dried at room temperature. Each part was ground separately using a mill with rotary knives. The roots were extracted sequentially at room temperature with hexane, ethyl acetate, dichloromethane, and methanol.

4.4 Microbiological test

Test microorganisms. The microorganisms tested were strains of *Escherichia coli*, *E. coli* multidrug resistant (MDR), *Salmonella typhi*, *Shigella boydii*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Yersinia enterocolitica*, nontoxic *Vibrio cholerae*, *Bacillus subtilis*, *Enterobacter aerogenes*, and *Enterobacter agglomerans*. The strains used for this study were provided by Dr. José Guillermo Avila of the Laboratory of Phytochemistry, UBIPRO, Facultad de Estudios Superiores, Iztacala, UNAM.

Bioassay. *In vitro* antibacterial activity was evaluated using the agar disk diffusion method in which the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by microdilution assay in 96-well plates. Disks of 6-mm-diameter sterilized filter paper with concentrations of the extract 2 and 3 mg/disk were prepared as follows: (1) the extracts and fractions were dissolved in dimethyl sulfoxide (DMSO) and diluted with distilled water to obtain concentrations of 133 and 200 mg/mL; (2) then, 15 mL was applied to each disk. The methodology proposed by Bauer et al. (1966) and

Cáceres et al. (1990) was followed. A pure culture of each of the bacteria tested was incubated at 37 °C for 24 h in 5 mL of 1% peptone water (pH 7.2). Afterward, the culture was adjusted to a MacFarland value of 0.5 and diluted in a proportion of 1:20 with 0.15 M saline solution. An aliquote of 100 mL of the bacteria solution was spread over Müller-Hinton agar in the Petri dish, and the disks with the extracts were placed equidistant over the agar surface. After maintaining the Petri dish for 25 min at room temperature, incubation was followed at 37 °C for 24 h. The diameter of each inhibition zone is expressed in millimeters.

4.5 Results and discussion

Activity of organic extracts obtained by liquid-liquid separation. The partition of the aqueous extract of the aerial parts with dichloromethane and microbiological evaluation of the fractions showed activity only in the organic fraction. At a concentration of 3 mg/disk, this fraction was active against all the tested strains with an inhibition halo that ranged from

9 to 20 mm. *Y. enterocolitica* and *S. typhi* were the most sensitive bacteria with halos of 20 and 18 mm, respectively. Also, antibacterial activity was detected in the organic fraction of the aqueous root extract and inhibited the growth of *Y. enterocolitica*, *B. subtilis*, all the tested *Salmonella* strains, *Staphylococcus aureus*, and *Staphylococcus epidermidis* with halos ranging from 7 to 11mm. *Escherichia coli*, *Enterobecter aerogenes*, and *Enterobecter agglomerans* were resistant to the root extracts. These extracts produced bactericidal effects on *Salmonella dublin* (MIC= 3.0 mg/mL) and *Salmonella gallinarum* that produced diarrhea in chickens as well as on *Salmonella typhi* and *Staphylococcus aureus* with MBC ranging from 5 to 7.5 mg/mL.

Antibacterial activity of the organic extracts from the roots. The hexane extract from thick roots (TkR) at a concentration of 3 mg/disk produced antibacterial activity against all 11 strains assayed; meanwhile, the hexane extract obtained from the thin roots (TnR) was active only against *B. subtilis*, *E. agglomerans*, *S. aureus*, *S. epidermidis*, and *S. boydii* at the two tested concentrations (Tables 1 and 2). This TkR extract inhibited the growth of *V. cholerae* with a halo of 32.5mm as well as that of *Y. enterocolitica*, *S. boydii*, *E. aerogenes*, *S. epidermidis*, and *S. typhi* with inhibition halos between 24.0 and 11.5 mm. The activity of the extract at a concentration of 2mg/disk ranged from 14.0 to 11.5 mm, and the most sensitive strains were *S. epidermidis*, *S. aureus*, *B. subtilis*, *S. boydii*, *S. typhi*, and *E. Coli* (Table 1). In general, the TnR extracts had less antibacterial activity than the TkR extracts (Table 2). Nonetheless, *S. aureus* was highly sensitive to TkR and TnR hexane extracts with halos of 17.5 and 12 mm, respectively. Even at a higher dose of TnR hexane extract, the activity maintained the same order (Table 2). Dichloromethane extracts were less active than the hexane extracts (Table 2). In comparison with TnR, TkR dichloromethane showed the broadest antimicrobial action. At a concentration of 3mg/disk, the bacteria *V. cholerae*, *S. aureus*, *S. boydii*, *Y. enterocolitica*, *B. subtillis*, and *S. epidermidis* were sensitive to TkR, whereas TnR inhibited only *B. subtilis* and *E. agglomerans*.

	Inhibition zone (mm)						
	Organic Extract						
		2 mg/disk			3 mg/disk		
Strains	H	DM	EA	M	H	DM	EA
B. subtilis	13.0	14.5	12.5	9.5	12.0	10.0	12.0
E. coli	9.0	-	-	-	11.5	-	-
E. coli MDR	-	-	-	-	10.5	-	-
E. aerogenes	-	-	7.5	-	12.5	-	7.5
E. agglomerans	-	-	-	-	10.5	-	-
S. aureus	13.5	11.0	11.0	-	17.5	16.5	11.0
S. epidermis	14.0	12.0	13.0	-	11.5	10.5	8.0
S. boydii	11.5	12.0	11.0	-	17.0	16.5	11.5
S. typhi	11.0	-	-	-	11.5	-	8.5
Y. enterocolitica	NT	NT	NT	-	24.0	13.5	17.0
V. cholerae	NT	NT	NT	-	32.5	30.0	26.5

H, hexane; DM, dichloromethane; EA, ethyl acetate; M, methanol; –, no inhibition, NT, not tested.

Table 1. Antibacterial activity of the organic extracts of the thick roots of *Piqueria trinervia*.

The halo diameters for the effective dichloromethane extract of TkR at the concentration of 3mg/disk ranged from 16.5 to 10 mm, with the exception of the 30mm halo produced by V. cholerae. The ethyl acetate extract from TkR showed an inhibitory effect with halos between 17.0 and 8.0 mm. This extract was effective against most of the tested strains. TkR was inactive against *E. coli*, *E. coli* MDR, and *E. agglomerans*, and TnR was inactive against *E. Coli* MDR and *E. agglomerans*. Methanol extracts from TkR and TnR were generally inactive with the exception of the inhibition of *B. subtilis*, which produced a 9-mm halo (Tables 1 and 2).

In the majority of the inhibition zones, no bacterial growth was observed, but in some inhibitions zones we detected traces of bacterial growth. The inhibition zones without growth are indicated in the tables with bold numbers.

	Inhibition zone (mm)						
	Organic Extract						
		2 mg/disk			3 mg/disk		
Strains	H	DM	EA	M	H	DM	EA
B. subtilis	12.5	8.5	12.5	9.0	11.5	9.5	10.0
E. coli	-	-	-	-	-	-	7.0
E. coli MDR	-	-	-	-	-	-	-
E. aerogenes	-	-	-	-	-	-	8.5
E. agglomerans	8.7	-	-	-	9.0	8.0	-
S. aureus	12.0	-	11.0	-	12.0	-	9.5
S. epidermis	11.5	10.5	11.0	-	10.5	-	8.0
S. boydii	9.5	-	9.0	-	10.5	-	9.0
S. typhi	9.5	-	-	-	-	-	8.5
Y. enterocolitica	NT	NT	NT	-	NT	NT	12.5
V. cholerae	NT	NT	NT	-	NT	NT	27.0

H, hexane; DM, dichloromethane; EA, ethyl acetate; M, methanol; –, no inhibition; NT, not tested.

Table 2. Antibacterial activity of the organic extracts of the thin roots of *Piqueria trinervia*.

Activity of TkR hexane fractions. Nine fractions were obtained by TLC chromatography (hexane-ethyl acetate, 8:2) of the hexane TkR extract. *B. subtilis* and *V. cholerae* were highly sensitive to most of the isolated fractions, and the halos of the effect strains ranged from 8 to 18 mm. The greatest growth inhibition of *B. subtilis* was produced by F2, while that of *V. cholerae* (30 mm) came from the polar residue at the application zone. A moderate activity was found with fractions F1–F3 against *S. aureus*, *S. epidermidis*, and *S. boydii* (8 to 12 mm). *E. aerogenes*, *E. agglomerans*, and *S. typhi* were resistant to all the fractions. *E. coli* and *E. coli* MDR were inhibited by F1 and F2.

Chemical analyses. The organic fraction (dichloromethane) of the aerial part is a light oil, and the hexane extract of the roots is a dark yellow oil. Both oils were submitted for GC-MS study. According to the electronic database of the equipment, it was possible to identify 20 compounds besides Piquerol A (Fig. 1). The numbers are the proportion of each compound in the oil. The compounds present in both oils are the phenol, which was obtained also by Jiménez-Estrada et al. (1996) by chemical reaction of the piquerol; the other compound is the carquejol. The antibacterial activity is attributed to these compounds, as the antibacterial activity of the phenols have been reported. The carquejol and the piquerol A (Fig. 1)

monoterpenes have a biogenetic structural arrangement characteristic of this plant; the destitution of the group on the six member ring is in the vicinal positions 5, 6. Thus, we assigned aromatic compounds the same substitution to the aromatic compounds.

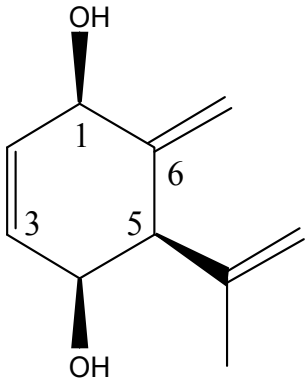

Fig. 1. Compound I: Piquerol A.

5. Conclusion

The organic portion of the aqueous extract prepared with the aerial part has highly activity and favors sustainable harvesting practices that would not damage the roots of plants in natural or cultivated populations. The extracts prepared with the thick root showed more activity with the hexane extract being the most active, followed by the ethyl acetate, dichloromethane, and methanol extracts, respectively. When the hexane extract of the thick root was subfractionated, the greatest antibacterial activity was retained in the residue at the application point and in the three most polar fractions. Based on these results, we conclude that P. trinervia exhibited antimicrobial activity.

6. Acknowledgments

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10

Effect of Sanitizers and Oregano Essential Oils to Reduce the Incidence of *Salmonella* and Other Pathogens in Vegetables

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México

1. Introduction

Consumption of fruits and vegetables has increased in the last years because form an important component in human nutrition, being rich sources of phytochemicals compounds that play a significant role in the health to helps prevent many degenerative diseases (González-Aguilar et al., 2008). However, as a consequence of inappropriate manipulation during their growth, development, harvesting, processing, distribution, retail sale, and final preparation, pathogenic microorganisms, may contaminate a product, thus increasing the risk of microbial diseases. In fact, the number of outbreaks and cases of illness caused by consumption of these produce has increased in the last years.

One of the most common disease-causing pathogens in fruits and vegetables is *Salmonella*. This reside in the intestinal tracts of animals, including humans, and are more likely to contaminate raw fruits and vegetables through contact with feces, sewage, untreated irrigation water or surface water (Beuchat, 1996; Wells & Butterfield, 1997). Outbreaks of salmonellosis have been linked to a diversity of fruits and vegetables including tomatoes (Hedberg et al., 1994; Wood et al., 1991), bean sprouts (Mahon et al., 1997; Van Beneden et al., 1999) and melons (Blostein 1991).

Washing is an important step that has been widely adopted by the industry to remove soils and microorganism from the surface (Sapers, 2003). Given that fresh and fresh-cut products are marketed as pre-washed and ready to eat, and not subject to further microbial killing steps, the development and proper application of sanitizing agents or antimicrobial

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compounds to remove microorganism's pathogen effectively is critical to ensure the safety of these produce (Ruiz-Cruz et al., 20006).

Chlorine has been widely used as a sanitizer during produce washing (Sapers, 2003). However, numerous studies have shown that chlorine used at concentrations permitted by the FDA lacks efficacy in removing human pathogens and spoilage microorganisms (Zhang & Farber, 1996). Additionally, chlorine may react with organic matter in water to form carcinogenic products (Paris et al., 2003). The inadequacies of chlorine as a sanitizer have stimulated interest in finding safer, more effective sanitizers (Ruiz-Cruz et al., 2006). Alternatives to chlorine, such as chlorine dioxide, peroxyacetic acid, acidified sodium chlorite and some essential oils have been already proposed (Beuchat, 1998). Acidified sodium chlorite as shown strong antimicrobial activity against *E. coli* O157:H7, *Salmonella*, *Listeria monocytogenes* and spoilage microorganisms on fresh-cut produce (González et al., 2004; Ruiz-Cruz et al., 2007). Peroxyacetic acid has also been shown to be effective against spoilage and pathogenic microorganisms (Weissinger & Beuchat, 2000). Others sanitizers such as chlorine dioxide and essential oils have been to be very effective in reducing the microbial populations of fresh-cut fruit and vegetable (Burt & Reinders, 2003; Selma et al., 2008).

Moreover, since water wash is often recycled, high organic matter content reduces the activity of sanitizers and increases the likelihood for contamination of fresh vegetables. For this case not all washing methods and washing solutions are equally effective. In order to ensure the safety of fresh produce, it is necessary to evaluate the efficacy of chemical sanitizers and natural antimicrobial compounds in water containing concentrations of organic matter to simulate commercial practices. In view of the importance of *Salmonella* as a cause of food-borne disease and vegetables as a vehicle for its transmission, in our researches are going to look alternatives chemical and naturals to eliminated *Salmonella* and others pathogens in different fruits and vegetables. The objective of this chapter is to evaluate the effectiveness of chemical sanitizers, such as chlorine (Cl, 200 ppm), chlorine dioxide (DC, 5%), peroxyacetic acid (PA, 80 ppm), and acidified sodium chlorite (ASC, 100, 250 and 500 ppm) and oregano essentials oils (OEO, 1 and 2.5 mM) for reducing populations of *Salmonella* and *E. coli* O157:H7 from inoculated cilantro, spinach, lettuce and jalapeño peppers under laboratory and simulated commercial processing wash water conditions.

2. Material and methods

2.1 Bacterial strains and media

Salmonella Typhimurium (ATCC 14028) and *E. coli* O157:H7 (ATCC 43890) were used in this study. Cultures of *Salmonella* and *E. coli* O157:H7 from freezer stocks were grown in tryptic soy broth (Difco Laboratories, Detroit, MI). To suppress growth of microorganisms naturally present on different vegetables, nalidixic acid-resistant strains were obtained and used in this study (Inatsu et al., 2005a; Inatsu et al., 2005b; Ruiz-Cruz et al., 2007). *Salmonella* and *E. coli* O157:H7 strains were adapted to grow on Luria-Bertani broth (LBB; Difco, Becton Dickinson, Sparks, MD) supplemented with 50 µg/mL nalidixic acid (LBB-Nal) and incubated at 37°C. To obtain pure cultures, a loop of *Salmonella* was streaked on Bismuth Sulfite agar (BS; Difco Laboratories, Detroit, MI), *E. coli* O157:H7 on Sorbitol MacConkey agar (SMAC; Difco Becton Dickinson, Sparks, MD). Each agar medium was supplemented with nalidixic acid (50 µg/mL) and plates were incubated at 37°C. After incubation, a single colony from each plate was

selected and inoculated into 10 mL of LBB-Nal (*E. coli* O157:H7 and *Salmonella*). Individual strains were grown in each broth at room temperature (30°C with constant agitation at 175 rpm. Cultures were transferred to each broth by loop at two successive 24 h intervals and one overnight (16-18 h) before they were used as inoculants. Cells of each strain were harvested by centrifugation (4,000 X *g*, 15 min) and washed with 2 vol of sterile phosphate-buffered saline and resuspended at a cell density of approximately 10^9 cfu/mL. Volumes of cell suspensions of *Salmonella* or *E. coli* O157:H7 were combined to create a two-strain mixture. The cells were added proportionally to tap water (laboratory conditions) and simulated commercial processing to obtain a dip inoculum solution of approximately 10^7 cfu/mL. The inoculum level was confirmed by replica plating onto selective agar after serial dilution in sterile phosphate-buffered saline, followed by incubation at 37°C for 24 h.

2.2 Vegetable preparation

Fresh cilantro, spinach, lettuce and jalapeño peppers were purchased from a local wholesale market in Cd. Obregón, Sonora, México, transported to the laboratory and used within 24 h following storage at 5°C. Produce were sorted to eliminate damaged, poor quality produce. Vegetable were washed in tap water at ambient temperature to remove residual soil. After washing, cilantro, lettuce and spinach were cut manually in 2 cm pieces and jalapeño peppers into longitudinal strips of 1 cm width and divided into individual 40 g portions contained in nylon mesh bags.

2.3 Inoculation procedure

Since the immersion process is a possible point of contamination in the food industry, dip inoculation is the most suitable method that can be used to simulate such a process (Beuchat et al., 2001). Spinach, lettuce, cilantro and jalapeño peppers were immersed in the inoculum solution (sample:inoculums ratio = 1:7 w/v) and kept under constant agitation for 30 min. After dipping, the samples were drained for 30 s. Samples were then placed into plastic containers and maintained for 1 h at room temperature until washed with the different treatments. Previous studies indicate that bacteria attach to leaf surfaces within 1 h of inoculation (Yang et al., 2003).

2.4 Treatment procedure

The following sanitizer treatments were evaluated for their efficacy in killing or reducing *E. coli* O157:H7 and *Salmonella* on fresh produce cilantro, jalapeño peppers, lettuce and spinach. The treatments were 200 ppm sodium hypochlorite of a commercial bleach preparation, with pH adjusted to 6.5 with HCl (Cloralex®, NL, México, 6% NaOCl), 80 ppm PA; pH 3.5 (Ecolab, St. Paul,Minn., U.S.A.), 100, 250 and 500 ppm ASC; pH 2.8, 2.6 and 2.4, respectively (Sigma-Aldrich, USA), DC 5%; pH 5.4 and 1 and 2 mM OEO (Sigma-Aldrich, USA). Untreated produce samples were used as control. For the simulated commercial water processing, was obtained by repeatedly dipping freshly shredded produce of known mass in a fixed volume of tap water (González et al., 2004; Ruiz-Cruz et al., 2007). Each mesh bag of inoculated produce were dipped into one sanitizer solution (sample to wash water ratio of 1:5 w/v) with contact time of 1 (ASC and OEO) and 2 min (Cl, PA and DC) with constant stirring. After dipping samples were drained for 30 s to remove excess water. Samples of produce weighing 30 g were packaged in ziploc bags and stored at 5°C for 7 days.

2.5 Procedures for microbial enumeration

Samples (10 g) were transferred aseptically into sterile stomacher bags, 90 mL of Dey-Engley (DE) neutralizing broth was added and samples were macerated. Homogenized samples were serially diluted by a factor of ten in sterile phosphate buffer saline. For each dilution, 1 mL was plated on each of sorbitol MacConkey agar and bismuth sulfite agar for *E. coli* O157:H7 and *Salmonella*, respectively and incubated at 37°C for 24 or 36 h. Agars for pathogenic bacterial growth were supplemented with 50 µg/mL nalidixic acid.

3. Results and discussions

Analysis of the fresh produce that had not been inoculated revealed the absence of *Salmonella* and *E. coli* O157:H7. Preliminary studies were conducted to determine the level of inoculum that could be retained on the surface of different produce. The amount of *Salmonella* and *E. coli* O157:H7 attached to the surface of spinach was 6.53 and 6.35 log cfu/g (Fig. 1), jalapeño peppers 6.52 and 6.4 log cfu/g (Fig. 2), cilantro 5.81 and 5.92 log cfu/g (Fig. 3) and lettuce 5 and 5.35 log cfu/g (Fig. 4), respectively.

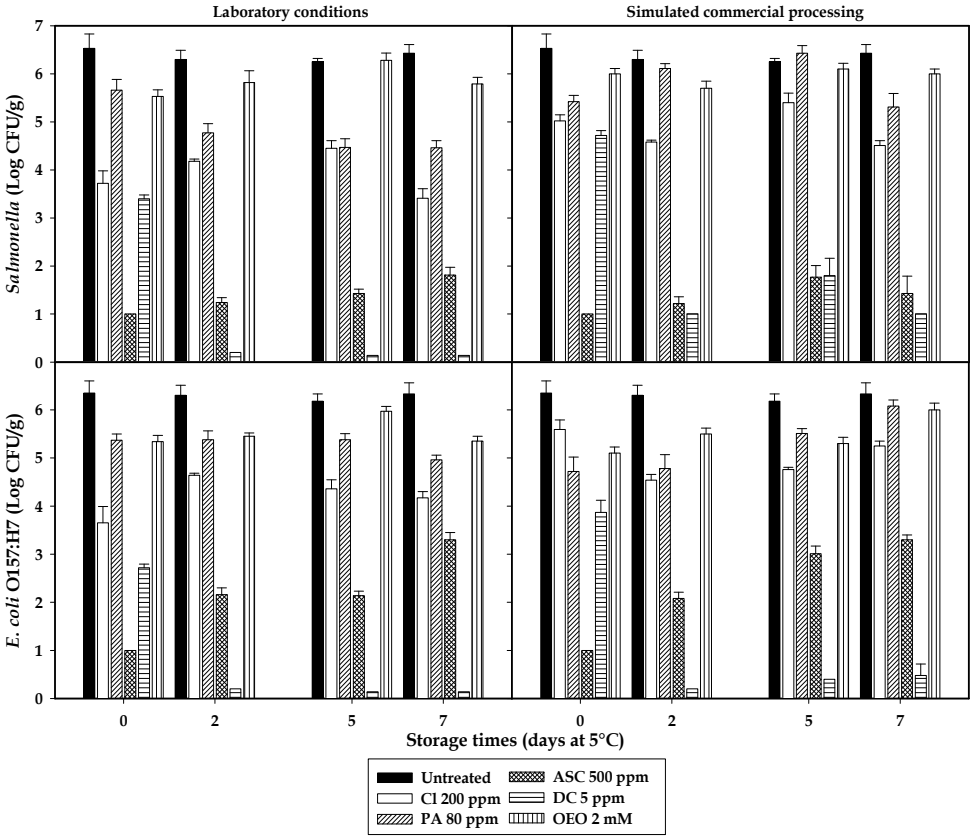

Fig. 1. Efficacy of sanitizers on the reduction of *Salmonella* and *E. coli* O157:H7 populations from artificially inoculated spinach. Bars represent the standard errors of the mean resulting from triplicate experiments. The limit of detection was 0.2 log cfu/g of produce.

All sanitizers reduced significantly (P≤0.05) *Salmonella* and *E. coli* O157:H7 compared with the control (untreated) on day 0, with reduction of 1 – 6 log cfu/g and all produce evaluated. The results of the decontamination for *Salmonella* and *E. coli* O157:H7 on spinach are shown in Fig. 1. The organic matter content in simulated commercial processing reduces the activity of the Cl and DC treatment. However, the effectiveness of Cl was significantly higher that PA and OEO with reductions of 2.5 – 2.7 log cfu/g, but lower that ASC and DC. ASC was the most effective treatment in reducing *Salmonella* and *E. coli* O157:H7. This sanitizer reduced the both pathogen populations to undetectable levels (with 10 cfu/g detection limit), achieving reductions of 5.53 and 5.35 log cfu/g of *Salmonella* and *E. coli* O157:H7, respectively, under both water conditions. The results are similar to those reported by other researchers (González et al., 2004; Ruiz-Cruz et al., 2007). Cells of both pathogens increased by 1.5-3 log cfu/g at the end of storage. On the other hand, interestingly sample treated with DC on tap water conditions lower after 2 days of storage at levels of 0.2-1 log cfu/g. This reduction could be due to damage to the cell by sanitizer and coupled with the cooling temperature, the cells failed to grow.

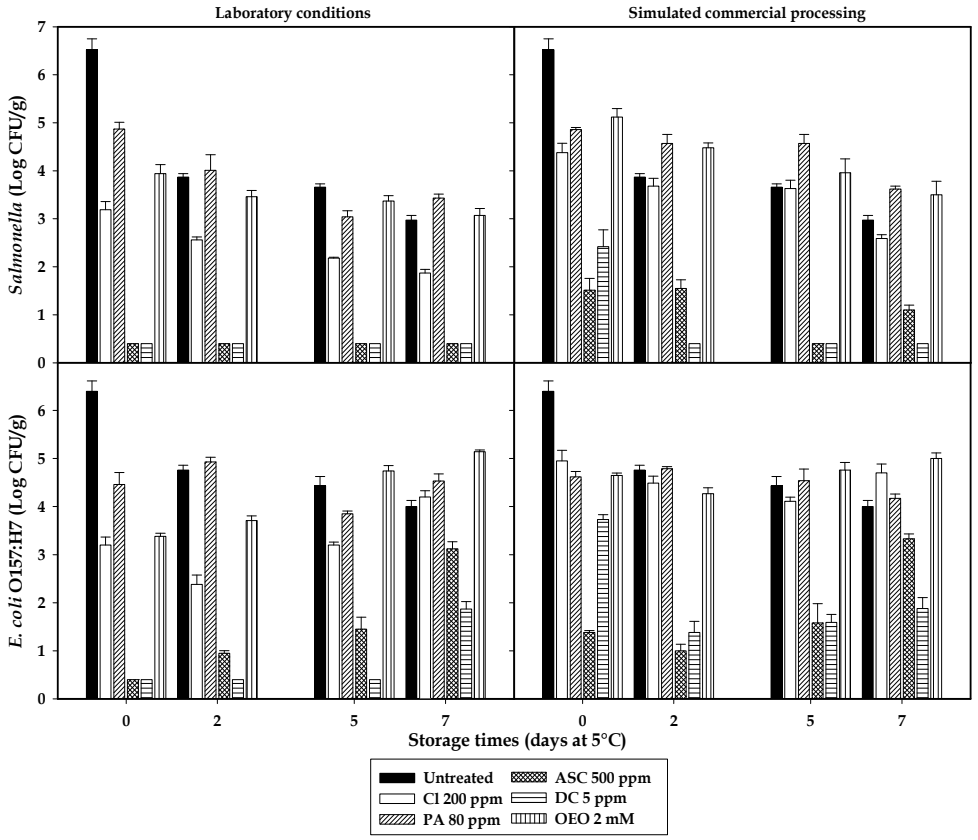

Fig. 2. Efficacy of sanitizers on the reduction of *Salmonella* and *E. coli* O157:H7 populations from artificially inoculated jalapeño peppers. Bars represent the standard errors of the mean resulting from triplicate experiments. The limit of detection was 0.2 log cfu/g of produce.

The use of ASC and DC showed better in inactivating *Salmonella* and *E. coli* O157:H7 on jalapeño peppers that the others sanitizers (Fig. 2). Populations were reduce to undetectable levels (0.4 log cfu/g) at day 0 only under laboratory conditions. Followed by Cl, OEO and PA with reductions of 2-3.2 log cfu/g. The efficacy of DC was affected significantly when was used in simulated commercial processing; however, also caused a higher reduction with 2.7-5 log cfu/g. In general the PA treatment was the least effective in reducing the pathogens; however it effectivity was not affected by the presence of organic matter and this treatment resulted in 1.6 to 2 log cfu/g reduction both pathogens. The cells of *Salmonella* were not recovered during storage time with DC were used under both water conditions. This indicates that the cell could be not retrieved in the agar plate, which caused a reduction of 6 log cfu/g. ASC caused the same effect but only under laboratory conditions. The results are similar to those reports by us previously researched with 250 and 500 ppm of ASC on shredded carrots (Ruiz-Cruz et al., 2007).

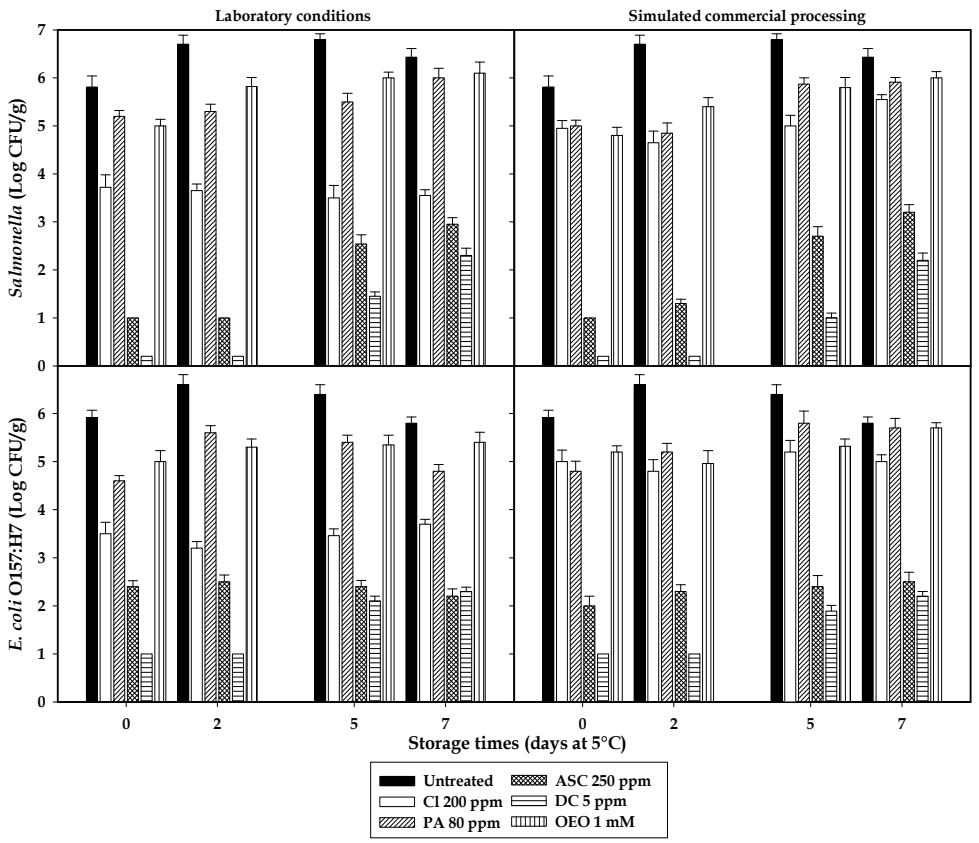

Fig. 3. Efficacy of sanitizers on the reduction of *Salmonella* and *E. coli* O157:H7 populations from artificially inoculated cilantro. Bars represent the standard errors of the mean resulting from triplicate experiments. The limit of detection was 0.2 log cfu/g of produce.

The efficacies of Cl, ASC and DC in reduced *Salmonella* and *E. coli* O157:H7 in cilantro were statistically different ($P \leq 0.05$), when compared with untreated and other treatments (Fig. 3). The effectiveness of Cl treatment was significantly higher than PA and OEO, but lower

than ASC and DC in both pathogens. DC and ASC were the most effectives treatments in reducing pathogens on day 0. DC treatments reduced *Salmonella* to undetectable levels (with 0.2 log cfu/g, detection limit), achieving reductions of 5.61 and 4.92 log cfu/g by *Salmonella* and *E. coli* O157:H7, respectively. ASC also caused a strong reduction of both pathogens with values of 4.81 and 3.52 log cfu/g of *Salmonella* and *E. coli* O157:H7, respectively. Allende et al. (2009) reported on reduction of more than 3 log cfu/g were used 100 ppm of ASC on fresh-cut cilantro. Ruiz-Cruz et al. (2007) observed that ASC treatment at 100, 250 and 500 ppm reduced the *Salmonella* and *E. coli* O157:H7 population on shredded carrots in a similar manner to the reduction of *Salmonella* and *E. coli* O157:H7 achieved with 250 ppm, in this study on fresh-cut cilantro. Our study previously, found that 1000 ppm of ASC affected overall quality of shredded carrots (Ruiz-Cruz et al., 2006). Moreover, we found that concentrations up of 250 ppm of ASC affected the quality of fresh-cut cilantro and the concentrations used in this study (250 ppm) maintained the quality of cilantro by 16 days at 5°C (data no show). Moreover, concentrations of ASC above 500 ppm affected the quality of different vegetable (spinach, jalapeño peppers and lettuce). These results confirmed that the effectiveness of ASC to maintain quality and reduce pathogen counts is influenced by the concentration of the sanitizer and the contact time.

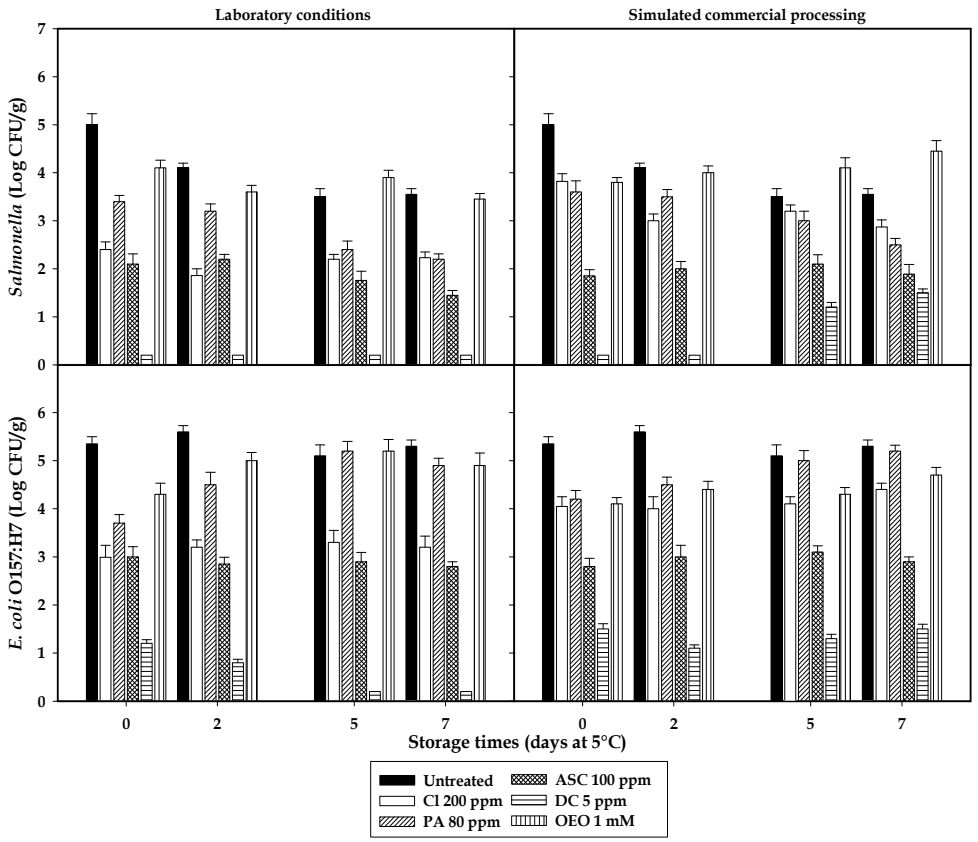

Fig. 4. Efficacy of sanitizers on the reduction of *Salmonella* and *E. coli* O157:H7 populations from artificially inoculated lettuce. Bars represent the standard errors of the mean resulting from triplicate experiments. The limit of detection was 0.2 log cfu/g of produce.

The reduction of *Salmonella* and *E. coli* O157:H7 on lettuce after washing with the different sanitizers were shown in Fig. 4. All treatments caused significant reductions in *Salmonella* and *E. coli* O157:H7 on day 0 under both water conditions compared with the control. Reduction of 1.2 log cfu/g in both pathogens in both water conditions were observed for the OEO treatment, this indicated that its effectivity was not affected by the organic matter. This reduction was maintained throughout the storage. The effectiveness of Cl treatment was significantly higher than PA and OEO, but lower than ASC and DC in reducing *Salmonella* and *E. coli* O157:H7, were used in tap water, causing a reduction of 2.6 and 2.35 log cfu/g, respectively. However, its effectivity was affected in reduced *Salmonella* under simulated commercial processing reducing only 1.18 log cfu/g. PA caused a reduction of 2.4 and 2.6 log cfu/g of *Salmonella* and 1.65 and 1.15 log cfu/g on *E. coli* O157:H7, under tap water and simulated commercial processing, respectively. Similar to the results on the DC treated cilantro, after treated lettuce with DC resulted in a high reduction of *Salmonella* to undetectable levels (0.2 log cfu/g, limit detection), achieving reductions of 4.8 log cfu/g.

4. Conclusions

ASC and DC were the most effective treatments in reducing *Salmonella* and *E. coli* O157:H7 in all produces at all concentrations evaluated. No viable cells of *Salmonella* and *E. coli* O157:H7 were recovered at concentrations of 5% of DC in spinach and lettuce. As well as *Salmonella* in jalapeño peppers treated with DC 5% and ASC 500 ppm, producing a bactericidal effect. However, cells were able to grow during storage, therefore this indicates the ability of the pathogens to adapt to adverse environments present in food is an interesting area that requires more investigation.

The results show that all sanitizers were capable of controlling growth of *Salmonella* and *E. coli* O157:H7 during storage time and can be used by washed these produce. ASC and DC was the most effective sanitizer and have the advantage of being more stable and preserve its efficacy in the presence of organic matter. However, further studies are needed to determine whether these sanitizers might have more lethal effects when lower levels of bacteria are present on produce.

5. Acknowledgments

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11

Antibiotic Resistance in *Salmonella*: A Risk for Tropical Aquaculture

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1. Introduction

Salmonelas are rod-shaped, non-spore-forming Gram-negative facultative anaerobes measuring 0.7-1.5 by 2-5 μm. With the exception of the serovars Gallinarum and Pullorum, salmonelas are motile organisms. They are classified according to morphology and staining pattern and are divided into serotypes and serovars based on their reaction to somatic (O) and flagellar (H) antigens (Bremer et al., 2003). According to Kumar et al.(2003), the genus *Salmonella* has over 2,000 serovars. Two of these—Saintpaul and Newport—have been isolated from seafood (Ponce et al., 2008).

The prevalence of specific *Salmonella* serovars is related to food type. Thus, the serovars Weltevreden and Rissen are predominant in seafoods, as shown by Kumar et al. (2009) in a study on the distribution and phenotypical characterization of *Salmonella* serovars isolated from samples of fish, crustaceans and mollusks from India.

High incidences of *Salmonella* in seafoods have been reported worldwide (Kumar et al., 2010; Asai et al., 2008) in association with outbreaks of fever, nausea, vomiting and diarrhea (Ling et al., 2002). Since *Salmonella* inhabits the intestinal tract of warm-blooded animals, its presence in aquaculture livestock is most likely due to the introduction of fecal bacteria into culture ponds (Koonse et al., 2005). In fact, in a study on *Salmonella* in shrimp, Shabarinath et al. (2007) concluded this pathogen is generally found in rivers and marine/estuarine sediments exposed to fecal contamination.

The quality of aquaculture products may be compromised by exposure to pathogens and biological or chemical contaminants. The latter include chemical agents commonly used in aquaculture, such as veterinary antibiotics, antiseptics and anesthetics. Few antibiotics have been adapted to or developed specifically for use in aquatic organisms. Thus, in Europe several classes of antibiotics may be used in aquaculture, including sulfonamides, quinolones, macrolides, tetracyclines and emamectin. This, however, poses a considerable risk of release of antimicrobials into the environment and eventually of the development of resistance in pathogenic bacteria (Fauconneau, 2002).

The second half of the 20th century saw two major events in the epidemiology of salmonellosis: the appearance of human infections caused by food-borne *S. enteritidis* and by *Salmonella* strains with multiple resistance (Velge et al., 2005). In fact, Angulo et al. (2000) suggested that the factors determining resistance to multiple antibiotics in strains of *S.* Typhimurium DT104 may first have developed in bacteria in the aquaculture environment, possibly as the result of the regular use of antibiotics in fodder.

The present study is a review of the literature on resistant *Salmonella* strains in aquaculture and an assessment of the risk this represents for human health. In addition, information was collected on the incidence of resistant *Salmonella* strains isolated from shrimp farm environments in Northeastern Brazil.

2. Methods of isolation, identification and evaluation of antibacterial susceptibility in *Salmonella*

2.1 Isolation and identification of *Salmonella*

Salmonella may be detected in samples from aquaculture environments using the traditional method described by Andrews and Hammack (2011). The method includes pre-enrichment of 25-g aliquots in lactose broth, selective enrichment in broth (*eg*, tetrathionate and Rappaport-Vassiliadis or tetrathionate and selenite cystine) and selective plating on MacConkey and Hektoen enteric agar. Typical *Salmonella* colonies grown during the selective enrichment stage are screened biochemically with triple sugar iron agar (TSI), lysine iron agar (LIA) or sulfide indole motility agar (SIM). Serotyping is done with commercially available antisera (Koonse et al., 2005), O:H polyvalent antiserum (Carvalho et al., 2009) or somatic (O), flagellar (H) and capsular (Vi) antisera (Kumar et al, 2009).

In addition, molecular biology techniques may be used for rapid detection of *Salmonella* in foods: TaqMan PCR (Kimura et al., 1999), PCR amplification of a 152-bp segment of the gene *hns* (Kumar et al., 2003), real-time PCR (Malorny et al., 2004), PCR, dot blot hybridization, RAPD and ERIC-PCR (Shabarinath et al., 2007), PCR amplification of the gene *invA* (Upadhyay et al., 2010) and uniplex and multiplex PCR (Raj et al., 2011).

2.2 Antibiogram, MIC and plasmid curing

The phenotypical susceptibility of *Salmonella* to antibiotics may be determined by the method of disk diffusion on Muller-Hinton agar (Kha et al., 2006). When testing salmonellas from aquaculture environments, the selection of antibiotics depends on the origin of the isolates, but usually covers a range of families, including the tetracyclines, sulfonamides, quinolones, macrolides and aminoglycosides (Ponce et al., 2008; Carvalho et al., 2009). The classification of bacteria according to susceptibility or resistance to antibiotics is based on the criteria of the Clinical and Laboratory Standards Institute (CLSI, 2009). The antibacterial resistance index (ARI) may be calculated following the recommendations of Jones et al. (1986). Multiple antibacterial resistance (MAR) may be calculated using the methodology described in Krumperman (1983).

Antibacterial susceptibility may also be estimated by determining the minimum inhibitory concentration (MIC) based on macrodilution of Mueller-Hinton broth (CLSI, 2009).

Salmonella strains with phenotypical profile of antibacterial resistance may be submitted to plasmid curing in Luria-Bertani broth supplemented with acridine orange dye at 100 μg mL^{-1}. The method makes it possible to determine whether resistance stems from chromosomal or plasmidial elements (Molina-Aja et al., 2002).

2.3 Determination of resistance genes and plasmid profile

Polymerase chain reaction (PCR) has been used to detect genes encoding resistance to tetracycline in *Salmonella* strains from fish farms. Restriction enzymes used in PCR include *Sma*I (for detecting the gene *tetA*), *Sal*I (for *tetC*), *Sph*I (for *tetB*, *tetD* and *tetY*), *Eco*RI (for G) and *Nde*II (for *tetE*, *tetH* and *tetI*) (Furushita et al., 2003).

The extraction of plasmidial DNA from salmonelas is usually done by alkaline lysis, as proposed by Birnboim and Doly (1979), with or without modification, or with acidic phenol, as described by Wang and Rossman (1994). For small plasmids, the extraction product may be submitted to electrophoresis in 1% agarose gel following the protocol of Akiyama et al. (2011). The protocol for electrophoresis of mega-plasmid DNA molecules in 1% agarose gel is described in Ponce et al. (2008).

3. Results

3.1 *Salmonella* in tropical aquaculture

Salmonelas are recognized worldwide as one of the main etiological agents of gastroenteritis in humans. Despite variations in the regulation of microbiological quality of foods around the world, the largest importers of seafoods only buy products completely free from *Salmonella*, based on the claim that salmonelas are not part of the indigenous microbiota of aquatic environments and that, therefore, the presence of salmonelas in aquatic organisms is associated with poor sanitation and inadequate hygiene practices (Dalsgaard, 1998).

Several studies published in the 1990s reported *Salmonella* in shrimp farming environments in tropical countries. Reilly and Twiddy (1992) found *Salmonella* in 16% of their shrimp samples and 22.1% of their pond water and sediment samples collected on farms in Southeast Asia. Weltevreden was the most abundant *Salmonella* serovar identified, followed by Anatum, Wandsworth and Potsdam. According to the authors, the incidence of *Salmonella* was higher in ponds located near urban areas and, not surprisingly, the bacterial load increased during the rainy season. Bhaskar et al. (1995) detected *Salmonella* in 37.5%, 28.6% and 37.4% of shrimp, sediment and water samples, respectively, collected from semi-intensive grow-out ponds in India.

In contrast, despite detecting high indices of thermotolerant and total coliforms, Dalsgaard et al. (1995) found no *Salmonella* in water, sediment and shrimp samples from sixteen different penaeid shrimp farms in Thailand.

Hatha and Rao (1998) reported only one *Salmonella*-positive sample out of 1,264 raw shrimp. They believed the presence of the bacteria was due to pond contamination from different sources, including the use of untreated fertilizer of animal origin. Likewise, Hatha et al. (2003) found the incidence of *Salmonella* to be low in shrimp farm products exported by India.

Koonse et al. (2005) investigated the prevalence of *Salmonella* in six major shrimp-producing countries in Southeast Asia (n=2), Central Asia (n=1), Central America (n=1), North America

(n=1) and the Pacific (n=1). In four of these countries, *Salmonella* was detected in 1.6% of shrimp samples, and two serovars were identified (Paratyphi B var. Java and Weltevreden Z6). The authors highlighted the need to control or eliminate potential sources of fecal matter polluting the water bodies adjacent to the grow-out ponds.

In Brazil, the microbiological quality of shrimp (*Litopenaeus vannamei*) farmed in Ceará was evaluated by Parente et al. (2011) and Carvalho et al. (2009), both of whom detected *Salmonella* in shrimp and water samples (Table 1). The authors associated the presence of salmonelas with discharge of fecal matter into the respective estuaries where the farms are located. The detection of *Salmonella* in estuaries in Ceará is not an isolated finding. Farias et al. (2010) found salmonelas in samples of the bivalve *Tagelus plebeius* collected in the estuary of the Ceará river and identified the serovars Bredeny, London and Muechen. Similar findings were reported by Silva et al. (2003) in a study on *Salmonella* in the oyster *Crassostrea rhizophorae* obtained from natural oyster grounds in the estuary of the Cocó river, on the outskirts of Fortaleza, Ceará.

Country	Sample	N°	Sorovars	Source
Brazil	Water and Shrimp	3	*S.* ser. Saintpaul e *S.* ser. Newport	Parente et al. (2011)
Brazil	Fish	30	*S.* ser. Agona, *S.* ser. Albany, *S.* ser. Anatum, *S.* ser. Brandenburg, *S.* ser. Bredeney, *S.* ser. Cerro, *S.* ser. Enteretidis, *S.* ser. Havana, *S.* ser. Infantis, *S.* ser. Livingstone, *S.* ser.London, *S.* ser. Mbandaka, *S.* ser. Muenchen, *S.* ser. Newport, *S.* ser. Saintpaul, *S.* ser. Thompson, *S.* ser. O4,5:i:-, *S.* ser. O4,5:-:1,7, *S.* O:17	Ribeiro et al., 2010
Brazil	Water, Sediment and Shrimp	23	*S.* ser. Anatum, *S.* ser. Newport, *S.* ser. Soahanina e *S.* ser. Albany	Carvalho et al. (2009)
Vietnam	Shrimp	29	*S.* ser. Bovismorbificans, *S.* ser. Derby, *S.* ser. Dessau, *S.* ser. Lexington, *S.* ser. Schleissheim, *S.* ser. Tennessee, *S.* ser. Thompson, *S.* ser. Virchow, *S.* ser. Weltevreden, *S.* ser. II heilbron	Ogasawara et al. (2008)
India	Shrimp	54	*S.* ser. Bareilly, *S.* ser. Braenderup, *S.* ser. Brancaster, *S.* ser. Derby, *S.* ser. Kottbus, *S.* ser. Lindenburg, *S.* ser. Mbandaka, *S.* ser. Oslo, *S.* ser. Rissen, *S.* ser. Takoradi, *S.* ser. Typhi, *S.* ser. Typhimurium, *S.* ser. Weltevreden, *Salmonella* VI	Kumar et al. (2009)

*N°: number of positive samples.

Table 1. *Salmonella* in tropical seafood.

Thus, Shabarinath et al. (2007), who also detected *Salmonella* in shrimp, concluded that since salmonelas inhabit the intestinal tract of warm-blooded animals, their presence in rivers and in marine/estuarine sediments exposed to fecal contamination is not surprising.

Tropical fish species may also be infected with salmonelas (Ponce et al., 2008; Heinitz et al., 2000; Ogbondeminu, 1993); in fact, microorganisms of this genus have recently been associated with farmed catfish (McCoy et al., 2011).

3.2 Antimicrobial susceptibility profile of *Salmonella*

The use of antibiotics for prophylaxis in aquaculture not only favors the selection of resistant bacteria in the pond environment, thereby changing the natural microbiota of pond water and sediments, but also increases the risk of transferring resistance genes to pathogens infecting humans and terrestrial animals (Cabello, 2006). Thus, Le and Munekage (2005) reported high levels of drug residues (sulfamethoxazole, trimetoprim, norfloxacin and oxolinic acid) in pond water and sediments from tiger prawn farms in Northern and Southern Vietnam due to indiscriminate use of antibiotics.

According to Seyfried et al. (2010), autochthonous communities in aquatic environments may serve as a reservoir for elements of antibacterial resistance. However, the contribution of anthropic activities to the development of such reserves has not been fully clarified.

Holmström et al. (2003) reported the use, often indiscriminate, of large amounts of antibiotics on shrimp farms in Thailand, and concluded that at a regional scale human health and the environmental balance may be influenced by such practices. Adding to the impact, many of the antibiotics used for prophylaxis in shrimp farming are very persistent and toxic.

Heuer et al. (2009) presented a list of the major antibacterials used in aquaculture and their respective routes of administration: amoxicillin (oral), ampicillin (oral), chloramphenicol (oral, bath, injection), florfenicol (oral), erythromycin (oral, bath, injection), streptomycin (bath), neomycin (bath), furazolidone (oral, bath), nitrofurantoin (oral), oxolinic acid (oral), enrofloxacin (oral, bath), flumequine (oral), oxytetracycline (oral, bath, injection), chlortetracycline (oral, bath, injection), tetracycline (oral, bath, injection) and sulfonamides (oral).

Current aquaculture practices can potentially impact human health in variable, far-reaching and geographically specific ways. On the other hand, the increasing flow of aquaculture products traded on the global market exposes consumers to contaminants, some of which from production areas (Sapkota et al., 2008).

Antibacterial susceptibility in microorganisms associated with aquaculture livestock is an increasingly frequent topic in the specialized literature (Molina-Aja et al., 2002; Peirano et al., 2006; Akinbowale et al., 2006; Costa et al., 2008; Newaja-Fyzul et al., 2008; Dang et al., 2009; Del Cerro et al., 2010; Fernández-Alarcón et al., 2010; Patra et al., 2010; Vieira et al., 2010; Tamminem et al., 2011; Laganà et al., 2011; Millanao et al., 2011; Rebouças et al., 2011; Dang et al., 2011).

In this respect, salmonelas are one of the most extensively investigated groups of intestinal bacteria. Thus, in China salmonelas isolated from fish ponds were resistant to ampicillin

(20%), erythromycin (100%), cotrimoxazole (20%), gentamicin (20%), nalidixic acid (40%), penicillin (100%), streptomycin (20%), sulfanomides (40%), tetracycline (40%) and trimethoprim (20%) (Broughton and Walker, 2009).

Ubeyratne et al. (2008) detected *Salmonella* resistant to erythromycin, amoxicillin and sulfonamides in shrimp (*Penaeus monodon*) farmed in Sri Lanka. Likewise, Ogasawara et al. (2008) found salmonelas resistant to oxytetracycline and chloramphenicol in Vietnamese shrimp samples but concluded ARI values were not as high as in neighboring or developing countries.

Low ARI values were also reported by Boinapally and Jiang (2007) who in a single sample of shrimp imported to the US detected *Salmonella* resistant to ampicillin, ceftriaxone, gentamicin, streptomycin and trimethoprim. This is in accordance with published findings for shrimp in tropical regions, where the major exporters of farmed shrimp are located.

Zhao et al. (2003) evaluated the profile of antibacterial resistance in salmonelas isolated from seafood from different countries and found that most of the resistant bacteria came from Southeast Asia. The authors believe the use of antibiotics in aquaculture, especially in Southeast Asia, favors the selection of resistant *Salmonella* strains which may find their way into the US market of imported foods.

In Brazil, Ribeiro et al. (2010) reported an antibacterial resistance index of 15.1% among salmonelas isolated from an aquaculture system. The *Salmonella* serovars Mbandaka (n=1) and Agona (n=2) were resistant to tetracycline, Albany (n=1) was resistant to sulfamethoxazole-trimethoprim, and London (n=2) was resistant to chloramphenicol. In addition, Carvalho et al. (2009) collected samples from three penaeid shrimp farms in Ceará (Northeastern Brazil) and found *Salmonella* serovars Newport and Anatum to be resistant to tetracycline and nalidixic acid. Water and sediment samples collected in the vicinity of the three farms contained the *Salmonella* serovars Newport, Soahanina, Albany and Anatum, which were likewise resistant to tetracycline and nalidixic acid, suggesting the ponds were contaminated by water drawn from the estuaries.

Bacterial resistance in *Salmonella* may be of either chromosomal or plasmidial nature (Frech e Schwarz, 1999; Mirza et al., 2000; Govender et al., 2009; Tamang et al., 2011; Glenn et al., 2011). In bacteria, the acquisition and diffusion of resistance genes may be influenced by exchanges of DNA mediated by conjugative plasmids and by the integration of resistance genes into specialized genetic elements (Carattoli et al., 2003).

Evidence of plasmidial mediation of antibacterial resistance in *Salmonella* has been available since the 1970s and 1980s (Anderson e Threlfall, 1974; Frost et al., 1982). Thus, Anderson et al. (1977) detected three types of resistance plasmids in *Salmonella* strains from different countries. According to the authors, plasmids of the F_{Ime} type confer resistance to penicillin, ampicillin and streptomycin, whereas, for example, resistance to furazolidone in all *Salmonella* isolates from Israel was considered to be chromosomal. Mohan et al. (1995) determined the plasmid profile of *Salmonella* strains isolated from different regions in India and found a large diversity of small plasmids (2.7 to 8.3 kb) in strains resistant to ampicillin, chloramphenicol, kanamycin, streptomycin, sulphamethoxazole, tetracycline and trimethoprim.

In one study, salmonelas isolated from food animals were found to carry CMY-2, a plasmid-mediated AmpC-like β-lactamase (Winokur et al., 2001). Doublet et al. (2004) found *florR* (a florfenicol resistance gene) and bla_{CMY-2} plasmids to be responsible for resistance to wide-spectrum cephalosporines in salmonelas isolated from clinical samples, animals and foods in the US. The authors added that the use of phenicols in animal farming environments may place a selective pressure on organisms and favor the dissemination of bla_{CMY-2} plasmids. In addition, *florR* is known to confer cross-resistance to chloramphenicol.

Kumar et al. (2010) found evidence that tropical seafood can serve as vehicle for resistant salmonela strains, some of which resistant to as many as four antibiotics (sulfamethizole, carbenicillin, oxytetracycline and nalidixic acid). The authors also identified low-molecular-weight plasmids in the *Salmonella* serovars Braenderup, Lindenburg and Mbandaka.

Six isolates of *Salmonella* serovar Saintpaul from samples of shrimp and fish from India, Vietnam and Saudi Arabia presented one or more resistance plasmids of varying size (2.9 to 86 kb). One of these carried a *Incl1* plasmid (Akiyama et al., 2011).

As discussed above, the indiscriminate use of antibiotics in aquaculture is one of the major causes of the emergence of resistant bacteria in the environment. Several of the mechanisms of resistance in *Salmonella* have been investigated, especially with regard to beta-lactams (Alcaine et al., 2007) and quinolones (Piddock et al., 1998; Piddock, 2002)—two families of antibiotics widely used in aquaculture.

4. Conclusion

The growing incidence of *Salmonella* in tropical aquaculture environments is a worldwide concern which may have local impacts (in the culture area) or global impacts (considering the dynamics of the international seafood market). Human health and environmental balance are further threatened by the emergence of salmonelas resistant to antibiotics employed in farming, in some cases mediated by mobile genetic elements. The elimination of sources of fecal pollution from tropical areas used for aquaculture seems to be the main strategy for minimizing the risk of transference of salmonelas to foods destined for human consumption. As a final consideration, studies should be encouraged on the presence, antibacterial susceptibility and mechanisms of resistance in salmonelas occurring in tropical areas destined for culture of fish, crustaceans and mollusks.

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Part 3

Genetics

12

Reticulate Evolution Among the Group I *Salmonellae*: An Ongoing Role for Horizontal Gene Transfer

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1. Introduction

Salmonella enterica is responsible for 1.4 million cases of foodborne salmonellosis in the United States annually making it the number one causative agent of bacterial foodborne illnesses (CDC, 2007). Infection can occur after eating undercooked meat, poultry and eggs that have been contaminated with *Salmonella* (CDC, 2007). In recent years several outbreaks have occurred in the United States that were associated with *Salmonella* contamination of produce, the most recent being a *S. enterica* Saintpaul outbreak associated with tomatoes, jalapeño and serrano peppers that sickened over 1400 individuals (CDC, 2008). The movement of several serovars of *Salmonella* into previously naïve niches (*i.e.,* produce-growing environs) suggests that the pathogen is readily adapting to new environments. An understanding of the reticulate evolutionary mechanisms that underpin the acquisition and composition of the requisite genetic and phenotypic features of *Salmonella* is essential to more accurate risk assessment of this pathogen (Hohmann, 2001).

It is now widely accepted that horizontal gene transfer (HGT) has driven the emergence of more aggressive and virulent strains of *Salmonella* in the environment, on the farm, and in the food supply. Such assault by various salmonellae has fueled the in-depth examination of specific genotypes and conditions that permit reticulate evolutionary change and the rise of deleterious phenotypes (LeClerc et el., 1996; 1998; 1999; Cebula and LeClerc, 1997). The hypermutable phenotype represents one scheme by which reticulate evolution of the bacterial chromosome may occur (Trobner and Piechoki, 1984; Haber et al., 1988; Haber and Walker, 1991; LeClerc et al., 1996; Matic et al., 1997; Radman et al., 1999; Cebula and LeClerc; 2000; Funchain et al., 2000). Methyl-directed mismatch repair (MMR) defects, leading to a mutator or hypermutable phenotype, are found in more than 1% of the isolates within naturally-occurring populations of *Salmonella enterica* (LeClerc et al., 1996) and at even greater frequencies in the food supply where oxidative and other anti-microbial stressors are applied (Cebula et al., 2001). Up to 73% of the MMR defects found in feral settings are due to lesions within the *mutS* gene, resulting in increased nucleotide substitution rates, enhanced DNA transposition, and, perhaps most importantly, a relaxation of the internal barriers that

normally restrict homeologous recombination following HGT of foreign DNA (Cebula and LeClerc, 1997; Radman et al., 1999).

This latter role, as a major sentinel for recombination, led to a substantial focus on the genetics and evolution of the *mutS* gene and its adjacent sequences located at 63 min on the *Salmonella* chromosome (Brown et al, 2002; 2003; Kotewicz et al., 2003; 2003). Phylogenetic analyses of *mutS* alleles from strains of the SAR (*Salmonella* reference) collections (i.e., SARA, SARB, and SARC)—largely taken to represent the extent of genetic variability within the species (Boyd et al., 1993; 1996; Beltran et al., 1991)—have revealed striking levels of phylogenetic discordance between trees derived from *mutS* alleles and whole-chromosome trees of the same strains based on MLEE (multilocus enzyme electrophoresis) analysis (Brown et al., 2002, 2003). These differences were interpreted as numerous examples of HGT among *mutS* alleles in *Salmonella.* Similar observations have been made among sequences abutting the *mutS* gene in *Salmonella, E. coli,* and *Shigella* spp (Kotewicz et al., 2002; 2003; Brown et al., 2001b). Our laboratory showed previously that the 61.5 min *mutS-rpoS* region retains a novel and highly polymorphic 2.9 kb sequence in the genome of all *E. coli* O157:H7 strains, *Shigella dysenteriae* type 1, and several other *E. coli* strains (LeClerc et al., 1999) but not in *Salmonella enterica* (Kotewicz et al., 2003). This highly polymorphic stretch of DNA (previously coined the *mutS-rpoS* "unusual region") is varied in its distribution among enteric bacterial lineages and is absent in others entirely (Kotewicz et al., 2003). Sequence analysis of the region revealed an IS1 insertion element in place of the *prpB* gene in *S. dysenteriae* type 1 suggesting the existence of a recombinational crossover in the *mutS-rpoS* region for this strain (LeClerc et al., 1999). Evidence for additional crossovers in the same region were also obtained for other *E. coli* strains (Brown et al., 2001b). These findings support the notion that HGT helped forge current relationships among *Salmonella* and other enteric pathogens in this region and throughout numerous other locales in the *Salmonella* chromosome.

Indeed, as evidenced from global efforts involving whole-genome sequencing, microarray, and multi-locus sequence typing, the substantial impact that HGT has played in structuring the chromosome of *Salmonella enterica* is now indisputable (Porwollik and McClelland, 2003; Fricke et al., 2011; Kelly et al., 2009; Hall, 2010). Previous estimates indicate that at least one-quarter of the *Salmonella* genome may have been forged through HGT and reticulate evolutionary events (Porwollik and McClelland, 2003), although this number seems conservative from current views. In addition to the 61.5 min region surrounding *mutS*, HGT has played a key role in structuring many other regions of the *Salmonella* chromosome. Notably, SPI elements (*Salmonella* pathogenicity islands) have likely been acquired through HGT (Groisman and Ochman, 2000; Ochman et al., 2000; Hacker and Kaper, 2000; Baumler et al., 1997). For example, the SPI-1 pathogenicity island, comprising the genes encoding a type III secretion system, was probably acquired early in *Salmonella* evolution (Kingsley and Baumler, 2000; Li et al., 1995), yet several *inv–spa* alleles seem to have converged horizontally more recently between *S. enterica* groups IV and VII (Boyd et al., 1997; Brown et al., 2002). Additionally, type 1 pilin genes that encode fimbrial adhesins retain unusually low GC contents and aberrant DNA sequence phylogenies relative to other *fim* genes (Boyd and Hartl, 1999). Other studies focusing on numerous housekeeping gene loci have reported evolutionary histories for these genes that are strikingly decoupled from *S. enterica* strain history (Nelson and Selander, 1994; Thampapillae et al., 1994; Brown et al., 2002; Boyd et al.,

Christensen and Olsen, 1998; Groisman et al., 1992; Li et al., 1994; Liu and Sanderson, 1996; Nelson and Selander, 1994; Nelson et al., 1992; 1997).

The now incontrovertible connection between horizontal transfer and MMR gene evolution has led to the thesis that genetic exchange of *mutS* alleles could simultaneously quiet the mutator phenotype while rescuing adaptive changes from the population (LeClerc et al., 1996; Denamur et al., 2000). Consistent with this hypothesis, the *mutS* gene is evolutionarily scrambled by HGT in subspecies I *Salmonella enterica*. Our laboratories documented the prevalence of horizontal gene transfer (HGT) among strains of *Salmonella enterica* (Brown et al., 2002; 2003). In comparing across and within subspecies of *Salmonella*, a recombination gradient was noted wherein the incidence of HGT was inversely correlated with the genetic diversity separating individual strains. It appears that a genetic threshold exists that tolerates free exchange of sequences within a framework delimited by sequence variation and niche diversity of individual strains. We demonstrated this through identification of intragenic (patch-like) recombination as the primary outcome across disparate *Salmonella* subspecies and assortative (whole-allele) recombination which caused extensive reassortment of alleles among more genetically homogeneous populations of group I *Salmonella* pathogens, all sharing a common niche restricted to warm-blooded mammals.

A torrent of scientific information has accrued over the past decade to support the important role of HGT in the genetic and evolutionary diversification of *S. enterica* subspecies, serovars, and individual pathogenic clones (McQuiston et al., 2008; Octavia and Lan, 2006; Lan et al., 2009; Fricke et al., 2011). Our understanding in reconstructing the horizontal acquisitions of important features including those involved in virulence, drug resistance, and other adaptations that foster an enhanced fitness for *Salmonella* persistence in foods, animals, and people is expanding at a pace which we could not have foreseen even a decade ago (Sukhnanand et al., 2005). It is important to recall however that reticulate evolutionary pressures do not subside once selectively advantageous traits are gained. Rather, horizontal exchange likely continues to dapple the evolutionary landscape between even the most closely related salmonellae (Brown et al., 2003). Here, we provide results of several previously unreported phylogenetic studies that evidence (i) the continued role of HGT in the intra-operon shuffling of SPI-1 alleles among subspecies I *S. enterica* strains; (ii) the often under-appreciated role for HGT and recombination in the homogenization of allele structure in a closely related population of *S. enterica*; and (iii) the panmictic and reticulate nature of restriction-modification (R-M) genes among group I salmonellae. This last finding, noting free exchange of R-M (*i.e., hsd*) alleles, provides phylogenetic evidence of the compatibility of *S. enterica* subspecies I R-M complexes, likely accounting for the documented successful HGT of entire gene sequences among closely (*e.g.*, intra-subspecies) related strains as DNA exchange between strains that shared or recently shared common R-M alleles would not be subject to substantial restriction (Sharp et al., 1992).

2. Reticulate evolution in SPI-1 of *Salmonella enterica* subspecies I

Salmonella pathogenicity island 1 (SPI-1) specifies a type III secretion system essential for host cell invasion and macrophage apoptosis (Galan and Curtiss, 1989; Galan and Collmer, 1999). SPI-1 comprises a cluster of virulence genes (*e.g.*, the *inv/spa* gene cluster) that encode, in part, the "needle complex", a key delivery component for transporting virulence associated effector molecules into the host cell (Galan and Collmer, 1999). The

disparate phylogenetic distribution, lack of chromosomal synteny, and diverse base compositions of SPI-1 and its homologues indicate that these sequences were obtained independently across enteric species of bacteria. It is presumed that SPI-1 was present in the last common ancestor of all *Salmonella* lineages. Horizontal acquisition of the *inv/spa* gene cluster, however, is thought to have been a pivotal event for the emergence of *Salmonella* as a pathogenic species (Boyd et al., 1997; Groisman and Ochman, 2000). The gene complex lies adjacent to the polymorphic *mutS–rpoS* region of the chromosome. We and others previously presented phylogenetic evidence for intragenic recombination of sequences within several SPI-1 invasion loci (Boyd et al., 1997; Brown et al., 2002), primarily among *S. enterica* subspecies IV and VII. However, in order to determine the extent to which HGT may have disrupted SPI-1 evolution across the more ecologically and genetically homologous group I salmonellae, we examined nine SPI-1 invasion loci from nearly half of the SARB reference collection of strains (Boyd et al., 1993), composed exclusively of subspecies I *Salmonella* serovars.

2.1 SPI-1 gene evolution is decoupled from *Salmonella* chromosome evolution

Using a cladistic approach (Forey et al., 1992; Allard et al., 1999; Bell et al., 2011), the nucleotide sequences from nine invasion gene sequences were subjected to phylogenetic analysis. The resultant invasion gene phylogenies were then compared to phylogenetic groupings from the *mdh* gene, a chromosomal anchor locus that is taken largely to reiterate chromosome evolution within subspecies I (Boyd et al., 1994) and MLEE (multi-locus enzyme electrophoresis), also applied here as a metric of strain/chromosome evolution for the group I salmonellae (Boyd et al., 1993). As shown in Fig. 1, strains composing single SARB *mdh* and MLEE lineages were, for the most part, distributed across disparate *inv/spa* gene clades for all nine invasion genes tested indicating that many of these strains, although linked tightly in chromosome evolution, retain invasion gene alleles with unrelated evolutionary histories, presumably as a result of HGT.

Evolutionary incongruence between *inv/spa* genes and the *Salmonella* chromosome was affirmed using the ILD (incongruence length difference) test, which evaluates the likelihood of a common evolutionary history between genes (Farris et al., 1995; LeCointre et al., 1998; Brown et al., 2001a). Seven of the nine invasion genes yielded significant ILD scores ($p < 0.05$), indicating that a hypothesis of congruence could be rejected for these strains and further reinforcing the discordance evident in the clade comparisons. The only exceptions were *invB* ($p = 0.08$) and *spaP* ($p = 0.59$), albeit both still retained cladistic signatures of HGT from broken clade structures in the tree analysis.

2.2 SPI-1 gene evolution is decoupled from *mutS* gene evolution

The *mutS* gene, downstream and adjacent to SPI-1 in *S. enterica*, has been shuffled extensively by HGT (Brown et al., 2003). In order to determine whether *mutS* may have been linked in the recombination now evident among SPI-1 genes, cladistic comparisons were made between *mutS* phylogeny and *inv/spa* gene phylogeny revealing substantial incongruence between *inv/spa* trees and *mutS* trees. Six of these comparisons are shown in the form of tanglegrams (Fig. 2). Again, strains composing SARB *mutS* clades were distributed across disparate *inv/spa* gene clades for all nine invasion genes tested, and seven of nine *inv/spa* genes were further

confirmed as discordant with *mutS* based on ILD testing. Taken together, these findings indicate that *inv/spa* gene sequences and *mutS* sequences from the same strains are decoupled in their evolution. These data suggest that reticulate evolution has repeatedly forged this contiguous region of the *Salmonella* chromosome such that different strains appear to have been affected by assortative (allelic) HGT between the two loci.

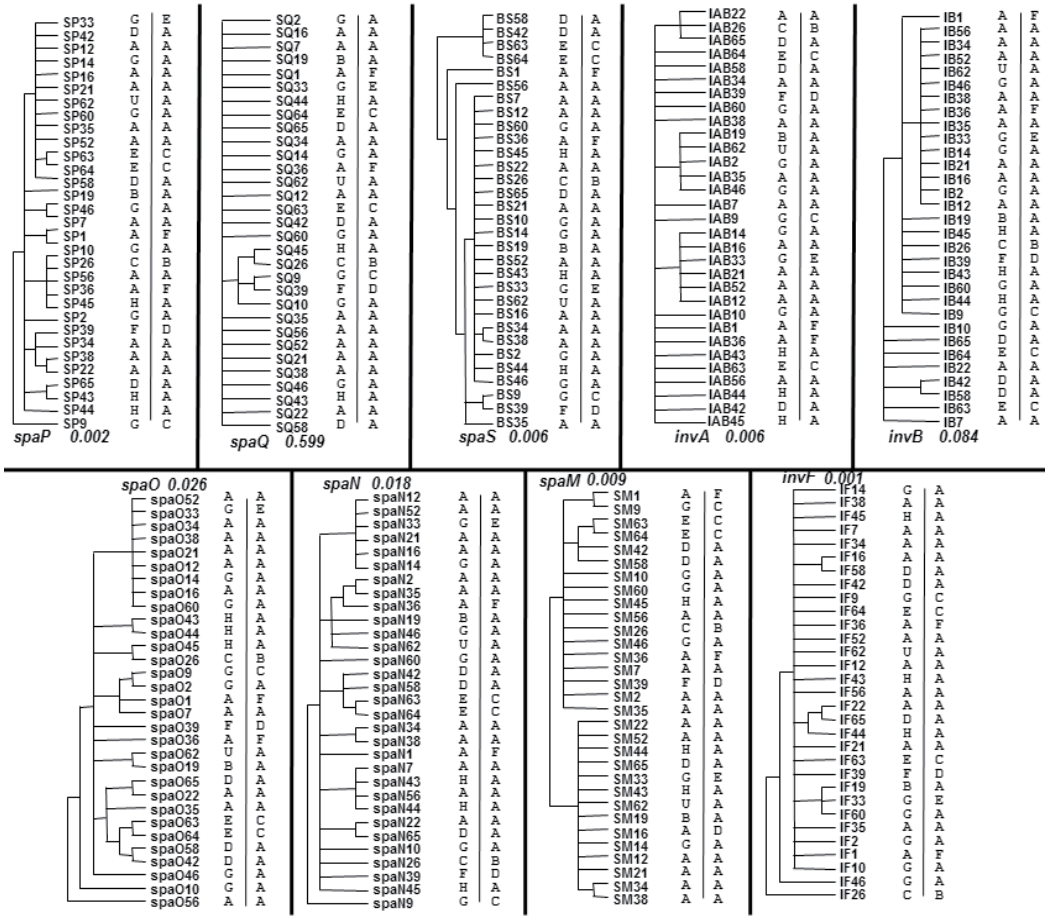

Fig. 1. Phylogenetic discordance between SPI-1invasion genes and the *Salmonella* chromosome. *mdh* and MLEE comparisons are shown to each of nine different *inv/spa* genes indicated. Identical letters denote strains from the same *mdh* or MLEE lineage. It is important to note that letters are only relevant to their respective data column and do not cross-over columns. The column to the left of the dividing line designates *mdh* clade assignments for the respective *S. enterica* strain while the column of letters to the right of the divider corresponds to MLEE clade assignments. The number at the base of each tree denotes the ILD score (p-value) relative to a comparison for congruence between the respective *inv/spa* gene and the *mdh* gene sequence alignment for the same strains. Trees shown were rooted using *S. bongori* as an outgroup. Nucleotide sequence alignments were performed using CLUSTAL X (Thompson et al., 1998). Most parsimonious trees were generated in PAUP* v.10 (Swofford et al., 2002).

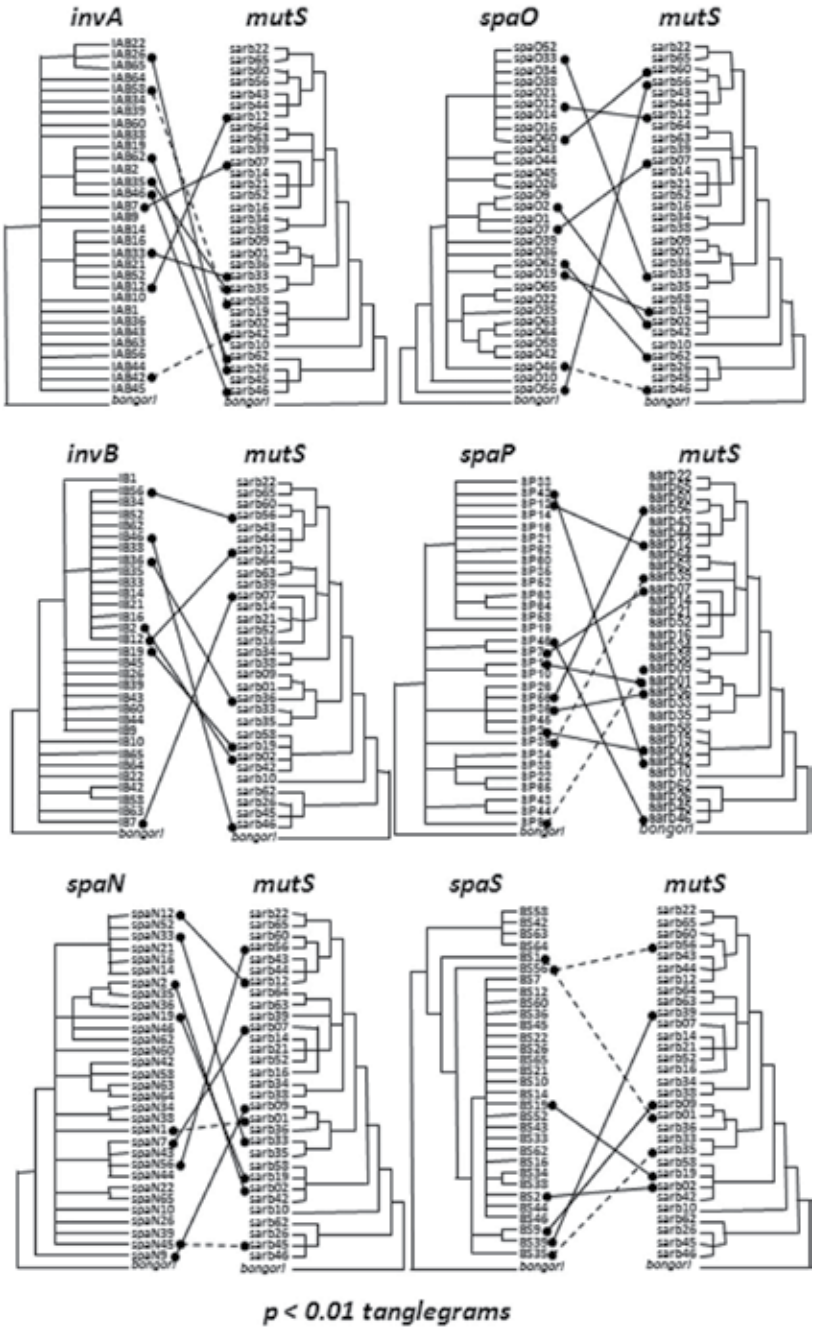

Fig. 2. Tanglegrams of several invasion gene and *mutS* revealing the phylogenetic incongruence between *inv/spa* genes and the *mutS*, which lies adjacent to SPI-1 on the *Salmonella* chromosome. Lines connect the discordant, potentially recombinagenic (incongruent) strains. *inv/spa* to *mutS* comparisons with an ILD score of $p < 0.01$ were displayed. Trees shown were again rooted using *S. bongori* as an outgroup taxa.

2.3 Intra-island HGT within the SPI-1 region of subspecies I *Salmonella* strains

In order to determine the presence and extent to which HGT has shuffled individual alleles within SPI-1 among more closely related subspecies I strains, a pairwise ILD approach was adopted wherein congruence was scored for individual comparisons of all nine of the *inv/spa* genes included in this study (Fig. 3). Several findings were noteworthy. Although no individual invasion gene showed unanimous evolutionary discordance with its neighbors, three *inv/spa* loci (*invA*, *invB*, and *spaP*) were incongruent ($p < 0.10$) with a significant majority of other genes. *invA* and *invB* showed discordance with all other loci except *spaN* and *spaQ*, while *spaP* showed discordance to all but *spaM* and *spaQ*. Conversely, with the exception of *spaQ*, no *inv/spa* gene was congruent with every other. Thus, a hypothesis of extensive intra-island shuffling begins to emerge with an evolutionary decoupling of individual invasion loci one from another. Additional tree comparisons buttressed this conclusion. Akin to the selfish operon theory (Lawrence and Roth, 1996), these data suggest that the SPI-1 region is a chromosomal mosaic, composed of *inv/spa* gene sequences that have converged within this island but with each retaining unique evolutionary paths.

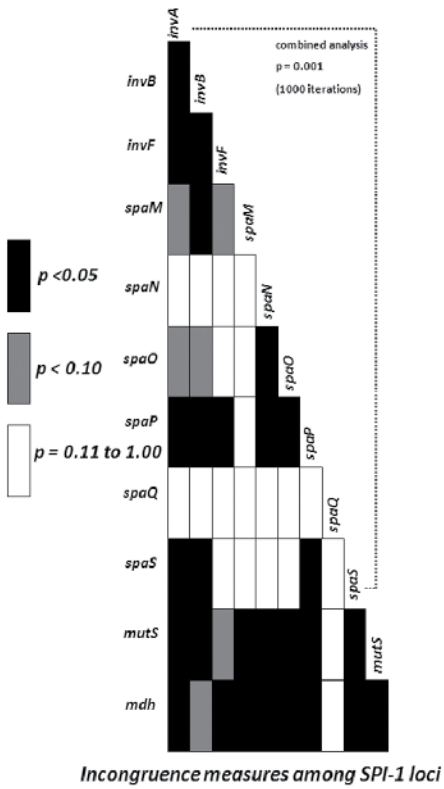

Fig. 3. ILD test results for intragenic comparisons among *inv/spa* invasion genes. ILD tests (Farris et al., 1995) were performed with 1000 partitions using the Partition Heterogeneity command in PAUP* v.10 (Swofford et al., 2002). A p-value of 0.05 or less allows for a rejection of the null hypothesis of congruence (vertical evolution) and accepts the alternative hypothesis of incongruence which is interpreted among bacterial phylogeny as evidence for HGT (LeCointre et al., 1998).

2.4 Key observations

i. The *inv/spa* complex of *S. enterica* subspecies I appears to have undergone extensive intra-island allelic shuffling due to HGT. This suggests that the SPI-1 region is a mosaic composed of SPI-1 gene sequences with distinct evolutionary origins.
ii. Invasion genes within this *Salmonella* population are not only decoupled phylogenetically from *mutS* and other flanking sequences but also from the chromosomes of group I *S. enterica* strains, suggesting that these genes have been re-assorted by HGT.
iii. Much of the recombination observed here appears to be assortative transfer, a finding that contrasts to the *inv* genes in *S. enterica* as a whole, where tree structure was largely intact with HGT limited mostly to subspecies IV and VII (Boyd et al., 1997; Brown et al., 2002).
iv. Allele shuffling appears to be most prominent within the subspecies I taxonomic boundary and not across other subspecies of *S. enterica*. This finding is consistent with a relaxed and compatible restriction-modification system among more closely related *Salmonella* strains (Brown et al., 2003).

3. HGT homogenizes the *mutS* gene among 'Typhimurium' complex strains

Here, we present phylogenetic and genetic analyses of *Salmonella* reference collection A (SARA), also known as the Typhimurium strain complex—the most homogeneous *S. enteric* reference collection, consisting solely of five closely related subspecies I serovars (Typhimurium, Paratyphi B, Muenchen, Saintpaul, and Heidelberg) (Beltran et al., 1991). Given the evolutionary similarity shared among these pathogens and trend noted previously that highlight the inverse relationship between *Salmonella* diversity and recombination, one would expect to observe an even greater role for HGT in the population structure of the *S. enterica* SARA collection of pathogens.

3.1 Cladistic evidence for horizontal exchange of *mutS* alleles among 'Typhimurium' complex strains

As was done for SPI-1 gene sequences, a phylogenetic tree was derived from 72 SARA *mutS* sequences and was compared to phylogenetic trees derived from multi-locus enzyme electrophoresis (MLEE) and *mdh* (malate dehydrogenase) gene sequences for the same strains. Phylogenies derived from horizontally exchanged sequences display evolutionary discordance (incongruence) when compared to *mdh* and MLEE trees. In the tree shown, six clades of *mutS* alleles were observed and compared to the distribution of four *mdh* and six MLEE multi-strain containing clades (Fig. 4). Two of the four SARA *mdh* clades were found to be displaced into multiple clades on the *mutS* tree. Two additional *mdh* clades were found to have converged into a single *mutS* clade, suggesting that HGT may have homogenized *mutS* diversity of these particular *mutS* lineages. Similarly, strains from five of the six MLEE lineages were displaced into separate clades on the *mutS* tree. The only exception was a single clade of MLEE SARA strains (A57, A58, A59, and A60), which was also found intact in the *mutS* tree except for the inclusion of SARA strain A56. Nonetheless, numerous examples of evolutionary discordance between the 1.1 kb *mutS* segment and the chromosome of the 'Typhimurium' complex strains indicate that horizontal exchanges of *mutS* alleles have accumulated during the rather shallow radiation of even these highly homogeneous group I pathogens. As an aside, it was

noteworthy that full-length *mutS* alleles were horizontally transferred among SARA *S. enterica* strains, lending further credence to a model for R-M compatibility among closely related *S. enterica* serovars and strains.

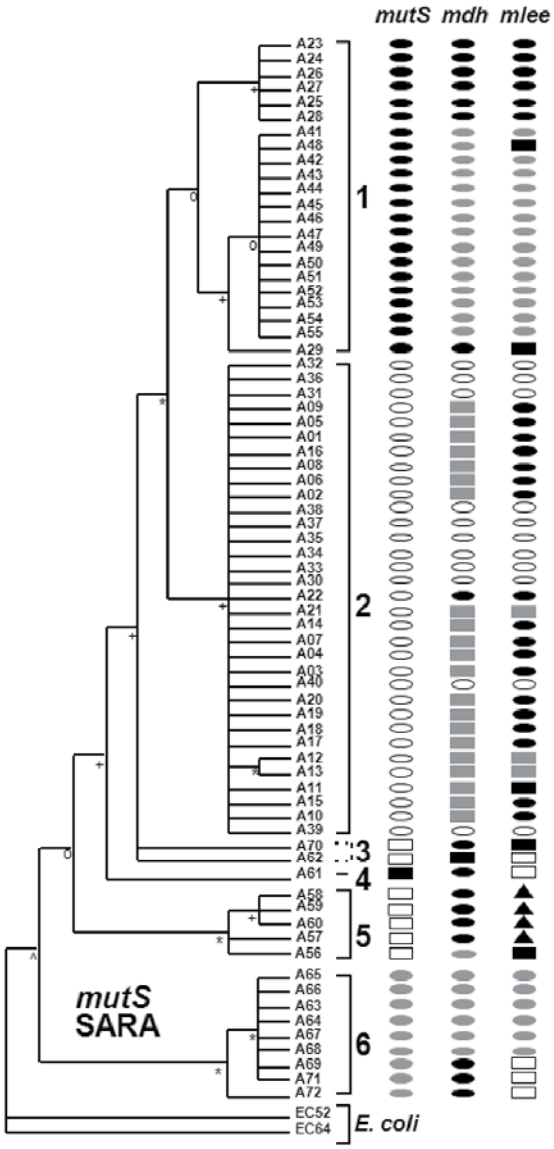

Fig. 4. Most-parsimonious relationships of SARA *mutS* alleles. *mutS* clades are bracketed and numbered to the right of the tree. Distributions of *mutS*, *mdh*, and MLEE clades are presented in column form. Note that strains originating from the same clade retain a common shape and common internal shading. Bootstrap nodal support values (Felsenstein et al., 1985) are presented on the *mutS* tree as follows: ^, 76-100%; *, 51-75%; +, 26-50%; o, 1-25%. In this case, *mdh* and MLEE are taken to represent the evolution of the strain in general (Boyd et al., 1994; Beltran et al., 1991). The tree shown is rooted with two *E. coli* outgroups.

3.2 Homogenization of *mutS* sequence diversity among *S*. Typhimurium and *S*. Heidelberg strains

Curiously, a single clade in the SARA *mutS* tree was found to comprise three distinct *Salmonella* serovars. In this clade, every strain representing *S*. Typhimurium (n=21) and *S*. Heidelberg (n=11), along with a single strain of *S*. Saintpaul, converged into a single evolutionary lineage of *mutS* alleles. In the SARA *mdh* tree (Fig. 5), *mdh* alleles for these same SARA serovars formed three disparate clades in the tree such that *S*. Typhimurium strains clustered only with other *S*. Typhimurium and *S*. Heidelberg strains only with other *S*. Heidelberg. *S*. Saintpaul strains formed a single lineage at the tip of the tree with strains of *S*. Muenchen and a single *S*. Paratyphi B. It should be noted that these distinct clades retained substantial statistical support with bootstrap values around 90% (Felsenstein, 1985). Thus, phylogenetic comparison of *mutS* and *mdh* sequences supported the notion that these serovars have converged into a single *mutS* clade, possibly as a result of the repeated HGT of only one or a few preferred *mutS* alleles.

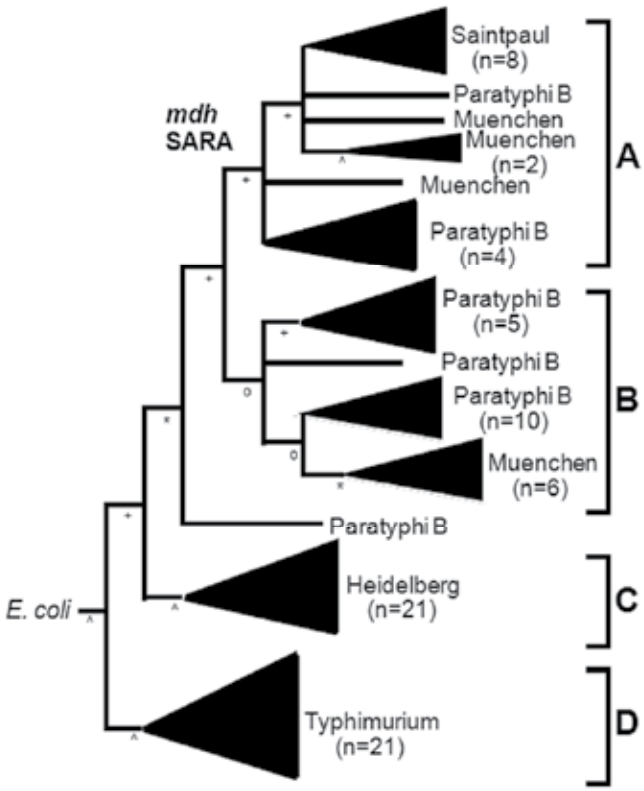

Fig. 5. Phylogenetic tree revealing the most-parsimonious relationships of SARA *mdh* alleles. *mdh* clades are bracketed and lettered while SARA serovars are labeled to the right of the tree. For sample sizes greater than one, multiple strains of the same serovar are depicted as a cone on the tree terminal nodes. Note that strains originating from the same clade are designated by a common bracket and letter. Bootstrap nodal support values are presented on the *mdh* tree as follows: ^, 76-100%; *, 51-75%; +, 26-50%; o, 1-25%. Note the bifurcations between specific clusters in the tree, signaling sequence diversity among distinct serovars using the *mdh* gene.

In order to further investigate the genetic structure of this converged clade, we examined *mutS* sequence homogeneity across the strains composing this lineage as well as the remaining *mutS* alleles of the SARA collection (Fig. 6). Evaluation of polymorphic positions in the *mutS* alignment revealed several findings consistent with homogeneous clade structure surrounding these serovars. First, five substitutions were observed across the entire 1,115 bp sequence for all 33 strains that define this *mutS* clade (#2). Second, with the exception of the polymorphism at position 913 in SARA strains 12 and 13, no clade #2 substitution was retained by more than one strain. Thus, none of the substitutions present within this clade partitioned any member serovar from another. The near structural uniformity of this clade at the nucleotide level further suggests that HGT has homogenized *mutS* alleles among these particular serovars. This is consistent with the thesis of Dykhuizen and Green (1991) who reminded that recombination can not only diversify the genome but can also homogenize it as well.

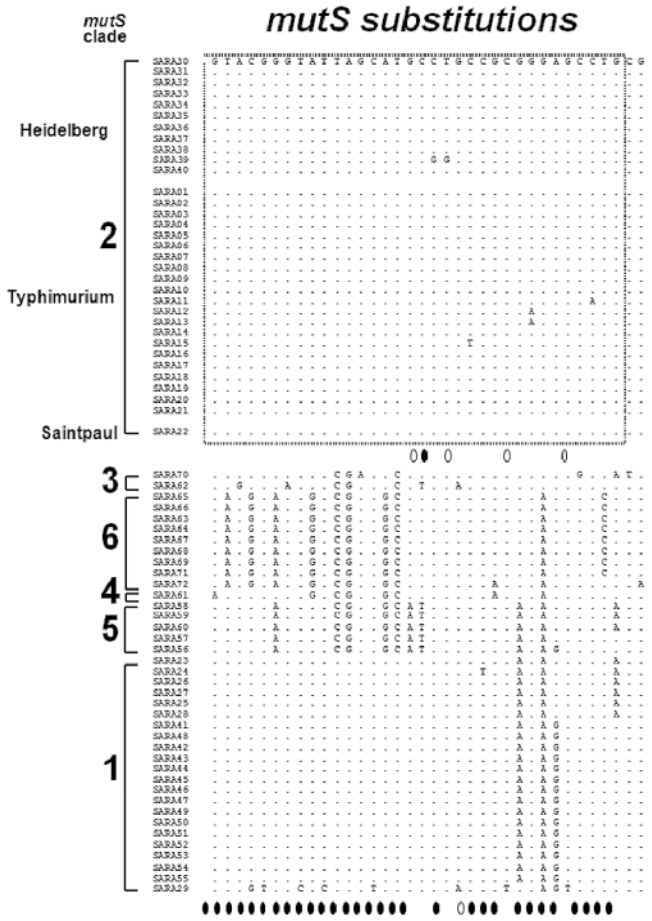

Fig. 6. *mutS* nucleotide sequence homogeneity among *S. enterica* serovars Typhimurium, Heidelberg, and a strain of Saintpaul. Periods indicate exact nucleotide identity to the reference sequence at the top of the alignment while listed nucleotides represent actual substitutions. The synonymous/nonsynonymous status (blackened ovals indicate synonymous change) of each substitution is noted below the alignment. Nucleotide sequences were generated using a PCR-based approach and automated CE-sequencing technology.

3.3 Distinct roles for HGT across various taxonomic tiers of *S. enterica*

With the inclusion of the SARA analysis reported here, we have been able to define varying roles for HGT across three taxonomically distinct populations of *S. enterica* (SARA, B, and C) (Fig. 7). Within *S. enterica* as a whole, a model for HGT begins to emerge that tolerates near-free HGT among closely-related subspecies I strains. As genetic divergence increases across serovars, however, the extent of HGT appears to decrease. The analysis reported here suggested two unique findings for SARA, the most genetically monomorphic population.

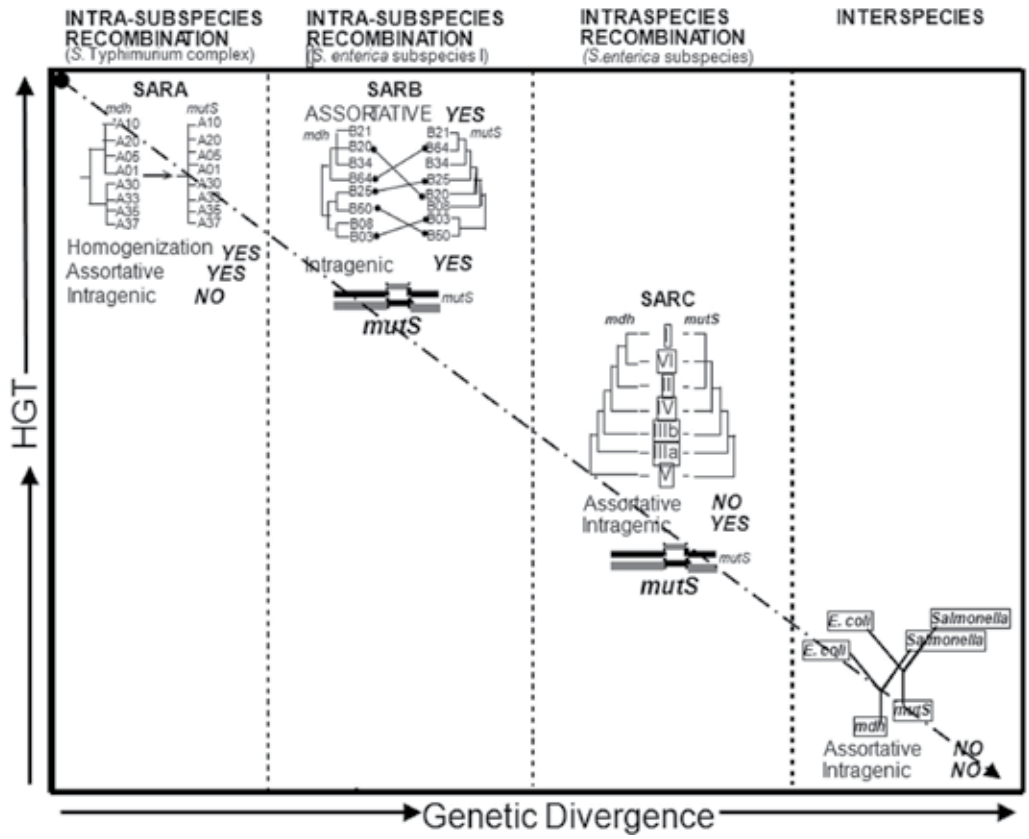

Fig. 7. Model for the frequency and effects of HGT among various taxonomic tiers of *Salmonella enterica*. Graphic representation of the various effects of HGT on the taxonomically distinct SARA, SARB, and SARC strain collections as well as an interspecies comparison. The *S. enterica* collections are plotted relative to genetic divergence versus the extent of HGT observed. Specific effects and trends associated with the HGT occurring at each taxonomic level are noted below each of the *Salmonella* populations shown.

First, SARA revealed evidence for a substantial convergence of *mutS* alleles between distinct serovars suggesting, that, recombination can have a homogenizing effect on sequence diversity in this population. Second, despite yielding numerous examples of assortative (allelic) exchange, SARA appears to be—at least from a phylogenetic perspective—refractory to intragenic (mosaic) HGT within the *mutS* gene. Thus, the SARA and SARB groups seem

to have been influenced more extensively by HGT than SARC possibly because they are not so diverged that exchange is inhibited due to extreme niche or R-M (restriction-modification) system variability. Moreover, it is also possible that much of the HGT among SARA strains have gone undetected here since identical alleles would leave no phylogenetic footprint following an exchange event.

3.4 Key observations

i. Horizontal gene transfer of *mutS* alleles in *Salmonella* appears to play a prominent role in the evolutionary structure of the five closely-related serovars representing the SARA ('Typhimurium' complex) collection, a finding consistent with extensive HGT that has been documented among subspecies I serovars in general (Brown et al., 2003).
ii. Cladistic analysis of SARA strains revealed the first example of a substantial convergence of *mutS* alleles from disparate serovars into a single clade. This suggests that HGT is homogenizing allele diversity among certain *Salmonella* strains and serovars—an observation reminiscent of allele homogenization observed for the *E. coli polA* gene (Patel and Loeb, 2000).
iii. Among closely related 'Typhimurium' complex strains, *mutS* alleles appear to have shuffled largely as single units rather than in intragenic segments. One explanation for this might be a more recent evolutionary divergence of the five serovars composing the highly homogeneous 'Typhimurium' strain complex. Alternatively, recombination of highly homologous mosaic segments of the *mutS* gene would do little to obscure phylogeny and likely go undetected in these analyses.
iv. Retrospective comparison of SARA HGT patterns with that of SARB and SARC strains yields a gradated model for HGT whereby different taxonomic tiers of *Salmonella* are subject to different HGT effects. The differences appear coupled to the extent of genetic diversity that defines these three different "tiers" of *Salmonella* population structure.

4. HGT among restriction-modification (R-M) genes of subspecies I salmonellae

The restriction and modification (R-M) system is a defense mechanism developed by bacteria to protect the bacterial genome from invasion by foreign DNA (Bullas et al., 1980). Foreign sequences entering the cell are cleaved by restriction enzyme(s), while the bacterial DNA itself is modified by methylase(s), thus providing protection from its own restriction enzyme (Murray, 2000). R-M systems are composed of genes that encode a specific restriction endonuclease and modification methylase. There are several types of R-M systems, namely type I (*e.g.*, *Eco*KI), type II (*e.g.*, *Eco*RI), and type III (*e.g.*, *Sty* LTI) (Barcus et al., 1995). Types of R-M systems are classified on the basis of their composition and cofactor requirements, the nature of the target sequence, and the site of DNA cleavage with respect to the target sequence (Murray, 2000; Naderer et al., 2002).

Compatibility of R-M systems among strains was proposed as one explanation to account for contrasting recombination rates (Brown et al., 2003). In this model, compatible R-M complexes would permit the successful transfer of larger gene segments among closely related *Salmonella* pathogens; crosses between strains with identical R-M systems would not be subject to restriction (Sharp et al., 1992). A gradation in the size limits of DNA segments exchanged would depend on the polymorphic character of R-M systems in natural strains.

Here, we investigate this model by examining the molecular evolutionary relationships of *hsd* genes encoding R-M complexes among closely related pathogenic *Salmonella* strains (*i.e.*, the 'Typhimurium' complex). If, indeed, *hsd* alleles are freely exchanged themselves among strains that display a substantial tolerance for HGT and recombination of diverged DNA sequences, then an explanation accounting for observed tolerance to extensive HGT begins to emerge for *S. enterica* group I serovars.

4.1 Evidence for HGT of R-M alleles among *Salmonella enterica* group I strains

DNA sequences from three *hsd* type I R-M genes were subjected to cladistic analysis. The resultant invasion gene phylogenies were then compared to phylogenetic groupings from the *mdh* gene and from the *Salmonella* MLEE data. Cladistic comparisons of *hsd* genes to markers of stable *Salmonella* chromosome evolution revealed several findings, and the data for *hsdS* is shown (Fig. 8). For *hsdS* section S1, SARA 56 is removed from neighboring strains when compared to *mdh* or *mutS*. For *hsdS* section S2, the collapsing of numerous clades into a single conserved clade was observed. It should be noted that such collapsing was observed in many of the trees reported here and suggests that HGT may be homogenizing *hsd* alleles. Moreover, a distinct allele that has no homology with its sister allele in a neighboring clade can be seen on the tree. Finally, the *hsdS* section S3 tree breaks up clades from both MLEE and *mdh*. In addition, this tree has three distinct allele types that can be seen phylogenetically, as in the case of S2.

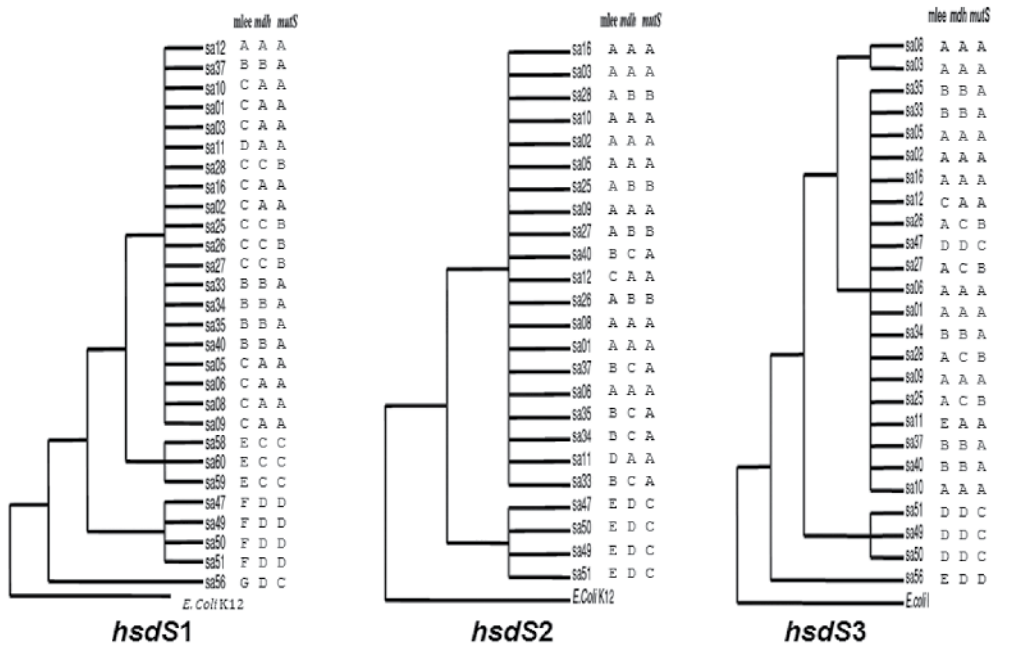

Fig. 8. Phylogenetic trees constructed for three segments comprising the *hsdS* gene, *hsdS*1, *hsdS*2, and *hsdS*3. Each respective gene tree is rooted with an *E. coli* outgroup and compared to MLEE, *mdh* and *mutS* clade patterns. Identical letters signal a common clade origin in the *mdh*, MLEE, or *mutS* datasets. Note that the letter designations from the *mdh*, MLEE, and *mutS* bar columns are independent of each other.

Compatibility among R-M systems has been proposed to account for the extensive levels of HGT documented among subspecies I *Salmonella* pathogens. Since the *mutS* gene appears to have been shuffled among this group of strains, we examined the phylogenetic relationship of *mutS* to type I R-M *hsd* genes. Incongruence was observed between *hsd* genes and *mutS* phylogeny, suggesting that patterns of HGT for *hsd* alleles differ from those for *mutS* alleles. *hsd* segments S1 and S3 each retained at least one incongruent strain between these gene phylogenies. In addition, several *hsd* genes collapsed divergent *mutS* clades into single *hsd* lineages in the trees. For instance, three *mutS* clades composed a single *hsdM* clade, a pattern that held true for other *hsd* genes, including *hsdS* segments 2 and 3. These data indicate distinct roles for HGT between most R-M genes and *mutS*. Nonetheless, observed homogenization of type I R-M loci among subspecies I *Salmonella* strains suggests they are compatible systems, allowing additional genes like *mutS* to be transferred within this population in its entirety.

4.2 Evidence for intra-operon HGT of R-M alleles

Intra-operon evolutionary incongruence between *hsd* genes was further examined using the ILD (incongruence length difference) test, which evaluates the likelihood of a common evolutionary history between genes. The ILD comparisons yielded more notable incongruence between genes than did the tanglegram analysis, suggesting that small patches of sequence within individual genes may be responsible for much of the observed incongruence. Intragenic patterns of HGT have been noted previously for more diverse subspecies (Brown et al., 2002). In the ILD comparisons (Fig. 9), eight of the ten *hsd* data set comparisons yielded significant

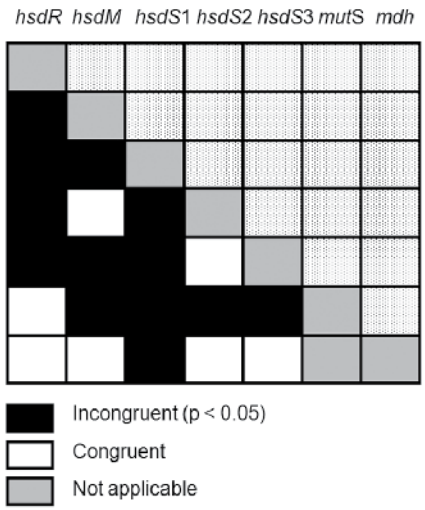

Fig. 9. Pairwise incongruence length difference (ILD) test results for several genes of the Type I Restriction – Modification system in *Salmonella*. ILD tests were performed with 1000 partitions using the Partition Heterogeneity command in PAUP*v.10 (Swofford et al., 2002). Pairwise ILD comparisons were made among the three *hsd* genes R, M, and S including the three sub-regions that were amplified from *hsdS* (*i.e.*, S1, S2, and S3). As in Fig. 3, a p-value of 0.05 or less allows for a rejection of the null hypothesis of congruence (vertical evolution) and accepts the alternative hypothesis of incongruence which is interpreted among bacterial phylogeny as evidence for HGT (LeCointre et al., 1998).

