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Bacterial Infectious Diseases Annual Volume 2023

Edited by Katarzyna Garbacz and Tomas Jarzembowski

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Aims and Scope of the Series

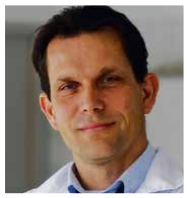
This series will provide a comprehensive overview of recent research trends in various Infectious Diseases (as per the most recent Baltimore classification). Topics will include general overviews of infections, immunopathology, diagnosis, treatment, epidemiology, etiology, and current clinical recommendations for managing infectious diseases. Ongoing issues, recent advances, and future diagnostic approaches and therapeutic strategies will also be discussed. This book series will focus on various aspects and properties of infectious diseases whose deep understanding is essential for safeguarding the human race from losing resources and economies due to pathogens.

Meet the Series Editor

Dr. Rodriguez-Morales is an expert in tropical and emerging diseases, particularly zoonotic and vector-borne diseases (notably arboviral diseases), and more recently COVID-19 and Monkeypox. He is the president of the Publications and Research Committee of the Pan-American Infectious Diseases Association (API), as well as the president of the Colombian Association of Infectious Diseases (ACIN). He is a member of the Committee on Tropical Medicine, Zoonoses, and Travel Medicine of ACIN. Dr. Rodriguez-Morales is a vice-president of the Latin American Society for Travel Medicine (SLAMVI) and a member of the Council of the International Society for Infectious Diseases (ISID). Since 2014, he has been recognized as a senior researcher at the Ministry of Science of Colombia. He is a professor at the Faculty of Medicine of the Fundacion Universitaria Autonoma de las Americas, in Pereira, Risaralda, Colombia, and a professor, Master in Clinical Epidemiology and Biostatistics, at Universidad Científica del Sur, Lima, Peru. He is also a non-resident adjunct faculty member at the Gilbert and Rose-Marie Chagoury School of Medicine, Lebanese American University, Beirut, Lebanon, and an external professor, Master in Research on Tropical Medicine and International Health, at Universitat de Barcelona, Spain. Additionally, an invited professor, Master in Biomedicine, at Universidad Internacional SEK, Quito, Ecuador, and a visiting professor, Master Program of Epidemiology, at Diponegoro University, Indonesia. In 2021 he was awarded the "Raul Isturiz Award" Medal of the API and, the same year, the "Jose Felix Patiño" Asclepius Staff Medal of the Colombian Medical College due to his scientific contributions to the topic of COVID-19 during the pandemic. He is currently the Editor in Chief of the journal Travel Medicine and Infectious Diseases. His Scopus H index is 55 (Google Scholar H index 77) with a total of 725 publications indexed in Scopus.

Meet the Topic Editors

Katarzyna Maria Garbacz, MD, is an Associate Professor at the Medical University of Gdańsk, Poland and she is the head of the Department of Oral Microbiology at the Medical University of Gdańsk. She has published more than 50 scientific publications in peer-reviewed journals. She has been a project leader funded by the National Science Centre of Poland. Prof. Garbacz is a microbiologist working on applied and fundamental questions in microbial epidemiology and pathogenesis. Her research interest is in antibiotic resistance, host-pathogen interaction, and therapeutics development for staphylococcal pathogens, mainly *Staphylococcus aureus*, which causes hospital-acquired infections. Currently, her research is mostly focused on the study of oral pathogens, particularly *Staphylococcus spp*.



Tomasz Jarzembowski was born in 1968 in Gdansk, Poland. He obtained his Ph.D. degree in 2000 from the Medical University of Gdańsk. After specializing in clinical microbiology in 2003, he started studying biofilm formation and antibiotic resistance at the single-cell level. In 2015, he obtained his D.Sc. degree. His later study in cooperation with experts in nephrology and immunology resulted in the designation of the new diagnostic method of UTI, patented in 2017. He is currently working at the Department of Microbiology, Medical University of Gdańsk (GUMed), Poland. He is a member of the steering committee of the Gdańsk branch of the Polish Society of Microbiologists and a member of ESCMID. He is also a reviewer and a member of the editorial boards of several international journals.

Contents

Preface

Never before has there been such a critical need for knowledge of drug resistance among top bacterial pathogens and a precise approach to their diagnosis and treatment. The incidence of infections caused by multidrug-resistant (MDR) bacteria is on the rise, presenting one of the most significant global medical challenges. Multidrug-resistant bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and ESKAPE pathogens, continue to pose a threat to humans. The Bacterial Infectious Diseases Annual Volume 2023 provides an update on infectious diseases such as tuberculosis, listeriosis, campylobacteriosis, and ESKAPE infections. The volume covers selected aspects of epidemiology, introduces new diagnostic methods, and discusses therapeutic options that offer promising opportunities for patients.

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Chapter 1

The Threat of Methicillin Resistant *Staphylococcus aureus* (MRSA) in the Aquatic Environment via Wastewater Generated from Healthcare Facilities

Abimbola Olumide Adekanmbi, Ridwan Olamilekan Adesola, Adedoyin Olutoyin Adeyemi and Chisom Chinyere Mbionwu

Abstract

In most developing countries of the world and few advanced ones, wastewater are discharged into the environment without any form of treatment, thus exposing the general public to hazardous chemicals, residual antibiotics, heavy metals and so many antimicrobial compounds. This chapter deals with the threat posed by methicillin resistant *Staphylococcus aureus* (MRSA) introduced into the aquatic ecosystem via wastewater generated from the operations of healthcare facilities. It focuses more on the microbiology and composition of wastewater from the hospital environment, and the role they played as a stimulant for the development of resistance in bacteria, while also emphasizing their roles as important reservoirs of MRSA in the aquatic environment. The epidemiology of MRSA in wastewater discharge from low-middle and high -income countries was examined, with another dig at the public health significance of these organisms in the water environment. The concluding part dwells heavily on the management and control strategies from the authors' perspective, and this includes the one-health approach and the enactment of Government policies to control the indiscriminate discharge of untreated wastewater from the healthcare settings into receiving water bodies.

Keywords: wastewater, hospital wastewater (HWW), healthcare facilities, methicillin resistant *Staphylococcus aureus* (MRSA), aquatic environment

1. Introduction

Wastewater is any water whose quality has been lowered as a result of anthropogenic influence, and could be from several sources including agricultural, domestic, pharmaceutical, and hospitals. Wastewater from the hospital environment comes from various places hence the composition could vary. These places include the

surgical areas, administrative blocks, laundries, laboratories, wards and the kitchens [1]. These wastewater contain a lot of hazardous and potentially dangerous chemicals compared to the urban wastewater. Most of these compounds are persistent and potentially toxic and could include radionuclides, disinfectants, antiseptics, quaternary ammonium compounds (QAC), solvents, remnants of drugs, and some antimicrobial compounds at various concentrations [2–4]. Verlicchi et al. [5] in a review, reported that the concentrations of antibiotics, analgesics and metals (micropollutants) in HWW are between 4 and 150 times higher than in urban wastewater, making HWW a hub of several toxic agents. In addition to this, hospital wastewater (HWW) is also a repository of antibiotic resistant organisms and resistance genes, as reported in a study by Adekanmbi et al. [6] on the diversity of resistant *Escherichia coli* and their genes in wastewater of a University Health care center. Wastewater and other receiving water bodies receiving input of HWW are pre disposed to pathogenic organisms, making them a threat to the existence of aquatic organisms and also the health of the human population. In this chapter, we try to explore the threat posed to the aquatic environment via the release of methicillin resistant *Staphylococcus aureus* and proffer some solutions in curbing the menace.

2. Wastewater from healthcare systems (composition and microbiology)

Wastewater is any water from different processing and manufacturing operations such as pharmaceuticals, agriculture, domestic sources, and healthcare centres, whose quality have been tainted by a high level of anthropogenic influence [7]. The hospital wastewater is however quite different from wastewater from other sources because it contains a vast number of micro- and macro- pollutants, which have been discharged from the different departments of hospitals including laboratories, theaters, laundries, research units and other notable sections [8]. Most of the pollutants in questions range from pharmaceuticals, chemical compounds, metals, media remnants, antibiotics, disinfectants, radioactive isotopes, and stock cultures [9]. In some instances, some pharmaceuticals present in hospital wastewater have been implicated in causing the disruption of the endocrine system, impairment of the reproductive system and sex reversal in some aquatic species [10]. The discharge of this wastewater has led to the build-up of nutrients in the receiving aquatic ecosystem, leading to eutrophication [7].

In many countries of the world, wastewater from the healthcare settings are discharged into sewage channels without any form of pre-treatment, after which it undergoes treatment in the municipal wastewater treatment plants, but in most instances, the treatment is not sufficient to remove these pollutants from the wastewater [11]. Another worrisome situation is the fact that pharmaceutical compounds present in the wastewater could undergo transformation and form conjugates, whose toxicity could be higher than that of the parent metabolite [12].

The HWW can act as an ideal medium for the proliferation of various classes of microorganisms e.g. viruses, bacteria, fungi and other parasites. Wastewater generated from the operations of the healthcare facilities is also a hub of bacteria showing resistance to several classes of antibiotics and antibiotic residues, which could cause an inhibition of the sensitive bacteria, thereby causing an elevation of the population of the resistance bacteria in the receiving water channels. These resistant bacteria could also act as vectors for the transmission of genes, or serve as vehicles and reservoirs for the proliferation of antibiotic resistance genes (ARG), that could pose a potential threat to public health [13].

HWW poses a very challenging threat to humans, society, hospital employees, patients, public health and the environment at large, as it has been implicated in the spread of infective diseases, and could also be a vehicle in terms of contagiousness [14]. A very worrisome constituent of HWW are the residual drugs. When medications are consumed by patients, the drugs are not fully metabolized by the human body, and residual concentrations are excreted into sewage or other receiving water sources via urine and feces. These residual quantities of these antimicrobial compounds act as stimulants for the onset of resistance in the microflora of the water or wastewater, thus leading to an increase in the population of potentially pathogenic strains of organisms [15]. These pathogenic organisms, which could be viruses, bacteria, algae, yeasts, protozoa, parasites and sometimes bacteriophages could survive for a long time in the receiving soils or water, and could eventually find their way into the food chain, causing infectious diseases and so many health risks to humans [16].

2.1 Wastewater from the healthcare facilities: a stimulant for antibiotic resistance

The treatment procedures for wastewater do not completely remove antibiotics, which increases the amount of antibiotics in aquatic ecosystems [17, 18]. The observed amounts of many antibiotics in surface water range from 0.001 to 484 g/L globally [19]. According to studies, the use of antibiotics in aquatic environments is linked to an increase in the number of antibiotic resistant bacteria (ARB) and the emergence of resistance genes [20]. Horizontal gene transfer (HGT) and mutagenesis in bacteria are influenced to a large extent by sub-inhibitory doses/concentration of antibiotics in aquatic systems [21]. As a result, a number of pathogenic microorganisms have developed resistance to the most potent medicines, and it is unclear how quickly new antibiotic resistant microorganisms emerge. Antibiotic resistant microorganisms disseminate resistance genes in the environment and transmit them to the following generation [22].

Clinical sewages have long served as important sources of antibiotic resistance determinants in aquatic ecosystems due to the usage of antibiotics in hospitals, particularly due to the excretion of their powerful forms into the environment [23]. Previous research revealed that HWW contains significant amounts of microorganisms and antibiotic drug residues, which has the ability to exert selective pressure on the spread of antibiotic resistant bacteria [24]. Consequently, compared to other wastewater systems, such as urban sewage systems, HWW is likely to pose greater hazards of the spread of ARGs [25, 26]. Therefore, hospitals and other healthcare settings are considered as one of the leading polluting sectors around the world [27]. Hospital wastewater treatment facilities in particular are regarded and best defined as the epicenter and major location for the spread of antibiotic resistance that could endanger public health if water is reused [28, 29].

Large amounts of antibiotics and other substances can impose selective pressure at low concentrations (below therapeutic levels) in pharmaceutical wastewaters from pharmaceuticals and healthcare wastewater [30, 31]. Studies have demonstrated that wastewaters produced during the manufacture of pharmaceuticals are reservoirs of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs), and they may hasten the potential horizontal transfer of environmental resistance determinants across the endogenous microbial community [30, 32, 33].

Antibiotic-resistant bacteria (ARB) and their functional metabolites, as well as resistance genes (ARG), are frequent and pervasive pollutants in many ecosystems as a result of years of antibiotic misuse and overuse [34, 35]. Excreta from people and

animals, as well as wastewater, are acknowledged and documented as primary sources of the microorganisms and chemicals mentioned above [34]. Despite the fact that treatment methods can lower pathogen concentrations in wastewater, wastewater treatment plant effluents do not generally show a significant reduction of ARB and ARG, and a lot of these pathogens, possibly from hospitals, are released into the recipient waterways [28, 36].

According to recent reports, the environment contains other key sources of antibiotic resistance, such as animal farms, wastewater treatment facilities [WWTPs] [32, 37, 38]. Since antibiotic resistance genes are now regarded as environmental contaminants, it is evident that their future dissemination must be prevented [39, 40]. To enable this, it is necessary to clarify their potential reservoirs, particularly those found in the environment. A major global public health concern is the prevalence of antibiotic resistance genes (ARGs) in the environment due to the extensive use of antibiotics in healthcare systems, agriculture, and breeding [41, 42]. Long-term trends show that the use of antibiotics is increasing, thus stagnation or decline is not anticipate [43]. To prevent the establishment of resistance, research is already pointing to the potential use of novel medications in combination with nanotechnologies. Different nanomaterials having antibacterial properties based on carbon, titanium, silver, or gold are used in several new technologies [44].

In low- and middle-income countries (LMIC), where many hospitals either lack wastewater treatment plants or have inadequate ones, the hazards of such clinical illnesses may be more serious. To make matters worse, surface water is frequently used for home and agricultural uses, or even ingested untreated, especially in rural regions [23]. Antibiotic resistance genes are disseminated in such waters and have been reported to be more widespread in environmental non-pathogenic microbial populations than was originally believed [45, 46]. According to reports, these resistances spread among bacterial populations in two main ways: vertical gene transfer (during bacterial cell division) and horizontal gene transfer, or conjugation, transformation, and transduction, supported by mobile genetic elements (MGEs) [47]. The diversity, distribution, and future of ARGs in urban water systems are still unknown, despite the fact that antibiotic resistance is widely acknowledged as a major danger to human public health [48].

Regulations on sludge/sewage emission criteria have been adopted globally since the 1980s in an effort to reduce the harm caused by effluent after it is discharged [49]. However, only a few countries (such as France and Italy) have set up laws governing hospital wastewater (HWW) treatment before release [50]. Unfortunately, no emission standard for wastewater has required the biological safety assessment of ARGs [51]. The current condition results in a significant biosafety risk of ARGs from HWW, which has been largely disregarded by legislation and contemporary wastewater treatment facilities [51].

2.2 Wastewater from healthcare facilities: a reservoir for the introduction of MRSA into the aquatic ecosystem

Hospital wastewater poses a significantly greater environmental threat than urban effluent [52]. The hospitals use an enormous quantity of water each day, ranging from 400 to 1200 liters, and produce a sizeable amount of wastewater each day in their operations [53]. These facilities are also renowned for the wide range and high rates of wastewater pollution in aquatic environments [54]. Hospitals are well known for their excessive and ongoing usage of antibiotics [55]. According to Bui *et al*. [56], between 30 and 90% of antibiotics are not absorbed by the human body; and as a result, they

are discharged directly into effluent and build up in wastewater treatment systems. A significant global concern shared by many scientific researchers is the possibility that bacteria could come into contact with antibiotics and develop antibiotic resistance due to this situation [57]. To develop efficient solutions to stop the emergence and spread of this issue, a worldwide strategy is necessary.

Most antibiotics used are excreted into wastewater, where they may sustain or impose selective pressure on microorganisms culminating in resistance development [58]. In wastewater, antibiotic resistant bacteria and genes are frequently found in more significant numbers and concentrations than in surface water [59]. Additionally, wastewater can foster the development of a diverse bacterial community that serves as a breeding ground for bacteria that are resistant to antibiotics. As a result, it has been hypothesized that wastewater treatment facilities contribute to the spread and evolution of antibiotic-resistant microorganisms. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a significant issue globally as a nosocomial pathogen. Still, less is known about its incidence in non-clinical settings, such as wastewater, and what role wastewater has in spreading and developing MRSA in aquatic ecosystems. *Staphylococcus aureus* is a bacterial pathogen linked to various human infections, such as skin infections, pneumonia, and septicemia [60]. Due to the strains' frequent resistance to one or more antibiotics, particularly methicillin, infections caused by these bacteria can be challenging to cure. Since its discovery in 1960, infections caused by methicillin-resistant *S. aureus* (MRSA) have predominantly been linked to hospital settings and are called hospital-acquired MRSA [61]. MRSA, just like extended spectrum β-lactamase (ESBL)-producing Enterobacterales, has historically been a cause of nosocomial infections, but is now becoming common place even in non-clinical settings [62]. On top of that, MRSA can be found in wastewater from sewage treatment facilities (STPs) [63]. *Staphylococcus aureus*, including MRSA, has been the subject of numerous surveys in Europe and the United States as an indicator bacterium in wastewater and river water [64].

2.3 Epidemiology of MRSA in wastewater from healthcare facilities in low-middle- and high-income countries

Antimicrobial-resistant bacteria are increasingly causing environmental water pollution issues on a global scale [65]. In addition to making antibiotic treatment challenging, the appearance and spread of Antimicrobial-resistant bacteria have become a significant issue for hospitals and other healthcare facilities [66]. This is because it increases the danger of epidemics and severe outbreaks of infectious illnesses. The World Health Organization (WHO) released a list of 12 particular groups of antimicrobial resistant bacteria (AMRB). Methicillin-resistant *Staphylococcus aureus* (MRSA) is listed as a high-priority bacterium among these AMRB, following carbapenem-resistant *Acinetobacter baumannii*, carbapenem-resistant *Pseudomonas aeruginosa*, and carbapenem-resistant/third generation cephalosporin-resistant Enterobacterales that are listed as critical priorities [67]. One of the most significant aspects of the epidemiology of MRSA is the global onset and dissemination of the disease caused by these pathogens. As seen in **Table 1**, many countries have reported the spread of all MRSA subtypes. MRSA is one of the most common nosocomial pathogens currently and is known to be more prevalent in hospital settings. The Centre for Disease Control and Prevention (CDC) reported that MRSA is a serious concern to public health due to its rising frequency in hospitals, the general population, and animals, as well as its transmission between people and animals, infection rates, resistance, and therapeutic challenges [77].

Year of study	Prevalence	Source	Countries	Region	Reference
2022	94–96%	Hospital effluent and Healthcare facility effluent	Japan	High-income country	[67]
2021	97%	Hospital effluent	Japan	High-income country	[68]
2013	70–81%	Regional hospital and Metropolitan hospital	Australia	High-income country	[69]
2019 to 2020	37.5%	Hospital wastewater	Japan	High-income country	[70]
2012	10%	Hospital wastewater	Ethiopia	Low-income country	[71]
2020	100%	Hospital wastewater	Bangladesh	Low-middle-income country	[72]
2015	90%	Hospital effluent	India	Low-middle-income country	[73]
2017	11%, and 8%	Raw and Treated hospital sewage water	Iran	Low-middle-income country	[74]
2019 to 2020	46.9%	Hospital wastewater	Portugal	High-income country	[75]
2015	47%	Hospital wastewater	Iran	Low-middle-income country	[76]
2015	53%	Hospital wastewater	Iran	Low-middle-income country	[76]

Table 1.
Prevalence of MRSA in wastewater from healthcare facilities from different countries.

2.4 Public health challenges of MRSA in the aquatic environment

The ubiquitous bacteria- *Staphylococcus aureus* - causes a wide range of infections, from minor skin infections to serious and potentially fatal invasive diseases [78]. The bacterium could invade the skin, mucosal membranes, and internal organs and cause severe sickness in both humans and animals, including septicemia, osteomyelitis, endocarditis, respiratory tract infection, and suppurative infections of the skin [79]. Additionally, *S. aureus* is one of the main causes of mastitis in cattle [80]. The environment of hospitals and the humans are both well suited to *Staphylococcus aureus*. It is a major factor in endocarditis, bacteremia, osteomyelitis, and infections of the skin and soft tissues. *S. aureus* swiftly became a major cause of infections related to health care as hospital-based medicine took off [81].

The treatment of bacterial illnesses relies heavily on antibiotics. As the number of bacteria that are resistant to antibiotics increases, there are less and fewer drugs that can effectively combat certain infections. Bacteria develop resistance mostly by horizontal gene transfer and genetic alterations, which allow infections to flourish in the environment while antibiotics are present [82, 83]. Hospitals and other settings are increasingly encountering multiple drug resistant strains, causing hazardous illnesses for people [84].

Within the next 30 years, the world's population is expected to reach 10 billion, and agricultural production is expected to rise by 70%. This will further put pressure

on freshwater supplies [85]. Nearly 50% of the world's population use contaminated water sources to irrigate crops, and it is estimated that 20 million hectares are watered with wastewater [86]. Many cities throughout the world with historically low rainwater collection have used wastewater in agriculture for generations. It is also becoming a more important alternative source of water in nations mostly affected by water scarcity, particularly in those that depend on agriculture for a living. Re-using untreated wastewater is one of the few accessible options to the sophisticated procedures used in the majority of wastewater treatment facilities in high-income nations for many low-income countries [87]. High levels of pathogens, pharmaceuticals, heavy metals, plastic additives, and other contaminants can be found in wastewater, and these contaminants might negatively affect plant growth when wastewater is used for their irrigation [88].

Pharmaceuticals, personal care items, antibiotic residues, antimicrobial resistant bacteria (ARB), and antimicrobial resistance genes (ARGs) are contaminants of particular concern [89]. Antibiotics have been detected in treated wastewater effluent and ARB/ARGs can withstand or even proliferate at treatment plants [90, 91]. Wastewater irrigation can result in ongoing antibiotic exposure for the irrigated crops, which can cause the establishment of resistant bacteria. ARGs can be transferred between native soil communities and wastewater bacteria via irrigation-delivered ARB in wastewater [92].

The following vegetables can be eaten raw: carrot, radish, cucumber, tomato, cabbage, lettuce, coriander, and. Food-borne illnesses could occasionally develop from improper washing and peeling, which can act as a vehicle for several germs. Numerous microorganisms can infect people through the oral route [93]. For instance, since lettuce is not processed before consumption, all of the (resistant) bacteria that are present in it may be directly ingested by consumers [94]. Because they are necessary components of our diet and are frequently eaten raw or with minimal preparation, fruits and vegetables can be a major source of human pathogens [95]. Market garden items are frequently thought to be contaminated by irrigation water [96]. The fact that they come from market gardeners who have already engaged in self-medication by treating infections brought on by their working tools, can explain the presence of multidrug resistant *Staphylococcus aureus* [97].

Surface waters have been noted as possible antibiotic and antibiotic resistance reservoirs [98, 99]. In numerous countries, research have found genes and pathogens associated with antibiotic resistance in lakes, rivers, streams, ponds, and estuaries. A possible risk of human exposure to resistant bacteria exists since some of the surface waters that contain antimicrobial resistance genes (ARGs) are used for recreational purposes [100]. Antibiotic residues, heavy metals, natural processes, and climate change are the causes of antibiotic resistance in surface waterways, whereas healthcare facilities, wastewater, agricultural settings, food, and wildlife populations are the main vehicles [101, 102]. For hospitals and other medical facilities, the formation and spread of antimicrobial resistance has become a critical issue since it makes antimicrobial treatment challenging and raises the danger of epidemics and severe outbreaks of infectious illnesses [103].

Penicillin provided temporary relief, but in the 1940s, resistance developed through the β-lactamase gene *blaZ*. Around 1960, the first semi-synthetic anti-staphylococcal penicillins were created, and within a year after its initial clinical application, methicillin-resistant *S. aureus* (MRSA) was discovered [81]. Since an emergence of these strains has been noted, the World Health Organization (WHO) now recognizes MRSA as a high-priority pathogen [104].

Resistance to methicillin and oxacillin is caused by the acquisition of a gene that encodes a PBP2 homolog termed PBP2a that is resistant to drug activity [105, 106]. The peptidoglycan produced when an MRSA strain is cultured in the presence of β-lactams is not well cross-linked. If the MRSA strain is exposed to β-lactams, one outcome of this is that the peptidoglycan has higher proinflammatory effects, which may lead to pathology during infection [107]. The *mecA* gene, which is part of a family of various but connected staphylococcal chromosomal cassette (SCC) elements, encodes PBP2a [105, 108] while *MecC*, a unique PBP2a with just 63% residue identity to *MecA*, was recently found. In Europe, it mostly affects one lineage of MRSA [109]. Some MRSA strains have spread across the globe, while others are endemic to specific geographic areas [110]. In the original MRSA strains, drug exposure is the causative factor for the *mecA* gene to be expressed. MecIR regulatory proteins, which are related to the BlaIR proteins that govern *blaZ* expression, are in charge of it [105, 106].

Antibiotic-resistant *S. aureus* has also been isolated from municipal water supplies both domestically and internationally [111, 112], in the hospital [69], and agricultural wastewaters/sewage [112, 113], depicting potential sources of contamination of the human environment. According to estimates, 1.8 billion people, mostly in underdeveloped nations, drink contaminated water [114]. Humans are exposed to contaminated surface water as a result of contaminated rivers and lakes' role in the release, mixing, and persistence of antibiotic-resistant bacteria (ARB) and their antibiotic resistance genes (ARGs) [115].

2.5 Management and control strategies

The World Health Organization (WHO) has urged countries to create a Global Action Plan on Antimicrobial Resistance, a framework for an action plan for AMRB, and has argued that comprehensive measures should be taken to assess and resolve the issues involving AMRB, taking into account their interactions among people, animals, and the environment based on the fundamental principle of One Health [116]. These countermeasures include Clarification of the pollution status and an evaluation of the environmental danger of MRSA in aquatic environments: For life to exist, there must be water. Additionally, more medicines and residues are discovered in the ambient compartment. Water pollution is one of the critical issues associated with water, along with water scarcity and floods. Drug residues and resistant microbes can be found in large quantities in hospital wastewater, but the situation is much more severe in numerous countries. In LMIC, where drug use is more prevalent, the Sustainable Development Goals are critical [117]. However, according to the United Nations [102], over 80% of the world's wastewater is dispersed into the environment without sufficient treatment. Water recycling is likely necessary to address water shortages and advance a circular economy, but the quality of the recycled water must be ensured. As part of the Registration, Evaluation, Authorization, and Restriction of Chemicals Program (ECREARCP) of the European Commission, ecotoxicological evaluations based on bioaccumulation tests are crucial for determining the environmental risk of chemical compounds. In order to assess the risk that prospective water pollutants pose and, consequently, whether specific restrictions should be imposed, the Surface Water "Watch List" under the Water Framework Directive (WFD) is a system used in Europe. For the benefit of other regions of the world, especially the LMIC, this list should be updated every 2 years and made available.

