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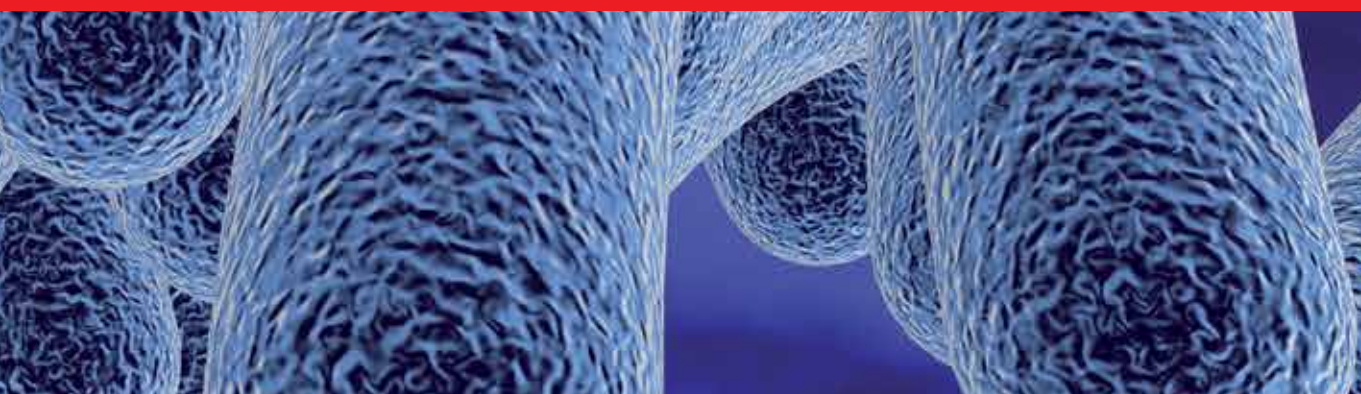
Salmonella

A Re-emerging Pathogen

Edited by Maria Teresa Mascellino



SALMONELLA - A RE-EMERGING PATHOGEN

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Meet the editor

Maria Teresa Mascellino completed her MD degree at the age of 25 in Rome in 1980 and earned her specialization in Clinical Microbiology from Sapienza University of Rome (Italy). She works as an aggregate professor in the Department of Public Health and Infectious Diseases. She has published about 110 papers in reputed journals and has been serving as an editorial board member of repute. She is the editor of the book *Bacterial and Mycotic Infections in Immune-Compromised Hosts* from OMICS Group. She is a reviewer for important scientific journals as well as for international research projects. She has attended many national and international conferences as speaker presenting relevant research topics. She is a member of scientific societies such as ESCMID, ASM, and New York Academy of Science.

Contents

Preface

This book concerns the particular aspects and features of *Salmonella* infections. *Salmonella* once regarded as an endangered bacterium and generally susceptible to the most common antibiotics such as chloramphenicol, tetracycline, and fluoroquinolones has changed its characteristics over time becoming the etiologic agent of many pathological processes other than the classic diseases (typhus, paratyphus, and food-borne infections) and also showing more specific and aggressive resistance mechanisms toward antibiotics. As a matter of fact, its involvement in cancer development, in inflammatory process, and in immune pathogenesis is now well known. For this reason, we state that *Salmonella* may be considered a re-emerging pathogen.

This book specifically deals with the epidemiology and the spread of this bacterium in animals, with the virulence and pathogenesis, the antimicrobial resistance through both mutation and plasmid mediation, as well as the biotechnology and the medical engineering.

Salmonella is widespread all over the world, and millions of human cases are reported every year resulting in thousands of deaths. Salmonellosis is then one of the most common and economically important food-borne zoonotic diseases in humans. The usual reservoir of *Salmonella* is the intestinal tract of the wide range of domestic and wild animals. The study of epidemiology results to be the basis for better understanding all the aspects related to *Salmonella* infection ranging from disease sources, virulence, prevention, host susceptibility and specificity, risk factors, vaccination, etc. The connection between this infection and the immunodeficiency is quite common because salmonellosis may occur as unapparent infection or in acute and fatal diseases in debilitated hosts.

The presence of bacterium flagellum with its multifunctional tasks could be involved in the pathology and virulence of *Salmonella* as well as the production of biofilm that is able to protect the bacteria from the antibiotic action allowing them to survive and to exercise their pathogenicity in the infected individuals.

The host-pathogen interaction that plays a crucial role in *Salmonella* invasion and disease progression is reported in the book in detail. Topics on virulence factors such as the *Salmonella* pathogenicity islands (SPIs) and the genes located on the virulent plasmids have been deeply treated. The plasmid-encoded genes are involved in serum resistance and fimbriae production that determines the formation of surface filamentous structures promoting the adhesion to the small intestine and to Peyer's patches.

The most interesting topic of this book concerns the prevalent involvement of *Salmonella enterica* in both tumor development (such as gallbladder and colon cancers) and conversely its cure by some attenuated strains. These two aspects of the infection result to be crucial in the

management of this microorganism that on one hand, it is able, through the inflammatory process, to induce DNA injury and cellular proliferation then contributing to the tumor growth, and on the other hand on the contrary, it shows through the antitumor innate and adaptive immune response an oncolytic activity.

The antibiotic resistance is another important issue in the management of *Salmonella*, especially the fluoroquinolone resistance has been deeply discussed evaluating the possible correlation to the bacterial fitness and virulence. This can be defined a multifactorial process. The resistance (R) mechanisms are carefully reviewed, and the R level is reported to be increased by the plasmid-mediated quinolone resistance genes, which could horizontally transfer the resistance from strain to strain. The emerging problem of antimicrobial-resistant *Salmonella* in fresh produce is correlated to the human transmission consequently leading to serious consequences for the individual's health. Introducing a program able to act on the produce contamination seems to be appropriate because this practice could be able to avoid or to reduce food-borne pathogens in the farm products.

Lastly, an intriguing topic regarding the medical engineering is worth being taken into account. The use of modified *Salmonella* strains for possible application of a vaccine but mainly for the cancer treatment is intriguing. The described experiments performed in mice for the validity of the engineered microorganisms in the tumor management are very fascinating. The combined associations between antibiotics, radiotherapy, and TNF (a potent antitumor molecule) with modified *Salmonellae* result to be very interesting.

Acknowledgment

First of all, I wish to thank the IntechOpen for offering me the opportunity to write the present monograph dealing with *Salmonella* infections as well as all the authors who with their competence and expertise have contributed to the draft of this book leading to important innovations and up-to-date topics.

But I mainly wish to thank the director of the Department of Infectious Diseases at Sapienza University of Rome, Prof. Vincenzo Vullo, for his continuous support and his valuable suggestions but above all for giving me the possibility to carry on this project.

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Section 1

Morphology and Epidemiology

Chapter 1

Salmonella Flagellum

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Additional information is available at the end of the chapter

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Abstract

Flagella-driven motility contributes to effective bacterial invasion. The bacterial flagellum of *Salmonella enterica* is a rotary motor powered by an electrochemical potential difference of protons across the cytoplasmic membrane. The flagellum is composed of several basal body rings and an axial structure consisting of the rod as a drive shaft, the hook as a universal joint and the filament as a helical propeller. The assembly of the axial structure begins with the rod, followed by the hook and finally the filament. A type III protein export apparatus is located at the flagellar base and transports flagellar axial proteins from the cytoplasm to the distal end of the growing flagellar structure where their assembly occurs. The protein export apparatus coordinates flagellar gene expression with assembly, allowing the hierarchy of flagellar gene expression to exactly parallel the flagellar assembly process. The basal body can accommodate a dozen stator complexes around a rotor ring complex in a load-dependent manner. Each stator unit conducts protons and pushes the rotor. In this book chapter, we will summarize our current understanding of the structure and function of the *Salmonella* flagellum.

Keywords: bacterial flagellum, motility, rotary motor, self-assembly, gene expression, torque generation, type III protein export

1. Introduction

Salmonella is well known as a zoonotic pathogen, which causes gastroenteritis. Motility of *Salmonella* assists in reaching an appropriate site for invasion and enhances the infectivity. The bacterial flagellum is a long filamentous organelle responsible for motility. *Salmonella* swims in liquid environments and moves on solid surfaces by rotating flagella. In addition, the flagella also facilitate bacterial adhesion and biofilm formation. *Salmonella* has several

flagella on the cell surface. Each flagellum consists of tens of thousands of flagellin molecules, allowing host cells to acquire both innate and adaptive immune responses to flagellin. Toll-like receptor 5 recognizes flagellin to activate the host immune system. Thus, the flagellum is also a considerable target to detect bacterial pathogens [1, 2].

The flagellum consists of basal body rings and an axial structure consisting of the rod, the hook, the hook-filament junction, the filament and the filament cap (**Figure 1**). The basal body rings are embedded within the cell membranes and act as a rotary motor powered by the trans-membrane electrochemical gradient of protons, namely proton motive force (PMF). The rod is directly connected to the basal body MS ring and acts as a drive shaft. The filament works as a helical propeller to propel the cell body. The hook exists between the rod and filament and functions as a universal joint to smoothly transmit torque produced by the motor to the filament. A type III protein export apparatus is located at the base of the flagellum to construct the axial structure beyond the cell membranes. A dozen stator units surround the basal body rings. The stator unit acts as a proton channel to couple the proton flow through the channel with torque generation. The flagellar motor regulates the number of active stator units in the

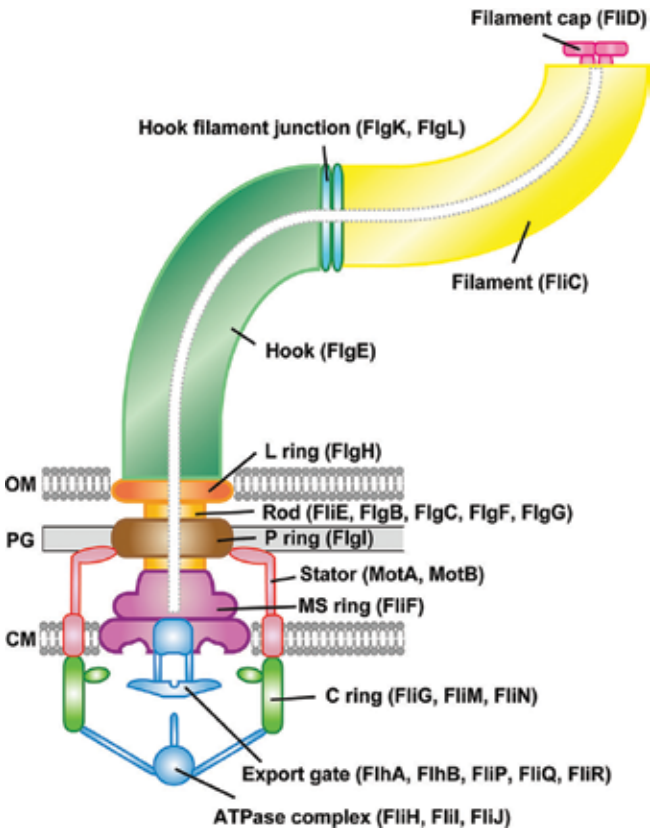

Figure 1. Schematic diagram of the bacterial flagellum. The name of each part and the component protein(s) is shown in black letters. OM: outer membrane, PG: peptidoglycan layer, CM: cytoplasmic membrane.

motor in response to changes in the environment [3–6]. In this chapter, we describe our current understanding of the structure and function of the *Salmonella* flagellum.

2. Structure of the flagellum

2.1. Basal body

The basal body consists of the C ring, the MS ring, the P ring and the L ring and the rod. The C, MS, P and L rings are located in the cytoplasm, the cytoplasmic membrane, peptidoglycan layer and outer membrane, respectively (**Figure 1**). FliF self-assembles into the MS ring in the cytoplasmic membrane [7]. Recently, it has been shown that a C ring protein FliG is required for efficient MS ring formation [8]. FliG, FliM and FliN assemble into the C ring onto the cytoplasmic face of the MS ring (**Figure 2**) [9]. The MS-C ring complex acts as a rotor of the flagellar motor. A stator protein MotA interacts with the C-terminal domain of FliG ($FliG_C$) [10], allowing the motor to spin at the maximum speed of about 300 revolutions per second.

The flagellar motor rotates in both counterclockwise (CCW) and clockwise (CW) directions. The C ring acts as a structural switch to change the direction of flagellar motor rotation [6].

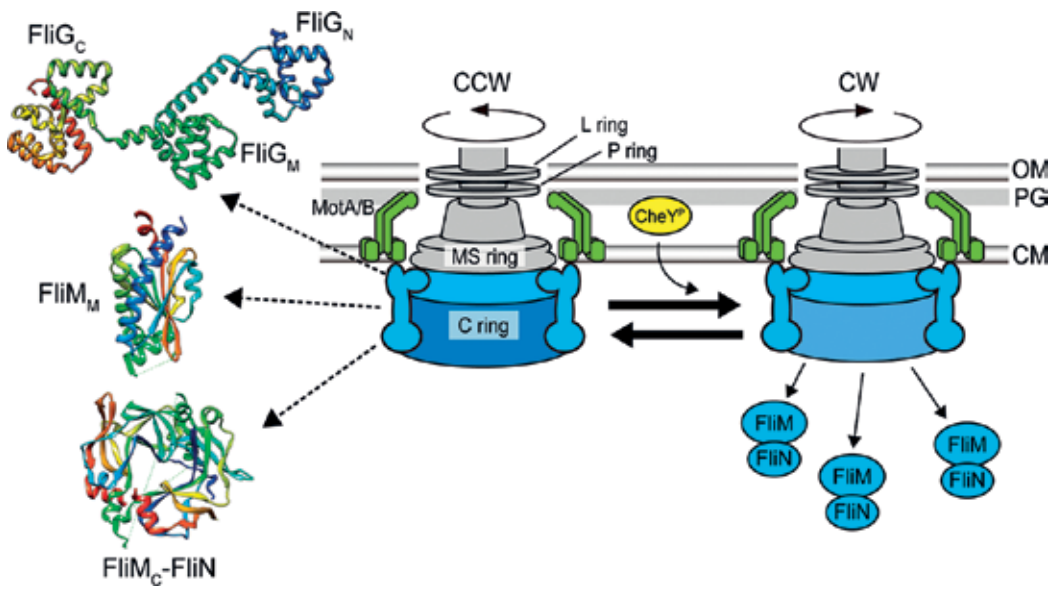

Figure 2. Structure and dynamic of the C ring. The C ring consists of FliG, FliM and FliN. FliG consists of three domains: $FliG_N$, $FliG_M$ and $FliG_C$. Since $FliG_C$ interacts with the stator protein MotA, $FliG_C$ is located at the upper part of the C ring. FliM binds to FliG through an interaction between $FliG_M$ and the middle domain of FliM ($FliM_M$) to form the continuous wall of the C ring. FliN binds to the C-terminal domain of FliM ($FliM_C$) to form the FliM/FliN complex. $FliM_C$ and FliN together form a spiral structure at the bottom of the C ring. The binding of CheY-P switches the direction of motor rotation from counterclockwise to clockwise directions and induces the dissociation of several FliM/FliN complexes from the C ring. Cα ribbon representations of FliG (PDB ID: 3HJL), $FliM_M$ (PDB ID: 2HPN) and the $FliM_C$-FliN fusion (PDB ID: 4YXB) are shown. CM, cytoplasmic membrane; PG, peptidoglycan layer; OM, outer membrane.

Phosphorylated CheY (CheY-P), which acts as a signaling molecule in a signal transduction network responsible for chemotaxis, binds to FliM and FliN, thereby inducing highly cooperative remodeling of the FliG ring structure. As a result, the motor can spin in the CW direction [11]. The FliG/FliN complex binds to the FliG ring through an interaction between $FliG_M$ and FliM to form a continuous wall of the C ring [12]. The CheY-P binding to FliM and FliN also induces the dissociation of several FliM/FliN complexes from the FliG ring, indicating that the C ring is a highly dynamic structure (**Figure 2**) [13].

FlgI assembles into the P ring around the rod. FlgI self-assembles into the L ring on the P ring to form the LP ring complex. Since the LP ring complex acts as a molecular bushing, the friction between the rod and the inner surface of the LP ring is postulated to be very small [5].

The rod is a helical structure consisting of three proximal rod proteins, FlgB, FlgC and FlgF and the distal rod protein FlgG [5]. Recent high-resolution structural analysis of the FlgG polyrod by electron cryomicroscopy and helical image analysis have shown that the FlgG rod is composed of 11 protofilaments [14]. FlgG consists of domains D0, Dc and D1, arranged from the inner to the outer part of the FlgG rod structure (**Figure 3A**). The N- and C-terminal α-helices form a coiled coil in the D0 domain to stabilize the entire rod structure [14]. Residues 46–63 in the Dc domain make the FlgG rod straight and rigid and so the rod can act as a drive shaft [14].

FliE is a basal body protein that interacts with FlgB [15]. Since FliE is the first export substrate to be transported by a type III protein export apparatus [16], FliE is thought to form the junction connecting the MS ring and the rod [15].

2.2. Hook

About 120 subunits of the hook protein FlgE form the hook structure at the tip of the rod. The hook is a short, curved tubular structure made of 11 protofilaments [17]. The hook protein is composed of four domains, D0, Dc, D1 and D2, arranged from the inner to the outer part of the hook structure (**Figure 3B**) [17]. The D0, Dc and D1 domains of FlgE are highly homologous to those of FlgG, thereby allowing the hook to be directly connected to the rod [17]. The axial packing of the subunits in the outer part of the tube made of the D1 and D2 domains is relatively loose [17]. The curvature and twist of the supercoiled structures presumably depend on the direction of intermolecular D2-D2 interactions along the protofilaments in the outermost part of the hook structure [18], and domain Dc plays a critical role in the polymorphic transformation of the supercoiled form of the hook structure [19]. The N- and C-terminal α-helices form a coiled coil in the inner core domain D0 in a way similar to the rod [17].

2.3. Hook-filament junction

FlgK and FlgL together form the hook-filament junction structure at the distal end of the hook structure. When these two proteins are missing, flagellin cannot form the flagellar filament at the hook tip and hence is excreted into the culture media [20]. So, the junction is a buffer structure to connect the hook and filament with distinct mechanical characteristics [21].

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2.4. Filament

S. enterica has two distinct flagellin genes, *fliC* and *fljB*. About 30,000 subunits of flagellin form the filament at the tip of the hook-filament junction zone. The filament is a tubular structure made of 11 protofilaments in a way similar to the rod and hook. Flagellin consists of four domains, D0, D1, D2 and D3 (**Figure 3C**). Domains D0 and D1 form the inner and outer tubes of the concentric double-tubular structure, respectively. Hydrophobic interactions between domains D0 make the filament structure mechanically very stable. Domains D2 and D3 form the outer part of flagellin in the filament [22, 23].

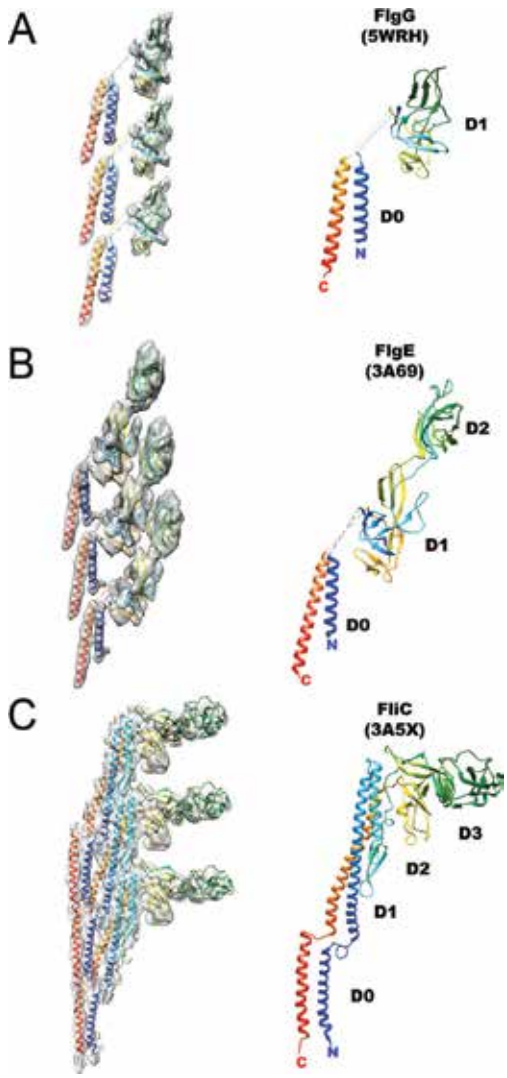

Figure 3. Protofilament structures of the rod, hook and filament. (A) Three subunits of the hook cut out from the EM density map (EMDB-6683) and an atomic model of FlgG (PDB ID: 5WRH) are shown. (B) Three subunits of the hook cut out from the EM density map (EMDB-1647) and a crystal structure of FlgE (PDB ID: 3A69) are shown. (C) Three subunits of the filament cut out from the EM density map (EMDB-1641) and an atomic model of FliC (PDB ID: 3A5X) are shown.

The filament switches between two distinct left- and right-handed supercoiled forms. When each motor spins in CCW direction, several left-handed helical filaments form a flagellar bundle, thereby allowing the cell to smoothly swim in liquid media. Quick reversal of the motor to CW rotation produces a twisting force that transforms the left-handed to the right-handed helical form in a highly cooperative manner. As a result, the flagellar bundle is disrupted and so the cell tumbles and changes the swimming direction [3]. The supercoiled filament forms can be produced by combinations of two distinct conformations and packing interactions of the L- and R-type protofilaments [24]. It has been proposed that conformational change of the β-hairpin in domain D1 is postulated to be responsible for the switching between the L- and R-type filaments [25].

2.5. Filament cap structure

The filament cap is composed of five copies of FliD and exists at the growing end of the filament to facilitate filament assembly [26, 27]. The FliD cap consists of a pentagonal plate domain as a lid and five axially extended leg-like domains [28]. Since there is a symmetry mismatch between the FliD cap with the five-fold rotational symmetry and the helical subunit array of the filament with 11 protofilaments, this symmetry mismatch is postulated to drive filament formation [28].

2.6. Type III protein export apparatus

Component proteins of the axial structure are transported via a type III protein export apparatus into the distal end of the growing flagellar structure [29]. The protein export apparatus has been visualized to be located at the flagellar base by electron cryo-tomography (ECT) and subtomogram averaging (**Figure 4**) [30–32]. The export apparatus is composed of a PMF-driven transmembrane export gate complex made of FlhA, FlhB, FliP, FliQ and FliR, and a cytoplasmic ATPase ring complex consisting of FliH, FliI and FliJ [29]. These proteins are highly homologous to those of the injectisome of pathogenic bacteria, which are involved in direct injection of virulence effector proteins into eukaryotic host cells [33]. Interestingly, the entire architecture of the cytoplasmic ATPase ring complex looks very similar to F-type and A-type rotary ATPases [34–36]. In addition, FlgN, FliS and FliT act as flagellar type III export chaperons to facilitate the export of their cognate substrates [29].

FliP forms a homo-hexamer [37]. FliO is required for efficient FliP ring formation although it is not essential for flagellar protein export [37]. FliQ and FliR are associated with the FliP ring [37], suggesting that FliP, FliQ and FliR together form a core structure of the export gate complex. FlhA and FlhB bind to the FliO/FliP/FliQ/FliR complex [37]. FlhA is also associated with the MS ring [37]. FlhA forms a homo-nonamer through its C-terminal cytoplasmic domains named $FlhA_C$ [8, 31]. FliO, FliP, FliQ and FliR are required for efficient assembly of nine FlhA subunits into the export gate complex inside the MS ring, suggesting that the assembly of the export gate complex begins with FliP ring formation with the help of FliO, followed by the assembly of FliQ, FliR and FlhB and finally that of FlhA [8, 37].