ILD scores ($p < 0.05$) such that a hypothesis of congruence could be rejected for these intragene comparisons. The only exceptions were the *hsdS2-hsdM* comparison ($p = 1.00$) and the *hsdS3-hsdS2* comparison ($p = 1.00$). It is noted that, with the exception of *hsdR*, all of the *hsd* data matrices were also incongruent with *mutS*. When examined in total, the data suggest that the Type-1 R-M operon is a mosaic comprising *hsd* gene sequences that have converged evolutionarily within this operon, but with each possessing a unique phylogenetic path.

It was also noteworthy that *hsdS* segments S2 and S3, however, retained groups of alleles that shared little or no identified homologies. That is, *hsdS2* yielded two unique sequence cassettes, one of which was found in strains of serovar *S.* Paratyphi B in the SARA complex. *hsdS3* yielded three distinct cassette types within the alignment, all of which shared no homology with their counterparts. A cassette retained by SARA strain 56 showed homology to an *hsdS* variant in *E. coli*, suggesting that this sequence has resulted from HGT between these lineages. The other unique cassette, retained by SARA strains 49, 50, and 51, showed no homology to any other *hsd* sequence, indicating that it may be been transferred into *S.* Paratyphi B from a yet unidentified source. The examples of unique cassette formation within this gene reinforce the role that HGT has played in the intra-operon and intragenic evolution of the Type-1 R-M gene system. These data also reveal the exchangeable nature of *hsd* gene sequences in these loci as a result of HGT.

4.3 Key observations

i. These findings demonstrate several instances for the three *hsd* loci encoding the type I R-M operon in *Salmonella* to be decoupled phylogenetically from the chromosomes of group I *Salmonella* strains (*i.e., mdh* and MLEE), suggesting that certain alleles from these genes have been shuffled by HGT between closely related *S. enterica* strains.
ii. The *hsd* operon of S. enterica subspecies 1 appears to have undergone intra-operon structuring due to HGT, producing an evolutionary mosaic in the *hsd* region.
iii. The lack of homology within *hsdS* indicates that these specific segments may have been acquired from distantly related bacterial species. An aberrant GC content for *hsdS* of 41%, a value far removed from an average value for enteric bacterial genomes of 56%, reinforces this conclusion.
iv. The data demonstrate that HGT has been a common occurrence in *hsd* gene evolution and point to a genetic compatibility among closely-related salmonellae for exchange of *hsd* alleles that appears to resemble a panmictic genetic structure among these closely related strains. This may explain, in part, why *Salmonella* known to share homologous genomes and common niches more freely exchange DNA.

5. Discussion and conclusions

In summary, substantial phylogenetic evidence has been presented for the horizontal transfer of *mutS* alleles within a pathogenically homogeneous group of subspecies I *Salmonella enterica* pathogens. Of note, is the observation that *mutS* clades appear to be undergoing homogenization within the 'Typhimurium' strain complex as a result of the repeated HGT of only a few preferred alleles. Moreover, examination of R-M loci revealed that subspecies I *Salmonella* readily exchange *hsd* genes. These findings support the notion that R-M compatibility may be, in part, responsible for the substantial tolerance of HGT and recombined DNA between subspecies I strains.

An overwhelming body of evidence has been compiled that documents the reticulate evolutionary nature of the *Salmonella mutS* gene and its surrounding sequences. In an analysis of nearly 200 different strains documented here and in several previous reports over the past decade (LeClerc et al., 1998; Brown et al., 2002; 2003; Kotewicz et al., 2002; 2003), our laboratory has demonstrated the extent, the chromosomal effects, and the evolutionary history of HGT events that have scrambled this part of the genome in *S. enterica*. Exhaustive phylogenetic comparisons have been brought to bear on *mutS* sequences using various chromosomal markers including MLEE, rDNA, several individual housekeeping genes, and an *a priori* prior agreement MLST data based on concatenation of a three-gene supermatrix (Brown et al., 2002). Puzzling then was a later report that argued a more nonremarkable evolutionary pattern for *mutS* stating, "*mutS* is not more recombinogenic than the other genes" (Octavia and Lan, 2006). The authors based this conclusion solely on a modest 15 strain set of subspecies I salmonellae. Albeit, it remains to be seen to what extent additional homogeneous *Salmonella* populations retain the phylogenetic vestiges of horizontally transferred *mutS* and *hsd* alleles. Whatever the final outcome, it is apparent that horizontal transfer has played a prominent role in the current evolutionary structure of *mutS* and many other genes with virulence, resistance, stress-response, and general housekeeping function, all underscoring recombination as a key mechanism in the generation of genetic diversity among these closely related salmonellae.

Roughly two decades ago, *Salmonella enterica* was regarded as one of only a few eubacterial species that maintained a "truly clonal" evolutionary structure (Selander *et al.*, 1990; 1996; Reeves et al., 1989). Today, armed with whole-genomic analysis, it is now clear that horizontal transfer has shaped and honed unique evolutionary histories for numerous genes, operons, and islands within the *Salmonella* chromosome. With the complete genome sequences of dozens of *Salmonella* now available and countless more underway, such analyses of congruence should aid in determining the extent to which recombination has disrupted clonality throughout the entire *Salmonella* chromosome. Certainly, a greater recognition of precisely how HGT has forged the genomes of *Salmonella* pathogens should enhance the accuracy of risk assessment strategies for these bacteria as well as provide avenues for better detection and characterization of this devastating foodborne pathogen.

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13

The Importance of Mobile Genetic Elements in the Evolution of *Salmonella*: Pathogenesis, Antibiotic Resistance and Host Adaptation

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1. Introduction

Since the divergence from *Escherichia coli*, more than 100 million years ago, *Salmonella* has acquired by lateral gene transfer a repertoire of genes that confers a set of physiological features that define its particular ecological niche (Ochman and Groisman 1994; Ochman et al., 2000). Some of these chromosomally-encoded genes can be considered as part of the "core genome" of *Salmonella* (i. e. genes present in all the strains), whereas some other chromosomally-encoded genes are part of the "accessory genome" (i. e. genes present in some of the strains) along with mobile genetic elements such as plasmids, bacteriophages, transposons and integrons. In this chapter we review the role that mobile genetic elements have played in *Salmonella* evolution, particularly in pathogenicity attributes, antibiotic resistance and host adaptation.

2. Pathogenicity islands

Pathogenicity islands are large genomic regions that are present in pathogenic variants but less frequently present in closely related non-pathogenic bacteria. They often carry virulence-associated genes, have a G+C content that differs from that of the rest of the chromosome, are frequently associated with tRNA genes and are flanked by repeated sequences (Dobrindt et al., 2004). *Salmonella* pathogenicity islands (SPIs) are large gene cassettes within the *Salmonella* chromosome that encode determinants responsible for establishing specific interactions with the host, and are required for bacterial virulence in a given animal. Like other pathogenicity islands, SPIs generally have a G+C content lower (between 37 and 47%) than the rest of the bacterial chromosome (about 52%), and are often inserted into tRNA genes. Therefore, SPIs have likely been acquired by horizontal transfer from bacteriophage or plasmids of unknown origin, and they are highly conserved between the different *Salmonella* serovars. It is sometimes unclear how certain DNA regions are designated as pathogenicity islands. Small DNA regions (often single genes), which are acquired horizontally, are numerous and their distinction from pathogenicity islands is in some cases arbitrary (Marcus et al., 2000). More than twenty SPIs have been described (Sabbagh et al., 2010). Most SPIs have become part of the core set of genes of *S. enterica* and

encode species-specific traits. A smaller subset of SPIs is limited to certain subspecies or serovars. These SPIs harbor genes associated with DNA mobility and are likely to represent more recent acquisitions (Hensel 2004). SPI1 and SPI2 both encode type III secretion systems (TTSS), which mediate the respective virulence phenotype by translocating bacterially-encoded proteins into the host cell cytoplasm (Hansen-Wester and Hensel 2001). TTSS are used by many bacterial pathogens to deliver virulence factors to the host cell and interfere with or subvert normal host cell signaling pathways. They consist of many components, including more than twenty proteins, some of which are homologous to those involved in flagellar assembly. Effector proteins generally require specific chaperones which prevent incorrect folding, degradation and premature association, and may even aid delivery of the effector into host cells. These systems are highly regulated, and proteins are only secreted when the bacteria sense specific environmental cues (Marcus et al., 2000; Cornelis 2006). A remarkable observation is the functional interaction of SPI1 and SPI2 with further loci encoding effector proteins, including SPI5 and some mobile DNA elements (Hensel 2004).

3. *Salmonella* genomic island

A chromosomal island called *Salmonella* genomic island 1 (SGI1) was initially described in the epidemic multiple-drug-resistant (MDR) *Salmonella enterica* serovar Typhimurium phage-type DT104 (Boyd et al., 2001). In this respect, phage-typing is carried out by infecting a *Salmonella* isolate with a number of phages listed in the phage-typing scheme for a specific serovar (Kropinski et al., 2007). The present phage-typing scheme for Typhimurium consists of 34 phages and identifies 207 phage types, referred to as definitive types (DT) (Anderson et al., 1977). Since the original identification of SGI1 in Typhimurium DT104, variants of SGI1 have been described in a wide variety of *Salmonella* serovars (Levings et al., 2005). The 43 kb SGI1 contains 44 open reading frames (ORFs), many of them without homology to known gene sequences. In all the serovars, SGI1 showed the same chromosomal location. In the first part of the island, a number of ORFs showing homology to plasmid-related genes are present. The 13 kb-antibiotic resistance gene cluster is located near the 3′ end of SGI1 (Levings et al., 2005). The MDR cluster confers resistance to streptomycin and spectinomycin, sulfonamides, chloramphenicol and florfenicol, tetracyclines, and beta-lactam antibiotics. The G+C content for the MDR cluster is 59%, while for the rest of the SGI1 it is 49%, as compared with 52% for the Typhimurium chromosome, suggesting a mosaic structure (Boyd et al., 2001). SGI1 is an integrative mobilizable element which contains a complex class 1 integron (see below) named In104, located within the antibiotic resistance cluster located at the 3′ end of the island (Boyd et al., 2001; Doublet et al., 2005; Mulvey et al., 2006). In 2005, Doublet et al. reported that SGI1 could be conjugally transferred from an *S. enterica* donor to *E. coli* recipient strains where it integrated into the recipient chromosome in a site-specific manner (Doublet et al., 2005). First, an extra-chromosomal circle of SGI1 was formed, and this circular intermediate was transferred in the presence of an IncC helper plasmid, which provided the mating apparatus. This study demonstrated that the mobilization of SGI1 probably contributes to the spread of antibiotic resistance genes between *S. enterica* serovars and possibly to other bacterial pathogens (Doublet et al., 2005). As predicted by Doublet et al. (2005), a variant of SGI1 has been reported in *Proteus mirabilis* (Boyd et al., 2008). Moreover, a recent report showing the ability of IncA/C plasmids to mobilize SGI1 has implications for the worldwide spread of these MDR elements (Douard et al., 2010).

4. Antimicrobial resistance and virulence plasmids

A substantial amount of the variation in bacteria is due to the presence of plasmids (Levin and Bergstrom 2000). Plasmids are part of the flexible genome, which is defined by the high plasticity and modularity of its genetic elements and high rates of gene acquisition and loss (Heuer et al., 2008). They are typically composed of conserved backbone modules coding for replication, maintenance and transfer functions as well as variable accessory modules. The capture of genetic modules by plasmid backbones can increase phenotypic diversity and thereby increment the chances of responding to uncertain environmental changes or exploit an opportunity for niche expansion (Souza and Eguiarte 1997; Frost et al., 2005; Heuer et al., 2008; Norman et al., 2009). Often, antimicrobial or heavy metal resistance, or virulence factors that allow their bacterial host to adapt to changing environments are encoded by plasmids. Plasmids are classified according to incompatibility (Inc) groups, that are based on the inability of plasmids with the same replication or segregation mechanisms to co-exist in the same cell (Couturier et al., 1988). Plasmids of *Salmonella enterica* vary in size from 2 to more than 200 kb. The best described plasmids are the so-called virulence plasmids present in some serovars. Another group of high molecular weight plasmids are responsible for antibiotic resistance, which are in most of the cases conjugative, contributing to the spread of genes in bacterial populations (Rychlik et al., 2006). The low molecular weight multi-copy plasmids are widespread in *Salmonella*, but are less studied and are referred as cryptic, although some of them have been shown to increase resistance to bacteriophage infection due to the presence of modification systems (Rychlik et al., 2006).

Eight *Salmonella* enterica serovars harbour a large (50-285 kb) plasmid named the *Salmonella* virulence plasmid, containing the *spv* operon, which is a major determinant of virulence in their specific hosts (Gulig et al., 1993; Chiu et al., 2000; Fierer and Guiney 2001). In addition to the *spv* operon, other plasmid genes are involved in virulence. The *rsk* and *rck* genes are required for serum resistance, and *traT*, a surface exclusion protein for plasmid transfer, is also responsible for serum resistance (Chu and Chiu 2006). Within a single serovar some strains can carry the virulence plasmid while others not (Olsen et al., 2004). Despite many common properties shared by these plasmids, each virulence plasmid seems to be specific to its serovar, but the outcome of the infection in different animal hosts may be variable. For example, Typhimurium strains that harbour the virulence plasmid are highly virulent to mouse, but there is lack of evidence of an association between the carriage of virulence plasmid and the bacteremia caused in humans (Chiu et al., 2000). Whether the virulence plasmid is necessary to produce systemic infections in humans has been subject of intense debate. Some authors claim that there is lack of evidence of an association between the carriage of the virulence plasmid and human bacteremia (Chiu et al., 2000). Other authors suggest that *spv* genes promote the dissemination of Typhimurium from the intestine (Fierer 2001). In recent reports contrasting results have been found. We studied more than 100 Typhimurium strains isolated from human and food-animal sources in Mexico, and found that only 30% of the strains harboured the plasmid (Wiesner et al., 2009). The presence of the virulence plasmid was significantly associated with human isolates, but only one of the six isolates recovered from patients with systemic infection had the virulence plasmid. Our data support the notion that the virulence plasmid has a role in host adaptation (Baumler et al., 1998); however, it was not consistent with the view that it is associated with systemic infection in humans (Wiesner et al., 2009). In a recent study, Litrup et al. (2010) analyzed 21 Typhimurium strains isolated from patients with mild and sever infections with the aim of

correlate genomic content with the outcome of disease. They used a DNA microarray targeting 281 known virulence factors, and found that the presence or absence of the virulence plasmid did not correspond to disease symptoms (Litrup et al., 2010). On the other hand, Heithoff et al. (2008) found that all the Typhimurium strains isolated from animals or humans with bacteremia possessed the virulence plasmid, while 34% of the strains isolated from human gastroenteritis lacked the plasmid (Heithoff et al., 2008). These contrasting results highlight the complex nature of specific host-pathogen interactions, and call to avoid making generalizations since the diversity of environmental (biotic and abiotic), host, and bacterial genetic conditions may produce different outcomes.

Large antimicrobial resistance plasmids are of public health concern. The global scene is that the therapeutic options for MDR microbes are reduced, periods of hospital care are more extended and costly and, in some cases, the strains have also acquired increased virulence and enhanced transmissibility. Realistically, antibiotic resistance can be considered a virulence factor (Davies and Davies 2010). Resistance mechanisms are pandemic and create an enormous clinical and financial burden on health care systems worldwide (Davies and Davies 2010). The resistance genes found in *Salmonella* are closely related to, or are indistinguishable from, those found in other bacteria, including not only members of the *Enterobacteriaceae* but also distantly related bacteria. It is most likely that *Salmonella* acquired these genes from other bacteria, and probably *Salmonella* strains also play a role in the further dissemination of these resistance genes to other bacteria (Michael et al., 2006). Frequencies of conjugative transmission in nature are probably several orders of magnitude higher that those observed under laboratory conditions, and occur readily in networks of multi-host interactions (Dionisio et al., 2002; Sorensen et al., 2005; Davies and Davies 2010).

The IncA/C plasmids exemplify the problematic of resistance plasmids in *Salmonella*. They have attracted the attention of the research community due to their ability to acquire antimicrobial resistance traits and to mobilize across geographical and taxonomical borders (Fricke et al., 2009). Recent comparative studies have addressed the evolutionary relationships among the IncA/C plasmids from *Salmonella enterica*, *Escherichia coli*, *Yersinia pestis*, *Yersinia ruckeri*, *Vibrio cholera*, *Photobacterium damselae* and *Aeromonas salmonicida* (Welch et al., 2007; Kim et al., 2008; McIntosh et al., 2008; Pan et al., 2008; Fricke et al., 2009; Call et al., 2010). The genomic comparison of seven IncA/C plasmids showed that these plasmids share a common backbone, including the origin of replication and a conjugative plasmid transfer system (Welch et al., 2007; Fricke et al., 2009). Several loci containing antimicrobial resistance determinants are distributed along the plasmids, and are integrated at few sites within the conserved plasmid backbone; they are generally located as resistance gene arrays, composed of resistance genes and mobile genetic elements such as insertion sequences, transposons or integrons (Fricke et al., 2009). For example, in the IncA/C plasmids of *Yersinia pestis* and *Salmonella* Newport, a Tn*21* transposon is inserted in a similar location but some nucleotide divergence is evident and its orientation is reversed (Welch et al., 2007; Fricke et al., 2009). These studies suggest an evolutionary model in which each IncA/C plasmid diverged from a common ancestor, through processes of stepwise integration events of horizontally-acquired resistance genes arrays (Welch et al., 2007; Fricke et al., 2009).

Over the last decade, increasing attention has been focused on plasmids that harbour the antimicrobial resistance gene $bla_{\text{CMY-2}}$, which encodes an AmpC-type beta-lactamase that hydrolyzes third-generation cephalosporins (Bauernfeind et al., 1996; Zhao et al., 2001;

Carattoli et al., 2002; Wiesner et al., 2009; Wiesner et al., 2011). In *Salmonella enterica,* bla_{CMY-2} is frequently carried by IncA/C or IncI1 plasmids (Bauernfeind et al., 1996; Carattoli et al., 2002; Hopkins et al., 2006; Lindsey et al., 2009). In a recent study, Call et al. (2010) analyzed five *E. coli* and *Salmonella* Newport IncA/C plasmids carrying bla_{CMY-2}, and showed that although they share a common ancestor with the *Yersinia* and *Photobacterium* plasmids, they are genetically distinct (Call et al., 2010). In a population study we found that IncA/C plasmids were associated to the Mexican Typhimurium ST213 genotype. We determined that the bla_{CMY-2} gene was carried in IncA/C plasmids, and genetic variability was observed using a plasmid typing scheme, targeting ten conserved regions in IncA/C plasmids (Wiesner et al., 2009; Wiesner et al., 2011). The Typhimurium bla_{CMY-2}-bearing IncA/C plasmids possessed most of the accessory elements found in other *Salmonella* and *E. coli* plasmids (Call et al., 2010), but also more than half contained a class 1 integron (*dfrA12-orfF-aadA2*). The screening of the total Mexican Typhimurium population showed the presence of another IncA/C plasmid harboured by ST213 strains, yet lacking bla_{CMY-2}. These plasmids also carried antibiotic resistance determinants, but they shared only three of the ten genetic markers used to study the IncA/C plasmids, and were smaller than the bla_{CMY-2}-bearing IncA/C plasmids (100 vs. 150-160 kb). Nevertheless, the nucleotide sequences of the regions shared with the bla_{CMY-2}-bearing IncA/C plasmids were identical, suggesting that the bla_{CMY-2}-bearing plasmids could be the result of the insertion of DNA modules into this smaller precursor plasmid (Wiesner et al., 2009; Wiesner et al., 2011). The general agreement from the analysis of the genetic structure of the IncA/C group,is that plasmid evolution progresses faster through the insertion/deletion of DNA stretches rather than by point mutations (Welch et al., 2007; Kim et al., 2008; Fricke et al., 2009; Call et al., 2010; Wiesner et al., 2011).

Large resistance plasmids circulate among microbial populations in distinct environmental niches, even in the absence of antibiotic selective pressure. In other environments the target of the selective pressure could be, for example, mercury resistance carried by many transposons, such as Tn*21* (Liebert et al., 1999; McIntosh et al., 2008). Regardless of the primary selective agent, the complete battery of resistance determinants will be maintained, imposing a global health risk (Liebert et al., 1999; Frost et al., 2005; Welch et al., 2007; McIntosh et al., 2008; Pan et al., 2008; Davies and Davies 2010). Another worrisome situation is the emergence of *Salmonella* virulence-(antibiotic) resistance plasmids. Several studies had reported large hybrid virulence-resistance plasmids in serovars Typhimurium, Choleraesuis and Enteritidis, isolated from Spain, Italy, Czech Republic, Taiwan and the United Kingdom (Chu et al., 2001; Guerra et al., 2001; Guerra et al., 2002; Guerra et al., 2004; Villa and Carattoli 2005; Chu and Chiu 2006; Herrero et al., 2008a; Herrero et al., 2008b; Hradecka et al., 2008; Herrero et al., 2009; Rodriguez et al., 2011). In some of the studies it was demonstrated that the hybrid plasmids were conjugative, which may lead to their spread to new recipients and allow the co-selection of the antibiotic and virulence genes, representing a hazard to human and animal health (Fluit 2005).

5. Integrons

Integrons are assembly platforms that incorporate genes by site-specific recombination and convert them to functional genes by ensuring their correct expression. They are composed of three key elements: a gene encoding an integrase, a primary recombination site, and a promoter that directs the transcription of the captured genes. The integrase can recombine discrete units of circularized DNA known as "gene cassettes"; they are transcribed only

when captured into an integron, since most of them lack a promoter. Integration occurs downstream of the resident promoter, at the primary recombination site, allowing the expression of the genes in the cassette (Figure 1). The integron inserted gene cassettes share specific structural characteristics and contain an imperfect inverted repeat at the 3′ end, called the "59-base element". This site functions as a secondary recognition site for the site-specific integrase, and can further integrate gene cassettes. The ability to capture disparate individual genes and to physically link them in arrays suitable for co-expression is a trait unique to integrons, and theoretically facilities the rapid evolution of new phenotypes (Stokes and Hall 1989; Recchia and Hall 1995; Fluit and Schmitz 1999; Holmes et al., 2003; Fluit and Schmitz 2004; Mazel 2006; Boucher et al., 2007; Joss et al., 2009).

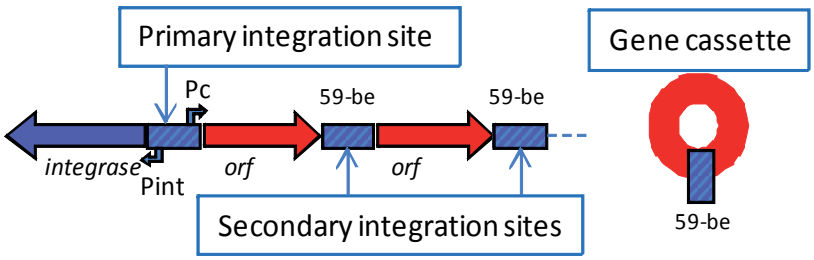

Fig. 1. Diagrammatic representation of the basic features of an integron. Integrons consist of the gene for the integrase, the promoters for the expression of the integrase (Pint) and the gene cassettes (Pc), and the primary recombination site, where the cassettes are integrated. Gene cassettes consist of a single promoter-less gene and a recombination site known as a 59-base element (59-be), which functions as a secondary recognition site for the site-specific integrase, and can further integrate gene cassettes (Stokes and Hall 1989; Hall and Collis 1995; Levesque et al., 1995). An integron carrying an array of two inserted cassettes, and a free circularized gene cassette is shown.

Analyses on the diversity of gene cassettes in environmental samples have shown a great diversity of predicted genes, suggesting that essentially any DNA-encoded function may be contained within a gene cassette (Stokes et al., 2001; Holmes et al., 2003). However, the main part of the gene cassettes found in mobile integrons from cultured bacteria contain antibiotic-resistance determinants (Levesque et al., 1995; Fluit and Schmitz 1999, 2004). Class 1 integrons are found extensively in clinical isolates, and most of the known antibiotic resistance gene cassettes belong to this class (Fluit and Schmitz 2004). Although gene cassettes conferring resistance to nearly every major class of antibiotics have been identified, there are some antibiotic resistance determinants that are preferentially associated to integrons, such as streptomycin, trimethoprim, sulfafurazole, and the early aminoglycosides (White et al., 2001; Fluit and Schmitz 2004). Moreover, it has been reported that MDR is associated significantly with the presence of integrons (Leverstein-van Hall et al., 2003; Fluit 2005; Wiesner et al., 2009). Integrons themselves are not mobile although they can be part of transposons, which are capable of moving from one carrier replicon to another (Fluit and Schmitz 1999; Liebert et al., 1999). A well-known example are the integrons found in Tn*21* and related transposons. These transposons generally are located on plasmids, which further enhances the spread of gene cassettes (Fluit and Schmitz 1999; Liebert et al., 1999). The association of integrons with mobile elements and resistance genes has led to their rapid dispersal among various bacteria found in environments exposed to antibiotics (Martinez-Freijo et al., 1999; White et al., 2001; Boucher et al., 2007).

Since the discovery of the importance of integrons in the dissemination of antibiotic resistance, many studies have addressed the presence of integrons in *Salmonella*. At the moment of writing there were more than 300 research papers regarding integrons in *Salmonella* (http://www.ncbi.nlm.nih.gov/pubmed). An overview of these studies shows that integrons have been detected in many different *Salmonella* serovars (i. e. Agona, Albany, Anatum, Braenderup, Branderburg, Bredeny, Brikama, Derby, Dublin, Emek, Enteritidis, Eppendorf, Goldcoast, Grumpensis, Hadar, Haifa, Heildelberg, Infantis, Javiana, Kedougou, Kentucky, Kingston, Krefekd, Mbandaka, Muenster, Newport, Panama, Paratyphi B, Rissen, Rough, Saintpaul, Schwarzengrund, Stanley, Senftenberg, Tees, Tshiongwe, Typhimurium, Virchow, Weltevreden, Wien and Worthington), that were isolated from diverse sources (i. e. animal feed, beef, chicken, camel, environment, feline, foodstuff, goat, horse, human, milk, pork and turkey), and from countries all around the world (i. e. Albania, Algeria, Brazil, China, Chile, England, Ethiopia, Germany, Great Britain, Iran, Ireland, Italy, Japan, Lithuania, Mexico, Portugal, Slovak Republic, Spain, Thailand, The Netherlands, United States and Vietnam). Among the most studied cases are the chromosomally-located integrons present in the SGI1. There is a great diversity of integron cassettes detected in *Salmonella*, most of them encoding antibiotic resistance genes, carried in a wide variety of cassette arrays. It is noteworthy that similar cassette arrays are found in different *Salmonella* serovars isolated from different sources and distant countries, and even in other bacterial species. For example, in the study of the integrons present in a Mexican Typhimurium population (Wiesner et al., 2009), we found that the two most abundant integrons (*dfrA12-orfF-aadA2* and *dfrA17-aadA5*) were reported for other *Salmonella* serovars (Anatum, Branderup, Brikama, Enteritidis, Mbandaka, Rissen and Saintpaul) and in other *Enterobacteriaceae* (Lindstedt et al., 2003; Antunes et al., 2006; Su et al., 2006; Molla et al., 2007; Zhao et al., 2007). More surprising was that these integrons were also found in different species of the Gram-positive genera *Staphylococcus* isolated in China (Xu et al., 2008), providing evidence of the successful spread of these integrons around the world and across bacterial phyla (Wiesner et al., 2009).

6. Bacteriophages

Although bacteriophages carrying antibiotic resistance genes have rarely been identified (Davies and Davies 2010), their role in the dissemination of virulence factors has been widely documented (Boyd and Brussow 2002). Bacteriophage-encoded virulence factors can convert their bacterial host, in a process known as phage conversion, from a non-pathogenic strain to a virulent strain or a strain with increased virulence. The phage-encoded proteins involved in lysogenic conversion provide mechanisms to invade host tissues, avoid host immune defenses, and damage host cells (Boyd and Brussow 2002). The extra genes present in prophage genomes which do not have a phage function, but may act as fitness factors for the bacteria, are termed "morons". The moron-encoded genes are not required for the phage life cycle. Their expression is controlled by an autonomous promoter and, thus, can be expressed while the rest of the prophage genes remain silent (Hendrix et al., 2000). Morons enhance phage replication indirectly since moron-encoded functions enhance fitness of the lysogen. This hypothesis provides the theoretical framework for phage-mediated horizontal transfer of fitness factors between bacteria (Hendrix et al., 2000). The ecological success of a lysogenic bacterium contributes to the dissemination of phage genes, providing a case of co-evolution of viruses and bacteria. It has been hypothesized that the driving force behind the emergence of new epidemic clones is the phage-mediated re-assortment of virulence and

fitness factors, optimizing the *Salmonella*-host interaction (Mirold et al., 1999; Figueroa-Bossi et al., 2001; Brussow et al., 2004). The contribution of phages to *Salmonella* evolution is one of the best documented cases, and many phage-encoded virulence factors have been documented (Table 1). In particular, the functional biology of many phage-encoded genes has been studied in detail for Typhimurium strains.

Phage	Gene	Protein	Function in virulence
Fels-1	*sodC-III*	Superoxide dismutase	Intracellular survival
Fels-1	*nanH*	Neuraminidase	Intracellular survival
Gifsy-1	*gogB*	Type III efector	Involved in invasion
	gipA	IS-like	Critical for survival in Peyer´s patches
Gifsy-2	*sseI (gtgB)*	Type III efector	Involved in invasion
	sodC-I	Superoxide dismutase	Intracellular survival
	gtgE	Type III efector	Required for full virulence
Gifsy-3	*sspH1*	Type III efector	Involved in invasion
	pagJ	*phoPQ*-activated gene	Bacterial envelope for invasion
Fels-1 and Gifsy-2	*grvA*	Antivirulence gene	Decreases the pathogenicity in the host
P22	*gtrB*	Glucosyl transferase	O-antigen conversion
	gtrA	Flippase	O-antigen conversion
SopEΦ	*sopE*	Type III efector	Involved in invasion
ε34	*rfb*	Glucosyl transferase	Altering antigenicity

Table 1. Bacteriophage-encoded virulence factors of *Salmonella enterica* (Figueroa-Bossi et al., 2001; Boyd and Brussow 2002; Porwollik and McClelland 2003; Ehrbar and Hardt 2005; Kropinski et al., 2007).

Prophages contribute significantly to the diversity among *Salmonella* strains (Boyd and Brussow 2002), and different Typhimurium strains harbor distinct sets of prophages (Figueroa-Bossi et al., 2001; Mirold et al., 2001; Mmolawa et al., 2002). Most of them belong to the P2 family (SopEΦ, Fels-1, and Fels-2) or the lambda family (GIFSY-1, GIFSY-2, GIFSY-3 and P22). Several of the Typhiumurium prophages encode the so-called type three effector proteins, which are injected by the bacterium into animal cells via a type three secretion system (TTSS) (Ehrbar and Hardt 2005). These effector proteins manipulate signal transduction pathways of the cells, which provoke a strong intestinal inflammation and diarrhea. The SopE effector, encoded by SopEΦ, is one of the better studied cases. It is injected into the intestinal cells by the TTSS encoded by SPI-1, and its expression is co-regulated with other genes. The proper timing of SopE expression and delivery into the host cell depends on the regulatory circuits of SPI-1 (Mirold et al., 1999; Brussow et al., 2004; Ehrbar and Hardt 2005). Since the earlier studies, Mirold et al. (1999) demonstrated that SopEΦ is capable of infecting a range of Typhimurium strains (Mirold et al., 1999). In an experimental study, it was demonstrated that the lysogenic conversion of the laboratory Typhimurium strain ATCC14028 with SopEΦ provided increased enteropathogenicity compared with the wild-type strain (Zhang et al., 2002). Thus, it was shown that the horizontal transfer of phage-mediated genes may contribute to the emergence of more pathogenic epidemic clones. Moreover, Mirold et al. (2001) provided evidence for the

transfer of the SopE cassette between lambda and P2-like phages families (Mirold et al., 2001). They proposed that the transfer of virulence factors between phages increases the flexibility of the re-assortment of effector protein repertories, by circumventing restrictions imposed by immunity functions or the occupancy of the attachment sites by resident prophages (Mirold et al., 2001). By this mean, phages would contribute a great deal to the evolution of bacterial pathogens, and might explain the rapid emergence of new epidemic clones and the ability of *Salmonella* to adapt to a broad range of hosts.

The development of genome-based methods such as microarrays, and the tools to compare complete genome sequences, has opened a new era in the study of *Salmonella* evolution. Several studies have addressed the importance of bacteriophages in the evolution of *Salmonella*, and the role of prophage-encoded virulence factors in pathogenicity. Comparison of complete genomes have pointed out that the prophage content is one of the most important differences between genomes of *Salmonella* serovars, and specially among strains within a single serovar (McClelland et al., 2001; Parkhill et al., 2001; Porwollik et al., 2002; Porwollik et al., 2004; Thomson et al., 2004; Hermans et al., 2005; Cooke et al., 2007; Vernikos et al., 2007; Litrup et al., 2010). The detection of prophage sequences has been recently developed and proposed as a tool for the subtyping of strains (Hermans et al., 2006; Cooke et al., 2007). Recent population studies are supporting the notion that the prophage content is one the most dynamic part of the genome, indicating that phage integration/excision are frequent events shaping *Salmonella* genome evolution (Hermans et al., 2006; Cooke et al., 2007; Drahovska et al., 2007; Matiasovicova et al., 2007; Cooke et al., 2008; Litrup et al., 2010). These observations are in agreement with the phage remnants found in the genomes of *Salmonella* (McClelland et al., 2001; Parkhill et al., 2001; Porwollik et al., 2002; Chan et al., 2003; Porwollik and McClelland 2003; Matiasovicova et al., 2007).

7. Host adaptation

More than a decade ago, Baumler (1998) postulated that the genus *Salmonella* evolved in three phases (Baumler 1997; Baumler et al., 1998). The first phase involved acquisition of SPI1 by an ancestral lineage to all *Salmonella*, since it is present in all phylogenetic lineages of the genus *Salmonella* but absent from *E. coli* and other enterobacteria. In the second phase, the split of *S. enterica* from *S. bongori* involved the acquisition of SPI2, which is not present in *S. bongori* serovars (Ochman and Groisman 1996). Finally, the lineage of *S. enterica* branched into several phylogenetic groups. The formation of *S. enterica* subspecies *enterica* (I) involved a dramatic expansion in host range: while *S. bongori* and *S. enterica* subspecies II, IIIa, IIIb, IV, VI and VII are mainly associated with cold-blooded vertebrates, members of *S. enterica* subspecies I are most frequently isolated from avian and mammalian hosts. The host adaptation of *S. enterica* subspecies I to warm-blooded vertebrates, characterized the third phase in the evolution of virulence in the genus *Salmonella*. The immune system of higher vertebrates is more developed and organized than that of cold-blooded vertebrates. The common ancestor of subspecies I, II, IIIb and VI acquired mechanisms of flagellar antigen shifting (diphasic condition), which is thought to play a role in adaptation to warm-blooded hosts (Li et al., 1995; Baumler et al., 1998; Porwollik and McClelland 2003). The mechanism of phase shifting is amazing and involved the acquisition of the *fljBA* operon, which contains *hin*, encoding for a recombinase that catalyzes the reversible inversion of a segment of the chromosome containing the promoter for the *fljBA* operon. In one orientation, the promoter directs the transcription of the *fljA* (repressor of *fliC*) and *fljB* (phase 2 flagellin)

genes, inducing the repression of *fliC* (phase 1 flagellin). In the other orientation, *hin*, *fljB* and *fljA* are not expressed and *fliC* is expressed (Zieg et al., 1977). A schematic representation of *Salmonella* evolution is presented in Figure 2 (Silva and Wiesner 2009).

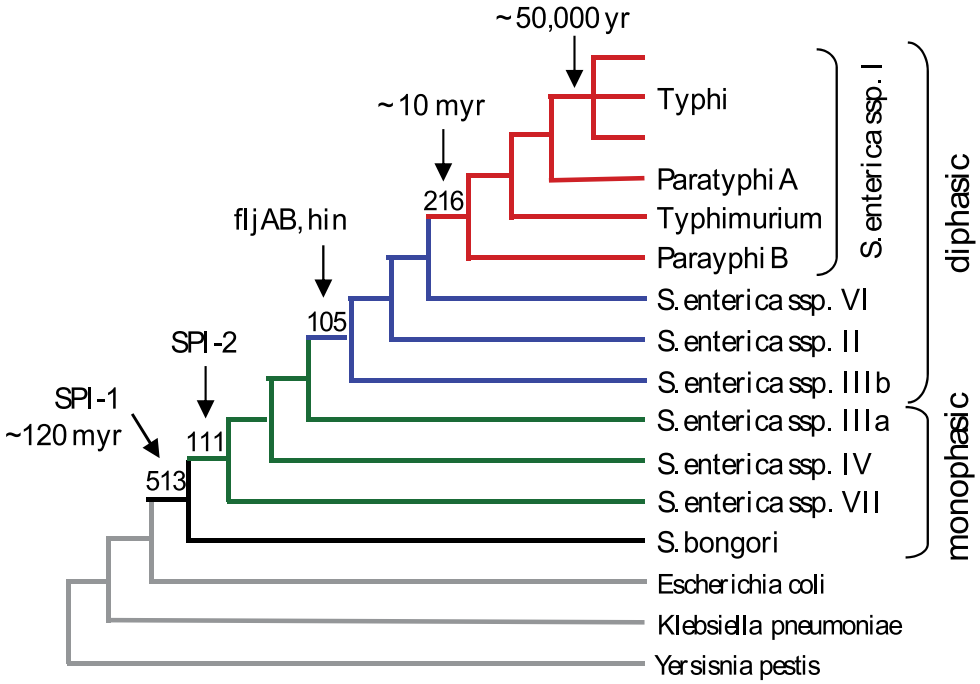

Fig. 2. Schematic representation of *Salmonella* evolution. The cladogram shown is a modified version of that proposed by Porwollik & McClelland (2003), and published by Silva & Wiesner (2009). The number of genes acquired at crucial steps in *Salmonella* evolution is indicated on the nodes along with prominent examples (Porwollik et al., 2002). During the divergence of *E. coli* and *Salmonella*, about 120 million years (myr) ago (Ochman and Wilson 1987), more than 500 genes were acquired, including *Salmonella* pathogenicity island 1 (SPI-1). In the divergence between *S. bongori* and *S. enterica* more than 100 genes were acquired in the *S. enterica* lineage, including those of the SPI-2 (Ochman and Groisman 1996). Along the diversification of the *S. enterica* lineage, about 100 genes were acquired by the common ancestor of subspecies IIIb, II, IV and I, among them were the phase shifting genes (*fljBA* and *hin*), required to confer the diphasic condition to *Salmonella*. During the evolution of subspecies I, which is the most diverse of the subspecies and adapted to warm-blooded vertebrates, more than 200 genes were acquired. Kidgell et al. (2002) estimated that the time of divergence among serovars was around 10 myr, and that the last common ancestor of serovar Typhi existed about 50,000 yr (Kidgell et al., 2002).

However, not everything is said about *Salmonella* evolution. The report of serovar Senftenberg human clinical isolates lacking SPI-1 (Hu et al., 2008) is an example of subsequent loss of genetic determinants during the diversification of *Salmonella*. Another example is the loss of the diphasic trait in some of the serovars of subspecies I (e. g. Typhi) and II. They have reverted to the monophasic condition, usually by loss of expression of phase 2 flagella. In Enteritidis and serovar 4,5,12:i:- the phase 2 flagellin gene (*fljB*) has been

deleted rather than merely silenced (Selander et al., 1996; Echeita et al., 2001). Likewise, in a study analyzing *E. coli* and *Salmonella* genomes, Retcheless and Lawrence (2007) found that their chromosomes diverged over a 70 million year period, and that the regions flanking SPI1 and SPI2 diverged more recently, suggesting that they did not promote the separation of *E. coli* and *Salmonella* (Retchless and Lawrence 2007).

The distribution of pathogenicity islands, fimbrial operons, and capsular biosynthesis genes among *S. enterica* suggests that during evolution, new combinations of virulence determinants arose through multiple horizontal transfer events, a process which may have driven the development of host adaptation. In addition, deletion events and sequence divergence by point mutations were likely among the events which contributed to changes in the host range of *S. enterica* serovars (Baumler et al., 1998; Porwollik and McClelland 2003). The *S. enterica* subspecies I serovars form a group of pathogens that differ widely in their spectrum of host range within mammals and birds. For classification purposes, they can be categorized into three different groups: broad-host range, host-adapted, and host-restricted serovars. For example, serovars Typhi, Gallinarum, and Abortusovis are host-restricted serovars that are associated with systemic disease in humans, fowl and ovine hosts, respectively. Serovars Dublin and Cholerasuis are host-adapted serovars that are often associated with systemic disease in cattle and pigs, respectively, but can cause disease in other animals. Typhimurium and Enteritidis are broad-host range serovars capable of causing systemic disease in a wide range of animals, but are usually associated with gastroenteritis in a broad range of phylogenetically unrelated host species (Baumler et al., 1998; Kingsley and Baumler 2000; Rabsch et al., 2002). However, even within a single serovar there are differences in host range. For example, Rabsch *et al*. (2002) showed that two Typhimurium variants (DT2 and DT99) were almost exclusively associated with pigeons during decades, over a wide geographic range, indicative of a narrow host range; while other Typhimurium variants, such as DT104, are truly broad-host-range, thus circulating in cattle, swine, poultry and humans. Therefore, it may be more accurate to describe serovar Typhimurium as a collection of variants that vary significantly in their host range and degree of host adaptation. One possible mechanism by which such variants arise is through phage-mediated transfer, of a small number of host-specific virulence factors (Rabsch et al., 2002; Porwollik and McClelland 2003; Brussow et al., 2004; Porwollik et al., 2004; Ehrbar and Hardt 2005; Vernikos et al., 2007). There is currently no genetic explanation for the phenotype of host adaptation; it is unlikely that a single locus will be found to be responsible for this complex biological trait. Instead, a combination of multiple genes is likely to contribute to the overall virulence phenotype (Fierer and Guiney 2001).

8. How much lateral transfer occurs in natural populations?

In this chapter, we have provided extensive evidence on the importance of lateral transfer of genetic information in the evolution of *Salmonella*. Compiling evidence on the role of gene loss and acquisition in the origin of the genus *Salmonella* has been presented in several evolutionary studies (Groisman and Ochman 1997; Lawrence 1999; Porwollik et al., 2002; Lerat et al., 2005; Retchless and Lawrence 2007; Vernikos et al., 2007), exemplified by the acquisition of pathogenicity islands (Ochman and Groisman 1996; Porwollik and McClelland 2003; Hensel 2004). Likewise, the evolutionary processes shaping the genetic structure within serovars and host-adapted ecotypes involve in many cases lateral transfer events, such as prophage insertions (Porwollik and McClelland 2003; Thomson et al., 2004;

Hermans et al., 2006; Cooke et al., 2007; Vernikos et al., 2007). The selective pressure that antimicrobial drugs have imposed on the survival of *Salmonella* probably has increased the acquisition of resistance determinants, often carried by mobile genetic elements that are acquired by lateral transfer. These processes are the result of a long evolutionary history of adaptation to changing environments and hosts. However, we do not want to leave the misconception that the amount of lateral transfer is so rampant that there are no limits to genetic exchange in *Salmonella* populations.

Since more than two decades ago, the clonal nature of Salmonella species was documented by several studies based on multilocus enzyme electrophoresis analysis (Beltran et al., 1988; Reeves et al., 1989; Selander et al., 1990; Boyd et al., 1996; Spratt and Maiden 1999). Evidences for the clonal structure of *Salmonella* include the global distribution of certain genotypes, the congruent relationships between isolates derived from several housekeeping genes, and the robust subspecies structure (Reeves et al., 1989; Boyd et al., 1996; Selander et al., 1996; Falush et al., 2006). In the past decade, multi-locus sequence typing (MLST) studies, analyzing *Salmonella* populations for epidemiological purposes, showed concordant results with the studies based on enzyme electrophoresis, which support the view that *Salmonella* has a clonal population structure. Among these results are the almost strict association between multilocus genotype and serovar, the low genetic diversity within serovars, and the maintenance of old globally-distributed clones (Sukhnanand et al., 2005; Harbottle et al., 2006; Tankouo-Sandjong et al., 2007; Wiesner et al., 2009). The clonal nature of *Salmonella* populations was observed in our study based on a survey of more than 100 Mexican Typhimurium strains. MLST and macrorestriction fingerprints by pulsed-field gel electrophoresis were used to address the core genetic variation, and genes involved in pathogenesis and antibiotic resistance were selected to evaluate the accessory genome. SGI1 was found in a defined subset (16%) of the strains. They were in a compact cluster conformed by strains belonging to the second most abundant genotype (ST19), and in most of the cases they also carried the *Salmonella* virulence plasmid (Figure 3). On the other hand, the strains with the most abundant genotype (ST213) lacked *Salmonella* virulence plasmid or SGI1, but in most of the cases carried a multiple-drug resistant (MDR) IncA/C plasmid. The ST19 isolates carrying the virulence plasmid were significantly associated with the human host, whereas ST213 isolates were more frequently isolated from animal sources, indicating that the distinct accessory genes carried by these genotypes are probably involved in the interaction with the host (Wiesner et al., 2009). No strain carrying both the *Salmonella* virulence plasmid and the MDR IncA/C plasmid, nor hybrid virulence-resistance plasmids, was detected. We concluded that, in the Mexican Typhimurium population, the association between distinct core and accessory genes creates a structure of genetic subgroups within the population, which could be due to the existence of barriers to genetic exchange among subgroups (Wiesner et al., 2009).

It is intriguing why if *Salmonella* evolution is marked by lateral transfer events and genome rearrangements, the genetic structure of populations seems to be extremely clonal. It is possible that in an evolutionary time scale (millions of years) there were several occasions where the mismatch repair system was impaired and large scale recombination events occurred and marked the genomes of diverse *Salmonella* lineages (Taddei et al., 1997; Matic et al., 2000; Didelot et al., 2007). However, in the ecological time scales (thousands of years to decades) the recombination events are rare.

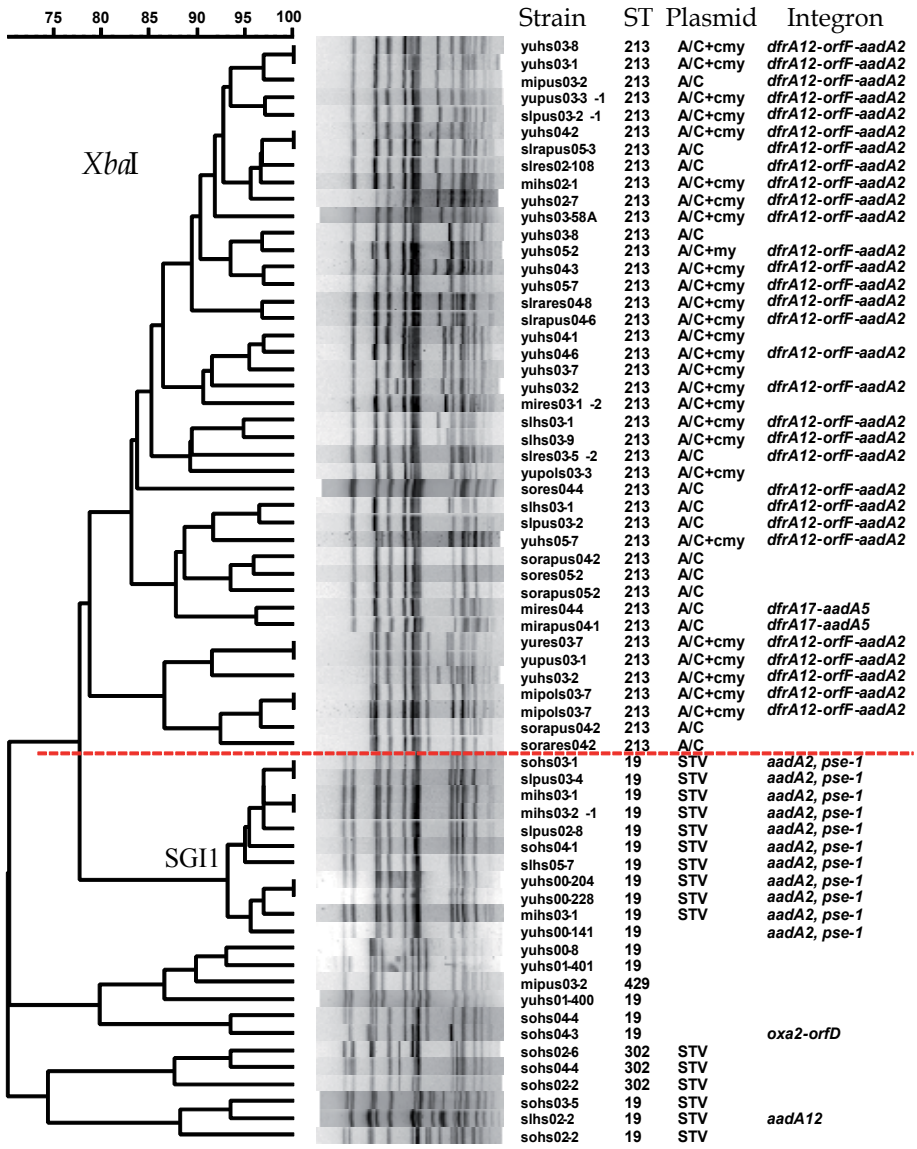

Fig. 3. Dedrogram depicting the genetic relationships between 65 representative Mexican Typhimurium strains, and the associations among core and accessory genes. The data were obtained from Wiesner et al. (2009) and Wiesner et al. (2011). The *Xba*I restriction fingerprints were clustered by the UPGMA algorithm using Dice coefficients. The first column contains the strain designation. The second column displays the multi-locus sequence type (ST) for each strain. The plasmid column indicates if the strains harbored the *Salmonella* virulence plasmid (STV), the bla_{CMY-2}-bearing IncA/C plasmid (A/C+cmy) or the smaller bla_{CMY-2}-lacking IncA/C plasmid (A/C). The last column shows the integrons carried by the strains. The horizontal dashed line separates the ST213 strains from the remaining STs. SGI1 indicates the cluster containing the strains with the *Salmonella* genomic island.

9. Concluding remarks

Mobile genetic elements are key to understanding *Salmonella* evolution and ecology. Actually there is a wealth of information regarding the record of horizontal gene transfer in *Salmonella*; for a revision we recommend the review of Porwollik & McClelland (2003) and the paper of Vernikos *et al*. (2007). In *Salmonella* many of the virulence factors are present as part of pathogenicity islands on the chromosome, yet some virulence factors are encoded on plasmids or bacteriophages. Their composition, presence or absence determines at large differences in pathogenicity and host range between serovars and strains (Fluit 2005). We certainly need to expand our vision, in order to integrate the knowledge about the great variety of genetic virulence determinants in *Salmonella* and the antibiotic-resistance "tool kit". A vision is emerging regarding different molecular routes that determine the plasticity of the accessory genome, which are subject to intricate interactions that we still do not completely understand. Complex interactions among pathogenicity islands, bacteriophages, plasmids and other mobile elements, such as transposons and integrons, are increasingly being evidenced. For example, the transfer of the resistance phenotypes associated with Typhimurium DT104 SGI1, by phage-transduction experiments, suggest the involvement of phages in the mobilization of SGI1 (Lawson et al., 2002b; Fluit 2005). In another example, derivatives of the original Typhimurium DT104 clone have emerged over the more than 20 years after the first report. Different phage-types, such as DT12 or DT120, are indistinguishable from the DT104 clone by genotyping, suggesting that they arose from DT104 through changes that led to different phage susceptibility patterns (Fluit 2005). These shifts in phage-type are probably due to the gain or loss of prophages. More than three decades ago, Threlfall et al. (1978) showed that changes in phage-types can be the result of the loss or acquisition of plasmids (Threlfall et al., 1978). Likewise, in another study related to Typhimurium DT104 and SGI1, some strains showed to be phage-untypeable or DT104B, and it was found that these strains carried a larger plasmid (140 kb) resulting from the recombination between a resistance and virulence plasmid (Guerra et al., 2002). In these latter cases, the changes in the plasmid content could be associated with the loss of determinants required by phages (Fluit 2005), such as surface lipopolysaccharides (Lawson et al., 2002a). Of particular concern is the increasing number of reports of co-integrates of resistance and virulence plasmids, envisioning that new *Salmonella* strains will arise posing a threat to public health (Fluit 2005).

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14

Salmonella as a Unique Tool for Genetic Toxicology

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1. Introduction

In molecular biology and genetics, mutations are described as sudden and spontaneous or induced changes in a genomic sequence (Brown, 2007). They have wide effects on all living organisms from bacteria with a single prokaryotic cell construction to multicellular and eukaryotic organisms including human being with high-level cellular differentiation. Mutations occur also in the genomic materials (DNA or RNA) of viruses and affect their functionality (Hartl & Jones, 1998; Lewin, 2004). When a mutation happens, it can basically result in several different types of change in DNA (or RNA for some viruses) sequences; these can have no effect, alter the product of gene, and prevent the gene from functioning properly or completely. Alterations in the product of gene and partial or total loss of gene function generally result in a disadvantageous situation for the organism, which cause various symptoms and ailments affect the maintenance of life (Brown, 2007). Previous studies made to understand the relations between mutations and their negative effects on human being clearly showed that some diseases, such as most forms of cancer, heart disease and mental disorders, have a partly or completely genetic basis closely related to mutagenesis (Bertram, 2000; Alberts et al., 2002; Lodish et al., 2007). Therefore, recent investigations have mainly focused on mutation classification, understanding mutagenesis mechanisms, determination of mutagenic agents and prevention strategies (Cox, 1976; Albertini et al., 1990; Davidson et al., 2002; Akiyama, 2010; Evans et al., 2010; Gulluce et al., 2010; Lynch, 2010; Waters et al., 2010; Lange et al., 2011; Loeb, 2011; Pao & Girard, 2011). Thus, the identification of substances capable of inducing mutations has become an important procedure in safety assessment. In the research studies, mutations can be divided in two main groups to get more comprehensive results according to their size. First group is described as gene mutations, where only single base is modified, or one or a relatively few bases are inserted or deleted (Brown, 2007). Other one consists of chromosome mutations, which are including chromosome breaks, large deletions, rearrangements, or gain or loss of whole chromosome (Hartl & Jones, 1998; Lewin, 2004).

Mutation test systems also divide in long-term and short-term systems according to obtaining of the results (Wickramasnghe, 1979; Mortelmans & Zeiger, 2000; Zeiger et al., 2005). The long-term mutagenicity tests, which use *in vivo* researches with various experimental animals, give more reliable results than short-term mutagenicity test systems. However, they are not preferred as beginning test systems due to their high cost and long time requirements, where mutagenic potential of many synthetic and natural chemicals are checked (Wickramasnghe, 1979; Gulluce et al., 2010). In these studies, the short-term test

systems, which eliminate disadvantages of the long-term test systems, are more suitable and acceptable. Many short-term studies result in gaining reliable and alternative data under controlled *in vitro* conditions. Another important advantage is that short-term mutation test systems are not only correlated with other short-term test systems, but also long-term systems (Mortelmans & Zeiger, 2000). Thus, the combinations of the mutagenicity test systems play a key role to get more meaningful results.

The bacterial mutation assays are known as most important short-term systems in order to determine mutagenic and antimutagenic potential of natural or synthetic chemicals related to gene mutations (Ames et al., 1973a, 1973b; Maron & Ames, 1983; Mortelmans & Riccio, 2000; Mortelmans & Zeiger, 2000). The *Salmonella* bacterial reverse mutation assay is one of the simplest, the most meaningful and acceptable short-term mutagenicity and antimutagenicity test systems. The test was initially developed by Ames in 1971. Therefore, it is also called as the Ames mutagenicity assay or the Ames/*Salmonella* mutagenicity assay (Ames et al., 1973a, 1973b; Maron & Ames, 1983; Zeiger, 2004, 2010). The main advantages of the assay, which employs mutant *Salmonella typhimurium* tester strains as model prokaryotic organisms, can be ordered as inexpensive applications enable studying a large number of test materials, quickly resulting (approximately 48 hours) allows making replicates in a short time, divers tester strains with several gene mutations allow to research the molecular effect mechanism of test materials, additional mutations in each strain result in more sensitivity such as *rfa* or *uvrB* and mesophile character of *Salmonella* allows to study several test materials affective at human body temperature. Furthermore, combination of the cytochrome-based P450 metabolic oxidation system, which usually consists of a 9000×g supernatant fraction of a rat liver homogenate (S-9 microsomal fraction), with the *Salmonella* mutagenicity test allows to determine some mutagenic agents, which are biologically inactive unless they are metabolized to active forms (Ames et al., 1973b; Mortelmans & Zeiger, 2000; Zeiger et al., 2005). Thus, the assay is used world-wide in genetic toxicology laboratories as a beginning mutation test to determine mutagenic and antimutagenic potentials of various chemicals.

The present study includes an introduction to use of *Salmonella* strains in genetic toxicology, principles of *Salmonella* bacterial reverse mutation assay, the most popular assay procedures with explanatory figures and clues for experimental design.

2. Scientific background

2.1 Mutations and their effects on living organisms

Genetic materials of all living organisms are dynamic structures that change and rearrange many times as a result of cumulative effects of mutations. Mutations, described as sudden and spontaneous or induced changes in a genomic sequence, are classified in two main groups depend on their physical effect sizes on the genome (Hartl & Jones, 1998; Lewin, 2004; Klug et al., 2005; Brown, 2007). First group is large-scale mutations in chromosomal level, including numerical and structural anomalies. Numerical anomalies are also called as aneuploidy, means an abnormal number of chromosomes. The most known examples for aneuploidy are monosomy (missing a chromosome from a pair), nullisomy (missing a pair of chromosomes), trisomy and polysomy (having one or more than two chromosomes of a pair). Down and Turner syndromes are important examples related to chromosomal anomalies in humans. An individual with Down syndrome has a developmental disorder caused by having three copies of chromosome 21. Therefore, it is also called as Trisomy 21.

Another disorder is Turner syndrome, an example of a monosomy where the individual is born with only one X chromosome (Klug et al., 2005).

Structural anomalies of the large-scale mutations include deletions, duplications, inversions and translocations. A deletion is a loss of one or more pieces from a chromosome after DNA breaks induced by various physical or chemical agents (Klug et al., 2005). Genetic information loss together with deletions causes serious disorders in humans, for example, Wolf-Hirschhorn syndrome, also known as deletion 4p syndrome, and Jacobsen syndrome, also known as deletion 11q syndrome (Hirschhorn et al., 1965; Jacobsen et al., 1973). Duplication is described as a phenomenon that a chromosome has extra copies of a chromosomal region, which may affect phenotype by altering gene function and transcriptional dosage (Zhang, 2003; Mao & Pevsner, 2005). Because most embryonic processes requires sensitively balanced protein levels, many duplications lead to developmental defects such as *Bar* eye mutation in *Drosophila* and Charcot-Marie-Tooth disease in humans (Sutton, 1943; Latour et al., 1997). An inversion type chromosomal mutation occurs when a portion of the chromosome breaks off, 180° rotates and reattaches, resulting in an inverted genetic material. There is little knowledge about the linkage between inversions and disease formation, and it is believed that many affect mechanisms of inversions directly associated with deletions. Juvenile Polyposis of Infancy, a rare genetic disorder, is a good example for a disease evolved by the cumulative effects of inversions (a paracentric inversion in 10q) and deletions (a deletion in 10p) (Gimelli et al., 2003; Antonacci et al., 2009; Vargas-Gonzales et al., 2010). The last group of the structural anomalies is translocations, defined as an exchange of segments among the non-homologues chromosomes. Several forms of cancer, leukemia and lymphoma are the best known disorders related to translocations (Li et al., 1999; Kurzrock et al., 2003; Anton et al., 2004). Figure 1 illustrates structural chromosome mutations.

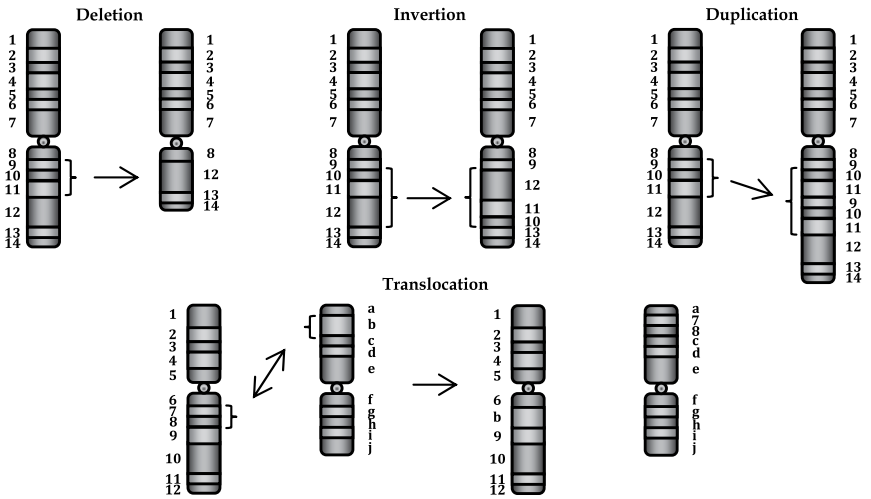

Fig. 1. Structural chromosome mutations

Small-scale mutations, also known as gene mutations, include three main groups: point mutations, which are the most common type of the gene mutations and replace one nucleotide with another, insertions, which add one or a few extra nucleotides into the DNA, and deletions, which remove one or a few nucleotides from the DNA (Brown, 2007).

Point mutations are also divided into two categories as transitions and transversions. Transitions, which are purine-to-purine or pyrimidine-to-pyrimidine changes (A↔G or C↔T), are more common type of the point mutations than transversions, which are purine-to-pyrimidine or pyrimidine-to-purine changes (A↔C, A↔T, G↔C or G↔T) (Brown, 2007).

Contrary to "small-scale" word in their names, these mutations can cause wide-range significant changes in genomes and phenotypes of living organisms with mutated genetic materials. For example, a point mutation may result in a synonymous change that causes forming a new codon specifying the same amino acid as the unmutated codon, a non-synonymous change that causes a missense mutation where a new codon specifies a different amino acid from the unmutated codon, a nonsense mutation where the change converts an amino acid specifying codon into a termination codon, or a readthrough mutation where the change converts a termination codon into an amino acid specifying codon. Except synonymous changes, also called as silent mutations because the mutated gene codes for exactly the same protein as the unmutated gene, the other three types of point mutations have significant impacts on the genome and related phenotypes by effecting amino acid sequence of the coding protein (Hartl & Jones, 1998; Alberts et al., 2002; Lewin, 2004; Brown, 2007; Lodish et al., 2007). The effects of point mutations on the coding region of a gene are shown in Figure 2.

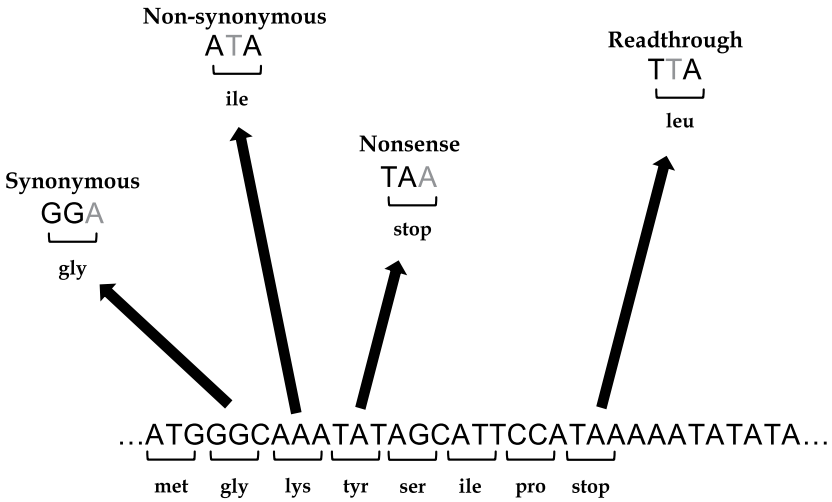

Fig. 2. The effects of point mutations on the coding region of a gene (Brown, 2007).

Insertion and deletion types of small-scale mutations affect the coding capabilities of the gene in a different way. It is defined as a frameshift mutation, caused by addition or deletion of a number of nucleotides that is not evenly divisible by three from a DNA sequence. Because codons consist of three nucleotides, an insertion or deletion type mutation can disrupt the reading frame, resulting in a completely different translation from the unmutated gene. Thus, insertion or deletion mutations generally have more significant effects on the protein function than the point mutations because the translated protein have completely different sequence from the mutated point to the end. An exception occurs that the number of inserted or deleted nucleotides is three or a multiple of three, which results in addition or deletion of one or more codons (Alberts et al., 2002; Lewin, 2004; Brown, 2007; Lodish et al., 2007). Figure 3 illustrates two possible effect mechanisms of the insertion or deletion type mutations on the coding region of a gene.

Fig. 3. Two possible effect mechanisms of the insertion or deletion type mutations on the coding region of a gene (Brown, 2007).

Phenotypic results of mutations can be deleterious or advantageous for the affected organism. Many hereditable disorders are either directly caused by the mutations or indirectly associated with the mutagenesis. Cancer formation can be given as a satisfactory example to demonstrate deleterious effect of mutations. Recent studies clearly showed that there is a strict connection between mutagenesis and the formation of the several cancer types (Davidson et al., 2002). In this manner, mutations provide a unique resource for all tumors that show genomic instability with few exceptions. On the other hand, mutated organisms can gain various advantages as a result of mutagenesis. Gain of antibiotic resistance in microorganisms and HIV/AIDS protective mutations on the SDF1, CCR5 and CCR2 genes in the human genome are well examples for the advantageous mutations (Stephan et al., 1998; Galvani & Slatkin, 2003; Apostolakis et al., 2005). These are also very important for evaluation of the organisms. Therefore, mutagenesis can be considered as one of the most important evolutionary sources. For example; simple sequence repeats (SSRs, also called microsatellites and minisatellites) are defined as advantageous mutators in adaptive evolution. Recent studies showed that temperature compensation of circadian rhythm in *Drosophila*, adaptive divergence among barley and wheat populations, social behavior in voles, skeletal morphology in domestic dogs and sporulation efficiency and cell adhesion in yeast are closely related to SSRs, which are mutation-prone DNA tracts composed of tandem repetitions of relatively short motifs (Kashi & King, 2006).

2.2 The causes of mutations

Mutations are divided into spontaneous and induced alterations according to their formation sources. Spontaneous mutations arise from replication errors due to defective replication enzymes and alternative tautomer forms of nucleotide bases. These are rare types of mutations. On the other hand, induced mutations, common types of mutations, are caused by various mutagens. In the molecular mechanism of induced mutations, a physical or chemical mutagen reacts with the DNA strand, causing a structural change that affects the base-pairing capability of the altered nucleotide. The most important types of physical

mutagens are ultraviolet radiation of wavelength 260 nm, ionizing radiation and heat shock. However, base analogs such as 5-bromouracil (5-bU), deaminating agents such as nitrous acid, alkylating agents such as ethylmethane sulfonate (EMS) and intercalating agents such as (ethidium bromide) are the most well-known chemical mutagen classes (Brown, 2007).

Chemical mutagens are more frequent agents because thousands of natural or synthetic chemicals have been introduced for daily use in many areas including medicine, pharmacy, food and cosmetics. The count of new chemicals is increasing day-by-day, and each chemical can be considered as a potential mutagen before tested. Therefore, many test systems for detecting of chemical mutagens have been developed and frequently used in the laboratories around the world (Zieger, 2000; World Health Organization [WHO], 2007).

2.3 The mutagenicity and antimutagenicity test systems

The deleterious effects of mutations enforce the determination of mutagenic chemicals. There are many assay systems for this purpose, and a new chemical is tested for mutagenic potential before introduced to use. The main groups of the assay systems are long-term and short-term assay systems (Wickramasnghe, 1979; Zeiger et al., 2005; Mortelmans & Zeiger, 2000).

The long-term assay systems mainly include *in vivo* applications performed with experimental animals. These are the most comprehensive and reliable test systems. However, the long-term assay systems are not preferred as the beginning mutation test systems due to their high—cost and time consuming properties (Wickramasnghe, 1979; Zeiger et al., 2005).

On the other hand, the short-term assay systems mainly include *in vitro* assays performed with bacterial strains, cytological cell-line cultures and biotechnology based applications. Relatively inexpensive and time-saver nature of the short-term assay systems makes them good candidates for preliminary mutagen determination studies performed with fairly huge numbers of synthetic or natural chemicals. Furthermore, these assays can identify substances inhibiting mutagens and mutations (called as antimutagens) with some modifications. Therefore, a mutagenicity test system can be also considered as antimutagenicity test system (Ames et al., 1973a, 1973b; Wickramasnghe, 1979; Fenech, 2000; Maron & Ames, 1983; Mortelmans & Riccio, 2000; Mortelmans & Zeiger, 2000; Zeiger et al., 2005; Rossi et al., 2007; Ozbek et al., 2008a, 2008b; Gulluce et al., 2010).

2.4 Ames/*Salmonella* test system

The Ames/*Salmonella* test system, also called as Ames test, was developed by Ames *et al.* in the beginning of 1970s. The test, which employs histidine auxotroph *Salmonella* strains originated from *Salmonella typhimurium* LT-2 by chemical and radiation induced mutations, was initially designed as a spot test for determination of mutagenic chemicals, then as a more sensitive method: plate incorporation test (Ames et al., 1973a, 1973b; Maron & Ames, 1983; Gee et al., 1994; Mortelmans & Zeiger, 2000; Tijs, 2008).

In the molecular mechanism of the test system, a tester strain carries a unique gene mutation at the histidine operon that makes the strain histidine-dependent to grow, and a mutagenic chemical reacts with the mutated site resulting in a reverse mutation. Thus, the strain regains histidine production ability and the bacterial cells can grow in the absence of histidine. Therefore, the test is often referred as a reversion assay (Ames et al., 1973a, 1973b; Maron & Ames, 1983; Gee et al., 1994; Mortelmans & Zeiger, 2000; Tijs, 2008).

After its introduction to the scientific world, Ames test has been widely accepted as a short-term bacterial test system for determining chemicals that can cause gene mutations. The test has many advantages for identification of the chemicals that cause gene mutations. These advantages can be listed in:

- Short-term resulting: allows making replicates and obtaining more reliable results in a short duration. It takes only about 48 hours.
- Low-cost: allows studying a large number of test materials inexpensively.
- Various tester strains with several gene mutations: enable to research the molecular effect mechanism of test materials
- Additional mutations and genetic alterations: allow gaining more sensitivity for various chemicals.
- Mesophile character of *Salmonella* strains: enables to study mutagenic potential of the chemicals at human body temperature.

Apart from all the maintained advantages, Ames/*Salmonella* test system is very versatile, and many modifications has been developed to determine mutagenic potencies of various materials such as environmental chemicals, environmental mixtures, body fluids, foods, drugs and physical agents. The most common assay procedures are the spot test: a primal method for determination of chemical mutagens, the standard plate incorporation method: an easily resulting and more comprehensive method than the spot test, the pre-incubation method: developed for performing more effective studies with lower volumes of test materials, the desiccator assay modifications: developed to study volatile materials and gases, and the modified *Salmonella* microsuspension assay (Kado): a highly sensitive method for testing the materials that are available only in small amounts (Kado et al., 1983; Hughes et al., 1987; Zeiger et al., 1992; Araki et al., 1994; Mortelmans & Zeiger, 2000; Tijs, 2008).

Although *Salmonella* has prokaryotic cell structure, combination of the cytochrome-based P450 metabolic oxidation system with the Ames/*Salmonella* test system allows determining some mutagenic agents, which are biologically inactive unless they are metabolized to active forms (Ames et al., 1973b; Mortelmans & Zeiger, 2000). Moreover, all the procedures of the test system can be altered to identify antimutagenic agents, inhibit mutagenesis and protect the organisms against deleterious effects of the mutagens, with some modifications (Nagabhushan et al., 1987; Bala & Grover, 1989; Edenharder et al., 1999; Edenharder & Grünhage, 2003; Ozbek et al., 2008a, 2008b; Gulluce et al., 2010) (see 3.8).

3. Material and methods

3.1 Supplies and equipment

The following items are required for performing the Ames/*Salmonella* mutagenicity and antimutagenicity protocols.

3.1.1 Supplies

- Magnetic stir bars
- Sterile glass test tubes (100×16 mm) and racks
- Sterile microbiological loops
- Sterilizing membrane filters (0.2 μm)

- Sterile syringes (5, 10 and 50 ml)
- Sterile Petri dishes (100×15 mm)
- Disposable spectrophotometer cuvettes
- Solvents, reagents, media and positive control chemicals
- General laboratory glassware (bottles, flasks and graduated cylinders)
- Dispensers for delivering top agar, buffer and S-9 mix to the test tubes
- Sterile cryogenic storage vials for freezing down permanent and working cultures
- General laboratory safety items (biohazard waste bags, goggles or protective eye wear, gloves, lab coats)
- Glass pipettes (1, 2, 5 and 10 ml), automatic micropipettes (adjustable volumes up to 200 and 500 µl) and pipette tips

3.1.2 Equipment

- Autoclave
- Manual or electronic colony counter
- Spectrophotometer for monitoring cell density
- Centrifuge (up to 8000 rpm)
- Liquid and solid waste disposal
- Magnetic stirrers
- Desiccator and vacuum pump
- Balances
- Biological/chemical safety cabinet equipped with gas line for keeping aseptic techniques while inoculating cultures
- Ultra-low temperature freezer set at -86 °C or liquid nitrogen tank for long term storage of frozen permanent cultures
- Refrigerator (4 °C) and freezer (-20 °C)
- Water purification system to generate distilled water
- Water bath set at 43 °C to 48 °C to maintain temperature of top agar
- Incubator for incubating the agar plates
- Shaking incubator for incubating the liquid cultures and growing the overnight cultures
- Boiling water bath or microwave oven for melting top agar

3.2 Reagents and media

Glucose solution (10% w/v): The solution is used as carbon source for the GM agar plates. Dissolve 100 g dextrose (D-glucose) in 700 ml of distilled water by stirring on a magnetic stirrer. Add additional water to bring the final volume to 1000 ml and distribute in 50 ml aliquots. Autoclave 121 °C for 20 min and store at 4 °C.

Vogel-Bonner medium E (VB salts 50×): The solution is used as salt source for the GM agar plates. Add 10 g magnesium sulfate ($MgSO_4 \cdot H_2O$), 100 g citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$), 500 g potassium phosphate dibasic (K_2HPO_4) and 175 g sodium ammonium phosphate ($Na_2NH_2PO_4 \cdot 4H_2O$) in the order indicated to 650 ml of warm water making sure that each salt is dissolved thoroughly by stirring before adding the next salt. Add additional water to bring the final volume to 1000 ml and distribute in 20 ml aliquots. Autoclave 121 °C for 30 min and store at room temperature in the dark.

GM agar plates: The medium is used as bottom agar for mutagenicity and antimutagenicity assays. Add 15 g agar to 900 ml of distilled water and autoclave for 30 min at 121 °C. When cooled to approximately 65 °C, add 20 ml of sterile Vogel-Bonner medium E and mix well; then add 50 ml of sterile glucose solution and mix thoroughly. Pour nearly 25 ml of the medium into sterile 100×15 mm petri dishes and store at 4 °C for several weeks by packing with sealed plastic bags after solidified. Note that the plates should be warmed up to room temperature and examined for excess moisture before use. Put the plates with too much moisture overnight in an incubator set at 37 °C prior to use.

Histidine/biotin solution (0.5 mM): The solution is used to supplement top agar with adequate biotin and a trace amount of histidine. Dissolve 124 mg D-biotin and 96 mg L-histidine·HCl in 1000 ml of boiling water. Sterilize the solution by filtration through a membrane filter with 0.2 μm pore size or autoclaving for 20 min at 121 °C. Store at 4 °C in a glass bottle.

Top agar supplemented with histidine/biotin: The solution is used to apply the bacteria, chemicals and buffer or S9 mix to the bottom agar. Dissolve 6 g agar and 6 g sodium chloride (NaCl) in 900 ml of distilled water by heating. Add 100 ml of histidine/biotin solution (0.5 mM) and dispense 200 ml aliquots in screw-cap bottles. Autoclave for 20 min at 121 °C and store at room temperature in the dark. Melt the top agar in a microwave oven or boiling water bath before use.

Nutrient broth: Oxoid nutrient broth no. 2 or Difco nutrient broth can be used to grow the tester strains overnight. Follow the manufacturer's instructions for preparing the medium. Dispense 50 ml in Erlen Meyer flasks with 125 ml capacity or 5 ml in 100×16 mm test tubes, autoclave for 20 min at 121 °C and store in the dark at room temperature.

Nutrient agar plates: The medium is used for streaking newly received cultures for single colonies, checking crystal violet sensitivity due to presence of *rfa* mutation and testing viability of bacteria. Add 15 g agar to 1000 ml of nutrient broth medium and dissolve by heating. Autoclave for 20 min at 121 °C. After cooled to 65 °C, pour nearly 25 ml of the medium into sterile 100×15 mm petri dishes and store at 4 °C by packing with sealed plastic bags.

Sodium phosphate buffer (0.1 mM – pH 7.4): The solution is used to perform mutagenicity and antimutagenicity assays in the absence of metabolic activation. In the first step, prepare Reagent A (0.1 M sodium phosphate monobasic solution: 13.8 g $NaH_2PO_4 \cdot H_2O$ in 1000 ml of distilled water) and Reagent B (0.1 M sodium phosphate dibasic reagent: 14.2 g $Na_2HPO_4 \cdot H_2O$ in 1000 ml of distilled water). After that, mix 120 ml of Reagent A and 880 ml Reagent B and swirl well. Adjust pH to 7.4 using Reagent A/B and dispense 100 ml aliquots in screw-cap bottles. Autoclave for 30 min at 121 °C and store at room temperature in the dark.

Metabolic activation system (S-9 mix): The solution is used to perform mutagenicity and antimutagenicity assays in the presence of metabolic activation. Moltox metabolic activation system products can be used. Follow the manufacturer's instructions for preparation and storage of the related solutions.

Biotin solution (0.01%, w/v): The solution is used to prepare enriched GM agar plates for biotin auxotrophy check. Dissolve 10 mg D-biotin in 100 ml of boiling distilled water. Sterilize using a membrane filter with 0.2 μm pore size and store at 4 °C.

Histidine solution (0.5%, w/v): The solution is used to prepare enriched GM agar plates for histidine auxotrophy check. Dissolve 500 mg L-histidine in 100 ml of distilled water. Autoclave for 15 min at 121 °C and store at 4 °C.

Ampicillin solution (0.8%, w/v): The solution is used to prepare enriched GM agar plates for examining presence of plasmid pKM101 in several tester strains such as TA97, TA98, TA100 and TA102. Dissolve 8 mg ampicillin in 100 ml of warm (65 °C) distilled water and sterilize using membrane filter with 0.2 µm pore size. Store at 4 °C.

Tetracycline solution (0.8%, w/v): The solution is used to prepare enriched GM agar plates for examining presence of plasmid pAQ1 in TA102. Dissolve 8 mg tetracycline in 100 ml of 0.02 N hydrochloric acid (HCl) and sterilize using membrane filter with 0.2 µm pore size. Store at 4 °C in the dark due to the light sensitivity of tetracycline.

Enriched GM agar plates: Each medium contains essential nutrients and antibiotics for the strain check and preparation of stock cultures' master plates.

- Biotin plates (B): Prepare GM agar medium. After autoclaving, add 8 ml of sterile biotin solution (0.01%, w/v), mix well and pour nearly 25 ml of the medium into sterile 100×15 mm petri dishes.
- Histidine plates (H): Prepare GM agar medium. After autoclaving, add 8 ml of sterile histidine solution (0.5%, w/v), mix well and pour nearly 25 ml of the medium into sterile 100×15 mm petri dishes.
- Biotin/histidine plates (BH): Prepare GM agar medium. After autoclaving, add 8 ml of sterile biotin solution (0.01%, w/v) and 8 ml of sterile histidine solution (0.5%, w/v), mix well and pour nearly 25 ml of the medium into sterile 100×15 mm petri dishes.
- Biotin/histidine/ampicillin plates (BHA): Prepare GM agar medium. After autoclaving, add 8 ml of sterile biotin solution (0.01%, w/v), 8 ml of sterile histidine solution (0.5%, w/v) and 3 ml of ampicillin solution, mix well and pour nearly 25 ml of the medium into sterile 100×15 mm petri dishes.
- Biotin/histidine/tetracycline plates (BHT): Prepare GM agar medium. After autoclaving, add 8 ml of sterile biotin solution (0.01%, w/v), 8 ml of sterile histidine solution (0.5%, w/v) and 0.25 ml of tetracycline solution, mix well and pour nearly 25 ml of the medium into sterile 100×15 mm petri dishes.
- Biotin/histidine/ampicillin/tetracycline plates (BHAT): Prepare GM agar medium. After autoclaving, add 8 ml of sterile biotin solution (0.01%, w/v), 8 ml of sterile histidine solution (0.5%, w/v), 3 ml of ampicillin solution and 0.25 ml of tetracycline solution, mix well and pour nearly 25 ml of the medium into sterile 100×15 mm petri dishes.

Crystal violet solution (0.1%, w/v): The solution is used to confirm the presence of the *rfa* mutation in all of the tester strains. Dissolve 100 mg crystal violet in 100 ml of distilled water. Mix well and store at 4°C in an amber glass bottle to protect against light.

All reagents and solutions, reported here, have been previously described by Mortelmans and Zeiger (2000).

3.3 Bacterial strains

Salmonella typhimurium TA1535, TA1537, TA1538, TA97, TA98, TA100, TA102 and TA104 are the most common tester strains used in the Ames/*Salmonella* test system. All strains are histidine auxotroph because of a mutation in the histidine operon. The tester strains also have additional mutations and genetic alterations that provide more sensitivity for chemical mutagens. These are *uvrB*, *rfa* mutations and introduction of pKM101 and pAQ1 plasmids.

The *uvrB* mutation, which is present in all strains except TA102, arise from a deletion type mutation through the *uvrB-bio* genes that eliminates the accurate DNA repair and makes the cells biotin dependent. All strains have the *rfa* mutation that affects the bacterial cell wall, resulting in a defective lipopolysacharide layer that provides more permeability to bulky chemicals. Existence of pKM101 plasmid in TA97, TA98, TA100, and TA102 provides ampicillin resistance and sensitivity for chemical and induced mutagenesis associated with error-prone recombinational DNA repair pathway. TA102 strain also has multicopies of pAQ1 plasmid carrying *hisG428* mutation, which provides tetracycline resistance and sensitivity for detection of DNA cross-linking agents (Mortelmans & Zeiger, 2000). Table 1 presents genotypes of the tester strains.

Strain	DNA Target	*uvrB*	*rfa*	Plasmid	Reversion Event
TA1535	*hisG46* -G-G-G-	+	+	-	Base-pair substitutions
TA1537	*hisC3067* -C-C-C- +1 frameshift	+	+	-	Frameshifts
TA1538	*hisD3052* -C-G-C-G-C-G-C-G- -1 frameshift	+	+	-	Frameshifts
TA97	*hisD6610* -C-C-C-C-C-C- +1 frameshift	+	+	pKM101	Frameshifts
TA98	*hisD3052* -C-G-C-G-C-G-C-G- -1 frameshift	+	+	pKM101	Frameshifts
TA100	*hisG46* -G-G-G-	+	+	pKM101	Base-pair substitutions
TA102	*hisG428* TAA (ochre)	-	+	pKM101 pAQ1	Base-pair substitutions
TA104	*hisG428* TAA (ochre)	+	+	-	Base-pair substitutions

Table 1. Genotypic properties of the most common *Salmonella* tester strains.

There are also additional tester strains (TA7001-7006 and TA7041-7046 series), which developed by Gee et al. (1994), to identify specific transitional and transversional base-pair substitutions induced by various mutagenic agents. Table 2 presents genotypic properties of these strains.

Strain	DNA Target	*uvrB*	*rfa*	Plasmid	Reversion Event	Amino acid change
TA7001	*hisG1775*	+	+	pKM101	AT → GC	Asp-153 → Gly-153 (GAT → GGT)
TA7002	*hisC9138*	+	+	pKM101	TA → AT	Ile-217 → Lys-217 (ATA → AAA)
TA7003	*hisG9074*	+	+	pKM101	TA → GC	Val-153 → Gly-153 (GTT → GGT)
TA7004	*hisG9133*	+	+	pKM101	GC → AT	Gly-169 → Asp-169 (GGG → GAT)
TA7005	*hisG9130*	+	+	pKM101	CG → AT	Ala-169 → Asp-169 (GCG → GAT)
TA7006	*hisC9070*	+	+	pKM101	CG → GC	Arg-163 → Gly 163 (CGA → GGA)
TA7041	*hisG1775*	+	-	pKM101	AT → GC	Asp-153 → Gly-153 (GAT → GGT)
TA7042	*hisC9138*	+	-	pKM101	TA → AT	Ile-217 → Lys-217 (ATA → AAA)
TA7043	*hisG9074*	+	-	pKM101	TA → GC	Val-153 → Gly-153 (GTT → GGT)
TA7044	*hisG9133*	+	-	pKM101	GC → AT	Gly-169 → Asp-169 (GGG → GAT)
TA7045	*hisG9130*	+	-	pKM101	CG → AT	Ala-169 → Asp-169 (GCG → GAT)
TA7046	*hisC9070*	+	-	pKM101	CG → GC	Arg-163 → Gly 163 (CGA → GGA)

Table 2. Genotypic properties of *Salmonella* tester strains developed by Gee et al. (1994).

The test system performed with TA700x tester strains is called as AMES II (Kamber et al., 2009). The set of TA7041-7046 strains is not suitable to test mutagenic and antimutagenic potential of chemicals due to lack of *rfa* mutation and their instable genotypes.

3.4 Positive control chemicals

Chemicals divide in two groups according to their affect mechanisms. These groups are direct and indirect acting positive controls.

Many direct acting agents has been introduced as positive controls because of their high specificity for the tester strains. The most common direct-acting positive control chemicals for Ames/*Salmonella* test system are listed in Table 3.

2-Aminoanthracene (2-AA; CAS# 613-13-8), 2-Aminofluorene (2-AF; CAS# 153-78-6) and Aflatoxin B1 (AFB1; CAS# 1162-65-8) are frequently used indirect-acting positive controls that requires metabolic activation before react with the *Salmonella* tester strains (Mortelmans & Zeiger, 2000; Ozbek et al., 2008b; Limem et al., 2010).

Chemical	Mechanism of Genotoxicity	Tester Strain	Reference	CAS#
4-Nitro-*o*-phenilenediamine (4-NPD)	Teratogenic and intercalating agent	TA1538 TA98	Ben Sghaier et al., (2010) Kaur et al., (2010) Mortelmans & Zeiger (2000)	99-56-9
4-Nitroquinoline 1-oxide (4-NQO)	Causing DNA lesions	TA1538 TA100 TA98	Oh et al., (2008) Ozbek et al., (2008a) Brennan & Schiestl (1998)	56-57-5
9-Aminoacridine (9-AA)	DNA intercalating agent	TA1537 TA97	Gulluce et al., (2010) Miadokova et al., (2009) Mortelmans & Zeiger (2000)	90-45-9
Methyl methane sulfonate (MMS)	Alkylating agent	TA102 TA104	Mortelmans & Zeiger (2000) Dellai et al., (2009) Zahin et al., (2010)	66-27-3
Mitomycin C (MTC)	DNA cross-linker and alkylating agent	TA102	Biso et al., (2010) Mortelmans & Zeiger (2000) Zhang et al., (2011)	50-07-7
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)	Alkylating agent	TA100	Caldini et al., (2005) Duh et al., (2009) Oh et al., (2008)	70-25-7
Sodium azide (NaN_3)	L-azidoalanine mediated base substitution	TA1535 TA100	Bulmer et al., (2007) Gulluce et al., (2010) Mortelmans & Zeiger (2000)	26628-22-8

Table 3. The frequently used direct-acting chemicals for the *Salmonella* tester strains.

3.5 Genetic analysis of the *Salmonella* tester strains

When a new strain received, its genotypic characteristics (*his, rfa* and *uvrB-bio*), spontaneous mutation rate and the presence of pKM101 and pAQ1 plasmids should be checked before preparation of frozen cultures for long term storage. For this purpose, follow these steps:

3.5.1 Inchoative stages for genetic analysis

- Add 1 mL of sterile nutrient broth to rehydrate the lyophilized culture.
- Transfer 10 µL of the rehydrated culture to nutrient agar plate and strake the inoculum to get individual colonies that serve as main sources for the genetic analysis of the tester strains.
- Transfer the rest portion of the rehydrated culture to 4 mL of nutrient broth. This broth culture serve as a back-up point in case of there is no growth on the nutrient agar plates.
- Incubate the cultures overnight at 37 °C. Then, check the agar plates and broth cultures for bacterial growth.

- At least two purification steps should be made to get more reliable results. Pick one healthy looking colony and streak it again on nutrient agar plates or GM agar plates supplemented with excess of biotin and histidine. If the tester strain carries pKM101 and/or pAQ1 plasmids, GM agar plates should be also supplemented with ampicillin and/or tetracycline, respectively. However, growth of the tester strains on the supplemented GM agar plates takes more time (approximately 48 h) than nutrient agar plates; it is recommended because using of them reduces contamination risks.

3.5.2 Genetic analysis

Five mandatory steps for all strains and additional one or two steps for plasmid carrying strains should be made to perform the best reliable genetic analysis. For this purpose, inoculate 5 mL of nutrient broth with a single colony after purification steps, and incubate the culture overnight 37 °C. Then, follow these steps for a complete strain check:

- In the 1st step, streak a loop of the overnight culture on the surface of a GM agar plate supplemented with excess of biotin, which demonstrates the histidine dependence (*his* mutation) of all the *Salmonella* tester strains. After an incubation period at 37 °C for 24-48 h, there should be no growth on the plate (see Figure 4a).
- In the 2nd step, streak a loop of the overnight culture on the surface of a GM agar plate supplemented with excess of histidine, which demonstrates the biotin dependence (*bio* mutation) of all the *Salmonella* tester strains except TA102 strain. After an incubation period at 37 °C for 24-48 h, there should be no growth on the plate. Due to lack of the *bio* mutation, TA102 strain can be growth on a GM agar plate supplemented with excess of histidine (see Figure 4b).
- In the 3rd step, streak a loop of the overnight culture on the surface of a GM agar plate supplemented with excess of biotin and histidine, which demonstrates the biotin and histidine dependence (*bio* and *his* mutations) of all the *Salmonella* tester strains. After an incubation period at 37 °C for 24-48 h, there should be growth on the plate (see Figure 4c).
- In the 4th step, streak a loop of the overnight culture on the surface of a GM agar plate supplemented with excess of biotin and histidine. Place a sterile filter paper disk in the middle of the plate and apply 10 µL crystal violet solution (0.1%, w/v) onto the disk. After an incubation period at 37 °C for 24-48 h, all strains show a zone of growth inhibition surrounding the disk, which demonstrates the presence of *rfa* mutation (see Figure 4d).
- In the 5th step, streak a loop of the overnight culture on the surface of a GM agar plate supplemented with excess of biotin and histidine. Unseal the top and cover the half of the plate with sterile aluminum foil. Expose the plate to a low level of UV irradiation for a short time (approx. 8-10 seconds) that kills the *uvrB* strain but not its isogenic DNA repair proficient strain. After an incubation period at 37 °C for 24-48 h, there should be normal growth on the non-exposed part of the plate but not on the exposed part. It demonstrates the presence of *uvrB* mutation. It is known that the source of *uvrB* mutation, a deletion mutation, also covers the biotin gene region. Therefore, if a strain shows a positive *bio* mutation result in the 2nd step, there is no need to check the presence of the *uvrB* mutation for this strain (see Figure 4e).
- In the 6th step, streak a loop of the overnight culture on the surface of a GM agar plate supplemented with excess of biotin, histidine and ampicillin. After an incubation period at 37 °C for 24-48 h, there should be growth on the plate, which demonstrates the

presence of pKM101 plasmid in the tester strains TA97, TA98, TA100, TA102 and TA104 (see Figure 4f).

- In the 7th step, streak a loop of the overnight culture on the surface of a GM agar plate supplemented with excess of histidine and tetracycline. After an incubation period at 37°C for 24-48 h, there should be growth on the plate, which demonstrates the presence of pAQ1 plasmid in the tester strain TA102 (see Figure 4f).

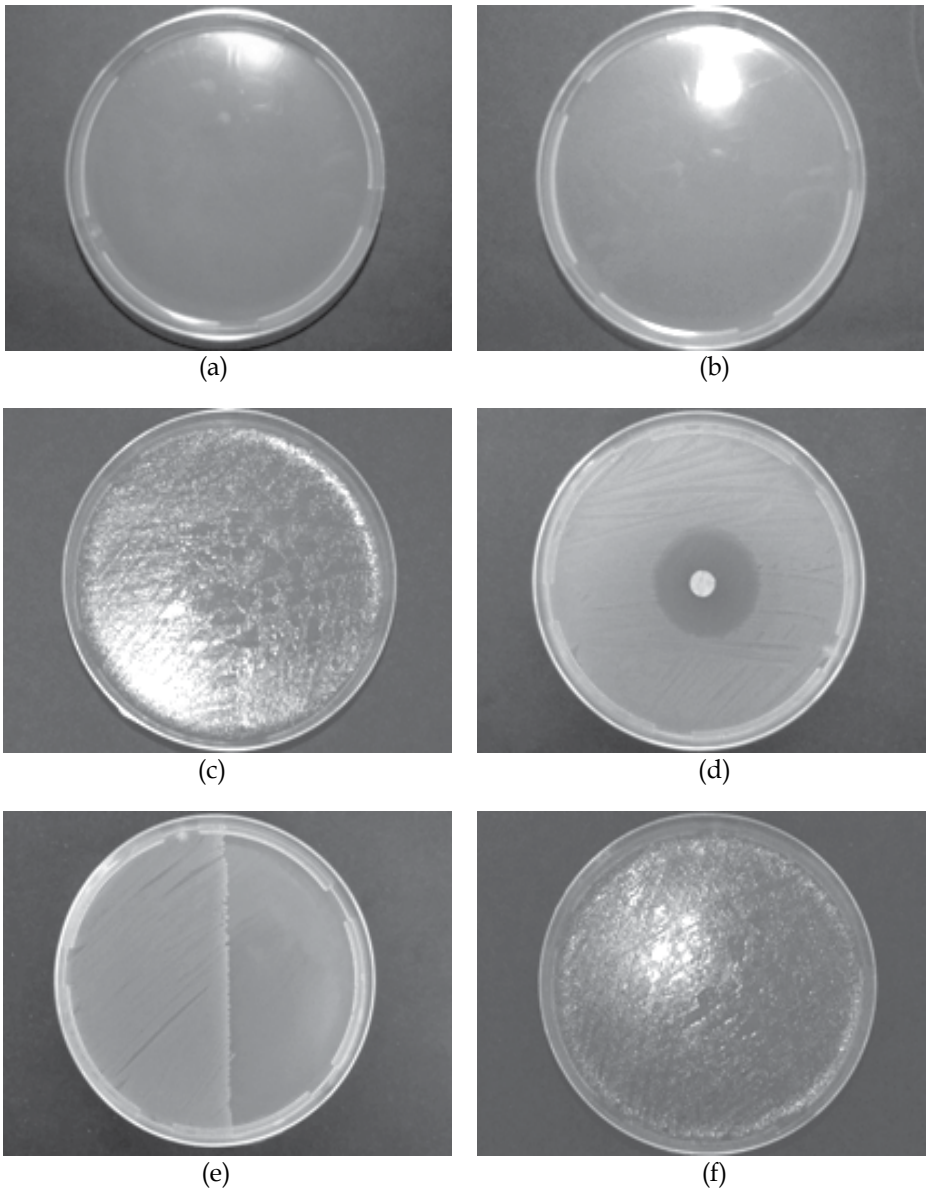

Fig. 4. Demonstration of (a) histidine, (b) biotin and (c) biotin/histidine dependence of the *Salmonella* tester strains, and presence of (d) the *rfa* mutation, (e) the *uvrB* mutation, (f) pKM101/pAQ1 plasmids.

3.5.3 Spontaneous mutation rates

Each laboratory has its characteristic spontaneous mutation rates for the all tester strains, and these values show a wide-range variation among the laboratories. Therefore, the spontaneous mutant frequency should be determined for all strains and recorded. It serves as historical control values provide choosing suitable strains for mutagenicity and antimutagenicity assays. Table 4 shows a sample of acceptable control values for the most common *Salmonella* tester strains.

Strain	Number of revertants	
	Without metabolic activation	With metabolic activation
TA97	75-200	100-200
TA98	20-50	20-50
TA100	75-200	75-200
TA102	100-300	200-400
TA104	200-300	300-400
TA1535	5-20	5-20
TA1537	5-20	5-20
TA1538	5-20	5-20

Table 4. Spontaneous revertant control values for the most common *Salmonella* tester strains (Mortelmans & Zeiger, 2000).

3.6 Long term storage of the tester strains

The *Salmonella* tester strains should be stored in a freezer at -80 °C or liquid nitrogen. Healthy looking single colonies should be chosen to prepare the frozen stock cultures. Dimethylsulfoxide (DMSO) or glycerol is suggested as cryoprotective agent. The final concentration of the cryoprotective should be at least 10% (v/v) (Mortelmans & Zeiger, 2000).

3.7 Viability assay and determination of test concentrations

Cytotoxic properties of the test materials toward the *Salmonella* tester strains should be determined before performing mutagenicity and antimutagenicity assays. The viability assay includes observations for *Salmonella* colonies on plates after 48 h incubation at 37 °C. Following three main characteristics for the tester strains should be taken into account.

- Thinning of the background lawn
- Absence of background lawn
- Presence of pinpoint non-revertant colonies

These characteristics indicate toxic levels of the test chemicals, and applicable dose ranges should be determined by repeating of the viability assay with lower concentrations of the test chemicals (Mortelmans & Zeiger, 2000).

3.8 Mutagenicity and antimutagenicity assays

Various test procedures for Ames/*Salmonella* test system have been developed to determine mutagenic and antimutagenic potency of synthetic and natural chemicals. These procedures mainly are based on the physical properties or quantity of the test chemical. For example; the desiccator assay has been developed for gases and volatile substances, and Kado assay allows studying the chemicals in small amounts. However, the standard plate incorporation method is the most common application procedure of the Ames/*Salmonella* test system (Kado et al., 1983; Hughes et al., 1987; Zeiger et al., 1992; Araki et al., 1994; Mortelmans & Zeiger, 2000; Tijs, 2008).

3.8.1 The standard plate incorporation method

The method consists of exposing the tester strains to the test chemical directly on a glucose agar plate. The main advantages of the method can be listed in giving easy, reproducible, reliable and comprehensive results.

Follow these steps for performing mutagenicity assay (Mortelmans & Zeiger, 2000):

1. Steps taken prior to performing the experiment
   - Inoculate the tester strain from frozen culture into 5 mL of nutrient broth and incubate the new culture overnight at 37 °C.
   - Prepare an appropriate number of labeled GM agar plates and sterile tubes for each test chemical.
   - Prepare metabolic activation system and keep on ice until use.
   - Prepare chemical dilutions.
   - Melt top agar supplemented with 0.05 mM histidine and biotin and maintain at 43 °C to 48 °C.
2. Add following items respectively into sterile glass tubes maintained at 43 °C and mix well each addition*.
   - 2 mL of molten top agar
   - 0.5 mL of S-9 mix (for the test performed with metabolic activation system) or buffer (without activation)
   - 0.05 mL of the test chemical dilution
   - 0.05-0.10 mL overnight culture of the tester strain (approx. $1\text{-}2\times10^8$ bacteria per tube – A_{540} 0.1-0.2)
3. Mix well the tubes and pour onto the surface of GM agar plates
4. When the top agar is solidify, invert and incubate the cultures at 37 °C for 48 h
5. Count the colonies after incubation and express the results as the number of revertant colonies per plate.

*Notes: This step includes two additional groups which are negative controls and positive controls. The negative controls do not include 0.05 mL of the test chemicals, but include the solvent at equal quantity. Positive controls also do not include 0.05 mL of the test chemicals, but include the suitable positive mutagen solution for the tester strain at equal quantity.

The procedures of mutagenicity assay are all applicable to the antimutagenicity assay. The only procedural difference is the addition of the suitable positive mutagen solution to the all test chemical groups (Nagabhushan et al., 1987; Bala & Grover, 1989; Edenharder et al., 1999; Edenharder & Grünhage, 2003; Ozbek et al., 2008a, 2008b; Gulluce et al., 2010).

4. Conclusion

In conclusion, the mutant *Salmonella* strains are beneficial for humanity contrary to their pathogenic wild-type strains. The histidine auxotrophic *Salmonella typhimurium* strains, object of the present study, provide a possibility to determine natural and synthetic chemicals with mutagenic properties. Similarly, these are also valuable for identification of antimutagenic chemicals after minor technical modifications. When a chemical, precious for industrial or health applications, is found or synthesized, determination of its genotoxic properties has a great importance. In this perspective, the Ames test allows making relatively cheap and reliable applications resulting in a short time.

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Part 4

Immunology

15

Salmonella enterica Serovar Enteritidis (SE) Infection in Chickens and Its Public-Health-Risk Control Using an SE Vaccine in Layer Flocks

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1. Introduction

Food poisoning caused by *Salmonella enterica* serovar enteritidis (SE) became a major public health problem in the middle of the 1980s, and several years were required to identify that the main causative food material was chicken eggs (Altekruse S. et al. 1993, [a),b)]CDC 1990, Cogan TA et al., Cowden JM et al. 1989, Henzeler DJ et al. 1994, Humphrey TJ 1994, Kusunoki J et al. 1996, Lin FY et al. 1988, Shivaprad HL et al. 1990, St Louis ME et al. 1988). Since CDC had firstly-reported the main causative origin of SE food born disease being shell eggs (CDC. 1987), shell eggs as a causative food have attended (Hogue A. et al. 1997, Humphrey TJ et al. 1991, Rodrigue DC et al. 1990,). World status of SE outbreaks at around 1999 is well-reviewed in the book of "*Salmonella enterica* serovar enteritidis in human and animals". (Saeed AM. Ed. 1999. Iowa State University Press). SE-contaminated chicken eggs are indistinguishable from non-contaminated eggs in appearance. As the sensory elimination of SE-contaminated chicken eggs was shown to be impossible, greater importance has been attached to the control of SE contamination in the egg production step ([a),b)]CDC 1990, Okamura M et al. 2001, Rodrigue DC et al. 1990, and Stevens A et al. 1989, Thomas RD 1989). The development of live and inactivated SE vaccines has been investigated because SE contamination of chicken eggs remained even after various hygienic countermeasures were taken on layer farms, and SE vaccine administration was started in the 1990s in Western countries. However, SE vaccines have only recently been recognized as an important tool to reduce SE-contaminated chicken egg production on layer farms.

Regarding efficacy evaluation of SE vaccines, the effect of live SE vaccine was understood as competitive elimination (Barrow PA et al. 1991, Hassan JO et al. 1997, Nasser TJ et al. 1994, and Parker C 2001), but many questions remained regarding inactivated SE vaccine (Davies R et al. 2003, Gast RK et al. 1992, Okamura M et al. 2007). The question concerning efficacy was whether the vaccine can eliminate SE which colonizes the digestive tract and reproductive organs even after elevating resistance in blood and parenchymal organs by SE antigen inoculation. For example, 100% elimination of gastrointestinal SE could not be achieved immediately after challenge in the chickens administrated with an inactivated SE vaccine, and also orally ingested bacteria proliferated in the gastrointestine. Moreover, concerning the

mode of infection of pullorum disease (*Salmonella enterica* serovar pullorum infection) in chickens as a model (Gwatkin R. 1948, Shivaprasad HL. 2000), SE infection normally manifests no clinical symptoms and natural resistance levels rise in chickens aged 3 weeks or older, at which time inactivated SE vaccine becomes administrable, and SE colonization in the gastrointestine is very limited. Accordingly, it was considered that inactivated SE vaccine is unnecessary for chickens aged 3 weeks or older because of enhanced resistance to SE infection and the effect of inactivated SE vaccine is not useful. However, considering that the ultimate objective of inactivated SE vaccine administration to chicken flocks is to reduce SE-contaminated chicken egg production, we performed studies assuming that SE contamination of chicken eggs can be prevented by employing inactivated SE vaccine through a mechanism different from those of vaccines preventing chicken diseases, and epidemiologically clarified that inactivated SE vaccine administration in layer farms reduced the number of SE-contaminated chicken eggs to 1/260 and the isolation frequency to 1/10 as shown in Table 1 (Summary of field study on SE isolation incidence and bacterial No. in the presence and absence of SE bacterin application in four layer farms. [a)]Toyota-Hanatani Y et al. 2009). We also confirmed a high epidemiological risk-reducing effect of inactivated SE vaccine administration to flocks. Furthermore, we investigated the active component of inactivated SE vaccine and identified that the important activity is located at the flagellar g.m. antigen site (SEp 9) and the other components do not induce potent specific antibody production.

Incidence/Percentage	**Vaccination status with inactivated SE vaccine**	**Result**	**bacterial No. or Mean (MPN/100mL) ±SE a)**
Bacterial No.	Yes	<2 to <8	2.5±0.1**
	No	<2 to >1,600	674±4.1
Incidence	Yes	14/571	2.45%**
	No	10/40	25%

**p<0.01 a) Mean ± Standard error

Table 1. Summary of field study on SE isolation incidence and bacterial No. in the presence and absence of SE bacterin application in four layer farms. ([a)]Toyota-Hanatani Y 2009. et al.) This table summarizes SE detection using more than 20 kg of liquid egg in four laying houses where inactivated SE-vaccinated and -unvaccinated chickens were housed for three years. As shown in this table, SE was isolated at up to 8 MPN (Most Probable Number) per 100 ml from inactivated SE-vaccinated chickens and over 1,600 per 100 ml from some non-vaccinated flocks. The inactivated SE vaccine significantly reduced public health risks. Material and Method; Four layer farms were monitored using over 20 kg liquid eggs for 3 years according to the method described in Fig. 1. In this duration, the vaccination status with SE bacterin was mixed (Vaccinated and un-vaccinated flocks were there in each farms).