2.5.1 Surveillance

As part of the hospital infection control program, surveillance must be conducted regularly and a recognized component of the clinical governance process. For benchmarking purposes, surveillance data should be gathered and published consistently, using agreed-upon case definitions and specialty activity denominators, with case mix stratification. Most hospital staff should easily understand surveillance data, which should be discussed often at hospital senior management committees and in regional infection control training. The results of microbiological tests conducted for clinical purposes, as well as the results of those investigations conducted for screening purposes, should all contain MRSA monitoring if there are any requirements in the hospital state rules.

2.5.2 Screening

MRSA carriage should be actively screened for in hospital wastewater and patient samples, and the findings should be connected to a focused strategy for isolation and facility cohorts. It is essential to routinely check hospital wastewater that poses a high MRSA risk. The infection control team must conduct local screening of the hospital wastewater, discuss it with the necessary clinical teams, and receive approval from the pertinent hospital management structure. This will affect the risk status of each hospital wastewater, the local prevalence of MRSA in the wastewater, and the propensity for MRSA to be present in the wastewater.

2.5.3 Antibiotic stewardship

Many hospital staff members need to be made aware of antibiotics' use and side effects. Identifying key personnel in charge of monitoring antibiotic resistance and consumption, prescriber education, and the effects of antibiotics in wastewater on aquatic life and the community are critical elements of implementing antibiotic stewardship programs in healthcare facilities. The prevalence of MRSA in hospital wastewater can be decreased by avoiding inappropriate or excessive antibiotic therapy and prophylaxis, making sure that antibiotics are administered at the correct dosage and for the right amount of time, and only the proper antibiotics in cases of infection.

2.5.4 One health approach

One Health Approach is founded on the idea that treating each issue separately will prevent us from understanding the interconnectedness of human, animal, and environmental health. We need a comprehensive approach to grasp these domains' interdependence to tackle complicated public health concerns. However, what impact would One Health have on our healthcare system? The community's dynamic microbiological inputs from people and animals are included in hospitals as incubators. The use of antibiotics places selection pressure on these incoming microbial populations, causing a change to a more significant proportion of resistant organisms. Hospital-associated multidrug-resistant organisms (MDRO) can colonize people while they are in the hospital (both patients and staff). A feedback loop is initiated when they are released back into the community. The hospital serves as a surveillance site and a multiplier for resistant organisms and infections due to MDRO acquisition and infection, which further emphasizes the need to describe community- and hospital-based

risk factors that affect the hospital environment. By considering the interaction between the patient, the hospital equipment, the hospital environment, and the role of the community, a One Health approach may help create unique research and multimodal intervention approaches. This includes well-known local risk factors for MRSA colonization in hospital wastewater, such as patients, pet ownership, or residing in an area with an active livestock industry. The complexity of hospital infection control warrants a multidisciplinary approach. An integrated approach is required to direct research avenues and public policy mediation.

3. Conclusion

This write-up further outlines the public health challenge associated with the discharge of untreated wastewater from healthcare facilities into the environment. This act not only introduce potentially pathogenic microorganisms into the environment, it also serve as a medium for the dissemination of the antibiotic resistant strains of which MRSA is a major threat. There is an urgent need to put mitigation protocols in place to prevent a potential public health breakdown as a result of the unwholesome act of discharging wastewater into receiving water bodies and the environment at large without any form of treatment. Relevant agencies in countries affected by this menace should wake up to their responsibilities and carry out enlightenment campaigns to educate hospitals and the populace on the danger inherent in such practices. A stitch in time saves nine.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 2

War against ESKAPE Pathogens

Safiya Mehraj and Zahoor Ahmad Parry

Abstract

ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter species*) are the prominent reasons of Healthcare-Acquired Infections (HAIs) with multidrug resistance against vancomycin, carbapenem, methicillin, and extended spectrum β-lactamases (ESBL). Multidrug resistance develops owing to inappropriate prescription, poor quality pharmaceuticals, patient non-compliance, and use of antimicrobials as growth promoters. The worst is the fact that resistance development and spread are continuous processes to the extent that present times are times of extensively drug resistant and totally drug resistant pathogens (confirmed worldwide). These dangerous pathogens pose global threat of the magnitude to the extent of reversing the situation to pre-antibiotic era as they have left majority of efficient antibiotics futile and estimates show expected death rates are 10 million/year by 2050. Considering this global havoc due to ESKAPEs intensive research from academia and industry is going on with significant success about the causes, mechanisms, spreading ways, and most importantly the novel/alternative strategies to combat them all. Substitute therapies such as combination use of antibiotics or immunomodulators/adjuvants with antibiotics, nanoparticles, antimicrobial peptides (AMPs), AMPs with antibodies, star polymers, and structurally nano-engineered antimicrobial peptide polymers (SNAPPs) all these aspects are well discussed and reviewed here.

Keywords: *Acinetobacter*, *Enterococcus*, *Enterobacter*, *Enterobacterales*, *Klebsiella*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, antibiotic resistance, multidrug resistance

1. Introduction

Antimicrobial resistance (AMR) is a wide-ranging global menace and declared by World Health Organization (WHO) as one among top 10 global public health concerns. WHO made a nerve-racking forecast that by the year 2050, infections due to drug-resistance, mainly heightened through the Misuse and overuse of antimicrobials [1], will exterminate approximately 10 million people per annum that will go up in flames of financial catastrophe and in turn entail severe poverty upon millions of people [2]. ESKAPE pathogens are a faction of bacteria including Gram-positive and Gram-negative bacteria, namely, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and

Enterobacter species. With the appearance and widespread of antibiotic-resistant pathogens due to diverse mechanisms of resistance acquired by bacteria, intimidate our capacity to treat infections particularly frightening is the hurried worldwide stretch of multi-resistant and pan-resistant bacteria (also known as "superbugs") causing infections which are untreatable with the already accessible antimicrobials. Bacterial genome analysis made a remarkable conclusion that there is a scarcity of effective antimicrobials as more than 20,000 impending resistant genes have been reported [3] and the number is predictable to be higher in the coming years. In both the developing and developed countries the ESKAPE bug infections are growing in a similar manner [4, 5]. The possible reasons accountable for the widespread of AMR in the community and hospitals is the malnourishment, poor sanitation practices that are responsible for the preamble of antibiotics which are not metabolized into the environmental milieu through animal and human waste [6], unsystematic use of various antibiotics in agricultural practices that comprises of growth promoters and likewise in animal and human medicines [7, 8], in developing countries, the improper regulation over the contradict antibiotics as they are effortlessly accessible without proper medical prescription [9], poor hygienic conditions. Physicians recommend mammoth number of antibiotic combinations devoid of taking into account its side effects. With an over view to combat AMR, the disease which can be treated easily with a single dose antibiotic regimen, is compelled to be treated with high dose combinations to which the bacterium is not susceptible, and the inadequate and overuse of antimicrobial therapy in humans, animal farming, and agriculture is the main driver of AMR [10]. Among ESKAPE pathogens, vancomycin-resistant Enterococcus (VRE), extended-spectrum β-lactamase producing (ESBL) *Escherichia coli,* and methicillin-resistant *Staphylococcus aureus* (MRSA), are frequently seen. And they have gained popularity as they wield resistance in healthcare set-ups against various antimicrobial agents. A correlation of resistance linking the frequency of biofilm formation with host-immune responses has already been recognized [11, 12]. The various resistance conferring mechanisms in bacteria to approximately existing antibiotic classes are extensively studied and described in various literatures [13–15]. The possible mechanisms for resistance include altered permeability of membrane, antibiotic degradation by enzymes, efflux pumps over expression that abolish antimicrobials actively [16–18]. In countries reporting to the Global Antimicrobial Resistance and Use Surveillance System (GLASS), the frequency of ciprofloxacin resistance, which is used to treat urinary tract infections, speckled from 4.1% to 79.4% for *Klebsiella pneumoniae* from 8.4% to 92.9% for *Escherichia coli* [19]. In *E. coli*, the Resistance to antibiotic fluoroquinolone, used for urinary tract infections, is extensive [20]. Carbapenem resistant Enterobacteriaceae (i.e., *E. coli, Klebsiella*, etc), responsible for causing life-threatening infections, colistin seems to be the merely last choice treatment [21]. Whilst in several countries, bacteria resistant to colistin causing infections have been detected for which there is no efficient antibiotic treatment at present [22]. In the community as well as in health-care facilities the *Staphylococcus aureus bacteria which is a* component of our skin flora is a general cause of infections. People with drug-sensitive infections are less prone to death as compared to People with methicillin-resistant *Staphylococcus aureus (MRSA*) infections which are 64% more expected to die (WHO report 2021). A new AMR indicator, In the SDG monitoring framework was incorporated in the year 2019, which monitors the rate of various bloodstream infections due to two

distinct antibiotic resistant pathogens: Resistance of *E. coli* to third generation cephalosporins (3GC), and methicillin-resistant *Staphylococcus aureus* (*MRSA*). In 2019, the data provided to GLASS on blood-stream infections owed to MRSA the median rate observed for MRSA was 12.11% (Interquartile Range {IQR} 6.4–26.4) by 25 countries, areas, and territories and the data provided by 49 countries on blood-stream infections due to *E. coli* resistance to cephalosporins third generation was 36.0% (IQR 15.2–63.0) and the data was still at halt to be presented nationally (WHO report 2021). The control and management of gonorrhea is halted by the extensive spread of highly variable and resistant strains *Neisseria gonorrhoeae*, Extended-spectrum cephalosporin (ESC) ceftriaxone which is injectable is the only left behind empiric monotherapy for gonorrhea in various countries [23, 24]. Widespread antibiotic resistance emerged to various classes of antibiotics like penicillins, macrolides, tetracyclines, fluoroquinolones, sulphonamides, and early generation cephalosporins has increased dramatically [6, 25–27]. There is a surge in Antibiotic resistant *Mycobacterium tuberculosis* strains. As per WHO report 2021, 1.5 million people died owing to Tuberculosis (TB). Almost half a million new cases of rifampicin-resistant TB (RR-TB) were identified globally, among which the majority have multi-drug resistant TB (MDR-TB), a form of tuberculosis resistant to the two most potent anti-TB drugs [28]. In present Scenario, at least 700,000 deaths annually are caused due to drug-resistant infections, the World Health Organization published a report in 2019 stating that, if no action is taken, the figure is expected to increase exponentially to 10 million deaths annually by 2050, surpassing cancer, diabetes, and heart disease, as the primary catastrophic cause of death in humans [29]. Hence, stern actions are required to curtail the widespread of strains resistant to antimicrobials as they impose a key challenge to global public health. Therefore, antibiotics in conjunctions, antimicrobial peptides (AMPs), nanomaterials, phages, synthetic chemicals, photodynamic light therapy and integrated multi-omics have been surfaced as an substitute method [30, 31]. AMPs with backbone of amino acids are the host defense peptides which are natural and can be used as a potential alternative candidate to the existing conventional antimicrobials responsible for resistance [32]. Despite of being a powerful weapon to eradicate resistance these AMPs also face drawbacks like: proteolytic susceptibility, toxicity, poor profile of pharmacokinetics, etc. Encapsulating these AMPs in the development of nanomaterials and nanocarriers helps in increasing efficiency of AMPs at the target site and decreasing the cytoxicity and degradation [33, 34]. Due to potential therapeutic efficacy and momentous advantages, structurally nanoengineered antimicrobial peptide polymers (SNAPPs) and the star polymers are used to carry the AMPs [35]. Antimicrobial peptides (AMPs) with diverse mechanisms of action (MOA) and effective antimicrobial activities are measured as significant substitute to solve the problem of multidrug resistance [36].

2. Bacterial structure and antimicrobials mechanism of action

The bacterial cytoplasm is strewn with DNA material and ribosomes, however there are no structured organelles. DNA is single and thread like in appearance, and is compactly folded and organized so that its length which is 1000 times that of the cell itself can be accommodated. DNA gyrase prevents tangling of the DNA

molecule and pedals during DNA replication with regard to folding and supercoiling. Quinolone antibiotics inhibit the DNA synthesis by inhibiting the activity of DNA gyrase; rifampin also hinders the DNA replication process by inhibiting DNA-dependent RNA polymerase [37]. For vital functioning of the cell the chromosomal DNA contains the genetic blueprint; nevertheless, extra chromosomally DNA might also subsist in the cell in the appearance of plasmids. Plasmids are separate from the chromosomes and are circular bodies of double-stranded DNA containing genes that encode for diverse traits, comprising of antimicrobial resistance. In a process of conjugation the plasmids might be transferred from one bacterium to another by means of sex pili [38]. Ribosomes which are nucleoproteins containing the DNA blueprint, allied with long chains of messenger RNA (mRNA) for the process of protein synthesis. In order to allow the amino acids to get linked and initiate protein synthesis, the 30S ribosomal subunit reads the mRNA code, that signals transfer RNA (tRNA) molecules, that carry amino acids, so as to attach to both the 50S and 30S subunits. This process is intervened by the antimicrobials. For instance, 30S subunit gets attached by antibiotic-aminoglycosides so that the erroneous amino acids get inserted into the protein [39]. The 50S ribosomal subunit gets reversibly attached by the macrolides, clindamycin and tetracycline that in turn halt the linking of amino acids. These antibiotics—macrolides, clindamycin and tetracycline are bacteriostatic, even though in some of the bacterial strains macrolides might be bactericidal [40]. The cytoplasm is surrounded by the plasma membrane which acts as the main permeability barrier for the cell. Gram-negative, Gram-positive and fungi all possess this cytoplasmic membrane and rarely few lipophilic, small substances can infiltrate this lipid bilayer, antibiotics—erythromycin and aminoglycosides in order to make their way to ribosomes must cross this lipid bilayer. The cytoplasmic membrane is surrounded by the cell wall that comprises of a sugar (polysaccharide) backbone which is cross-linked by the peptide bonds, the polymer thus formed is mucopeptide, the Peptidoglycan is the precise mucopeptide present in the cell wall. Penicillin-binding proteins and various enzymes that are implicated in synthesis of cell wall are the attachment sites for antibiotic-penicillin [41]. Transpeptidase is the essential PBP, that catalyzes the ultimate cross-link between peptide and sugar in the peptidoglycan molecule, and this cross-link is indispensable for a robust bacterial cell wall. The peptidoglycan and cell wall synthesis is inhibited by β-lactam antibiotics—{cephalosporins, carbapenems, penicillins, monobactams} that bind to the transpeptidase and lead to cell lysis and cell death by triggering the release of bacterial autolysin, are effectual only in opposition to actively dividing bacteria [42, 43]; the tolerance phenomenon wherein mutant bacteria that are lacking autolysins stay susceptible to the β-lactams growth inhibition effect however are resistant to the process of lysis and killing [44]. If antibiotic—tetracycline which is a bacteriostatic agent is given concomitantly, antagonism might be seen. D-alanine gets attached by the vancomycin which is bactericidal against actively dividing bacteria and inhibits the activity of transpeptidase to complete the ultimate cross-linking in the synthesis of peptidoglycan. [45–47]. Cell wall synthesis is also intervened by the antimicrobial—Teicoplanin that get fastened to the nascent Peptidoglycan chain via terminal D-residues, and in this manner inhibiting the cross-linking steps which are crucial for unwavering synthesis of cell wall [48]. When used in combination

with an aminoglycoside, vancomycin becomes effective against *Enterococcus faecalis* [49]. β-lactams and vancomycin which are effective against puncturing of the cell wall are usually synergistic in combination with an aminoglycoside by allowing its way into the cytoplasm so as to target its residues in opposition to enterococci; [50, 51]. The Gram-positive and Gram-negative bacteria diverge in their cell walls as in Gram-negative bacteria there is an extra outer membrane to the cell wall peptidoglycan layer, in Gram-positive bacteria the peptidoglycan layer is thicker; and in Gram-negative bacteria the periplasmic space is present between the cell wall and the outer membrane [52]. Gram-negative bacteria possess the mixed hydrophilic and lipophilic properties in outer membrane that acts as an efficient barricade against various antibiotics. Nevertheless, Porins are the small pores that expand throughout the membrane and permit effortless course for small molecules which are hydrophilic in nature, for instance aminoglycosides, into the periplasmic space. The transport of aminoglycosides across the remaining cell membrane needs electron transport, energy, and oxygen; the absence of these requirements turn bacteria into resistant strain [53]. Likewise, acidic and anaerobic conditions inside abscesses guide towards the less activity of aminoglycosides [48]. The higher the drug concentration of aminoglycosides correlates to efficient rate of microbe killing thus acts as swiftly bactericidal [39]. Gram-negative bacteria is intrinsically resistant to vancomycin which is a big molecule to be passaged via too small porins. The tightly adhered and packed lipopolysaccharide molecules in the outer membrane that turn it somehow hydrophilic obstacles the entry of pencillin like lipophilic molecules. While as amoxicillin and Ampicillin are effectively active against Gram-negative bacteria as they are less lipophilic than penicillin G [54, 55]. Gram-positive bacteria on the contrary are more defenseless to antimicrobial attack as compared to Gram-negative bacteria. β-Lactamase and various exoenzymes that are secreted peripheral to the cell wall of bacteria are inadvertently secreted into the periplasmic space found only in Gram-negative bacteria. The enzyme-β-lactamase will competently render antimicrobial inactive prior to reaching the cell wall as concentration of antimicrobial is low. In this way, Gram-negative bacteria can scrimp and save on the quantity of β-lactamase to be secreted so as to become more effective. On the other side, Gram-positive bacteria should generate large quantities of enzyme, as they secrete the same into the exterior environment, where concentrations of antimicrobial is too high. The folate metabolism is inhibited at two steps by the combinatorial antimicrobial therapy trimethoprim-sulfamethoxazole, that is harmful to bacteria as they need to synthesize their own folate from the precursor—para-aminobenzoic acid [56, 57]. It is postulated that by escalating permeability of bacterial membrane, which renders seepage of bacterial contents, the antimicrobial—polymyxins may exert their inhibitory effects [55]. The daptomycin which is a cyclic lipopeptide causes depolarization of membrane and ultimate death of the bacterium by apparently thrusting its lipophilic tail into the bacterial cell membrane [58]. Hence, the various antimicrobials that are used for the infection treatment caused by bacteria may be categorized according to their key mechanism of actions, and the possible four major modes of action as mentioned aforesaid are as: (i) intervention with synthesis of nucleic acid (ii) inhibition of protein synthesis, (iii) intrusion with synthesis of cell, and (iv) metabolic pathway inhibition (**Figure 1**).

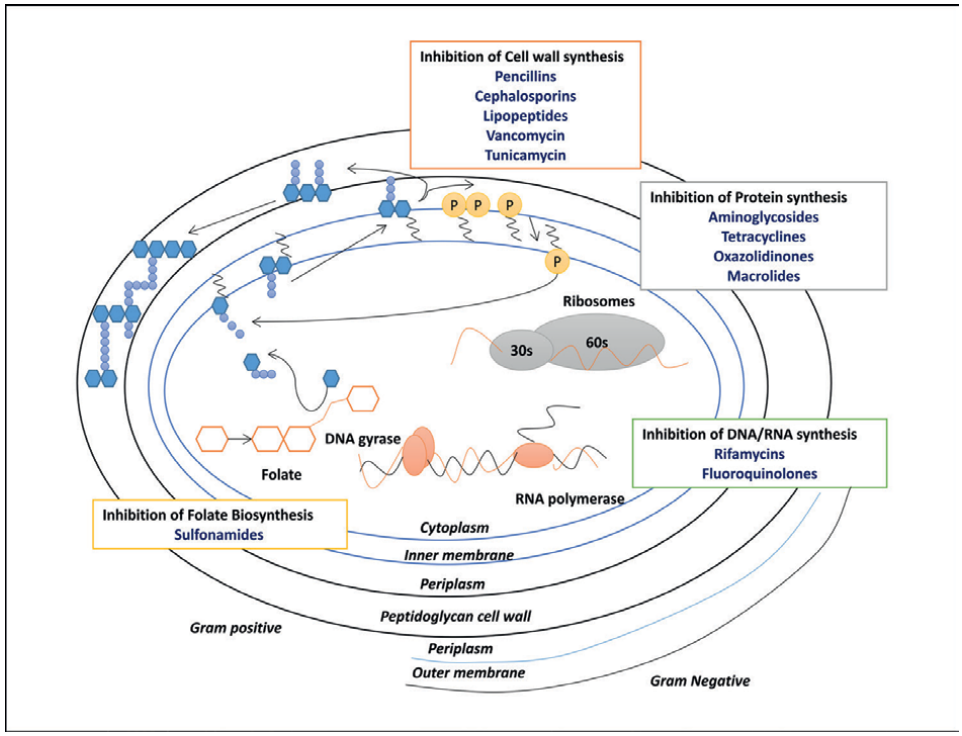

Figure 1.
Schematic representation of major mechanisms of action widely used by antibiotics: antibiotics are medications used to treat bacterial infections. They work by interfering with the growth and reproduction of bacteria, thereby helping the body's immune system to eliminate the infection. There are several major mechanisms of action employed by antibiotics: (i) inhibition of cell wall synthesis: many antibiotics, such as penicillins and cephalosporins, target the synthesis of bacterial cell walls. They inhibit the enzymes involved in building the cell wall, weakening it and causing the bacteria to burst due to osmotic pressure, (ii) inhibition of protein synthesis: antibiotics like macrolides (e.g., erythromycin) and aminoglycosides (e.g., gentamicin) interfere with bacterial protein synthesis. They bind to the bacterial ribosomes, blocking the translation process and preventing the synthesis of essential proteins needed for bacterial growth and reproduction, (iii) inhibition of nucleic acid synthesis: certain antibiotics, such as fluoroquinolones (e.g., ciprofloxacin) and rifampin, target the replication and transcription processes of bacterial DNA or RNA. They interfere with the enzymes involved in nucleic acid synthesis, preventing bacteria from replicating their genetic material and inhibiting their ability to reproduce, (iv) disruption of cell membrane function: some antibiotics, such as polymyxins (e.g., colistin) and daptomycin, disrupt the integrity and function of bacterial cell membranes. They interact with the lipids in the cell membrane, leading to its destabilization and leakage of cellular components, ultimately causing bacterial cell death, (v) inhibition of metabolic pathways: antibiotics like sulfonamides (e.g., sulfamethoxazole) and trimethoprim target specific metabolic pathways in bacteria. They inhibit enzymes involved in the synthesis of essential metabolites, such as folic acid, which bacteria need for survival and reproduction.

3. Antimicrobial resistance: intrinsic, adaptive, and acquired

Bacterial attains antimicrobial resistance and can be as: intrinsic, adaptive, or acquired [59].

3.1 Intrinsic resistance

Intrinsic resistance is the resistance which bacteria can attain due to its inherent properties. For instance impermeability in the outer membrane of Gram-negative bacteria cell envelope is responsible for the glycopepetide resistance. Gram-positive

bacteria are intrinsically less resistant as compared to Gram-negative bacteria due to the presence of outer membrane (OM) in the Gram-negative bacteria, that obstacles the entry of antimicrobials to reach the target site by acting as permeability barrier [58]. Composition of OM, which is an asymmetric bilayer is of phospholipids (internal leaflet), and lipopolysaccharides (LPS, external leaflet) [60, 61]. lipopolysaccharides characteristically includes a short-core oligosaccharide, lipid A, and an O-antigen that can be a stretched polysaccharide. Lipooligosaccharides (LOS) as an alternative of LPS is possessed by some of the Gram-negative microbes for example by members of the genera *Haemophilus*, *Campylobacter jejuni*, *Neisseria.* LPS and LOS share the analogous lipid A structures, but LOS is devoid of the O-antigen units and as such the oligosaccharide is constrained to 10 saccharide units [62]. Small hydrophilic molecules achieve entrance easily via speckled porins on the OM, while as hydrophic molecules passive diffusion is comparably slow. Hydrophilic antimicrobials which are larger in size are debarred efficiently. For example: Despite of being a choice of treatment against methicillin-resistant *S. aureus* (MRSA), Vancomycin—glycopeptide antibiotic which is comparatively larger in size is ineffective against Gram-negative bacteria as it is unable to infringe the Outer membrane permeability barrier. *P. aeruginosa* is resistance against various classes of antimicrobials and also against biocides which are used in disinfectants as it displays number of antibiotic efflux pumps on its surface, additionally the absence of non-specific porins through which antibiotics can permeate via OM [63–67]. Lack of the antibiotic target is the another means of intrinsic resistance to antibiotics. For instance the antibiotics—daptomycin, lipopeptidolactone, which are otherwise effective against vancomycin-resistant *S. aureus* (VRSA), vancomycin-resistant enterococci (VRE), and MRSA, so far are ineffective against Gram-negative bacteria [25, 68]. Gram-positive cytoplasmic membrane has considerably elevated fraction of phospholipids which are anionic than that of Gram-negative bacteria; the composition variance lowers the Ca^{2+}-mediated insertion efficiency of daptomycin antibiotic into the cytoplasmic membrane and thereby decreases the bactericidal efficiency of the antibiotic [69, 70].

3.2 Adaptive resistance

Resistance to one or more antimicrobial agents that is induced by the various environmental stimuli (e.g., nutrient conditions, pH, stress, growth state, sub-inhibitory levels of antibiotics, concentrations of ions). Adaptive resistance is transitory on contrary to intrinsic and acquired resistance. Once the inducing stimuli is impassive, adaptive resistance allows bacteria to react more hastily to the antimicrobial challenges, and usually reverts it back to the original state [59, 71–73].

Adaptive resistance is probably the outcome of epigenetic changes, those results from the change in the gene expression in retort to the changes in environment which in turn is responsible for the formation of irreversible phenotypes. For adaptive resistance to take place, it has been proposed that DAM methylase causing DNA methylation which is responsible for various gene expression profiles that are diverse in the bacterial population and possibly provide epigenetic inheritance of gene expression and heterogeneity for the occurrence of adaptive resistance [71, 74]. Meticulously, modulation in the porins and in the expression of efflux pumps have been concerned with the appearance of adaptive resistance [71, 75]. The elevated resistance with regard to the environmental signal might possibly not be reversed once the signal is retrieved and leads to the steady enhancement of minimum

inhibitory concentration (MIC) with time, when comparing the differences in the effectiveness of an antibiotic *in vitro* and *in vivo*, the adaptive resistance phenomenon may be responsible for the same and can be involved in the antimicrobial treatment failure in the clinics [16, 76]. The capability of microbial populations to propagate in the existence of antimicrobials sub-inhibitory levels via adaptive resistance may permit for enduring and efficient mechanisms of resistance to develop [26, 73]. In response to the external environmental changes the bacteria are facilitated to modify their behavior by the extra mechanisms of adaptive resistance, which is more seen in the persister and biofilm development. Quorum sensing process that is driven by the secretion of various small signaling molecules that allows the microbes to commune is the driver for the biofilm formation. Bacteria are much more resistant within a Biofilm when compared to the free swimming bacteria [77]. If, for instance the initial signal may possibly be approximately translated as: "Is there anybody?", the succeeding revealing of a suitable quorum (cell density) would elicit a amend in the memo to: "Let's reconcile downward and structure a population". At this stage, the bacteria will underwent a significant change from the free swimming, planktonic form distinctive of an acute infection, to the Biofilm mode found in chronic and infections (device-related) due to altered gene expression [78, 79]. In comparison to planktonic microorganism the biofilms which are attached to the surface, and sheathed by a polymer matrix, as whole communities of microorganisms, leave bacteria thousand times more resistant to antimicrobials [77, 80]. Biofilms allows the microorganisms to withstand in exceptionally callous environments as they become more resistant to host immune defenses, biocides, and sheer force [81]. The capability of antimicrobials to inhibit the required cellular proteins for microbial growth is reduced in the subpopulations of cells referred as persisters that stop dividing actively and enter into a quiescent state [82].

