Molecular and Phenotypic Characterization of Virulent, Multidrug-Resistant, and Biofilm-Forming *Salmonella* spp. in Retail Fish from Noakhali Markets, Bangladesh

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List of Abbreviations

Abbreviation	Full Form
AMR	Antimicrobial Resistance
ARG	Antimicrobial Resistance Gene
AST	Antibiotic Susceptibility Test
BPW	Buffered Peptone Water
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
CDC	Centers for Disease Control and Prevention
DNA	Deoxyribonucleic Acid
EU	European Union
MIU	Motility Indole Urease Test
MR	Methyl Red
VP	Voges–Proskauer
PCR	Polymerase Chain Reaction
TAE	Tris-Acetate-EDTA Buffer
TSB	Tryptic Soy Broth
TSI	Triple Sugar Iron
WGS	Whole Genome Sequencing
HEA	Hektoen Enteric Agar
SS	*Salmonella–Shigella* Agar
RV	Rappaport–Vassiliadis Medium
SCA	Simmons Citrate Agar
LIA	Lysine Iron Agar
invA	Salmonella invasion gene A
hilA	Hyperinvasive locus gene A
fimA	Fimbrial subunit gene A

blaTEM-1	Beta-lactamase TEM-1
blaCTX-M-1	Extended-Spectrum Beta-lactamase CTX-M-1
tetA	Tetracycline Resistance Gene A
sul1	Sulfonamide Resistance Gene 1
AMP	Ampicillin
CTX	Cefotaxime
CAZ	Ceftazidime
FEP	Cefepime
IMP	Imipenem
MRP	Meropenem
AZM	Azithromycin
TE	Tetracycline
DO	Doxycycline
CIP	Ciprofloxacin
LE	Levofloxacin
OFX	Ofloxacin
COT	Trimethoprim–Sulfamethoxazole
C	Chloramphenicol

List of Symbols

Symbol	Meaning / Description
°C	Degree Celsius
%	Percentage
v/v	Volume per volume
g/L	Grams per liter
mL	Millilitre
h	Hour(s)
rpm	Revolutions per minute
bp	Base pairs
–	Indicates absence/negative
+	Indicates presence/positive
n	Number of samples
µL	Microlitre
g	Gram
→	Direction or transformation

Abstract

Fish is a widely consumed protein source in Bangladesh, yet retail markets often operate without adequate microbiological monitoring, increasing the risk of foodborne infections. *Salmonella* spp., a major zoonotic pathogen, is frequently associated with aquatic environments and poses a significant public health threat, particularly when antimicrobial resistance (AMR) and virulence factors converge. A total of 100 fish samples from six major retail markets in Noakhali, Bangladesh, were examined for the presence of *Salmonella*. Presumptive isolates were identified based on cultural and biochemical characteristics, followed by molecular confirmation using PCR targeting invA. Virulence genes (hilA, fimA), antimicrobial susceptibility against 14 antibiotics, AMR genes (blaTEM-1, blaCTX-M-1, tetA, sul1), and biofilm-forming ability were also assessed to evaluate pathogenic and resistance profiles. Among 145 presumptive isolates, 24 (16.55%) were confirmed as *Salmonella* spp. All 24 isolates carried the invA gene, while 95.8% and 79.2% possessed hilA and fimA, respectively, indicating substantial virulence potential. Antibiogram profiling revealed multidrug resistance, including complete resistance to ceftazidime (100%) and high resistance to ampicillin (75%) and levofloxacin (50%). PCR detection showed universal carriage of blaTEM-1 (100%), along with blaCTX-M-1 (50%), tetA (29.2%), and sul1 (29.2%). Biofilm assays demonstrated diverse adherence capacities, with several isolates classified as strong biofilm formers, suggesting enhanced environmental persistence and increased risk of cross-contamination. The identification of virulent, multidrug-resistant, and biofilm-forming Salmonella spp. in retail fish markets highlights significant risks to public health and food safety in Bangladesh. The coexistence of virulence genes, AMR determinants, and biofilm-forming capacity underscores the urgent need for improved hygiene practices, regulated antibiotic use in aquaculture, and enhanced surveillance of foodborne pathogens. Although serotyping and whole genome sequencing (WGS) could not be performed due to limited funding and time constraints, these advanced analyses are planned for future work to better understand the genetic diversity and epidemiology of circulating strains.

Keywords: *Salmonella*; Antimicrobial resistance; Virulence genes; Biofilm formation; Whole genome sequencing; Fish samples; Food safety; Retail markets; Public health risk.

Chapter 1

Introduction

1.1 Introduction

Pathogenic microbial contamination from food is a significant concern, as microbiological foodborne infections and illnesses are closely related to food safety. Variations in contamination severity are contingent upon the quantity of contaminated food consumed and the individual's susceptibility to the microorganisms [1]. The opportunistic pathogenic characteristics of the microorganisms have made them critical, despite their natural presence in food [2]. Contamination can occur at any point, from where it originates to the manufacturing and retail chain, and consumption of fish and fish products can lead to various types of bacterial illnesses [3]. Pathogens can be transmitted from fish to humans through contact with an infected individual in a similar environment. The rate of transmission may be influenced by the season, the manner in which fish are handled, individual dietary patterns, and cross-contamination [4], [5].

In Noakhali District, key markets such as Pouro Bazar, Sonapur Bazar, Maijdee Bazar, Chowmuhani Bazar, and Datterhat serve as important hubs for the trade of various fish species, including Giant tiger prawn (*Penaeus monodon*), Giant freshwater prawn (*Macrobrachium rosenbergii*), Banana prawn (*Penaeus merguiensis*), Indian white prawn (*Penaeus indicus*), Bombay duck (*Harpadon nehereus*), and Pabda catfish (*Ompok pabda*). The fish species collected for this study are popular for their unique taste and nutrition, among all the other varieties of local fish in the Noakhali retail markets [6].

P. monodon, the largest commercially available shrimp, is distinguished by its dark coloration and banded carapace and is a major export commodity for Bangladesh [7]. *M. rosenbergii*, the giant river prawn, is a freshwater species recognized for its large size and elongated chelae [8]. The popularity of *P. merguiensis* is driven by the relatively low-cost culture methods practiced by coastal communities, offering a profitable livelihood with minimal management expense [9]. *P. indicus* is commonly available due to its consistent supply from local coastal fisheries and its high demand among consumers and traders in the region [10].

H. nehereus, locally known as loitta, is highly valued both fresh and dried for its unique taste and nutritional benefits, including high protein and Omega-3 content, which contributes to cardiovascular health. Its dried form is an affordable, traditional source of protein, especially consumed during pre-monsoon and monsoon months when fresh fish availability declines due to fishing restrictions [11]. *O. pabda* is highly demanded due to its remarkable taste and high

nutritional value, and is very popular as a rich source of proteins, omega-6 fatty acids, omega-3, minerals, and vitamins [12].

The marketing and distribution of local fish in Noakhali involves multiple stakeholders, including farmers, local forias, depot owners, auctioneers, commission agents, wholesalers, and retailers. The fish trade provides livelihoods for thousands of people, with daily supplies in major markets ranging from several tons, depending on the season and market size [6]. Despite their economic significance, the handling and processing of fish in these markets often occur under suboptimal sanitary conditions, with inadequate infrastructure, limited access to ice, and poor packaging facilities cited as persistent challenges.

The safety of fish and fish products is a growing concern due to the potential for contamination with pathogenic bacteria. Recently, fish products have been recognized as reservoirs of bacterial pathogens associated with human diseases, including *Mycobacterium* spp., *Streptococcus iniae*, *Photobacterium damselae*, *Vibrio alginolyticus*, *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Vibrio cholerae*, *Erysipelothrix rhusiopathiae*, *Escherichia coli* O157: H7, *Aeromonas* spp., *Salmonella* spp., *Shigella* spp., *Staphylococcus aureus*, *Listeria monocytogenes*, *Clostridium botulinum*, *Clostridium perfringens*, *Campylobacter jejuni*, *Delftia acidovorans*, *Edwardsiella tarda*, *Legionella pneumophila*, and *Plesiomonas shigelloides* [13]. These bacteria are recognized as major causes of foodborne illnesses worldwide, capable of causing severe gastrointestinal diseases in humans [14], [15].

A study revealed that these microorganisms are commonly present on fish surfaces, such as skin and gills, and inside the fish in areas like the digestive tract and internal organs, including the kidney, liver, and spleen. However, outbreaks linked to bacterial infections, biotoxins, histamine, viruses, and/or parasites have been linked to fish and fish products, particularly raw or undercooked food [16].

Salmonella is the primary bacterial cause of fish-associated disease outbreaks worldwide, and it is the second most common cause of foodborne disease in the European Union (European Food Safety Authority and European Centre for Disease Prevention and Control) [3], [17]. Although it is not a natural constituent of fish, it is present in a variety of sources, including farm-to-retail stores, fresh fish and fishery products, and imported and domestic fish. It can be introduced to fish by cross-contamination through contaminated water and improper handling [18]. *Salmonella* causes salmonellosis, characterised by stomach discomfort, nausea, diarrhoea,

vomiting, and fever [19], [20]. It is a gram-negative bacterium, mesophilic, facultatively anaerobic, non-spore-forming rod, and motile. It can also survive for long periods in the environment [21]. They often contaminate eggs and poultry [22]. Research results have shown the isolation of *Salmonella* spp from food samples in Bangladesh: milk, chicken meat, beef, and street foods such as fuska, sugarcane juice, borhani, deep-fried and fried snacks, quick lunch items, pickles, fruit chutney, baked items, spicy sour hot snacks, tamarind water, and plain drinking water purchased from food vendors across the country [23], [24], [25]. Food vendors are very common in Bangladesh, and they usually push their ready-to-eat foods along the streets, in schools, at industrial sites, and around the markets. People, especially the young population, highly patronize them without questioning the hygiene of the food peddlers and the overall safety of the food. However, the high prevalence of *Salmonella* in local fish of cultured and natural ponds of the Noakhali region reflects broader challenges in food safety management [26].

Antibiotics are used to effectively prevent and treat bacterial infections [27]. Farmers, in the greater Noakhali region, use antibiotics (beta-lactam group, oxytetracycline, erythromycin, etc.) and other chemicals to control the diseases of fish, along with pond preparation [28]. The overwhelming use of antibiotics has developed antibiotic resistance in fish pathogens, with the emergence of antibiotic-resistant bacteria in aquatic environments. It has been reported that after administration, many antibiotics persist in the sediment and in the aquatic environment for a long time, which affects the sedimentary microbial community [29], [30]. In addition, the presence of antibiotic-resistant genes (ARGs) in these microorganisms can help them [31], [32], [33]. Therefore, finding effective and environmentally friendly methods for disease prevention in aquaculture is crucial to mitigate these risks [34], [35].