In previous studies on the mode of SE infection in flocks, the mode of infection of pullorum disease (PD) bacteria in chickens (Table 2. Mode of SE infection in chickens and chicken flocks) was referred to and investigated as a model in the SE vaccine development. However, a recent study and our survey results suggested that SE contamination/infection of chickens disseminates through mouse-mediated transmission between hen houses (Davies RH et al. 1995, Henzler DJ et al. 1992), and not by vertical infection as in PD infection model (Yamane Y et al. 2000). Unlike SE infection of mice and humans (Guiney DG

et al. 1995), SE infection of individual chickens occurs as opportunistic infection excluding that immediately after hatching ([a),b)]Bohez L et al. 2007, and 2008, Dhillion AS et al. 2001, and Roy TP et al. 2001), and the infection is not systemic and manifests no symptoms in infected chickens. Based on previous study results reported, it is suggestive that SE ingested by a chicken evades the chicken's immune system by changing substances expressed on its surface, which may be the essence of Salmonella infection

Mode of infection	Concept of mode	Examples and explanations
1. Vertical transmission	In chicks SE Infection occurred from infected parent chickens via embryonated eggs. PEQAP research data said this mode infection might be positioned at less than 5 % in all the infection cases in chicken flocks.	Infection mode of pullorum disease(PD) in chickens (PD is asymptomatic in adult chickens, but is highly lethal in chicks.) Thus, an antibody test was conducted in adult chickens to successfully eradicate positive ones.
2. Horizontal infection	Between chicken flocks (This route is more common according to the PEQAP survey)	Common route (SE may be transmitted by mice.)

Table 2. Mode of SE infection in chickens and chicken flocks. (Summarized by Ohta H)At first, many poultry farmers considered that SE was vertically transmitted. This is because both Salmonella pullorum and SE carry O9, O12, and O1 (belong to Salmonella D group and have the same antigenicity except for the presence of flagellar antigen) as bacterial antigens. Thus, many poultry farmers had tried that they could take measures by conducting a SE contamination (antibody) survey in breeding chickens to remove positive chickens. However, the PEQAP and our surveys demonstrated that SE infection was transmitted among laying flocks and that contamination was limited in growing and breeding farms. Thus, we considered that the inactivated SE vaccine might be effective.

2. Historical changes in the concept of 'food safety' with the recent emergence of SE food poisoning

2.1 History of occurrence of SE-contaminated chicken egg-induced SE food poisoning

Outbreaks of SE food poisoning, not previously noted, frequently occurred worldwide after 1980, mainly in Western countries, and became a social problem (Davison S et al. 2003, Stevens A et al. 1989). Before 1980, Salmonella food poisoning was mainly caused by *Salmonella enterica* serovar Typhimurium (ST), and so this species was mainly studied. Countermeasures against ST-induced food poisoning were taken to avoid hygiene problems of cooking facilities in many cases, and actions rarely reached the management of food material production. However, outbreaks of SE-induced food poisoning occurred in the middle of the 1980s in Western countries, and studies and study result-based countermeasures for not only ST but also SE food poisoning became necessary. The most surprising evidence with SE outbreaks for epidemiologists is that the outbreaks are

sometimes caused with several number of SE not caused with numerous SE (Foley SL et al. 2008). At the beginning, contaminated food products and materials in SE food poisoning were unclear, and actions were mainly taken involving only cooking facilities, similarly to measures taken for ST. Many cooking facilities tried to eliminate 'inappropriate food materials (food materials detectable by sensory evaluation, such as those which had started decomposition and color change)', and complete hygiene measures were taken at cooking facilities. However, the occurrence of SE food poisoning did not stop. Around the end of the 1980s, researchers started to point out that chicken eggs were very likely to be contaminated by SE ([a),b)]CDC 1990, Rodrigue DC et al. 1990, Steven A et al. 1989, Thomas RD 1989). In poultry industry in United Kingdom, a poultry association consisting of eggs producers, feed suppliers, eggs traders, egg-packing sections joined together to establish an egg sanitary standard like as HACCP (Hazard analysis and critical control point) system, so called Red Lion Code. On the other hand, SE-contaminated chicken eggs were vigorously studied, and several tens of thousands of chicken eggs were individually tested for SE contamination, and the incident of SE contamination eggs originated from SE infected poultry flocks also reported to be about 10 folds increasing compared with those of ordinary shell eggs (Humphrey TJ et al. 1994). In the U.S., it has been said that several million chicken eggs were individually tested for SE. These are unbelievable numbers compared to that in the current SE test, and our studies are based on the efforts of researchers at that time, to which I express my respect.

2.2 Why did SE-contaminated chicken eggs become a public health issue?

Since very few chicken eggs on layer farms are contaminated, reportedly, several in 10,000 eggs, SE-contaminated chicken eggs were not recognized as the cause of SE food poisoning earlier, as described above. However, many retroactive surveys suggested that the main cause was chicken egg-mediated SE contamination. On the other hand, layer farm-related test facilities performed SE tests based on the mode of PD infection as the model, and this was mainly performed for breeding flocks and chicks. Nearly 100% of samples from layer breeder farms were negative, and the isolation rate from breeding flocks and just hatched chicks was not high enough to explain the occurrence of SE food poisoning in humans. It was revealed that growing chickens and chicks are rarely contaminated with SE, which was markedly different from the retroactive survey results of food poisoning. Accordingly, chicken egg producers and chicken salmonellosis researchers believed that the frequency of SE-contaminated chicken eggs is very low, SE contamination of eggs on layer farms is not the main cause, and food poisoning occurs due to SE contamination of chicken eggs during distribution or at the consumer level due to inappropriate classic hygiene management. The detection and removal of SE-contaminated chicken eggs by employing sensory tests were considered possible at that time, and some people strongly believed that detection and removal at cooking facilities using the conventional method were possible. However, many retroactive surveys (Fris C et al. 1995, Henzler DJ et al. 1994, Kusunoki J et al. 1996) revealed that SE contamination was present in eggs that appeared normal as well as on layer farms, which led to recognizing that measures to reduce SE-contaminated chicken eggs are necessary and the safety control of food products and materials should be facilitated by producers, distributors, and consumers in unity. However, considerable time was necessary to spread the necessity of taking actions to reduce SE-contaminated chicken egg production to people related to chicken egg production.

1. Characteristics of farms with frequent SE contamination
1. Large scale (many chickens)
2. Many mice
3. Multiage flocks
4. Many poultry houses
5. EP center established side by side
2. Age (days) when SE infection occurs more commonly
1. After transfer to egg collection poultry farm and during laying peak (170-200 days old)
2. After laying peak
3. Newborn to 10 days old
4. 10 days old to transfer to egg collection poultry farm (in rare cases)

*Most SE infections occur in 1) and 2).

Table 3. Characteristics of SE infection in layer flocks (Biomune's seminar on SE vaccine in Japan in 1996, based on PEQAP research data)

In the northeastern U.S., severe SE contamination occurred in the latter half of the 1980s. Thus, egg consumption markedly decreased because of harmful rumors among consumers. Poultry farm associations, Pennsylvania government, U.S. federal government, etc. established the Pennsylvania Egg Quality Assurance Program (PEQAP) to survey SE contamination in egg-collecting farms in Pennsylvania. In addition, SE contamination rates of eggs and measures, such as inactivated SE vaccine, were evaluated. As shown in this table, SE infection spread in egg-collecting farms, in which mice played an important role. In addition, the inactivated SE vaccine reduced SE-contaminated eggs, but did not effectively eradicate them.

In the 1980s to early 1990s, chicken eggs were reported to be the main cause of SE food poisoning by various media, resulting in a marked reduction of chicken egg consumption. The SE issue put chicken eggs in a disadvantageous sales situation: egg consumption decreased not due to the reports of scientists, but because consumers and egg sellers (supermarkets) considered that eggs were produced by large layer farms performing no hygiene control. Layer farms in North East America, and England and Wales specified as SE-contaminated regions instantaneously lost more than 50% of chicken egg consumption, so-called 'damage caused by harmful rumors' (Davison S et al. 2003). The layer farm industry and instructing administrative agency were surprised and started joint studies involving layer farms, universities, and related administrative agencies. Based on the study results, the administrative agency adopted various measures for chicken egg production and distribution including legal action. For example, in response to this situation, Pennsylvania State in the U.S. vigorously surveyed the actual state of SE contamination on layer farms, investigated the mode of SE contamination, and evaluated various countermeasures. The survey results of the Pennsylvania Project (PEQAP) concerning the mode of SE infection of chickens (Hogue A et al. 1997) revealed that no SE infection was observed in chickens in the growing period, and most cases of infection occurred after chicks were introduced into layer hen houses (Table 3. Characteristics of SE infection in layer flocks unpublished data). The point emphasized was the presence of mice playing an important role in SE transmission between hen houses as above mentioned. Based on these epidemiological study findings, the U.S. government took administrative action by establishing a law which allows selling only sterilized egg liquid prepared from eggs collected from SE-contaminated hen houses (Table 4 US-FDA egg safety rule; US Federal Register 2009. 74: 33030-330101.). In Japan, labeling chicken eggs sold in

packages with a date (laid, packaged, or sell-by date) is required. Administrative actions have been taken against SE food poisoning in many countries. In which "Red Lion Code" is involved. The history of the recent emergence of SE food poisoning emphasized the necessity of analyzing the cause of food poisoning in the processes of chicken egg production through distribution and consumption (Schroeder CM et al. 2006). For analysis and the control of health damage risks of not only chicken eggs but also all food products, identification and evaluation of possible risks in each step of production, distribution, and consumption and investigation of countermeasures while considering the cost-effectiveness have been established as " risk analysis concept" and applied to the problem of SE-contaminated chicken eggs as one case. However, no basic concept for the control of bacterial food poisoning has been established, and many epidemiological studies are still necessary.

Testing or Procedure	FDA
Chicks	NPIP SE Clean breeders
Pullet testing	14 to 16 weeks
Requirement for pullet + manure	Egg testing of 4 sets of 1000 eggs at 2 week intervals
Layer testing	40w and 4-6 weeks after molt completion
Egg testing if manure positive	1000 eggs at 2 week intervals, 4 submissions
Diversion to pasteurization required for egg+ flocks	Yes
Return to shell market allowed	Yes after a completed set of 4 submissions of 1000 eggs every 2 weeks
Egg testing after initial egg test set	None if negative first set; once a month if were previously egg positive
C&D of manure or egg + houses	Wet or dry cleaning
Vaccination required	None
Biosecurity plan	Required
Rodent Control Plan and Records	Required
Fly Control Plan and Records	Required

(personal information from Dr. Lozano F)

Table 4. US-FDA egg safety rule (Established by USDA, 2009)

2.3 History of SE vaccine

Live ST vaccine was used as SE control in large-scale state layer farms in Former Eastern Europe before the reunification of East and West Germany as described below. The safety and efficacy of this live ST vaccine for SE control were investigated, and several preparations are still used now.

Regarding inactivated SE vaccine, I would like to introduce the history of its first appearance in the world. The in-house vaccine system was established in the U.S. in the 1980s. In this system, farms which isolated the pathogen were allowed to use an inactivated vaccine for the infectious disease not included in highly pathogenic infectious diseases of animals, such as legal infectious diseases, and approval was granted to in-houses vaccine manufacturers. An in-house vaccine manufacturer produced a vaccine using an SE strain isolated from a layer farm,

and the vaccine reduced the SE isolation frequency on the farm. This was the first preparation of inactivated SE vaccine. The world's first state approval was granted for a vaccine which showed efficacy in the field, not prepared through establishing an evaluation method in a laboratory and then confirming the efficacy in the field. Subsequently developed inactivated SE vaccines were produced following the first inactivated SE vaccine as the standard.

3. Discussion on the usefulness of SE vaccine administration to chickens

3.1 Situation at the time of early approval of inactivated SE vaccine

The world's first approval of inactivated SE vaccine by the administrative authority was granted to Layermune SE (Biomune Co., Kansas) in the U.S.A. in 1992. Since then, inactivated SE vaccine has been discussed with regard to not only the efficacy but also many other aspects. Discussions have mainly concerned doubt regarding the efficacy, and, secondly, vagueness of the objective of use. Generally, the objective of animal and human vaccines is the prevention of clinical problems of vaccinated animals and humans, but SE infection manifests no clinical symptoms in chickens excluding newborn chicks, causing no economic damage. For newborn chicks, there is no time for vaccination because infection occurs before inactivated SE vaccine exhibits an immunological effect. Accordingly, inactivated SE vaccine is administered to chickens developing no clinical problems, and the objective is only to reduce the public health risk (reduction of SE-infected chicken egg production). Chickens are vaccinated for a disease manifesting no clinical symptoms, but the effect of the vaccine has to be investigated in these chickens. Economically, inactivated SE vaccine has to be administered to individual chickens, requiring considerable human labor and expense for purchasing the vaccine. Since SE infection causes no direct economic damage, vaccine administration to chickens on farms requires the high-level motivation of vaccine users. The first inactivated SE vaccine was a new type of vaccine, i.e., emergence of a high-cost vaccine slightly stressful to vaccinated animals and not preventing disease in the animals white leghorn chickens (Mizumoto N et al. 2004).

We also investigated the efficacy of inactivated SE vaccine employing various challenge tests. In one of the tests, SE was orally challenged 3 or 4 weeks after inactivated SE vaccine administration, and SE was re-isolated from the gastrointestine and parenchymal organs. Concretely, 3-week-old SPF chickens were vaccinated at the normal dose and orally challenged with food poisoning-derived SE at a high bacterial count after 4 weeks (at 7 weeks of age), and the bacteria were re-isolated from the cecum after 1-7 days. SE was isolated from nearly 100% of chickens despite the vaccine having been administered. When the number of challenged bacteria was reduced to a moderate count, the number of isolated bacteria was significantly decreased in many animals in the vaccinated group, but the results were not stable. In chickens subjected to the test at 5 or 7 weeks of age, the isolation frequency after challenge (at 11 weeks of age) was markedly lower in the control non-vaccinated group. Accordingly, a large number of chickens are necessary to perform the challenge test at this age, which is not routinely possible. In this laboratory test, a significant reduction of the intestinal bacterial count was observed, but complete disappearance of the bacteria from the gastrointestine has not been confirmed within a couple of weeks.

3.2 Situation at the time of the initiation of our study

In 1990, we were informed of SE-contaminated chicken egg production on layer farms covered by our veterinary care activity. We administered bacteriostatics and organic acids

on large-scale layer farms, hoping to avoid a decline in consumption, which occurred in America and England, but no effect was obtained. Thus, we performed a field epidemiological study of the efficacy of inactivated SE vaccine (Yamane Y et al. 2000). Inactivated SE vaccine was administered to flocks on a large-scale layer farm with apparent SE contamination. Eggs (500 kg) were broken in a liquid egg plant, 1,000 ml was sampled from the liquid egg batch, and 400 ml was subjected to SE isolation. The isolation rate was compared between the vaccinated and non-vaccinated groups. The results are shown in Fig. 1. (Fig. 1 Number of SE isolates and SE isolation frequency of SE-contaminated chickens and inactivated SE-vaccinated chickens in the same poultry house). Furthermore, our study confirmed horizontal infection of 4 industrial poultry farms (Table 5. SE samples monitored and their results with 4 integrated layer companies (1996-1998)). Based on those results, it was considered necessary for the positivity rate on plate agglutination with pullorum disease-diagnostic antigen to be 90% or higher in the SE-inoculated group (0% in the non-inoculated control group), while vaccination of SE-contaminated farms significantly reduced the number of bacteria isolated from liquid egg samples from 500 kg or more of eggs compared to that from non-vaccinated chickens (Table 6 Evaluation criteria for the inactivated SE vaccine (Layermune SE) in field chickens). In addition, the requirement of the number of isolated bacteria from chicken feces was set at 1 CFU or lower per 1 g in the inactivated SE vaccine-treated group. Later, similar results were obtained in the test using 20 kg of eggs (about 320 eggs). We partially demonstrated these established values epidemiologically after more than 10 years ([a])Toyota-Hanatani Y et al 2009).

Vertical section for SE monitor	Sample materials	Result monitored	Memo
Breeder farms	1) Several swabs 2) Manure 3) Sera to detect antibody 4) Workers feces	No detection at all	Using SE cell antigen coated ELISA
(Hatchery)	1) Swab 2) Worker feces	No detection	
Feed mile	Any protein source	No detection	
Growing	Like as breeder	No detection	
Laying	1) Swabs 2) Manure 3) Dusts 4) Liquid eggs 5) Workers feces 6) Water in EP center	No detection A few positive A few positive Several positive No detection Detectable	

Table 5. SE samples monitored and their results with 4 integrated layer companies (1996-1998) (Yamane Y et al. 2000. modified). Our severance studies (Yamane Y et al. 2000) summarizes the results of SE tests conducted in breeding farms, feed mills, and EP centers for three years. As shown in this table, no vertical transmission (infection from laying to adult chickens) occurred. The infection was repeated within a laying poultry houses. Materials and Methods; See Fig. 1.

		Case 1			Case 2		
		Age	**SE(MPN)**	**ELISA[C]**	**Age**	**SE(MPN)**	**ELISA**
Year1	Jan.	237	○	0 %	181		
		260	○		252	●(>1600)	0 %
				0 %			
		350	○				
				0 %			45 %
	Jul.			20 %	349	●(NT)	
		455	●(NT)	20 %			28 %
							35 %
					447	●(NT)	
		552	●(39)	15 %			20 %
Year2	Jan.						
				35 %			25 %
				25 %			10 %
				25 %			
				40 %			
	Jul.				729	Replaced	
		881	△				
		911	▲	35 %			
					218	△	
		937	Replaced		245	(<2)	
					259	△	
Year3	Jan.						
		168	△		316	△	
		239	△		386	△	
		302	△				
					435	△	

Fig. 1. Number of SE isolates and SE isolation frequency of SE-contaminated chickens and inactivated SE-vaccinated chickens in the same poultry house (Yamane Y et al. 2000)
SE isolation in field chickens before and after inoculation of the inactivated SE vaccine (Layermune SE) (Four cases are shown in the reference. Two cases are shown here.) (Filled circles indicate SE isolation). The number indicates the number in ( ) of SE isolates.
The detection rates of SE antibodies in unvaccinated chickens by ELISA coated with SE cell antigen was 0-40% for Case 1 and 0-45% for Case 2. To our experiences, the antibody positive rate of 400-500-day-old chickens inoculated with the inactivated SE vaccine (at 300-400 days after vaccination) was about 70-100%. Inaccurate administration of SE bacterin may induce the antibody positive rate of inactivated vaccine to be further decreased. Thus, vaccination and field infection cannot be distinguished at antibody level. The number and frequency of SE isolates decreased in the vaccinated group.

Material &Methods: An industrial layer farm was monitored. SE isolation was done using liquid eggs samples originating from 500 kg of shell eggs. And then most probable number (MPN) per 100 m was determined. For detection of specific antibody in the sera of the flocks, an ELISA coated with SE cell antigen was used.

Test item	Method	Procedures	Criteria
Antibody response	RPA	Twenty chickens were examined at 4 weeks after vaccination.	≥90%: Markedly effective <90%~≥80%: Effective <80%: Non effective
Antibody response	ELISA	Same as above	Same as above
Bacterial isolation	Bacterial isolation	500 kg of eggs are collected from the vaccinated group. The eggs are broken and cultured within 48 hours after collection.	≤10MPN/100mL: Markedly effective (if materials from the unvaccinated group of the same farm showed ≥1,600 MPN/100 mL)

Table 6. Evaluation criteria for the inactivated SE vaccine (Layermune SE) in field chickens (application form for the reexamination of this formulation in Japan, provided by CAF Laboratories)
The effectiveness of the formulation (Layermune SE) in Japan is evaluated based on this table. The formulation was effective in all the 12 chicken groups by an antibody test. However, SE-contaminated farms could not be surveyed by bacterial isolation.

3.3 Risk of misjudging inactivated SE vaccine-treated chickens as SE-infected chickens

We had a problem in handling inactivated SE vaccine in our field facilities: inactivated SE vaccine-treated chickens and SE-infected chickens showed the same serological reaction (Table 7. Production of antibodies against SE bacterial antigens in inactivated SE-vaccinated and -unvaccinated chickens). Inactivated SE vaccine is generally administered at about 80 days of age. In chickens treated with a commercial inactivated SE vaccine, the anti-bacterial cell antigen-antibody positive rate determined using commercial antigen solution for the diagnosis of PD, or SE cell antigen coated ELISA reaches nearly 100% within about 120 days of age and then slowly decreases and reaches 20-60% at about 300 days of age, whereas the positive rate in SE-infected chickens is about 5-70%. We attempted to distinguish SE-infected from inactivated SE vaccine-treated chickens because eggs laid by inactivated SE vaccine-treated chickens are misjudged as those laid by SE-infected chickens, if the 2 chicken groups of SE infected and vaccinated cannot be distinguished. Thus, we investigated specific antibodies present only in chickens with 'inactivated SE vaccine treatment' described below (Fig. 4. Detection of specific antibodies in sera against SE cell antigen and SEp9 on oral SE administration to field white leghorn chickens) (Mizumoto N et al. 2004).

Group	Positive rate (references)	Test methods**	(References)
Inactivated SE vaccine	Vaccination	ELISA	
In the laboratory	At 30-40 dpv: 95-100%	RPA	
	: ≥ 90%		
In field	300~400 days old: 70~100%	ELISA	
SE infected group (Field group)	Shipping to slaughterhouse (about 700 days old): 0-15%	ELISA	(Mizumoto N et al. 2004, Sunagawa H et al. 1997, Yamane Y et al. 2000)
	Induced molting (400-500 days old): 0~45%	ELISA	

* Age of vaccination: around 80 days old
** ELISA: Indirect method with SE cell antigen coated.
RPA: rapid plate agglutination with diagnostic for pullorum disease antigen.

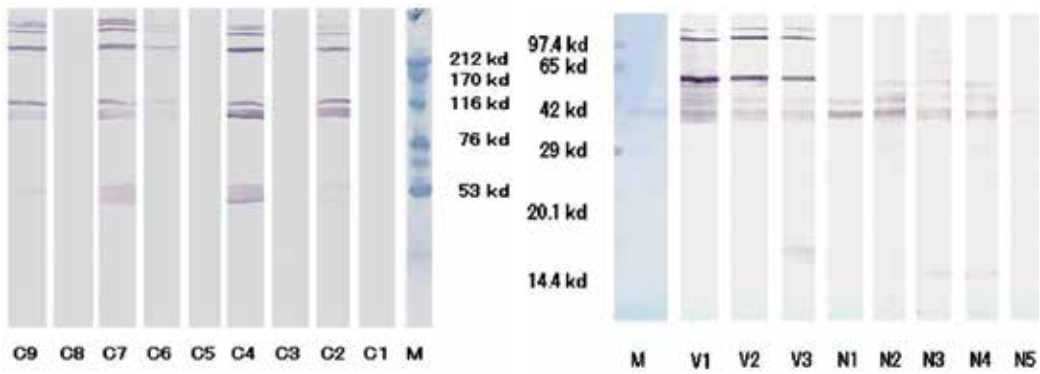
Table 7. Production of antibodies against SE bacterial antigens in inactivated SE-vaccinated and -unvaccinated chickens (summarized by our research group)

Almost all the 3-week-old or older chickens inoculated with the inactivated SE vaccine were positive at around four weeks by both ELISA (coated with SE cell antigen) and RPA. Subsequently, the positive rate decreased at 250 days or later after inoculation. The positive rate in the ELISA coated with the g.m. antigen of SE was shown above 80% up to about 700 days old. On the other hand, SE-contaminated chickens showed the similar positive rates as those of inactivated SE-vaccinated chickens in ELISA coated with SE bacterial antigen and RPA. Generally, the positive rate of SE-contaminated chickens is lower than that of inactivated SE-vaccinated chickens. However, an antibody test cannot distinguish these 2 groups, because some SE-contaminated chickens show higher positive rate.

3.4 Active component of inactivated SE vaccine (main Fli C antigen: SEp 9)

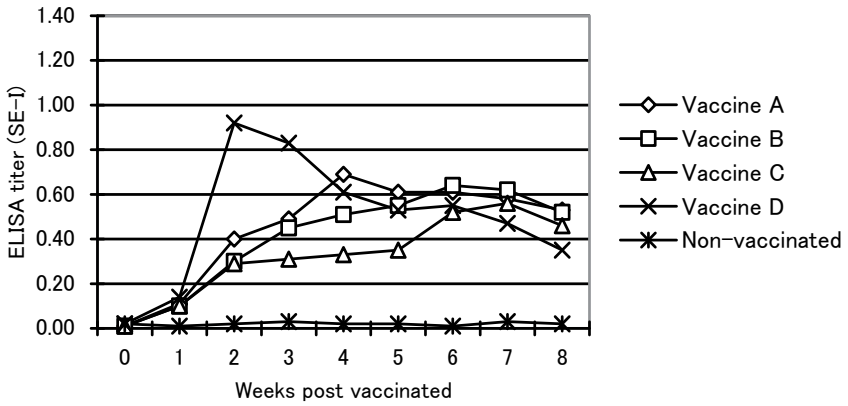
Using sera from inactivated SE vaccine-treated and SE-infected chickens, we compared the production of antibodies against the SE cell antigen to investigate differences between the sera. A strong reaction with a 53-kDa polypeptide (Fli C) (Namba K et al. 1997) was observed in all serum samples from inactivated SE vaccine-treated chickens, but rare reaction with a specific antigen was noted in SE-infected chicken-derived serum samples (Fig. 2. Western blotting with sera from SE-infected and inactivated SE-vaccinated chickens using formalin-treated SE antigens (surface antigens)). Fli C is considered to be strongly antigenic as inactivated SE vaccine. When the SE-specific polypeptide (g.m. antigen) in Fli C (Van Asten AJ et al. 1995, and Yap LF et al. 2001) was prepared by genetic engineering and reacted with serum from inactivated SE vaccine (Layermune SE)-treated chickens, strong reactivity was noted, but SE-infected chicken-derived serum did not react with g.m. antigen. When the specific antibody reaction was investigated in sera from chickens treated with other vaccines sold in Japan (oil adjuvant vaccine 3 and aluminum hydroxide gel vaccine 1), a specific antibody reaction with g.m. antigen was noted in the serum of oil adjuvant vaccine-treated chickens (Fig. 3.

Production of specific antibodies against commercial inactivated SE vaccines SE cell and SEp9 antigens). In an experiment, the inoculated chickens with SE induces antibody against SE cell antigen but not SEp 9. In field poultry flocks, inactivated SE vaccine administration was confirmed a long period persistency of specific antibody level against SEp 9 until 700 days of age (Fig. 5. Positive rates of g.m.-specific antibodies in the yolks derived from field chickens inoculated with the inactivated SE vaccine).

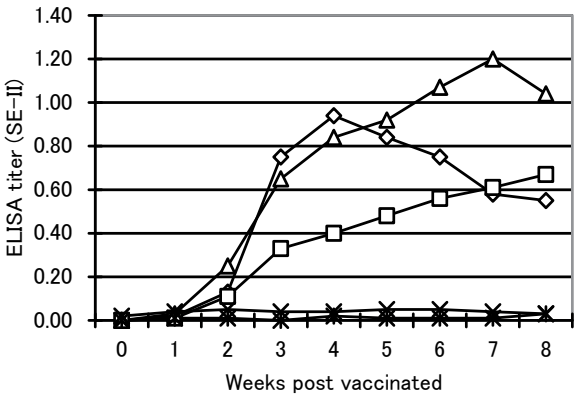


(a) (b)

Fig. 2. Western blotting with sera from SE-infected and inactivated SE-vaccinated chickens using formalin-treated SE antigen (surface antigen) (Nakagawa Y et al. reported by Japanese)
Figure 2a shows the reactivity of sera from 3-week-old SPF chickens which received oral SE administration (C1~9, M: marker protein), examined by Western blotting (SDS-PAGE) with SE surface antigen. Fig. 2b shows Western blotting with the same antigen using sera from 3-week-old SPF chickens inoculated with the inactivated SE vaccine (Layermune SE) (at 4 weeks after inoculation) (V1~3) or from those from which SE was isolated from naturally-infected-field flocks (N1~5; 710 days old).
Fig. 2a shows that light antibody response against 53 kDa (Fli C of SE) was noted in two chickens (one chicken at 2 weeks) and no band against Fli C (53 kDa polypeptide) was noted in all the nine SE-intraoral inoculated chickens. As shown this figure, one of 2 responded band at week post inoculation (wpi) was continued by 2 wpi but not by 4 wpi. Thus, the responsive antibody was considered to be IgM antibody. In another our report, a 53 kDa band was not detected in 4-week-old SPF chickens and 300-day-old field chickens, which received SE administration, but was detected in molting-induced chickens (Mizumoto N et al. 2004, Piao Z et al. 2007). Thus, the antibody against the 53 kDa polypeptide after SE inoculation is suspected no invasion into the internal organs.
Fig. 2b shows strong bands against the 53 kDa polypeptides and its dimer (98 kDa) in inactivate SE-vaccinated chickens. However, in chickens from which SE could be isolated, a weak band could be detected at around 42 kDa, but no band could be detected at 53 kDa.
Materials and Methods: For antigen preparation, SE was treated with formalin and centrifuged at 2000 g for 20 min. Then the supernatant was further centrifuged at 10,000g for 60 min and the precipitate dissolved in a buffered saline. The antigen was used in this analysis. The sera for SE infected chickens were prepared from the chickens inoculated with SE at the age of 3 weeks, and were weekly bled individually for this study. To the "vaccine sera", SPF chickens were injected with Layermune SE at the age of 3 weeks and bled 4 weeks post injection. The sera were designed as vaccine sera.



(a)



(b)

Fig. 3. Production of specific antibodies SE cell (deflagellated) and SEp9 antigens (Nakagawa Y et al. Japanese report)
(a; Antibody response to SE cell antigen, b; Antibody response to SEp9)
Four commercial inactivated SE vaccines (Vaccine A to D) were used to inoculate five 3-week-old SPF chickens/group to examine the responsiveness to SE cell antigen and SEp9. Results shown in Fig. 3A and 3B were obtained. No response was noted in unvaccinated chickens. The inactivated SE vaccine responded to SE cell antigen in all the chickens. Notably, the antibody response of the formulation with aluminum gel used as adjuvant rapidly increased and then decreased. On the other hand, the antibody response to SEp9 was specific to each vaccine. However, this may have resulted from vaccine lot-variation. Further studies are needed to make a conclusion. Notably, there was no response to the formulation with aluminum gel used as adjuvant.

When the levels of antibodies against inactivated SE vaccine-induced SE cell antigen and flagella were compared, as shown in Table 8. (Table 8. Detection of SE-specific antibodies by

Age (day)	SE Ⅰ		SE Ⅱ	
	Mean E value±2SD	Positive (%)	Mean E value±2SD	Positive (%)
125	0.66 ± 0.36	100	1.37 ± 0.76	100
330	0.26 ± 0.18	60	0.77 ± 0.41	100
550	0.29 ± 0.16	65	0.49 ± 0.34	100
650	0.35 ± 0.23	65	0.47 ± 0.39	95

n=20/group (chicken groups in a farm where molting is induced once at 450 days old)

Table 8. Detection of SE-specific antibodies by ELISA coated with SE cell antigen (SE-I) or the g.m. antigen (SEp 9; SE-II) in inactivated SE-vaccinated chickens (Nakagawa Y et al. Japanese report)

In this survey, 20 chickens were randomly extracted from inactivated SE (Layermune SE)-vaccinated chickens (applied at about 80 days old). The positive rates of specific antibodies against serum SE-I and -II and mean antibody titer (E value) were examined in these flocks. As a result, the positive rates of specific antibodies against bacterial antigen (SE-I antigen) were 100% in 125-day-old chickens and 60% in 330-day-old chickens. Subsequently, these positive rates remained at the same levels. The positive rate of specific antibodies against the g.m. antigen (SEp9) gradually decreased, but remained at a high level of positive ratio.

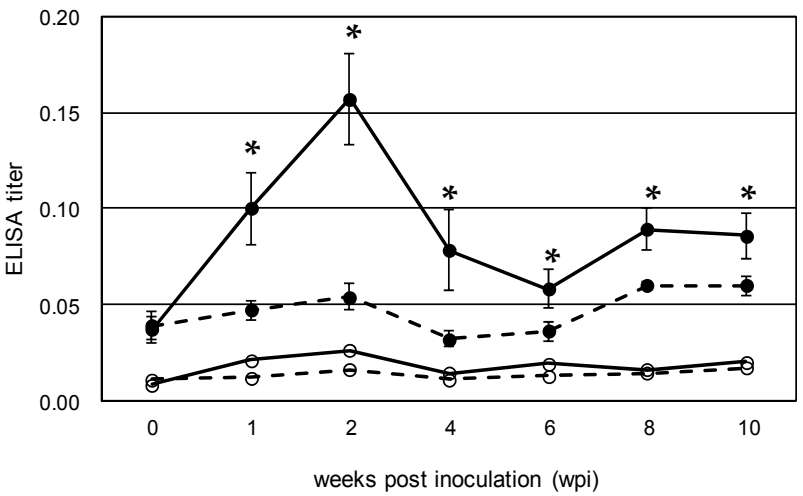


Fig. 4. Detection of specific antibodies in sera against SE cell antigen and SEp9 on oral SE administration to field white leghorn chickens (Mizumoto N et al. 2004)

SE was orally administered to 500-day-old laying chickens. Specific antibodies against SE bacterial antigen were produced in the blood. However, no specific antibody against SEp9 was produced. Specific antibodies were similarly detected in yolks. (The dashed line indicates an antibody level against SEp9, and the solid line indicates an antibody level against an SE cell antigen.) The symbol of closed circles means the antibody level in sera obtained from inoculated chickens. The open circle means the ones from not-inoculated chickens. The yolk antibody responses obtained from same birds were shown similar pattern as this figure.

ELISA coated with SE cell antigen (SE-I) or the g.m. antigen (SEp 9; SE-II) in inactivated SE-vaccinated chickens), the anti-CE cell antigen antibody level was high at 120 days of age about 50 days after vaccination, the antibody positivity rate was 50-60% at 300 days of age

(220 days after vaccination), and the rate was retained thereafter. In contrast, g.m. antigen (SEp 9)-antibody level was maintained at a high level until 700 days of age (about 620 days after vaccination). An experimental inoculation with SE in SPF chickens showed similar response (Fig. 4. Detection of specific antibodies in sera against SE cell antigen and SEp9 on oral SE administration to field white leghorn chickens). This tendency of the presence of specific antibody in egg yolk was observed (date not shown).

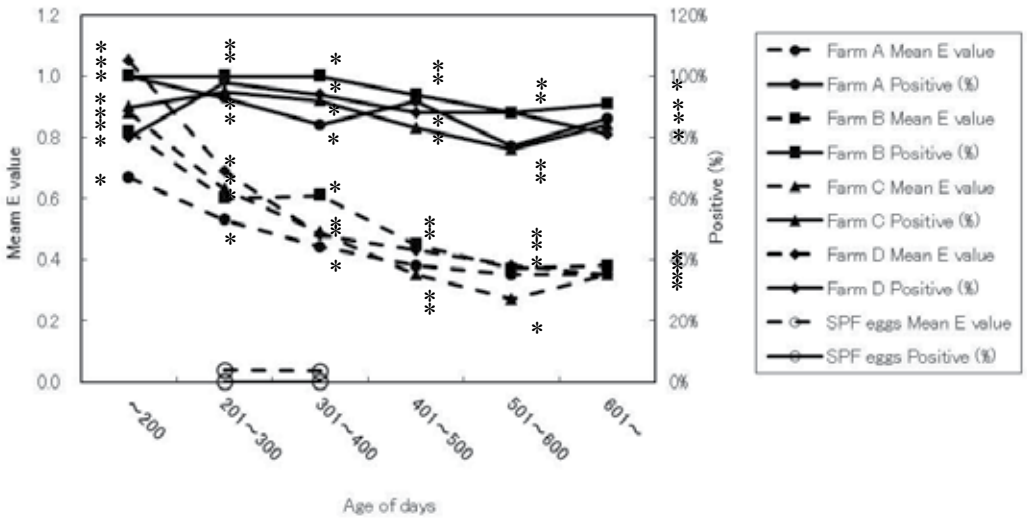


Fig. 5. Positive rates of g.m.-specific antibodies in the yolks derived from field chickens inoculated with the inactivated SE vaccine (Publishing elsewhere by Nakagawa Y et al.)
In this study, the inactivated SE vaccine (Layermune SE) was used to inoculate about 80-day-old chickens in six farms. Ten eggs were randomly collected once in two months from 150-700-day-old chickens of each farm. Mean antibody titers (positive: ≥ 0.1 E values) against the g.m. antigen (SEp9) in yolks were determined. These chickens were giving an induced molting for about 40 days after day 450. During this period, eggs were not sampled. The determination with specific antibody to g.m. antigen was done according to the method described by Mizumoto N et al. 2004.
The mean positive rate of the farms was about 88%. The positive rates were above 80% in all the farms. Thus, about 700-day-old chickens carried antibodies against SEp9. Antibodies against SEp9 markedly decreased the number of SE isolates in the gastrointestinal tract). In addition, antibodies against SEp9 inhibited SE isolation from eggs in the report. Proper vaccination prevented SE infection for a long time.
Thus, specific antibodies remained in chickens inoculated with the inactivated SE vaccine, even after molting was induced once, as examined by SEp9-coated ELISA. The specific antibodies could be detected also in yolks.

3.5 Immunogenicity of SEp 9

A high specific antibody production level was noted in antibodies against a flagellar component, Fli C, in inactivated SE vaccine-treated chickens, as described above. The SE-

specific region in Fli C is g.m. antigen (SEp 9), and the antigen was assumed to be effective as the antigenic site of inactivated SE vaccine (Toyota-Hanatani Y et al. 2008, and [b)]Toyota-Hanatani Y et al. 2009), for which we prepared SEp 9 antigen by genetic engineering and investigated the efficacy of SEp 9 vaccine. Since no international method (challenge test model) has been established for efficacy evaluation of inactivated SE vaccine, we analyzed tissue reactions at the vaccine administration site in vaccinated chickens.

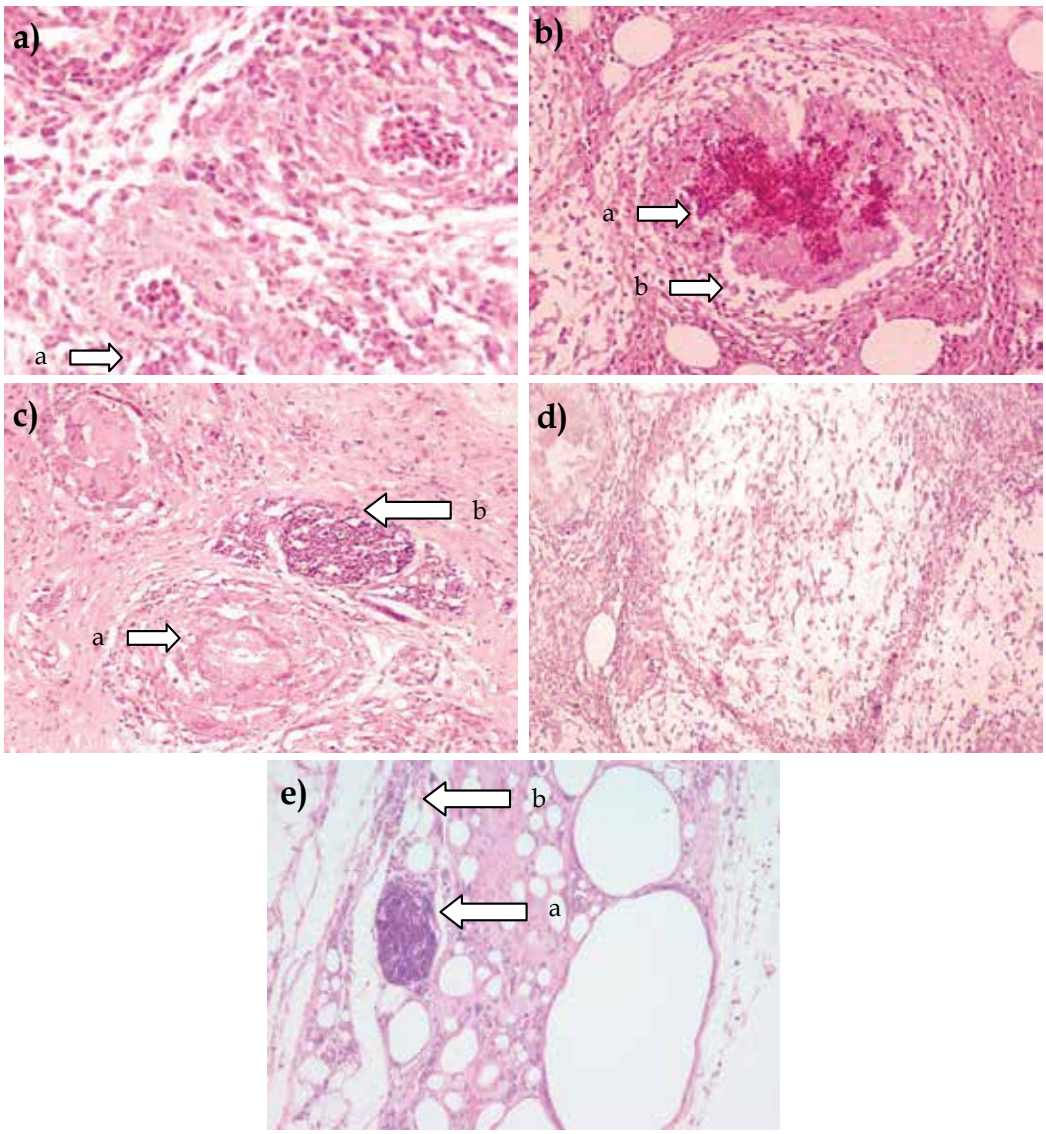


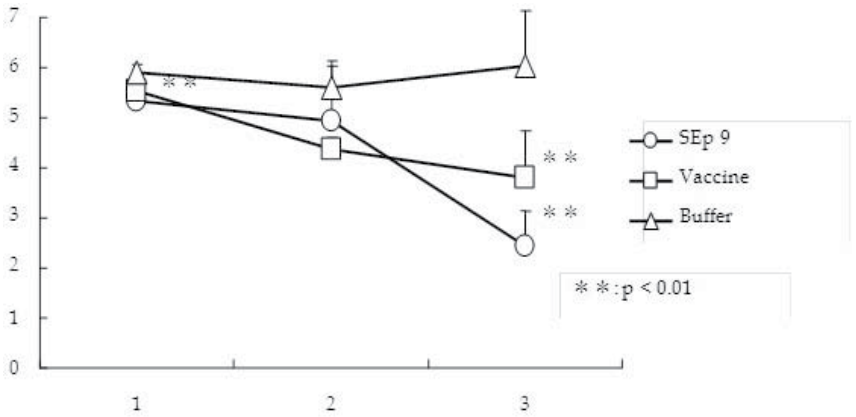
Fig. 6. Histological reactions at the inoculation with f the inactivated SE vaccine or the g.m site of Fli C ([b)]Toyota-Hanatani H et al. 2008)

We investigated a kinetic of histological reactions at the inoculation site of commercial inactivated SE vaccine or SEp 9 antigen. In the inoculation site (7a) at one week post vaccination (wpv), many histocytes were infiltrating, and hyperplastic connective issues are

shown (arrow a). However, tissue images, such as oil cyst, were not observed. In (7b) at 2wpv, necrosis (arrow a), surrounded by granulomatous structures (arrow b), was observed in the middle of inflammatory response. Polynuclear cells appeared in some granulomatous structures. Oil cyst was also observed. These images indicate that the antigen and oil ingredients were actively excluded from the vaccine, suggesting the establishment of specific immunity. At 4 wpv (7c), severe necrosis at 2 weeks became smaller, and the inflammatory response resolved (arrow a). In addition, peripheral lymphoid node structures (arrow b) appeared near the disappearing necrosis, suggesting active antibody production. At 6 wpv (7d), hyperplastic connective tissues also disappeared. Of the tissue reactions in the vaccination site, the characteristic responses during specific immune reaction are the emergence of polynuclear, which surrounded the granulomatous structure, and peripheral lymphoid node like structure. Thus, the inoculation site of SEp9 antigen was histologically examined at four weeks. As shown in Figure 7e, a lymphoid node like structure (arrow a) and a small number of polynuclear cells (arrow b) appeared in the SEp9 inoculation site. Thus, we concluded that SEp9 could induce specific immunity in chickens.
Materials and Methods; A commercial inactivated SE vaccine was injected and weekly taken tissue sample at the injected site, and then fixed and stained as usual (HE staining, X50).

The general time course of histological changes at the inoculation site with inactivated SE vaccine (oil-adjuvant-type) is shown in Fig. 6 (Histological reactions at the inoculation with f the inactivated SE vaccine or the g.m site of Fli C); nonspecific inflammation characterized by marked monocyte infiltration was noted after 1 week, and perivascular granulomatous changes were noted at 2 weeks including the appearance of multinucleated giant cells. At 3 weeks after vaccination, lymphocyte clustering showing a lymph node-like structure, considered to be an antibody production site, was noted. These reactions then slowly disappeared. In granulomatous changes accompanied by multinucleated giant cell infiltration observed after 2 weeks, cellular reactions of delayed hypersensitivity were noted (Table10. Characteristics of histological lesions at the inoculation site in the chicken applied with commercial SE vaccine (4wpi)). The tissue reactions at the SEp 9-administered site were similar to those induced by commercial inactivated SE vaccine, confirming anti-SEp 9-specific antibody production (Table 11 Production of specific antibodies in chickens inoculated with the inactivated SE vaccine or the g.m. site of Fli C).

When SEp 9-treated and non-treated chickens were orally challenged with SE, gastrointestinal SE was significantly decreased in the SEp 9-treated group compared to that in the non-vaccinated group, and the number of isolated bacteria was decreased similarly to that in the commercial inactivated SE vaccine-treated group (Fig. 7. Challenge test in chickens inoculated with the inactivated SE vaccine or the g.m site of Fli C). Although it is not clarified why the specific immunity induced by SEp 9 injection in chickens is able to reduce SE colonization in gastrointestinal organs, we have suspected that the induced immunity may affect SE yielding lower colonization ability SE. For example, the amount of a fibrin molecular, 21 kDa polypeptide, might be reduced on surface resulting from the induced specific immunity without SE-proliferation reduction. This is because the isolation level at 1 week post challenge in Fig. 7 (Challenge test in chickens inoculated with the inactivated SE vaccine or the g.m site of Fli C) does not show different bacterial level between SEp 9 injection and non-injection groups, even though statistical difference is observed. To this point, we will attempt to further clarify the mechanisms of lower SE-colonization in SEp 9-injected birds.



weeks post challenge (wpc)

Fig. 7. Challenge test in chickens inoculated with the inactivated SE vaccine or the g.m site of Fli C (Toyota-Hanatani Y et al. 2009)

SEp9 induced specific immunity. Subsequently, a challenge test was conducted in SEp9-inoculated chickens. The results are shown in this figure. When buffer alone was used for inoculation, the number of SE isolates did not decrease, but remained constant. The number of SE isolates decreased in chickens, inoculated with a commercial inactivated SE vaccine or SEp9, with aging.

Material and Methods; a buffered saline, SEp 9 and Layermune SE were twice-injected with mixture with an oil adjuvant, respectively. Four weeks post injection from final application, those chickens were orally challenged with SE Y 24 strain, and SE isolation was performed from intestinal samples.

Type of Bird	Type of Vaccine	Vaccination Age	Route of Administration	Program Advantages
Breeders	Live	1 day old 7 Weeks old	Coarse Spray Drinking Water	Broad Protection Selective Competitive Exclusion
	Killed	12-14 Weeks of Age 18- 20 Weeks of Age	Subcutaneous Subcutaneous	Strong Maternal Immunity
Layers	Live	1 day old 7 Weeks old	Coarse Spray Drinking Water	Broad Protection Selective Competitive Exclusion
	Killed	10-12 Weeks of Age	Subcutaneous	Strong Immunity
Broilers	Live	1 Day old Coarse Spray or Drinking Water	Coarse Spray or Drinking Water	Strong Immunity

Table 9. Recommended Salmonella vaccination programs in poultry.

Indicator No.	Activation result of	Characteristics in histological observations	Immunological properties
1	Cellular immunity	Granular formation (lumps) with epithelioid cells	Type 4 hypersensitivity (Pellertier M et al. 1984. Uthoaisangssok S et al. 2002)
2	Humoral immunity	Perivascular accumulation with lymphocytes	Activated B-lymphocytes
3	Non-specific immunity	Hyperplastic connective tissue, infiltration of non-specific immune cells	Early or late non-specific immune reaction

Table 10. Characteristics of histological lesions at the inoculation site in the chicken applied with commercial SE vaccine (4wpi) (supplementary data by Toyota-Hanatani Y et al. 2008).
This table shows three categories of reactions characteristic of tissue images on inoculation of a commercial inactivated SE vaccine: (1) cellular immunity, (2) hormonal immunity, and (3) nonspecific reaction. All the above reactions were observed in SEp9-inoculated chickens at 4 weeks. Supplementary data from other studies by Toyota-Hanatani Y et al. were also discussed in this table.
Granulomatous reaction, observed at 2-4 weeks after inoculation of the inactivated SE vaccine, was considered to be the same tissue reaction as tuberculin reaction. We considered it to be cellular type IV (delayed) hypersensitive reaction. This might be a process of developing cellular immunity.

Immunizing antigen	Tested chickens	Production of antibodies against g.m. (SEp 9)	
		Antibody positive conversion	Mean value in ELISA
g.m.(SEp 9) antigen	4	4	0.84
De-flagellated SE antigen	4	0	0.00
Buffer	4	0	0.00
Inactivated SE vaccine	4	4	0.83

Table 11. Production of specific antibodies in chickens inoculated with the inactivated SE vaccine or the g.m. site of Fli C (supplementary data by Toyota-Hanatani Y et al. 2008)
This table shows specific humoral immunity induced by the g.m. antigen site (SEp9). As shown in the table, specific antibodies were produced when SEp9 was used to inoculate chickens with adjuvant. However, no specific antibodies against SEp9 were produced when SE cell antigen was used. Importantly, the g.m. antigen site has high immune induction capacity in chickens because a small amount of antigen (100 μg/bird; about 30 μg/bird of not involving GST) induces specific immunity.
In another study where SEp9 in buffer was used to inoculate chickens (un-published data), specific antibodies were produced. Thus, specific immunity can be induced even without adjuvant.
Materials and Method; See Fig. 7

3.6 The details of attenuated live *Salmonella* vaccines for poultry

The first live *Salmonella* vaccine for poultry was a *Salmonella enterica* Serovar Gallinarum (SG) developed in the early 1950's (Williams SH.et al. 1956). This attenuated SG rough strain called 9R has been used in many countries around the world for the control of fowl typhoid. However, interference with official *Salmonella* control and eradication programs using serological methods has limited the wider use of this attenuated strain in addition to scattered field reports of excessive attenuation and reversion to virulence. The development of paratyphoid live attenuated *Salmonella* vaccines is an advancement and reinforcement to the use of inactivated vaccines for *Salmonella* control programs in the poultry industry. These new attenuated live *Salmonella* vaccines elicit cell-mediated, mucosal and humoral immune responses (Gomez-Duarte. et al. 1999, Roy Curtiss R 3rd et al. 1996, Kulkarni KK et al. 2008, Ashraf S et al. 2011). In addition, new recombinant DNA technology permits the expression in *Salmonella* serovar strains of protective antigens from unrelated bacterial, viral or parasitic pathogens.

There are two common approaches which have been applied in the development of the new paratyphoid live *Salmonella* vaccines. One of them is the genetic manipulation through recombinant technology selecting virulence genes to be deleted in selected *Salmonella* serovars. The other approach is the manipulation of the media used for *Salmonella* propagation resulting in a metabolic drift mutation reducing the activity of essential enzymes and the bacterial metabolic regulatory systems resulting in slower propagation cycles under natural infection conditions and this prolonged generation time cause reduced bacterial multiplication within the host at a significant rate. Consequently, when the genetically or chemically attenuated *Salmonella* strain is administered to the birds, the modified bacteria lives long enough to stimulate an immune response in chickens before to be eliminated within few weeks after administration of the vaccine. Currently, two paratyphoid serovars are commercially available as live attenuated vaccines: ST and SE.

It is considered that the genetic deletion of selected virulent genes induced a more attenuated recombinant *Salmonella* serovar strains compared with the chemically induced metabolic mutants, which still have residual enzymatic activity and more invasivity inducing a stronger immune response.

Epidemiological markers (Specific antibiotic resistance or sensitivity patterns) are included in the development process of these live vaccines to be able to differentiate the new construct or mutant from similar wild bacterial serovars in case of a field combined infection.

The field use of these new live attenuated *Salmonella* vaccines has advantages and precautions to observe when administered to the chickens. The advantages of these live vaccines are: mass administration, different routes of administration (Drinking water, coarse spray), selective competitive exclusion and broader spectrum of immunity. Among the precautions to be observed are: Not compatible with antimicrobials, no water chlorination when administered in the drinking water, careful handling by the operator to protect the worker from self-infection. Different recommendations on the use of the attenuated live *Salmonella* vaccines may be found in the literature to obtain the best protection against field challenge in a specific environment. Short duration of immunity of the live attenuated vaccines may require 2 to 3 applications every 6 to 10 weeks to obtain a more solid protection. The combined administration of live and inactivated *Salmonella* vaccines provides broader and long lasting immunity, especially in breeders to transfer strong maternal immunity to the progeny. (Table 9. Recommended *Salmonella* vaccination programs in poultry).

3.7 SE vaccine in the future

The current live and inactivated SE vaccines have advantages and disadvantages. Live vaccine is readily administrable to newborn chicks, but inactivated SE vaccine cannot be administered before 3 weeks of age. The detail potency mechanisms with live vaccine has not been clarified yet, and concerns over causing public health problems are always present: the possibility of back mutation of the vaccine production strains of SE and ST (such as reversal of pathogenicity) or mutation to a pathogenic strain cannot be completely ruled out, and, accordingly, live vaccine is not applicable for laying chickens as described above. Currently, inactivated SE vaccine is manufactured using the whole cell body containing endotoxin, which may induce stress in chickens, although this is slight.

To overcome these problems, the development of a subunit or vector vaccine comprised of active components of SE is awaited, and many researchers may have started research and development.

4. Marked usefulness of inactivated SE vaccine administration to flocks for reducing the human health risk

4.1 Reduction of SE contamination risk of chicken eggs by inactivated SE vaccine

We have surveyed the reduction of the SE contamination risk of chicken eggs by employing inactivated SE vaccine on field layer farms for a prolonged period. Herein, we report the study results.

Four-year surveys were performed on 4 field layer farms (a total of 2,300,000 chickens maintained in 37 hen-houses). Records of SE isolation from liquid eggs were analyzed. Some chickens in these layer farms were treated with inactivated SE vaccine as a trial before analysis, and all chickens were vaccinated in the 4th year of analysis.

The mean numbers of SE isolated from liquid eggs (MPN/100 mL) in the vaccinated and non-vaccinated groups were 2.5±0.1 and 674.8±162.9, respectively, and the isolation frequencies were 2.45 and 25%, respectively, showing that the isolation frequency was reduced to 1/10 in the vaccinated group. In addition, no SE was isolated after vaccination of all chickens in the 4th year (0 of 257 samples), as described above.

It was clarified that the use of inactivated SE vaccine on layer farms significantly reduced the number of SE isolated from SE-contaminated eggs and the isolation frequency.

4.2 Risk reduction by inactivated SE vaccine on risk analysis

As described above, inactivated SE vaccine decreased the mean number of SE contaminating eggs as a food product to about 1/260 and the isolation frequency to 1/10. These occurred on SE-contaminated farms when vaccinated and non-vaccinated chickens were mixed. When these were simply compared with the number of orally ingested SE and the incidence of patients reported by the [a), b)]WHO and FAO-US, the incidence of SE patients in healthy subjects was estimated to be decreased to 1/100 or lower.

The 4 farms involved in our study on the reduction of SE contamination of liquid eggs by inactivated SE vaccine were large-scale farms maintaining 350,000-950,000 chickens. These were windowless farms and high-level general hygiene control was also performed.

Accordingly, similar surveys should be conducted on floor feeding and loose housing layer farms, and the risk-reducing effect of SE vaccine should be investigated based on the combined results at national and community levels. In previous reports, the frequency of SE isolation from feces was reduced by about 70% in regions which applied live and inactivated SE vaccines individually or in combination ([a),b)] WHO FAO-US, 2002). The accumulation of individual epidemiological surveys and studies may lead to the effective control of SE food poisoning.

5. Re-consideration of the mode of SE infection in chickens

5.1 Mode of SE infection on farms and in flocks

Many points regarding the mode of SE infection on layer farms were unclear around 1990. Layer farm veterinarians referred to the mode of infection of PD (vertical infection), considering that SE also infects in this mode, and prepared an SE detection and monitoring system. Briefly, the mode of SE infection was considered as follows: SE infects breeding chickens and the infection transmits to chicks through breeding eggs (eggs raised to chickens). Some chicks die, but latent infection occurs in survivors and these chicks grow and lay SE-contaminated eggs. Accordingly, they considered that the antibody test in breeding chickens and SE test in chicks after hatching are important, and did not attach greater importance to SE tests of grown chickens, especially laying hens. Moreover, they considered that inactivated SE vaccine is ineffective for chicks after hatching, and only bacteriostatics and analogous agents are effective. The Pennsylvania Egg Quality Assurance Project (PEQAP) of the U.S.A. actively performed field SE contamination surveys to investigate this hypothesis, and found several new facts, as described above (Davison S et al. 2003, Henzler DJ et al. 1998), Hogue A et al. 1997, Lin FY et al. 1988, Stevens A et al. 1989). The points particularly attracting attention in the PEQAP report are a very low infection frequency in newborn chicks, although contamination occurred, and the absence of SE contamination in raising houses. However, SE contamination was observed most frequently after transfer to layer hen houses over 180 days of age. Even though new episodes of SE contamination occurred thereafter (after the laying peak), the frequency was very low.

SE sensitivity of chickens is schematically presented based on the study results reported by PEQAP and our experience in Fig. 8 (Age-dependent susceptibility of chickens against SE colonization). Chicks are very sensitive to SE infection immediately after hatching, but the sensitivity rapidly decreases. No clinical symptoms develop over the growth and egg-laying periods, but the sensitivity rises around the initiation of sexual maturation (100-120 days of age). In layer hen houses, the frequency of SE contamination is high, elevating the infection risk of chickens. It is considered that most SE infection of chickens occurs after transfer to layer hen houses (around 115 days of age) over the peak laying period (around 180 days of age). The sensitivity of layer hens slightly decreases thereafter but then slowly rises with aging. SE sensitivity may be enhanced when induced molting is performed during this period, but these chickens are already infected immediately after transfer to layer hen houses. Therefore, the infection rate is not actually elevated by induced molting, although the sensitivity is high. Considering SE sensitivity and SE control of layer flocks and economic damage, chicks infected immediately after hatching may be culled because they develop clinical symptoms. The period after transfer to layer hen houses over the egg-laying peak is the most important for hygienic SE control because chickens are highly sensitive to

SE but infection is unclear. The survey results of PEQAP well reflected this condition. Therefore, how hygiene control is performed during this period (after transfer to layer hen houses over the egg-laying peak) is important, and inactivated SE vaccine can be administered corresponding to this high contamination risk period.

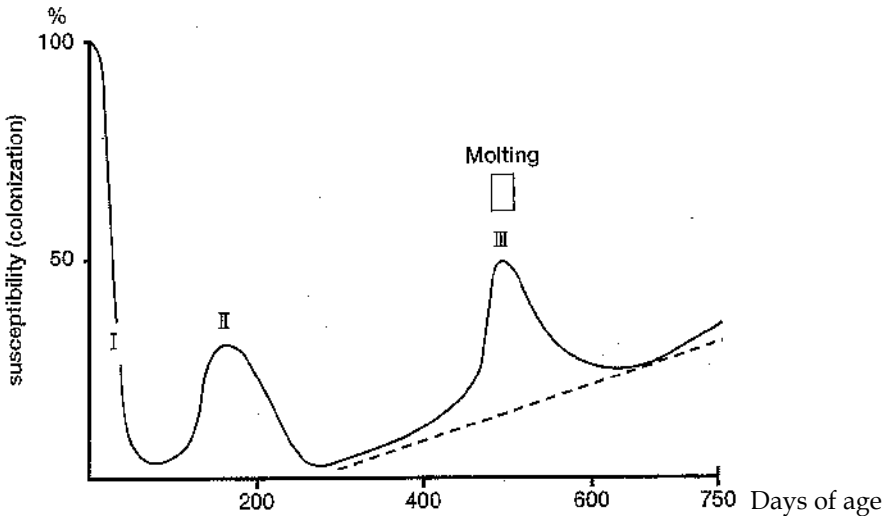


Fig. 8. Age-dependent susceptibility of chickens against SE colonization (Ohta H et al. presented in 2nd Symposium of the Germany-Japan veterinary association 1998)

The susceptibility of chickens to SE infection changed with aging. Oral SE administration killed almost 100% of chicks before feeding. However, the death rate rapidly decreased after feeding. No death was usually noted in 2-3-week-old chickens even after administering 10^9 FFU/bird. However, SE colonized in the intestine only for a short time. However, chickens became more susceptible to SE infection at around 100 days old when the reproductive organ developed. SE infected and colonized in the intestine for a long time in chickens of 50-100% laying age (145-180 days old). Subsequently, chickens gradually became susceptible with aging. Molting chickens were more susceptible to SE infection. More susceptible chickens were not necessarily more vulnerable to SE infection. SE infection risks became higher during the stage II (145-180 days old) in the figure, causing environmental SE contamination condition. Thus, chickens of this age group were more susceptible to SE infection. These facts were taken into consideration in the measures taken in the U.S.

5.2 SE infection of chickens

The epidemiological mode of infection of chickens is described above, but how does it occur in individual chickens? Generally, SE is orally ingested. Regarding experimental SE infection of chickens, Bohez et al. and other study-groups actively investigated pathogenicity in young chickens as above mentioned, and observed that the pathogenicity manifestation mechanism was similar to that in mice and systemic sepsis occurred and resulted in death at a high rate. We also obtained similar results (data not shown). In contrast, pathogenicity was rarely observed and the course was asymptomatic when grown chickens and layer hens were infected. Weakened chickens were observed in very rare cases, but the presence of other factors, such as stress, is generally considered for these cases, and SE infection alone is

considered to induce no morbidity. It has been considered most SE strains are not actually pathogenic for chickens. Therefore, it is unclear what roles are played in chickens by the genes, components, and molecules reported to exhibit pathogenicity in mice.

However, unlike other *Salmonella* species, such as ST, SE infection shows high tropism for intestinal and reproductive organ epithelial cells in chickens, and the colonization rate in the chicken intestine is high (Mizumoto N et al. 2005, Okamura M et al. 2007). Regarding tropism, there has been no report on differences in tropism for epithelial cells of SE and other *Salmonella* species in other animal species, but SE shows high species specificity for chickens. When the oviduct surface was contacted and colonized by various *Salmonella* species, the number of colonizing bacteria of SE strains was the highest and the number decreased in the other of *S*. Agona, *S*. Typhimurium, *S*. Heidelberg, *S*. Harder, *S*. infantis, and *S*. Montevideo. The high tropism of SE for chickens is an interesting study subject. For example, if SE-contaminated chicken eggs serve as the main cause of SE food poisoning resulting from the acquisition of species specificity for chickens by many currently isolated SE strains, this property of SE will be a major epidemiological study subject, i.e., it explains the sudden emergence of appearance of chicken egg-mediated SE food poisoning caused by SE contaminating chicken farms in the 1980s.

6. Proposal for food safety based on the history of emergence and decline of SE food poisoning

We selected 2 topics concerning chickens and SE infection in this chapter. One was the usefulness of inactivated SE vaccine administration to chickens to reduce the public health risk. The other was the introduction of some of our studies on SE infection of chickens. In the first topic, the history was described in some detail because a description of the historical background is necessary to understand why we wanted to describe the history of the emergence of chicken egg-induced food poisoning. In the 1980s, the production, distribution, and consumption of food products and materials became global. Safety standards became necessary for mass production, international distribution, and the selling of food products and materials, with which SE food poisoning occurred and rapidly spread in Western countries and then declined. However, this declined incidence has recently tended to slightly re-increase in some countries, suggesting that it is time to review SE control from the basics. Together with the history of overcoming the BSE problem of beef, the history of emergence and control of SE food poisoning contributes to establishing the concept of 'risk analysis of health damage by foods'.

In the control of food poisoning before 1980, hygienic measures were mainly taken in the steps after cooking, but analysis of SE-contaminated chicken eggs led to a new concept of food poisoning control: SE infection of chickens should be prevented although no clinical symptoms develop in chickens excluding chicks immediately after hatching. The problem of food poisoning seems to widely extend over the world. This is of course due to large-scale distribution and consumption of food products and materials, but it may also be due to failure of inheriting food culture in countries throughout the world. Previously, sensory elimination of problematic food products and materials was performed in each home as 'food culture', but this may not have been passed on in modern society in many countries. Studies on food poisoning are required to closely investigate the safety of the globalized

production, distribution, and consumption of food products and materials. These worldwide changes in food culture are a background to the emergence and decline of SE-contaminated chicken egg-induced food poisoning.

A large part of the text was also devoted to the usefulness of inactivated SE vaccine in this chapter. The first vaccine approved by the US government does not completely stop SE proliferation in the gastrointestine after SE challenge, and the bacterial count rather increases transiently. The previous concept of vaccine for chicken diseases was the inhibition of clinical symptoms and bacterial proliferation after challenging the pathogen, but SE infection does not induce clinical symptoms in chickens excluding chicks immediately after hatching. In other words, inactivated SE vaccine is administered for asymptomatic SE infection, and stress load of vaccination gives no advantage to farms. The use of inactivated SE vaccine was initiated in response to demands from consumers, to which layer farms had strong resistance, and the usefulness was frequently questioned. In this situation, we investigated the usefulness of inactivated SE vaccine.

Our study demonstrated that inactivated SE vaccine is very useful with regard to the inhibition of SE-contaminated chicken egg production, unlike conventional vaccine for chicken diseases. Although SE temporarily proliferated in the chicken gastrointestine on the SE challenge test, the production of SE-contaminated chicken eggs was markedly inhibited. Specific immunity against flagellar components plays a central role in the inhibition, and, particularly, specific immunity against g.m. antigen is assumed to play a major role. Unfortunately, the mechanism of the effect of flagellar component-specific immunity has not been clarified, and so remains to be investigated.

Reportedly, the current inactivated SE vaccine may induce stress in some cases. The development of vector vaccine with the insertion of flagellar components inducing no stress is underway, and may be realized in the near future.

Some of our study results on SE infectious disease in chickens were introduced in this chapter. SE infection of chickens may be opportunistic infection, unlike infections of mice and humans. However, SE infection of chickens was not regarded as opportunistic infection in previous studies. SE infection of chickens has been investigated employing the mode of PD infection or partially employing the mode of SE infection in mice and humans as a model, but we have been considering that it is appropriate to basically regard *Salmonella*-induced infectious disease as 'opportunistic infection' or less pathogenic 'indigenous bacteria'. Although it causes food poisoning in humans and may result in death, it is very rarely fatal in chickens. For fatal cases, other factors may be the major cause, such as hot conditions in summer. SE infection causes no damage to chickens, but there is no doubt that SE-contaminated chicken eggs cause food poisoning in humans, although the mode of SE infection in chickens cannot be fully explained.

We attempted to describe live SE vaccine. However, I could not draw the efficacy of the live vaccine for the applied flocks in their whole the life.

Responses of SE to stimulation by chickens were confirmed as the lacy phase changed to the colonial phase when the bacteria entered the intestine, but other responses are slightly unclear. In our study, marked colonization (tropism) of the reproductive organs by SE was noted, compared to that by other *Salmonella* species, but enumeration of these facts will not lead to studies in the future. Thus, we selected 2 topics in this field as study subjects

(working hypotheses). Various conversions occur in SE and these are important to evade the chicken's immune system and become indigenous. We selected these conversions as one topic. The other is the colonization mechanism of SE on layer farms which should be deduced based on the history. The colonization may have resulted from the facts that flagellar antigens are not expressed in chickens and chickens do not respond to the fimbrial components. These two mechanisms have been well-known for a long time, but it may be essential to analyze the mode of SE infection of chickens. SE infection of chickens is a never-ending study area.

7. Acknowledgement

First of all, we apologize that this chapter mainly describes our field experiences, with little reference to literature. This chapter is comprised of not only studies performed mainly by us but also joint studies with many researchers, and contains fewer descriptions referring to the literature. Many contents of this chapter were those of cooperative studies performed by Osaka Prefectural University and CAF Laboratories. We would like to thank for the persons related to this publishing program, to be given a chance to write this chapter.

We express our respect to Dr. Joan D. Leonard of Biomune Co., U.S.A., who launched the world's first inactivated SE vaccine, for her courage and fine studies, and we deeply appreciate having an opportunity to perform studies using this vaccine. We are also grateful to Dr. Fernando Lozano of Ceva/Biomune Co. for providing us with global information concerning SE vaccine.

We are grateful to the many researchers who performed cooperative studies with us, especially the following persons:

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Dr. Eiichiroh Baba, formerly of Osaka Prefectural University
Dr. Kazumi Sasai, Osaka Prefectural University

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16

Salmonella as Live Carrier of Antigens in Vaccine Development

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1. Introduction

Attenuated *Salmonella* strains have been extensively used as live carriers of heterologous antigens. In animal models they elicit strong mucosal and systemic immune responses to passenger antigens of a broad variety of pathogens. *Salmonella* has several theoretical advantages over other vaccine vector systems. Among them, especially attractive is the bacterial ability to interact with mucosal and systemic compartments of the immune system and deliver passenger antigens directly to antigen presenting cells (APC) when administered by the oral route. However, despite the promising results in animal models, clinical trails have been disappointing and more research is needed in order to understand the protective immunity mechanisms and solve the main drawbacks of vaccine design. Herein, we summarize the accumulated experience using *Salmonella* as live carrier emphasizing the role of passenger antigen localization into different bacterial compartments. This is an important factor determining the type and quality of the host immune response. The evidence suggests that antigens located on the bacterial surface induce higher antibody responses whereas those located in the cytoplasm elicit better cellular immunity. In order to display recombinant antigens on the bacterial surface we have used outer membrane (OMPs) or autotransporter proteins. Mice immunized with *Salmonella* strains expressing the main B cell epitope from the *Plasmodium falciparum* circumspozoite protein, presented better antibodies response when placed on the bacterial surface as a fusion proteins with OMPs or autotransporters, than the whole recombinant protein located in the bacterial cytoplasm.

2. *Salmonella* as live carrier of antigens

Vaccination is the public health intervention intended to control infectious diseases with the best cost-effective ratio. Among the number of different approaches for vaccine development, live vectors stand as one of the most promising options. They may be defined as attenuated microorganisms which produce heterologous recombinant proteins (or bear plasmids DNA with eukaryote transcription machinery) and elicit immune response to the passenger antigen. Extensive research in the last decade in the fields of molecular biology,

bacterial genetics and immunology has significantly accelerated the use of a number of microorganisms as vaccine vectors. Several pathogenic bacteria can be engineered for attenuation and expression of foreign proteins originally encoded by other microorganisms. Although several attenuated or commensal nonpathogenic bacteria have been employed to express passenger antigens (Kottonet al. 2004; Medinaet al. 2001), enteric Gram negative bacteria, especially *Salmonella*, have been preferred because they are easy to manipulate. However, these vaccine candidates raise safety concerns associated with the release of genetically modified microorganisms into the environment. (Abd Elet al. 2007; Kottonet al. 2004).

An ideal vaccine has to fulfill not only immunogenicity, efficacy and safety requirements, but also has to be inexpensive, stable at room temperature and easy to administer (Polandet al. 2010). Bacterial carriers specifically *Salmonella* strains, attenuated by genetic engineering, have several advantages over other vaccine types because they can be administered by the oral route and are more stable at room temperature than other vectors, such as viruses. Recent research have developed more stable strains, one example is a live attenuated *S.* Typhi Ty21a vaccine treated by foam drying, a modified freeze drying process, formulated with trehalose, methionine, and gelatin, is stable for approximately 12 weeks at 37°C and its derivatives expressing foreign antigens, such as anthrax, are immunogenic (Ohtakeet al. 2011).

Persistent and effective immune responses require initial activation of innate immunity. Bacterial pathogen associated molecular patterns (PAMPS) recognized by pattern recognition receptors (PRRs) in macrophages, dendritic cells (DC) or epithelial cells (ECs) determine the nature and extent of the adaptive response through membrane associated and soluble cytokine signaling. For example, Toll-like receptors (TLRs) TLR4 and TLR2 deficient mice immunized with *S* Typhi porins, which have been found to elicit maturation of $CD11c^{+}$ conventional DC, showed impaired B-cell response, characterized by reduction of IgG antibody titers to porins, specially of the IgG3 isotype (Cervantes-Barraganet al. 2009). Other authors have investigated the role of TLRs responses to an attenuated *S.* Typhimurium BRD 509 expressing the saliva-binding region (SBR) of *Streptococcus mutans*. Using TLR2, TLR4 and MyD88 deficient mice, they demonstrated that the induction of a serum IgG2a (type 1 response) to the passenger antigen involved TLR2 signaling, whereas the response to *Salmonella* involved signaling through TLR4 (Salamet al. 2010). Thus, antigen specific T and B cells are activated in a coordinated manner to achieve optimal primary and secondary immune responses. The induction of memory is a key characteristic of an effective vaccine (Chenet al. 2010; Medinaet al. 2001). It has been demonstrated that primary effector T cell activation to Salmonella depends on the innate function of B cells (though TLR signaling mediated by MyD88) whereas the induction of T cell memory mediated by antigen specific presentation by the BCR (Barret al. 2010). Moreover, the cell wall of Gram negative bacteria promotes Th1 responses. For instance, an *Escherichia coli* strain expressing an ovalbumin (OVA) allergenic peptide on the bacterial surface, as a fusion protein with the *S.* Typhi OmpC porin, reduced the lung inflammatory response in mice allergic to OVA, with a significant decrease of IL-5 mRNA and induction of IFN-γ mRNA in cells from bronchio alveolar lavages and specific anti-OVA IgE reduction (Yepezet al. 2003).

Several routes of immunization have been assessed with *Salmonella* live carriers. The oral route elicits protective immunity. For instance, mice immunized orally with *S.* Typhiumurium or *S.* Typhi expressing the fullength *B. anthracis* protective antigen (PA)

were protected against a lethal challenge with aerosolized *B. anthracis* spores (Stokeset al. 2007). However, the intranasal route is more immunogenic. Indeed, mucosal immunity is most effectively induced when antigens are delivery directly in mucosa, i.e. by oral, intranasal, intrarectal, or intravaginal routes. (Galenet al. 1997). The eye conjunctiva has also demonstrated to be a feasible administration route. Attenuated *Salmonella* vaccine strains administered by eyedrops induced LPS-specific antibodies and protection to the oral challenge with virulent *Salmonella* in mice. Eyedrop vaccinations do not deliver antigens into the CNS as noted with the intranasal route.(Seoet al. 2010)

A needleless delivery system has been developed recently, consisting in a micro wave controlled explosion which disrupts the skin barrier. This system was used to immunize mice with a *S.* Typhimurium vaccine strain pmrG-HM-D (DV-STM-07) with the idea to place the bacteria in the epidermis where resident Langerhans cells may uptake them more efficiently and present the bacterial antigens to lymphocytes (Jagadeeshet al. 2011)

There are a number of attenuated *S.* Typhi strains with defined mutations constructed by genetic engineering. Some have been tested in humans demonstrating immunogenicity and acceptable safety profile, such as *S.* Typhi Ty800, which is mutated in *phoP/phoQ.(Hohmannet al. 1996) or* M01ZH09, a *S.* Typhi (Ty2 aroC$^-$ssaV$^-$) ZH9 (Tranet al. 2010). *Other* attenuated *Salmonella* strains have disrupted the *aroC* and *aroD genes*. The interruption of the biosynthetic pathway of aromatic metabolites results in a bacterial nutritional dependence on *p*-aminobenzoic acid and 2,3-dihydroxybenzoate, substrates not available to bacteria in mammalian tissues (Hoisethet al. 1981). As result, the *aro*-deleted bacteria are not able to proliferate within mammalian cells. However, the organisms survive intracellularly long enough to stimulate immune responses. Inactivation of either *aroC* or *aroD* independently results in attenuation, but deletions in both genes reduce the possibility of virulence restoration by recombination. Two vaccine strains harboring deletion mutations in *aroC* and *aroD* have been evaluated as candidate live oral vaccines in adult volunteers (Bumannet al. 2010; Gonzalezet al. 1994; Tacketet al. 2000; Tacketet al. 2007).

Attenuated *S.* Typhi vaccines have been engineered to express and deliver passenger antigens (proteins and DNA encoded) of a number of pathogens, as the measles virus hemagglutinin, the *Bacillus anthracis* protective antigen (PA), the *Plasmodium falciparum* circumsporozoite surface protein (tCSP), the nucleocapsid (N) protein of severe acute respiratory syndrome-associated coronavirus (SARS-CoV), or the HPV16 L1 protein (L1S).(Chinchillaet al. 2007; Frailleryet al. 2007; Galenet al. 2004; Luoet al. 2007; Pasettiet al. 2003)

3. Influence of passenger antigen location on the immune response

There is increasing evidence that expression level and antigen location determine vaccines efficacy. It is important to achieve passenger antigen expression in the desired bacterial compartment under constitutive or inducible conditions, in order to regulate antigen production. (Bumann 2001; Galenet al. 2001; Kurlandet al. 1996; Pathangeyet al. 2009). Insufficient expression interferes with the immune response to passenger antigens and there is a general notion that high antigen production by live vectors may result in better immune response. Therefore, the production of antigens from high copy number plasmids is apparently the best designing approach. However, excessive expression drives to increased metabolic load, plasmid loss and toxicity (Galenet al. 2001; Pathangeyet al. 2009) Antibody

responses to antigens delivered by S. Typhi live vectors are inversely related to the metabolic burden imposed by antigen production, and may be improved when antigens are expressed from low-copy-number plasmids and exported out of the cytoplasm (Galenet al. 2010). Three solutions are proposed to solve this problem: 1) chromosomal integration of heterologous genes, 2) On/off recombinant protein production by using *in vivo*-inducible promoters, and 3) Plasmid stabilization systems. Although both strategies are intended to limit heterologous gene expression, the second strategy has the additional advantage that protein is preferentially produced at the appropriate host environment, such as acidic vacuoles in macrophages. In addition, when plasmid stability is maintained in the absence of antibiotics, there is flexibility for the introduction of a variety of passenger genes without the need to use chromosomal integration systems.

Thus, S Typhi ZH9 (Ty2 Delta aroC Delta ssaV) producing the B subunit of *Escherichia coli* heat-labile toxin or hepatitis B virus core antigen from the bacterial chromosome using the *in vivo* inducible ssaG promoter, stimulated potent antigen-specific serum IgG antibodies to the heterologous antigens (Stratfordet al. 2005).

S. Typhimurium aroA (STM-1) expressing *Mycoplasma hyopneumoniae* antigens form plasmid or chromosomal systems were administered to mice. Whereas no significant immune response was detected with the plasmid based expression, systemic IgM and IgG responses were detected with the chromosomal integration system which used strong promoters (Maticet al. 2009).

A plasmid maintenance system has been tested in *S.* Typhi CVD 908-htrA consisting in the deletion of genes encoding catalytic enzymes and addition of random segregation function of multicopy plasmid (Galenet al. 2010). Other option to achieve plasmid stability relies on the development of plasmid trans- complementation of lethal deletions in the live vector. Thus, plasmids encoding the single-stranded binding protein (SSB), an protein involved in DNA replication were used to transform *S.* Typhi CVD 908-htrA and CVD 908, and used to deliver anthrax toxin from *Bacillus anthracis* as a foreign antigen in mice (Galenet al. 2010).

A dual system to achieve increased antigen expression was developed by chromosomal integration of the T7 RNA polymerase gene (T7pol) in *S.* Typhi CVD908. The T7pol gene was amplified from *Escherichia coli* BL21(DE3) and inserted by homologous recombination in the bacterial chromosome under the control of the inducible nirB promoter. The resulting strain, S. typhi CVD908-T7pol, was able to trans-complement two plasmids bearing the luc or the lacZ reporter genes controlled by the T7 promoter under anaerobic culture conditions (Santiago-Machucaet al. 2002)

Other factor influencing protein expression efficiency include differences of codon usage between the native passenger gene and that host chromosome. Although so far exclusively applied to *Escherichia coli*, codon harmonization may provide a general strategy for improving the expression of soluble, functional proteins during heterologous host expression (Angovet al. 2011)

It has been suggested that passenger antigens delivered by attenuated *Salmonella* strains induce better systemic and mucosal immune responses when displayed on the bacterial surface (Chenet al. 2000; Leeet al. 2000; Ruiz-Perezet al. 2002). A variety of surface display systems have been described (Samuelsonet al. 2002). The most widely used have been fimbria and outer membrane proteins (OMPs), including porins and autotransporters. (Kjaergaardet al. 2002; Klemmet al. 2000; Krameret al. 2003; Rizoset al. 2003)

Passenger fusion proteins (peptides-flagellin) have demonstrated to enhance the immunogenicity of vaccine peptides (Newtonet al. 1989; Newtonet al. 1991a; Newtonet al. 1991b; Stocker 1990; Stockeret al. 1994). In these models the heterologous peptide is fused in-frame to the central hypervariable domain of *Salmonella* FliCd flagellin, which is derived from *S.* Müenchen and expressed by an attenuated *S.* Dublin strain. The chimeric flagellins are exported to the bacterial surface where the subunits assemble into the flagellar shaft without a significant impact on bacterial motility and host tissue colonization (Newtonet al. 1989; Stockeret al. 1994). Nonetheless, previous results showed that the genetic fusion may not enhance antigen-specific antibody responses in mice immunjized by the oral route with recombinant *S.* Dublin (De Almeidaet al. 1999; Sbrogio-Almeidaet al. 2001). Interestingly, the genetic background of both the mice and the *Salmonella* strains affected the immunogenicity of flagellins (Sbrogio-Almeidaet al. 2004). Indeed, recent evidence indicates that *Salmonella* flagellin administered by the oral route may trigger immunological tolerance in healthy mice, although the precise mechanism underlying this response remains unknown (Sanderset al. 2006)

However, in some cases exported, secreted proteins may induce stronger antibody response. (Galenet al. 2001).

Table 1 shows some examples of the influence that heterologous protein location in *Salmonella* has on the type of immune response, which are described as follows.

Antigen displayed	Location in bacterial carrier	Immune response	Reference
TGEV C and A epitopes	Fimbria and outer membrane (MisL)	Humoral	(Chenet al. 2007)
NS3 Dengue Virus	Outer membrane (MisL)	CTL	(Luria-Perezet al. 2007)
Ea1A y EaSC2 ***Eimeria stiedae***	Cytoplasm	CTL	(Vermeulen 1998)
LTB ***E. coli***	Periplasm	Humoral and cellular	(Takahashiet al. 1996)
SERP and HRPII ***P. falciparum*** **antigens**	Outer membrane (OmpA)	Humoral	(Schorret al. 1991)
p60 ***L. monocytogenes***	Cytoplasm	CTL	(Gentschevet al. 1995)

Table 1. Influence of heterologous protein location in *Salmonella* carrier, on the immune response.

Attenuated *S.* Typhimurium CS4552 (crp cya asd pgtE) was constructed expressing transmissible gastroenteritis virus (TGEV) C and A epitopes fused to the passenger domain of the MisL autotransporter or to the 987P FasA fimbriae subunit under the control of in vivo-induced promoters. The antibody response between both expression systems was compared. Mice vaccinated with the recombinant bacteria displaying the antigens in fimbriae presented the highest level of anti-TGEV antibodies with the epitopes expressed in fimbriae. This result suggests that polymeric display could induce better immune responses towards specific epitopes (Chenet al. 2007).

The second example is a *S.* Typhimurium SL3261 producing a fusion protein designed to destabilize the phagosome membrane and allow a dengue epitope to reach the cytosol. The fusion protein was displayed on the bacterial surface though MisL and the passenger alpha domain contained a fusogenic sequence, a NS3 protein CTL epitope from the dengue virus type 2 and a recognition site for the protease OmpT. The passenger antigen was released to the milieu, processed through the MHC class I-dependent pathway and simulated cytotoxic T lymphocytes (CTLs).(Luria-Perezet al. 2007)

Eimeria stiedae antigens Ea1A and EaSC2, a parasite refractile body transhydrogenase and a lactate dehydrogenase, respectively were expressed in *S.* Typhimurium, and used to immunize chickens. The challenge with the parasite demonstrated oocyst output reduction related with $CD4^+$ and $CD8^+$ T cells activation (Vermeulen 1998)

The fourth example in **Table 1** is a comparison between the immune response elicited in mice immunized with native *Escherichia coli* enterotoxin (LT) or with *S.* enteritidis expressing the heat-labile toxin B subunit (LT-B) in the bacterial periplasm. Both antigens elicited mucosal IgA antibodies directed to different LT-B epitopes, and serum IgG antibodies to the same immunodominant LT-B epitopes. The same single T-cell epitope was recognized by immune lymphocytes purified from mice immunized with either antigen (Takahashiet al. 1996)

Immunogenic epitopes of the *Plasmodium falciparum* blood stage antigens SERP and HRPII were expressed on the surface of the attenuated *S.* Typhimurium SR-11 strain as fusion proteins with OmpA from *Escherichia coli*. Mice immunized orally with the bacterial recombinants produced anti-SERP and anti-HRPII IgG and IgM antibodies. (Schorret al. 1991)

Finally, an attenuated *S.* Dublin aroA strain which secretes an active listeriolysin from *Listeria monocytogenes* is partially released from the phagosome into the cytoplasm after uptake by J774 macrophage cells. This is an attractive approach to evoke CTLs responses to passenger antigens through the MHC class I-dependent antigen processing pathway (Gentschevet al. 1995)

4. Display of antigens on the bacterial surface

Following, some of our experience with cytosolic versus surface display of antigens will be described with more detail. The same antigen and live vector were used with the only difference in the bacterial compartment where the antigen was expressed.

It has been demonstrated that *Salmonella typhi* OMPs, porins, and particularly OmpC induce protective immune response in a murine model of infection (Gonzalezet al. 1993; Gonzalezet al. 1995; Isibasiet al. 1988; Isibasiet al. 1992; Isibasiet al. 1994). Porins are OMPs which conform diffusion channels for low molecular weight molecules into the bacterial cell. The tertiary structure is a barrel conformed by 16 anti parallel β sheets with external and internal loops. These external loops have permissive regions were heterologous peptides can be inserted (Vegaet al. 2003; Yepezet al. 2003; Zenteno-Cuevaset al. 2007)

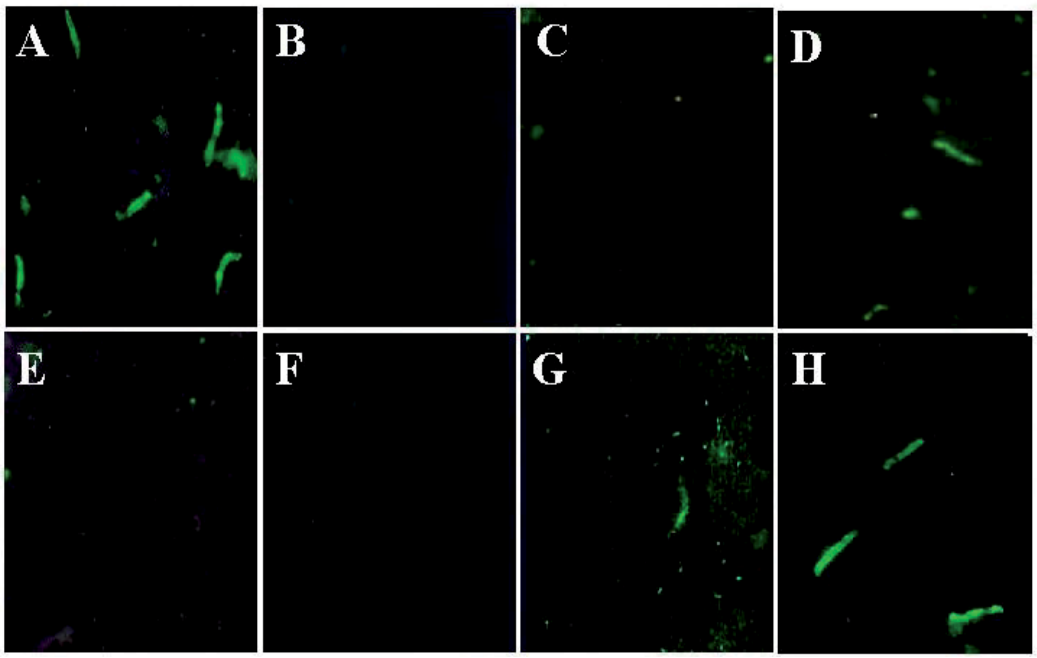
Considering that the major B cell epitope from the *Plasmodium falciparum* circumpsporozoite protein (CSP), the Asp-Ala-Asp-Pro (NANP) repeating sequence, has been inserted in permissive sites of *Pseudomonas aeruginosa* OMP OprF (Wonget al. 1995) we decided to introduce the NANP encoding sequence in the of *S.* Typhi OmpC porin. The NANP3 *P.*

falciparum CSP was inserted in the predicted external loop 5. A site directed mutagenesis was achieved by an overlapping PCR in two amplification rounds. In the first amplification two products were generated separately from plasmid pST13 (bears the complete *S.* Typhi OmpC porin and was kindly donatd by Dr. Felipe Cabello Felipe C. Cabello, New York Medical College) a 5` *omp*C moiety bearing the NANP3 sequence in the 3`end, and a 5´moiety with the NANP3 sequence in the 5´end. In the second amplification both moieties were used to generate a fusion product which was digested and religated to pST13, resulting plasmid pST13-NANP. Functionality of the hybrid *omp*C-NANP gene was assessed by Northern blot using RNA obtained from *Escherichia coli* UH312 transformed with pST13 or pST13-NANP. The autoradiography revealed a more intense band in *Escherichia coli*-pST13 as compared with the same strain transformed with pST13-NANP. Thus, suggesting that the *omp*C-NANP hybrid gene is transcribed less efficiently than the *omp*C native gene.

Protein extracts were obtained from *Escherichia coli* UH302 and *Salmonella typhi* CVD908 transformed with pST13 or pST13-NANP, and the OmpC-NANP fusion protein expression was estimated by SDS-PAGE. When the porinless *Escherichia coli* UH302 strain was transformed with pST13, produced large amounts of the 36 kDa protein. Nevertheless, when transformed with pST13-NANP a faint 36 kDa band was observed. The lower protein production found in the strains transformed with pST13-NANP is consistent with the Northern blot analysis. No differences in OmpC expression was observed between the *Salmonella typhi* CVD908 strains transformed with pST13 or pST13-NANP

Groups of five BALB/c mice were immunized with *Escherichia coli* UH302 transformed with pST13 or with pST13-NANP and *S.* Typhi CVD908, CVD908-pST13-NANP, CVD908ΩCSP (bears the whole CSP integrated in the bacterial chromosome)(Gonzalezet al. 1994), CVD908ΩCSP-pST13-NANP. Seven days after the last immunization, antibodies against *Plasmodium falciparum* sporozoites were assessed by IFA. Neither preimmune sera nor sera from mice immunized with *E. coli* UH302-pST13 or *Salmonella typhi* CVD908 recognized sporozoites, and some structures suggesting sporozoites were observed with sera from mice immunized with *E. coli* UH302-pST13-NANP (**Figure 1**). Interestingly, *S. typhi* CVD908ΩCSP (cytosolic CSP expression) was unable to induce antibodies against the parasite and similarly to *E. coli* UH302-pSt13-NANP, sera from mice immunized with *S. typhi* CVD908-pST13-NANP (epitope surface expression) showed some structures resembling parasites. Nevertheless, only mice immunized with *Salmonella typhi* CVD908ΩCSP-pST13-NANP (both surface and cytosolic expression) clearly depicted sporozoites comparable to the positive controls reveled with 2A10 antibody (**Figure 1**). These data were consistent with the measurement of antibodies by ELISA. Serum of BALB/c mice immunized with S. Typhi CVD908, CVD908ΩCSP, CVD908-pST13-NANP, and CVD908ΩCSP-pST13-NANP were collected, and antibodies to (B)4MAPs (a branched peptide containing the NANP) sequence were determined. *S.* Typhi CVD908ΩCSP, which produce a cytosolic CSP from a chromosomal integrated gene, did not elicit measurable antibodies under the experimental conditions for this experiment (1:25 to 1:250). *S.* Typhi CVD908-pST13-NANP, which displayed the NANP epitope on the bacterial surface, elicited mild antibody response (titer 1:25), whereas *S.* Typhi CVD908ΩCSP-pST13-NANP, which produced the epitope on the bacterial surface and the whole CSP in the cytosol, raised the highest antibody response (titer 1:200) after both o.g or i.p immunization (**Figure 2**). Taken together these data suggest, as stated earlier, that the epitope expressed on the bacterial surface may exhibit antigenic

identity with the native CSP in *P. falciparum* sporozoites and that both surface and cytosolic expression elicited better antibody response.

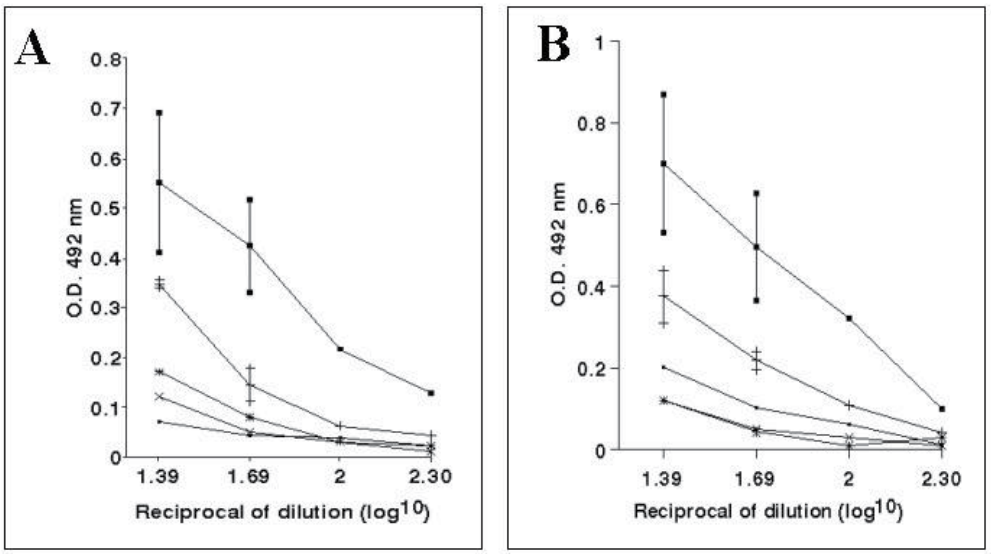


A) Positive control, sporozoites revealed with the 2A10 monoclonal antibody (which recognizes the (NANP)n repeat of Plasmodium falciparum circumsporozoite surface protein); **B)** Negative control, preimmune sera; **C)** *Escherichia coli* UH302,-pST13; **D)** *Escherichia coli* UH302-pST13-NANP; **E)** *Salmonella typhi* CVD908; **F)** *Salmonella typhi* CVD908ΩCSP; **G)** *Salmonella typhi* CVD908-pST13-NANP; **H)** *Salmonella typhi* CVD908ΩCSP-pST13-NANP.

Fig. 1. Antibodies against *Plasmodium falciparum* sporozoites elicited by the immunization of BABL/c mice with bacterial strains expressing the NANP epitope on the bacterial surface were assessed by immunofluorescence assay. The antibody response was compared between strains with cytosolic expression of CSP, surface expression of $(NANP)_3$, or both surface and cytosolic expression.

Western blot was performed revealing OmpC with a rabbit hyperimmune anti-OmpC serum or a cocktail of monoclonal antibodies against OmpC. The rabbit anti-OmpC serum showed two clear bands in the *Salmonella typhi* strains due to cross reactivity with other porin (probably OmpF, which is 35KDa). *Escherichia coli* UH302-pST13 and pST13-NANP revealed a single band. The cocktail of monoclonal antibodies against OmpC revealed a single band and demonstrated that native OmpC and the OmpC-NANP fusion protein display similar molecular weight. It is important to notice that the western blot from these protein extracts using monoclonal antibody 2A10, which recognizes the *Plasmodium falciparum* CSP, failed to recognize the $(NANP)_3$ epitope in the fusion OmpC-NANP protein. Nevertheless, the flow cytometry performed with *Escherichia coli* UH302 transformed with pST13 or pST13-NANP using the same 2A10 monoclonal antibody revealed that *Escherichia coli* UH302-pST13-NANP displays the chimeric OmpC with the *Plasmodium falciparum*

NANP epitope on the bacterial surface. These data could be explained by the low expression levels of the ompC-NANP fusion protein, but may be related to conformational changes in the SDS-PAGE.



A). Antibodies against $(B)_4$MAPs in mice immunized orogastrically with *Salmonella typhi* strains as described elsewhere (Gonzalezet al. 1998) assessed by ELISA; **B)**. Mice immunized intraperitoneally with (•) CVD908; (+) CVD908-pST13-NANP; (∗) CVD908ΩCSP; (■) CVD908ΩCSP-pST13-NANP; (×) Preimmune sera.

Fig. 2. Comparison between the antibody response against $(B)_4$MAPs, a tetramer branched synthetic peptide containing $(NANP)_3$ in each of the four branches (kindly donated by Dr. Elizabeth Nardin, Department of Medical and Molecular Parasitology. New York University School of Medicine, New York, NY), elicited in mice immunized with *Salmonella typhi* CVD908 expressing the CSP in the cytosol, the $(NANP)_3$ epitope on the bacterial surface or from both bacterial compartments.

Finally, we will describe some experience with autotransporters for autodisplay of antigens. Autotransporters belong to a family of OMPs, which lack the requirement of specific accessory molecules for secretion through the outer membrane. These proteins bear all necessary signals encoded within the polypeptide itself. They contain a C-terminal domain, (β-domain or translocator domain) which allows the N-terminal α passenger domain to cross from the inner membrane to the periplasmic space. The α-passenger domain is flanked by an N-terminal signal sequence responsible for initial export into the bacterial perplasmic space by a *sec* dependent mechanism. Once in the periplasmic space the C-terminal translocator β-domain forms a barrel and inserts in the outer membrane, and the N-terminal passenger α passenger domain travels through the central pore to the external milieu where exerts its biological function. Once on the surface, the final fate of the N-terminal passenger α passenger domain is determined by the presence of autoproteolytic mechanisms or surface proteases, which cleavage and release the α passenger domain to the external environment. (Finket al. 2001). More than 40 proteins with autotransporting properties have

been characterized (Desvauxet al. 2004; Hendersonet al. 2001). Due the relative simplicity of their transporting mechanism, the β-domain from several autotransporters has been employed translocate and display recombinant passenger proteins on the surface of enterobacteria. We already reported the use of MisL (another member of the AIDA-subfamily) to express foreign immunogenic epitopes on the surface of gramnegative bacteria (Luria-Perezet al. 2007; Ruiz-Olveraet al. 2003; Ruiz-Perezet al. 2002).

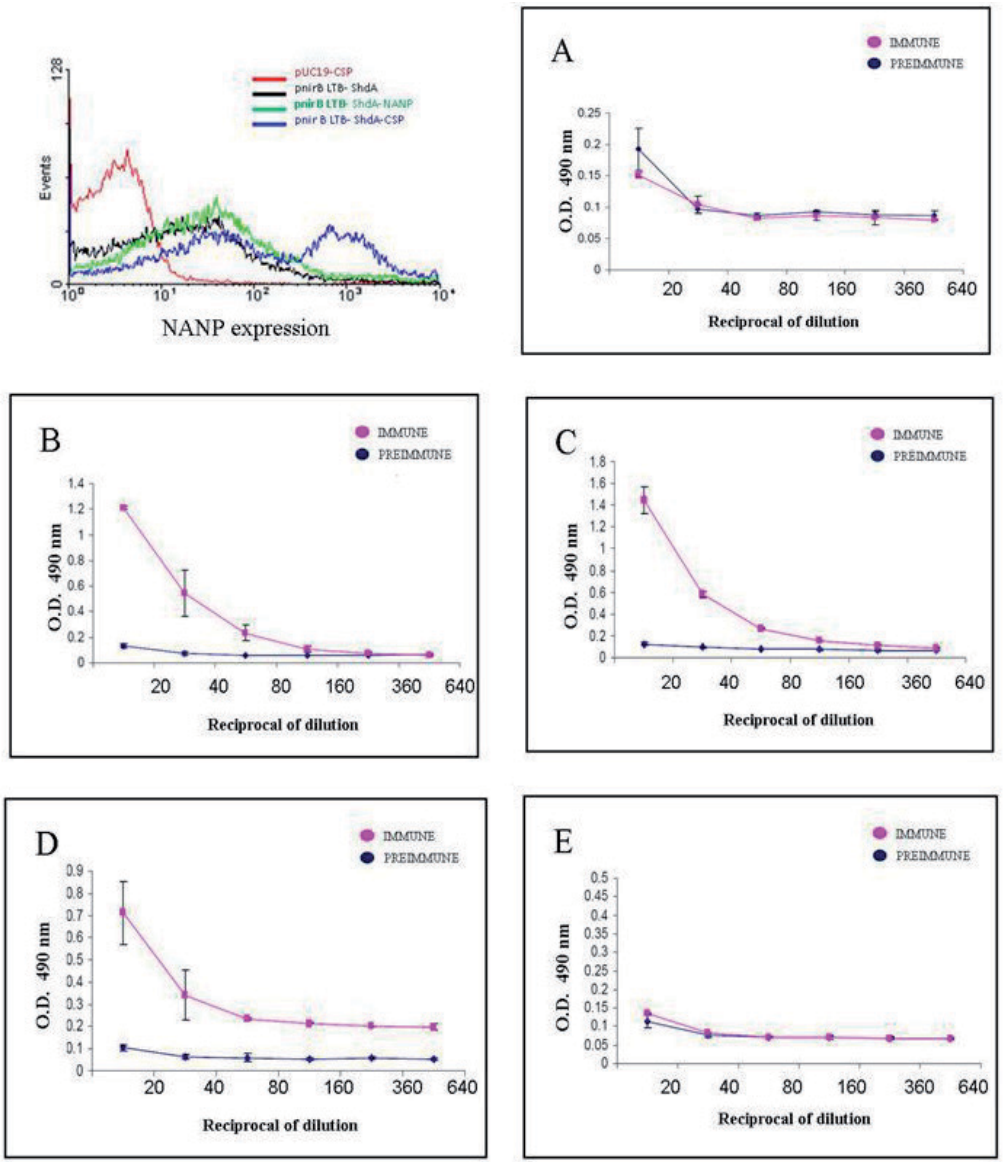
ShdA is other large autotransporter, (Desvauxet al. 2004) identified in *S. enterica* subespecies (Kingsleyet al. 2000), with similar structure to AIDA-I, TibA, and MisL, therefore it has been included also in the AIDA-subfamily. The α-domain is an adhesin (Kingsleyet al. 2000) that mediates bacterial colonization in the host cecum, the main reservoir for *S.* Typhimurium during infection in mice (Kingsleyet al. 2002). In fact, the inactivation of *shdA* produces bacterial number and bacterial permanence in the intestinal mucosa (shedding reduction) (Kingsleyet al. 2000; Kingsleyet al. 2002). The extracellular matrix protein fibronectin is a receptor for the ShdA passenger domain. This was demonstrated by a ShdA–GST (glutathione *S*-transferase) fusion protein which bound fibronectin *in vitro* in a dose dependent manner and was partially inhibited by anti-fibronectin antibodies, suggesting that other receptors may also play a role in ShdA-mediated adherence to the intestinal mucosa (Kingsleyet al. 2004).

Several autotransporters (Maureret al. 1999) require a link region between the α and β domains for autodisplay. This minimal translocation unit (TU) is necessary to allow folding of the passenger α -domain (Oliveret al. 2003). The role of TU in ShdA still remains to be show. Since autotransporters are able to display heeterologous peptide substituting the α -domain they have been used for the construction of bacterial whole-cell absorbents, study receptor-ligand interactions surface display of random peptide libraries and vaccine development (Lattemannet al. 2000).

We describe here an example of the latter application exposing the NANP immunodominat epitope from *Plamodium falciparum* CSP on the surface of *Salmonella* using an autotransporter. We generated a series of NANP-ShdA fusion proteins containing the β-domain and different truncated α-domains forms under the control of *nirB* promoter (Chatfieldet al. 1992), using the technical approach described elsewhere (Ruiz-Perezet al. 2002).

The flow cytometry **in Figure 3** presents the summary of several assays performed to identify the minimal α-domain amino acid strand necessary for translocation through the ShdA β-domain. *S.* Typhimurium SL3261 was transformed with plasmids bearing different truncated α-domain forms fused to three repeats of NANP [(NANP)3] or the complete CSP. NANP expression on to the surface of the bacteria was determined with a monoclonal antibody. We identified that the minimum translocation unit necessary to translocate the epitope is conformed 16 residues in the α-domain. Interestingly only around 45% of the bacterial strains expressed the antigen on their surface.

BALB/c mice were immunized with different S. Typhimurium SL3261 expressing the full length CSP or the (NANP)3 epitope on the surface and compared with a strain producing the antigen in the bacterial cytosol **(Figure 3 A-E)**. As expected, the strain expressing only ShdA did not elicit antibodies. The strains expressing the NANP or the CSP elicited good antibody response **(Figure 3 B-C)**, whereas the strain producing the CSP in the cytosol was unable to elicit antibodies **(Figure 3 E).** An additional control, autotransporter MisL expressing the NANP epitope, was able as well to elicit antibodies **(Figure 3 D)**.



Serum antibody response elicited by immunization with *Salmonella enterica* serovar Typhimurium SL3261 transformed with differents plasmids. BALB/c mice were immunized o.g. as described elsewhere (Gonzalezet al. 1998) (A) pnirB LTB- ShdA (negative control) (B) pnirB-LTB NANP ShdA; (C) pnirB-LTB CSP ShdA.; (D) pnirB-LTB NANP MisL; (E) pUC19 CSP. Groups of 5 BALB/c mice were immunized orally with two doses of $1x10^{10}$ C.F.U. (15-day interval) of the *Salmonella SL3261* strain transformed with different plasmids. IgG levels were determined one week after last immunization by ELISA as previously described (González et al., 1998). Each graphic represents the serum IgG from one mouse.