3.3 Acquired resistance

A bacterium attains resistance by either mutation or via horizontal gene transfer—from an exogenous source the attainment of new genetic material. The three mechanisms by which horizontal gene transfer can occur [16, 83]. (i) Conjugation: is almost certainly imperative mechanism of horizontal gene transfer. The genetic material is transferred from one cell to another by sex pillus formation by which plasmid is taken by recipient cell from the donor cells. Single plasmid has assembly of various multiple resistance genes that are mediated by mobile genetic elements (integrons, Insertion Sequence Common Region—ISCR-elements, and transposons). In a single conjugation incident these multiple resistant genes facilitate the transport of multidrug resistance. (ii) Transduction: the transfer of the genetic material is transferred among a recipient and donor bacterium by a bacteriophage. (iii) Transformation: In a recipient bacterium Free DNA fragments from a dead bacterium enter and get integrated into its chromosome via genetic recombination. Rarely bacteria are transformable naturally.

Gram-negative bacteria also exhibit explicit acquired molecular mechanisms of resistance to antibiotics [6, 84]. These are classified as: (1) inactivation/modification of antibiotic, (2) abridged antibiotic uptake, (3) antibiotic target alteration, (4) augmented antibiotic efflux. To provide high level of resistance against a specific antimicrobial, in maximum incidents, more than a few of these mechanisms coalesce (**Figure 2**).

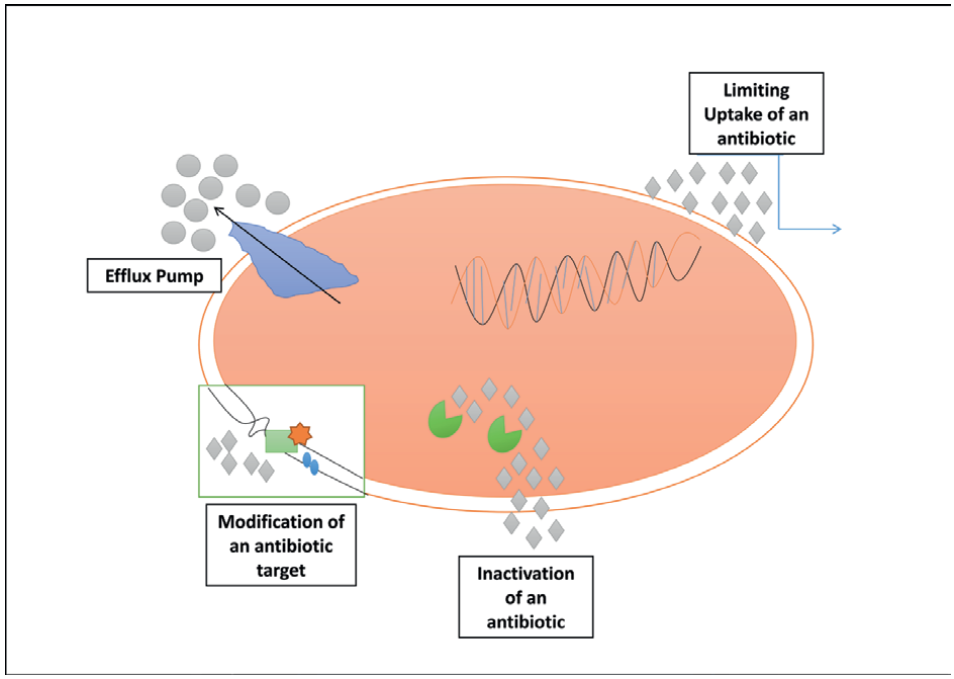

Figure 2.
Schematic representation of general antibiotic resistance mechanisms: antibiotic resistance is a phenomenon where bacteria and other microorganisms develop the ability to withstand the effects of antibiotics. There are several mechanisms through which bacteria can acquire antibiotic resistance. Here are some of the common mechanisms: (i) mutation: bacteria can undergo genetic mutations that result in changes to their DNA, including genes responsible for antibiotic susceptibility. These mutations can alter the target site of the antibiotic, making it less effective. Additionally, mutations can lead to the production of enzymes that inactivate or modify the antibiotic, rendering it ineffective, (ii) efflux pumps: bacteria can possess efflux pumps, which are specialized proteins that pump antibiotics out of the bacterial cell before they can exert their effect. These pumps act as a defense mechanism by expelling the antibiotic from the cell, reducing its concentration and rendering it less effective, (iii) enzymatic inactivation: bacteria can produce enzymes that chemically modify or degrade antibiotics, rendering them inactive. For example, β-lactamase enzymes are responsible for the breakdown of β-lactam antibiotics, such as penicillins and cephalosporins, (IV) altered permeability: bacteria can modify the structure of their outer membrane or cell wall, reducing the permeability of antibiotics into the cell. This prevents the antibiotics from reaching their target sites and reduces their effectiveness.

4. Inactivation of antimicrobials: hydrolysis of β-lactam antibiotics is catalyzed by β-lactamase enzymes

The most often prescribed antibiotics are β-lactams. These antibiotics inhibit the cell wall synthesis by inhibiting the transpeptidase enzymes (penicillin binding proteins; PBPs) that are involved in peptidoglycan strand cross-linking. Autolytic endogenous enzymes under these circumstances are activated via a two-component system VncR/S {is one of the two component systems (TCSs) and is composed of a response regulator 'VncR' and a sensor histidine kinase 'VncS'}, which predisposes the bacterial cell towards osmotic rupture and destabilizes the cell wall [85]. β-Lactam ring is an essential component of β-lactam antibiotics. β-Lactamase enzymes render this ring (four-membered) of β-lactam antibiotics prone to deactivation and hydrolysis so as to overcome these antibiotics. β-Lactamase enzymes are having different activity profiles and are highly diversified. The chief categories of these enzymes are

carbapenems, cephalosporins, monobactams, penicillins, and cephamycins [84]. Regrettably, β-lactam antibiotics resistance is prevalent and escalating swiftly. New Delhi metallo-β-lactamase 1{NDM-1}, is the recently discovered β-lactamase enzyme that is capable of rendering inactive the last line of carbapenem antimicrobials and is almost resistant to all β-lactam antibiotics. NDM-1 is contemplated to have its origin from New Delhi and its swift wide-reaching spread was precipitated by medical tourism [86, 87]. β-Lactam antibiotics are now frequently used in combination with β-lactamase inhibitors (sulbactam, tazobactam, and clavulanate) with an aim to combat the widespread increased issue of bacterial β-lactamases so as to protect the β-lactam antibiotics from hydrolysis and subsequent deactivation [88].

5. Antimicrobial efflux: antibiotic efflux pumps lessen the level of antibiotics inside the cell

Antibiotic efflux pumps are proteins that act by reducing the concentration of antibiotic to sublethal/subtoxic levels by extruding antibiotics from the bacterial cell (periplasm), an intriguing characteristic feature of these efflux pumps is their capability to extrude an extensive variety of different compounds that are structurally diverse [65, 89–92]. This substrate promiscuity is the ensuing development of multidrug resistance in clinical aspects [65, 66, 93, 94]. Antibiotic efflux pump is recognized as the first line defense of the cell mainly in the adverse conditions wherein bacteria is challenged with an antibiotic, the momentary up-regulatory expression of efflux pumps takes place, which lowers the concentration of antibiotic to sub-lethal levels in the cell, which permits the cell survival till a particular mechanism of resistance is achieved. As a result, an active drug efflux pump is mutually sufficient and necessary for the selection of novel drug-resistant mutations [95–98]. Clinically pertinent levels of AMR are conferred by efflux pumps of the resistance-nodulation division (RND) family in Gram-negative bacteria [15, 65, 99]. In Gram-negative bacteria, these span the outer membrane (OM), periplasm, and the inner membrane (IM) to extrude the antibiotics and are complexes as protein assemblies—tripartite in nature [100]. The tripartite drug efflux complexes: MexA-MexB-OprM and AcrA-AcrB-TolC transporters from *P. aeruginosa* and *E. coli* respectively, are the best-studied. The outer membrane proteins TolC/OprM, permit the antibiotic to get transported to the outside of the cell, and the inner membrane fusion proteins AcrB/MexB, also referred as periplasmic adaptor proteins drive out antibiotics from the periplasm or from the cytoplasm by make the most of the proton motive force [100–102].

6. Distorted outer membrane permeability—drop in antibiotic uptake

The dissemination of small hydrophilic antibiotics, for example β-lactams, via outer membrane (OM) of Gram-negative bacteria occurs through porins [103]. As the OM of Gram-negative bacteria acts as first line of defense and permeability barricade. These porins are characterized by a pore {aβ-barrel structural motif} with a inner region which is hydrophilic in nature. Porins either wield substrate specificity or are diffusion porins (non-specific). For instance: ferric enterobactin protein (FepA), which is an iron acquisition porin possess an extra 'plug' domain, which autonomously increases the conscription of the precise cargo [104]. On the basis of interaction and size of the molecule/compound with an inwardly folded loop (loop 3)

which contains charged residues, the diffusion porins are capable to limit cargo [61, 75, 105]. For the intrinsic level of antimicrobial resistance in Gram-negative bacteria the properties of constitutively articulated porins are immensely important. For instance: intrinsic level of resistance to a variety of distinctive antibiotics in *P. aeruginosa is* much higher as compared to the Enterobacteriaceae. As *P. aeruginosa* expresses 'slow' porins with condensed diffusion rates and does not produce lofty permeability classical porins [61]. *P. aeruginosa* expresses numerous explicit porins due to its large genome size, these explicit porins permit the diffusion of small, definite nutrients, while as antibiotics which are bulkier-{Cephalosporins} are not allowed to pass through, and are deactivated by β-lactamase hydrolysis after developing insensitivity [105].

Porins can develop acquired resistance through these possible mechanisms: (i) mutations that renders non-functional via various modifications (for instance: in PenB porin, the amassing of two negatively charged amino acids in the channel-constricting loop 3 of *N. gonorrhoeae* consequences out in drastically condensed permeation of antibiotic-{penicillin} [61, 105, 106]. (ii) Mutations down-regulating the porin expression (for instance: β-lactam resistance to *E. coli* is conferred by the loss of OmpF), and (iii) substitution of small channel size porin with large sized porin (for instance: OmpK36 replaces the large channel porin OmpK35 and is responsible the *K. pneumoniae* isolates resistance to various β-lactams.

In AMR, there is a considerable relationship linking antibiotic-efflux and reduced outer membrane permeability. Collectively, these two mechanisms impart resistance to various classes of antimicrobials such as aminoglycosides, choloramphenicol, erythromycins, tetracyclines fluoroquinolones, etc. Nevertheless, antimicrobial resistance is frequently multi-dimensional, and relies on various molecular mechanisms that operate concurrently. Increased antibiotic efflux and reduced permeability together with the various mechanisms such as target alteration and drug modification confers antimicrobial resistance to the antibiotics mentioned above.

6.1 Antimicrobial modification

Alteration by enzyme alteration of the antibiotic-aminoglycoside is an imperative example of antibiotic modification that is currently the common mechanism of resistance clinically. Gram-negative bacteria (*A. baumannii*, *P. aeruginosa*, and *Enterobacteriaceae* ssp.,) causing infections are treated clinically by aminoglycoside as in treatment of carbapenem-resistant Enterobacteriaceae ssp. causing uncomplicated Urinary Tract Infections [107]. Aminoglycosides act by binding to the 30S ribosomal subunits, 16S rRNA-aminoacyl site where it leads to the misinterpretation of the genetic code and translation inhibition and interferes with the protein synthesis and thereby exert antimicrobial activity [108, 109]. Nevertheless, the aminoglycoside structure left them susceptible to alterations by various enzymes such as aminoglycoside *O*-nucleotidyltransferases (ANTs), aminoglycoside *O*-phosphotransferases (APHs), and aminoglycoside *N*-acetyltransferases (AACs), that can alter the antimicrobial and render it ineffective [110]. The consequential altered antibiotic wherein various aminoglycoside modifying enzymes (AMEs) intercede adenylation, phosphorylation, or aminoglycoside acetylation rendered aminoglycoside with decreased target avidity. AMEs are encoded by genes which are generally positioned in mobile genetic elements (MGEs) allowing them to competently disseminate among bacteria. Through this mechanism, almost all medically significant bacteria can reveal resistance to aminoglycoside [111]. Chloramphenicol resistance is mainly inferred by

the enzymatic acetylation of the antibiotic. In an extensive range of bacterial species various chloramphenicol acetyltransferases (CATs) have been described [112].

6.2 Antibiotic target alteration

When antibiotic has no longer any activity against target as the antibiotics target is changed it is referred as—target alteration. Various classes of antibiotic resistance are caused by this mechanism and are very common. Gram-negative and Gram-positive resistant bacterial strains causing infections are treated nowadays with fourth-generation fluoroquinolones [113]. Here we will discuss the alteration of the target of fluoroquinolones antibiotic. Epidemiological verification suggests a sturdy association between resistance to antibiotic—fluoroquinolones and various other exigent resistance phenotypes. (e.g., *K. pneumoniae* are concurrently resistant to fluoroquinolones producing elevated levels of extended-spectrum β-lactamase (ESBL) [114]. These antibiotics target vital bacterial enzymes, exclusively type II topoisomerases (topoisomerase IV and gyrase) therefore, intervening with the process of DNA replication.

Fluoroquinolones result in the fragmentation of DNA and eventually cell death by interacting with the DNA–topoisomerase complex [115]. Fluoroquinolone affinity for binding is altered by the mutations in the genes *gyrA and gyrB-* (particularly *gyrA*) that led to the substitution of amino acids in the structure of proteins and results in drug resistance [116, 117]. The chromosomal mutations in the bacterial topoisomerase IV and/or gyrase genes is the cause of Quinolone resistance [117].

Likewise the commonest mechanism for resistance to linezolid is due to the gene mutation encoding the domain V of the 23SrRNA. The add up of the alleles which are mutated correlates with the raise in Minimum Inhibitory Concentration (MIC), as bacteria possess various copies of the 23SrRNA genes. Linezolid resistance has been also related to the mutations in L3 and L4 {ribosomal proteins} which margin the binding site of antibiotic—linezolid [118]. In the development of resistance to various antibiotics—streptogramin B, lincosamide and, macrolide, implication of 23SrRNA mutations have been reported [119]. The resistance in the β subunit gene of RNA polymerase is typically accountable for resistance to rifampicin [120, 121]. Similarly, in various bacteria which are of clinical importance, the resistance to sulfonamides, and trimethoprim is due to the recombinational changes/mutations in the dihydropteroate synthase (DHPS) gene or the dihydrofolate reductase (DHFR) gene respectively [122]. Resistance to antibiotics—clindamycin, linezolid, and chloramphenicol is due to the 23SrRNA methylation by an enzyme which is encoded by the cfr gene [18, 112, 123]. Cross-resistance to lincosamides, macrolides, and streptogramin B is due to the 23SrRNA methylation by enzymes, which are encoded by a number of erythromycin ribosome methylase {erm} genes [119].

7. Strategies to combat antimicrobial resistance include

7.1 Use of non-essential target inhibitors

To date, among the various promising approaches that are used to curtail the antibiotic resistance is using antibiotic adjuvants which will hit targets that are non-essential in bacteria. There is a decline in investment by various pharmaceutical companies with regard to the new antibiotic drug discovery in the last few years [124].

The scientific challenges strive towards the fact that since the "golden age" just two new antibiotic classes have made their way into the clinics. Numerous bacteria by now possess the resistance mechanisms against the diverse antibiotics which are in the developmental phase are derivatives of previously accepted antibiotics [125]. To target non-essential pathways so as to reduce the rate of antibiotic resistance the promising success has been achieved with the combinatorial approach of antibiotics or with antibiotic "adjuvants" [126]. Drug combinations and synergy are coming up as appealing line of attack against MDR bacteria and possibly protect the existing antibiotics via the use of adjuvants. Amoxicillin and clavulanic acid combination is so far success story wherein clavulanic acid acts as β-lactamases inhibitor having fragile antibacterial activity and Amoxicillin is an effective β-lactam rendered inactive by β-lactamases, The Augmentin, that was the preeminent-selling antibiotic in 2001 is a result of this union comprising of an antibiotic "adjuvant" together with an antibiotic. Antibiotic adjuvants are molecules that are capable to improve the antibiotic activity thereby minimizing or jamming the mechanism of resistance though they are themselves with fragile or no antibacterial activity. These can expand the antibiotics spectrum of activity by suppressing the intrinsic resistance. In literature, it has been reported that the Gram-negative bacteria causing infections are treated by the usage of Gram-positive selective antibiotics. Where toxicity is a concern this proves to be a good strategy (e.g., colistin). Antibiotic adjuvants render antibiotic molecules potent even at lower doses via enhancing the bacterial susceptibility [127]. Till date, to obstruct the antibiotic resistance three main antibiotic adjuvants have been developed:

7.1.1 Efflux pumps inhibitors {EPIs}

Efflux pumps inhibitors {EPIs} are tiny molecules which are capable to fasten efflux pumps and obstruct their extrusion movement. Efflux pumps can be inhibited by adding drug substrate with new functional group that will impede detection, Intervening with the expression of efflux gene, ability to obstruct the channel and transfer machinery of the pump is disjoined [128, 129]. Various studies that are carried so as to recognize the substrates of efflux pumps and their inhibitors. From accessible antibiotics, the first EPIs were discovered accidentally, the reserpine is the popular one that inhibit the NorA multi-drug transporters, lowering the MIC values by elevating the fluoroquinolone intracellular concentration [130].

Till date, MP-601, is the only documented inhibitor that is presently administered in patients with cystic fibrosis or ventilator-associated pneumonia or as an aerosol [131, 132]. Dipeptide amide, named phenylalanine-arginine-β-naphthylamide is the EPI lead compound that inhibits numerous but not all RND efflux pumps. In ample range of bacteria this have been found to enhance or restore the activities of diverse classes of antimicrobials, which comprises of chloramphenicol, 4-fluoroquinolones, and macrolides [133]. Nevertheless, phenylalanine-arginine-β-naphthylamide and its derivatives are toxic to be included in therapy [134]. Phenothiazine derivatives are other molecules with efflux pumps' inhibition activity and various efforts have been employed to optimize them for therapeutics, phenothiazines enhanced the antibiotic activity of various classes, counting azithromycin, erythromycin, and levofloxacin. This EPIs class are allied to interfere at the inner membrane of the bacteria with the proton gradient [135]. Both *in vitro* and *in vivo*, *M. tuberculosis* efflux pumps activity has been reported to be inhibited by EPIs [136]. Thioridazine (TZ) derivatives with already known anti-tuberculosis drugs, showed efflux inhibitor activity jointly with

the synergistic effect both *in vitro* and with human monocyte-derived macrophages which are infected. In multi-drug-resistant bacterial isolates, Quinolines showed antibiotic efflux inhibition. Certainly, it has been shown that several quinoline derivatives are competent of enhancing the Antibiotic activity via the efflux transporters inactivation: AcrAB-ToIC (RND family) [137]. Studies reported, this class of compound showed synergy with antibiotics: including chloramphenicol, tetracycline, and norfloxacin, in Gram-negatives isolates of *E. aerogenes* and *K. pneumoniae* [138]. In salmonella enterica, chlorpromazine also inhibits AcrB, by indirectly exerting synergistic activity by modulating acrB gene expression [139].

Consequently, it is promising to substantiate that efflux inhibition might direct to a multiplicity of optimistic results by: (i) enhancing the activity of antibacterial drugs subjected to efflux, (ii) maintenance of the antibiotic concentration at the remedial dose, and (iii) reducing the treatment period by limiting multi-drug tolerance [140, 141].

7.1.2 β-Lactamase inhibitors

Antibiotic penicillin hydrolysis by enzyme lactamases was the first mechanism of lactam resistance reported in Gram-positive bacteria. Lactam antibiotics mechanism of action involves the transpeptidases inactivation which is utmost for the final biosynthesis of cell wall in bacteria. In order to protect the cell wall, bacteria synthesize the lactamases that are capable for hydrolyzing lactam-based antibiotics and the degree of hydrolysis depends on the form and β-lactamases number formed by the bacteria. For antibiotic activity the key element is the β-lactam ring, for the reason of its electrophilicity, for acylating the penicillin-binding proteins (PBPs) irreversibly. PBPs are accountable for peptidoglycan synthesis that is liable for maintenance of the bacterial cell wall structural integrity. Till date, discovery of hundreds of β-lactamases are capable with identical action. The difference in affinity for various substrates is due to difference in their amino acid sequences. Commonly, two different methods for the classification of β-lactamases are: one is based on characterization of structures-Ambler classification and, the other one is based on a functional characterization—Bush and Jacoby classification [142, 143]. In therapeutic, several β-lactams antibiotics are used and has led to the synthesis of specific β-lactamases class, referred as extended-spectrum β-lactamases (ESBL), that hydrolyzes maximum β-lactam antimicrobials, and are particularly delineated in Enterobacteriaceae-{including *K. pneumoniae*, *P. mirabilis*, and *E. coli*} [144]. The family of β-lactamases, which are most versatile with broader spectrum activity and these β-lactamases identify approximately all hydrolysable β-lactams, while as most are resistant to the inhibition by all viable commercially β-lactamase inhibitors [145]. In order to surmount the β-lactamase-mediated resistance to β-lactams, two possible strategies are opted: (i) selective β-lactamase inhibitors (BLIs) developed and to be used in combination with a β-lactam antibiotic, and (ii) development of stable β-lactamase—antibiotics {e.g., carbapenems and cephalosporins which are stable towards β-lactamases hydrolysis [146]. The significant step in the antibacterial discovery field is the discovery of *Streptomyces clavuligerus* secondary metabolite—clavulanic acid, which is able to inactivate many β-lactamases, therefore, the association of amoxicillin and clavulanic acid is the first development in the β-lactam-β-lactamase inhibitor amalgamation in the form of Augmentin, [147] further led by the prefacing of other combinations.

After the clavulanic acid discovery, a crusade in medicinal chemistry was initiated with an aim to synthesize various penicillanic acid sulfones having inhibitory activity

against β-lactamase. Tazobactam and sulbactam among these were commercialized productively. Both possess the similar activity spectrum as that of clavulanic acid. In combination with piperacillin, tazobactam is used with the recent ceftolozane and cefoperazone for nosocomial infections, comprising the ones caused by MDR *P. aeruginosa* [148]. For worldwide use, ampicillin and sulbactam is combined and an additional synergy against anaerobic bacteria is achieved with cefoperazoe [149, 150]. These compounds in broad if administered alone do not show any antibacterial activity. With some exceptions MIC of clavulanic acid alone against *N. gonorrhoeae* is 1 μg/mL [151]. Sulbactam is ineffective against MDR strains and has MIC in the range of 10 and <8 μg/mL against wild-type *Burkholderia cepacian* and *Acinetobacter spp.* respectively [152]. After two decades of space, following the discovery of β-lactamase inhibitors, a new class of non-β-lactam β-lactamase inhibitors arose, which are based on the diazabicyclooctane (DBO) scaffold, avibactam is the first inhibitor from this class which possess higher activity spectrum in comparison with clavulanic acid, and approved for therapeutic usage with ceftazidime in combination. Likewise, the combination development (e.g., aztreonam-avibactam or ceftaroline-avibactam combinations) is ongoing [88, 153, 154]. In combination with Imipenem, relebactam (MK7655, 23) and Nacubactam (RG6080, 22) are DBOs under development. The relebactam activity is same as that of avibactam spectrum of activity [155]. RG6080 (formerly OP0565) like other DBOs is having inhibitory spectrum of activity and against enteric bacteria also exhibits some intrinsic antibacterial activity [156]. To target microbes synthesizing carbapenemases, synthetic non β-lactam β-lactamase inhibitors, a new class of inhibitors that are made up of boronic acids including RPX7009 in combination with meropenem is developed. Widespread β-lactams resistance in on surge, particularly in Gram-negative organisms [157–159].

At the present time, to tackle the resistance developing new β-lactamase inhibitors is the most pursuing challenge which will endow with defense for the almost many antibiotics that are used in clinical therapeutics, at least for the current time.

7.1.3 Baiting outer membrane: {outer membrane permeabilizers}

Specifically, the antibiotics hit target(s) inside the cells, exerting their antimicrobial action in therapy that is used presently. Outer membrane which acts as the defense, shelter the Gram-negative bacteria and is composed of porins and polyanionic lipopolysaccharides, which hinders the entry of xenobiotics antibiotics, as a result of complex wall that is responsible for reduced efficacy of antibacterials, at the outer membrane level mostly stirring strains which are resistant generally adopt mutation in proteins, Therefore, there is a need to develop the antibiotics that pass through the bacterial membrane [105]. In this regard, to deal with the bacterial resistance, the outer membrane (OM) acts as a potential target, ability to develop new effective classes of antibiotics can be enhanced by knowing the bacterial cell wall [160].

Depending on the small molecules chemical nature, the antibiotics use two strategies to penetrate the bacterial cell wall: (i) antibiotics {e.g., β-lactams, phenicol antibiotics, and fluoroquinolones) are hydrophilic molecules that take benefit of their capability to interact with peculiar porins and diffuse via active transport mechanism; (ii) antibiotics {e.g., rifampicin and macrolides} are transported via mechanism of passive transport across the lipid bilayer [61, 105].

