Prevalence, Molecular Characterization and Antibiotic-Resistance Pattern of *Salmonella* spp. In Aquaculture Supply Chain in Bangladesh

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A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOTECHNOLOGY

Department of Mathematics and Natural Science
BRAC University
February, 2025

Declaration

I hereby declare that the thesis entitled 'Prevalence, Molecular Characterization and Antibiotic-Resistance Pattern of *Salmonella* spp. In Aquaculture Supply Chain in Bangladesh' is my own work done under joint supervision of Dr. Mohammed Badrul Amin, Associate Scientist, Laboratory of Food Safety and One Health, Nutrition and Research Division (NRD), International Centre for Diarrheal Disease Research, Bangladesh (icddr,b) and Dr. Iftekhar Bin Naser, Associate Professor, Department of Mathematics and Natural Science.

It has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

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CERTIFICATE OF APPROVAL

We hereby declare that the thesis entitled 'Prevalence, Molecular Characterization and Antibiotic-Resistance Pattern of *Salmonella* spp. In Aquaculture Supply Chain in Bangladesh' is from the student's own work and effort, and all other sources of information used have been acknowledged. This Thesis has been submitted with our approval.

Supervisor	**Supervisor**	**Program Coordinator**	**Chairperson**
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Acknowledgment

At very first, all my praises, gratitude, and thanks to Almighty Allah for his benevolence and blessing which has kept me wealthy and enabled me to work on my research project. I would like to give my cordial gratitude to my respective supervisor, Dr. Iftekhar Bin Naser, Associate Professor, Department of Mathematics and Natural Science, BRAC University, for his continuous support, helpful and inspirational advice helping me to go ahead and do my research project work completely. I was also privileged to thank Dr. Mohammed Badrul Amin, Associate Scientist, Laboratory of Food Safety & One Health, Nutrition Research Division, icddr,b for his expertise, and valuable insights, and for allowing me to continue my thesis at the Laboratory of Food Safety & One Health, Nutrition Research Division (NRD), icddr,b. Besides this, A very special thanks to Kazi Injamamul Hoque, Senior Research Officer; Ajrin Sultana, Senior Research Assistant; Laboratory of Food Safety & One Health, Nutrition Research Division (NRD), icddr,b. for their constant supervision, constructive criticism, and invaluable encouragement were crucial in shaping and completing my work.

The significant contributions made by David Mondol, Medical Technologist of the Laboratory of Food Safety & One Health, icddr,b, fill my heart to the brim with gratitude. My knowledge and skills have been enhanced by his kind instruction on the fundamental laboratory protocols for my thesis work. I am also very grateful to all the employees and students of the Laboratory of Food Safety & One Health, Nutrition Research Division (NRD) , icddr,b for their continuous support and suggestions. Finally, cordial thanks to my parents, family members, and all of my friends (Rubaiya, Salman, Tasmia, Sharif) and well-wishers for their encouragement, and inspiration throughout the period of this thesis period.

The author

February, 2025

Ethics statement

While conducting the thesis, I was committed to uphold the highest ethical standards in all aspects of my actions and decisions. My commitment to ethical practices underscores my dedication to trust, responsibility, and the pursuit of moral excellence.

Abstract

Salmonella represents a persistent global health threat, primarily causing food-borne infections through contaminated animal-derived products, including poultry, eggs, and fish. In developing countries like Bangladesh, *Salmonella* is increasingly found as a diarrhea-causing agent. Despite its significance, systematic detection and monitoring of *Salmonella* within Bangladesh's aquaculture supply chain remains poorly understood, necessitating a comprehensive investigation into its prevalence and characteristics. The study examined 794 samples, a total of 400 fish samples (Pangas and Tilapia species) fish tank water (n=206) and cutting board swabs(n=188) were collected from various points along the supply chain including ponds(n=120), wholesale markets(n=100), and retail markets(n=574). Standard microbiological culture methods and molecular identification techniques were employed, with PCR analysis targeting the invasion *(invA)* marker gene. The investigation included detection of clinically relevant virulence genes *(avrA, ssaQ,* s*odC1, bcfC)* and antimicrobial susceptibility testing against 20 antibiotics using the Kirby-Bauer Disc Diffusion method. Analysis revealed that 32% (258/794) of fish samples tested positive for *Salmonella* species, with the highest incidence observed in retail markets 37%(n=211/574) followed by wholesale markets 23%(n=23/100) and ponds 20%(n=24/120).For environmental samples, *Salmonella* spp. Was detected in 35% of fish tank water samples and 38% of cutting board swab samples. Nalidixic acid and Tetracycline showed 10-11% resistance in both fish samples where as other antibiotics (Gentamycin, Pipeperacillin/Tazobactum, Meropenem, Ertapenem showed resistance within (0-3)% as well as Cefepime showed 0% resistaNce. Virulence gene analysis identified *bcfC* as the most prevalent gene (77%), followed by ssaQ (74%), avrA (69%) and sodC1(46%). The findings highlight the significant burden of *Salmonella* species within Bangladesh's Tilapia and Pangas aquaculture value chain. The study emphasizes the critical need for implementing a comprehensive One Health approach that integrates public health, aquaculture sector practices, and environmental health considerations. These results underscore the importance of developing and implementing effective risk reduction strategies in Bangladesh's aquaculture sector to ensure public health safety and sustainable aquaculture practices.

Dedication

I dedicate this piece of work to

My beloved parents

&

My younger brother

whose endless support and belief in me made this possible.

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Chapter 1
Introduction

Introduction

1.1 *Salmonella* spp. as public health concern

Food-borne infection which is occurred by *Salmonella* puts a threat for public health globally, which can extend the financial burden on both developed or developing nations through the cost of disease surveillance, prevention, and treatment measures, with gastroenteritis being the most prevalent manifestation follows by bacteremia and enteric fever (Crump et al.., 2004; Majowicz et al, 2010). Salmonellosis stands for describing enteric infection caused by *Salmonella* bacteria except *Salmonella* Typhi and *Salmonella* Paratyphi species. *Salmonella* is a worldwide pathogen which puts impact upon both humans and animals, it also has zoonotic effect. This bacteria is morphologically gram negative, flagellated, facultative anaerobic bacillus structure and classified by two species named *Salmonella bongori* and *S. enterica*. *Salmonella* is categorized by the capability of causing specific pathologies for humans. these categories are Typhoidal *Salmonella* and Non-typhoidal *Salmonella* (NTS). Additionally, Typhoidal *Salmonella* causes typhoid fever which is a severe systemic illness, as well as NTS is responsible for a wide range of infections, including gastroenteritis and bacterimia, which are less severe than typhoid fever (Hendriksen et al., 2011). Non-typhoidal *Salmonella* infections can be developed from the consumption of contaminated food (Gianella, 1996) , *Salmonella enterica* is a highly virulent species that has 2600 serovars or serotypes. *Salmonella enterica* is the most significant subspecies of *Salmonella*. All of the serovers (99%) have the potential to cause infectious disease in both humans and animals (Issenhuth -jeanjen et al.) *Salmonella* spp. Is able to infect human being by the process of food production and consumption cycle, but initially through the consumption of animal derived, but they are not limited to poultry products and aquatic foods. So it can be claimed that contaminated foods can be the strongest and primary source of the transmission of this bacterial pathogen. (Jajere, 2019).

From the perspective of public health, *Salmonella* spp. Is the most responsible bacteria which can cause intestinal disease in human, which is claimed by World Health Organisation (WHO). *Salmonella* ranked the second main reason of FTDs in both European Union and the United States. *Salmonella* typhi and paratyphi A are the most frequently encountered pathogen for bloodstream infections in children above two months of age in Bangladesh. These two

serotypes, nearly two-thirds are positive for all blood culture in laboratories. (Saha et al., 2017). The Centre for Disease Control and Prevention (CDC) has confirmed that recent *Salmonella* outbreak is maintaining a connection with fishes in four states of USA.

1.1.1 Environmental effect in water and fish

Salmonella can be found in different aquatic environment like pond, river, seas and fresh reservoirs (Polo et al., 1999). *Salmonella* is naturally found in the gastrointestinal tracts of vertebrates, which refers it is their primary habitat (Woodword et al, 1997). Of feces are excreted by infected animals, the pathogens can be transmitted into aquatic systems through sewage charges, heavy rainfalls and the surfaces of the runoffs.(Sha et al, 2013). It is scientifically pointed that enteric bacteria like *Salmonella*, can survive in aquatic ecosystems, as well as these bacteria can contaminate the water and puts a potential risk of transmission to aquatic animals like fishes. *Salmonella* has been detected in fish supply chain Among various countries. (Gelaw and Teshome, 2021; Fernandes et al., 2018;Havelaar et al. 2013) *Salmonella* also can infect the outer part of fishes' body such as skin and gill. Cross contamination can be occurred during the steps of fish handling, processing, storage and commercialization, since hygienic practices are not followed accordingly, sometimes equipment and surfaces are not properly sterilized due to the production chain. Although it is not common incident where the presence of *Salmonella* species has been found in both finned and scaly fishes. (S er al. ;Da et al., 2005) fish is a staple food among the consumers and a rich source of protein, however, fish muscles do not contain any kind of microorganisms in it. (Noboslaveskijj et al., 2016;Karunasgar, 2015;Austin, 2006). On the other hand, fishes are spontaneously exposed to microorganisms which are present in water, sediment, including pollutants and wastages (Sa et al., 2004). Microflora of external surfaces of the fishes can be harmed by microorganisms. The digestive tract of fishes may come in contact with water and food containing microorganisms. Colonization of microorganisms may occur in primary stage, like during egg and larval phases, and can continue thoroughout the fishes' growth. (Austin, 2006;Olafsen, 2001).

1.1.2 Current Situation of Pangas and Tilapia production in Bangladesh

Bangladesh is considered one of the top fish producing countries worldwide, modestly due to its various water resources and in the 2020-2021 FY, the total fish production reached 4. 621 million MT (DoF, 2022). Moreover, annual production of cultured Pangas increased from 155 tons in 2010-2011 to 395 thousand tons in 2021-2022 fiscal year. In 2022, Tilapia production was about to increase by 2-4 percent. China, Latin and certain American countries were in challenges for high costs, limited inputs, and demand instability, trade volumes remained stable in the first half of 2023. (FAO, 2024). Bangladesh also ranked as major Tilapia producers among other international market producers. Tilapia remained popular than other fishes in the international market for the reasonable cost in the international market due to stable supply. (FAO, 2023). Bangladesh ranked as 5th position in the aquaculture production worldwide as well as owned the position in 4th in tilapia production, also got 3rd position in Asia. (The State of World Fisheries and Aquaculture, 2022). Consumers in Bangladesh purchase pangasius fish approximately 1-5 times a month. Medium-income consumers tend to buy the highest amount of pangasius fish per month.

1.2 Historical overview of *Salmonella* spp.

Tilapia and Pangasius are two of the most widely farmed finfish species in Bangladesh, playing a crucial role in both local food security and the national economy. Despite their importance, the aquaculture sector in Bangladesh faces ongoing food safety challenges, primarily due to microbial contamination issues. This contamination not only poses health risks for domestic consumers but also restricts access to lucrative international markets, where food safety standards are stringent. Throughout the production process and supply chain, fish can become contaminated by harmful microorganisms, including antibiotic-resistant bacteria, potentially arising from farming practices, handling, and processing conditions. Addressing these contamination risks is essential to ensuring the safety and sustainability of Bangladesh's aquaculture industry. (Islam et al.; 2024)

*Salmonella*e are bacteria that are gram-negative, motile, nonsporulating, and have a straight-rod shape. Daniel E. Salmon, an American veterinarian, was the first to isolate *Salmonella* choleraesuis from pigs with hog cholera in 1884, after whom the genus *Salmonella* is name.

*Salmonella*e are facultative intracellular pathogens capable of surviving in diverse environments. Their ability to adjust to different environmental conditions poses a serious danger to the food industry. Pathogenic *Salmonella* species are able to navigate with the help of their peritrichal flagellum. (Eng et al. ;2015) *Salmonella* consists of straight, non-spore forming rods that are of medium size, typically ranging from 0. 7 to 1. 5 micrometers in width and 2. 0 to 5. 0 micrometers in length. These bacteria are Gram-negative, lack a capsule, and are not acid-fast; however, they can be easily stained using standard dyes like methylene blue or carbol-fuchsin. *Salmonella* is a genus of rod-shaped gram-negative bacteria belonging to the family Enterobacteriaceae. This genus is divided into two species: *Salmonella enterica* and *Salmonella bongoli*.

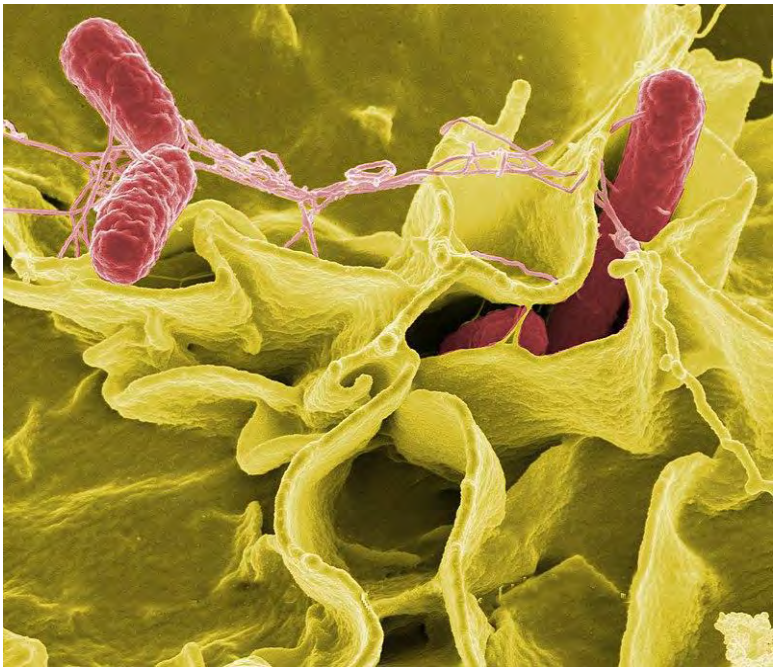


Figure 1: Salmonella spp. (Ryan et al. 2004)

Some species are included in the *Salmonella* genus*: Salmonella enterica* and *Salmonella bongori. S. enterica*is split into seven subspecies: *enterica (I) , salamae (II) , arizonae (IIIa) , diarizonae (IIIb) , indica (IV) , houtenae (VI) ,* and some serovars previously in group IV (VII. Subspecies I strains cause 99% of human salmonellosis cases. The genomes of two *S. enterica* strains have been sequenced, enabling comparisons with other *Salmonella* strains for host range and virulence differences. (Winfield et al. ;2004)