In Bangladesh, shrimp is the second largest export product, where most are farmed, and the two main species are black tiger shrimp (*Penaeus monodon,* Fabricius, 1798) and giant freshwater prawn (*Macrobrachium rosenbergii*, de Man, 1879) [36], [37]. In 2022, around 251,964 MT of shrimp were produced in Bangladesh, where farmed shrimp accounted for 55.34% [38]. For exporting shrimp, the processing plant of Bangladesh always follows the international rules for maintaining shrimp quality, such as bacterial status, antibiotic residue, and the presence of filth and dirt. On the other hand, shrimp used to be sold in the domestic market of Bangladesh are not checked for bacterial quality, as well as antibiotic residues [1].

In the US, European Union (EU), Australia, New Zealand, and Hong Kong, the legal limit for *Salmonella, Listeria monocytogenes*, and *Vibrio cholerae* in 25 g of raw or cooked shrimp is zero [39]. In 2018 and 2019, shrimp from Bangladesh, India, and Indonesia that were imported into the US were rejected due to the presence of *Salmonella* spp. [40]. On the other hand, the status of *Salmonella, and* the identification, isolation, and antibiotic resistance pattern of other pathogenic bacteria from fish of cultured and natural ponds have already been reported [26]. However, the microbiological status of local fish found in the retail market in Noakhali, Bangladesh, has not been reported yet for food safety purposes. For the above reasons, this study aimed to investigate the bacterial quality (occurrence of *Salmonella, Shigella, E. coli* O157: H7, and other pathogenic bacteria) of fresh fish available for purchase for human consumption from retail markets of Noakhali, Bangladesh.

1.1 Aims and Objectives

Aim

The primary aim of this project is to characterise the presence of antibiotic resistance genes in *Salmonella*, *Shigella*, and *E. coli* O157: H7 within local fish samples collected from various fish markets in the Noakhali district, Bangladesh.

Objectives

1. Collecting local fish samples from different regional markets across Noakhali zila, ensuring representative sampling from diverse sources.

2. Performing microbiological enrichment using Buffered Peptone Water (BPW) and Rappaport Vassiliadis (RV) broth for the selective enrichment of *Salmonella.*

3. Selective plating of fish samples on Hektoen Enteric Agar (HEA), *Salmonella-Shigella* (SS) Agar, for the targeted isolation of *Salmonella.*

4. Identifying and characterising bacterial isolates based on colony morphology, biochemical tests, and confirmatory molecular techniques.

5. Antibiotic susceptibility testing of the identified microbial strains from the different fish samples from retail markets across the Noakhali region.

6. Antibiotic resistance gene identification of the microbial strains that have previously been identified as resistant to specific antibiotics.

7. Determine the prevalence and distribution of *Salmonella* in local fish samples from different markets within Noakhali district.

8. Assess the potential public health risks associated with the consumption of local fish contaminated with these pathogenic bacteria in the study area.

9. Provide recommendations for improved hygiene practices and food safety interventions in fish handling and marketing across the Noakhali region of Bangladesh.

1.2 Literature Review

1.3.1 Outbreak of Foodborne Diseases

The outbreak of foodborne disease is defined as the occurrence of two or more cases of similar illness due to the consumption of food [41]. Foodborne illness can be caused by bacteria, viruses, and parasites. Foodborne outbreaks have been reported with significant morbidity worldwide and pose a risk towards the human population. Concurrently, in less developed countries, diarrheal diseases are the primary reason for mortality [42]. On a global scale, diarrheal disease has caused 3% mortality [43] and should be a great concern. In industrialised countries, foodborne diseases are not rare because 30% of the global population experiences foodborne diseases each year. Incidence rates have been reported to be 1210 cases per 100,000 inhabitants in France, 2600 cases per 100,000 in the United Kingdom, and more than 25,000 cases per 100,000 inhabitants in Australia and the United States [44]. In general, the frequent sources of foodborne outbreaks are meat, dairy products, eggs and vegetables, whereas the common agents of foodborne outbreaks are *Salmonella typhi*, *Staphylococcus aureus*, *Escherichia coli* and *Clostridium perfringens* [45].

1.3.2 Socioeconomic Impact of the Fisheries Sector

The fisheries sector is one of the most productive and dynamic industries, which has a tremendous potential for future development in the agrarian economy of Bangladesh. Fisheries and aquaculture are the second-largest export industry and the most critical contributors to export earnings in Bangladesh [46]. Bangladesh produces and exports a diverse range of fish and fishery products in around 60 countries of the world [47] and the major export countries

of Bangladeshi fish and fishery products are the European Union (EU), the USA, and Japan [48].

The fisheries sector can also contribute to the pro-poor goals directly by providing employment (as fishers and other related trades) and a source of livelihood. The entire fisheries sector supports the livelihoods of more than 18 million people in the country directly and indirectly [49]. About 1.4 million women depend on the fisheries sector for their livelihoods through fishing, farming, fish handling & processing [50]. There is a close connection between agricultural growth and economic development [51]. Countries neglecting expansion in agriculture cannot boost their economy when the agricultural sector starts to improve; automatically, the export of the country increases. Thus, revenue begins to rise and strengthens the country's economic development.

1.3.3 *Salmonella* as an Emerging Hazard in Aquaculture and Fish Supply Chains

From harvesting to the consumer's table, fish products may be contaminated with various kinds of hazards, some of which are natural and others are introduced by the handlers. There are serious safety concerns about raw fish due to the presence of biological (bacteria, viruses, parasites) and chemical hazards [52]. Fish is not a natural reservoir for *Salmonella,* but acts as a vehicle for its transmission to humans, raising public health concerns [53]. The prevalence of Salmonella varies by type of fishery product and region. For instance, contamination rates reported include 1.2% in shrimp, 2.5% in marine fish, 8.5% in freshwater fish, and around 2.7% in shellfish [54]. Contamination of fish products with *Salmonella* is a major public health concern. The occurrence of *Salmonella* in fish, either in fresh or marine waters, has normally been associated with faecal contamination of the area from which they were harvested [55]. The use of poultry litter and co-rearing with other animals (poultry, cattle, pigs) in fish farms increases the microbial load, thus raising the risk of fish contamination by *Salmonella* [53].

1.3.4 Salmonellosis

Salmonellosis is a major cause of bacterial enteric illness in both humans and animals [56]. Infection with *Salmonella* in humans and animals primarily causes self-limiting gastrointestinal infections with mild to moderate symptoms, including fever, abdominal cramps, and diarrhoea [57]. More severe clinical outcomes, including death, may occur in cases of bacteremia or enteric fever (typhoid), which is often characterised by severe headaches and high fever but no diarrhoea. The symptoms are usually self-limiting and typically resolve within two to seven days [58]. In a small percentage of cases, septicemia and invasive infections of organs and

tissues can occur, leading to diseases such as osteomyelitis, pneumonia, and meningitis [59]. People who are very young, very old, or immunocompromised are most susceptible to these severe manifestations of salmonellosis, which typically require antimicrobial therapy [60]. The number of *Salmonella* human isolates reported to the Centres for Disease Control has been steadily increasing since 1977, and in 1983, there were over 38,000 *Salmonella* isolates [61]. Despite improvements in sanitation and careful monitoring of food processing, large outbreaks of salmonellosis continue to occur when food becomes contaminated [61]. In humans, the clinical pattern of salmonellosis can be divided into four disease patterns, namely enteric fever, gastroenteritis, bacteremia, and other complications of nontyphoidal salmonellosis, as well as chronic carrier state [62]. *Salmonella typhi* causes typhoid fever, whereas *Paratyphi* A, B, and C cause paratyphoid fever with symptoms that are milder and a lower mortality rate [62]. Both serotypes are solely human pathogens; infection typically occurs due to ingestion of food or water contaminated with human waste. In recent years, antibiotic-resistant strains have been isolated in most endemic areas, particularly Southeast Asia, India, Pakistan and the Middle East [63]. According to [62], roughly 10% of patients may relapse, die or encounter serious complications such as typhoid encephalopathy, gastrointestinal bleeding and intestinal perforation [64], [65]. Relapse is the most common occurrence, probably due to persisting organisms within the reticuloendothelial system (RES). Typhoid encephalopathy, often accompanied by shock, is associated with high mortality. Slight gastrointestinal bleeding can be resolved without blood transfusion, but in 1 to 2% of cases, it can be fatal if a large vessel is involved. Intestinal perforation may present with abdominal pain, rising pulse and falling blood pressure in those who have the disease. Hence, it is very serious in 1 to 3% of hospitalised patients [64], [65]. Nontyphoidal salmonellosis or entercolitis is caused by at least 150 *Salmonella* serotypes, with *Salmonella typhimurium* and *Salmonella enteritidis* being the most common serotypes in the United States [62]. Infection always occurs via ingestion of water or food contaminated with animal waste rather than human waste. The emergence of multidrug-resistant *S. typhimurium* DT104 has been associated with outbreaks related to beef contamination and resulted in hospitalisation rates twice that of other foodborne salmonellosis [66], [67]. Ciprofloxacin is often administered at the first sign of severe gastroenteritis, whereas ceftriaxone is given to children with systemic salmonellosis. In production animals like swine, the treatment is usually contraindicated, but when necessary, it can be given via injection with several treatment alternatives based on considerations such as withdrawal time. Antibiotic treatment is usually not advised except for rare cases because it can prolong the presence of bacteria in the stool [66], [67]. About 8% of untreated cases of salmonellosis result in

bacteremia, a serious condition in which bacteria enter the bloodstream after crossing the intestinal barrier [63], [68]. It has been associated with highly invasive serotypes such as Choleraesuis or Dublin. Bacteremia caused by *Salmonella* should be taken into account in cases of fever of unknown origin. Patients with bacteremia and other complications should be treated with antibiotics [63], [68]. Salmonellosis can be spread by chronic carriers who potentially infect many individuals, especially those who work in food-related industries [62]. Factors contributing to the chronic carrier state have not been fully explained, on average, nontyphoidal serotypes persist in the gastrointestinal tract from six weeks to three months, depending on the serotypes [62]. According to [62], only about 0.1% of nontyphoidal *Salmonella* cases are shed in stool samples for periods exceeding one year. The authors further explain that about 2 to 5% of untreated typhoid infections result in a chronic carrier state. Up to 10% of untreated convalescent typhoid cases will excrete S. typhi in faeces for one to three months, and between 1 and 4% become chronic carriers excreting the microorganism for more than one year [63], [65].