A new strategy to improve the antibiotic entrance capacity is the use of permeabilizers that act as antibiotic adjuvants to enhance the permeability membrane

propensity. Permeabilizers act by capturing cations in the outer layer, and interacts with polyanionic lipopolysaccharides and thereby destabilizes the bacterial membrane wall. As a result the OM can be easily crossed over by xenobiotics {antibiotic}. Polymyxin—for instance polymyxin-B, cationic peptides, aminoglycosides, colistin, polyamines, or cationic cholic acid derivatives, are membrane permeabilizers [161, 162]. New substitute strategies for designing novel small molecules that can enhance antibiotic dissemination across the membrane, and increasing intracellular concentration, is in great demand [163]. With regard to same, various chemosensitizers (e.g., antimicrobial peptides, surfactants, detergents, etc.) have been proposed that are enable to interrupt protein activities in the membrane (e.g., membrane channels and porins) [164, 165]. In order to fight with resistant strains, the classical antibiotics are used in combination and administered with these classes of antibiotic "adjuvant" [166, 167]. It has been reported recently that on *E. coli* membrane, glycine basic peptide (GBP) exhibits concentration dependent antibacterial activity and leads to cell fragmentation, GBP is a cationic peptide that works by disturbing the ion-channel and membrane barrier of *E. coli*, which results in the ion loss {Mg^{2+}, Ca^{2+}, and K^{+}} and also enhanced the susceptibility of *E. coli* to rifampicin and erythromycin which are otherwise unable to cross the OM of Gram-negative bacteria [168]. The menadione in another study revealed that in combination with aminoglycoside class of antibiotics it showed synergy and reduced the MIC of these antibiotics [169].

7.2 Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) are interesting antibiotic class which is endowed with antibiotic adjuvant potential. Multicellular organisms naturally produce peptides (AMPs) which are amphiphilic in nature comparatively small in size (10–50 amino acids) with cationic charge and acts against pathogenic bacteria during infections as the first line of defense in opposition to microbes. AMPs proposed mechanism of action is their capability of forming amphipathic α-helix or short β-sheet structures, thereby destabilizing the bacterial outer membrane [170, 171]. AMPs cationic residues forms the electrostatic interaction with the bacterial anionic cell wall and targets it by diverse mechanisms so as to obstruct and hamper the development of resistance. It also leads to the disintegration or permeabilization of the bacterial cell wall by insertion of hydrophilic subunits. They also form pores on the bacterial membrane and leads to the death of microbe [172, 173]. The cationic short amphipathic antimicrobial peptides act by immunomodulatory action and direct cell killing. The three crucial steps that are involved in AMP mediated cell killing are as attraction, attachment, and insertion of peptide. The process of attraction is electrostatic in nature between the negatively charged surfaces units and charged anionic/cationic peptides. The bacterial polysaccharide surface must be infiltrated by these peptides and adhere with the teichoic and lipoteichoic acid from Gram-positive bacteria or lipopolysaccharide from the Gram-negative bacteria in the attachment step. Attachment is followed by the peptide insertion. AMPs cause cell membrane disintegration by pore formation in the bacterial cell membrane and are explained by {The 'Carpet model', 'Barrel-stave' and 'Toroidal-pore'} (**Figure 3**) [174]. Negatively charged cell membrane and the peptides are electrostatically bonded and is spread all over in the 'Carpet model,' (**Figure 3a**). The lipidic fraction is aligned by the hydrophobic region, the inside portion of pore is hydrophilic in the 'Barrel-stave' model (**Figure 3b**). The peptides which penetrate leads towards lipidic portion twisting so as to give a structure of pore in the 'Toroidal pore' model (**Figure 3c**) [175]. By metabolic modulators, intracellular killing activity

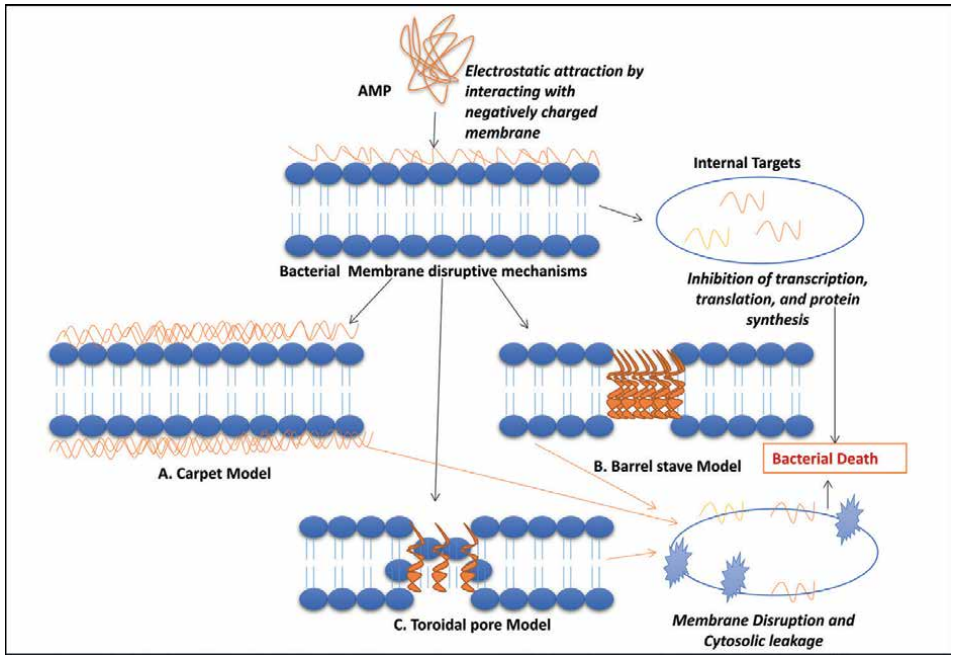

Figure 3.
Schematic representation of membrane disruptive and non-membrane disruptive mechanisms of antimicrobial peptides (AMPs): a. Carpet model: in the carpet model, AMPs bind to the surface of microbial membranes and disrupt their integrity by forming a "carpet" of peptides. This disrupts the packing of lipids in the membrane, leading to the formation of transient pores. The carpet model suggests that the peptides do not form well-defined channels but rather cover the membrane surface, causing leakage of intracellular components and ultimately cell death. b. Barrel stave model: according to the barrel stave model, AMPs insert themselves into the lipid bilayer of the microbial membrane to form transmembrane channels. The peptides assemble together in a "barrel" fashion, with their hydrophobic regions embedded in the lipid bilayer and their hydrophilic regions facing the aqueous environment. This model suggests that the peptides create stable channels that span the membrane, allowing ions and molecules to flow across. The channels formed by the peptides disrupt the membrane's electrochemical balance, leading to cell death. c. Toroidal pore model: the toroidal pore model proposes that AMPs induce the formation of toroidal pores in the microbial membrane. In this model, the peptides interact with the lipid bilayer, causing local curvature and bending of the membrane. The peptides form a toroidal structure, where both the peptides and the lipid head groups curve inward, creating a pore-like structure. This pore allows the passage of ions and molecules, disrupting the membrane potential and leading to cell death. It's important to note that these models represent simplified representations of the complex interactions between AMPs and microbial membranes. The exact mechanisms of action may vary depending on the specific AMP and the target microorganism. Additionally, recent research suggests that multiple models may operate simultaneously or in a sequential manner to exert the antimicrobial effects of AMPs. These models provide valuable insights into how AMPs function and can aid in the design and development of new antimicrobial therapies. However, it's important to continue research in this field to gain a deeper understanding of the intricacies of AMP-membrane interactions.

is exerted by AMPs. These activate bacterial apopotosis behavior by autolysin upregulation {e.g., N-acetylmuramoyl-L-alanine} via acting as DNA replication modulators—Buforin II, Inhibition of enzymatic activity by drosocin, apidaecin, histatins, Inhibition of DNA, RNA, and synthesis of protein by pleurocidin, dermaseptin, Human Neutrophil Peptide-1 (HNP-1), Human Neutrophil Peptide-2 (HNP-2). Bacteria are showing resistance to AMPs, similar to the conventional antimicrobials through the mechanism of cell surface bacterial alteration via discharging enzymes that are proteolytic and thereby results in the hydrolysis of peptides {for instance: by forming capsular body *K. pneumoniae* hinders the AMPs penetration, and by incorporating basic groups like D-ala *S. aureus* that changes the overall charge of surface towards low negative, the increased resistance in *S. aureus* towards AMPs is

due to the occurrence of enzymes which are proteolytic in nature (metalloproteinase-(aureolysin)) and occurrence of active efflux transporters. As in *Salmonella* spp., by altering the lipid A portion and outer membrane protein modulation as in *Yersinia enterocolitica* is also responsible for AMPs resistance [176]. AMPs are proteolytically degraded by *Enterobacteriaceae* so as to exert resistant mechanism, and thereby limit the penetration of AMPs by defending the cell surface of bacteria. The diverse genes in *Enterobacteriaceae*, encoding for AMPs resistance are as PmrAB, PhoPQ, and RcsBCD Phosphorelay system and are signaling pathways. In *Enterobacteriaceae* spp. the release of protease by the OM is the main cause of AMP disintegration. In *P. aeruginosa,* complex formation of AMPs with exopolysaccharides. In *K. pneumoniae*, capsule polysaccharides formation, O-polysaccharide modification in the OM [177] are responsible for the shield formation in the cell surface of bacteria against AMPs. The research has further been augmented in case of AMPs with the widespread of antimicrobial resistance. AMPs are very important with regard to the enhancing the penetration of certain antibiotics [178, 179]. Several drug delivery systems with novelty were executed to deliver AMPs in order to lessen their resistance. List of various antimicrobial peptides from different sources that are under clinical trials presently are mentioned in **Table 1**.

AMPs from humans					
S. no.	**Source**	**Peptide name**	**Amino acid number**	**Anti-bacterial activity**	**References**
1.	Human neutrophils	Cathelicidins	30	F, G–, G+	[180]
2.	Human neutrophils	A Defensins	12-80	F, G–, G+	[181]
3.	*Homo sapiens*	Human Histatin 8	12	F, G–, G+	[182]
4.	Neutrophils (*Homo sapiens*)	LL37	37	F, G–, G+	[183]
From insects					
1.	*Acalolepta luxuriosa*	Acaloleptin	71	G+, G–	[184]
2.	*Drosophila melanogaster*	Andropin	34	G+	[185]
3.	*Apis mellifera*	Apidaecin IA	18	G–	[186]
4.	*Hyalophora cecropia*	Cecropin	37	G–	[187]
5.	*Aedes aegypti*	Defensin-α	40	G+, G–	[188]
6.	*Drosophila melanogaster*	Drosomycin	44	F	[189]
7.	*Holotrichia diomphalia*	Holotricin	43	G+, G–	[190]
8.	*Sarcophaga peregrine*	Sapecin-α	40	G+, G–	[191]
9.	*Tenebrio molitor*	Tenicin 1	43	G+, G–	[192]
10.	*Podisus maculiventris*	Thanatin	21	G+, G–	[193]
From animals					
1.	*Androctonus australis*	Androctonin	25	F, G–, G+	[194]
2.	Bovine Neutrophils	Bactenecin	12	G–, G+	[195]
3.	*Rana brevipora porsa*	Brevinin	24	G–, G+	[196]

AMPs from humans					
S. no.	**Source**	**Peptide name**	**Amino acid number**	**Anti-bacterial activity**	**References**
4.	*Bufo bufo gargarizans*	Buforin II	21	F, G−, G+	[197]
5.	*Cupiennius salei*	Cupiennin	35	G−, G+	[198]
6.	*Phyllomedusa sauvagii*	Dermaseptin S1	34	G−, G+	[199]
7.	*Lycosa carolinensis*	Lycotoxin	27	G−, G+	[200]
8.	*Tachypleus tridentatus* (Horseshoe crab)	Tachyplesins	17	G−	[201]
From microorganims					
1.	*Lactococcus lactis*	Nisin	34	G+	[202]
2.	*Trichoderma viride*	Alamethicin	20	G+	[203]
3.	*Enterococcus*	Enterocin	70	G+, G−	[204]
4.	*Staphylococcus hominis* MBBL 2-9	Hominicin	21	G+, G−	[205]
5.	*Bacillus subtilis*	Ericin S	32	G+	[206]
6.	*Lactobacillus plantarum*	Plantaricin A	26	G+, G−	[207]
7.	*Carnobacterium piscicola*	Carnobacteriocin B2	48	G+, G−	[208]
8.	*Leuconostoc pseudomesenteroides*	Leucocin A	37	G+, G−	[209]
9,	*Bacillus subtilis*	Subtilin	32	G+	[209]
10.	*Pyrularia pubera*	Pyrularia thionin	47	G+, G	[210]
11.	*Escherichia coli* AY25	Microcin J25	21	G−	[211]
12.	*Bacillus brevis*	Gramicidin A	15	G+, G−	[212]
13.	*Pediococcus acidilactici* PAC-1.0	Pediocin PA-1/ AcH	44	G+	[213]
14.	*Leuconostoc mesenteroides*	Mesentericin Y105	37	G+	[214]
15.	*Carnobacterium piscicola* LV17B	Carnobacteriocin BM1	43	G+, G−	[215]
16.	*Bacillus subtilis* A1/3	Streptin 1	23	G+	[216]
17.	*Planomonospora alba*	Planosporicin 24	24	G+, G−	[217]
18.	*Lactobacillus gasseri* LA39	Gassericin A	58	G+, G−	[218]
19.	*Clostridium beijerinckii* ATCC 25752	Circularin A	69	G+, G−	[219]
20.	*Carnobacterium divergens* V41	Divercin V41	43	G+	[220]
21.	*Listeria innocua* 743	Listeriocin 743A	43	G+	[221]
22.	*Lactobacillus plantarum* C19	Plantaricin C19	37	G+	[222]

AMPs from humans					
S. no.	**Source**	**Peptide name**	**Amino acid number**	**Anti-bacterial activity**	**References**
23.	*Enterococcus faecium* P13	Enterocin P	44	G+	[223]
24.	*Bacillus subtilis*	Subtilosin A	35	G+, G−	[224]
25.	*Lactobacillus plantarum* A-1	Plantaricin ASM1	43	G+	[222]
26.	*Bacillus licheniformis*	Lichenin	12	G+, G−	[225]
From plants					
1.	Latex of rubber trees	Hevein	43	F	[226]
2.	Wheat endosperm	Purothionins	45	G+, G−	[227]

F, fungus; G+, Gram-positive; G−, Gram-negative.

Table 1.
List of antimicrobial peptides from different sources that are under clinical trials presently (https://clinicaltrials.gov/, NIH).

7.3 Phage-based therapy

Phage-based therapy, also known as bacteriophage therapy, is an innovative approach to combat antimicrobial resistance (AMR). Bacteriophages are viruses that specifically infect and kill bacteria. They have been recognized as a potential alternative to antibiotics in the battle against bacterial infections, particularly those caused by antibiotic-resistant bacteria [228]. The rise of antimicrobial resistance is a major global health concern, as it reduces the effectiveness of traditional antibiotics, making it challenging to treat certain infections [229]. Bacteriophages, being highly specific to particular bacterial strains, can potentially overcome some of the limitations of broad-spectrum antibiotics and help address AMR in several ways:

Specificity: Phages target specific bacterial species or strains, leaving beneficial bacteria and the human body's microbiota largely unaffected. This specificity reduces the risk of disrupting the natural microbial balance in the body.

Diversity: Phagechigh level of genetic diversity. This diversity means that new phages can be isolated and selected to target emerging antibiotic-resistant strains of bacteria.

*Self-replicatin*g: Once a suitable phage is identified, it can replicate within the infected host bacterium, leading to an exponential increase in the number of phages, which can improve treatment efficacy.

Co-evolution: Phages can evolve alongside bacteria, potentially countering bacterial resistance mechanisms through natural selection.

Safety: Phages are generally considered safe for human use, as they are naturally present in the environment and have co-evolved with bacteria.

Biofilm disruption: Phages can penetrate and disrupt bacterial biofilms, which are protective structures that make bacterial infections difficult to treat with conventional antibiotics.

Phage therapy is an evolving field, and its integration into mainstream medical practice requires continued research, investment, and collaboration between

scientists, clinicians, and regulatory bodies. As research progresses, phage-based therapy could become a valuable tool in the fight against antimicrobial resistance and help address the growing global health threat posed by antibiotic-resistant infections.

8. Novel nano formulation approaches for AMPs

Generation, development of new antimicrobials, or AMPs development is considered as a novel way to tackle the emergence and widespread resistance to the known conventional antibiotics by several microorganisms, AMPs were potentially effective in curbing the antimicrobial resistance as compared to the conventional antibiotics. However, AMPs face various problems {e.g., proteolytic degradation, Nonspecific interactions, less stability, selectivity and inadequate *in vivo* activity which render AMPs ineffective to exercise its feat as hampered to arrive at target site}. In order to curtail the problem associated with delivering AMPs alone, attempts are made by researchers for delivering AMPs via developing formulation systems which are novel. AMPs targeting in direct application with alternative ways comes with AMP encapsulation into various nanocarrier. Diverse encapsulated AMPs developed to target AMR includes carbon nanotubes, novel polymeric & lipidic nanoparticles, cubosomes, microspheres, micelles, polymersomes, dendrimers, nanocapsules, and additional colloidal delivery systems. AMPs loaded in nano carriers can assist in combating proteolysis, curbing pitiable bioavailability, or toxicity & susceptibility adhered with AMPs alone. Encapsulated AMPs are delivered to the intracellular pathogens or into the cells which are infected via these nano formulations that act as transporters. Moreover, functional polymer conjugated with AMPs provides new functionalities, improves selectivity by reducing toxicity and acts with potential antimicrobial activity [230]. For the purpose of translating the AMPs and its various formulations from bench to bedside the development of polymer conjugation and novel nano-formulations come up with broad new avenues. While as, very few AMPs and its formulations are actually translated into the clinical trials [231–236].

Besides nanocarriers, researchers also attempted to work on various diverse nanomaterials which are novel showing less susceptibility to develop antibiotic resistance. These novel nanomaterials are structurally nanoengineered antimicrobial peptide polymers (SNAPPs) and star peptide polymers [237]. As proved by *in vitro* and *in vivo* studies, these star-shaped polymers are constructive in microbial carnage, in comparison to the conventional antibiotics, these act through diverse pathways and are less toxic, making them more effective and accepted than the conventional nanocarriers [36].

9. Nanostructured polymeric antimicrobial peptides

Exploiting the line of attack of SNAPPs or polymeric peptides which are nanostructured has revealed efficient activity against both Colistin MDR (CMDR) *A. baumannii* and ESKAPE bugs. Involving the action as: apoptotic cell death pathway initiation, destabilizing outer membrane, and interruption of ionic movement crossways the cell membrane. In the occurrence of SNAPPs prototype (S16) [238] sub-micron levels no wild mutation were observed in *S. aureus* multiplication even after 600 generations, enlightening these SNAPPs hinder resistance.

Commercially developed functional AMPs stereospecific structures are developed by using ROP-NCA (ring-opening polymerization N-carboxy anhydride) technique [239]. In a latest study, ROP-NCA have been utilizing valine (hydrophobic) and lysine (cationic) as amino acid residues, SNAPPs were developed. Likewise, to augment the solubility in water the structures were synthesized with poly(amidoamine) PAMAM dendritic arms using lysine to valine ratio of 2:1 [240]. Elevated Minimum Bactericidal Concentration (MBC) in opposition to *E. coli* has been reported in the structures possessing homolysine residues. In contrast to the host defense peptides which directly circumvent the ESKAPE bugs by bacterial pathway, SNAPPs immunize the mammalian cells against ESKAPE pathogens and CMDR by effecting both bacterial as well as utilizing diverse indirect pathways. By escalating the neutrophil infiltration mechanism the aforesaid indirect pathway is exhibited [35, 241]. Utilization of alpha-amino acids via NCA-ROP techniques are other strategies used to develop AMPs. Even at the lowest MICs against *C. albicans, P. aeruginosa*, *Serratia marcescens*, and MRSA Antimicrobial peptides were found to be highly susceptible consisting of phenylalanine, lysine in the ratio of 15:10 and lysine (hydrophilic moiety), leucine and phenylalanine as the hydrophobic moiety in the ratio of 10:7.5:7.5 [242–244]. Owing to the nanostructures, localization of the charges increases efficacy of the AMPs by bacterially induced peptide aggregation, which are formulated as SNAPPs. Polymers which are cationic in nature are chosen as with bacterial surface they exhibit electrostatic interactions. Protonated polyesters, polyethyleneimines, polyarylamides, and polymethacrylates are examples of few cationic polymers which are synthesized. By changing the length of carbon chain of the functionalities side group for the development of various polypeptide libraries gave comprehensive idea that these are potentially efficient against a broad spectrum of Gram-negative and Gram-positive bacteria and also curtail the formation of Biofilm particularly against *E. coli* and *S. aureus* [245, 246].

10. A ray of hope-star polymers

Using diverse polymeric structures in the approach of novel delivery system which evolved extremely with the purpose to improve the biocompatibility, stability, and therapeutic efficacy of the antibiotics. With the purpose to improve antibiotic delivery the various noteworthy approaches undertaken are as nanoparticles, polymeric carriers which are hydrophobic and hydrophilic, and targeting moieties, towards antibacterial therapy from gene delivery, In the field of biomedical applications, star polymers has achieved significance for novel delivery system. Star polymers consist of arms which are linear (contrasting dendrimers with branched arms) and form simpler structures and characteristics such as biocompatibility, simpler structure (lower viscosity solution), and introduction of functional groups are gaining attention in the biomedical research field. In order to execute cell-specific targeting, star polymers with multifunctional central part having at least three macromolecular chain can fasten to a targeting moiety [247, 248]. With the widespread emergence of AMR (ESKAPE bugs), this strategy gained focus wherein linear start polymers are integrated with an antibiotic and has resulted in the improvement of antimicrobial therapy. Which includes attachment of antibacterial groups or AMPs that are polycationic (e.g., poly(2-dimethylaminoethyl methacrylate), star polymers which are poly(2-(dimethylamino) ethyl methacrylate) PDMAEMA based are susceptible to *E. coli* (MIC < 250 μg/ml, 99% in 2 h) [247, 248]. Studies relevance with regard to

the inclusion of AMPs inside these star polymers has demonstrated augmenting of improved encapsulation characteristics via this process and star polymer's compartmentalized functionalities that gave rise to the idea of functionalized stars with stereospecificity. Ring-opening polymerization technique is adopted for the generation of these polymers which are star-shaped. Core-cross linked stars (CCS) also referred as Stereospecific stars, were synthesized by ring-opening polymerization technique of amino acid poly (ε-Z-L-lysine) N-carboxy anhydride (NCA), which serves as the macromolecular initiator or arm, following by the adding of the L-cystine (agent poly cross-linking). Water solubility of the CCS is enhanced by the deprotection of the arms, additionally improved the biocompatibility of star polymers [246, 249–251].

11. Caragenins

"Caragenins", a new-fangled adjuvant class, developed so as to surmount the aforesaid concerns associated with the AMPs usage; these are cationic steroidal antibiotics (CSA), in which an aminoalkyl function substitutes the sterol core structure's alkoxy groups. This substitution in the structure makes "Caragenins" resistant to the proteases because they can be produced in larger amounts as their structure is devoid of peptidic bonds. Furthermore, CSA are able to complex with phospholipids and are capable to stably get incorporated into the membranes [252, 253]. CSA are positively charged and interact to the negatively charged membranes (protozoa, bacteria, fungi, and viruses) via electrostatic force of attraction and through the disruption of the membrane leading to cell death [254, 255]. Synthesis of caragenins such as CSA-8 and CSA-13 was in a way so that they imitate the physico-chemical properties of cationic structural of AMPs, with a comparable mode of action, that is based on (i) stimulation of bacterial membrane swift depolarization and (ii) improved permeabilization in the outer membrane of Gram-negative bacteria. In a specific order so that CSA-8 and CSA-13 make bacteria more prone to susceptibility towards antibiotics (e.g., erythromycin antibiotic when used alone against *K. pneumoniae* resistant strain the MIC is reported as 70 μg/ml, but the combination of erythromycin with CSA-8 compounds decreases the MIC value to 1 μg/ml. Anti-microbial activity of CSA-13 was analyzed on carbepenem resistant strains. It has also been reported that the combination of CSA-13 with antimicrobials, the synergy was attained with tobramycin-35% and colistin-55%, on the contrary there was no observation of antagonism [253, 256]. Wide-ranging research is required for the development of this type of antibiotic adjuvant class with an aim to enhance the absorption, distribution, metabolism, and excretion (ADME) profile of these molecules, in order to allow them to enter clinical trials and finally make entry into market.

12. Conclusion

Antimicrobial resistance poses a widespread threat to patients, health care systems and overall global economy. Using diverse mechanisms of action bacteria develop resistance and multi-drug resistance (MDR) is now the rule rather than the exception. The key driver for the emergence of resistance is the extensive use of antibiotics. A major concern with regard to the control of infectious disease is the dearth of the antimicrobial agents. ESKAPE bugs are becoming self-reliant as they are destroying antimicrobial delivery stratagem. Several drug delivery systems that are novel

and copy the peptides natural bacteriolytic action have been reported involving Antimicrobial peptides, via incorporation of these peptides into nano-carriers and into star-shaped polymers. The ultimate structure and architecture of the star polymers is well described by the SNAPPs that show supplementary apoptotic mechanism switched as they make entrance into the bacteria and therefore, show promising future in curtailing AMR. Using ROP technique, AMPs synthesis techniques are in confines. Nevertheless, widespread research is required for AMPs synthesis so as to yield reproducible and cost-effective outcomes. The future ambition for the upcoming research to verify a series of therapeutic activities will be the modulation of the functionalities on the star polymers surface. Moreover, the production and the antimicrobials use are perplexed with a very complex network of stakeholder interests that extends well beyond the boundaries of medicine. Specifically, the immense majority of antimicrobials are fed to animals and, in various countries, the antimicrobials therapeutic in humans is regulated poorly. This imposes a colossal selection pressure on microbiota in various ecosystems that will unavoidably result in a few bacterial genotypes competent of surviving. The antimicrobial resistance mechanism of defense have been chosen during evolution can readily be dispersed into other ecological compartments, including pathogens, by sophisticated HGT mechanisms. Therefore, the problem of antimicrobial resistance cannot be dealt simply by the introduction of new antimicrobials. It requires the combined efforts of governmental organizations, regulatory agencies, health-care professionals, veterinarians, agricultural specialists, educators, researchers, and stakeholders to retain the therapeutic benefit of antimicrobials for efficient control of infectious diseases. In order to combat the widespread it will require the multidisciplinary efforts so as to limit the extensive antibiotic use and to implement avoidance and control measures to limit transmission of these dangerous pathogens.

Conflict of interest

The author(s) declare that there are no conflicts of interest.

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Chapter 3

Tuberculosis Diagnosis: Updates and Challenges

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Abstract

Tuberculosis (TB) is caused by a single infectious agent, *Mycobacterium tuberculosis,* and a public health concern due to increased cases of drug-resistance and high mortality rates. Rapid identification of tuberculosis is necessary for its early treatment and to prevent the emergence of drug-resistant strains. For effective management of patients, rapid, cost-effective, and point-of-care (POC) diagnostic methods are required. The commonly used screening and identification methods are clinical examination, radiography, sputum smear microscopy, culture method, serological method, and tuberculin skin test. In addition, several molecular methods such as NAAT based GeneXpert, loop-mediated isothermal amplification (LAMP), line probe assay (LPA), whole genome sequencing (WGS) and other non-invasive methods of lateral flow urine lipoarabinomannan assay (LF-LAM) and eNose assays are developed. Sputum smear microscopy, Xpert MTB/RIF, and LED-Fluorescence microscopy (LED-FM) are the preferred methods to use in peripheral laboratories. The non-invasive methods of tuberculosis diagnosis are more beneficial in patients from whom collecting sputum sample is difficult particularly in children and HIV co-infected patients. Molecular methods can simultaneously identify the pathogen, *M. tuberculosis*, and mutations in drug-resistance genes. Even though, many advanced methods are currently available, accurate and affordable diagnostic method for tuberculosis is still challenging. Here, we review and highlight the uses and challenges of currently available conventional and advanced diagnostic methods of tuberculosis screening and diagnosis.