1.2.1 Structural Features and Host-Pathogen Interactions of *Salmonella*

Salmonella is by and large not recognized as being part of the typical microflora in aquaculture situations, and their presence in fish is hence seen as a sign of poor standards of process hygiene and sanitation. Appropriately, most countries will not acknowledge *Salmonella* in any fish product. In spite of the fact that a few thinks about propose that *Salmonella* may be ubiquitous in tropical aquaculture environments, advance studies are required to affirm this. (Dalsgaard, 1998)

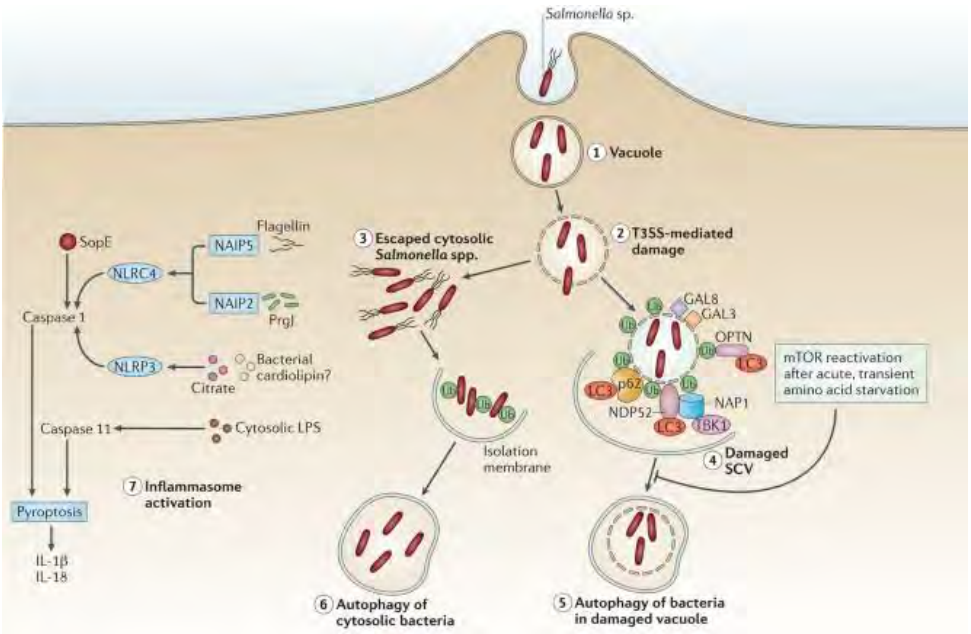


Figure 2: Salmonellae interactions with host processes. (LaRock et al. 2015)

Salmonella enterica is a highly diverse genus of bacteria comprising over 2, 600 serotypes, including notable pathogens such as *Salmonella* Typhi, the causative agent of typhoid fever, and *Salmonella* Enteritidis, commonly linked to foodborne illnesses. (Talib et al ;2022) These bacteria are non-spore-forming, non-capsulated, and facultatively anaerobic. They typically exhibit a rod shape, measuring approximately 2-4 µm in length and 0. 5-0. 6 µm in diameter. (Lamichhane et al.;2024)

The flagellar antigens are unstable when exposed to heat and alcohol, while somatic antigens are more stable and integral to the bacterial cell wall. *Salmonella* primarily resides in the

intestinal tract of humans and animals. While some serotypes coexist harmlessly as commensals, others can cause diseases ranging from mild gastroenteritis to severe systemic infections like typhoid fever. Transmission usually occurs through the ingestion of contaminated food or water. (Caldwell et al. 1966)

This genus, part of the *Enterobacteriaceae* family, is divided into two species: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further subdivided into various serotypes, including *Salmonella* Typhi, responsible for typhoid fever, and *Salmonella* Enteritidis, often linked to foodborne illnesses. These bacteria exhibit versatility in their habitat, being able to colonize various hosts, including humans, animals, and even plants. (Cheraghchi, et al. 2014).

1.2.2 Structural Diversity & Antigenic Architecture of *Salmonella enterica* Complex

Salmonella enterica is a highly diverse genus of bacteria comprising over 2, 600 serotypes, including notable pathogens such as *Salmonella* Typhi, the causative agent of typhoid fever, and *Salmonella* Enteritidis, commonly linked to foodborne illnesses. These bacteria are non-spore-forming, non-capsulated, and facultatively anaerobic. They typically exhibit a rod shape, measuring approximately 2-4 μm in length and 0. 5-0. 6 μm in diameter.

Most serotypes of *Salmonella* possess motility facilitated by peritrichous flagella. However, some strains, like *Salmonella gallinarum* and *Salmonella pullorum*, are non-motile. Additionally, many strains produce type I pili, which are crucial for adhesion to host cells. The flagellar antigens are unstable when exposed to heat and alcohol, while somatic antigens are more stable and integral to the bacterial cell wall. (Kipper et al. 2024)

Salmonella enterica is a highly diverse genus of bacteria comprising over 2, 600 serotypes, including notable pathogens such as *Salmonella* Typhi, the causative agent of typhoid fever, and *Salmonella* Enteritidis, commonly linked to foodborne illnesses. These bacteria are non-spore-forming, non-capsulated, and facultatively anaerobic. They typically exhibit a rod shape, measuring approximately 2-4 μm in length and 0. 5-0. 6 μm in diameter.

1.3 Occurrence and Transmission of *Salmonella* in Fish Production Systems

Salmonella spp. has been found at various stages of the fish production. Researchers generally agree that *Salmonella* spp. is not native to aquatic environments, despite being found in water, fish, and aquaculture products. Research has found little information about the contamination of fish by *Salmonella* spp., as studies have shown that fish can carry the bacteria for short periods without showing any symptoms of the disease. As a result, *Salmonella* spp. has been found in both the internal organs and the gills and skin of fish, leading to a higher chance of cross-contamination during various stages of production and distribution due to poor hygiene practices or insufficiently sanitized equipment, surfaces, and utensils. (Porto, 2023). Although *Salmonella enterica* serotypes are among the best-studied bacterial pathogens, the field still has a long way to go, especially given that (i) they cause significant morbidity and mortality worldwide (ii) they have a wide host range but unknown causes due to infection of different hosts causes different diseases. *Salmonella*: from Pathogenesis to Therapeutics. The *invA* gene for instance affects the host cell by delivery of type III secreted effectors, for mutant phenotype, and is also essential for invasion of epithelial cells genetic diagnosis of *Salmonella* species (Fekry, Ammar & Hussien, 2018). The presence of HilA gene in *Salmonella* is essential for the expression of the type III secretion system (TTSS) components, and it encodes the central regulator HilA (Borges et al. 2013). This gene (HilA) is required to induce apoptosis of macrophages and *invA*de epithelial cells (Borges et al. 2013).

1. 3. 1 *Salmonella* Transmission Dynamics in Water and Food Pathways

Comparing the spread rates of *Salmonella* through water and food sources reveals intriguing insights. Water, particularly irrigation water, acts as a reservoir and vector for *Salmonella*, facilitating its spread to plants and, subsequently, the human food chain. (Threlfall et al. ;2002) The bacterium's ability to form biofilms and survive in various water environments enhances its persistence and spread, leading to contamination of fruits, vegetables, and other crops.

Conversely, food sources, especially contaminated meat, poultry, eggs, and dairy products, serve as direct vehicles for *Salmonella* transmission to humans. The consumption of contaminated food products is the primary route of infection, leading to outbreaks of foodborne illnesses. The prevalence of *Salmonella* in food sources is often higher due to direct

consumption by humans, which provides a conducive environment for the bacteria to thrive and cause infections. While both water and food sources play significant roles in the spread of *Salmonella*, the direct consumption of contaminated food products poses a more immediate threat to human health. (Talib et al. ;2022) Water sources, however, contribute to the broader environmental persistence and distribution of the bacterium, underscoring the need for comprehensive monitoring and control measures across the entire food production and supply chain. This multifaceted approach is essential to mitigate the risks posed by *Salmonella* and ensure food safety and public health.

1. 4 Epidemiological Patterns and Host Adaptation of *Salmonella*

The adaptability and pathogenicity of *Salmonella* are particularly concerning. For instance, their presence in irrigation water and soil can lead to the contamination of fresh produce, posing significant public health risks. (Liu et al.;2018) Their ability to form biofilms and survive in diverse environmental conditions underscores the need for stringent monitoring and control measures in food safety and public health initiatives.

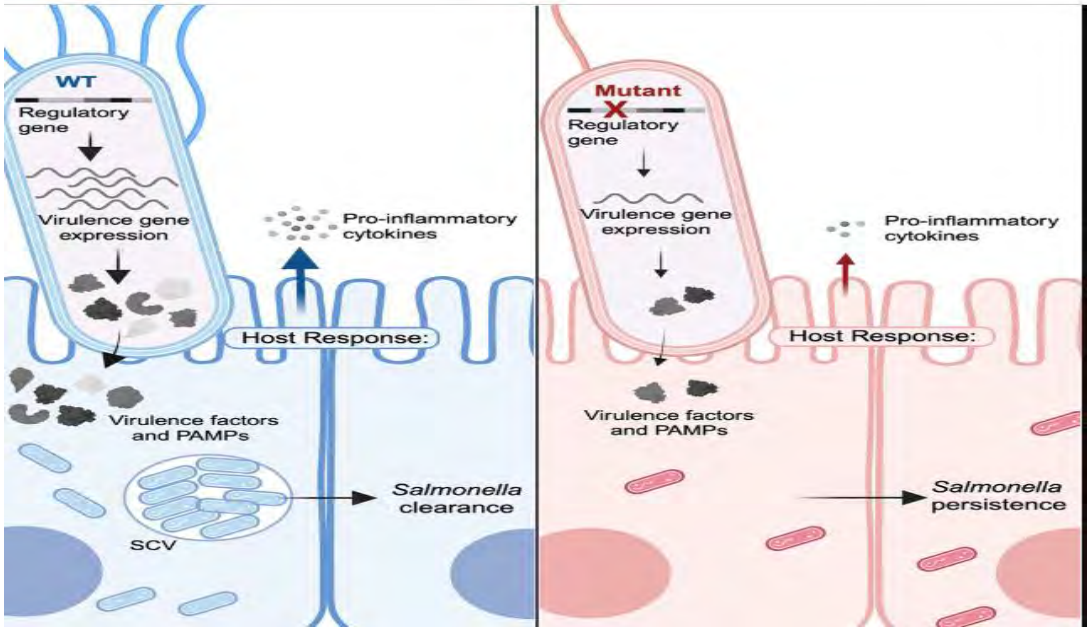


Figure 3: Persistat Salmonella in Humans (Grote et al. 2024)

Salmonella's capacity to navigate various barriers and different cells has been well-documented. Non-typhoidal *Salmonella* (NTS) is often transmitted through contaminated

foods, animals, animal products, and manures, causing gastrointestinal disorders, localized infections, and bacteremia, particularly in immune-compromised individuals, such as those with malaria, malnourished children, and HIV patients. Despite ongoing treatment and preventive strategies, millions of new typhoid infections are reported globally each year. (Bell et al.; 2021)

Moreover, *Salmonella* Typhimurium is not limited to human and animal hosts but also uses plants as alternative hosts. Infections in humans, animals, and plants raise questions about their host specificity. The bacteria's ability to form biofilms on plant roots, colonize emerging lateral roots, and survive in soil and on plant surfaces further complicates control efforts.

Salmonella enterica serotype Choleraesuis is recognized for its significant pathogenicity in humans, often leading to severe systemic infections such as bacteremia and mycotic aneurysms. This serotype is particularly challenging due to its resistance to multiple antibiotics, including ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole, and fluoroquinolones. The emergence of antibiotic-resistant strains necessitates vigilant monitoring and the development of new therapeutic strategies.

Salmonella are carried in irrigation water or from other sources interact with plants, survive or persist on these intermediate hosts have become an interesting topic in recent years. Studies indicate *Salmonella* can be internalized into tomato plants through different routes and may possibly colonize and employ the plant as an alternative host. The formation of biofilm-like structures on the surface of roots, the colonizing regions of emerging lateral roots and wounded tissues has been reported. Moreover, *Salmonella* inoculated into soil or blossoms can be recovered from the fruit at low internal levels, suggesting its ability to colonize and internalize tomato plants, and however, survival ability with different serovars varies in the soil and in different parts of the plants. *Salmonella* was also recovered from tomato fruits after it was introduced into the plant by stem injection or by flower inoculation. (Liu, 2018)

1.4.1 Clinical Impact and Cross-Species Transmission of Non-typhoidal *Salmonella*

NTS is often reported as an important foodborne pathogen causing gastrointestinal disorders, different localized infections, and bacteremia. These bacteria can develop worse conditions in immunosuppressive humans, especially malaria-infected patients, malnourished children, and human immunodeficiency virus (HIV) patients. Notwithstanding treatment and preventive strategies being implemented, millions of new typhoid infections are being reported globally every year. Interestingly, *S.* Typhimurium not only infects humans and animals but also can use plants as their alternative hosts. Infections in humans, animals, and plants by *Salmonella* have raised questions about their host specificity. (Talukder et al. ;2023) *Salmonella* carried by fish and other aquaculture products as well as by fresh produce irrigated with dirty water has been indicated as a vehicle for a growing number of enteric disease outbreaks Use of feces-contaminated water to irrigate the produce has been reported to lead contaminating of soil and vegetables with *Salmonella* for an extended period of time (Traoré et al. ; 2015)

1.4.3 Pathogenic Colonization and Plant-Microbe Interactions of *Salmonella* in Agricultural System

The pathogenicity and spread rate of *Salmonella* among food and water sources present critical challenges in public health and food safety. *Salmonella* bacteria, carried in irrigation water or from other sources, interact with plants and persist on these intermediate hosts, posing significant risks. Studies indicate that *Salmonella* can be internalized into tomato plants through various routes, potentially colonizing and employing the plant as an alternative host (Liu, 2018). The formation of biofilm-like structures on the surface of roots, colonizing regions of emerging lateral roots, and wounded tissues has been observed. Moreover, *Salmonella* inoculated into soil or blossoms can be recovered from the fruit at low internal levels, suggesting its ability to colonize and internalize tomato plants. The survival ability of different serovars varies in the soil and in different parts of the plants.

In food sources, particularly among reptiles, avian species, and mammals, including humans, non-typhoidal *Salmonella* (NTS) is usually transmitted through contaminated foods, animals,

animal products, and manures. Studies have found associations between *Salmonella*-contaminated fruits and vegetables with food poisoning, highlighting its pathogenicity. NTS often causes gastrointestinal disorders, localized infections, and bacteremia, particularly in immunosuppressive individuals such as malaria-infected patients, malnourished children, and HIV patients (Alenazy, 2022). Despite treatment and preventive strategies, millions of new typhoid infections are reported globally each year, interestingly, *Salmonella typhimurium* not only infects humans and animals but also uses plants as alternative hosts.