1.3.5 Origin and History of *Salmonella* as Multiple Species

Salmonella is named after an American bacteriologist, D. E. Salmon, who first isolated *Salmonella choleraesuis* from porcine intestine in 1884 [69]. The organism was originally called "*Bacillus choleraesuis*", which was subsequently changed to "*Salmonella choleraesuis*" by Lignieres in 1990 [70]. Kauffmann proposed that each serovar be considered a separate species [71]. That is why *Salmonella* serovars identified after 1966 were designated mainly by their antigenic formula, and multiple species within the genus *Salmonella* were generally accepted. However, [70] stated that some clinically important *Salmonella* identified before had been given specific names either according to the disease and/or the animal from which the organism was isolated, such as *S. typhi* and *S. typhimurium* or by the geographical area where the strain was first isolated, e.g., *S. london* and *S. panama*. These names had been used for a number of years and, therefore, were adopted without being amended into the new antigenic formula system [70]. Due to the complexity of multiple *Salmonella* species, it was proposed that the genus *Salmonella* be subdivided into three species: *S. choleraesuis* (the type species), "*S. thphosa*" (*S. thyphi*), and "*S. kauffmannii*," with the last containing all the other serovars [72]. Later, "*Salmonella enterica*" was proposed by Kauffmann and Edwards to encompass all *Salmonellae* [73]. In 1966, a similar three-species model was proposed, with "*Salmonella enteritidis*" representing all serovars other than *S. typhi* and *S. choleraesuis* [74]. Another proposal in 1970 recommended that Kauffmann's "subgenera" be considered a species, i.e., "*S.*

kauffmannii" for "subgenus" I, *S. salamae* for "subgenus" II, *S. arizonae* for "subgenus" III, and *S. houtenae* for "subgenus" IV [75]. Serovars of "*S. Kauffmannii*" would be designated by their species names followed by that of their serovar (e.g. "*S. kauffmannii"* serovar *typhi*), and serovars of the other three species would be designated by their species names followed by their antigenic formulae [70]. In 1973, on the basis of DNA-DNA hybridisation experiments, it was demonstrated that all *Salmonella* strains should belong to a single species [76]. In 1982, on the basis of numerical taxonomy and DNA relatedness studies, the name "*Salmonella choleraesuis*" was proposed for the single *Salmonella* 15 species, and six subspecies were defined [77]. These authors also proposed that the name of serovars should be used without italicisation or underlining (e.g. *Salmonella choleraesuis* subsp. *choleraesuis ser. typhimurium*). In 1989, a single exception was described: one of the subspecies, *Salmonella choleraesuis* subsp. *bongori*, was separated from the other subspecies as a unique *Salmonella* species due to differences demonstrated by DNA relatedness studies [78]. Because of confusion caused by using "choleraesuis" as a name for both a species and a serovars, in 1986 "*Salmonella enterica*" was proposed again as the type species of *Salmonella* by the subcommittee of *Enterobacteriaceae* of the International Committee on Systematic Bacteriology at the XIV International Congress of Microbiology [79]. The proposal was formally made to the Judicial Commission of the International Committee of Systematic Bacteriology by [77] of the WHO collaborating centre. A study further explained that the epithet "entrerica" was recommended because it has not been used previously for a serovar. They also proposed that the seven subgenera of *Salmonella* be referred to as subspecies (subspecies I, II, IIIa, IV, V, and VI) [70]. Subgenus III was divided into IIIa and IIIb by DNA similarity and phenotypic characteristics. The suggestion was accepted by the Centres for Disease Control and Prevention (CDC) and other experts and laboratories, but denied by the Judicial Commission due to concerns that the status of *Salmonella* serovar *Typhi* might be overlooked [80], [81]. *S. choleraesuis* was thus returned as the legitimated type species pending an amended request for an opinion [82]. To comply with this ruling and also to support the proposal by [77], [83] made an amended request to use "*Salmonella enterica*" as the type species of *Salmonella* and reserve the name "*Salmonella typhi*" to reflect its clinical importance. In 2002, the Judicial Commission carefully discussed the request by [83] and issued an opinion (the Judicial Opinion 80) which finally approved that from January 2005, "*Salmonella enterica*" would replace "*Salmonella choleraesuis*" to become the type species of the genus *Salmonella* [77]. Furthermore, an accompanying commentary was written to help the bacteriologists better interpret both the nomenclatural and taxonomic consequences of Opinion 80 [84]. According to the ruling of the

Judicial Commission, the genus *Salmonella* consists of two species, "*Salmonella bongori*"and "*Salmonella enterica*". The latter includes six subspecies: "*arizonae*", "*diarizonae*", "*enterica*", "*houtenae*", "*indica*", and "*salamae*" [85]. In 2005, a new species, "*Salmonella sub-terranea,*" was approved by the Judicial Commission [86]. Names of some medically important *Salmonella* serovars, such as "*Salmonella typhi*", "*Salmonella typhimurium*" and "*Salmonella enteritidis*", have been used frequently, and in 2000, it was proposed that these names be conserved [83]. However, this proposal was not granted by the Judicial Commission. Furthermore, the proposal to raise "*Salmonella choleresuis* subsp. *choleraesuis* serovar *Paratyphi A*" to a new species, "*Salmonella paratyphi*", was not granted by the Judicial Commission [87]. The WHO collaborating Centre for Reference and Research on Salmonella at the Pasteur Institute, Paris, France, is responsible for updating the scheme [85]. Every year, newly recognised serovars are reported in the journal Research in Microbiology. In the latest report published in 2004, there were a total of 2,541 serovars in the genus *Salmonella* [88].

1.3.6 Classification and Nomenclature

Historically, *Salmonella* had been named based on the original places of isolation, such as *Salmonella London* and *Salmonella Indiana*. The nomenclature system given below was replaced by the classification based on the susceptibility of isolates to different selected bacteriophages, which is also known as phage typing. Phage typing is generally employed when the origin and characteristics of an outbreak must be determined by differentiating the isolates of the same serotype. It is very reproducible when international standard sets of typing phages are used. More than 200 definitive phage types (DT) have been reported so far. For example, *S. typhimurium* DT104 designates a particular phage type for *Typhimurium* isolates [62], [89], [90]. Epidemiologic classification of *Salmonella* is based on the host preferences. The first group includes host-restricted serotypes that infect only humans such as *S. typhi*. The second group includes host-adapted serotypes, which are associated with one host species but can cause disease in other host serotypes, such as *S. pullorum* in avian. The third group includes the remaining serotypes. Typically, *Salmonella enteritidis*, *Salmonella typhimurium* and *Salmonella heidelberg* are the three most frequent serotypes recovered from humans each year [62], [91]. The genus consists of two species: the first is *S. enterica*, which is divided into six subspecies (**Figure 1.1**): *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *indica*; and the second is *S.bongori* (formerly called *S. enterica* subsp. *bongori*) [92]. Under the modern nomenclature system, the subspecies information is often omitted, and

culture is called *S. enterica* serotype *typhimurium*, and in subsequent appearances, it is written as *S. typhimurium*. This system of nomenclature is used nowadays to bring uniformity in reporting [65], [90]. Kauffmann-White scheme classifies *Salmonella* according to three major antigenic determinants composed of flagellar H antigens, somatic O antigens and virulence (VI) capsular K antigens. This was adopted by the International Association of Microbiologists in1934. Agglutination by antibodies specific for the various O antigens is employed to group *Salmonellae* into the 6 serogroups: A, B, C1, C2, D and E. For instance, *S. paratyphi* A, B, C and *S. typhi* express O antigens of serogroups A, B, C1 and D, respectively. Therefore, further classification of serotypes is based on the antigenicity of the flagellar H antigens, which are highly specific for *Salmonella* [65] (**Figure 1.1**). In brief, O antigens are lipopolysaccharide (LPS) of the outer bacterial membrane. They are heat stable, resistant to alcohol and dilute acids. H antigens are heat-labile proteins associated with the peritrichous flagella and can be expressed in one of two phases. K antigens, which are heat-sensitive carbohydrates, are produced by *Salmonella* serovars that express a surface-bound polysaccharide capsular antigen [65], [67].

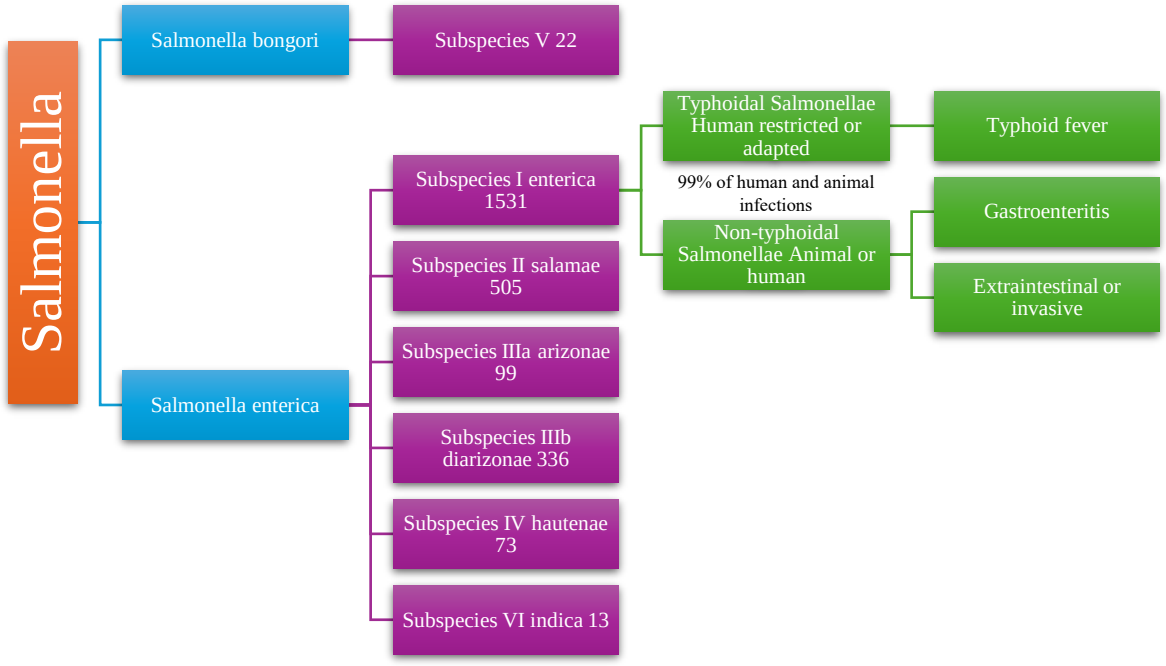

Figure 1.1: Classification of *Salmonella* and disease caused by *Salmonella* [56]**.**

The scientific classification of Salmonella was described by [93] as follows:

Domain: Bacteria

Kingdom: Monera

Phylum: Proteobacteria

Class: Gamma Protobacteria

Order: Enterobacteriales

Family: Enterobacteriaceae

Genus: *Salmonella*

1.3.7 General Characteristics of *Salmonellae*

Salmonellae are Gram-negative, short and plump-shaped rods, non-capsulated, non-spore-forming, facultative anaerobes and aerobic bacteria of the family Enterobacteriaceae [94] (**Figure 1.2 and 1.3**). *Salmonella* classification has changed several times, yet still, it's not stable. The genus *Salmonella* was formerly divided into two main species: *Salmonella bongori* and *Salmonella enterica*. Nonetheless, a new species, *Salmonella subterranea,* was discovered and validated [86]. Among these species, *Salmonella enterica* is further classified into six subspecies: *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), and *S. enterica* subsp. *indica* (VI). Previously, *S. bongori* was considered subspecies V, but later was recognized as a separate species. Fermentation of certain materials, such as malonate, dulcitol, sorbitol, d-tartrate, mucate, galacturonate, salicine, ONPG, and lactose, as well as the production of certain enzymes, such as β-glutamyl-transferase, 3-gelatinase, or β-glucuronidase, allows a distinction among the various species and subspecies [77]. Again, the genus comprises over 2500 serovars, classified into three discrete types of surface antigens: somatic (O), flagellar (H), and capsular (VI). Approximately 99% of these serovars belong to *S. enterica,* and almost 60% of them are in *S. enterica* subsp. *enterica*. The average DNA sequence similarity between *Salmonella* serotypes is 96-99% [95].