Keywords: tuberculosis, GeneXpert, LAMP, LPA, whole genome sequencing

1. Introduction

Until the coronavirus (COVID-19) pandemic, tuberculosis (TB) was the leading infectious disease, ranking above HIV/AIDS. Tuberculosis is an ancient bacterial infection and genetic evidence indicates that the causative infectious agent, *Mycobacterium tuberculosis* (*Mtb*), is infecting humans for more than 40,000 years and have originated from animal domestication [1]. Tuberculosis is a communicable disease and *Mtb* spreads through air droplets during coughing and sneezing. The global prevalence of TB in 2019 is approximately 10 million people. Global TB report of World Health Organization (WHO) has reported 5,946,816 pulmonary tuberculosis (PTB) patients, 206,030 patients with MDR/rifampicin-resistant (RR), and 12,350

Test	Principle/ Technology	Sensitivity (%)	Specificity (%)	Target setting	Comments
Chest X-Rays	Imaging	87	89	Secondary & tertiary centres	Even though used as screening tool for PTB, etiological agent cannot be identified. Used to differentiate primary & secondary TB.
Sputum smear microscopy	Ziehl-Neelsen (Z-N) staining & microscopic detection of bacilli	32–94	50–99	Peripheral & reference labs	Cannot differentiate *Mtb* and other acid fast bacilli
LED-fluorescence method	Auramine staining & detection by fluorescent microscope	52–97	94–100	Peripheral & reference labs	
TB *Detect* kit	BioFM-Filter-based sputum concentration & detection by kit method.	~55	88	Peripheral & reference labs	Biosafe & equipment-free method
Conventional culture method	Growth on Lowenstein–Jensen (LJ) media & identification by colony morphology & biochemical tests	93	>99	Secondary & tertiary centres	Longer turnaround time
BACTEC Mycobacterium Growth Indicator Tube (MGIT) 960 system	Liquid culture method with drug susceptibility testing	89 (smear +ve) 73 (smear −ve)	>99	Reference labs	*Mtb* identification requires additional laboratory tests
Tuberculin skin test (TST)	Host immune reaction to *Mtb* in body	87–98	74–96	Secondary & tertiary centres	False +ve in BCG vaccinated, NTM infected & high endemic regions Low sensitivity in immune-compromised individuals

Test	Principle/ Technology	Sensitivity (%)	Specificity (%)	Target setting	Comments
Serological tests	Detection of *Mtb* mycolic acid components & inflammatory biomarkers	87–92	72–83	Peripheral & reference labs	Results may vary depending upon host metabolic and disease states
Interferon gamma release assay (IGRA)	Immune response against *Mtb* antigen in blood	QFT (75–84)	QFT (75–91)	Secondary & tertiary centres	Not recommended to predict active TB and treatment decisions
Lateral Flow urine lipoarabinomannan (LF-LAM)	Antigen (mycobacterial cell wall components) detection in urine	44	92	Reference labs	Cross reacts with other mycobacterial species & fungi
Xpert MTB/RIF	NAAT qPCR	98 (smear and culture +ve)	99 (smear and culture –ve)	Distric or sub-district labs	Limited utility in resource-limited settings
Loop-mediated isothermal amplification (LAMP)	NAAT	76–80	97–98	Reference labs	Simple method to use in resource-limited settings & high endemic regions

Key: PTB – pulmonary tuberculosis, TB – tuberculosis, Mtb – M. tuberculosis, LED - light emitting diode, BCG - Bacillus Calmette Guerin, NMT - nontuberculous mycobacteria, QFT - QuantiFERON-TB Gold, NAAT - nucleic acid amplifcation test, qPCR – quantitative PCR.

Table 1.
Commonly used tests for screening and diagnosis of TB and detection of drug resistance.

patients with extensively drug-resistant (XDR) TB globally in 2019 [2]. The cases of pulmonary tuberculosis are higher than extrapulmonary cases. The majority of TB patients (about 90%) are found to be adults, with more cases being men than women [3]. Globally, among four people, approximately one demonstrates an immunological reaction to *Mtb* infection and they may either remain dormant or progress to an active infection. Patients who are infected with TB but not having significant signs of the active disease were previously defined to have latent TB and more recently changed to TB infection. Tuberculosis can affect the lungs, which is named pulmonary tuberculosis (PTB), or other organs, named extrapulmonary tuberculosis (EPTB) [4].

WHO recommends to utilize the TB screening tests initially to screen high-risk individuals and further identification of pulmonary tuberculosis for rapid diagnosis and early treatment initiation [5, 6]. Rapid and feasible diagnostic methods are required to screen and diagnose active TB cases, HIV positive patients, workers having current or past history of silica exposure, identify cases in high endemic

regions and having limited access to healthcare facilities [7]. Systematic diagnostic methods to screen and diagnose TB include physical examination for signs and symptoms, Chest-X Rays, conventional culture method followed by antibiotic susceptibility testing, molecular tests such as Xpert MTB/RIF [7]. WHO recommended TB diagnostic methods include light emitting diode (LED) microscopy method, BACTEC Mycobacterium Growth Indicator Tube (MGIT) 960 system, Xpert MTB/RIF, lateral flow urine lipoarabinomannan assay (LF-LAM), loop-mediated isothermal amplification (LAMP), line probe assay (LPA). Sputum smear microscopy, Xpert MTB/RIF, and LED-Fluorescence microscopy (LED-FM) are the preferred methods to use in peripheral laboratories. A summary of commonly used screening and diagnostic tests is presented in **Table 1** [8]. Even though, several advanced diagnostic methods are available, rapid and accurate diagnosis of TB is still challenging [9], particularly in regions with high endemic TB. In this review we provide an overview of various currently available methods to screen and diagnose tuberculosis.

2. Clinical diagnosis

The clinical manifestations of tuberculosis occur only in 5–10% of infected patients. In majority of TB patients, pulmonary tuberculosis is reported which affects mostly the lower respiratory system. The common clinical signs of pulmonary tuberculosis include hemoptysis, productive and prolonged cough, low-grade fever, loss of appetite, fatigue, night sweats, malaise, and weight loss [10]. Tubercle bacilli can also infect other body sites such as lymph nodes, kidneys, bone, joints, and meninges and is called extrapulmonary TB and its clinical signs and symptoms depend on the body sites being affected [11].

3. Radiography

Chest X-Ray (CXR) is a common diagnostic tool that differentiates primary and secondary TB. Primary TB is manifested by the presence of a single lesion in the middle or lower-right lobe and enlarged draining lymph nodes. CXR shows endogenous reactivation in the apical site and typical lymph nodes with multiple secondary tubercles. In addition, miliary lesions spread throughout the lungs [12]. Even though Chest X-Ray detects pulmonary TB, it cannot identify its etiological agent.

4. Advances in microscopy

Ziehl–Neelsen (ZN) stain is a traditional and common method and the sample is termed "smear positive" or "smear negative", based on the presence or absence of Acid Fast Bacilli (AFB). The sensitivity of traditional ZN stain is lower and requires bacillary load of 5000–10,000 CFU/ml in sputum. In addition, it cannot differentiate *Mtb* and other acid-fast bacilli. The sensitivity of microscopic detection of *Mtb* is improved with fluorescence using carbolfuchsin and flourochromes such as Auramine-rhodamine which has been widely supported by WHO [8].

Recent developments in light emitting diode technology (LED) have increased the utility of fluorescent microscopy. LED microscopes use fluorescent stains

and are more sensitive in pathogen detection. To maximize the identification, multiple sputum specimens can be collected and examined on the first visit itself, rather than asking the patient at a later date. WHO has endorsed front-loading, or so-called 1-day diagnosis under defined programmatic condition [13]. 'TB*Detect*' kit is a bio-safe and fluorescent microscopy filter (BioFM-Filter) based method and increased the diagnostic efficiency of smear microscopy and LED method due to increased performance, feasibility and safety considerations. TB*Detect* kit concentrates sputum by filtration using BioFM-Filter. The sensitivity of TB*Detect* is 20%, LED-Fluorescence microscopy (LED-FM) is 16.1% and ZN microscopy is 16% [14]. This equipment-free TB*Detect* kit is more potential in TB diagnosis and has more utility in routine laboratory settings. However, when compared with Xpert MTB/RIF for examining pulmonary and extrapulmonary TB in specimens of sputum, urine, gastric aspirates, and others, LED-FM has less sensitivity in *Mtb* detection [15].

5. Advances in culture

All acid-fast bacilli are not *M. tuberculosis* and for definitive identification of pathogen, sputum smear microscopy and culturing of *Mtb* on suitable medium are required [16]. Sputum culturing is a sensitive method which can detect viable bacilli as low as 10 to 100 in volume of a few tenths of an ml. Culture method is more sensitive than sputum smear microscopy, in which sputum sample must have at least 5000 AFB/ml to get a positive result. The tubercle bacilli can be cultured on Lowenstein-Jensen (L-J) medium, egg-based medium and Ogawa's medium [17]. The L-J medium has glycerol that improves *Mtb* growth, but not *Mycobacterium bovis*, whereas sodium pyruvate enhances *M. bovis* and few strains of drug-resistant *Mtb* culture in the medium [18].

WHO recommends the use of dual medium, solid medium (e.g. Lowenstein–Jensen or Middlebrook 7H11) and liquid medium (e.g. for use with the BACTEC Mycobacterium Growth Indicator Tube (MGIT) 960 system) to increase the sensitivity, specificity and avoid contamination and reduce turnaround time. The use of liquid culture medium in identification and drug-susceptibility testing (DST) was endorsed by WHO in 2007. BACTEC™MGIT™960 system contains liquid Middlebrook 7H9 medium that detects the increasing fluorescence signals, every 60 minutes, automatically as oxygen is consumed by growing bacilli. Oxygen quenches the fluorescent compound present at the bottom of MGIT. Growing *Mtb* uses oxygen in the MGIT and subsequently, the fluorescent compound is detected [19]. Currently used two models of BACTEC system are BACTEC460 and BACTEC MGIT960. BACTEC MGIT960 is a user-friendly system with having non-radiometric and continuous signal monitoring system and is more advanced than BACTEC460 [20]. The MGIT technology yields result in less than 8 days. Addition of Streptomycin, INH, rifampicin and ethambutanol at critical concentrations allows Automated MGIT technology to detect drug susceptibility. Mycobacterial culture remained the gold standard method for detection and drug susceptibility testing [4]. However, in MGIT method species identification of *Mtb* requires additional laboratory tests, and hence its utility is limited. Micro MGIT system is more advanced as it does not need any special instrument other than UV lamp for fluorescence detection and hence utility of Micro MGIT is more in resource-limited settings [21].

6. Serological tests

Several commercially available antibody-based TB diagnostic tests are on the market, but clinical validation and current test performance are poor. Serum biomarkers are considered potential in diagnosing TB. Devising specific and accurate biomarkers which are consistent in different HIV status, ethnicities, and sites of TB infection is difficult; however, C-reactive protein, interferon-γ, interferon-γ inducible protein-10, fibrinogen, α2-macroglobulin, matrix metalloproteinase-9, transthyretin, complement factor H, and tumor necrosis factor-α have shown as potential biomarkers with 92% sensitivity and 72% specificity in detecting TB [22]. Trehalose esters of mycolic acids of *Mtb* cell wall lipids have been used in serological tests to diagnose PTB and the assay showed 87% sensitivity and 83% specificity [23]. If commercial methods are developed for these biomarkers, the serum assay would be rapid and effective in determining whether the patient needs further diagnostic testing. Most of these biomarkers are inflammatory markers and vary widely among patients depending upon their metabolic and disease states. Even though WHO is recommending against using commercial serological assays, antibodies, and combinations of antigens in the test panel improved performance of TB screening tests [24].

7. Interferon gamma release assays

Past or current *Mtb* infection can be detected by measuring T-cell mediated interferon-gamma that are secreted following subsequent stimulation with specific *Mtb* antigens. QuantiFERON-TB Gold In-Tube assay (QFT-GIT, Cellestis Ltd., Australia) and the T-SPOT.TB (Oxford Immunotec, UK) are the two commercially available interferon-gamma release assay (IGRA) kits. These assay kits detect *Mtb* infection in blood samples by detecting plasma levels of immune cells secreted gamma interferon. The interferon gamma-specific *Mtb* antigens include early secretory antigenic target-6 (ESAT-6), culture filtrate protein 10 (CFP-10), and tuberculosis 7.7 antigens. The blood sample is considered TB positive if the gamma interferon levels are above a specified threshold [13, 25]. However, previous studies conducted in children and adults across the globe have reported that these two IGRA assays cannot differentiate latent *Mtb* infection from active tuberculosis disease and are less efficient than tuberculin skin test (TST). IGRA-based QIAreach QFT is a new and simplified version of QFT-PLUS. QIAreach QFT provides qualitative analysis using a fluorescence lateral flow reader, transportable, user-friendly and does not require well-trained personnel [26]. However, few reviews and meta-analyses have reported neither IGRA nor TST is highly accurate in predicting active tuberculosis. IGRA is not recommended for treatment decisions for suspected cases of TB instead UK National Institute for Health and Clinical Excellence guidelines and WHO suggest using IGRA as a supportive screening test in diagnostic laboratories particularly in low-and middle-income countries having high tuberculosis and HIV-infected patients [27, 28]. A negative result of IGRA assay may not definitively exclude active TB infection in a high-risk group. Compared to TSTs, IGRAs are more specific in infections caused by non-tuberculosis mycobacteria but they are wrongly marketed as a confirmatory TB test despite their limited clinical utility. IGRAs are more expensive and need tedious sample processing for accuracy in results. Even though IGRA evaluation studies and its recommendations required many years, effort, funding, and resources across the globe, it did not have a major contribution to successful tuberculosis control [13]. A recent meta-analysis

has shown that heparin-binding hemagglutinin (HBHA) is a latency-associated *Mtb* antigen and HBHA-induced IGRA can be a promising diagnostic test to differentiate latent and active TB [29]. IGRA testing is also a cost-effective screening method [30].

8. Tuberculin skin test

Tuberculin skin test (TST) was introduced around 100 years ago but is still in use for an initial TB screening to detect *Mtb* exposure in many countries. In TST purified protein derivative (PPD) tuberculin is injected intradermally and after 48–72 h the induration is measured (≥5 mm is considered positive) at the injected site to detect the individual's immune response. The accurate TST result depends on well-experienced personnel for intradermal injection of PPD and its interpretation. The test is a simple and suitable method to detect *Mtb* exposure in geographical areas with rare TB cases, the test may be false positive in individuals from high endemic areas, vaccinated to Bacillus Calmette Guerin (BCG) and infected with non-tuberculous mycobacteria. However, TST has low sensitivity in immune-compromised patients [31].

9. GeneXpert

The most significant development toward tuberculosis diagnosis was NAAT-based GeneXpert. It is a real-time PCR-based multifunctional, automated, point-of-care (POC), user-friendly diagnostic system. In GeneXpert *M. tuberculosis* complex and rifampicin (RIF) resistance (targets *rpoB* gene for RIF resistance and associated *M. tuberculosis*–specific flanking regions) can be simultaneously detected in 2 h of time. The sensitivity of GeneXpert is higher than the sputum smear microscopy and culture method. The assay has utility in detecting extra pulmonary tuberculosis with sensitivities of 53–95%. In addition to high sensitivity, the assay provides high specificity without any cross-reaction with nontuberculous mycobacteria. Among children having pulmonary tuberculosis, GeneXpert rapidly detects all the smear positive and 61% of smear-negative samples after two induced sputum samples. GeneXpert is successfully used for routine screening of patients before antiretroviral treatment [13].

Even though GeneXpert detects RIF resistance in initial multicentre evaluation with high sensitivity and specificity, few studies have reported that *rpoB* gene sequencing and other methods have detected false-positive RIF resistance in areas of low RIF resistance prevalence [32, 33]. Although GeneXpert is a long-awaited development in TB diagnosis, it may not be feasible in settings with a lack of infrastructure for working on real-time PCR and computers. The requirement of annual maintenance of equipment is another hindrance. To overcome the challenges, corrective measures are introduced, including the revision of diagnostic device software and redesigning cartridge oligonucleotide probes and the newer software and combination of the oligonucleotide probes, called G4 version cartridge is released [34].

As sputum smear microscopy and culture methods are having several limitations, WHO recommended Xpert (MTB/RIF or MTB/RIF Ultra) or Truenat (MTB or MTB Plus) in TB suspected individuals. These biomolecular diagnostic tests are also recommended to identify extra pulmonary TB and pediatric TB cases. This cartridge system is nucleic acid amplification tests (NAATs) based method and within 2 h of turnaround time detects the presence of *Mtb* DNA as well as mutations

in *rpoB* which is a rifampicin drug resistance-associated gene [35–37]. Xpert assays are successful in diagnosing PTB in adults with 89% sensitivity and 99% specificity. The Xpert MTB/RIF Ultra assay is less specific than Xpert MTB/RIF assay as it fails to differentiate between dormant and active TB DNA samples. In addition, these assays are less sensitive in detecting *Mtb* in children and patients co-infected with HIV and extrapulmonary TB [38–40]. To overcome the limitations of high cost, and need of an uninterrupted power supply and to make these methods affordable in rural areas, several smaller and battery-operated technologies are in process. Currently, GeneXpert-Omni (Omni;Cepheid) is a promising, cost-effective, portable tool for widespread use in peripheral healthcare settings. It ensures point-of-care and is portable hence reducing the cost and time involved in transporting the specimens to central laboratories.

In addition to Omni, Cepheid is developing Xpert MTB/XDR assay to cover the detection of resistance to INH, FQL, ethionamide (ETH), and SLID. Similar to other Xpert assays, it is also a NAAT-based assay detecting 16 clinically significant mutations associated with resistance in 90 minutes. When compared to phenotypic drug sensitivity testing (pDST), it has 94% sensitivity and 100% specificity in detecting drug resistance [41]. Several large-scale multicentre clinical trials are currently ongoing in establishing it as a follow-on test to existing methods of Xpert MTB/RIF and MTB/RIF Ultra. The diagnostic performance of Xpert MTB/XDR needs to be improved for the early identification of drug resistance and shorter drug regimens.

10. Loop-mediated isothermal amplification (LAMP)

LAMP is a rapid, easy, inexpensive, and highly specific NAAT-based method used to diagnose infectious diseases. It utilizes different sets of primers (minimum 4) which can identify the target sequence by recognizing distinct regions (minimum 6) of target gene giving high amplification efficiency with a sensitivity of 76–80% and 97–98%. It is a single-step amplification-based strand displacement reaction of approximately 15–60 min at a constant temperature of 65°C and subsequent amplicon detection by visual inspection of incorporated fluorescence [8]. It is a simple method and does not use any expensive reagents or equipment for result interpretation and can be used as a rapid diagnostic tool in resource-limited settings and high endemic regions [42, 43]. For diagnosing EPTB, LAMP assay has higher sensitivity [44].

11. Line probe assay

Line probe assay (LPA) is a rapid PCR-based method that amplifies DNA from *Mtb* and immobilization of oligonucleotides on a strip. In the presence of gene mutations, immobilized oligonucleotides emit a colorimetric signal indicating isoniazid or rifampicin drugs resistance as well as drug-sensitive strains in the sample [45]. LPAs are efficient to detect drug-resistant strains of *Mtb* in smear-positive samples [46] and has optimal diagnostic accuracy in smear-negative TB cases [47]. WHO has endorsed LPA as the initial detection method of multidrug-resistant TB (MDR) for isoniazid and rifampicin resistance in both pulmonary and extrapulmonary TB patients [48]. LPA-based commercial products for TB drug resistance include INNO-LiPA Rif TB Kit (Innogenetics, Zwijndrecht, Belgium), GenoType MTBDRplus (Hain Lifesciences-Bruker, Nehren, Germany), and Nipro NTM + MDRTB II (Osaka, Japan).

Fluoroquinolone (FLQs) resistance can be detected by a more sensitive and new-generation LPA method (GenoType MTBDRsl version 2.0; Hain Lifesciences-Bruker) [8]. SL-LPA MTBDRs1 ((Hain Lifescience, Germany) is a second-generation line probe assay. MTBDRs1 (version 1.0) detects mutations in *gyrA*, *rrs*, *embB* genes and version 2.0 detects additional mutations in *gyrB* and *eis* promoter region. Even though LPA is a cost-minimizing method [49], its accuracy varies, and WHO has limited its utility in XDR-TB surveillance [48].

12. ddPCR (digital droplet PCR)

Droplet digital PCR (ddPCR) is a newly emerging technology and is being utilized in recent developments. In ddPCR, sample is diluted and partitioned into several hundred and millions of reaction chambers (**Figure 1**) [50]. Each separate chamber contains single or more copies of target sequence while rest do not contain target sequence and provides higher sensitivity than qPCR, detects single copy of DNA, and provides absolute quantification of gene expression [50]. This technique is used as a reference method in absolute quantification and detection of mutant DNA of drug-resistant subpopulations of *Mtb* [51]. The ddPCR has a higher sensitivity than quantitative or qPCR and *Mtb* infection can be detected in sputum and blood specimens of pulmonary and extrapulmonary TB patients [52, 53]. Though ddPCR detects a single copy of DNA per sample, is potent in absolute quantification, and works for both sputum and blood samples, it is prohibitively expensive and requires an uninterrupted power supply [25].

13. CRISPR

Combined use of Clustered Regularly InterSPaced Repeats or CRISPR and CRISPR ASsociated nuclease 9, Cas9 is an approach involving a programmable

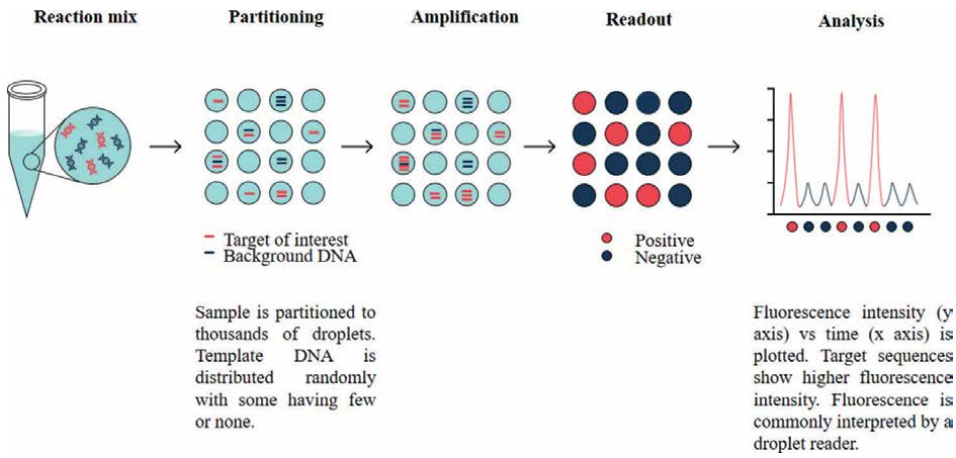

Figure 1.
Schematic Representation of principle of droplet digital PCR: Sample containing target sequence is partitioned into several droplets (in the magnitude of thousands) and then amplified separately. Each of these droplets are read by a dedicated droplet reader and fluorescence intensities are measured (indicative of positive/negative reactions) KEY: DNA – deoxyribonucleic acid.

enzyme which cuts DNA at specific sites (**Figures 2** and **3**) [25]. CRISPR is potential in detecting pediatric TB [54]. This test is a highly sensitive method with single-copy DNA detection, requires less sample requirement and a short turnaround time than Xpert method for detecting both pulmonary and extrapulmonary TB [55, 56]. CRISPR associated enzymes are used in SHERLOCK (specific-high sensitivity enzymatic reporter unlocking) platform which detects a single copy of RNA or DNA. This isothermal-based SHERLOCK technology can be used in places where electricity or portable readers are not available [57]. The combined LAMP and CRISPR-Cas12b detection method are more efficient in smear-negative paucibacillary TB patients [58].

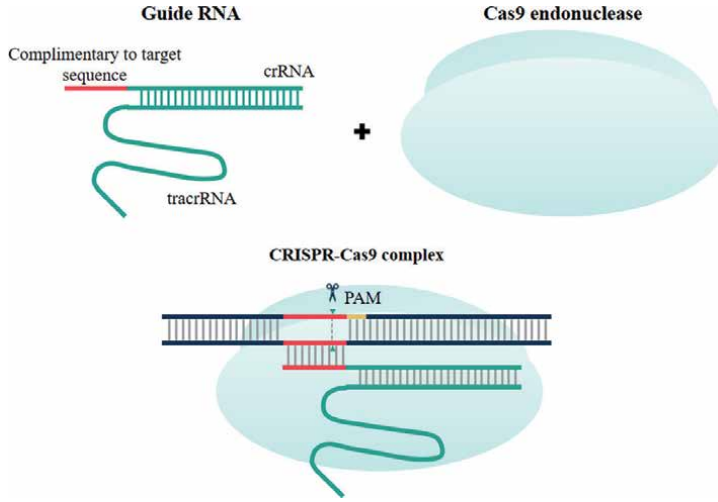

Figure 2.
Schematic Representation of the CRISPR/Cas9 system: GuideRNA (gRNA) binds with Cas9 enzyme to form a complex. Cas9 endonuclease nicks the DNA a few bases upstream to a Protospacer Adjacent Motif (PAM) and mediates the cleavage of target DNA regions which are complementary to the gRNA. Key: RNA – ribonucleic acid, CRISPR/Cas9 - CRISPR (clustered regularly interspaced short palindromic repeat)-associated protein 9, crRNA - CRISPR RNA, tracrRNA - trans-activating crRNA.

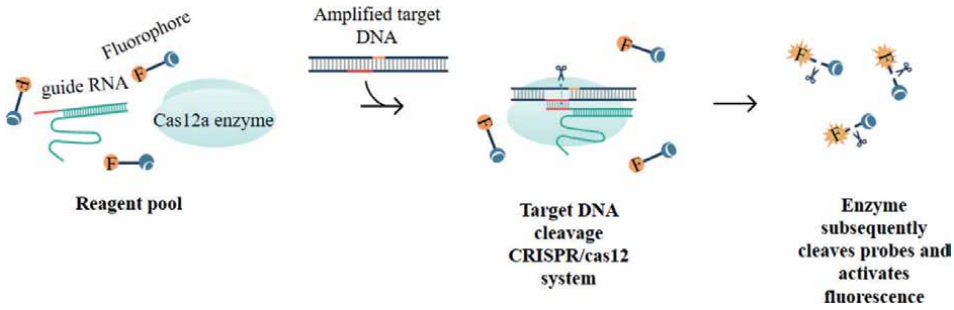

Figure 3.
Schematic Representation of the CRISPR/Cas12a system: CRISPR RNA binds with Cas12a enzyme and forms a complex. Fluorescent probes (C) are in the periphery binding to quenchers (Q) with an oligonucleotide. The enzyme cleaves the target DNA and subsequently probes which activates the fluorescent signals. Key: CRISPR/Cas12a - CRISPR (clustered regularly interspaced short palindromic repeat)-associated protein 12a, DNA – Deoxyribonucleic acid.