1.4.4 Clinical Manifestations and Epidemiology of *Salmonella* Infections

Salmonella primarily inhabits the intestines of humans and animals. While some serotypes are harmless, others cause a spectrum of diseases ranging from mild gastritis to severe systemic infections. Infection typically results from ingesting contaminated food or water. The facultative anaerobic nature of *Salmonella* enables it to thrive in both aerobic and anaerobic environments, contributing to its status as a prevalent foodborne pathogen globally, leading to gastroenteritis outbreaks. (Fàbrega, et al. ;2023)

Epidemiological studies highlight that food supplies, particularly those of animal origin such as poultry, are principal carriers of *Salmonella* contamination to humans. The formation of biofilms on food surfaces facilitates infection transmission. As significant intestinal pathogens, *Salmonella* species cause gastroenteritis and typhoid fever. Non-typhoidal *Salmonella* serovars, such as *S.enterica* serovar Typhimurium, account for most foodborne diseases worldwide, while *S.enterica* serovar Typhi is responsible for substantial morbidity and mortality associated with typhoid fever. (Teklemariam et al.; 2023)

1.4.5 Host-Pathogen Interactions of *Salmonella*

Extensive research has focused on the pathogenic properties of *Salmonella*, including its ability to penetrate the intestinal barrier and, for typhoidal serotypes, replicate within host macrophages. Recently, studies have increasingly examined the interactions between *Salmonella* and the gastrointestinal microbiota, highlighting the bacterium's complex relationship with its host's internal environment. (Ahmer, 2011) Integrating this information into a broader context reveals that *Salmonella*'s pathogenicity in humans is primarily due to its

adaptability to various environments, biofilm formation, and utilization of diverse hosts. The interaction of *Salmonella* with food sources, water, and environmental samples illustrates its complex lifecycle and widespread infection potential. The variability in pathogenicity and survival strategies among different serotypes underscores the necessity for comprehensive approaches to control and prevent *Salmonella*-related illnesses, thereby ensuring food safety and public health. (Mkangara et al. ;2023)

This analytical overview emphasizes the need for integrated measures to mitigate the impact of *Salmonella*, considering its adaptability, biofilm-forming capabilities, and the significant public health risks it poses through contaminated food and water. Addressing these challenges requires robust monitoring and innovative strategies to control this pervasive pathogen effectively.

1. 5 Antimicrobial Resistance of *Salmonella* as a Global Burden

Antimicrobial resistance (AMR) is a global challenge that poses a significant threat to public health. As bacteria and other microorganisms evolve to become resistant to the drugs designed to kill them, it is becoming increasingly difficult to treat infections effectively. This phenomenon has emerged as one of the leading health threats of the 21st century, with the potential to cause up to 10 million fatalities annually by 2050 if left unchecked. {Ferri et al. 2017) The mechanisms by which antimicrobial resistance develops are complex and multifaceted. Bacteria can employ various strategies to overcome the effects of antibiotics, such as modifying the drug target, altering cell permeability to restrict drug entry, actively expelling the drug, or even developing entirely new metabolic pathways that circumvent the need for the targeted antibiotic. Understanding these diverse mechanisms is crucial for devising effective strategies to combat the spread of AMR. Addressing the AMR crisis requires a multifaceted approach involving healthcare providers, policymakers, researchers, and the general public. Improved surveillance and data collection are necessary to accurately assess the extent of AMR, particularly in areas with limited resources and insufficient data. Developing new antibiotics, promoting the responsible use of existing ones, and implementing infection prevention and control measures are all key components in the global fight against the growing threat of antimicrobial resistance.

1.5.1 Antimicrobial Resistance and Typhoid Fever: A Global Health Threat

Antimicrobial resistance (AMR) is a significant global health challenge, with bacterial AMR alone estimated to cause over 1. 27 million deaths annually. Typhoid fever, a serious bacterial infection caused by *Salmonella enteric* serovar Typhi, contributes to this burden, leading to over 11 million cases and 100, 000 deaths each year. South Asia bears the brunt of the disease, accounting for 70% of the global burden. The rise of AMR is linked to various factors, including the misuse and overuse of antibiotics in both human and animal health. In developing countries like Bangladesh, inadequate healthcare monitoring, unsanitary food production practices, and widespread antibiotic use in agriculture further exacerbate the problem. *Salmonella* spp., the bacteria causing typhoid, is increasingly resistant to crucial antibiotics like cephalosporins and fluoroquinolones. This resistance is concerning as it limits treatment options and is associated with the use of fluoroquinolones in food animals. Addressing AMR requires a multi-pronged approach, including improved antibiotic stewardship, enhanced surveillance and monitoring, and stricter regulations on antibiotic use in agriculture. (Naghavi, 2024)

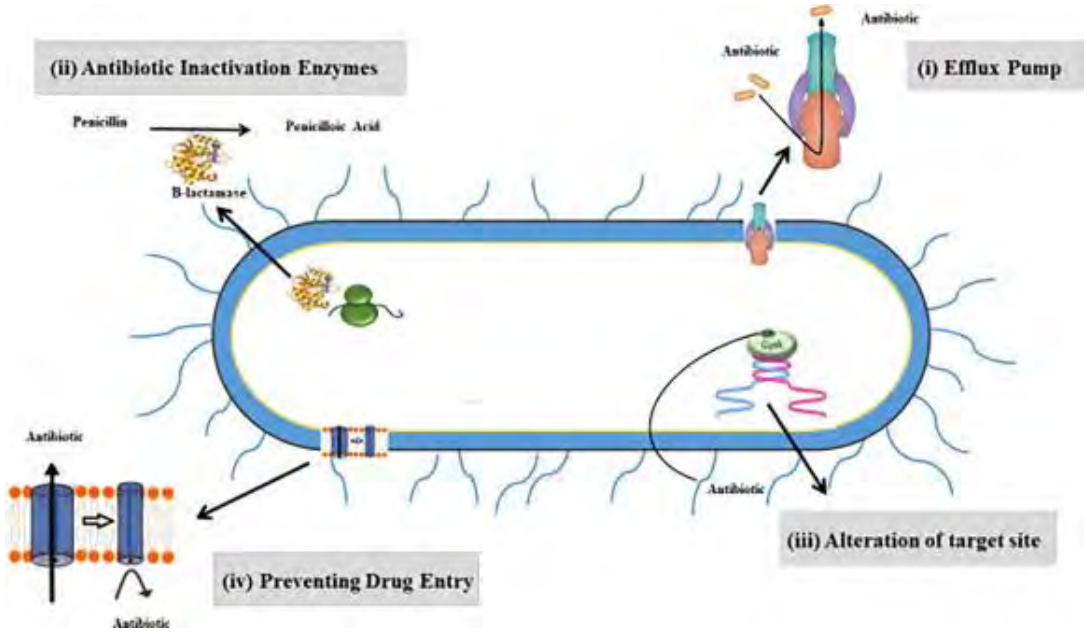


Figure 4: Antimicrobial resistance mechanisms in pathogenic bacteria (Alenazy et al. 2022)

1. 5. 2 Antimicrobial Resistance in *Salmonella* spp. in Bangladesh

Salmonella enterica is a common bacterial pathogen in the commercial chicken production industry in Bangladesh. A significant proportion of *Salmonella* isolates found in this industry are resistant to multiple antimicrobial drugs, indicating widespread misuse and overuse of antibiotics. This highlights the urgent need for improved antibiotic stewardship practices in the poultry sector to prevent the emergence and spread of antibiotic-resistant strains. (Siddiky et al. 2021).

1. 5. 3 Multidrug-Resistant *Salmonella* in South Asia

Multidrug-resistant (MDR) *Salmonella*, particularly *S*.Typhi and *S*.Paratyphi, is prevalent in South Asia, with higher rates observed in countries like India, Pakistan, and Vietnam compared to China, Indonesia, and Laos. *S*.Paratyphi exhibits a greater resistance to fluoroquinolones than *S*.Typhi, with high rates of resistance to nalidixic acid reported in Pakistan, India, and Vietnam. (Ochiai et al. 2008)

1. 6 Ecological Versatility in *Salmonella* species:

Most serotypes of *Salmonella* possess motility facilitated by peritrichous flagella. However, some strains, like *Salmonella gallinarum* and *Salmonella pullorum*, are non-motile. Additionally, many strains produce type I pili, which are crucial for adhesion to host cells. The flagellar antigens are unstable when exposed to heat and alcohol, while somatic antigens are more stable and integral to the bacterial cell wall (Kipper et al., 2024).

Salmonella primarily resides in the intestinal tract of humans and animals. While some serotypes coexist harmlessly as commensals, others can cause diseases ranging from mild gastroenteritis to severe systemic infections like typhoid fever. Transmission usually occurs through the ingestion of contaminated food or water (Ortiz-Solà et al., 2022). This genus, part of the Enterobacteriaceae family, is divided into two species: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further subdivided into various serotypes, including *Salmonella* Typhi, responsible for typhoid fever, and *Salmonella* Enteritidis, often linked to foodborne illnesses. These bacteria exhibit

versatility in their habitat, being able to colonize various hosts, including humans, animals, and even plants (Smith, 2016).

The adaptability and pathogenicity of *Salmonella* are particularly concerning. For instance, their presence in irrigation water and soil can lead to the contamination of fresh produce, posing significant public health risks. Their ability to form biofilms and survive in diverse environmental conditions underscores the need for stringent monitoring and control measures in food safety and public health initiatives (Ortiz-Solà, 2022).

1. 7 Disease Spectrum and Transmission Dynamics of *Salmonella* Infections

Salmonella's capacity to navigate various barriers and different cells has been well-documented. Non-typhoidal *Salmonella* (NTS) is often transmitted through contaminated foods, animals, animal products, and manures, causing gastrointestinal disorders, localized infections, and bacteremia, particularly in immunocompromised individuals, such as those with malaria, malnourished children, and HIV patients (Marchello et al., 2021). Despite ongoing treatment and preventive strategies, millions of new typhoid infections are reported globally each year. Moreover, *Salmonella* Typhimurium is not limited to human and animal hosts but also uses plants as alternative hosts. Infections in humans, animals, and plants raise questions about their host specificity. The bacteria's ability to form biofilms on plant roots, colonize emerging lateral roots, and survive in soil and on plant surfaces further complicates control efforts.

Salmonella enterica serotype Choleraesuis is recognized for its significant pathogenicity in humans, often leading to severe systemic infections such as bacteremia and mycotic aneurysms (Gray et al., 1996). This serotype is particularly challenging due to its resistance to multiple antibiotics, including ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole, and fluoroquinolones. The emergence of antibiotic-resistant strains necessitates vigilant monitoring and the development of new therapeutic strategies (Bolton et al., 1999).

1.7.1 Contamination Sources, Disease Mechanisms, and Control Strategies of *Salmonella* spp.

The primary habitat for *Salmonella* is the intestines of humans and animals. While some serotypes exist as harmless commensals, others cause a range of diseases from mild gastritis to severe systemic infections. Infections typically occur through the ingestion of contaminated food or water. (Nwabor, 2015). The bacterium's facultative anaerobic nature allows it to thrive in both aerobic and anaerobic environments, contributing to its role as a common foodborne pathogen worldwide, leading to outbreaks of gastroenteritis. (Acheson, et al. ;2001)

The great majority of sporadic cases and outbreaks of salmonellosis is caused by contaminated food beside frequently mentioned foods like poultry, pork, or egg products, the role of ready-to-eat products is increasingly recognized. This is of particular concern when contamination with *Salmonella* cannot be excluded and the food is consumed without further heat treatment. Spices and herbs as source of *Salmonella*-related foodborne diseases. {Dawoud et al. 2017)

Epidemiological studies indicate that food supplies, especially of animal origin such as poultry, are the main carriers of *Salmonella* contamination to humans. The formation of biofilms on food surfaces facilitates the transmission of infections. (Joseph et al. 2001) *Salmonella* species are significant intestinal pathogens, causing gastroenteritis and typhoid fever. Non-typhoidal *Salmonella* serovars like *S. enterica* serovar *typhimurium* are responsible for the majority of foodborne illnesses globally, whereas S. *enterica* serovar Typhi causes significant morbidity and mortality through typhoid fever. (Oludairo, 2022) Research has extensively studied the pathogenic properties of *Salmonella*, including its ability to penetrate the intestinal barrier and, for typhoidal species, to replicate within host macrophages. Recent studies have also intensified focus on the interaction between *Salmonella* and the gastrointestinal microbiota, highlighting its complex relationship with the host's internal environment.

Connecting this information to the broader overview, *Salmonella*'s pathogenicity in humans is due to its ability to adapt to different environments, form biofilms, and utilize various hosts. The interaction between *Salmonella*, food sources, water, and environmental samples demonstrates the bacterium's complex life cycle and its potential to cause widespread infections. The variability in pathogenicity and survival strategies among different serotypes emphasizes the need for comprehensive approaches to control and prevent *Salmonella*-related illnesses, ensuring food safety and public health.

1. 8. A Comprehensive Study of *Salmonella* Serotypes and Their Impacts

Salmonella enterica is a significant pathogen responsible for a wide range of foodborne illnesses globally. Among its various serotypes, *Salmonella enterica*s erovar - and *Salmonella enterica* serotype Derby are notable for their genetic diversity and phenotypic characteristics3. This overview aims to synthesize key findings from recent studies on these serotypes, highlighting their genetic traits, pathogenicity, and implications for public health. (Hayward et al. ; 2013)

Salmonella enterica serovar 4, [5], 12:i:- is recognized as a monophasic variant of S. *enterica* serovar Typhimurium. Research has shown that this serotype harbors a 94-kb virulence plasmid common to Typhimurium, contributing to its pathogenicity4. This serotype has been isolated from various sources, including humans, cattle, swine, chickens, birds, meat, and river water, indicating its widespread presence and potential for causing infections. (García et al. ;2014)

The presence of plasmids carrying resistance genes and virulence factors, such as the *Salmonella* pathogenicity island (SPI) gene avrA and the fimbrial gene bcfC, further underscores its pathogenic potential. (Awosile, 2018) Molecular subtyping has revealed multiple distinct clones, suggesting that foods of animal origin, particularly pork, are significant sources of human infection. The genetic diversity and phenotypic characteristics of these serotypes highlight the complexity of This comprehensive overview underscores the importance of continued research and surveillance to address the evolving threat posed by *Salmonella enterica*, leveraging insights from genetic studies to inform public health. (Eichelberget al. ;1999)

1.9 Whole-Genome Sequencing and the Genetic Underpinnings of *Salmonella* invasion

Salmonella, a significant pathogen responsible for numerous foodborne illnesses worldwide, employs a variety of genetic mechanisms to invade host cells and establish infections. Among the critical invasion genes in *Salmonella*, invasion protein A (*invA*) is pivotal in initiating the attack on intestinal epithelial cells. The *invA* gene is crucial for the delivery of type III secreted effectors into host cells, facilitating the *invA*sion process. (Borges et al. ;2013) This gene is essential not only for the *invA*sion of epithelial cells but also for the accurate genetic diagnosis of *Salmonella* species. The role of the HilA gene in *Salmonella* is equally vital. HilA is

indispensable for the expression of the components of the type III secretion system (TTSS) , acting as the central regulator. This gene is crucial for inducing apoptosis in macrophages and enabling the invasion of epithelial cells. (Ellermeier et al. ; 2003) The presence and function of HilA highlight the sophisticated mechanisms *Salmonella* employs to manipulate host cell processes and evade immune responses.