The cytoplasmic ATPase ring complex is composed of six copies of the FliH homo-dimer, six copies of the FliI ATPase and one copy of FliJ [34–36]. The C-terminal domain of FliH

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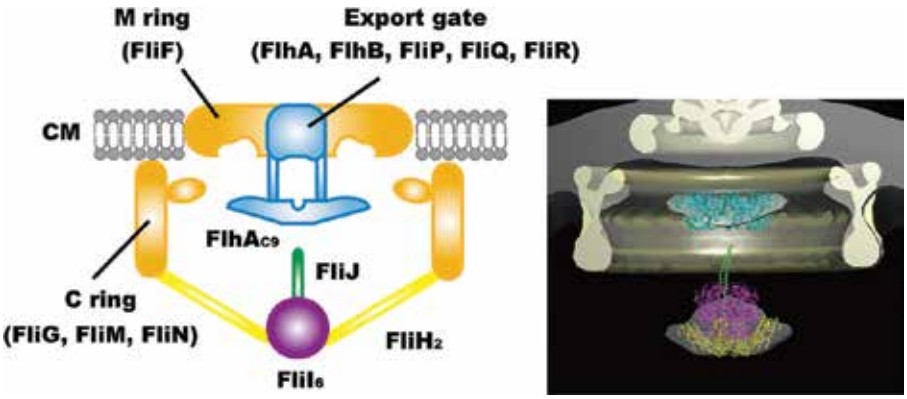

Figure 4. *In situ* structure of the flagellar type III export apparatus. A schematic diagram of cytoplasmic portions of the basal body (left panel). Name of each part of the basal body and component protein(s) are shown. Superposition of a cryoEM density map of isolated basal body on and docking of the atomic models of the $FlhA_{C9}$ ring and the $FliH_{12}$-$FliI_6$-FliJ ATPase ring complex and into the density map of *in situ* basal body (right panel). Cα ribbon representations of $FlhA_C$ (PDB ID: 3A5I), the $FliH_2FliI$ complex (PDB ID: 5B0O) and FliJ (PDB ID: 3AJW) are shown.

($FliH_C$) binds to the N-terminal domain of FliI ($FliI_N$) [38, 39]. FliJ binds to the center of the FliI homo-hexamer [35]. Interactions of the N-terminal domain of FliH ($FliH_N$) with FliN and FlhA anchor the ATPase ring complex to the flagellar base [40–42]. FliH and FliI also exist as the $FliH_2FliI$ complex in the cytoplasm [38]. The $FliH_2FliI$ complex binds to export substrates in complex with flagellar export chaperones [43, 44] and efficiently brings export substrates and chaperone-substrate complexes from the cytoplasm to the export gate complex [45].

FlgN, FliS and FliT are flagellar export chaperones specific for FlgK and FlgL, FliC and FliD, respectively [29]. They bind to the type III export apparatus proteins and facilitate docking and subsequent unfolding of their cognate substrates at the docking platform made of nine copies of $FlhA_C$ [46–48]. FlgN, FliS and FliT adopt a highly α-helical structure and undergo their helical rearrangements coupled with the association with and dissociation from their binding partners during protein export [49–51].

The flagellar type III protein export apparatus utilizes ATP hydrolysis by the FliI ATPase and PMF across the cytoplasmic membrane to drive flagellar protein export [52, 53]. The transmembrane export gate complex acts as a proton/protein antiporter to couple the proton flow through the proton channel of the export gate complex with protein export [54, 55]. FlhA forms part of a proton channel in the export gate complex [56]. ATP hydrolysis by the cytoplasmic ATPase ring complex is postulated to activate the export gate complex to drive flagellar protein export in a PMF-dependent manner [57].

2.7. Stator complex

The stator complex of the flagellar motor is composed of four copies of MotA and two copies of MotB [58]. The $MotA_4MotB_2$ complex acts as a proton channel to couple the proton flow with torque generation. MotA consists of four transmembrane helices, two short periplasmic loops and two extensive cytoplasmic regions. MotB consists of an N-terminal cytoplasmic

region, a single transmembrane helix and the C-terminal periplasmic domain termed $MotB_C$. The transmembrane helix of MotB forms a proton channel along with the transmembrane helices 3 and 4 of MotA [59]. A highly conserved aspartic acid residue, Asp-33 of MotB, which is located near the cytoplasmic end of its transmembrane helix, is involved in proton translocation [60]. $MotB_C$ binds to the peptidoglycan layer, allowing the $MotA_4MotB_2$ complex to act as an active stator unit in the flagellar motor [61]. The flagellar motor can accommodate a dozen $MotA_4MotB_2$ complexes around the MS-C rotor ring complex [62]. The $MotA_4MotB_2$ complexes alternate between localized and freely diffusing forms in response to changes in the environment such as PMF and external load [63, 64]. This indicates that a dozen $MotA_4MotB_2$ complexes do not permanently bind to the peptidoglycan layer.

3. Flagellar gene expression and assembly

3.1. Flagellar assembly

Flagellar assembly proceeds from more proximal structures to more distal ones [65]. FliF and FliG together assemble into the MS ring in the cytoplasmic membrane. During MS ring formation, FlhA, FlhB, FliP, FliQ and FliR together assemble into the transmembrane export gate complex with the help of FliO. Then, the FliM/FliN complex binds to FliG to form the C ring on the cytoplasmic face of the MS ring, followed by the assembly of the $FliH_{12}$-$FliI_6$-FliJ ring complex through interactions of $FliH_N$ with FliN and FlhA. Upon completion of the type III protein export apparatus at the flagellar base, FliE is translocated across the cytoplasmic membrane by the protein export apparatus and assembles at the periplasmic surface of the MS ring. Then, FlgB, FlgC, FlgF and FlgG assemble in this order to form the rod. Then, the LP ring complex forms around the rod. Upon completion of the basal body, FlgD forms the hook cap at the rod tip to support the assembly of FlgE into the hook structure. When the hook reaches its mature length of about 55 nm in *Salmonella*, the hook cap is replaced by FlgK. FlgK and FlgL self-assemble at the hook tip in this order to form the junction structure. Then, FliD forms the filament cap at the tip of the junction to promote the assembly of FliC into the filament that grows up to 15 μm long.

3.2. Flagellar gene expression

More than 70 genes are required for flagellar formation and function in *Salmonella*, and are organized into a transcriptional hierarchy of three promoter classes [66]. At the top of the hierarchy is the *flhD* master operon (class 1) which encodes two genes *flhD* and *flhC* that are required for the expression of class 2 and 3 operons. FlhD and FlhC together form the $FlhD_4FlhC_2$ complex to act as a transcriptional activator that drives the transcription from class 2 promoters. The class 2 genes encode proteins required for the structure and assembly of the hook-basal body (HBB). Also present in this class are the *fliA* gene whose product acts as a flagellum-specific sigma factor (σ^{28}) necessary for the transcription from class 3 promoters, and the *flgM* gene, of which product acts as an anti-sigma factor to inhibit the σ^{28} activity of FliA during HBB assembly. The class 3 operons contain genes required for flagellar filament formation, motility and chemosensory signal transduction [66].

3.3. Coordinating flagellar gene expression with assembly

The hierarchy of flagellar gene expression exactly parallels the flagellar assembly process [66]. The flagellar type III protein export apparatus couples the activation of class 3 genes with flagellar filament assembly. During HBB assembly, FlgM binds to FliA in the cytoplasm and prevents FliA from acting as σ^{28} to drive the transcription from the class 3 promoters [67]. Upon completion of HBB assembly, the protein export apparatus switches its export specificity from the hook protein FlgE to those required for filament formation, thereby terminating hook assembly and initiating the secretion of FlgM from the cytoplasm to the culture media. As a result, σ^{28} can transcribe the class 3 genes [68].

At least, two flagellar proteins, namely FlhB and FliK, are involved in export specificity switching of the flagellar type III protein export apparatus [69, 70]. The C-terminal cytoplasmic domain of FlhB ($FlhB_C$) acts as an export switch to switch substrate specificity of the protein export apparatus from FlgE to FlgM [71]. FliK is secreted via the protein export apparatus into the culture media during hook assembly [72] and acts an infrequent molecular ruler to determine the hook length of about 55 nm in *Salmonella* [73]. The N-terminal region of FliK ($FliK_N$) has the molecular ruler function [73] whereas the C-terminal domain of FliK ($FliK_C$) is responsible for the interaction with $FlhB_C$ to catalyze the export specificity switch [74].

4. Load-dependent energy coupling mechanism of flagellar motor rotation

The flagellar motor regulates the number of active stator units around a rotor ring complex in response to changes in external load [64]. $MotB_C$ acts as a structural switch to drive the assembly-disassembly cycle of the $MotA_4B_2$ complex in response to the load change [75]. A highly conserved Asp33 residue of MotB is involved in the load-dependent proton translocation mechanism of the $MotA_4B_2$ complex [76]. Highly conserved Arg90 and Glu98 residues in the cytoplasmic loop between transmembrane helices 2 and 3 of MotA ($MotA_C$) interact with highly conserved Asp289 and Arg281 residues in $FliG_C$, respectively [10]. It has been shown that the M76V, Y83H, A145E and E155K mutations in $MotA_C$ considerably affect load-dependent assembly and disassembly dynamics of the $MotA_4B_2$ complex. These suggest that the $MotA_4B_2$ complex itself acts as a load sensor and that $MotA_C$ acts as a load sensor that can detect changes in external load to regulate not only the number of active stator units in a motor but also its proton channel activity [77].

A plug segment consisting of residues 53 to 66 in $MotB_C$ suppresses undesirable proton leakage through a proton channel of the $MotA_4B_2$ complex prior to stator assembly into a motor [78]. Since the $MotA_C$-FliG interaction is also responsible for efficient assembly of the $MotA_4B_2$ complex around the rotor ring complex [79, 80], it has been proposed that this interaction induces the detachment of the plug segments from the proton channels, allowing $MotB_C$ to bind to the peptidoglycan layer. As a result, the $MotA_4B_2$ complex becomes an active stator unit to couple the proton flow with torque generation.

5. Conclusion

The flagellar type III protein export apparatus ensures the well-ordered export of flagellar proteins, thereby coupling flagellar gene expression with assembly. The export apparatus utilizes the energy derived from ATP hydrolysis by the FliI ATPase and PMF to efficiently couple the proton influx through the proton channel of the export gate complex with protein translocation into the central channel of the growing structure. But it remains unknown how the export apparatus coordinates flagellar protein export with assembly and how flagellar proteins are unfolded and transported by the export apparatus in a PMF-dependent manner. We are to look into these processes in much more detail to fully understand these intricate mechanisms.

The $MotA_4B_2$ complex is a load-sensor to regulate the number of active stators in a motor in response to external load change. To clarify the load-dependent energy coupling mechanism of the flagellar motor, we need to investigate more precise measurements of flagellar motor dynamics by biophysical techniques combined with genetic and biochemical approaches.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 2

Salmonellosis in Animals

Serpil Kahya Demirbilek

Additional information is available at the end of the chapter

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Abstract

Salmonella has long been recognized as an important zoonotic pathogen of economic importance in animals and humans. The prevalent reservoir of *Salmonella* is the intestinal tract of a wide range of domestic and wild animals which may conclude in a diversity of foodstuffs of both animal and plant origin becoming infected with faecal organisms either directly or indirectly. In spite of mounting concerns about other pathogens in recent years, *Salmonella* remains among the leading causes of food-borne disease throughout the world. Lots of both domestic and wild animals are infected by *Salmonella* spp., mostly harboring the bacteria in their gastrointestinal tracts with no obvious signs of illness. Therefore, *Salmonella* are usually present in faeces excreted by healthy animals and many times pollute raw foods of animal origin through faecal contact during production and slaughter. The organism may also be transmitted through direct contact with infected animals or humans or faecal contaminated environments. Infected food handlers may also act as a source of contamination for foodstuffs. Because of increasing antibiotic resistance of organism and companion animals, animals are important source of *Salmonella* infection for human. The organism can be monitored and precautions should be taken regularly by new technological methods.

Keywords: salmonellosis, animals, zoonosis

1. Introduction

Salmonella enterica subspecies *enterica* can be separated into more than 2400 antigenically different serovars and the pathogenicity of most of these serovars is unspecified. The greater number of incidents of salmonellosis in humans and domestic animals originated from relatively few serovars and these can be separated into three groups on the basis of host prevalence. Host-specific serovars are the first group. These typically result in systemic disease in a small number of phylogenetically connected species. For example, *S. enterica* serovar

Abortus ovis, serovar Paratyphi and serovar Pullorum are almost exclusively associated with systemic disease in sheep, fowl and humans, respectively. Host-restricted strains are the second group. These are mainly connected with one or two closely related host species but may also unusually result with disease in other hosts. For instance, *S. enterica* serovar Choleraesuis and serovar Dublin are generally associated with severe systemic disease in pigs and ruminants, respectively [1]. Nevertheless, these serovars are possibly efficient of infecting other animal species and humans. The third group comprises of the extensive *S. enterica* serovars, such as Infantis and Enteritidis that usually induce gastroenteritis to a large extent of unrelated host species. Obviously the nature and rigidity of *Salmonella* infections in different animal species varies hugely and is affected by many factors including the *Salmonella* serovar, dose, age, strain virulence, host animal species, immune status of the host and the geographical region [2].

Salmonella enterica subsp. *enterica* remains a main cause of infection and disease in human and animals worldwide. Much of the public health and economic problem originated from diseases or infected animals carriage. In Europe, animal salmonellosis as a cause of human infection became increasingly important as agricultural production started to intensify after World War II. In the 1950s, the rapid intensification of the poultry industry in numerous countries was supported by importation of dried fish meal from South America which comprised many *Salmonella* serovars. So, non-typhoidal salmonellosis is one of the leading causes of acute bacterial gastroenteritis in the USA, responsible for an estimated 1.4 million cases of illness annually. Widespread commercial distribution of contaminated foods can sometimes involve huge numbers of consumers in *Salmonella* outbreaks. For example, a 1994 S. Enteritidis outbreak associated with ice cream in the USA affected 224,000 people. *Salmonella* outbreaks can particularly have severe consequences for highly vulnerable populations in facilities such as day care centres and nursing homes [3, 4].

Although the genus *Salmonella* consists of more than 2400 serovars, most human cases of salmonellosis in the USA are caused by 5–8 serovars. United States (US) Centers for Disease Control and Prevention (CDC) reported that approximately 60% of human cases were caused by *Salmonella enterica* ser Enteritidis (24.7%), S. ser Typhimurium (23.5%), *S* ser Newport (6.2%) and *S* ser Heidelberg (5.1%). These same four serovars represented 46.4% of the isolates from nonhuman sources that year. Also serotypes are changing with time, for example, CDC reported that many of *Salmonella* serotypes decreased in incidence compared with 2012, infections caused by serotype 4, [5],12;I:- continued to rise [5].

Salmonella ser Enteritidis infections are mostly seen with fresh shell eggs and egg products, in which the bacteria contaminate the interior essences of the egg through transovarial infection. *Salmonella* ser Enteritidis infects the ova or oviduct of the hen's reproductive tract, which causes contamination of the albumen, vitelline membrane and possibly the yolk. Internal contamination of the egg's content performs egg-sanitizing practices, which focus on decreasing pathogen contamination on the eggshell surface, ineffective.

Salmonella Typhimurium definitive phage type DT104 appeared in the early 1990s as the dominant type of *Salmonella* spp. Most isolates have chromosomally encoded resistance to

five antimicrobials, specifically sulfonamides, chloramphenicol, ampicillin, streptomycin and tetracycline (R-type ACSSuT). There is sign that some penta-resistant DT104 strains are also evolving resistance to quinolones and trimethoprim [6]. Evidence in Europe indicates that the emergence of DT104 in cattle was the harbinger to its spread to other animals used for food production [2].

Although DT104 is currently the dominant penta-resistant clone of *S* ser Typhimurium, many other phage types (DT29, DT204, DT193 and DT204C) of this serovar have also be seen with multi-drug resistance. Understanding the causes that influence the emergence of these prevalent serovars of *Salmonella* spp. and the factors leading to the distribution and persistence of *Salmonella* spp. in animals is beneficial for the occurrence of effective intervention strategies to decrease human exposure to *salmonellae* [7].

Forms of livestock production and movement are varying as the world is changing. Advanced wages in the West conclude in increased production and importation of poultry meat and processed products from countries in South America and Asia. An improved standard of living in many countries is attended by increased meat ingestion, chiefly pork and poultry but also beef and dairy yields. Regulation of meat production in many countries is improving but there are presently large problems of antibiotic resistance which is enhancing a global problem. Poor control and hygiene conclude in the transmission of many microorganisms of which *Salmonella* is just one. Other changes connected with increasing living standards in world contain the increasing importance of companion animals in people's lives which are adequately recognized as sources of infection. Correlated to global changes in trade and human populations, improvements in technology have allowed us to obtain an unprecedented understanding of the biology of *Salmonella* [7].

However, many aspects of *Salmonella* biology and infection biology remain tantalizingly unresolved after the last 10 years of research, and more than 50 years after Professor Buxton's book [8] acted, such that the *Salmonella* should stay the centre of worldwide investigation activity for many more years. In many details the study of this organism is now a global project. Shrinking investigation budgets in the West have been changed with increasing concern in those countries with increasing budgets and where a value of the animal and public health *Salmonella* problem is increasing [7].

2. Infection in animals

Salmonella infections occur in lizards, snakes and turtles (including tortoises), in birds such as parrots, canaries, finches and pigeons and in mammals such as dogs and cats. They are less common in small caged animals. In dogs, cats and reptiles, infection may be unapparent and *salmonellae* can be found in the faeces of normal animals. These organisms can live happily in the intestine of some animals. They are called carrier animals. *Salmonella* infections most often cause enteritis and diarrhoea. The bacteria can also invade the body to cause septicaemia. This invasion results in fever that commonly accompanies the enteritis caused

by *Salmonella* infection. Affected animals are lethargic, do not eat and have diarrhoea. The diarrhoea is often not distinguishable from that caused by other microbes. The diarrhoea may be profuse and normally house-trained dogs and cats may become incontinent and foul the house unintentionally. In birds, the illness can be less apparent and may only be seen as pasting of the vent.

Very young, old or immunosuppressed animals or birds may be severely affected by the dehydration accompanying the diarrhoea, develop septicaemia or even die. Survivors may have diarrhoea for a time, but most go on to recover completely. Any recovering animal may be a carrier for a varying length of time. The organism can live in the gut lining in small numbers and within local lymph nodes, particularly in the lymphoid areas such as the caecum of birds. Persistence inside the animal can lead to reappearance of infection if the animal develops a different disease [9].

3. *Salmonella* infections in the domestic fowl

Four diseases induced by *Salmonella* are significant in poultry; pullorum disease caused by *Salmonella enterica* serovar Pullorum, fowl typhoid (FT) caused by *S*. Gallinarum, paratyphoid caused by several serovars and subspecies of *Salmonella* most particularly *S*. Typhimurium, *S*. Enteritidis, S. Infantis to name a few and arizonosis caused by *S. enterica* subsp. *arizonae* [7]. The poultry's specific *S. enterica* serovars Gallinarum and Pullorum have mostly been eradicated from the industries of Europe and North America. Nevertheless, in parts of the world with less developed industries, and especially in systems with poor biosecurity, these serovars still represent larger threats to bird health and welfare. Even though chickens are the normal hosts of *S. enterica* serovars Gallinarum and Pullorum, natural outbreaks induced by these serovars have been explained in turkeys, guinea fowl and other several species. There are many sources of infection in poultry containing vertical transmission, contaminated feed and the environment. Asymptomatic excreting of *Salmonella* from the intestine causes the contamination of eggs concluding in vertical transmission. As soon as after hatching, oral intake by the chicks results in very high numbers of *Salmonella* in the gut and great shedding in the faeces. This causes rapid horizontal spread around the hatchery [2].

Domestic fowl compose one of the largest reservoirs of *Salmonella* and is significant as a risk to public health through consumption of polluted eggs and meat. Arizonosis caused by *S. enterica* subsp. *arizonae* is an egg-transmitted infection mainly of young turkey poultries that still happens sporadically in commercial flocks and which may as well infect and unusually induce disease in chickens or other species of birds. Reptiles can be a reservoir of *S. arizonae* for birds and for man. The bacteria to place in the ovary and oviduct of breeder turkeys and the poults hatched from infected breeders develop disease. The disease is described by diarrhoea with pasting of faeces in the vent, huddling near the heat source, anorexia and boosted mortality sometimes accessing 50% [10].

4. *Salmonella* infection in poultry

Poultry products are frequently identified as important sources of *salmonellae* that cause human illness. An estimated 182,060 Americans became infected with S. Enteritidis during 2000 after consuming contaminated eggs [11]. Approximately 80,010 of S. Enteritidis outbreaks occurring in the USA between 1985 and 1999 with an identified food source were attributed to eggs [12]. Eating contaminated chicken has also been identified as a significant risk factor for S. Enteritidis infection [13]. Illustrating the importance of poultry as a reservoir for the transmission of *salmonellae* to humans, many of the serotypes that are most prevalent in humans (such as S. Typhimurium and S. Enteritidis) are also found common in poultry [4].

The ability of *Salmonella* to cause disease in poultry is closely related to the infecting serovar and the age and genetic background of the bird. Fowl typhoid (FT) is a disease caused by *S. enterica* serovar Gallinarum that is usually transmitted by the oro-faecal route and mainly affects adult birds [2]. The first described outbreak of FT was characterized by high mortality, especially during the first 2 months of the outbreak [7]. The pullorum disease (PD) is caused by *S. enterica* serovar Pullorum, is egg transmitted and occurs primarily in the first few days of life, high numbers of dead-in-shell chicks are seen (white bacillary diarrhoea). The ability of serovars other than Gallinarum and Pullorum to cause disease is relatively poorly understood [2].

Poultry may be infected with a wide variety of *Salmonella* serovars with the infection largely confined to the gastrointestinal tract with faecal excretion [7]. *S. enterica* serovar Typhimurium is primarily known for producing clinical salmonellosis in very young birds. Mortality rates vary enormously, from less than 10% to more than 80% in severe outbreaks. Resistance to infection develops rapidly over the first 72 hours of life and has been attributed to maturation of macrophages and the development of a commensal flora in the gut leading to competitive exclusion of *Salmonella* [7]. Strains of *S. enterica* serovar Enteritidis are also highly virulent for young chicks [14]. *S. enterica* serovar Enteritidis, and in particular strains of phage type 4 (PT4) can also cause asymptomatic and chronic infections in older birds including commercial layers and broiler breeders [15–17]. Epidemiological data demonstrate a clear association between food poisoning caused by serovar Enteritidis PT4 and the consumption of undercooked eggs [18]. The extent to which egg contamination occurs before or after egg formation is unclear [2].

Many *S. enterica* serovars have been associated with food poisoning in humans, however the potential for such serovars to infect poultry has been little studied in controlled experiments. A chick isolate of *S. enterica* serovar Kedougou colonized the gut, but did not intrude on the mucosa of tentatively infected day old chicks [19]. Likewise, strains of serovars Heidelberg, Senftenberg, Infantis, Montevideo and Menston all expeditiously colonized the intestines of youth birds, but were less invasive than a strain of serovar Typhimurium [20]. Lately, the virulence of various different serovars of *Salmonella* was evaluated in day old specific pathogen-free chicks. The host-specific serovar Pullorum affirmed to be the most virulent, pursued by the omnipresent serovars Typhimurium and Enteritidis. Three out of the four strains of serovar Heidelberg made low levels of mortality, whereas birds infected with isolates of Kentucky, Hadar and Montevideo all lived. Nevertheless, these latter serovars all colonized

the intestines expeditiously and caused a reduction in body weight, showing that subclinical *Salmonella* infections can even be harmful to bird health, welfare and productivity [21]. The reasons why such serovars are clearly much less virulent in chicks, yet retain the ability to induce human food poisoning are not seen [2].