Fig. 3. Flow cytometry analysis of strains of *Salmonella enterica* serovar Typhimurium SL3261 transformed with differents plasmids. Plasmid pUC19 CSP corresponding to cytosolic form of antigen, whereas pnir B LTB ShdA-CSP and pnir B LTB ShdA-NANP corresponding to antigen display on the bacterial surface.

5. Conclusion

In summary, there is increasing evidence that antigen location in live bacterial carrier vaccines, in this case attenuated *Salmonella* strains is an important factor determining the type of immune response elicited to the passenger antigen.

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17

Neutrophil Cellular Responses to Various *Salmonella typhimurium* LPS Chemotypes

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1. Introduction

The first line of defense against invading bacteria is provided by the innate immune system, and polymorphonuclear leukocytes (PMNL) contribute to bacterial clearance by uptake and intracellular killing of microbes. Lipopolysaccharides (LPS, endotoxin), a major component of the outer membranes of Gram-negative bacteria, is shed into the environment and acts as a highly potent proinflammatory substance. About 15—25% of the bacterial surface in *Salmonella typhimurium* was found to be covered by LPS (Mühlradt et al., 1974). LPS initiates the cascade of pathophysiological reactions called endotoxin shock. LPS released from Gram-negative bacteria induces a strong priming of superoxide production (Guthrie et al., 1984) and facilitates the rapid elimination of the bacteria. However, an excessive activation of neutrophils could be self-destructive in septic shock. A number of mediators, such as cytokines, nitric oxide and eicosanoids, are responsible for most of the manifestations caused by LPS. The toxic and other biological properties of LPS are due to the action of endogenous mediators, which are formed following interaction of LPS with cellular targets (Galanos & Freudenberg, 1993). Biological activities of LPS have been well established, but some uncertainty remains regarding to the responses to various LPS chemotypes.

LPS are phosphorylated glycolipids that possess complex chemical structures (Müller-Loennies et al., 2007). LPS are composed of covalently linked structural domains: lipid A, an oligosaccharide core, and O- polysaccharide (or O- antigen) (Raetz & Whitfield, 2002). Lipid A is the minimal biologically active unit of LPS and is thus called the 'endotoxic principle' of LPS. The full chemical structures of lipid A from *E. coli* and *Salmonella enterica* serovar Typhimurium (S. Typhimurium) were identified in 1983, and the similarity of their structures was proved (Takayama et al., 1983; Alexander & Rietschel, 2001, review). Lipid A

is the hydrophobic portion of the molecule. The hydrophilic polysaccharide portion may be further subdivided into the O-specific and the core oligosaccharide. Bacteria which contain an O- polysaccharide have a smooth colony appearance when grown on agar plates and therefore this type of LPS is referred to as smooth(S)-type LPS. The outer parts of LPS (O-polysaccharide) interact with the host immune system. Westphal and al. established that the O-polysaccharide component contained the serologically active determinants (the species-specific bacterial O-antigen) (Westphal, 1978; Westphal & Luederitz, 1961). Currently, based on O-antigens (O-polysaccharides), *Salmonella* strains have been classified into over 50 serogroups (Fitzgerald et al., 2007).

The presence of O-antigen in LPS is irrelevant for bacterial invasion of epithelial cells; in contrast, a core structure is necessary for adhesion and subsequent entry of S. Typhimurium into epithelial cells (Bravo et al., 2011). Mutant bacteria (rough mutants) produce LPS with short oligosaccharide chains but not O- polysaccharide. Chemical analysis of LPS from such *Salmonella* mutants distinguished Ra from Re chemotypes: Ra describes the largest core structure and Re was assigned to the smallest core structure. LPS from rough mutants, so-called Ra, Rb, Rc, Rd and Re LPS, mainly differ in the length of the core oligosaccharide, while the lipid-A portion is assumed to be identical. The chemical structures of *Salmonella* LPS have been investigated in many details (Olsthoorn et al., 1998; Perepelov et al., 2010).

Neutrophil-mediated innate host defense mechanisms include phagocytosis of bacteria. Upon activation, polymorphonuclear leukocytes (PMNL, neutrophil), produce signicant amounts of leukotriene B4 (LTB4) in addition to several cytokines and inammatory mediators, and thus recruit other neutrophils to the site of inammation. LTB4 is one of the most potent chemotactic compounds produced in macrophages and neutrophils (Toda et al., 2002). Stimulation of leukotriene B4 synthesis in PMNLs plays a role in stimulation of phagocytosis and bacterial killing (Mancuso et al., 2001). The key enzyme of LT synthesis in neutrophils is 5-lipoxygenase (5-LO), which metabolizes arachidonic acid (AA), first to 5S-hydroperoxyeicosatetraenoic acid (5-HPETE), and then to leukotriene A4 (LTA 4) (Samuelsson, 1983). Unstable LTA4 intermediate is converted to 5S,12R-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid (leukotriene B4, LTB4) and (non-enzymatically) to its isomers. The 5-LO metabolite LTB4 is a proinflammatory mediator that activates neutrophils, thus changing their shape and promoting their binding to endothelium by inducing the expression of cell-adhesion molecules. The localization of leukocytes to the site of inflammation results in endothelial and other tissue damage, i.e. metabolites of 5-LO contribute to the multiple organ injury and dysfunction during inflammatory process (Collin et al., 2004; Cuzzocrea et al., 2003; 2004). Any modulation of the activity of PMNL is a potential cause of the altered immune response to infection. The phagocytosis of microorganisms by PMNL is enhanced by LPS. And though *Salmonella*-LPS related complications have been successfully blunted with 5-LO inhibitors (Matera et al., 1988; Altavilla et al., 2009), little is known about phagocytosis and 5-LO products regulation by LPS chemotypes.

Effects of structurally different LPS types upon neutrophil functions were examined. Ruchaud-Sparagano et al. (Ruchaud-Sparagano et al., 1998) investigated the mechanisms of LPS action by examining the effect of smooth and rough chemotypes of LPS in stimulating neutrophil beta2 integrin activity and fMLP-induced respiratory burst. They reported just kinetic differences in the action of rough LPS and smooth LPS: rough LPS acts more rapidly

than S-LPS to cause functional alterations in neutrophils. Similar results were obtained on neutrophils in whole blood: again just kinetic difference was observed between R- and S-LPS in the expression of cell surface receptors CD11b and CD11c on neutrophils (Gomes et al., 2010). Nevetheless, the rough mutant as well as S LPS differ in some distinct physico-chemical properties. Due to these differences, it was found a lower fluidity of S LPS chemotype than Ra and Re mutants (Luhm et al., 1998). It was established that the bioactivity of LPS was dependent on the length of their core oligosaccharides, and endotoxin-induced cytokine secretion decreased with decreasing sugar moiety (and increasing fluidity) in the order S ≥ Ra>Rc>Re LPS (Luhm et al., 1998). Comparative evaluation of the endotoxic properties of LPS preparations by using the LAL assay showed that endotoxic activity of the rough Re mutant SL1102, the rough Ra mutant TV119, and the smooth strain SH4809 of *Salmonella* Typhimurium increased in the order S < Ra < Re (Shnyra et al., 1993).

When neutrophils were challenged with *Salmonella minnesota* smooth-strain and rough-strain mutants (Ra, Rb2, RcP-, Rd1P- and Re) as well as with lipid A, in the case of luminol-dependent chemiluminescence (respiratory burst), lipid A was the most potent stimulus, with the response decreasing as molecular complexity increased, with S- LPS equally potent as Ra LPS (Pugliese et al., 1988). An oxygen-independent system in the antimicrobial effects of neutrophils is also sensitive to LPS chemotype. As the carbohydrate content of the mutant LPS decreased, the bacteria became less resistant to the oxygen-independent bactericidal activity of neutrophils (Okamura & Spitznagel, 1982). Based on these data, one can conclude that there are qualitative as well as quantitative effects of the carbohydrate moieties of LPS. We report here that various LPS forms from *Salmonella typhimurium* bacteria significantly differ in their ability to influence adhesion, phagocytosis as well as formation of 5-LO products, and reactive oxygen and nitrogen species in human neutrophils.

2. Materials and methods

Zymosan A from *Saccharomyces cerevisiae*, lipopolysaccharides from *Salmonella* enterica serovar Typhimurium (the source strain for smooth form is ATCC 7823, rough strains from *Salmonella* typhimurium TV119 (Ra mutant) and SL1181 (Re mutant)), N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME), staurosporine from *Streptomyces sp.* were from Sigma (St. Louis, MO, USA and Steinheim, Germany). *S.* Typhimurium virulent strain C53 was a kind gift of Prof. F. Norel (Pasteur Institute, France) (Kowarz et al., 1994). Bacteria were grown in Luria–Bertani broth and washed twice using physiological salt solution with centrifugation at 2000 *g*. The concentration of the stock suspension was 1×10^9 CFU/mL. The bacteria were opsonized with 5% fresh normal human serum (NS) from the same donor whose blood was used for preparation of neutrophils. NS was prepared by clotting and centrifugation of fresh whole blood at room temperature. In some experiments, the NS was decomplemented by heat inactivation for 30 min at 56°C (heat inactivated serum, HIS). Nitrate/Nitrite fluorometric assay kit was from Cayman Chemical (Ann Arbor, MI, USA). Ficoll-Paque was purchased from Pharmacia (Uppsala, Sweden). Human serum albumin, fraction V (HSA) was from Calbiochem (La Jolla, CA, USA). Hepes and *o*-phenylenediamine were from Fluka (Deisenhofen, Germany). Phosphate buffered saline (PBS) was purchased from Gibco (Paisley, UK, Scotland, UK). Dextran T-500 was from Pharmacosmos (Holbaek, Denmark). High-pressure liquid chromatography (HPLC) solvents were purchased from Chimmed (Moscow,

Russia). Prostaglandin B2 was from Cayman Chemical Company (Ann Arbor, USA). Hank's balanced salt solution (with calcium and magnesium but without phenol red and sodium hydrogen carbonate, HBSS), HBSS modified (without calcium, magnesium, phenol red and sodium hydrogen carbonate), Dulbecco's PBS (with magnesium, but without calcium), cytochrome *c* from horse heart were purchased from Sigma (Steinheim, Germany).

2.1 Human neutrophil and red blood cell (RBC) isolation

PMNLs were isolated from freshly drawn EDTA-anticoagulated donor blood by standard techniques, as previously described (Sud'ina et al., 2001). Leukocyte-rich plasma was prepared by sedimentation of RBCs with 3% dextran T-500 at room temperature. Granulocytes were purified by centrifugation of leukocyte-rich plasma through Ficoll-Paque (density 1.077 g/mL) followed by hypotonic lysis of the remaining RBCs. PMNLs were washed twice with PBS, resuspended at 10^7/mL (purity 96–97%, viability 98–99%) in Dulbecco's PBS containing 1 mg/mL glucose (without $CaCl_2$), and stored at room temperature. RBCs were isolated from EDTA-anticoagulated donor blood by sequential centrifugation (at 1100 rpm) and washing with PBS. After three washes, the cells were resuspended at 2.7×10^9/mL in PBS and stored at room temperature.

2.2 Preparation of collagen-, fibronectin- or HUVEC-coated surfaces

Plastic tissue-culture 24-well plates (Corning Incorporated, Corning, NY, USA) were coated with 75 μg/ml type I collagen or 15 μg/ml fibronectin for 24h. Prior to use, the protein coated surfaces were washed, incubated for 1 h in PBS with 0.1% human serum albumin, and then thoroughly washed with PBS. Human umbilical vein endothelial cells (HUVEC), passages 1–3, were maintained in medium 199 containing 10% fetal calf serum (FCS), 3.5 units/ml heparin (Fluka, Deisenhofen, Germany), 50 μg/ml endothelial cell growth factor (ICN, Ohio, USA), 10 U/ml penicillin and 10 mg/ml streptomycin. The cells were passaged using trypsin-EDTA solution (500 BAEE units trypsin and 180 mg EDTA/ml in PBS), and seeded on 24-well plates (Galkina et al., 2004). One day before the experiments, the monolayers were washed and medium was replaced with the same medium containing 2% FCS, rather than 10 %.

2.3 Preparation of lipopolysaccharides (LPS) solutions and opsonized zymosan (OZ)

Lipopolysaccharides from *Salmonella enterica* serovar *typhimurium* were solubilized in PBS (1 mg/ml) by vortexing, heated in a water bath to 60°C for 30 min, cooled to room temperature, and subjected to one more cycle of heating to 60°C and cooling to room temperature. Zymosan A particles from *Saccharomyces cerevisiae* were suspended in PBS and boiled for 5 min. After cooling to room temperature, the prepared suspension was washed with PBS and opsonized by adding 20-30 % freshly prepared autologous human normal serum for 30 min at 37°C, washed 3 times with PBS and resuspended in the Hank's balanced salts medium containing 10 mM Hepes (HBSS/Hepes).

2.4 PMNL adhesion assay

Myeloperoxidase activity was used to measure PMNL attachment under static conditions to collagen or HUVEC adsorbed on to plastic surfaces. For measuring PMNL adhesion, HUVECs grown in 24-well plates were washed once with HBSS. PMNLs (10^6/well) were

added to a coated 24-well culture plate in 500 µl of HBSS/Hepes medium. After 30 min of incubation with or without the additives in a CO_2 incubator at 37°C to allow neutrophil adherence, wells were washed twice with 500 µl of PBS solution for removal of non-adherent PMNLs. The extent of adherence was measured after the addition of detergent and a myeloperoxidase substrate, as described (Schierwagen et al., 1990; Sud'ina et al., 1998). A solution (300µl) of 5.5mM *o*-phenylenediamine and 4mM H_2O_2 in buffer (67mM Na_2HPO_4, 35mM citric acid and 0.1% Triton X-100, pH5) was added to each well, and after 5 min the reaction was stopped by the addition of an equal volume of 1M H_2SO_4. Standard dilutions of PMNLs with or without tested compounds were used for calibration.

2.5 Phagocytosis experiments

PMNLs (5×10^6/ml) were placed into 6-well plates (2 ml/well) containing collagen- of fibronectin-coated coverslips for 30 min of incubation with tested compounds. Then 0.25 mg/ml of opsonized zymosan (OZ) was added for another 5 min. The cells were gently washed with PBS, and then fixed for 30 min in HBSS medium modified, with 10 mM HEPES and 2.5% glutaraldehyde. After gentle washing with PBS, the samples were examined by phase contrast microscopy. The number of OZ particles ingested was counted and the data were expressed as a phagocytic index, which was derived by multiplying the portion of PMNLs containing at least one ingested target by the mean number of phagocytosed targets per positive PMNL. Data were obtained from ~ 100 cells per coverslip.

2.6 Scanning electron microscopy

Cells were fixed for 30 min in 2.5% glutaraldehyde, postfixed for 15 min with 1% osmium tetroxide in 0.1 M cacodylate (pH 7.3), dehydrated in an acetone series, critical-point dried with liquid CO2 as the transitional fluid in a Balzers apparatus, sputter-coated with gold–palladium, and observed at 15 kV with a Camscan S-2 (Tescan, USA) or JSM-6380 (JEOL, Germany) scanning electron microscope.

2.7 Nitrite measurement

Nitric oxide, derived from the conversion of L-arginine to L-citrulline, reacts with molecular oxygen to form nitrite and nitrate (Moncada & Higgs, 1993). NO production was measured as total nitrite concentration in the sample after enzymatic conversion of nitrate to nitrite by nitrate reductase. A highly sensitive fluorometric assay for nitrite measurements, which is based on the acid-catalyzed ring closure of 2,3-diaminonaphtalene (DAN) with formation of highly fluorescent product 2,3-aminonapthotriasole in the presence of nitrite, was used to probe PMNLs for NO production (Nath & Powledge, 1997). For this purpose, PMNLs (2×10^7/ml) were incubated with compounds tested for 30 min, then OZ was added for the next 30 min, reaction was stopped by centrifugation (400g, 10 min) and supernatant was filtered though 10 000 Mr cutoff microcentrifuge filters (Millipore corporation, USA) at 14 000g for 30 min at room temperature. The ultrafiltration step was necessary to remove any trace amounts of zymosan particles and hemoglobin which may be present in PMNL samples due to red cells contamination, which strongly interferes with the fluorescent measurements (Misko et al., 1993). Nitrite measurements in the prepared supernatants were performed in triplicate using Nitrate/Nitrite fluorometric assay kit (Cayman Chemical, Ann Arbor, MI,

USA) according to manufacturer's protocol at excitation and emission wavelengths of 360 and 430 nm, respectively, by plate reader Infinite 200 (Tecan Group Ltd., Mainz, Germany). All compounds added to PMNLs were tested for their autofluorescence within the spectrum region in the assay buffer.

2.8 Superoxide measurement

PMNL incubations on collagen- and fibronectin-coated surfaces were performed as described for PMNL adhesion assay. 50 μM cytochrome c, tested compounds and 300 u/ml superoxide dismutase (SOD), were added (as indicated) to the medium prior to the cells. The plates were incubated at 37 °C for 30 min, then OZ was added or not for another 30 min. The incubation was stopped by cooling to 4°C, and cytochrome c reduction was measured as the increase in Δ550/535 (the change in the ratio of absorbances at 550 and 535nm). Reduction of 10 μM cytochrome c produced an increase in Δ550/535 of 0.18 absorbance unit.

2.9 Assay of reactive oxygen species

The formation of active oxygen by neutrophils stimulated with phorbol 12-myristate 13-acetate (PMA), LPS chemotypes and OZ was monitored by measuring luminol-enhanced luminescence as described earlier (Sud'ina et al., 1991). Chemiluminescence was monitored in a 1251 LKB luminometer, using 1 μM luminol. Measurements were made every 5 min over a 30 min period at 37°C.

2.10 Incubations for studies of arachidonic acid (AA) metabolism and leukotriene (LT) synthesis

PMNLs suspension (2×10^7 cells) was incubated in 6 ml HBSS/Hepes medium at 37 °C with or without agents tested for 30 min, and then stimulated by the addition of opsonized Salmonella (OS) or OZ for 20 min. The incubations were stopped by addition of an equal volume of methanol at -20°C with prostaglandin B2 (PGB2) as an internal standard. The samples were stored at -20°C. The denatured cell suspension was centrifuged (at 2000 rpm), which yielded supernatants designated as water/methanol extracts.

2.11 Lipoxygenase product analysis

The water/methanol extracts were purified by solid-phase extraction using C18 Sep-Paks (500mg), which was conditioned first with methanol, then with water. 5-LO metabolites were extracted with 1.4 ml methanol, the samples were evaporated, redissolved in 35 μl methanol/water (2:1) and chromatographed by reversed-phase HPLC. The purified samples were injected into a 5 μm Nucleudur C18 column (250 mm×4.6 mm; Macherey-Nagel, Dueren, Germany). The products were eluted at 0.7 ml/min in a linear gradient from 30 to 100% solvent B: the eluents consisted of methanol/acetonitrile/water/acetic acid/triethylamine in the ratios (solvent A) 25/25/50/0.05/0.08 and (solvent B) 50/50/0/0.05/0.04, and elution was monitored using a UV detector at 280 nm and 238 nm. Products of the 5-LO pathway that were measured included leukotriene B4 (LTB4), 5-hydroxyeicosatetraenoic acid (5-HETE), 20-hydroxy-LTB4 (ω-OH-LTB4) and iso-LTB4 [5(S),12(S,R)-dihydroxy-all-trans-eicosatetraenoic acids], identified by their co-elution with

authentic standards. The respective extinction coefficients and their ratios to that of the internal standard were used to quantify products.

2.12 Statistics

Statistical analysis was performed using the Student's t-test. Statistical significance was assumed, where probability values of less than 0.05 were obtained. Results are reported as mean ± SD of the data of at least three independent experiments.

3. Results and discussion

Neutrophils are professional phagocytes and the first line of defense of innate immune system at bacterial challenge (Borregaard, 2010). Circulating lipopolysaccharides released from bacteria may activate neutrophils. LPS elicit wide spectra of biological responses in human body. When activating an immune response, they may produce pathologically imbalanced immune response, - that is why they are called "endotoxins". Depending on a dose, they may stimulate antigen-specific immune response, and they are added as accompanying agents in vaccination (Baldridge et al., 1999; Gereda et al., 2000). High levels of endotoxins cause endotoxic shock (Rietschel et al., 1996). LPS induce numerous cellular signals. In the focus of the current work, we should stress on NO formation and leukotriene synthesis. Superproduction of NO in sepsis results in the disturbed blood flow (Li & Forstermann, 2000), which originates from contractile disfunction of smooth muscle cells and impaired PMNL chemotaxis (Lopez-Bojorquez et al., 2004). LPS interaction with leukocytes signal to phosphorylation of phospholipase A2 and arachidonic acid (AA) release from cell membranes (Doerfler et al., 1994). AA and its methabolites are the compounds with high biological activity.

In this work, we investigated effects of *Salmonella* enterica serovar typhimurium LPS species of various chemotypes (from deep rough Re mutant consisting of the lipid A and the KDO (3-deoxy-D-manno-oct-2-ulosonic acid) residues, rough Ra mutant with complete core, and S form) on cellular responses of human PMNLs. LPS chemotype structures are schematically presented in Fig.1.

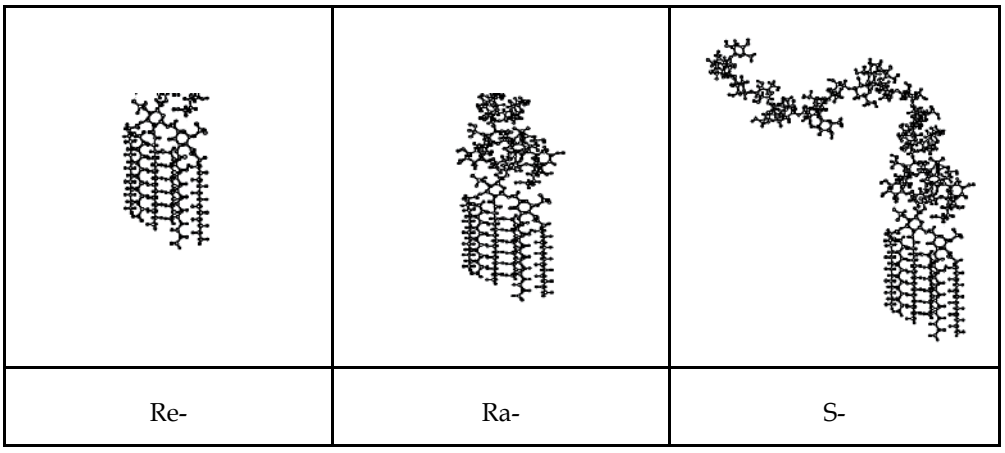
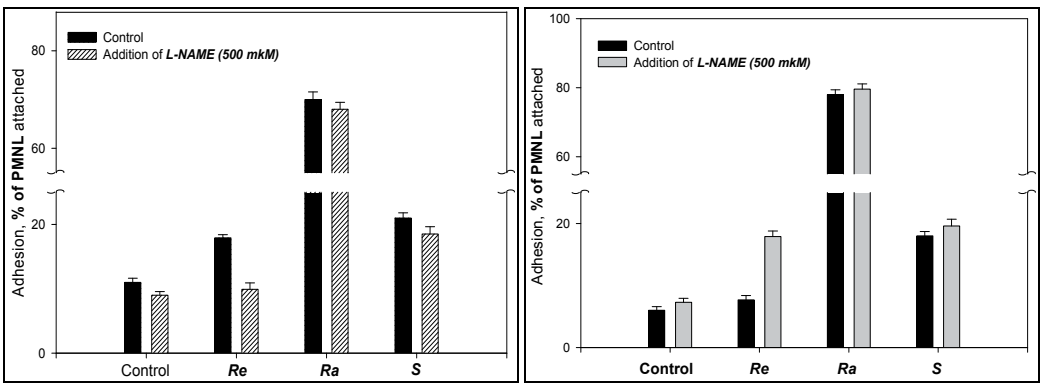


Fig. 1. Schematic presentation of LPS molecules: Re-, Ra- and S-LPS.

3.1 Neutrophil adhesion

Regulation of neutrophil adhesiveness is generally considered to be a key element in the development of inflammatory reactions. Neutrophils are known to spread on a protein-coated surface, a process that has been interpreted as "frustrated" phagocytosis. To elucidate whether Salmonella LPS of various chemotypes selectively influenced the number of adherent neutrophils, an adhesion study was performed. PMNL adhesion to collagen-coated surface was crucially increased by Ra LPS (Salmonella LPS from Ra mutant TV119). The effect was slightly lower on the surfaces coated by endothelial monolayer (Fig.2).



PMNL adhesion to HUVEC-coated surfaces PMNL adhesion to collagen-coated surfaces

Fig. 2. PMNL attachment to HUVEC- and collagen-coated plastic surface determined as described in Methods after 30 min incubation without (control) or with various LPS forms, and 500 μM L-NAME. Cell attachment has been expressed as a percentage of PMNLs adhered in relation to the total number of PMNLs added.

Priming of PMNLs with the LPS chemotypes induced cell activation including NO and superoxide release, as well as an increase in intracellular calcium concentration. The experiments with nonselective NOS inhibitor L-NAME at 500 μM demonstrated that only Re LPS (Salmonella LPS from Re mutant SL1181) was sensitive to NOS inhibition (Fig.2). The antioxidant agent diphenileleiodonium (DPI) that inhibits NADPH oxidase-mediated ROS formation, and also inhibits other flavo-enzymes such as NO synthase and xanthine oxidase (Wind et al., 2010), did not affect LPS-induced PMNL attachment (data not shown). LPS- induced intracellular calcium concentration varied in the order Ra ≥ S > Re (Zagryazhskaya et al., 2010). Taking into account the slight sensitivity of PMNL attachment to NO synthesis inhibitors, we can propose that the divalent cation requirements for the Mac-1 and LFA-1-dependent processes of adhesion (Graham & Brown, 1991; Wright & Jong, 1986) may limit the role for NO and superoxide in the specificity of these LPS chemotypes in PMNL adhesion, in the serum-free medium.

The addition of heat inactivated normal serum (HIS) markedly decreased neutrophil adhesion, but the selective prominent increase of the neutrophil attachment induced by Ra LPS chemotype was evident (Fig.3). It has been published that serum enhanced LPS-induced production of nitric oxide in J774.1 and BAM3 macrophage-like cell line (Ohki et al., 1999). Human serum albumin is known to increase iNOS expression in the lung of rats (Jakubowski et al., 2009). In our assay 500 μM L-NAME partially reversed the effect of

serum (Fig.3), which supported the hypothesis that nitric oxide mediates smoothing out the specificity of various LPS chemotypes in the presence of serum. NO is known to reduce adhesion molecules expression on neutrophils (Kubes et al., 1991; 1994; Banick et al., 1997; Kosonen et al., 1999), and we observed decreased attachment in the presence of serum (Fig.3). We propose that in the serum-containing medium, along with other factors, NO also plays a role in the specificity of LPS chemotypes in PMNL adhesion.

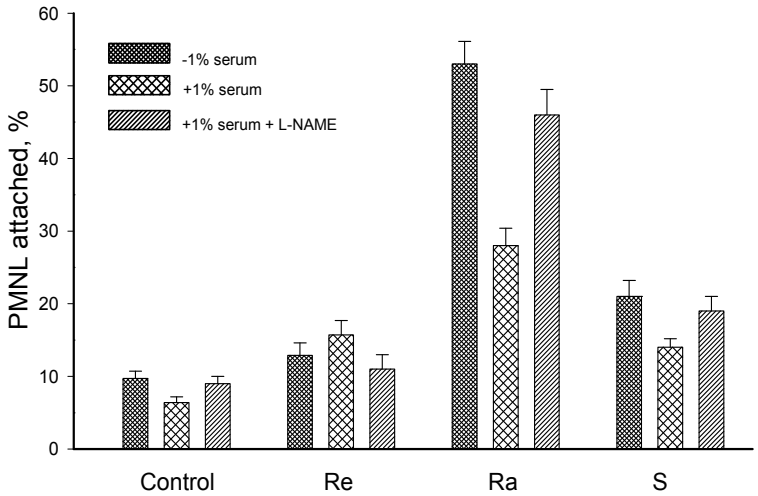


Fig. 3. Effect of serum on PMNL attachment, induced by LPS chemotypes. PMNLs ($5x10^5$/well) were added to a collagen-coated 24-well culture plate in 500 µl of HBSS/Hepes medium, without (control) or with 1% serum, 5 µg/ml LPS and 500 µM L-NAME. After 30 min of incubation in a CO2 incubator at 37 °C to allow leukocyte adherence, wells were washed twice with 500 µl of PBS solution, for removal of non-adherent PMNLs. The extent of adherence was measured after the addition of detergent and a myeloperoxidase substrate, and has been expressed as a percentage of PMNLs adhered in relation to the total number of PMNLs added.

3.2 NO and ROS formation in neutrophils

NO release is an important endogenous regulatory mechanism of inflammatory response. Human neutrophils were evaluated for their ability to generate nitric oxide more than 30 years ago (Schmidt et al., 1989; Wright et al., 1989). Nitric oxide synthase (NOS) enzymes in neutrophils were characterised, and the data on the expression of NOS isoforms are contradictory (Amin et al., 1995; Carreras et al., 1996; Greenberg et al., 1998; Cedergren et al., 2003; de Frutos et al., 2001; Molero et al., 2002; Saini et al., 2006; Chatterjee et al., 2007; 2008). The level of NO synthesis in PMNLs is comparable to one in endothelial cells, and therefore contributes significantly to the amount of NO in circulation (Miles et al., 1995; Wright et al., 1989). It is proposed that NO synthesis in neutrophils is of great physiological significance, as it modulates neutrophil function at sites of inflammation. NO participates in activation of a newly described mechanism of immune defense as formation of neutrophil extensions (cytonemes), when neutrophils do not phagocyte, but bind bacteria extracellularly (Galkina et al., 2009).

Lipopolysaccharides are well known for their ability to elicit the release of NO from eukaryotic cells including macrophages, neutrophils, and endothelial cells (Jean-Baptiste, 2007; Titheradge, 1999; Tsutsui et al., 2009). Endotoxemia is often associated with increased NO (Evans et al., 1993; Szabo et al., 1993; Gomez-Jimenez et al., 1995). NO is a unique "messenger". The biological half-life of NO is rather long - several seconds (Lancaster & Ignarro, 2002), and this molecule easily passes through cell membranes, and can interact with transition metals forming nitrosyl complexes and influencing activity of many enzymes (Korhonen et al., 2005). A reaction of particular biological relevance is the reaction of NO with superoxide with the formation of OONO- (peroxynitrite, PN) (Beckman et al., 1990). Concentration of superoxide increases up to 0.1 μM during inflammatory responses (Zweier et al., 1989), but the spectrum of reactive oxygen/nitrogen species depends on the balance of NO and superoxide within the local chemical environment (Jourd'heuil et al., 1999; 2001).

Conditions for production and release of NO in human PMNLs are still largely unknown. We recently published a paper on the influence of various LPS differing in their chain length on NOS activity in opsonized zymosan stimulated human PMNLs. We observed significant difference between Re and Ra forms of S. Typhimurium LPS in the capacity to induce NO release: Re LPS was twice more potent than Ra LPS (Zagryazhskaya et al., 2010). It is known that LPS activates protein kinase C (PKC) in macrophages and PMNLs. Increased PKC activity may inhibit NOS activity and staurosporine was shown to reverse this inhibition (Muniyappa et al., 1998). Therefore, we tested if staurosporine (St), the nonselective PKC inhibitor, could influence the specific LPS effects on NO synthesis, observed in our studies. The results are presented in Fig. 4.

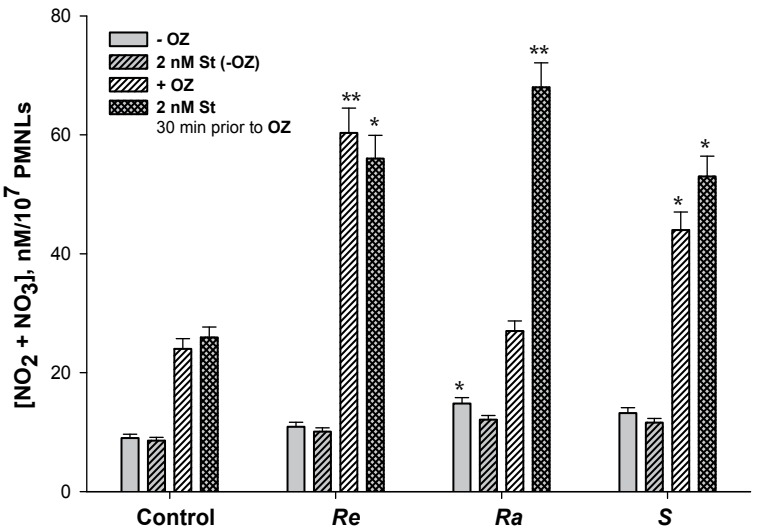


Fig. 4. Modulation of nitrite production in PMNLs by staurosporine. PMNLs (2×10^7/ml) were incubated for 30 min at 37 °C in the presence or absence of additives, (as specified): 5 μg/ml of different LPS forms, 2 nM staurosporine (St) and then stimulated for 30 min with 2 mg/ml OZ. * $P < 0.05$ vs corresponding control. ** $P < 0.01$ vs corresponding control.

Staurosporine (1-2 nM) inhibited a small increase in nitrate/nitrite level, produced by Ra and S LPS in the absence of OZ, slightly increased NO production caused by OZ alone, and partially reversed NO synthesis in OZ-stimulated PMNLs, primed by various LPS. Ra LPS form, which produced the minimal increase in NO synthesis (and even decrease in some experiments), caused the maximal NO production in the presence of staurosporine, Re and S LPS were less active. L-NAME, NOS inhibitor, significantly decreased NO production in the presence of St (data not shown) indicating staurosporine influence on NOS activity in human PMNLs, primed with LPS. These experiments demonstrated significant difference in NO production between LPS species and confirmed the role for NO in the specificity of LPS chemotypes.

LPS-priming of phagocytic leukocytes leads to nicotinamide adenine dinucleotide (NADPH) oxidase activation and potent generation of reactive oxygen species (ROS) upon stimulation (Curnutte & Babior, 1974; Drath & Karnovsky, 1975), and this process is often referred to as the respiratory burst. We determined the capacity of various LPS species to modulate superoxide anion (O2-) production measured as cytochrome c reduction, as well as ROS production measured as luminol-dependent chemiluminescence, in PMLNs prior to or without their activation by OZ. The most potent O2- production was detected in Ra-primed cells in which we observed approximately 5-fold increase in the production level detected in control cells (Zagryazhskaya et al., 2010). It is noteworthy that activation of the cells with OZ dramatically increased O2- generation in both LPS-primed and control cells, while relative values of the LPS effects were diminished. In luminol-dependent chemiluminescence, again, the efficacy pattern Ra > S > Re was found (Fig. 5). Ra LPS was the most potent chemotype in ROS and O2- release from LPS-treated PMNL.

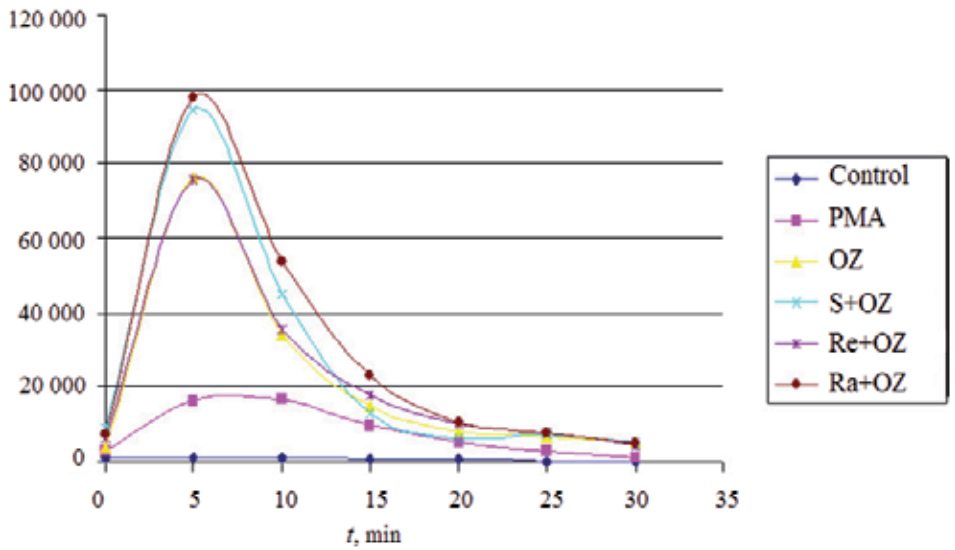


Fig. 5. Effect of various LPS forms on ROS formation in PMNLs, as recorded by luminol-enhanced luminescence (y-axis, arbitrary units). PMNLs were incubated at 37 °C in the presence or absence of additives, as specified: 100 nM PMN, 5 μg/ml of different LPS forms, 0.2 mg/ml OZ.

The addition of heat inactivated normal serum (HIS) decreased superoxide release, but the effect of Ra LPS chemotype was still maximal (Fig.6). Supposedly, serum increased LPS-induced NO release, which neutralized superoxide and resulted in smoothing out the specificity of various LPS chemotypes (Fig.6.).

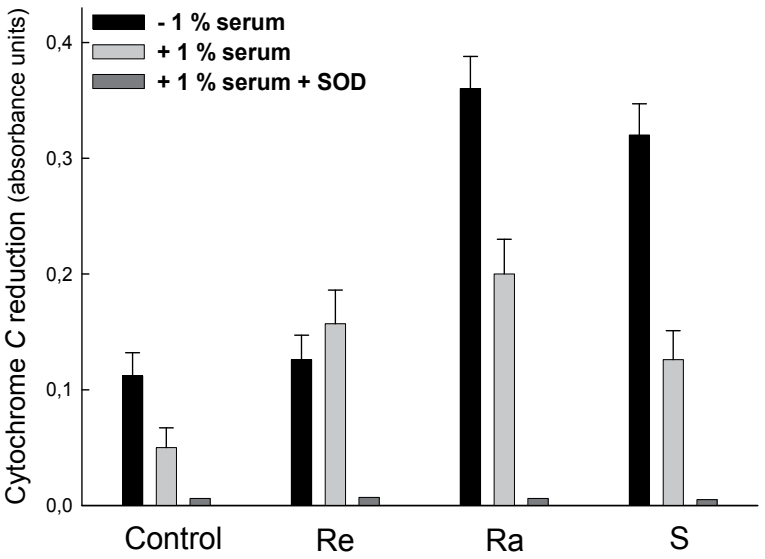


Fig. 6. Effect of serum on LPS-induced superoxide production in human PMNLs. 10^6 PMNLs/ml were incubated for 30 min at 37 °C without (control) or with 5 µg/ml LPS on a collagen-coated surface, in the medium without (- 1% serum) or with 1 % serum (heat inactivated human serum). When indicated, 300 u/ml superoxide dismutase (SOD) were added. Superoxide production was measured as cytochrome c reduction, as described in Methods.

3.3 NO levels dictate the signaling pathway to phagocytosis and LT synthesis in PMNL

We addressed the role for NO in phagocytosis of opsonized zymosan (OZ) influenced by various LPS chemotypes. OZ was prepared by incubating zymosan particles (dried cell walls of Saccharomyces cerevisiae) with autologous serum. Using phase-contrast microscopy we determined the phagocytic index in the cells exposed to 1 µg/ml of LPS species (Re, Ra, S) from *Salmonella* enterica serovar typhimurium for 30 min prior to OZ addition (for additional 5 min). The role for NO in distinct effects of various LPS chemotypes is clearly evident in phagocytosis of OZ by PMNL (Fig. 7).

Ra LPS mutant caused maximal increase in the index as compared to the control measurement. The S- and Re- forms were less effective than the Ra-form and the resulting pattern of their efficacy can be presented as Ra >S >Re.

In Fig. 8 scanning electron microscopy photos illustrate phagocytosis of OZ by untreated PMNLs (Control + OZ) and cells preincubated for 30 min with 1 µg/ml Ra LPS (Ra LPS + OZ). Scanning electron microscopy studies revealed that LPS-treated neutrophils engulfed simultaneously more particles of opsonized zymosan (OZ) than control cells (Fig.8).

We investigated how the LPS forms augment neutrophil phagocytosis in the presence of NOS inhibitor (500 μM L-NAME). L-NAME produced an increase in the effects of Re and S LPS chemotypes and furthermore attenuated the difference between effects of various LPS chemotypes (Fig.7). These data pointed out that the potency of different LPS chemotypes to activate neutrophil phagocytosis is largely due to their ability to induce NO synthesis.

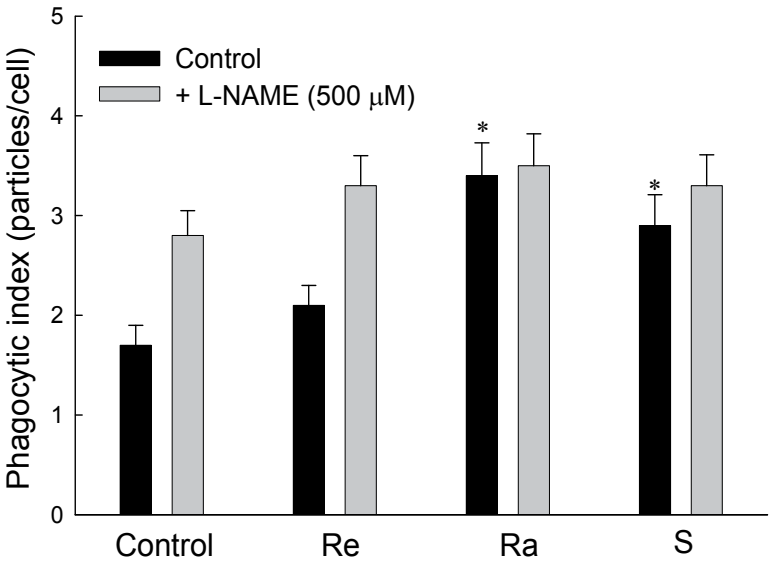


Fig. 7. Effect of LPS chemotypes on OZ uptake by PMNL. Phagocytic index was assessed by light microscopy 5 min after OZ addition to PMNLs, pretreated for 30 min at 37 °C without (control) or with 1 μg/ml of different LPS species and 500 μM L-NAME. * $P < 0.05$ vs corresponding control.

The phagocytosis of zymosan is a good experimental model to study leukotriene synthesis in PMNLs. Leukotrienes constitutes a family of inflammatory mediators, being formed in PMNL during phagocytosis of bacteria or zymosan particles. The opsonization of zymosan with normal serum resulted in enhanced activation of LT synthesis in PMNLs (Fig.9). LPS-priming of neutrophils further increased LT synthesis, with the effect increasing in the order Re < S < Ra LPS, as we published recently (Zagryazhskaya, et al., 2010). 100 μM and 500 μM L-NAME decreased the specificity of LPS chemotypes (Zagryazhskaya, et al., 2010).

In contrast, 10 μM diphenileleiodonium chloride (DPI) emphasized the effects of Re and S LPS (Fig 10). The antioxidant agent diphenileleiodonium inhibits NADPH oxidase-mediated ROS formation, and also inhibits other flavo-enzymes such as NO synthase and xanthine oxidase (Wind et al., 2010). In our experiments, inhibition of NO release attenuated the specificity of LPS chemotypes, but when we simultaneously inhibited NO and ROS formation, the chemotypes demonstrated the most prominent variation of their effects on LT synthesis (Fig.10). We suggest that LT synthesis is regulated by various LPS chemotypes via multiple mechanisms, and peroxynitrite is involved in this regulation. The specificity of LPS species is mainly dependent on nitric oxide generation induced by LPS. The published data concerning NO interaction with 5-LO admit various interpretations however, the most

recent findings support the inhibitory effect of NO on 5-LO synthetic capacity (Coffey et al., 2000). Furthermore, we revealed that minimal NO synthesis facilitated OZ uptake, adhesion, LT and O2- production, as it was observed in the cells primed with Ra LPS. As soon as we inhibited NOS with L-NAME, the other LPS forms, Re and S chemotypes, exhibited comparable capacity to stimulate OZ uptake, LT and O2- production.

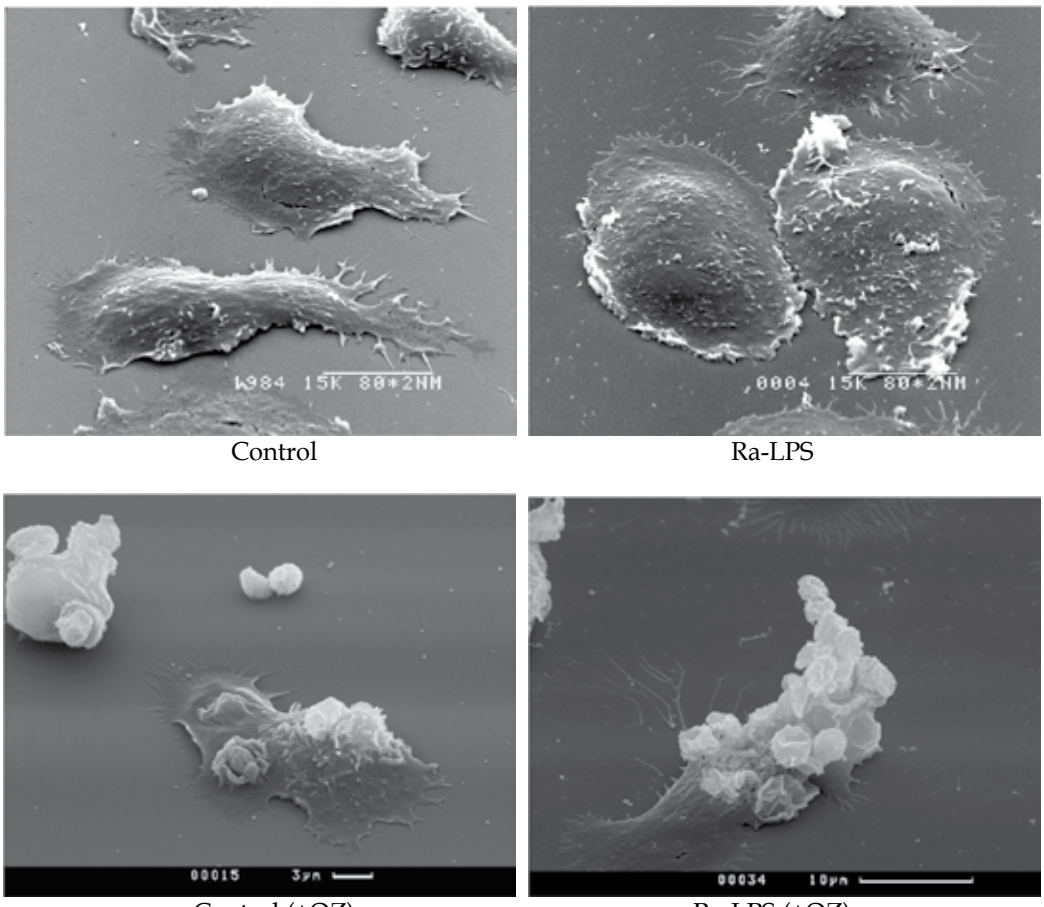


Fig. 8. Scanning electron microscopy of PMNLs untreated (control) or treated with 1 μg/ml Ra-LPS for 30 min (Ra-LPS). OZ uptake in untreated PMNLs (Control + OZ) and PMNLs exposed to Ra-LPS chemotype (Ra-LPS + OZ).

NO can inhibit 5-lipoxygenase directly (Coffey, et al., 2000) and via activation of soluble guanilate cyclase (Coffey, et al., 2008). Peroxynitrite (PN), formed by NO and superoxide, can cause inhibition of 5-LO (Coffey, et al., 2001), as well as 5-LO activation by increasing 'peroxide tone' of the cell (Goodwin et al., 1999; Ullrich & Kissner, 2006). Complex interplay between NO, superoxide and PN is obviously involved in fine regulation of LT synthesis. When we inhibited both NO and PN in incubations with DPI, we revealed huge activation of LT synthesis in Ra- and S- LPS primed cells (Fig 10).

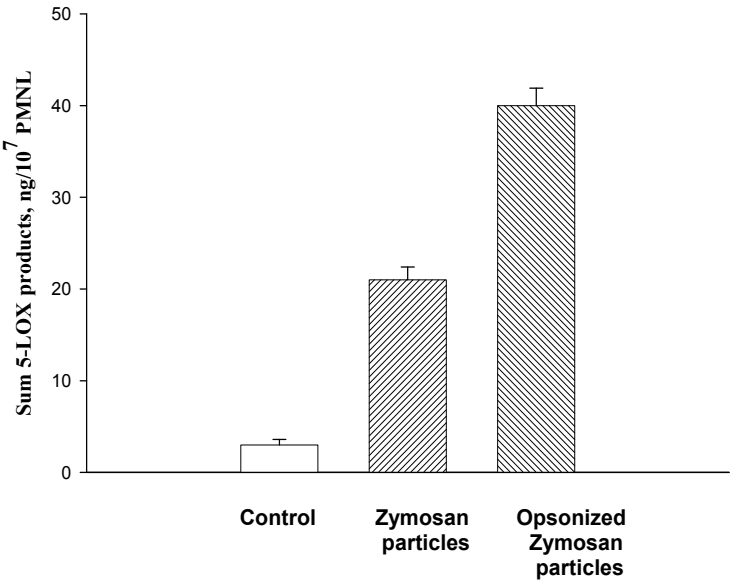


Fig. 9. Opsonized zymosan (OZ) activates LT synthesis in PMNLs. PMNLs suspension (2 × 10^7 cells) was incubated at 37 °C for 30 min, then zymosan particles and opsonized zymosan particles were added where indicated for 20 min. Products of the 5-LO pathway were determined by HPLC, as described in Materials in Methods.

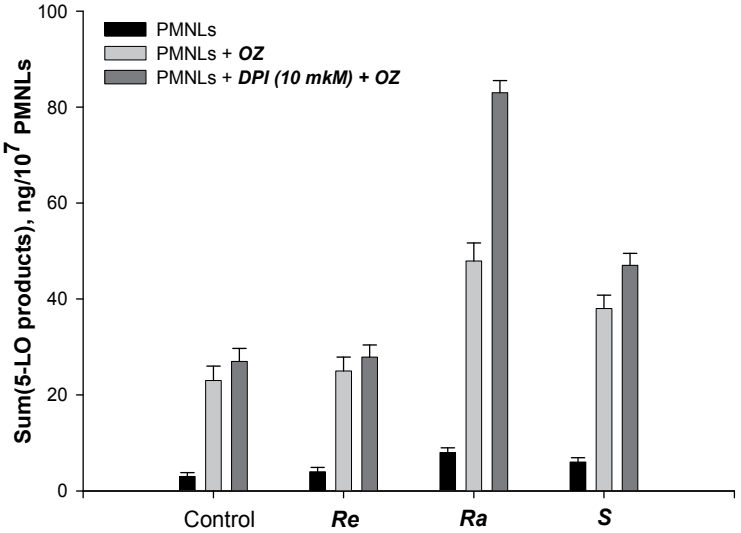


Fig. 10. 5-LO products synthesis in PMNLs is selectively regulated by LPS chemotypes. PMNLs (2 × 10^7) were treated for 30 min at 37 °C without (control) or with 5 μg/ml of various LPS forms and 10 μM diphenileleiodonium chloride (DPI), and then stimulated for 30 min with 2 mg/ml OZ.

Red blood cells are known to consume NO (Romero et al., 2006). In the vascular space, where phagocytes are relatively rare, particles that have been opsonized by complement are

immobilized to the surface of red blood cells for further clearance by phagocytes (Pilsczek et al., 2005). When we added RBC in incubations with neutrophils, we found higher effects of LPS on LT synthesis in the presence of red blood cells. This effect was observed in PMNL interaction with OZ (Fig.11) and with opsonized bacteria (Fig.12). Establishing which mechanisms of NOS and NADPH-oxidase activation and signaling are essential for phagocytosis and 5-LO activation is the next objective.

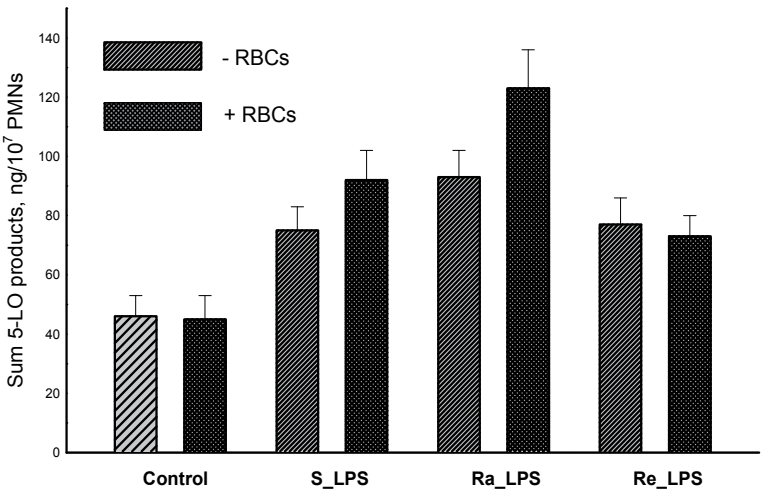


Fig. 11. 5-LO products synthesis in PMNLs is selectively regulated by LPS chemotypes in the presence of red blood cells (RBC). PMNLs (2×10^7) without or with RBC (5×10^7) were preincubated for 30 min without (control) or with 5µg/ml LPS, then stimulated for 20 min with 2mg/ml OZ.

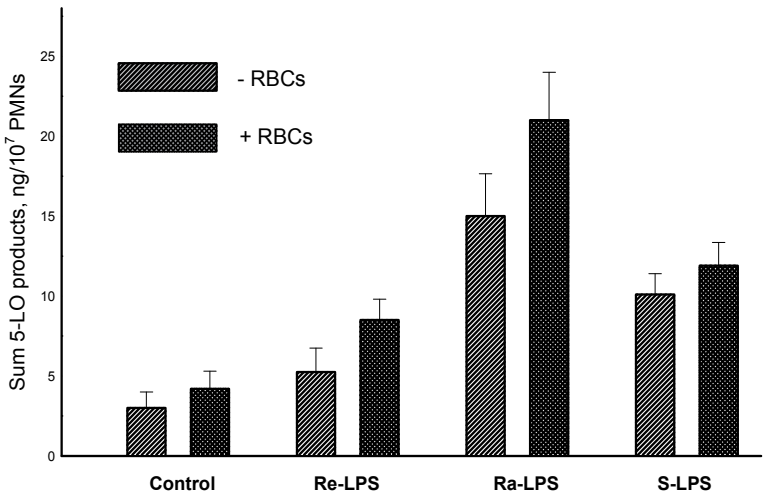


Fig. 12. 5-LO products synthesis in PMNLs is selectively regulated by LPS chemotypes in the presence of red blood cells (RBC). PMNLs (2×10^7) without or with RBC (5×10^7) were preincubated for 30 min without (control) or with 5µg/ml LPS, then stimulated for 20 min with $3x10^8$ OS (opsonized *S. Typhimurium* cells).

4. Conclusion

The regulation of neutrophil adherence, phagocytosis and leukotriene synthesis by various LPS chemotypes from S. Typhimurium has received little attention in scientific literature, which prompted us to this study. We presented data on the regulation of neutrophil cellular responses by three LPS species from *Salmonella enterica* serovar *typhimurium* with different increasing chain lengths, namely Re mutant SL 1181 (lipid A + 2 KDO residues), Ra mutant TV 119 (comprising lipid A and complete core) and S - form smooth LPS which possesses all three main components of endotoxin structure (lipid A, core and O-antigen). Our investigation supports the hypothesis that NO plays a crucial role in regulation of LPS-induced phagocytosis and leukotriene synthesis in neutrophils. High levels of endogenous NO inhibit 5-LO activity and leukotriene synthesis, and erythrocytes constitute an important 'sink' for NO and its product peroxynitrite. When excess NO is consumed by red blood cells, we found distinct and significant priming of neutrophils by LPS chemotypes. We conclude that LPS and red blood cells mediate activation of leukotriene synthesis in PMNL using NO release as intra- and intercellular regulatory mechanism. These data with LPS chemotypes contribute to the understanding of the basic factors involved in the regulation of neutrophil responses to LPS.

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18

Use of Isolation and Antibody Detection for *Salmonella* Assessment

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1. Introduction

1.1 *Salmonella* in pigs

Salmonella infections of swine are of concern for two major reasons. The first is the clinical disease (salmonellosis) in swine that may result, and the second is that swine can be infected with a broad range of *Salmonella* serovars that can be a source of contamination of pork products. The genus *Salmonella* is morphologically and biochemically homogeneous group of Gram-negative, motile, non-spore-forming, facultative anaerobic bacilli with peritrichous flagella (Griffith et al., 2006). According to their biochemical characteristics it is divided in two species *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further divided in six subspecies. Regarding their antigenic structure of somatic (O), flagellar (H) and capsular (Vi) antigens they are divided in serovars. Traditionally the serovars of *subspecies enterica*, which account for more than 99.5% of isolated *Salmonella* strains, have names, while all the others are named by their antigenic formula only (Grimont and Weill, 2007). Final differentiation within serovars is carried out by phage typing, plasmid profiling, restriction endonuclease analysis and resistance patterns. Serovars Typhimurium, Derby, Saintpaul, Infantis, Heidelberg, Typhisuis and Choleraesuis may all occur in pigs (Taylor, 2006).

The reservoir for *Salmonellae* is the intestinal tract of warm-blooded and cold-blooded animals. *Salmonellae* are hardy and ubiquitous bacteria that multiply at 7-47° C; survive freezing and desiccation well; and persist for weeks, months, or even years in suitable organic substrates. The bacteria are readily inactivated by heat and sunlight as well as by common phenolic, chlorine, and iodine disinfectants. Ability to survive in the environment, as well as prolonged carrier states in innumerable hosts ensures the widespread distribution of this genus worldwide (Griffith et al., 2006).

Pigs usually get infected through oral intake of the organism. After infection, animals can become carriers in the tonsils, the intestines and gut-associated lymphoid tissue (Wood et al., 1989; Fedorka-Cray et al., 2000). Most of the time, carriers are not excreting the bacteria

but under stressful conditions, re-shedding may occur. In this way, carriers are permanent potential source of infection for other animals and humans. Stress factors can occur during the fattening period, but also prior to slaughter, for instance during transport to the slaughterhouse or during the stay in the lairage (Seidler et al., 2001; Rostagno et al., 2010). Along the slaughter line, several steps can be critical for *Salmonella* contamination, removal of the pluck set and meat inspection procedures (De Busser et al. 2011). During these steps, the carcass can be contaminated with faeces and bacteria can be spread all over the carcass and to subsequent carcass.

After tracing the *Salmonella* data from the colon content isolated in the slaughterhouse back to the herd level, it was estimated that 40% of the herds were *Salmonella* positive at the moment of slaughter. A high level of herd contamination was also found in the Netherlands with 23% of the herds *Salmonella* positive sampled on the farm (van der Wolf et al., 1999) and in the UK with 63% positive farms (Davies et al., 1999). For interpretation of our data, it has to be kept in mind that the pigs with positive colon content and/or mesenteric lymph nodes in the slaughterhouse could have been infected on the farm and during transport or during the waiting period in the lairage before slaughtering. There are indeed indications that the contamination could already be detected in the faeces and the mesenteric lymph nodes as early as 3 h after infection (Fedorka-Cray et al., 1994). Especially the lairage and the high contamination level of the slaughterhouse environment are probably the major source for *Salmonella* infections prior to slaughter (Hurd et al., 2001; Swanenburg et al., 2001). Hurd et al. (2002) demonstrated that rapid infection during transport, and particularly during holding, is a major reason for increased *Salmonella* prevalence in swine: a sevenfold higher *Salmonella* isolation rate and twice as many different serovars were observed from pigs necropsied at the abattoir than from those necropsied on the farm.

There is currently an explosion of investigational activity related to issue of food safety, including *Salmonella* contamination of variety of foods. Salmonellosis is considered to be one of the most common food-borne illnesses in humans. There has been an increased public awareness of microbiological hazards of food and improved monitoring. Over the recent years, salmonellosis has been the second most commonly reported zoonoses in the European Union, accounting for 151,995 recorded human cases in 2007 (EFSA, 2009b) and 131,468 in 2008 (EFSA, 2010). Although *Salmonella* contamination of poultry and beef products exceeds that of pork, *Salmonella* control programs in swine will continue to be a primary focus of food safety initiatives. *Salmonella* reduction programs are becoming commonplace, with long-range goals to include the production and marketing of *Salmonella*-free pork products. Numerous dynamic programs are in place utilizing hazard analysis and critical control point (HACCP) principles (Griffith et al., 2006). Those programs, that have been in place for sufficient period of time, such as the Danish program, have significantly reduced the rate of *Salmonella* infection in pork products (Nielsen et al, 1995). Fortunately, most of the methods useful for pre-harvest *Salmonella* reduction in swine populations are related to sound management practices that also improve the overall health of swine operation.

Reduction of *Salmonella enterica subsp. enterica* (*Salmonella*) prevalence in the pig industry will be set as a target at the EU level and it is believed to significantly contribute to the protection of human health. The specific reduction target will be based upon the results of

a quantitative microbiological risk assessment on *Salmonella* in slaughter and breeder pigs as well as cost-benefit analyses, all conducted at the EU level. According to the Regulation EC-2160/20032, protection of human health from food-borne zoonotic agents is an issue of paramount importance. Farm-to-fork control programs will probably be needed to ensure a reduction of the prevalence of specified zoonoses and zoonotic agents. Moreover, Member States will have the responsibility to establish effective national control programs adjusted for the country-specific characteristics, including the disease burden and the financial implications for stakeholders. Results of the EU baseline survey on the prevalence of *Salmonella* in lymph nodes of slaughter pigs showed a wide range of prevalences in EU countries, from 0% to 29% infected pigs (EFSA, 2008). These findings suggest that country tailored surveillance-and-control strategies should be designed aiming to achieve the targets in a cost-effective way, assuring human-health protection (Baptista et al., 2010).

Bacteriological isolation methods are used to detect *Salmonella* positive pigs and to identify the *Salmonella* serovars, but because of the low sensitivity of bacteriological faecal or intestinal examination *Salmonella* positive pigs can be missed (Bager et al., 1991). Another method to screen pigs for *Salmonella* is detection of *Salmonella* serum antibodies. The *Salmonella* –LPS-ELISA (*Salmonella*-ELISA) has been developed in Denmark (Nielsen et al., 1995) and in The Netherlands (Van der Heijden et al., 1998). The setup of the *Salmonella*-ELISA is based on a mixture of lipopolysaccharides (LPS) from two *Salmonella* serovars and should theoretically detect 95% of *Salmonella* serovars (Baggesen et al., 1997). From field studies it became clear that the *Salmonella*-ELISA detects antibodies against serovars Typhimurium and Infantis more effectively than other *Salmonella* serovares (Basggsen et al., 1997). Experimental studies to investigate the feasibility of this method for other *Salmonella* serovars have not been carried out yet (Van Winsen et al., 2001).

Results from direct diagnostic methods (bacteriology) and indirect diagnostic methods (serology) cannot be compared easily. The actual shedding of *Salmonella* indicates true infection and transmission, whereas the positive serology indicates also silent transmission within the herd (Van Winsen et al., 2001). The two *Salmonella* ELISA´ s have been shown to be useful to screen herd or groups that are possibly infected with certain serovars but are of no use to judge individual animals (Nielsen et al., 1995; Van Winsen et al., 2001). The EU baseline study in fattening pigs showed that due to the diversity of tests and cut-off points, used by the 9 Member States (MSs) that chose to collect meat juice samples, no group level prevalence can be estimated. The sensitivity and specificity of these tests is not precisely known and in most MSs, some inconclusive results were reported. The sero-prevalence amongst these 9 MSs was estimated to have been from as low as 2.2% (lower boundary of 95% CI, classifying inconclusive results as negative) in Sweden to as high as 41.6% (upper boundary of 95% CI, classifying inconclusive results as positive) in Cyprus (EFSA, 2008). Community reference laboratory for *Salmonella* received from this study 60 meat juice samples per participating Member State and additionally tested them to evaluate possible comparison of results between member States. Four different ELISA kits were used by Member States and considerable discrepancies between Member States' results and the results of Community Reference Laboratory were found (Berk, 2008).

Danish *Salmonella* scheme categorised pig farms in four levels from 0 to 3. Once a month, all herds were assigned to official *Salmonella* level (1, 2 or 3) according to the results from the

preceding 3 months. Level 1 included herds with low acceptable prevalence of *Salmonella*, Level 2 included herds with a moderate still acceptable prevalence of *Salmonella*, and Level 3 included herds with a high unacceptable prevalence (Alban et al., 2002). Farm category must be a result of several consequential serological testing (two or three) in different period (monthly or four times per year) which is for determination of "serological salmonella index" in monitoring schemes in EU members differently regulated. Number of samples from each farm is also important for estimation of seroprevalence for *Salmonellae.* In Danish *Salmonella* control program the sampling has been simplified into 60, 75 or 100 samples per herd per year depending on herd size after revision of their program in 2001. Also cut off for tested samples has been reduced from OD 40 % to OD 20 % which increases the number of seropositive samples approximately two times. Level 1 herds have an index of <40, Level 2 herds have an index between 40 and 70, and Level 3 herds have an index >70. A Level 0 category is currently being evaluated for herds in which the seroprevalence is 0 for 3 consecutive months. Three months results of the prevalence were weighed 0.2: 0.2: 0.6 where the immediate month is counting three times as much as the previous months. Producers are interested to be introduced in level 0 where herd is seronegative for *Salmonellae* in certain period (Alban et al. 2002; Benchop et al., 2008). Beginning in 2002, Germany initiated a voluntary *Salmonella* control program similar to the Danish one, and the United Kingdom introduced the Zoonoses Action Plan (ZAP) *Salmonella* monitoring program, also based on meat juice ELISA. The Netherlands and Belgium are considering similar programs (Nielsen, 2002). Presently, there is no national *Salmonella* monitoring program for pig producers in the United States or Canada. Sera collected as part of the National Animal Health Monitoring System (NAHMS) Swine 2000 Study being evaluated with the DME conducted at Iowa State University, Ames, Iowa (Turney, 2003). The Norwegian *Salmonella* surveillance and control programme (NSSCP) was launched in 1995 and has been approved by the EU (EFTA Surveillance Authority Decision No. 68/95/COL of 19 June 1995) as the background for accepting testing meat, meat products or live animals for *Salmonella* before it is allowed to enter Norway from EU member countries. The program covers activities directed towards both live animals (cattle, pig and poultry) and meat (cattle, pig, sheep and poultry) and is designed similarly to the Swedish and Finnish *Salmonella* control programmes (Hopp et al., 1999). The program includes systematic sampling in the breeding herds (BH) and random sampling of carcasses at the abattoirs in order to identify infected carcasses originating from BH, IH (integrated herds) and FH (finishing herds). The sample sizes have been calculated so that a prevalence of 5% in any breeding herd and 0.1% in the total population can be detected, assuming a diagnostic test sensitivity of 100% (Sandberg et al., 2002).

The control program was based on the assumption that there was an association between serological reaction and bacteriological *Salmonella* prevalence. This association has been described (Nielsen et al., 1995; Stege et al., 1997; Christensen et al., 1999; Sørsen et al., 2000). The general conclusion of these studies was that the serological test was effective mainly at herd-level and especially well suited to detect high prevalence herds. A central question is how to describe the association between serology and bacteriology, because the serological results from a herd may be interpreted differently (Alban et al. 2002).

In 2008 there were 43,124 breeding pigs and 432,011 fattening pigs in Slovenia, reared on 34,725 holdings. Pig production in 2010, which includes only pigs, slaughtered in slaughterhouses in Slovenia, was 241,332 for year 2010. Number of breeding pigs was 30,345

which were on 4,373 farms. From these farms there were 3,296 farms with five or less than five breeding sows. All these farms are one-site farms, which means, that all categories of pigs from breeding pigs till fatteners are located on one site. All pigs were raised indoor (Statistical office of the Republic of Slovenia, 2011).

Seroprevalence of *Salmonella* in Slovenia is low. Comparison of the seroprevalence between large and small farms shows that the number of positive breeding swine and fatteners are higher at the large farms than in small farms. The seroprevalence of fatteners from small farms was 0.1 and of breeding sows was 0.3. The seroprevalences of pigs from large farms were higher; the seroprevalence of fatteners was 0.3 and of breeding sows was 0.68 (Stukelj et al., 2004). In our Serology laboratory we tested annually 270 to 375 serum samples. Our tested farm could be classified into the level 1 according to revised Danish surveillance-and-control program for *Salmonella*. In our preliminary study we randomly selected 100 samples out of 375 tested in 2007 which would be the number of tested samples for that herd size according to Danish program. Seroprevalence to *Salmonellae* for year 2007 for mentioned farm was for all tested samples 12.8% for OD 40% and 24% for OD 20%. For randomly selected samples for the same year the prevalence was 7.5 % for OD 40% and 17% for OD 20%. We also compared results after testing with classification with weighted three months seroprevalence. Prevalence from all tested sera in the first three months in 2007 was 8% for OD 40% and 14% for OD 20%. In randomly selected samples for the same months prevalence was 7.5 % for OD 40% and 10% for OD 20%. Results from testing of all the samples and results for randomly selected samples show only differences in percentages but the classification level of the farm remains the same (Stukelj et al., 2009).

1.2 EU baseline studies of the prevalence of *Salmonella* in pigs

1.2.1 EU baseline study on the prevalence of *Salmonella* in slaughter pigs

To obtain an overview of the *Salmonella* prevalence in pigs in EU Member States (MSs) two baseline studies on the prevalence of *Salmonella* in slaughter and breeding pigs were conducted. The baseline study in slaughter pigs started on the 1st October 2006 and lasted till the 30th September 2007. Tested slaughter pigs were selected in slaughterhouses that together accounted for 80% of pigs slaughtered within each Member State (MS), which constituted the survey target population. Twenty-five EU MSs participated in the survey. Norway participated on a voluntary basis.

Slaughtered pigs with a live weight between 50 kg and 170 kg and their carcasses were randomly sampled in slaughterhouses representing at least 80% of MSs' total production of slaughtered pigs. The samples to take were stratified by the slaughterhouses' capacity (throughput) in the year 2005 and by the month. The day on which the samples were taken was also randomly chosen from all days of the month of sampling as was the slaughtered pig or its carcass from all scheduled pigs to slaughter on the selected slaughter day. From a selected slaughter pig at least 5 ileo-caecal lymph nodes weighing at least 15 grams were collected on a mandatory basis. The number of pigs to sample was 384 minimum and 2,400 maximum and was calculated for each MS. In addition, in order to assess the contamination of slaughter pig carcasses, 13 MSs (Austria, Belgium, Cyprus, Czech Republic, Denmark, France, Ireland, Latvia, Lithuania, Poland, Slovenia, Sweden and The United Kingdom) voluntarily sampled each at least 384 carcasses belonging to

the slaughtered pigs of which lymph nodes were taken. This additional sampling was done by swabbing the surface of the carcass in a standardized way, after evisceration and before chilling. Moreover, 9 MSs (Cyprus, Denmark, France, Ireland, Lithuania, Slovenia, Sweden, The Netherlands and The United Kingdom) voluntarily collected a muscle sample (to extract meat juice) or a blood sample from all pigs selected for lymph node sampling for antibody detection examination. Samples were taken by the competent authority in each MS or under its supervision.

The EU live pig population totalled 160 million heads in 2005. The largest population was in Germany, 17% of the EU live pig population. Seven MSs (Germany, Spain, Poland, France, Denmark, The Netherlands and Italy) accounted for 74% of the total EU population. Conversely, several MSs had very small live pig populations. The EU slaughtered pig population totalled 240 million heads in 2005. The largest population was in Germany, 20% of the EU slaughtered pig population. Eight aforementioned MSs plus Belgium, accounted for 81% of the total EU slaughtered pig population. Conversely, several MSs had very small slaughtered pig populations.

The cleaned validated dataset comprised data on 19,159 slaughter pigs. On the sample-level the dataset contained 18,663 samples of lymph nodes, 5,736 carcass swabs and 5,972 serological samples originating from 25, 13 and 9 MSs, respectively. The dataset also included data on 408 lymph node samples from Norway. For slaughter pigs and of lymph node samples some invalid lymph node test results were excluded. A total of 934 slaughterhouses in the EU and nine in Norway were sampled, varying from three in Cyprus and Luxembourg to up to 400 in Poland (EFSA, 2008).

Observed prevalence of slaughter pigs infected with *Salmonella* spp. in lymph nodes

It is important to note that the absence of any *Salmonella* from the tested samples does not imply that a MS is *Salmonella* - free, as firstly the detection method has a sensitivity of less than 100%, so false negative results are plausible. Secondly, the prevalence within the MS may be too low for even one positive animal to be detected with the sample size that was used. *Salmonella* spp. was found in 24 out of the 25 MSs providing data on lymph node samples of slaughter pigs. No lymph node tested positive in Finland, whereas one pig tested positive in Norway. The observed EU-level prevalence was 10.3% (95% CI: 9.2; 11.5). The unweighted prevalence (10.8%) was included in the CI 95%. Within MSs, the prevalence varied between 0.0% and 29.0%. Serovar Typhimurium was isolated in all the 24 MSs reporting positive results for *Salmonella* in lymph nodes. One pig tested positive in Norway. The observed EU-level prevalence was 4.7% (95% CI: 4.1; 5.3). The unweighted prevalence (4.2%) was included in the CI 95% CI. At the MS-level, the observed prevalence was highest in Luxembourg (16.1%). Serovar Derby was isolated in 20 MSs. No lymph node tested positive for Derby in Cyprus, Estonia, Finland, Lithuania, Sweden and in Norway. The observed EU-level prevalence was 2.1% (95% CI: 1.8; 2.6). The unweighted prevalence (1.8%) was included in the CI 95% CI. At the MS-level, the observed prevalence was highest in France (6.5%). Serovars of *Salmonella* other than Typhimurium and Derby were found in lymph nodes of slaughter pigs from 24 MSs. The observed EU-level prevalence was 5.0% (95% CI: 4.4; 5.7). The unweighted prevalence (5.6%) was included in the CI 95%. At the MS-level, the observed prevalence was highest in Greece (17.2%).

The EU prevalence of 10.3% can be interpreted as showing that one in ten pigs slaughtered in the EU was infected with *Salmonella* when slaughtered. This infection may have arisen on the farm of origin or at any time during transport to slaughter or lairage. About half of the MSs had a *Salmonella* prevalence in lymph nodes above the EU average, while the other half had prevalence below the EU mean. This was also the case for serovar Typhimurium, but less true for Derby and for serovars other than these latter two, for which fewer MSs had figures above the EU mean. It is noteworthy that although there was a large variation in the slaughter pig *Salmonella* prevalence, the serovar distribution was not remarkably varying between the MSs, because two specific *Salmonella* serovars, Typhimurium and Derby, accounted for a major part of the positive findings at the EU-level and for most *Salmonella*-positive MSs. All 24 *Salmonella*-positive MSs isolated *Salmonella* Typhimurium and 20 detected *Salmonella* Derby. These two serovars are common serovars found in *Salmonella* infection cases in humans, and are both amongst the ten most frequently reported serovars in humans (EFSA, 2008).

Observed prevalence of carcasses contaminated with *Salmonella* spp.

Salmonella spp. was found in 11 out of the 13 MSs providing data on surface swabs-sampling of carcasses. No carcass swabs tested positive in Slovenia and Sweden. The observed 13 MS-group level prevalence was 8.3% (95% CI: 6.3; 11.0). At the MS-level, the observed prevalence was highest in Ireland (20.0%). For this 13 MS-group the observed prevalence of slaughter pigs infected with *Salmonella* spp. in lymph nodes was estimated as 9.6% (95% CI: 8.2%; 11.1%). Thus, one in 12 pig carcasses produced in this group of 13 MSs was contaminated with *Salmonella*. This estimation cannot as such be extrapolated to the level of the EU, because this group of MSs may not be representative for all MSs. One group of participating MSs had a prevalence above the weighted average (Belgium, France, Ireland and the United Kingdom), and the other one below the average (Austria, Cyprus, Czech Republic, Denmark, Latvia, Lithuania, Poland). This was the case for *Salmonella* spp., for serovar Typhimurium, and to a lesser extend for Derby. It was not the case for serovars other than the two latter ones.

Serovar Typhimurium was isolated in 10 MSs reporting positive results for *Salmonella* in carcass swabs. No carcass swabs tested positive in Latvia, Slovenia and Sweden. The observed 13 group-level prevalence was 3.9% (95% CI: 2.8; 5.5). At the MS-level, the observed prevalence was highest in Ireland (11.7%). Serovar Derby was isolated in 10 MSs. No carcass swabs tested positive in Cyprus, Slovenia and Sweden. The observed 13 MSs group-level prevalence was 2.6% (95% CI: 1.7; 3.9). At the MS level, the observed prevalence was highest in France (5.9%). Serovars of *Salmonella* other than Typhimurium and Derby were found on carcass swabs from 11 MSs. No carcass swabs tested positive in Slovenia and Sweden. The observed 13 group level prevalence was 2.3% (95% CI: 1.6; 2.5). At the MS-level, the observed prevalence was highest in France (4.8%).

It is again noteworthy that although there was a large variation in the prevalence of *Salmonella* contaminated carcasses, the serovar distribution was not remarkably varying between these MSs, because two specific *Salmonella* serovars, Typhimurium and *D*erby, accounted for a major part of the positive findings at the EU-level and for most *Salmonella*-positive MSs. The contamination of the carcasses occurred in the slaughterhouse and may have been due to infection within the pigs or from the slaughterhouse environment. For this 13-MS group the carcass swab *Salmonella* spp. prevalence appears to be similar to the

lymph node prevalence. At the MS-level, the prevalence of contaminated carcass swabs tended to be similar or lower than the prevalence of slaughter pigs infected with *Salmonella* spp. in lymph nodes in 11 of the 13 MSs. Conversely, in two MSs (Belgium and Ireland) the prevalence of contaminated carcass swabs seemed higher than the prevalence of infected lymph nodes. However, sample size calculations have not been predicated for such comparison.

In this survey the carcass swab represents the closest sampled point to the exposure of the consumer, at the beginning of the food chain. Thus, since the imperative for control of *Salmonella* in pigs is the protection of public health, there is an argument that the carcass swab is the most appropriate measure of those utilised in this survey. Further, individual MSs might choose whether intervention at the farm, the slaughterhouse or some combined strategy afforded the best option for their particular circumstances (EFSA, 2008).

Observed prevalence of slaughter pigs with antibodies against *Salmonella*

Amongst the 9 participating MSs, two used the Salmotype Pig Screen® ELISA by Labor Diagnostik Leipzig, three MSs used the HerdCheck Swine *Salmonella*® ELISA by IDEXX, two MSs used an in house ELISA, one MS used the VetSign Porcine *Salmonella*® ELISA by Guildhay, and one MS used both the Salmotype Pig Screen® ELISA and the HerdCheck Swine *Salmonella*® ELISA. The NRLs used the cut-off of their choice. Eight MSs reported their results as relative optical densities (OD%) and one MS reported his results in S/P ratio (sample value related to positive control value). It was difficult to estimate the real seroprevalnece because of some inconclusive results, which could be counted as positive, negative or missing.

Seroprevalence (presence of *Salmonella* antibodies in meat juice or in sera) is a measure of the prior exposure of the pig to *Salmonella* infection. Due to the diversity of tests and cut-off points employed by the 9 MSs that chose to collect these samples, no group level prevalence can be estimated. The sensitivity and specificity of these tests is not precisely known and in most MSs, some inconclusive results were reported. The seroprevalence amongst these 9 MSs was estimated to have been as low as 2.2% (lower boundary of 95% CI, classifying inconclusive results as negative) in Sweden to as high as 41.6% (upper boundary of 95% CI, classifying inconclusive results as positive) in Cyprus.

The future value of testing of serological samples probably lies in their application within a MS for surveillance purposes and identification of positive herds, since these tests are relatively cheap, sample collection is straightforward and can be done by a slaughterhouse technician and in the case of meat samples, can be frozen for transport and batch testing. However, it should be recalled that these samples are poor predictors of the *Salmonella* status of the individual pig or carcass. This was further underpinned by the survey concordance-discordance results, at the MS-level, between the test for *Salmonella* spp. using lymph nodes and meat juice and sera samples. These analyses results revealed no to low agreement (EFSA, 2008).

Frequency distribution of *Salmonella* serovars in lymph nodes and carcass swabs

The serotyping of *Salmonella* isolates was mandatory according to the technical specifications of the survey. At least one isolate from each positive sample was to be typed

according to the Kaufmann-White Scheme. Results from any sample where the serovar information was not available for any isolate were excluded from the final dataset. In total there were 2,600 *Salmonella*-positive lymph node samples. Two different *Salmonella* serovars were isolated from three *Salmonella*-positive lymph nodes. Eighty-seven different serovars were isolated from the lymph nodes of slaughter pigs across the EU. Serovars Typhimurium and Derby were highly predominant. Serovar Typhimurium was the most frequently reported serovar from the slaughter pigs' lymph nodes in EU and Norway, isolated in 40.0% of the *Salmonella* positive slaughter pigs, and reported by all (24) MSs having found *Salmonella* positive slaughter pigs and by Norway. The next common reported serovar was Derby, isolated from 14.6% of the positive slaughter pigs. Serovar Derby was also the second serovar most commonly isolated in terms of number of reporting MSs (20). Serovars Rissen and monophasic 4,[5],12:i:- were the third and the fourth most frequently recovered serovars, with an isolation rate in lymph nodes of 5.8% and 4.9%, respectively. Serovar Rissen was isolated in five MSs and *S.* 4,[5],12:i:- in eight MSs. Serovar Enteritidis was the fifth most common reported serovar and recovered in 19 MSs, in particular in Cyprus, Estonia, Poland and Slovenia where it was the most frequently isolated serovar in lymph nodes.

There were a total of 387 carcasses testing positive for *Salmonella* by surface swab-sampling in the 13 MSs. Thirty different serovars were isolated on the surface of the slaughter pig carcasses. Serovar Typhimurium was the most frequently recovered serovar from the surface of the slaughter pig carcasses in EU, representing 49.4% of the *Salmonella* positive carcasses. The second most frequent serovar was Derby (24.3% of the positive carcasses). The three next most frequent serovars were Infantis, Bredeney, and Brandenburg (3.4%, 2.1% and 1.8% of the positive carcasses, respectively). Serovar Typhimurium was the dominant serovar in 10 MSs. In Austria and in Poland, serovar Derby was isolated as frequently as Typhimurium.

A greater diversity of *Salmonella* serovars were isolated from lymph nodes than from carcass swabs, although there were five serovars that were only isolated from carcass swabs. Firstly, carcass swabs were collected from fewer MSs and secondly, the overall prevalence of *Salmonella* positive swabs was lower than that of lymph node samples within those MSs that tested both. The number of bacteria that may be collected from a carcass is also likely to be lower than the number found in the lymph node of an infected pig except in case of extreme contamination. Finally, the presence of *Salmonella* on a carcass swab may reflect post-slaughter contamination with serovars that exist in the slaughterhouse environment as well as infection originating from within the slaughtered pigs.

Serovar Typhimurium was isolated in all of the 24 MSs that found *Salmonella* in lymph node samples and in Norway. It was the most frequently isolated serovar in all MSs except Bulgaria (Derby), Cyprus (Enteritidis), Estonia (Enteritidis), Italy (Derby), Latvia (Brandenburg), Poland (Enteritidis), Slovenia (Enteritidis) and Slovakia (Derby). In six of these 8 MSs, serovar Typhimurium was the second most common serovar to be isolated whilst in Bulgaria, serovar Infantis was the second most prevalent serovar and in Latvia, where Derby came second. Serovar Typhimurium has long been recognised in many European countries as a common serovar amongst pigs although it has a wide host range and has also been isolated from domesticated mammals and poultry species. Overall, *S.* Typhimurium accounted for 40% of the serovars isolated in the survey.

In 18 of 24 MSs that isolated *Salmonella* from lymph nodes, serovar Derby was amongst the top three serovars to be isolated. In Spain and Portugal, serovar Derby was ranked fourth whilst it was not detected in Cyprus, Estonia, Lithuania or Sweden. It is widely recognised as a common serovar in pigs although it does occur in other livestock species. It accounted for 14.6% of the S*almonella* isolated in this survey.

A wide range of other serovars were also detected, many in very low numbers. Serovar Enteritidis, which is usually associated with poultry, was found in 19 MSs and from 4.9% of all lymph node samples. It was as noted above, the most common isolate in Cyprus, Estonia, Poland, and Slovenia and the second most frequent isolate from Austria, Czech Republic, and Hungary. Serovar Enteritidis is the most frequent cause of human salmonellosis in the EU.

It can further be mentioned that *S.* Typhimurium and *S.* Derby were the most frequent serovars both in lymph nodes and on the surface of carcasses, suggesting that the serovars that exist in the slaughterhouse environment come mainly from the infected pigs that are slaughtered there. Overall, this survey demonstrates a wide variation in the distribution of *Salmonella* serovars in slaughter pigs and the presence of two dominant serovar in this species (EFSA, 2008).

Interpretation of the results from each of the three used survey tests

Salmonella infection results from ingestion or occasionally inhalation of viable bacteria. In pigs, infection within the intestinal tract may be followed by invasion of the cells of the gut and thence, infection is established in the intestinal lymph nodes. It is possible for pigs to ingest material containing *Salmonella* and for this to be in passive transit through the gut without actively establishing infection. Infected pigs may become carriers and excrete *Salmonella* in their faeces intermittently. Therefore, the presence of *Salmonella* within the lymph node is incontrovertible evidence that a pig is infected, as it is very unlikely that *Salmonella* can be isolated from lymph nodes of uninfected pigs and false positive results are rare. However, the test sensitivity is not 100% and there may therefore be false negative results. *Salmonella* excretion by carrier pigs is thought to be provoked by stress and may occur as the pigs are loaded and transported to the slaughterhouse. It is possible for pigs to become infected and for that infection to be transferred to the intestinal lymph nodes in a matter of hours. Therefore, a positive lymph node result may reflect infection on the farm of origin or during transport or lairage. The longer the duration of the transport and lairage phases, the more contaminated the environment during those phases, and the more stressful the conditions that are experienced, the greater the risk of infection occurring after departure from the farm.

Presence of *Salmonella* on carcass swabs reflects the surface contamination of the carcass. Although this may occur during transport or in the lairage, normal slaughterhouse practices including passing pigs through a scald tank and singeing to remove bristles act to reduce *Salmonella* contamination. Presence of *Salmonella* infection in the pig need not result in carcass contamination unless e.g. there is faecal leakage from the anus or the gut is accidentally nicked during processing. *Salmonella* may also survive in slaughterhouse environments, especially in equipment that is difficult to clean thoroughly. Poor hygiene in a slaughterhouse or amongst staff may also result in contamination of carcasses and one

contaminated carcass may touch others, resulting in cross-contamination. Thus, the prevalence of positive carcass swabs is a product of the risk of infection within a pig, the risk that the infection is released to the exterior and the risk of cross-contamination from other carcasses or the slaughterhouse environment. It is predictable that presence of *Salmonella* in the gut is not completely associated with carcass contamination. It is also important to consider that the presence of *Salmonella* infection in the intestinal lymph nodes, which are removed from the carcass and are not consumed, may only represent a limited public health threat whilst a contaminated carcass is likely to be a greater risk to public health as the carcass is the start of the food chain.

Salmonella infection stimulates an immune response and circulating antibodies can be detected in blood, serum or meat juice. As antibodies persist beyond the time of infection, unsurprisingly a positive serological result is a poor indicator of current infection. Infection during transport to a slaughterhouse or in lairage does not result in a seropositive reaction, as there is insufficient time for a detectable immune response to occur before death. However, the prevalence of seropositive pigs does give a good estimate of the lifetime exposure to *Salmonella*. Therefore, it may be a valuable tool for surveillance of *Salmonella* infection on farms as part of a control programme (EFSA, 2008).

Conclusions

The main conclusions made by reporting team were:

- The survey provides valuable data for risk managers on the prevalence and distribution of *Salmonella* in EU MSs, and results are suitable to be used for setting targets for the reduction of the frequency of the *Salmonella* infection in slaughter pigs in the EU.
- Three tests were used in the survey: bacteriological tests of lymph nodes and of carcass swabs and a test for antibodies. *Salmonella* prevalence in lymph nodes reflects the infection of the pigs at the level of the primary production (i.e. on the farm and during subsequent transport and lairage). *Salmonella* contamination of the carcass may derive from the infection within the pig or from the slaughterhouse environment, whereas the presence of antibodies reflects past exposure of the pigs to *Salmonella*.
- The observed prevalence of slaughter pigs infected with *Salmonella* spp. varied widely amongst MSs.
- A large variety of serovars of *Salmonella* were isolated from ileo-caecal lymph nodes of slaughter pigs in the EU.
- A more limited range of serovars was identified on the surface of carcasses.
- With regard to seroprevalence, the observed estimates in slaughter pigs varied among the 9 participating MSs. However, these seroprevalence estimates are not directly comparable because of different tests and different thresholds used within participating MSs. No prevalence was therefore estimated at the MS-group level. Credible estimate of prevalence amongst these MSs varied from as low as 2% to as high as 42% (EFSA, 2008).

1.2.2 EU baseline study on the prevalence of *Salmonella* in holdings with breeding pigs

European Union Baseline survey on the prevalence of *Salmonella* in holdings with breeding pigs was carried out at farm level to determine the prevalence of *Salmonella* in pig breeding

holdings. The herds were randomly selected from holdings constituting at least 80% of the breeding pig population in a Member State.

Sampling took place between January 2008 and December 2008. A total of 1,609 holdings housing and selling mainly breeding pigs (sows or boars of at least six months of age kept for breeding purposes) (breeding holdings) and 3,508 holdings housing breeding pigs and selling mainly pigs for fattening or slaughter (production holdings) from 24 European Union Member States, plus Norway and Switzerland were included in the survey. In each selected breeding and production holding, fresh voided pooled faecal samples were collected from 10 randomly chosen pens, yards or groups of breeding pigs over six months of age, representing the different stages of production of the breeding herd (maiden gilts, pregnant pigs, farrowing and lactating pigs, pigs in the service area, or mixed). The pooled samples from each holding were tested for the presence of *Salmonella* and the isolates were serotyped.

The overall European Union prevalence of *Salmonella*-positive holdings with breeding pigs was 31.8% and all but one participating Member State detected *Salmonella* in at least one holding. Twenty of the 24 Member States isolated *Salmonella* in breeding holdings and at European Union level 28.7% of the holdings were estimated to be positive for *Salmonella*. This prevalence varied from 0% to 64.0% among the Member States. The estimated European Union prevalence of breeding holdings positive to serovar Typhimurium and to serovar Derby was 7.8% and 8.9%, respectively. Twenty-one of the 24 Member States isolated *Salmonella* in production holdings and at the European Union level 33.3% of the production holdings were estimated to be positive for *Salmonella*. This prevalence varied from 0% to 55.7% among the Member States. The estimated European Union prevalence of production holdings positive for serovars Typhimurium and Derby was 6.6% and 9.0%, respectively. For the two non-Member States, Switzerland detected *Salmonella* in both breeding and production holdings while Norway did not detect any *Salmonella* in its surveyed holdings.

The number of different *Salmonella* serovars isolated in breeding holdings and production holdings across the European Union was 54 and 88, respectively. Serovar Derby was the most frequently isolated serovar in both breeding and production holdings, detected in 29.6% and 28.5% of the *Salmonella*-positive holdings, respectively. The next most commonly isolated serovar was serovar Typhimurium accounting for 25.4% and 20.1% of *Salmonella*-positive breeding holdings and production holdings, respectively. These serovars were also commonly found in the EU-wide baseline survey of fattening pigs at slaughter in 2006-2007. The next most frequently reported serovars were serovars London, Infantis and Rissen both in breeding and production holdings and each accounted for approximately 7% of the positive holdings, in each type of holding. Also *Salmonella* isolates with the incomplete antigenic formula 4,[5],12:i:-, which are likely to be related to the recent emergence of monophasic serovar Typhimurium, were reported by several Member States.

Salmonella infection in breeding pigs may be transmitted to slaughter pigs through trade and movement of live animals and contamination of holding, transport, lairage and slaughter facilities. This may lead to *Salmonella*-contamination of pig meat and consequently to human disease. Further studies in surveillance and control methods for *Salmonella* in breeding pigs

as well as in the public health importance of consumption of meat from culled breeding pigs are recommended. Also investigations on the epidemiology of monophasic serovar Typhimurium would be welcome. The results of this survey provide valuable information for the assessment of the impact of *Salmonella* transmission originating from holdings with breeding pigs as a source of *Salmonella* in the food chain. These baseline prevalence figures may be used for the setting of targets for the reduction of *Salmonella* in breeding pigs, to follow trends and to evaluate the impact of control programmes (EFSA, 2009a).

1.3 Objectives of our investigation

The objectives of our investigations were to obtain an overview on *Salmonella* prevalence in pigs in Slovenia, which was part of EU Baseline study on the prevalence of *Salmonella* in slaughter pigs in 2008. Within this study Slovenia was one of the 9 countries that voluntarily included also detection of antibodies against *Salmonella* in meat juice. To assess the suitability of antibody detection for *Salmonella* we had previously monitored one of our big holdings already in 2007 (Stukelj et al., 2009).

2. Baseline study on the prevalence of *Salmonella* in slaughter pigs in Slovenia

2.1 Materials and methods

2.1.1 Pigs and holdings

In the EU baseline study on the prevalence of *Salmonella* in slaughter pigs 440 pigs from 178 holdings in Slovenia were tested. Almost a half of the pigs (212 or 48% of all tested) originated from small holdings (163 or 92% of all tested), which were represented in this study by only one to three pigs. For *Salmonella* isolation intestinal lymph nodes (minimum 15 grams) and carcass surface swabs were collected at slaughter and a piece of either diaphragm or neck muscles were collected for detection of antibodies in meat juice. Samples were sampled by official veterinarians and proceeded to Veterinary Faculty, National Reference Laboratory for *Salmonella*.

2.1.2 Detection of *Salmonella*

Isolation and identification were performed according to ISO/FDIS 6579, Annex D: 2007. We used Buffered peptone water (Biolife) for pre-enrichment, enrichment on Modified semisolid Rappaport-Vasilliadis agar (MSRV, Biocar) and plating on Xylose-lisine-desoxicholat agar (XLD, Biolife) and Rambach agar (Merck). *Salmonella* suspicious colonies were identified biochemically either by API 20 E (Biomérieux) or Crystal Enteric/nonfermenter ID kit (BBL). Serovars were identified by slide agglutination with STATENS SERUM INSTITUT *Salmonella* antisera according to White-Kauffman-Le Minor scheme (Grimont and Weill, 2007).

2.1.3 Antibody detection

The diaphragm samples were stored in plastic bags in freezer at -18° C. Before testing with ELISA, bags were taken from the freezer and the diaphragm samples were thawed, the

angles of the plastic bags were cut and the meat juices from each bag were poured over to the micro tubes. The samples were prepared for further testing.

The Swine Salmonella Antibody IDEXX ELISA allows rapid screening for the presence of antibodies to three *Salmonella enterica* serogroups indicating swine herds' exposure to the bacteria. The assay is designed to detect antibodies to *Salmonella* in swine serum, plasma and meat juice. LPS antigen (serogroups O:4 (B), O:7 (C1) and O:9 (D1)) is coated on 96-well plates.

The presence or absence of antibody to *Salmonella* in the sample was determined by relating the absorbance value at 650 nm of the unknown to the positive control mean by calculating the sample to positive (S/P) ratio. In many countries and/or laboratories the results are calculated in OD% referring to a set of standard sera, defined according to the Danish Mix-ELISA system. To obtain a result comparable to this OD% scale, a correlation factor has been experimentally determined. The S/P value was divided by this factor to give an approximate OD% value. Samples with OD% equal or grater than 40% (S/P = 1. 0) were considered positive in general screening, and samples with OD% equal or grater than 20% (S/P = 0. 5) were considered positive in more stringent screening.

2.2 Results

2.2.1 Prevalence of *Salmonella* in pigs and holdings

All the carcass swabs tested negative for *Salmonella*. From lymph nodes of 28 pigs (6.36%) from 18 holdings (10.11%) we isolated *Salmonella enterica* subsp. *enterica*, belonging to 13 serovars, including four of five serovars of public health importance: Enteritidis (7 pigs from 6 holdings), Typhimurium (3 pigs from 3 holdings), Virchow (2 pigs from 2 holdings) and Infantis (1 pig from 1 holding). All the serovars belonged to sero-groups O:4 (B), O:7 (C1) and O:9 (D1), which are covered by IDEXX ELISA, used for antibody detection. From some bigger holdings, represented by 11 to 65 pigs, we isolated two to four different serovars.

For antibody detection we used two criteria: OD 40% and OD 20%. In IDEXX ELISA at OD 20% 91 (20.68%) pigs from 45 (25.28%) holdings tested positive. At OD 40% 48 (10.91%) pigs from 25 (14.04%) holdings, tested positive. This means that 52.75% of pigs positive at OD 20% from 55.56% serologically positive holdings reacted with high antibody titres.

Of 178 holdings in 102 (57.30%) holdings, represented by 165 (37.50%) pigs, all pigs tested negative in both tests. Another 165 pigs (37.50%) originating from 16 positive holdings (either by culture or ELISA) tested negative in both tests. All together 110 (25.00%) pigs from 50 (28.09%) holdings tested positive either by culture or ELISA and 9 (8.18%) of these 110 pigs from 6 holdings (12.00%) tested positive in both tests. Of 18 holdings positive by culture, 13 (72.22% of positives) were represented by 122 pigs (27.73% of all pigs). Of these 122 pigs 67 (54.92%) tested positive either by culture or ELISA or both and 55 (45.08%) tested negative both by culture and ELISA. Of 5 (27.78%) holdings with pigs testing positive only by culture and represented by 9 pigs (2.05% of all pigs), 6 pigs were positive by culture and 3 negative. Percentage of positive pigs (either by culture or ELISA) within holdings varied considerably. In the holdings represented by at least 10 pigs the range of ELISA positive pigs at OD 20% was from 5.00% to 53.33% with the average 22.91% and at OD 40% it was from 0.00% to 53.33% with the average of 15.46%. The results of culture and ELISA positive pigs and holdings are presented in Figure 1 and 2 respectively.

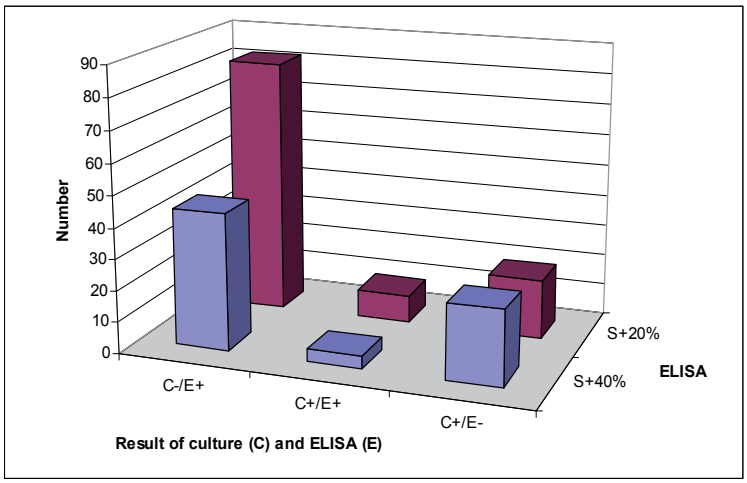

Fig. 1. Results of culture and ELISA positive pigs (N = 110).

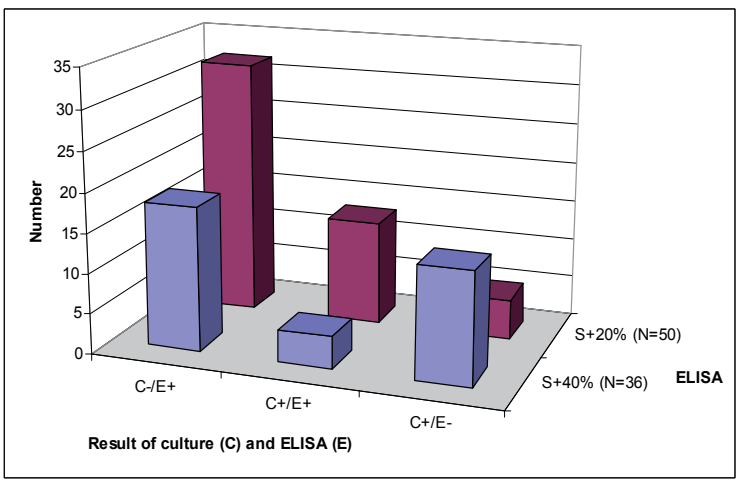

Fig. 2. Results of culture and ELISA positive holdings (N = 50).

2.2.2 Suitability of diaphragm and neck muscle meat juice

We also compared the results of ELISA of meat juice from diaphragm and neck muscles. For 9 pigs we did not have the data on the sampling site, so the results of altogether 439 pigs were processed. Meat juices of 304 (70.53%) pigs were from diaphragm and 127 (29.47%) from neck muscles. From diaphragm 71 (23.36%) meat juices were positive and from neck muscles 17 (13.39%). In the chi-square test the difference was statistically significant ($t = 4.88$, $P < 0.05$). Since different holdings were represented by different number of pigs, we compared also holdings from which only samples of diaphragm muscles, only neck muscles or both were tested. We had data for 175 holdings, of which 132 (75.43%) were represented only by diaphragm meat juices, 34 (19.43%) by neck muscle juices and 9 (5.14%) by both meat juices. Of the holdings with only diaphragm meat juices 35 (26.52%) had at least one positive pig. Of the holdings with only neck muscles juice 7 (20.59%) had at least one

positive pig. Of the holdings with both juices 5 (55.56%) had at least one positive pig. Since the number of holdings with both juices was low and the proportion of pigs with either of juices varied greatly within holdings, we compared only holdings with one type of juice. In the chi-square test the difference was not significant (t = 0.24, P <0.05). The comparison of diaphragm and neck muscles' ELISA are presented also in Figure 3.

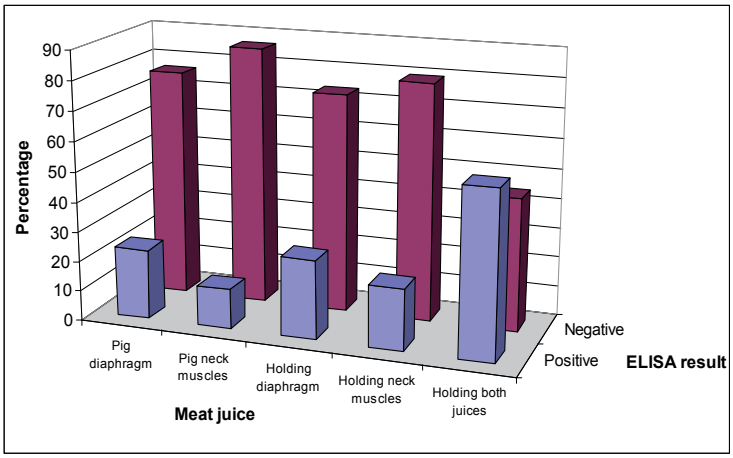

Fig. 3. Comparison of pig and holding meat juices' results.

2.2.3 Comparison of ELISA and culture

We found no correlation between culture and ELISA results. In the chi-square test the difference between them was statistically highly significant (t = 39.523, P< 0.005). We present the results of culture and ELISA in the holdings represented with at least 10 pigs in the Figure 4.

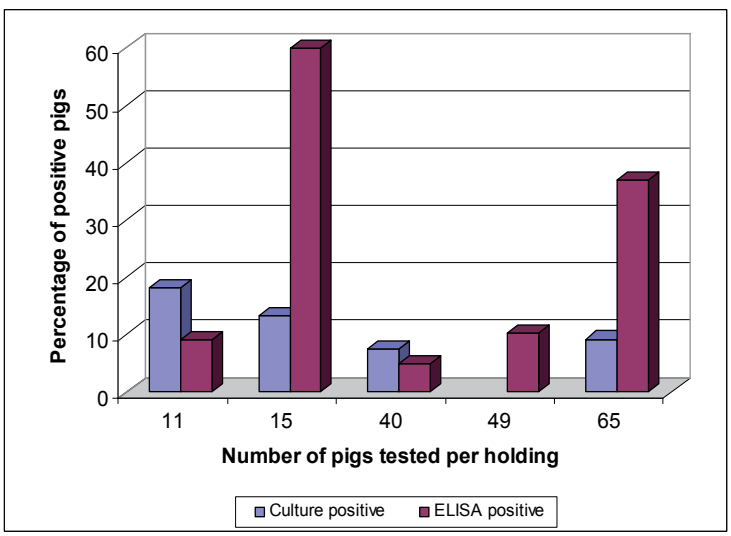

Fig. 4. Comparison of ELISA and culture.

2.2.4 Sampling for *Salmonella* reduction program

What happens if we do not have enough results over the whole year is presented in the figures 5, 6 and 7. In the figure 5 we present the results of holding A. The sampling covers all the twelve month, but the number of samples is too small, so the results are not reliable enough. We isolated three different serovars of *Salmonella*. The first was serovar Virchow in December and the next was Derby in January. In January and February high levels of antibodies were detected. Till August when serovar Coeln was isolated, there were no isolates. Its effect on seroconversion can not be estimated due to low number of samples.