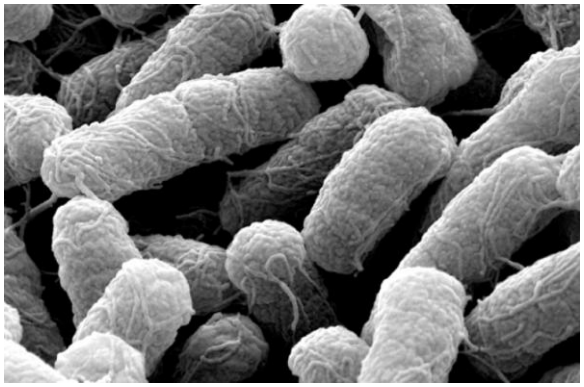

Figure 1.2: *Salmonella* spp. [96].

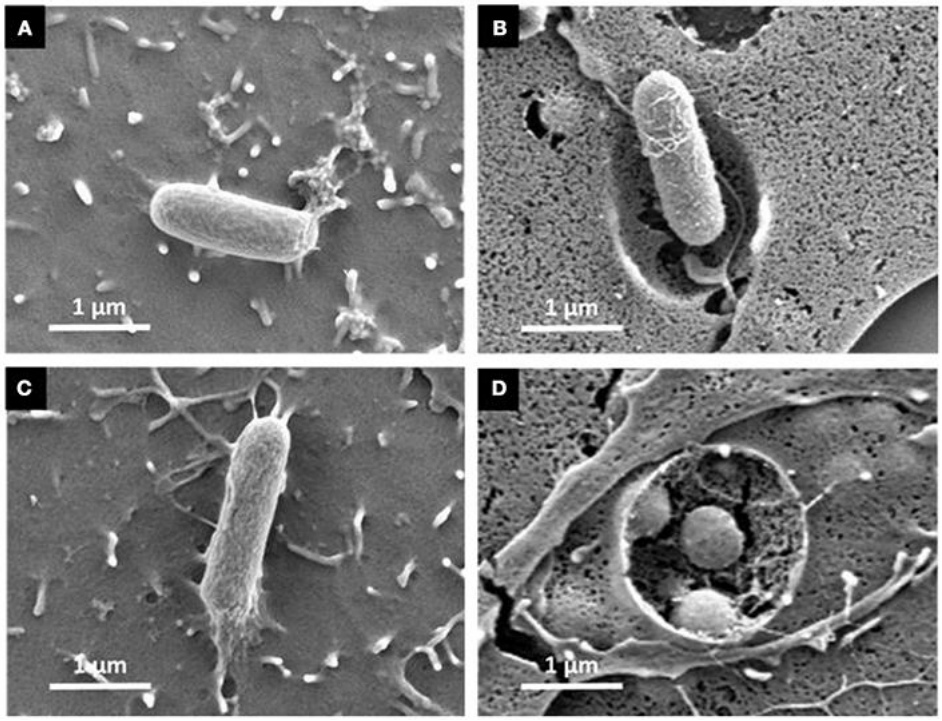

Figure 1.3: Electron micrograph of *Salmonella* [97].

1.3.8 Reservoir and Transmission

Salmonella contamination in fish and fish products occurs throughout the farm-to-fork process, primarily from environmental sources and exacerbated by poor hygiene during processing [98]. The initial contamination of the aquaculture environment (water and sediment) often comes from contaminated source water or agricultural runoff containing animal waste (including feces from poultry fed with contaminated feed, and wild birds/pests) [99]. Live fish, being transient carriers, acquire the bacteria by ingesting contaminated water or feed, leading to colonization, mainly in the gastrointestinal tract [53], [100]. This contamination is then carried into the processing plant during harvesting [101]. Crucially, the risk escalates post-harvest through secondary contamination events like cross-contamination between raw and ready-to-eat products, contact with contaminated equipment or ice, or transfer from infected food handlers [102]. The final risk to human health, resulting in salmonellosis, occurs when the consumer prepares the product and either practices insufficient cooking (allowing bacteria to survive) or causes cross-contamination in the kitchen to other foods [103] (**Figure 1.4**).

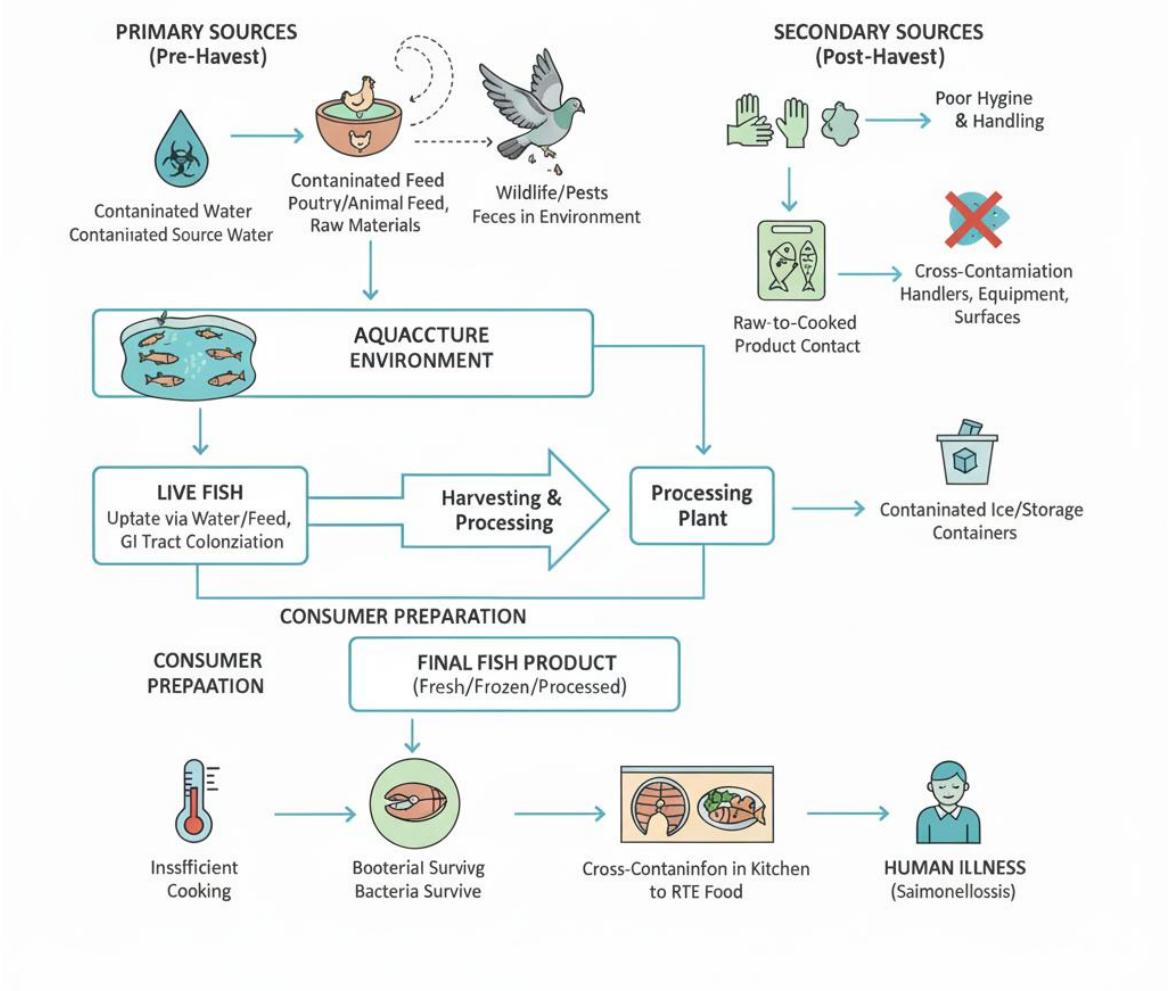

Figure 1.4: Farm-to-fork transmission route of *Salmonella* to fish and fish products.

1.3.9 Virulence Factors

The outcome of a Salmonella infection is determined by the status of the host and the status of the bacterium. The status of the bacterium is determined by the so-called virulence factors, which are described as follows [104].

1.3.9.1 *Salmonella* Pathogenicity Islands (SPIs)

The majority of virulence genes of *Salmonella* are clustered in regions distributed over the chromosome called *Salmonella* pathogenicity islands [105].The SPIs are of major importance for the virulence of *S. enterica*. Hallmarks of *Salmonella* virulence, such as cell invasion, intracellular survival, and the production of Vi antigen capsule, are encoded by SPIs. Until recently, more than 10 SPIs have been identified on the *Salmonella* chromosome, but SPI-1 and SPI-2 are central for the pathogenesis of *Salmonella* infections [106]. All types of *S. enterica* have two large clusters of genes known as *Salmonella* Pathogenicity Island one and two. *Salmonella* Pathogenicity Island one encodes genes necessary for invasion of intestinal

epithelial cells and induction of intestinal secretory and inflammatory response [107]. *Salmonella* lacking a functional SPI-1 Type three secretion system are unable to invade epithelial cells and induce cytokine synthesis [108]. *Salmonella* Pathogenicity Island 2 encodes genes essential for intracellular replication and necessary for the establishment of systemic infection beyond the intestinal epithelium [109]. The function of the SPI-2 encoded Type III secretion system is required to protect the pathogens within the *Salmonella*-containing vacuole (SCV) against the effector functions of innate immunity. It has been reported that SPI-2 prevents the localisation of the phagocyte oxidase [110] and the inducible nitric oxide synthases to the SCV [111].