5. *Salmonella* infections in cattle

Salmonella infections are an important cause of mortality and morbidity in cattle and subclinically infected cattle are frequently found. Cattle thus constitute an important reservoir for human infections. There have been numerous reviews over the years [22] increasingly reporting about multi-drug resistant strains [23] as well as the importance of *Salmonella* for the food industry. Interestingly, despite decades of research into salmonellosis, the disease and its public health consequences are not really resolved [7]. Salmonellosis occurs worldwide in cattle and has been associated primarily with serovars Dublin and Typhimurium. Other serovars are sporadically associated with bovine infections [2]. During the period 1968–1974, Sojka *et al*. [1] recorded the isolation of 101 different *Salmonella* serovars, usually at a low prevalence, detected annually in cattle [7]. Salmonellosis reached a peak in the British cattle industry in the 1960s with over 4000 incidents in 1969 [1, 2]. In the USA, 48% of the 730 *Salmonella*, other than S. Dublin and S. Typhimurium, isolated from cattle were represented by 7 serovars [24]. In the UK, in 2009, there was 10 *Salmonella* reports of non-GB origin reported from cattle, these included *S*. Typhimurium DT104, *S*. Mbandaka, *S*. Anatum and *S*. Dublin, clearly showing that importation of new strains remains a constant risk [7].

In the recent times, there has been a sharp reduction in the number of *Salmonella* outbreaks and over the last 5 years there have been only 400–500 cases annually, with similar numbers of events caused by *S. enterica* serovar Typhimurium and serovar Dublin in adult cattle and calves. *S. enterica* serovar Dublin and serovar Typhimurium are endemic in northern Europe, despite the divisions of these serovars vary. The origin of most outbreaks of salmonellosis in cattle is possibly faecal to oral contact. Infected cattle may excrete up to 108 CFU *Salmonella*/g of faeces and pollution of the environment in the nearness of other animals is a potent source of infection. Subclinical discharge of *Salmonella* aggravates the problem of pollution. Cattle that discharge an active *Salmonella* infection but show no clinical symptoms (often convalescing animals) are known as "active carriers". These may spread *Salmonella* constantly in quantity greater than 105 cfu/g of faeces and thus can be determined by routine bacteriological examination. Active carriage is commonly the sequel to clinical enteritis or systemic infection, and infected animals may excrete *Salmonella* for years or as well for life. "Passive carriers" are immunized animals that swallow *Salmonella* with feed and subsequently pass them in their faeces with no active infection of the intestines. Hence, when eliminated from a dirty environment these animals will stop excreting *Salmonella*. "Latent carriers", *Salmonella* remains subclinically in the tissues but is just randomly excreted in faeces [2]. Excretion may be initiated by stress, for example, at parturition. Understanding the biology of this true "carrier state" is likely to be key to ultimately controlling this important pathogen in cattle and may also provide insight into, for example, the asymptomatic carriage of *S. enterica* serovar Typhi by humans [7].

The spread of *S. enterica* serovar Dublin to reproductive tissues is not well understood and may originate either from a systemic infection or possibly from faecal contamination of the vagina. Adult survivors of *S. enterica* serovar Dublin infections often become latent carriers, a state which may last for life. The outcome of infection with other serovars seldom results in the latent carrier state although active excretion may continue for years. The reasons for this remain unclear [2].

6. *Salmonella* infections in pigs

The organism now known as *Salmonella enterica* serovar Choleraesuis was first isolated from pigs by [25], when they considered it to be the cause of swine fever (hog cholera). The ability of *Salmonella* to cause disease in pigs depends on numerous factors including the infecting serovar and the age of the pig. Regional variation in salmonellosis incidence is loosely correlated to pig density, husbandry practices and co-mingling of pigs [7]. The serovars of *Salmonella* associated with clinical disease in pigs can be divided into two groups: the host-restricted serovars typified by *S.* Choleraesuis and the ubiquitous serovars typified by S. Typhimurium. Then the existence of *S*. Choleraesuis has diminished dramatically and it is now only isolated sporadically. In contrast, this serovar stays a major threat to the pig industry in the USA. The fall of serovar Choleraesuis in the UK was not linked with any specific intervention measure. It was later understood that a diversity of antigenically distinct *S. enterica* serovars can be isolated from pigs, some of which are of zoonotic as they transferred through the food chain and farm environment to humans, where they typically cause acute but self-limiting gastroenteritis [8]. *S*. Typhimurium is the most usual serovar isolated from pigs both in Europe and in the USA. Likewise, S. Derby has a strong linked with pigs on both sides of the Atlantic Ocean, and for the past 20 years it has been the second most predominant serovar in pigs in the UK. Oral ingestion is thought to be an important route of infection as *Salmonella* are shed in high numbers in the faeces of clinically infected pigs.

Consistent results are only received applying a lower portion if the gastric pH is first neutralized with antacids [26]. This showed that the low pH of the stomach is a productive barrier to infection by *Salmonella*. Aspiration of infected material into the upper respiratory tract is another possible route of infection. Pneumonia is a general feature of S. Choleraesuis infections in pigs [27] and several works have shown that pigs can be experimentally infected by intranasal inoculation. Pigs infected with S. Choleraesuis via the intranasal route improve more severe clinical signals than those infected via the oral route [28]. Together these observations indicate that the tonsils and lungs are likely to be significant sites of invasion. Clinical salmonellosis in pigs is standardly of two forms; septicaemia caused by host limited *S. enterica* serovars such as Choleraesuis, and enterocolitis originated by broad host limit serovars such as Enteritidis. Unsurprisingly, weaned pigs that are intensively reared are most often influenced by *Salmonella* infections. Like other host-specific serovars, S. Choleraesuis has the capacity to induce disease in both young and older animals, whereas S. Typhimurium typically lead to disease in pigs aged between 6 and 12 weeks, but seldom in adult animals. In older animal, subclinical infections with S. Typhimurium are frequent, leading to high transmission rates if active carrier animals are not detected. S. Choleraesuis typically cause septicemic forms of infection. S. Typhimurium typically causes enterocolitis [2].

A year-long work during 2006–2007 determined *Salmonella* in the ileocaecal lymph node of 21.2% of pigs at slaughter in the UK, with S. Typhimurium by far the most dominant serovar. This correlated to a usual across Member States of the European Union of 10.3% [29]. European Community-wide it is estimated that 10–20% of human non-typhoidal salmonellosis may be linked to pigs [30]. In the USA, the most common serovars isolated from pigs during the National Animal Health Monitoring Survey in both 2000 and 2006 were Typhimurium, Derby, Agona, Typhimurium-Copenhagen and Heidelberg, three of which were also in the top five serotypes isolated from humans in the same period [31]. The number of investigation of some other serovars has developed during the last 20 years, but it is not understood whether this is the result of better monitoring or whether it indicates increased disease or environmental prevalence. It is evident that the problem of *Salmonella* in pigs is not limited geographically, and this is valuable considering the range of global trade in pork as personal countries are no longer isolated from world events [7].

7. *Salmonella* infections in sheep

In most countries of the world with a large sheep population, including the UK, Australia, New Zealand and the USA, sheep salmonellosis is apparently rare and does not represent a relevant economic issue. Disease distribution and prevalence of infections due to ubiquitous serovars is typically seasonal and associated with animal movement and shipping [32, 33]. Exposition to prolonged environmental stress, including cold, poor nutrition and concurrent diseases, might be important to activate latent infection and *Salmonella* shedding in faeces [33].

Serovar Abortus ovis strains, being host restricted to ovines, are expected to be introduced into a flock by an infected sheep and transmitted by the faecal-oral route [34]. There is no convincing proof of bacterial spread by water, feed or other host's faeces. Therefore, precaution has to be taken when transferring animals from a flock with history of infection into non-infected ones. Particularly, while many authors have published faecal shedding of culturable infectious bacteria up to 3 months following abortion [35], S. Abortus ovis DNA has also been detected in faeces up to 12 months from abortion [36], suggesting that sheep may be long-term asymptomatic carriers. Experimental infection studies have demonstrated that sheep may become infected by the conjunctival and vaginal routes [34, 35], but their significance in natural transmission has not been evaluated. Due to serovars Dublin, Abortus ovis and others induce pneumonia in young lambs, infection of grazing animals because of the nasal path might also be possible and respiratory secretion may distribute the infection to other individuals. High bacterial load in aborted foetuses and discharged placenta, elimination of bacteria with vaginal emissions following abortion and by scouring lambs are the main source of transmission throughout a flock during the lambing season [36].

Examination of slaughter-age healthy sheep and identification of *Salmonella* species have been often reported in the past few years, due to public health concerns of these serovars entering the human food chain [37]. Ovine salmonellosis might be an important zoonotic reservoir for human infection and a number of studies have reported food-borne transmission to humans [30–40].

8. *Salmonella* infections in horses

By the 1950s, *Salmonella enterica* serovar Abortus equi had disappeared from the USA following widespread use of bacterin and other control measures. The non-host adapted serovar S. Typhimurium was first recognized as a cause of colitis in 1919 [41] and has since dominated globally as a cause of equine salmonellosis. Antibiotic usage in combination with stressors associated with hospitalization has proved to be potent influences in increasing susceptibility of the horse to invasion by *Salmonella* spp. and in selection of resistant strains. Anorexia, antimicrobial administration, intestinal surgery and marked changes in diet increase the susceptibility of horses to *Salmonella* challenge [42].

Salmonella Abortus equi, the cause of equine paratyphoid, is the sole *Salmonella* host adapted for equids. A notable feature of the epidemiology of equine salmonellosis in the USA has been the rise and fall in incidence of infection by specific serovars. This may result in growing of herd immunity and/or reduction of virulence of the specific serovar. The latter may be conducting by the choosing pressure of antibody as herd immunity progresses. Topical spikes in the rate of isolation of particular serovars is often correlated with nosocomial outbreaks in local veterinary hospitals where in there is improved transmission. Control methods including closure of affected facilities will decrease the number of new cases finally providing to disappearance of the epidemic serovar.

The widespread dispersion of *Salmonella* spp. in wild and domestic animals and their environment is an important barrier to the persistence of a *Salmonella*-free horse population on a farm or following admission to a veterinary hospital. The origin of infection is often not understand in the first stages of an outbreak and so primary control efforts must be focused on rigid isolation of clinically problematical animals with diarrhoea or colic or those known to be shedding *Salmonella* spp. control measures on farms differ in some significant considerations from what are needed in a hospital environment [7].

9. *Salmonella* infections in dogs and cats

Carriage of *Salmonella* in dogs and cats may be asymptomatic, with intermittent shedding. Disease occurs intermittently, and ranges from mild to severe gastroenteritis, with occasional occurrence of abortion, systemic spread or septicaemia [43]. Recovered animals may shed *Salmonella* for several weeks, and chronic carriage with periods of recrudescence is possible. The challenges joined with making a diagnosis of bacterial associated diarrhoea in the lack of objective advices for faecal testing and the fact that identical isolation ratio have been found for presumed bacterial entero pathogens in some populations of animals with and without diarrhoea [44]. Both selective and non-selective serovars have potential for zoonotic spread, and may also be important in the emergence of antimicrobial resistance in the bacterial population [45]. Most of the infections were clinically silent, but mild diarrhoea without fever developed in only nine dogs from one kennel. Latest studies have demonstrated dogs eaten raw meat diets can go on to shed the organism in the faeces for a while time. Twenty-eight research dogs were entered to detect the prevalence of *Salmonella* shedding after ingestion of

a *Salmonella*-contaminated commercial raw food diet meal [46]. Cats have also been detected to carry *Salmonella*. Studies of the prevalence of *Salmonella* shedding in normal, asymptomatic cats have identified a prevalence typically of between 0.8 and 2.1% in cats [47, 48]. The epidemiology, prevalence, clinical signs, diagnosis and pathological findings and sources of salmonellosis in 100 cats in Scotland and England during 1955–2007 were reported [49]. Of the 49 isolates, 28 (57%) were from kittens less than 6 months of age. From the point of their function in the transmission of salmonellosis, cats were discovered to be the most abundant ecological section (125 of all samples positive) in a 2-year investigation of the circulation of *Salmonella* on 12 pig production units in the USA [50]. In addition, the presence of cats on the farm was identified as a significant risk factor for outbreaks of clinical salmonellosis on Dutch dairy farms [51]. Tauni and Osterlund [52] reported an outbreak of S. Typhimurium in cats and humans connected with infection in wild birds in Sweden in 1999. A total of 62 ill cats were investigated. Altogether were anorectic and lethargic, 31% had diarrhoea and 57% were vomiting. It was thought similar that salmonellosis was passed on from cats to humans, but there were just a few such cases. These studies indicate that *Salmonella* shedding is comparatively sporadic in cats and that clinical signals such as diarrhoea are not trusted predictors of whether a cat is potently shedding enteric organisms. Nevertheless, when infection does happen, cats may take part in a significant role in the transmission of the organism. That is, the prevalence of *Salmonella* spp. in healthy dogs and cats is very similar to the prevalence in diarrhoeic dogs and cats while the prevalence in stray or kennelled dogs and cats is often higher. The prevalence of *Salmonella* infection in kennelled or stray cats and dogs is often excessive. Most events of salmonellosis in dogs and cats are subclinical. Following contact to *Salmonella*, the organism is usually discharged by the host's immune system. Nevertheless, in a small rate of cases the organism may continue leading to the formation of a transmitter state. A small percentage of cases of human salmonellosis are related to contact with infected dogs and cats.

10. *Salmonella* infections in exotic pets

Reptiles are known to release *Salmonella* frequently [53] and reptile-associated salmonellosis has been recognized as an emerging zoonosis. From the epidemiological point of view [54] and in addition to an earlier recommendation ('Reptile-Associated Salmonellosis', RAS, [55] we suggest to call this particular type of epidemic 'Reptile-Exotic-Pet-Associated Salmonellosis' (REPAS). The primary statement for this proposal is that past several years the approach of trading reptiles has changed substantially and this will likely continue in the future. The particular risk of *Salmonella* dissemination from reptiles to humans is not due to European wild species but, as outcome of this study also demonstrate, at present is mainly due to 'exotic' imported reptile species. Moreover, following new investigations *Salmonella* shedding is higher in reptiles kept in captivity in comparison to wild reptiles [53, 56] and 'pet' reptiles are apparently in closer contact to humans. These arguments justify the inclusion of 'exotic pet' into the term describing the problem. The risk to human health connected with the reptile pet market has been highlighted recently [57] and the exact definition of the problem using REPAS might be significant to contribute the problem in education and support the European Commission to contribute suggestions to harmonize animal welfare and public health [7].

Each year infections are also obtained through direct or indirect animal contact in homes, farm environments, veterinary clinics, zoological gardens, or other public, professional or private settings. Clinically infected animals may propagate a higher prevalence of shedding than seemingly healthy animals, but both can exhibit *Salmonella* over long periods of time. Also, environmental contamination and indirect dissemination through contaminated food and water may complex control efforts. The public health risk varies by mammals, birds and reptile species, age group, husbandry practice and health status [58]. A study from Canada conducted between 1994 and 1996 illustrated the potential problem of reptile-associated salmonellosis for the first time. In 2011, a 13-month-old child from Austria passed away on the transport to the hospital with vomiting and diarrhoea. A multi-state outbreak in the USA in 2008 was associated with pet turtle exposure. In nearly half of the 135 cases, children ≤5 years were affected. This outbreak was the third turtle-associated outbreak since 2006 [59].

11. *Salmonella* detection

Diagnosis is based on the identification of the *Salmonella* either from faeces or from tissues collected aseptically at necropsy, environmental samples or rectal swabs, feedstuffs and food products; prior or current infection of animals by some serovars may as well be detected serologically. If reproductive organs are infected, abortion or conceptus occurs, it is essential to culture vaginal swabs, placenta, foetal stomach contents and embryonated eggs. Organism may be identified using a diversity of techniques that may include pre-enrichment to resuscitate sublethally damaged *salmonellae*, enrichment media that comprise inhibitory substances to inhibit competing organisms, and selective agars to differentiate *salmonellae* from other enterobacteria. Various biochemical, serological and molecular tests can be used to the pure culture to allow for a reliable verification of an isolated strain. Organism has antigens named somatic (O), flagellar (H) and virulence (Vi), which may be identified by special typing sera, and the serovar may be assignated by reference to the antigenic formulae in the Kauffman-White scheme. Many laboratories may require to send isolates to a reference laboratory to ensure the full serological identity and to verify the phage type and genotype of the strain, where suitable [60].

Serological tests should be carried on a statistically representative sample of the population, but results are not at all times signifier of active infection. In the laboratory, the tube agglutination test is the procedure of choice for export and diagnostic plans for samples from all species of farm animals. Enzyme-linked immunosorbent assays are usable for some serovars and may be used for serological diagnosis and observation, especially in pigs and poultry. Vaccination may risk the diagnostic worth of serological tests [60].

Since some of the common serovars such as *S*. Enteritidis and *S*. Infantis not only solely induce human infections but are also important livestock colonizers, the *Salmonella* subclassification needs more discriminative methods than serotyping. During the past 50 years, phage typing gets a very worthful device for epidemiological aims. The scheme for *S*. Typhimurium developed by Felix in 1956 (England) played a big role in many outbreak investigations and the

S. Enteritidis scheme from Ward [61] and Lalko/Laszlo [62] has been invaluable in the investigation of egg- and poultry-associated outbreaks that have been accomplished worldwide from the 1980s till today [7].

In 1929, White developed a typing scheme consisted on this antigenic chancing, which was afterwards changed by Kauffmann. This investigation allowed the separation of *Salmonella* into serovars. In 1934, the first Kauffmann-White scheme comprising 44 serovars was reported by Kauffmann and the *Salmonella* Subcommittee [63].

Phage typing supplies a worthful epidemiological work for greater sub-distinction of different serovars and is of exceptional importance in outbreak research. At the NRC, this method has been accomplished for serovars Enteritidis, Typhimurium and some others. Moreover, molecular techniques such as ribotyping (for *S*. Enteritidis) and pulsed field gel electrophoresis (PFGE) (for S. Typhimurium and others) are utilized to presumed outbreak isolates [7].

Whole of the methods; the gold standard diagnostic method for *Salmonella* is culture.

- Culture.

The culture techniques and media that may result best in a specific diagnostic condition subject to a variety of factors, including the *Salmonella* serovar, type and source of specimens, practice of the microbiologist, animal species of origin, availability of selective enrichment and selective plating media. *Salmonella* determination by bacteriological methods generally requires 5–11 days, and samples with low numbers of *Salmonella* cells, generally seen in subclinically infected chickens, may give false-negative results. The increasing application of external quality assurance programmes has led to larger use of international standard methods, such as ISO 6579:2002; [64] while this has not been validated for faecal and environmental samples and was intended for foodstuffs and feeding stuffs. Latest years a standard method for determination of *Salmonella* from primary animal production has been developed and assessed, and an ISO method (ISO 6579:2002 Annex D) has now been accepted (ISO, 2002). The core of the standard method is pre-enrichment in buffered peptone water, enrichment on modified semi-solid Rappaport-Vassiliadis (MSRV) and isolation on xylose-lysine-deoxycholate (XLD) and an additional plate medium of choice. This method has also been demonstrated to be greatly effective for animal feed and meat products, and is simpler and less costly than the full ISO method [61].

- Immunological and nucleic acid recognition methods.

Numerous alternative *Salmonella* detection methods have not been fully validated for faecal and environmental samples, although progress has been made [65, 66] and are more suited for analysis of human foodstuffs where inhibitors of the PCR reactions are not so problematic even though there is a role for quick methods in test and release of batches of *Salmonella*-free animal feedstuffs. The quick methods are generally more costly than conventional culture, but can be economically convenient for screening materials where a low prevalence of transmission is expected or where materials, such as feedstuffs, are held pending a negative test. An enrichment/IMS method associated with ELISA or PCR can identify most transmission within 24 hours but faecal and environmental samples can be problematic for quick methods. At present none of the quick methods has been proved to be acceptable for direct detection of *Salmonella*

so non-selective or selective enrichment stages are necessary [67]. Standardly, this introduces more actions and operator time in the detection procedure. For DNA-based methods, inhibition of the PCR reaction by components of the test sample substance, particularly in the case of faeces, is problematic and needs appropriate DNA extraction techniques and controls to determine inhibition, which may reduce the sensitivity of the test in some cases [65]. Quick isolation methodologies may also be linked with sophisticated detection systems, such as biosensors [68]. There are many variations and developments in rapid methods for *Salmonella* detection, but none has been shown to satisfactorily replace culture in all circumstances [60].

Salmonella enterica subspecies *enterica* is an interesting pathogen varying in its pathogenesis and virulence in different animal species. Some serovars have a broad host range and typically cause subclinical intestinal infections and/or acute enteritis. In contrast, host-restricted and host-specific serovars have narrower host ranges and associated infections tend to be of the more severe systemic form. By targeting the intestines and/or reproductive tracts of animals, *Salmonella* are disseminated between animals in high numbers concluding in maximum levels of disease and transmission. High costs are met annually by public health services and farming industries in monitoring and trying to control *Salmonella*. Knowledge of the pathogenesis of *Salmonella* infections in divergent animal species would support to discover measures to hinder the spread of these pathogens between animals. The mechanisms of pathogenicity of a *S. enterica* serovar have been mainly studied in rodent models of infection. However, the behaviour of these microorganisms in one particular animal species is not necessarily predictive of its behaviour in another host species. Therefore, the application of modern molecular genetics to strains of defined virulence, together with infection studies in natural target animal species will enable a more comprehensive understanding of the determinants *Salmonella* serovar host-specificity and of the biology of these pathogens in individual animal species.

S. Enteritidis, S. Infantis, S. Typhimurium and lots of serovars are most commonly connected with human illness. Human S. Enteritidis cases are most frequently related with the consumption of contaminated eggs and poultry meat, while S. Typhimurium cases are mostly associated with the consumption of contaminated poultry, pig and bovine meat. In animals, subclinical infections are common. *Salmonella* may easily spread between animals in a herd or flock without detection and animals may become intermittent or persistent carriers. All animal and human perform the below precautions to prevent from companion animals and other food-associated Salmonellosis. Clean and disinfect utensils such as food dishes, feed foods that are more likely to be free from *Salmonella* such as processed foods, for example, those that are tinned, packaged or bagged. If you are buying a pet ensure that it is healthy first, keep dogs away from carrion, animal faeces and prevent them from drinking suspected contaminated water as far as possible, consider any case of diarrhoea as a potential source of infection for other animals, make sure that diarrhoea is treated properly, always disinfect after cleaning up diarrhoea, consider all diarrhoeas in your pet as potentially infective, dispose of diarrhoea safely, wrapped and double polythene bagged into a bin, washed down the lavatory, burned or buried in a safe place after disinfection, disinfect the contaminated area, wash your hands after handling your pet at all times, do not allow infected pets to come into contact with young children, old people or those already ill and keep infected dogs away from food preparation area.

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Section 2

Virulence and Pathogenesis

Chapter 3

Virulence System of *Salmonella* with Special Reference to *Salmonella enterica*

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Additional information is available at the end of the chapter

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Abstract

Virulence system of *Salmonella* is very complex as many genes are involved in contributing the virulence of *Salmonella*. Some of the genes are involved in enhancing the invasion of organism in host defense system; some are playing their role in survival and replication of organism inside the host, while some genes are involved in the production of molecules that produce the clinical symptoms of the disease. Broadly, we can classify virulence genes into two categories: genes that are located on the virulence contributing plasmid like *spvc* gene and genes that are chromosomal in nature like *stn*. On chromosome, virulence genes are located in various clusters, which are known as *Salmonella* pathogenicity islands and till today seventeen pathogenicity islands have been identified. The genes located on these pathogenicity islands produce several effector molecules, which assist in invasion, replication and survival of *Salmonella* inside the host. The role of plasmid is still not very clear, but it is presumed that the genes located on virulence plasmids affect the intracellular growth of *Salmonella* in macrophages. Though lot of research work has been carried out to understand the virulence regulation system of *Salmonella*, still many questions are to be answered to decode the virulence regulation of *Salmonella*.