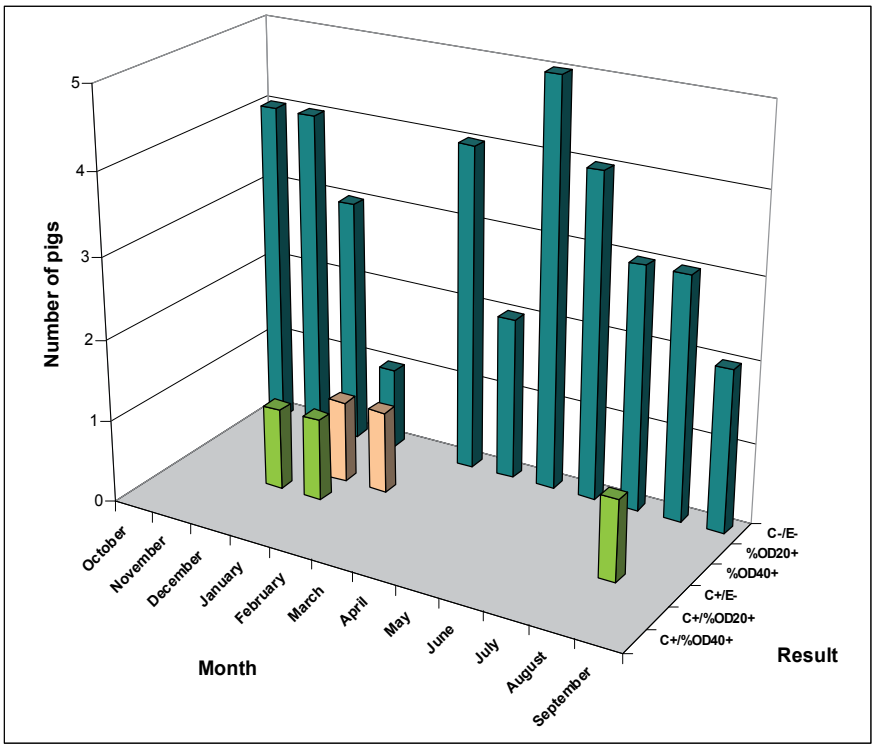

Fig. 5. Results of holding A over the year (N = 40 pigs tested; C = culture, E = ELISA).

In the holdings B and C there was no sampling in the second half of the study (spring and summer). In the holding B only 7 months were covered, and in the holding C only 6 months. In the holding B we found only some seroconversion, but no positive culture for *Salmonella* (Figure 6).

In the holding C we found seroconversion and culture positive pigs, but we didn't have an overview over the whole year. In October it seems that seroconversion remained from previous infection. In October we isolated serovar Choleraesuis var. Decatur, in November serovar Heidelberg and in December serovar Enteritidis. In February we isolated serovar Infantis and in March Enteritidis. From April to September, when the rate of *Salmonella* infection in humans is usually the highest, there were no samples. Some seroconversion was detected in October, which increased till January when the highest number of pigs positive at %OD 40 was detected. (Figure 7).

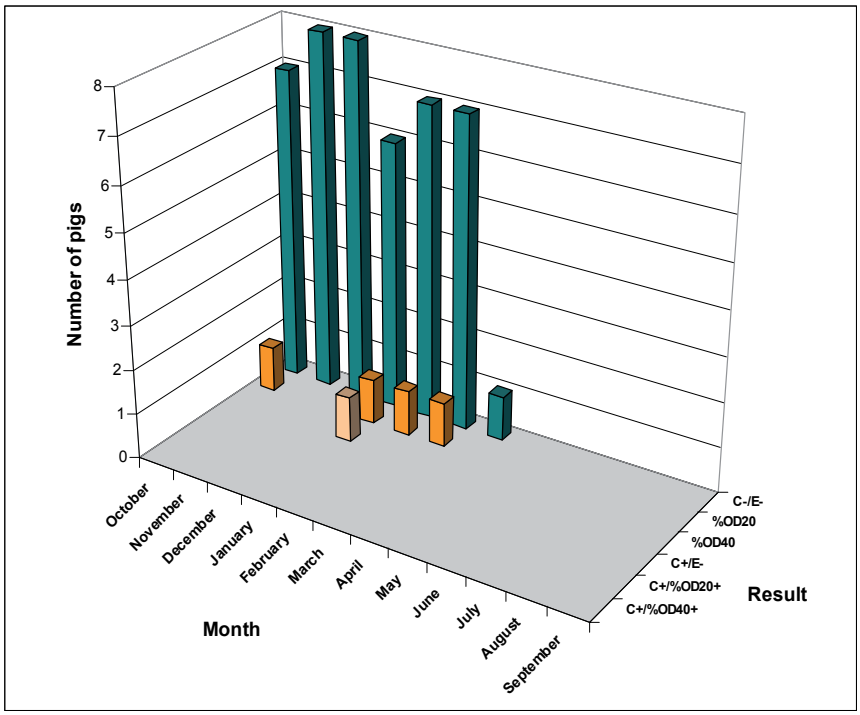

Fig. 6. Results of holding B over the year (N = 49 pigs tested; C = culture, E = ELISA).

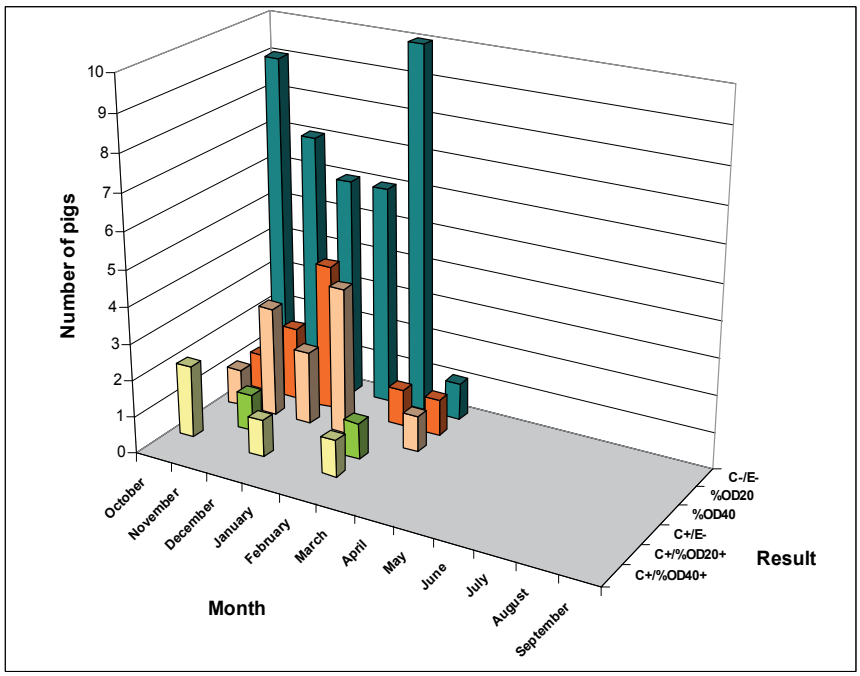

Fig. 7. Results of holding C over the year (N = 65 pigs tested; C = culture, E = ELISA).

2.3 Discussion

Current surveillance of *Salmonella* in pigs involves intensive and expensive scheme for herd classification. Isolation of *Salmonella* from lymph nodes is believed to reflect long-term exposure at herd level, but might indicate infection during transport and lairage. Bacteriological techniques for *Salmonella* detection are reported to have very high specificity (up to 100%) but sensitivity is low (Funk et al., 2000). In the EU Interlaboratory comparison study food II (2007), organized by CRL-*Salmonella*, the sensitivity of use of MSRV and XLD media depended on serovar and number of colony forming units (cfu) in samples. It ranged from 54.3% for 10 cfu of serovar Enteritidis to 100.0% for 50 cfu of serovar Typhimurium (Kuijpers et al., 2008). Besides, *Salmonella* are usually localized focally, so thorough homogenisation of samples is important. In faecal and dust samples competitive microflora might also lower the recovery of *Salmonella*.

ELISA detects specific antibodies against *Salmonella* and therefore it indicates past or resent exposure and different serological stages. According to producers manual the IDEXX Swine *Salmonella* Ab ELISA test specificity is 99.4%. Among *Salmonella* negative herds, some might be misclassified due to small number of pigs tested per holding - in 126 (70.79%) holdings in our Baseline study only one. More herds would be expected to be positive, if more samples had been collected. To improve herd test sensitivity, more samplings of herds would have been desirable (Baptista et al., 2010). CRL-*Salmonella* organized comparability of different ELISAs on the detection of *Salmonella* spp. antibodies in meat juice and serum. Ten national reference laboratories participated, using four different ELISA kits. The kits were designed to detect antibodies against *Salmonella* serogroups O:4 (B) and O:7 (C1) and one of them also against O:9 (D1). Laboratories used different OD% cut-off values from 15% to 40%. The comparison of results between laboratories was difficult. In nine of ten NRLs the results were significantly different from the results of CRL (Berk et al., 2008). Similar results were observed by Vico who compared three commercial enzyme-linked immunosorbent assays for meat juice samples. When these three kits were used in the same herd, the results deffered substantially. Thus caution is advised if it is decided to use these assays for herd health classivication in *Salmonella* control programs (Vico et al., 2010).

In our study, the seroprevalence at cut-off 20 OD% was in pigs 20.7% and in holdings 25.3%. The seroprevalence for cut-off 40 OD% in pigs was 10.9% and in the holdings 14%. Results from the bacteriological testing showed 6.4% positive pigs from 10.1% positive holdings. The results of statistical analysis showed the poor correlations between serology and bacteriology in pigs and in holdings.

In the instructions for sampling in the baseline study both meat juices from neck muscles and diaphragm were treated as equivalent. Our results also show that at the holding level there were no significant differences, although at pig level the difference seemed significant. We attribute this to the difference of seroprevalence between holdings and the differences in the numbers of pigs tested per single holding.

EU Member States approach the problem of reduction of *Salmonella* prevalence in pigs with different reduction programs. They categorize holdings in categories regarding seroprevalence. Danish *Salmonella* surveillance and control program in slaughter pigs was introduced in 1995 and started with cut-off for positive serology result 40 OD%. In August 2001 a new assignment was introduced which among others included reduction from cut-

off 40 OD% to 20 OD% in the interpretation of the individual meat juice sample results (Alban et al., 2002). German *Salmonella* surveillance and control program in slaughter pigs classified herds in three categories: I (0-20%), II (20-40%), III (<40%) by their percentage of yearly positive samples, which was re-calculated quarterly. The number of participating herds increased continuously since the start of the monitoring program, with regional differences in the degree of participation. In the forth quarter of 2008, 81.9% of the herds were allocated to category I, 14.0% to category II and 4.0% to category III. However, the prevalence of *Salmonella* tended to decrease in herds that participated over of long period (Merle et al., 2011). In Slovenia only one holding sent samples monthly, so it was used in our preliminary study. Seroprevalence to *Salmonellae* for year 2007 for the mentioned holding was for all tested samples 12.8% for OD 40% and 24% for OD 20%. For randomly selected samples for the same year the prevalence was 7.5 % for OD 40% and 17% for OD20 % (Stukelj et al., 2009). In the baseline study in 2008 this holding was represented by 9 pigs, sampled in four months over summer and only one pig tested positive at OD 20%. The example of the three holdings (A, B, C) from the baseline study clearly shows the necessity of monthly testing of relevant number of pigs. In Danish *Salmonella* surveillance and control program herds with annual kill less than 100 pigs were excluded; they were considered insignificant, because of pigs from such herds only constituted around 1% of the total number of pigs slaughtered at the time of study. Also relatively more animals would need to be sampled to estimate the prevalence in these herds with an acceptable precision. The minimal number of tested pigs was 60 per year (Alban et al., 2001). In the baseline study 168 holdings (94.4% of all tested) in Slovenia were represented only by 1-5 pigs. Also in Slovenia such a program for monthly testing with relevant number of pigs would be appropriate but adapted to the high percentage of small herds. Sandberg et al. reported that the unit for testing should be the herd rather than the individual animal. The sampling should focus on the larger herds that supply most of the meat in the market and on the herds that distribute sows and piglets to other herds and can thus contribute to the spread of *Salmonella* among herds (Sandberg et al., 2002).

Sørensen et al. reported that they found no linear association between the proportion of positive lymph nodes and herd serology. In general, the highest proportion of positive pigs was observed for finishers originating from herds with seroprevalences varying from 61-70% (Sørensen et. al., 2004). CRL-*Salmonella* came to the same conclusion that there is no correlation between antibody levels and detection of *Salmonella* from lymph-nodes and carcass swabs (Berk et al., 2008). Österberg et al. reported that the seroconversion and excreting of *Salmonella* were serovar and dose-dependent. Pigs inoculated with levels of 10^6 and 10^9 cfu of serovar Derby produced specific antibodies, while pigs inoculated with 10^3 cfu of Derby or serovar Cubana produced no detectable antibody levels (Österberg et al., 2009). Within our study we compared individual pigs instead of serological statuses of the farm to *Salmonella*. The *Salmonella* prevalence in lymph nodes in individual pig is irrespective of herd serology. The presence of *Salmonella* in the lymph nodes may be caused by an infection so early in the pig's life that the serological response is no longer there, but bacteria has remained in the lymph nodes, or the pig has been infected very recently (Sørensen et. al., 2004). We, too, had cases, where pigs were culture positive and serologically negative. The *Salmonella* bacteria probably related to infection in the herd, but this infection may have occurred several weeks or months prior to slaughter. If the aim is to monitor the *Salmonella* prevalence in the herd, than herd serology is better indication than in

caecal lymph nodes. Additionally, the presence of *Salmonella* in intestinal lymph nodes has a negligible impact on food safety as they are neither cut nor eaten. Usually leaking of intestinal content is more likely and more dangerous cause of carcass contamination with *Salmonella* and other enteric pathogens, so the technology and the way of handling pigs and their carcasses in slaughterhouse is very important. The results of De Busser et al. indicate that the lairage area is primary source of *Salmonella* in slaughter pigs and the carcass contamination originates from the environment rather than from the pig (inner contamination) itself (De Busser et al., 2011). Despite this, some countries used analyses of caecal lymph nodes to measure the *Salmonella* prevalence in pigs and herds.

The strong correlation between bacteriological findings and herd serology indicates that despite the fact that most *Salmonella* infections are silent in pigs, they nevertheless undergo an infectious process that results in immune response. The question is how *Salmonella* should be measured? Bacteriological measures as well as the measure of antibodies are strongly correlated. Therefore, four bacteriologically tested sites (carcass surface, pharix, lymph nodes, caecal content) and herd serology can in principle be used. The results of the study conducted by Sørensen et. al. demonstrated a strong association between herd serology measured by use of Danish mix - ELISA and the presence of *Salmonella* in caecal - contents, or carcass surface, and in pharynges, but not in caecal lymph nodes. This applies to Danish conditions where the transport time and duration of lairage is short (Sørensen et. al., 2004). The transport time and duration of lairage is short also in Slovenia, so similar measures would be indicated. In this study we tested by culture only lymph nodes and compared the results with serology. The results were not comparable, which was also expected from other studies (Sørensen et. al., 2004). This can also be expected in the case that *Salmonella* remains only in intestine and is occasionally excreted in environment.

3. Conclusion

For food safety assurance both approaches can be valuable. Antibody detection is an indicator of possible previous or on-going *Salmonella* infection in a herd, while *Salmonella* detection by culture in faeces, lymph nodes or animal environment indicates possible threat of food contamination, especially with serovars of public health importance. To obtain a reliable overview of the *Salmonella* prevalence in individual holdings regular monthly testing of relevant number of pigs is mandatory. Hygienic measures during pig production in holdings and in food production in slaughterhouses and food production plants are the key to reduction of *Salmonella* problem in humans.

4. Acknowledgment

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19

The Central Nervous System Modulates the Immune Response to *Salmonella*

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1. Introduction

Salmonella infection induces an immune response, the first and principal element of which is a local activation in the intestine. This intestinal response and the systemic response of the immune system have multidirectional interactions with the nervous and endocrine systems (Berczi and Szentivanyi 2003). The central nervous system (CNS) signals the immune system via hormonal and neural pathways, and the immune system signals the CNS through various cytokines. Whereas most information regarding these interactions is related to functions of the systemic immune response (Berczi, Nagy et al. 1981; Chrousos 1995; Madden and Felten 1995; Elenkov, Wilder et al. 2000; Webster, Tonelli et al. 2002; Berczi and Szentivanyi 2003), much less is known about the interactions between the hypothalamus, the pituitary, and local gastrointestinal immune reactions (Berczi, Nagy et al. 1981; Ottaway 1991; Bienenstock 1992; Chrousos 1995; Madden and Felten 1995; Elenkov, Wilder et al. 2000; Webster, Tonelli et al. 2002; Berczi and Szentivanyi 2003; Campos-Rodriguez, Quintanar-Stephano et al. 2006).

The CNS regulates the intestinal immune system through the three divisions of the autonomic nervous system: sympathetic, parasympathetic, and enteric. By signals sent along sympathetic and parasympathetic fibers, the CNS controls the enteric nervous system (ENS),which in turn regulates gastrointestinal functions, including immune functions (Cooke 1986; Ottaway 1991; Gonzalez-Ariki and Husband 1998; Bueno 2000; Spiller 2002). Moreover, the CNS regulates the mucosal immune system through the hypothalamic–pituitary–adrenal (HPA) axis, an essential part of whichis glucocorticoid production (Sternberg 2001; Webster, Tonelli et al. 2002; Jarillo-Luna, Rivera-Aguilar et al. 2008).

Ongoing research to clarify the bidirectional communication between the immune and central nervous systemshas in part been carried out byproducing electrolytic or pharmacologic lesions in several areas of the brain, such basal ganglia, striatum, hypothalamus, hippocampus and thalamus, and then observing the resulting immune response. This approach has been used in our recent studies (Campos-Rodriguez, Quintanar-Stephano et al. 2006; Rivera-Aguilar, Querejeta et al. 2008; Quintanar-Stephano, Abarca-Rojano et al. 2010) to observe the effect of brain lesions on the immune response to *Salmonella* and one of its main components, lipopolysaccharide (LPS). It has been found that brain lesions modify the number and functions of lymphocytes in the spleen, thymus and blood (Jankovic and Isakovic 1973; Payan, McGillis et al. 1986).

The aim of this chapter is to describe the effects of CNS lesions on the immune response to *Salmonella*. The mechanisms are explored by which these lesions affect the systemic and intestinal immune responses. Since the production of intestinal IgAis fundamental in the protection against *Salmonella* invasion, an evaluation is made of the role of neurotransmitters, glucocorticoids and neuroendocrine molecules in the regulation of such production.

2. Hypophysectomy and neurointermediatepituitary lobectomy reduce the humoral immune response to *Salmonella enterica* serovar Typhimurium

The hypothalamus induces the secretion of anterior pituitary hormones, and in this way the CNS can have both an immunostimulatory and immunosuppressor effect. In this sense, the immune response is stimulated mainly by the release of growth hormone (GH) and prolactin (PRL) (Berczi, Nagy et al. 1981; Block, Locher et al. 1981; Nagy and Berczi 1981; Berczi, Nagy et al. 1984; Edwards, Yunger et al. 1991; Nagy and Berczi 1991; Edwards, Arkins et al. 1992; Nagy and Bercz 1994; Madden and Felten 1995; Berczi and Szentivanyi 2003), and inhibited by the hypothalamic-pituitary-adrenocortical (HPA) axis, which causes the release of adrenocorticotropin (ACTH), which in turn stimulates the secretion of adrenocortical glucocorticoids (Chrousos 1995; Sternberg 2001; Webster, Tonelli et al. 2002). This increase of circulating glucocorticoids (GCs) is caused when the HPA axis is activated during many bacterial and viral infections.

In vivo, we have demonstrated that arginine vasopressin (AVP) released from the posterior pituitary affects humoral and cell mediated immune responses (Organista-Esparza, Tinajero-Ruelas et al. 2003; Quintanar-Stephano, Kovacs et al. 2004; Quintanar-Stephano, Organista-Esparza et al. 2004; Quintanar-Stephano, Chavira-Ramirez et al. 2005; Quintanar-Stephano, Organista-Esparza et al. 2005; Quintanar-Stephano, Abarca-Rojano et al. 2010). Regarding *Salmonella enterica* serovar Typhimurium (*Salmonella typhimurium*) infection, there is experimental evidence that pituitary hormones have a protective effect (Edwards, Yunger et al. 1991; Edwards, Ghiasuddin et al. 1992). For instance, the increased susceptibility to intraperitoneal *Salmonella typhimurium* infection found in hypophysectomized (HYPOX) rats is countered by GH treatment, which restores normal resistance. In intact rats and mice, GH and PRL enhance resistance to *Salmonella typhimurium* infection through an increase in phagocytosis and intracellular destruction of bacteria by peritoneal macrophages. *Salmonella* or other challenges to the immune system, such as immobilization stress and burn injury, increase the levels of GCs, which in turn increase bacterial translocation from the gastrointestinal tract to the mesenteric lymph nodes (Jones, Minei et al. 1990; Fukuzuka, Edwards et al. 2000; Dunn, Ando et al. 2003).

All of the aforementioned suggests that anterior and posterior pituitary hormones participate as stimulating factors in the control of systemic and intestinal immune responses to *Salmonella*. To further explore this idea, we investigated the systemic and intestinal immune responses in HYPOX and neurointermediate pituitary lobectomy (NIL) rats orally infected with nonlethal doses of *Salmonella typhimurium* (Campos-Rodriguez, Quintanar-Stephano et al. 2006).The most relevant results are that the kinetics of intestinal *Salmonella* elimination in sham-operated (SHAM), HYPOX and NIL groupswas similar with no clinical signs of salmonellosis and no mortality.However, nine days after inoculation, the number of *Salmonella typhimurium* cells in Peyer's patches and spleens of HYPOX and NIL groups was higher than in the sham-operated group ($P < 0.001$) (Fig. 1), andthere were a greater number of bacteria in HYPOX than NIL animals ($P < 0.01$). The fact that the total or partial ablation of the hypophysis increased susceptibility to infection after oral inoculation with *Salmonella typhimurium* means that the pituitary gland is required for protection against infection by intraperitoneal *Salmonella* inoculation (Edwards, Yunger et al. 1991).

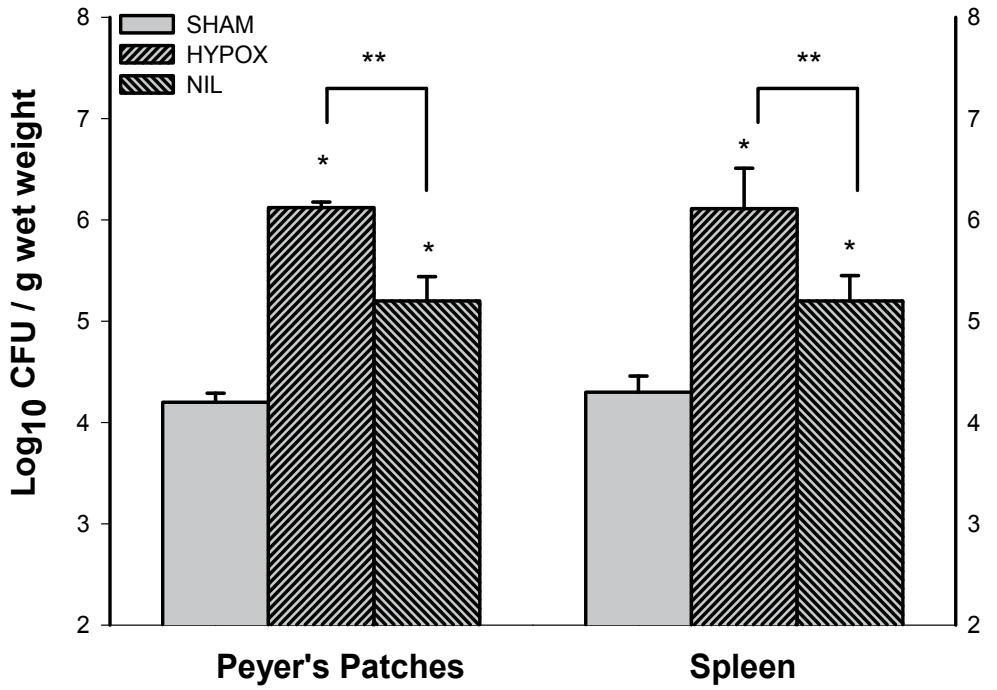

Fig. 1. Persistence of serovar Typhimurium infection in Peyer's patches and spleen. Sham-operated (SHAM), HYPOX, and NIL groups were orally infected and sacrificed 9 days postinoculation. Tissues were aseptically removed and processed for bacterial quantification. Data are expressed as means SD of results from four to six rats per group. In Peyer's patches and spleens, bacterial counts were significantly higher in HYPOX and NIL groups than in the sham-operated group (*, $P < 0.001$) and significantly higher in the HYPOX group than in the NIL group (**, $P < 0.01$). (From Campos-Rodriguez et al. Hypophysectomy and Neurointermediate Pituitary Lobectomy Reduce Serum Immunoglobulin M (IgM) and IgG and Intestinal IgA Responses to *Salmonella enterica* Serovar Typhimurium Infection in Rats. Infect Immun. 2006; 74(3):1883-1889).

Most pituitary hormones directly or indirectly modulate inflammatory/immune responses. For example, adrenocorticotropin increases the secretion of GCs, which in turn stimulate the immune function at physiological doses (Munck and Naray-Fejes-Toth 1992; Reichlin 1993; Chrousos 1995; Wiegers and Reul 1998; Sapolsky, Romero et al. 2000). GH, PRL, TSH and -endorphin produced in the anterior pituitary as well as the AVP released from the posterior pituitary have immunopotentiating and proinflammatory properties (Heijnen, Kavelaars et al. 1991; Navolotskaya, Malkova et al. 2002; Klein 2003). Therefore, the differences between NIL and HYPOX rats may be related to the amount of hormones that regulate the immune response located in the anterior and posterior pituitary. Another possible factor is that the partial or total removal of the pituitary may affect the activity of phagocytes, the principal cells of the innate immunity involved in killing *Salmonella typhimurium* (Mittrucker and Kaufmann 2000; Kirby, Yrlid et al. 2002). It has been demonstrated that peritoneal macrophages from HYPOX rats have an impaired tumor necrosis factor alpha response to *in vitro* lipopolysaccharide stimulation and are less effective in killing *Salmonella typhimurium* than those derived from rats with intact pituitaries (Edwards, Lorence et al. 1991). GH injections enhanced resistance of both intact and HYPOX rats following a challenge with *Salmonella typhimurium* (Edwards, Lorence et al. 1991; Edwards, Ghiasuddin et al. 1992). The enhanced resistance is correlated with the ability of peritoneal macrophages from these animals to generate toxic oxygen metabolites, such as superoxide anion and hydrogen peroxide (Edwards, Ghiasuddin et al. 1992). In addition, GH activates human monocytes for enhanced reactive oxygen intermediate production *in vitro* (Warwick-Davies, Lowrie et al. 1995; Warwick-Davies, Lowrie et al. 1995; Navolotskaya, Malkova et al. 2002).

An analysis of the secretion of intestinal IgA specific to outer membrane proteins of *Salmonella* shows that the titers of the specific intestinal IgA response was significantly lower in HYPOX and NIL animals than in the sham-operated group ($P < 0.001$, Fig. 2), and was also lower in the HYPOX than NIL rats ($P < 0.001$)(Campos-Rodriguez, Quintanar-Stephano et al. 2006).The fact that HYPOX induced a more marked decrease in the humoral immune responses to outer membrane proteins of *Salmonella typhimurium* than NIL suggests that the hormones melanocyte stimulating hormone (MSH), AVP, and oxytocin from the neurointermediate pituitary lobe may affect adaptive immune responses. The direct anti-inflammatory effects of MSH on immunocytes have been described previously (Catania and Lipton 1993; Blalock 1999; Luger, Scholzen et al. 2003; Taylor 2003). Since NIL eliminates the intermediate lobe— the main source of pituitary -MSH— an increased inflammatory response to *Salmonella typhimurium* infection may be expected. However, our results show that -MSH from the intermediate pituitary lobe is not involved in the immune response to *Salmonella typhimurium* infection. Further experiments are required to test this possibility.

Furthermore, in the aforementioned study levels of IgG and IgM were also significantly lower in the HYPOX and NIL animals than in the sham-operated group (Fig. 2), and in HYPOX rats than in the NIL group (Campos-Rodriguez, Quintanar-Stephano et al. 2006). The cause of these reduced humoral immune responses may be the decreased secretion of the neurointermediate pituitary hormones. In previous experiments, we found that in NIL rats there are decreased humoral and cell-mediated immune responses, including: (i) decreased hemagglutination, IgG and IgM responses to sheep red blood cells (Organista-Esparza, Tinajero-Ruelas et al. 2003; Quintanar-Stephano, Kovacs et al. 2004), (ii) decreased contact hypersensitivity to dinitrochlorobenzene (Quintanar-Stephano, Kovacs et al. 2004), and (iii) protection against EAE (Quintanar-Stephano, Chavira-Ramirez et al. 2005). In

agreement with these previous findings, our results suggest that the higher colonization of the Peyer's patches and spleens and the decreased IgG, IgM, and IgA responses to *Salmonella typhimurium* may be due to AVP deficiency in the NIL animals. In another study we found that in both HYPOX and NIL rats, there was a decrease in the IgM response to the LPS of *Salmonella typhimurium* (Quintanar-Stephano, Abarca-Rojano et al. 2010). These results support the view that hormones from both pituitary lobes play an important stimulatory/modulatory role in both humoral and cell-mediated immune responses.

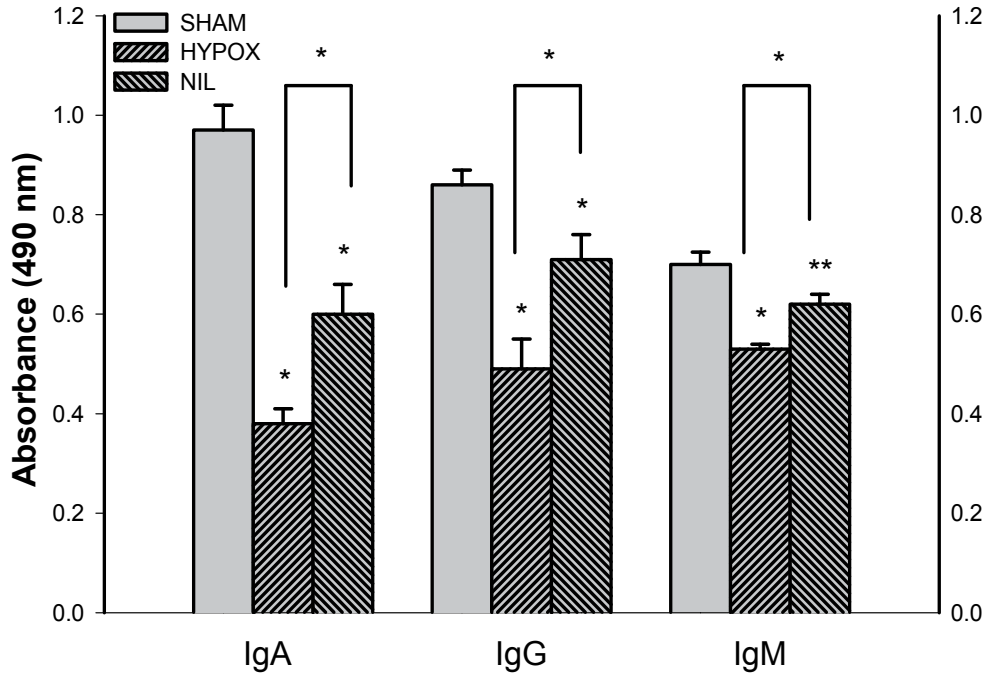

Fig. 2. Intestinal IgA and serum IgG and IgM response to *Salmonella typhimurium*. Intestinal IgA or serum IgG and IgM antibodies were quantified by ELISA using *Salmonella typhimurium* surface antigens. Serum and gut samples were obtained 9 days postinoculation. The samples were assayed in triplicate, and the titers were expressed as the absorbance at 490 nm. Data are expressed as means SD of results from four to six rats per group. The immunoglobulin levels were significantly lower in HYPOX and NIL groups than in the sham operated group (*, $P < 0.001$; **, $P < 0.01$) and significantly lower in the HYPOX group than in the NIL group (*, $P < 0.001$). (From Campos-Rodriguez et al. Hypophysectomy and Neurointermediate Pituitary Lobectomy Reduce Serum Immunoglobulin M (IgM) and IgG and Intestinal IgA Responses to *Salmonella enterica* Serovar Typhimurium Infection in Rats. Infect Immun. 2006; 74(3):1883-1889).

Finally, intestinal elimination of *Salmonella typhimurium* HYPOX and NIL rats was similar to that seen in sham-operated animals. However, it is known that HYPOX animals develop an increased susceptibility to intraperitoneal *Salmonella typhimurium* infection, and that GH and PRL treatments protect the rats against the disease (Edwards, Yunger et al. 1991; Edwards, Ghiasuddin et al. 1992). Similarly, PRL increases resistance to infection in normal mice after intraperitoneal inoculation of *Salmonella typhimurium* (Di Carlo, Meli et al. 1993; Meli, Raso et al. 1996).

Since the immune responses are PRL and GH dependent and no pituitary hormones are produced in the HYPOX animals, how can the formation of anti-*Salmonella typhimurium* IgG, IgM, and IgA immunoglobulins be explained? Perhaps part of the answer lies in an unpublished study with HYPOX animals. After surgery a gradual increase was observed in the plasma PRL levels, which after 7 to 9 weeks post-operation reached 50% of the levels of this hormone found in intact animals (Nagy and Berczi 1991; Quintanar-Stephano and A. Organista- Esparza, unpublished). Although the source of this non-pituitary PRL is not known, one possibility is from T lymphocytes (Draca 1995; Stevens, Ray et al. 2001). The fact that HYPOX rats had a higher number of *Salmonella typhimurium* cells in Peyer's patches and spleen than sham operated and NIL rats suggests that the low serum IgG and IgM and intestinal IgA immunoglobulin levels in HYPOX rats may be due to the insufficient immune-stimulating effect of the non-pituitary PRL (Nagy and Berczi 1991). However, further studies are needed to evaluate this suggestion.

In summary, it can be concluded that through different mechanisms, hormones from both the anterior and neurointermediate pituitary lobes play an important role in the control of systemic and gastrointestinal immune responses to *Salmonella*. However, more experiments are needed to establish the interactions between the hypothalamo-neurohypophysial (AVP) and immune systems.

3. Striatum modulates the humoral immune response to LPS and outer membrane proteins of *Salmonella enterica* serovar Typhimurium

The striatum is implicated in movement and learning (Costall, Naylor et al. 1972; Pycock 1980; Graybiel 1995), and there is increasing evidence that it is involved in the modulation of immune responses, although such evidence is contradictory. Bilateral electrolytic lesions of the caudate nucleus of rats do not reduce the intensity of cell-mediated immune responses or the production of antibodies to bovine serum albumin (BSA) (Jankovic and Isakovic 1973). On the other hand, such lesions result in a reduction of the antibody immune response to sheep red blood cells (SRBC) (Devoino, Alperina et al. 1997; Devoino, Cheido et al. 2001; Nanda, Pal et al. 2005; Rivera-Aguilar, Querejeta et al. 2008). In addition, the destruction of dopaminergic neurons in the substantia nigra or dopaminergic terminals in the caudate nucleus by in situ injection of 6-hydroxydopamine decreases the antibody response and impairs cell-mediated immunity (Deleplanque, Vitiello et al. 1994; Devoino, Alperina et al. 1997; Devoino, Cheido et al. 2001; Filipov, Cao et al. 2002).Furthermore, bilateral lesions of nigrostriatal pathways induced by systemic injections of the neurotoxin 1-methyl-4-phenyl-1,2,3,6- tetrahydropymidine reduce the number of leukocytes, alter lymphocyte populations,decrease proliferation of T lymphocytes induced bymitogens or alloantigens, and modify the synthesis of cytokines (Renoux, Biziere et al. 1989; Bieganowska, Czlonkowska et al. 1993; Shen, Hebert et al. 2005; Engler, Doenlen et al. 2009).

These findings suggest that the nigrostriatal dopaminergic system has an immunostimulatory effect on the humoral and cell-mediated immune response. To test the hypothesis that GABAergic medium-sized spiny neurons in the striatum modulate the humoral immune response, in rats with a bilateral lesion of the striatum provoked by the injection of quinolinic acid we analyzed this response to several antigens (both T-independent and T-dependent antigens), including LPS and outer membrane proteins of *Salmonella typhimurium*. Quinolinic acid produces axon-sparing lesions that result in a loss of GABAergic medium-sized spiny neurons (MSP) in the striatum, while the dopaminergic

terminal network originating from cell bodies in the substantia nigra remains unchanged (McGeer and McGeer 1976; Schwarcz, Whetsell et al. 1983; Beal, Kowall et al. 1986).

3.1 Bilateral lesion of the striatum decreases the humoral immune response to TNP-LPS

The serum levels of IgG and IgM antibodies anti-trinitrophenol-lipopolysaccharide (TNP-LPS)(Fig. 3, panel A and B), and the IgA antibodies anti-TNP-LPS in intestinal fluid (Fig. 3, panel C) were significantly lower in rats with a bilateral lesion of the striatum compared with the control group ($P < 0.01$). These results show that the lesions of the striatum had a prolonged effect on the immune response to this T-independent antigen, indicating that the striatum modulates this type of humoral immune response. On the contrary, a bilateral lesion of the striatum increased the humoral immune response to T-dependent antigens (ovoalbumin, lysozyme and bovine serum albumin).

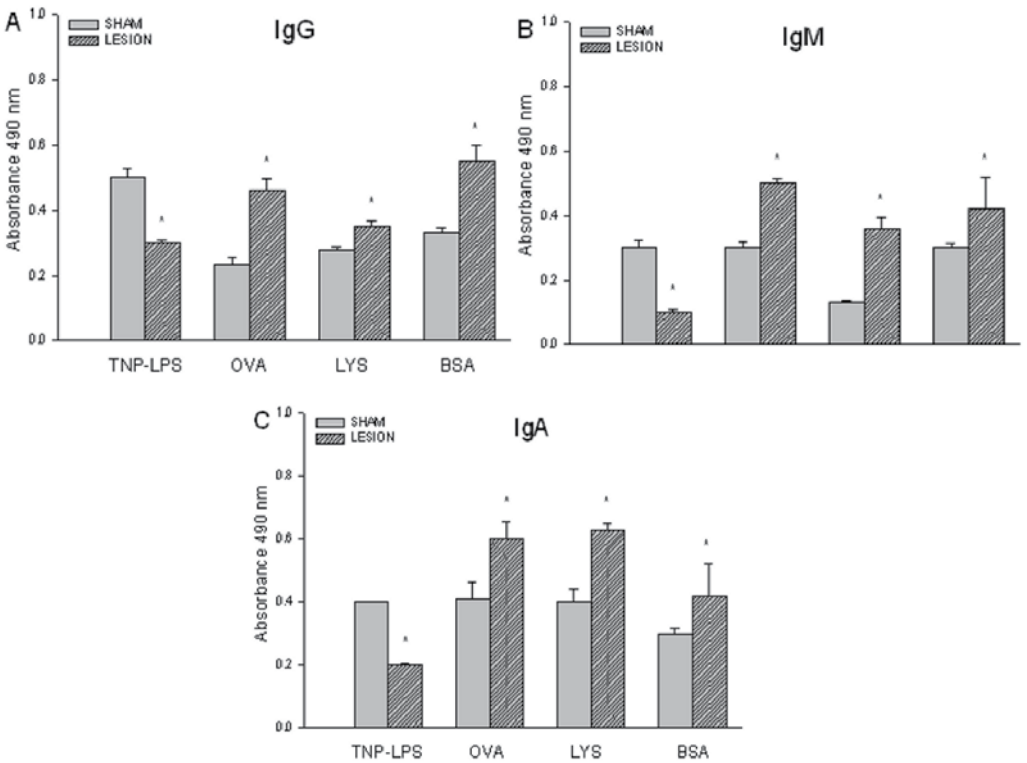

Fig. 3. IgG, IgM and IgA response to T-independent and T-dependent antigens in rats with bilateral lesion of striatum. The antibody response to TI and TD antigens was analyzed in rats that had been lesioned 25 days before immunization. The serum IgM and IgG levels as well as the intestinal IgA levels to the T-independent antigen (TNP-LPS) were significantly lower in lesioned rats than in the sham-operated rats (*$P < 0.01$). On the contrary, the antibody levels to all the T-dependent antigens (OVA, lysozyme, and BSA) were significantly higher in the lesioned group than in sham operated group (*$P < 0.01$). (From Rivera-Aguilar et al. Role of the striatum in the humoral immune response to thymus-independent and thymus-dependent antigens in rats. ImmunolLett 2008;120:20-28).

Although themechanisms by which the lesion of the striatal MSP neurons leads to a decrease in the immune response to TNP-LPS (TI type 1 antigen) are not known, it is likely that they are related to defects in B lymphocyte activation. In fact, the number of IgM+ B cells in the marginal zone of the spleen was significantly lower in lesioned rats than in the control group.However, the mechanisms by which striatal lesions reduce the population of B cells in the spleen are at the present unknown, as are the mechanisms involved in the maturation, selection and long-term survival of immature peripheral B cells (Thomas, Srivastava et al. 2006).

We also found that striatal lesions caused a reduction in the expression of the gene for caveolin-1 and in the number of lymphocytes caveolin-1+ in the spleen (Fig. 4). Caveolin-1, expressed on B-lymphocytes, down-regulates tyrosine phosphorylation of Btk, a molecule that participates in B-cell activation and signaling (Vargas, Nore et al. 2002; Medina, Williams et al. 2006). Caveolin-1 deficient mice have a reduced response to both type 1 and type 2 thymus-independent antigens, but have a normal response to thymus-dependent antigens (Medina, Williams et al. 2006). Therefore, it is possible that the reduced response to TNP-LPS is caused by the decreased expression of caveolin-1 in B cells that respond to TI antigens.

3.2 Bilateral lesion of striatum increased the humoral immune response to outer membrane proteins (OMP) of *Salmonella enterica* serovar Typhimurium

To evaluate whether the increase in the humoral immune response to protein antigens was a general effect in rats with striatal lesions, we analyzed the IgG immune response to outer membrane proteins of *Salmonella typhimurium*. The levels of IgG in serum were significantly higher in rats with a bilateral lesion than in sham-operated animals (Table 1, $P < 0.001$). These results support the idea that a bilateral lesion of striatum increases the humoral immune response to T-dependent antigens.

Group	SHAM	Lesion of CN	P
Saline	0.100 ± 0.050	0.091 ± 0.060	
10^7*	0.282 ± 0.008	0.519 ± 0.023	< 0.001 ‡
10^8*	0.524 ± 0.020	0.650 ± 0.050	0.004 ‡

* 10^7 or 10^8 CFU of *Salmonella enterica* Serovar Typhimurium were administered i.p 7 days before the serum was collected and the titers were determined by ELISA. The data are presented as mean ± standard deviation of the absorbance at 490 nm (n = 4-6 rats per group).
‡ Difference in IgG levels between sham-operated and rats with lesion of striatum were significant as determined by the non-paired Student *t* test. Representative results from two independent experiments are shown.

Table 1. IgG antibody response to proteins of *Salmonella enterica* Serovar Typhimurium rats with bilateral lesion of striatum

The mechanisms involved in the increase of the immune response to OMP and other TD antigens in rats with a bilateral lesion in striatum are not clear. One possibility is that cytokines produced by the inflammatory cells in the injured area of the brain increase the antibody production. However, we did not find inflammatory cells in these areas and the expression of mRNA for cytokines did not increase (Fig. 4, panel A). Another possibility is

that alterations in the HPA axis contribute to the observed changes in the humoral immune response. Nevertheless, we did not find any increase in the expression of mRNA for prolactin in the hypophysis of rats with a bilateral lesion.

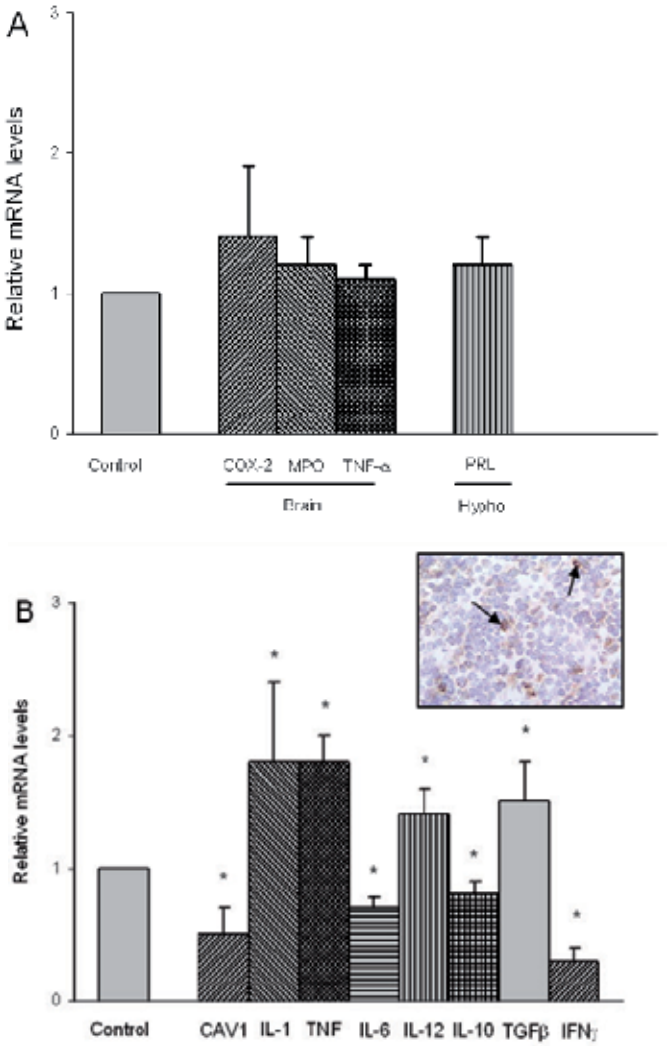

Fig. 4. Real-time RT-PCR analysis: (A) expression of genes in brain and hypophysis. Samples were collected 15 days after bilateral lesion of striatum and the mRNA expression of cyclooxygenase (COX)-2,myeloperoxidase (MPO), tumor necrosis factor-alpha (TNF-α), and prolactin (PRL) was measured by real-time RT-PCR. (B) expression of genes in spleen. The expression of caveolin-1 (CAV1), TNF-α, Interleukin (IL)-6, IL-12, IL-10, Transforming growth factor-beta (TGF-β), Interferon-gamma (IFN-γ) was measured by real-time RT-PCR, as detailed in materials and methods. Data represent the mean ± S.D (n = 6). *P < 0.05 compared with sham rats. Insert: immunolocalization of caveolin-1 positive cells in the splenic marginal zone, 400×. (From Rivera-Aguilar et al. Role of the striatum in the humoral immune response to thymus-independent and thymus-dependent antigens in rats. ImmunolLett 2008;120:20-28).

Because the response to OMP requires CD4+ T cells and cytokines,we analyzed that population aswell as the expression of genes for cytokines in the spleen. The number of CD4+ T cells in the spleen was significantly higher in lesioned rats than in the control group, and that increase could explain the augmented immune response to TD antigens (Table 2). Although the mechanism by which striatal lesions increase the number of naïve CD4+ T cells in the spleen is unknown, one possibility is that high corticosterone levels promote the migration of lymphocytes from the blood to the spleen, as occurs from the blood to other tissues (Dhabhar 2001). The other possibility, that the population of CD4+ T cells was activated by antigens and costimulators, is ruled out by the fact that CD4+ T cells did not express the gene for interleukin (IL)-2 , since this cytokine is produced by activated CD4+ T cells (Jenkins, Khoruts et al. 2001).On the other hand, the increase in their number can be explained by a greater migration of CD+ T cells from the blood into the spleen, although the mechanism of this possible migration remains unclear.

Spleniccell	**SHAM**	**Lesion of Striatum**	**P**
IgM +	34 ± 3	25 ± 3	< 0.001‡
IgG +	5 ± 0.4	5 ± 0.6	1.0
CD4+	5 ± 0.4	8 ± 1.2	< 0.05‡

The data are presented as mean ± standard deviation of the number of positive cells for IgM, IgG, Caveolin-1, and CD4+ (n = 4-6 rats per group).
‡ Differences in number of cells between sham-operated and rats with lesion of striatum were significant as determined by the Student *t* test. Representative results from two independent experiments are shown.

Table 2. Lymphocytes and Caveolin-1+ cells in the spleen of rats with bilateral lesion of striatum

Whereas in lesioned rats the expression of genes for IL-1, tumor necrosis factor (TNF), IL-12 and transforming growth factor-beta (TGF-β) increased, the expression of genes for IL-6, IL-10 and interferon-gamma (IFN-γ) decreased (Fig. 4, panel B). Although this pattern of cytokine production could contribute to the activation of the immune system (Trinchieri 1998; Pestka, Krause et al. 2004), further studies are needed to elucidate the role of cytokines in these changes. However, the fact that CD4+ T cells did not express the gene for IL-2, and that an increased expression of the gene for TGF-β was foundprobably explains the higher synthesis of IgA antibodies observed in lesioned rats, since TGF-β stimulates the production of IgA antibodies (Li, Wan et al. 2006).

Finally, the higher corticosterone levels found in lesioned rats compared with the control group (221. 8±53 ng/ml versus 24.6±12 ng/ml; mean ± S.D.; $P < 0.001$) could contribute to the changes observed in the immune response. Since glucocorticoids have opposite effects on the TI and TD antibody responses (Addison and Babbage 1981; Garvy and Fraker 1991), high corticosterone concentrations may depress TI responses and stimulate TD responses. In addition, given that physiological glucocorticoid concentrations enhance immunoglobulin production *in vitro* and *in vivo* (Ambrose 1964; Halliday and Garvey 1964; Fauci, Pratt et al. 1977; Gonzalez-Ariki and Husband 1998), the rise in corticosterone levels that we found might explain the increase in the immune response to TD antigens. However, pharmacological studies are required to elucidate the role of glucocorticoids in mediating the effects of striatal lesions on immune function.

In summary, our results indicate that striatal GABAergic medium-sized spiny neurons probably modulate the humoral immune response to *Salmonella* outer membrane proteins (OMP) through mechanisms related to the function of B and T cells, the expression of caveolin-1, and andchanges in serum levels of corticosterone.

4. Pathways for the CNS regulation ofthe immune response to *Salmonella* in the intestinal mucosa

In the intestinal mucosa, main site of entry of *Salmonella*, the CNS may regulate the immune response to *Salmonella* by modulating the activity of the HPA axis and the activity of the autonomic nervous system.

4.1 Role of the HPA axis in the immune response to *Salmonella*

The activity of the hypothalamus-pituary-adrenal axis results in the release of the corticotropin-releasing factor (CRF), the adrenocorticotropin hormone (ACTH) and glucocorticoidsinto the circulatory system (Wilder 1995; Webster, Tonelli et al. 2002; Charmandari, Tsigos et al. 2005; Gunnar and Quevedo 2007). Glucocorticoids released from the adrenal gland are delivered to the intestinal mucosa through blood circulation. Glucocorticoids inhibit mucosal inflammation through activation of glucocorticoid receptors present on epithelial cells and intestinal lymphocytes (Boivin, Ye et al. 2007; Jarillo-Luna, Rivera-Aguilar et al. 2008; Fujishima, Takeda et al. 2009; Resendiz-Albor, Reina-Garfias et al. 2010). Also, GCs increase bacterial translocation from the gastrointestinal tract to the mesenteric lymph nodes (Jones, Minei et al. 1990; Fukuzuka, Edwards et al. 2000; Dunn, Ando et al. 2003).

4.2 Role of the autonomic nervous system in the immune response to *Salmonella*

The CNS can modulate the activity of the autonomic nervous system (the adrenergic and cholinergic nervous system) and evoke the neuronal release of norepinephrine (NE), acetylcholine (ACh) and other neurotransmitters in peripheral tissues, including the intestinal mucosa (Felten, Felten et al. 1987; Kulkarni-Narla, Beitz et al. 1999; Kohm and Sanders 2001; Tracey 2002; Green, Lyte et al. 2003; Pavlov, Wang et al. 2003; Sternberg 2006; Sanders and Kavelaars 2007; Schmidt, Xie et al. 2007; Chrousos 2009; Kvetnansky, Sabban et al. 2009). These mediators may influence the function of the intestinal mucosa and its associated surface bacterial populations.

4.2.1 Sympathetic nervous system and the immune response to *Salmonella*

The sympathetic or adrenergic division of the autonomic nervous system is associated with a dual mode of regulation of inflammatory responses (Hasko and Szabo 1998; Elenkov, Wilder et al. 2000). Epinephrine (adrenaline), secreted from the adrenal medulla, and norepinephrine (noradrenaline), which is both secreted from the adrenal medulla and released from sympathetic nerve axons, modulate the release of cytokines and inflammation through adrenoceptors on immune cells (Hasko and Szabo 1998; Elenkov, Wilder et al. 2000).

There is strong immunohistochemical evidence for catecholaminergic innervation of Peyer's patches, the inductive sites for mucosal immunity and the main entry site for *Salmonella*. In

addition, adrenergic receptors are expressed on neurons, epithelial cells and other cellular components of the intestinal mucosa (Kulkarni-Narla et al. 1999) (Nijhuis, Olivier et al. ; Kulkarni-Narla, Beitz et al. 1999; Green, Lyte et al. 2003; Chiocchetti, Mazzuoli et al. 2008; Lyte, Vulchanova et al. 2011).

Norepinephrine (NE), released within the intestinal wall during activation of the sympathetic nervous system, has a wide variety of actions at the intestinal mucosa. Norepinephrine participates in the host-S*almonella* interaction by enhancing the growth of Salmonella *enterica* and other enteropathogens, such as enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) and *Yersinia enterocolitica* (Freestone, Haigh et al. 2007; Green and Brown 2010; Lyte, Vulchanova et al. 2011). This same neurotransmitter substancealters mucosal attachment, and therefore the invasiveness, of serovars of *Salmonella enterica* by acting on cells of the intestinal mucosa that express adrenoreceptors (Green and Brown 2010). In this same study, the electrical stimulation of enteric nerves increased *Salmonella typhimurium* internalization in ileal mucosa explants from swine (Schreiber, Price et al. 2007). These results suggest that enteric catecholaminergic nerves modulate *Salmonella* colonization of Peyer's patches at the earliest stages of infection, in part by altering epithelial uptake of bacteria(Brown and Price 2008).Furthermore, NE apparently activates the expression of virulence-associated factors in *Salmonella typhimurium*, including flagella-mediated motility (Bearson and Bearson 2008; Moreira, Weinshenker et al. 2010), and Type III protein secretion (Rasko, Moreira et al. 2008; Moreira, Weinshenker et al. 2010).Currently, the cellular mechanisms underlying these neurally mediated effects on *Salmonella* internalization in the intestinal mucosa are undefined.It has been proposed that catecholamines may regulate the sampling function of Peyer's patches in the control of the entry of pathogenic microbes or immune processing of the same at these intestinal sites (Green and Brown 2010).

4.2.2 The parasympathetic nervous system and the immune response to *Salmonella*

Efferent vagus nerve fibers innervate the small intestine and proximal colon of the gastro intestinal tract(Altschuler, Ferenci et al. 1991; Altschuler, Escardo et al. 1993),suggesting the possibility that cholinergic activity may modulate immune cells residing in, or recruited to, the densely innervated bowel wall(Van Der Zanden, Boeckxstaens et al. 2009). In fact, current knowledge indicates that the vagus nerve provides an important bi-directional communication circuit by which the brain modulates inflammation (Tracey 2002; Pavlov, Wang et al. 2003).

The presence of bacterial infection and inflammation can be detected by the sensory (afferent) vagus nerve and communicated to the nucleus tractus solitarus in the brainstem medulla oblongata. Neural communication between this other brainstem nuclei and "higher" brain structures, including the hypothalamus, are associated with the generation of brain-derived anti-inflammatory output through the efferent vagus nerve, which inhibits pro-inflammatory cytokine release and protects against systemic and mucosal inflammation. As acetylcholine is the principle parasympathetic neurotransmitter,this vagal function has been termed "the cholinergic anti-inflammatory pathway" (Borovikova, Ivanova et al. 2000; Tracey, Czura et al. 2001; Tracey 2002; Pavlov, Wang et al. 2003; Pavlov and Tracey 2005; Pavlov and Tracey 2006; Bonaz 2007; Gallowitsch-Puerta and Pavlov 2007; Tracey 2007; Tracey 2010).

Information about the role of the parasympathetic system and the immune response to S*almonella* is scarce. In one study, in *Salmonella typhimurium*-stimulated groups, inflammatory pathological changes were seen in ileum and the mesenteric lymph node. Whereas *Salmonella* induced a decrease in the level of CD4+ T cells in peripheral blood, such levels were restored to normal by a subdiaphragmatic vagotomy. The vagus nerve is involved in the transmission of abdominal immune information to the brain during *Salmonella typhimurium* infection, and it plays an important role in the maintenance of the immune balance of the organism (Wang, Wang et al. 2002). In another study, the specific inhibition of acetylcholinesterase (AChE), the enzyme that degrades ACh, rendered animals more resistant to infection by a virulent strain of *Salmonella typhimurium*, which correlated with the efficient control of bacterial proliferation in spleen. Immunologically, inhibition of AChE enabled the animals to mount a more effective systemic (inflammatory and anti-microbial) response, and to secrete higher levels of interleukin-12, a key T helper type 1-promoting cytokine. Thus, in one model of Gram-negative bacterial infection, cholinergic stimulation was shown to enhance the anti-microbial immune response leading to effective control of bacterial proliferation and enhanced animal survival (Fernandez-Cabezudo, Lorke et al.).

Currently, there is no evidence that the cholinergic anti-inflammatory pathway inhibits or enhances the immune response to *Salmonella* in the intestinal mucosa. However, taking into account that the anti-inflammatory activity of the cholinergic nervous system is based on cholinergic signals that are linked to macrophages and other innate immune cells, whichare central to the control of *Salmonella* infection, it is likely that the cholinergic nervous system attenuates the inflammatory response to *Salmonella* (Jones and Falkow 1996; Mittrucker and Kaufmann 2000; Wick 2004).

5. Neuroendocrine regulation of intestinal IgA and protection against *Salmonella*

Glucocorticoids, catecholamines and acetylcholine regulate the secretion of Intestinal IgA, which in turn plays a key role in protecting against *Salmonella* infection.Therefore; this molecule may mediate the effects of the CNS on the immune response to this bacterium.

Secretory immunoglobulin A (S-IgA) is the most abundant intestinal immunoglobulin. By binding to antigens, such as microbes and toxins, S-IgA prevents them from attaching to or penetrating the mucosal surface (Mowat 2003; Fagarasan and Honjo 2004; Kaetzel 2005; Cerutti and Rescigno 2008; Macpherson, McCoy et al. 2008; Brandtzaeg 2009). IgA is secreted into the intestinal lumen due to the cooperation of local plasma cells with epithelial cells. The polymeric IgA (pIgA) secreted by plasma cells diffuses through the stroma and binds to the polymeric immunoglobulin receptor (pIgR) on the basolateral surface of the epithelial cells to form the pIgA–pIgR complex, which in turn is translocated to the apical surface of epithelial cells, where it is cleaved and secreted into lumen as S-IgA (Norderhaug, Johansen et al. 1999; Kaetzel 2005).

In the intestinal lumen, S-IgA protects against infection by inhibiting *Salmonella* adhesion to epithelial cells and M cells and the penetration of this bacterium into deeper tissues (Michetti, Mahan et al. 1992; Michetti, Porta et al. 1994; Mittrucker and Kaufmann 2000; Matsui, Suzuki et al. 2003). However, little is known about the neuroendocrine regulation of intestinal IgA (Schmidt, Eriksen et al. 1999; Schmidt, Xie et al. 2007; Reyna-Garfias, Miliar et al. 2010).

5.1 Glucocorticoids and IgA

Glucocorticoids have several diverse effects on the production and secretion of IgA in the intestine. They have been shown to increase or decrease intestinal IgA levels, effects which may be species-dependent (Alverdy and Aoys 1991; Spitz, Ghandi et al. 1996; Reyna-Garfias, Miliar et al. 2010; Lyte, Vulchanova et al. 2011). Other studies have demonstrated that GCsreduce the number of IgA-producing cells in Peyer's patches of mice (Martinez-Carrillo, Godinez-Victoria et al. 2011), decrease the number of intraepithelial lymphocytes (IEL) in the proximal small intestine of mice (Jarillo-Luna, Rivera-Aguilar et al. 2007; Jarillo-Luna, Rivera-Aguilar et al. 2008; Reyna-Garfias, Miliar et al. 2010),andincrease the levels of mRNA for pIgR in the proximal duodenum of suckling rats (Li, Wang et al. 1999).Thus,it may be through the liberation of GCs that the CNS regulates the production and secretion of intestinal IgA specific to *Salmonella*.

5.2 Noradrenaline and IgA

Although the intestinal tract is a major site for mucosal immunity and is extensively innervated, little is known about the adrenergic regulation of enteric S-IgA secretion.Norepinephrine stimulates S-IgA secretion by acting through alpha-adrenergic receptors in the colonic mucosa, and in this way may enhance mucosal defense *in vivo* (Schmidt, Xie et al. 2007)]. This neurotransmitter also significantly increases pIgR mRNA expression and intestinal IgA concentration (Reyna-Garfias, Miliar et al. 2010). The increased expression of pIgR might contribute to an increasedsecretion of S-IgA in the gut, andthus a greater protectionagainst pathogens including *Salmonella*. A sympathectomy decreases the number of IgA-positive lamina propria cells in the weanling rat (Gonzalez-Ariki and Husband 2000). Furthermore, NE has been found to slightly increase the number of IgA-immunoreactive cells in the intestinal wall of marathon runners (Nilssen, Oktedalen et al. 1998).Finally, we have found that catecholamines reduce the number of IgA-producing cells in Peyer's patches of mice(Martinez-Carrillo, Godinez-Victoria et al. 2011), decrease the number of IEL in the proximal small intestine of mice (Jarillo-Luna, Rivera-Aguilar et al. 2008), increase the IgA concentration in rat small intestine(Reyna-Garfias, Miliar et al. 2010), and reduce the intestinal IgA concentration in mice (Jarillo-Luna, Rivera-Aguilar et al. 2007).

Although the effect of noradrenaline or adrenaline (catecholamines) on the production of IgA antibodies specific to *Salmonella* has not been studied, it is possible that the release of these molecules by the activation of the sympathetic-adrenal medullary axis may modify the production and secretion of intestinal IgA specific to *Salmonella*.

5.3 Acetylcholine and IgA

Some data indicate that intestinal secretion of immunoglobulin A is stimulated by the muscarinic effect of cholinergic agonists, which suggest that the basal secretion of immunoglobulin A may be influenced by the parasympathetic nervous system (Wilson, Soltis et al. 1982; Freier, Eran et al. 1987; Freier, Eran et al. 1989; Schmidt, Xie et al. 2007). However, there is no information about the role of the parasympathetic nervous system in the secretion of IgA during infections by *Salmonella*.

6. Neurotransmitters and neuroendocrine molecules: Substance P, cholecystokinin, Somatostatin and the Macrophage migration inhibitory factor (MIF)

Apart from the immune regulatory role of the classic neurotransmitters, acetylcholine and norepinephrine, both the sympathetic and parasympathetic subdivisions of the autonomic nervous system include several subpopulations of neurons that express several neuropeptides related to the modulation of the immune response. In this sense,corticotropin-releasing hormone (CRH), neuropeptide Y (NPY), somatostatin, and galanin are found in postganglionic noradrenergic vasoconstrictive neurons, whereas vasoactive intestinal peptide (VIP), Substance P (SP), and calcitonin gene-related peptide are found in cholinergic neurons (Charmandari, Tsigos et al. 2005; Kvetnansky, Sabban et al. 2009).

There are even some gut neuropeptides, including SP, neuropeptide Y and neurotensin, that possess inherent antimicrobial activity (Brogden, Guthmiller et al. 2005). The role of neuropeptides and their receptors in the inflammatory response to *Salmonella* and other invasive pathogens has scarcely been analyzed.

6.1 Substance P

Substance P participates in the intestinal immune response to *Salmonella* in several ways. Oral infection with *Salmonella* increases SP and neurokinin A mRNA precursors, and the expression of substance P receptors in Peyer's patches, lymph nodes and spleen (Bost 1995; Kincy-Cain and Bost 1996; Pothoulakis and Castagliuolo 2003). Substance P increases resistance to *Salmonella* by improving the activity of macrophages, and increases the production of IFN-γ and IL-12, which are part of the initial response to *Salmonella* that helps limit bacterial growth and dissemination (Kincy-Cain, Clements et al. 1996; Kincy-Cain and Bost 1997; Weinstock 2003). Thus, it is postulated that SP and its receptor may contribute to the mounting of a coordinated early immune response against *Salmonella* infection (Pothoulakis and Castagliuolo 2003; Weinstock 2003).

6.2 Somatostatin

Somatostatin (SOM) exerts an active role in the regulation of mucosal inflammatory responses (Pothoulakis and Castagliuolo 2003). SOM released from neuronal and non-neuronal cells distributed throughout the gastrointestinal tract may modulate la inflammatory response to *Salmonella* infection by inhibiting the release of pro-inflammatory cytokines such as IL-10 and IL-8 from intestinal epithelial cells (Chowers, Cahalon et al. 2000; Pothoulakis and Castagliuolo 2003).

6.3 Macrophage migration inhibitory factor (MIF)

The cytokine macrophage migration inhibitory factor (MIF) exerts a multitude of biological functions. Notably, it induces inflammation at the interface between the immune system and the HPA axis (Flaster, Bernhagen et al. 2007). The role of MIF in infectious diseases has scarcely been studied. MIF-deficient (MIF(-/-) knockout mice do not control an infection with wild-type *Salmonella typhimurium*. Increased susceptibility is accompanied by decreased levels of IL-12, IFN-γ, and tumor necrosis factor alpha, and markedly increases of IL-1β levels. Additionally, compared with control animals, infected MIF (-/-) mice show

elevated serum levels of nitric oxide and corticosterone. These results suggest that MIF is a key mediator in the host response to *Salmonella typhimurium*. Not only does MIF promote development of a protective Th1 response, but it also ameliorates disease by altering levels of reactive nitrogen intermediates and corticosteroid hormones, which both exert immunosuppressive functions (Koebernick, Grode et al. 2002). Epithelial MIF from cultured cells was found to be released predominantly from the apical side after *Salmonella* infection (Maaser, Eckmann et al. 2002).

6.4 Effect of these molecules on the production and secretion of IgA

The aforementioned molecules, in addition to their functions in the innate and cellular immune responses, affect the production and secretion of intestinal IgA. For example, the intravenous or intra-arterial injection of gut neuropeptides cholecystokinin, substance P and somatostatin increase S-IgA secretion in isolated loops of the rat small intestine and vascularly-perfused segments of the swine ileum (Wilson, Soltis et al. 1982; Freier, Eran et al. 1987; Freier, Eran et al. 1989; Schmidt, Xie et al. 2007).

7. Conclusion

CNS can regulate the immune response to *Salmonella* by the activation of both the HPA axis and the autonomic nervous system (including the sympathetic, parasympathetic and enteric divisions). Hormones, neurotransmitters, neuropeptides and neuroendocrine molecules mediate the effects of the CNS on the systemic and intestinal immune responses. In the intestinal mucosa, the CNS may modify the synthesis and secretion of IgA, which protects against the invasion by *Salmonella*.

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Porins in the Inflammatory and Immunological Response Following *Salmonella* Infections

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1. Introduction

Today numerous information are available on the molecular mechanisms activated by *Salmonella* and its components during the interaction with host cells and in determining the disease state. The molecular mechanisms of how *Salmonella* enter host cells and function as an intracellular pathogen are under intense investigation. Much progress has been made in identifying the bacterial factors that mediate invasion. Once *Salmonella* enters a cell, it remains within a membrane-bound vacuole and does not appear to fuse with lysosomes, the outcome of infection is determined both by bacterial and host factors, including the virulence of *Salmonella* strain, and the ability of the host to respond with an inflammatory and immunological reaction. The host response involves multiple cells that are resident at the site of infection or infiltrate from the circulation. Induction of an array of cytokines occurs in response to infection of macrophages with live *Salmonella* and after exposure to various *Salmonella* components including lipopolysaccharide (LPS), and porins. Of the different biologically active components present in Salmonella, LPS and porins are the most potent inducer of host response. LPS and porins present an intrinsic biological activity on cell involved in the inflammatory response and also on other types of cells; moreover they are immunogenic molecules against which the organism raises the humoral and cellular response. The LPS molecule is the most studied. Techniques previously used in the extraction of LPS from the endotoxin had greatly favoured the study of this portion of the macro-complex ignoring the protein fraction, allowing the identification of most of the effects of endotoxin with those of LPS. Subsequent extraction techniques for membrane proteins then allowed the study of the protein fraction, which was extracted globally in the endotoxin. Later experiments suggested that a chemical subunits of LPS, lipid A, was the actually toxic moiety and that the O-specific chain found on LPS was not involved in the toxic effect. The endotoxin-associated protein (EP) consists of a complex of 4-5 major proteins that range in size from 10 to 35 KDa. Originally considered to be a superfluous carrier of LPS, EP is now recognized to have potent biological activities, some of which are unique (Mangan et al., 1992). For example, EP is a

powerful mitogen for C3H/HeJ mouse and human lymphocytes which are hyporesponsive to LPS. Among the proteins associated with LPS, the techniques actually used for the extraction of native proteins from the cellular membranes allowed the isolation of outer-membrane proteins (OMPs). LPS and OMPs are released by different bacteria during both in vitro and in vivo growth and this release is significantly enhanced when the bacteria are lysed following exposure to antibiotics or human serum. Molecular complexes of LPS and OMPs, together with the other molecules which constitute the external surface of Gram-negative bacteria, are released as outer membrane vesicles (OMVs). OMVs are formed by blebbing and pinching of segments of the bacterial outer membrane.

2. Outer membrane and porins

The cell envelope of Gram-negative bacteria (Figure 1) is composed of two distinct membranes, the inner plasma membrane and the outer membrane. The peptidoglican layer is located between the two membranes and this area between the plasma membrane and the outer membranes is referred to as the periplasmic region. The outer membrane is composed of phospholipid and protein as is the cytoplasmatic membrane. The outer leaflet is occupied by about 45% lipopolysaccharide (LPS). The phospholipid is localized almost exclusively in the inner layer of the outer membrane bilayer. In *Salmonella enteric serovar Typhimurium,* the phospholipid composition of the outer membrane consists predominantly of phosphatidylethanolamine, phosphatidylglycerol and cardiolipin. LPS consists of a hydrophobic membrane anchor, lipid A, a short core oligosaccharide, and an O antigen that may be a long polysaccharide. The lipid A is rather well conserved among Gram-negative bacteria. The core oligosaccharide and O-antigen, if present, is the most variable part of LPS and shows even a high degree of variability between different strains of the same species. In bacteria the number of porin copies was determined to be up to 100.000 for cell. Porins form β-barrels with 14, 16 or 18 strands, all of which are connected by extraplasmic loops and periplasmic turns with the particularly long loop L3 folded inside the barrel. Porins of 16 strands are called general or non-specific porins and form pores allowing the diffusion of hydrophilic molecules, showing no particular substrate specificity; while 18-strands porins are substrate specific porins. The best-studied examples is the sucrose-specific porin ScrY from *S. typhimurium*. The three-dimensional structures of this specific porin has been elucidated, (Forst et al., 1998). ScrY forms homotrimers whose monomers consist of 18-stranded antiparallel β-barrels. As was found with the general porins, the third loop, L3, folds back inside the β-barrel. A peculiar feature of ScrY is the presence of a 70-residue-long N-terminal extension, which hangs out into the periplasm in the form of a parallel triple-stranded coiled-coil.

An homology based 3D structural model for the porin OmpC from *S. typhimurium* was built to understand the possible unique conformational features of its antigenic loops with respect to other immunologically cross reacting porins. The homology model was built based on the known crystal structures of the *E. coli* porins OmpF and PhoE. The resulting model was compared with other porin structures, having β-barrel fold with 16 transmembrane β-strands, and found that the variable regions are unique in terms of sequence and structure (Arochiasamy et al., 2000).

Recently, a structural model for a 50kDa antigen protein of *S. enterica serovar Typhimurium* was also built by Siew-Choong et al. (Yee et al., 2011). The characteristic of the built model also resembles the structure of known transmembrane proteins in other Gram-negative bacteria (S.

Galdiero et al., 2003). It shows a similar structure as the TolC transmembrane channel protein with the combination of β-barrel domain projecting from the membrane, across the periplasmic space with α-helical domain and the equatorial domain (mixed of β-sheets/α-helices). The upper part of the structure is open and could provide solvent access, while the lower part is narrowed. The structure shows that it may be an ion channel whose conductance depends on the open or close conformation at the lower end, which is similar with the characteristic of TolC and its analogues. The surface exposed loops might act as a "lid" to access into the top end of the β-sheets domain. The β-barrel domain consists of 16 strands, which is within the number of strands that has been characterized for other bacterial outer membrane proteins (S. Galdiero et al., 2007). The 40 Å long axis of the β-barrel domain fits into a lipid bilayer membrane which is typically 30 Å. As for other porins the base of the β-barrel is mainly composed of aromatic residues, specifically phenylalanine and tyrosine. These residues are usually found in a typical β-barrel membrane protein to define the inner edge of a lipid bilayer of membrane. The lower part of the built structure is the left twisted antiparallel-helices barrel, which is involved in the control of the opening or closing of the access.

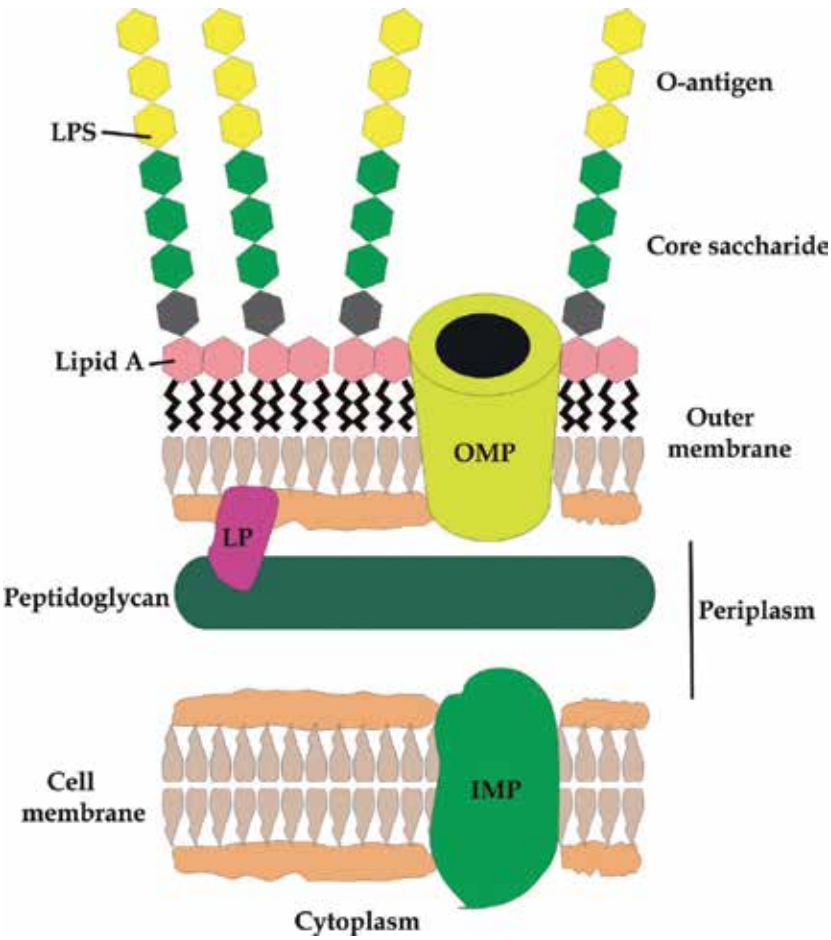

Fig. 1. Depiction of Gram-negative cell envelope. IMP, integral membrane protein; LP, lipoprotein; LPS, lipopolysaccharide; OMP, outer membrane protein.

3. Biological activity of porins

The release of porins at the infection site, whether secreted during growth or derived from the lysis of the bacterial cells, involve the host defence and influence the course of enterobacterial diseases.

Porins of *S. enterica serovar Typhimurium* added to macrophage cultures in vitro are able to modify several macrophage functions. The superficial hydrophobicity of macrophages adhering to the slide, as measured by the average contact angle monolayers, shows an increase of about 15-20%. The phagocitic index of macrophage treated with porins was found to be significantly lower. The same reduction was observed in the intracellular killing of macrophages treated with porins (Tufano et al., 1984). Porins inhibit phagocytosis by activating the adenylate cyclase system (Di Donato et al., 1986). The effect of *Salmonella* typhimurium porins was also studied on human polymorphonuclear leukocytes (PMNs). Labelled porins were shown to bind to the PMNs, and could be completely displaced by unlabeled porins. The binding caused modifications of membrane integrity and of the physico-chemical characteristics of the PMN surface, e.g. decreased oxidative burst, decreased hydrophobicity and altered cell morphology. The porins acted as both chemotaxins and chemotaxinogens. When PMNs were preincubated with porins their migration in the presence of commonly used chemoattractions (serum activated by zymosan or N-formyl-L-methionyl-L-leucyl-L-phenylalanine) was inhibited (Tufano et al., 1989).

S. enterica serovar Typhimurium porins injected into the paws of rats, induced a dose dependent edema which was maximal at 2 to 3h and still present at 5h. Edema was unaffected in animals which had their complement levels depleted, demonstrating that inflammation was not associated with complement activation; however it could be decreased by indomethacin and by dexamethasone. Rat peritoneal cell incubated with porins released histamine but little prostacyclin, suggesting that porins have little ability to induce the prostenoid producing enzyme cicloxygenase II (F. Galdiero et al., 1990).

Porins were also shown to kill D-glucosamine-sensitized LPS-responsive and LPS-unresponsive mice. A 100 μg amount of porins was sufficient to kill 80-90% of animals. But lethal effect of the porin preparation could be completely blocked by pre-administration of a neutralizing antiserum to TNF-α but was not abolished by polimixin B indicating that LPS did not contribute to the biological responses. Porins were also pyrogenic in rabbits and elicited a localized Swartzman reaction when used as the sensitizing and eliciting agent (F. Galdiero et al., 1994).

4. Porins interaction with host cell membranes and signal transduction

The cell ability to sense external stimuli and to react by initiating a program of expression often involves propagation of a cell surface-initiated signal along a specific pathway of protein kinases whose ultimate targets are nuclear transcription factors (Figure 2). These pathways consist of a cascade of biochemical events that include phosphorylation of a variety of kinases, that in turn modulate other factors that control gene expression. Porins purified from *S. enterica serovar Typhimurium* induce tyrosine-phosphorylation in THP-1 cells and in C3H/HeJ macrophages. After porin stimulation a pattern of tyrosine-phosphorilated proteins appeared in the soluble cytoplasmic fraction, the membrane fraction and in the

insoluble protein fraction. The events of tyrosine protein phosphorylation were present in macrophage from LPS-hyporesponsive C3H/HeJ mice stimulated with porins, while they were markedly reduced where the macrophage where stimulated with LPS-(R). Among the most prominent tyrosine phosphorylated bands in porin-stimulated cells there is a number of proteins with a molecular mass that is similar to that of the family of tyrosine/serine/threonine protein kinases. Mitogen-activated protein kinase (MAPK) cascade are among the best known signal transduction systems and play a key role in the regulation of gene expression as well as cytoplasmic activities.

MAPKs have been shown to be involved in the regulation of cytokine responses. MAPKs are activated upon phosphorylation of both tyrosine and threonine residues by MAPK kinase (MEK). These enzymes participate in cell signalling pathways leading to AP-1 and NF-κb activation following porin stimulation of cells. Raf-1 was also phosphorylated in response to the treatment of U-937 cells with porins; the porin-mediated increase in Raf-1 phosphorylation is accompanied by the phosphorilation of MAPK kinase 1/2 (MEK 1/2), p38, ERK 1/2 and C-Jun N-terminal kinase. P38 signalling pathway mainly regulates AP-1 and NF-κb activation in cells treated with *S. enterica serovar Typhimurium* porins. The transcriptional factor AP-1 is composed of multiple protein complexes formed between the protein products of proto-oncogenes C-fos and C-jun and their related gene family members. The fos family consists of C-fos, the gene for Fos-related antigen-1 (Fra-1), Fra-2 and Fas-B and its naturally truncated form Fas B2; the Jun family consists of C- jun, Jun B and Jun-D. The AP-1 family of transcription factors consists of homodimers and heterodimers of these subunities; the different complexes regulate their abilities to transactivate or repress transcription. AP-1 composition may change in the cell as a function of time and stimules; the binding affinity for a given target DNA sequence is determined by the different AP-1 dimer combinations and the context of the surrounding sequences. In U937 cells treated with porins from *Salmonella*, different complexes including C-Jun and Fra-2 subunits appeared. While in cells treated with LPS, the stimulus leads to AP-1 complexes containing Jun D, C-Fos and C-Jun, stimulation by porins induces AP-1 complexes containing Fra-2 in addiction to the other subunits (M. Galdiero et al., 2002). The formation of different complex represent a further difference between stimulation with LPS and stimulation with porins to be added to other observations: that cytokine release after stimulation with porins begins after 120 min and continues for 5 to 6 hours, whereas cytokine release following LPS stimulation begins after 30 min. and decrease at 120 min (M. Galdiero et al., 1995).

Porins trigger multiple synergistic signal transduction pathways, including protein kinase A (PKA), proteine kinase C (PKC), NT proteine tyrosine kinase (NT-PTKs). The role of PKC in signal transduction in mouse macrophages stimulated by *S. enterica serovar Typhimurium* porins is reported by Gupta S et al., (Gupta et al., 1999). Their experiments showed that porin activation of macrophages results in the increased inositol-triphosphate and intracellular Ca2+ mobilization: there is a translocation of PKC to the membrane which is accompanied by nitric oxide release.

Several polypeptide ligands use the JAK-STAT molecules in signal transduction (Darnell et al., 1994). The family of transcription factors called STAT (signal transducers and activators of transcription) have been found to be activated by the Janus Kinases (JAKs) that are associated with the cytokine receptor components (Kisseleva et al., 2002) . In resting cells the

STATS, when phoshorylated by the JAKs, dimerize via their SH2 domains and traslocate in the nucleus, where they interact with specific DNA sequences and transactivate the associated genes. The Jak/Stat signalling pathway plays a fundamental role in response to infection and in sepsis (Scott et al., 2002) In vitro experiments on U937 cells demonstrate a complex indirect mechanism of STAT-1 and STAT-3 activation after stimulation of cells with porins. The treatment with porins did not results in increase of JAK phosphorylation although STAT-1 and STAT-3 activation was observed. The activation of STAT-1/STAT-3 by porins can occur through the activation of MAPK and possibly other PTKs but not through JAK activation (M. Galdiero et al., 2006).

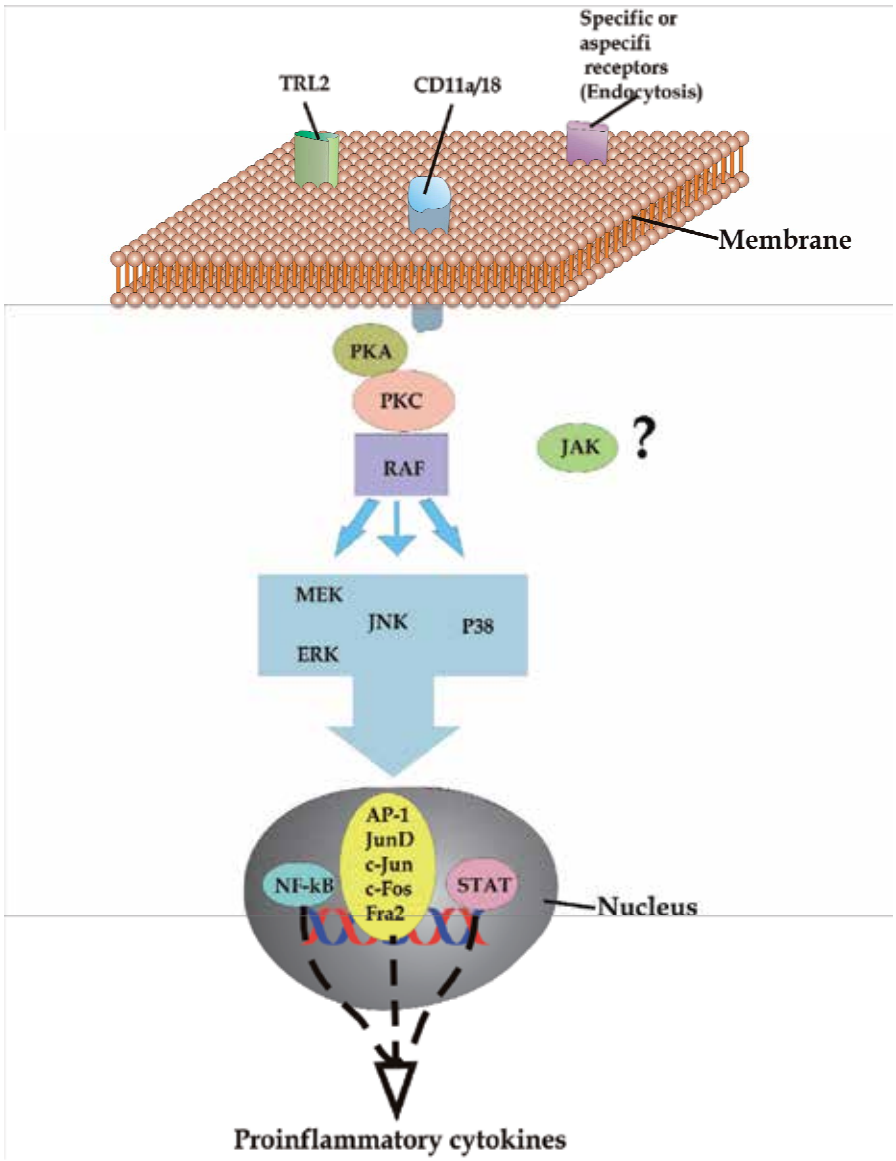

Fig. 2. Porin signal transduction pathways

5. Cytokines release by porins

Many of the pathophysiologic mechanisms of Gram-negative bacterial infections are due to bacterial surface components acting on cells directly or via mediators such as cytokines. Cytokines are polypeptides that exert a wide spectrum of biological effects, including haematopoietic, metabolic, inflammatory and immunologic homeostasis. Among the large family of cytokines, porins by *S. enterica serovar Typhimurium* induce the release of TNF-α, IL-1, IL-6, and TGF by macrophages and IL-4 and IFN-γ by lymphocytes. The role played by porins in the production of cytokines derives from the comparison with LPS-S e LPS-R extracted by the same strain of *S. typhimurium*. Porins at 1 μg/ml induce the greatest release of TNF-α, IL-1α and IL-6 by monocytes and IL-4 by lymphocytes, while porins at 5μg/ml induce the greatest release of IFN-γ by lymphocytes. The R-form of LPS (LPS-R) induces the greatest release of TNF-α and IL-1α by monocytes when used at 1 μg/ml concentration. At concentration of 5 and 10 μg/ml, respectively, LPS-R induce the maximal release of IL-6 from monocytes and the maximal release of IL-4 from lymphocytes. The S-form of LPS (LPS-S) induces the greatest release of TNF-α, IL-1α and IL-6 by monocytes and that of IL-4 by lymphocytes when used at a concentration of 1 μg/ml. Porins (5 μg/ml) induce the release of IL-8 by THP-1 cells after 24h of stimulation (Vitiello et al., 2004). The level of IL-8 in THP-1 cells stimulated with porins was comparable to that induced in response to 1 μg/ml of LPS-R.

While CD-14, CD-11/18 and Toll receptors 2 and 4 appears to be very important LPS signal transducer, porin-specific receptors are still unknown. Therefore, it is possible that porin stimulation is not due to binding to specific receptors, but the consequence of the perturbation of the cell membrane lipoproteic phase, induced during adsorption or porin penetration. CD-14 is a glycosyl-phosphatidyl inositol linked 55 kDa protein present on the surface of monocytes and polymorphonuclear leucocytes, and it function as the cell surface receptor for LPS and several surface components of Gram-positive bacteria. CD14 is also found as a soluble protein (sCD14) in human serum. CD14 lacks transmembrane and cytokine-binding domains and is not believed to have intrinsic signalling capabilities. Toll-like receptors 4 (TLR4) appears to be very important LPS signal transducer. It's thought that, also, Toll-like receptor 2 (TLR2) functions as a signal transducer upon LPS binding by CD14. TLRs make up a family of evolutionary conserved pattern recognition molecules that are important signal transducers for the induction of mammalian innate immunity responses, including cytokine responses. The best characterized TLRs to date are TLR2 and TLR4. TLR2 is involved in the recognition of a wide assay of bacterial products, including peptidoglycan, lipopeptides, zymosan and bacterial lipoproteins, whereas TLR4 is activated by LPS. CD 14 acts as a abroad specificity coreceptor that can enhance cell activation induced by TLR4 or TLR2 agonists. Data from *Haemophilus influenzae* (Hib) porin and from neisserial porin P or B indicate that porins from different bacteria may be recognized by TLR-2 (M. Galdiero et al., 2004). The Hib porin-induced TNF-α and IL-6 production was eliminated in macrophages from TLR2 or MyD88 deficient mice. In contrast, macrophages from LPS hyporesponsive C3H/HeJ mice which are defective in TLR4 function, responded normally to Hib porin. Neisserial porin adjuvant activity was mediated by surface expression of B7-2 and class 2 major histocompatibility complex on B cells by TLR-2-dependent mechanisms; the presence of the adaptor molecule MyD88 was also required.

CD11/18 (M. Galdiero et al., 2004) integrin may also participate in LPS signalling. This family of receptors are heterodimeric cell surface glycoproteins composed of a CD11 and a

CD18 subunit. The release of TNF-α, IL-6 and IL-8 by THP-1 cells stimulated by porins is independent of CD14, but is partially dependent on CD11/18 integrins. *S. enterica serovar Typhimurium* porins enhance the synthesis and release of IL-6 in U937 cells regulating the transcriptional activity of IL-6 gene by nuclear transduction of NF-κB. The characterization of the human IL-6 promoter revealed a highly conserved control region of 300bp upstream of the transcriptional initiation site that contains the elements necessary for its induction by a variety of stimuli commonly associated with acute inflammatory or proliferative states. In particular, electrophoresis mobility shift assay, as well as promoter deletion and point mutation analysis, revealed the presence of an NF-κB binding element. In U937 cells stimulated by *Salmonella* porins, NF-κB is able to enhance IL-6 gene promoter activity. Activation of this nuclear factor may be responsible for porin induced expression and release of IL-6 (Finamore et al., 2009).

These observations allow to outline a specific ability of porins that is expressed by stimulation of the cell surface, signal transmission, activation of nuclear factors, activation of gene promoters and finally release of cytokines.

6. Transmigration of leukocytes following porin activation

It has long been recognized that *Salmonella* provokes un intense intestinal inflammatory response, consisting largely of neutrophil migration across the epithelial lining of the intestine (M. Galdiero et al., 1999); this inflammatory event manifests as an epithelial dysfunction, namely diarrhea. In an in vitro model, Mc Cornick et al (McCormick et al., 1995) showed that *S. typhimurium* tran-epithelial signaling to polymorphonuclear neutrophils (PNM) plays a direct and substantial role in stimulating enteritis in humans. Leukocyte-endothelial cell interaction both in vivo and in vitro are active multistep processes. The initial adhesion of circulating leukocytes to vascular endothelium is induced by interaction of constitutively functional leukocyte homing receptors with regulated endothelial cell ligands. During inflammation a dramatic increase of endothelial cell surface molecule expression occurs that support the adhesion of circulating leucocytes. Bacteria or bacterial products may constitute important inducers of surface molecule expression on endothelial cells. LPS-S induce adhesion of leucocytes to endothelial cells as potent as that induced by IL-1β (Takeuchi et al., 1967) *S. typhimurium* porins and LPS-R are able to induce the release of s-E-selectin and sICAM-1 from human umbilical vein endothelial cells (HUVEC) and also were to up-regulate the surface expression of E-selectin and ICAM-1 on endothelial cells (Donnarumma et al., 1996).

Treatment of the HUVEC with either porins or LPS in the form S or R increased the transmigration of different leukocyte populations, in particular that of neutrophils; transmigration increased remarkably during the simultaneous stimulation of endothelial cells by IL-1β together with either porins or LPS (M. Galdiero et al., 1999). Porin treatment caused transmigration that lasted several hours longer than that caused by LPS and further increase the activation of those cells already activated by IL-1β. Consequently, the in vivo activity of the two molecules shows an effect prolonged in time. In vitro, the simultaneous stimulation of endothelial cells with IL-1β and either porins or LPS causes overlapping effect leading to a very high migration index. Neutrophil transmigration was partially inhibited by monoclonal antibodies (MoAb) binding to E-selectine; the transmigration of lymphocites and monocytes was partially inhibited by MoAbs anti-VCAM-1; the transmigration of

neutrophils, lymphocytes and monocytes was partially inhibited by MoAb anti-ICAM1. Monocyte and granulocyte transmigration was, also, inhibited by MoAbs binding to CD11a/CD18 and CD11b/CD18. Lymphocyte transmigration was inhibited by MoAbs CD11a/CD18 and not by CD11b/CD18. Therefore, porins may constitute important inducers of surface molecule expression on endothelia cells. This ability makes these molecules particularly important in the inflammatory process during *Salmonella* infections.

7. Porin immunogenicity

Porins demonstrate immunogenic and adjuvant properties. Heat denaturable surface components play a role in inducing protection to *S.enterica serovar Typhimurium* infection in mice (Isibasi et al., 1994). The protective role of the outer membrane proteins or of porins from Neisseria (Melancon et al., 1983), *Salmonella* (Muthukkmar et al., 1993), Haemophilus and Vibrio genus has been shown. Antiporin antibodies have been demonstrated to be bactericidal and opsonic (Isibasi et al., 1994); patients with pelvic inflammatory disease (PID), who recover spontaneously have high levels of antiporins antibodies showing that the presence of serum antibodies to neisserial porins may correlate with protection against PID. Several studies have been recently performed with porins extracted from *N. meningitidis, N. gonorrhoeae* and *H. influenza* (Massari et al., 2003; Song et al., 1998; Wetzler et al., 1996). Porin vaccines have also been developed to protect against *S. typhimurium* infection. Porins are excellent antigens, efficiently stimulating humoral and cell-mediated immune response of the host immune systems which could play a role in the protection against the disease. *S. enterica serovar Typhimurium* porins are also able to induce expression of CD86 on antigen–presenting cells (Massari et al., 2002). Macrophages from mice immunized with porins and infected later with Salmonella, express 53% more B7 versus control macrophages and can therefore support a host–protective immune response.

Complement is an important arm of innate immune defenses against invading pathogens. Complement activation leads to the deposition of C3 fragments, which can enhance opsonophagocytosis of microbes. Porin contribute to complement activation mainly through the classical pathways in an antibody-indipendent manner. All of the porins tested to date have been shown to bind fragment C1q, the first component of the classical pathway of the complement system. The effect of porins purified from *S. enteric serovar Tiphimurium* on the complement system was investigated both in vitro and in vivo. Incubation of porins with either human or guinea pig serum resulted in the consumption of the total complement activity when an amount of porins ranging from 8 to 10 μg per 100 ml of serum was used. The activation of complement system was temperature dependent, suggesting an active process rather than passive adsorption of the complement components by porins. In addition, the activation had a fast kinetic and proceeded mainly through the classical pathway. This conclusion is supported by the consumption of C1s and C4 in normal human serum treated with porins and also by the depletion of C3 activity in the C1s-deficient serum which was marked only when purified C1s was added to the serum before incubation with porins. Injection of 100 μg of porins into guinea pigs induced profound complement consumption at 6 h post-injection that persisted up to 12 h (F. Galdiero et al., 1984). Also porin Omp K 36 from *Klebsiella pneumonia* interact with C1q.

The cloning of the genes encoding the outer membrane proteins has facilitated the production of pure porins free from other bacterial antigens for investigation as potential

products for protective responses. The protection against bacterial infection is correlated to the presence of the antibodies with the ability to activate complement mediated killing of bacterial cells. The amminoacid sequences of the superficial loops of porin are highly variable in different strains and are the regions actually responsible for stimulation the production of bactericidal antibodies (Snapper et al., 1997). The role of surface loops of porin in immunological responses has been also studied in Haemophilus. Protein P2 of H. influenzae is a homotrimeric porin, which constitutes approximately one-half of the total outer membrane protein and it is an important target of the immune response to Haemophilus. P2 contains 16 transmembrane regions with β-sheet conformation and 8 suface-exposed loops. Analysis of sequences of P2 genes indicates that the transmembrane regions are relatively conserved among strains while considerable heterogenicity exists in surface-exposed loops (Qi et al., 1994). Challenging with whole bacterial cells resulted in a prominent antibody response directed at the P2 molecule. Analysis of the antibodies to whole organisms, and peptides corresponding to each of the eight loops of P2 by immunoassay revealed that bacterial antibodies were prevalently specific for loop 5, a highly variable region, and for loop 6, a conserved surface exposed loop.

Infection of mice with *S. typhimurium* is widely accepted as a valuable experimental model for human typhoid fever. Oral infection with *S. enteric serovar Typhimurium* induces a strong T helper 1 (Th1) response that is responsible for the CD4+ T-cell-mediated protection. Cytokines such as IFN-γ and TNF-α released by Th1 cells activate bactericidal pathway in macrophages. Moreover, CD 4+ T lymphocytes help B lymphocytes to produce antibodies and salmonella-specific CD8+T lymphocytes. Various studies have demonstrated that porins induce a Th1 response (Gupta et al., 1999, M. Galdiero et al., 1998) reported that porins of *S. enteric serovar Typhimurium* elicit Th1 response in the host. In fact, porins appear to stimulate T cell proliferation in the presence of macrophages incubated with dead bacteria, live *Salmonella* infected macrophages stimulated a minor proliferation compared to dead *Salmonella* incubated macrophages. Furthermore infection with live *Salmonella* induced the loss of accessory molecules, such as B7 and ICAM-1 on macrophages. OMPA of *S. enteric serovar Typhimurium* activate dendritic cells and enhances Th1 polarization (Lee et al., 2010). Others studies demonstrate that purified porins are able to induce a different response to that induced by the porins present on the *S. typhimurium* cell surface. Porin treated or orally infected mice show anti-porin antibodies with bactericidal activity. The complete adaptive transfer of resistance to *S. typhimurium* infection is achieved only using splenic T cells from survivor mice after experimental infection. After stimulation with specific antigen in vitro CD4+ cells from porin-immunized mice released large amounts of IL-4, while CD4+ cells from *S. typhimurium* infected mice predominantly secreted IFN-γ. Limiting dilution analysis showed that infection resulted in a higher precursor frequency of IFN-γ producing CD4+ T cells and a lower precursor frequency of IL-4 producing CD4+ T cells, while immunization with porins resulted in a higher precursor frequency of IL-4 producing cells and a low frequency of IFN-γ producing cells.

Analysis of polymerase chain reaction-amplified cDNA from the spleens of infected mice demonstrated that IFN-γ, IL-2 and IL-12 mRNA were found 5 days after in vitro challenge and increased after 15 days; IL-10 expression was rarely present after both 5 and 15 days, while IL-4 mRNA expression was not detected. In porin-immunized mice, the IL-4 mRNA expression increased after 15 days, IFN-γ mRNA expression decreased after 15 days, while IL-2, IL-10 and IL-12 mRNA remained relatively unchanged (M. Galdiero et al., 1998) Other

works demonstrated that subletal doses of live *S. typhimurium* give rise to an IFN-γ dominant Th1–like immune response whereas heat-killed bacteria generate an IL-4 dominant Th2-like response (Thatte et al., 1993). Therefore, during experimental oral infection with *S. typhimurium* in mice a T-lynphocyte differentiation occurred, leading to prevalent TH1 response, while the immunization with isolated porins did not induce in vivo a similar pattern of differentiation. During the initial phase of infection with virulent strains which express large amounts of porins on their surface, *Salmonella* are present in the bloodstream and are resistant to complement-mediated lysis (Munn et al., 1982); bacterial cell is able to survive and entry into macrophages and therefore is resistant to bactericidal anti-porin antibodies. In this phase of infection, cell-mediated immunity is important for protection against typhoid fever. Transfer of immunity experiments have demonstrated that CD4+ cells, CD8+ cells and serum are all required to protect mice from challenge with virulent *S. typhimurium* (Mastroeni et al., 1993).

8. Conclusion

Porins from several Gram-negative bacteria, including Salmonella, play a fundamental role in the host-pathogen interaction, eliciting diverse biological proinflammatory activities and immune responses.

Porins present an intrinsic biological activity when interacting with eukaryotic cells, but also behave as antigens stimulating specific immune responses. Porins of *Salmonella* have endotoxin-like effects such as lethal action, the ability to elicit a local Shwartzman reaction, to activate the complement system and pyrogenicity. Furthermore, the porins stimulate pro-and anti-inflammatory cytokine synthesis and release. It has been established that protein tyrosine phosphorylation plays a central role in porin mediated transduction processes. Signal transduction pathways and transcriptional activation known to occur during immune cell activation have been widely investigated in cells stimulated by porins. Bacterial porins also may constitute important inducers of surface molecule expression on endothelial cells and contribute to endothelial transmigration of leucocytes. The protective role of porins from *Salmonella* and other bacteria has been demonstrated. Anti-porin antibodies have been shown to be bactericidal and opsonic; patients with pelvic inflammatory disease who self cure present high levels of antiporins antibodies. Porins also play an important role in the development of cellular immunity. During experimental oral infection with *S. typhimurium* in survivor mice a T-lymphocyte differentiation occurred leading to a prevalence of the Th-1 response, while the treatment with purified porins did not induce in vivo a similar pattern of differentiation. Transfer of immunity experiments have demonstrated that $CD4^+$, $CD8^+$ cells and serum are all required to protect naive mice from challenge with virulent *S. typhimurium*. These studies have led to the establishment of a multiplicity of targets for novel therapies.

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21

Serology as an Epidemiological Tool for *Salmonella* Abortusovis Surveillance in the Wild-Domestic Ruminant Interface

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1. Introduction

Salmonella sp, are opportunistic pathogens that can infect a wide range of hosts, including man (Murray, 1991). The increasing numbers of *Salmonella* infections reported in the last decades reveal an important health problem of considerable socio-economic impact (Kapperud et al., 1998). Salmonellosis has been reported in 85% of food-borne bacterial enteritis in humans from Spain (Pérez-Ciordia, et al; 2002), and *Salmonella* sp, are increasingly recorded in animals (Echeita et al.; 2005).

Unlike other *Salmonella* species, *Salmonella enterica* subspecies *enterica* serovar Abortusovis (S. Abortusovis) is adapted to sheep, and considered to be host specific (Jack, 1971). Discarded as a zoonotic pathogen, its importance lies in the economic losses that occur in ovine production systems in regions that depend on sheepherding (Pardon et al., 1988; Sojka, et al., 1983). It has been most frequently associated with ovine salmonellosis in ovine flocks from Europe and the Middle East, causing abortion outbreaks, stillbirths, and illness in lambs infected at birth (Jack, 1968; Pardon et al., 1988). These mainly result from the epidemic behavior of the disease, which is most recognized when the organism is newly introduced into a flock, because abortion storms reach high proportions. In endemic scenarios it also causes abortions in up to 50% of the ewes in a flock, usually during the first pregnancy, as in newly introduced (González 2000).

Available epidemiological data show a limited distribution for Abortusovis serovar. It is considered rare in most countries and regions of the world except in Europe, where it is particularly common, with reported cases in France, Spain, Germany, Cyprus, Italy, Switzerland, Russia, and Bulgaria, southwest England and Wales and also in Western Asia (Jack., 1968; Echeita et al., 2005; Valdezate et al., 2007). In northern Spain it has been considered to be among the major etiological agents of ovine abortion (González, 2000) , but it is also spread through 11 Spanish provinces, where 20 different clones have been identified in fifty-five field strains collected from epidemic abortions or neonatal mortality episodes affecting different ovine flocks during the period 1996–2001 (Valdezate et al., 2007).

The infection can appear in naive flocks by means of animal carriers such as new sheep replacements, contact with other animals in seasonal migration, wild and carrion birds, or

rodents (Valdezate et al., 2007). Sensitive animals acquire the infection by ingestion of food and water contaminated by vaginal discharges, placenta, aborted foetus (liver and stomach contents), and infected newborn. Furthermore, in some conditions, faeces, milk and respiratory secretions can correspond to infectious material. Other routes of acquisition include respiratory and conjunctival routes (Jack., 1971).

From the third month of pregnancy, this pathogen induces abortion, in the absence of other clinical symptoms (Jack, 1971), but it is sometimes preceded by depression, uncertain walking, mucous vaginal discharge and diarrhoea. Following this, ewes seem to be healthy or show transient fever, but sometimes ewe mortality occurs from septicaemic complications like anorexia, acute metritis, enteritidis and peritonitis that result from placental retention (5–7% of cases) (Astorga et al., 2000), differing from infection causes by Dublin and Typhimurium serovars. In addition, neonatal mortality of lambs is frequent with living muttons at term which are non-viable and die within a few hours of birth from septicaemia. Occasionally, lambs appear to be healthy but die during the first month, showing signs of enteritis, pneumonia or polyarthritis. Conversely, the infection is asymptomatic in non-pregnant ewes and rams (Uzzau et al., 2001).

Within a flock, *S.* Abortusovis is maintained by effective transmission from infected to susceptible sheep through the oral, conjunctival, or respiratory routes, while venereal infections appear to be of minor importance (Uzzau et al., 2001). Spread to other susceptible populations is mostly the consequence of commercial translocations of asymptomatic carriers. The dissemination of *S.* Abortusovis by food, water, birds or other mammals, has been traditionally considered as negligible. But as well as the host-specificity to sheep of *Salmonella* Abortusovis, its adaptation to other mammals can be discussed: mice and rabbits can be experimentally infected, and it has occasionally been isolated from goats and rabbits.

Wild ruminants can act as asymptomatic carriers of pathogenic *Salmonella* serovars (Cubero et al., 2002; Renter et al., 2006), and some serovars can also cause clinical disease in deer species (Foreyt et al., 2001; McAllum et al., 1978). However, this bacteria has not been related to abortion in non domestic species.

Many European countries face difficulties in controlling *S.* Abortusovis disease because there are no ways to diagnose all infected animals (Lantier et al., 1983). In this sense, it is essential to consider sampling procedures (e.g. type of samples, sampling frequency) according to the objectives of the testing program, clinical findings, level of detection or precision of prevalence estimates required, cost and availability of sampling resources and laboratory facilities. In recent years a standard method for detecting *Salmonella* from primary animal production has been developed and evaluated, and an ISO-method (ISO 6579:2002 Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp., Annex D) has now been adopted), and diagnosis procedures have been well defined (OIE, 2010).