1.3.9.2 Pathogenesis

Salmonellosis in the human host is generally associated with *Salmonella enterica* subspecies *enterica*, and acute infections can present in one of four ways: enteric fever, gastro-enteritis, bacteremia, and extra-intestinal (EI) focal infection. As with other infectious diseases, the course and outcome of the infection are dependent upon a variety of factors, including inoculating dose, immune status of the host, and genetic background of both host and infecting organism [112]. Broadly speaking the *Salmonella enterica* from human infections can be subdivided into two groups: the enteric fever (typhoidal) group and non-typhoidal *Salmonella* (NTS), which typically cause gastroenteritis but can cause invasive disease under certain conditions [113]. All *Salmonella* infections begin with the ingestion of organisms in contaminated food or water [114], [115]. The infectious dose of *Salmonella* varies from 103 to 106 colony-forming units. This variability probably reflects the ability of *Salmonellae* to resist the low pH of the stomach, a powerful component of host defense [115]. After leaving the stomach, *Salmonella* must traverse the mucosal layer overlaying the epithelium of the small intestine. After crossing the mucosal layer overlaying the intestinal epithelium, *Salmonella* interacts with both enterocytes and Microfold cells (M-cells) [114]. The organisms are rapidly internalized and transported into submucosal lymphoid tissue, where they may enter the systemic circulation. *Salmonella* also has the ability to induce non-phagocytic epithelial cells by a process known as bacterial-mediated endocytosis. This process involves the formation of large membrane ruffles around the organism and cytoskeleton rearrangement [114]. *Salmonella* is then internalized within bound vacuoles through which organisms" transcytose from the apical to the basolateral surface [116]. Once it crosses the intestinal epithelium, *Salmonella* serotypes that cause systemic infections enter macrophages, and migration of infected macrophages to other organs reticulo-endothelial systems, probably facilitates the

dissemination of bacteria in the host [112] (**Figure 1.5**). Gastroenteritis due to NTS may persist with fever, nausea, vomiting, abdominal pain, and symptoms may continue for over a week. In contrast, the early symptoms of enteric fever are often vague and may include a dry cough, severe headache, anorexia, fever, and a tendency to constipation rather than diarrhoea [65]. If enteric fever is not treated on time, serious complications like haemorrhage from ulcers can occur during the third week of illness, or perforation of the Peyer's patches (PP) can cause generalised peritonitis and septicemia; these are the most typical causes of death in typhoid fever.

1.3.10 Use of Antibiotics in Fish

Antibiotics are used widely and in vast quantities to ensure the health and promote the growth of livestock, poultry, and fish reared for food production. The fact that greater quantities are used in healthy animals than in unhealthy humans is a cause for serious concern, particularly as some of the same antibiotics are involved, and food animals have been shown to carry resistant human pathogens. Some countries have banned the use of antibiotics as growth promoters, but the practice remains widespread. Legislation and regulation with enforcement are needed to control the use of antibiotics for these purposes in many countries [117].

Approximately 50% of all antibacterial agents used annually in the EU are given to animals [118]. Amoxycillin, Ciprofloxacin, Tetracycline, Nitrofurantoin, Sulfamethaxol, and various other antibiotics were used in fish in Bangladesh [26]. Aminoglycosides, fluoroquinolones, Ionophores, Penicillins, Sulfonamides, Tetracyclines, and other groups of antibiotics were used in livestock production. These antibiotics are not only used in veterinary indications for therapy and prevention of bacterial infections, but may also be added continuously to animal feeds to promote growth, increase feed efficacy, and decrease waste production. The antibiotics used for this purpose are commonly called feed savers, antimicrobial growth promoters, or performance enhancers (APE).

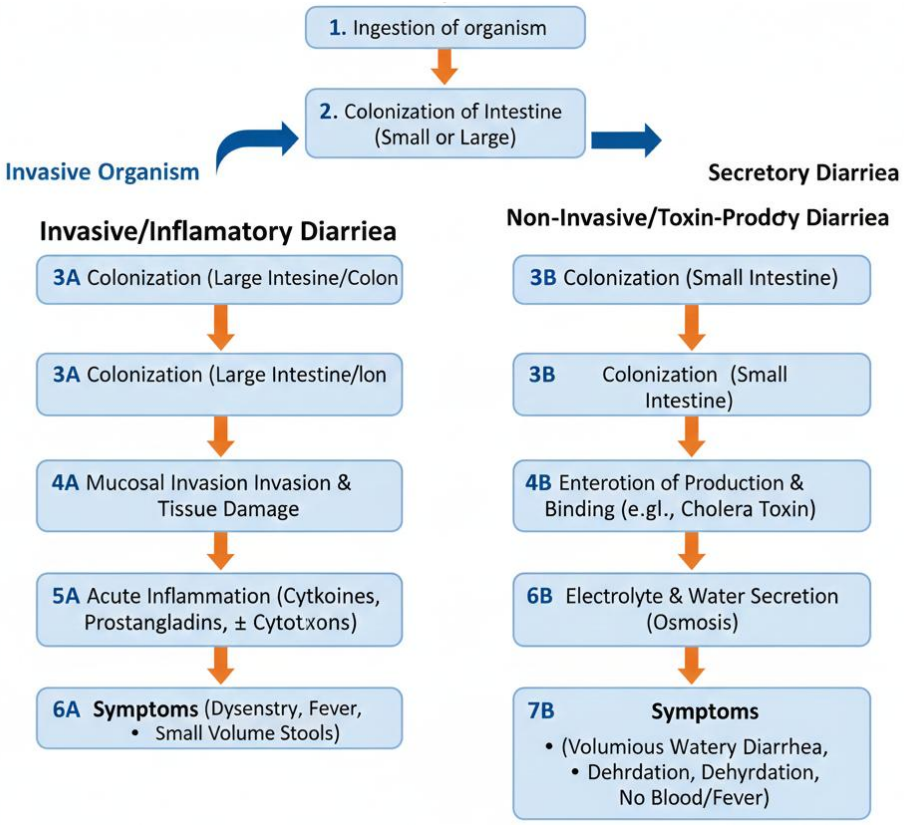

Figure 1.5: Scheme of the pathogenesis of *Salmonella* [119].

1.3.10.1 Antibiotic Resistance

The resistance of *Salmonella* to a single antibiotic was first reported in the early 1960s [120]. Since then, the isolation frequency of *Salmonella* strains resistant to one or more antibiotics has increased in Saudi Arabia, the United States, the United Kingdom, and other countries of the world. This is due to the increased and uncontrolled use, as well as easy accessibility to antibiotics in many countries of the world [121], [122]. Emerging resistance in *Salmonella typhi* has been described especially in Africa and Asia, and the appearance of Salmonella typhimurium DT104 in the late 1980s raised the main public health concern, thereby threatening the lives of infected individuals [120], [121]. [123] According to an analysis, multi-resistance occurred in *Salmonella* serotypes including Albany, Anatum, Havana, London, and Typhimurium [123]. The resistance towards the traditional first-line antibiotics such as ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole defines multidrug resistance (MDR) in *Salmonella enterica* [124]. This is of great concern because the majority of infections with MDR *Salmonella* are acquired through the consumption of contaminated foods of animal origin, such as swine and chicken eggs. [125] mentioned that cephalosporin- and fluoroquinone-resistant strains of *S. choleraesuis* have been identified in swines in Taiwan and Thailand. Apart from that, antibiogram testing by [126] revealed *Salmonella* isolates from

chicken eggs in the marketing channel and poultry farms in North India were resistant to bacitracin, colistin, and polymyxin-B. Due to the use of antibiotics for the promotion of growth and prevention of disease in food animals, there is an increase in human salmonellosis cases caused by foodborne MDR *Salmonella* nowadays [127]. This indiscriminate and injudicious use of antibiotics in any setting, especially in food animals worldwide, should be monitored to reduce the transfer risk of MDR *Salmonella* to humans [128]. Finally, there is a need for continuous surveillance and sharing of antimicrobial susceptibility data for *Salmonella* among countries worldwide [129] to ensure the effectiveness of control programs.

1.3.10.2 Molecular Mechanisms of Antibiotic Resistance and Resistance Genes

Bacteria may manifest resistance to antibacterial drugs through a variety of mechanisms. Some species of bacteria are innately resistant to ≥I class of antimicrobial agents. Of greater concern are cases of acquired resistance, where initially susceptible populations of bacteria become resistant to an antibacterial agent and proliferate and spread under the selective pressure of use of that agent [130]. Several mechanisms (**Figure 1.6**) of antimicrobial resistance are readily spread to a variety of bacterial genera [130].

1. At first, the organism may acquire genes encoding enzymes, such as β-lactamases, that destroy the antibacterial agent before it can have an effect.
2. Next, mutation and modification in the target of the antimicrobial agent, which reduces the binding of the antimicrobial agent.
3. Then, bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell before it can reach its target site and exert its effect.
4. Following that, bacteria may acquire several genes for a metabolic pathway which ultimately produces altered bacterial cell walls that no longer contain the binding site of the antimicrobial agent, or bacteria may acquire mutations that limit access of antimicrobial agents to the intracellular target site via down-regulation of porin genes.
5. The last event may occur through one of several genetic mechanisms, including transformation, conjugation, or transduction. Through genetic exchange mechanisms, many bacteria have become resistant to multiple classes of antibacterial agents, and these bacteria with multidrug resistance (defined as resistance to ≥3 antibacterial drug classes) have become a cause for serious concern.

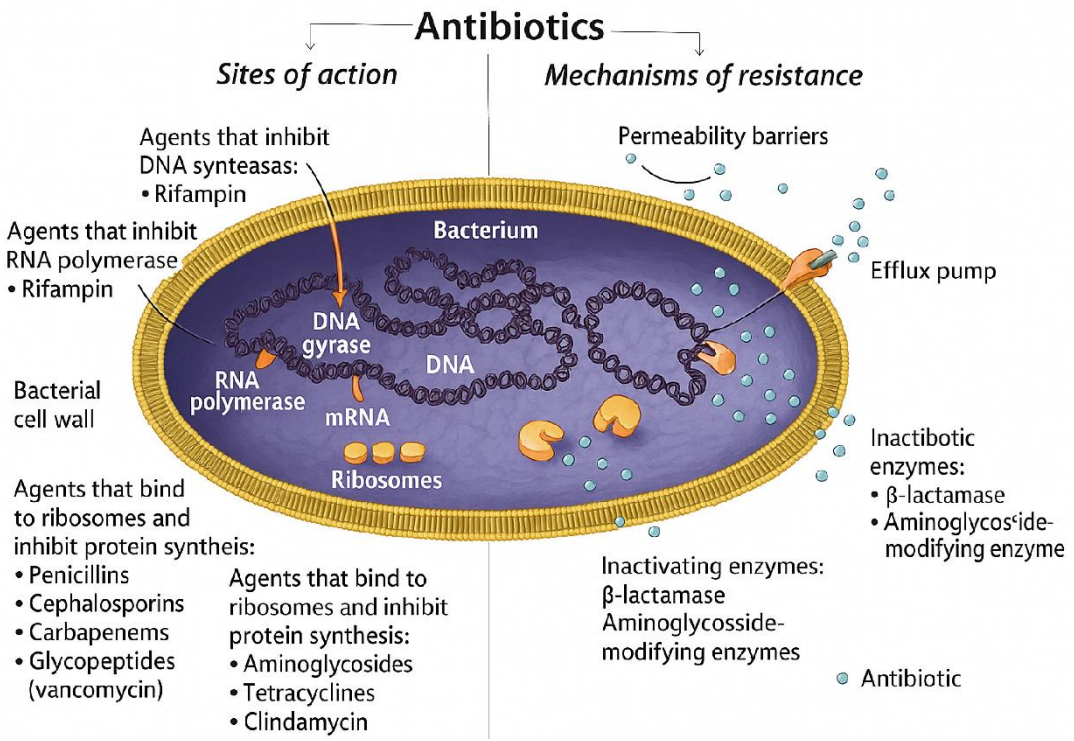

Figure 1.6: Sites of action and potential mechanisms of bacterial resistance to antimicrobial agents [131].