Keywords: *Salmonella*, virulence, genes, plasmid, *Salmonella* pathogenicity islands

1. Introduction

The genus *Salmonella* was discovered by Daniel Elmer Salmon with his assistant Theobald Smith in 1885. Smith isolated a new species of bacteria from ill pig and named *Salmonella* Choleraesuis. The genus *Salmonella* is Gram-negative, non-spore forming, rod-shaped bacteria

belonging to family Enterobacteriaceae. The size varies 2–5 μm in length from 0.4 to 1.5 μm in diameter. They are facultative anaerobes and show peritrichous motility. These are intracellular pathogen leading to different clinical manifestations in humans and animals [1–3]. According to Kauffmann, white scheme genus *Salmonella* consists of two species: *S. enterica* and *S. bongori*. *Salmonella* enterica is subdivided into six subspecies: (1) *S. enterica* sub sp. *salamae*; (2) *S. enterica* sub sp. *arizonae*; (3a) *S. enterica* sub sp. *diarizonae*, (3b) *S. enterica* sub sp. *houtenae*; (4) *S. enterica* sub sp. *indicia*; (5) *S. bongori* [4]. Most of the *Salmonella* isolates that cause disease in human and animals belong to *S. enterica* subspecies enterica. Alternatively, *S. enterica* strains can be classified on basis of their antigens (O and H) into 67 sero groups and 2557 serovars like *Salmonella typhimurium*, *S. enteritidis*, etc. *Salmonella* causes two types of diseases in human being typhoid fever and non-typhoidal salmonellosis. Typhoid fever is caused by *S. typhi* and *S. paratyphi* clinical manifestations include fever, headache, abdominal pain, and transient diarrhea, which may result in fetal respiratory, hepatic, spleen, or neurological damage. Mortality ranges from 10 to 20% in untreated cases [5, 6]. Non-typhoidal *Salmonella* (NTS) cause diarrheal disease in humans. *S. typhimurium* and *S. enteritidis* are two major serovars contributing non-typhoidal Salmonellosis. Mortality rate due to NTS is as high as 24% in developing countries where *Salmonella* infection is the major cause of childhood diarrhea morbidity and mortality [7]. After the infection host may act as the carrier for a long duration (over 10-week postinfection). These carriers are characterized by symptom-free conditions and can act as reservoirs and hence contribute to the propagation of disease. Antibiotics are used for the treatment of salmonellosis. Commonly used antibiotics are fluoroquinolones, trimethoprim-sulfamethoxazole (TMP-SMZ), ampicillin or expanded-spectrum cephalosporins. Development of multiple drug resistance has become very common phenomena among the isolates which are mainly contributed by dissemination of dominant resistance clone or by dissemination of strains carrying drug-resistant plasmids [8–10]. Therefore, the rational use of antibiotics is very important to overcome the problem of development of multiple drug resistance in *Salmonella* [9, 11].

1.1. Pathogenesis of *Salmonella enterica*

Ingestion of contaminated food or water is the major cause of the disease. After ingestion, once the organism reaches in the stomach to overcome the acidic pH of the stomach. *Salmonella* activates acid tolerance response, which maintains the intracellular pH of *Salmonella.* After entering in the small intestine, organism adheres to intestinal epithelial cells. The adherence of organism with intestinal cells provokes the signaling pathway which results into cytoskeletal rearrangements and disruption of epithelial brush border and leads to the formation of membrane ruffles that engulf adherence bacteria in large vesicles called *Salmonella*-containing vacuoles (SCVs) [12]. Production of several proinflammatory cytokines such as TNF and IL-8 is increased in intestinal cell and initiates recruitment and migration of phagocytes into the intestinal lumen [13]. To overcome lysosomal enzymes of host endocytic pathway, *Salmonella* direct changes in host endocytic trafficking system. *Salmonella* induces the formation of F-actin meshwork around the bacterial vacuoles, which is important for maintenance of the integrity of vacuole membrane. For replication of bacteria, SCV migrates to the peri-nuclear position in close proximity to Golgi apparatus [14]. *Salmonella* induces the formation of long filamentous membrane

structure called as *Salmonella*-induced filaments (SIFs) which may play important role in increasing availability of the nutrient in SCV. Once *Salmonella* invades intestinal epithelium, they are transported by dendritic cells (Antigen presenting cells) through the bloodstream to various organs like the liver, spleen. In these target organs, bacteria replicate more efficiently.

1.2. Virulence genes of *Salmonella*

The genes encoding the virulence factors of *Salmonella* may be divided into two major categories, that is, genes, which are located on chromosomes, (like stn) [15–17] mainly *Salmonella* pathogenicity islands (SPIs) [18] and genes which are located on the virulence plasmid. In *Salmonella* seventeen SPIs (SPI-1 to SPI-17) have been identified which contribute to the virulence of *Salmonella* [19] along with several genes like Spv operon which are located on the plasmid.

1.3. *Salmonella* pathogenicity Islands

Genes located in SPI-1 encode for several proteins, which are involved in the invasion of epithelial cells by mediating cytoskeletal rearrangement. These effector molecules are translocated into the host cells by type III secretion system (T_3SS-1), which is composed of several operons. The prg/org and inv./spa operon encode the effector protein. SPI-2 Island: mainly contribute to replication and survival of bacteria inside the host cell (epithelial cell and macrophages). SPI-2 mainly contains four groups of genes contributing to the virulence of *Salmonella*: ssa, the gene encoding for T_3SS-2; ssr: encoding for regulators; ssg: encoding the chaperones and ssc: encoding the effectors. SPI-3 encodes for proteins, which are involved in both initial attachment and long-term persistence and survival during systemic phase of infection. SPI-4 contains six ORF under the control of single Operon and plays their role during the initial interaction with intestinal epithelium and long-term persistence. SPI-5 is involved in accomplishing several pathogenic proven during infection [18, 20]. Apart from this, other pathogenicity islands have been identified in few serovars of *Salmonella*. These pathogenicity islands also contribute to the virulence of *Salmonella.*

1.3.1. SPI-1

The size of SPI-1 is approximately 40 Kb and the GC content of SPI-1 is significantly lower than the average G + C content of *Salmonella* genome. SPI-1 encodes for a type III secretion system (T_3SS) that mediates the contact-dependent translocation of complex sets of effector proteins into eukaryotic host cells [21]. SPI-1 produces two subsets of effector protein one subset mediates the invasion of non-phagocytic cells by *Salmonella* by modification of active cytoskeleton system of host cell while the second subset is associated with entero-pathogenesis and inflammation of intestinal epithelium cells **(Table 1).** Genes of the SPI-1 show some sequence similarity with *E. coli* and *Shigella*, and this leads to a hypothesis of that SPI-1 is a rather ancient acquisition gained at the separation of the genera *E. coli* and *Salmonella* from the common ancestor [22].

Effector protein	Major function
Sip A	Rearrangement of cytoskeletal system of non-phagocytic cells and recruitment of neutrophils
Sip B	Nucleation of actin protein and translocation of other effector proteins/molecules
Sip C	Translocation of effector molecule
SOP A	Recruitment of immune cells and secretion of fluid in intestinal lumen
SOP C	Recruitment of Neutrophils and secretion of fluid in intestinal lumen
SOP D	Recruitment of Neutrophils and secretion of fluid in intestinal lumen
SOP E and spt P	Rearrangement of cytoskeletal of host cells
Iae P	Post translational modification of effector proteins of type III secretion system
Inv B	Act as chaperone
Avr A	Inhibition of apoptosis in epithelial cell, Inhibition of macrophage pyroptosis
Sic A Sic P	Act as chaperone

Table 1. Major virulence determinants of SP-I of ***Salmonella***.

1.3.2. SPI-2

The size of SPI-2 locus is approximately 40 Kb in size, and it is composed of two different regions. The larger region of approximately 25 Kb which is present only in *S. enterica* is involved in systemic pathogenesis. It encodes for second type three secretion systems of *Salmonella*. Another smaller region of approximately 15 Kb in size was detected in *S. bongori a*nd encodes the tetrathionate reductase (Ttr) involved in anaerobic respiration [23] **(Table 2)** .

1.3.3. SPI-3

The size of SPI-3 locus is approximately 17 Kb and GC content range 47–48%. The major virulence determinants of the SPI-3 locus are Mgt CB (Magnesium transport system), Mis L and Mar T. Mgt CB are required for the adaptation of *Salmonella* in nutritional limitation conditions of the intra-phagosomal habitat. Mis L (anti-transport protein of SPI3) is very similar to the AIDA-1 auto transporter and involved in the process of adhesion to epithelial cells. Mar T (Transcriptional activator of Mis protein) has resemblance with Tax R (Toxin gene regulator) of *Vibrio cholerae* and involved in activation of Mis L auto transport protein [24]. Though there is the high degree of sequential variation in SPI-3 among the various serovars of *Salmonella* but SPI-3 was found to be conserved between *S. typhi* and *S. typhimurium*. Even among the other serovar Mgt CB region of SPI-3 was found to be conserved.

1.3.4. SPI-4

The size of SPI-4 locus is approximately 27 Kb. Though the role of SPI-4 in *Salmonella* virulence is still not very clear, SPI-4 contributes for several putative virulence factors such as putative type I secretion system and Sic E which involve in the process of adhesion to epithelial cells. SPI-4 was found to be conserved among various serovars of *Salmonella* [24, 25].

Virulence determinant	Functions
Ssa B	Disruption of Golgi apparatus and Lysosomes, Inhibition of SCV-lysosome fusion
Ssa E	Acts as chaperone
Ssc A	Acts as chaperone
Ssc F	SCV perinuclear migration, microtubule bundling and SIF formation
Sse G	SCV perinuclear migration and SIF formation
Ttr genes	Tetrathionate respiration and outgrowth in the intestine
SPi C	Disruption of vesicular transport
SIF A	*Salmonella* containing vacuole membrane integrity
$SsPH_2$	Cytoskeleton rearrangements
SrFT	Apoptosis
Ssej	Cytoskeleton rearrangements
Pip B	Targeting to *Salmonella* induced filaments
SOP D_2	Targeting to *Salmonella* induced filaments/late endosomes

Table 2. Major virulent determinants of SPI-2 and its function.

1.3.5. SPI-5

The size of SPI-5 locus is approximately 7.6 Kb. It encodes the effector proteins for both the T_3SS encoded by SPI-1 and SPI-2. It encodes for Pip A and Pip B. Pip A contributes in the development of systemic infection while Pip B is involved in the accumulation of lipid rafts and is a translocated effector of SPI-2 encoded T_3SS under the control of Ssr AB two-component systems [26].

1.3.6. SPI-6

The size of SPI-6 is approximately 59 Kb and it has been identified in *S. typhi* and *S. typhimurium*. SPI-6 contains saf gene coding for fimbriae and pag N gene encoding for invasion protein. Deletion of this region did not affect the systemic pathogenesis but reduced the invasion of bacteria in tissue-cultured cells. SPI-6 was detected in *S. enterica* subspecies I, and some of the portion of SPI-6 that was identified in subspecies III b, IV, and VII.SPI-6 has shown sequential homology with the genome of *P. aeruginosa* and *Y. pestis* [25].

1.3.7. SPI-7

The size of SPI-7 is approximately 133 Kb, and it is specific to *S. typhi*, *S. dublin* and *S. paratyphi*. This region encodes for Vi antigen (capsular exo-polysaccharides) SPI-7 contains pil gene cluster, which encodes for putative virulence factors. The genetic organization of SPI-7 is very complex and composed of several horizontally acquired elements. It contains few genes of conjugative plasmid-like *tra* and *sam.* Though sequential homology with SPI-7 has been reported in few other bacteria like *Xanthomonas axonopodis* and *Pseudomonas aeruginosa* the loss of Vi antigen from the isolates of *S. typhi* suggests the instability of SPI-7 [27].

1.3.8. SPI-8

The size of the SPI-8 locus in 6.8 Kb and it has been identified in *Salmonella typhi*. The genes located in these islands encode for putative virulence factors, but the exact function has not reported so far.

1.3.9. SPI-9

The size of SPI-9 locus is 16,281 bp and it encodes for virulence factors of type I secretion system and RTX like protein.

1.3.10. SPI-10

The size of SPI-10 is 32.8 Kb. SPI-10 contains a cryptic bacteriophage within it. It encodes for several virulence factors which contribute to Sef fimbriae. Sef fimbriae are restricted to few serovars like *S. typhi* and *S. enteritidis*. The role of cryptic bacteriophage is still not clear.

1.3.11. SPI-11 and SPI-12

These SPIs were identified in *Salmonella choleraesuis*. The GC content of SPI-11 is 41.32%. Though the putative proteins encoded by these SPIs contribute to *Salmonella* virulence, yet the exact roles of these proteins are still not very clear.

1.3.12. SPI-13 and SPI-14

These SPIs were identified in *S. gallinarum*. SPI-13 is composed of 18 ORFs, while SPI-14 is composed of 6 ORFs. These SPIs are not present in *S. typhi* and *S. paratyphi* A but reported in *S. enteritidis* and *S. typhimurium*. The mechanism action of proteins encoded by these SPIs is not clear yet.

1.3.13. SPI-15, SPI-16 and SPI-17

These SPIs were identified in *S. typhi* and showed association with t-RNA genes. SPI-16 and SPI-17 encode for the proteins involved in LPS modification. The role of effecter proteins of SPI-15 is still not clear.

Apart from pathogenicity islands of *Salmonella*, few other isolates like *Salmonella* genomic island I which plays a significant role in the multiple drug resistance of *Salmonella*. Moreover, high pathogenicity island (HPI), which has been well characterized in *Yersinia enterocolitica* and *Y. pseudo-tuberculosis*, has been identified in few serovars of *Salmonella*.

2. Plasmids and their role in virulence of *Salmonella*

Plasmids have been found only in few serovars of *Salmonella* belonging to subspecies I. The size of virulent plasmid varied from 50 to 90 Kb and have been called serovar-specific plasmids (Silva, 2017) The virulent plasmid of *Salmonella* are important for bacterial multiplication in the reticulo-endothelial system of the warm-blooded vertebrate. Spv region (7.8 Kb)

is necessary to confer the virulent phenotype of plasmid other regions are involved in other functions such as biosynthesis of fimbriae of the plasmid [28]. The exact role of the virulent plasmid in pathogenesis is unclear. Evidence exists that spv genes enable *S. typhimurium* to infect the liver and spleen by increasing the rate of replication within the host cells. Virulent plasmid affects the intracellular growth in macrophages but not in non-phagocytic cells.

Salmonella virulence plasmids are low copy number, stable, and nonconjugative plasmids. They contain two independent replicons rep B and rep C which function independently. Despite low copy number (1–2 copies), plasmids of *Salmonella* are very stable and Par VP region is responsible for the partition of the plasmid. Some of the plasmids of *Salmonella* contain more or less complete tra operon, whereas others have suffered the major deletions in tra operon. The presence of tra operon suggests that *Salmonella* ancestors acquired the virulence plasmid by conjugation and that divergence has occurred during the evolution of various serovars [29].

2.1. Gene organization on SPV region of plasmid

SPV must be written in uppercase when referring to regions and in lowercase when referring to genes (*spv*). This rule has to be followed through the whole text. Please check). In *Salmonella* subspecies I, SPV region is present on the virulent plasmid but in some other subspecies like II, IIIa, and VII the homologous region is present on the chromosome. The SPV region is composed of five genes *spv* R, A, B, C, D. *spv* R acts as regulator protein and binds to the promoter of *spv* A. Though the expression of SPV R protein is self-regulated, some factors like σ^s (product of rpos gene) and H-Ns protein also play important role in the regulation of spv operon. Expression of rpos is induced after entry of *Salmonella* into macrophages or epithelial cell. Therefore, the expression of spv genes in response to intracellular signal supports the view that the virulent plasmid may play a role in the multiplication of *Salmonella* as an intracellular parasite (Silva, 2017). Gene spvA encodes for 28 kDa protein, which is found on the outer membrane. The function of SPVA is still not clear as the mutation in SPVA does not reduce the virulence of *Salmonella*. SPVB (66Kda) is found in two fractions. The small amount of SPV B is found in the inner membrane while the larger fraction in cytoplasmic. SPV B sequence shows a certain degree of similarity to all toxin of *Vibrio cholerae* (Accessory cholera enterotoxin) which acts as ion transporter across the cell membrane and contribute to diarrhea. SPV B is absolutely essential for virulence of *Salmonella* and mutation in spv B gene resulted in the loss of virulence. SPV C is a cytoplasmic protein of 28 KDa while SPV D (25 kDa) is exported outside the cell. Mutations in *spv* C and *spv* D genes caused the various defect in *Salmonella* virulence [30, 31].

2.2. Plasmid-encoded genes involved in serum resistance and fimbriae

The pef (plasmid-encoded fimbriae locus contains four genes (pef B C D1). In *Salmonella* Typhimurium pef genes carried on multicopy plasmid determine the formation of surface filamentous structures. Pef mediates adhesion to the small intestine. Adhesion mediated by PEF is different from induced by chromosomally encoded by long polar fimbriae (lpf), which promote the adhesion of *Salmonella* to Peyer's patches. Three virulence plasmid genes have been reported to be involved in serum resistance. These are tra T, rck and rsk. Tra T, 27 kDa protein which is encoded by transfer region of plasmid confers weak serum resistance. The exact mechanism of serum resistance contributed by Tra T protein is not clear, but it has been

observed that after continuous passage for 20 generations tra T mediated resistance was lost. In some serovars like *S. enteritidis*, *S. dublin* and *S. choleraesuis*, tra T gene was found to be absent. The rck gene has been detected in *S. typhimurium* and *S. enteritidis*, and it is located near pef genes on the plasmid. The rck gene encodes for 19 kDa protein that is inserted in the outer membrane after the cleavage of the leader sequence and inhibit the polymerization of the C9 protein of complement and contribute to serum resistance. Rck has also been found to be involved in the invasion of epithelial cells. Another gene rsk is a regulatory element able to bind the replication protein, Rep A. It is found to be involved in regulation of integration of plasmid on the chromosome, which not only increases the susceptibility to serum, but also the log time of culture grown in minimal medium (Silva, 2017) .

2.3. Regulation of virulence in *Salmonella*

Virulence system of *Salmonella* is very complex and more than 300 genes have been reported to play their role in contributing the virulence of *Salmonella*. There are 14 regulators including PhoP/PhoQ, Spv R, RpoS, Omp R/Env z, and Hfq are involved in regulation of virulence system of *Salmonella*. *Salmonella* is a facultative, intracellular pathogen, and PhoP/PhoQ is an important sensor for extracellular and intracellular life [32, 33]. Two major events of *Salmonella* virulence host invasion and intracellular proliferation are regulated by genes located in SPI-1 and SPI-2 respectively. Type III secretion system plays a major role in the invasion of the host cell by *Salmonella*. The biological function of T_3SS is the translocation of proteins from bacterial cytoplasm into the host cell, thus, functioning as the molecular syringe. On interaction with the host epithelial cell, T_3SS of SPI-1 triggers and facilitate the invasion of the host cell. The two major structural components of T_3SS are base structure and needle structure in the inner rod that forms the connection between cytoplasm and host cell membrane. Major structural genes of T_3SS of SPI-1 includes prg HIJK, spa MOPORS and inv. ABCDEFGH along with regulatory protein of T_3SS. The assembly of SPI-1, T3SS starts from the base and inner ring structure is assembled by Prg H and Prg K proteins followed by cytoplasmic export machinery, which is composed of Inv A, Inv C, SPo P, SPoQ, SpaR, and SPaS proteins. The outer ring structure is composed of Inv G and Inv H protein, remains connected with inner ring structure, and is stabilized with the aid of regulatory protein Inv J. The needle and inner structure are made up of Prg J and Prg I subunits [34].

T_3SS system secretes many effector proteins through the needle of secretion systems such as SIP ABC and SOP ABCDEP. SoPE and SoP E_2 act as guanine nucleotide exchange factor (GEFs) for small GTPase Cde 42 and Rae. Additional SPI-1 translocated effectors of *Salmonella* affect actin dynamics during the invasion process. SIP A and C bind and stabilize actin dynamics and cause actin rearrangement via their distinct actin binding and actin nucleating domains that result in membrane ruffling. SIP C along with SoP E direct fusion of the exocytic vesicle with plasma membrane for the expanding ruffle or phagocytic cup. SIP D/SoP B of SIP-I also alter the actin cytoskeleton through manipulation of phosphoinositides. This increases the elasticity to facilitate remodeling of plasma membrane associated with *Salmonella* entry. SIP D is also involved in sealing plasma membrane invaginations to form bona fide vacuoles. After invasion, SIP P act as GTPase-activating protein for Cdc42 and Rae1, thereby inactivating these G proteins and returning the cell morphology into normal. SIP P is also involved in

triggering of membrane ruffling. Membrane ruffling is characterized by rearrangement of the cell membrane and cytosol such that the bacteria are surrounded by the host cell and internalized followed by formation of *Salmonella*-containing vacuole (SCV). As the SCV matures, it migrates to basal membrane and *Salmonella* interacts with macrophages associated with Peyer's patches. SoP B manipulates the surface of SCV and assists in inhibition of fusion of SCV with late endosomes [35]. This inhibition helps *Salmonella* to avoid being killed by normal phago-lysosomal processing pathways. The SCVs play an important role in survival and transportation of *Salmonella* within the phagocytic cells during the enteric phase of infection. Once *Salmonella* has formed SCV, the genes of SPI-2 T_3SS system expressed. A number of environmental factors have been associated with induction of these genes through OmpR/Env Z regulatory system. These factors include low osmolarity, low pH and low level of certain nutrients [36] The major function of effector proteins of SPI-T_3SS are disruption of vesicular transport and formation of *Salmonella*-induced filaments (SCF) is still not clear, but they may play their role in intracellular replication of *Salmonella*. To facilitate systemic phase of infection *Salmonella* present in immune cells (macrophages) of the intestine is carried to other organs of the body like liver, spleen, etc. [37]. Dendritic cells are mainly involved in transportation and spread of *Salmonella* in various parts of the body. In dendritic cells, *Salmonella* does not replicate but remain viable. Genes encoded by SPI-2 T_3SS appear to suppress antigen presentation by dendritic cells which limit the immune response by host cells [38]. The metabolic activity of dendritic cell possessing *Salmonella* is significantly reduced and a combination of reduced metabolic activity and immune-suppression contribute the persistence of *Salmonella* in the host cell. Dendritic cells express the antigens of *Salmonella* which further activate T and B cell immune response. Macrophages containing *Salmonella* are transported to Liver and Spleen by reticulo-endothelial where *Salmonella* replicate and multiply more efficiently. In the liver, Kupffer cells are activated by the presence of *Salmonella* and try to neutralize the bacteria with oxidative free radicals, nitric oxides as well as enzymes active in acidic pH. The survived bacteria invade hepatocyte and cause cellular death by apoptosis. The bacteremic phase of the disease is characterized by dissemination of organism in the spleen, bone marrow, and gall bladder where it can replicate and survive for the longer duration.

3. Conclusion

Salmonella is an enteric pathogen who has versatile abilities to invade and survive in host system. It contains more than 300 genes which contribute in various aspects of virulence such as adhesion, invasion, and replication. *Salmonella* has evolved a complex which not only hosts immune system but also coordinates the various genes for providing a suitable environment for invasion and proliferation of *Salmonella*. *Salmonella* pathogenicity islands (SPIs) along with the virulence plasmids play an important role in survival and proliferation of bacteria in host system. SPI-1 along with SPI-4 is involved in primary stage of disease that is adhesion and invasion of intestinal mucosa. SPI-2 is referred for growth and survival of bacteria inside the host cell during systemic phase of disease. SPI-3 and SPI-5 play a dual role in pathogenesis as their protein are involved in invasion and intracellular survival. An extremely complex gene regulation and expression system are involved in various aspects

of virulence like invasion, replication, Quorum sensing, and 14 regulators are involved in regulation of virulence. In spite of large number of regulators reported to influence the virulence gene expression, the role of many regulators and genes is still not very clear. Therefore, further studies are needed to decode and understand the complex and interesting virulence gene system of *Salmonella.*

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Chapter 4

Infection by *Salmonella enterica* Promotes or Demotes Tumor Development

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Additional information is available at the end of the chapter

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Abstract

Cancer is a disease that claims the lives of millions of people every year around the world. To date, multiple risk factors that may contribute to its development have been described. In recent years, a factor that has been associated to cancer development is the presence of bacterial infections that could contribute to its occurrence not only by favoring the inflammatory process, but also through the release of proteins that trigger tumorigenesis. One of the bacterial species that have recently generated interest due to its possible role in cancer development is *Salmonella enterica*. Nevertheless, for more than a decade, attenuated strains of *Salmonella enterica* have been proposed as a treatment for different neoplasms due to its bacterium tropism for the tumor microenvironment, its oncolytic activity and its ability to activate the innate and adaptive immune responses of the host. These two facets of *Salmonella enterica* are addressed in detail in this chapter, allowing us to understand its possible role in cancer development and its well-documented antitumor activity.