The advent of whole-genome sequencing (WGS) has significantly advanced our understanding of the genetic basis of pathogenicity in *Salmonella*, particularly in relation to the *invA* gene. WGS allows for comprehensive analysis of the entire genetic makeup of *Salmonella* strains, enabling the identification of key virulence factors and resistance genes. By examining the complete genome, researchers can pinpoint mutations and genetic variations that contribute to the bacterium's invasive capabilities. (Shanmugasamy, 2011) The *invA* gene, identified through WGS, has been shown to play a critical role in the bacterium's ability to invade host cells. The type III secretion system (TTSS) , regulated by *invA*, is a sophisticated molecular apparatus that injects bacterial effector proteins into host cells, manipulating their functions to the benefit of the pathogen. This system is essential for the *invA*sion and subsequent intracellular survival of *Salmonella* within host cells. {Amini et al. ;2010)

1. 10 Research Gap

Aquaculture products like fish are increasingly recognized as potential vectors for transmitting enteric diseases, particularly Salmonellosis (Lynch et al., 2009; Olgunoğlu, 2012). Studies have detected human pathogenic *Salmonella* in various tissues of fish, highlighting the contribution of municipal and domestic waste to bacterial contamination in aquatic environments, such as the Bakkhali River Estuary in Bangladesh (Jahan et al., 2019). While some research has focused on isolating and identifying bacteria like *E. coli* from fish farms and markets using conventional methods (Ava et al., 2020) , there is a need for studies employing molecular techniques for more accurate identification. Previous research on *Salmonella* prevalence in fish from Bangladeshi markets (Seel et al., 2016) has often been limited in scope, such as only testing susceptibility against a small panel of antibiotics. Other studies have revealed significant *Salmonella* contamination in raw and dried fish samples from markets in Dhaka, Bangladesh (Nur et al., 2020) , and multidrug-resistant strains have even been found in houseflies from fish markets, suggesting a wider spread of contamination (Sobur et al., 2019). Research focusing on shrimp farms in specific districts of Bangladesh has also

demonstrated the presence of *Salmonella* in water, pond sediment, shrimp, and equipment using traditional bacteriological methods (Faridullah et al., 2016). Furthermore, studies examining various fish species from different sources (processing plants, markets, and stores) have revealed varying levels of *Salmonella* contamination, with markets showing the highest prevalence (Begum et al., 2015). Beyond fish and shrimp, investigations into *Salmonella* prevalence and antibiotic resistance have been conducted on other food products in Bangladesh (Siddiky et al., 2021; Alam et al., 2020; Mridha et al., 2020; Hassan et al., 2019) , indicating a broader concern about food safety and public health.

In essence, the existing research highlights the widespread presence of *Salmonella* in aquaculture products and related environments in Bangladesh, with concerns about multidrug resistance. However, there is a need for more comprehensive studies utilizing advanced techniques to fully understand the scope of the problem and inform effective control strategies. Existing research on *Salmonella* prevalence and antibiotic resistance in Bangladesh is limited by small-scale studies focused on select markets. This raises concerns about the generalizability of findings to the entire country. To gain a more accurate understanding of the situation, comprehensive research encompassing a wider range of markets and utilizing diverse antimicrobial agents is crucial. Such studies are essential for developing effective strategies to combat antibiotic resistance and prevent the spread of pathogens in retail settings. Enteric fever, caused by typhoid and paratyphoid bacteria, poses a significant health burden globally, particularly in low- and middle-income countries like Bangladesh (GBD 2017 Typhoid and Paratyphoid Collaborators, 2019). Characterized by poor sanitation, hygiene practices, and limited access to clean water, Bangladesh faces a high prevalence of enteric fever, with salmonellosis identified as a major zoonotic bacterial disease (Samad, 2011).

Given that fish is a dietary staple and primary protein source in Bangladesh, understanding the presence of *Salmonella* and its antibiotic resistance patterns within the fish supply chain is vital for assessing potential human health risks. Currently, however, this information remains largely unknown.

1. 11 Objectives

This study aims to investigate the prevalence, distribution, and antibiotic resistance profiles of *Salmonella* spp. in Pangas and Tilapia fish along the aquaculture supply chain, ultimately determining the potential sources of contamination at the consumer level.

* Isolate and identify *Salmonella* spp. from fish samples obtained from retail markets, wholesale markets, and grower's ponds.

* Assess the antibiotic susceptibility of the isolated *Salmonella* spp. against clinically relevant antibiotics.

* Investigate the pathogenic properties of the identified *Salmonella* spp. by detecting virulence genes.

Chapter 2
Materials and Methods

Materials and Methods

2.1 Sampling Site and Sample Collection

A total of fish (n=400) were collected from ponds, wholesale markets, and retail markets for this study. Of the collected specimens, water samples (n=206) , swab samples (n=188) were included, Sampling was conducted in two shifts (Morning and evening) for both wholesale and retail markets.. Each sample was placed in a labeled polythene bag inside an ice box to maintain a cool temperature (4°C). The samples were transported to the laboratory promptly and processed immediately upon arrival.

Pangs and Tilapia have been collected from various areas of Bangladesh for the research work of Pangas and Tilapia aquaculture value chain of the city of Dhaka, Bangladesh Pond samples were from outside Dhaka city. But wholesaler market and retailer market sample were collected from Dhaka city.

Figure 5: Fish Sample Collection from Retailer (A) , Wholesaler (B) , Pond (C) samples

2.1.1Fish Sample processing

Fishes were processed aseptically using sterile scissors. 10g flesh were collected along with skin for further experimental design. 10g of samples were mixed with 100ml Buffered Peptone Water (BPW). Then it was put into the stomacher Mixer at 1000 rpm for one and a half minutes.

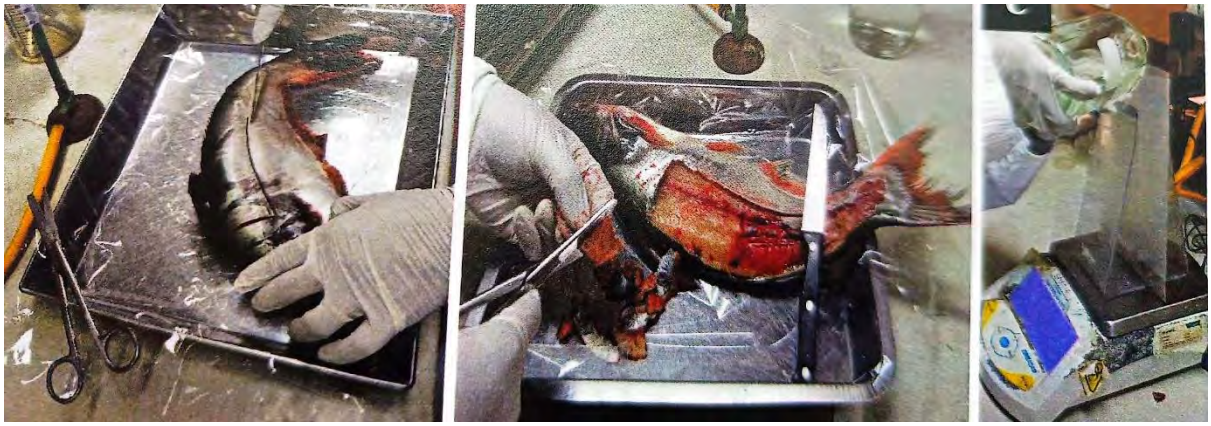


Figure 6: Fish Sample Processing

2.1.2 Fish water sample processing:

Water samples were collected from a wholesale fish market. Twenty milliliters of each sample were filtered through a Millipore filter machine. The filtered paper was then transferred into 50 milliliters of BPW (enrichment broth) , vortexed, and incubated at 37°C. Subsequently, one milliliter of the incubated culture was transferred to RAPPAPORT SOY BROTH(RVS)broth and incubated at 42°C for 24 hours, while another one milliliter was transferred to Muller-Kauffmann Tetrathionate Novobiocin broth. MKTTn broth and incubated at 37°C for 24 hours.

A B

Figure 7: Fish Water Sample Processing (A. peptone water, B. Filter paper placing into pepotone water

2.1.3 Swab Sample Processing:

Samples were collected from a 10 × 10 cm² square cutting board using sterile cotton swabs. The swabs were immediately immersed in buffered peptone water (BPW) and transported to the laboratory in a cooling box. Upon arrival, the samples were vortexed thoroughly, and serial dilutions were prepared by transferring 1 mL of the sample into 9 mL of BPW (1:10 dilution). The diluted samples were then incubated at 37°C for 18-24 hours under aerobic conditions.

These are the areas from where the samples were collected for interpreting the prevalence of *Salmonella* spp. in the aquaculture value chain.

Wholeseller (5)	**Retailer (18)**	**Pond (10)**
Jatrabari	Mohakhali Bazar	Mymensingh
MerulBadda	Karwan Bazar	Narsingdi
Swarighat	Banani Bazar	Comilla
BGB Market, Gabtoli	Gulsan 2 Bazar	Khulna Pottiyaghata, South
Abdullahpur	Moghbazar	Rajshahi, Baya
	Kachukhet Bazar	
	Middle Badda Bazar	
	Natunbazar	
	Shewrapara Bazar	
	Bashabo Bazar	
	Azampur Bazar	

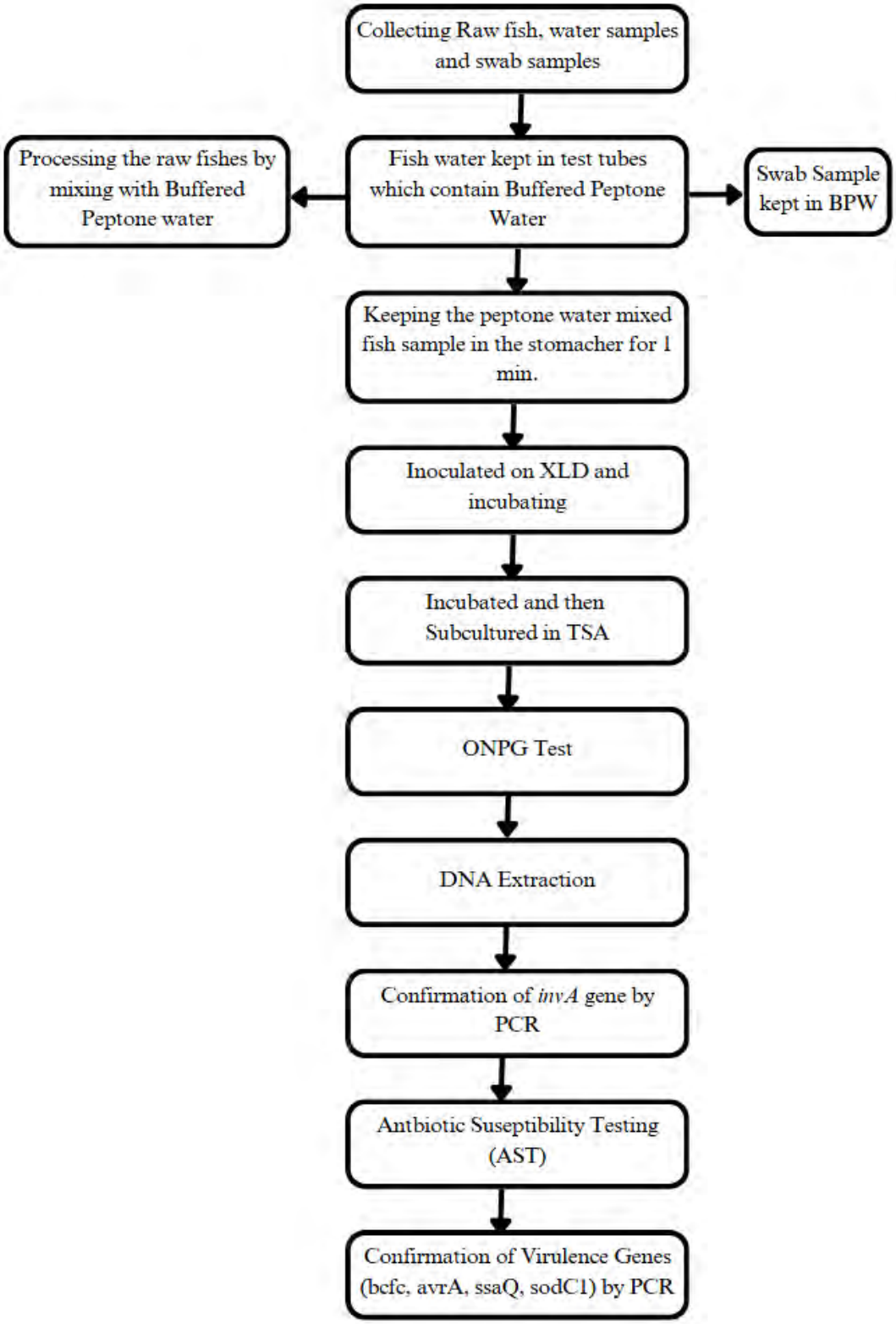
	Newmarket Bazar	
	Hatirpul Bazar	
	Kaptan Bazar	
	Fakirapul Bazar	
	Mirpur bazar	
	Khilgao Bazar	
	Kathalbagan Bazar	
	Zigatola Bazar	

	Hazaribagh Bazar	
	Mirpur 6 no Bazar	
	Lalbagh Bazar	
	Duaripara Bazar	
	Rupnagar R/A bazar	
	Goipbag Bazar	
	Mirpur 11 no. Bazar	
	Mirpur 12 no Bazar	

	Naya Bazar	
	Thatari Bazar	
	Jarabari Bazar	

Table 1: Areas of Sample Collection

2.2 Designing of the Whole Method:



2.3 Enrichment in Selective Liquid Medium

For the pre-enriched homogenized samples, two enrichment broths are required : Muller-Kauffman Tetrathionate-Novobiocin Broth (MKTTn) and Rappaport-Vassiliadis Soya Peptone broth (RVS). From the pre-enriched BPW broth, two transfers were made: 0. 1 mL was transferred to 10 mL RVS broth, and 1 mL was transferred to 10 mL MKTTn broth. Both mixtures were homogenized by vortexing. 1000 µL of pre-enriched samples were mixed into 10 mL of MKTTn and RVS broth. The samples with MKTTn were incubated at 37°C, and those with RVS were incubated at 42°C. Both were incubated for 24 hours. After the enrichment, Xylose Lysine Deoxycholate media was used for culturing the samples.