Chapter 2

Materials and Methods

2. Materials and Methods

The study was aimed to identify the burden of *Salmonella* among fish samples from retail markets in Noakhali, Bangladesh. To fulfil the objectives, the study was designed to isolate and identify *Salmonella* isolates phenotypically and genotypically from different fish samples. The materials methods described in the chapter refer to procedures and operations that are of relevance to the study as a whole. All media compositions, chemicals, reagents, and apparatus used in this study are listed in Appendices I, II, and III, respectively. **Figure 2.1** illustrates the whole work plan of the presented investigation.

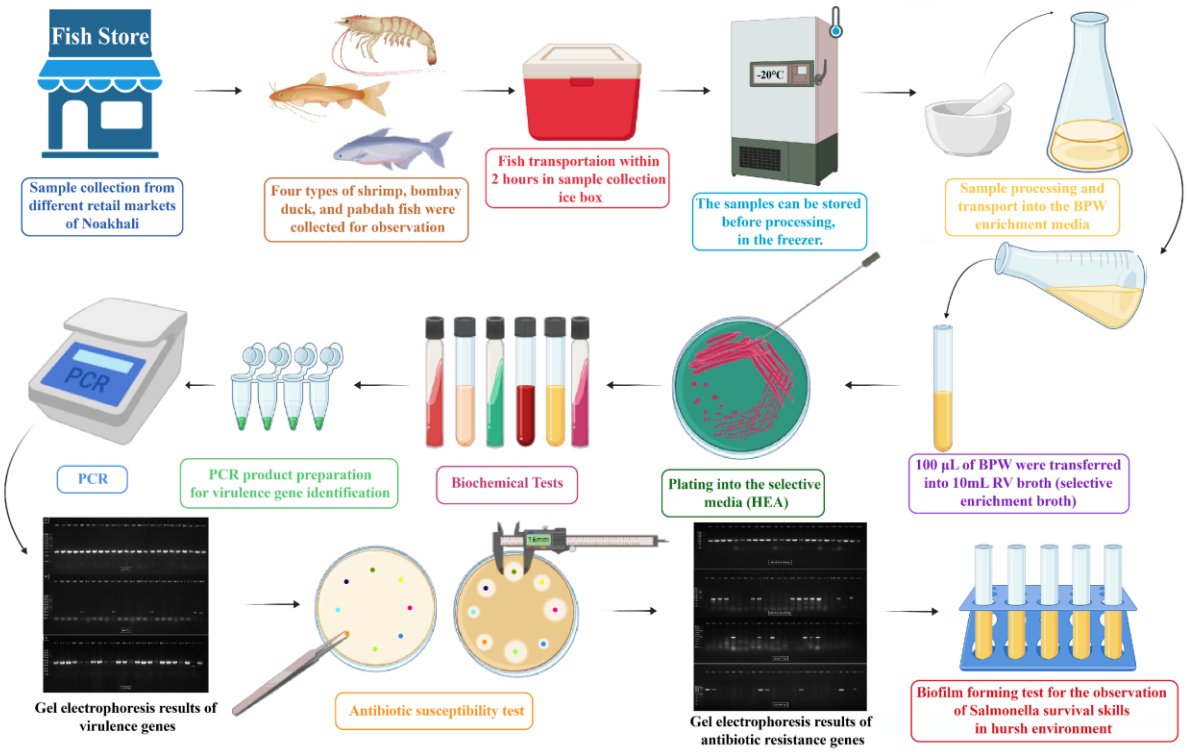

Figure 2.1: Sequential analysis of fish samples for phenotyping and genotyping of *Salmonella* spp.

2.1 Collection of Fish Samples

2.1.1 Sample Collection Site

A total of 100 fish samples were collected from different retail markets (**Table 2.1**) in the Noakhali district of Bangladesh from 26th February 2025 to 14th August 2025 (**Figure 2.2**). Samples were collected randomly.

Table 2.1: List of Sample Collection Sites

Serial No.	Retail Markets
1	Sonapur Bazar
2	Kalitara Bazar
3	Dotter Haat
4	Pouro Bazar
5	Maijdee Bazar
6	Chowmuhuni Bazar

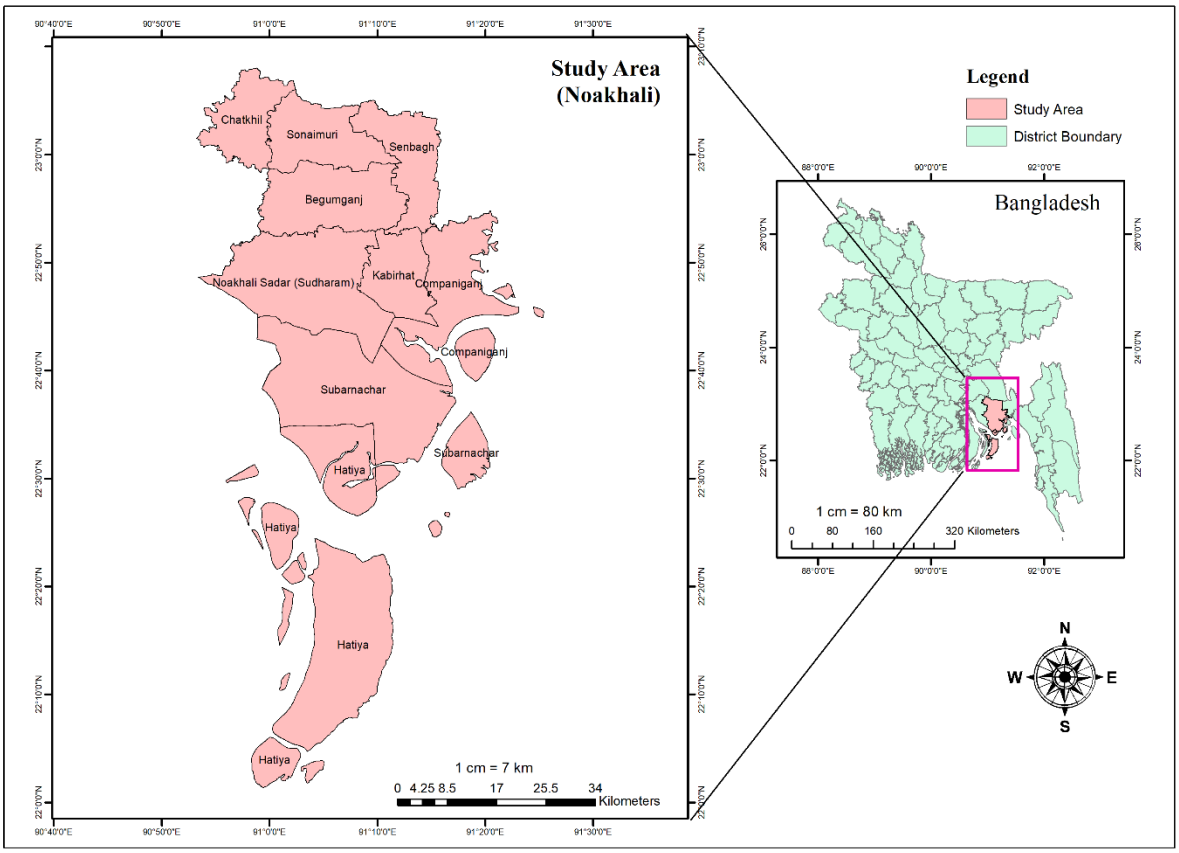

Figure 2.2: Location of sample collection area for the identification of *Salmonella* from different retail markets of Noakhali, Bangladesh.

2.1.2. Transportation of the Samples

Each of the samples was brought using sterile plastic zipper bags to avoid cross-contamination, within a cooler box, as shown in **Figure 2.3**. Transportation was completed immediately (within approximately 1 hour) to the Department of Microbiology laboratory at Noakhali

Science and Technology University, Sonapur, Noakhali, for microbiological analysis. After reaching the lab, the samples were kept at −20°C until analysis. Frozen shrimp were thawed within 12 h at 2–5°C.

Figure 2.3 (A) Sample collection, and (B) transportation in the thermal ice box.

2.1.3 Types of Samples

A total of 6 types of fish samples were collected, such as *P. monodon* (n=15), *M. rosenbergii* (n=15), *P. merguiensis* (n=10), *P. indicus* (n=10), *H. nehereus* (n=25), and *O. pabda* (n=25) (**Figure 2.4**).

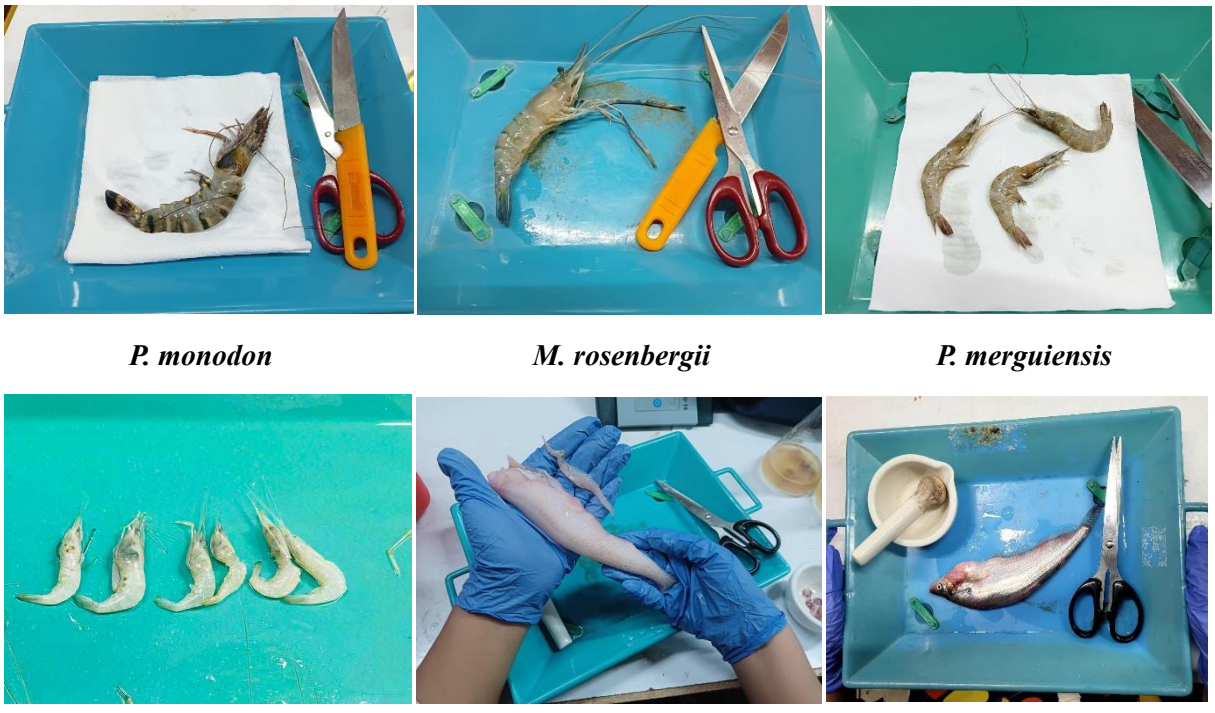

Figure 2.4: Different samples collected from retail markets.