Keywords: *Salmonella*, cancer, live-attenuated bacterial vector, tumor selectivity, immunotherapy

1. Introduction

In recent years, cancer has become a worldwide public health problem, and millions of people die of this disease every year in the world [1]. Despite the efforts made to understand the mechanisms involved in carcinogenesis to better develop new therapeutic strategies, the cure

for cancer remains unsolved. Among the causes that have been associated with cancer origin and development, it is found physical and chemical agents as well as biological processes such as inflammation [2], this inflammation has been associated with the presence of infectious biologic agents; these may be viral like human papilloma virus associated to cervical cancer [3], or bacterial like *Helicobacter pylori* in the development of gastric cancer [4], or *Escherichia coli* (*E. coli*) in the development of colon cancer [5]. In this context, *Salmonella enterica* has also been associated with the development of neoplasms that affecting the gastrointestinal tract such as gallbladder cancer [6] and colon cancer [7]. On the other hand, since more than a decade, attenuated strains of *Salmonella enterica* have been evaluated as adjuvants in the treatment of different neoplasms [8], including colon cancer [9] due to its great affinity for tumor tissue [10, 11], its oncolytic activity and the induction of the innate and adaptive immune response against the tumor [12].

The role of *Salmonella enterica* in cancer is a provocative issue to debate, for that reason, in this chapter, we document these two facets of *Salmonella enterica* as a promoter of the development of gastrointestinal tract neoplasms and as a bacterium with antitumor activity and with potential use in cancer treatment.

2. Infection by *Salmonella enterica* and colon cancer

Salmonella enterica genus comprises a wide range of bacteria, including species such as *Salmonella typhi* and *Salmonella paratyphi*, for which natural host is human and *Salmonella typhimurium*, which has mouse as its natural host [13]. The fact that *S. typhimurium* causes the same type of infection in the mouse than in the human has allowed us to understand in great detail the pathogenicity and immunogenicity of these bacteria [14]. Nevertheless, the infection by *Salmonella enterica* has recently begun to be associated with the development of neoplasia of the gastrointestinal tract such as colon cancer [7] and gallbladder cancer [6].

The role of *Salmonella enterica* infection in cancer development is currently under investigation. *Salmonella enterica* capacity to modulate host's inflammatory response [15], contributing to neoplasm development has been documented, showing that chronic inflammation induced by bacterial infection causes DNA damage and increases cell proliferation and migration, factors associated with cancer development [16]. Likewise, it has been suggested that at least two proteins of *Salmonella enterica* could trigger the development of colon cancer; the first one, the typhoid toxin, a cyclomoduline similar to *E. coli* CDT protein [17]; which increases cell survival and is capable of favoring dysbiosis [18], a process known as a risk factor for developing inflammatory bowel disease and colon cancer [19]; the second protein of *Salmonella enterica* is the effector protein AvrA, secreted via the type 3 secretion system [20], and that has been detected in stool samples obtained from patients with colon cancer [21].

AvrA is a multifunctional protein. On the one hand, AvrA is responsible for decreasing the inflammatory response by inhibiting signaling pathways such as the one induced by NF-κB [22] or suppressing the secretion of cytokines such as IL-12, IFN-γ and TNF-α [23] as well as inhibiting IL-6

transcription and increasing IL-20 transcription [24]. On the other hand, AvrA would favor tumor formation in the intestinal epithelium by activating cell proliferation pathways such as Wnt/β catenin pathway [25], associated with colon cancer [26], through two post-translational modifications, β catenin phosphorylation (activation) and deubiquitination of it (decreasing degradation) [7]. Also, AvrA activates Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway [27], which also plays an important role in carcinogenesis because it is involved in apoptosis regulation, cell proliferation and differentiation as well as on the inflammatory response [28]. In addition, AvrA has acetyl transferase activity and one of its targets is p53 [29]; when it is acetylated, it causes cell cycle arrest and apoptosis inhibition by decreasing proapoptotic proteins such as Bax [30]. AvrA mechanisms are summarized in **Figure 1**.

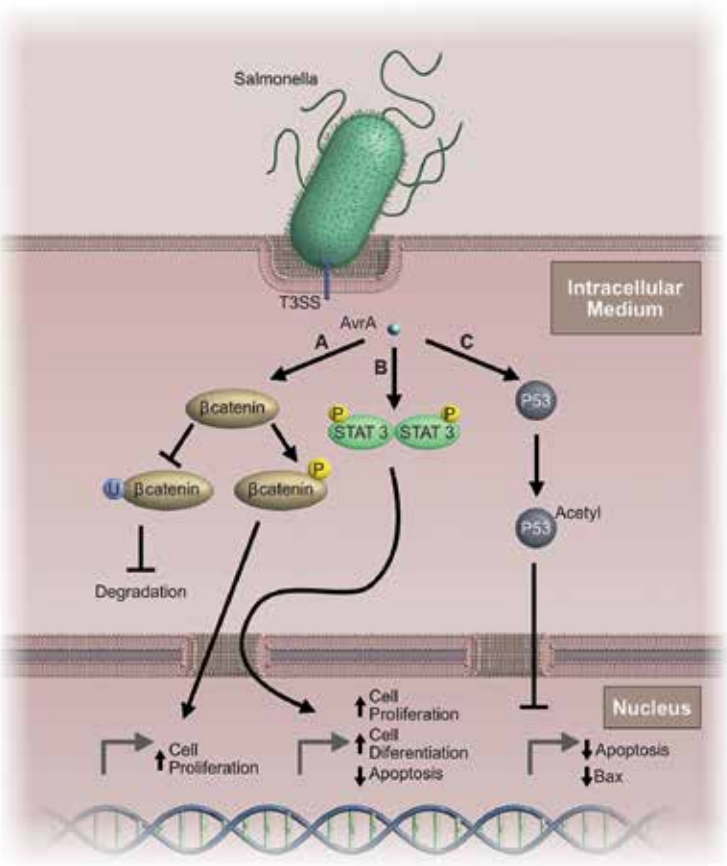

Figure 1. Oncogenic activity of the *Salmonella enterica*. Once AvrA is released and internalized via the type 3 secretion system of *Salmonella enterica*, it exerts its oncogenic effect by modulating the following signaling pathways (1) phosphorylation and deubiquitination of β catenin, promoting cellular proliferation [7, 25], (2) STAT3 phosphorylation, fostering cell proliferation and differentiation as well as decreasing apoptosis [27] and (3) acetylation of the p53 transcription factor that decreases apoptosis by transcriptional downregulation of proapoptotic proteins such as Bax [29].

3. Infection by *Salmonella enterica* and gallbladder cancer

Gallbladder cancer is the main type of neoplasm that affects the bile ducts. Even though the incidences of this neoplasm is low worldwide compared to other types of cancer that affect the gastrointestinal tract, the high incidence in some geographic regions like South America [31, 32] and Southeast Asia [33, 34] have generated a particular interest on studying the causes that contribute to the development of this type of neoplasm on these population.

The main risk factor for developing gallbladder cancer is cholelithiasis, gallstone formation (GSD), which favors the inflammatory process and damage to the epithelium [35]. Likewise, a second risk factor that has begun to be associated with the development of this neoplasia is the infection with *Salmonella enterica* [33], which enters the gallbladder directly from the bloodstream or through the bile [36]. Interestingly, a high incidence of *Salmonella enterica* has been reported in geographic regions where there is a higher number of gallbladder cancer cases [6], and several studies have shown its presence in biopsies of patients with gallbladder cancer [32, 37–39], where different serotypes of *Salmonella enterica* such as *S. typhi*, *S. paratyphi*, *S. typhimurium* and *S. choleraesuis* have been found [37].

To date, there is a little information about how an infection with *Salmonella enterica* would participate in the development of gallbladder cancer. One of the main proposed mechanisms is the induction of chronic inflammation in the gallbladder [40], which is recurring in patients with cholelithiasis [39]. Since *Salmonella enterica* can go unnoticed for years, and it has the ability to form biofilm on gallstones constituted by cholesterol [38]; the inflammation would increase immune cell recruitment, including activated macrophages expressing COX-2 [41], which is an enzyme that plays a role in the development of tumors in the gastrointestinal tract [42, 43]. In addition, the inflammatory process causes alterations in the *TP53* gene, increasing the risk to develop gallbladder cancer [44]. Lastly, in another study, it was shown that infection with *S. typhimurium* in cell lines and gallbladder organoids produces malignant transformations, by activating the MAPK and AKT pathways, which were associated with the development of gallbladder tumors in a murine model [6].

According to the data presented earlier, infection with *Salmonella enterica* could be a factor associated with the development of neoplasms in the gastrointestinal tract, where the chronic inflammatory process induced by the bacteria, as well as some of its effector proteins would be responsible for triggering the tumor process. However, more studies are needed in order to better understand the role of *Salmonella enterica* in carcinogenesis.

4. Antitumor activity of *Salmonella enterica*

Contrary to carcinogenesis induction, infection by bacteria such as *Salmonella enterica* facilitates the elimination of tumor cells [11]. The use of bacteria and their derivatives to treat cancer was first documented by William Coley over a century ago, using "Coley's Toxin," a compound of *Streptococcus pyogenes* and *Serratia marcescens* extract intended for the treatment of patients with sarcoma, carcinoma, lymphoma, melanoma and myeloma [45]. Since 1976,

subsequent studies led to the use of the attenuated strain of *Mycobacterium bovis* (Bacillus de Calmette-Guérin, BCG) administered intravesically as immunotherapy against superficial transitional cell bladder carcinoma [46].

To date, *Salmonella enterica* is one of the most studied bacteria in the fight against cancer [11]. Results of a phase I clinical trial with *S. typhimurium* strain VNP20009 showed that the bacterium does not lead to severe adverse effects and it is well tolerated by patients with metastatic melanoma, metastatic renal carcinoma, carcinoma of the head and neck and esophageal adenocarcinoma [47–49]. The mechanisms implicated in the ability of *Salmonella enterica* to eliminate tumors remain under scrutiny, but its tropism for the tumor microenvironment, its oncolytic activity and its ability to activate the innate and adaptive immune responses of the host have been documented (**Figure 2**).

4.1. Tumor selectivity of *Salmonella enterica*

For over a decade, the use of live-attenuated strains of *Salmonella enterica* as a therapeutic alternative against cancer [8, 11] has been favored by this bacterium's ability to effectively and selectively colonize the tumor microenvironment [8, 12]. Several studies have described

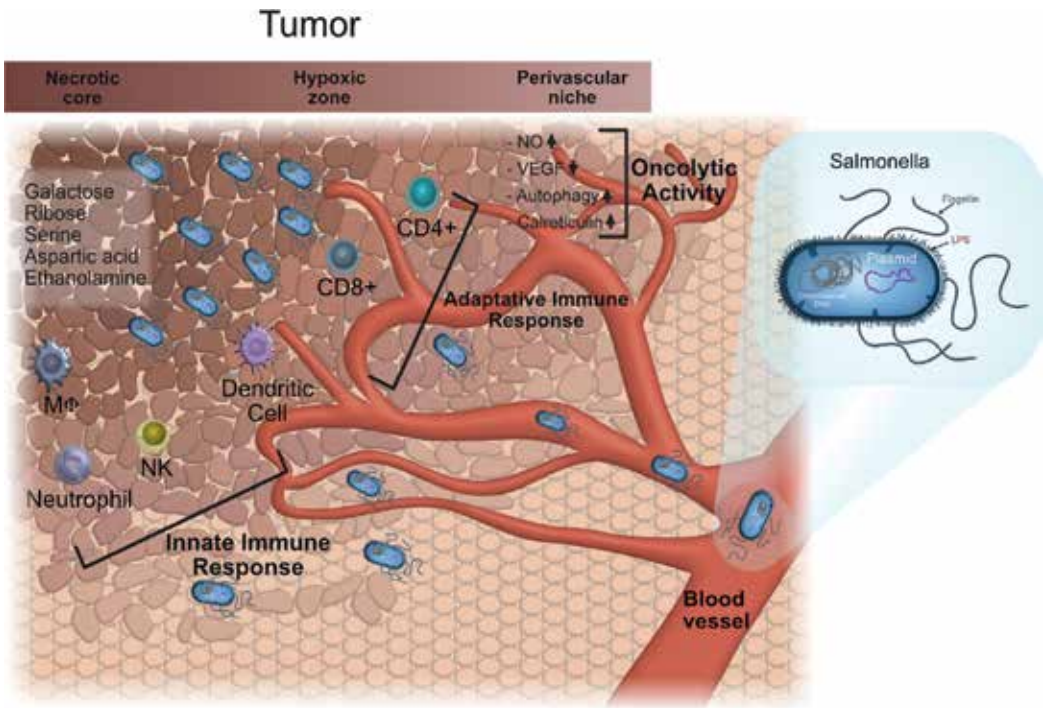

Figure 2. ***Salmonella enterica*** **selectivity for the tumor, oncolytic activity and induction of immune response.** Once *Salmonella enterica* reaches the tumor tissue, attracted by molecules such as aspartate, serine, ribose/galactose [50, 51] and ethanolamine [53], it induces its antitumor oncolytic activity promoting cell death via nitric oxide production [74], decreased angiogenesis [78], autophagy activation [79, 80], activation of immunogenic death [81] and activation of the innate and adaptive antitumor immune responses [74, 87, 89–91].

how *Salmonella enterica* infects and replicates within tumors in murine models in a 1:1000 ratio compared to normal tissue [10]. Although the mechanisms of tumor selectivity are still controversial, *in vitro* studies mimicking the tumor microenvironment have shown that *Salmonella enterica* migrates to the tumor tissue due to attraction by certain molecules such as amino acids and carbohydrates that allow the bacteria to arrive and penetrate the tumor tissue and then direct to the necrotic area [50, 51]. In addition, ethanolamine, a molecule found in elevated concentrations in different types of neoplasia [52], has also been found to act as a chemotactic agent because the deletion of the *eutC* gene (part of the operon encoding the enzyme ethanolamine-ammonia-lyase (EAL) which metabolizes ethanolamine [53]) in *Salmonella enterica*, decreased its colonization in a murine model of breast cancer [54].

Other studies have referred that *Salmonella enterica* migration involves motility proteins such as the CheA/CheY system [50, 51, 55], proteins fliA, fliC and flgE [56] and the *motAB* gene, the flagellar motor of the bacteria [54]. The *Salmonella enterica* metabolic pathways of aromatic amino acids (*aroA*) and purines (*purA*) are also relevant since mutations in these metabolic pathways lead to decreased recruitment in tumor tissue [56, 57].

On the other hand, the microenvironment in the tumor characterized by (1) hypoxia [58], (2) acidity [59] and (3) necrosis contributes to bacterial proliferation [11]. The permanence of *Salmonella enterica* in tumor tissue may be fostered by low macrophage and neutrophil activity [60], suppression of the immune response mediated by cytokines such as TGF-β, and the difficult access of anti-Salmonella antibodies and factors of the complement pathway due to the irregular growth of blood vessels in the tumor [61].

4.2. Oncolytic activity of *Salmonella enterica*

Several studies have documented the antitumor activity of *Salmonella enterica* in murine cancer models, including lung cancer [62], carcinoma of the colon [57, 63], prostate cancer [64], T-cell metastatic lymphoma [65] and B-cell lymphoma [66], among others. In these studies, *Salmonella enterica* inhibited tumor growth and its metastases, while also increasing the lifespan of the mice. These results are consistent with reports in murine models of xenotransplants of breast cancer [67] and prostate cancer [68, 69], using auxotrophic strains of *S. typhimurium* such as the A1 strain (deficient in leucine and arginine synthesis) and the A1-R strain (deficient in leucine and arginine synthesis but with a greater capacity to eliminate tumor cells); these do not cause any injuries in the host because the bacterium has greater affinity for the tumor tissue [67]. Other studies have shown that the A1-R strain inhibits the formation of metastases in bone of murine breast cancer models [70] as well as metastases from osteosarcoma [71], pancreatic cancer [72] and dorsal spinal cord gliomas [73].

Although the mechanisms through which *Salmonella enterica* induces tumor cell death are still under study, some proposed mechanisms involve: (1*) apoptosis induction via nitric oxide (NO) production* [74]: NO, the product of nitrate and nitrite degradation (generated by the hypoxic tumor microenvironment) [75] via *Salmonella enterica* nitrate reductase (NirB) [76], could induce the intrinsic apoptotic pathway [77]. (2) *Decreased angiogenesis: Salmonella enterica* inhibits the expression of the transcription factor HIF-1α and thus, the decrease in vascular endothelial growth factor (VEGF) [78]. (3) *Autophagy activation through the AKT/mTOR pathway:* the presence of *Salmonella enterica* in the tumor decreases phosphorylation of the

proteins AKT and mTOR and increases the expression of Beclin-1 and LC3 (microtubule-associated protein 1A/1B-light chain 3) [79, 80], thus promoting autophagy. (4) *Induction* of *immunogenic cell death (ICD):* this type of cell death could be caused by calreticulin (CRT) [81], a protein in the endoplasmic reticulum, when secreted by the cell participating in ICD [82], which increases due to the presence of *Salmonella enterica* in tumor tissue. Other mechanisms involved in tumor cell elimination and fostered by S*almonella enterica* include the induction of the innate and adaptive immune response, as described later.

4.3. Activation of the innate antitumor response by *Salmonella enterica*

The immune response generated against *Salmonella enterica* once it has entered the host [83, 84] plays an important role in tumor recognition due to the recruitment of immune response cells in the tumor and its metastases [85, 86]. In the tumor microenvironment, *Salmonella enterica* induces the reversal of the suppressor environment by facilitating the expression of soluble mediators such as inducible nitric oxide synthase (iNOS) and interferon-γ (IFN-γ), molecules that promote antitumor activity and inhibit the expression of immunosuppressive factors such as arginase-1, interleukin-4 (IL-4), transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF) [8, 87]; also, *Salmonella enterica* decreases the activity of myeloid-derived suppressor cells (MDSCs) [88] and promotes the recruitment of natural killer (NK) cells [89], neutrophils [74], macrophages [87] and T [90] and B lymphocytes [91]. The first studies describing the immunotherapeutic antitumor properties of *Salmonella enterica* were reported by Kurashige S. et al.; whereby with the use of mini cells (vesicles with no genomic DNA) obtained from *S. typhimurium* and administered to a murine sarcoma model [92] and T-cell lymphoma [93], and macrophage activity was restored in the tumor microenvironment and helped eliminate the tumor.

Some studies have documented the ability of *Salmonella enterica* to induce the activation of the inflammasome during the early stages of bacterial colonization, via type NOD receptors (NLR) [94], favoring interleukin-1β (IL-1β) and TNF-α activation [95], and increasing the levels of proinflammatory cytokines and decreasing those of anti-inflammatory cytokines [86] in the tumor microenvironment. The antitumor efficacy of *Salmonella enterica* is further promoted by the induction of the immune response via TLR-MYD88 signaling, thus establishing that cytokine production and modulation may result from the activation of toll-like receptors (TLRs) in the tumor tissue [96].

It is known that bacterial components of *Salmonella enterica*, such as lipopolysaccharide (LPS), flagellin and the CpG sites are recognized by the TLRs, and lead to activation of the signaling pathways inducing the innate and adaptive immune responses. In this context, the interaction of the LPS from *Salmonella enterica* with TLR4 has been shown to contribute to decreased tumor growth and to the recruitment of neutrophils and macrophages [97]. Likewise, the interaction of *Salmonella enterica* flagellin with TLR5 prevented the development of metastases in a murine melanoma model [98]. These results were consistent with the use of a TLR5 agonist used in a murine lymphoma model in which the antitumor effect was associated to the activation of $CD8^+$ lymphocytes and NK cells [99]. Subsequent studies using TLR4 and TLR5 knockout (KO) mice have confirmed their role in the antitumor response mediated by S*almonella enterica* [100].

The antitumor effects, to which TLRs have been associated, are the recruitment of cells such as macrophages, NK cells, T and B lymphocytes, resulting from increased TNF-α level due to TLR4 activation by LPS [95, 101]. The increased TNF-α would therefore promote bleeding from the blood vessels of the tumor and allow the infiltration by immune response cells [102] that would eliminate the tumor cells. Further, the presence of *Salmonella enterica* in tumor tissue increases the amount of immune response cells in the spleen [81], which subsequently migrate to the tumor and contribute to its eradication.

4.4. Induction of the antitumor adaptive immune response by *Salmonella enterica*

Some studies have described that the adaptive immune response induced against *Salmonella enterica* antigens is one of the mechanisms eliminating tumor cells. Tumor cells infected with *Salmonella enterica* and that present these antigens of the bacteria are eliminated by cytotoxic T lymphocytes; this has been documented in the elimination of solid and non-solid tumors [85, 89].

Salmonella enterica contributes to the reversal of tumor immune tolerance by decreasing the number of Treg lymphocytes (CD4+ CD25+) in tumor tissue [103] due to the effects of LPS and the Braun lipoprotein (Lpp) of *Salmonella enterica* [104], and the decreased levels of indoleamine 2, 3 dioxygenase 1 (IDO1) (enzyme participating in tryptophan metabolism and associated with the development of immune tolerance by T lymphocytes) [105, 106], precluding the formation of kynurenine and thus favoring the proliferation of T lymphocytes capable of recognizing and eliminating the tumor [79]. Aside from reversing immune tolerance and promoting the recruitment of immune response cells in the tumor microenvironment, *Salmonella enterica* also induces the activation and maturation of T lymphocytes [107], probably as a result of the induced overexpression of gap junction proteins such as connexin 43 (Cx43) [108]; this protein plays a role in B and T lymphocyte activation [109] as well as in antigen presentation to DC [110], thus allowing the transfer of tumor cell preprocessed antigens to DC for their adequate presentation by MHC class I [108], thus generating a specific antitumor response.

Studies conducted by Shilling et al. [111] showed that the *in vitro* activation of DC purified from mice, with cytoplasmic fractions of *S. typhimurium* and with heat shock proteins from tumor cells, prevented tumor formation after regrafting. Further, they showed that activated dendritic cells tended to preferentially localize in the tumor. These studies were consistent with the reports published by Avogadri F et al., which observed that the intravenous administration of *Salmonella enterica* favored cross-presentation of tumor antigens to DC, inducing the activation of $CD8^+$ lymphocytes capable of recognizing the tumor [86]. Studies conducted by Grille et al. demonstrated that the administration of *Salmonella enterica* to a murine B-cell lymphoma model induced a local and systemic antitumor response, with the recruitment of $CD8^+$ and $CD4^+$ lymphocytes in the tumor and the presence of specific antibodies directed against the tumor cells [89].

5. Conclusion

The aforementioned data document the duality of the infection caused by *Salmonella enterica*, in which the chronic inflammation promoted by this bacterium induces DNA injury, and some proteins of the bacterium increase cellular proliferation and migration and decrease the

cell death, all these factors are associated with the development of cancer. On the other hand, infection with attenuated strains of *Salmonella enterica* promotes the elimination of tumor cells via intrinsic mechanisms that induce an oncolytic effect on the tumor cell while simultaneously promoting antitumor innate and adaptive immune responses; it appears to be an excellent candidate as a therapeutic alternative against cancer [8].

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Conflict of interest

The authors have no conflict of interest to declare.