The identification of the *S.* Abortusovis is based on the isolation of the organism; when infection of the reproductive system, abortion or conceptus occurs, it is necessary to culture fetal stomach contents, placenta and vaginal swabs. Nevertheless, few epidemiological surveys have been able to be carried out owing to the low number of available *S.* Abortusovis isolates (Valdezate et al., 2007).

In this sense, serology can improve the study of natural infections through identification of infected herds, rather than to confirm infected individual animals; although repeated herd tests can be used as an aid to detect chronic carriers. Antibodies to *S.* Abortusovis may become undetectable in some sheep 2–3 months after abortion. Flock diagnosis of *S.* Abortusovis in sheep can be performed by serological tests conducted on a statistically representative sample of the population, but results are not always indicative of active infection (OIE, 2010). These include serum agglutination test (SAT), hemagglutination inhibition, complement fixation, indirect immunofluorescence, gel immunodiffusion, and enzyme–linked immunosorbent assay (ELISA) (Davies, 2004).

In Spanish mountain rural areas extensive-grazing farming and game management practices favor the establishment of multispecies assemblies with a high diversity of herbivore species that share pastures and forests. There, sheep can be considered as primary hosts of *Salmonella* Abortusovis, but the potential dissemination of this agent to other possible sensible ruminant species has not been considered in epidemiological studies, even though specific antibodies have been found in Spanish ibex (*Capra pyrenaica*), fallow deer (*Dama dama*), European mouflon (*Ovis aries*) and red deer (*Cervus elaphus*) from Southern Spain (Pérez, 2007; León et al., 2002). In the Serranía de Cuenca domestic ruminant flocks find sympatric conditions for grazing with wild populations of red deer, fallow deer, roe deer (*Capreolus capreolus*), European mouflon and Spanish ibex, were introduced for big game hunting purposes from 1960 to 1979, and have successfully colonized the territory 30 years later.

2. Objectives

The aim of this study was to identify antibody responses against *Salmonella* Abortusovis in sheep flocks by means of the serum agglutination test (SAT), and evaluate the possibility of adapting it to official veterinary programs sampling efforts. A further aim was to evaluate the potential application of this test for screening *Salmonella* infections in cattle herds and wild ruminant populations. We therefore estimated the risk factors of sero-conversion against specific antigens of *Salmonella* Abortusovis in wild and domestic ruminants, and analyzed the statistical association between the sero-epidemiological indexes of each population and the related environmental conditions in the grazing pastures of the "Serranía de Cuenca Regional Game Reserve", where traditional shepherding constituted the core of the economic activity as in other many regions of Castile La-Mancha, Spain.

3. Material and methods

3.1 Study area

The Serranía de Cuenca Natural Park is one of the best conserved mountainous areas of Castile-La Mancha, Spain. The study area is situated in the centre-east of the Iberian Peninsula (40º 12´- 40º 28´ N / 1º 51´- 2º 03´ W), attached to the limits of the "Serranía de Cuenca Regional Game Reserve". It has a Mediterranean climate tempered by the altitude and barrier effect from the local orography to the wet winds from the west that confer a temporal semi-damp hydrologic pattern (annual rainfall varies between 600 and 1.000 mm, which is more intense from November to January), with long periods of drought during the hottest months (normally July and August). The climatic conditions that occur are typical for the Mediterranean Mountains, with temperate weather (Mean temperatures between 7'5 and

12'5ºC), cold winters (Mean temperatures fall below the 0ºC between 60 to 120 days) and dry, but not excessively hot, summers (law-ranking decree 99/2006). Vegetation is dominated by forests: Special importance is given to those constituted primarily by *Pinus nigra* Subsp. *Sazlmanii*, and the Oromediterranean forests of *Pynus sylvestris*. The latest, integrate the climax vegetal community at altitudes that limit the Mediterranean forests (*Quercus faginea* and *Q. ilex*), or on not suitable soils for the Sabin (*Juniperus sabina*), while Mediterranean scrubland (*Crataegus monogyna*, *Ligustrum vulgare*, *Viburnum lantana*, *Rhamnus saxatilis*) and pastures (*Poo-Festucetum istricis* association) present limited extensions (Peinado et al., 1985).

The "Serranía de Cuenca Regional Game Reserve"is an open area of 25.850'9 hectares covering eleven public mountains with free access to people, an open private property (El Maillo) and a fenced private property (Valsalobre), where human activities are restricted to traditional non-intensive use, such as eco-tourism, wood production, extensive farming, and hunting. In the core of this territory the human activities, including farming, are not allowed in a valley of 910 hectares, known as "El Hosquillo", which is partially fenced for its administration as Experimental Game Park. In contrast, game ungulates share the forest's pasturelands with cattle and small-ruminants herds in the surrounding protected areas.

3.2 Ruminant populations

The bio-climatic conditions in the region traditionally favor extensive ruminant farming system, specially focused on summer-grazing migratory merino-sheep flocks, for wool production (Cava, 1994). During recent decades this system has been modified by different social and economic influences that have led to a current orientation of the production to lamb meat, employing mixed raced animals for such purpose. On the other, goats have always played a self-sufficient role in the human communities of the Serranía de Cuenca and are usually maintained in the sheep flocks as guides or stepmothers, but are not of relevant economic importance. In contrast, beef productions are gaining importance, and the traditional local use of bovines as working elements is no longer practised. During the years 2.003-2.005 permissions for grazing in the "Serranía de Cuenca Regional Game Reserve" allowed the herding of 12.881 domestic ruminants in flocks classified by species composition in: 19 ovine, 14 ovine-caprine, and 6 bovine (Martín Atance, 2.009).

Big game hunting was historically practised in the area until the autochthonous wild ruminant populations became extinct, at the end of the 19th century. In order to encourage this activity, red and fallow deer were introduced in the "Serranía de Cuenca Regional Game Reserve" from 1960 to 1966, the European mouflon from 1974 to 1977, and Spanish ibex from 1976 to 1979 (Rojo Arribas, 2007). In 2001 the free ranging population sizes estimated in the Game Program of the "Serranía de Cuenca Regional Game Reserve" were: 850 red deer, 617 fallow deer, 300 mouflon and 230 Spanish ibex, in 25,724 hectares; and in semi-captivity: 209 red deer, 32 fallow deer, 91 mouflon, and more than 11 Spanish ibex at the fenced hectares in "El Hosquillo" Game Park (Martínez & Verona, 2002).

3.3 Collection and preparation of serum samples

The sampling of domestic ruminants herds that grazed at the study area were collected in two annual Livestock Sanitary Surveys: In 2,004, 241 blood samples were collected from cattle (5 herds), and 1,196 from sheep (13 herds), and in 2.005 samples were taken from 166

cattle (3 herds), and 2,543 from sheep (27 herds) in the course of Official Veterinary Programs. Sampling of wild ruminants was performed from 2,003 to 2,006 on 885 free ranging animals hunted in the "Serranía de Cuenca Regional Game Reserve", and from 225 live animals captured in the Game Park.

Blood samples were taken from the jugular veins in live animals, and from the heart in hunter-harvested wild ruminants. The samples were placed in test tubes and sent to the laboratory under refrigeration (4ºC). They were then centrifuged and the serum was frozen at –80ºC.

Animals were classified into four age groups: young (under 1 year), juveniles (1–2 years), adults (2-9 years), and old (over 9 years).

3.4 Serological procedures

We employed the Serum agglutination test (SAT) to evidence specific antibodies to *Salmonella* Abortusovis O antigen in 5,256 sera from 7 ruminant species: 3,739 from sheep, 556 from fallow deer, 407 from cattle, 314 from red deer, 211 from European mouflon, 21 from Spanish ibex and 8 from roe deer.

SAT were performed according to standard procedures (Pardon et al., 1983; Lindberg, & Le Minor, 1984; Sanchis et al., 1985; Sanchis et al., 1991; OIE, 2010). They were adapted to the microtitre format and used to determine somatic and flagellar titres of specific antibodies induced by *Salmonella* enteritidis subsp. enteritidis serovar Abortusovis natural infections. Antigens used were a serogroup B (O) antigen (Salmonella O Antigen -2840-56-3 Difco®) and a serogroup C (H) antigen (Salmonella Flagelar H Antigen -2846-56-7 Difco®). Negative (serum and normal saline) and positive (Standard sera Salmonella O Anti-sera Grupo B, Difco®, and Salmonella Flagellar Poli Anti-sera Difco®, respectively) controls were included in each test run as confirmatory method for quality control of SAT. Normal saline solution (0'85%) and bi-distilled water Mili-Q were also employed in test procedures.

Sera were screened at dilutions of 1:10 to 1:1.280; 50 µl ml of (O) antigen and added 100 µl of serum pre-diluted to 1:10. The plates were covered by a film and shacked automatically (40-50 rpm) for 5 minutes at 37º C. They were then incubated at the same temperature, without movement, for 18 hours. Sera that presented a positive reaction (from 1:20) were retested with (H) antigen.

To interpret the results of each test run the control wells were examined first, in order to confirm absence of agglutination in the negative controls, and agglutination in the positive controls. Agglutinations appeared as a "matt" or "carpet" at the bottom of the positive O antigen control wells and loose, woolly or cottony in the case of H antigens. In each sample, the highest dilution of serum that produced a positive agglutination was taken as titre.

Samples that held titres over 1:20 to both antigens were considered as sero-positive to *Salmonella* Abortusovis. The sera that reacted at 1:10 against any of the antigens were considered as doubtful, and as negative when agglutination was not observed.

3.5 Statistical tools and definition of epidemiological indexes

In order to describe the immune reactions observed, interpreted as previous exposure to *Salmonella* Abortusovis, and analyze the factors that could contribute to explain the

variations observed between animals and populations, we performed statistical analysis (Caughley, 1977; Crawley, 1993; Petrie & Watson, 1999; Siegel, 1956; Daniel, 1993) suited to the methodological principles of Epidemiology (Thrusfield, 1990; Goldberg, 1994).

As a descriptive study, the main objective was to determine the patterns of sero-conversion presentations. The information obtained from every sample was registered in qualitative terms (presence or absence of sero-conversion), according to the evidence of previous exposure to *Salmonella* Abortusovis; and in quantitative terms related to the intensity of immune recognitions (titre values). The intensity of immune responses in animal groups was expressed by means of the Geometric Mean Titre (G.M.T.), calculated as described by Thrusfield (1995), and in order to measure the variability within groups we have used the standard deviations of the Inverse function of the titres.

The temporal relationships between host, agent, and environmental factors influence the risk of disease. They have been considered here in association to the Prevalence term, defined as the proportion of a population affected by a disease at a given point in time, and interpreted as the probability of a subject in a defined population being diseased at a particular annual sampling campaign. Prevalence is a function of both the incidence (frequency of new cases in a population) and duration (time to recovery of a disease). Also, the relationship between prevalence and incidence will be greatly influenced by the persistence of a detectable antibody following infection, where sero-positivity defines cases of disease.

As an analytical study, our objective was to understand the "natural history" of *Salmonella* Abortusovis. For this purpose we investigated the interrelatedness of the humoral response phenomenon within the biological system, selecting the segments of the chain of infection where the interactions performed betwen hosts and the environment could be measured.

We have used the risk to express the probability of sero-conversion occurrence following a particular exposure to S. Abortusovis. The differences in immune recognition between groups were respected to the frequency of the presence or absence of potential risk factors by means of a cohort study to evaluate the hosts' risk factors. Here, we considered those endogenous characteristics that could influence the immune response of individual ruminants: species and breed (genetic constitution); age and gender (Goldberg, 1994).

Our field study in th e"Serranía de Cuenca Natural Park" measured sero-positive and sero-negative animals considering their exposure to all the known and unknown environmental factors present in their natural environment. The quantification of sero-conversion cases occurrence was performed by counts of sero-positive individuals and expressed as a fraction of the number of animals that held a similar condition (= population at risk). From a mathematical perspective, frequencies were expressed through static measures as proportions and ratios (Thrusfield, 1990; Goldberg, 1994).

Finally,to obtain further data to understand the epidemiology of this infectious disease we evaluated the distribution of the agent in the ecosystem and the factors that could influence the modes of transmission between its compartments (Scott & Smith, 1994). As environmental risk factors we considered those not identifiable with the host or agent but related to the animals comprising individual populations, where they live under similar

conditions of clime, management and nutrition. As empirical research, it involved the measurement of variables, estimation of population parameters and statistical testing of hypotheses by comparisons between groups.

In order to estimate the magnitude of an association between a putatively causal factor and sero-conversion and to assess if there was potential for a cause-effect relationship between a single or multiple risk factors and sero-conversion, we used Generalized linear models (GLM; McCullagh & Nelder 1989) to measure and represent the statistical interaction of response variables (dependents), such as abundance of sero-conversion (prevalences and frequencies),with environmental predictors (independent variables).

Statistical analysis were performed with Microsoft EXCEL 2000© (1985-1999, Microsoft Corporation, USA), Statistica 6.0® (1984-2001 Statsoft, EE.UU.), and EpiInfo 3.3.2 (Center for Disease Control, USA, 2005) integrated epidemiological statistics package. The analysis of the risk factors for the infection was calculated by the Pearson Chi-square test without correction and the Fisher exact test. We considered the value of two tailed *P* in all analyses. The level of significance was set at $P \leq 0.05$. The association of risk factors and infection were quantified by the analysis of the odds ratio (OR) using Cornfield 95 % confidence limits. Finally, we included the statistically significant factors in General Linear Models to establish which variables act as predisposing factors.

4. Results

Antibodies to *Salmonella enterica* Serogorup B Somatic (O) were not found in 4,318 (82.1 %) samples and were classified as sero-negative SAT reactions. We observed coloured films on the surfaces of the well at 1/10 dilutions in 716 (13.6 %) sera, that were considered as doubtful and, at higher titres 222 (4.2 %) were identified as sero-positives.

Results showed that frequency of immune reactions (titres ≥ 1:10) against somatic antigen of *Salmonella* Abortusovis (applicable to others included in B serogroup) were higher bovines (RF = 36.8%) than in sheep (RF = 19.73%), fallow deer (RF = 7.55%), mouflon (RF = 9.47%), or red deer (RF = 1.59%) (Table 1; Figure 1).

Moreover, specific antibodies to *Salmonella* Abortusovis H antigen were revealed by SAT among the 939 sera that showed titres of 1:10 or higher to the O antigen (Cattle = 150; sheep = 738; fallow deer = 42; red deer = 5; mouflon = 4). Results showed that frequency of immune reactions (titres ≥ 1:10) against flagellar antigen of *Salmonella* Abortusovis were higher in mouflon (RF = 75%) and domestic ovine (RF = 74.39%), than in fallow deer (RF = 54.76%), cattle (RF = 30-66%), or red deer (RF = 0%) (Table 1; Figure 2).

The relative intensity of reactions in this species against each antigen was estimated by the Mean Geometric Titer, and only considering sero-positives (titers ≥ 1:20). Immune responses to O antigen were higher in cattle (MGT = 1:39.2), than in fallow deer (MGT = 1:23.7), sheep (MGT =1:22), or red deer and European mouflon (MGT =1:20). Other ways, responses to H antigen were higher in ovines (MGT = 1:30.6) and mouflon (MGT= 1:25.2), than cattle or fallow deer (MGT = 1:20), and were not detected in red deer (Table 1).

The interpretation of serological results obtained in both techniques for *Salmonella* Abortusovis serological diagnosis was performed with attention to positive reactions (titres

≥ 1:20) to both antigens O and H. This condition was only found in 150 (2.8%) serum samples: 145 (3.85%) ovine sera, 3 mouflon sera (0.95%), and 2 fallow deer sera (0.35%) (Table 1; Figure 3).

Species	Somatic antigen "O"(group B)					Flagellar antigen H (group C)				
		Titre					Titre			
	n	1:10	> 1:10			n	1:10	> 1:10		
		AF	AF	%	MGT		AF	AF	%	MGT
C	407	111	39	9,58	39,2	150	0	46	30,6	20
S	3739	567	171	4,57	22,0	738	128	421	74,3	30,68
RD	314	4	1	0,32	20	5	0	0	0	-
FD	556	34	8	1,44	23,7	42	21	2	54,7	20
RoD	8	0	0	0,00	-	0	0	0	-	-
SI	21	0	0	0,00	-	0	0	0	-	-
M	211	1	3	1,42	20	4	0	3	75,0	25,19
T	5256	716	222	4,22	-	939	149	490	51'7	-

AF: absolute frequency; GMT: mean geometric titre; C: cattle; S: sheep; RD: red deer; FD: fallow deer; RoD: roe deer; SI: Spanish ibex; M: mouflon; T: Total.

Table 1. Frequencies of sero-positives and Mean Geometric Titres aganist S Abortusovis Somatic and flagellar antigens obtained by Serum Agglutination Test

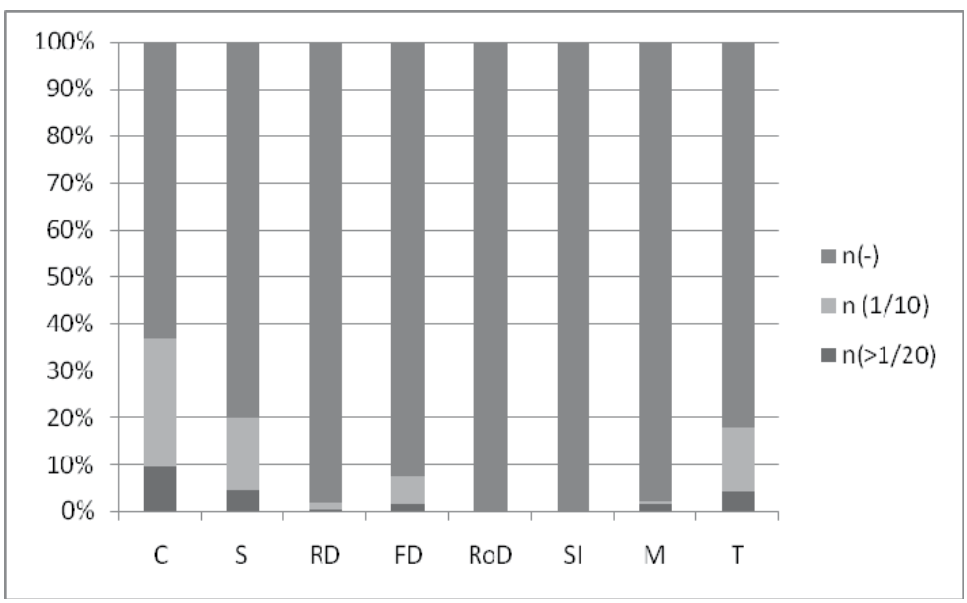

Fig. 1. Percentage of sero-positive, doubtful and sero-negative reactions against O Antigen

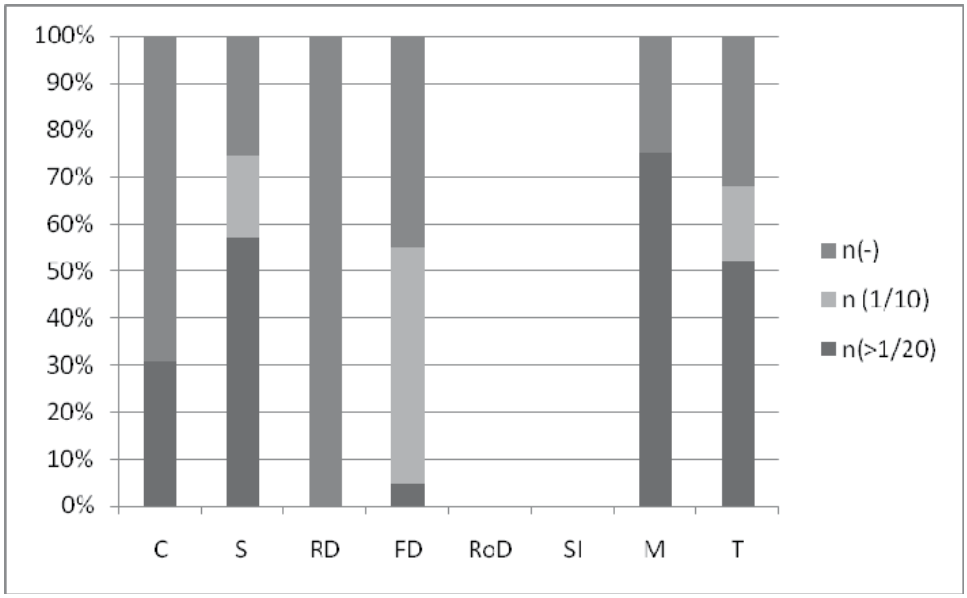

Fig. 2. Percentage of sero-positive, doubtful and sero-negative reactions against F Antigen

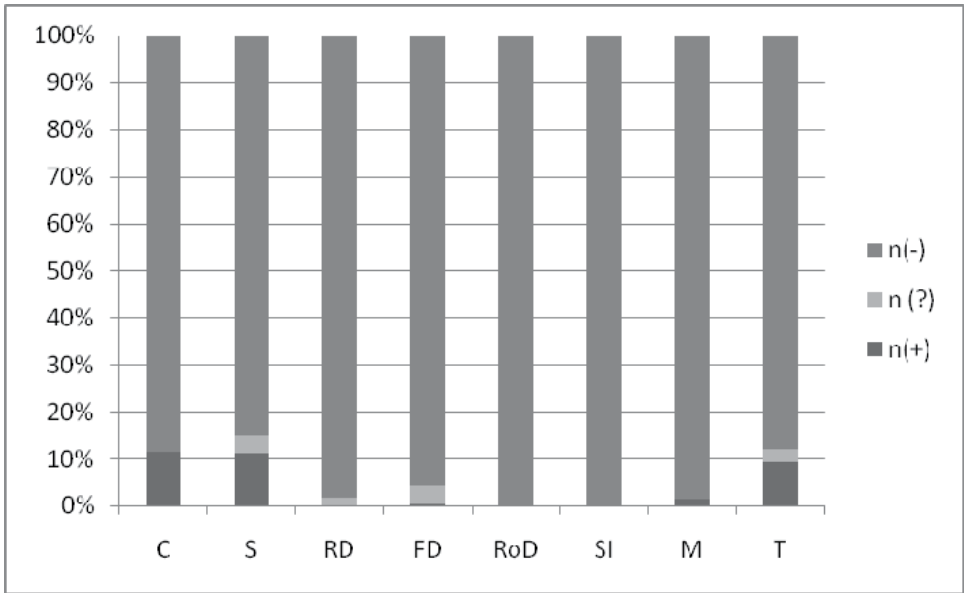

Fig. 3. Percentage of sera catalogued as sero-positive, doubtful and sero-negative against *Salmonella* Abortusovis

Statistical relationship between the sero-conversion cases and individual factors of the animals was explored by means of risk factors analysis. Statistical risk to sero-conversion was observed between sexes in domestic sheep ($X^2 = 42.37$; $p < 0.001$), that was higher for males compared to females (Relative risk = 3.85; IC95%: 2.5-5.8 vs. RR = 0,26; IC95%: 0.1-0.3) (Table 2).

Species	TOTAL			♀		♂		φ (IC 95%)		X^2	*p*
	Nº	(+)	(-)	(+)	(-)	(+)	(-)	♀			
C	361	0	361	0	290	0	71	-	-	-	-
S	3249	139	3110	116	2974	23	136	0,26 (0,1-0,3)	3,85 (2,5-5,8)	42,37	0,00
RD	314	0	314	0	184	0	130	-	-	-	-
FD	533	2	531	2	307	0	224	-	-	-	-
RoD	8	0	8	0	5	0	3	-	-	-	-
SI	21	0	21	0	9	0	12	-	-	-	-
M	211	3	208	1	141	2	67	0,24 (0,02-2,6)	2,12 0,3-44,6)	1,60	0,20

(+) Positive. (-) Negative. Risk ***φ.-*** *Odds ratio.* AF: absolute frequency; GMT: mean geometric titre; C: cattle; S: sheep; RD: red deer; FD: fallow deer; RoD: roe deer; SI: Spanish ibex; M: mouflon; T: Total.

Table 2. Risk associated to sex in the studied species.

Among domestic ruminants, yearling sheep showed a high risk of sero-conversion to *S.* Abortusovis (p=0'01, φ=2'27) respect to older animals (p=0'14, φ=0'6). In the same way. the results for mouflon sheep, revealed a significantly high *Odds ratio* in yearling animals (φ = 7'58), that was followed by a decreasing value (φ = 3'12 in the next age group (2 to 4 years); estimates in the rest of the cohorts considered were not able to be performed due to the scarce absolute number of sero-positive animals. (Table 3).

By the same way, the juvenile age group seems to be an important risk factor for sero-conversion in domestic sheep (OR = 1'39; $IC_{95\%}$:1'13-2'71) and mouflon (OR = 7,6; $IC_{95\%}$:0,73-77,18), compared to other age categories (X^2 = 5'6; *p* = 0'01 and X^2 = 3,8; *p* = 0,049, respectively) (Table 3).

Group age	Specie	Cases			Probability		Risk	
		+	-	RF (%)	X^2	p	φ	$IC_{95\%}$
Young	Sheep	8	77	9'4	5'6	0'01	2'27	1'15-4'49
	Cattle	0	66	0'0	-	-	-	-
Adult	Sheep	131	3004	4'3	2'16	0'14	0'6	0'3-1'19
	Cattle	0	177	0	-	-	-	-
Old	Sheep	0	29	0	-	-	-	-
	Cattle	0	118	0	-	-	-	-

RF: Relative frquency. *Odds ratio*

Table 3. *Odds ratio* (φ) for sero-conversion to *S.* Abortusovis in the different age groups.

Sero-prevalence values in sheep flocks were estimated in 2004 and 2005. The frequency of affected flocks in the first year reached 100% (13/13), with sero-prevalences ranging from 4.3% to 22.0% (12-33± 5.9%) and also low MGT (1:23.25 ± 1:5.57). The following year sero-positive sheep were found only in the 33.33% of the sampled flocks (9/27), and sero-prevalence values in the same flocks ranged from 1.06% to 7.9% (3.0±2.14%) with very low MGT (1:0.99±7.91). The results obtained in the Serranía de Cuenca show a decline phase of an epidemic outbreak.

Mean sero-prevalence values in each flock and grazing area were estimated and used as indexes to evaluate association of sero-conversion with management factors, and spatial relationships with environmental conditions, respectively. In the correlation matrix analysis built to evaluate management factors in sheep flocks a significant trend to seroconversión was observed in association with lambing intensities of 3 parturitions each 2 years (r = 0.4013; p = 0-028), but this could not be confirmed by ANOVA ($F_{(7,23)}$= 1.19; p = 0.34).

The spatial distribution of infected flocks in the "Serranía de Cuenca Regional Game Reserve" Pasture Areas conditioned a wide distribution of sero-positive sheep, restricted only by their own flock permissions, with mean sero-prevalences of 4.2 ± 3.04% (Range: 0 to 9.57%). These sero-prevalence values were associated with the relative extension of *Pinus nigra* within each area, by means of Lineal Regression Models: "PRV OA Salm" = 1.852 + 0.07 * "Pn" (R^2 = 0.33; p = 0.039).

The scarce evidence of specific antibodies against *Salmonella* Abortusovis in other ruminant populations prevents evaluating the potential host-pathogen interactions in the lesser sampled species (roe deer and Spanish ibex). Furthermore, frequencies of sero-positives in mouflon (3/211, 1.4 %) and fallow deer (2/555. 0.3%) populations were low and not intense (Range of titres: 1:20-1:40), and immune responses were absent in red deer, as in cattle (Table 4).

RD (n = 314)	**FD** (n = 555)	**RoD** (n= 8)	**SI** (n = 21)	**M** (n = 211)
0	2 (0.36)	0	0	3 (1.4)

RD: red deer; FD: fallow deer; RoD: roe deer; SI: Spanish ibex; M: mouflon.

Table 4. Frequency of sero-positives in wild ruminant species.

5. Discussion

Different *Salmonella* serovars have been isolated from a wide variety of vertebrates, including European wild ungulates, but bacterial isolation is not sufficient, in itself, for a diagnosis of salmonellosis (Mörner, 2001; Nielsen et al, 1981).

Diagnosis of salmonellosis should be based on culture and identification of the bacteria, together with clinical and pathological evidence (Olsen et al, 2003; Threlfall & Frost, 1990; Valdezate et al., 2007; Van der Zee & Huis In't Veld, 2000). In the case of paratyphoid abortion diagnosis, this can be compromised, because the use of common bacteriological procedures is not an advisable option, and clinical signs or lesions are not specific enough (González, 2000; Beuzón et al., 1997; Linklater, 1983). In such situations, serological methods, such as SAT on microplates, may also be used to identify infected flocks or herds, rather than to identify individual infected animals (Pardon et al., 1988; González, 2000; OIE, 2010).

For detailed epidemiological investigations strain identifications are necessary (OIE, 2010). Traditionally, the identification of S. Abortusovis has relied on the use of antisera against O and H antigens (Brenner, 1984; Vodas and Martinov, 1986), and currently molecular characterization techniques like ribotyping, plasmid profiling, and IS*200* fingerprinting can be performed successfully to identify different and predominant Abortusovis genotypes (Nikbakht, et al.2002; Schiaffino et al. 1996; Nastasi, et al. 1992). The use of this method led to the identification of two different strains in sheep flocks from Cuenca province between

1996 and 2001, but this epidemiological surveillance system is limited by the low number of Abortusovis isolates available (Valdezate et al., 2007).

On the other hand, detection of specific immune responses to S. Abortusovis can provide further evidence of infection, but little is known about the duration of effective immunity following *Salmonella* infections, and positive results cannot always be interpreted as indicative of active infections (OIE, 2010; Brennan et al., 1994; 1995). Animals that have been infected recently would, in all probability, eventually be detected serologically by an appropriate monitoring programme throughout the life of the flock/herd, but there are often cost limitations to the application of effective monitoring programmes (OIE, 2010).

The agglutination test is considered the preferred method in export and diagnostic purposes for samples from all species of farm animals (Davies, 2004; OIE, 2010). Our research demonstrated the possibility of performing it alongside the analysis scheme established in the brucellosis official eradication program. SAT proved to be easily adaptable to the routine diagnostic procedures in "Albaladejito" Veterinary Laboratory as well as being an economical method of performing simultaneous analyses of large numbers of samples. In this sense, we must argue for the potential adoption of this method by official veterinary programs if further studies are to be carried on.

In order to define the level of detection or accuracy of prevalence in the testing program we must consider the lower sensitivity of SAT in comparison with ELISA tests (Berthon, et al., 1994, Sting et al., 1997; Veling et al., 2000), and the conditions relating to sampling procedures regarding the dynamics of immune responses aganist *Salmonella* Abortusovis (OIE, 2010). For most animals, a significant increase in agglutination titres could be observed from day 5 after inoculation (Lantier, 1987), but care in interpreting the serological results has to be taken if this test is performed after abortion, as antibody levels fall and may become undetectable 2-3 months later (Davies, 2004; González, 2000). Thus, our serological results should be interpreted as punctual sero-frequencies or sero-prevalences.

The wide frequency of O-agglutinating sera found among the domestic (bovines RF = 39.2%; ovines RF=22 %) and wild ruminants (fallow deer RF =23.7%; mouflon RF = 20%: red deer RF = 20%), sampled in the "Serranía de Cuenca Regional Game Reserve" indicated wide immune recognition of *Salmonella* between 2003 and 2006, but these immune responses may respond to other group B *Salmonella* infections, whatever the O antigen (Bernard et al., 2002). In fact, results obtained in cattle and red deer against group C H antigen were not correlated, in any case, to the respecting anti-O titres. In this sense, although serological responses can be demonstrated against both flagellar and somatic antigens, it has been stated that it is advisable to restrict the search to anti-H agglutinins, as these are more specific, reach higher titres and have a precocity and persistence similar to those against O antigen (Pardon et al., 1988; González, 2000).

The frequencies obtained in the group C H-antigen SAT showed very high values among the ovines (mouflon RF = 75%; sheep RF = 74.39%), but were also found in a high percentage of fallow deer (RF = 54.76 %), and cattle (RF= 30.66%). However, many samples presented a low titre to group B O antigen (1:10), but reached high agglutination titres to H antigen, and were not considered as specific responses against *S.* Abortusovis. These were recorded in a limited percentage of the sheep analysed (3.85%), mouflon (0.95%), and fallow

deer (0.35%), thus restricting the potential host range. In order to improve the knowledge of *Salmonella* infections in each species it would be advisable to include other antigens in the serological screening.

The presence of humoral immune specific recognition of *S.* Abortusovis in sheep from the "Serranía de Cuenca Regional Game Reserve" is further evidence of paratyphoid abortion in the province of Cuenca (Valdezate et al., 2007). Besides possible controversial discussions about prevalence values estimated under different methods (Jack., 1968; Uzzau et al., 2000; Valdezate et al., 2007), the characteristic epidemic behaviour of paratyphoid abortions leads to differences of incidence and importance of S. Abortusovis infection between sheep flocks, countries and periods of time (González, 2000; Giannati-Stefanou et al., 1997). In this sense, the use of SAT can improve sheep abortion surveillance by identifying infected flocks (OIE, 2010), but also by indicating epidemiological trends, as may be suggested by the significant differences found among sero-epidemiological indexes (frequency of affected flocks, MGT, and sheep sero-prevalence) estimated in consecutive sampling campaigns, which clearly indicate a decreased epidemic phase of infection.

In addition, the main affection of yearlings and recently purchased sheep is usual after paratyphoid abortion storms (González, 2000). This situation is also suggested by the results obtained in the risk factor analysis. The odds ratio for sero-conversion in sheep showed a statistical association to host factors such as sex or age, with higher risk in males and yearlings. These findings could respond to different predisposing physiological conditions of lambs, ewes, and rams, but also to environmental factors related to management practices. At this point, it is necessary to consider that in the "Serranía de Cuenca Regional Game Reserve" extensive grazing system these sheep groups maintain different farming conditions, such as transport, herding, housing or nutrition, that respond to the conditions of the reproduction scheme in each flock (Martín Atance, 2009).

The communal grazing practices in the "Serranía de Cuenca Regional Game Reserve" may allow a wide dissemination of infection between flocks, but differences in the extension of Pinus nigra forests within each grazing area can modulate the variance of the mean herd-sero-prevalence values estimated ($R^2 = 0.33$; $p = 0.039$). In our opinion, this finding could indicate the effect of the environment on the host conditions, such as nutritional stress in carrier hosts, but this hypothesis needs to be confirmed by specific research (González, 2000; Linklater et al., 1991).

The detection of SAT specific responses in mouflon and fallow deer allows us to hypothesize about the possible sensitivity to infection of both species, or even a potential adaptation of similar strains to this hosts. In this sense, it would be recommendable to favour research into the genetic and physiological differences of *Salmonella* isolates from sheep and compare them to others of wildlife origin in order to test the basis for *Salmonella* enterica evolution in relation to hosts (Bäumler et al., 1997; Hensel, 2004; Popoff & Le Minor, 2005). Nevertheless, the scarce amount of evidence suggests that wild ruminants play a minor role in the epidemiological cycle of paratyphoid abortion in the "Serranía de Cuenca Regional Game Reserve", as has been suggested in Andalusia, where sero-positive Spanish ibex, mouflon, fallow deer and red deer have been detected at higher rates (Arenas et al., 1993; Cubero et al., 2002; Pérez, 2007; León Vizcaíno et al., 1980; León Vizcaíno et al., 1992; León Vizcaíno et al., 1994; León Vizcaíno et al., 2002).

These results indicate the epidemiological role of sheep as primary hosts of S. Abortusovis, and the absence of natural niches of infection among other ruminant populations. However, the presence of specific antibodies in European mouflon and fallow deer could be indicative of the infection in this populations and the presence of chronic carriers that might help to disperse this agent.

6. References

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Part 5

Pathogenesis

22

Animal Models for *Salmonella* Pathogenesis: Studies on the Virulence Properties Using *Caenorhabditis elegans* as a Model Host

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1. Introduction

Studies associated with host-pathogen interaction and the principal mechanisms of pathogenesis including the systems adopted by the host for its defense has always been a topic of interest to the scientific community that primarily deals with pathogenic microbes causing human diseases. More importantly the post-genomic era has set a milestone in basic and applied science research by proper identification and validation of potential human "disease-causing" or "disease-associated" genes. Although the host-pathogen interaction is a complex biological system (Huffman et. al., 2004) it is equally important to understand the characteristic features of microbes and their respective hosts that always may not culminate into a disease process. So present day researchers are also working with microbes that may exist within hosts without causing any obvious disease, and at the same time trying to explore why some microbes only cause disease in certain hosts. Besides, a wide range of microbes those are pathogenic to the mammals, like bacteria and fungi, have also been known to manifest diseases in simple non-vertebrate hosts as well. In order to understand a pathogen, researchers would preferably screen the microbe's genome at length to identify all its virulence genes. On the other hand, screening in mammalian experimental hosts, such as mice, rats, or other mammals *per se*, sometimes seems unfeasible since they would be required in large numbers and thus quite expensive. In recent days use of simple non-vertebrate hosts, such as the round worm *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and the plant *Arabidopsis thaliana*, are becoming common for convenience in investigating the virulence strategies adopted by several mammalian pathogenic microbes (bacteria and fungi) (Sifri et al., 2005). Also uses of these model organisms are in great practice to comprehend the conserved molecular pathways that are related to human diseases caused by microbial pathogens.

There is a wide variety of pathogens (both bacteria and fungi) that are known to affect the human health including animals. Amongst them those that have been extensively studied with different laboratory model hosts are, Gram-negative bacteria *Burkholderia*, *Pseudomonas,*

Salmonella, Serratia and *Yersinia*; Gram-positive bacteria *Enterococcus, Staphylococcus* and *Streptococcus*; and the fungus *Cryptococcus neoformans*. However there are some that are not pathogenic to mammals, but pose as insect pathogens, like *Bacillus thuringiensis* and the nematode-specific *Microbacterium nematophilum*.

Caenorhabditis elegans (commonly known as *C. elegans*) is a free living soil nematode that feeds on bacteria. Under laboratory conditions *C. elegans* feeds on *E. coli* (strain OP50). In order to study the effects of pathogenic strains on animals, researchers have started to use *C. elegans* as an excellent and a convenient model to explore the bacterial pathogenesis on animals by making the worms feed on the pathogens. Amongst the different categories of pathogens that are known to infect the worms in the same manner as in humans / animals, *Salmonella* is one that has been broadly studied in the worm system. The present chapter shall focus the attribute of the nematode *C. elegans* as a convenient model to study host pathogen interaction with special emphasis to *Salmonella*.

2. Caenorhabditis elegans as a simple model system to study human diseases

For the past few decades, the free-living bacteriovorous nematode *Caenorhabditis elegans* (Caeno, recent; rhabditis, rod; elegans, elegant) has emerged lately as a powerful model for study of developmental genetics, neurobiology and aging. Ever since it was introduced by Sydney Brenner, this simple multicellular eukaryote has been studied intensively with comprehensive genotypic and phenotypic information now available. This free-living nematode has the following features: small body length (1.5 mm adults), quick generation time (three days), large brood size (approximately 300 progeny per gravid adult), short lifespan (~3 weeks), ease of maintenance, reduced cost, a small genome (one half that of *Drosophila melanogaster*), ability to be stored for long periods by freezing, and the fact that it is a simple and genetically tractable model have made this nematode species an ideal model to study longevity and process of ageing (Ewbank, 2002; Houthoofd et al., 2003; Kurz et al., 2007), and as well as a model organism for molecular and developmental biology. Moreover, under the microscope, the unique transparent body of the worm allows one to observe many biological processes, including organogenesis, behavior, and as well as, pathogenesis. The life cycle is short, temperature dependent and consists of embryogenesis (development from fertilization to hatching) and post-embryonic development that has four larval stages separated by molts followed by the adult stage (Figure 1). Also, at every larval stage a new cuticle of stage-specific composition is secreted and the older one is shed. In L1 larvae, the nervous system, the reproductive system, and the digestive tract begin to develop, and this is completed by the L4 stage. Sometimes these nematodes can adopt another non developmental stage known as the Dauer stage instead of the normal third larval stage (Cassada and Russell, 1975). Entry into dauer is induced by stress like high temperature, starvation or overcrowding at the second molt (Fig. 1).

In 1998, *C. elegans* became the first metazoan to have a completely sequenced genome (The *C. elegans* Sequencing Consortium, 1998). More than 40% of the human disease genes have been predicted to have orthologs in the *C. elegans* genome. Overexpressing human genes in specific cell types in *C. elegans* using tissue- or cell- specific worm promoters, or studying the differential gene expression patterns of worms at the transcriptional level by

microarray analysis may well reveal the gene expression profiles (up- or down-regulated) for the worms. This helps the identification of certain members of the signaling cascades that are activated in diseases and may well act as candidates for drug therapies. Double-stranded RNA interference (dsRNAi) has also been another modern approach to study human diseases by treating worms with dsRNAs thus inactivating the function of specific *C. elegans* gene orthologs (Fire et al., 1998; Tabara et al., 1998; Timmons et al., 2001). Up to date several genomic data on human and animal microbial pathogens that are shown to harm and kill nematodes have been created. With these vast resources of genetic information, there is always a growing need for simple and innovative ways to study microbial virulence strategies and assay the role of individual genes to pathogenesis. Since both the host (i.e. *C. elegans*) and pathogens are amenable to genetic analysis and high throughput screening, in each of these pathosystems the worm has been successfully utilized both for the identification of microbial virulence factors and as well as the worm's immune-defense mechanisms.

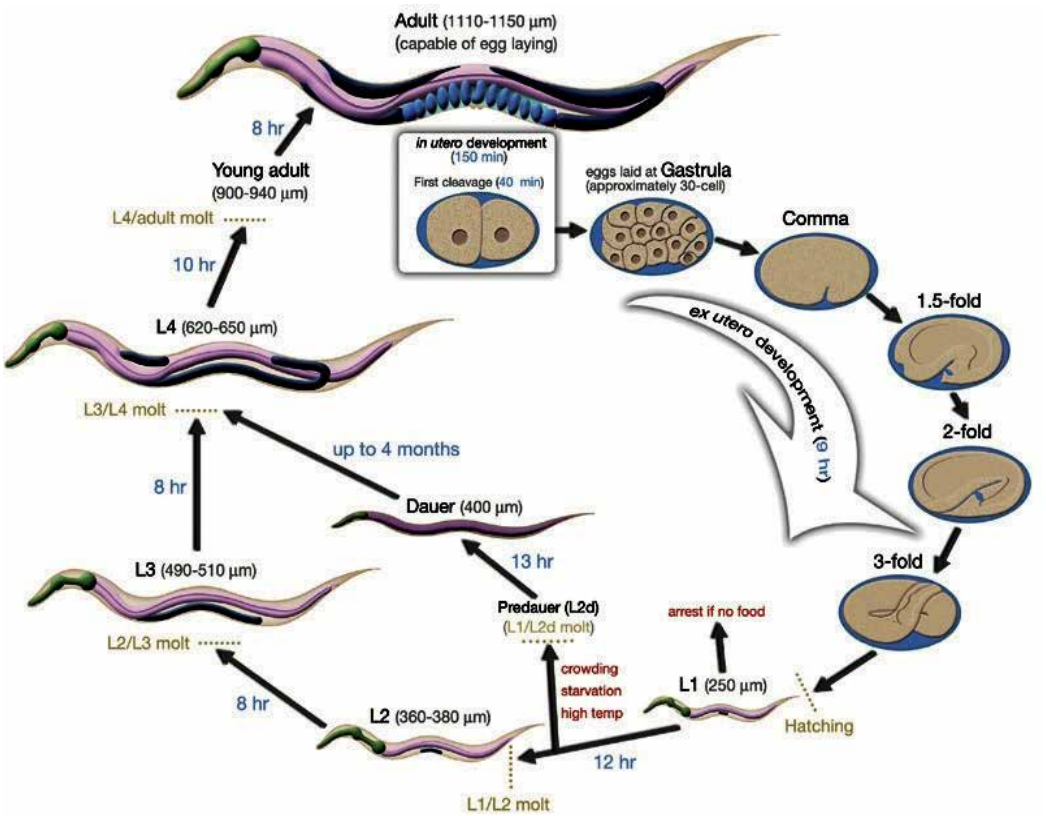

Fig. 1. Life cycle of *C. elegans*. Life cycle consists of four larval stages. At the first larval stage the life cycle can be interrupted by the "dauer" stage which is triggered by increased pheromone levels that result due to food scarcity, overcrowding and temperature (Courtesy: Introduction to *C. elegans* anatomy, Handbook-Hermaphrodite; http://www.wormatlas.org)

3. Bacteria as a food source for *C. elegans*: Effect of *Salmonella* and other pathogens as food

C. elegans has a life span of approximately two weeks at room temperature when fed on *Escherichia coli* OP50 bacteria (Brenner, 1974) grown on Nematode Growth Medium (NGM) agar (Garsin et al., 2001). However, when *C. elegans* is fed on other human pathogens, they exhibit a range of significant defects, like shorter life spans. Several human pathogens, including *Pseudomonas aeruginosa, Salmonella typhimurium, Serratia marcescens*, *Staphylococcus aureus*, *Vibrio cholerae,* and *Burkholderia pseudomallei*, kill *C. elegans* when supplied as a food source, and a diverse array of bacterial virulence factors have been shown to play a role in both nematode and mammalian pathogenesis (Aballay et al., 2000; Kurz & Ewbank, 2000; Labrousse et al., 2000; Tan et al., 1999). An important feature of *Pseudomonas aeruginosa*, a Gram-negative pathogen, is known to kill *C. elegans* and more particularly, under different media conditions. The *P. aeruginosa* strain PA14 kills *C. elegans* by "slow-killing" (in few days) or even by "fast killing" (few hours) (Tan et al., 1999). *Vibrio cholerae,* another Gram-negative bacterium kills *C. elegans* within few days (~ 5 days) by a "slow-killing" process (Vaitkevicius et al., 2006). A marked decrease in the life span was observed in worms feeding *V. vulnificus* as opposed to the regular food of *E. coli* OP50. In many cases, the intestines of the worms were found to get distended with clumps of pathogenic microorganisms that accumulate within it and probably happen to be primary cause of early deaths (Dhakal et al., 2006). Normally, the pharyngeal grinder of the worm efficiently disrupts the *E. coli* and essentially no intact bacterial cells are found within the intestinal lumen. However, virulent bacterial strains like *V. vulnificus* or *V. cholerae* have been shown to accumulate in both pharynx and the lumen of the worm intestines (Vaitkevicius et al., 2006; Dhakal et al., 2006) as evidenced under fluorescent microscope for the GFP-labeled bacterial strains.

Salmonella is a gram-negative enteric bacterium that represents a major public health problem. *S. enterica* colonizes the *C. elegans* intestine as reported (Aballay et al., 2000; Labrousse et al., 2000). *S. enterica* serovar Typhi causes typhoid fever, a severe systemic infection. *S. enterica* serovar Typhimurium is known to be lethal to mice, causing a typhoid-like disease, and in humans it causes nonfatal infection restricted to the gastrointestinal tract and thus had been studied in the mouse model for systemic infections. When worms were exposed to *S. enterica* for only 3 h, then removed to plates seeded with OP50, there was significant early death. Invasion of host cells is an essential aspect of *Salmonella sp*. pathogenesis in mammalian systems, but *S. enterica* does not appear to invade *C. elegans* cells. Many novel strategies have been devised for understanding its mode of action and its interactions with host cells (Lee & Camilli, 2000; Chiang et al., 1999). The finding that *Salmonella* is capable of infecting *C. elegans*, and that genes important for its full pathogenicity in vertebrates also play a role during infection of *C. elegans*, opens the possibility of taking a new genetic approach to study *Salmonella.*

Salmonella, a gastrointestinal tract pathogen of humans, is responsible for approximately 2 million - 4 million cases of enterocolitis every year in the United States (Tampakakis et al., 2009). During infection, *S. enterica* serovar Typhimurium has the propensity to compete with normal intestinal flora. *Candida albicans* is another opportunistic human fungal pathogen that usually resides in the gastrointestinal tract and on the skin as a commensal and can also cause life-threatening invasive disease. Besides, both these organisms are pathogenic to the nematode *C. elegans*, causing a persistent gut infection and eventually leading to death of the worms (Fig. 2). Mylonakis and his group developed *C. elegans* as a polymicrobial infection

model to assess the interactions between *S.* Typhimurium and *C. albicans* (Tampakakis et al., 2009). They reported that when *C. elegans* is infected with *C. albicans* and *S. enterica* serovar Typhimurium, *C. albicans* filamentation is inhibited. They further utilized the host, *C. elegans*, to identify the antagonistic interaction between two human pathogens that reside within the gastrointestinal tract.

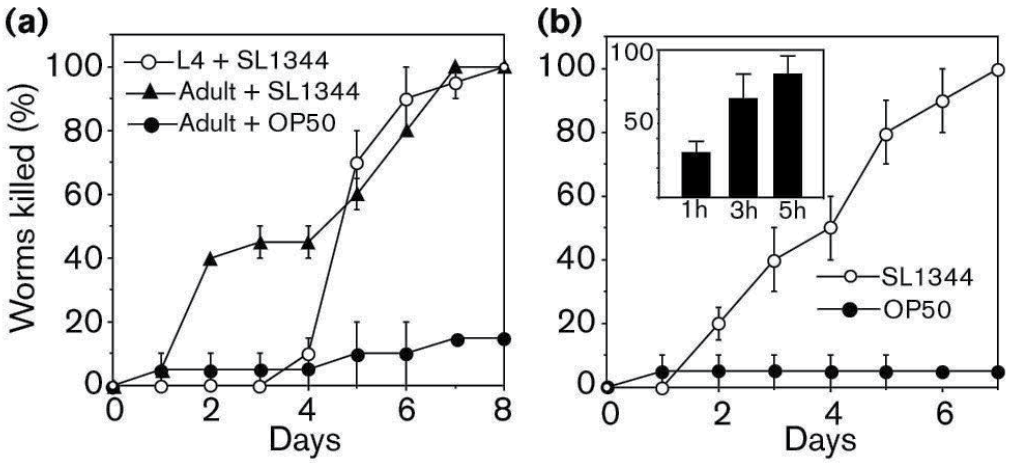

Fig. 2. *S. typhimurium* kills *C. elegans*. (a) L4 stage (open circles) or 1-day-old adult hermaphrodite (solid triangles and solid circles) worms fed either on *S. typhimurium* SL1344 (solid triangles and open circles) or on *E. coli* OP50 (solid circles). (b) *C. elegans* fed on *S. typhimurium* SL1344 (open circles) or *E. coli* OP50 (solid circles) for 5 h, then shifted to *E. coli* OP50. The inset shows the percentages of dead worms after transfer to OP50-containing plates after feeding for 1, 3 or 5 h on SL1344 (Courtesy Aballay et al., 2000).

4. Intestine as the store house of bacterial infection in *C. elegans*

Numerous bacteria infect the intestine of *C. elegans*. In many cases, the intestine becomes inflated; but it is not clear whether this is due to physical pressure exerted by the growing pathogen or as a physiological response of the nematode. The standard laboratory food, i.e. *E. coli* OP50 and *Cryptococcus laurentii* (Tan et al., 1999; Garsin et al., 2001; Mylonakis et al., 2002) does not colonize wild-type *C. elegans*, but various pathogens do. For example, *Enterococcus faecalis,* a gram-positive bacteria, colonizes in *C. elegans* and kills very rapidly (Garsin et al., 2001). It was eventually shown that genes involved directly or indirectly with the quorum-sensing system are involved in killing (Sifri et al., 2002). On the other hand, *Pseudomonas aeruginosa* can kill *C. elegans* rapidly by toxin-mediated mechanisms or slowly in an infectious process. In the "slow killing" model, bacteria colonize the intestine, but within a day exposure to the bacteria, no strong disease symptoms were observed (Tan et al., 1999). With continued exposure, the worms gradually cease pharyngeal pumping, become immobile, and eventually die. Moreover, large quantities of live bacteria like *Salmonella enterica*, *Burkholderia cepacia*, *Serratia marcescens*, *Staphylococcus aureus, Vibrio vulnificus*, *V. cholerae*, and *C. neoformans* are known to kill worms by colonization (Aballay et al., 2000; Garsin et al., 2001; Mylonakis et al., 2002; Kothe et al., 2003; Kurz et al., 2003; Rhee et al., 2006; Vaitkevicius et al., 2006). A screen of 960 transposon insertions in *S. enterica* produced 15 mutations with reduced killing of *C. elegans*, of which only some were virulent

(Tenor et al., 2004). Although bacterial colonization is greatly correlated with worm killing, it is not adequate for killing. For instance, aerobically grown *Enterococcus faecium* although accumulates to high levels, it does not kill (Garsin et al., 2001). *S. enterica*, *S. marcescens*, and *E. faecalis* are pathogens also known to cause persistent infections (Aballay et al., 2000; Labrousse et al., 2000; Garsin et al., 2001; Kurz et al., 2003) in *C. elegans* in contrast to *Pseudomonas aeruginosa* and *S. aureas*.

Different strains of *Salmonella*, such as *S. typhimurium* as well as other *Salmonella enteric* serovars including *S. enteritidis* and *S. dublin* are all effective in killing *C. elegans* (Aballay et al., 2000). When worms are placed on a lawn of *S. typhimurium*, the bacteria have been shown to accumulate in the intestinal lumen and the nematodes die over the course of several days (Fig. 3). This killing in particular requires direct contact with live bacterial cells. Interestingly, the worms die in the same manner even when placed on a lawn of *S. typhimurium* for a relatively short period of time (3–5 hours) before transfer to a lawn of *E. coli*, their natural food. A high titer of *S. typhimurium* still persists in the *C. elegans* intestinal lumen for the rest of the worms' life even after their transfer to an *E. coli* lawn. Killing is directly correlated with an increase in the titer of *S. typhimurium* in the *C. elegans* lumen. Even a small inoculum of *S. typhimurium* has been shown to be enough to establish a persistent infection *C. elegans* which is probably due to the presence of *C. elegans* intestinal receptors to which bacteria might adhere (Fig. 4).

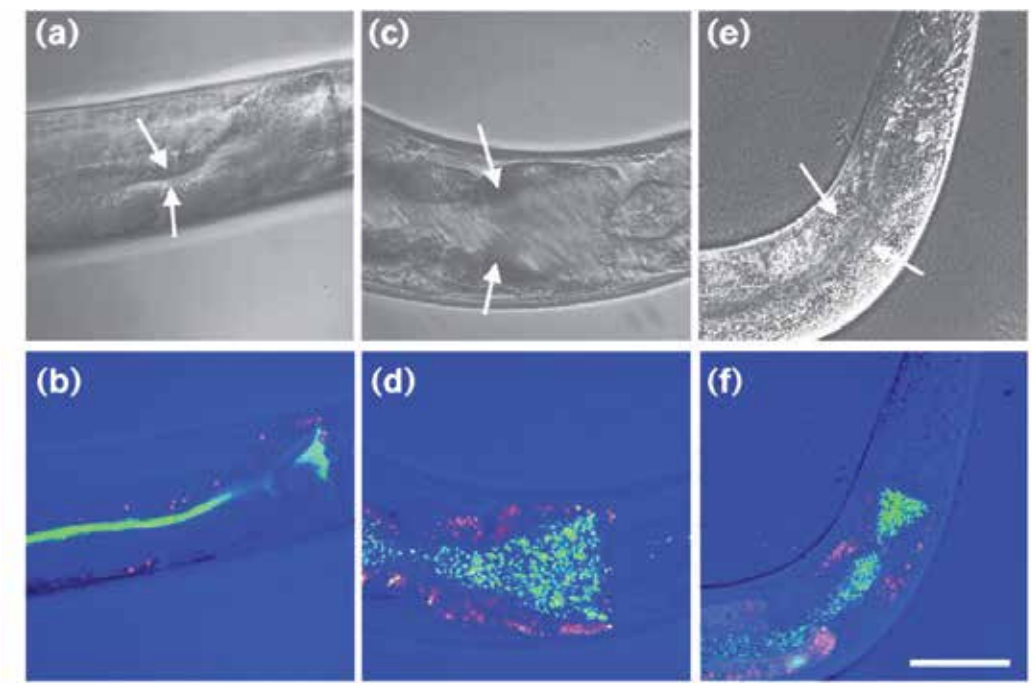

Fig. 3. Bacterial colonization of the *C. elegans* intestine. Confocal images showing young adult hermaphrodite worms fed on (a,b) *E. coli* DH5α–GFP for 72 h, (c,d) *S. typhimurium* SL1344–GFP for 72 h, or (e,f) *P. aeruginosa* PA14–GFP for 24 h. (a,c,e) Transmission images showing the intestinal margins (indicated with arrows). (b,d,f) Merged images showing bacterial fluorescence (green channel) and the gut autofluorescence (red channel). Scale bar, 50 μm (Courtesy Aballay et al., 2000).

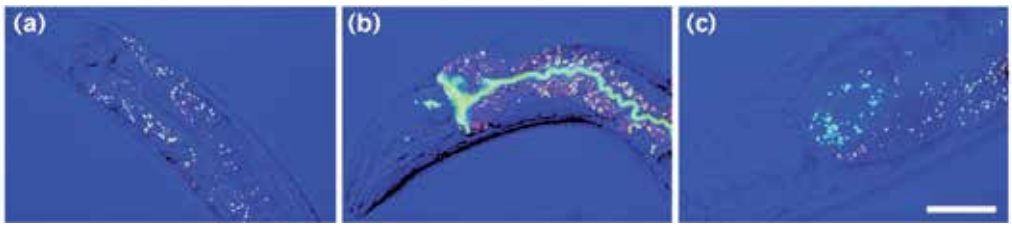

Fig. 4. *S. typhimurium* colonizes the worm intestine. Young adult worms were fed on (a) *E. coli* DH5α–GFP or (b, c) *S. typhimurium* SL1344–GFP for 5 h and then transferred to *E. coli* OP50 for (a, b) 24 h or (c) 96 h. Scale bar, 50 μm (Courtesy Aballay et al., 2000).

Bacterial proliferation and persistence can be easily determined by monitoring the worms in due course under microscope for the presence of GFP-labeled bacteria. In particular, pathogenic strains expressing green fluorescent protein (GFP) are therefore extremely useful in examining the fate of such microbes upon ingestion by the worms (Fig. 5). A virulent strain of

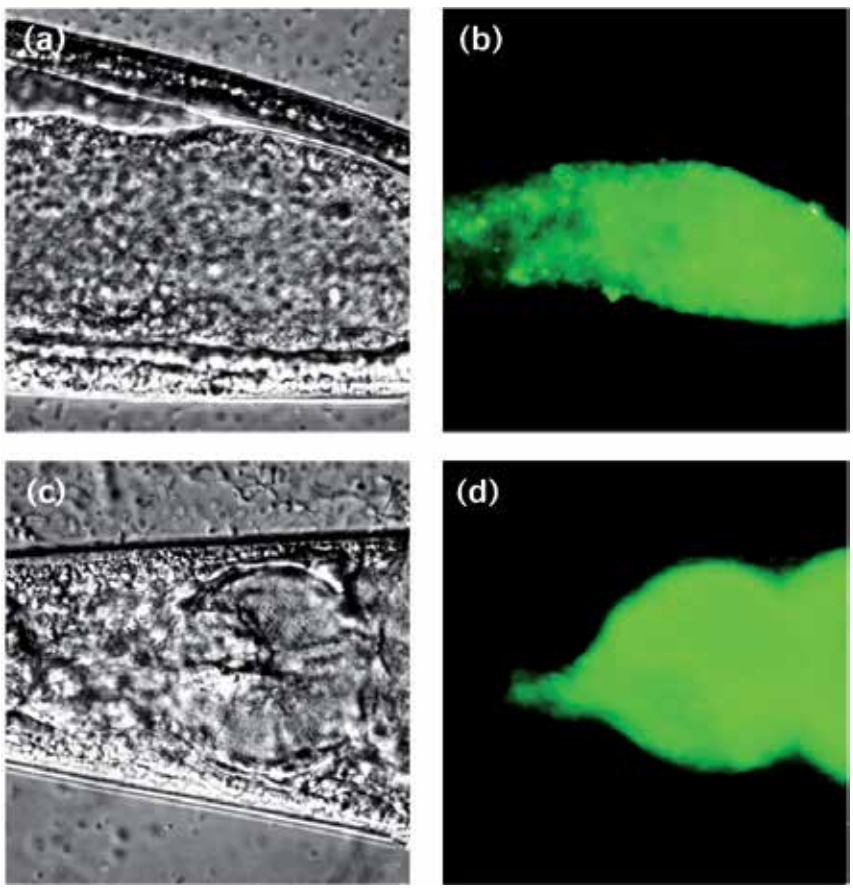

Fig. 5. Accumulation of *S. typhimurium* within the intestine and pharynx of *C. elegans.* (a,c) Nomarski and (b,d) fluorescence photomicrographs of the (a,b) posterior and (c,d) anterior of a worm after contact for 5 days with GFP-expressing *S. typhimurium*. The intestine and terminal bulb of the pharynx are seen to be full of intact bacteria (Courtesy Labrousse et al., 2000).

*S. typhimurium e*xpressing GFP (12023 ssaV–GFP) is known to kill *C. elegans* as the wild-type strain. The grinder which is located in the terminal bulb of the pharynx of the wormsnormally breaks bacteria (Albertson & Thomson, 1976). However, with increasing infection, the number of *S. typhimurium* significantly increases beyond the terminal bulb and gradually starts to mount up within the intestinal lumen (Fig. 5). Increase in the intestinal lumen of the worms is accompanied by the decrease in the volume of the intestinal cells. Nonetheless, the cells of the terminal bulb of the pharynx get progressively destroyed and their place is taken up by bacteria. Also worms with defective grinders have been found to be more susceptible to *Salmonella* infection and therefore less resistant to the pathogenic effects. For example, *phm-2* worm mutants possess abnormal terminal bulb and therefore are more susceptible to bacterial attack than the N2 worms (Fig. 6) (Labrousse et al., 2000).

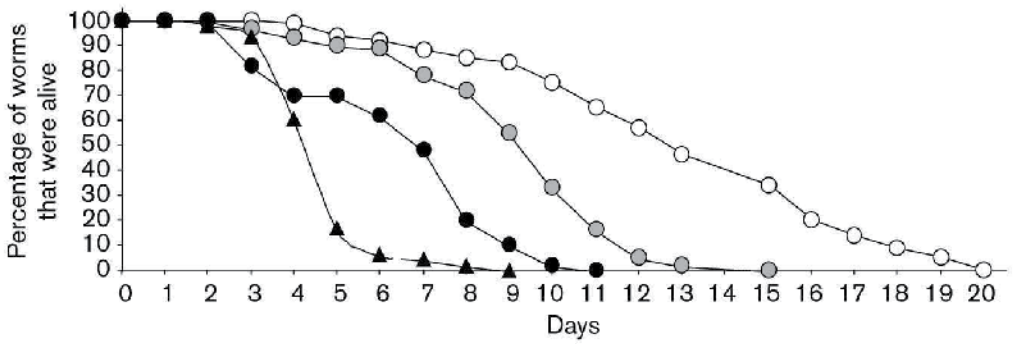

Fig. 6. Survival of *C. elegans* fed on *E. coli* and *S. typhimurium*. Wild-type worms (circles) *or phm-2* mutants (triangles) fed on *E. coli* strain OP50 until the larval L4 stage and then kept on OP50 (open circles), or transferred *to S. typhimurium* strain 12023 (black symbols), or after 8 h Thimerosal sterilization and returned to OP50 (grey circles). Dead worms were scored accordingly. (Courtesy Labrousse et al., 2000).

5. Assessment of pathogenicity of microbes to *C. elegans*

Both genetic and environmental factors play an important role in determining the virulence of a pathogen. Host mortality assays are generally performed to assess the pathogenicity of the microbes. This is generally done by measuring the time (TD50: time to death for 50% of the host) required by the microbe to kill a fixed percentage of host (Mahajan-Miklos et al., 1999; Garsin et al., 2001). As already mentioned earlier, *S. enterica* serovar Typhimurium colonizes the nematode intestine (Aballay et al., 2000; Labrousse et al.,2000). Adult worms transferred plates seeded with *S. enterica* and incubated at 25° C, the TD50 was shown to be was 5.1 days, compared to 9.9 days for control animals fed on *E. coli* OP50 (Aballay et al., 2000). When worms were exposed to *S. enterica* for merely 3 h, then removed to OP50, there was a significant early death in the worm population suggesting the pathogenic effect of *S. enterica* on *C. elegans*. Although invasion of host cells is an essential aspect of *Salmonella* sp. pathogenesis in higher animal systems, yet it has been demonstrated that *S. enterica* does not appear to invade *C. elegans* cells.

Salmonella pathogenicity islands -1 and -2 (SPI-1 and SPI-2), PhoP and a virulence plasmid are required for the establishment of a persistent infection (Alegado & Tan, 2008). It was observed that the PhoP regulon, SPI-1, SPI-2 and spvR are induced in *C. elegans* and isogenic strains lacking these virulence factors exhibited significant defects in the ability to persist in the worm intestine. *Salmonella* infection also led to induction of two *C. elegans* antimicrobial genes, *abf-2* and *spp-1*, which operate to limit bacterial proliferation. Thus resistance to host antimicrobials in the intestinal lumen has been found to be a key mechanism for *Salmonella* persistence. Apart from genetic factors there are environmental factors, such as, the composition of the media on which the pathogen is grown that has been shown to have influence on the host's mortality rate. For example, *Escherichia coli* OP50, which is non pathogenic otherwise can be rendered pathogenic almost as pathogenic as *Enterococcus faecalis* when it is grown on brain heart infusion (BHI) agar (Garsin et al., 2001). *Salmonella enterica* strains grown on NGM are rendered infectious depending on their serotypes (Table 1).

Strain	Growth media	Pathogenicity status	References
S. enterica ser. Paratyphi	NGM	Non-pathogenic	Aballay et al. , 2000
S. enterica ser. Typhi	NGM	Non-pathogenic	Aballay et al. , 2000
S. enterica ser. Dublin	NGM	Infectious	Aballay et al. , 2000
S. enterica ser. Enteritidis	NGM	Infectious	Aballay et al. , 2000
S. enterica ser. Typhimurium	NGM	Infectious	Aballay et al. , 2000, Labrousse et al. (2000)

Table 1. Effect of media on *C. elegans* exposed to *Salmonella* (Adapted from Alegado et al., 2003).

6. *C. elegans* inherent immune response to *Salmonella* infection

Innate immunity consists of a variety of defense machinery used by metazoans to avert microbial infections. These nonspecific defense responses used by the innate immune system in animals are governed by interacting and intersecting pathways that not only directs the immune responses but also governs the longevity and responses to different stresses. Even though ample research on *C. elegans* immune response is still ongoing, yet there has not been enough information on the worms' innate immune response towards bacterial pathogens in contrast to the fruit fly, *Drosophila,* and mammals where a fundamental feature like Toll signaling pathway exists. For example, isolation of a strain carrying a mutation in *nol-6*, which encodes a nucleolar RNA-associated protein in *C. elegans* or RNAi-mediated depletion of *nol-6* as well as other nucleolar genes led to an enhanced resistance to *S. enterica* mediated killing that was associated with a reduction of pathogen accumulation. These results also demonstrated that animals deficient in *nol-6* are more resistant to infections by Gram-negative and Gram-positive pathogens signifying that

nucleolar disruption activates immunity against different bacterial pathogens (Fuhrman et al., 2009). Studies also indicated that nucleolar disruption through RNAi ablation of ribosomal genes resulted in an increased resistance to pathogen that requires P53/CEP-1. Thus from the reports it is quite evident that *C. elegans* activates innate immunity against bacterial infection in a *p53/cep-1*-dependent manner (Fig. 7). Furthermore, *C. elegans* mutants which exhibited reduced pathogen accumulation (Rpa), displayed enhanced resistance to *S. enterica*-mediated killing (Fig. 8).

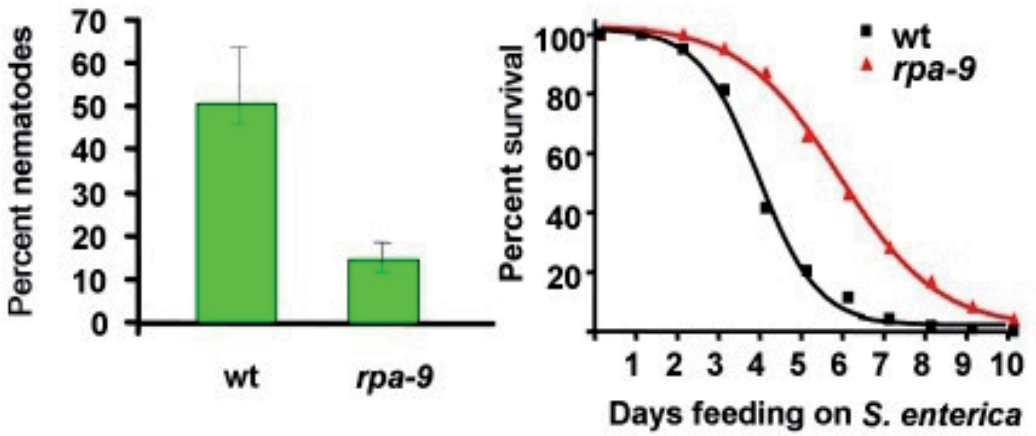

Fig. 7. *rpa-9* mutants are resistant to both *S. enterica* accumulation and *S. enterica*-mediated killing (Courtesy Fuhrman et al., 2009).

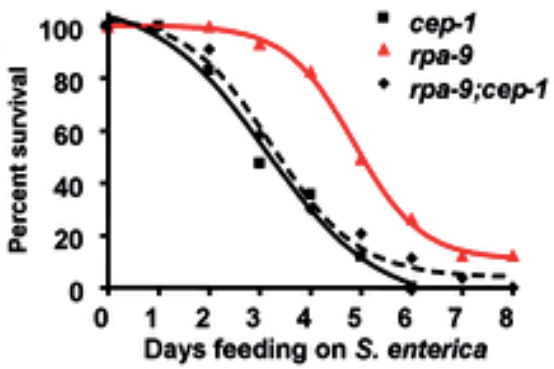

Fig. 8. *rpa-9* mutation activates immunity against *S. enterica* in a p53/cep-1– dependent manner. (Courtesy Fuhrman et al., 2009).

To date different molecular approaches, including forward genetics screens and RNAi have facilitated the identification of certain signaling pathways involved in the response of *C. elegans* to infection. For example, *Salmonella enterica* serovars is also known to trigger programmed cell death (PCD), and *C. elegans* cell death (ced) mutants have been shown to be more susceptible to *Salmonella*-mediated killing (3) (Aballay et al., 2003). *Salmonella*-elicited PCD was shown to require p38 mitogen-activated protein kinase (MAPK)

encoded by the *pmk-1* gene. On the other hand inactivation of *pmk-1* by RNAi blocked *Salmonella*-induced cell death. *C. elegans* innate immune response triggered by *S. enterica* was thus shown to require intact lipopolysaccharide (LPS) and is mediated by a MAPK signaling pathway. Besides innate immunity in *C. elegans* is known to be regulated by neurons expressing NPR-1/GPCR, a G-protein-coupled receptor related to mammalian neuropeptide Y receptors that functions to suppress innate immune responses (Styer et al., 2008).

With regard to the conserved Toll signaling, *C. elegans* too possesses a toll-signaling pathway comparable to the innate immunity found in *Drosophila* or mammals. As opposed to the fly and mammalian tolls, *C. elegans tol-1* (the *C. elegans* homolog of Toll) was previously stated to be required for the worm development and recognition of pathogens but not important for resistance to the pathogens (Pujol et al., 2001). However, later evidences subsequently support that TOL-1 is required to prevent *Salmonella enterica* invasion of the pharynx, which comprise one of the first barriers against pathogens in *C. elegans*. It was also illustrated that TOL-1 is required for the correct expression of ABF-2, which is a defensin-like molecule expressed in the pharynx, and heat-shock protein 16.41 (HSP-16.41), which is also expressed in the pharynx, and is part of a HSP superfamily of proteins required for *C. elegans* immunity. Thus, TOL-1 has been shown to have a direct role in *C. elegans* defence against pathogens (Tenor & Aballay, 2008).

7. Influence of probiotic bacteria on *Salmonella*-infected *C. elegans*

Probiotic bacteria have been defined as living microorganisms that exert useful effects on human health when ingested in sufficient numbers. Lactic acid bacteria (LAB) are the most frequently used probiotic microorganisms. LAB have been found to have a wide range of physiological influences on their hosts, including antimicrobial effects, microbial interference, supplementary effects on nutrition, antitumor effects, reduction of serum cholesterol and lipids, and immunomodulatory effects. Lactobacilli and bifidobacteria fed worms were shown to display increased life span and resistance to *Salmonella* clearly showing that LAB can enhance the host defense of *C. elegans* by prolonging the life span (Ikeda et al., 2007). Hence the nematode may once again emerge out as an appropriate model for screening useful probiotic strains or dietetic antiaging substances.

8. Role of NRAMPs and autophagy in bacterial infection

The *C. elegans* intestine also presents many advantages because this system can mimic the host–pathogen interactions that occur specially during phagocytosis. Macrophages play a pivotal role in the resolution of microbial infections via the process of phagocytosis. Nramp1 (Natural resistance-associated macrophage protein-1) is a functionally conserved iron-manganese transporter in macrophages and manganese, a superoxide scavenger, which is required in trace amounts and functions as a cofactor for most antioxidants. Nramp homologues, *smf*s, have been identified in the nematode *C. elegans* (Bandyopadhyay et al., 2009). We have demonstrated that hypersensitivity to the pathogen *Staphylococcus aureus*, an effect that was rescued by manganese feeding or knockdown of the Golgi calcium/manganese ATPase, *pmr-1*, indicating that manganese uptake is essential for the innate immune system. Reversal of pathogen sensitivity by

manganese feeding suggested a protective and therapeutic role of manganese in pathogen evasion systems thus proposing that the *C. elegans* intestinal lumen may mimic the mammalian macrophage phagosome and thus could be a simple model for studying manganese-mediated innate immunity. Similar experiments with *Salmonella enterica* in the near future may open more possibilities in favor of utilizing the nematode intestine as a model for manganese-mediated innate immunity.

Autophagy, a lysosomal degradation pathway, plays a crucial role in controlling intracellular bacterial pathogen infections. Jia et al., (2009) showed the outcome of autophagy gene inactivation by feeding RNAi techniques on *Salmonella enterica* serovar Typhimurium infection in *C. elegans*. Genetic inactivation of the autophagy pathway increased bacterial intracellular replication, decreased animal lifespan, and resulted in apoptotic-independent death. In *C. elegans*, genetic knockdown of autophagy genes abrogates pathogen resistance conferred by a loss-of-function mutation, *daf- 2(e1370)*, in the insulin-like tyrosine kinase receptor or by overexpression of the DAF-16 FOXO transcription factor. Therefore, autophagy genes play an essential role in host defense *in vivo* against an intracellular bacterial pathogen and mediate pathogen resistance in long-lived mutant nematodes.

9. *C. elegans* as a target for drug discovery

By means of genomics technologies, *C. elegans* is growing into a prominent model organism for functional characterization of novel drugs in biomedical research. In fact many biomedical discoveries, for example diabetes type 2 diseases, depression (relating to serotonergic signaling) or the neurodegenerative Alzheimer's disease have been made for the first time using the worms. The simple body plan of the worms has always made it an appropriate model for the fastest and most amenable to cost-effective medium/high-throughput drug screening technologies. Besides, *C. elegans* has always been a better choice over *in vitro* or cellular models to study drug-reporter interaction and in doing so monitoring the actual behavioral responses of the animals. Conventionally, antimicrobial drug discovery has brought about screening candidate compounds directly on target microorganisms (Johnson & Liu, 2000). In order to discover such novel antimicrobials, a series of antibiotics are therefore being screened to identify those that help in the survival of the worms or markedly reduce the number of bacteria colonizing the nematode intestine. For such high throughput screening of compound libraries, conventional agar-based infection experiments in *C. elegans* are later assessed in liquid media contained in standard 96-well microtiter plates for carrying out the curing assays. Interestingly, these simple infection systems may allow one to screen nearly 6,000 synthetic compounds and more than 1000 natural extracts. Moreover, the *in vivo* effective dose of many of these compounds was significantly lower than the minimum inhibitory concentration (MIC) needed to prevent the growth of the pathogens *in vitro*. More importantly, many of the compounds and extracts had not as much of affect on in bacterial growth *in vitro*. Screening synthetic compound libraries and as well as extracts of natural products for substances that cure worms from bacterial persistent infection allows one to identify compounds that not only blocks pathogen replication *in vitro* but in addition identifies virulence of the pathogen, may kill it, or may augment the host's immune response. Nevertheless, activities of some these compounds or extracts are considerably high only in whole animal assay *in vivo*, and hence the rationale for using a whole-animal screen in a drug discovery program.

10. Closing remark

Attention must be given to the *C. elegans* natural bacterial food, pathogens and their virulence factors. A better understanding about the dietary behavior and the natural pathogenic organisms of the *C. elegans* shall open the gates for more information about this worm. Besides, the introduction of genomics and combinatorial chemistry has firmly enabled one to make use of defined targets to identify new antibiotics. The nematode *C. elegans* has undoubtedly proven to be a simple model for studying the interaction between microbial pathogens and host factors, and further examining the roles of specific gene products to virulence and immunity. It is apparent that there are conserved pathogenic genes involved in *C. elegans* killing and mammalian pathogenesis. An important experimental advantage of *C. elegans* as a model to study bacterial pathogenesis is that genetic analysis may as well be carried out in both the pathogens and in the host, simultaneously, a process termed as "interactive genetic analysis." It would undoubtedly be more useful to further focus on the characterization of chemical suppressors of virulent factor expressions or secretions as candidate novel antibiotics, taking *C. elegans* as the model. Additionally the worm model would also be useful to address questions with regard to the pathophysiology of worm death in case of lethal infections and further extend to identify the groups of virulent factors that are important in *C. elegans* killing.

The various categories of experiments so far carried out has provided a proof-of-principle that screening experiments may be useful in identifying new bacterial virulence factors, not only in *Salmonella*, but perhaps other pathogens that are able to cause a persistent infection in *C. elegans*, such as *S. aureus* (Sifri et al., 2003). Until date several loci have been identified from screens not having direct implication in *Salmonella* virulence. Thus, a saturating genome-wide screen would be extremely fruitful in identifying the predominance of *Salmonella* genes that are required for persistent infection in *C. elegans*, some of which could also be important for pathogenesis in other hosts.

11. Acknowledgment

All publications and figures referred in this chapter have been cited to justify the theme of the present review article. We are deeply indebted to all the authors of the original papers. At the same time we sincerely regret for those references that have been left out unintentionally.

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23

Insect/Bacteria Association and Nosocomial Infection

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1. Introduction

The genus *Salmonella* belongs to the family Enterobacteriaceae, a group of bacteria normally found in the intestine of the hosts. More than 2,500 serotypes have been identified in the *Salmonella enterica* complex (Popoff et al., 2004). *Salmonella* species are classified as Gram-negative, rod-shaped, facultatively aerobic bacteria that have mobility in liquid environments and reproduce at temperatures ranging from 5°C to 47°C and pH from 4.5 to 9.0 (Varnan & Evans, 1991). *Salmonella* species produce hydrogen sulfide, are oxidase, indol, and Voges-Proskauer negative, catalase, citrate, lysine-ornithine, decarboxylase, and glucose positive, also presenting other carbohydrates fermentation with acid and gas liberation (Le Minor, 1984). Although the dispersion of these microorganisms is limited due to their incapacity to sporulate and sensitivity within the pasteurization temperature range (Varnan & Evans, 1991; D'Aoust, 2000), they are resistant to desiccation and freezing and are able to survive in the environment for several years (Tortora et al., 2005).

A variety of human foods of plant and animal origin have been identified as vectors of transmission of *Salmonella*. A study with inoculation of *Salmonella* in a tomato plantation soil evidences the risk of human contamination through ingestion of plant foods (Barak & Liang, 2008). According to Hirsh (2003), apparently healthy animals can develop diseases caused by *Salmonella* because of stress factors, such as sudden alterations of the environment temperature, water and food deprivation, overpopulation, gathering animals of different lots, and inappropriate antimicrobial use. As contaminated meat is the most frequent source of human disease caused by *Salmonella*, this type of food is of particular interest concerning epidemiological studies (Gatto et al., 2006). The genus *Salmonella* was found in 20% of the commercialized chicken samples in Malaysia (Rusul et al., 1996). Human disease outbreaks caused by *Salmonella* are generally associated to egg, chicken, and pork consumption (Castagna et al., 2004). Inappropriate food storage also represents an important cause of proliferation and dissemination of these microorganisms (Murmann et al., 2005). *Salmonella enteritidis* and *Salmonella typhimurium* were the most prevalent serotypes involved in foodborne infections registered in the world (Van Der Wolf et al., 2001).

2. Epidemiology

The acute symptoms of the infection caused by *Salmonella* are fever, migraine, nausea, and dysentery. Depending on the patient profile, the extraintestinal chronic form can evolve into sepsis (CDC, 1999; Wilson & Whitehead, 2004; Loureiro et al., 2010). *S. enterica* serotype Blockley is related to up to 29% of the cases of arthritis described in the literature (Dworkin et al., 2001). An aggravating factor in diseases caused by *Salmonella* is the evolution of resistant types due to uncontrolled antibiotic use both in domesticated animals and humans. Antibiotics and chemotherapics are chemical compounds that can inhibit bacterial growth (Cromwell, 1991), but can result in microorganism resistance if used indiscriminately, disregarding bacterial specificity or the minimum inhibitory concentration (Wannmacher, 2004). Therefore, Silva et al. (2004) consider that the use of antimicrobials in bird diets is of great importance to break the cycle of bird disease caused by *Salmonella*. In cases of continuous or prolonged treatment with high-dose antibiotic in humans, instead of curing the patient the infection rates can increase (Barza & Travers, 2002; Wannmacher, 2004). Studies demonstrated that the rates of *Salmonella* multi-drug resistance have increased considerably in recent years (Haneda et al., 2004; Chiu et al., 2006; Carneiro et al., 2008; Pesquero et al., 2008). Bacterial resistance can lead to increased virulence and consequently increased morbidity and mortality of infected people (Mølbak, 2005).

Salmonella resistance to antibiotics has been related to the presence of plasmids. Approximately 30 low-molecular-weight plasmids have been identified in *S. enteritidis* (Rychlik et al., 2001). Plasmids described in *S. enterica* serovar typhi isolates (Boyd et al., 2003) confer pathogenicity to these bacteria (Baker & Dougan, 2007). Some plasmids simultaneously confer resistance and virulence to *Salmonella*. This bacteria-plasmid association presents epidemiological relevance, because a process of recombination with *Salmonella* provides it with advantages to survive in a hostile environment and chances to evolve a new genetic lineage (Majtan et al., 2006).

Antibiotic resistance genes are frequently located within transposons, but they can also be found in the form of gene cassettes captured and clustered in integrons and thereafter mobilized to spread resistance among other organisms (Fluit, 2005). In *S. typhimurium*, antibiotic resistance depends on integrons more frequently associated to a genomic island located on the bacterial chromosome (Tosini et al., 1998). There are two categories of integrons, one represented by repeated direct sequences (IS) and the other represented by inverted sequences (IR). The first integron category, widely distributed in *Salmonella*, consists of two conserved sequences, regions 50 Cs and 30 Cs, which carry the gene int I to the integrase protein and the gene sul I of resistance to the sulfonamide, respectively (Guerra et al., 2002).

Some plasmids are responsible for the phage conversion, which permits bacteria to resist phage infection. For example, the pOG670 plasmid of 54 kb, belonging to the group of incompatibility X (IncX), present in *S. enteritidis*, is capable of converting phages types 1 and 4 into type 6a, and phage 8 into type 13 (Ridley et al., 1996). In *S. abortus-equi*, a plasmid of 85 kb that codifies resistance to toxic heavy metals (chromium, arsenic, cadmium, and mercury) was described. This plasmid was also proven to encode genes that allowed the use of citrate and conferred ß-lactam antibiotic resistance (Ghosh et al., 2000).

Guiney et al. (1995) and Roudier et al. (1992) found serotypes of non-typhoid *Salmonella*, associated with extraintestinal disease, possessing virulent plasmids (spvC) that contain virulence genes (spvC), important for the induction of a systemic and lethal infection. Fierer et al. (1992) reported plasmids in *Salmonella* in 76% of 79 samples of human blood and in 42% of 33 human stool samples. Both in animals and humans, bacteria of the genus *Salmonella* are more frequently found in systemic infections compared with enteric ones. According to Fierer et al. (1993), non-typhoid *Salmonella* pathogenicity is related to the presence of plasmids.

The high susceptibility of hospitalized children to nosocomial infections was attributed to antimicrobial resistance of *S. enterica* (Fonseca et al., 2006). Some disease outbreaks caused by *Salmonella* serotypes resistant to antibiotics have been registered in pediatric settings worldwide and can provoke the death of newborn babies (Pessoa-Silva et al., 2002; Bouallègue-Godet et al., 2005). Diseases caused by *Salmonella* represent 10% to 15% of the acute gastroenteritis cases all over the world (Jay, 2005). Of the 4,012 disease outbreaks of enteric infections that occurred in England and Wales, *Salmonella* was the most frequent microorganism, responsible for 22% of the cases (Guard-Petter, 2001). Estimates indicate that *Salmonella* is responsible for one third of the cases of foodborne illnesses in the US, corresponding to 2-4 million cases a year (Andrews et al., 1992), causing economic losses of about 4 billion dollars annually (Mead et al., 1999). In South American countries, the prevalence of *Salmonella* infections is low (2.5%) compared with the US, although considered one of most important epidemiological illnesses (Franco, 2003).

3. Insect vectors

The transformation of natural ecosystems into urban areas and crop fields results in changes in animal, plant, and microorganism biodiversity and dynamics. In a broad sense, the simplification of the environment reduces biological diversity but, on the other hand, it favors the population growth of other species of bacteria (Fowler, 1983). Opportunistic animals that benefit from human presence are called synanthropic and considered pests if they cause damages to human health and the economy. The current context of worldwide social economic development contributes to environmental deterioration, facilitating horizontal and vertical transmission of illnesses through vectors, mainly insects (Pongsiri & Roman, 2007).

Vectors are organisms that contribute to the dispersion of pathogens by carrying and transmitting them (Purcell & Almeida, 2005), and are known as intermediate or definitive hosts, respectively when the pathogenic organism is carried externally or internally to the vector body. Transmission between hosts can occur indirectly by pathogen spread in the environment, normally through feces and/or secretions, and also due to physical contact, when the pathogen is adhered to the surface of the vector body, or directly by inoculation of the pathogen in the host body through the vector bite. Triatomine species (Hemiptera: Reduviidae) increase human transmission rate of the protozoan *Trypanossoma cruzi* by depositing feces contaminated with infecting metacyclic trypomastigotes on the host face skin near the insect bite.

Insects are among the most diversified, abundant, and widely dispersed animals in the world. They represent more than 50% of all species living on the planet, 71% of animal

species, 74% of invertebrates, and 87% of arthropods (Lewinson & Prado, 2002). This success is mainly due to their morphologic characteristics, such as locomotion appendices (legs and wings), exoskeleton, small body size, and metamorphosis. Insects can use specialized types of sexual and asexual reproduction, such as parthenogenesis, pedogenesis, and neoteny, as reproductive strategies (Gullan & Cranston, 2008). The most representative insect orders regarding species richness and abundance are Coleoptera (beetles), Diptera (flies and mosquitoes), Hymenoptera (bees, wasps, and ants), Lepidoptera (butterflies and moths), and Hemiptera (chinch bugs, aphids, whiteflies, mealybugs, and cicadas).

Some species of blood-feeding insects found a direct food source in humans, and many of them are vectors of microorganisms that cause important diseases in tropical and subtropical countries, such as malaria, yellow fever, typhoid fever, dengue fever, filariasis, leishmaniasis, chagas disease, and sleeping sickness. Another group of synanthropic insects indirectly benefits from human foods found in crop fields, or stored, industrialized, and prepared in households, or rejected food waste in landfills and sewage. These insects are known as phytophagous, granivorous, parasites, saprophagous, coprophagous, and generalists, constituting the great majority of insects associated with humans in urban centers and rural areas. Among these insects, the species that are carriers of pathogenic microorganisms from contaminated environments to human food, medical instruments, and kitchen utensils deserve special attention of public health authorities. Therefore, the participation of insects in the transmission of bacteria has been investigated aiming to reduce the occurrence of enteric disease outbreaks in hospitals.

Arthropods are common in hospital environment (Gazeta et al., 2007) and the main vectors of pathogenic microorganisms that infect humans are cockroaches, ants, and flies because of their contact with human feces and other contaminated materials. Sewage and landfills are major sources of pathogenic microorganisms and lack of investments in basic sanitation is a serious public health problem (Andreoli & Bonnet, 2000; Bastos et al., 2003). Cockroaches (Blattodea) are dorsoventrally flattened body insects that eat preferentially decayed vegetable substances. They have specific enzymes and endosymbiont microorganisms that assist them in cellulose digestion and essential amino acid synthesis (Hirose & Panizzi, 2009; Louzada, 2009). *Blattella germanica* and *Periplaneta americana* are cosmopolite cockroach species adapted to urban environments, specially sewage, bathroom, and kitchen, where they find abundant food and absence of predators. Microbiological analyses of cockroaches captured in hospitals and residences have identified these insects as vectors of more than 80 recognized bacteria species belonging to 51 genera, many of them resistant to antimicrobials, posing health risk to the already weakened interns (Table 1). *Salmonella* was found in nine out of twelve studies with cockroaches in hospitals and residences in the world. Although *Salmonella* multiplication might occur in the intestines of the insects (Klowden & Greenberg, 1977), Gram-negative bacteria are more commonly found in their cuticle, facilitating the dispersion of pathogens (Mpuchane et al., 2006).

Ants are eusocial insects that live in colonies with one or more queens and thousands of workers. The latter need to collect food for their own maintenance and to feed both the immature and adult individuals of the colony, including the queen. Through chemical orientation, workers can cover great distances searching for food and return to the nest indicating the way for the other members of the colony. Their feeding habit is varied (Brandão et al., 2009), but the species adapted to urban environments are characterized by the

consumption of a generalist diet, mobility of colonies, polygeny, monomorphism, and reduced body size (Passera, 1994). *Monomorium pharaonis*, *Paratrechina longicornis*, *Tapinoma melanocephalum*, and species of *Pheidole* and *Solenopsis* are the main ants already found in hospital environments (Fig. 1), and the literature registered 50 bacteria species belonging to 31 genera associated to these and other ant species (Table 2, Fig. 2). Species of the genus *Staphylococcus, Escherichia, Pseudomonas, Enterobacter, Bacillus, Streptococcus*, and *Klebsiella* are the most frequent bacteria associated with ants in Brazilian hospitals (Fig. 3). *Salmonella* was found associated only with *Monomorium pharaonis* (Beatson, 1972) and a species of *Pheidole* (Pesquero et al., 2008) carries bacteria presenting antimicrobial resistance (Carneiro et al., 2008).

Diptera constitutes a great order of insects that present small body size, fly fast, and have a variety of feeding habits, including necrophagy and coprophagy, during the immature phase. When walking over feces and corpses to deposit eggs, female flies acquire and carry pathogenic bacteria to the interior part of residences, restaurants, and hospitals, contaminating mainly foods and kitchen utensils. *Musca domestica* (Muscidae) is a very common cosmopolite and synanthropic species in urban zones, and together with five other species of flies, is associated to 12 bacteria species belonging to 11 genera (Table 3). *Musca domestica* is the most studied fly considered a vector of *Salmonella* that occurs in some countries (Bolaños-Herrera, 1959; Bidawid et al., 1978; Arnold, 1999; Olsen & Hammack, 2000; Mian et al., 2002; Oliveira et al., 2006; Cardozo et al., 2009; Prakash & Tikar, 2009; Butler et al., 2010; Choo et al., 2011).

Cockroaches	Bacteria	Reference
Blattella germanica	*Enterobacter* *Enterococcus* *Escherichia coli* *Haemophilus* *Klebsiela* *Pseudomonas* *Shigella* *Staphylococcus* *Streptococcus*	Salehzadeh et al. (2007)
Periplaneta *Blatta*	*Salmonella bovismorbificans* *Salmonella oslo* *Salmonella typhimurium*	Devi & Murray (1991)
Blattella germanica	*Salmonella enteritidis*	Ash & Greenberg (1980).
Periplaneta americana *Blattella germânica* *Supella longipalpa* *Blatta lateralis* *Polyphaga aegyptiaca* *Arenivaga roseni* *Parcoblatta*	*Salmonella*	Fathpour et al. (2003)

Cockroaches	Bacteria	Reference
cockroaches	*Aeromonas* *Escherichia coli* *Citrobacter freundii* *Enterobacter cloacae* *Klebsiella pneumoniae* *Proteus mirabilis* *Proteus vulgaris* *Pseudomonas aeruginosa* *Salmonella* *Serratia marcescens* *Staphylococcus aureus* *Staphylococcus faecalis* *Staphylococcus epidermidis*	Tatfeng et al. (2005)
Periplaneta americana	*Enterobacter* *Escherichia coli* *Klebsiella* *Proteus vulgaris* *Salmonella* *Serratia* *Shigella* *Staphylococcus aureus* *Staphylocoques epidermidis* *Streptococcus*	Lamiaa et al. (2007)
Blattela germanica	*Bacillus cereus* *Escherichia coli* *Salmonella* *Shigella flexneri* *Staphylococcus aureus*	Tachbele et al. (2006)
Periplaneta americana *Blattella germânica*	*Acinetobacter* *Alcaligenes faecalis* *Arizona* *Bacillus* *Citrobacter freundii* *Enterobacter aerogenes* *Enterobacter agglomerans* *Enterococcus* *Escherichia coli* *Hafnia alvei* *Klebsiella pneumoniae* *Proteus mirabilis* *Proteus vulgaris* *Providencia rettgeri* *Pseudomonas* *Salmonella typhimurium* *Shigella* *Staphylococcus* *Yersinia*	Fakoorziba et al. (1910)

Cockroaches	Bacteria	Reference
Periplaneta americana	*Enterobacter aerogenes* *Escherichia coli* *Citrobacter freundii* *Hafnia alvei* *Salmonella* *Serratia liquefaciens* *Staphylococcus*	Miranda & Silva (2008)
Periplaneta americana *Blatta orientalis*	*Cedecea davisae* *Cedecea lopagei* *Cedecea neteri* *Citrobacter diversus* *Citrobacter freundii* *Edwardsiella ictaluri* *Edwardsiella tarda* *Enterobacter aerogenes* *Enterobacter agglomerans* *Enterobacter asburiae* *Enterobacter cloacae* *Enterobacter gergoviae* *Enterobacter sakasakii* *Escherichia balttae* *Escherichia coli* *Escherichia hermanii* *Escherichia vulneris* *Ewingella americana* *Hafnia alvei* *Klebsiella oxytoca* *Klebsiella ozanae* *Klebsiella pneumoniae* *Klebsiella rhinoscleromatis* *Klebsiella terrigena* *Kluyvera ascorbata* *Moraxella uretharlis* *Morganella morganii* *Obesumbacterium* *Proteus mirabilis* *Proteus myxofeceins* *Proteus penneri* *Proteus vulgaris* *Providencia rustigianii* *Pseudomonas aeruginosa* *Pseudomonas maltophilia* *Pseudomonas pseudoalkaligenes* *Rahnella aquatilis* *Salmonella* *Serratia liquefaciens* *Serratia marcescens* *Serratia odotifera*	Chaichanawongsaroj et al. (2004)

Cockroaches	Bacteria	Reference
Supella supellectilium	*Achromobacter* *Acinetobacter calcoaceticus* *Aeromonas hydrophila* *Alcaligenes faecalis* *Buttiauxella agrestis* *Cedecea* *Citrobacter diversus* *Citrobacter freundii* *Enterobacter aerogenes* *Etnterobacter agglomerans* *Enterobacter amnigenus* *Enterobacter cloacae* *Enterobacter sakazakii* *Escherichia adecarboxylata* *Escherichia coli* *Klebsiella oxytoca* *Klebsiella pneunoniae* *Kluyvera* *Proteus mirabilis* *Pseudomonas aeruginosa* *Pseudomonas cepacia* *Pseudonmonas paucimobilis* *Pseudomnonas fluorescens* *Pseudomonas maltophilia* *Pseudomonas stutzeri* *Serratia marcescens* *Serratia liquefaciens* *Staphylococcus aureus*	Le Guyader et al. (1989)
cockroaches	*Actinomyces randingae* *Alcaligenes faecalis* *Arthrobacter cumminnsii* *Aureubacterium* *Bacillus* *Brevibacterium* *Burkholderia vietnamiensis* *Buttiauxella* *Citrobacter* *Corynebacterium* *Enterobacter* *Erwinia* *Escherichia coli* *Hafnia* *Kauri rosea* *Kigali* *Klebsiella* *Kluyvera*	Mpuchane et al., (2006)

Cockroaches	Bacteria	Reference
	Leuconostoc	
	Microbacterium	
	Micrococcus	
	Proteus	
	Providencia ruttier	
	Pseudomonas	
	Rhodococcus australis	
	Rhodococcus rhodochrous	
	Salmonella typhimurium	
	Serratia	
	Shigella	
	Spingobacterium thalpophilum	
	Staphylococcus	
	Stenotrophomonas maltophillia	
	Streptococcus	
	Tsukamurella inchonensis	
	Vibrio metschnikovii	
	Xanthomonas	

Table 1. Registry of occurrences of vector species of the order Blattaria and types of transported bacteria.

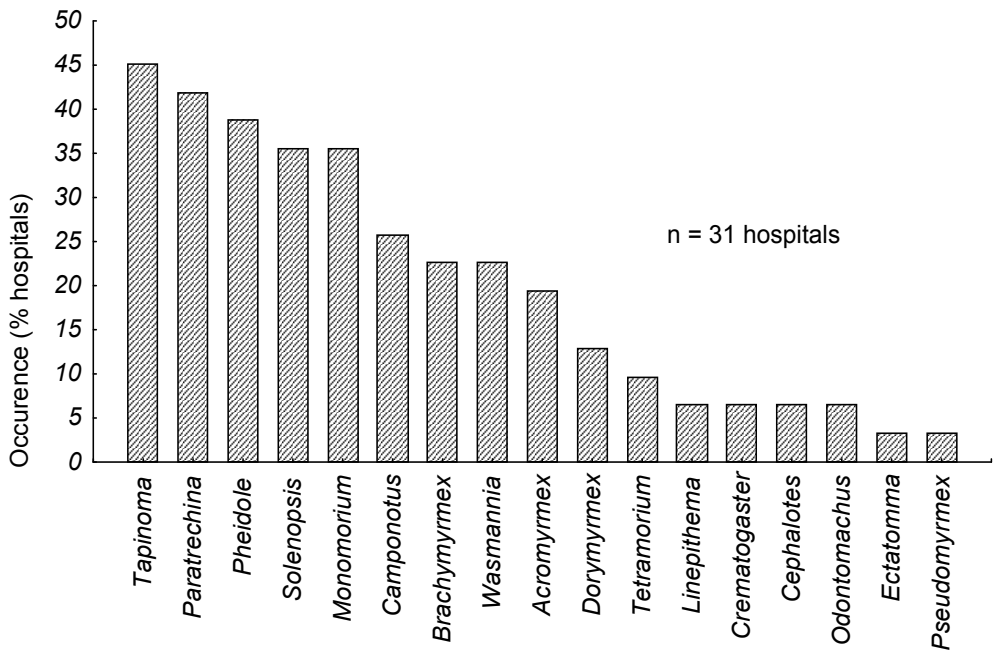

Fig. 1. Occurrence of ants in hospitals.

Ant	Bacteria	Reference
Acromyrmex	*Pseudomonas aeruginosa* *Staphylococcus* *Streptococcus faecalis*	Santos et al. (2009)
Brachymyrmex	*Enterococcus* *Streptococcus agalactiae*	Lise et al. (2006)
Camponotus	*Corynebacterium diphtheria* *Corynebacterium jeikeium* *Streptococcus*	Lise et al. (2006); Santos et al. (2009)
Camponotus vittatus	*Bacillus* *Staphylococcus*	Rodovalho et al. (2007)
Linepithema humile	*Escherichia coli* *Streptococcus*	Santos et al. (2009)
Monomorium pharaonis	*Acinetobacter haemolyticus* *Aeskovia* *Clostridium* *Corynebacterium* *Listeria monocytogenes* *Planococcus* *Pseudomonas aeruginosa* *Pseudomonas luteola* *Salmonella* *Sphingobacterium* *Sphingomonas paucimobilis* *Staphylococcus intermedius* *Stenotrophomonas maltophilia* *Streptococcus bovis* *Enterobacter agglomerans* *Enterococcus faecalis* *Enterococcus faecium* *Gemella haemolysans* *Klebsiella pneumoniae* *Streptococcus acidominimus* *Staphylococcus lugdunensis*	Lise et al. (2006); Moreira et al. (2005); Beatson (1972)
Odontomachus	*Enterococcus* *Escherichia coli* *Pseudomonas aeruginosa* *Staphylococcus* *Streptococcus*	Santos et al. (2009)

Ant	Bacteria	Reference
Paratrechina longicornis	*Acinetobacter haemolyticus* *Alcaligenes faecalis* *Alcaligenes sylosidans* *Bacillus* *Burkholderia cepacia* *Citrobacter diversus* *Comomonas acidoverans* *Corinebacterium* *Enterobacter aerogenes* *Enterobacter agglomerans* *Enterobacter cloacae* *Escherichia coli* *Gemella haemolysans* *Gemella morbillorum* *Klebsiella pneumoniae* *Proteus mirabilis* *Providencia alcalifaciens* *Pseudomonas fluorescens* *Pseudomonas putida* *Pseudomonas stutzieri* *Serratia marcescens* *Serratia rubidae* *Staphylococcus aureus* *Staphylococcus cohnii* *Stenotrophomonas altophilia*	Lise et al. (2006); Moreira et al. (2005); Fontana et al. (2010); Tanaka et al. (2007)
Pheidole	*Aeromonas* *Enterococcus* *Escherichia coli* *Klebsiella* *Pseudomonas aeruginosa* *Salmonella* *Staphylococcus* *Streptococcus*	Santos et al. (2009); Pesquero et al. (2008); Carneiro et al., (2008)
Pheidole megacephala	*Acinetobacter baumannii* *Bacillus* *Escherichia coli* *Pseudomonas aeruginosa* *Serratia liquefaciens* *Shigella sonnei* *Staphylococcus aureus*	Fontana et al. (2010)

Ant	Bacteria	Reference
Solenopsis	*Enterococcus* *Staphylococcus* *Streptococcus*	Santos et al. (2009)
Solenopsis globularia	*Bacillus* *Staphylococcus*	Fontana et al. (2010)
Solenopsis saevissima	*Corynebacterium* *Enterococcus* *Neisseria* *Pseudomonas luteola* *Staphylococcus saprophyticus* *Stenotrophomonas maltophilia*	Lise et al. (2006)
Tapinoma melanocephalum	*Acinetobacter baumanni* *Alcaligenes faecalis* *Bacillus* *Burkholderia cepacia* *Corinebacterium* *Enterobacter aerogenes* *Enterobacter amnigenus* *Enterobacter cloacae* *Enterococcus faecalis* *Escherichia coli* *Gemella morbillorum* *Hafnia alvei* *Klebsiella oxytoca* *Klebsiella pneumoniae* *Pseudomonas aeruginosa* *Pseudomonas fluorescens* *Staphylococcus saprophyticus* *Sphingomonas paucimobilis* *Staphylococcus aureus* *Staphylococcus epidermidis* *Staphylococcus equorum* *Staphylococcus saprophyticus* *Staphylococcus simulans* *Staphylococcus warneri* *Streptococcus viridans*	Lise et al. (2006); Moreira et al. (2005); Fontana et al. (2010); Tanaka et al. (2007); Teixeira et al. (2009); Rodovalho et al. (2007); Santos et al. (2009)
Wasmannia auropunctata	*Pseudomonas aeruginosa* *Staphylococcus* *Streptococcus*	Santos et al. (2009)

Table 2. Registry of occurrences of vector species of the family Formicidae (Hymenoptera) and types of transported bacteria.

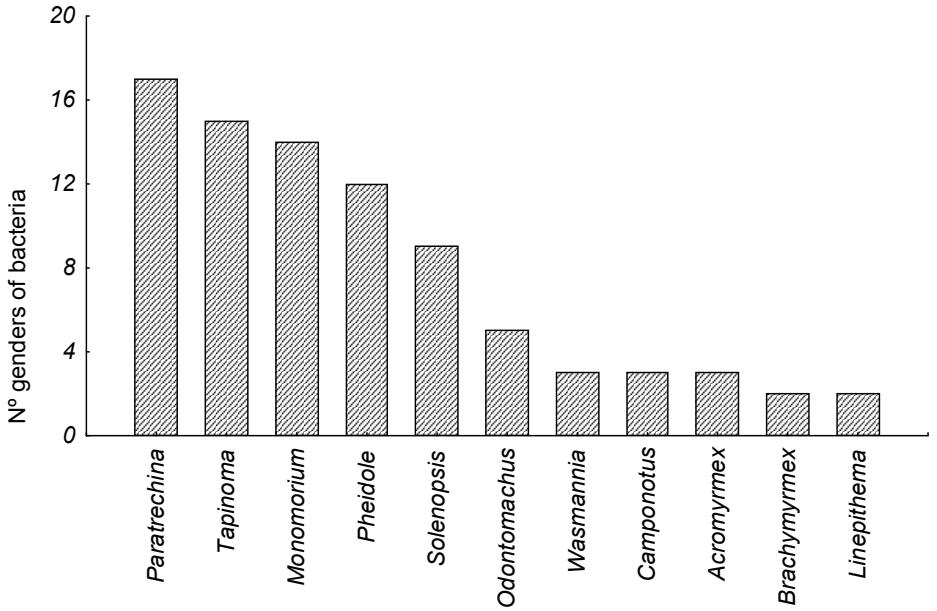

Fig. 2. Occurrence of bacteria genera per ant genus in hospitals.