2.2 Processing of Fish Samples

At first, the external surfaces of the samples were cleaned with 70% (v/v) alcohol. For shrimp samples, each 10 g sample (brain, leg, muscle, and intestine) was crushed into small pieces in a sterile mortar, then added to 90 mL of BPW and incubated overnight at 37◦C (**Figure 2.5**). For Bombay duck and pabda catfish, the intestines, gills, and muscles were collected for processing. After incubation, 0.1 mL of the mixture was added to 10 mL of RV medium for the specific enrichment of *Salmonella*. RV medium was incubated in a shaking water bath at 120 rpm at 42°C for 24 h [132].

Figure 2.5: (A) Sample processing, **(B)** Blending, and **(C)** Enrichment of the sample.

2.3 Isolation and Identification of Bacterial Isolates

2.3.1 Inoculation and Incubation of Selective Media

A 3 mm loopful of RV broth was streaked on premade HEA and SS agar plates for culturing isolates. The enriched RV medium was vortexed before inoculating the loop into the broth. Incubation of the HEA and SS petri dishes was done for 24 h at 35°C. Based on their morphology (size, shape, and color), bacterial colonies were identified as *Salmonella* after incubation. For further purification, a single, distinct colony, which was either blue-green or translucent green, with or without a black center, on an HEA plate, was subsequently streaked onto another fresh HEA plate and is believed to be a colony of *Salmonella* spp. On the other hand, a black-centered colony on an SS agar plate was identified as *Salmonella*. These colonies were further streaked on HEA plates for pure culture.

2.3.2 Microscopic Analysis

Microscopic studies, such as size, shape, and colour, were performed using the Gram staining procedure.

2.3.2.1 Gram Staining

Presumptive isolates were Gram-stained as follows: a smear of an 18–24-hour colony of the presumptive isolate was made on a grease-free glass slide and heat-fixed over the Bunsen flame. It was then covered with crystal violet for about 1 minute and washed with water. The smear was there after covered with Gram's iodine for about 1 minute and then washed with water. Decolonization of the smear was carried out by adding acetone and gently agitating for 10-30 seconds. It was then washed with water, neutralized with safranin for 10-30 seconds, rewashed with water, and allowed to dry. The Gram-stained slides were then viewed under a microscope using the oil immersion objective (100X).

2.3.3 Biochemical Identification of the Isolates

Some biochemical tests were performed according to the methods as described in "Manual of Methods for General Bacteriology [133]". Following biochemical tests: Triple Sugar Iron (TSI), Motility Indole Urease (MIU), Simmon's Citrate Agar (SCA), Oxidase, Catalase, MR-VP, Lysine Iron Agar (LIA) tests were performed.

2.3.3.1 Triple Sugar Iron (TSI) Test

The TSI agar test determines the ability of a microorganism to ferment sugars and produce hydrogen sulfide. TSI agar medium contains lactose and sucrose in 1% concentrations and glucose in 0.1% concentration. To facilitate observation of carbohydrate utilization patterns, TSI agar medium is made with a slant and a butt. TSI agar medium also contains sodium thiosulfate and ferrous sulfate for the detection of hydrogen sulfide production, which is indicated by blackening of the medium.

Procedure

- With a sterilized straight inoculation needle, first touch the top of a well-isolated colony, and then inoculate TSI Agar by stabbing through the center of the medium to the bottom of the tube, and then streak on the surface of the agar slant.
- The cap was left on loosely, and the tube was incubated at 35°C in ambient air for 18 to 24 hours.

If lactose (or sucrose) is fermented, a large amount of acid is produced, which turns the phenol red indicator yellow both in the butt and in the slant. Some organisms generate gases, which create bubbles/cracks in the medium. On the other hand, if lactose is not fermented, but the small amount of glucose is oxygen-deficient, the butt will be yellow. Still, on the slant, the acid (less acid as the media in the slant is minimal) will be oxidized to carbon dioxide and water by

the organism, and the slant will be red (alkaline or neutral pH). Conversely, if neither lactose/sucrose nor glucose is fermented, both the butt and the slant will be red. The slant can become a deeper red-purple (more alkaline) due to the production of ammonia from the oxidative deamination of amino acids. The H_2S production is determined by the black colour of ferrous sulfide [134].

2.3.3.2 Motility Test

Some bacteria are motile because of the presence of flagella. To observe the presence or absence of flagella, a motility test is done [135].

Procedure

- For this test, semisolid agar medium (SIM) containing tubes were inoculated by selected isolates.
- The tubes were incubated at 37°C for 2-3 days
- After incubation, the tubes were compared with an uninoculated tube.
- The bacteria were considered positive for motility if there was turbid growth spreading from the stab line in the media.

2.3.3.3 Indole Test

The presence of tryptophanase allows an organism to separate indole from the amino acid tryptophan. The enzyme tryptophanase is necessary for the deamination and hydrolysis of the amino acid tryptophan [136].

- The test organisms were cultured in test tubes having 3 ml of peptone water containing tryptophan at 37°C for 48 hours.
- Then 0.5 ml of Kovac's reagent was gently run down the side of the test tube so that it forms a ring between the medium and the ether layer, and was observed for the development of color of the ring.

Positive: Development of a brilliant red colored ring indicated indole production.

Negative: If the red-colored ring is not formed, then the test is indole negative.

2.3.3.4 Urease Test

Urease activity (urease test) is detected by growing bacteria in a medium containing urea and using a pH indicator such as phenol red. When urea is hydrolyzed, ammonia accumulates in the medium and makes it alkaline [137].

$$(NH_2)_2CO + 2H_2O \rightarrow 2NH_3 + CO_2 + H_2O$$

Procedure

- 24-hour pure cultures were inoculated in urease broth for 48 hours, maintained at a temperature of 37°C, and then the colour was observed.

Positive: The colour changes from light yellow to pink, indicating the test is positive.

Negative: No colour change is considered a negative result.

2.3.3.5 Simmons Citrate Test

The citrate test determines the ability of microorganisms to utilize citrate as the sole source of carbon and energy. Simmons citrate agar medium used for this test contains sodium citrate as the carbon source, inorganic ammonium salts ($NH_4H_2PO_4$) as a nitrogen source, and the pH indicator bromophenol blue. When microorganisms utilize citrate, the ammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH turns the indicator bromophenol blue from green to blue above pH 7.6. No colour change indicates a negative result [138].

2.3.3.6 Oxidase Test

The oxidase test is an essential differential procedure that should be performed on all gram-negative bacteria that are to be identified. The test reagent, N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride, acts as an artificial electron acceptor for the enzyme oxidase. The oxidase test can identify organisms that produce the enzyme cytochrome oxidase, and it is beneficial for categorizing organisms into groups during the initial stages of identification. Cytochrome oxidase is a participant in the chain of electron transport. It transfers electrons from a donor molecule to oxygen [139]. This test was done to determine the ability of bacteria to produce cytochrome oxidase. To determine this,

- Some area of a Whatman filter paper was soaked with the Oxidase reagent.
- A loopful of 24 hours pure culture bacteria was streaked on it using a sterile glass needle.

Positive: The appearance of purple color over the bacteria is a positive result.

Negative: Delayed reactions are negative.

2.3.3.7 Catalase Test

The purpose of the test was to find out the microbes that could convert hydrogen peroxide (H_2O_2) into oxygen and water.

$$2H_2O_2 + \text{Catalase} \rightarrow 2H_2O + O_2$$

The quick development of bubbles indicates this response. Using a wooden applicator stick or sterile inoculating loop, a tiny organism from a well-isolated 18–24-hour colony was deposited onto a microscopic slide inside a petri dish for this test. One or two drops of 3% H_2O_2 were applied to the organism on the microscopic slide using a dropper or Pasteur pipette [140].

Positive: Production of bubbles (oxygen) within 5-10 seconds of the addition of reagents indicates positive for catalase activity.
Negative: If rising bubbles are not observed, then the result is negative.

2.3.3.8 Methyl Red (MR) Test

The methyl red test is used to detect whether the microbe performs a specific type of fermentation called mixed acid fermentation when supplied with glucose. Some bacteria ferment glucose and produce large quantities of acidic end products that lower the pH of the medium below 5.0. The addition of pH indicator methyl red is used to detect this acidity (methyl red is red at pH 4.4 or below and yellow at pH 6.2 or above). If red color develops within a few seconds, the bacterium is MR-positive. When the medium remains yellow, it indicates the bacterium is MR-negative.

Procedure:

- 5 ml of MR-VP broth was added to all test tubes (2 test tubes/isolate).
- All the test tubes were autoclaved and allowed to cool.
- All the bacteria to be tested were inoculated into half of the test tubes, each containing 5ml MR-VP broth, and incubated at 37°C for 24 hours.
- After the incubation period, five drops of the Methyl red reagent were added to the test tubes (labeled as MR test) to check the medium pH, and the results were recorded.

2.3.3.9 Voges Proskauer (VP) Test

The Voges-Proskauer test is used to determine if an organism produces acetylmethylcarbinol from glucose fermentation. If present, acetylmethylcarbinol is converted to diacetyl in the presence of a-naphthol, strong alkali (40% KOH), and atmospheric oxygen. The diacetyl and quinidine-containing compounds found in the peptones of the broth (MR-VP broth) then condense to form a pinkish-red polymer. Appearance of a pink-red color at the surface of the broth is indicated as a positive result, and no pink-red color appearance suggests a negative result.

Procedure:

- All the bacteria to be tested were inoculated into 5 ml MR-VP broth and incubated at 37°C for 24 hours.
- After the incubation period ended, six drops of 5% a-naphthol were added to each test tube, and the tubes were shaken. Immediately, two drops of 40% KOH were added, and the test tubes were shaken again.
- After 15 minutes, the cultures' colours were examined, and the results were recorded.

2.3.3.10 Lysine Iron Agar (LIA) Test

The LIA test is used to differentiate gram-negative bacilli based on their ability to decarboxylate or deaminate lysine and to produce hydrogen sulfide (H_2S). In the test, organisms are inoculated by stabbing the butt and streaking the slant. If the organism produces lysine decarboxylase, the butt turns alkaline purple after initially becoming acidic yellow due to glucose fermentation, indicating lysine decarboxylation. If the organism deaminates lysine, the slant turns red due to a reaction involving ferric ammonium citrate. Hydrogen sulfide production is indicated by black precipitate in the medium.

Thus, LIA allows detection of lysine decarboxylation (purple butt and slant), lysine deamination (red slant), and H_2S production (black precipitate), which helps differentiate enteric bacteria such as Salmonella and Shigella based on these metabolic traits [141].

2.3.4 Preservation of the Isolates

Each isolate was streaked aseptically on Luria-Bertani (LB) medium plate and was incubated overnight at 37°C for single colony formation. After incubation, single colonies for each isolate were inoculated into LB broth and incubated for 24 hours at 37°C. After overnight incubation, 80 µL culture broth was suspended thoroughly with 20% glycerol in a sterile Eppendorf tube and kept at -20°C. Duplicate sets of Eppendorf tubes were prepared for each isolate and maintained at -80 °C.