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Section 3

Antimicrobial Resistance

Chapter 5

Salmonella and Antimicrobial Resistance in Fresh Produce

Agnes Kilonzo-Nthenge and Winnie Mukuna

Additional information is available at the end of the chapter

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Abstract

Contamination of fresh produce with *Salmonella* may occur during any point from fork to table. It may occur during produce production, harvest, processing, and transportation. Fresh produce has been recognized as a common source for *Salmonella* since the bacteria has the ability to attach and internalize in produce. *Salmonella* has been isolated from produce including mangoes, cantaloupe, cucumbers, alfalfa sprouts, and lettuce. Bacteria from fresh produce include a number of opportunistic human pathogens which may be resistant to several antibiotics. Antimicrobial resistant bacteria may have the potential to make their way over to fresh produce through contaminated irrigation water and manure applied to agricultural fields. *Salmonella* resistant to antibiotics including vancomycin, erythromycin, ampicillin and penicillin has been isolated from vegetables. With the increasing foodborne illness associated with fresh produce, there is a lot of emphasis on good agricultural practices (GAPs) to validate that farms are producing fresh produce in the safest means possible. With proper education and training on GAPs, produce growers will be able limit the occurrence of *Salmonella* and other foodborne pathogens in fresh produce.

Keywords: *Salmonella*, fresh produce, antimicrobial resistance, good agricultural practices

1. Introduction

The demand for fresh produce in the United States is intensifying, in part, due to their nutritional value and consumer health awareness [1]. Studies have shown that consuming more fruits and vegetables can lead to a more productive and healthier lifestyle [2]. Despite the health benefits attained from fresh produce, microbial safety of fresh produce continues to be a major challenge as these foods are consumed raw, and are known for spreading infectious

foodborne diseases [3]. About 48 million people in America get sick, 128,000 are hospitalized, and 3000 die from consuming adulterated fresh fruits and vegetables every year [4]. Fresh produce has been recognized as a common source for *Salmonella* since the bacteria has ability to attach and internalize in produce [5]. Fresh produce can become contaminated anywhere along the farm to plate continuum [6]. According to FDA [7], contamination with pathogenic bacteria may be directly or indirectly through contact with animals or insects, soil, water, dirty equipment, and human handling. Most *Salmonella* infections are caused by poultry products; however, it is estimated that fruit and vegetables are implicated in about 50% of *Salmonella* illnesses [8]. *Salmonella* outbreaks are frequently linked with animal products; however there have been outbreaks related to fresh produce, particularly in the United States [9]. *Salmonella* has been isolated from produce such as mangoes [1], cantaloupe [10], cucumbers [11], alfalfa sprouts and lettuce [12, 13]. The rise of antimicrobial resistance is thought to be as a result of excessive use of antibiotics in agriculture. Most antibiotics are used for treating animal and plant diseases in agriculture [14].

2. *Salmonella* and produce

2.1. *Salmonella* outbreaks

Fresh produce is ever more contributing to the consumer diet, an inclination that has been paralleled by an intensification in foodborne illnesses. Globally, many fresh produce linked outbreaks have occurred over the last few years including *Salmonella* outbreaks. Between 2012 and 2015, there were 596 *Salmonella* outbreaks which resulted in 13,765 illnesses, 2136 hospitalizations, and 18 deaths [11]. Within the same time period, 68 outbreaks were associated with lettuce and these outbreaks caused 1293 illnesses, 136 hospitalizations, and 3 deaths. *Salmonella* Typhimurium and *Escherichia coli* O15:H7 are the most significant foodborne pathogens that cause outbreaks through the consumption of contaminated fresh produce [15]. In October 2015, a multi-state outbreak of *Salmonella* Poona occurred in the United States. The outbreak was associated with 14-day shelf life cucumbers and it resulted to over 150 hospitalizations, 3 deaths and 671 confirmed cases of the outbreak in 34 states [4]. In 2008 and 2011, *Salmonella enterica* serovars outbreaks were linked to imported cantaloupes from Honduras and Guatemala [16]. Another foodborne disease outbreak was caused by *Salmonella* Newport and *Salmonella* Typhimurium. This outbreak was linked to cantaloupes in United States from a cantaloupe production and packaging operation in Indiana [16]. In 2008, a large outbreak of *Salmonella* Saint Paul took place in the United States and was linked to the consumption of jalapeño and serrano peppers [17]. Tomatoes have also been implicated in many *Salmonella* outbreaks [18]. In 2007, fresh herbs retailed in the UK exposed an international outbreak of *Salmonella* infection connected to tainted basil from Israel that involved at least 51 individuals from England, Wales, Scotland, Denmark, the Netherlands and the USA [19]. *Salmonella, E. coli* O157:H7, and *Listeria monocytogenes* have been associated with illnesses linked to produce from USA, Finland and Denmark [20]. Several produce-related outbreaks associated with *Salmonella* have also been global, for example *Salmonella* Saintpaul in fresh peppers from North America, *Salmonella* Senftenberg in tomatoes from Europe and North America, *Salmonella* Weltevreden in alfalfa sprouts from Europe, and *Salmonella* Thompson in arugula from Europe [21].

2.2. Emerging antimicrobial resistant *Salmonella* in fresh produce

Antibiotic resistance has been recognized as a global health problem and as the uppermost health challenges facing the twenty-first century [22]. The emergence of antimicrobial resistant (AMR) bacterial in foods [23] including fresh produce has become a challenge and a major public health concern worldwide. Antimicrobial resistance is responsible for 2 million illnesses and 23,000 deaths yearly in the US, with over $20 billion as direct health-care costs and $35 billion in lost productivity [4]. Previous studies have identified antibiotic resistant bacteria on vegetable products at harvest or at the retail level [14, 24]. AMR is an emerging problem worldwide and antimicrobial usage in animal production is understood to be a contributing factor [25]. Fecal material from food animals, humans, and animals often contain bacteria that are resistant to some antibiotics [26]. It is reported that extensive use of antimicrobials in agriculture expose antimicrobial-resistant bacteria to humans through contaminated food products [27]. It is also documented that antibiotic resistant bacteria has been identified in animal waste, wastewater, river sediments, and farmland soil [28]. Antimicrobial resistant bacteria may be disseminated to the environment through farm waste, and may reach humans through the consumption of contaminated foods of animal origin, water, and vegetables [29]. Leafy greens are contaminated with antibiotic-resistant bacteria from animal and human sources during production and harvesting [30]. Consumption of fresh produce, particularly raw fresh produce, represents a route of direct human exposure to resistant microorganisms.

Salmonella is an important cause of foodborne infections and some species are becoming increasingly resistant, creating it more challenging to treat patients with severe infections [31]. The occurrence of ARM in *Salmonella* has become a major concern in food safety [31]. The contamination of food by *Salmonella* is an international concern due to contamination and antimicrobial resistance rates in imported food products [32, 33]. According to Wadamori et al. [34], *Salmonella* resistant to antibiotics including vancomycin, erythromycin, ampicillin and penicillin has been isolated from vegetables. Most common multidrug resistance phenotype of *Salmonella* is reported to confer resistance to ampicillin and streptomycin [35]. Antibiotic resistant *Salmonella* has also been detected in fresh vegetable at the retail level [36]. Brazilian ready-to-eat (RTE) salad vegetables have been associated with resistant *Salmonella* enterica isolates [37].

Several studies on antimicrobial resistance in animal-producing environments have been conducted [37]. However limited publications are prevailing on whether vegetables or the environment where they are produced has the potential to act as a reservoir of antimicrobial resistance [38]. According to Sjölund-Karlsson [39], several studies on antimicrobial resistance of *Salmonella* from humans, food animals, and retail meats have been conducted, whereas limited research on *Salmonella* associated with fresh produce is less common. It is essential to understand the nature of fresh produce safety challenges, origins of antimicrobial resistant bacteria, contamination pathways, risk factors to the consumer, and approaches to exclude or reduce the occurrence of *Salmonella* and other contaminants. There is a dire need to conduct more research and determine the origins of antimicrobial resistance in fresh produce.

2.3. Educational programs and good agricultural practices (GAPs)

Leafy green vegetables are the highest priority in terms of fresh produce safety from a global perspective [40]. During the period from 1996 to 2006, many countries implicated leafy greens

as a primary vehicle of concern implicated in *Salmonella* foodborne illnesses [40]. The application of GAPs is broadly accepted as the most significant measure in reducing *Salmonella* and other foodborne pathogens in fresh produce. GAPs are important not only for production but also to minimize food safety risks [41]. GAPs are voluntary guiding principles concentrating on best agricultural practices for fresh produce production that validate the production, packing, handling, and storage of produce. GAPs reduce the potential risks of microbial contamination in fresh fruits and vegetables.

With the increasing foodborne illness associated with fresh produce, there is a lot of emphasis on good agricultural practices to verify that farms are producing fruits and vegetables in the safest means possible. GAPs should be used as a control measure in fighting food safety threats within the fresh produce chain, while good hygienic practices (GHP) should be the second important measure for produce growers to use in concurrence with GAP [42]. A fresh produce grower's current food safety knowledge is often shaped by their knowledge of contamination hazards associated with the production of fresh produce, the sources of microbial threat and the impact caused by the hazard [43]. A study by Kilonzo-Nthenge [36] indicated about 64% of the farmers instituted hygiene practices on their farms. From this study, many produce farmers are faced with many challenges in produce production. These include limited knowledge of GAPs and finding food safety denoted information on produce safety. Growers' limited familiarity with GAPs implies a need for food safety education, which trained Extension educators should deliver [36].

Recent outbreaks and changes in consumer demands have prompted the writing of new regulations that establish standards for produce safety. However, these regulations do not cover all produce farms. There is a need to support every farmer in the produce industry. Large growers can fairly easily absorb the costs and annual audit fees associated with GAPs program; however, limited-resource farms often do not pursue these programs due to the costs, which can be exorbitant. Many growers are also not aware on risk factors on their farms and therefore, risk communication is critically needed to persuade produce growers to take appropriate actions and safe practices to avoid and reduce foodborne pathogens farms. Fresh produce farmers need additional education and training on implementing GAPs to prevent the spread of foodborne pathogens. With proper education and training on GAPs, produce growers will be able to shift their focus from responding to contamination to preventing it.

There is a need for cooperative Extension Programs to develop curriculums that can be given to county Extension agents to present to local producers. In addition tailored food safety plans and GAPs should be developed for small-scale fresh produce growers. The new agriculture marketplace require produce growers who are not only aware but also highly knowledgeable in food safety as it relates to their fresh produce.

2.4. *Salmonella* and the farming environment

Several groups of microorganisms can contaminate fruits and vegetables at any point throughout the food supply chain. Fresh produce is contaminated with pathogenic bacteria directly

or indirectly through contact with animals or insects, soil, water, dirty equipment, and human handling. The application of manure and other animal wastes in organic fresh produce production has the potential to contaminate produce with pathogenic bacteria including *Salmonella, Listeria,* and *Escherichia coli* O157:H7. Antibiotic resistant bacteria have been identified in animal waste, wastewater, river sediments, and farmland soil [28]. The challenge arises when these pathogens are antibiotic resistant bacteria. Antimicrobial resistant bacteria may have the potential to make their way over to fresh produce through contaminated irrigation water and manure applied to agricultural fields [44]. Resistant bacteria have the ability to colonize fresh fruits and vegetables in a number of ways including direct use of antibiotics during cultivation, use of contaminated irrigation water; hence a public health issue.

2.5. International food trade and *Salmonella*

The antimicrobial resistance has become a global concern as geographic borders among countries have become less discrete due to increasing global trade. Given that no country is self-sufficient in the supply of food, trade in overall is essential to ensure access to food products. For example, increasing global trade agreements and the demand for fresh produce have led to a significant growth in U.S. produce imports. Increasing global connectivity trade can facilitate the introduction of both antimicrobial resistant and pathogenic bacteria to a country through food imports. A rapidly growing universal trade in agricultural food products has significantly enabled the introduction of new *Salmonella* serovars within the geographical boundaries of importing countries. Imports allow a continuing and abundant supply of fresh produce in the U.S., however antimicrobial resistant bacteria may diffuse to the country as a result of contaminated produce from other countries. In a previous report, *Salmonella* (3.48 and 0.58%) was positive for imported and U.S. grown produce, respectively [45]. Evidence shows that fresh produce trade has the potential to disseminate antibiotic resistant bacteria between countries; a noble example is the 2005 nationwide outbreak of multidrug resistant *Salmonella* Typhimurium DT104B in Finland which was due to contaminated lettuce imported from Spain [46]. *Salmonella* has been isolated from various types of fresh produce including cantaloupe, cilantro, cucumber, leafy green, pepper, and tomatoes from Honduras [47].

Antimicrobial resistant bacteria may have the potential to make their way over to fresh produce through contaminated irrigation water and manure applied to agricultural fields. The utmost threat to the consumer is when vegetables and fruits are consumed without being washed. Practicing good agricultural practices (GAPs) on farms and good handling practices on farms and homes is often recommended to elude *Salmonella* in fresh produce.

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Chapter 6

Fluoroquinolone Resistance in *Salmonella*: Mechanisms, Fitness, and Virulence

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Additional information is available at the end of the chapter

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Abstract

Fluoroquinolones are highly effective broad-spectrum antibiotics usually used for the treatment of human and animal infections, including salmonellosis. Fluoroquinolones act against *Salmonella* by inhibiting their DNA replication. However, several zoonotic serotypes of *Salmonella* have developed resistance or are less susceptible to fluoroquinolones. *Salmonella* presents its resistance by substituting amino acids within the topoisomerase subunits, overexpression of multidrug efflux pumps, or decreasing the expression of outer membrane porins. The resistance level is further increased with the plasmid-mediated quinolone resistance genes which could horizontally transfer the resistance from strain to strain. The development of resistance in *Salmonella* shows that it is a multifactorial process and the acquisition of fluoroquinolone resistance might have significant influences on the bacterial fitness and virulence. Due to the high level resistance against fluoroquinolones that has been observed in *Salmonella*, care needs to be taken to avoid misuse and overuse of this important class of antibiotics to minimize the occurrence and dissemination of resistance.

Keywords: fluoroquinolone, *Salmonella*, resistance, mechanism, fitness, virulence

1. Introduction

Zoonotic *Salmonella* infections are common causes of foodborne human infections worldwide [1]. Typhoid fever and gastroenteritis are the two main subtypes of salmonellosis. Typhoid fever, caused by *Salmonella* Typhi and Paratyphi, is a generalized infection and is fatal in about 10% of cases. The symptoms are usually very severe and show serious sequel. On the other hand, gastroenteritis is a localized infection of the gut leading to diarrhea, fever, nausea, and

headaches and usually caused by all other zoonotic *Salmonella* serotypes [1, 2]. Antimicrobial therapy is indicated in case of generalized infection with life-threatening situation. Presently, fluoroquinolones are the drug of choice for having the high level of clinical efficacy against most of the enteric pathogens including *Salmonella* [3, 4]. Probably, both human and veterinary uses have significantly contributed to the emergence of *Salmonella* strains with reduced susceptibility to fluoroquinolones [5–7]. In this chapter, the updates on the development and mechanisms of fluoroquinolone resistance in *Salmonella* and also the fitness and virulence changes after acquiring resistance are introduced.

2. Resistance

2.1. Mechanism of resistance

The genetic basis of fluoroquinolone resistance in *Salmonella* is the mutations in DNA gyrase (topoisomerase II) and topoisomerase IV, which are the intracellular targets of this class of antibiotics (**Figure 1**) [4, 8, 9]. Other mechanisms which contribute to the resistance of *Salmonella* to fluoroquinolone are overactivation of multidrug efflux pumps and decreased outer membrane permeability [10, 11]. In some clinical isolates of *Salmonella*, plasmid-mediated quinolone resistance (PMQR) genes also confer low-level quinolone resistance (**Figure 1**). Thus, the development of fluoroquinolone resistance in *Salmonella* is an endpoint result of the accumulation of several biochemical mechanisms [12].

2.1.1. Target mutations in DNA gyrase and topoisomerase IV

The quinolone resistance in *Salmonella* was firstly attributed to point mutations in the *gyrA* gene coding for the subunit A of DNA gyrase. In *Salmonella*, a single-point mutation in the quinolone resistance-determining regions (QRDRs) of the *gyrA* gene, which have been clustered in a region of the protein between amino acids 67 and 106 [4], could mediate resistance to nalidixic acid and decrease susceptibility to ciprofloxacin [13]; however, for higher-level resistance to fluoroquinolones, the bacteria must attain additional mechanisms [14].

The most prevalent amino acid changes in nalidixic acid-resistant strains are Ser-83 (to Leu, Thr, Phe, Tyr, or Ala) and Asp-87 (to Gly, Lys, Asn, or Tyr) [6, 15–23]. In high-level resistant clinical *S. enterica* serovar Typhimurium isolates (e.g., MIC of ciprofloxacin, 32 μg/mL), double mutations at both residues 83 and 87 have been commonly observed [24]. Other than Ser-83 and Asp-87 amino acid substitution mutations at GyrA, *Salmonella* strains also have mutations at Ala-67 (to Pro), Gly-81 (to Ser, Asp, Cys, or His), and Leu-98 (to Val) (**Figure 2A**) [16, 18, 25]. Previously, Eaves et al. identified the mutations at Ala131 and Glu133 which are outside of the QRDR [26] which may have different types of mechanisms conferring resistance. Different serotypes may have different mutation positions in the *gyrA* gene. As reported by Giraud et al., the substituting amino acids at Ser83 and Asp87 were not equally distributed among different serotypes, and mutation in Asp87 prevailed in serovars Hadar and Kottbus and mutation at Ser83 were more prevalent in serovars Newport, Virchow, and Typhimurium

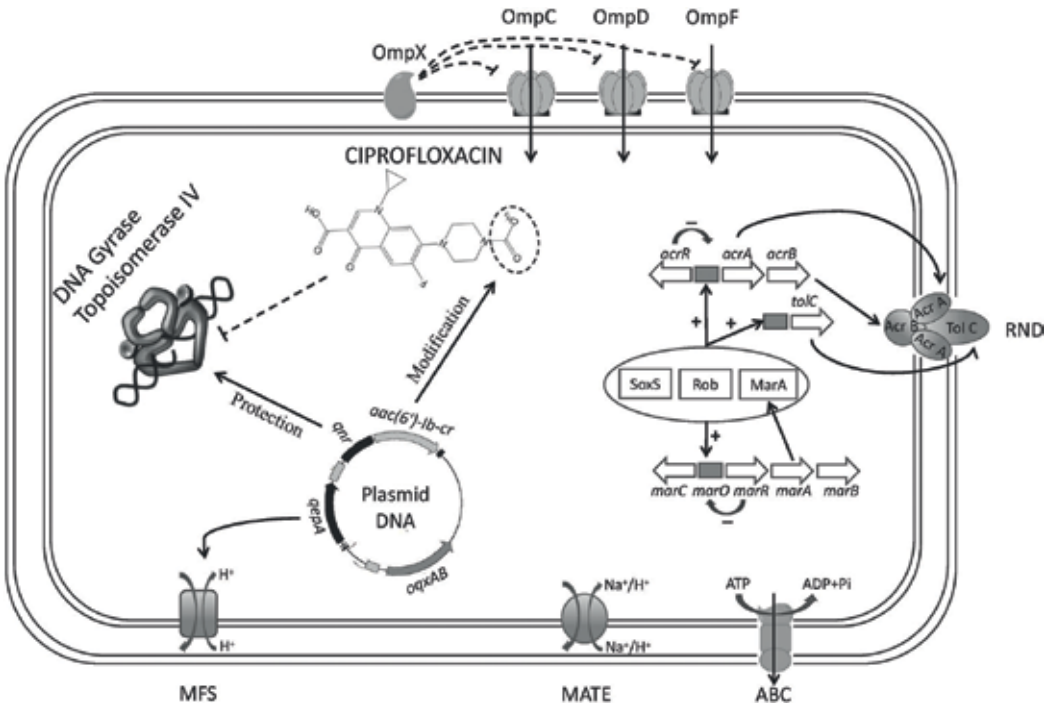

Figure 1. Mechanisms of quinolone resistance. Chromosomal mutations within the QRDRs of the genes encoding the subunits A and B of DNA gyrase and topoisomerase IV structurally change the target protein, reducing its drug-binding affinity. Chromosomal mutations lead to reduced outer membrane permeability and also increased expression of efflux pumps. Plasmid-encoded quinolone-resistant genes can produce Qnr target protection proteins and AAC(6′)-Ib-cr acetyltransferase variants capable of modifying certain quinolones or QepA and OqxAB efflux pumps that actively extrude quinolones. The global regulatory proteins MarA, SoxS, and Rob are primarily responsible for activation of *acrAB* and *tolC* transcription.

[16]. These findings were further supported by the results documented by Allen and Poppe, who reported that all the *S*. Bredeney strains tested have a Ser83Tyr substitution, while all the *S*. Senftenberg strains tested have a mutation of Asp87Gly [27]. The acquisition of mutations in *gyrA* may play an important part in the dissemination of *Salmonella* of particular serotypes [28]. The source of the strains may also be the cause of differences in the distribution of mutations. Lindstedt et al. reported that the *S*. Hadar strains from Southeast Asia harbored mutation at Ser83, while *S*. Hadar strains from Southern Europe and North Africa have mutations at Asp87. They further explained that the differences might be due to the exposure of sublethal concentrations of quinolones in East Asia and Europe/North Africa [29]. In human strains of *S*. Typhimurium DT104 [22, 30] and farm animal isolates of *S*. Hadar and *S*. Montevideo [31], Asp87Asn was the most frequently detected mutation site, while Asp87Gly as the most common mutation in their panel of veterinary *Salmonella*, as reported by Piddock et al. [21]. In contradiction to these findings, the study of Griggs et al. documented that mutation at Ser83 is very common in veterinary isolates of *S*. Newport strains [18]. Strains having different substitutions at codons 83 and/or 87 and some other additional resistance mechanisms always show different susceptibility levels to quinolones. It might be due to the fact that sometimes

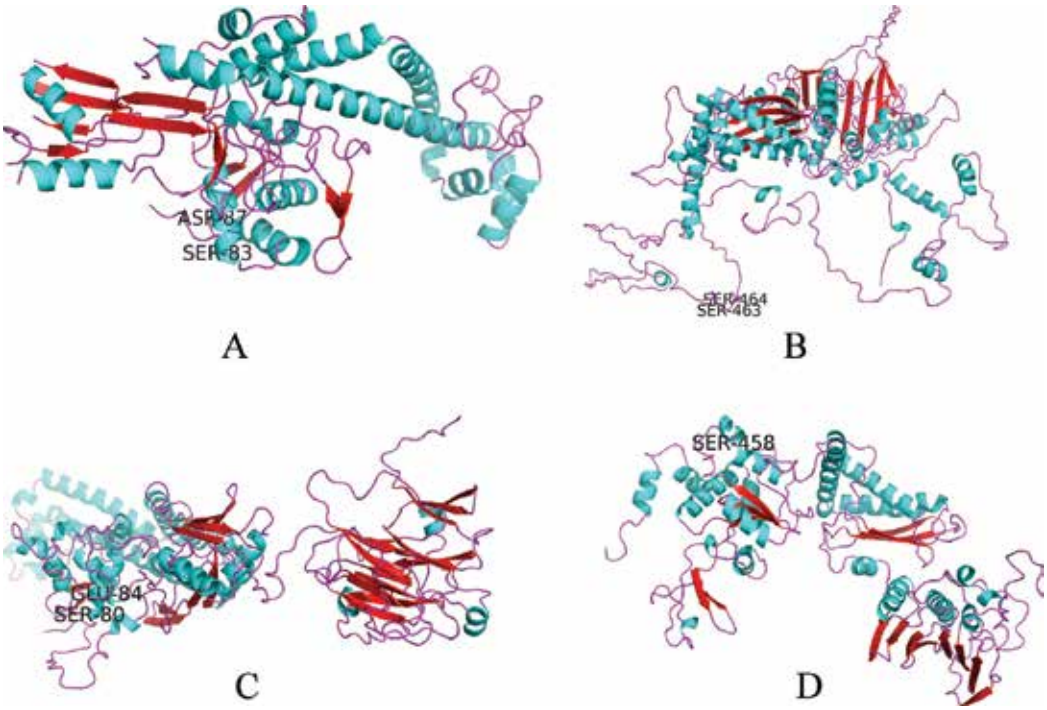

Figure 2. Homology modeling and the amino acid mutations of the subunit A (A, GyrA) and subunit B (B, GyrB) of DNA gyrase and subunit C (C, ParC) and subunit D (D, ParE) of the topoisomerase IV in *Salmonella*.

the same codon may have different substitutions which alter the binding capacity of quinolones to the DNA- gyrase complex. As reported by Levy et al., during selection process the nature of the FQ determines the *gyrA* mutation spectra [32]. For instance, selection with enrofloxacin appeared more likely to select for Ser83Phe substitutions, whereas selection with ciprofloxacin favored recovery of Asp87Gly mutants [16, 18, 22, 25, 26, 32, 33]. Levy et al. concluded that the emergence of quinolone resistance is usually because of the mutant strains being defective in methyl-directed mismatch repair [32].