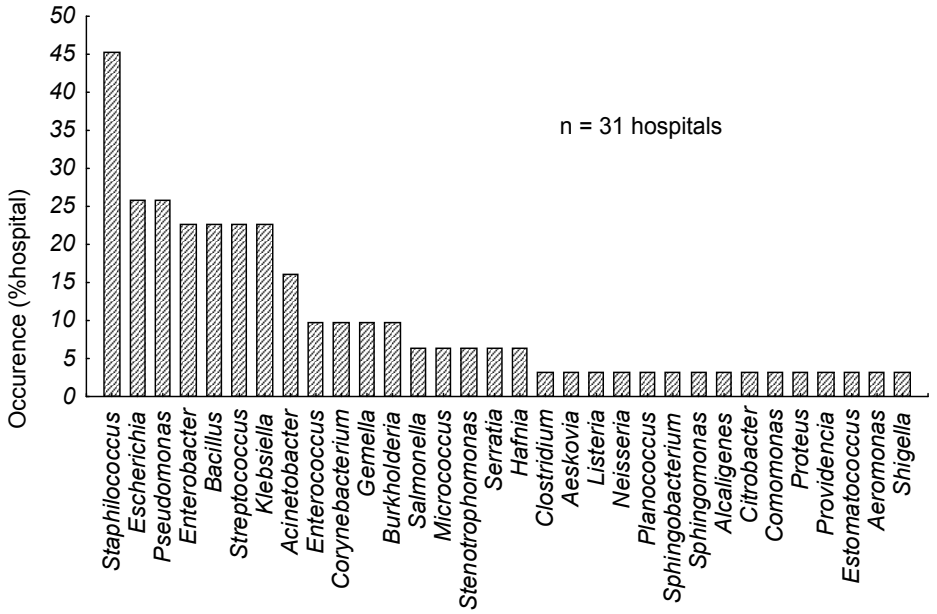

Fig. 3. Occurrence of bacteria genera in hospitals.

Fly	Bacteria	Reference
Hydrotaea aenescens *Musca domestica*	*Salmonella*	Olsen & Hammack (2000)
Chrysomya megacephala *Musca domestica*	*Citrobacter* *Enterobacter* *Escherichia coli* *Klebsiella* *Morganella* *Proteus mirabilis* *Pseudomonas* *Salmonella agona*	Oliveira et al. (2006)
Musca domestica	*Salmonella* *Shigella*	Bolaños-Herrera (1959)
Musca domestica *Fannia caniculares* *Muscina stabulans* *Phaenicia sericata*	*Salmonella* *Shigella*	Bidawid et al. (1978)
Musca domestica	*Salmonella enteritidis*	Mian et al. (2002)
Musca domestica	*Escherichia coli* *Salmonella typhi* *Shigella flexneri* *Yersinia enterocolitica*	Béjar et al. (2006)
Musca domestica	*Campylobacter* *Salmonella*	Choo et al. (2011)

Table 3. Registry of occurrences of vector species of the Order Diptera and types of transported bacteria.

4. Conclusion

The presence of any serotypes of *Salmonella* in any types of food is a reason to classify them as improper for consumption in the international market. Animals contaminated with *Salmonella* destined to human feeding cannot show clinical signals of the illness (Castagna et al., 2004). Therefore, industries that deal with products of animal origin must implement quality control strategies with the purpose of guaranteeing food safety. The main insect vectors of *Salmonella* are cockroaches and flies, but ants also represent a potential risk. Therefore, governments must invest in the construction of modern hospitals equipped with some devices capable of avoiding the entrance and permanence of vectors, particularly these three types of insects.

5. References

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24

The *Salmonella* Pathogenicity Island-1 and -2 Encoded Type III Secretion Systems

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1. Introduction

1.1 General aspects

Salmonellae are motile, facultatively anaerobic, Gram-negative rods measuring 0.3-1.5 by 1.0-2.5 μm in size. The genus Salmonella was named for Dr. Daniel Salmon, a veterinary bacteriologist at the United States Department of Agriculture (USDA) (Gast, 2003, Salyers & Whitt, 2002). The *Salmonella* species are closely related to *Escherichia*, *Yersinia*, and *Shigella*, and contain a circular chromosome approximately 4.7 Mbp in size with an overall GC content of 52% (Marcus, *et al*., 2000, Salyers & Whitt, 2002, Thomson, *et al*., 2008). The genus *Salmonella* lies within the kingdom Eubacteria, class Gammaproteobacteria, order Enterobacteriales, and family Enterobacteriaceae. *Salmonella* is divided into two species, *Salmonella bongori* and *Salmonella enterica*. Within *Salmonella enterica* there are 6 subspecies: *salamae*, *arizonae*, *diarizonae, houtenae*, *indica*, and *enterica* (Tindall, *et al*., 2005). These subspecies can be further classified into approximately 50 serogroups based on their lipopolysaccharide (LPS) O antigen component (Sabbagh, *et al*., 2010). *Salmonella enterica* subspecies *enterica* finds its niche in warm-blooded animals and is the primary species associated with human infections. *S. bongori* and other *S. enterica* subspecies are more commonly associated with cold-blooded animals, and in some cases can cause disease in these animals (Brenner, *et al*., 2000).

Salmonella enterica subspecies *enterica* can be further divided into over 2500 serovars based on their flagellar (H) antigen and LPS O antigen structures (Brenner, *et al*., 2000, Coburn, *et al*., 2007, Sabbagh, *et al*., 2010, Tindall, *et al*., 2005). For the purposes of this document, serovars within *Salmonella enterica* subspecies *enterica* (e.g., Enteritidis, Typhimurium, and Typhi) will be identified by an italicized S, followed by the serovar name (e.g., *S.* Enteritidis, *S.* Typhimurium, and *S.* Typhi). Many serovars are host-adapted, and tend to cause life-threatening systemic disease in their host. For example, *S.* Typhi and *S.* Paratyphi cause systemic disease in humans and some primates, while *S.* Gallinarum and *S.* Pullorum produce systemic disease in chickens, *S.* Dublin causes systemic disease in cattle, and *S.* Choleraesuis in pigs. In contrast, many serovars are non host-adapted and tend to cause

gastroenteritis in many different host species. *S.* Typhimurium and *S.* Enteritidis are the most well-known examples and are able to cause different disease outcomes in various host species (Barrow, 2007, Boyle, *et al*., 2007, Lax, *et al*., 1995, Spreng, *et al*., 2006, Zhang & Mosser, 2008). *S.* Typhimurium and *S.* Enteritidis are able to induce a systemic infection in mice, young calves, chicks, and piglets. However, they are also able to colonize poultry and adult cattle without symptoms (Barrow, 2007, Boyle, *et al*., 2007, Lax, *et al*., 1995, Spreng, *et al*., 2006, Zhang & Mosser, 2008). In humans, infection with either of these serovars results in a self-limiting gastroenteritis (salmonellosis) involving fever, diarrhea, and abdominal pain. In rare cases, typically in the very young or immunocompromised, the infection can become systemic and lead to hospitalization and even death. A very small proportion of humans with salmonellosis can develop reactive arthritis (previously referred to as Reiter's syndrome), which is initially characterized by joint pain, eye irritation, and pain during urination (Boyle, *et al*., 2007, Cogan & Humphrey, 2003, Townes, 2010).

1.2 Human disease, animal reservoirs, and modes of transmission

Infections by *S. enterica* are one of the most common causes of bacterial food-borne gastroenteritis (food poisoning) in the world, along with *E. coli* and *Campylobacter* infections (WHO, 2007). Of the *S. enterica* serovars, *S.* Enteritidis and *S.* Typhimurium are the leading cause of salmonellosis in humans in most countries. *S.* Enteritidis and *S.* Typhimurium are passed to humans primarily via consumption of contaminated poultry meat, water, and eggs. *S.* Enteritidis is more often associated with salmonellosis acquired from eggs, as it has a greater tendency to colonize eggs and reproductive organs of poultry than *S.* Typhimurium (Gantois, *et al*., 2008). Because chickens mostly do not show symptoms of disease, entire flocks can become colonized quite quickly and shed bacteria in their feces for extended periods of time (Catarame, *et al*., 2005, Clavijo, *et al*., 2006, Penha Filho, *et al*., 2009, Van Immerseel, *et al*., 2005). Loss of consumer confidence in products because of *Salmonella* contamination can result in substantial economic loss to the poultry industry. Additionally, human cases of salmonellosis place a significant burden on the health care system (Boyle, *et al*., 2007). There are approximately 1.4 million cases of salmonellosis per year resulting in about 15,000 hospitalizations and 400 deaths per year in the United States of America (USA) (USDA-ERS, 2009). Around 95% of these cases are caused by consumption of contaminated food products, and *S.* Enteritidis is responsible for at least 15% of these cases. *S.* Enteritidis is the second most commonly isolated serovar in North America after *S.* Typhimurium, while *S.* Enteritidis is number one in the European Union (EU) (Barrow, 2007, Callaway, *et al*., 2008, Cogan & Humphrey, 2003, Foley & Lynne, 2008, Vieira, 2009).

1.3 Virulence factors

1.3.1 Flagella

Flagella are complex motility structures found in members of Prokarya, Archaea, and Eukarya (Gophna, *et al*., 2003). The presence of flagella has been associated with virulence in many pathogens, including *Salmonella,* which usually expresses between five and ten flagella at random positions on the cell surface (Parker & Guard-Petter, 2001, van Asten & van Dijk, 2005). However, there is conflicting evidence for the contribution of flagella to

virulence in *S.* Enteritidis. Flagellar mutants have been shown to be less proficient in colonizing eggs than wild-type *S.* Enteritidis (Cogan, *et al*., 2004). In 20-day-old chickens, Parker *et al.* (Parker & Guard-Petter, 2001) observed that disruption of flagella (by deletion of transcriptional regulator FlhD) caused enhanced invasiveness upon oral challenge. Other studies have shown that *S.* Enteritidis strains with deletions in major flagellar genes had decreased adherence to chicken intestinal explants and human intestinal epithelial cell lines, suggesting that flagella are important in adherence of *S.* Enteritidis to intestinal epithelial cells prior to invasion (Allen-Vercoe & Woodward, 1999, Dibb-Fuller, *et al*., 1999). Allen-Vercoe *et al.* (Allen-Vercoe, *et al*., 1999) also demonstrated that flagella-defective strains were recovered at lower numbers than the wild-type strain from the spleens and livers of 1-day-old chicks after oral challenge, implicating a role for flagella in invasion. This group also showed that flagellar mutants performed similarly to the wild-type strain in colonization of the ceca of 1- and 5-day-old chickens following oral challenge. However, when mutant strains were given in conjunction with wild-type *S.* Enteritidis in a competition experiment, there was much greater shedding of the wild-type strain than the mutants, suggesting that flagella do provide a competitive survival advantage (Allen-Vercoe & Woodward, 1999).

1.3.2 Fimbriae

Fimbriae, or pili, are typically 2-8 nm in width and extend 0.5-10 μm from the cell surface. Fimbriae play an important role in many bacteria, including biofilm formation and the persistence of bacteria in the environment, as well as contribute to colonization and invasion of the host. Many fimbriae are conserved between the *Salmonella* serovars, while some are unique. As each fimbria is typically specific to a given host receptor, the differences in fimbrial distribution among serovars may contribute to host specificity. There are many known and predicted fimbrial operons in *S.* Enteritidis (Gibson, *et al*., 2007, Sabbagh, *et al*., 2010, van Asten & van Dijk, 2005). Since fimbriae are not the subject of this review, detailed description of the different fimbrial types and their proposed roles is not included.

1.3.3 *Salmonella* pathogenicity islands

Pathogenicity islands were first identified in uropathogenic *E. coli* (UPEC) in the late 1980s, and have since been described in a wide variety of bacteria (Blum, *et al*., 1994, Hacker, *et al*., 1997, Schmidt & Hensel, 2004). Pathogenicity islands have been identified in both Gram-negative and Gram-positive species, and are associated with plant, animal, and human pathogens, as well as non-pathogenic bacteria. They typically harbour large clusters of genes (10 – 200 kb) related to virulence and/or survival and fitness, and have a different GC content in comparison to the rest of the genome. Pathogenicity islands can often be mosaic in structure and are often bordered by transposon insertion sequences and direct repeats, as well as bacteriophage genes, indicating that their insertion into the genome occurred via single or multiple horizontal gene transfer events (Hacker & Kaper, 2000, Schmidt & Hensel, 2004). To date there have been 21 *Salmonella* pathogenicity islands (SPIs) identified; a brief description of each of these islands is listed in Table 1.

SPI	Size (kb)	Function	Reference
SPI-1	40.2	T3SS - Invasion of the intestinal epithelium	(Marcus, *et al*., 2000, Thomson, *et al*., 2008, van Asten & van Dijk, 2005)
SPI-2	39.8	T3SS - Systemic infection of mice, survival in intestinal epithelial cells and macrophages	(Marcus, *et al*., 2000, Thomson, *et al*., 2008, van Asten & van Dijk, 2005)
SPI-3	16.6	MgtC and B Mg^{2+} transporter, MisL T5SS Implicated in intramactraphage survival Certain components important *S.* Typhimurium infection of mice, calves and/or chicks	(Blanc-Potard, *et al*., 1999, Morgan, *et al*., 2004, Schmidt & Hensel, 2004, Thomson, *et al*., 2008, van Asten & van Dijk, 2005)
SPI-4	25.0	T1SS (*siiCDF*) and large non-fimbrial adhsin SiiE Co-regulated with SPI-1 Important for membrane ruffling and entry of polarized epithelial cells in conjunction with the SPI-1 T3SS Implicated in *S.* Typhimurium infection of calves	(Gerlach, *et al*., 2008, Morgan, *et al*., 2004, Sabbagh, *et al*., 2010, Thomson, *et al*., 2008)
SPI-5	6.6	SPI-1 T3SS effector SopB and its chaperone PipC SPI-2 T3SS effectors PipA and PipB; PipD Important for *S.* Dublin induced enteritis in cattle Important for *S.* Typhimurium systemic infection in chicks	(Marcus, *et al*., 2000, Morgan, *et al*., 2004, Thomson, *et al*., 2008, Tsolis, *et al*., 1999, van Asten & van Dijk, 2005, Wood, *et al*., 1998)
SPI-6	17.6	The *saf* finbrial operon of chaperone usher class A T6SS and *tcf* fimbrial operon that are absent in *S.* Enteritidis Up to 44 kb in other serovars	(Blondel, *et al*., 2009, Sabbagh, *et al*., 2010, Thomson, *et al*., 2008, van Asten & van Dijk)
SPI-7	Absent	Vi capsule biosynthetic genes; type IV fimbrial operon SopE in *S.* Typhi Only present in *S.* Typhi, *S.* Paratyphi & some *S.* Dublin Largest PI identified so far, size varies between serovars Up to 134 kb	(Sabbagh, *et al*., 2010, Seth-Smith, 2008, Thomson, *et al*., 2008, van Asten & van Dijk, 2005)
SPI-8	Absent	Resistance to bacteriocins Absent in *S.* Enteritidis and *S.* Typhimurium 6 – 8 kb in other serovars	(Sabbagh, *et al*., 2010, Thomson, *et al*., 2008, van Asten & van Dijk, 2005)

SPI	Size (kb)	Function	Reference
SPI-9	16.3	T1SS, and a RTX-like protein The RTX protein is complete in *S.* Enteritidis, but not *S.* Typhimurium	(Thomson, *et al*., 2008, van Asten & van Dijk, 2005)
SPI-10	10.0	*Sef* fimbrial operon in *S.* Enteritidis Larger in other serovars (up to 33 kb)	(Sabbagh, *et al*., 2010, Thomson, *et al*., 2008, van Asten & van Dijk, 2005)
SPI-11	6.7	PagC, PagD and MsgA important for survival of *S.* Typhimurium in macropahges	(Sabbagh, *et al*., 2010, Thomson, *et al*., 2008)
SPI-12	5.8	SPI-2 T3SS effector sspH2 Important for full virulence of *S.* Typhimurium in mice	(Haneda, *et al*., 2009, Sabbagh, *et al*., 2010, Thomson, *et al*., 2008)
SPI-13	25.3	Important for systemic infection in mice by *S.* Typhimurium	(Haneda, *et al*., 2009, Shi, *et al*., 2006, Thomson, *et al*., 2008)
SPI-14	6.8	Electron transfer and putative regulatory genes	(Sabbagh, *et al*., 2010, Thomson, *et al*., 2008)
SPI-15	Absent	5 hypothetical proteins Not present in either *S.* Enteritidis or *S.* Typhimurium	(Sabbagh, *et al*., 2010, Thomson, *et al*., 2008)
SPI-16	3.3	LPS modification High homology to SPI-17	(Sabbagh, *et al*., 2010, Thomson, *et al*., 2008)
SPI-17	3.6	LPS modification; high homology to SPI-16 Present in *S.* Enteritidis and *S.* Typhi, but not *S.* Typhimurium	(Sabbagh, *et al*., 2010, Thomson, *et al*., 2008)
SPI-18	Absent	In *S.* Typhi encodes 2 genes for the cytolysin HlyE and the invasion TaiE Not present in either *S.* Enteritidis or *S.* Typhimurium	(Sabbagh, *et al*., 2010, Thomson, *et al*., 2008)
SPI-19	14.1	T6SS likely non-functional in *S.* Enteritidis as most of island has been deleted Up to 45 kb in other serovars	(Blondel, *et al*., 2009, Thomson, *et al*., 2008)
SPI-20	Absent	T6SS Only identified in *Salmonella enterica* subsp. *arizonae* 34 kb	(Blondel, *et al*., 2009, Thomson, *et al*., 2008)
SPI-21	Absent	T6SS Only identified in *Salmonella enterica* subsp. *arizonae* 55 kb	(Blondel, *et al*., 2009, Thomson, *et al*., 2008)

Table 1. *Salmonella* pathogenicity islands

2. Type III secretion systems

Type III secretion systems (T3SSs) act as 'injectisomes' and are used by bacteria to deliver effector proteins directly into the cytoplasm of host cells. The first T3SS was isolated in 1998 from *S.* Typhimurium (Kubori, *et al*., 1998), although it was was initially thought to be an intermediate complex of the flagellar system (Journet, *et al*., 2005, Moraes, *et al*., 2008). These structures have since been detected in numerous pathogenic bacterial species. All T3SSs share significant genetic and protein homology and can be divided into five phylogenetic groups: **1)** the Ysc group (such as the plasmid-encoded T3SSs of *Yersinia* species and *Pseudomonas aeruginosa*); **2)** the Hrp1 group (plant pathogens *Pseudomonas syringae* and *Erwinia* species); **3)** the Hrp2 group (such as the mega-plasmid-encoded T3SSs of the plant bacteria *Ralstonia* and *Xanthamonas* species, and one of the T3SSs of *Burkholderia* species); **4)** the Inv/Mxi/Spa group (the SPI-1,T3SS of *Salmonella enterica*, the chromosomally-encoded T3SS of *Shigella,* the non-functional ETT2 T3SS of enterotoxigenic *Escherichia coli* [ETEC], and the second T3SS of *Burkholderia* species); and **5)** the Esa/Ssa group (the locus of enterocyte effacement [LEE] T3SS of ETEC, the SPI-2 T3SS of *Salmonella enterica*, the chromosomally-encoded T3SS of *Yersinia* species, and the plasmid-encoded T3SS of *Shigella* species) (Foultier, *et al*., 2002, He, *et al*., 2004). T3SSs are typically encoded on large pathogenicity islands, located either within the chromosome or on a plasmid. T3SSs are mainly found in pathogenic Gram-negative bacteria; however, there are a few exceptions. For instance, T3SSs have been found in the *Chlamydia/Verrucomivrobia* super-phylum that does not resemble either Gram-negative or Gram-positive bacteria. As well, there are a few examples of non-pathogenic symbiotic bacteria of plants having T3SSs, and even a T3SS used for virulence by unicellular Protozoa (Gophna, *et al*., 2003, Tampakaki, *et al*., 2004). Flagella are associated with both pathogenic and non-pathogenic bacteria and are most often not involved in direct virulence as T3SSs are. However, there are cases where the flagellar apparatus is responsible for the secretion of virulence factors. For instance, the flagellar apparatus of *Campylobacter jejuni* is essential for virulence and secretes *Campylobacter* invasion antigens (Cia), and the flagellar system of *Bacillus thuringiensis* can secrete the virulence factors hemolysin BL and phosphatidylcholine-preferring phospholipase C (Journet, *et al*., 2005).

2.1 Flagella

The flagellum of *Salmonella enterica* is made up of 22 structural proteins, six cytoplasmic proteins, four structural chaperones, and three regulatory proteins (Fig. 1). The structure consists of a C ring (FliG, FliM and FliN) and an MS ring (FlgF and FliF) embedded in the cytoplasmic (inner) membrane. An ATPase is located on the cytoplasmic side of the apparatus (FliI). The P ring (FlgI) is located in the peptidoglycan layer and the L ring (FlgH) is in the outer membrane. A rod spanning the two bacterial membranes, made up of FliF, connects the inner membrane and outer membrane rings; other proteins (FliE, FlgB, FlgC, FlgF, and FlgG) are associated with the basal body. A type three secretion (T3S) apparatus is located within the basal body structure (FliO, FliP, FliQ, FliR, FlhA, FlhB, FliH, and the FliI ATPase). The motor-stator (MotA and MotB), which is the driving force for motion, is also located within the basal body. MotA is located within the inner membrane and connects to MotB, which extends into the periplasm. The FlgE hook protein extends from the L/P rings. FliK acts as a 'molecular ruler' to control the length of

the hook. The hook is followed by the hook-filament junction (FlgK and FlgL) and the long filament is comprised of either FliC or FljB flagellin; *Salmonella* encodes both proteins, but they are never expressed at the same time. This differential expression may aid *Salmonella* in escaping the host immune defenses by antigenic variation, and/or contribute to host specificity. Finally, the filament is topped off by the FlgD cap (Aizawa, 2001, Liu & Ochman, 2007, Macnab, 2004, McCann & Guttman, 2008, Morgan, *et al*., 2004, Pallen & Matzke, 2006, van Asten & van Dijk, 2005).

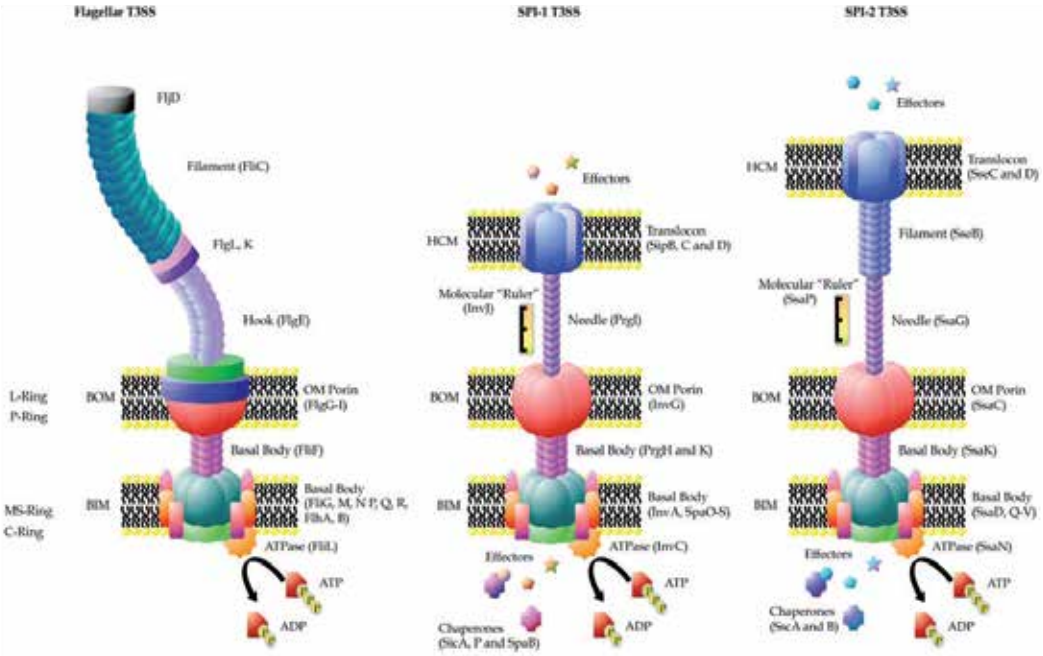

Fig. 1. Schematic representation of the flagellar apparatus, SPI-1, and SPI-2 T3SSs of *Salmonella.* The molecular organization of the flagellar system is depicted above on the left, the SPI-1 T3SS in the middle, and the SPI-2 T3SS on the right. Stoichiometry of proteins was followed where known. Adapted and modified from (Moraes, *et al*., 2008, Pallen, *et al*., 2005, Tampakaki, *et al*., 2004).

2.2 *Salmonella* pathogenicity island-1 type III secretion system

In *S.* Enteritidis, SPI-1 is 40.2 kb in length and has a GC content of 47% (Marcus, *et al*., 2000, van Asten & van Dijk, 2005). SPI-1 contains 41 genes encoding a T3SS, T3SS regulatory genes, T3SS effectors, and a metal transport system (Fig. 2) (Schmidt & Hensel, 2004, Thomson, *et al*., 2008). SPI-1 is important for cell invasion of intestinal epithelial cells as well as apoptosis of macrophages (Galán, 2001, Mills, *et al*., 1995, van der Velden, *et al*., 2000). *S.* Typhimurium strains defective for InvC (a major structural component of the SPI-1 T3SS) have a 50% higher lethal dose when given orally to Balb/c mice, but perform similarly to wild-type strains when given intraperitoneally, indicating a role for SPI-1 in colonization and invasion during the initial phase of infection, but not during the systemic phase (Galán & Curtiss, 1989).

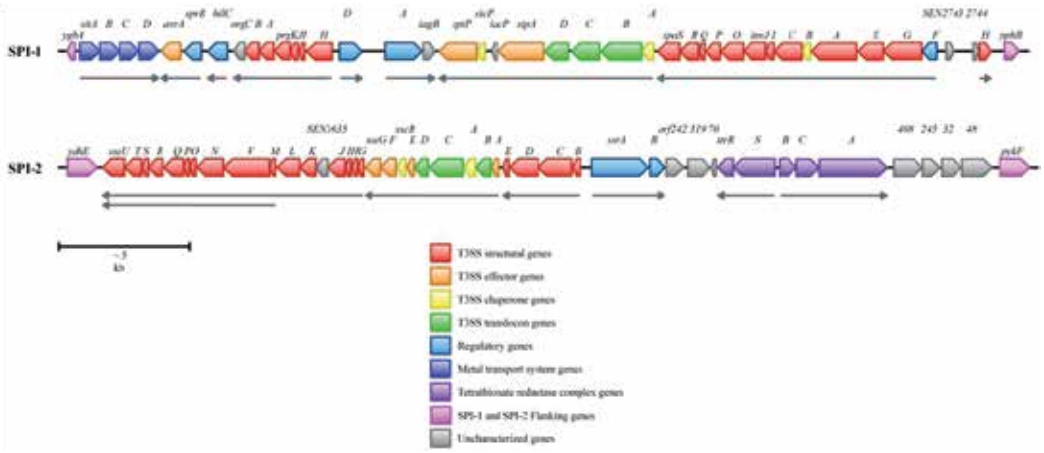

Fig. 2. Genetic organization of *Salmonella* pathogenicity islands 1 and 2. The organization of the ~40kb regions of the *S.* Enteritidis chromosome harbouring SPI-1 and SPI-2 is shown above. Gray arrows represent known or predicted transcriptional units and genes are coloured based on the function of the encoded protein. Based on the published *S.* Enteritidis genome sequence (Thomson, *et al*., 2008).

2.2.1 Structural components and effectors of the *Salmonella* pathogenicity island-1 type III secretion system

The basal body of the SPI-1 T3SS (Fig. 1) is composed of an inner membrane ring formed by PrgH and PrgK, many inner membrane proteins (SpaP, SpaQ, SpaR, SpaS, and InvA), an ATPase (InvC) and an outer membrane secretin (InvG). Extending from the outer membrane secretin is the needle formed by PrgI, topped by the translocon made up of SipB and SipC (Moraes, *et al*., 2008). The SPI-1 T3SS is responsible for the secretion of a specific set of effectors. AvrA, SipA, SipB, SipC, SipD, and SptP are all encoded on SPI-1, while the genes encoding GogB, SopE, SopE2, and SspH1 are located on lysogenic bacteriophages in the genome. The gene for SopB is located on SPI-5, and the genes for SopA, SopD, SlrP, SteA, and SteB are located elsewhere within the chromosome. GogB, SlrP, SspH1, SteA, and SteB are also secreted by the SPI-2 T3SS (Abrahams & Hensel, 2006, Bernal-Bayard & Ramos-Morales, 2009, Salyers & Whitt, 2002, Thomson, *et al*., 2008).

2.2.2 Assembly and regulation of the *Salmonella* pathogenicity island-1 type III secretion system

The assembly of the SPI-1 T3SS proceeds in a similar manner to the assembly of the flagella. The inner membrane and outer membrane rings are formed first in a sec-dependent manner, followed by the association of the rings and formation of the remaining basal body components, including the ATPase. Formation of the needle and translocon is T3S-dependent, and needle length is controlled by InvJ, which acts as a 'molecular ruler' (Deane, *et al*., 2010, He, *et al*., 2004, Moraes, *et al*., 2008).

Expression of the SPI-1 T3SS is regulated by many environmental and genetic signals. Environmental signals include pH, osmolarity, the presence of bile, magnesium concentration, and the presence of short chain fatty acids (Altier, 2005). The preferred

invasion site of *Salmonella* is the M-cells of the distal small intestine. When bile is present, indicating the beginning of the small intestine, or when short-chain fatty acids are present, which are produced by microflora of the large intestine, SPI-1 expression is repressed. These environmental signals indicate that the bacterium is not near its preferred site of entry. SPI-1 expression is induced at near neutral pH, and high osmolarity (Altier, 2005, Garmendia, *et al*., 2003). In the presence of high iron, the ferric uptake regulator (Fur) acts to increase the expression of HilD (a SPI-1 regulator, discussed further in the following text) in an unknown manner. Once in the *Salmonella* containing vacuole, SCV, where there is limited iron, this indirect activation of HilD by Fur is stopped (Altier, 2005, Ellermeier, J. R. & Slauch, 2008). See Fig. 3 for a diagram of the interaction of the regulation pathways outlined below.

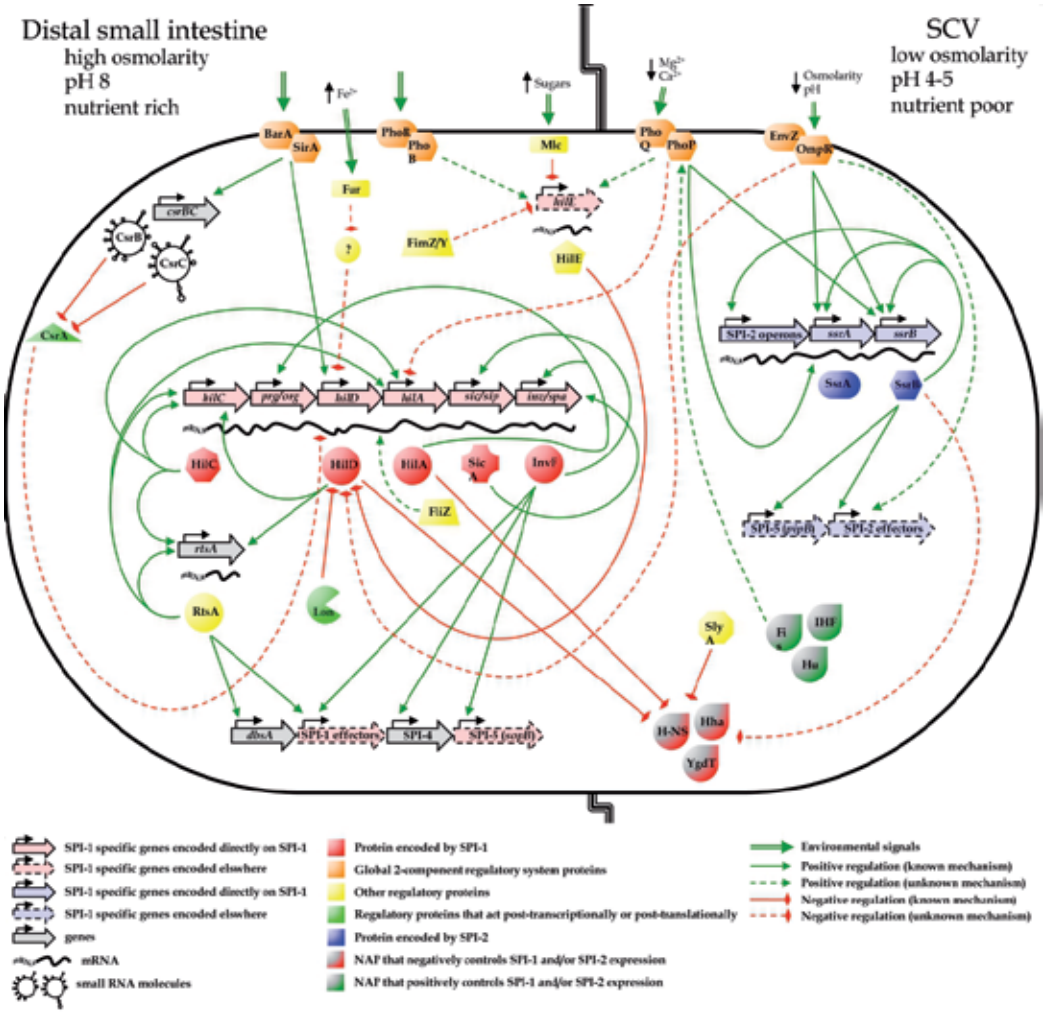

Fig. 3. Regulation of *Salmonella* pathogenicity islands 1 and 2. The major modes of SPI-1 and SPI-2 regulation are depicted above; see text for details (Sections 2.2.2, 2.3.2, and 2.4).

Nucleoid associated proteins (NAPs) affect supercoiling of deoxyribonucleic acid (DNA), and are thus able to alter gene expression. The NAPs Hha and H-NS both repress transcription of many genes, including *rtsA* and the SPI-1 gene *hilA* under conditions of low osmolarity (Altier, 2005, Olekhnovich & Kadner, 2007, Rhen & Dorman, 2005). Hu, IHF, and Fis are also NAPs, and are important for expression of SPI-1 genes (Altier, 2005, Fass & Groisman, 2009).

PhoP/PhoQ and BarA/SirA belong to two-component global regulatory systems that respond to environmental conditions. In low magnesium conditions, for example within the SCV, PhoP can act to negatively regulate HilA, leading to down regulation of the SPI-1 T3SS. SirA positively regulates HilA, by regulating the expression of HilD (Altier, 2005, Ellermeier, J. R. & Slauch, 2007, Hacker & Kaper, 2000, Hueck, 1998). BarA/SirA also controls the *csr* system. CsrA can bind messenger ribonucleic acid (mRNA) at their ribosomal binding site, thus stabilizing, or alternatively, reducing, translation of SPI-1 T3SS proteins, likely at the level of HilD. CsrB and C are small RNA molecules that bind and stop the action of CsrA. BarA/SirA activate CsrB and C, keeping CsrA levels in check. Optimal levels of all three molecules are needed for proper expression of SPI-1. The EnvZ/OmpR system senses osmolarity and has been proposed to regulate HilD post-translationally. The PhoP/PhoQ and PhoR/PhoB systems can activate expression of HilE, which then acts to repress expression of SPI-1 genes, through direct binding to HilD. The type 1 fimbriae regulators FimZ and FimY have also been shown to negatively regulate transcription of SPI-1 genes, likely through activation of *hilE*, while the flagella regulator FliZ positively regulates expression of HilA post-transcriptionally (Altier, 2005, Ellermeier, J. R. & Slauch, 2007). Mlc is a global regulator that detects the presence of sugars such as glucose and mannose, whereby Mlc can repress expression of *hilE* when sugars are readily available, such as in the small intestine (Lim, *et al*., 2007). The Lon protease (controlled by DnaK and σ^{32}), negatively regulates SPI-1 by degrading HilD in response to the stress of the SCV environment (Matsui, *et al*., 2008).

HilA belongs to the OmpR/ToxR family of transcriptional regulators, while InvF, HilC, and HilD are in the AraC/XylS family. Each of these genes are located on SPI-1 (Fig. 2) (Hacker & Kaper, 2000, Schmidt & Hensel, 2004). Expression of HilD is likely induced by environmental conditions, and leads to expression of HilC and RtsA. RtsA and HilC can also activate expression of themselves, and each other. RtsA activates *hilA* expression directly, as well as the expression of *slrP*, a SPI-1 T3SS effector, and *dsbA*, which is required for assembly of the T3SS. HilC and D act to derepress transcription of *hilA* and *rtsA* by relieving silencing by H-NS and Hha. HilA is then free to activate transcription of the *prg/org* and *inv/spa* operons (including *invF*). RtsA, HilD, and HilC can also activate transcription of the *inv/spa* operon independently of HilA, but to a lower degree than HilA. InvF activates transcription of the *sic/sip* (including *sicA*) operon of SPI-1, as well as genes within SPI-4 and SPI-5 (Altier, 2005, Ellermeier, J. R. & Slauch, 2007, Hacker & Kaper, 2000, Olekhnovich & Kadner, 2007, Rhen & Dorman, 2005, Schmidt & Hensel, 2004). SicA is the chaperone for the translocator proteins SipB and C. Once the translocon components have been secreted, SicA is free and can activate expression of *invF,* creating a positive feedback loop of secreted effector gene expression once the SPI-1 T3SS is fully formed (He, *et al*., 2004, Rhen & Dorman, 2005).

2.3 *Salmonella* pathogenicity island-2 type III secretion system

In *S.* Enteritidis, SPI-2 is 39.8 kb in length with a GC content of 43%. SPI-2 is important for survival within the *Salmonella* containing vacuole (SCV) and the systemic phase of infection (Cirillo, *et al*., 1998, Karasova, *et al*., 2010, Ochman, *et al*., 1996, Shea, *et al*., 1996). There are 44 genes encoded on SPI-2 including a T3SS, T3SS regulatory genes, T3SS effectors, and a tetrathionate reductase system (Fig. 2) (Schmidt & Hensel, 2004, Thomson, *et al*., 2008).

2.3.1 Structural components and effectors of the *Salmonella* pathogenicity island-2 type III secretion system

The SPI-2 T3SS (Fig. 1) is composed of an inner membrane ring that, in conjunction with many other inner membrane proteins, makes up the basal body. These include SsaD, SsaR, SsaS, SsaT, SsaU, and SsaV, along with the cytoplasmic ATPase SsaN. The outer membrane secretin is made up of SsaC, and is connected to the inner membrane components via SsaJ. A small needle, composed of SsaG, extends from the outer membrane secretin and is extended by a larger filament, made up of SseB. In comparison, many other T3SSs do not have a filament extension. The end of the filament is comprised of the translocon proteins SseC and SseD (Aizawa, 2001, Moraes, *et al*., 2008, Tampakaki, *et al*., 2004). SsaP, which acts as a 'molecular ruler', controls the length of the needle (Wilson, 2006).

The SPI-2 T3SS has been shown to secrete many effectors (GogB, PipB, PipB2, SifA, SifB, SopD2, SseF, SlrP, SseG, SseI, SseJ, SseK1, SseK2, SseL, SspH1, SspH2, SteA, SteB, and SteC), although most of their functions are still unknown at this time. Some of the genes encoding these proteins are located directly on the chromosome in the SPI-2 region, but some are located elsewhere on the chromosome, within lysogenic phages (*e.g*. Gifsy-1, -2 and -3) or on the *Salmonella* virulence plasmid. Although GogB, SlrP, SspH1, SteA, and SteB are secreted by the SPI-2 T3SS, these proteins are also known to be secreted by the SPI-1 T3SS (Abrahams & Hensel, 2006, Bernal-Bayard & Ramos-Morales, 2009, Salyers & Whitt, 2002). The functions of these effectors in *Salmonella* pathogenesis are described in section 3.

2.3.2 Assembly and regulation of the *Salmonella* pathogenicity island-2 type III secretion system

Similar to the SPI-1 T3SS and flagellar apparatus, the SPI-2 T3SS is assembled in a step-wise manner involving first the insertion of the inner membrane ring and outer membrane secretin in a sec-dependent manner. Association of the inner membrane and outer membrane rings, placement of further basal body components and recruitment of the ATPase takes place, followed by the subsequent assembly of the rest of the apparatus (Brutinel & Yahr, 2008, Deane, *et al*., 2010, He, *et al*., 2004).

Expression of the SPI-2 T3SS is also regulated by many environmental and genetic signals. The preferred replication site of *Salmonella* is within the SCV of macrophages, and environmental signals that mimic the environment of the SCV, such as low magnesium concentration and an acidic pH between 4 and 5, are SPI-2-inducing (Cirillo, *et al*., 1998, Fass & Groisman, 2009, Rathman, *et al*., 1996, Rhen & Dorman, 2005). As with the SPI-1 T3SS, expression of SPI-2 genes is affected by the global two-component regulatory systems PhoP/PhoQ and EnvZ/OmpR. Under conditions of low magnesium and calcium PhoP induces SPI-2 gene expression by

direct interaction with the *ssrB* gene, and post-transcriptional action on SsrA. In the presence of low osmolarity and acidic pH, OmpR can directly bind both the *ssrA* and *ssrB* promoters, activating transcription. OmpR can also act in conjunction with SsrB to activate transcription of the non-SPI-2-encoded effector SseI (Deiwick, *et al*., 1998, Fass & Groisman, 2009, Feng, *et al*., 2003, Garmendia, *et al*., 2003, Walthers, *et al*., 2007).

SPI-2 encodes its own two-component regulatory system, SsrA/SsrB. SsrB is able to bind to all SPI-2 promoters, including those of *ssrA*, *ssrB*, and many effectors located outside of SPI-2 (Fass & Groisman, 2009, Walthers, *et al*., 2007). As with SPI-1, H-NS silences the expression of SPI-2 genes by binding directly to many SPI-2 promoters. This binding can be relieved by the SPI-1 protein HilD under certain conditions, such as stationary phase growth in LB, and may also be relieved by SsrB and/or SlyA (Bustamante, *et al*., 2008, Fass & Groisman, 2009, Walthers, *et al*., 2007). The NAPs Hha and YdgT can also repress transcription of SPI-2 genes. Fis, a NAP that is able to bind the promoter regions of *ssr* and *ssa* operons, is also important for expression of SPI-2 as well as SPI-1 genes. Proper levels of Fis are important for activation of *ssrA*, and Fis may also induce SPI-2 gene expression indirectly through controlling expression of PhoP. IHF, another NAP, is also important for expression of both SPI-2 and SPI-1 genes (Fass & Groisman, 2009). Some of the mechanisms controlling regulation of SPI-2 are outlined in Fig. 3.

2.4 Cross-talk between the *Salmonella* flagellar and pathogenicity island-1 and -2 type III secretion systems

The complex regulation of the T3SSs ensures that each system is only expressed under the correct conditions. Expression of multiple versions of each T3SS simultaneously would be energetically expensive, so coordinated expression of the three systems under the specific conditions where each system is required is desirable. Global regulation by two-component regulatory systems that sense divalent cation concentrations, osmolarity, and pH are, in part, responsible for the changes in expression between the flagellar, the SPI-1, and the SPI-2 T3SSs. The SPI-1 T3SS is preferentially expressed within the distal small intestine, which has low oxygen, high osmolarity, a pH of 8, a high concentration of divalent cations, and is rich in nutrients. The environment of the SCV is much different, having low osmolarity, a low concentration of divalent cation concentration, a pH between 4 and 5, and is nutrient poor. In these conditions, the SPI-2 T3SS is preferentially expressed (He, *et al*., 2004).

The BarA/SirA system positively regulates expression of SPI-1 genes, but negatively regulates expression of flagellar genes. Therefore, in environmental conditions that activate BarA/SirA, the SPI-1 T3SS will be expressed while the flagellar system is downregulated. RtsA and RtsB have also been proposed to be involved in the switch from expression of flagella to expression of the SPI-1 T3SS. RtsA is important for SPI-1 expression, while RtsB represses expression of flagellar genes by interfering with the *flhDC* promoter (Ellermeier, C. D. & Slauch, 2003). In conditions of low divalent cation concentration, PhoP suppresses expression of SPI-1 genes while activating expression of SPI-2 genes. This ensures that once in the SCV, when the SPI-1 T3SS is no longer needed for invasion of non-phagocytic cells, the SPI-2 T3SS expression is induced while the SPI-1 T3SS is downregulated (Rhen & Dorman, 2005).

Interspecies and interkingdom quorum sensing may also be involved in regulating expression of these three systems. In the presence of host norepinephrine there is an upregulation of flagellar genes in *S.* Typhimurium (Bearson & Bearson, 2008). *S.* Typhimurium encodes a putative regulatory protein, YhcS, which has high amino acid similarity to QseA of *E. coli*. QseA activates expression of the LEE T3SS by *E. coli* in response to autoinducer 3 (AI-3) quorum sensing molecules produced by intestinal flora, as well as epinephrine and norepinephrine produced by the host. YhcS may act similarly to QseA in *E. coli* by activating expression of either (or both of) the SPI-1 or SPI-2 T3SSs (Bearson & Bearson, 2008, Choi, *et al*., 2007, Karavolos, *et al*., 2008). As mentioned previously, under certain growth conditions HilD can relieve H-NS-mediated repression of SPI-2 genes (Bustamante, *et al*., 2008). This may account for the fact that SPI-2 is expressed to some extent along with SPI-1 in the intestinal lumen, and that SPI-1 is expressed for a short time in macrophages before the complete switch to SPI-2 expression. The expression of the SPI-2 T3SS before invasion of intestinal epithelial cells would allow the bacteria to ready itself for the SCV environment. Furthermore, the expression of the SPI-1 T3SS is important for inducing macrophage apoptosis during the initial stage of infection while the bacteria is replicating, and before spread to the rest of the body. Some of the interplay between regulation of SPI-1 and SPI-2 can be visualized in Fig. 3.

2.5 Evolution of the type III secretion system

The flagellar systems of Prokarya are completely different from those of Archaea and Eukarya, suggesting that they evolved convergently into structures serving the same function (Gophna, *et al*., 2003, Liu & Ochman, 2007). However, prokaryotic flagellar systems with a chemotaxis apparatus that controls changes in the direction of motion share their chemotaxis system with archaeal flagellar systems (Liu & Ochman, 2007, Pallen & Matzke, 2006). As some members of Prokarya do not have this chemotaxis system, it may have been acquired by horizontal transfer from a member of Archaea or may have been present for sensing environmental signals before the diversification of Prokarya and Archaea, and has since been lost in some prokaryotic families. While the flagellar systems of Prokarya maintain many of the same genes and proteins among members, they can be quite diverse in their function. For instance, the flagella of *Spirochaetes* are located in the periplasm, while *Vibrio* species express both polar and lateral flagellar systems that share a chemotaxis transduction system but use different motive forces (Na^+ or H^+). Most of the flagellar proteins that serve the same function are homologous, however, not all flagellar system proteins are conserved among all bacterial species. For example, the flagellar structures of Gram-positive bacteria do not have the L and P rings (which would be located in the outer membrane of Gram-negative bacteria). *Spirochaetes* do not have the L and P ring either, as their flagella are located in the periplasm. Some of the structural genes (*flgH*, *flgI*, *fliD*, *fliE*, and *fliH* specifically) are missing in some bacteria; this could indicate a later evolution of these genes combined with limited horizontal transfer, or be an example of sporadic loss of genes from some bacterial families. The latter explanation seems more likely in this case as there are many families of bacteria that contain these genes, and only a few who are lacking (Liu & Ochman, 2007).

The flagella phylogenetic tree is directly related to that of the bacterial speciation genetic tree based on 16S ribosomal RNA. This suggests that flagella have been in existence since

before the diversification of bacteria, and have been maintained throughout vertical evolution (McCann & Guttman, 2008). Liu and Ochman propose that the entire flagellar system is actually evolved from a single gene. They suggest, based on sequence similarities, that all of the flagellar genes arose from random duplications and reassortments of a single precursor gene in the ancestor of modern bacteria (Liu & Ochman, 2007). This seems quite unlikely; although there may be sequence similarities between an inner membrane component and an outer membrane component, this does not mean that they are related on an evolutionary scale. Convergent evolution is a more likely explanation for this, in which two different proteins have evolved to serve a similar function, in this case to be embedded in the bacterial membrane.

Unlike flagellar systems, the T3SS phylogenetic tree is not related to that of 16S ribosomal RNA, suggesting that T3SSs were acquired at some point after the diversification of bacteria, and evolved via horizontal transfer events (Foultier, *et al*., 2002, Gophna, *et al*., 2003, Liu & Ochman, 2007, Nguyen, *et al*., 2000). T3SSs are encoded on large pathogenicity islands, while flagellar genes are encoded on the chromosome (Hueck, 1998, Macnab, 2004, van Asten & van Dijk, 2005). It is thought that SPI-2 may have arrived in two separate events, with the *ttr* operon arriving first, followed by the rest of SPI-2 (Marcus, *et al*., 2000).

The effectors of T3SSs are highly variable between species of bacteria, and are quite often encoded on different regions of the chromosome than the pathogenicity island-encoded T3SSs. The effectors and their evolution will not be discussed here, but information on this topic can be found in a recent review (Stavrinides, *et al*., 2008). In general, there are about ten core proteins of the flagellar T3S apparatus and the injectisome T3SSs that are highly similar in gene sequence, amino acid sequence, and function (Fig. 1.). For the purposes of this discussion, the flagellar system will be compared only with the two *Salmonella* T3SSs, with homologous proteins given in the order flagella/SPI-1/SPI-2. These homologous proteins are: the cytoplasmic ATPase (FliI/InvC/SsaN), the T3S apparatus (FliH/PrgH/SsaK, FliN/SpaO/SsaQ, FliP/SpaP/SsaR, FliQ/?/SsaS, FliR/SpaR/SsaT, FlhB/SpaS/SsaU and FlhA/InvA/SsaV), part of the connecting rod (FliF/PrgK/SsaJ), and the needle/hook 'molecular ruler' (FliK/InvJ/SsaP) (Blocker, *et al*., 2003, Desvaux, *et al*., 2006, He, *et al*., 2004, Tampakaki, *et al*., 2004, Wilson, 2006).

The structure of the flagellar apparatus and T3SSs begin to differ more markedly starting at the outer membrane (besides the motor-stator which is only present in the basal body of the flagellar system). The MS ring of the flagellar system is larger than that of the outer membrane secretin of the T3SS (Aizawa, 2001). The secretin of the T3SS belongs to the same family of proteins that make up the T2SS and T4SS secretins, and the pore used by filamentous phages, suggesting that filamentous phages either introduced this type of protein to bacteria, or acquired it from them (Nguyen, *et al*., 2000). The T3SS needle is straight and thin, as is its filament, although the filament is slightly larger, and notably rigid. The flagellar hook apparatus is larger and curved, and its filament is quite long and flexible. These structures lack significant amino acid and genetic homology, but do share helical symmetry, and overall assembly mechanisms. The flagellum contains approximately 5.6 subunits of flagellin per turn, with an axial rise of 4.7 Å. To compare, the filament of the LEE T3SS in *E. coli* contains 5.5 subunits of EspA per turn, and has an axial rise 4.6 Å (Aizawa, 2001, Journet, *et al*., 2005, Snyder, *et al*., 2009, Tampakaki, *et al*., 2004). The inner diameter of the T3SS filament is between 2 and 3 nm, similar to the inner

channel of the flagellum (Blocker, *et al*., 2003, Journet, *et al*., 2005, Tampakaki, *et al*., 2004). The action of the 'molecular rulers' is likely different as well. It has been proposed that the method for measuring hook length in flagella is more 'measuring cup'-like than 'molecular ruler'-like. Journet suggests that the motor-stator switch area of the flagellum acts as a measuring cup, filling with FliK. FliK acts as an accessory to the hook protein, and is secreted at the same time. Once the 'cup' empties of FliK, the apparatus switches its secretion preference from the hook protein (FlgE) to the flagellin protein (FliC or FljB), completing assembly of the flagellum. In contrast, the InvJ and SsaP proteins (similar to the 'molecular rulers' of other bacterial T3SSs) act more like a ruler. It has been suggested that dimers of these proteins are located outside the cell, with one attached to the outer membrane, and the second extending from that. Once the needle complex (PrgI/SsaG) reaches the same height as the InvJ/SsaP dimer, the T3SS switches to secretion and assembly of the translocon of SPI-1 (SipB, C, and D) or filament of SPI-2 (SseB) (Journet, *et al*., 2005).

Another key area in which the T3SSs and flagellar systems differ is in their chaperones. Although both systems tend to have specific chaperones for specific proteins, the T3SS proteins are recognized by their chaperones at an N-terminal region, while flagellar system chaperones bind at the C-terminal region (Liu & Ochman, 2007). Although the flagellar and T3SS chaperones are different, in some cases the three systems can secrete each other's proteins. For example, both the SPI-1 and SPI-2 T3SSs can secrete the flagellar protein FliC, while the flagellar system can secrete the SPI-1 T3SS effector proteins SptP and SopE, if the SptP and SopE chaperones are absent, and in some instances effectors from T3SSs of other bacterial species (Journet, *et al*., 2005, Tampakaki, *et al*., 2004).

3. Pathogenesis of *Salmonella*

Salmonella can enter host cells in at least two ways. The first involves uptake into phagocytic cells (macrophages), while the second is more complicated and involves the action of the SPI-1 T3SS on non-phagocytic cells. After attachment to epithelial cells, the SPI-1 T3SS induces membrane ruffling by secreting effectors into the host cell to trigger cytoskeleton rearrangement. Once inside the epithelial cell, some of these same effectors 'switch off' the membrane ruffling, returning the host cell membrane to its original state (Ibarra & Steele-Mortimer, 2009, Ly & Casanova, 2007, Salyers & Whitt, 2002, Waterman & Holden, 2003). Entry into the host cell (epithelial or macrophage) results in the bacteria being encased within an SCV. While the goal of many intracellular pathogens would be to escape this vacuolar space into the cell cytoplasm, *Salmonella* takes advantage of this space and remains in the SCV (Bhavsar, *et al*., 2007, Ibarra & Steele-Mortimer, 2009, Salyers & Whitt, 2002).

Once inside the SCV, the SPI-2 T3SS is expressed and begins secreting effector proteins, which are used to manipulate the intracellular environment (Cirillo, *et al*., 1998, Ibarra & Steele-Mortimer, 2009, Ramsden, *et al*., 2007). Approximately one hour after entry into the host cell, the SCV switches from early endosomal markers, such as early endosome marker 1 (EE-1), to late endosomal/lysosomal markers, such as lysosomal-associated membrane protein-1 (LAMP-1) and lysosomal glycoproteins (lgps). One important factor that the SCV acquires during this switch is the V-ATPase, which facilitates the acidification of the SCV.

This acidification is an important factor for the induction of *Salmonella* virulence/survival genes (Abrahams & Hensel, 2006, Bhavsar, *et al*., 2007, Ibarra & Steele-Mortimer, 2009, Kuhle & Hensel, 2004, Ramsden, *et al*., 2007, Salyers & Whitt, 2002). Another important factor for *Salmonella* survival within host cells is iron acquisition. *Salmonella* releases two siderophores for sequestering Fe^{2+} from the host cell, enterobactin and salmochelin (Ibarra & Steele-Mortimer, 2009). As the SCV matures, it moves along host cell microtubules towards the Golgi apparatus. This process is dependent on many effectors, including SifA, SifB, SopD2, SseF, SseG, SseI, SseJ, SseL, PipB, and PipB2 (Abrahams & Hensel, 2006, Ibarra & Steele-Mortimer, 2009, Ramsden, *et al*., 2007). SsaB is also important in blocking the fusion of the SCV with lysosomes during this process, which would result in bacterial killing (Kuhle & Hensel, 2004). Movement along the microtubules involves recruitment of a dynein-dynactin motor complex by SifA, SseF, SseG, and PipB2. PipB2 interacts with the motor protein kinesin, while the other three proteins have also been shown to be responsible for keeping the SCV localized to the Golgi apparatus in an unknown manner. These proteins are also very important in *Salmonella*-induced filament (sif) formation, which will be discussed in the following paragraph. SCV membrane integrity is important, and is controlled by a number of SPI-2 T3SS effectors, including SspH2, SseI, SteC, and the *Salmonella* virulence plasmid-encoded protein SpvB. The interaction of these proteins with host filamen and actin causes the formation of an actin-mesh around the SCV (Abrahams & Hensel, 2006, Kuhle & Hensel, 2004, Ramsden, *et al*., 2007). Another function of the SPI-2 T3SS may be to stop the formation of the NADPH phagocytic oxidase (phox) and inducible nitric oxide synthase (iNOS) on the SCV membrane, ultimately resulting in protection of *Salmonella* from reactive oxygen and nitrogen species (ROS and RNS, respectively) (Abrahams & Hensel, 2006, Coburn, *et al*., 2005, Salyers & Whitt, 2002). A superoxide dismutase encoded by the Gifsy-2 lysogenic phage helps *Salmonella* survive the oxidative burst, which involves production of ROS and RNS by phagocytic cells that can damage bacteria, and is therefore important for bacterial survival within the SCV (Ibarra & Steele-Mortimer, 2009, Salyers & Whitt, 2002).

The maturation/movement process of the SCV can take around 4 to 6 hours. At this point, when the SCV has been altered to suit the bacteria, *Salmonella* begin to replicate (Abrahams & Hensel, 2006, Finlay & Brumell, 2000). Replication of *Salmonella* is associated with the formation of sifs. Sifs have similar markers to the SCV, and many of the same proteins are responsible for their formation/membrane integrity (SifA, SifB, SseF, SseG, SseJ, SseL, SspH2, SpvB, PipB, and PipB2). The SPI-1 effector SipA has also been shown to be important in sif formation. These sifs extend from the SCV towards the host cell membrane, and other SCVs, if there are multiple SCVs in one cell (Ibarra & Steele-Mortimer, 2009, Kuhle & Hensel, 2004, Ramsden, *et al*., 2007). The AvrA effector secreted by the SPI-1 T3SS deubiquitinates both IκB-α and β-catenin, which stabilizes the proteins and results in the continued repression of NFκB-mediated gene transcription. This delays apoptosis of intestinal epithelial cells, thereby allowing *Salmonella* to survive within them for longer (Bernal-Bayard & Ramos-Morales, 2009, Bhavsar, *et al*., 2007, Grassl & Finlay, 2008, Ibarra & Steele-Mortimer, 2009). SlrP also mediates ubiquitination of certain host proteins including Thioredoxin-1 (Trx1). Trx1 can activate the NFκB transcription factor, and has functions among other host cell proteins as well. Binding of SlrP to Trx1 stops its action, which under some conditions can lead to apoptotic cell death, although the exact mechanisms of this need to be studied further (Bernal-Bayard & Ramos-Morales, 2009, Bhavsar, *et al*., 2007,

Ramsden, *et al*., 2007). SspH1 can also inhibit NFκB transcription (Ibarra & Steele-Mortimer, 2009, Kuhle & Hensel, 2004, Ramsden, *et al*., 2007).

3.1 Role of the *Salmonella* pathogenicity island-1 and -2 type III secretion systems in the chicken model of infection

Contaminated poultry and eggs remain a major source of food poisoning caused by *Salmonella*. As the majority of work on *Salmonella* to date has been done in mice, we examined the role of this bacterium in a chicken model of *S*. Enteritidis infection developed using select strains isolated from chickens. We were particularly interested in the role of the SPI-1 and SPI-2 encoded T3SSs of *S*. Enteritidis in the various stages of infection and colonization of birds, as the SPI-1 encoded T3SS has been found to be important for entry into intestinal epithelial cells, and the SPI-2 encoded T3SS has been found to be highly important in the later stages of infection in mice, particularly after *Salmonella* has been vacuolised. To examine this, chickens were challenged orally with 10^6-10^9 *Salmonella* bacteria. In co-challenge trials using 35-day-old chickens we showed that although a *S*. Enteriditis wild-type strain was only slightly more competitive in colonizing the ceca than mutants defective in SPI-1 and SPI-2, the systemic spread of both of these mutant strains to the liver and spleen was significantly less successful than that of the wild-type strain (Desin, *et al*., 2009, Wisner, *et al*., 2010). Colonization of the gut was nearly 100% for both wild-type and mutants, whereas *Salmonella* was detected in the liver and spleen in approximately 30% of the birds in these trials. In order to acheive a higher percentage of chickens that were systemically infected, we performed colonization experiments with younger leghorn chickens hatched from specific pathogen-free (SPF) eggs and orally challenged 7 days post-hatch. With respect to colonization of the ceca we found no statistically relevant differences between the wild-type and SPI-1 or SPI-2 mutant strains. However, although 100% of the younger birds developed a systemic infection when challenged with the wild-type strain, challenges with mutant strains devoid of functional SPI-1 and/or SPI-2 T3SSs resulted in a clearly delayed, and less severe, systemic infection. Interestingly, at the end of the test period, 4 days post-challenge, the systemic presence of both the mutant and the wild-type strains was found to be decreasing. From these findings, it is evident that both the SPI-1 and SPI-2 pathogenicity islands are important for the fast and efficient invasion and systemic spread of *Salmonella* in chickens. However, the data obtained 3 and 4 days post-challenge indicates that SPI-1 and SPI-2 are not the only factors needed for systemic dissemination, and that other virulence factors may compensate for the loss of SPI-1 and SPI-2.

4. Concluding remarks

Most of the animal studies in the context of bacterial T3SS-related pathogenicity have been performed with *S. Typhimurium* in mice. Based on our findings, however, we see that not all the results obtained from those experiments can be directly transposed to other hosts. Our *in vitro* and *in vivo* results based on a chicken model of *S. Enteritidis* infection demonstrated that both SPI-1 and SPI-2 play an important role in invasion and systemic spread, although they do not seem to be essential. Further studies will be necessary to understand the full spectrum of virulence mechanisms of different *Salmonella* strains in various host organisms, including humans.

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Part 5

Novel Techniques

25

Immunoimmobilization of Living *Salmonella* for Fundamental Studies and Biosensor Applications

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1. Introduction

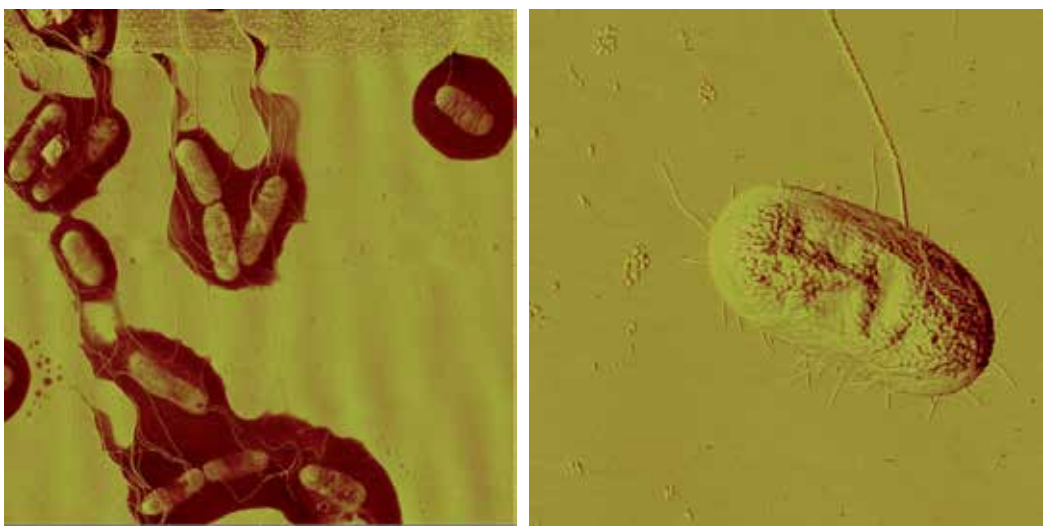
Currently, there is no technique available to probe an individual bacterium in its physiological environment for a prolonged period of time to determine its response to environmental stimuli or to conduct measurements on it. Controlling and manipulating individual bacteria will facilitate fundamental studies of such bacterial characteristics as morphology, adhesion, biomineralization and mechanical properties under physiological conditions. Access to specific individual cells will open new research frontiers in areas such as differentiation among the individual offspring of a predetermined bacterium (Arnoldi et al. 1998; Chao et al. 2011; Gao et al. 2011). In many applications, it is necessary to immobilize bacteria on flat substrates or particles. For example, there have been many reports on using living bacteria as sensors for environmental monitoring because of their low cost, fast growth, easy genetic modification and handling, and sensitivity to a wide variety of environmental stimuli (Kuang et al. 2004; Mbeunkui et al. 2002; Premkumar et al. 2002). It is often necessary to immobilize living bacteria on a designated area of a detecting surface to build a practical sensor. Reliable, controllable and efficient immobilization of bacteria is crucial for the success of pathogen detection. Typically, a captured bacterium triggers an event that converts the capturing process into a signal which is detectable by optical, electrochemical, mechanical or other means (O'Kennedy et al. 2009). The technique developed by our group appears to be very promising for these applications. Before we describe our methodology here, we will give a brief review of the previous approaches and methods for the immobilization of bacteria on material surfaces.

1.1 Nonspecific immobilization of bacteria through physical adsorption or entrapment

The majority of reported immobilization approaches utilize either the nonspecific adsorption of bacterial cells on charged surfaces by means of electrostatic forces or the physical entrapment of cells in gel or micro-holes.

1.1.1 Physical absorption

Typically, bacterial surfaces are negatively charged under physiological conditions for most Gram-positive and Gram-negative species. Thus, it is possible for bacteria to adhere to a positively charged substrate prepared by modifying the surface using positively charged polymers or silanes. Various bacteria have been attached to substrates decorated with polylysine (Rozhok et al. 2006; Rozhok et al. 2005), polyethyleneimmine (Razatos et al. 1998), amino-terminated silanes (Arnoldi et al. 1998), gelatin (Doktycz et al. 2003) and alginate (Polyak et al. 2001). However, unlike eukaryotic cells, bacterial cells are still very challenging to immobilize reliably and reproducibly under their physiological conditions using positively charged polymers. For example, none of the experiments we report here could be done using this approach. This is mostly because, in contrast to a eukaryotic cell, only a very small fraction of a bacterial cell surface can come into close enough contact with a charged substrate surface to adhere, preventing the bacterium from attaching to the surface effectively. Additionally, many bacterial species, including *Salmonella*, have a layer of capsular extracellular polymeric substances (EPS) covering their outer surface, as shown in Fig. 1A (Suo et al. 2007), which further weakens interactions with and adhesions to the substrate surface.



(a) (b)

Fig. 1. AFM phase images of *S.* Typhimurium showing the detailed structures of (A) flagella and capsular EPS (scan size: 20 μm), and (B) a single bacterium with its fimbriae and part of its flagellum (scan size: 4 μm).

1.1.2 Physical entrapment

Because bacterial motion can be significantly slowed in viscous media or micro-cavities, living bacteria can be physically entrapped in hydrogels or inside microwells. Micro- (Xu et al. 2007) and macro-contact printing (Weibel et al. 2005) have been employed to transfer live bacteria onto the surface of a nutrient-rich matrix such as agarose or hydrogel. Bacterial microarrays have also been prepared by loading individual bacterial cells into microwells (2.5 μm wide, ~3 μm deep) at the distal end of an optical fiber bundle by centrifuge (Brogan and Walt 2005). A bacterial array printed onto porous nylon has also been reported

(Heitkamp and Stewart 1996), in which cells were physically entrapped in the pores of a special nylon substrate in close contact with a nutrient medium. Akselrod et al. reported three-dimensional heterotypic arrays of living cells in hydrogels created by means of high-precision (submicron accuracy) time-multiplexed holographic laser trapping (Akselrod et al. 2006). However, this technique has limited applications in practice, as, besides the need for a trapping laser, excessive exposure to laser light may cause photodamage to the cells; furthermore, arrays are expected to merge in a few hours because of cell division. The entrapment methods suffer from slow response times, low loading rates into microwells and easy detachment from surfaces.

2. Antibody-mediated immobilization of bacteria

Another approach to bacterial immobilization takes advantage of the interaction between a receptor and an appropriate ligand on a bacterial surface. Many different types of receptors can be used for this purpose, including enzyme receptors, nucleic acid receptors, polysaccharide receptors (lectins) (Gao et al. 2010) and antibodies against bacterial surface antigens. Since receptors often recognize specific types of ligands on a bacterial surface, such immobilization could achieve a high degree of specificity and efficiency. In this chapter, we focus on antibody-mediated immobilization, referred to as immunoimmobilization. Readers can refer to a recent review for the current status of the field of immobilization using a broad spectrum of receptors (Velusamy et al. 2010b).

The large variety of bacterial surface antigens and corresponding antibodies offers a number of choices for immunoimmobilization, which could be highly specific for a given species. This approach has been used to detect *Salmonella* (Table 1) (Mantzila et al. 2008; Oh et al. 2004) and other bacterial pathogens (Byrne et al. 2009; Skottrup et al. 2008; Velusamy et al. 2010a). Efficient capturing is always desired for bacterial detection, since it will facilitate converting captured pathogens into a detectable signal and, most importantly, a higher capture efficiency will result in a higher sensitivity (lower detection limit). Extensive research has been reported on the development of new detection methods that involve converting an already captured pathogen into an output signal by optical, electrochemical, mechanical or other means (O'Kennedy et al. 2009). However, there has been little study of how to enhance the capture efficiency. In fact, poor immobilization of bacterial cells is often observed. For example, only 2% surface coverage of the bacteria was achieved for a sensor using *E. coli* to monitor environmental toxicity (Premkumar et al. 2001).

In order to achieve reliable and efficient immunoimmobilization, the substrate should be decorated with a dense layer of an antibody which targets the most abundant antigen on the bacterial surface. This requirement draws attention to the two most critical aspects in immunoimmobilization: optimization of the surface chemistry to maximize the antibody density on the substrate surface and selection of an antibody which targets the appropriate bacterial surface antigen such as fimbriae (Fig. 1B).

2.1 Surface chemistry

The surface chemistry for linking antibody molecules is similar to that which has been popularly used to prepare protein microarrays and protein-modified resin (Hermanson et al. 1992). However, in order to achieve a high immobilization efficiency, a substrate for

bacterial immobilization should have a larger number of antibody molecules on the surface, with the paratope of each antibody molecule pointing away from the substrate. It would also be desirable for the antibody molecules to have sufficient freedom of movement to orient themselves in a proper binding direction towards the bacterial antigens.

As shown in Fig. 2A, thiolated tethers including 16-mercaptohexadecanoic acid (MHA) and 11-mercapto-undecanoic acid (MUA) have been commonly used to activate gold and silver surfaces. The carboxyl terminal of MHA and MUA can link to amino groups after activation. Silanes with an active terminal are widely used for silicon oxide, silicon nitride, glass, indium tin oxide (ITO), aluminum, titanium and steel surfaces. In Fig. 2, two popular silanes are shown, aminopropyltriethoxylsilane (APTES) (Fig. 2B) and (3-glycidoxypropyl)-trimethoxysilane (GOPTMS) (Fig. 2C). APTES is further modified with *N*-(3-maleimidopro-pionyloxy)succinimide (BMPS), a short cross-linker, to provide an active maleimido terminal to link to the cysteine residue of antibodies. The glycidyl terminal can react with amino or hydroxyl groups of an antibody. Another popularly used linkage is the biotin-avidin (streptavidin/neutravidin) system (Fig. 2E), which involves the covalent linking of biotin to antibodies followed by the binding of biotin-labeled antibodies to an avidin layer on the substrate (Taitt et al. 2004). Antibodies can also be linked to substrate surfaces through protein A/G/L (Fig. 2D), which specifically binds to the Fc region of IgG (Choi et al. 2008; Gao et al. 2006).

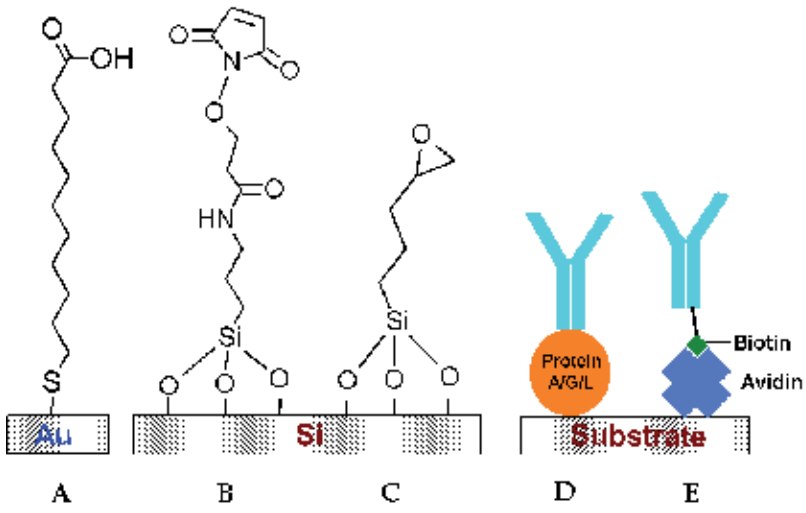


Fig. 2. Chemistry of antibody linkage

Many short cross-linkers are commercially available for linking antibodies; some examples are shown in Fig. 2. An antibody attached to a substrate through one of these cross-linkers forms a dense two-dimensional (2-D) monolayer on the substrate in which the paratopes of the individual antibody molecules are randomly oriented and the individual antibody molecules have very limited freedom of movement for reorientation. A short cross-linker works well with a high-purity antibody against a bacterial surface antigen: this gives a fairly high expression level, and a satisfactory immobilization of bacteria can be achieved. However, when the antibody is not pure or has a low affinity, or when the bacterial surface antigen has a

low expression level, it becomes necessary to boost the antibody binding probability by aligning the antibody molecules so that their paratopes point away from the substrate and using long cross-linkers to obtain the necessary degree of freedom of movement.

When linked through flexible tether molecules with lengths varying from tens to hundreds of nanometers, the antibody molecules will form a three-dimensional (3-D) network on the substrate surface. Such tethers provide the necessary degree of freedom of movement for an antibody to access a larger fraction of the bacterial surface and increase the immobilization efficiency. These tethers can be constructed from brush polymers, dendrimers (Han et al. 2010), certain peptides and block copolymers. Because of their flexibility and polydispersity, these highly branched tethers will maximize the loading of antibody molecules onto the substrate surface in a 3-D network, hence increasing the number of antibody molecules per unit area to more than can be linked by short tethers forming a 2-D network.

Poly(ethylene glycol) (PEG) has been widely used for surface modification since the early 1990's, and a variety of PEG-based cross-linkers are now commercially available. The aqueous solubility and flexibility of PEG make these linkers ideal for antibody molecules. It should be noted that most available PEG cross-linkers can only link one antibody molecule to a terminal, so it is expected that surfaces modified by such PEG cross-linkers will be covered by only one monolayer of antibody.

2.2 Antibody-antigen selection

Antibody-mediated immobilization works in a complex environment, such as growth medium, blood or a food sample, which simplifies the sample preparation. However, previous work using antibodies against whole bacterial cells often resulted in low immobilization efficiency (Premkumar et al. 2001; Rozhok et al. 2005). A general guideline for antibody selection is to use antibodies targeting antigens on the surface of the bacterium (the outer membrane of Gram-negative and the peptidoglycan layer of Gram-positive bacteria). Antigens inside bacterial cells or embedded in cell wall components usually should be avoided since it is impossible for antibody molecules to reach them in living bacteria. A systematic evaluation and comparison of the immobilization efficiencies of selected antibody-antigen pairs associated with common bacterial surface antigens is still needed.

We have evaluated the immobilization efficiencies of IgG antibodies against four different types of surface antigens of *S.* Typhimurium and *E. coli*: lipopolysaccharides (LPS), flagella, fimbriae, and a capsular protein (Suo et al. 2009a). The results show that, with the exception of the capsular protein, all the surface antigens tested can in principle be targeted to achieve some degree of immobilization and that the immobilization efficiency is correlated to multiple factors, especially to the choice of antibody-antigen pairs.

2.2.1 Method

The immobilization efficiency is defined by the number of immunoimmobilized bacteria per unit area within a specific time period for a specific concentration of bacteria in the medium. The antibody solution is deposited onto an activated silicon substrate as small droplets. Because of the specificity of the antibody-antigen interaction, bacterial cells are immobilized only inside the antibody-modified areas. Therefore, a sharp separation of the bacteria-covered areas from those which are not covered is expected. An optical image focused on the antibody-coated areas is used to determine the immobilization efficiency.

2.2.2 Anti-fimbria antibodies

Antibodies against various fimbriae have been tested in our lab, and they usually result in very efficient immobilization. We have studied the immobilization efficiency of both engineered and wild-type strains of *S.* Typhimurium and *E. coli* using antibodies against various types of fimbriae, including K88ab (F4), K88ac (F4), K99 (F5), 987P (F6), F41 and CFA/I. An example of two CFA/I-expressing strains (*S.* Typhimurium Δ*asd*::*kan*^{R} H71-pHC and *E. coli* H681-pBBScfa) being immobilized on a silicon substrate modified with anti-CFA/I is given in Fig. 3A,B. A sharp boundary can be observed separating the bacteria-covered area from the area that is not covered, indicating the high specificity and efficiency of the antibody binding. The cell coverage within the antibody-modified area approached a dense monolayer. The cell density of *E. coli* H681-pBBScfa was slightly lower than that of *S.* Typhimurium Δ*asd*::*kan*^{R} H71-pHC, because of the lower CFA/I expression level of the *E. coli* strain relative to that of the *S.* Typhimurium strain. The areas outside the antibody-modified regions (upper right-hand sides of the panels in Fig. 3) serve as a negative control for evaluaing the immobilization efficiency. Usually no cells or only sparsely attached cells were observed in these control areas. We conducted additional control experiments on similar substrates using no antibody and using an irrelevant antibody (anti-cytochrome *c*), for which no immobilization was observed.

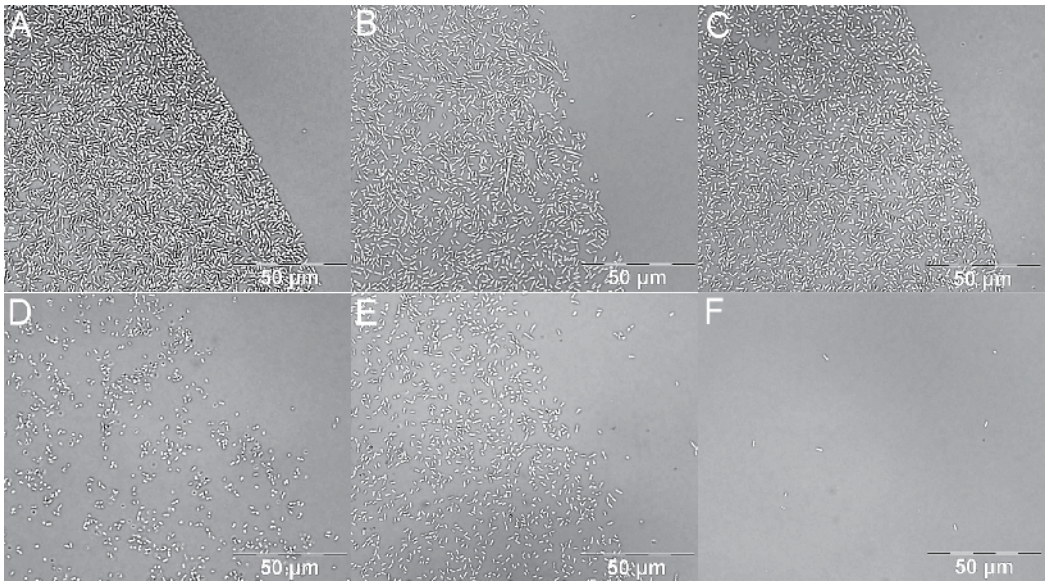


Fig. 3. Immobilization of bacteria using antibodies targeting various bacterial surface antigens: (A) *S.* Typhimurium *Δasd::kan*^{R} H71-pHC on substrate modified with anti-CFA/I, (B) *E. coli* H681-pBBScfa on substrate modified with anti-CFA/I, (C) *S.* Typhimurium *motA3::cat* H683-pTP2fliC (with flagella motion paralyzed) on substrate modified with anti-flagellin (notice that all the bacteria are lying down), (D) *S.* Typhimurium H647 (with active flagella) on substrate modified with anti-flagellin (notice that almost all the bacteria are standing up) (E) H647 on substrate modified with anti-O4 antigen (sc52224), and (F) *S.* Typhimurium *Δasd::kan*^{R} H71-pF1 on substrate modified with anti-F1.

2.2.3 Anti-flagellin

The efficiency of immobilization using anti-flagellin and anti-LPS was tested on *S.* Typhimurium H647, which expresses flagella but lacks CFA/I fimbriae. As expected, this strain could not be immobilized on substrate modified with anti-CFA/I antibody. However, H647 could be immobilized on substrates modified with anti-flagellin (Fig. 3C,D), although with a relatively low cell density as compared with CFA/I fimbriae. In spite of the fact that anti-flagellin shows a reasonably strong affinity to purified *S.* Typhimurium flagella (data not shown), the coverage density of H647 could not be improved by increasing the incubation time (Suo et al. 2009a). We speculate that the low cell coverage density of H647 is a result of the high-speed rotary motion of the flagella, which can be as high as 10,000 rpm at 35°C (Magariyama et al. 2001), hindering antibody-antigen interactions. Preliminary results showed that the immobilization efficiency could be enhanced while using the same anti-flagellin by paralyzing the flagella motion, as shown in Fig. 3C for *motA3*::*cat* H683-pTP2fliC. Notice in Fig. 3D that almost all the bacteria are standing up, most likely because of the flagella motion, as opposed to lying down when their flagella are paralyzed (Fig. 3C).

2.2.4 Anti-LPS

The antibodies against *S.* Typhimurium LPS showed the anticipated results, in that only one of the four antibodies tested demonstrated efficient immobilization of H647. LPS is an important amphiphilic molecule extending out from the bacterial outer membrane. It is composed of three covalently linked domains: lipid A, core antigen (oligosaccharide) and O-antigen (polysaccharide) (Raetz 1996). The lipid A is embedded in the outer membrane lipid bilayer, and hence it is expected that it would be difficult for an antibody to recognize it in a living bacterium. Our observations support this expectation, as the antibody against lipid A failed to provide any immobilization of *S.* Typhimurium. The saccharides, including both the core antigen and O-antigen, protrude from the phospholipid bilayer of the outer membrane and, therefore, can serve as potential targets for immunoimmobilization. Our experiments also showed no successful immobilization for the antibody against the core antigen. This implies that the core antigen is shielded by the O-antigen, preventing antibody-antigen interactions.

About 67 types of O-antigens have been identified for *Salmonella* serovars (Grimont and Weill 2007). These O-antigens are long chains of polysaccharides with a total length of up to 40 repeating units, typically with three to six sugars in each repeating unit (Raetz 1996). The sugar composition and the alteration of linkages among the sugars determine the serogroup to which a specific strain belongs (Selander et al. 1996). We tested three commercially available antibodies targeting *S.* Typhimurium O-antigen (Santa Cruz Biotech. Inc., Santa Cruz, CA): sc52221, sc52223 and sc52224. The latter two antibodies (sc52223 and sc52224) are specific to the O4-antigen. Only one antibody (sc52224) showed successful immobilization (Fig. 3E). *S.* Typhymurium usually contains O-antigens 4, 5 and 12 (Grimont and Weill 2007), all of which share a common tetrasaccharide repeating unit (with different sizes) given by α-D-mannose-1→2-α-L-rhamnose-1→3-α-D-galactose trisaccharide, to which an abequose is αl,3 linked (Curd et al. 1998; Weintraub et al. 1992). So far there is not enough information on the O-antigen structure of H647, and we infer that H647 may not contain O-4 antigen. H647 is a recombinant strain constructed by complementing an *asd* mutation strain H683 with an asd^+ plasmid (Ascon et al. 1998). There are results indicating the asd^+ plasmid may interfere with the expression of cell wall components including LPS in our experiments. Based on the fact that only one of the two monoclonal O4-specific antibodies showed

positive results in immobilization experiments, we hypothesize that H647 altered its O-antigen structure to a different group, O-9 (D1), which has a repeating unit of α-D-mannose-1→2-α-L-rhamnose-1→3-α-D-galactose trisaccharide to which a tyvelose is αl,3 linked (Curd et al. 1998). This is a reasonable hypothesis considering that this structure is very similar to serogroup O-4 in that tyvelose differs from abequose only in the 3-D orientation of their OH groups at the 2 and 4 positions. Although more experiments are needed to verify this hypothesis, the results demonstrate that the antibodies targeting the O-antigen can be used for bacterial immobilization. The high specificity of anti-O-antigens can be used for rapid serotyping of *Salmonella* (Cai et al. 2005).

2.2.5 Anti-capsular protein

A polyclonal antibody raised against the proteinaceous capsular antigen (F1-antigen) was tested for immobilizing *S.* Typhimurium. F1-antigen, originally discovered for *Yersinia pestis*, can form a dense amorphous capsule that covers the bacterium (Friedlander et al. 1995; Titball and Williamson 2001). *S.* Typhimurium strain Δ*asd*::*kan*^{R} H71-pF1 was constructed to express F1-antigen as a model bacterium. The expression of F1 antigen was confirmed by immunofluorescence and Western blot analysis (Yang et al. 2007). It was expected that such a capsular proteinaceous antigen would have a relatively strong interaction with the corresponding antibody. However, Δ*asd*::*kan*^{R} H71-pF1 cells could not be immobilized on the substrate premodified with anti-F1 antigen (Fig. 3F). Most likely this is because the F1 capsule (Fig. 1), unlike CFA/I fimbriae, is not tightly bound to the bacterial cell wall and, therefore, can easily slough off from the surface of the bacterium, leading to the failure of bacterial immobilization.

2.3 Other factors

Other factors, such as the clonal type, the purity of the antibody, the antigen expression level, and the incubation time, also contribute to immobilization efficiency. The optimization of all these factors will lead to a very low detection limit. For example, we can readily detect *S.* Typhimurium or *E. coli* at a concentration of 10^3 to 10^4 CFU/ml.

3. A platform for studying an individual living bacterium

Immunoimmobilization offers access to controlling and manipulating living bacteria at the single-cell level. With this technique, it is possible to deploy a single living bacterium at a designated location, and then conduct measurements on the living bacterium with optical, mechanical, electrochemical and other tools. Combining these with PCR techniques, it is also possible to investigate the bacterial genomes of individual cells. Below, we present the preparation of bacterial micropatterns and the study of the mechanical properties of living *S.* Typhimurium.

3.1 Preparation of bacterial micropatterns

During the past decades there have been great advances in micro-electromechanical systems (MEMS) and nano-electromechanical systems (NEMS) technologies. Most of the techniques, such as microcontact printing, focused ion beam (FIB) etching, micro-plotting, e-beam lithography and dip-pen nanolithography (DPN), can be used to prepare micro- and nano-scale patterns on a flat substrate (Salaita et al. 2007).

The successful preparation of bacterial patterns relies mainly on the preparation of high-quality antibody micropatterns, which can be made using two general approaches: (1) modifying the substrate surface chemically to form a chemical micropattern in which the antibody will bind only inside or outside the modified areas and (2) depositing the antibody directly onto designated locations on an activated substrate surface. Most patterning techniques, such as FIB etching, DPN, e-beam lithography and microcontact printing, fall into one of these categories. The advantage of the first approach is the convenience of preparing patterns at nanoscale resolution (except for microcontact printing, which has a practical resolution of around 1 μm (Huck 2007)), which makes it possible to prepare single-cell arrays. However, it is difficult to represent multiple antibodies on the substrate using this approach since there are fairly limited chemical linkages available for antibody differentiation. The second approach (direct deposit approach) often refers to the microplotting method, which uses antibody solutions as ink for preparing antibody microspots on various substrates. We have tried two different microplotters whose antibody pattern sizes reached down to 25 μm in diameter. The antibody spot size is related to many factors, including the tip size, the viscosity of the protein solution, the hydrophobicity of the substrate surface and the moving speed of the tip of the microplotter (Larson et al. 2004), but generally it is very difficult to obtain spots smaller than 25 μm. The microplotting approach has a great advantage in that there is, in principle, no upper limit on the number of antibodies that can be represented in the microarray. For both approaches, it is necessary to passivate the substrate areas to prevent the nonspecific absorption of bacterial cells. In the first approach, the passivation is done by modifying the substrates using PEGlyated tethers (Lahiri et al. 1999) before patterning the surface with antibodies. In the microplotting methods, the passivation is done by post-exposing the protein micropatterns to BSA or milk proteins before exposing them to bacterial cultures.

Once prepared, an antibody micropattern is incubated with bacterial cultures. The bacterial cells are immobilized only on the antibody-patterned areas and thus form bacterial micropatterns. In Fig. 4, micropatterns of living *S.* Typhimurium prepared using FIB, microplotting and DPN are presented as examples. As can be seen in Fig. 4A, a cellular resolution of bacterial patterning is achieved in FIB patterning. Some of the line thicknesses in the patterns, ~1 μm, are comparable to the dimensions of the bacteria, and the bacteria are concentrated along these narrow lines; very few cells are observed outside the lines. Fig. 4B shows well-defined circular patterns obtained by microplotting. Single-cell resolution is best

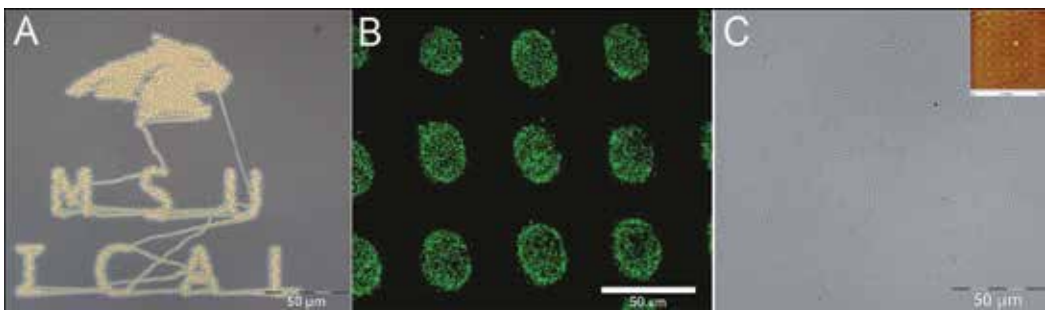


Fig. 4. Micropatterns of *S.* Typhimurium prepared by incubating the bacterial culture with antibody micropatterns fabricated using (A) FIB etching, (B) microplotting, and (C) DPN. Inset of panel C: A DPN pattern on gold substrate before antibody linking (scan size, 20 μm).

demonstrated by the microarray in Fig. 4C, which was prepared using the DPN method. An array of 16×16 antibody spots (each submicron in diameter) was placed on a chip using a sharp AFM tip. Notice that almost all the antibody spots captured at least one bacterium, most of the time multiple bacteria. This shows that immunoimmobilization, applied properly, is an extremely efficient technique.

3.2 Bacterial cells remain bioactive while immunoimmobilized

The question of whether or not immunoimmobilized microorganisms will maintain bioactivities such as cell division is highly relevant. An excessive number of antibodies surrounding a bacterium will eventually alter its behavior and perhaps even kill it. The technique used in immunoimmobilization is a fairly mild treatment of the bacterium that is immobilized. All the antibodies are bound to the surface; hence, only those bacteria that are near the surface of the substrate will interact with the antibodies. Of the many antibody molecules (~thousands per μm^2) evidence points to only a handful (tens per bacteria) interacting with the organism and keeping it bound to the surface. The evidence for this is that an immobilized bacterium still moves around with a considerable degree of freedom; in fact, when necessary it stands up, as mentioned above and also shown below, indicating that it is held to the surface by a small number of antibody-antigen pairs. This fact enables organisms to maintain their usual bioactivities, while giving rise to secondary activities triggered by the process of immobilization. We have seen evidence of such activity in the excessive production of flagella by bound bacteria as compared to that of the planktonic variety of the same bacteria (Suo et al. 2009a). Below we give some examples of the bioactivities of immobilized bacteria.

3.2.1 Immobilized bacteria are capable of division

Our results, as well as those of previous studies, indicate that immunoimmobilization does not hinder such physiological activities as the cell division (Suo et al. 2008), gene expression or bioluminescence of bacteria at the locations of their immobilization (Premkumar et al. 2001).

Immunoactivated areas exposed to low concentrations of bacteria (10^3-10^4 CFU/ml) for long periods of times (~20 hr) become populated by living bacteria for two reasons: (1) As time passes, some bacteria, even at low concentrations, are eventually captured by the antibodies within the activated areas. (2) The offspring of the captured bacteria start populating the antibody-activated areas (Fig. 5) because they too are captured at these locations following cell division, adding to the density of immunoimmobilized bacteria. Experiments with low cell concentrations are currently in progress, and the results will be published elsewhere.

It should be noted that once the surface is covered by a monolayer of live cells, the bacterial patterns on the antibody-modified areas do not change physical dimensions as a result of cell division. After the activated area is fully covered with bacteria, excess bacteria are released into the medium and become planktonic. This feature is not available for patterns prepared by embedding (Mbeunkui et al. 2002; Polyak et al. 2001; Premkumar et al. 2002; Weibel et al. 2005; Xu et al. 2007) or physical entrapment (Kuang et al. 2004). For these patterns, bacteria are held on the substrates or inside the microwells with weak forces, and the cells do not stay fixed to the patterned areas for long periods of time, causing the eventual disintegration of the pattern.

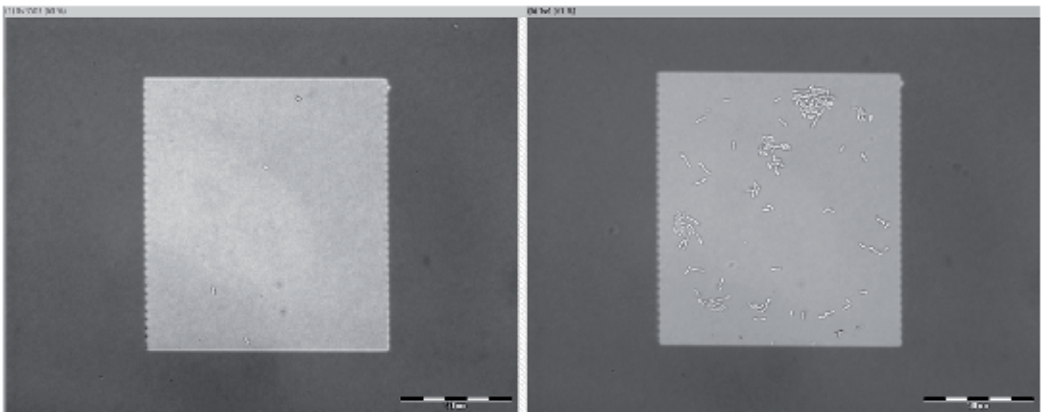


Fig. 5. Division of immobilized bacterial cells: (A) Four cells were captured inside the antibody-modified area (the bright square) when the substrate was incubated with a *S.* Typhimurium culture of 10^4 CFU/ml and (B) more cells were observed after the sample was incubated in cell-free medium for 20 hr at room temperature.

3.2.2 Crowded bacteria stand up

A surprising result is that bacteria initially immobilized in a lying-down orientation take a standing-up orientation as their density increases (Fig. 6A,B). This standing-up orientation of crowded cells has also been confirmed by laser scanning confocal microscopy (LSCM) images (Fig. 6C,D). Images (A) and (B) correspond to a sample incubated in growth medium at 37°C for 3 and 15 h, respectively. Notice that all the cells that were lying down in panel (A) appear to stand up in panel (B), corresponding to the increased bacterial density (see also panel (D)). Fig. 6C shows an LSCM image of a sample incubated in growth medium for

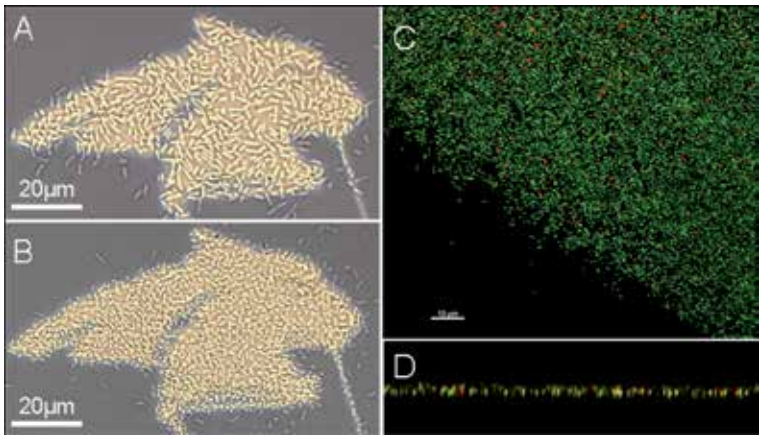


Fig. 6. Reorientation of immobilized *S.* Typhimurium cells due to surface population density. (A) Sample incubated in growth medium at 37°C for 3 h. (B) Same sample, incubated in growth medium at 37°C for 15 h. (C) LSCM image of a sample incubated in growth medium for 15 hr and then stored in PBS buffer at 4°C for 6 h and stained with viability stains. (D) Reconstituted *Z*-section image of the immobilized cells in (C).

15 hr and then stored in PBS buffer at 4°C for 6 h and stained with viability stains, which indicated that a majority of the cells were alive. The reconstituted Z-section image of the immobilized cells (Fig. 6D) further confirms that a majority of the cells took a standing-up orientation when crowded. Although at this time this behavior is not well understood, it might be related to the depletion of nutrients at the crowded bacterial positions and to the struggle of the bacteria to move away from their immobilized positions. This hypothesis is supported by the observation of an excess number of flagella produced by the immobilized bacteria (Suo et al, *Langmuir* 2008), presumably in an effort to free themselves from their positions.

3.2.3 Biofilm formation

The immobilized bacteria retained their cellular functions, including division, and continued to divide until a dense monolayer of bacteria filled the patterned area of the surface (Suo et al. 2008). We observed that biofilm started to form on the substrate after the initial two weeks. We incubated fifteen identical antibody-modified silicon chips with *S.* Typhimurium Δ*asd*::*kan*^{R} H71-pHC to form the initial bacterial patterns, and then all of the chips were rinsed with PBS and transferred to non-flow cell-free growth medium at room temperature. These silicon chips were prepared such that the patterned area was modified with anti-CFA/I fimbriae and the rest of the area was passivated using PEG molecules to prevent nonspecific attachment (Suo et al. 2008). After a predetermined incubation period, one silicon chip was taken out and rinsed with PBS to remove the unattached and loosely attached cells, then imaged under the optical microscope to determine the bacterial content on the substrate surface and its viability. The bacterial patterns were maintained for about two weeks, after which a bacterial biofilm started to form on the substrate, both inside the antibody-modified area and in the PEG-covered areas (Suo et al. 2008).

3.3 Investigation of mechanical properties of living *Salmonella*

We measured the physical properties of living *S.* Typhimurium using an atomic force microscope (AFM) at room temperature (Suo et al. 2009b). In a previous study of bacterial turgor pressure, a fairly gentle pressure, not enough to break the bacterial cell wall, was applied to bacterial cells (Yao et al. 2002). A fairly common belief is that a bacterium will lyse and die if its cell wall suffers severe mechanical damage, such as being punctured by an AFM tip. Our results show not only that *S.* Typhimurium cells can survive such damage, but also that the cells are still capable of self-replication.

3.3.1 Puncturing curves

A layer of *S.* Typhimurium cells in their exponential growth phase was immobilized in well-defined square patterns on a silicon wafer with ~20 cells within each square. Such immobilization lends itself to the continuous observation of the same group of cells under a light microscope before and after they are subjected to AFM probing. A sharp AFM tip was brought into contact with a live *S.* Typhimurium cell, and the loading force on the tip was increased until the tip punctured the cell wall (Fig. 7A). This was marked by a sharp decrease in the cantilever deflection value from ~150 nm to ~35 nm (inset in Fig. 7B). The tip was pushed continuously until it stopped penetrating the cell, which was interpreted as the

tip contacting the hard silicon surface underneath the organism. When the loading force reached a preset value (i.e. ~4 nN) the tip was lifted to ~2 µm above the cell surface in order to start another puncture cycle at an adjacent location on the organism. This puncturing process was repeated at a rate of 1 Hz or 2 Hz until each cell within a 7 × 7 µm² or 10 × 10 µm² area had undergone ~20 or ~40 puncturing events per µm². Each puncturing event generated a pair of force vs. displacement curves, or "puncturing curves" (Fig. 7B). These curves reveal a variety of information, including the true height of a live bacterium, its mechanical properties under physiological conditions, the pressure required to puncture the cell wall for a given tip geometry, and amount of cell deformation (indentation) before the cell wall is punctured. The inset shows the raw cantilever deflection versus displacement curves from which the puncturing curves were obtained by subtracting the cantilever deflection from the z-piezo displacement using a MatLab code developed by our group.

When a sharp tip punctures the cell wall of a Gram-negative bacterium it tears the three-layer cell wall structure before entering the bacterial cytoplasm. The pressure required to tear, or puncture, the cell wall is determined from the puncturing curve. The distance (~800 nm) between where the tip makes contact with the bacterial surface and where it touches the substratum is the measure of the true height of the bacterium in its physiological medium. The maximum penetration of F ≈ 2 nN at ~100 nm suggests that the bacterium deformed under the pressure exerted by the sharp tip and that the bacterial surface was indented by ~100 nm just before the tip punctured the cell wall. The modulus of elasticity of the living cell at the initial contact and the turgor pressure of the organism can be determined from the early part of the loading vs. tip penetration curve, using simple models such as the Hertzian model (Yao et al. 2002) or more complicated ones (Arnoldi et al. 2000).

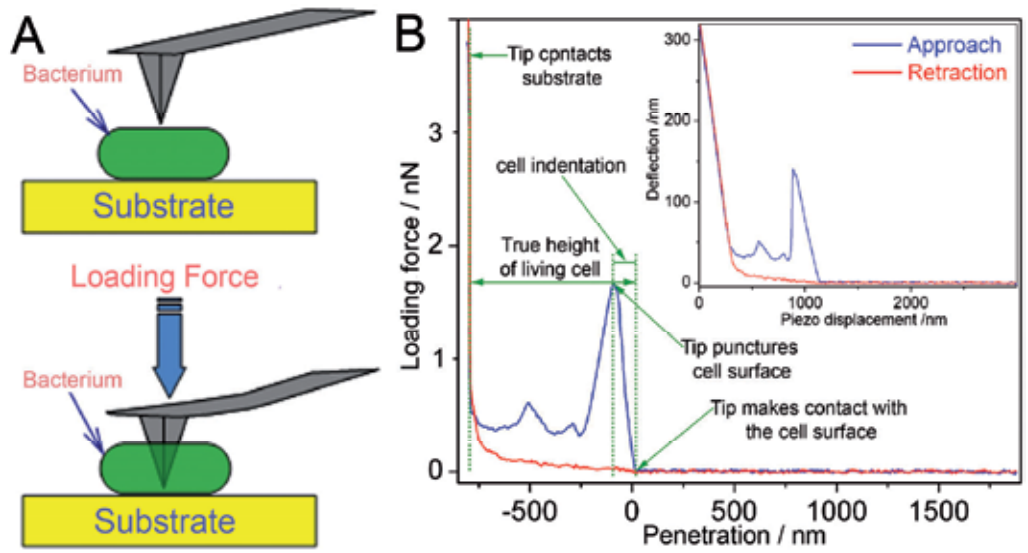


Fig. 7. (A) Schematics of puncturing experiments and (B) a typical puncturing curve

The pressure, *P*, required to penetrate the bacterial cell wall can be determined from $P = F/\pi r^2 \approx 5.0 \pm 0.8$ atm where *r* refers to the radius of the AFM tip. In our experiments, the average modulus of elasticity was calculated to be $0.4 \pm 0.2 \times 10^6$ Pa for living *S.* Typhimurium

cells in growth medium or in PBS buffer. This is comparable to results reported for *P. aeruginosa* and *E. coli* (Ingraham and Marr 1996; Yao et al. 2002).

The fine structures in the penetration range from ~250 nm through ~600 nm are ascribed to the resistance the tip experienced as it penetrated the cytoplasm of the organism. These forces are related to the tip geometry and, more importantly, to the tip aspect ratio. The force the tip experiences when it is pushed into the cytoplasm can be traced to two sources: (1) vertical resistance from the cell membranes, particularly from the peptidoglycan layer, during the initial 50 to 200 nm indentation (depending on the tip radius), until the tip overcomes this resistance and punctures the cell wall, and (2) lateral resistance as the tip tears the peptidoglycan layer. A tip with a high aspect ratio, such as a "spike tip" with a conical geometry, will give virtually no fine structures after the initial puncturing. A lower aspect ratio tip (pyramid tip) will tear the cell wall much more as it pushes into the cytoplasm and will introduce more severe cell damage than a spike tip (Suo et al. 2009b).

The common feature among all the puncturing curves is that there was very little or no resistance as the tip was pulled back from the cytoplasm of the bacteria (red line in Fig. 7B). This lack of resistance during tip retraction implies that there are only weak tip/cell wall interactions once the tip breaks the cell wall. The lipids, which are in continuous contact with the tip, offer no resistance to the motion of the tip as it is withdrawn from the cytoplasm.

3.3.2 Bacteria maintain their viability after being punctured multiple times

The size of the puncture hole while a tip penetrates a bacterium depends on the tip radius, the aspect ratio of the tip and the depth of penetration. However, there is no evidence of damage due to a puncture hole left behind from a puncturing event. It appears that puncture holes self-repair so that bacterial integrity and functionality are maintained, which is supported by the observation that punctured cells are capable of cell division, as is shown in Fig. 8. In this experiment, the colony of bacteria encircled by the blue dashed square in Fig. 8B was imaged before and after the puncturing. The data show that there was a one-to-one correspondence between the bacteria inside the blue square and the bright features in the AFM force-volume image. The light pixels in the dark background mark the locations of the punctured bacteria with a density of 20 puncturing events per square micron. Fig. 8B corresponds to the optical image acquired immediately after the puncturing experiment. We then kept the bacteria in their growth medium while taking images of the same location at one-minute intervals for 100 minutes. The effect of puncturing on the viability of *S.* Typhimurium was also studied using viability dyes. The majority of cells that had been punctured multiple times were able to divide, and there was no statistical difference found in survival rate between the punctured and the un-punctured cells, regardless of tip geometry or puncturing density varying between 20 and 40 puncturing events per μm^2.

It is also notable that the puncturing curves for dead *S.* Typhimurium cells were markedly different from those for living cells (Suo et al. 2009b). *S.* Typhimurium cells killed using glutaraldehyde did not show the force maximum associated with the puncturing event for live bacteria. Further analysis of the puncturing curves of dead cells revealed that dead bacteria shrink by about 40% and also appear to be softer, with an elastic modulus of ~0.13 ± 0.07 MPa in PBS as opposed to an elastic modulus of ~0.50 ± 0.10 MPa for living bacteria.