Figure 8: MKTTn and RVS broth for enrichment

One microliter of enriched sample was streaked onto that agar media and incubated for 24 hrs. *Salmonella* spp. was identified by the morphological structures where it appeared as red colonies with black centers and sometimes only in red-colored colonies. On BGA agar, *Salmonella* appears as red colonies

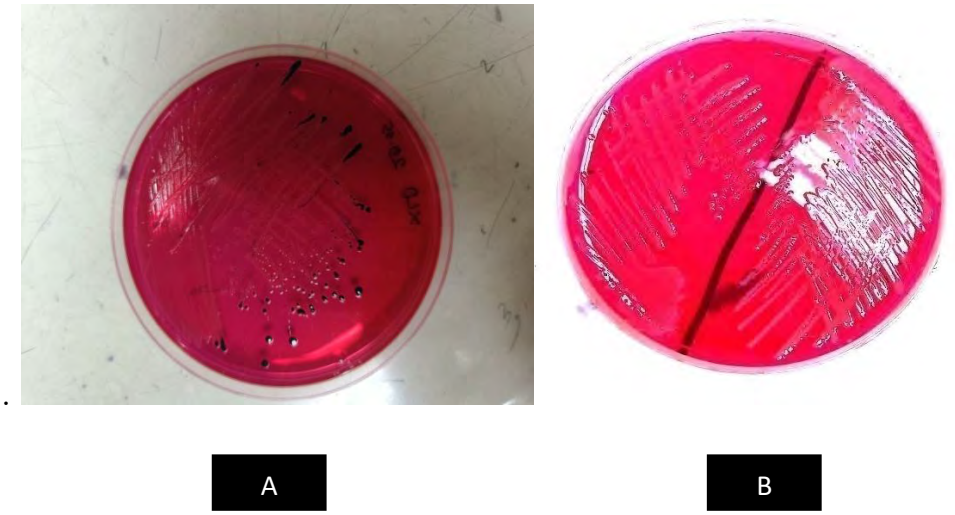


Figure 9: Salmonella spp. Colonies on XLD (A) and BGA (B) agar plate

2.3.1 Controls

Salmonella Typhimurium ALM 40 served as the positive control. *E. coli* ATCC 25922 served as the negative control.

2.3.2 Ortho-nitrophenyl -beta -D-galactopyranoside (ONPG) test for *Salmonella* spp.

A colony of *Salmonella* spp. was picked from the XLD agar plate using a loop and placed into small vials containing 100 microliters of normal saline. The bacteria were initially cultured in general Tryptone Soya Agar (TSA) media. Then the ONPG disc was placed in each vials and incubated for 24 hours. In this biochemical test, *Salmonella* spp will not produce any color, but if a yellow-colored hue occurs the isolates can be identified as something other than *Salmonella*. This ONPG test is used as a screening test for *Salmonella* spp. But it's not specific to this bacteria. also, further testing is required for identification.

Figure 10: Glass vials ready to incubate 100 micro-litre of normal saline, one suspected Salmonella colony and one ONPG Disc.

2.4 DNA extraction

2.4.1 Materials and equipment

- Eppendorf
- Autoclaved Distilled Water
- Burner
- Loop
- Heat block machine
- ice
- Centrifuge Machine

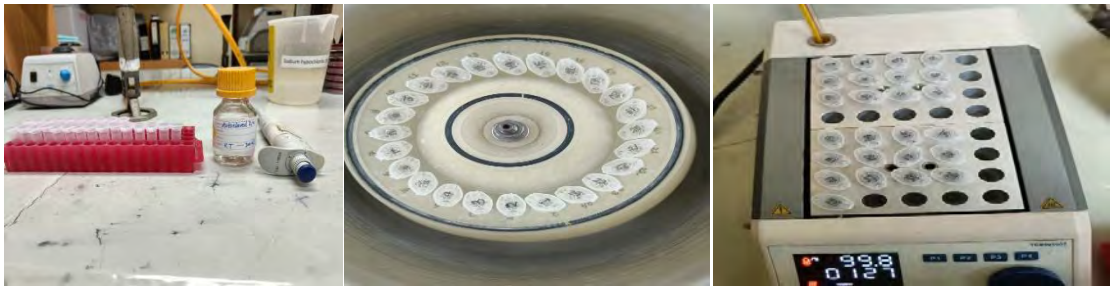


Figure 11: Arrangement of DNA Extraction

Molecular detection is started by DNA extraction of *Salmonella* spp. By the process of heat extraction method,, 300µL of Autoclaved Distilled Water (ADW) was aliqoted into 1. 5 ml Eppendorf tubes, and 1-2 colonies were put into the ADW containing Eppendorf. The

colonies were taken from an XLD agar plate. The mixture was vortexed to ensure the colonies were well-mixed with the water. The tubes were then heated in a heat block at 100°C for 10 minutes. Tubes were kept in ice for 15 minutes before centrifugation. The tubes were centrifuged at 6000 rpm for 6 minutes. 120 microliters of supernatant were transferred in a fresh 1. 5 microliter Eppendorf tube, and then they were incubated in -20°C.

2.4.2 Polymerase Chain Reaction (PCR) to detect virulence gene:

Master mix was made with relevant reagents mentioned below in the table. The supernatant was then placed into PCR tubes, and the template DNA was mixed with the master mix for each PCR process.

Reaction Volume (15µL)	Reagents	End Concentrations
10. 94µL	Filtered Deionized The tubes were then heated in a heat block at 100°C for 10 minutes. minutes. Water	
1. 5µL	PCR Buffer	1x Buffer
0. 24µL	d-NTP (10mM)	200µM
0. 6µL	Primer *invA*1 (10Pmole/µL)	. 5Pmol/ µL
0. 6µL	Primer *invA*2 (10Pmole/µL)	. 5Pmol/ µL
. 12	Taq polymerase	1 unit
1. 0µL	Template DNA	

Table 2: Measurement and Reagents of PCR Mastermix

2.4.3 Mastermix Preparation

Reagents	Amount
Filtered Deionized Water	14. 85µL
PCR Buffer	2. 5µL
d-NTP (10mM)	0. 5µL
Forward Primer (10pmole/µL)	1. 0µL
Reverse Primer (10pmole/µL)	1. 0µL
Taq Polymerase (5U/µL)	0. 15µL
Template DNA	5. 0µL

For initiating the PCR mechanism, invasion gene (*invA*) was used for the confirmation of the *Salmonella* genus. After that, the tubes were placed in the PCR thermal cycler, and the conditions were set accordingly. Prior to operating the equipment, pre-denaturation was carried out at 94 °C for 1 minute as per the established conditions. 94 °C for 30 seconds for the process of separating DNA strands, 68°C for 30 seconds for annealing the primers, and 7230 seconds at °C for the elongation.

Singleplex PCR was used for identifying the virulence genes. The reactions were performed targeting theavrA, ssaQ, sodC1 and bcfc which were located on a fimbrial cluster. The system of incubation for all target virulence genes, only except bcfc, was set in 95°C for 1 min, and lastly, it was performed in 72 °C for 4 mins. Annealing temperature was set in 53°C for the gene bcfc. The annealing temperature was 53 °C. For initiating the PCR mechanism, the *invA*sion gene (*invA*) was used for the confirmation of the *Salmonella* genus.

Primer	Virulence Gene	Sequence	Size & Temperature	Reference
inv-F INV-R	*invA*	5'-CGCGGCCCGATTTTCTCTGGA-3' 5'-AATGCGGGGATCTGGGCGACAAG-3	321bp	Khan et al., 2000)
bcfc-F bcfc-R	bcfc	5'-ACCAGAGACATTGCCTTCC-3' 5'-TTCTGATCGCCGCTATTCG-3	467bp (53°C)	Weening et al., 2005
ssaQ-F ssaQ-R	ssaQ	5'-GAATAGCGAATGCGAATGAAGAGCGTCC-3' 5'-CATCGTGTTATCCTCTGTCAGC-3'	677bp (58°C)	(Soto et al)
avrA-F avrA-R	avrA	5'-CCT GTA TTG TTG AGC GTC TCG-3' 5'-AGA AGA GCT TCG TTG AAT GTC C-3'	422bp (58°C)	(Collier-Hyams et al. 2002)
sodC1-F sodC1-R	sodC1	5'-CCAGTGGAGCAGGTTTACG-3' 5'-GGTGCGCTCATCAGTTGTTC-3'	424bp (58°C)	Fang et al., 1999;Her

Table 3:Primer Sequences of Specific Genes

2.4.4 Agarose Gel Electrophoresis

To clearly visualize the PCR products, agarose gel was used. To prepare the agarose gel, 1. 5 gm of agarose powder was mixed with 100 ml of autoclaved distilled water, then mixed well by gently shaking the bottle, and then placed it in the oven for 1 minute and 30 seconds.

After moving it out of the microwave, the outer surface of the bottle was rinsed with cold water to cool it, and then poured into the gel doc system. 5 microliters of PCR products were loaded onto the well mixed with Midori Green Safe DNA, and the gel documentation was collected using the Fastgene FAS-DIGI gel documentation system. The 100 Bp markers also known as the ladder to interpret the size of the amplification product.

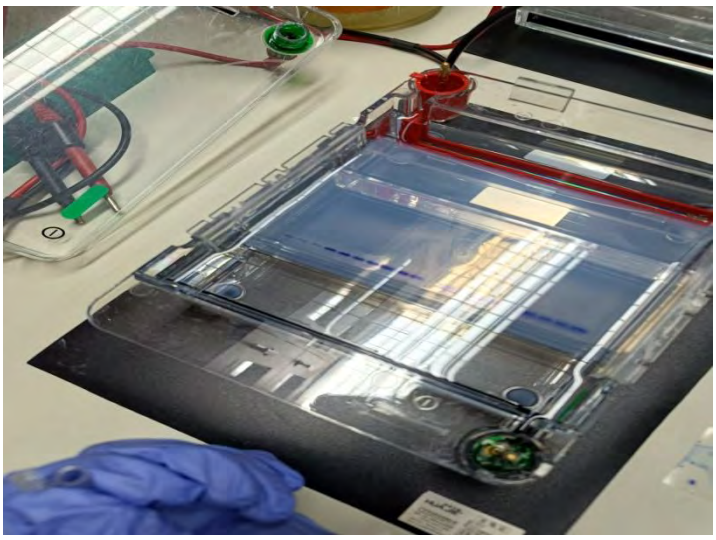


Figure 12: Agarose Gel Loading

2.5 Antibiotic Susceptibility Testing (AST) of confirmed *Salmonella* spp. :

Antimicrobial Susceptibility Testing (AST) was performed to confirm the isolation of *Salmonella* spp. McCconkey agar plates were prepared for this test, and then the subcultured plates were incubated at 37°C overnight. A suspension was prepared with 3 ml of normal saline, which was then mixed with a few *Salmonella* spp. colonies isolated from XLD plates, using a sterile loop. It was then stirred to mix the colonies with the saline solution and then compared with the standard McFarland optical density. The suspension was placed aseptically on Muller Hilton agar (MHA) plates. Twenty-one antibiotic discs were placed on the MHA at equal distances. By measuring the length with the mm portion of a ruler, the zone of inhibition was determined. 20 antibiotic agents were used for the antibiotic susceptibility test of *Salmonella*

spp. resistance, susceptibility, and intermediate condition were interpreted according to the chart of the Clinical Laboratory Standards Institute (CLSI, 2020)

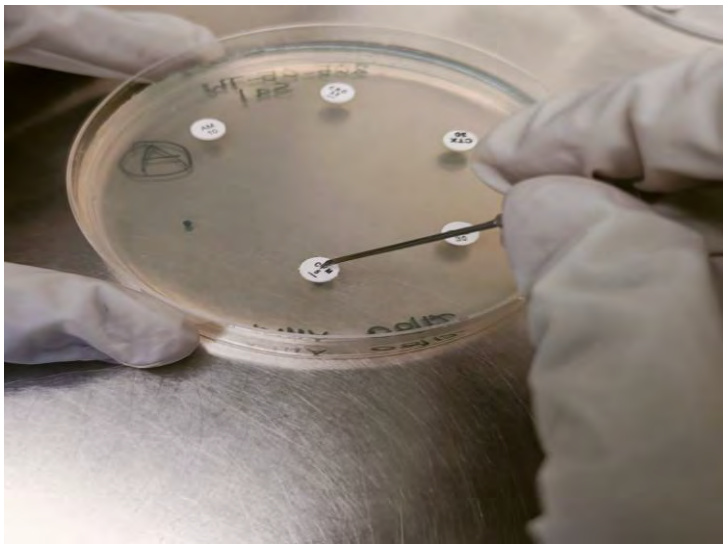


Figure 13: Antibiotic discs placed on MHA plate

Antibiotic Class	Antibiotic Agents	mg
Aminoglycosides	Gentamycin (CN)	10mg
Antipseudomonal pennicillin+b-lactamase inhibitors	Piperacillin-Tazobactum (TZP)	110mg
Carbapnem class	Meropenem (MEM)	10mg
Cephalpsporins class	Cefotaxime (CTX) Ceftazedime (CAZ) Cefixime (CFM) Cefepime (FEP)	30mg 30mg 5mg 30mg
Cephamycins	Cefoxitin (FOX)	30mg
Fluoroquinonoles	Ciprofloxacin (CIP)	5mg
Folate Pathway Inhbitors	Sulfamethoxazole (SXT)	25mg
Monobactums	Aztreonam (ATM)	30mg
Nitrofurans	Nitrofurantion (F)	300mg
Peninnicilin	Mecillinam (MEL)	25mg
Phenicols	Chlorumphenicol	30mg
Quinonoles	Ciprofloxacin (CIP) Naidixic Acid (NA)	5mg 30mg
Tetracycline class	Tetracycline	30mg

Table 4: Antibiotic Agents among Different Antibiotic Classes

2.5.1. Statistical Analysis

Data collected in this study were organized using Microsoft Excel (MS-2016) and analyzed with SPSS software (IBM SPSS-29.0). Descriptive statistical analysis was employed to determine the prevalence and associations between multidrug-resistant (MDR) isolates and virulence gene profiles. Statistical significance was established at a level of $p < 0.05$.