14. MicroRNA detection

MicroRNAs have an active role in several biological processes and are used as biomarkers in diagnosis, treatment, and prognosis of a wide range of diseases including tuberculosis. Circulating mRNAs are consistent among individuals, stable, and least influenced by endogenous RNAse activity. MicroRNA expression studies in plasma samples of pulmonary TB patients and normal individuals have identified smiR-769-5p, miR-320a, and miR-22-3p as potent plasma-based biomarkers in TB diagnosis and miR-320a levels were significantly higher in drug-resistant TB [59]. In addition, plasma miRNA levels of hsa-miR-29a-3p, hsa-miR-155-5p, and hsa-miR-361-5p were found to be significantly upregulated in active tuberculosis compared to normal individuals. This plasma-based detection method is a convenient way of diagnosing TB in a population where it is difficult to obtain sputum, especially in pediatrics and extrapulmonary TB cases [60]. These circulating plasma miRNAs can further enable the differential diagnosis of latent and active TB. Diagnostic performance of miRNA can be increased by integrating serum miRNAs with diagnostic models developed by miRNA characteristics and electronic health records (HERs) [61, 62] and bioinformatics analysis [63]. Even though miRNA-based detection of TB is convenient in diagnosis in children, the method is challenging to adopt in resource-limited settings. In addition, the efficacy of the test depends on the correct sequence of miRNA. A considerable number of miRNAs are identified in children TB cases and only 7% of them are considered significant for the test [64].

15. Handheld electronic nose model

The handheld electronic nose model is a point-of-care and portable model and it can be used for tuberculosis screening in remote rural areas and health care settings and to rule out TB test in vulnerable populations [65]. This device detects infection *via* the presence of volatile organic compounds (VOC). The Aeonose (eNose BV, Zutphen, Netherlands) is an example of such model and this device has sensors and pre-concentrator. The patient needs to breathe through Aeonose *via* a disposable mouthpiece for 5 minutes. Breath data will be generated on the laptop and analyzed through the website of eNose. The preliminary results showed poor sensitivity and specificity in suspected tuberculosis patients. The portable and short turnaround time for results (< 5 minutes), makes the model more suitable in rural areas [66, 67].

16. Raman spectroscopy

Raman Spectroscopy (SR) is another diagnostic tool used to diagnose cancer and bacterial infections. It is a portal device and can be used in rural areas [68]. The phenomenon of Raman scattering is used to identify unique molecular markers of bacteria upon excitation with a particular wavelength of light. A combination of Surface-Enhanced Raman Spectroscopy (SERS) with a bead-beating module of a lab-on-a-chip (LOC) device can successfully differentiate Mycobacterium tuberculosis complex (MTC) and nontuberculous mycobacteria (NTM) [69]. SR with less cost, and short turnaround time is potential enough to diagnose active tuberculosis and latent tuberculosis [70]. The combination of SR with an optical microscope is useful

in non-destructive identification of a single bacterial cell. SR is a rapid, user-friendly, non-invasive method for identification of pathogens. In addition, integration of machine learning can make SR a more effective TB screening method [71]. A recent study on developing PCR-based SERS has reported it can rapidly distinguish TB positive rifampicin-resistant and TB positive rifampicin-susceptible patients [72].

17. Artificial intelligence

The application of artificial intelligence (AI) along with historical methods of tuberculosis diagnosis such as Chest-X rays and smear microscopy is rapidly increasing and minimizes human errors in the interpretation of results [73, 74]. AI technology is in its initial stages which need to be validated with a large number of sample sizes. Depending upon the population used such as HIV co-infected patients or pediatrics, the results of AI vary widely [75]. Further, AI is under consideration by WHO as a technique to diagnose tuberculosis. In addition, the result images can be transferred via mobile phone technology from rural settings to server site and data can be analyzed with AI [76]. Artificial intelligence can be used to diagnose pulmonary and extrapulmonary TB [77] and to predict drug-resistant and drug-susceptible *Mtb* strains [78].

18. Electrochemical biosensor platform

Arginine film-based biosensor platform is a new PCR-free method used in the detection of tubercle bacilli. IS6110 gene is used as a biomarker. The probe corresponding to IS6110 gene will be immobilized on the biosensor platform and hybridized with a sputum sample or isolated DNA sample. The target gene will be identified based on electrochemical analysis using the principle of pulse voltammetry and methylene blue reduction signal measurement. The biosensor is a portable device with high sensitivity and selectivity for TB diagnosis [79]. An electrochemical device (EC) can be integrated to loop mediated isothermal amplification (LAMP) PCR for rapid detection of tubercle bacilli. LAMP-EC functions with a screen-printed graphene electrode (SPGE), redox probe, and a portable potentiostat for diagnosis of tuberculosis. The biosensor is a portable device. DNA isolation and hybridization with the sputum sample can be performed at room temperature, therefore, it can be used in rural areas [80].

19. Whole genome sequencing (WGS)

Whole genome sequencing (WGS) data has 96% concordance with culture-based drug sensitivity testing. It provides comprehensive detail of *Mtb* whole genome and genotypic sensitivity data to most drugs used in the treatment of MDR-TB. The correlation between genotypic results of WGS and phenotypic sensitivities is yet fully explored. WGS ideally can detect all the mutations in the genome and their functional characteristics [81]. This technique can be applied for genotypic and phenotypic characterization of organisms and profiling of drug susceptibility [82, 83] including detecting mutations in new drugs such as bedaquiline and delamanid [84]. The performance of WGS technology is further enhanced by incorporating a novel method

'SplitStrains' which helps to analyze WGS data of patients having mixed infections [85]. Due to high cost, the need of robust technologies, and technical expertise, initially, there was limited WGS utility in low-income countries [86]. In some countries, it is used as an important tool for case diagnosis and formulation of public health policies to trace TB contact cases in outbreak [87]. Even though NAAT and LPA methods are rapid, feasible, and accessible, detection of mutations in regions other than rifampicin resistance-associated gene *rpoB* is challenging. It is reported that 95% of resistance is due to mutations in this region, and WGS with the advanced genomics data on TB resistance can develop as a revolutionary to tailor TB treatment of each patient [4].

20. Flow cytometry assays of the M. tuberculosis: specific T-cell responses

The functional profile of T-cell mediated *Mtb*-specific responses to active disease and latent *Mtb* infection can be detected using polychromatic flow cytometry. Tuberculosis-specific flow cytometry panel comprises markers such as CD3, CD4, and CD8 which determine T-cell lineage, and interleukin 2, interferon-gamma, and tumor necrosis factor-alpha (TNF-a) antibodies as cytokine functional profile. TNF-α specific CD4 T cells are reported as a predictive marker to differentiate between active disease and the latent *Mtb* infection. The CD4 T-cell marker has higher sensitivity of 100% and specificity of 96% [88]. Even though flow cytometry is a highly sensitive method, antiretinal antibodies, and associated $CD4^+$ T cells were not found significant in latent TB-associated uveitis or sarcoid uveitis patients [89].

21. Urine-based diagnostic tests

Urine samples ensure a non-invasive method of detection assays and are easy to collect from both adults, particularly in HIV-coinfected TB patients and children. Commercial methods are available to detect tuberculosis in urine samples. Unlike NAAT detecting *Mtb* DNA, lateral flow urine lipoarabinomannan (LF-LAM) test detects *Mtb* infection by identifying lipopolysaccharide of mycobacterial cell walls in urine samples. Even though commonly not used in many countries, LF-LAM is recommended to use in HIV-coinfected patients. LAM test helps early detection of TB and lowers TB deaths in people living with HIV (PLHIV) [90, 91]. It can be often used in low-resource settings and it is beneficial in patients in whom obtaining sputum samples is difficult. It has 42% sensitivity in HIV-coinfected having TB symptoms. However, the specificity is less as it cross-reacts with other mycobacterial species and fungi. Thus currently, it is recommended to use as an initial screening test in rural healthcare centers of high endemic areas with TB infection [92, 93].

Few studies reported lower sensitivity of LAM in patients with non-HIV infections and moderate to higher specificity in patients coinfected with HIV and having advanced immunodeficiency [94]. Many tuberculosis diagnostic tests are less sensitive in HIV-infected patients having advanced immunodeficiency but the sensitivity of LAM enzyme-linked immunosorbent assay (ELISA) is high even at lower CD4 lymphocyte cell counts. In most TB patients with CD4 cell count <50 cells/μl detectable amount of LAM antigen in urine is reported and it can be tested using TB-LAM Ag urine dip-stick assays. This advanced assay is a point-of-care lateral flow, low-cost ($3.50 per test), and highly specific in patient with advanced HIV-associated immunodeficiency [13].

22. Conclusion

Diagnosis of tuberculosis needs a rapid with possible reporting on the same day of sample collection and making a quick therapy decision. Current existing methods of the tuberculin skin test, smear microscopy, immunological test, and conventional PCR method still face several challenges for optimal diagnosis. Tuberculin skin test shows false positive results in certain populations such as patients with prior BCG vaccine, children with *Mtb* infection, HIV co-infected patients. Sputum AFB stain is a quick and easy method, it is not a confirmatory for *Mtb* as nontuberculous mycobacteria also take up the stain and even symptomatic patients may remain undiagnosed or undergo delayed diagnosis. Volatile Organic Compounds (VOC), LF-LAM and eNose assays in *Mtb* patients have some future potential. These non-invasive and cost-effective tests are useful in children or critically ill patients.

Conventional diagnostic methods are used as routine diagnostic methods, however, WHO recommends next-generation of NAATs as they provide fast and reliable results as point-of-care diagnostics in peripheral healthcare settings. Advanced methods of GeneXpert MTB/RIF (GX) are highly sensitive and specific one-step PCR-based methods with a short turnaround time of 2 h. NAAT-based GeneXpert is routinely used in clinical settings in many countries. In summary, in the current technological development, WHO recommends POC-NAATs (2nd generations) in addition to GeneXpert MTB/RIF and peripheral laboratories and the WGS method in at least reference laboratories in near future for TB diagnosis.

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Conflict of interest

The authors declare no conflict of interest.

Authors contributions

Both the authors contributed for the idea of the manuscript, literature review and data analysis. First draft was written by PK and critically revised by KSS. Both the authors read and approved the final version of the manuscript.

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Chapter 4

Recent Advances in the Detection of *Listeria monocytogenes*

Puja Adhikari, Nkurunziza Florien, Shagun Gupta and Ankur Kaushal

Abstract

Listeria monocytogenes is the third-most severe pathogen causing a yearly outbreak of food poisoning in the world that proliferates widely in the environment. Infants, pregnant mothers, and immuno-compromised people are at high risk. Its ability to grow in both biotic and abiotic environments leads to epidemics that infect 5 out of 10 people annually. Because of the epithelial adhesion (by E-cadherin binding), it can suppress immune cells and thrive in the gastrointestinal tract till the brain through blood flow (E-cadherin). Microbial culture is still used as a gold standard, but takes a long time and often yields false positive results due to incompetence and temperature variations. Therefore, in order to treat it rather than using broad spectrum antibiotics, a standardized time-saving and highly specific technology for early detection is very important. It has been observed that the production of a particular antibody is delaying (so does the detection process) as a result of the inadequate understanding of the pathophysiology of the bacteria. This book chapter provides a brief summary of a pathogen as well as the scientific advances that led to its identification more easily.

Keywords: *Listeria monocytogenes*, culture, antibiotics, gastrointestinal tract, E-cadherin, PCR, biosensor

1. Introduction

Listeria monocytogenes is a gram-positive, rod-shaped, facultative anaerobic ubiquitous bacterium consisting of a mucopolysaccharide capsule, and has a low G + C content [1–8]. It is motile between 10 and 25°C and measures about 0.4–1.5 μm in length [8], it does not produce spores. The genus *Listeria* includes six species: *L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri*, and *L. grayi*. Among them, *L. monocytogenes* (frequently available), *L. ivanovii* (often), and *L. seeligeri* (rare) are potentially pathogenic [2, 6, 8, 9]. Consuming contaminated foods such as unpasteurized dairy products, raw meats, frozen foods, pre-packaged foods, environmental factors, sporadic cases of listeriosis, and outbreaks of the disease are the main causes of *L. monocytogenes* infection [1, 3, 7–14]. *L. monocytogenes* infection can cause septicaemia, meningitis, encephalitis, spontaneous abortion, fever, and self-limiting gastroenteritis in immune-compromised conditions [1, 3, 6–8, 11, 13, 15, 16]. It is commonly accepted that bacteria, after passing the intestinal barrier, travel through

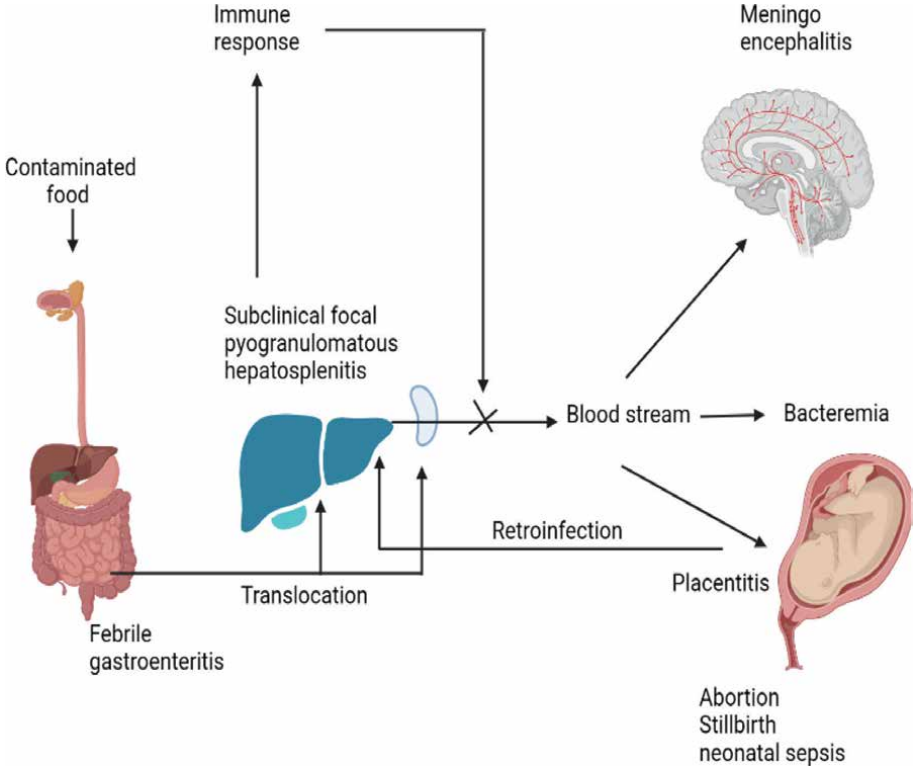

Figure 1.
Route of infection of Bacteria.

the blood and lymph to the liver, where they reproduce in hepatocytes, as well as the spleen. After that, bacteria can spread via the bloodstream and reach the brain and placenta (**Figure 1**).

To detect it and reduce the growth of *Listeria* various immuno-based and molecular techniques has been developed and modified. Modifications were made according to the report published by researcher in different time intervals. Starting from the gold standard culture, ELISA, PCR, NMR, NGS to biosensor was used. Though there is high chance of false positive result, culture is given the preferences to carry forward other techniques to justify the research. The purpose of this book chapter was to give a quick overview of *L. monocytogenes* and the advancement in available detection techniques. From culture based technique, immuno-based technique, molecular technique till sensor development had been discovered by different scientists in different time intervals and their advancements had been made as per the understanding of pathophysiology and virulence factor taking part in infection.

2. History and epidemiology

The Judicial Commission on Bacteriological Nomenclature and Taxonomy approved its use in 1940 and gave it the generic name *"Listeria"* in the sixth edition of Bergey's Manual of Determinative Bacteriology whereas Murray et al. proposed the name *"monocytogenes"* for the species, indicating that infected mono-gastric animals have a

high concentration of monocytes in their peripheral blood [4]. Though the first culture was done from a meningitis patient in France in 1921; the official human infection was reported by Nyfeldt from Denmark in 1929 [8, 17]. In 1979 contaminated vegetables affected 23 patients admitted to Boston hospital, in 1981 consumption of contaminated coleslaw affect numerous people. Contaminated milk (1983), contaminated manure used to grow cabbage in 1989, similar cases were observed till 1990s. In 2000 consumption of raw food and in 2001 consumption of contaminated hot dog resulted in 21% mortality [7]. 333 Food recalls made by US, Department of Agriculture (USDA) with Food and Drug Administration from 2002 to 2006 revealed that 32.4% of the most leading cases are from *Listeria monocytogenes* [18]. Study carried out by European Center for Diseases Prevention and Control together with European Food and Safety Authority in 2004–-2006, revealed that most *Listeria monocytogenes* positive cases found in meat and fish products [19]. Another outbreak was reported from CANADA in 2008 where 57 confirmed cases and 22 deaths that were due to the consumption of delicatessen meat [20, 21]. In 2010, 23.6% deaths had observed as stated by survey carried out by world health Organization [17], besides 78% were hospitalized with no deaths whereas in 2011 66% died, in 2013 17% died which if counted on average was 18% from the time range of 1998–2016. In 2018, 2 people died due to listeriosis [22]. According to the Centre for Disease Control and Prevention (CDC) it was estimated that 1600 people get listeriosis each year, and about 260 die. The recent outbreak as of June 8, 2022 reported from Florida was associated with contaminated ice-cream [23]. And presently it is no longer only a problem to humans but also to animals and various food items were confirmed to transmit this pathogen [24]. To summarize the outbreaks of different time intervals after 2008 have been summarized in **Table 1**.

Serial no.	Source	Year and cases	Contaminated Brand	References
1	Cantaloupes	2011, out of 147 persons infected, 33 deaths and 1 miscarriage.	NA	CDC [23]
2	Ricotta Salata Cheese	2012, out of 22 infected, 4 death, and 1 miscarriage.	Frescolina Marte brand of Italy	CDC [23]
3	Cheese	2013, out of 6 infected, 1 miscarriage and 1 death	Les Frères, Petit Frère, and Petit Frère with Truffles cheeses made by Crave Brothers Farmstead Cheese Company of Waterloo, Wisconsin, U.S..A	CDC [23]
4	Dairy Product	2014, 8 infected among them 5 were pregnant and 1 death	Roos Foods, Kenton, Delaware, U.S.A	CDC [23]
5	Cheese	2014, out of 5 infected, 1 death and 1 transmitted to new born and 3 cases of pregnancy.	Oasis Brand, Inc. U.S.A	CDC [23]
6	Bean Sprout	2014, out of 5 infected and 2 deaths.	Wholesome Soy Products, Inc. Sprouts of Chicago	CDC [23]
7	Commercial Apple Caramel	2014, out of 35 infected 1 miscarriage, 11 face problem during pregnancy and 3 suffered from meningitis.	Bidart Bros. of Bakersfield, California, Happy Apples, California Snack Foods and Merb's Candies	CDC [23]

Serial no.	Source	Year and cases	Contaminated Brand	References
8	Ice cream	2015, out of 10 infected, 3 death and 10 hospitalized.	Blue Bell Creameries Ice Cream	CDC [23]
9	Soft Cheese	2015, out of 30 infected, 3 death, 1 miscarriage and 28 hospitalized	Central Valley Cheese, Inc. Turlock, California. Karoun Diaries, Inc.	CDC [23]
10	Frozen vegetables	2016, out of 9 infected 3 death	CRF Frozen Foods of Pasco, Washington	CDC [23]
11	Raw Milk	2016, out of 2 cases reported, 1 death	Miller's Organic Farm in Bird-In-Hand, Pennsylvania	CDC [23]
12	Packaged Salads	2016, out of 19 infected, 1 death and 19 hospitalized	Dole Ohio Facility	CDC [23]
13	Vulto Creamery Soft Raw Milk Cheese	2017, out of 8 infected 2 death and 8 hospitalized where 1 was newly born..	Vulto Creamery of Walton, New York	CDC [23]
14	Deli Ham	2018, out of 4 infected, 1 death and 4 hospitalized.	Johnston County Hams, Inc. in Smithfield, North Carolina	CDC [23]
15	Pork products	2018, out of 4 cases, 4 hospitalized with no deaths	C. Corporation of Houston, Texas, Long Phung Food Products	CDC [23]
16	Deli sliced meats and cheese	2019, out of 10 infected, 1 death and 10 hospitalized.	Deli Sliced products	CDC [23]
17	Hard boiled eggs	2019, out of 8 infected, 1 death and 5 hospitalized.	NA	CDC [23]
18	Enoki Mushrooms	2020, out of 36 infected, 4 death and 31 hospitalized.	H and C Food, Inc. Guan's Mushroom Co. And Sun Hong Food. Inc.	CDC [23]
19	Deli Meats	2020, out of 12 infected, 1 death and 12 hospitalized.	Due to Italian style meat like salami, mortadella and prosciutto but supplier was not identified	CDC [23]
20	Queso Fresco	2021, out of 13 infected, 1 death and 12 hospitalized.	EI Abuelito Cheese Inc.	CDC [23]
21	Fully cooked chickens	2021, out of 3 infected, 1 death and 3 hospitalized.	Tyson Foods Inc., Jet's Pizza, Casey's General Store, Marco's Pizza, Little Caesars, and Circle K.	CDC [23]
22	Fresh Express Packaged Salads	2021, out of 10 infected, 1 death and 10 hospitalized.	Fresh Express, U.S.A	CDC [23]
23	Dole Packaged Salads	2021, out of 18 infected, 3 death and 16 hospitalized.	Dole	CDC [23]
24	Ice Cream	2022, Out of 23 infect3ed 1 death and 22 hospitalized.	Big Olaf Creamery Ice Cream	CDC [23]

Table 1.
Summarized table for the outbreaks of Listeria monocytogenes occurring from 2011 to 2022.

Serial number	Classification	References
Kingdom	**Bacteria**, Cavalier-Smith, 2002-bacteries, bacteria, bacterias	[25]
Subkingdom	**Postbacterial,** Cavaler-Smith, 2002	
Phylum	**Firmicutes** corrig/**Baccillota**, Gibbons and Murray, 1978	
Class	**Bacili**, Ludwig et al.,2010	
Order	**Bacillales**, Prevot, 1953	
Family	**Listeriaceae,** Ludwig et al., 2010	
Genus	*Listeria*, Pirie, 1940	
Species	*L. monocytogenes* (Murray et al., 1926) Pirie, 1940)	

Table 2.
Classification of Listeria monocytogenes.

3. Classification

It is classified on the basis of its character devised on culture media in different time intervals by different scientists and shown in **Table 2**.

4. Serotyping

L. monocytogenes has thirteen distinct serotypes. The majority of sporadic cases of listeriosis are tied to just three *L. monocytogenes* serotypes: 1/2a, 1/2b, and 4b, whereas serotype 4b is linked to nearly all outbreaks [1, 3]. While serovars 1/2a, 1/2b, and 4b appear to be similarly dispersed in Canada and the United States, serovars 4b predominate throughout the majority of Europe [16]. Several molecular subtyping methods were used.

5. Virulence factor

The *haemolysin* (*hly*) gene was the first virulence factor discovered in *Listeria*'s cellular structure, providing new insight into the intracellular and host-pathogen interactions during listeriosis [8, 16]. With the help of internalin A expressed on the cell surface of the bacteria, and epithelial cadherin (E-cadherin) expressed on the surface of epithelial cells [26, 27], *L. monocytogenes* is able to adhere to the host cell, evade immune response, and spread by penetrating the epithelial layers of the inner lining of the digestive tract [1, 28]. The proteins *actA*, Phospholipases (*PlcA* and *PlcB* (discovered in 1962)) are required for intracellular actin-based motility and cell-to-cell dissemination where the invasion protein *InlB, LLO* and *PlcA* encourage escape from the phagocytic vacuole, and *PlcB* promotes invasion [8, 13, 29]. The 10-kb virulence locus contains a group of these genes.

The *p60* protein, expressed by the *iap* gene, differs in each species of *Listeria* and contains 484 polypeptides but its specific role of it is not determined due to the fatality associated with the *iap* gene mutation. In one study it was found to be associated with intestinal invasion; binding with $CaCO_2$ and murine hydrolase [30]. In addition, *L. monocytogenes* contains metal ions that, when administered to infected mice in salt

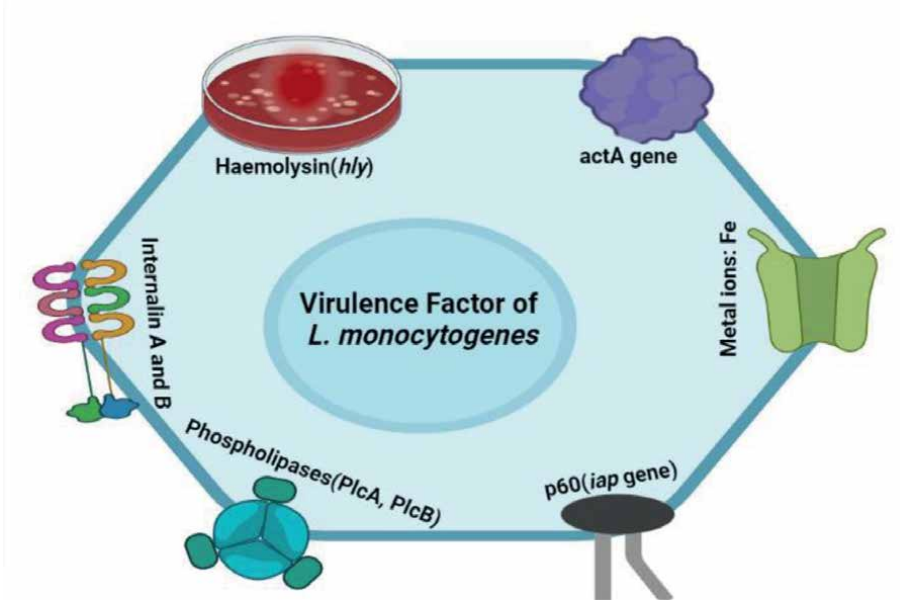

Figure 2.
Virulence factors of Listeria.

form, it will not only reduce the lethal dose but also increase bacterial proliferation rates in the liver and spleen and synthetic media (**Figure 2**) [8].