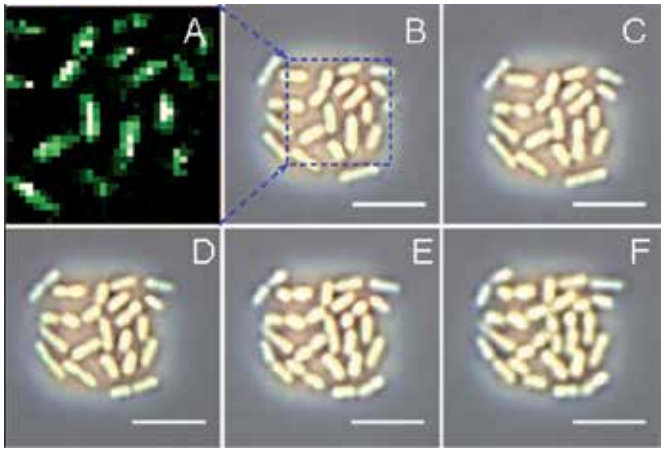


Fig. 8. Viability test: *S.* Typhimurium cells divide after being punctured multiple times. (A) An AFM force-volume image with bright spots showing the locations of the punctured bacteria. These spots have a one-to-one correlation with the cells shown in the blue dashed square of the optical image in panel B. Panels B-F show time-lapse images of the same group of bacteria taken 0, 25, 50, 75, and 100 min after the puncturing experiment, respectively. Cell division is highlighted by red, olive, and black ovals. Scale bar: 5 μm. Reprinted from Suo et al, *Langmuir* 2009 with permission from ACS

A model has been proposed to explain how bacteria survive the harsh damage done to their cell walls by an AFM tip. A detailed description of this model can be found elsewhere (Suo et al. 2009b). Briefly, the survival of punctured cells is owed to the remarkable features of the phospholipid bilayer that surrounds the cytoplasm of each bacterium. Phospholipid bilayers, at temperatures above the glass transition temperature, are composed of highly dynamic, fluid-like phospholipid molecules decorated with cross-membrane proteins (Osborn et al. 1972; Voet and Voet 1995). We believe that phospholipid molecules, including cross-membrane proteins with their hydrophobic regions intersecting the bilayers, undergo a reconfiguration (in a ns time frame) in response to AFM tip penetration (in a ms time frame). As the tip moves into or out of the cytoplasm, the fast-responding lipid membrane and membrane proteins are always in close contact with the tip surface and continuously seal against leakage into or out of the cytoplasmic and perhaps even the periplasmic regions regardless of the tip geometry. Apparently, a torn peptidoglycan layer is not a serious threat to cell viability.

4. Immunosensors for rapid detection of bacterial species

The rapid detection of microbial pathogens at a contamination site is critical for preventing the spread of the disease-causing microorganisms before the disease becomes epidemic. Diseases caused by water- and food-borne pathogens have been a serious threat to public health. A comprehensive examination of all the pathogen detection studies conducted over a period of 20 years showed that 38% were related to the food industry, 18% to clinical analysis, 16% to water and the environment and 27% to other areas (Lazcka et al. 2007), which emphasizes the importance of biosensor applications in these fields. *Salmonella* and *E. coli* were the most commonly detected pathogens in the aforementioned samples, with percentages of 33% and 27%, respectively (Turner 2011).

Bacterial pathogens are generally detected and identified using either a polymerase chain reaction (PCR) or antibody-based techniques (Velusamy et al. 2010a). The PCR approach offers an accurate determination of pathogens at the genomic level, but requires a proper design of primers targeting specific genes (Alexa et al. 2001; Holoda et al. 2005; Malorny et al. 2009). Antibody-based assays focus on the detection of bacterial antigens, and it is possible to detect multiple pathogens in a single assay using microarray techniques (Cai et al. 2005; Choi et al. 2008). Antibody-based techniques usually involve two events: capturing of the targeted pathogen on the sensor surface and follow-up signal generation. Efficient capturing is always desired, since it will facilitate converting captured pathogens into a detectable signal and, most importantly, a higher capture efficiency will result in a higher sensitivity (lower detection limit). Extensive research has been reported on the development of new detection methods that involve converting an already captured pathogen into an output signal by optical, electrochemical, mechanical or other means (O'Kennedy et al. 2009). During the past decade considerable advances were made in detecting pathogens by coupling immunological techniques with chemical and electronic actuators and techniques based on chemoluminescence (Wolter et al. 2008), electrochemical impedance (Geng et al. 2008), surface plasmon resonance (SPR) (Zordan et al. 2009), quartz crystal microbalance (QCM) (Adanyi et al. 2006; Boujday et al. 2008; Hirst et al. 2008) and wave guides (Adanyi et al. 2006). However, there has been little study of how to enhance the capture efficiency.

There have been many reports on the detection of *Salmonella* using antibody-based methods in the past decade, and in Table 1 we summarize these reports. The traditional detection of *Salmonella* spp. in food industries using differential growth media usually takes days to weeks (Amaguafia and Andrews 2000; Eaton et al. 2005). Detection based on immunoimmobilization or other antibody-based methods requires much less time, as evidenced by Table 1, showing typical assay times for these methods between 20 min and 6 hr. Such assays significantly reduce the time and effort needed for *Salmonella* detection and can possibly be used as on-site analysis in the critical initial stage of food poisoning.

As shown in Table 1, there have been many efforts to sense bacteria captured on an antibody-modified surface, ranging from the rather "traditional" method (ELISA) to new techniques such as microcantilevers. Most reports claim a detection limit close to 10^3 cells/ml, which could produce meaningful measurements in real applications, considering the infectious dose for human salmonellosis is around 10^3 CFU (Blaser and Newman 1982). Many different antibodies have been used, including commercial antibodies and antibodies prepared by the authors. It is desired for the readers to know the source, specificity, clonal type and purity of the antibodies used. However, it is not uncommon for inadequate information to be provided in this respect. For example, the term "anti-*Salmonella*" is often used without further specification.

Microarrays prepared using multiple antibodies are particularly efficient in the detection and identification of *Salmonella*. Cai et al. constructed a 8×15 model array for the identification of 20 common *Salmonella* serovars and evaluated the use of 117 target and 73 nontarget *Salmonella* strains (Cai et al. 2005). A total of 35 polyclonal antibodies against O-antigens or H-antigens (flagella) were used for serotyping *Salmonella* strains. Microarrays have been prepared using antibodies targeting multiple bacterial species. Choi et al. prepared a microarray containing four monoclonal antibodies against *E. coli* O157:H7, *S.* Typhimurium, *Yersinia enterocolitica*, and *Legionella pneumophila* (Choi et al. 2008). One problem with an antibody microarray is that generally the signal intensity of each antibody spot varies as a function of both the antibody concentration and the strain. This is actually

	Sensing element	Method	Detection limit	Medium	Assay time	Ref.
S. Infantis, S. Berta, S. enteritidis, S. Thompson, S. Typhimurium, S. Agona, S. Braenderup, S. Heidelberg, S. Dublin	paramagnetic beads coated with specific antibodies	immunomagnetic separation and enzyme immunoassay	10^4–10^6 CFU/ml	poultry environ mental samples	48 h	(Leon-Velarde et al. 2009)
S. Typhimurium (ATCC 53648)	anti-*S.* Typhimurium mAb; physical absorption	sandwich ELISA; electrochemical detection (chronoamperometry)	5×10^3 cells/ml	spiked chicken meat	n.a.	(Salam and Tothill 2009)
	anti-*S.* Typhimurium mAb; covalent linking		20 CFU/ml			
S. Typhimurium LT 2	anti-*Salmonella* magnetic beads; anti-*Salmonella*-HRP (rabbit pAb)	electrochemical magneto-immunosensing	5×10^3 CFU/ml	pure culture	50 min	(Liebana et al. 2009)
			7.5×10^3 CFU/ml	milk diluted 1/10 in LB		
			0.108 × CFU/ml	spiked milk, pre-enriched		
S. Typhymurium	mAb	ellipsometry	10^3 CFU/ml.	pure culture	n.a.	(Bae et al. 2005)
S. Typhimurium	goat IgG against *Salmonella* CSA-1	fluorescence resonance energy transfer based method	10^3 cells/ml.	pure culture	n.a.	(Ko and Grant 2006)
			10^5 CFU/g	spiked pork	5 min	
S. Typhimurium (ATCC 14028)	AP labeled anti-*Salmonella*, Ab-coated magnetic microbeads	immunomagnetic separation and immuno-optical absorption	2.2×10^4 CFU/ml	chicken carcass rinse	2 h	(Liu et al. 2001)
			2×10^4 CFU/ml	pure culture		
S. enteritidis isolated from food samples	HRP-labeled pAb	microcantilever	10^5 CFU/ml	pure culture	40 min	(Ricciardi et al. 2010)
S. Typhimurium	biotinylated anti-*Salmonella* CSA-1	surface plasmon resonance	1×10^6 CFU/ml	spiked chicken carcass rinse	n.a.	(Lan et al. 2008)
S. enteritidis	mAb MO9; anti-S. enteridis	piezoelectric immunosensor	1×10^5 cells/ml	pure culture	35 min	(Si et al. 2001)
S. Typhimurium (ATCC 14028)	anti-*Salmonella* CSA-1	quartz crystal microbalance	10^5 and 10^7 cells/ml	pure culture	hours	(Su and Li 2005)

	Sensing element	Method	Detection limit	Medium	Assay time	Ref.
S. Typhymurium	pAb, polyvalent somatic O antibody	quartz crystal acoustic wave device	10^2 to 10^{10} cells/ml	pure culture	3 h 40 min	(Olsen et al. 2003)
S. paratyphi A S. enteriditis S. Typhimurium	mAb MO2, MO4 and MO9	quartz crystal microbalance	6×10^4 cells/ml 6×10^4 cells/ml 8×10^4 cells/ml	pure culture	40 min	(Wong et al. 2002)
S. Typhi (strain SKST), a clinical isolate	pAb flagellar and mAb	sandwich ELISA	10^4-10^5 CFU/ml	pure culture	6 h	(Kumar et al. 2008)
			10^2 CFU/ml	spiked milk, vegetable /meat/ chicken rinse		
S. Typhimurium (KCCM 11806)	mAb against S. Typhimurium	surface plasmon resonance	10^2 CFU/ml	pure culture	n.a.	(Oh et al. 2004)
S. Typhimurium	rabbit pAb against *Salmonella*	Faradic impedimetric immunosensor	10^5 CFU/mL	spiked milk	2 h	(Mantzila et al. 2008)
S. Typhimurium	mAb against *S.* Typhimurium	microarray, fluorescence staining	n.a.	pure culture	n.a.	(Choi et al. 2008)
>*20 Salmonella* serovars	35 antisera against O- and H- antigens	microarray, fluorescence staining	n.a.	pure culture	n.a.	(Cai et al. 2005)
S. Typhimurium (ATCC 14028), heat killed	anti-*Salmonella* goat pAb (capture Ab); pAb HRP-anti–*Salmonella* (detection Ab)	microarray, microfluidics, chemiluminescence	2×10^7 cells/ml	pure culture	18 min	(Karsunke et al. 2009)
			3×10^6 cells/ml		13 min	(Wolter et al. 2008)
S. Typhimurium (ATCC 14028), heat killed	Antibody-labeled microspheres	flow cytometry	2.5-500	pure culture	180 min	(Dunbar et al. 2003)
S. Typhimurium (ATCC 14028 and wild type)	anti-*S.* Typhimurium mouse mAb, rabbit pAb	multiplexed assay, chemiluminescence	10^4 - 10^5 cells/ml	spiked human fecal and beef samples	60 min	(Magliulo et al. 2007)
S. Typhimurium, heat killed	rabbit pAb anti-*Salmonella* sp. (capture Ab), anti-S. Typhimurium LPS, mAb (detection Ab)	flow-through fluorescence assay	8×10^4	spiked food and fecal samples	15 min	(Taitt et al. 2004)
			8×10^3		60 min	

pAb: poyclonal antibody; mAb: monoclonal antibody; n.a.: not available; CSA: common structural antigens; HRP: horseradish peroxidase; AP: alkaline phosphatase.

Table 1. Antibody-based detection of *Salmonella*

an expected result, considering the immobilization efficiency is affected by multiple factors, including the substrate surface chemistry; the purity, clonal type and affinity of the antibody; the type and expression level of the bacterial antigen; the incubation duration; and the medium. Since microarrays employ multiple antibodies it is important to evaluate their cross-reactivities to avoid false positive results. One example is the work by Rivas et al. evaluating the binding capacities and cross-reactivities of 200 different antibodies for the detection of environmental toxins (Rivas et al. 2008). Our work focuses on determining how these factors affect the efficiency of capturing pathogenic bacteria. When coupled with microfluidics techniques, antibody-based detection can lead to miniaturized and automated detectors, which are in great demand for field applications (Karsunke et al. 2009; Wolter et al. 2008). Our work on using immunoimmobilization for biosensor applications has just been submitted for publication. Besides the direct capturing of bacterial cells, microarrays prepared using antibodies, proteins or carbohydrates have been used to detect pathogenic bacteria including *Salmonella*. These microarrays do not target the bacterial cell, and further reviews can be found in Bacarese-Hamilton's work (Bacarese-Hamilton et al. 2002).

5. Summary and outlook

We have presented the immobilization and manipulation of *Salmonella* cells on flat substrates in order to conduct tests on individual bacteria and to develop a biosensor technology based on capturing living organisms. Such immobilization is achieved using antibodies covalently tethered to flat substrates such as silicon wafers in order to capture living bacteria in their physiological environment**.** The immobilization process takes advantage of the specific interaction between an antibody and the corresponding antigen on a bacterial surface. Thus, the method offers a high immobilization efficiency with a rate of initial attachment of over 100 microorganisms per minute per 100×100 μm^2 area for a bulk concentration of microorganisms of ~2×10^6 CFU/ml. Because the surfaces to which the targeted organisms are tethered are highly polished and flat, the detection of these organisms is highly efficient: a single bacterium in a field with an area of 200×200 μm^2 can easily be imaged under optical microscope. The key to the success of immunoimmobilization is the combination of proper selection of antibody-antigen pairs and optimization of the surface chemistry for antibody linkage. If the surface chemistry is designed carefully, living bacterial cells can be immobilized on a variety of substrates, including silicon wafer, glass, gold and steel. Our work of the last 5-6 years on *Salmonella* and *E. coli* suggests that the most efficient and reliable immunoimmobilization involves a limited number of specific surface antigens such as pili, flagella or O-antigens and the corresponding antibodies. Bacterial cells immobilized in this way are linked robustly enough to be tethered to their locations but still maintain their viability and functionality without any noticeable hindrance. The technique provides a promising platform for the *in situ* investigation of individual or small groups of localized bacterial cells in their natural physiological environments, which offers substantial promise for the future. For example, our work has proven that multiple puncturings of the cell wall of a bacterium by means of an AFM tip does not kill the organism. Until this technique was published, many prominent microbiologists had believed that the puncturing process would undoubtedly kill the organism. This new phenomenon opens up the possibility of introducing macromolecules or nanoparticles into the cytoplasm of an individual living bacterium and observing the response of the bacterium to the intrusion. This possibility is a fertile ground for new science, and only time will show how fruitful.

The high efficiency and specificity of immunoimmobilization can also be utilized in biosensor technology for the rapid detection and identification of pathogenic species in field applications and the sorting of specific species from mixed consortia. To sum up, immunoimmobilization as described in this chapter has great potential both in fundamental and in practical applications.

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New Options for Rapid Typing of *Salmonella enterica* Serovars for Outbreak Investigation

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1. Introduction

A number of different serovars of *Salmonella enterica* are often implicated in human non-typhoidal outbreaks. Globally, serovars Typhimurium and Enteritidis are often the causative agent of such outbreaks, but other serovars can also be significant (Table 1). Some serovars such as Infantis and Virchow are routinely linked to outbreaks of gastroenteritis. The consumption of raw food products or poor food handling practices and/or storage procedures is often a catalyst for such outbreaks (Behravesh et al., 2010).

Accurate monitoring and tracking of specific *Salmonella* strains is of paramount importance, especially during an outbreak scenario. Large scale outbreaks such as the multistate *S.* Saintpaul outbreak in the U.S.A. in 2008 that implicated peppers as the source of contamination (Behravesh et al., 2010) highlight the need for high resolution testing procedures to enable confident identification of the source(s) of the outbreaks. Incorrect identification of potential sources will delay controlling and limiting the spread and health impact of outbreaks. Typing methods must be in place must not only identify an outbreak strain but also distinguish that strain from closely related but genetically distinct strains of the same serovar. However, many serovars of *Salmonella enterica* can appear clonal, making differentiation of strains difficult. Consequently, a high resolution typing system is required to distinguish individual strains within a serovar.

S. enterica can be subdivided into over 2,500 serotypes and, this, while useful in initial identification does not provide any further information. Classical methodologies such as bacteriophage (phage) typing can provide further subdivision within a serovar. Bacteriophage typing is a widely used phenotypic method for differentiation of clinically significant *Salmonella* serovars including Typhimurium, Enteritidis and Virchow. However, phage typing is a specialist methodology and is often unavailable to laboratories undertaking routine surveillance of *Salmonella*. Furthermore, particular phage types may dominate I a region over a period of time. This potentially makes identification of an outbreak strain difficult. Pulsed-field gel electrophoresis (PFGE) has routinely been employed for subtyping serovars and, where applicable, subtyping phage types of serovars such as Typhimurium, Enteritidis and Virchow. More recently Multiple-Locus Variable-number tandem-repeats (VNTR) Analysis (MLVA) (Lindstedt et al., 2003) and Multiple Amplification

of Phage Locus Typing (MAPLT) (Ross & Heuzenroeder, 2005) have been developed as PCR-based methodology for rapid, high resolution subtyping of *Salmonella* serovars.

Serovar	Year(s)	Associated food product	Country
Agona	2008	Processed cereal products	U.S.A.
Anatum	2006	Herbs (basil)	Denmark
Bareilly	2010	Bean sprouts	U.K.
Bovismorbificans	2001	Fast food outlets	Australia
Braenderup	2008	Egg product	Japan
Chester	1999	Cuttlefish chips	Japan
Derby	2006	Restaurant food	Japan
Havana	1998	Alfalfa	U.S.A.
Hvittingfoss	2005	not determined	Australia
Infantis	1999	Poultry	U.S.A.
Kedougou	2008	Infant formula	Spain
Montevideo	2007–2008	Various	Japan
Ohio	2005	Pork	Belgium
Oranienburg	1999	Cuttlefish chips	Japan
Potsdam	2002	Restaurant salad dressing	Australia
Saintpaul	2008	Raw produce	U.S.A.
Saintpaul	2009	Alfalfa	U.S.A.
Schwarzengrund	2006	Dry dog food	U.S.A.
Senftenberg	2007	Herbs (basil)	U.K.
Singapore	2004	Sushi	Australia
Tennessee	2006	Peanut butter	U.S.A.
Virchow	1997-1998	Sun-dried tomatoes/garlic	Australia
Virchow	2004-2009	Various	Switzerland

Table 1. Examples of non-typhoidal or Typhimurium and Enteritidis *S. enterica* serovars implicated with foodborne gastroenteric outbreaks

MLVA targets loci harbouring short tandem repeat sequences, using PCR with the product analysed for fragment length by capillary electrophoresis (Lindstedt et al., 2003) and separates isolates based on the number of tandem repeats in each locus. For *S.* Typhimurium, five loci have been described and a protocol for analysis described by The Institut Pasteur (www.pasteur.fr/recherche/genopole/PF8/mlva/). Loci for other serovars of interest have been described including Enteritidis (Boxrud et al. 2007; Malorney et al., 2008; Ramisse et al., 2004), Typhi (Lui et al., 2003) and Infantis (Ross & Heuzenroeder, 2008) although an agreed MLVA protocol for these serovars is yet to be ratified.

MAPLT is a multiplex PCR-based approach which detects prophage loci located within the *Salmonella* genome (Ross & Heuzenroeder, 2005). The assay is a binary method and is based on the presence or absence of particular loci. Depending on the design of primers for each locus, prophage PCR products can be simply detected by agarose gel electrophoresis, or they may be detected by capillary sequencing in the same manner as MLVA, or by real-time PCR. MAPLT primers have been described for serovars Infantis (Ross & Heuzenroeder, 2008), Typhimurium (Ross et al., 2009) and Enteritidis (Ross & Heuzenroeder, 2009).

While both methods usually provide resolution equivalent to that generated by PFGE, often particular loci within an assay do not provide sufficient allelic diversity for maximum isolate separation. For example, it has been reported that a number of different sized fragments for the plasmid-based MLVA locus STTR-10 in a range of Typhimurium isolates (Lindstedt et al., 2004). Conversely, specific definitive phage types (DTs) of serovar Typhimurium were found to have little or no allelic variation for this locus (Ross & Heuzenroeder, 2005). Routine analysis of human, food and environmental isolates of a range of Typhimurium phage types including untypable and **r**eactive-**d**oes **n**ot **c**onform (RDNC) isolates suggest that both STTR-9 and STTR-3 display much reduced allelic diversity compared to the other three loci (STTR-5, STTR-6 and STTR-10) (Ross & Heuzenroeder, unpublished data). This is particularly evident within specific definitive types. MAPLT data generated by our laboratory shows a similar phenomenon where some prophage loci are found in >95% of *Salmonella* isolates or are completely absent. For example, $gtrC_{ST64T}$ is generally found in many *S.* Typhimurium tested but the $gtrA_{ST64T}$ was rarely detected in the same group of isolates (Ross & Heuzenroeder, 2005).

Salmonella enterica serovar Virchow (*S.* Virchow) is a relatively less common serovar, showing a prevalence to certain geographic regions. In recent years *S.* Virchow has ranked among the top 10 serovars among human isolates in countries located in the African, European, Oceania, Latin American and Caribbean regions (Galanis et al., 2006). Australia is one country where *S.* Virchow has always been endemic, particularly in the Australian state of Queensland and has ranked among the ten most common serovars derived from human source since as early as 1991 (Australian Salmonella Reference Centre [ASRC], 1999-2009). *S.* Virchow is a ubiquitous organism that can be detected in various food animals and environmental sources such as chickens, pigs, horses and sewage sludge (ASRC, 1999-2009). However poultry and poultry-related products were reported to be the most prevalent reservoir in a number of countries. Over a ten year period to 2009, the majority of *S.* Virchow isolates received and serotyped by the ASRC were from poultry and eggs. In the United Kingdom *S.* Virchow has been linked to chickens and chicken-related products (Threlfell et al., 2002; Willocks et al; 1996). *S.* Virchow is a public health concern as a significant causative agent of food-borne gastroenteric outbreaks and severe extra-intestinal infections. Poultry are the main, but not exclusive reservoir of this serovar (Adak & Threlfall, 2005; Maguire et al., 2000; Semple et al., 1968). While some reported outbreaks in different countries were poultry-associated other food sources implicated in *S.* Virchow outbreaks included sun-dried tomatoes and processed milk products (Bennett et al., 2003; Taormina et al., 1999; Uresa et al., 1998). Systemic *S.* Virchow infections in young children have also been reported in Australia and the United Kingdom (Ispahani & Slack, 2000; Messer et al., 1997).

The current international phage typing scheme for *S.* Virchow was developed in 1987 and comprises 13 typing phages (Chamber et al., 1987). Fifty-seven lysis patterns or phage types have been identified (Torre et al., 1993). Phage types (PTs) 8 and 26 are the most predominant phage types in the UK consisting of 50% of isolates (Torre et al., 1993). Australia and Spain are the other two countries routinely using phage typing routinely. In Spain from 1990-1996 the most frequently isolated *S.* Virchow phage types were PTs 8, 19 and 31 (Martín et al., 2001), whereas in Australia the same period the most common phage types were PTs 8, 31 and 34 (ASRC, 1999-2009). These results demonstrate the important role of phage typing in the global surveillance of the *S.* Virchow population. It also indicates that PT8 is a global phage type predominating in endemic countries, whereas PTs 26 and 34

seem to be geographically specific to the UK and Australia respectively (Sullivan et al., 1998). In addition phage typing acts as a long-term epidemiological typing tool revealing any changes in incidence of *S.* Virchow phage types within a particular source. With respect to *S.* Virchow in Australia, no significant changes in the incidence of phage types were observed from human source in the decade to 2009 where PT8 was the most prevalent phage type (>50%) (ASRC, 1999-2009). In contrast, there were noticeable changes in the *S.* Virchow population in chickens and eggs based on the *S.* Virchow isolates received by ASRC. Even though PT8 was most commonly isolated from chickens and eggs in most years during the same period of time, the proportion of PT8 within these sources has decreased from 81.9% in 2000 to 35.5% in 2009 (ASRC, 1999-2009).

This chapter describes how specific data from MLVA and MAPLT can be combined into a single composite assay, thereby maximizing the resolving power of the assay for closely related isolates. The two classical typing methods, PGFE and phage typing provide a benchmark for determining the efficacy of MLVA and MAPLT, both individually and as a composite methodology. Previously published data for the two most significant non-typhoidal serovars, Typhimurium and Enteritidis have been re-analysed to determine the most variable loci for each protocol and single assays containing these loci have been identified for each serovar. The addition of phage typing data for serovar Enteritidis has also been taken into consideration to determine whether loci selection can be influenced by phage type. MLVA and MAPLT protocols have been developed for serovar Virchow with comparisons with PFGE. A single MLVA/MAPLT hybrid assay for *S.* Virchow has been developed and described here for the first time.

2. Materials and methods

2.1 *S.* Virchow strains and culture conditions

A total of 43 epidemiologically unrelated *S.* Virchow isolates were used for the development of MLVA and MAPLT assays. The isolates were provided by the ASRC, Institute of Medical and Veterinary Science, Adelaide, South Australia. The isolates represented a cross-section of the most commonly identified phage types submitted to the ASRC and were originally isolated throughout Australia between 2005 and 2008. Serotyping and phage typing of all *S.* Virchow isolates had previously been undertaken by the ASRC. Unless otherwise stated, all isolates were routinely cultured either on XLD agar medium or in bovine heart infusion broth (BHI) (Oxoid) at 37°C.

2.2 MLVA of *S.* Virchow

MLVA was undertaken utilizing primer sets previously described for Typhi (Liu, et al., 2003), Typhimurium (Lindstedt et al., 2003; Lindstedt et al., 2004) and Enteritidis (Boxrud et al., 2007; Ramisse et al., 2004) Primer sets targeting specific MLVA loci were selected based on their ability to differentiate within particular serovars of *Salmonella*. The touchdown PCR reaction and thermal cycler conditions were the same as those previously described (Ross & Heuzenroeder, 2005). Confirmation of fragment lengths as determined by genotyping was undertaken by nucleotide sequencing of selected isolates using Big Dye Terminator, version 3-1 (Applied Biosystems, Foster City, Calif.). Both genotyping and nucleotide sequencing were performed on an Applied Biosystems 3700 DNA Analyser. Data were entered into

BIONUMERICS v4.61 software (Applied Maths, Kortrijk, Belgium) as numerical values (fragment lengths in base pairs (bp) and negative PCR results entered as '0'). Dendrograms depicting the genetic similarity of isolates as determined by their MLVA profiles were generated using the categorical multi-state coefficient with zero tolerance and clustering by UPGMA utilising BIONUMERICS v4.61software (Applied Maths).

2.3 MAPLT of *S.* Virchow

Phages were induced from *S.* Virchow isolates as previously described (Ross & Heuzenroeder, 2008). Ten microlitres of each phage suspension were spotted onto lawns of epidemiologically distinct *S.* Virchow indicator isolates, allowed to dry and incubated at 37°C until plaquing could be observed. Phages that generated different lysis profiles (Fig. 1.) were selected for DOP-PCR to detect different phage sequences. DNA was extracted from phage and DOP-PCRs were undertaken as previously described (Ross & Heuzenroeder, 2009). Unique bands (Fig. 2.) were extracted from agarose gels and cloned into the vector PCRs 4-TOPO and transformed into TOPO One Shots TOP10 chemically competent *E. coli* cells (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Amplification of cell lysates using the TOPO primers was followed by sequencing PCR, undertaken with Big Dye Terminator v3-1 (Applied Biosystems, Foster City, CA). Characterization of sequence data was subsequently performed with KODON v3.5 (Applied Maths) and sequences compared with genomic library data for phage identification.

MAPLT analysis was undertaken with the primer combinations derived from prophages ST64B and P22 as published previously (Ross & Heuzenroeder, 2005), as well as loci identified by DOP-PCR from S. Virchow-derived prophages (Table 2). Amplification conditions using touchdown PCR and subsequent analysis were carried out as described previously (Ross & Heuzenroeder, 2005). MAPLT profiles for the *S.* Virchow isolates were determined based on the presence or absence of PCR product for all loci tested.

Fig. 1. Detection of different *S.* Virchow-derived bacteriophages by comparing plaquing patterns on lawns of *S.* Virchow isolates V15, V11 and V09 (as examples). By detecting differences in these patterns, potentially genetically different phages can then be isolated and identified by DOP-PCR and sequencing. This method results in a range of MAPLT primers that can detect a broad range of phage sequences in *S.* Virchow.

2.4 PFGE of *S*. Virchow

The protocol for PFGE was based on that of Maslow et al., (1993) as modified by Ross & Heuzenroeder (2005). Agarose-embedded *Salmonella* DNA and the *Staphylococcus aureus* strain NCTC 8325 marker DNA (Tenover et al., 1995) were digested overnight with the restriction endonucleases XbaI and SmaI, respectively (New England BioLabs Beverley, MA). The PFGE running conditions in the BIO-RAD CHEF-DR III System and subsequent comparisons of band profiles were undertaken as described previously (Ross & Heuzenroeder, 2005) using the GELCOMPAR II program (Applied Maths).

2.5 Data analysis

Comparison of the discriminatory power of all typing methods was undertaken using Simpson's index of diversity (Hunter & Gaston, 1988).

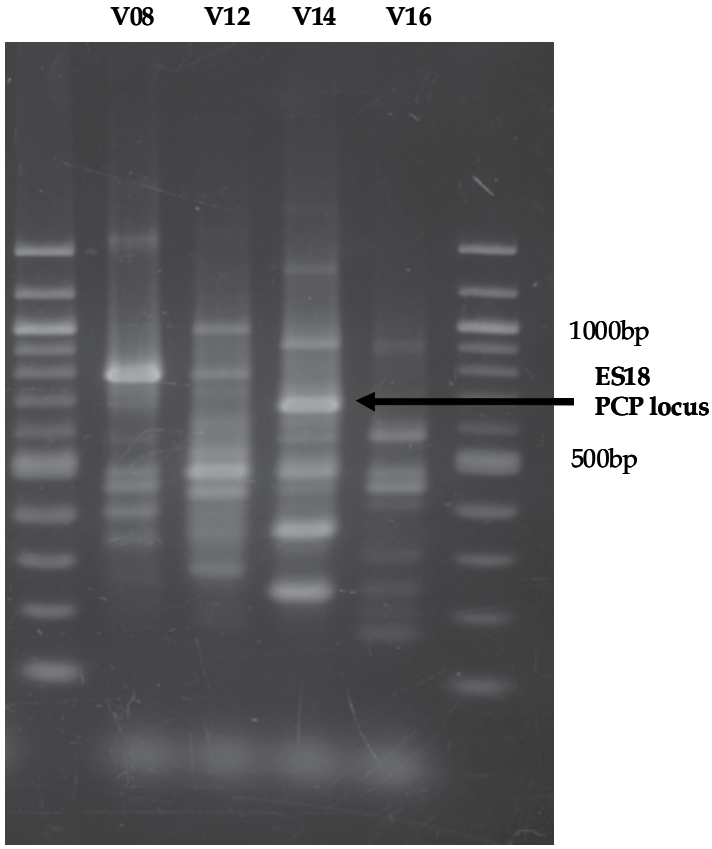


Fig. 2. DOP-PCR amplified phage DNA from *S*. Virchow isolates (V08, V12, V14 and V16). Individual bands were excised, cloned and sequenced to identify phage (see text for details). Phage from *S*. Virchow isolate V08 contained Fels2 sequences, V14 contained sequences from phage ES18 and V16 contained phage sequences from P186. The band containing the ES18 portal capsid protein sequence (PCP) is indicated as an example. No phage sequence was analysed from isolate V12 at time of publication. Molecular weight marker (first and last lanes) is a 100kb ladder.

Table 2. Primers for MAPLT analysis of *S.* Virchow

Phage	Gene or locus	Encoded proteins	Primers	Location infragment genomes[(i)]	Sizes (bp)
P22	*nin*B	ninB protein	ninBF1: AACCTTTGAAATTCGATCTCCAGC ninBR1: CTTCGTCTGACCACTTAACGC	16512 to 16891	380
ES18	*gene 9*	putative coat protein	PCPF: TGGAACGCACAGCATGATGC PCPR: GGACTGCACCTGAATATTCGG	6368 to 6853	486
Fels2	STM2736	CII protein	Fels2cIIF: TGTATGGAAACGGCAGCCAG Fels2cIIR: GTCACAACATGGCGAAGCTG	2875360 to 2875723	363
Gifsy-1	STM2608	Terminase large subunit	Gifsy1AF: GATCACGCATCCATTATGTTCAC Gifsy1AR: TATTCCCGTACCGCTTACCAC	2756675 to 2757449	775
186	*cII*	CII protein	P186cIIF: GACATAGCGGGATTAGTCTGC Fels2cIIR: GTCACAACATGGCGAAGCTG	23499 to 23892	394
18b	gene P	Endolysin	P186PF: TCACCGATTACAGCGACCAC P186PR: TGGTGACCAGCTTTTCGAGAC	7877 to 8200	324
ST64B	SB04	Putative portal protein	SB04F: TGTCATACGACACCTATACCG SB04R: TGTTCTGCACCATGTGCAATG	2513 to 3298	786
P7	*sit*	Putative injection transglyosylase	P7sitF: TGACCTTGATCGCGTACTCAC P7sitR: TAGCCACCAGGAGACATCTG	44668 to 45368	701
V16[(i)]	DOP13.7	Possible tail fibre protein	13.7F: CGGTTAGCTCCGTGGTTAAG 13.7R: TAGCCACCAGGAGACATCTG	not described	441

[(i)] Gene or locus accession numbers as follows:
P22: GeneBank accession no: AF217253
ES18: GenBank accession number AY736146
Fels2: GenBank accession number AE006468 (Prophage sequence of *Salmonella* Typhimurium strain LT2 from 2844427 to 2879233)
Gifsy-1: GenBank accession number AE006468 (Prophage sequence of *Salmonella* Typhimurium strain LT2 from 2844427 to 2879233)
186: GenBank accession number U32222.1
ST64B: Genbank accession number AY055382
P7: GenBank accession number AF503408
[(ii)] Unidentified prophage loci in *S.* Virchow isolate V16

3. Results

3.1 Composite data for *S*. Typhimurium

Ten loci comprising seven MAPLT and three MLVA sites were selected for analysis in the development of a combined MAPLT/MLVA protocol; $c1_{ST64B}$ $SB06_{ST64B}$, $SB26_{ST64B}$, $SB28_{ST64B}$, $SB46_{ST64B}$, gene 9_{ST64T}, $gtrC_{ST64T}$, STTR-5, STTR-6 and STTR-10. A dendrogram was generated reflecting analysis by this method (Figure 3). A total of 29 different profiles were generated. As previously observed, *S*. Typhimurium DT126 isolates were distinct from DT108, DT12 and DT12a isolates. The overall Simpson's Index of Diversity (DI) value for all non-DT126 isolates was 0.91, compared with previously published values of 0.83 for MLVA and 0.41 for MAPLT (Ross, et al., 2009). The Simpson's Index of Diversity (DI) value for the DT126 isolates was not calculated as most of these isolates were derived from two outbreaks and therefore would have skewed any statistical analysis due to their clonality.

3.2 Composite data for *S*. Enteritidis

Based on previously published data (Ross & Heuzenroeder, 2009), a combined MAPLT/MLVA was devised based on the most variable loci from each assay. Consequently a universal protocol targeting the following ten loci was devised; $SB40_{ST64B}$, $SB21_{ST64B}$, $SB28_{ST64B}$, $SB46_{ST64B}$, $gtrA_{ST64T}$, $gtrB_{ST64T}$, STTR-3, STTR-5, SE-1 and SE-2. These ten loci can be initially used where no phage typing data is available. Where phage typing data is available, improved separation within a phage type can be achieved. For example, our data shows that, instead of locus $SB21_{ST64B}$, the substitution of the ST64T gene *9* locus at the 5′ end (*g9*:5′) (Ross & Heuzenroeder, 2005) improves separation of phage type 26 isolates (Figure 4a) while the composite assay for the phage type 4 isolates indicated that the ten universal loci described above were suitable for this phage type (Figure 4b). The addition of ST64B *immC* gene *c1* improved separation of the *S*. Enteritidis RDNC isolates and isolates unable to be typed (ut) by phage typing (isolate designations RDNC- and Eut- respectively) (Figure 4c). Simpson's Index figures for the combined MAPLT/MLVA assay and comparisons to the previously published data for individual assays are provided in Table 3.

PT	MAPLT	MLVA	Composite	PFGE
26	0.87 (14)	0.89 (17)	0.99 (21)	0.66 (6)
4	0.83 (10)	0.85 (10)	0.99 (19)	0.48 (4)
ut/RDNC	0.98 (23)	0.96 (20)	0.99 (25)	0.89 (11)

Table 3. Comparative Simpson's Index values for *S*. Enteritidis phage types

Simpson's Index data for separate PFGE, MLVA and MAPLT analyses previously published (Ross and Heuzenroeder, 2009) Figures in brackets are the number of different profiles generated by each assay.

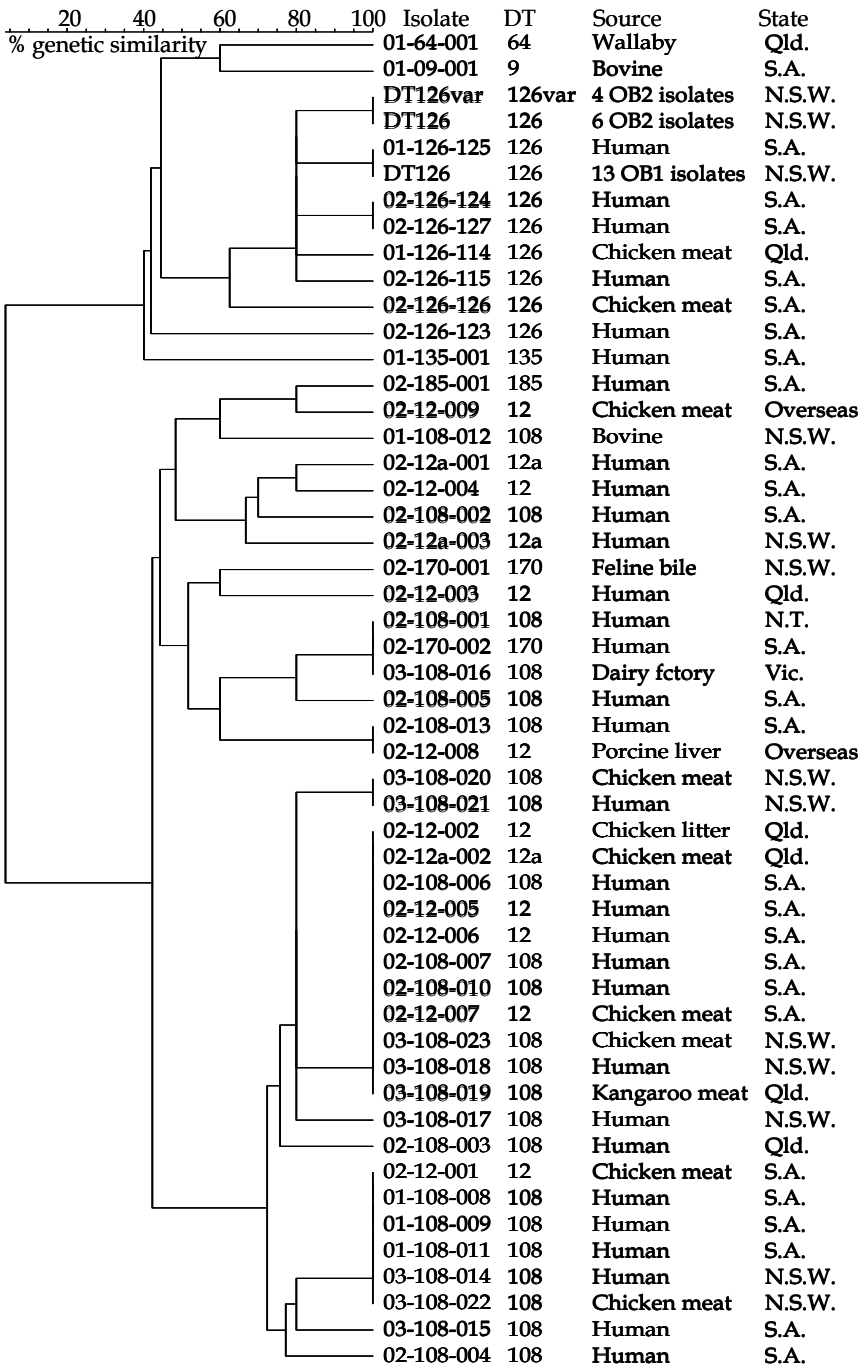


Fig. 3. Dendrogram showing genetic similarity of *S.* Typhimurium isolates. Abbreviations for states are: N.S.W. New South Wales, N.T. Northern Territory, Qld. Queensland S.A. South Australia, Vic. Victoria, W.A. Western Australia.

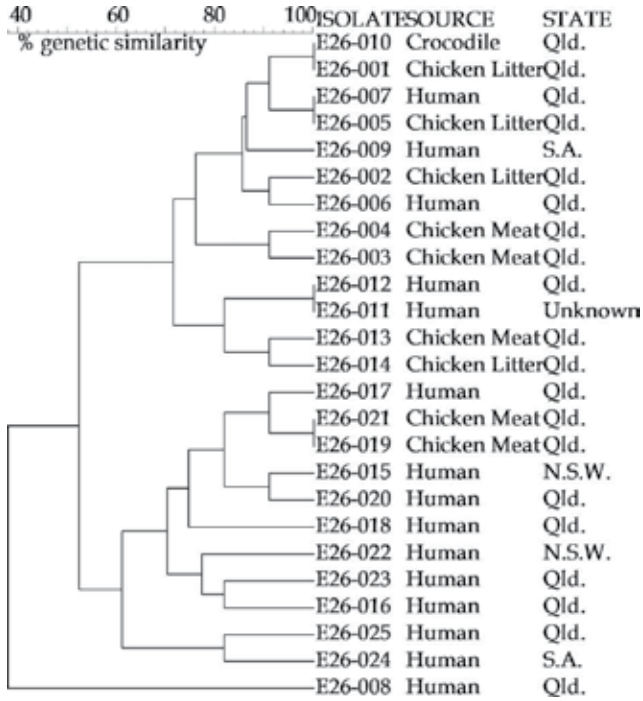


Fig. 4a. Dendrogram of *S. Enteritidis* PT26 analysed with composite MAPLT/MLVA data. No further information available for isolate E26-11

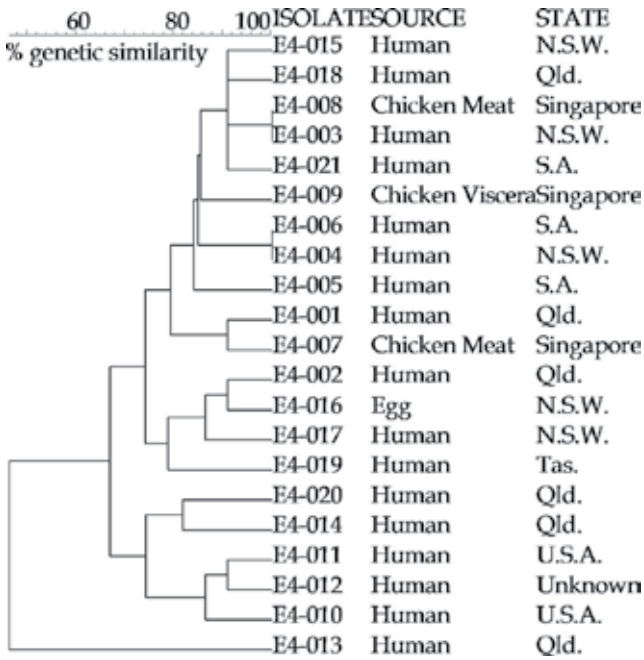


Fig. 4b. Dendrogram of *S. Enteritidis* PT4 analysed with composite MAPLT/MLVA data. All Australian states except where indicated.

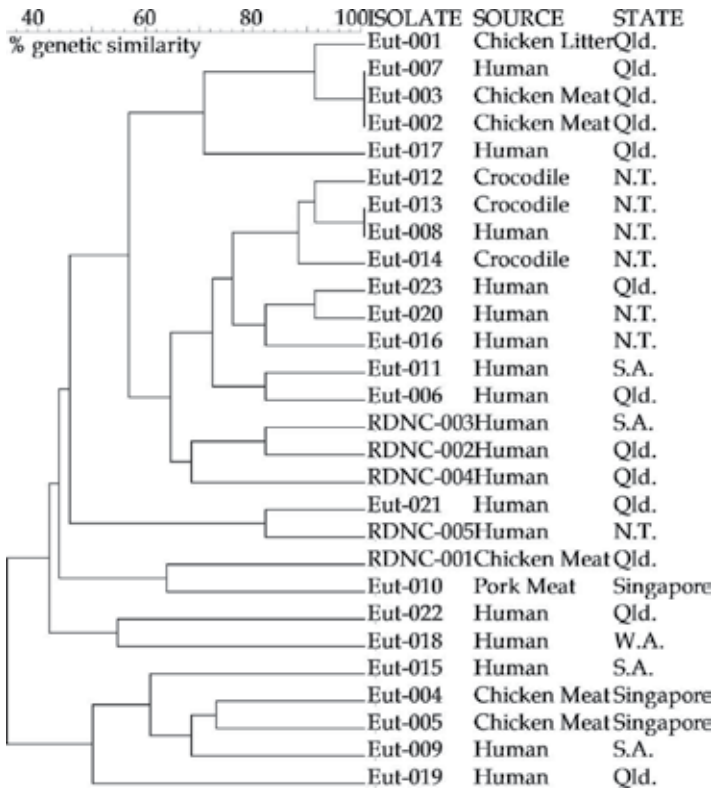


Fig. 4c. Dendogram of untypable and RDNC *S.* Enteritidis isolates analysed with composite MAPLT/MLVA data.

3.3 *S.* Virchow

PFGE analysis of *S.* Virchow divided the 43 isolates into 17 different profiles (Fig. 5). There was no distinct correlation between PFGE profile and phage type. For examples PFGE profiles 1, 3, 9 and 10 were generated from isolates with different phage types. Similarly, isolates of some phage types (17, 19, 31 and 36var1) produced PFGE profiles with 2 to 6 band differences between isolates, indicating that isolates within these phage types could exhibit an extensive genetic diversity. This study included a large proportion of PT8 isolates due to its predominance among all phage types seen in Australia. From the twenty-five PT8 isolates 15 (60%) generated PFGE profile 2. Nearly all PT8 isolates (14 out of 15) had the same MAPLT profile.

MAPLT analysis identified a number of loci derived from various bacteriophages which were useful in distinguishing between *S.* Virchow isolates. Nine MAPLT loci were subsequently chosen for *S.* Virchow differentiation based on the variability of frequency of these loci across the 43 isolates.

Using 15 MLVA primer sets previously described for a range of *S. enterica* serovars, only MLVA locus STTR-5 provided any allelic variation in the 43 *S.* Virchow isolates. The range of fragment sizes for this locus (based on the primer sequences of Lindstedt, et al., 2003) was 217bp (Fig. 6) to 271bp. There was no observed correlation between STTR-5 fragment size and phage type and in particular for PT8 the predominant type.

A composite MAPLT/MLVA dendrogram based on 9 MAPLT loci and the MLVA locus STTR-5 was generated (Fig. 6). This combination significantly improved the separation of the 43 *S.* Virchow isolates both in terms of diversity and number of different profiles generated (Table 4). More importantly, the differentiation of PT8 isolates was improved considerably using the combined method (DI = 0.88) in comparison to PFGE (DI = 0.59).

	MAPLT	MLVA	Composite	PFGE
Number of primers	9	13	10	na
Number of profiles	14	8	23	17
Simpson's DI	0.81	0.79	0.94	0.84

na not applicable

Table 4. Diversity of 43 *S.* Virchow isolates as determined by each method. Composite data based on combined MAPLT and MLVA primers; see Fig. 6 for details.

4. Discussion

The adoption of rapid, high resolution PCR-based typing assays such as MLVA and MAPLT for fine discrimination of closely related isolates of *Salmonella* may provide an alternative to phenotypic assays and current molecular methods such as PFGE. As more data is obtained it is obvious that there are sufficient differences in bacterial genome structure and prophage populations between different serovars of *Salmonella enterica* to necessitate development of such assays on a serovar by serovar basis. While PFGE is not limited by this issue, the development of PCR-based assays for specific serovars of interest is worthwhile due to the likelihood of improved discrimination of isolates and the ease of sharing data between interested laboratories and health authorities.

The combination of separate MAPLT and MLVA data into a single composite assay can provide superior discrimination of isolates than that obtained by either assay alone, as well as by PFGE. In the case of serovar Typhimurium, one of the most significant causative agents of non-typhoidal *Salmonella*-induced gastroenteritis, we have demonstrated that closely related phage types such as DT108 and DT12 can be separated by either PCR-based method, but combining the most variable loci into a single assay provides what may be the optimal separation of isolates. Furthermore, it should be noted that there was no correlation between phage type and clustering by MAPLT and/or MLVA. As mentioned previously the index of diversity for the DT126 isolates was not determined due to the clonality of the outbreak isolates clustering more tightly than would be seen with a group of epidemiologically-unrelated isolates. This however, demonstrates the ability of these PCR-based assays for discriminating outbreak isolates from closely related but epidemiologically distinct strains.

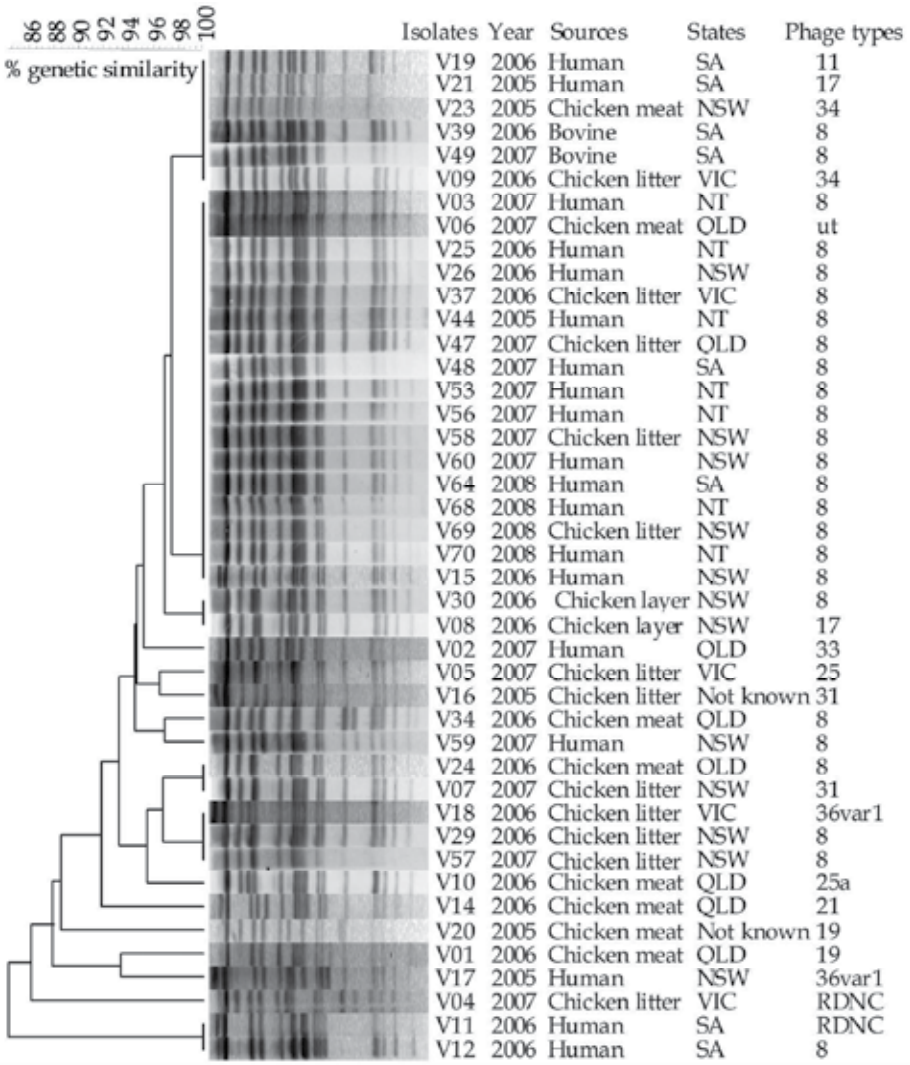
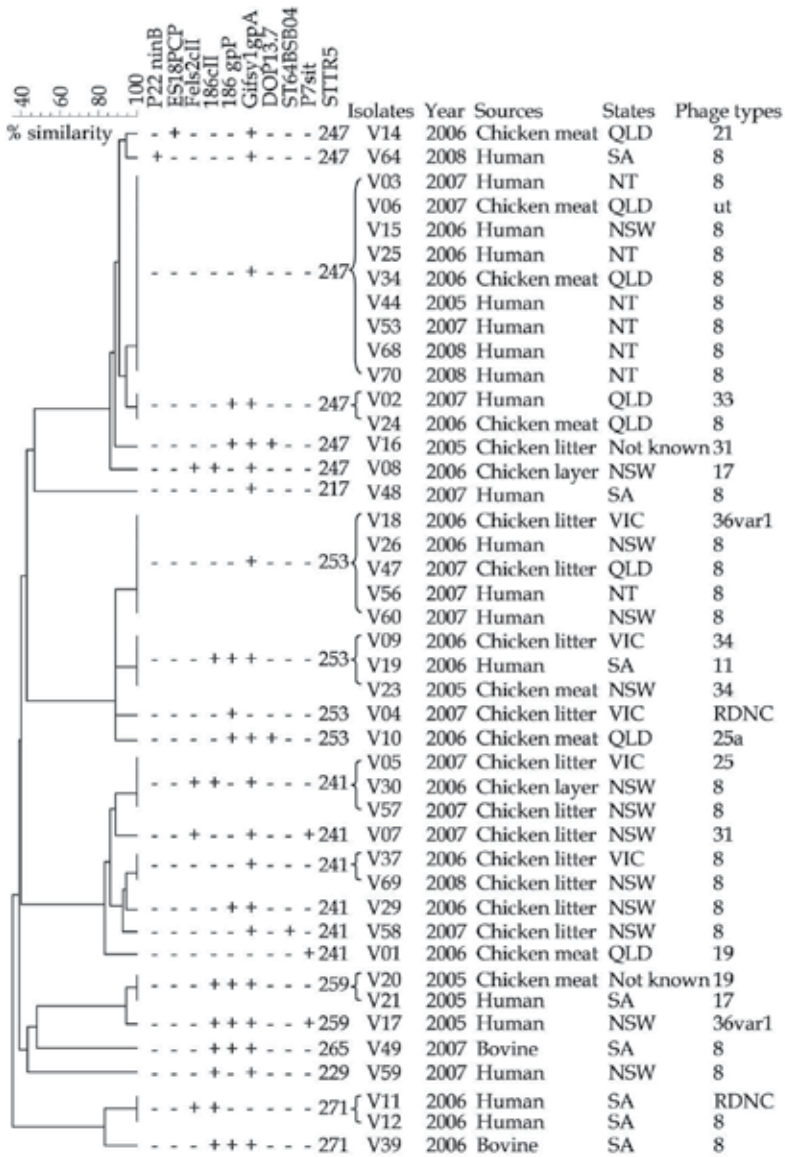


Fig. 5. Pulsed-field gel electrophoresis of 43 *S.* Virchow isloates.

While separate assays may need to be developed for different serovars with unique sets of primers, it is possible that individual loci may provide extra discrimination for particular phage types within a serovar. It has previously been reported that MLVA locus SENTR2 (locus STTR-7 as previously described by Lindstedt et al., 2003) may be useful for improved detection of differences within sample groups of both *S.* Enteritidis PT4 and PT8 isolates (Malorney et al., 2008). The data for *S.* Enteritidis presented here further supports this concept. While 10 primers sets formed the basis of a composite MAPLT/MLVA assay for this serovar (as demonstrated for the PT4 isolates), different MAPLT-derived loci proved useful for maximising isolate discrimination (see Fig. 4). This information is more relevant where phage type data is available and pre-selection of primers can be ascertained. However, even in the absence of the phage typing data, the assay may include primers for these extra loci as a matter of course.



+ MAPLT locus detected by PCR, - MAPLT locus not detected.
Fragment sizes for MLVA locus STTR-5 based on primer locations described by Lindstedt et al., (2003).

Fig. 6. A dendrogram based on composite MAPLT/MLVA data as described in section 3.3. All abbreviations for Australian states as per Fig. 3.

Development of MAPLT and MLVA as well as a composite assay for serovar Virchow has identified the importance of total genomic data being available in genome libraries such as Genbank (www.ncbi.nlm.nih.gov). While a number of suitable MAPLT loci were identified from a range of different prophages isolated from the *S.* Virchow strains with the exception of locus STTR-5, previously described MLVA loci were found to be either homologous in terms of fragment length or not detected by PCR and thus do not provide allelic variation

within this serovar. Access to total genomic data on different serovars and strains would facilitate searches for tandem repeat loci that may be unique to that serovar. It is also likely that more than one total genome per serovar may need to be sequenced to enhance the likelihood that most or all MLVA loci present in that serovar are detected. For example, many *S*. Typhimurium strains do not harbour the plasmid-based STTR-10 locus (Ross & Heuzenroeder, unpublished data). Development of a MLVA assay based solely on the sequence data of one of these isolates may have resulted in one of the most variable MLVA loci being excluded from any devised MLVA protocol. In the case of the development of a MLVA assay for *S*. Enteritidis, the genomes of two separate isolates of this serovar, LK5 and a phage type 4 isolate (as well as S. Typhimurium LT2) were analysed (Boxrud et al., 2007). Consequently, we conclude that the genomes of suitable *S*. Virchow isolates may need to be completely sequenced to identify unique tandem repeat loci that provide suitable allelic variation for a MLVA assay. In the interim however, MAPLT loci has provided excellent separation of the *S*. Virchow isolates while the inclusion of STTR-5 into a composite assay enhanced separation of the isolates, in particular the PT 8 isolates.

The use of PCR-based methodology can be quite useful in outbreak situations where the source of the outbreak must be quickly identified to stop or restrict the spread of the pathogen in the community or environment. Their usefulness is based on the high resolution capabilities, the relatively short time frame required for obtaining data and the simplicity for data sharing. It has been noted that because some MLVA loci of a strain can exhibit subtle mutations in tandem repeat number during the course of an outbreak, some subjective interpretation of data in conjunction with other epidemiological data may be necessary for accurate identification. Boxrud et al., (2007) has suggested that "interpretive criteria that account for genetic variability of MLVA patterns analogous to the Tenover criteria used for PFGE may need to be developed". In Australia, laboratories collaborating in MLVA of *Salmonella* have agreed that minor variations such as one tandem repeat change at two separate loci may not be significant, especially if epidemiological information supports the conclusion. A study on *S*. Typhimurium DT9 isolates involved in an outbreak in South Australia in 2007 revealed MLVA allelic variability in human-derived isolates that were linked to the outbreak (Ross et al., 2011). Local outbreaks of both DT9 and DT108 during 2011 have also indicated that variability in the three loci STTR-5, STTR-6 and STTR-10 can appear during the course of the outbreak (Ross et al., unpublished data). These variations however, did not prevent the rapid identification of the likely food source of the outbreak. As yet, a comparison with the stability of MAPLT loci in these isolates has yet to be determined.

With the development of PCR-based protocols being undertaken there is a need to ensure consistency of loci identification and nomenclature as well as clear guidelines for data generation. It has been noted that a single locus may be given more than one designation by different laboratories, leading to potential confusion. One example of this has been alluded to previously in this discussion; the naming of the MLVA locus as either STTR-7 or SENTR2 by different laboratories. As the name STTR-7 was documented first we have adopted this description and suggest all subsequent references to this locus be made in accordance with this nomenclature. A different example is where the sequence of a tandem repeat has been published in either direction by two different laboratories. *S*. Enteritidis MLVA locus SE-2 described by Boxrud et al., (2007), was later described as SENTR6 and published in the reverse direction. In both cases, using different nomenclature for identical loci can generate

confusion and unnecessary work for researchers during assay development and/or surveillance programmes.

Standardised guidelines for data generation and interpretation also need to be developed. We have already mentioned previously in the Introduction, guidelines for MLVA of *S.* Typhimurium published by The Institut Pasteur. Even so, there is still a lack of concordance in what constitutes an agreed tandem repeat sequence and whether single nucleotide polymorphisms in flanking tandem repeats disqualify them as being included in a tandem repeat analysis. This laboratory currently reports all *S.* Typhimurium MLVA patterns in terms of total sequence length of the five loci in base pairs in accordance with the primer sequences published by Lindstedt et al., (2003, 2004) and adopted and described in The Institut Pasteur website. This reporting method, lacking tandem repeat numbers, prevents any subsequent misinterpretation of data.

5. Conclusions

Both MAPLT and MLVA offer rapid PCR-based approaches for rapid, high resolution discrimination of phenotypically closely related but epidemiologically distinct *Salmonella* isolates. This level of discrimination is often at least equal to that offered by PFGE. Objective data generated by either PCR method can be easily shared between laboratories and appropriate jurisdictional health authorities for general pathogen surveillance purposes as well as the investigation and control of outbreaks. As either MAPLT or MLVA may be more suited for a particular serovar or, where applicable, phage type, a composite assay comprising multiplex primers from both individual assays targeting the most variable loci in a particular strain can provide the maximum level of isolate separation. This data in the form of universally agreed nomenclature, in combination with epidemiological information, would prove invaluable for detecting sources of outbreaks and thereby restricting their effects.

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27

Multiplex TaqMan Real-Time PCR (qPCR) Assay Targeting *prot6E* and *invA* Genes for Fast and Accurate Detection of *Salmonella* Enteritidis

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1. Introduction

Salmonella is an important foodborne pathogen causing significant public health concern, both domestically and internationally (Tirado and Schmidt, 2001; Scallan et al., 2011). According to the latest CDC report *Salmonella* infections affect millions of people every year accounting for 11%, 35% and 28%, of illnesses, hospitalizations and deaths, respectively of the total U.S. foodborne diseases caused by all known foodborne pathogens (Scallan et al., 2011). Among those non-typhoid salmonellosis, *S.* Enteritidis (SE) has emerged as a major egg-associated pathogen. SE transmission to humans has been linked mainly to consumption of contaminated foods containing undercooked eggs (Rabsch et al., 2000). Fresh shell-eggs can be contaminated easily with SE through cracks in the shell by contact with chicken feces or by transovarian infection (Snoeyenbos et al., 1969). Consequently, the increase of consumption of shell eggs and egg products per capita in the United States to approximately 249 eggs per year (American Egg Board, 2008) may have contributed, in part, to increases in foodborne outbreaks (Altekruse et al., 1997), including a large multistate SE outbreak of SE outbreak associated with eggs in the US in 2010.

Traditional culture methods for SE detection from shell eggs and liquid whole eggs consist of a series of steps including non-selective pre-enrichment, selective enrichment, and selective/differential plating, and finally biochemical and serological confirmation. The traditional microbiological method for SE isolation from liquid eggs is described in detail in Chapter MLG 4.05 "Isolation and Identification of *Salmonella* from Meat, Poultry, Pasteurized Egg and Catfish Products" by the United States Department of Agriculture (USDA) (http://www.fsis.usda.gov/PDF/MLG_4_05.pdf). This method is labor intensive and takes about one weeks to complete the analysis. Consequently, a need exists for the development and validation of faster screening and detection methods for this pathogen in eggs.

The use of PCR or real time PCR (qPCR) for specific pathogen detection in foods has increased in recent years. They are fast and reliable tools for the testing of contaminated foods and had helped in preventing outbreaks. In recent years, numerous methods based on *Salmonella* DNA detection (e.g. *invA* gene) either by conventional or real-time PCR have been developed

(Krascsenicsova et al., 2008; Malorny B et al., 2003; Wolffs et al., 2006). qPCR is faster, is more sensitive than conventional PCR, and provides real-time data avoiding the use of gels (Valasek and Repa, 2005). In particular, the *invA* gene represents a good candidate for *Salmonella* detection as it is present in all pathogenic serovars described to date (Rahn et al., 1992; Boyd EF et al., 1997). The product of this gene is essential for the organism's ability to invade mammalian cells and subsequently cause disease (Galan and Curtiss, III, 1991; Galan JE et al., 1992). In the case of SE in specific, several PCR and isothermal methodologies has also been developed targeting different genes (Seo et al., 2004; Malorny et al., 2007a; O'Regan et al., 2008; Hadjinicolaou et al., 2009). Although isothermal amplification techniques has some advantages over qPCR, such as increased detection limit and lower cost; still has the disadvantage that a single target can be used at a time and lacks internal control for monitoring possible inhibitors of the reaction that might exist in the food matrix analyzed.

In the present study we developed a fast and accurate qPCR assay for the specific detection of SE in eggs. This qPCR contained primers and probes to detect three different targets: the *invA* gene (*Salmonella* genus specific), the *prot6E* gene (SE specific), and the internal amplification control (IAC). A foreign internal amplification control (IAC) was incorporated into the assay with the aim of detecting potential inhibitors present in the matrix analyzed (eggs). *Salmonella* spp. detection in foods is usually achieved after food samples pre-enrichment approaches using overnight incubation (Feder et al., 2001). Consequently, this method described herein is intended as an initial screening of 24 h pre-enrichments for the presence of *Salmonella* in eggs. In turn, this method will dramatically decrease the time and effort required during standard microbiological testing, since only positive pre-enrichment samples will be processed further.

2. Materials and methods

2.1 Bacterial strains and media

Eleven *Salmonella enterica* serovar Enteritidis (SE) strains (CDC 2010K_1543, 13-2, SE12, 18579, 18580, 22689, SE10, SE26, 17905, SE22, and CDC_2010K_1441) (Table 1), were employed in this study for artificial contamination of eggs. Strain CHS44 was employed for determining the detection limit of the real-time PCR (qPCR) assay. These strains are from

Strain	Phage type	Location	Source
SE12	14b	ME	Egg follicle
18579	4	Mexico	poultry
18580	4	Mexico	poultry
22689	8	MD	Chicken breast
SE10	8	ME	Chicken ovary
SE26	13	TX	Chicken viscera
13-2	13	N/A	Chicken
17905	13a	N/A	Chicken
SE22	13a	ME	Poultry environment
CDC_2010K_1441	N/A	N/A	Egg outbreak USA 2010
CDC_2010K_1543	N/A	N/A	Egg outbreak USA 2010

Table 1. Characteristics of *S.* Enteritidis strains used in this study for artificial contamination of eggs.

the FDA, Center for Food Safety and Applied Nutrition (CFSAN), Division of Microbiology's culture collection. Strains were grown overnight in Luria-Bertani (LB) medium at 35°C with shaking (250 rpm). The inclusivity and exclusivity of the qPCR assay for SE was demonstrated with 186 SE (Table 2) and 97 non-SE strains belonging to the FDA's collection (Table 3). Further specificity was demonstrated with 32 non-*Salmonella* species (48 strains) from very closely related genera (Table 4).

***S.* Enteritidis strains**	**qPCR multiplex**		
	***invA* qPCR result**	***prot6E* qPCR result**	**IAC qPCR result**
54-5431	+	+	+
76-574	+	+	+
19755	+	+	+
22568	+	+	+
53-407	+	+	+
50-5306A	+	+	+
81-2625	+	+	+
78-1757	+	+	+
77-0424	+	+	+
36951	+	+	+
75-2325	+	+	+
60-2506	+	+	+
74-991	+	+	+
77-3493	+	+	+
76-2651	+	+	+
62-1976	+	+	+
77-1427	+	+	+
77-2659	+	+	+
75-199	+	+	+
76-2969	+	+	+
17912	+	+	+
75-1450	+	+	+
78-2938	+	+	+
31952	+	+	+
36388	+	+	+
76-1594	+	+	+
50-3079	+	+	+
50-5646	+	+	+
75-970	+	+	+
576709	+	+	+
639016-6	+	+	+

S. Enteritidis strains	qPCR multiplex		
	invA qPCR result	*prot6E* qPCR result	IAC qPCR result
607307-2	+	+	+
635290-58	+	+	+
640631	+	+	+
60738-9	+	+	+
629163	+	+	+
60-7307-6	+	+	+
622731-39	+	+	+
607308-16	+	+	+
607308-19	+	+	+
8a	+	+	+
98	+	+	+
415	+	+	+
13-1	+	+	+
23b	+	+	+
sz26	+	+	+
416 (pt4)	+	+	+
435 (pt4)	+	+	+
sz6	+	+	+
sz9	+	+	+
418	+	+	+
23a	+	+	+
pt23	+	+	+
421	+	+	+
sz10 (pt8)	+	+	+
436	+	+	+
414 (pt4)	+	+	+
419	+	+	+
CHS14	+	+	+
sz15 (pt8)	+	+	+
420	+	+	+
sz12	+	+	+
sz22	+	+	+
434	+	+	+
CHS44	+	+	+
426	+	+	+
13-3	+	+	+
60481	+	+	+

S. Enteritidis strains	qPCR multiplex		
	invA qPCR result	*prot6E* qPCR result	IAC qPCR result
chs15	+	+	+
chs39	+	+	+
60494	+	+	+
60562	+	+	+
13-2	+	+	+
30663	+	+	+
22689 (pt8)	+	+	+
18570	+	+	+
23711	+	+	+
22705	+	+	+
22599	+	+	+
22706	+	+	+
23698	+	+	+
23703	+	+	+
22581	+	+	+
22600	+	+	+
22606 (pt8)	+	+	+
22574	+	+	+
33944	+	+	+
22690 (pt8)	+	+	+
22619	+	+	+
22601	+	+	+
18580 (pt4)	+	+	+
18572 (pt4)	+	+	+
18671 (pt4)	+	+	+
22532	+	+	+
18577	+	+	+
18512	+	+	+
18575	+	+	+
17924	+	+	+
18511	+	+	+
18578	+	+	+
18579	+	+	+
17927	+	+	+
18510	+	+	+
17929	+	+	+
1793	+	+	+

S. Enteritidis strains	qPCR multiplex		
	invA qPCR result	*prot6E* qPCR result	IAC qPCR result
18568	+	+	+
18567 (pt4)	+	+	+
18518	+	+	+
13183	+	+	+
17912	+	+	+
18088	+	+	+
22621	+	+	+
17914	+	+	+
18514	+	+	+
17923 (pt8)	+	+	+
17921	+	+	+
17905	+	+	+
18509	+	+	+
17918	+	+	+
17919	+	+	+
17917	+	+	+
30661	+	+	+
18569	+	+	+
18081	+	+	+
22568	+	+	+
17930	+	+	+
22701	+	+	+
30658	+	+	+
18574	+	+	+
18516	+	+	+
18573	+	+	+
17931	+	+	+
22510 (pt8)	+	+	+
CDC_2010K_0895	+	+	+
CDC_2010K_0899	+	+	+
CDC_2010K_0956	+	+	+
CDC_2010K_0968	+	+	+
CDC_2010K_1010	+	+	+
CDC_2010K_1018	+	+	+
CDC_2010K_1441	+	+	+
CDC_2010K_1444	+	+	+
CDC_2010K_1445	+	+	+

S. Enteritidis strains	qPCR multiplex		
	invA qPCR result	*prot6E* qPCR result	IAC qPCR result
CDC_2010K_1455	+	+	+
CDC_2010K_1457	+	+	+
CDC_2010K_1543	+	+	+
CDC_2010K_1558	+	+	+
CDC_2010K_1559	+	+	+
CDC_2010K_1565	+	+	+
CDC_2010K_1566	+	+	+
CDC_2010K_1575	+	+	+
CDC_2010K_1580	+	+	+
CDC_2010K_1594	+	+	+
CDC_2010K_1725	+	+	+
CDC_2010K_1729	+	+	+
CDC_2010K_1745	+	+	+
CDC_2010K_1747	+	+	+
CDC_2010K_1791	+	+	+
CDC_2010K_1795	+	+	+
CDC_2010K_1808	+	+	+
CDC_2010K_1810	+	+	+
CDC_2010K_1811	+	+	+
CDC_2010K_1882	+	+	+
CDC_2010K_1884	+	+	+
SE12	+	+	+
SE26	+	+	+
SE22	+	+	+
CDC_07ST000857	+	+	+
CDC_08-0253	+	+	+
CDC_08-0254	+	+	+
02-0062	+	+	+
58-6482	+	-	+
59-365	+	-	+
54-2953	+	-	+
chs54	+	-	+
20036	+	-	+
20035	+	-	+
18845	+	-	+
32393	+	-	+
18685	+	-	+

S. Enteritidis strains	qPCR multiplex		
	invA qPCR result	*prot6E* qPCR result	IAC qPCR result
22558	+	-	+
20034	+	-	+
20037	+	-	+
23710	+	-	+
sz23	+	-	+
sz5	+	-	+
SE-10	+	-	+
Total (186)	186	170	186

Table 2. *Salmonella* strains used for testing SE inclusivity for the *prot6E/invA* multiplex TaqMan qPCR.

Salmonella subspecies and serovars	Strain Numbers	*prot6E* qPCR result	*invA* qPCR result	IAC qPCR result
S. enterica subsp. enterica (I)				
Typhimurium	14	-	+	+
I 4,[5],12:i:-	6	-	+	+
Typhimurium/DT104	4	-	+	+
Newport	1	-	+	+
Heidelberg	1	-	+	+
Typhi	1	-	+	+
4,5,12:b:-	1	-	+	+
Hadar	1	-	+	+
Brandenburg	1	-	+	+
Saphra	1	-	+	+
Rubislaw	1	-	+	+
Michigan	1	-	+	+
Urbana	1	-	+	+
Vietnam	1	-	+	+
Tornow	1	-	+	+
Gera	1	-	+	+
Fresno	1	-	+	+
Brisbane	1	-	+	+
Agona	1	-	+	+
Muenchen	1	-	+	+
Senftenberg	1	-	+	+
Muenster	1	-	+	+

***Salmonella* subspecies and serovars**	**Strain Numbers**	***prot6E* qPCR result**	***invA* qPCR result**	**IAC qPCR result**
Montevideo	1	-	+	+
Johannesburg	1	-	+	+
Javiana	1	-	+	+
Inverness	1	-	+	+
Cubana	1	-	+	+
Cerro	1	-	+	+
Alachua	1	-	+	+
S. enterica subsp. *Salamae* (II)				
II 58:l,z13,z28:z6	1	-	+	+
II 47:d:z39	1	-	+	+
II 48:d:z6	1	-	+	+
II 50:b:z6	1	-	+	+
II 53:lz28:z39	1	-	+	+
II 39:lz28:enx	1	-	+	+
II 13,22:z29:enx	1	-	+	+
II 4,12:b:-	1	-	+	+
II 18:z4,z23:-	1	-	+	+
S. enterica subsp. *arizonae* (IIIa)				
IIIa 41:z4,z23:-	1	-	+	+
IIIa 40:z4,z23:-	1	-	+	+
IIIa 48:g,z51:-	1	-	+	+
IIIa 21:g,z51:-	1	-	+	+
IIIa 51:gz51:-	1	-	+	+
IIIa 62:g,z51:-	1	-	+	+
IIIa 48:z4,z23,z32:-	1	-	+	+
IIIa 48:z4,z23:-	1	-	+	+
S. enterica subsp. *diarizonae* (IIIb)				
IIIb 60:r:e,n,x,z15	1	-	+	+
IIIb 48:i:z	1	-	+	+
IIIb 61:k:1,5,(7)	1	-	+	+
IIIb 61:l,v:1,5,7	1	-	+	+
IIIb 48: z10: e,n,x,z15	1	-	+	+
IIIb 38:z10:z53	1	-	+	+
IIIb 60:r:z	1	-	+	+

Salmonella subspecies and serovars	Strain Numbers	*prot6E* qPCR result	*invA* qPCR result	IAC qPCR result
IIIb 50:i:z	1	-	+	+
S. enterica subsp. *houtenae* (IV)				
IV 50:g,z51:-	1	-	+	+
IV 48:g,z51:-	1	-	+	+
IV 44:z4,z23:-	1	-	+	+
IV 45:g,z51:-	1	-	+	+
IV 16:z4,z32:-	1	-	+	+
IV 11:z4,z23:-	1	-	+	+
IV 6,7:z36:-	1	-	+	+
IV 16:z4,z32:-	1	-	+	+
S. enterica subsp. *Indica* (VI)				
VI 6,14,25:z10:1,(2),7	1	-	+	+
VI 11:b:1,7	1	-	+	+
VI 6,7:z41:1,7	1	-	+	+
VI 11:a:1,5	1	-	+	+
VI 6,14,25:a:e,n,x	1	-	+	+
S. enterica subsp. *houtenae* (VII)				
IV 40:g,z51:-	1	-	+	+
IV 40:z4,z24:-	1	-	+	+
S. bongori (V)				
V 48:i:-	1	-	+	+
V 40:z35:-	1	-	+	+
V 44:z39:-	1	-	+	+
V 60:z41:-	1	-	+	+
V 66:z41:-	1	-	+	+
V 48:z35:-	1	-	+	+
Total	101			

The nomenclatural system used is based on recommendations from the WHO Collaborating Centre for reference and research on *Salmonella*, 9th edition 2007.

Table 3. *Salmonella* strains used for testing SE exclusivity for the *prot6E/invA* multiplex TaqMan qPCR assay.

Organism	No. of strains	*prot6E* qPCR result	*invA* qPCR result	IAC qPCR result
Vibrio parahaemolyticus	4	-	-	+
V. vulnificus	1	-	-	+
Escherichia coli	9[a]	-	-	+
Enterobacter cloacae	1	-	-	+
E. aerogenes (ATCC 13048)	1	-	-	+
Cronobacter sakazakii (former E. sakazakii)	1	-	-	+
Yersinia enterocolitica	1	-	-	+
Y. pseudotuberculosis	1	-	-	+
Hafnia alvei	2	-	-	+
Morganella morganii	1	-	-	+
Edwardsiella tarda	1	-	-	+
Klebsiella pneumoniae	1	-	-	+
Proteus vulgaris	1	-	-	+
Pseudomonas aeruginosa	1	-	-	+
Serratia marcesans	1	-	-	+
Aeromonas hydrophila	1	-	-	+
Citrobacter freundii	1	-	-	+
C. koseri (ATCC 27028)	1	-	-	+
Staphylococcus aureus	1	-	-	+
Streptococcus faecalis	1	-	-	+
Bacillus subtilis	1	-	-	+
B. cereus	1	-	-	+
Listeria monocytogenes	1	-	-	+
L. innocua	1	-	-	+
Shigella sonnei	2	-	-	+
S. flexneri	2	-	-	+
S. boydii	2	-	-	+
S. dysenteriae	2	-	-	+
Achromobacter spp.	1	-	-	+
Providencia stuartii (ATCC 33672)	1	-	-	+
Proteus mirabilis	1	-	-	+
P. hauseri (*deposited as P.vulgaris*) (ATCC 13315)	1	-	-	+
Total	48			

[a] Five *E. coli* classes (virotypes) that cause diarrheal diseases were included: strain 10009 (enterotoxigenic, ETEC); strains 10010, 10015, 10016, 10017 and 10012 (enteroinvasive, EIEC); strain 10023 (enterohemorrhagic, EHEC); strain 10035 (enteropathogenic, EPEC) and strain ATM395 (enteroaggregative, EAEC).

Table 4. Organisms employed to assess the specificity of the *prot6E/invA* multiplex *TaqMan* qPCR assay for *S.* Enteritidis detection.

2.2 Preparation of SE inocula

Cultures of individual SE strains were prepared by transferring a loopful for three consecutive 24-h intervals to 10 ml of tryptic soy broth (TSB, Difco, Becton Dickinson) at 35 °C. SE cells from an overnight broth culture were centrifuged at 3,000 x g for 15 min at 4 °C. The pellet was washed twice with sterile 0.1% peptone water and re-suspended in sterile 0.1% peptone water. Serial dilutions of the suspension were prepared in sterile 0.1% peptone water to obtain the desired cell populations. The cell number in the inoculum was determined by plating 100µl dilutions (in sterile 0.1% peptone water) on TSA and incubating at 35 °C for 24 h.

2.3 Microbiological assay

All eggs were purchased from local grocery stores in College Park, MD. Analysis of liquid eggs was performed by following USDA procedure with some modifications. Shell eggs were broken by hands aseptically into sterile glass beakers. They were mixed well with a sterile stick by hands for about 2 minutes until it looked uniform. These liquid eggs were inoculated (day 1) at around 5 SE or at > 10^4 cells in 100 g. Each 100 g sample was placed into a 2-liter sterile glass beaker, mixed with 900 ml pre-enrichment broth. Five pre-enrichment broths were used for testing performance of pre-enrichments for SE recovery. They were TSB, TSB plus ferrous sulfate (TSB + Fe), universal pre-enrichment broth (UPB), nutrient broth (NB), and buffered peptone water (BPW). After 24 hr (day 2) pre-enrichment, 1 ml of each pre-enriched sample was transferred to 10 ml of selective enrichment media (Rappaport–Vassiliadis (RV) medium and Tetrathionate broth (TT) (Difco) and incubated for 24 h at 42 °C and 43 °C, respectively. On day 3, tube contents were vortexed for 10 sec, and 10 µl portions of the TT and RV media were streaked on bismuth sulfite (BS) agar, xylose lysine desoxycholate (XLD) agar, and Hektoen enteric (HE) agar and incubated at 35 °C for 24 h. On day 4, the plates were examined for the presence of typical *Salmonella* colonies. Typical colonies were confirmed with *Salmonella* agglutination test kit from BD.

2.4 Design of primers and standards for qPCR

All primers and probes (Table 5) employed in this study were purchased from IDT (Coralville, IA, USA). The targets for qPCR were *invA* gene and *prot6E* gene of SE. Primers and probes for *invA* assay were designed previously (Gonzalez-Escalona et al., 2009). Primers and probes for *prot6E* were designed using Beacon designer v.7 (PREMIER Biosoft, Palo Alto, CA). DNA from strain CHS44 was used to determine the *prot6E/invA* qPCR detection limit. DNA extraction was performed with the DNeasy kit as recommended by the manufacturer (QIAGEN). DNA concentration was determined using Qubit® 2.0 Fluorometer and Qubit™ dsDNA HS Assay Kit following manufacturer's instructions (Invitrogen). The numbers of copies of the qPCR standards were calculated by assuming average molecular masses of 680 Da for 1 nucleotide of double stranded DNA. The calculation was done with the following equation: copies per nanogram = (NL x 10-9)/ (n x mw), where n is the length of the SE strain P125109 complete genome (4,685,848 bp), mw is the molecular weight per nucleotide, and NL is Avogadro constant (6.02 x 1023 molecules per mol).

Target	Name	Sequence (5'-3')[a]	Reference
qPCR primers			
invA	invA_176F	CAACGTTTCCTGCGGTACTGT	(Gonzalez-Escalona et al., 2009)
	invA_291R	CCCGAACGTGGCGATAATT	
prot6E	prot6E-NGE-f	GTAGGTAGCCAGTATAAATC	This study
	prot6E-NGE-r	TCGGTTTCATAATCATTCC	
IAC	IAC-f	CTAACCTTCGTGATGAGCAATCG	(Deer et al., 2010)
	IAC-r	GATCAGCTACGTGAGGTCCTAC	
Probes			
	invA_Tx_208	TX-CTCTTTCGTCTGGCATTATCGATCAGTACCA-BHQ2	(Gonzalez-Escalona et al., 2009)
	prot6E-NGE-FAM	FAM-CACCACAAT/ZEN/ATGCGAATGAACCGT -BHQ3	This study
	IAC-Cy5	Cy5-AGCTAGTCGATGCACTCCAGTCCTCCT-Iowa BlackRQ-Sp	(Deer et al., 2010)

Table 5. Primers and probes employed in this study to detect *prot6E*/*invA* by qPCR. TX – Texas Red.

2.5 qPCR and data analysis

The qPCR reactions were carried out using the Platinum® Quantitative PCR SuperMix-UDG kit according to the specifications of the manufacturer (Invitrogen). This kit is a ready to use cocktail consisting of a 2X Reaction Mix (Platinum® Taq polymerase, 40 mM Tris-HCl, 100 mM KCl, 6 mM $MgCl_2$, 0.4 mM of each dNTP, 0.8 of dUTP, uracil DNA glycosilase (UDG) and stabilizers). Reactions were scaled down to a final volume of 20 µl. Additional $MgCl_2$ was added to the master mix to a final concentration per tube reaction of 5 mM. Also additional Platinum® Taq polymerase was supplied in order to have 2.5 final units per reaction. Final concentrations of primers in the qPCR mix were 200 nM for *invA* and *prot6E*, and 100 nM for IAC, respectively. Probes were added to a final concentration of 150 nM. qPCR and data analysis was performed on a Mx3005P QPCR System (Agilent Technologies, Inc., Santa Clara, CA) real-time PCR machine. qPCR conditions were as follows: an initial cycle of 2 min at 50°C for UDG incubation, a second cycle of 2 min at 95°C to activate the hot-start Taq polymerase and 35 cycles of denaturation at 95°C for 15 secs, primer annealing and extension at 60°C for 30 secs (the acquisition of dyes Cy5, FAM and Texas Red were performed at the end of this cycle). Two microliters of DNA IAC (10 pg -3,0 * 10^5 copies/2µl) was added to each qPCR reaction.

3. Results

3.1 Evaluation of the *prot6E/invA* multiplex qPCR TaqMan assay

The detection limit of the *prot6E/invA* qPCR was determined using 10-fold dilutions of DNA extracted from *S. enterica* Enteritidis strain CHS44. PCR primers specific for *prot6E* gene (prot6E-NGE-f and prot6E-NGE-r) and *invA* gene (invA_176F and invA_291R) were used (Table 5). Linear calibration curves with a correlation coefficient (R^2) of ≥ 0.99 and linear ranges of ≥ 5 orders of magnitude for both *prot6E* and *invA* were obtained (Fig. 1A and B). This corresponds to detection limits of about 40 genome copies for both *prot6E* and *invA* genes. The efficiency of the qPCR was ≥ 0.99 for both SE targets. The robustness of DNA IAC was observed for all dilutions tested (Fig. 1C). The inclusion of the DNA IAC (internal amplification control) did not affect amplification of either *Salmonella* gene target (Fig. 1C).

3.2 Specificity of the *prot6E/invA* qPCR TaqMan assay

The developed *prot6E/invA* qPCR assay showed 100% (186/186) and 91% (170/186) inclusivity for *invA* and *prot6E* target, respectively, after testing 186 SE strains (Table 2). The strains that rendered a negative result for presence of *prot6E* were: SE-10, 58-6482, 59-365, 54-2953, CHS54, 20036, 20035, 18845, 32393, 18685, 22558, 20034, 20037, 23710, sz23, and sz5. Furthermore, *prot6E/invA* qPCR showed 100% exclusivity, only SE was positive for *prot6E* target, while all *Salmonella* strains tested were positive for *invA* gene (Table 3). Specificity of the new multiplex *prot6E/invA* qPCR assay was examined by testing 48 non-*Salmonella* (Table 4), and was 100% specific for SE. These strains were chosen for specificity testing because many are close phylogenetic kin to the *Salmonellae* and, in several cases, are known to associate with the food supply. False negatives (inhibition of PCR reaction) were also ruled out through the use of a DNA internal amplification control (IAC).

3.3 Performance assessment of different pre-enrichment media for the recovery of SE from eggs using *prot6E/invA* qPCR and USDA microbial culture methods

The usefulness of the qPCR assay developed in this study for detecting SE in eggs was assessed by artificial contamination of eggs with SE. One hundred grams of pooled eggs were artificially contaminated with two different SE strains (CDC-2010K_1543 and 13-2) at high (10^6 CFU/100 g) and low (<10 CFU/100 g) levels (Table 6). We further tested the performance of 5 different pre-enrichment media for SE growth (BPW, TSB, TSB+Fe, NB, and UP). After 24 h, the pre-enrichments were used for detection of SE using both *prot6E/invA* qPCR and USDA *Salmonella* culture method (Chapter MLG 4.05 - "Isolation and Identification of *Salmonella* from Meat, Poultry, Pasteurized Egg and Catfish Products"; http://www.fsis.usda.gov/PDF/MLG_4_05.pdf). Un-inoculated egg samples were used as negative controls. One milliliter of pre-enrichment was boiled and used for qPCR amplification in triplicate. All artificially contaminated egg samples were positive for *Salmonella* using both *prot6E/invA* qPCR and the USDA methodologies (Table 6). We chose to show in the table only lower inoculation levels in order to highlight the sensitivity of this qPCR method. SE levels as low as 5 CFU/100 g were detected after 24 ± 2 h pre-enrichments. All pre-enrichment media showed fairly similar performances for SE recovery, save for NB which showed less growth after 24h, with SE levels 10-fold lower than other

media (Table 6). Absence of qPCR inhibitors was demonstrated by amplification of the IAC since IAC would not have been amplified had there been PCR inhibitors present in the samples analyzed (Table 6).

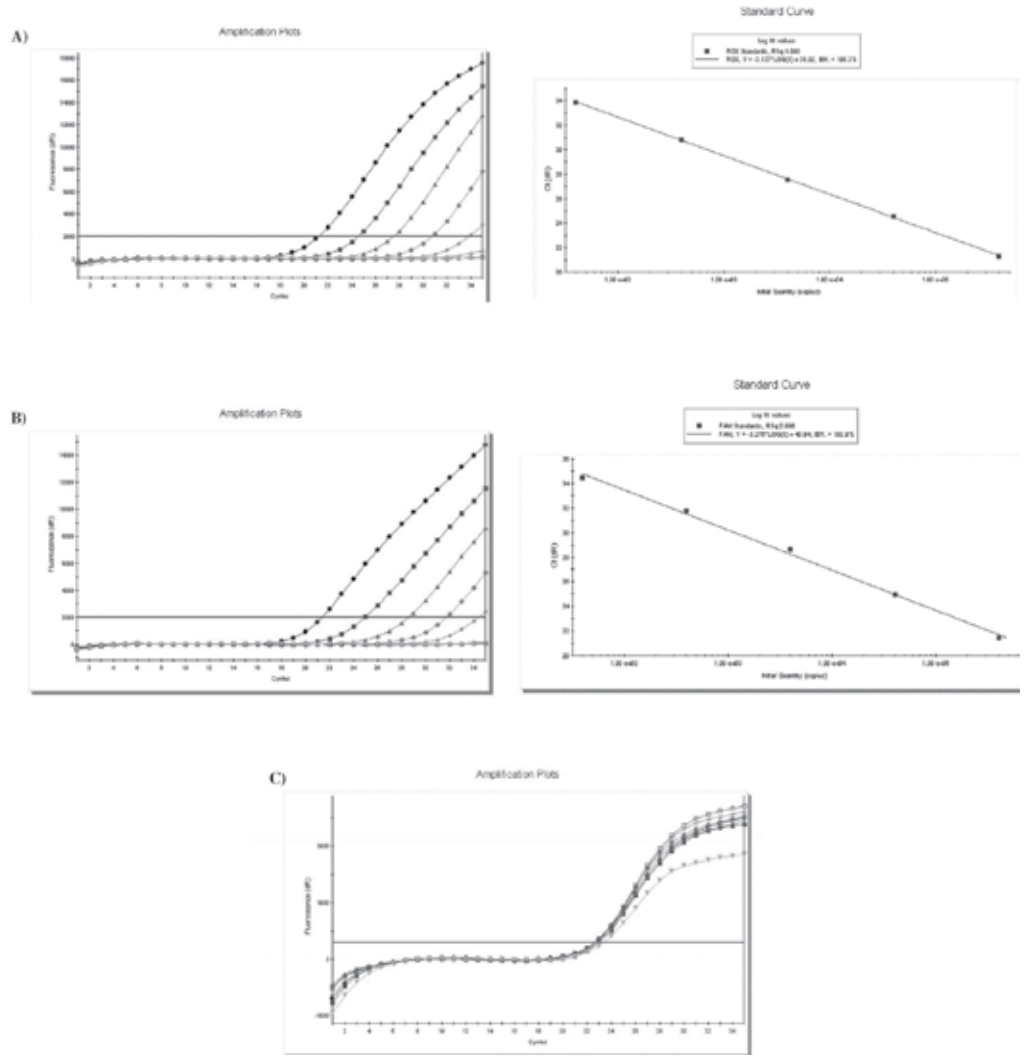

Fig. 1. Detection limit of the multiplex qPCR assay. Calibration curves were generated using 10-fold dilutions of CHS44 DNA (three replicates). A) Average *prot6E* amplification from dilutions 4.0*e10^6 - 40 CHS44 genome copies/reaction tube (FAM channel). B) Average *invA* amplification from dilutions 4.0*e10^6 - 40 CHS44 genome copies/reaction tube (ROX channel). C) Concurrent IAC amplification for each dilution (Cy5 channel). The Cq values were plotted against the nucleic acid target concentration (as copies per reaction for both DNA). The reaction efficiency (E) and R^2 values are also shown.

SE Strain	Pre-enrichment media	Minimum inoculation levels detected by USDA (CFU/100 g)	qPCR		
			prot6E (Cq)	*invA* (Cq)	IAC (Cq)
CDC-2010K_1543	BPW	5	+ (18.71 ± 0.79)	+ (19.98 ± 0.97)	+ (22.96 ± 0.41)
	NB		+ (22.69 ± 0.30)	+ (24.16 ± 0.38)	+ (22.31 ± 0.16)
	UP		+ (21.22 ± 0.55)	+ (20.12 ± 0.88)	+ (24.11 ± 1.30)
	TSB		+ (20.73 ± 0.90)	+ (22.22 ± 1.08)	+ (22.50 ± 0.42)
	TSB + Fe		+ (18.88 ± 0.30)	+ (20.32 ± 0.39)	+ (22.26 ± 0.66)
13-2	BPW	5	+ (17.70 ± 0.64)	+ (19.36 ± 0.72)	+ (22.69± 0.23)
	NB		+ (23.12 ± 1.13)	+ (24.39 ± 1.07)	+ (22.27± 0.32)
	UP		+ (19.63 ± 0.36)	+ (19.29 ± 0.79)	+ (23.83 ± 0.68)
	TSB		+ (20.24 ± 0.88)	+ (21.50 ± 0.62)	+ (22.42± 0.32)
	TSB + Fe		+ (19.43± 0.68)	+ (20.92 ± 0.82)	+ (22.20 ± 0.35)

Cq – Cycle quantification threshold, where the fluorescent is higher than the background.
+ = *Salmonella* positive by the method. In the case of IAC stands for positive signal for IAC.

Table 6. Pre-enrichment medium assessment for *Salmonella* Enteritidis (SE) recovery and detection using *prot6E*/*invA* multiplex qPCR and USDA culture method in pooled eggs artificially contaminated. Cq values are given in parentheses.

3.4 Application of the *prot6E/invA* qPCR assay for *Salmonella* Enteritidis detection in eggs artificially contaminated with different SE strains

After determining the effectiveness of the different pre-enrichment medias, we decided to employ TSB media as pre-enrichment for testing artificially contaminated eggs with 9 additional SE strains (Table 7). One of these strains lacked *prot6E* gene (SE-10). Eighteen pooled egg samples (100 g each) were artificially contaminated with high (~ 10^6 CFU/100 g) and low (<10 CFU/100 g) levels of SE and were analyzed as mentioned previously. After 24h, all artificially contaminated egg pre-enrichments were used for detection of SE using both *prot6E*/*invA* qPCR and USDA *Salmonella* culture method. Un-inoculated egg samples resulted in negative results by both *prot6E*/*invA* qPCR and USDA *Salmonella* culture method. On the other hand, all artificially contaminated egg samples were positive for

Salmonella using both *prot6E*/*invA* qPCR and the USDA methodologies except for SE-10 strain which was negative for *prot6E* (Table 7). Contrary to what is shown in Table 6, we showed the results of both high and low inoculation levels Table 7. SE levels as low as 2 CFU/100 g were detected after 24 ± 2 h pre-enrichments. As mentioned previously for the pre-enrichment media, the absence of qPCR inhibitors was demonstrated by observing no inhibition of the amplification of the IAC in every sample (Table 7).

SE Strain	Inoculation levels detected by USDA (CFU/100 g)	qPCR (Cq)		
		prot6E	*invA*	IAC
SE12	-	-	-	+ (23.33 ± 0.38)
	$1.15 * 10^4$	+ (23.17 ± 1.35)	+ (24.32 ± 0.68)	+ (23.08 ± 0.66)
	3	+ (23.32 ± 0.72)	+ (24.34 ± 0.96)	+ (23.72 ± 1.10)
18579	-	-	-	+ (24.11 ± 0.45)
	$1.24 * 10^4$	+ (23.56 ± 1.22)	+ (24.45 ± 0.29)	+ (22.91 ± 0.31)
	4	+ (24.47 ± 0.76)	+ (24.72 ± 0.81)	+ (23.57 ± 1.17)
18580	-	-	-	+ (23.45 ± 0.53)
	$0.68 * 10^4$	+ (22.49 ± 0.59)	+ (24.32 ± 0.64)	+ (22.85 ± 0.43)
	2	+ (22.51 ± 0.72)	+ (23.97 ± 0.60)	+ (22.70 ± 0.14)
22689	-	-	-	+ (23.35 ± 0.37)
	$1.35 * 10^4$	+ (21.77 ± 0.88)	+ (23.12 ± 0.85)	+ (22.51 ± 0.52)
	4	+ (23.83 ± 0.91)	+ (24.83 ± 0.81)	+ (24.42 ± 1.04)
SE10	-	-	-	+ (23.30 ± 0.36)
	$1.32 * 10^4$	-	+ (23.20 ± 0.43)	+ (23.01 ± 0.45)
	4	-	+ (24.61 ± 0.75)	+ (22.83 ± 0.11)
SE26	-	-	-	+ (23.04 ± 0.40)
	$1.53 * 10^4$	+ (21.44 ± 0.28)	+ (22.93 ± 0.43)	+ (22.40 ± 0.37)
	5	+ (23.70 ± 0.91)	+ (24.76 ± 0.69)	+ (23.23 ± 0.48)
17905	-	-	-	+ (23.35 ± 0.44)
	$1.52 * 10^4$	+ (22.14 ± 0.26)	+ (23.58 ± 0.55)	+ (22.20 ± 0.59)
	5	+ (23.00 ± 0.32)	+ (24.32 ± 0.27)	+ (22.63 ± 0.33)
SE22	-	-	-	+ (23.66 ± 0.37)
	$1.39 * 10^4$	+ (20.95 ± 0.46)	+ (22.44 ± 0.49)	+ (22.73 ± 0.17)
	4	+ (23.59 ± 0.28)	+ (24.90 ± 0.29)	+ (22.31 ± 1.05)
CDC_2010K_1441	-	-	-	+ (23.40 ± 0.49)
	$1.52 * 10^4$	+ (22.64 ± 0.24)	+ (23.97 ± 0.28)	+ (23.45 ± 0.90)
	5	+ (22.07 ± 0.80)	+ (23.27 ± 0.79)	+ (22.56 ± 0.02)

Cq – Cycle quantification threshold, where the fluorescent is higher than the background.
+ = *Salmonella* positive by the method. In the case of IAC stands for positive signal for IAC.
- = *Salmonella* negative by the method.

Table 7. *Salmonella* Enteritidis (SE) detection by *prot6E*/*invA* multiplex qPCR and USDA culture method in pooled eggs artificially contaminated. Cq values are given in parentheses.

4. Discussion

This study reports the development of a multiplex qPCR TaqMan assay that allowed for the fast and accurate detection of SE cells from eggs. The assay performed comparably to the traditional SE culture methods described in Chapter MLG 4.05 (USDA) for the detection of SE from meat, poultry, pasteurized egg and catfish products**.** The overall analysis took roughly 24 h, in contrast to the 5 days to 2 weeks that traditional microbiological culture methods often take. It is noteworthy that agreement between the qPCR and the two microbial culture methods was 100% for all artificially spiked samples.

This novel quantitative real-time PCR (qPCR) assay uses specific primers for the detection of *prot6E* and *invA* genes of SE with TaqMan probes. This assay also includes an internal amplification control (IAC) to detect potential PCR inhibitors that may be present in egg samples. It has become increasingly evident that there is a need for internal controls for PCR reaction, to rule out the presence of PCR inhibitors that can cause false negative results for *Salmonella*-positive samples (Hartman et al., 2005; Hoorfar et al., 2004). The inclusion of this internal control did not affect either the amplification or the detection limit of the qPCR assay. The qPCR developed here as opposed to an *invA* single target qPCR method (Feder et al., 2001; Malorny et al., 2004; Malorny B et al., 2003; Malorny et al., 2003; Bohaychuk et al., 2007; Gonzalez-Escalona et al., 2009) is able to detect specifically SE strains by the use of an SE specific marker (*prot6E*). Additionally it is capable to detect other SE that might lack the *prot6E* gene (Malorny et al., 2007a), such as the case for SE-10. The lack of *prot6E* in SE strains has been co-related with the absence of the SE virulence plasmid (~ 55 kb) (Malorny et al., 2007a). Due to the importance of that plasmid in SE virulence (Bakshi et al., 2003), without it SE has a diminish virulence, and such could be the case for SE-10 which was isolated from chicken. Moreover this assay has a further advantage in that it is an open formula assay, whereby no primers, no probes or IAC are patented or proprietary.

Among the most common gene targets used for SE detection by qPCR are: 1) Sdf1, a chromosomal fragment (Agron et al., 2001); 2) *sef*A, encoding for fimbrial antigen SEF14 (Seo et al., 2004); and 3) *prot6E*, encoding for a unique surface fimbriae (Malorny et al., 2007a; Clavijo et al., 2006). Sdf1 is highly specific for SE but is missing in SE phage types (PT) 6A, 9A, 11, 16, 20, and 27 and besides that were only tested on pure cultures (Malorny et al., 2007a). *SefA* gene is also present in all members of *S. enterica* serogroup D (Gallinarum, Pollorum, Dublin, Rostock, and Typhi, among others) which might lead to false positives results (Seo et al., 2004; Malorny et al., 2007a) and therefore it is not recommended for specific identification of SE. *prot6E* is present in the SE 60 kb virulence plasmid, which is present in most SE (>90%) (Chu et al., 1999; Helmuth and Schroeter, 1994; Clavijo et al., 2006) and therefore was our target of choice for SE specific detection by qPCR.

The detection limit of this qPCR assay was ~ 40 copies of genomic DNA. Usually 1 ml of pre-enrichment is boiled and 2 µl of supernatant is used for qPCR reaction. Thus, a population of approximately $4*10^4$ CFU/ml needs to be reached in the pre-enrichment to render a positive result. Commonly SE levels reach ~ 10^8 CFU/ml in the pre-enriched cultures. Therefore, this assay could be used for identification and/or quantification of SE cells in foods directly after pre-enrichment. It is also important to note, however, that in non-host environments, *Salmonella* persists most likely in a starved and highly stressed state. However, the addition of a requisite pre-enrichment step in culture media substantially increases cell number. Thus, a pre-enrichment culture provides an essential preliminary step in the application of this assay to the reliable detection of SE from eggs.

Rather than performing replicates of several inoculations with the same strain, we opted to spike the eggs in Table 7, with 9 different SE strains. This provided, in our opinion, a more powerful approach than simply repeating the experiment with the same strain multiple times as other investigators usually do. The ultimate goal of the assay is to detect different SE strains. Thus increasing the bio-complexity of testing provided a more thorough and rigorous challenge to the capability of the qPCR method to detect SE, in general. The IAC amplification was not affected in all the samples tested, however a possible failure in samples containing high levels of SE could be expected. That sort of possible failure is not un-expected given the competitive nature of this qPCR reaction, where primers and probes for *Salmonella* two targets are in excess. Thereby favoring SE targets instead of the DNA IAC. Nevertheless, it is important to emphasize that performance of the IAC in the presence of low DNA copy numbers or in the observed absence of SE was robust and reliable for each food sample analyzed, an imperative finding for any *Salmonella* detection qPCR assay (Malorny et al., 2007b).

In addition to being both effortless and reproducible, the use of ready to use mixtures, such as the one used in this study, facilitate performance of the assay. Likewise, conventional PCR methods are incapable of producing products with known identity (*i.e.* DNA sequence), subsequently failing to ensure proper specificity of PCR product(s) (Rahn et al., 1992; Malorny B et al., 2003; Malorny et al., 2003). Additionally, we employed TaqMan probes for our qPCR assay which had several advantages over the use of non-specific (although cheaper) SYBR Green I assays, including greater sensitivity and a probe-based sequence-specific verification of PCR product identity (Wittwer et al., 1997; Fey et al., 2004; Jacobsen and Holben, 2007).

In conclusion, we have developed a method that has the potential to be used as an initial screen for pre-enrichment cultures for SE without precluding the USDA culture method which is deemed necessary to yield a physical isolate that is acceptable to the regulatory process. This assay showed a high selectivity, accuracy and detection capacity. In addition, we believe that this assay will reduce the amount of samples, overall time, and effort expended in the laboratory since only positive samples will be further processed after the initial pre-enrichment step. As an added benefit, this is also a quantitative assay which allows for SE quantification in pre-enrichments or other samples. Last but certainly not least, the inclusion of the IAC makes it useful for rapid diagnosis of SE in foods directly. Moreover, in order to be applied extensively, collaborative studies should be conducted to assess the inter-laboratory reproducibility of this assay.

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Salmonella is an extremely diversified genus, infecting a range of hosts, and comprised of two species: enterica and bongori. This group is made up of 2579 serovars, making it versatile and fascinating for researchers drawing their attention towards different properties of this microorganism. Salmonella related diseases are a major problem in developed and developing countries resulting in economic losses, as well as problems of zoonoses and food borne illness. Moreover, the emergence of an ever increasing problem of antimicrobial resistance in salmonella makes it prudent to unveil different mechanisms involved. This book is the outcome of a collaboration between various researchers from all over the world. The recent advancements in the field of salmonella research are compiled and presented.

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