Chapter 3

Results

Results

3.1 Isolation of Presumptive *Salmonella* Colonies

Presumptive *Salmonella* colonies were identified as either red colonies or red colonies with a black center on XLD plate. Both colony types were selected for further testing to confirm the presence of *Salmonella* spp.

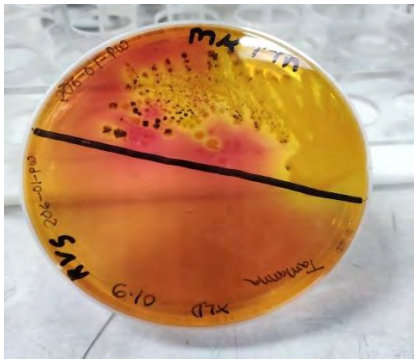


Figure 14: Yellow Colonies with Black Centers representing Salmonella spp. on XLD Plate

3. 2 O-nitrophenyl-Beta-D-galactopyranoside (ONPG) Test of Presumptive *Salmonella* spp.

Salmonella spp. typically yield negative results in the ONPG test. The lack of a yellow color change during incubation with an ONPG disc indicates the absence of beta-galactosidase activity, an enzyme commonly associated with *Salmonella*. Therefore, a negative ONPG test can serve as a presumptive indicator for the presence of *Salmonella* spp.

Figure 15: Result of ONPG test showing presumptive Salmonella spp. (No colour change in vials)

3.3 Molecular Detection of *Salmonella* spp. By Identification of invA Gene

To confirm that 258 among 794 isolates were indeed *Salmonella* bacteria, a DNA test was performed using the *invA* gene. The results showed that all 258 isolates had the *invA* gene, confirming that they belonged to the *Salmonella* genus.

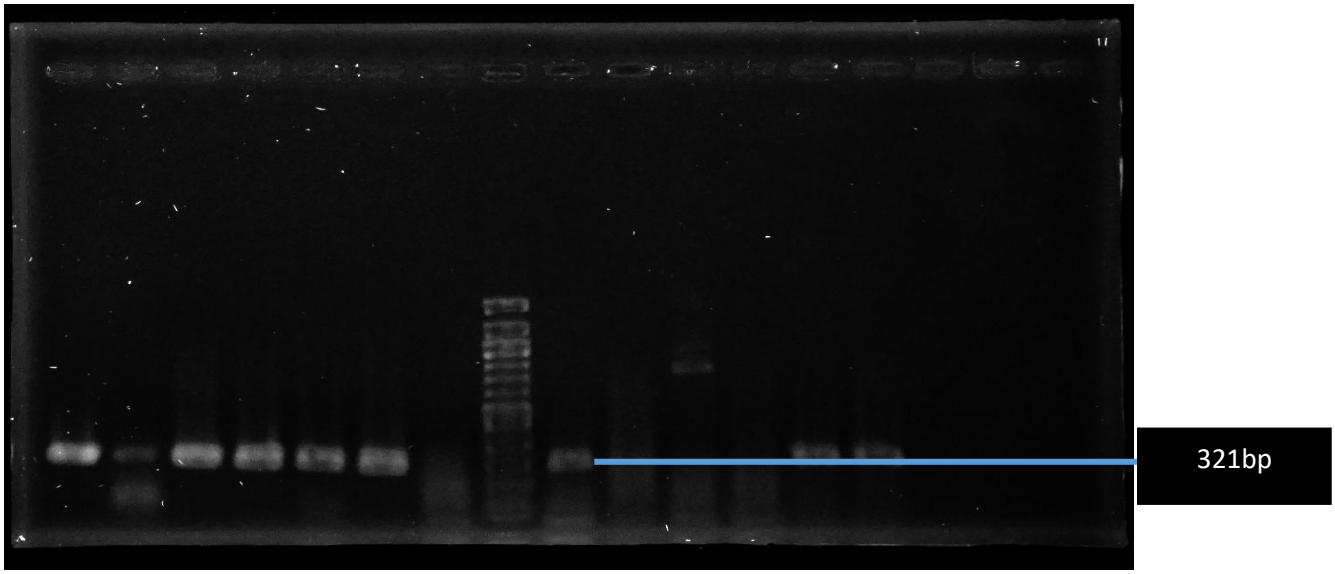


Fig 3.3: *invA* positive & *invA* negative isolates

3.4 Prevalence of *Salmonella* spp. In the variation of samples:

The study presented a prevalence of *Salmonella* species across the examined aquaculture supply chain. Of the total 794 fish samples analyzed, 32% (n=258) tested positive for *Salmonella* spp., in the marketed fish.

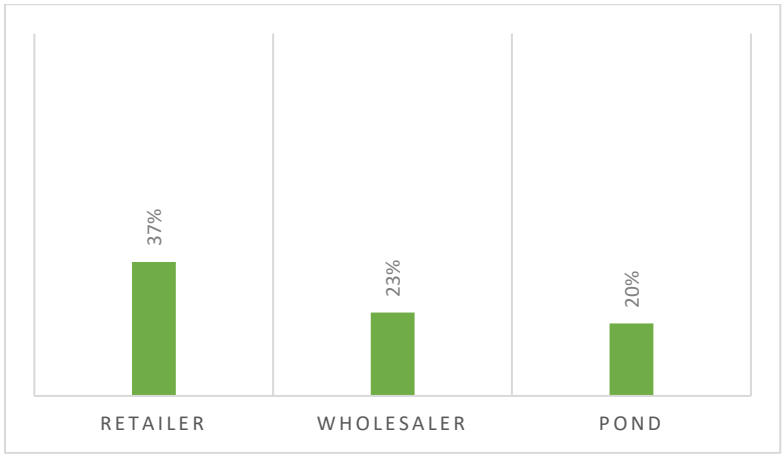


Figure 16: Prevalence of Salmonella spp. in market types

The bar chart shows the percentage distribution of *Salmonella* spp. prevalence among retailers, wholesalers, and ponds. The highest prevalence was observed at the retailer level (37%) , followed by ponds (29%) , and the lowest at the wholesaler level (23%). This indicates that retailers experience the greatest exposure to antibiotics compared to wholesalers and ponds.

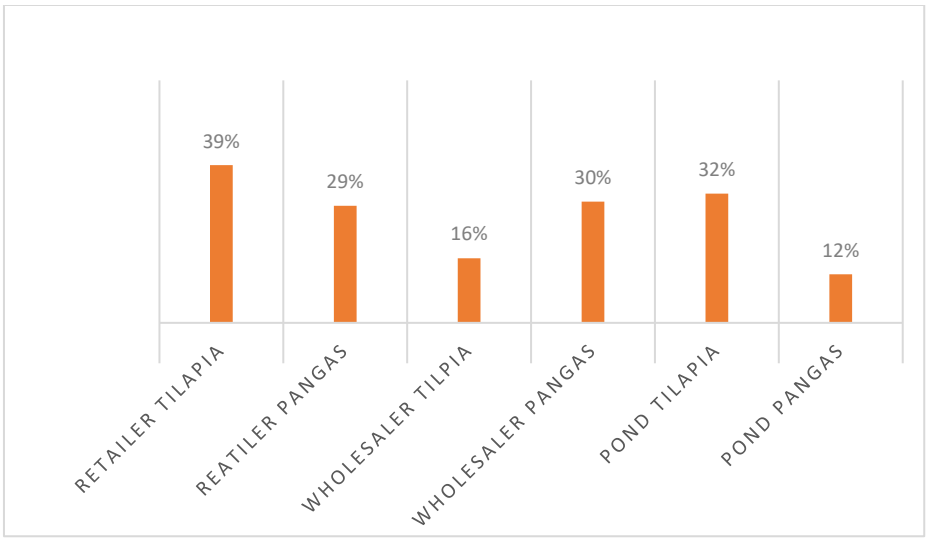


Figure 17: Prevalence of Salmonella spp. among sample types

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The bar chart displays the percentage distribution of different market participants involved in the trade of two fish species: Tilapia and Pangas. Among retailers, Tilapia accounts for 39%, which is higher than Pangas at 29%. For wholesalers, Pangas holds a larger share of 30% compared to Tilapia at 16%. Lastly, at the pond level, Tilapia has a higher percentage (32%) compared to Pangas (12%). Overall, Tilapia shows a stronger presence at the retailer and pond levels, whereas Pangas performs better at the wholesaler level.

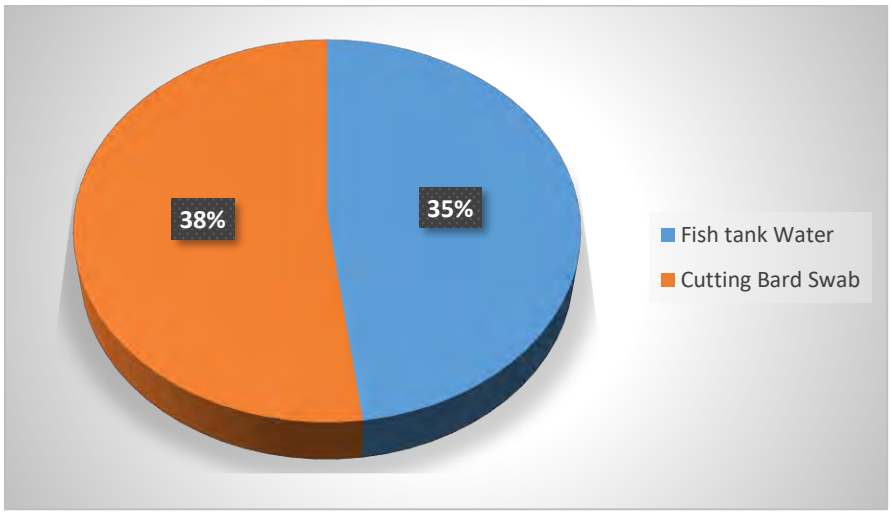

Figure 18: Salmonella spp. Prevalence in Environmental sample (water & swab)

It has shown that Fish Tank water has 35% *Salmonella* spp. Isolates and cutting board swab has 38% *Salmonella* spp. Positive isolates.

3.5 Detection of virulence Genes of *Salmonella* spp. Through Polymerase Chain Reaction (PCR):

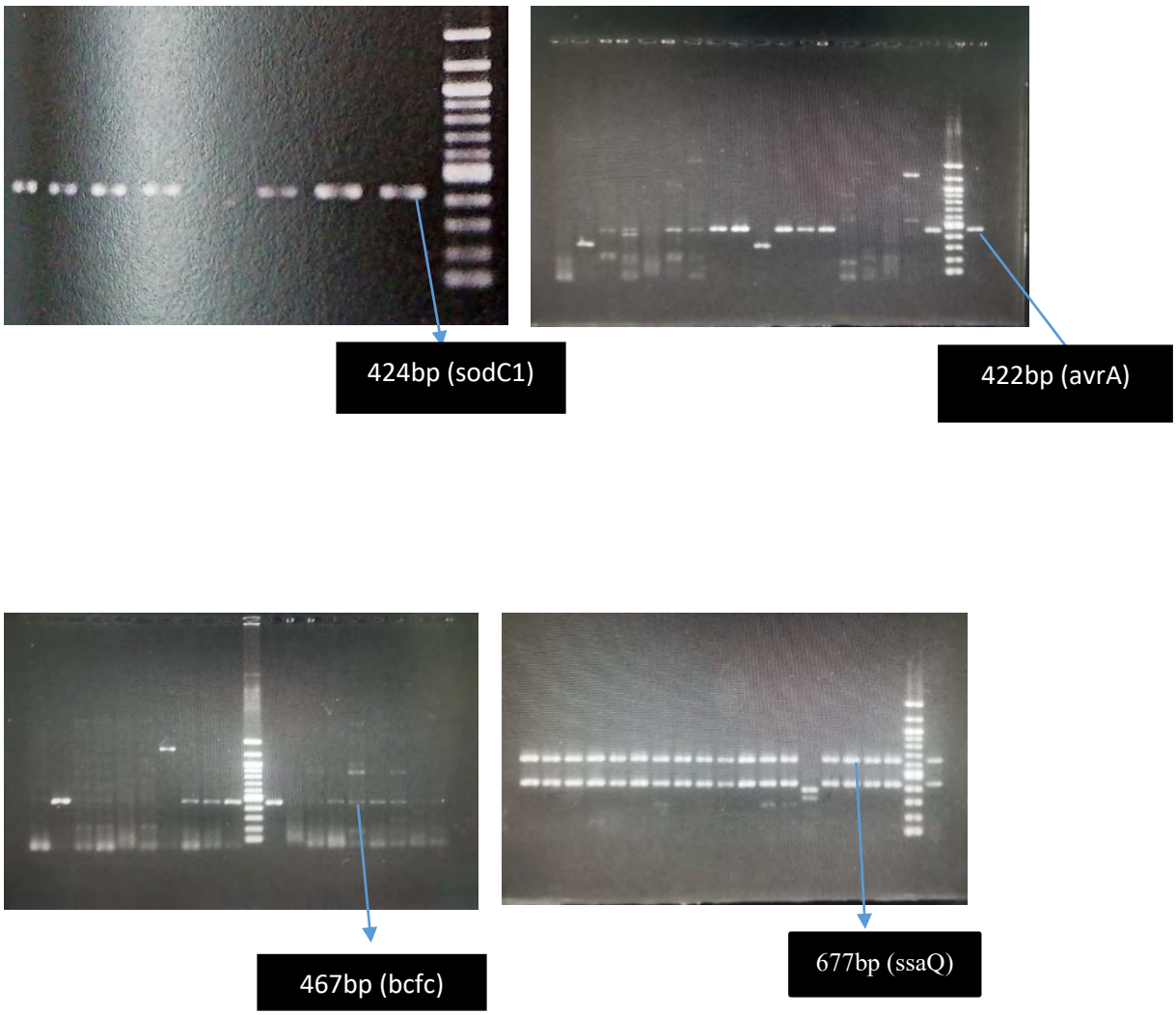

Figure 19: Gel Image of Virulence genes of Salmonella spp. (bcfc, avrA, ssaQ, sodC1)

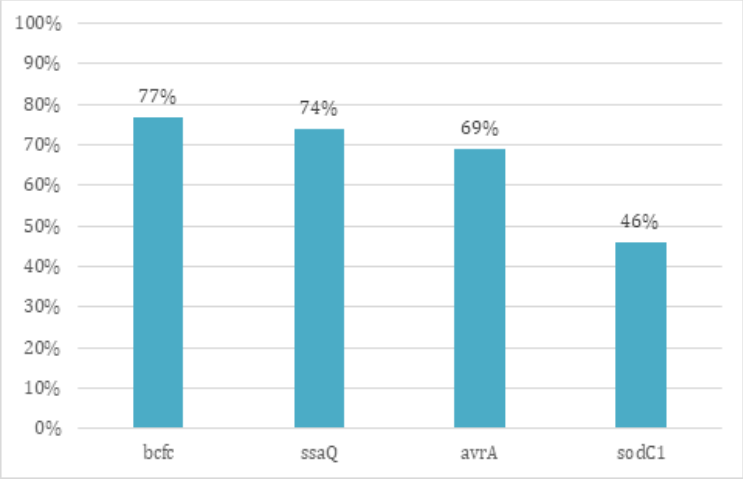

Figure 20: Virulence Genes for Salmonella spp. Positive Isolates.