As compared to *gyrA*, the mutations in *gyrB*, which encodes the B subunit of DNA gyrase, are less common (**Figure 2B**). Point mutation at codon 463 of *gyrB* with an amino acid substitution of Ser to Tyr has been reported in a quinolone-resistant post-therapy isolate of *S. enterica* serovar Typhimurium [34]. Complementation experiments provided evidence of the contribution of mutations in both *gyrA* and *gyrB* genes to the fluoroquinolone resistance [24]. For codon Ser464, it was considered as a mild spot since it was found altered (to Phe or Tyr) in a few independent FQ-resistant strains [33, 35–37].

The *parC* and *parE* genes of topoisomerase IV, which is the secondary target for quinolones, are homologous *gyrA* and *gyrB* in *Salmonella*. Generally, the quinolone-resistant mutations in *parC* occur at codon Ser80 and less frequently at codon Glu84 (**Figure 2C**). These codons are homologous to the Ser83 and Asp87 codons of DNA gyrase, respectively [33, 35, 38–40]. Studies showed that mutations in *parC* of *Salmonella* do not play an important role in quinolone

resistance as mutations in *gyrA* or they may only be required to achieve higher-level resistance [21, 23]. However, the experiment of transformation of *parC* mutants with wild-type *parC* shows an associated temporary reversal resistance to ciprofloxacin in *Salmonella* [37]. A study conducted by Piddock et al. reported that there are no *parC* mutants in 196 strains of veterinary isolates by using a Cip MIC of ≥0.5 mg/L as a cutoff value [21]. It was further supported by the study conducted by Giraud et al. who use in vitro and in vivo strains with Cip MICs of up to 16 mg/L [16]. Usually, mutant *parC* is detected in the *Salmonella* strains with two mutations in *gyrA*, while they have been observed in *E. coli* with only one *gyrA* mutation [24, 41–43]. In comparison to the strains without mutations, the Thr57Ser alone was able to increase the MIC of ciprofloxacin from 6 to 11 mg/L [38]. The Thr57Ser mutation which occurs outside the QRDR might have some different types of mechanism for quinolone resistance [29]. The substitution of amino acids (Ser458Pro) in *parE* of *Salmonella* was detected in human isolates from Hong Kong [38]. Mutations in ParE have been observed most rarely (**Figure 2D**) [44, 45].

2.1.2. Efflux pumps and porins

Different isolates may have same mutations in topoisomerases but present various quinolone-resistant phenotypes, other mechanisms such as overexpression of efflux pumps are also considered to contribute to the fluoroquinolone resistance [16]. Many studies have reported the contribution of overactivation of the efflux pumps to fluoroquinolone resistance in *Salmonella* (**Figure 1**) [11, 16, 40, 46].

In the past few years, many studies have been performed to investigate the role of efflux pumps to high- and low-level resistance in *Salmonella* [11, 40, 47]. The fluoroquinolone resistance level was decreased from 16- to 32-fold when the *acrB* gene (coding for the transporter) and *tolC* gene (coding for the outer membrane component of the efflux system) were inactivated or the AcrB efflux pump was inhibited by the inhibitor L-phenylalanine-L-arginine-β-naphthylamine (PAβN) [11]. AcrAB-TolC efflux system appears to be the main mechanism mediating quinolone resistance in *S.* Typhimurium DT104 strains with little contribution from *gyrA* mutations, while in *S.* Typhimurium DT204, both active efflux and accumulation of target gene mutations are required for the higher level of resistance to fluoroquinolone [47]. In a comparative study among the *S.* Typhimurium with *acrAB* operon mutation with its parent and AcrAB-overproducing strains, the results showed that the AcrAB efflux pump conferred significant resistance to a number of antimicrobials [48]. Giraud et al. reported that the resistance level of *S.* Typhimurium strains was strongly correlated with the expression of the AcrAB efflux pump [49]. In addition, the overexpression of efflux pumps (AcrEF and MdlAB) in a fluoroquinolone-resistant *Salmonella* Typhimurium strain S21was also reported by Chen et al., but they are not contributed to the elevation of MIC to fluoroquinolones [50]. However, another study reported that when the AcrAB is out of function, the AcrEF can be recruited to efflux fluoroquinolones [51]. It is generally observed that the level of the increase of the susceptibility of bacteria is dependent on the specific FQ antibiotic used [40].

The *soxRS* and *marRAB* operons are also present in *Salmonella* (**Figure 1**) [46, 52–56]. Recently, it came to know that the mutations in the *acrAB* and *acrEF* operons also play an important role in

FQ resistance. In an in vitro study conducted on FQ-resistant strain of *S.* Typhimurium, substitutions at amino acids Ile75 and Glu76 were described in *acrR*, which is the local repressor of *acrAB* [57]. A study of whole genome sequencing identified a mutation of Gln78Stp on *acrR* in a resistant clinical *S.* Choleraesuis strain with *acrAB* consistently overexpressed [58]. However, the author further found that this internal stop codon in *acrR* was also present in susceptible isolates, and it may be a genetic diversity in the Choleraesuis serotype rather as FQ resistance. Some studies have shown that strains with wild-type topoisomerase genes and *mar*, *sox*, or *acrR* regulatory loci, yet exhibit the low level of FQ susceptibility and overexpression of *acrAB*, suggest that some other regulators may be involved. The *ramA*, from *S. enterica* serovar Typhimurium and other enterobacteria (but is absent in *E. coli*), may be the regulator locus, whose product is homologous to the *acrAB* transcriptional activators SoxS and MarA [59]. Experimentally, overexpression of *ramA* in *S.* Typhimurium can lead to multidrug-resistant (MDR) phenotype, and the *ramA* might act by direct activation or MarA-controlled genes [60]. However, it was further reported by the authors that their MICs in 15 clinical strains were never affected by the inactivation of *ramA* and finalized that *ramA* was not a common MDR mechanism in *Salmonella* [60]. In a study by Koutsolioutsou et al. [53], during the clinical usage of fluoroquinolones, resistant *S.* Typhimurium emerged with a mutation in *soxRS* gene, whose overexpression leads to the increase of the resistance level [53]. Neither was *marA* induced by a number of antimicrobials, salicylate did also induce *marA* [61]. It has been found that the treatment of aspirin might lead to high plasma concentrations and induces MarA overexpression [62]. Coban et al. documented that the medication of aspirin and ibuprofen during clinical treatment of salmonellosis could lead to development of resistance [63].

It is thought that quinolones particularly hydrophilic ones penetrate the cells through porin [8]. But it is not clear yet whether the absence of OmpF has any role in decreasing the levels of quinolone accumulation in cells. In a study by Piddock et al., the decrease or absence of OmpF or any other OMP was not associated with the reduced accumulation of quinolones in several strains [63]. As described by Lewin et al. and Ruiz et al., in comparison of the nalidixic acid-resistant and acid-susceptible strains of *Salmonella*, no difference was found between the OMP [23, 64], and Giraud et al. also reported that the expression level of porins in their *S.* Typhimurium MAR mutants was not reduced [49]. In contradiction to the previous studies, Howard et al. reported substantially the reduced level of OmpF expression in a *S.* Typhimurium strain which was resistant to ciprofloxacin, and Toro et al. reported an isolate of *S.* Typhimurium that lacked OmpF and presented MAR phenotype [65, 66].

Some previous studies reported that in quinolone-resistant *Salmonella*, there is an alteration in the expression of outer membrane protein or lipopolysaccharide [17, 21, 49]. However, the role of these alterations in decreasing the outer membrane permeability and association with quinolone resistance is not clear. Although the role of lipopolysaccharide composition on the accumulation of quinolones has been studied in several bacterial species, it remains unclear, and sometimes contradictory results have been reported [67–70]. It has been assumed that in quinolone-resistant *Pseudomonas aeruginosa* isolates, the amount of lipopolysaccharide increases and forms a permeability barrier which acts preferentially against hydrophilic quinolones [68]. The lengthening of the O-chains in the quinolone- resistant *Salmonella* mutants also contributes to the reduction of permeability of the outer membrane [49].

2.1.3. PMQRs

Transferable nalidixic acid resistance had been sought unsuccessfully in the 1970s [71], and plasmid-mediated resistance was thought unlikely to exist since quinolones are synthetic compounds and adequate resistance can arise by chromosomal mutations [72]. However, a plasmid-mediated quinolone resistance (PMQR) mechanism was firstly reported by Martinez-Martinez et al. in 1998 [73], 31 years after nalidixic acid began to be used clinically and 12 years after modern fluoroquinolones were approved for use [74]. Presently, there are five Qnr families which differ in sequence (QnrA, QnrB, QnrC, QnrD, and QnrS) about 40% or more from each other [75]. In addition, the substitutions of amino acids within each family lead to numerous variants, e.g., with more than 20 alleles, and *qnrB* is the most varied [75]. The first PMQR that could transfer low-level ciprofloxacin resistance to a variety of Gram-negative bacteria was discovered in a multiresistant urinary isolate of *K. pneumoniae* from Alabama. After the responsible gene (*qnr* and later *qnrA*) was cloned and sequenced [76], *qnr* was soon found at low frequency on plasmids in Gram-negative isolates around the world [77]. The mechanism of Qnr protein is on the basis of protecting the quinolone target [4]. The *qnr* can encode for a 219 amino acid protein which belongs to pentapeptide repeat family and has the ability to bind to and protect both DNA gyrase and topoisomerase IV from fluoroquinolones [76, 78, 79]. Structural study of a pentapeptide repeat protein from mycobacteria (MfpA) that contributes to quinolone resistance revealed that it formed a rodlike dimer with surface charge and dimensions similar to double-stranded DNA and could thus act as a DNA mimic [80]. The Qnr protein might have similar structure with MfpA [80, 81], but it can only protect targets when the concentration of quinolones is very low [76, 81, 82], and it has a glycine residue which separates the Qnr protein into two parts. Generally, Qnr genes located on plasmids carrying multiresistant determinants, especially those having genes encoding extended-spectrum β-lactamases [83], e.g., *qnrA* and *qnrB*, are commonly found as a part of complex *sul1*-type integrons [84].

The production of a modified aminoglycoside acetyltransferase (AAC(6′)-Ib-cr) is another mechanism of resistance to ciprofloxacin. It can modify the drug and reduce the antimicrobial activity [85]. Based on an epidemiology study of human clinical strains, the detection frequency of the *aac(6′)-Ib-cr* gene varied from 0.4 to 34% [86] and mostly from *E. coli* and *K. pneumonia* strains. Recently, it has been identified in *Salmonella* spp. isolated from chickens in Japan and in *E. coli* of poultry origin in Spain or of pig origin in China [87–89]. The *aac(6′)-Ib-cr* gene is distributed worldwide, stable in the environment over time, and prevalent in both FQ-susceptible and FQ-resistant isolates [90].

A conjugative plasmid with a multidrug efflux pump OqxAB was detected in clinical *E. coli* strains isolated from swine, and it contributes to the resistance of olaquindox [91, 92]. Recently, Wong and Chen [93] reported that *oqxAB* was found in *Salmonella* spp. isolated from retail meats in Hong Kong and it confers resistance to multiple antibiotics (olaquindox quinolones and chloramphenicol). Other isolates characterized in this study carried the *qnrS* and *aac(6′)-Ib-cr* genes. Another important plasmid-mediated efflux pump (QepA) was found in a clinical strain of *E. coli* in Japan and presents MAR phenotype including aminoglycosides, fluoroquinolones, and broad-spectrum β-lactams [94].

PMQR genes facilitate the development of higher-level quinolone resistance and have been detected in various bacterial species in many countries around the world [77]. A previous study conducted on *Salmonella* (n = 1215) and *E. coli* (n = 333) isolates shows that six *qnrB* variants were found in 138 *qnrB*-positive isolates and majority of these isolated from turkeys [95]. Another study from Spain and Italy reported that the *qnrD* gene was identified in 22 *Salmonella* isolates of eight different serotypes [96]. A multiplex study about 107 strains of non-Typhi *Salmonella* isolated in the USA from 1996 to 2003 showed that *Salmonella* Bovismorbificans carried *qnrS1*, *qnrS2* was identified in *S.* Anatum, *qnrB2* was reported in *Salmonella* Mbandaka, and a new variant, *qnrB5*, was reported in seven *Salmonella* Berta isolates [84]. An international collaborative study conducted in 13 European countries showed that among isolates of *Salmonella enterica* of various origins (environment, food, humans, pigs, fowl, reptiles, sheep, turkeys), 59% (288/485) carried PMQR genes. The *qnrS1* gene was found in six isolates with one strain bearing the *aac(6′)-1b-cr* gene. *qnrB19* and *qnrD* genes were found in two and one isolates, respectively [85]. A survey conducted on 13 nalidixic acid-resistant *Salmonella* spp. strains isolated from food animals in Colombia from 2004 to 2007 shows that 30.8% of the strains were positive for *qnrB*, while *qnrB19* was found in all cases [97]. A study performed in the Henan Province of China reported that four *Salmonella enterica* isolates were slightly resistant to ciprofloxacin. These isolates were obtained from humans, and the resistance was transferable by a 4.3 kb plasmid bearing the *qnrD* gene. It increased the MIC of ciprofloxacin about 32-fold in *E. coli* [98]. The *qnrD* gene has been identified in 22 out of 1215 *Salmonella* isolates obtained from different European countries, being either of human or animal isolates [95].

2.2. Development of resistance

The order of the implementation of different mechanisms in the process of resistance development has attracted broad attention. The background of highly resistant isolates is not clear, and the parent-susceptible strain cannot be obtained; thus, multiple studies have attempted to use the in vitro multistep selections to trace the development of resistance [12]. In in vitro selection of FQ-resistant *E. coli*, the first-step mutants may have a mutation in *gyrA* [99], the second-step mutants show overexpression of efflux pumps and multiresistant phenotype, and the third-step mutants present further enhanced efflux expression and more mutations in the DNA gyrase or topoisomerase IV. In clinical isolates of *E. coli*, the development process seems to be the same, and several mutations are needed for the high resistance [41, 100]. The in vitro selection of high-level FQ-resistant *Salmonella* is also a multistep process [49], but the sequence of mechanisms may be different from *E. coli*, where active efflux caused by the overactivation of AcrAB efflux pump appears before mutation in the *gyrA* gene [49] and no mutations were detected in *parC* in the third-step mutants; only the further overexpression of AcrAB efflux pump was found.

The emergence order of each individual mechanism may somewhat depend on the particular bacteria strains to which the antibiotic is imposed [12]. Luria-Delbruck dogma reported that mutations may occur prior to the exposure of antimicrobials. Under the drug concentrations within the mutant selection window (MSW), which was defined by Drlica, the bacteria with

specific mutation can be selected [101]. In a parent-susceptible bacterial population, there may be two types of resistant bacteria, topoisomerase mutants and efflux mutants. The number of topoisomerase mutants is far less than the diverse efflux mutants, since only specific substitutions in target topoisomerase can increase resistance and may induce fitness cost in bacteria [102]. The efflux mutants usually mediate low-level FQ resistance; thus, for the drug concentrations near the bottom of the MSW, most of the selected mutants would be efflux mutants [101]. When the drug concentration increased, the topoisomerase mutants would be selected and become prevalent. In a treated animal, the drug concentration may be changed temporally and spatially, so that the highly resistant strains may be easily obtained. The initial efflux mutants facilitated the further step of selection of topoisomerase mutants. Mutations in *gyrA* are frequently detected in clinical-resistant *Salmonella* isolates, but the sequence of the mutation is not clear till now [16, 33]. There are also studies reported that the efflux mutations can be induced in *gyrA* mutants [49]. Olliver et al. revealed that the AcrEF efflux would be activated when the IS1 or IS10 elements were inserted in promoter regions. However, this phenomenon was only observed in *S.* Typhimurium DT204, but not in *S.* Typhimurium phage-type DT104 [51]. The efflux mechanisms would present in specific strain according to the characteristics of the IS elements [12].

In clinical settings, underdosing seems to be inevitable and tends to easily select for resistance [103]. It was supported by Giraud et al., who conducted an in vivo experiment on chicken, and the results showed that a single low dose of enrofloxacin was enough to select resistant isolates [16]. Fluoroquinolones are usually used for population medication of sick animals by feed or water. The variations of drug intake among each animal lead to the underdosing and selection for resistance. In addition, the salmonellosis in swine and poultry is usually self-limited without symptoms, when the fluoroquinolones are medicated for treating other diseases; *Salmonella* is also under the antibiotic pressure and resistance selection may occur [1].

3. Fitness

Understanding the fitness effects of antimicrobial resistance evolution is crucial for controlling the spread of resistance, as the fitness cost induced by antimicrobial resistance is one of the few biological features of resistant organisms that can be leveraged against them [104]. The FQ resistance in *Salmonella* is not as frequent as it is in other members of *Enterobacteriaceae.* It might be due to the different FQ resistance mechanisms in *Salmonella*, which may have a prohibitive fitness cost which restrains the spread of resistance [16, 105]. Nevertheless, the emergence and spread of highly resistant strains were observed in the early 1990s in Europe with *Salmonella enterica* serovar Typhimurium phage-type DT204 and presently reoccurred in various serovars, such as Typhimurium, Choleraesuis, or Schwarzengrund [38, 106, 107]. This strongly stresses the necessity of further surveillance of FQ resistance and the prudent use of FQs.

In contrast to the wealth of information available on the mechanisms leading to high-level fluoroquinolone resistance in *Salmonella*, few studies to date have investigated the fitness costs associated with this phenotype [105]. Data from these studies suggest that mutations in

antibiotic target genes and overexpression of multidrug resistance (MDR) efflux pumps have been associated with fitness costs, including reduced growth rates and virulence, which may limit the survival of resistant strains in the absence of antibiotic selective pressure [108–110]. However, stabilization of resistance can occur through the development of compensatory mutations that restore fitness without loss of the original level of resistance [111].

In vitro selected FQ-resistant *Salmonella* by Giraud et al. showed smaller colony size on solid media than the susceptible counterparts [16]. Further experiments indicated that FQ-resistant mutants selected in vitro or in vivo (chicken) varied dramatically in the level of resistance to FQs and the growth characteristics in culture medium and in chickens in the absence of FQ antimicrobials. The in vitro selected mutants were highly resistant to FQs, showed significantly reduced growth rate in culture medium, and could not colonize chickens. In contrast, the in vivo selected resistant isolates exhibited intermediate susceptibility to FQs, had normal growth in liquid medium (slow growth on solid medium), and were able to colonize chickens at the extent comparable to or lower than that of the wild-type strains [105]. The fitness was restored partly after several passages *in vitro* or *in vivo* without antibiotics [105]. Another study described the fitness costs associated with high-level fluoroquinolone resistance for phenotypically and genotypically characterized ciprofloxacin-resistant *Salmonella enterica* serotype Enteritidis mutants (104-cip and 5408-cip, MIC >32 g/ml) [112]. Mutants 104-cip and 5408-cip displayed altered morphology on agar and by electron microscopy, reduced growth rates, motility and invasiveness in Caco-2 cells, and increased sensitivity to environmental stresses. Microarray data revealed decreased expression of virulence and motility genes in both mutants. Reverted clones for mutant 104-cip were obtained from separate lineages after several passages on antibiotic-free agar. All fitness costs, except motility, were reversed in the reverted strains. The altered porin and lipopolysaccharide (LPS) profiles observed in 104-cip were reversed, and additional mutations in SoxR and ParC were observed in the reverted strain. Randall et al. reported that the disinfectant-exposed *S.* Typhimurium strains, although MAR, were less fit, were less able to disseminate than the parent strain, and were not preferentially selected by therapeutic antibiotic treatment [113].

However, using in vitro competition experiments, Baker et al. assayed the fitness of 11 isogenic *S.* Typhimurium strains with resistance mutations in the FQ target genes, *gyrA* and *parC* [104]. The results showed that in the absence of antimicrobial pressure, 6 out of 11 mutants carried a selective advantage over the antimicrobial-sensitive parent strain, indicating that FQ resistance in *S.* Typhimurium is not typically associated with fitness costs. Double mutants exhibited higher expected fitness cost as a result of synergistic epistasis, signifying that epistasis may be a critical factor in the evolution and molecular epidemiology of *S.* Typhimurium.

The measurement of fitness can also be influenced by a number of factors. In classical competition assays [114, 115], antimicrobial-susceptible and antimicrobial-resistant organisms are competed over many generations, and their sensitivity and resistance are noted at various stages; hence, the fitness of the resistant strain to the sensitive strain can be calculated from the population trajectories [116–118]. For competitive growth assay, the selection of relative strain is critically important [119, 120]. It would be difficult to measure the effect of a specific mutation when using imperfectly isogenic strains [112, 117, 121, 122]. The enumeration and

culturing of bacteria may also be inaccurate due to the spontaneous mutations after exposed to low concentrations of antibiotics. Usually, *S.* Typhimurium disseminate through the macrophages after invading the intestinal epithelial cells (M cells). Intracellular assay using epithelial cell or macrophage as models can provide a suitable method for measuring fitness in *S.* Typhi [123]. Nevertheless, the antibiotic exposure, uptake, and cellular replication and division would affect the experimental accuracy and reproducibility. The in vivo competition experiment using animals as models is a well-described method. But it is hard to control the brief duration of infection, which may result in small variations in bacterial numbers and generations [104].

4. Virulence

There is an increase in the knowledge about the virulence mechanisms of *Salmonella* which led to a broad study of the *Salmonella* pathogenicity islands (SPIs) [124, 125] and other virulence determinants, such as virulence plasmid, adhesins, flagella, and biofilm-related proteins [126–130]. These virulence factors are controlled by an extensively complicated regulatory system, which correlates and synchronizes all the elements [131].

Several studies have investigated the impact of acquisition of fluoroquinolone resistance on the virulence of *Salmonella*. In a classical study by Bjorkman et al. investigating the virulence of nalidixic acid-resistant strain of *Salmonella* Typhimurium, they found that the virulence was reduced after acquiring resistance, but compensatory mutations occurred rapidly to restore the virulence without losing the resistance [132]. Other studies showed that the *acrB* gene [133] and *tolC* gene [8] may associate with virulence in *Salmonella*. The *acrB* mutant showed a reduced ability to colonize the intestine of mice. The *tolC* mutant was a virulent factor for mice when administered by the oral route. Fabrega et al. [134] documented that the activation of efflux, production of biofilm, and bacterial fitness are interrelated. The FQ resistance was linked to the reduction of biofilm production and decreased expression of *csgB* gene. Giraud et al. [135] reported that the *ramRA* mutations may reduce the invasiveness ability of clinical FQ-resistant *S.* Typhimurium strains, but this is strain-dependent. In a registry-based cohort study performed by Helms et al. [136], in comparison with infections by pansusceptible strains, the infections with FQ-resistant *S.* Typhimurium was associated with a 3.15-fold higher risk of invasive illness or death within 90 days of infection.