6. Pathogenesis

Bacterial entry into cells and intra-cytosolic replication serve as a precursor to diffusion throughout tissues as well as a mechanism for evading numerous antibacterial host responses and replicating in a protected environment. Bacteria escape the internal vacuole of the host cell by releasing toxin listerolysin O after pore formation on host cells due to which it gains the ability of haemolysis [31, 32]. On the other hand, *actA* starts intracellular proliferation; E-cadherin present on the goblet cell's epithelial lining of intestinal villi [33] serves as a receptor for internalin, while InlB receptors include hepatocytes growth factor receptor, and GAGs (glycosaminoglycans) for the entry as depicted in **Figure 3**. *Listeria* was the only pathogen to use E-cadherin as a receptor for entrance till the discovery of fungus C*andida*. The fungus *Candida albicans* causes oropharyngeal and hematogenously disseminated candidiasis with an ability to penetrate oral epithelial cells and endothelium cells in vitro. Als_3 is a surface protein that permits fungi to enter host cells. It interacts with both E- and N-cadherin on epithelial and endothelial cells [34]. The three-dimensional structure of the N-terminal of ecto-domain of E-cadherin revealed the molecular details of the interaction between internalin and human E-cadherin. This structure demonstrated that internalin interacts via its leucine-rich repeats in a completely distinct form, thus forming the homotypic E-cadherin interactions [35].

The previously unrecognized crucial position of amino acid 16 [36, 37] proline showed specificity to E-cadherin as in guinea pig [38]. Hence, the cytoplasmic domain Ecad links via beta and alpha catenin [37]. Based on this structure, a

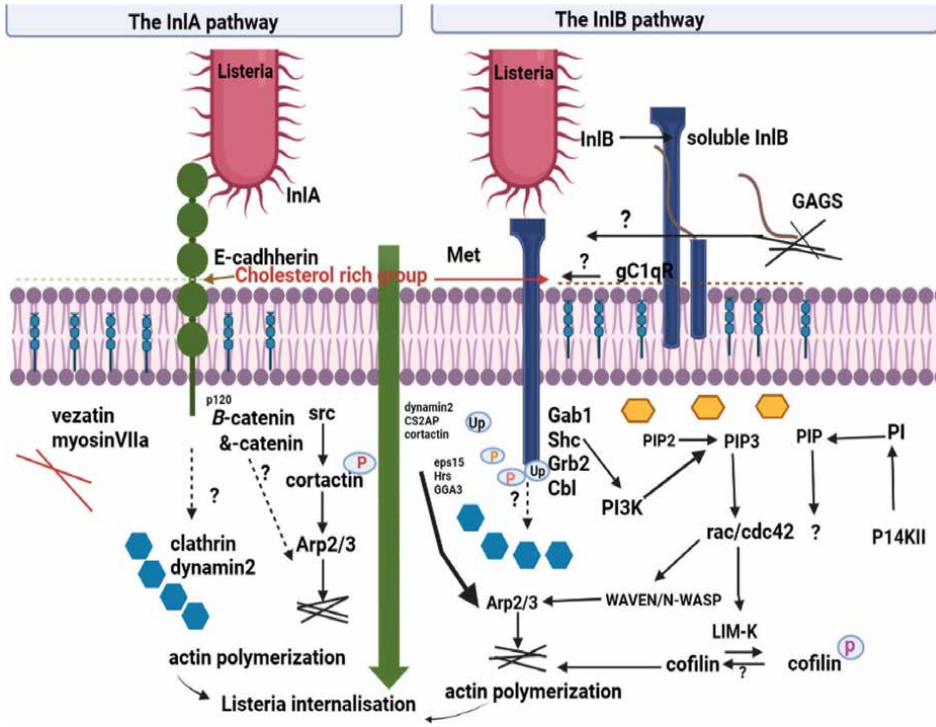

Figure 3.
Cellular signaling in Listeriosis.

"murinized" internalin has recently been designed and developed [39] to display the specific interaction with murine E-cadherin. The initial InlB receptor to be identified was gC1qR/p32 [40]. This intriguing molecule, which is very acidic and capable of trimerization, "sits" on a membrane [41]. It is mainly located in the mitochondria but can also be found in the nucleus and on the cell surface. In another case, it was found that gC1qR/p32 interacts with *Plasmodium falciparum*-infected red blood cells in contact with endothelial cells [42], InlB receptor, MET, a tyrosine kinase and the hepatocyte growth factor receptor (HGFR) [43]. HGFR and MET have been proposed crucial for *Plasmodium* invasion. *Plasmodium* invasion-induced hepatocyte injury results in the opening of cell structure to invasion. Met would then act as a signaling mediator [44]. Also, it was reported that the curved face of the *InlB* leucine-rich repeat region interacts with the first immunoglobulin-like domain of the MET stalk [a domain that does not bind HGF/S that allows *InlB* to get fixed for minute signal transmission [45]. MET activation requires C-terminal domains of *InlB* for heparin-mediated receptor clustering and robust signaling. In terms of structure, *InlB* differs from HGF. For *InlB*, the bacterium uses functional mimicry rather than structural imitation to take advantage of the properties of its receptors.

As the translocation of *L. monocytogenes* is fixed listerolysin O (*LLO*) triggered the host-tissue response allowing it to cross the intestinal barrier in an *InlA* dependent manner [33] where the absence of response to *Listeria* becomes consistent. After this, *Listeria* reaches the liver via the portal vein and got phagocytized by Kupffer cell including early necrostain-1-s-dependent death [46, 47]. Soon then, the monocytes get recruited and the type-1, type-2 inflammation takes place with the expression of

Serial no.	Factor affecting the growth of *L. monocytogenes*	References
1.	*pH*; suitable *pH* for its growth ranges from 4 to 9.4	[3, 51–54]
2.	Water activity; 0.90 lower the water lower is the chance of growth	[3, 51–53]
3.	Natural antimicrobial components; higher the quantity higher is the chance of growth of *Listeria*	[3, 52, 53]
4	Atmosphere; vacuum commission on pack for an aerobiosis, increased carbon Microbiological dioxide etc. extend the lag and generation times	[51–53]
5.	Metal/ Mineral ions; increase in concentration of metal ions decrease in growth of *Listeria* to no growth	[53]
6.	Temperature; −0.4°C to 45°C	[3, 51–54]
7.	High salt; $NaNo_3$, NaCl inhibit the growth of bacteria but can survive in salty environment	[3, 51–53, 55]

Table 3.
Different factors affecting the growth of Listeria monocytogenes.

Interleukin 33 and Interleukin 4. This phenomenon results in the restoration of liver haemolysis [48, 49] promoting the bacteria to flourish.

6.1 Factor affecting *Listeria monocytogenes*

In the case of *L. monocytogenes*, the main factors that contribute to its development and inhibition are *pH*, the composition of the food product, natural antimicrobial components, biological structures, temperature, atmosphere, competitive flora [2], metal ions, high salt [50], and water activity [51] which is discussed in detail in **Table 3**.

6.2 Recent advances in the detection of *Listeria monocytogenes*

It was said that to be infectious there must be 100 CFU/mL/g of *Listeria* in food. Due to delayed and non-specified symptoms, it is difficult to detect at an early stage. In Australia, it was observed that the 10 CFU in 25 g of packaged food cause listeriosis and 100 CFU/mL lead to the reoccurrence. So, scientists had developed several techniques to fulfill the need for a robust, sensitive and reproducible technique to detect *L. monocytogenes.* The most applicable and available detection techniques developed so, far are discussed below:

6.2.1 Culture-based techniques

According to a 2007 report by Lorber, the time-consuming yet precise cold enrichment method was created in the 1990s [56]. The FDA approved the Ottaviani et al., 1997 proposal to employ chromogenic medium (ALOA) for the identification of *Listeria* species [57]. Lecithin was hydrolysed, and the blue/green colonies appeared as a result of the dissociation of a chromogenic substrate by an enzyme β-D-glucosidase. Their colonies looked hazy halos. Following the confirmation of the bacteria, it was re-suspended in non-selective media and prepared for the 4–5 days long biochemical test. In addition, there used to be a high probability of false positive results, a need for several chemicals, media, and reagents [USA old 9], as well as a requirement of time and effort [58]. Hitchins and Valimaa et al. used the FDA-BAM technique to identify *Listeria* from dairy and seafood whose LOD was validated to be

less than 1 CFU/mL in 2013 [59] and 2015 [60], respectively. Valimaa et al. obtained comparable results from ISO 11290-1 method developed in 2004 where the LOD was 1 CFU/g. Later, Valimaa et al. discovered that the LOD was 1 CFU/g using the USDA-FSIS approach developed in 2013 [60]. The most probable number technique [61] was more sensitive than a chromogenic medium, according to Dwivedi et al. [61]. In order to quick identification of *Listeria*, demonstrated that the MPN-PCR technique was more promising than previous approaches [62].

6.2.2 Immuno-based techniques

Antigen-antibody biochemistry appears to be promising for the screening and diagnosis of a disease. To light this, Gasanov et al., reported in 2005 that an immunological technique had sensitivity greater than that of the conventional method, which is 10^5 cells/mL [6]. However, Diaz-Amigo, reported in 2010 that DNA is the most dependable basis to carry forward the immunological diagnosis, adding that the method is time-consuming for antibody preparation [63].

6.2.2.1 ELISA

In 2005, Bell and Kyriakides, discovered that the sandwich ELISA method was superior than culture to detect *Listeria* in food samples [64]. The LOD was found to be 10^5–10^6 CFU/mL when the anti-antigen was used to target the *Listeria* antigen [64]. Ueda and Kuwabara used the enzyme linked fluorescence assay, or ELFA, to analyze food samples in 2010 [65]. Based on the food sample's acidity and basicity, a LOD of 10^5–10^6 CFU/mL was found to be reliable. Malla et al. tested sera samples at a dilution of 1:200 for listeriosis using an indirect ELISA. Positive P/N ratios were set to greater than 2. Synthetic *LLO-2* peptide (0.40 g/well) and *rLLO* (0.50 g/well) were used as antigens during this method [66].

6.2.2.2 Immuno-magnetic separation

In 2006, Amagliani et al. introduced a method utilizing nanoparticles to combine a magnetic field with a concentrated amount of bacterial cells. This was done to boost the sensitivity of the detection technique [67]. In 2006 Yang et al., designed a prototype combining real time PCR to immuno based approach using rabbit anti-*Listeria* with immuno-magnetic nanoparticle coated beads for the detection of *hlyA* gene in milk sample and the LOD observed was $>10^2$ CFU/0.5 mL [68]. Similarly, in 2010 Walcher et al. used paramagnetic beads coated with endolysin-derived cell wall domain of *Listeria* from tainted raw milk. The LOD lies between 10^2 and 10^3 CFU/mL [69].

6.2.3 Molecular methods of detections

6.2.3.1 DNA microarrays

The *Listeria* virulence genes *inlB*, *plcA*, *plcB*, and *clpE* were discovered by Volkhov et al. in 2002 using DNA microarray. He reported that the *Listeria* result was positive using this method [70]. In 2003, Borucki and Call investigated serotype-specific probe differentiation by combining 585 genomic DNA (10 samples) mixed probes and found that it was successful for 29 probes [71]. After that, it was used as a confirmatory technique by Brehm-Stecher and Johnson to check the specificity of

polymorphism and PCR amplification. With a detection limit of 8 log CFU/mL [72], Bang et al. reported that 9/16 of the microarrays used to test artificially infected milk were positive. He emphasized that this approach was accurate and reliable. Despite being encouraged, it needs patience and has the potential to cross-hybridize, which could lead to a false test result [73].

6.2.3.2 PCR based methods

PCR has been widely used in molecular diagnostics as a potential tool for minute sample detection. A heat cycle in PCR required a set of specialized primers for the target amplification. The results are then analyzed using gel electrophoresis. The modifications that were made in order to detect *Listeria* using PCR are discussed below:

6.2.3.2.1 Conventional PCR

The use of primers in PCR makes it a potential method for identifying pathogens in a sample. Aznar and Alacron reported that the result obtained from PCR was positive for 56 out of 217 cases in naturally infected samples with a limit of detection of 1 CFU/g where only 17 was observed to be positive during culture. They employed primers designed to target the genes *hlyA*, *iap*, *inlB*, *inlA*, *16S*, and *23S rRNA*, as well as the proteins *phospholipase C*, *fibronectin-binding protein*, and *hypersensitivity protein* for the detection [74].

In cases of non-viable DNA amplification, Klein and Juneja [75] reported that the PCR method was a false positive. Reverse transcriptase PCR (RT-PCR) was used because mRNA has a short half-life and immediately disintegrates after cell death [72], to target the *hly, prfA,* and *iap* gene transcripts rather than DNA. The detection threshold was between (2.5×10^6 and 3×10^6) CFU/mL. In order to validate the technique, they used cooked beef that had been intentionally infected. They found that the analysis was sensitive to 1 CFU/g. However, Pan and Breidt used real-time PCR and achieved success with propidium monoazide and ethidium monoazide in amplification of dead cells, arguing against it as an efficient method for detecting bacteria in low numbers whose LOD was 10^3 CFU/mL [76].

6.2.3.3 Multiplex PCR

Multiplex PCR was described by Alarcon et al. [77] as a reliable, efficient, and time-saving technique for simultaneously detecting several organisms in contaminated samples. Samples with varying LOD as 57 CFU/ml of *Salmonella spp.,* 79 CFU/ml of *L. monocytogenes*, and 260 CFU/ml of *S. aureus* [73]. This strategy was employed by Lei et al. to identify six common food-borne pathogens in RTE food with LODs of 1–100 CFU/ml [78]. The MPCR method was introduced by Zhang et al., in 2009 and targets the *hly* gene of *L. monocytogenes*, the *nuc* gene of *S. aureus*, the *invA* gene of *S. enterica*, the *stx* gene of *E. coli,* and the *intimin* gene of *E. coli*, with a detection limit of 1 CFU/mL [79]. In 2006, Mustapha and Li stated that MPCR as non-specific for the similar sized amplicon and optimization [80].

6.2.3.4 RT-PCR (Real-Time PCR)

A three-day PCR-based assay was developed by Kaclikova et al. (2003) with a detection limit of 10° CFU per 25 g of food, which is equivalent to the standard EN

ISO 11290-1 or ISO 10560 methods of Listeria detection. LOD obtained was 1×10^4 CFU/mL [81]. Bhagwat et al. reported that the total viable count detected was $1.35 \pm 2 \times 10^8$ in the salad and $0.35 \pm 1.9 \times 10^8$ in broccoli. In which the limit of detection of L. monocytogenes was $1.74 \pm 1.1 \times 10^6$ in salad, $6.37 \pm 1.2 \times 10^3$ in broccoli and overall less than 1000 cells/mL [82]. A hly-IAC Q-PCR assay to detect *Listeria* was created in 2005 by Rodriguer and Lazaro et al., utilizing different concentrations to spike the sample, and the detection limit was determined to be 8 [83]. To expand the scope of the technique Berrada et al. [84], developed RTQ-PCR to quantify the fluorescence emitted by the spiked sample. The obtained LOD was $10–10^5$ CFU/mL [84]. In 2006, Fairchild et al. developed a detection method using SYBR green which shows in presence of non-target DNA and primer-dimer formation [85–89]. O'Grady et al. found that targeting the *ssrA* gene in naturally and artificially contaminated foods (milk products, meat, and veggies) resulted in a detection limit of 1–5 CFU/25 g/mL [90]. Therefore, he came to the conclusion that it was a smart strategy for the particular sample. In 2010 Suo et al. reported the result of a qRT-PCR assay with the detection limit of 18 CFU/10 g on naturally and artificially contaminated ground beef, chicken, turkey and pork [91].

6.2.4 Biosensor based techniques

A biosensor is the biological specimen analyzer using analyte as an object and an electrochemical set up as a transducer generating readable data. In 2004, BIA3000 was first used by Leonard et al., as a biosensor where he passed the antibody over a biosensor chip immobilized on polyclonal goat anti-rabbit Fab antibodies to detect *L. monocytogenes* [92]. Advancing the sensor platform Poltronieri et al. reported that the use of surface plasmon resonance to detect *L. monocytogenes* was promising with a detection limit of 10^2 CFU/mL [93]. In this platform Au-labeled secondary antibodies were used. On further advancement Banerjee and Bhunia reported the use of mammalian B-lymphocyte Ped-2E9 cell merged in collagen matrix as a sensing platform to detect listeriolysin O from the contaminated food sample with a detection limit of $10^2–10^4$ CFU/g [94].

In 2015, Lui et al. [95] developed a paper-based micro fluidic device that detects long DNA amplicons on the basis of hybridization reactions with a covalently immobilized DNA probe and biotin-labeled signal DNA strands, and chemiluminescent (CL) reactions catalyzed by a horseradish peroxidase (HRP) streptavidin conjugate. In this DNA biosensor, CL signals generated using a HRP-luminol-H2O2 system were heightened with p-iodine phenol (PIP) and detected with a CCD system. Under optical conditions, a linear range of 1.94×10^{-1} pmol/L to 1.94×10^4 pmol/L was achieved and the limit of detection was found to be 6.3×10^{-2} pmol/L [91]. Then, in 2022 Zhang et al. [96] and his team from China develop a portable paper-based multi-biocatalyst platform to identify *L. monocytogenes* by detecting multiple biomarkers at different levels: gene *hly* (nucleic acid), acetoin (small molecule metabolite), and LLO (protein). The integrating detections of the three biomarkers were successfully performed by two different modified working electrodes on a single paper-based multi-biocatalyst platform. The sensitive and reliable identification of *L. monocytogenes* was achieved using the portable paper-based multi-biocatalyst platform with a wider detection range (from 1.0×10^4 to 1.0×10^9 CFU mL^{-1}) and lower detection limit (10^4 CFU mL^{-1}) [97]. Similarly, in 2022 to further advance the sensing technology Du et al., developed a fluorescence-based dual recognition assembly using Fe_3O_4 @ZIF-8. The linear range of the detection of pure culture ranged from 1.4×10^1 to 1.4×10^7 CFU/mL, with the detection limit of 0.88 CFU/mL.

S. no.	Methods used	Source	Year	Specification	Limit of detection	References
1.	Bacterial culture	Food	1997	Blue/Green colonies of *Listeria* due to the dissociation of chromogenic substrate by an enzyme β-D-glucosidase.	4–5 days for the confirmation.	Ottavianii et al., [57]
2.	FDA-BAM	Dairy and Sea food	2013&2015	Specific to *Listeria* detection	1 CFU/mL	Valimaa et al. [59]
3.	ELISA	Food	2005	Able to detect other food pathogens too.	10^5–10^6 CFU/mL	Bell and Kyriakides [64]
4.	Immuno-magnetic separation	Milk	2007	Anti-listeria rabbit antibody was used with immuno-magnetic nanoparticles coated beads to detect hlyA gene	10^4 CFU/mL	Yang et al. [68]
5.	DNA Microarrays	Milk	2007	Antigen-labeled probe was used.	10^8 CFU/mL	Brehm-Stetcher et al. [72]
6.	Conventional PCR	Patient sample	2002 and 2003	Primers were used targeting *hlyA*, *iap*, *inlB*, *inlA*, *16S*, and *23S rRNA*, as well as the proteins *phospholipase C*, *fibronectin-binding protein*, and *hypersensitivity protein*.	101 CFU/mL	Aznar and Alacron [74]
7.	Multiplex PCR	Human	2004	Could detect other food-borne pathogen too. It was not limited to *Listeria*.	5.7×10^1 CFU/mL of *Salmonella spp.*, 7.9×10^1 CFU/mL of *L. monocytogenes*, and 2.6×10^2 CFU/mL of *S. aureus*	Alarcon et al. [77]
8.	Real Time PCR equivalent to standard EN ISO 11290-1 method	Food	2003	3 days PCR-based assays were developed to detect *Listeria* from 25 g of food.	10^4 CFU/mL	Kaclikova et al. [81]

S. no.	Methods used	Source	Year	Specification	Limit of detection	References
9.	RT-PCR	Food	2008	Target *ssrA* gene	1–5 CFU/mL	O'Grady et al. [90]
10.	qRT-PCR	Beef, Chicken, Pork	2010	qRT-PCR assay	10^2 CFU/mL	Suo et al. [91]
11.	BIA3000-Biosensor	Bacterial antigen	2004	Assembly was prepared immobilizing polyclonal goat anti-rabbit Fab antibodies	Resulted in minute detection	Leonard et al. [92]
12.	Surface plasmon resonance	Bacterial antigen	2009	Assembly was prepared immobilizing Au-labeled secondary antibodies	10^2 CFU/mL	Poltronieri et al. [93]
13.	Immuno-based sensor	mammalian B-lymphocyte	2010	Assembly was prepared with mammalian B-lymphocyte Ped-2E9 cell merged in collagen matrix.	10^2–10^4 CFU/mL	Banerjee and Bhunia [94]
14.	Single paper-based multi-biocatalyst	Bacteria	2022	Assembly was developed to identify *L. monocytogenes* by detecting multiple biomarkers at different levels: gene *hly* (nucleic acid), acetoin (small molecule metabolite), and LLO (protein).	10^4 CFU/mL	Zhang et al. [96]
15.	Dual recognition and highly sensitive detection of *Listeria monocytogenes*	Food	2022	Fe O @ZIF-8@ aptamer	0.88 CFU/mL	Du et al. [97]

Table 4.
Available diagnostics methods for the detection of Listeria monocytogenes.

Out of these methods, culture-based tests are usually preferred because of their availability, sensitivity, cost-effective and the 'gold standards' compared with other methods that are validated. To summarize the availability and development of detection methods is presented in **Table 4**.

7. Conclusion

Pregnant women, young children, and elderly individuals with suppressed immune systems are mostly affected by *Listeria monocytogenes*, a common and third-most lethal food-borne illness. The worldwide death rate is substantially worsened by its outbreak. Despite the use of suitable food processing methods, outbreaks spurred on by eating foods infected with *L. monocytogenes* inevitably occur. There is not a commercially available vaccination to prevent listeriosis at present. The molecular processes, interspecies interactions, and cross-domain interactions that affect *L. monocytogenes*' virulence, AMR, metabolic activity, and survival under stress as seen in the environment and host are not well explored. Numerous methodologies have been used until this point, notably DNA microarrays, PCR, immuno-based methods, culture-based methods, and ELISA. However, these methods have limits when it comes to the identification of this virus. Thereafter, it is necessary to put into effect a novel researcher's suggestion for a quick detection approach, such as biosensor-based techniques, which have high sensitivity and specificity as well as time savings and more research is required to determine the possible significance of bacterial communication in the control of the stress response in *L. monocytogenes.*

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Chapter 5

Campylobacteriosis in Sub-Saharan Africa

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Abstract

Research and clinical works have documented various species of campylobacter in Africa. Thermophilic campylobacter has been shown to be endemic in the sub-Saharan Africa (SSA) region, and the prevalence is suspected to be increasing. To define the geographic boundaries of SSA, the United Nations macrogeographic definition of Africa has been used in several studies https://unstats.un.org/unsd/methodology/m49/. According to this UN definition, sub-Saharan Africa is divided into East Africa, Central Africa, Southern Africa, and West Africa. The zoonotic potential of campylobacter has been extensively studied and documented in the SSA region. Children are the most affected by campylobacter infections, and the infections exhibit seasonal patterns. Research has shown varied sources of infection such as foods of animal and plant origins, as well as unpasteurized milk and water, but animal meat is the most common source of infection. This chapter will delve into finding more recent information on campylobacter in the region such as the species, their prevalence, virulence, and risk factors. It will also explore the options in management such as vaccines and recommended diagnosis therapeutic protocols in humans and animals.

Keywords: campylobacter, Africa, endemic, thermophilic campylobacter, diagnosis therapeutic protocols

1. Introduction

Campylobacter is a gram-negative, nonspore-forming, curved or spiral bacilli, which are oxygen-sensitive and prefer to grow under micro-aerobic conditions. Certain species are relatively thermotolerant and therefore are considered thermophilic. Such species include *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*), which are of critical importance to food safety, grow optimally at 42°C [1]. Campylobacter pathogen is common and endemic in sub-Saharan Africa (SSA) and causes gastroenteritis in animals and humans. The bacteria is highly infectious zoonotic pathogens and a major cause for the global human gastroenteritis infections with over 400 million cases reported annually in developing nations [2].

Assessment and quantification of the true burden of campylobacteriosis in the African context is hampered by the under-reporting of symptomatic diarrhea as well

as inadequate surveillance programs of foodborne illnesses, as well as the minimal attention to *Campylobacter* as a pathogen. During diagnosis and laboratory testing, *Campylobacter* is rarely considered among the top suspects of diarrhea therefore not investigated, diagnosed, and reported [3]. Owing to the thermotolerant nature of various species of *Campylobacter*, they can survive various temperatures of cooking and cause cross-contamination of foods and food products leading to human and animal infections.

Most campylobacter infections do not need to be treated with antimicrobial agents, since there is evidence of spontaneous recovery. However, in a subset of patients especially pediatric and geriatric patients, campylobacter may cause severe complications and increased risk for death and therefore requires treatment. Other groups who are vulnerable include especially in immune-deficient or immune-suppressed individuals [4]. The most common drugs of choice in the treatment of such infections including fluoroquinolones such as ciprofloxacin or macrolides such as erythromycin are currently used because of their large spectra activity on enteric pathogens [5].

The disease is endemic in all sub-Saharan countries with varying prevalence rates across the region. The main sources of campylobacter infections include meats and milk with the most common source being poultry meat and eggs. A review publication by Gahamanyi et al. [4] identified the highest prevalence in Nigeria among all age groups with the most prevalent species being *C. coli*.

2. *Campylobacter* virulence

Virulence refers to the propensity of an infectious agent to cause a disease. The proteins and the genes which have an important function in disease development are known as virulent factors or determinants. Virulence factors of campylobacter include toxins, adhesins, invasion factors, flagellum proteins for motility, iron acquisition factors chemotaxis, lipooligosaccharide (LOS) secretion systems and campylobacter polysaccharide (CPS), antigens, genes, and response to environmental and oxidative stress [6].

2.1 Motility

The *Campylobacter* motility system needs flagella and a chemotaxis-based system that regulates the movement based on conditions of the environmental. Chemotaxis is the capability to move toward environments which are favorable that contain higher nutrients concentration or lower concentration of toxicity [7]. Chemotaxis has implication in the virulence of various pathogenic bacteria, which relies on this process to invade hosts. The movement of motile bacteria can be controlled by different extracellular chemical gradients detected by transducer-like proteins (Tlps) also known as methyl-accepting chemotaxis proteins (MCPs). These external stimuli, which bind to, relay a signal to chemotaxis proteins in the cytoplasm, which initiate a signal transduction cascade resulting in directed flagellar movement. Motility is significant for survival under the various gastrointestinal tract conditions and for small intestine colonization [8]. Campylobacter has uncommon movement more so in viscous substances. This is because of the presence of one or two polar flagella and the helical cell shape. The former provides propulsive cell movement, while the helical shape ensures the corkscrew rotation [9].