The analysis of virulence genes detected by PCR in *Salmonella* spp. positive isolates highlighted varying prevalence rates. Among the identified genes, bcfc was the most prevalent, found in 77% of the samples. Following closely, the ssaQ gene showed 74% positivity rate, while avrA was identified in 69% of the samples. The least prevalent gene, sodC1, was present in 46% of the isolates. The bar chart clearly indicates that, across all *Salmonella*-positive samples from various market types and during both morning and evening shifts, the bcfc gene consistently showed the highest percentage at 78%. The ssaQ gene ranked second, and the avrA gene third in terms of prevalence.

Figure 21: Virulence Gene Rate in Sample (bcfc, ssaQ, avrA, sodC1)

The distribution of four *Salmonella* genes (*bcfC, ssaQ, avrA,* and *sodC1*) varied across different sample types. In Pangas, all genes were evenly distributed, ranging from 19-31%. Tilapia had similar proportions of all genes at around 25-26%. Swab samples showed slightly higher levels of *sodC1* at 29%, while the other genes were lower. In water samples, *sodC1* was the highest at 33%, and the other genes (*bcfC, ssaQ,* and *avrA*) were each at 28%.

3.6 Antibiotic Susceptibility Testing of confirmed *Salmonella* spp.

This study assessed the susceptibility of 258 *Salmonella* isolates to 21 different antimicrobial agents belonging to 13 classes.

Antibiotic Names	**FISH (Pangas, Tilapia)**	**Environmental (Cutting board swab & Fish tank water**
Getamycin	0%	1%
Pipperacillin/ tazobactam	1%	0%
Meropenem	2%	0%
Ertepenem	3%	0%
Cefotaxime	7%	6%
Ceftriaxone	4%	2%
Ceftazidime	2%	0%
Cefexime	10%	5%

Cefepime	0%	0%
Cefoxitin	5%	3%
Ciprofloxacins	6%	4%
Trimethoprim	7%	8%
Aztreonam	2%	0%
Nitrofurantions	7%	6%
Ampicillin	9%	8%
Mecillinam	5%	11%
Chloramphenicol	3%	6%
Nalidixic acid	10%	10%
Tetracycline	10%	11%

Table 5: Antibiotic resistance among fish and environmental samples

The table presents the prevalence of individual antibiotics detected in fish (Pangas and Tilapia) and environmental samples (cutting board swabs and water). In fish, cefixime, nalidixic acid, and tetracycline were the most frequently detected antibiotics, each at 10%, followed by trimethoprim (7%) , cefotaxime (7%) , and nitrofurantions (7%). Moderate levels of cefepime (6%) , cefoxitin (5%) , and chloramphenicol (3%) were observed, while getamycin and meropenem were absent or very low (≤2%).

In environmental samples, ampicillin (11%) , tetracycline (11%) , andchloramphenicol (6%) showed higher prevalence. Ciprofloxacin andtrimethoprimwere moderately detected at 8% and

6%, respectively. Low detection rates were observed for antibiotics such as cefixime (5%) , ceftriaxone (2%) , andaztreonam (2%).

Overall, tetracyclineandampicillinshowed similar high detection rates across bothsample types, while other antibiotics varied, with some showing greater prevalence in environmental samples (e. g., ampicillinand chloramphenicol) and others in fish (e. g., cefixime and nalidixic acid).

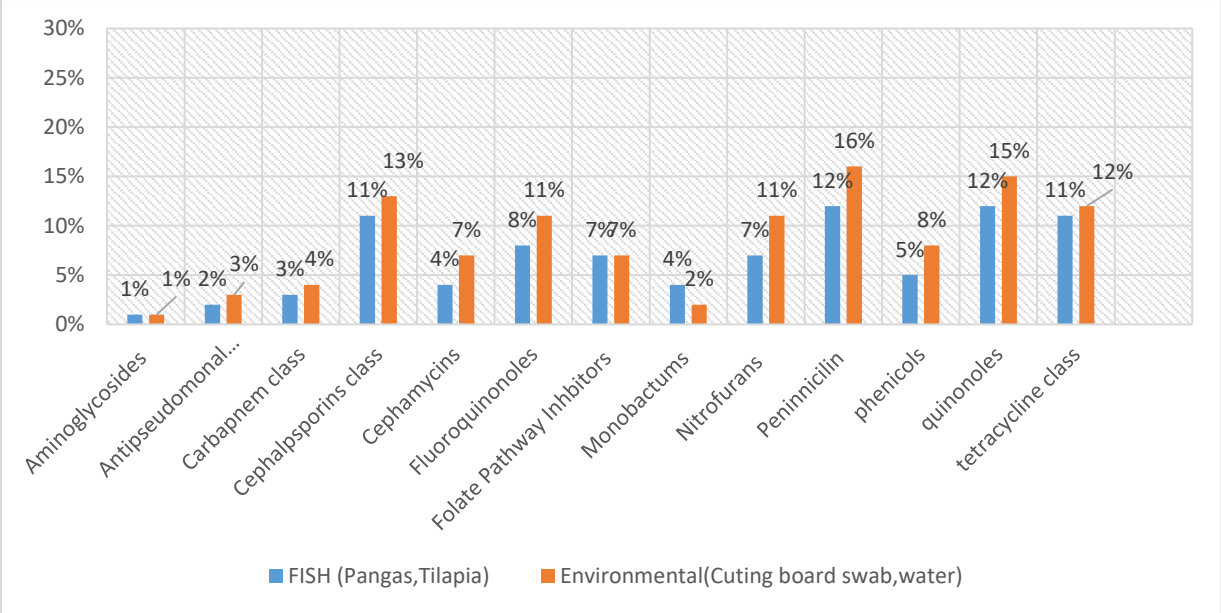

Fig 3.10: Prevalence of Antimicrobial Resistance in Fish and Environmental Samples according to class

The bar chart shows the prevalence of various antibiotic classes in fish (Pangas and Tilapia) and environmental samples (cutting board swabs and water). Aminoglycosides, antipseudomonal drugs, carbapenems, and monobactams were detected at low levels, ranging from 1% to 4%, with slightly higher rates in environmental samples. Cephalosporins (13%), penicillins (16%) , and quinolones (15%) had higher prevalence in the environment compared to fish, where their detection ranged from 11% to 12%. Cephamycins (7%) , fluoroquinolones (8%) , folate pathway inhibitors (7%) , nitrofurans (7%) , and tetracyclines (11%-12%) showed moderate levels in both sample types. Phenicols were slightly higher in the environment (8%) than in fish (5%). Overall, environmental samples exhibited a marginally higher prevalence of antibiotics, particularly in cephalosporins, penicillins, and quinolones.

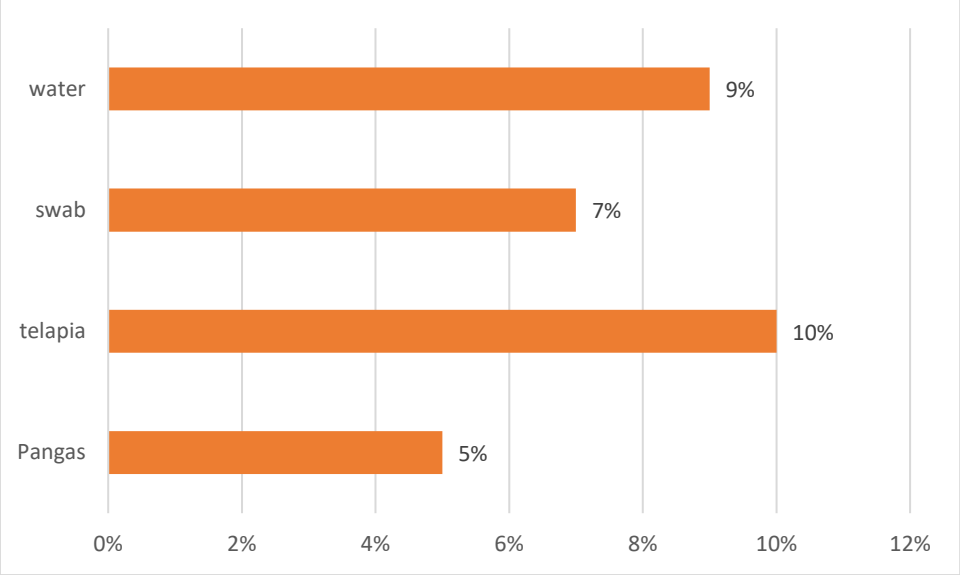

Figure 22: MDR (Multi Drug Resistance) Comparison in fish and Environmental Sample

Among the 258 *Salmonella* isolates tested, n=79 (30. 6%) exhibited multidrug resistance (MDR) patterns. The MDR rates varied across sample types, with tilapia showing 10% MDR prevalence (n=26), followed by water samples at 9% (n=23) , environmental swabs at 7% (n=18) , and Pangas fish samples showing the lowest MDR rate at 5% (n=12).

Chapter 4
Discussion

Discussion

The current study provides an investigation into the prevalence of *Salmonella* species within the aquaculture supply chain, highlighting significant microbial contamination in commercially important fish species. The findings reveal a notably high incidence of *Salmonella* contamination across different stages of production and distribution, which carries substantial implications for food safety and public health. The overall contamination rate of 32.4% (258 out of 794 fish samples) represents a considerable microbiological risk in the aquaculture sector. This prevalence is particularly concerning when considering the two primary fish species examined: *Pangasius* (*Pangasianodon hypophthalmus*) and *Tilapia* (*Oreochromis niloticus*). These species are not only economically significant but also widely consumed, which amplifies the potential public health implications of such widespread bacterial contamination. From a public health perspective, these findings underscore the critical need for enhanced microbiological monitoring, improved hygiene protocols, and potentially more stringent regulatory oversight in aquaculture production and fish handling processes. The widespread presence of *Salmonella* poses significant risks of foodborne illness, particularly for vulnerable populations. (Srivastava, Verma, Verma, & Singh, 2014)

Our study showed the highest prevalence in retail markets, which is 37%. The prevalence of *Salmonella* in *Pangasius* and *Tilapia* from Bangladeshi retail markets and aquaculture systems underscores a significant public health challenge. This study reveals alarming prevalence rates ranging from 25.3% to 36.5%, varying across different sampling locations. Specifically, in Dhaka's retail markets, *Salmonella* prevalence in fish samples was documented between 29.5% and 35.6%, with *Pangasius* and *Tilapia* showing contamination rates of 27.8–33.2% and 31.4–36.5%, respectively. (Amin et al., 2024) In addition, our examination of microbial water quality in aquaculture systems across Bangladeshi regions indicated water system contamination rates between 22.6% and 28.4%, alongside direct fish sample contamination of 25.3–32.1%. (Islam et al., 2021) The antibiogram study further revealed substantial multidrug resistance among isolates, highlighting complex resistance mechanisms and the presence of multiple antibiotic resistance genes. These results collectively illustrate the significant microbiological issues within Bangladeshi aquaculture, emphasizing the urgent

need for enhanced surveillance, improved hygiene protocols, and comprehensive strategies to mitigate foodborne health risks. (Begum, Salauddin, Hossain, & Begum, 2019)

The comprehensive analysis revealed a distribution of *Salmonella* spp. across different sample types and stages of the aquaculture supply chain, highlighting significant variations in bacterial contamination. The fish samples demonstrated variable prevalence, with Retailer *Tilapia* showing the highest contamination at 39%, followed by Retailer *Pangas* at 29%, while Wholesaler *Pangas* (30%), Pond *Pangas* (12%), and Pond *Tilapia* (32%) exhibited lower rates of *Salmonella* detection. Notably, the environmental samples presented an even more striking contamination profile, with environmental sources showing remarkably high *Salmonella* prevalence. Fish tank water showed 35% of *Salmonella*, while cutting board swabs showed 38% of *Salmonella* spp. This comparative analysis reveals a critical insight: environmental samples demonstrate substantially higher bacterial contamination rates compared to fish samples, suggesting that water and surface environments serve as potential reservoirs and transmission vectors for *Salmonella* in the aquaculture supply chain. The substantial *Salmonella* presence in environmental samples, significantly exceeding the fish sample prevalence rates, underscores the complex dynamics of bacterial transmission and the potential for cross-contamination throughout the production and distribution process. These findings emphasize the importance of comprehensive microbiological monitoring, not only of fish products but also of the surrounding aquatic environments and handling surfaces, as these environmental reservoirs appear to play a crucial role in the proliferation and spread of *Salmonella* species.

The comprehensive comparative analysis of *Salmonella* pathogenicity in fish samples and environmental water sources reveals key differences and interconnected characteristics, which are crucial for public health. Fish sample *Salmonella* displays host-specific invasion characteristics, higher genetic specificity, and a greater potential for systemic infection, indicating a direct threat to human consumption. On the other hand, water sample *Salmonella* shows enhanced environmental adaptability, broader genetic diversity, and persistent survival mechanisms. Environmental water samples have a broader prevalence strategy due to their extensive transmission potential and advanced stress resistance, while fish samples present an immediate human health threat through direct consumption. These findings underscore the necessity for rigorous microbiological monitoring of both fish products and

their surrounding environments. (Chatreman, Seecharran, & Ansari, 2020; Liu, Whitehouse, & Li, 2018)

For the *bcfc* and *ssaQ* genes, retailers accounted for the majority of isolates at 82% and 83%, while wholesalers and pond environments made up smaller proportions of 10% and 8%, respectively. This imbalance is further exacerbated in the *avrA* gene, where retailer contamination slightly decreased to 72%, but wholesaler and pond levels increased to 12% and 16%, respectively. The most alarming condition is observed in the *sodC1* gene, where retailer prevalence peaked at 82%, while wholesaler and pond levels decreased to 10% and 11%, respectively. This suggests a disproportionate increase in *Salmonella* prevalence at the retail stage, with rates approximately six times higher than those found in the earlier supply chain stages. This observation raises critical questions regarding the efficacy of food safety measures and quality control procedures implemented at the retail level.