5. Conclusions

Fluoroquinolones are one of the most valuable antibiotics used for the treatment of a variety of infections in both humans and animals, especially salmonellosis. However, the usage has led to the prevalence of FQ resistance among different serotypes of *Salmonella*, and ultimately the clinical efficacy has been compromised. To preserve the efficiency of fluoroquinolones, the drugs should be used prudently, the residues in foods need to be monitored, and comprehensive

surveillance should be implemented to the resistance of bacteria from both animals and humans. Efflux pump inhibitors can be applied as new therapeutics and combined with fluoroquinolones to minimize the emergence of high-level resistance in different pathogens, including *Salmonella*.

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Competing financial interests

The authors declare no competing financial interests.

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Section 4

Medical Engineering

Chapter 7

Salmonella and Biotechnology

Wonsuck Yoon

Additional information is available at the end of the chapter

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Abstract

Salmonella strains have been actively studied as live carriers of heterologous antigens for a possible vaccine application. Especially, *Salmonella Typhimurium*, a facultative anaerobe, has been adapted as an antitumor agent capable of preferentially proliferating within tumors and inhibiting their growth. To enhance the cancer therapeutic efficacy of *S. Typhimurium*, combinations of gene-directed protein or microRNA therapies and auxotrophic strains of *S. Typhimurium* have been generated by genetic engineering. Until now, the idea of using bacteria including *Salmonella* in the treatments of cancer and other diseases has been considered a novel approach. Here, we describe this application based on *Salmonella* engineering for treatments of cancer or atopic dermatitis.

Keywords: *Salmonella*, cancer, atopic dermatitis, therapy, delivery system

1. Introduction

Salmonella strains have been used to prepare attenuated vaccines. These bacteria invade epithelial cells and secrete the internal protein of bacteria. Thus, *Salmonella* strains have been adapted as carriers for delivery of a recombinant antigen, therapeutic protein, or functional plasmid. After invading the intestinal epithelium, a modified *Salmonella* strain survives and replicates within antigen-presenting cells such as macrophages, mast cells, and dendritic cells.

Salmonella induces strong mucosal and cell-mediated immune responses against recombinant antigens [1, 2]. Recombinant proteins expressed by *S. Typhimurium* can be secreted and recognized by host immune cells with or without lysis of the bacteria. However, *Salmonella* replication within a membrane-bound vacuole inhibits processing of a recombinant protein by antigen-presenting cells [3]. Therefore, in a genetically modified *Salmonella* strain, a method for effective delivery of a recombinant protein carried by bacteria into the host is needed for the development of an effective therapeutic strain.

Salmonella strains secrete recombinant proteins and introduce a heterologous protein into the extracellular environment. *Salmonella* strains use a type III secretion system (T3SS) to deliver cytoplasmic effector proteins into host cells [4]. In most T3SS-secreted proteins, *Salmonella* uses the N-terminal region for the signal for secretion of the target protein [5]. When several antigenic peptides are fused to the secretion domain of effector protein SopE of *S. Typhimurium* or YopE of *Yersinia enterocolitica*, the recombinant proteins are exported in a T3SS-dependent manner, resulting in activation of an immune response in mouse models [6, 7]. *S. Typhimurium* possesses two T3SSes encoded by *Salmonella* pathogenicity islands (SPIs) 1 and 2; SPI-1 is required for invasion of non-phagocytic epithelial cells, and SPI-2 for replication and survival in macrophages [4].

Salmonella has also been exploited as an antitumor agent that is capable of preferentially amplifying within a tumor and inhibiting its growth [8, 9]. In an effort to enhance therapeutic efficacy, this approach has been combined with a gene-directed enzyme/prodrug therapy [10]. For example, auxotrophic *S. Typhimurium* expressing prodrug-converting enzymes has been generated by transformation with a prokaryotic expression vector encoding herpes simplex virus thymidine kinase [11] or by chromosomal insertion of the *Escherichia coli* cytosine deaminase gene [12]. *Salmonella* has also been engineered for the transfer of prokaryotic and eukaryotic expression plasmids into host cells [13]. Oral administration of genetically modified *Salmonella* carrying a eukaryotic expression plasmid encoding interferon-gamma (IFN-γ) restores the production of this cytokine in the macrophages in mice [14]. When delivered orally to mice, *S. Typhimurium* carrying eukaryotic expression vectors encoding cytokines (i.e., interleukin-12 or GM-CSF) increases cytokine concentrations and exerts antitumor effects [15]. Thus, it should be feasible to use *Salmonella* strains transformed with eukaryotic expression vectors to deliver various effector molecules to cancer cells or skin inflammation sites, with the goal of enhancing therapeutic activity.

2. Medical application of *Salmonella* engineering

2.1. *Salmonella* strains are used as a carrier for delivery of a foreign protein or genetic material

Salmonella strains are considered good candidates as a vector for delivery of a foreign protein and/or plasmid(s). Attenuated *S. Typhimurium* strains are easy and cheap vector microbes to produce an antigen in comparison with any other synthetic protein delivery system and have been evaluated as vehicles for delivery of a plasmid expressing a heterologous antigen(s) to the host. Not only cytoplasmic expression of a recombinant protein in *S. Typhimurium* but also secretion or surface display of the target protein is a promising strategy for enhancing vaccine effects by improving recombinant antigen presentation in antigen-presenting cells. In one study, researchers used the T3SS signal from SipB, which possesses the domains for its secretion and outer membrane localization.

Many Gram-negative bacteria have a T3SS to deliver effector proteins into host cells, and the secretion signals of T3SS substrates have been used as carrier molecules for the delivery of foreign antigens or therapeutic molecules.

In an animal experiment, oral administration of attenuated *S. Typhimurium* bearing therapeutic plasmids showed that this strain secretes and surface-displays the SipB tetanus toxin and induces a strong antigen-specific immune response in mice.

Oral vaccination of mice with attenuated *S. Typhimurium* carrying T3SS-based delivery plasmids may increase the recombinant protein presentation in antigen-presenting cells, resulting in induction of recombinant protein-specific immune responses in mice. These findings suggest that the N-terminal domain of SipB can serve as a signal sequence for the surface display and secretion of heterologous proteins.

However, successful T3SS-mediated antigen delivery is restricted by several factors, including the size, folding, stability, and structure of a recombinant protein.

When a highly virulent *Salmonella* strain (*S. Typhimurium* UK-1) is transformed with a cytokine (IL-12)-expressing plasmid, this live, wild-type pathogen has been shown to work as a vaccine strain without any other biological or genetic attenuating processes.

Wild-type pathogenic *S. Typhimurium* UK-1 carrying an IL-12-expressing plasmid induces protection against a lethal dose of normal wild-type *Salmonella* [16]. These results also revealed that a wild-type *Salmonella* strain bearing a plasmid for secretion of IL-12 may be considered an alternative approach to the development of intracellular bacterial vaccines, without the inconvenience of time-consuming attenuation procedures.

2.2. Engineered *Salmonella* has therapeutic effects on cancer

In the field of anticancer therapeutic agents, biological modalities such as cell therapy, gene therapy, and antibody-related immunotherapy have been developed as possible candidates for cancer therapeutics. In addition to these new candidates, bacterial therapy is believed to be a promising technology of tumor treatments and tumor vaccines. This kind of bacterial therapy is safer, less expensive, and more versatile than other biological cancer treatments. These therapeutic bacteria could be produced cheaply. Moreover, *Salmonella* is thought to be a good anticancer therapeutic agent and has shown tumor-targeting properties and tumor-suppressing effects in some studies [17]. Tumor-targeted *Salmonella* has a tumor accumulation ratio greater than 1000:1 as compared to healthy tissues [9], and many research groups have used *Salmonella* strains for the development of anticancer agents [18]. In one study, a genetically engineered strain of *S. Typhimurium* expressing TNF-α was tested as a melanoma-suppressing agent. This *Salmonella* was attenuated for safety toward healthy cells and was specifically localized to and invaded various melanoma cells while bombarding them with tumor necrosis factor (TNF) proteins to induce tumor cell apoptosis. VEGF, p53, p19, IFNs, and other cytokines have been tested as tumor-suppressor proteins in *Salmonella* vector systems. Although systemically administered cytokines have short half-life and severe side effects after direct administration, cytokines are often used for regulation of the immune system and for tumor cell lysis [19]. For *Salmonella* cancer therapy, researchers engineered *Salmonella* expressing the TNF-α protein—a potent antitumor molecule that normally has limitations because of its side effects—to reduce the adverse effects via tumor-specific local immunotherapy [20].

In our test of *Salmonella*-based TNF-α therapy, the targeted recombinant TNF-α from bacteria did not induce histological changes in various tissues and cytokine upregulation such as severe inflammations after local administration of recombinant *Salmonella*. Some researchers reported that cytokine-expressing *S. Typhimurium* could act as a good biological anticancer agent without the cytotoxicity of high-dose cytokine administration. The production of genetically modified *Salmonella* would be convenient and easy, with a low cost and a short manufacture period; such biological anticancer agents are expected to have synergistic effects (bacterial cytotoxicity and immunostimulation by anticancer cytokines). In addition, bacteria can function as gene delivery shuttles for transporting recombinant gene vectors [11]. According to the latest studies, recombinant *Salmonella* produces 100 pg of TNF-α per 10^9 cells. TNF-α is not secreted into the normal environment, but is released into tumor cells after bacterial invasion of these cells. Accordingly, genetically modified *Salmonella* carrying a cytokine expression vector (e.g., *S. Typhimurium* expressing TNF-α) is known to express cytokines in melanoma cells and to suppress tumor growth in mice with melanoma, colon cancer, or breast cancer. Therefore, *S. Typhimurium* expressing TNF-α may serve as a therapeutic agent against various tumors and as an adjuvant to existing cancer therapies such as chemotherapy, radiotherapy, and immunotherapy. These findings suggest that *Salmonella* carrying a cytokine expression vector can act as a new, safe, and efficient anticancer agent. In particular, to determine the cytotoxic effects of genetically modified *Salmonella*, B16F10 melanoma cells were treated with *S. Typhimurium* expressing TNF-α. The *S. Typhimurium* expressing TNF-α invaded tumor cells at a higher invasion rate (50%) than did a naïve *Salmonella* strain and lysed the melanoma cells [21]. These results indicate that genetically modified *Salmonella* expressing TNF-α specifically lyses B16F10 melanoma cells in contrast to naïve *Salmonella* strains (**Figure 1a**) and that the engineered *S. Typhimurium* expressing TNF-α induces caspase activation for tumor cell lysis and inhibited tumor growth in tumor-bearing mice (**Figure 1b**).

Additionally, a *Salmonella*-based cancer therapy may not be compatible with antibiotics like gentamicin, and host immune responses and environments conducive to bacterial killing are

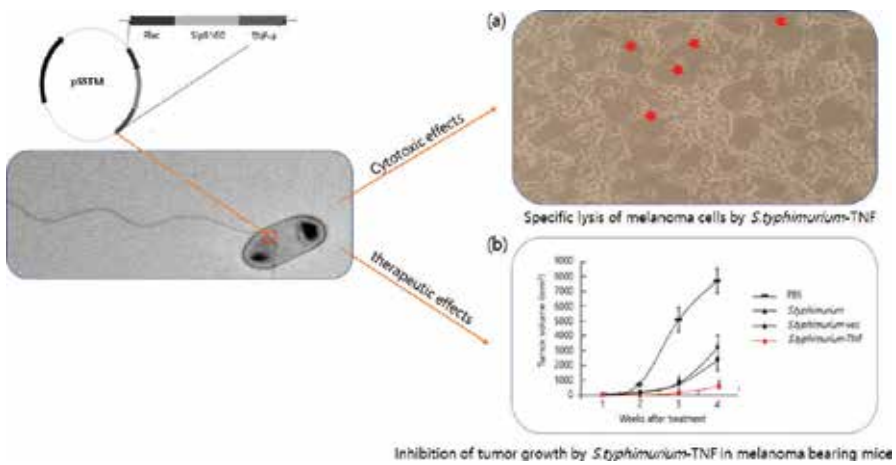

Figure 1. Construction and effects of the recombinant *Salmonella Typhimurium* containing TNF-α.

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likely to be disadvantageous for *Salmonella*-based cancer treatment. In some studies, researchers investigated the impact of antibiotics on a *Salmonella*-based cancer therapy. Tumor-bearing mice were treated with *Salmonella* expressing TNF-α and an antibiotic (gentamicin). Another group of mice was vaccinated with attenuated *Salmonella*, received a transplant of melanoma cells, and was then examined for the tumor inhibitory effect of *Salmonella* expressing TNF-α. In an in vivo assay, gentamicin did not interfere with *Salmonella*-mediated therapy of tumor cells (**Figure 2a**). In tumor-bearing mice, treatment with *Salmonella* and cisplatin also inhibited tumor growth (**Figure 2b**). In mice that were vaccinated with dendritic cells, host immune responses did not suppress tumor inhibition by *Salmonella* expressing TNF-α (**Figure 2c**). In treatment with *Salmonella* and radiation, *Salmonella* expressing TNF-α also inhibited tumor growth (**Figure 2d**). These results showed that the antitumor activity of subcutaneous treatment with *Salmonella* expressing TNF-α is not suppressed by antibiotics and host immune responses in mice.

Other studies were conducted on a vaccine based on recombinant *Salmonella* expressing human papilloma virus antigens [22]. This recombinant *Salmonella* was constructed from *Salmonella enterica* serovar *Typhimurium* expressing a fusion protein consisting of the SipB protein from *Salmonella* and the HPV16 E7 protein derived from human papillomavirus for tumor-suppressing effects. The genetically modified *Salmonella* expressing SipB-E7 was tested in a cervical cancer model. In cervical cancer TC-1-bearing mice, *Salmonella* expressing SipB-E7 induced cytotoxicity and slowed tumor growth after oral inoculation. Moreover, in the mouse model of cervical cancer, orally administered *Salmonella* expressing SipB-E7 induced cytokines IFN-γ and TNF-α and prolonged survival compared with the control group (naïve *Salmonella* or PBS-treated groups). These results revealed that *Salmonella* expressing fusion protein SipB160-E7 may be a candidate cancer therapeutic agent. Yoon et al. took advantage of a genetically engineered *Salmonella* strain as a candidate tumoricidal modality; to enhance tumor-suppressing effects, *S. Typhimurium* was designed to carry eukaryotic expression

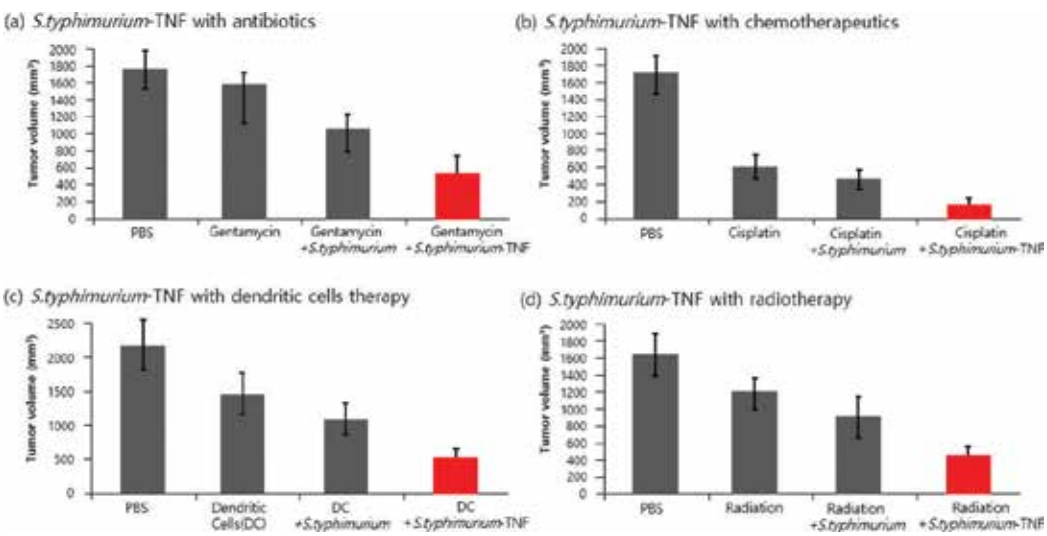

Figure 2. Tumour inhibition of *S. Typhimurium* containing TNF-α with antibiotics or vaccine or cisplatin.

plasmid expressing the Flt3 ligand (Flt3L) [23]. *Salmonella* carrying the Flt3L expression vector exerted antitumor effects against B16F10 melanoma cells in vitro. When the engineered *Salmonella* was injected locally into the tumor region, *S. Typhimurium* with the Flt3L expression vector inhibited tumor growth more effectively relative to control groups. Nonetheless, in the mice cured of melanoma after treatment with recombinant *Salmonella*, there was no induction of tumor immunity mediated by memory antitumor lymphocytes because there was no protective responses against a tumor rechallenge.

Compared to radiation alone, a combination therapy, *Salmonella* with γ-radiation, confers radiosensitization onto cancer cells by inducing apoptotic cell death [24]. *Salmonella* infection induces apoptosis via caspase 3 and Bcl2 in tumor cells. In addition, tumor growth is suppressed by this combined therapy pointing to possible new versions of radiation therapy against melanoma. Overall, cancer radiation therapy is significantly improved by the use of bacteria. For this reason, our findings indicate that bacteria may help to increase effectiveness of cancer radiation therapy in the future.

2.3. Engineered *Salmonella* induced therapeutic effects on atopic dermatitis

Salmonella-based therapy has been examined as a therapeutic agent for allergic diseases. Excessive Th2-biased immune responses are related to the pathogenesis of allergic diseases. Macrophage-derived chemokine (MDC) is directly related to Th2-associated atopic dermatitis, and MDC concentration is significantly elevated in the serum of patients. MDC has been studied as a marker of severity of atopic dermatitis. Yoon et al. tested genetically modified *Salmonella* as a gene therapy tool to treat atopic dermatitis with bacteria expressing specific microRNA [25]. To suppress the MDC gene for atopic dermatitis therapy, a *S. Typhimurium* strain was constructed that carries a plasmid expressing MDC microRNA. The engineered *Salmonella* strain bearing the microRNA-expressing plasmid (ST-miR-MDC) was used for an in vitro knockdown of MDC in human mast cells [26]. ST-miR-MDC was shown to significantly downregulate the MDC gene in activated human mast cells in vitro. In an atopic-like animal model, strain ST-miR-MDC downregulated IL-4 and IgE expression and upregulated IFN-γ. Strain ST-miR-MDC also suppressed Th17 in the atopic-like animal model (**Figure 3**).

In addition, orally administered strain ST-miR-MDC induced skin regeneration and hair regrowth in atopic-like mice, but control mice did not show these effects (**Figure 4a**). Pruritus

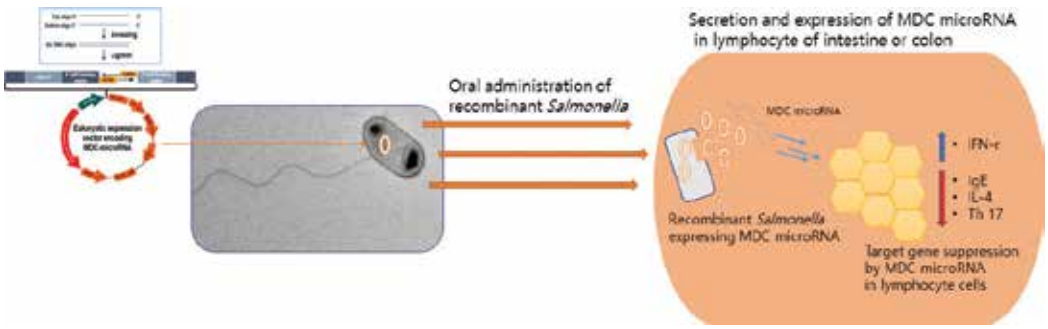

Figure 3. Construction of the recombinant *Salmonella Typhimurium* expressing miRNA-MDC.

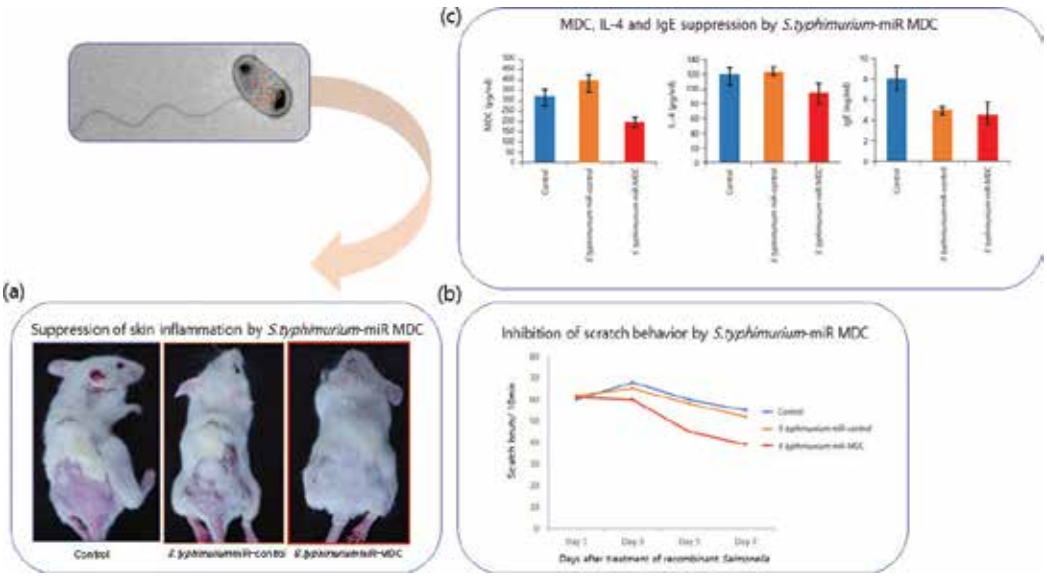

Figure 4. Improvement of the symptoms of atopic dermatitis by oral administration of *S. Typhimurium* miR-MDC in mice.

is one of the major symptoms of atopic dermatitis, and the ST-miR-MDC strain inhibited the scratching behavior of mice. The total scratching counts in the ST-miRCCL22-treated group were significantly lower than those among the mice treated with PBS or ST-miR-control (**Figure 4b**). This strain induced histological changes in the skin tissues of atopic-like mice after oral administration of the engineered *Salmonella*. Strain ST-miR-MDC reduced skin inflammation reactions and reduced cytokine IL-4, MDC, and IgE on mouse blood (**Figure 4c**).

These results indicate that *Salmonella* combined with a targeted microRNA delivery system may be a good candidate for the development of a therapeutic agent against atopic dermatitis.

3. Conclusions

To date, the idea of using bacteria, including *Salmonella* therapy, has been considered a novel approach. *S. Typhimurium* bearing a cytokine-expressing plasmid exerts an antitumor effect on melanoma or anti-inflammatory effects in an atopic-like mouse model.

The engineered *Salmonella* has been designed to target cancer cells, promote a tumor-suppressive environment, and increase the efficacy of existing cancer treatments, including chemotherapy, radiotherapy, and cell therapy.

Especially, *Salmonella* expressing microRNA has been used in vivo to knockdown a target gene and shows modulation of immune responses in a mouse disease model.

These results suggest that genetic engineering of *S. Typhimurium* may be an efficient method of delivery of cytokines or microRNA for therapeutic purposes.

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Edited by Maria Teresa Mascellino

This book deals with the microorganism *Salmonella*. This bacterium is well known for a long time, being involved in systemic (typhus and paratyphus infections) and nonsystemic diseases such as food poisoning. Major and minor *Salmonellae* are widespread worldwide in developing countries and industrialized areas, respectively. In 2015, about 3576 *Salmonella* strains have been isolated from human infections in Italy. *S. typhimurium* and *S. enteritidis* are the most prevalent serotypes and represent 80% of cases of infections over the last 10 years. The antibiotic susceptibility decrease over the last decades is a big issue in the management of this bacterium, once considered easy to treat. The use of antibiotic combinations in order to overcome the microorganism resistance should be hoped.

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