2.2 Adhesins

Campylobacter adhesion to the host intestinal epithelium is important for colonization. *C. jejuni* has a large number of different adhesins that individually or together mediate bacterial attachment to different cellular structures and various hosts [10]. Flagellum, outer membrane proteins (OMPs), and lipopolysaccharides (LPSs) are among the presumed adhesins. *Campylobacter* adhesion protein to fibronectin (CadF) attaches to epithelial cells fibronectin as the ligand. This adhesion stimulates a β-integrin receptor which triggers phosphorylation of the epidermal growth factor receptor. Erk1/2 signaling pathway is the activated one, and the GTPases Rac1 and Cdc42 are recruited and stimulated by Cia proteins, which begin the engulfing of *Campylobacter* via cytoskeleton reagent and membrane ruffling [10].

2.3 Invasion

After the bacterial adhesion to host cells intestinal membranes, *C. jejuni* invades the cells via endocytosis. Invasion process requires the *Campylobacter*-stimulated rearrangement of the cytoskeleton through microtubules and microfilaments [6]. Flagella are thought to have a second function in addition to that of motility designed to function as an export device type III secretion system (T3SS) in secretion of non-flagellar proteins during host invasion. It is also known that *C. jejuni* invasiveness in vitro is associated with de novo synthesis of entry-enhancing proteins and requires host cell signal transduction. Variants of the flagellin proteins such as flaA, flaB, flgB, and flgE genes have reduced invasiveness, while flaC and *Campylobacter* invasion antigens (Cia) gene products are important in colonization and invasion and are taken into the host cell's cytoplasm using this flagellar secretion system. Full invasion of INT-407 cells requires CiaC, while CiaI has a function in intracellular survival [9].

2.4 Toxin production

The bacterial invasion process does not appear to be solely responsible for the cytopathic effects associated with *C. jejuni* infection. Toxins are likely involved in the disease process. In *Campylobacter* only, one toxin cytolethal distending toxin (CDT) is a known toxin produced in *Campylobacter* and has DNAse activities which lead to damage of DNA. Cytolethal distending toxin functions in the host cell invasion and results to extended period of symptoms and persistence of infection. Formation of CDT is activated by many factors including quorum *sensing* and is synthesized after *C. jejuni* has invaded the intestinal epithelial membrane. The toxin consists of three subunits encoded by cdtA, cdtB, and cdtC gene, and gene products are needed for the toxin to be functionally active [9]. Once the toxin is inside the cell, cdtB results in DNA double-strand break and probably cell death [6].

2.5 Carbohydrate structures

Lipooligosaccharides (LOS) majorly O- and N-linked glycans and a capsule on the cell surface of the *Campylobacter* facilitate colonization and associated genes. The lipooligosaccharide molecule contains an oligosaccharide core and lipid A which have various roles, including, host cell adhesion, immune evasion, and invasion. Sialylation of the LOS increases invasive potency and lowers immunogenicity [11]. Polysaccharides have a central role in the host-bacteria interaction and are essential

for virulence and antigenicity. Cell surfaces of *C. jejuni* are covered by a polysaccharide capsule that enables survival, adhesion, and evasion of host immune system. Capsule mutants show decreased evasion process [7].

C. jejuni contains an N-linked glycosylation system controlling posttranslational changes of periplasmic proteins. Flagellin subunits are the only ones modified by O-linked glycosylation. N-linked glycosylation regulates evasion as the glycosyl molecules are immunodominant leading to slow generation of antibodies against the protein fraction [11]. N-glycans also protect *C. jejuni* surface proteins against protease enzymes of the gut. This explains why *Campylobacter lari* (PglB) mutants with a deficiency in the expression of oligosaccharyltransferase reveal slow growth in media supplemented with cecal contents [12].

3. *Campylobacter* prevalence in SSA

A wide variety of animals, including poultry, wild birds, sheep, cows, pigs, cats, and dogs, serve as natural reservoirs and source of transmission for *Campylobacter* [4]. As a result, *Campylobacter* colonization in various reservoirs creates a significant danger for human health due to the pathogen's release into animal waste, contaminated water sources, the environment, and food. In Africa, a sizeable share of the population keeps poultry or livestock and most cases both. Unfortunately, these animals are frequently kept and slaughtered in unhygienic and unsanitary settings; hence, the high rates of campylobacter reported in animal husbandry [13]. Information on prevalence of campylobacter is scanty in SSA region because of the cumbersome and expensive procedures involved in its isolation, although some studies have attempted to determine both human and animal prevalence.

According to results of a recent systematic review and meta-analysis, the species *C. jejuni* is the most common in sub-Saharan Africa [14]. These results are consistent with another systematic study done in West Africa in 2022 [13] in which it was shown that *C. jejuni* was the most frequently detected species compared to *C. coli,* with a prevalence rate of 52% and 30%, respectively. Likewise, it is the most prevalent campylobacter species found in food, and the one commonly associated with human campylobacteriosis is *C. jejuni* [15].

A review carried out by [4] reported prevalence of thermophilic *Campylobacter* in humans ranging from 9.6 to 62.7% on average in sub-Saharan Africa. Nigeria reported the highest prevalence of 62.7% in humans followed by Malawi (21%) then South Africa (20.3%). For *Campylobacter* infections in children under 5 years of age, Kenya reported 16.4% of her cases, followed by Rwanda (15.5%) and Ethiopia (14.5%). The mean prevalence among all age groups and the children under 5 years of age at the country level was 18.6% and 9.4%, respectively. The prevalence is within the ranges found in other low- and middle-income countries (LMICs) as shown by Coker et al. [16] study. The prevalence was, however, higher and lower than that reported from Korea and the USA, respectively [17, 18]. This difference could be explained by the fact that campylobacteriosis is hyperendemic in LMICs, perhaps as a result of poor sanitation and close contact between people and domestic animals [4].

According to a systematic review carried out by Hlashwayo et al. [14] in SSA, prevalence rate ranged from 0–100%. According to the review, species identified in various regions were *C. jejuni*, *C. coli*, *C. fetus* subsp. *venerealis*, *C. hyointestinalis*, *C. upsaliensis*, *C. fetus*, *C. fetus* subsp. *fetus*, *C. troglodytis* sp. nov, *C. sputorum* subsp.

Sputorum, C. lari and *C. f. venerealis* biovar *intermedius*. Like most other studies, the studies showed that *C. jejuni* and *C. coli* were the most prevalent species; while *C. hyointestinalis*, *C. sputorum,* and *C. troglodytis* were the least prevalent. Hlashwayo et al. [14] also reported that Western Africa recorded higher prevalence of *Campylobacter* species compared to other SSA regions. The high prevalence could be explained by transportation of unchecked poultry and other animals due to the presence of large market for live animals in the region [19].

Campylobacter subgroup analysis studies carried out in the same region by [13] recorded a pooled prevalence in poultry (39%, 95% CI: 27–52) higher than in other livestock (26%, 95% CI: 17–38). In poultry, the individual prevalence was estimated between 4–88% and 11–93% in livestock. Also, the same study recorded a pooled estimate of 10% (95% CI: 6–17) in humans with a lot high level of heterogeneity (I^2 = 98%).

Eastern Africa comes second in terms of percentage *Campylobacter* prevalence from studies [14]. Different animals have been screened for the presence of campylobacter prioritizing poultry and cattle. Isolates reported were *C. jejuni, C. coli, C. lari, C. upsaliensis, C. fetus*, and *C. troglodytis*. A 100% campylobacter prevalence was found in fecal materials from wild monkeys [14]. *C. fetus subsp. venerealis* has also been identified as the source of enzootic infertility in smallholder herds in this area.

In a systemic and meta-analysis review carried out by Zenebe et al. [20] in Ethiopia, the overall *Campylobacter* species prevalence was 10.2% (95% CI 3.79, 16.51) and heterogeneity was not observed across the included studies (I^2 0.01%; Q = 3.23, p = 1.00). Also, 75% of the studies reported *C. jejuni* and *C. coli* at the species level.

Middle Africa has the least data on campylobacter in SSA. *C. jejuni* and *C. coli* are the only species reported. A prevalence of 92.7% was reported from slaughtered chicken, highlighting the role of food animals in the epidemiology of campylobacter in the region just like other regions. This suggests that they may not be epidemiologically delinked or varied from other SSA regions.

In Southern African region, poultry and cattle have been studied more, while pigs, sheep, goats, and dogs have also been studied to a limited extent. *C. jejuni, C. coli, C. upsaliensis*, and *C. fetus* were the common isolates in the region. A higher prevalence of *C. jejuni* and *C. coli* was reported in diarrheic chicken and goats [21].

In overall, data on *Campylobacter* prevalence are limited due to the expensive procedures and capacity required for such studies compared to other bacteria.

4. Risk factors associated with campylobacteriosis

Generally, campylobacter presents itself in asymptomatic nature, forming natural commensals in the intestinal tracts of majority of animals but is more pronounced in birds. These animals act as natural reservoirs for the bacteria and more often a source of human infection by contamination through pathogen shedding in fecal matter [22]. Studies by Ogden et al. [23] faulted contaminated food products of animal origin as the source of human gastroenteritis.

4.1 Risk factors

There are numerous factors that predispose humans and animals to campylobacteriosis infections in SSA, and case-control studies have been done to quantify on their impacts to provide strategic and targeted control measures. These factors include:

4.1.1 Contaminated animal products

Contaminated animal products, especially poultry meat and unhygienic handling of food items, pose a human risk factor for sporadic campylobacter infections. Animal meat production patterns in SSA ranging from home slaughtering to unhygienic meat preparations in open fires and barbecues are channels for campylobacter transmission. Unhygienic slaughtering procedures and poor handling of meat and its products may provide an avenue for human contamination in abattoirs. The enteric nature of campylobacter species constitutes the risk of cross-contamination from the fecal matter of same or different animal during flaying and evisceration. Contamination can also occur through cross-contamination between hide and carcass or in situations where contaminated water is used to clean the animal carcasses [24].

A study in Tanzania that targeted screening of cattle in abattoirs for *Campylobacter* reported a 5.6% prevalence rate of thermophilic campylobacter with *C. jejuni* as the predominant strain [25]. Different studies have reported a prevalence range of between 5% and 89% of campylobacter in cattle [24, 26]. In 1999, Osano and Arimi [27] reported a 2% contamination level of campylobacter on carcasses in Kenya.

High levels of campylobacter carcass contamination in pigs have been reported in SSA. In Tanzania, pork carcasses have been recorded to have 10.16% levels of contamination Komba et al. [28]. This is similar to a study outside the SSA in Brazil Aquino et al. [29]. Other studies in other developing nations have shown high rates of pork contamination between 34 and 63.6% [30] and Malakauskas et al. [31].

Developed nations have recorded low levels of campylobacter contamination on pig carcasses, and this has been attributed to low levels of enteric campylobacter due to animal feeds, abattoir levels of hygiene, and carcass handling procedures [32, 33]. Unpasteurized milk and raw milk products are associated with campylobacter illness in developing nations. Contamination of milk often occurs during milking through fecal contamination of the animal's udders or udder infection. Poor personnel hygiene also contributes substantially in contamination of the udders [4]. In SSA, there are no strict legislations with regard to production and sale of milk to consumers so as to curb on unpasteurized milk contamination which is associated with campylobacter infections.

4.1.2 Domestic animal vectors

Animal contact is a major risk factor for acquiring human campylobacteriosis, and this is due to occupational exposure to animals in a slaughterhouse, pet shop, farm, or zoo. Animal contact also occurs during food handling/preparation or animal husbandry [34]. Most of the population living in rural areas and small farmers make up the largest population of animal keepers. Farm animals are mainly kept in free range systems, where there is close interaction between animals and humans, and thus an exposure to zoonotic pathogens is made possible [35]. Occupational exposure poses a potential risk factor, especially when biosecurity measures such as limited restriction to the animals or poultry housing and personnel security and hygiene are not followed. Cleaning and disinfection of animal housing prior to restocking and presence of a medicated footbath at the entrance plays a key role in the prevention of transfer campylobacter to the personnel house [36]. Children are found to be more susceptible to acquiring campylobacteriosis through animal contact during play than the general population. A study carried out by [34] reported a significant relationship between animal contact and acquiring campylobacteriosis.

4.1.3 Wild animal vectors

Wild birds are considered notable reservoirs of *Campylobacter* and often contaminate the environment through fecal droppings [37]. Children are at risk of ingesting campylobacter in the open playgrounds because of exposure to contaminated fecal matter. Open fields and playgrounds often act as natural habitats for wild birds and stray dogs in SSA and present a potential reservoir for campylobacter [35].

A case study by [38, 39] in New Zealand found a 12.5% positivity for *C. jejuni* in avian fecal samples from a children's playing field indicating the possibility of a high-risk factor in SSA [40]. Livestock manure and other uncovered waste are also very prominent sources of human and animal infections. Therefore, handling livestock manure and drinking untreated water pose a risk of health risk associated with campylobacter [41].

There is a wide range of natural reservoirs for campylobacter, including chickens and other poultry, wild birds, pigs, dogs, cats, sheep, and cows. Consequently, colonization of various reservoirs by campylobacter poses a significant risk to humans as the pathogen becomes contaminated in livestock waste and the environment [4]. Manure gets contaminated when the reservoirs shed the pathogens.

4.1.4 Contaminated water

Water bodies such as lakes and rivers have been associated with campylobacteriosis in SSA because of contamination with animal feces, draining of sewage effluent, discharge from slaughterhouses, and slurry that is used in agricultural farms. Campylobacter can remain infective in water for over 120 days [42]. In developing countries in SSA, water bodies act as sources of drinking water both for the animals and humans, providing platforms for bathing, swimming, and other water sports that can all act as routes for sporadic campylobacter contamination. Studies have also shown that rainwater can act as sources of campylobacteriosis through avian fecal contamination [43].

4.1.5 Age

Campylobacteriosis in children under the age of 5 years is common is SSA, and it is attributed to undesirable hygienic conditions and poor water sanitation systems. Poor maternal hygiene also predisposes young children to diarrhea infections through feeding, cleaning, and other routine childcare practices. Children are also at risk during outdoor play as they come closer to animal wastes on the environment and are often less keen to hand and body hygiene. Exposures to different environmental conditions influence children's risk to diarrhea [34].

A review article by [4] based on 33 articles showed that a young age is a high-risk factor for campylobacter infections in SSA. Kenya recorded the highest prevalence of campylobacteriosis at 16.4% in children under the age of 5 years. In Rwanda, the prevalence rate was 15.5%, while Ethiopia reported 14.5% on the same observation group.

4.1.6 Underlying diseases

Underlying diseases have been shown to act as predisposing factors for campylobacteriosis. Chronic conditions such as chronic gastrointestinal disease, gastric ulcers, celiac disease, liver disease, asthma, or diabetes have been associated with

campylobacteriosis in the different populations [4]. People with underlying diseases get immunocompromised, hence easily predisposed to campylobacteriosis [44]. For instance, human immunodeficiency virus (HIV)-infected patients with diarrhea are more likely to be infected with *Campylobacter* than uninfected individuals with diarrhea. In addition, the incidence of *Campylobacter*-related diseases is higher in HIV-infected patients than in the general population. Also, studies by [45] reported a high incidence of campylobacter-associated illness.

4.2 Campylobacteriosis diagnosis and species identification methods

4.2.1 Specimen collection

Appropriate clinical specimen types for *Campylobacter* testing are liquid or semi-soft stool and rectal or stool swabs. Though rare, *Campylobacter spp*. may also be recovered from specimens such as blood and tissue majorly liver for *C. lari*, and fetal uterine content in abortion caused *Campylobacter spp.* Specimens should be collected during the acute phase of the diarrheal illness before antibiotic treatment is initiated. Urine-free stool and swabs should be collected in a sterile, airtight container containing modified Cary-Blair (CB) transport medium [46]. Stools with evidence of blood, mucus, or pus are optimal. Rectal swabs are acceptable in infants and young children when feces are otherwise difficult to obtain; however, these are not acceptable specimen types for many culture-independent diagnostic tests (CIDTs)-based test platforms. Modified CB-moistened swabs provide good recovery of *Campylobacter*, though other swabs, including Amies, have also shown good recovery for campylobacter.

Typically, a single specimen is sufficient, particularly for the recovery of *C. jejuni* and *C. coli*. In cases of persistent diarrhea with a negative culture or any other time when initial testing does not provide a definitive pathogen, collecting a second specimen may be appropriate. Specimen rejection may be appropriate upon receipt of solid or formed stool, stool mixed with urine, dry swab or swab lacking visible evidence of stool, evidence of barium, leakage from the container, a frozen specimen, or a specimen submitted in expired or parasitic transport medium [47].

4.2.2 Transport and storage of isolates presumptive and confirmed

Campylobacter isolates may be submitted to Public Health Labs for confirmation and/or characterization. Post-culture, and for proper transportation, fresh campylobacter isolates (24 hours old) should be swabbed from a plate, placed in transport media (modified CB or Amies Transport Medium) and shipped on ice overnight or as a frozen bacterial culture in trypticase soy broth with 20% glycerol on dry ice. Isolates that are not preserved in glycerol should not be frozen or come into direct contact with ice packs, as this will reduce recovery. If isolates are to be submitted on solid media, Columbia agar with blood, Brain Heart Infusion (BHI) or Wang's should be used. Shipment on Trypticase Soy Agar is not preferred.

Prior to further testing, *Campylobacter* isolates should be held as frozen stocks (−60°C) in glycerol or maintained on fresh culture media with routine passage. It may be useful to store antibiotic resistant and outbreak-associated isolates for later reference and characterization, as per the laboratory's isolate retention policy. Frozen stocks should never be completely thawed. Instead, a small amount of the

stock should be partially thawed, removed, and the stock returned to the freezer as soon as possible. Repeated freeze-thaw cycles should be avoided [48].

4.2.3 Direct diagnosis

4.2.3.1 Culture-dependent diagnostic tests (CDTS) for the detection of Campylobacter

Campylobacter usually grows on most nonselective culture media, especially when enriched with blood. The majority of these media have been developed for isolation of *C. jejuni* and are rarely suitable for other species, hence limited application to veterinary samples. Five percent of blood agar is suitable for culturing *C. fetus* and *C. jejuni* of aborted ewe's samples. Pre-contamination filtration membrane is recommended to minimize contamination with other bacteria. Optimum atmospheric growth conditions are 55 oxygen, 10% CO_2, and 85% hydrogen and nitrogen and are artificially generated by commercial gas generating kits in conjunction with standard anaerobic jars use of tri-gas incubator [49].

4.2.3.2 Materials and reagents

- 5% sheep or horse blood agar plates
- 0.65 u millipore membrane filters
- Gas jars
- Gas packs—oxide gas generating packs for *Campylobacter.*

4.2.3.3 Procedure

Centrifuge macerated tissue/fecal/fetal stomach contents samples at 100 g for 10 minutes. Aseptically remove supernatant and incubate at 37°C for 1 hour. Place 0.65 um membrane filter on a surface of each isolating agar plate. Place between 5 and 10 drops of incubated supernatant fluid onto the center of each filter and incubate at 37°C for 1 hour. Remove and discard the filters and spread the filtrate over the agar surface. Allow the plates to dry. Incubate the plates on atmospheres of 5% O_2, 10% CO_2, and 85% H_2 or N_2 and examine by plate microscopy for typical colony of campylobacter species after 48–72 hours.

4.2.3.4 Results and interpretation

Following culturing on media containing blood at 37°C colonies will vary in appearance from small round and complex one. *Campylobacter fetus* are large and mucoid colonies. *C. jejuni* and *C. coli* will produce large and small colonies coexisting on plate which can be sub cultured for single colony isolation. Stains of *C. jejuni* can develop a metallic sheen over the culture surface. Generally, coccoid forms of *Campylobacter* are invariably due to long incubation periods and may be considered degenerative and nonviable; however, *C. jejuni* cultures produce coccoid forms before 48 hours. Hemolysis is not observed in blood agar.

4.3 Identification of campylobacter species

Campylobacter colonies on plate agar plates can be confirmed by gram stain, oxidase reaction, and catalase reaction. *C. fetus* and *C. jejuni* can be distinguished from other species by growth temperature studies antibiotic sensitivity, production of hydrogen sulphide, and hippurate hydrolysis. In gram stain, campylobacter cells are short gram-negative rods and have a distinctive curved or spiral appearance.

In oxidase test, 1% tetramethyl-p-phenalene diamine hydrochloride aqua solution is used, and it forms dark purple color within 10 seconds. In catalase test, *C. fetus, C. jejuni*, and *C. laridis* all possess catalase enzymes which catalyze release of oxygen from hydrogen peroxide. *Campylobacter fetus* will grow best between 25° and 37 but not at 42°. *C. jejuni, C. coli*, and *C. laridis* will grow at between 37 and 42 but not at 25°C.

4.4 Indirect diagnosis

4.4.1 Polymerase chain reaction

4.4.1.1 IQ-check campylobacter PCR technology

Polymerase chain reaction has advantage over the standard gold test of culturing, since culture diagnostic test for *Campylobacter spp*. is lengthy protocol [50]. The test is based on gene application and detection by real-time PCR [51]. The kit is ready to use PCR reagent containing oligonucleotides (primers and probes) for specific *C. jejuni, C. coli*, and *C. laridis* as well as DNA polymerase and nucleotides (IQ-check campylobacter PCR technology kit manual, BioRad). Detection and data analysis is optimized by Bio-Rad real-time PCR instrument called CFX 96 Touch Deep Well System. The test is used for qualitative detection of *Campylobacter* species in food products, environmental samples, fecal matters, and animal tissues.

The protocol involves sample enrichment, free DNA treatment, DNA extraction, real-time PCR, and data analysis. The sample enrichment step is a key in subculturing the contaminated samples to increase bacterial growth of *Campylobacter*. This protocol step is specific on type of sample diagnostics for food sample contamination, and n/10 g of sample is added into 9n/10 ml of supplemented Bolton broth in a stomacher bag with incorporated filter then incubated without shaking for 4 hours at 37 ± 1°C under micro-aerobic condition and transferred to 41.5 ± 1°C for additional 24 hours under microaerobic condition. For carcass rinsed sample, the carcass is rinsed in 40 ml of buffered peptone water for 1 minute, and the rinse of 30 ml is added to 30 ml of double-strength blood-free Bolton enrichment broth (2XBF-BEB), mixed gently, and then incubated for 24 hours at 42 ± 1°C under microaerobic conditions. For carcass swab after sponging carcass, 25 ml of 2XBF-BEB is added into it, mixed gently, and then incubated at 42 ± 1°C for 24 hours at microaerobic condition.

Fecal matters are homogenized into supplemented Bolton broth in stomacher bag with incorporated filter then allowed to decant at room temperature for 10 minutes. The next step is free DNA removal treatment. For DNA extraction, lysis reagent is aliquoted into wells of deep plates, enriched media sample added to the mixture and mixed by pipetting up and down until homogenized. The deep well is crossed by pre-pierced sealing. It is then heat-blocked at 95°C for 15 minutes and thereafter incubated under agitation at 1300 rpm at 95°C for 25 minutes. After that, it is vortexed at high speed for 2 minutes. The supernatant is then extracted and stored at 20°C and

always allowed to thaw,homogenize and then centrifuged at 12,000 g for 5 minutes before reusing.

The next step is real-time PCR. PCR involves preparing the mix containing application solution and the fluorescent probes. The volume of PCR mix needed depends on number of samples and controls to be analyzed, and at least one positive and negative control must be included in each PCR run. The application solution and fluorescent probes must be used within 1 hour after storage at 2–8°C. 45 ul PCR mix is aliquoted to each well of the plate, then 5 ul of DNA extract, negative control, and positive control are added to the corresponding wells. The wells of the PCR plates are sealed, centrifuged/ quick-spun to eliminate any bubble, then PCR plates are placed in thermocycler. To run PCR, the iQ check kits instructions should be followed in real-time PCR system guide. The PCR data analysis is done by CFX manager IDE software. Then data interpretation is done when the parameters have been set and the Cq values of each sample are interpreted. Positive and negative control sample results should always be verified before interpreting. Positive campylobacter samples have Cq values more than 10 FAM fluorophore (IQ-check campylobacter PCR technology kit manual, BioRad).

4.4.1.2 Real-time PCR

Different protocols of real-time PCR are applied for the detection of different species. In the detection of *Campylobacter jejuni*, primers and corresponding probes targeting hip O genes are used, while *Campylobacter coli* quantitative protocol of [52, 53] is used and adapted to the fast real-time PCR method by minor adaption (omitting 'G's at 3′-end) of forward primers. For other species, *C. lari* and *C. hyointestinalis* genus-specific 16S rRNA encoding DNA [54] region is targeted. To this purpose, the method by Lund et al. [55] is adapted to the fast real-time PCR method. To this end, the forward primer is elongated with three bases, a new reverse primer is designed, and the TaqMan probe is redesigned to contain the minor groove binding (MGB) quencher dye. Real-time PCR is performed on an Applied Biosystems 7500 thermal cycler, using the TaqMan® Fast Universal PCR Master Mix. For the real-time PCR, 5 ml of DNA, 10 ml of TaqMan® FastUniversal PCR Master Mix, 1 ml (10 pmol) of forward and reverse primers 1 ml (5 pmol) TaqMan probe are mixed, and 2 ml of DNase free water is added to a final volume of 20 ml. The cycling conditions consisted of 3 min at 95°C, followed by 40 cycles of 3 s at 95°C and 30 s at 60°C. Real-time data were analyzed with Applied Biosystems 7500 software (version 1.4). Upon completion of the run, a cycle threshold (Ct) is calculated and plotted against the log input DNA to provide standard curves for the quantification of unknown samples [56].

5. Conclusion

Campylobacter infection, or campylobacteriosis, is a challenge in the SSA, and the prevalence seems to be increasing with increasing surveillance and diagnosis. The disease seems to be more prevalent among the farming households and those living under lacking hygienic standards. Despite the disease being self-limiting, its impact, zoonotic potential, and cost cannot be ignored. With increased demand of animal protein for nutrition and this being a key source of infection, more work needs to be done with a focus of SSA in mind due to financial constraints and poverty. The work should be geared toward development of rabid diagnostics for this disease to enable early diagnosis and limit its effects on populations and economies.

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Despite the development of numerous antimicrobial agents, the successful eradication of bacterial infections remains a challenge, and bacteria continue to pose a major public health threat. It is estimated that, due to the increasing prevalence of antibiotic resistance, infectious diseases will become a leading cause of death by 2050. The current annual focus is on the challenges and advantages in the prevention and diagnosis of infections caused by ESKAPE pathogens. The book also includes a dedicated chapter on the diagnosis of tuberculosis, one of the leading infectious causes of death worldwide. Additionally, readers will find chapters describing achievements in the diagnosis and detection of common gastrointestinal tract pathogens, *Listeria monocytogenes* and *Campylobacter jejuni*, authored by experts in their fields.

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