The *avrA* gene is associated with *Salmonella*'s ability to invade and colonize host cells. It encodes a type III secretion system effector protein that helps *Salmonella* evade host immune defenses and establish a successful infection. The increased prevalence of the *sal_avrA* gene, as indicated in the data, suggests enhanced virulence potential of *Salmonella* strains, which could lead to more severe disease outcomes in humans. (Huehn et al., 2010) The *sodC1* gene encodes a copper/zinc superoxide dismutase enzyme, which helps *Salmonella* resist oxidative stress and survive within host phagocytic cells. The high prevalence of the *sodC1* gene, particularly at the retail level, indicates that *Salmonella* strains may be better equipped to withstand host immune responses and persist in the food supply chain, potentially increasing the risk of human infection. (Majeed, Kumarage, & Heo, 2024) The *sal_bcfC* gene is involved in *Salmonella*'s ability to attach to and colonize host cells, a crucial step in the infection process. The consistent presence of the *bcfC* gene across the different stages of the supply chain, as shown in the data, suggests that *Salmonella* strains possess a robust ability to adhere to and contaminate various food matrices, which could contribute to the widespread dissemination of *Salmonella* in the food system. The prevalence and distribution of these virulence genes in *Salmonella* isolates from different sources, including human health-relevant serovars, aquatic food, and poultry, highlight the significant public health and food safety concerns associated with *Salmonella* contamination. Continued monitoring and understanding of the virulence gene profiles of *Salmonella* strains can inform the development of more targeted

intervention strategies to mitigate the risks posed by this pathogen throughout the food supply chain. (Abdel Rahman, Nasef, & Hamed, 2017)

In the fish samples (*Pangasius* and *Tilapia*), there's a notable pattern of antibiotic resistance, with the highest resistance levels observed for Cefexime, Nalidixic acid, and Tetracycline, each at 10%. Ampicillin follows closely at 9%. The carbapenems (Meropenem and Ertapenem) and other broad-spectrum antibiotics like Ceftazidime show relatively low resistance rates (2–3%). Interestingly, there was no resistance observed for Cefepime and minimal resistance to Gentamycin, which is encouraging from a treatment perspective. The moderate resistance levels to commonly used antibiotics like Ciprofloxacin (6%) and Trimethoprim (7%) suggest careful consideration is needed when using these drugs for treatment.

The environmental samples (cutting board swab and fish tank water) show a slightly different resistance profile. The highest resistance levels were found for Mecillinam and Tetracycline (11% each), followed by Nalidixic acid (10%). It's noteworthy that several antibiotics that showed resistance in fish samples demonstrated zero resistance in environmental samples, including Meropenem, Ertapenem, Ceftazidime, and Aztreonam. However, Trimethoprim showed slightly higher resistance in environmental samples (8%) compared to fish samples (7%). This difference between fish and environmental samples could indicate that the resistance patterns are influenced by different selective pressures in these two contexts.

Antimicrobial resistance (AMR) is a serious and widespread global problem that affects the safety of the food supply and public health. In many regions, studies have demonstrated the presence of drug-resistant bacteria in seafood products. For example, Nile tilapia has been found to contain drug-resistant strains of bacteria such as *Aeromonas hydrophila* and *Pseudomonas entomophila*. (Preena, Dharmaratnam, & Swaminathan, 2020) In China, *Salmonella* serovars in retail seafood products are resistant to antibiotics such as ampicillin, tetracycline, and chloramphenicol, largely due to the overuse of these drugs in food production systems. (Yang et al., 2022) Similarly, in Bangladesh, multidrug-resistant *Salmonella* in street food and poultry are highly resistant to several antibiotics, including ampicillin and amoxicillin. In Tanzania, marine and freshwater fish have been reported to carry significant antibiotic-resistant bacteria, posing a risk to human health. (Hassan et al., 2018)

These findings highlight the complex and widespread nature of AMR in aquatic environments, largely due to the widespread and often indiscriminate use of antibiotics in aquaculture and other food production activities. Addressing this challenge requires concerted global efforts to enforce strict antibiotic control, improve surveillance programs, and develop effective control measures to minimize the spread of antibiotic-resistant bacteria. Such strategies are essential to ensure food security, protect public health, and maintain the effectiveness of antibiotics for future generations. By implementing comprehensive surveillance and appropriate management measures, we can work towards minimizing AMR in aquatic environments and the broader ecosystem.

The prevalence and diversity of antimicrobial resistance (AMR) genes across different environmental samples reveal a complex landscape of bacterial resistance mechanisms. The complex landscape of antimicrobial resistance (AMR) genes in *Salmonella* reflects a dynamic interplay of ecological, genomic, and environmental factors, as illuminated by recent comprehensive studies across diverse geographical contexts. Threlfall's seminal work on antimicrobial drug resistance highlights the persistent challenge of resistance mechanisms in food- and water-borne infections, while recent whole-genome sequencing analyses from Brazil and China provide nuanced insights into the molecular epidemiology of *Salmonella* resistance. (Rodrigues et al., 2020) The ubiquity of resistance genes, particularly *tet(A)*, echoes the findings in our current study, where this gene consistently emerged across multiple samples, suggesting a widespread adaptive strategy for tetracycline resistance. The Brazilian study's four-decade overview and the Hangzhou-based research underscore the heterogeneous distribution of resistance determinants, mirroring our observation of varying genetic diversity across different environmental samples. Multi-class resistance genes like *blaTEM-1* (β-lactam resistance), *aph* variants (aminoglycoside resistance), and *qnrS1* (quinolone resistance) demonstrate the sophisticated molecular mechanisms bacteria employ to survive antibiotic challenges. These findings collectively reveal that AMR gene prevalence is not merely a local phenomenon but a complex global narrative of bacterial adaptation, driven by intricate ecological pressures and horizontal gene transfer mechanisms that transcend individual environmental niches. (Yu et al., 2024)

It was found that the virulence genes *invA*, *hilA*, and *sseC* are closely associated with septicemia, playing crucial roles in systemic infections (Paolo D. et al., 2022). For enterocolitis, genes such as *fim*, *invA*, and *hilA* facilitate adhesion to and invasion of the intestinal

epithelium, contributing to the disease's pathogenesis (Gavin J. et al., 2023). Gastroenteritis, characterized by inflammation of the stomach and intestines resulting in diarrhea and abdominal pain, is linked to several virulence genes, including *hilA*, *hilC*, *hilD*, *acrB*, *acrD*, *acrF*, and *avrA* (Ali Hossain et al., 2021). Typhoid fever, on the other hand, is primarily associated with the genes *invA*, *ssaA-T*, and *invA-I*, which are significant for the invasion and survival of the bacteria within host cells (Ali Hossain et al., 2021). These findings highlight the diverse roles of specific virulence genes in causing various diseases.

The study of various studies provides a comprehensive understanding of the significance of the *invA* gene in *Salmonella* isolated from diverse sources, including meat, poultry, and fish. The *invA* gene consistently emerges as a crucial virulence factor across different *Salmonella* serovars, highlighting its fundamental role in the bacteria's pathogenicity and diagnostic importance. This study underscores the prevalent occurrence of the *invA* gene in *Salmonella* isolates from meat and poultry. The gene is essential for the invasion of epithelial cells, a key step in the infection process. Its detection in these food products signifies a potential risk for consumers, emphasizing the need for stringent monitoring and control measures in the food industry to prevent *Salmonella* outbreaks. The distribution of multiple *inv* genes (*invA*, *invB*, *invC*, and *invD*) in *Salmonella Typhimurium* and other serovars highlights the genetic diversity and complexity of these pathogens. (Karmi, 2013) Specifically, *invA* mutants of *Salmonella Typhi* demonstrate a deficiency in invading mammalian cells, reinforcing the gene's critical role in host invasion. This finding suggests that *invA* is a key determinant of *Salmonella*'s ability to cause disease, providing a target for future therapeutic interventions. (Uzairue & Shittu, 2023)

Molecular Detection of *invA* and *hilA* Virulent Genes in *Salmonella* Serovars Isolated from Freshwater Fish: The identification of *invA* and *hilA* genes in *Salmonella* isolates from freshwater fish further extends the understanding of these genes' roles in different environments. The presence of these virulence genes in aquatic sources implies potential transmission routes to humans, stressing the importance of monitoring water and fish products for *Salmonella* infection to safeguard public health.

This study between quinolone resistance and reduced mRNA expression of *invA* and *avrA* genes. While antibiotic resistance typically enhances bacterial survival, this reduction in virulence gene expression suggests a potential trade-off, where increased

resistance might compromise the bacteria's invasive capabilities. This observation could inform the development of novel antimicrobial strategies that exploit this vulnerability. Isolation and the consistent detection of the *invA* gene in *Salmonella* isolates from poultry farms highlights its widespread prevalence and diagnostic utility. The study reinforces the gene's role in the pathogenicity of *Salmonella*, serving as a reliable marker for identifying these bacteria in poultry and potentially preventing foodborne illnesses through early detection and intervention.

The *invA* gene is a pivotal component in the pathogenic mechanisms of *Salmonella* across various serovars and sources. Its consistent association with the bacteria's ability to invade host cells and cause disease underscores its importance in both clinical and food safety contexts. The detection and monitoring of *invA*, along with other virulence genes, can enhance our understanding of *Salmonella* epidemiology, inform public health strategies, and drive the development of targeted therapies to mitigate the impact of *Salmonella* infections.

Chapter 5

Conclusions and Recommendations

Conclusions and Recommendations

5.1 Conclusion

This study investigated the prevalence and antibiotic resistance of *Salmonella* bacteria in two popular fish species, Pangas and Tilapia, environmental samples (Fish Water and Cutting Board Swab) throughout the aquaculture supply chain in Bangladesh. It also explored the presence of virulence genes that contribute to *Salmonella*'s ability to cause illness. This research provides a comprehensive look at the occurrence, pathogenicity, and antibiotic susceptibility of *Salmonella* in these fish species within the Bangladeshi context.

The findings indicate that both Pangas and Tilapia fish are contaminated with potentially harmful *Salmonella* at various points in the supply chain. The level of contamination varied across different sample types and locations. Retail fish samples showed the highest *Salmonella* prevalence (37%) , likely because this is the final stage before consumption. Pond and wholesale samples had lower, but still significant, *Salmonella* prevalence (20% and 23%, respectively). Tilapia generally exhibited higher *Salmonella* contamination rates than Pangas in both pond and retail samples.

A concerningly high level of multidrug resistance was observed in *Salmonella* isolates from both fish species. Several virulence genes were identified, with bcfC being the most prevalent, followed by stn, ssaQ, and avrA. The isolates demonstrated resistance to multiple antibiotics, with A varying Multiple Antibiotic Resistance (MAR) index values. Importantly, the study revealed a statistically significant link between the presence of the virulence gene and multidrug resistance in the *Salmonella* isolates.

These results emphasize the urgent need for enhanced monitoring and control strategies to limit the spread of antibiotic-resistant *Salmonella* and minimize the risk of foodborne illnesses associated with contaminated fish in Bangladesh's aquaculture industry. This is crucial for protecting public health and ensuring the safety of fish products.

5.2 Recommendations

The detection of virulence genes in *Salmonella* emphasizes the need for ongoing research to better understand how *Salmonella* spreads and causes illness within the aquaculture industry.

This knowledge will be crucial for developing targeted interventions to prevent the emergence and spread of dangerous *Salmonella* strains.

Public health authorities should educate consumers about the potential risks of *Salmonella* contamination in fish and promote safe food handling practices. These practices include thoroughly cooking fish, proper storage, and careful handling of raw fish to minimize the risk of foodborne illness.

- **Nationwide Surveillance Program:**

It is strongly recommended that Bangladesh establish a nationwide surveillance program to monitor *Salmonella* prevalence and antibiotic resistance in aquaculture. This program should regularly sample fish farms, processing plants, and retail markets to pinpoint contamination sources and areas needing improvement. Educating farmers on responsible antibiotic use and promoting best practices, such as biosecurity and proper waste management, are also essential to minimize *Salmonella* contamination.

- **Further Risk Assessment and Regulatory Frameworks:**

Additional risk assessment studies are needed to evaluate the risks associated with fish consumption in Bangladesh. These findings can inform stronger regulations and promote fair trade practices while protecting consumer health and safety.

- **Antibiotic Resistance Monitoring and Research:**

Continuous monitoring of antibiotic resistance trends is crucial. Further research is necessary to understand the molecular epidemiology and pathogenesis of *Salmonella* in aquaculture. This will aid in developing targeted interventions to prevent the spread of pathogenic and antibiotic-resistant *Salmonella* strains

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Appendices

Appendix

Buffered Peptone Water

Component	Amount (g/L)	pH
Peptone	10.0	7.2 ± 0.2 @ 25°C
Sodium chloride	5.0	
Disodium phosphate	3.5	
Potassium dihydrogen phosphate	1.5	

Rappaport-Vassiliadis Soya Peptone Broth (RVS broth)

Component	Amount (g/L)	pH
Soya peptone	4.5	5.2 ± 0.2 @ 25°C
Sodium chloride	7.2	
Potassium dihydrogen phosphate	1.26	
Di-potassium hydrogen phosphate	0.18	
Magnesium chloride (anhydrous)	13.58	
Malachite green	0.036	

Muller-Kauffmann Tetrathionate-Novobiocin Broth (MKTTn)

Component	Amount (g/L)	pH
Meat extract	4.3	8.0 ± 0.2 @ 25°C
Enzymatic digest of casein	8.6	
Sodium chloride	2.6	

Component	Amount (g/L)	pH
Calcium carbonate	38.7	
Sodium thiosulphate (anhydrous)	30.5	
Ox bile	4.78	
Brilliant green	0.0096	

Xylose-Lysine-Deoxycholate (XLD) Agar

Component	Amount (g/L)	pH
Yeast extract	3.0	7.4 ± 0.2 @ 25°C
L-Lysine HCl	5.0	
Xylose	3.75	
Lactose	7.5	
Sucrose	7.5	
Sodium deoxycholate	1.0	
Sodium chloride	5.0	
Sodium thiosulphate	6.8	
Ferric ammonium citrate	0.8	
Phenol red	0.08	
Agar	12.5	

Tryptone Soya Agar (TSA)

Component	Amount (g/L)	pH
Pancreatic digest of casein	15.0	7.3 ± 0.2 @ 25°C
Enzymatic digest of soya bean	5.0	

Component	Amount (g/L)	pH
Sodium chloride	5.0	
Agar	15.0	

Mueller-Hinton Agar (MHA)

Component	Amount (g/L)	pH
Beef, dehydrated infusion from	300.0	7.3 ± 0.1 @ 25°C
Casein hydrolysate	17.5	
Starch	1.5	
Agar	17.0	

Normal Saline

Component	Amount (g/100 mL)	pH
Sodium chloride	0.85	-