2.4 Molecular Analysis of the Isolates

Today's technology allows us to identify any living thing to any level of precision, by using a single cell. This technology uses molecular probes that bind only to the DNA of targeted organisms, relies on gene amplification that multiplies the DNA of interest by a million-fold, and involves comparing specific pieces of DNA between organisms. Molecular identification

has two important advantages over conventional techniques of microscopic examination. Identification can be made using a very small amount of material, and is much more accurate than with previous methods - a species, a population, or even an individual can be identified. For these reasons, these are used in this study, which include the extraction and purification of DNA, DNA concentration measurement, Polymerase Chain Reaction (PCR), and assessment of the reproducibility of invA, hilA, and fimA primer specificity.

2.4.1 Extraction and Purification of DNA

The susceptible bacterial colonies were taken from pure culture stock, inoculated onto a nutrient agar (NA) plate, and incubated at 28°C for 24h. After incubation, a loopful of the isolate was inoculated on 1ml TSB and kept for overnight incubation at 37°C. Following that, the boiled DNA technique was utilised for the DNA extraction procedure [142]. The solution was centrifuged at 15,000 rpm for 5 minutes. Then the supernatant was discarded, and the sample was resuspended with 1mL sterilized distilled water and again centrifuged for 2 min at 15000 rpm. Next, the supernatant was again discarded, and 300 µL of PCR water was added to the pellet. After a thorough vortexing, the solution was incubated on the heat block machine at 100°C for 15 minutes. Then cold shock was performed at -20°C for 5 minutes. Next, the sample was again centrifuged at 15,000 rpm for 15 minutes. After the centrifugation, the supernatants were collected in separate sterile Eppendorf tubes and preserved in a refrigerator at -20°C [143]. This solution, containing the template DNA, was used in the preparation of PCR products for molecular identification.

2.4.2 DNA Concentration Measurement

After extraction, the DNA was quantified using Nano Drop Microvolume Spectrophotometers (Thermo Fisher, USA), ensuring accurate DNA concentration and purity measurement. The concentration of the DNA was obtained as ng/µL, and the purity was expressed in terms of the ratio of absorbance at 260 nm and 280 nm (OD 260/OD 280). According to a study, the pure DNA preparation has an OD 260/OD 280 value of 1.8 [144]. The DNA was stored at -20°C until further use.

2.4.3 Polymerase Chain Reaction (PCR)

Amplification of the desired gene in the selected isolates was carried out by polymerase chain reaction (PCR) for further analysis (**Figure 2.5**) [145].

2.4.3.1 Preparation of Reaction Mixture

The reaction mixture for PCR was prepared by mixing the specific volumes of the components in an appropriately sized tube in the order provided in **Table 2.2**. For a large number of reactions, a master mix without any template DNA was prepared and aliquoted into PCR tubes. At the end, a specific template was added to a properly labelled PCR tube. The PCR tube containing the reaction mixture and template DNA was capped properly and centrifuged briefly to spin down the contents. The PCR tubes were then placed in a thermal cycler (T100TM, Bio-Rad, USA), and the amplification parameters were set correctly (**Table 2.3** and **Figure 2.6**).

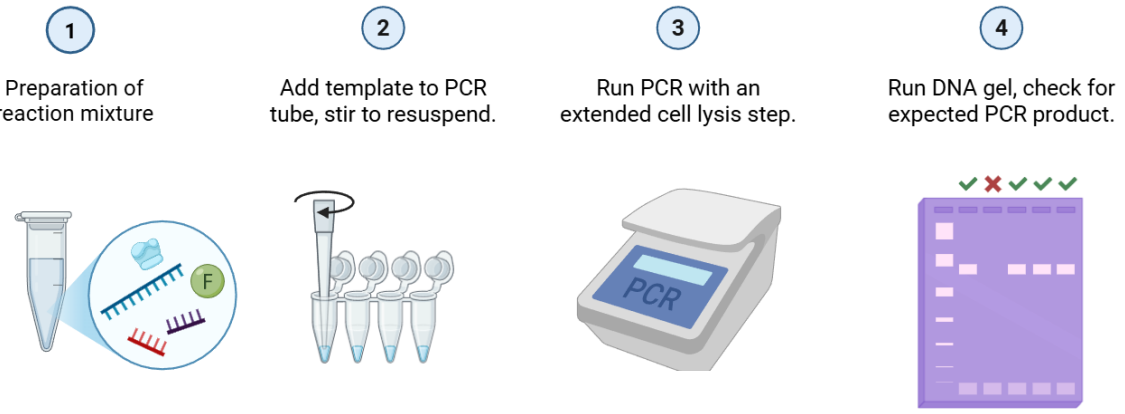

Figure 2.6: Schematic overview of the PCR workflow for template amplification (Created in https://BioRender.com).

Table 2.2: PCR reaction mixture preparation recipe.

Serial No.	Solution	Amount (µL)
1.	Mastermix	6.5
2.	Forward Primer	1
3.	Reverse Primer	1
4.	Nuclease Free Water	1.5
5.	Template DNA	3
Total	Reaction Volume	13

2.4.3.2 PCR Condition

The PCR protocol used to amplify virulence-associated genes (VAGs) followed a precise thermal cycling protocol. The PCR conditions at different stages are shown in **Table 2.3**.

Table 2.3: PCR conditions at different stages for invA, hilA, and fimA

Primer	Denaturation	Denaturation after the first cycle	Annealing	Extension	No. of cycles	Final extension
invA	94ºC (2 min)	94ºC (30sec)	59.8ºC (30sec)	72ºC (1min)	34 X	72ºC (5min)
fimA	94ºC (2 min)	94ºC (30sec)	51ºC (45sec)	72ºC (1min)	34 X	72ºC (7min)
hilA	94ºC (2 min)	94ºC (30sec)	51ºC (45sec)	72ºC (1min)	34 X	72ºC (7min)

After completion of the reaction, PCR tubes were stored at -20ºC until further analysis. The cycling profile for each primer target combination was optimized accordingly.

2.4.3.3 Primer Specificity

For the molecular identification of the isolate, three particular primers were selected [146], [147], [148]. **Table 2.4** lists the product size, forward and reverse primer sequence for each primer. Primers for the genes invA, hilA, and fimA were used in the individual reactions of the PCR. A final volume of 13 µL was used for PCR amplifications, which contained specific primer pairs, PCR mastermix, nuclease-free water, and template DNA.

Table 2.4: The list of common VAGs of *Salmonella* with their annealing temperature and product size.

Virulent-associated gene (VAG)	Primer sequence (5′–3′)	Annealing temp. (ºC)	Size (bp)
invA	F: GTGAAATTATCGCCACGTTCG	59.8	321
	R: TCATCGCACCGTCAAAGGAAC		
hilA	F: CGGAAGCTTATT TGCGCCATG	51	499
	R: GCATGGATCCCCGCCGGCGAG		
fimA	F: CCTTTCTCCATCGTCCTGAA	51	85
	R: TGGTGTTATCTGCCCGACCA		

2.4.4 Analysis of Amplicon by Agarose Gel Electrophoresis

A 1.5% gel was prepared by dissolving 0.6 g of agarose (CSL-AG100, Cleaver Scientific, USA) in 50 mL of 1X Tris-acetate EDTA (TAE) (Appendix II) buffer (T4415, Sigma, USA) by heating the mixture in a microwave until the agarose was completely dissolved. Then, 3 µL of ethidium bromide (EtBr) was added as a DNA dye to visualize the DNA. Once the gel cooled to approximately 60°C, it was carefully poured into a dual comb caster and left undisturbed for 15 minutes. After solidification, the gel was placed in an electrophoresis tank (BK-HET02, BIOBASE, China), and the PCR products were loaded onto the gel for analysis. For each well on the gel plate, 3 µL of the PCR products was loaded. Additionally, 3 µL of a DNA molecular

weight marker (100-bp DNA Ladder, New England Biolabs) was included to compare the molecular weights of the PCR amplicons. The samples were electrophoresed simultaneously by applying a voltage of 100 V to the gel for 30 min. The integrity of the DNA was evaluated by visualising it under UV light with a gel documentation system (Bio-Rad Gel Doc EZ Imager Documentation, Berkeley, California).

2.5 Antimicrobial Resistance (AMR) Profiling

2.5.1 Antimicrobial Susceptibility Test

Antimicrobial susceptibility testing was performed using the Kirby–Bauer disk diffusion technique, as described initially by Bauer et al. [149]. For the AMR profiling, the following antibiotics were used: Ampicillin (AMP-10), Cefotaxime (CTX-30), Ceftazidime (CAZ-30), Cefepime (FEP-30), Imipenem (IMP-10), Meropenem (MRP-10), Azithromycin (AZM-15), Tetracycline (TE-30), Doxycycline (DO-30), Ciprofloxacin (CIP-5), Levofloxacin (LE-5), Ofloxacin (OFX-5), Trimethoprim-sulfamethoxazole (COT-25), Chloramphenicol (C-30) (Oxoid Limited, England) (**Table 2.5**) on the Mueller-Hinton agar (**Appendix-I**) plates. The zone of inhibition measurement was taken in millimetres (mm) using the antibiotic zone scale. By the standards set forth by the Clinical and Laboratory Standards Institute (CLSI) (2025), classifications of resistant (R), intermediate (I), and susceptible (S) are identified using the resistance breakpoints described in **Table 2.5** [150].

Table 2.5: Antibiotics used for antibiotic susceptibility test (AST) of Salmonella spp. And their resistance breakpoints according to the CLSI guideline 2025.

Antibiotic classes	Name of antibiotic	Disk potency (µg)	Zone of inhibition in diameter (mm)		
			Susceptible (S)	Intermediate (I)	Resistance (R)
β-lactam	AMP-10	10	≥17	14-16	≤13
	CTX-30	30	≥26	23-25	≤22
	CAZ-30	30	≥21	18-20	≤17
	FEP-30	30	≥25	19-24	≤18
	IMP-10	10	≥23	20-22	≤19
	MRP-10	10	≥23	20-22	≤19
Macrolide	AZM-15	15	≥13	-	≤12
Tetracycline	TE-30	30	≥15	12-14	≤11
	DO-30	30	≥14	11-13	≤10
Quinolone	CIP-5	5	≥31	22-25	≤20
	LE-5	5	≥21	17-20	≤16
	OFX-5	5	≥16	13-15	≤12
Sulfonamide	COT-25	25	≥16	11-15	≤10
Phanicol	C-30	30	≥18	13-17	≤12

2.5.2 Antimicrobial Resistance Gene Identification

For the observation of the antimicrobial resistance gene, four particular primers were selected, depending on the antimicrobial profile of the isolates [151], [152], [153], [154]. **Table 2.6** lists the product size, forward and reverse primer sequence for each primer. Primers for the genes blaTEM-1, blaCTX-M-1, tetA, and sul1 were used in the individual reactions of the PCR. A final volume of 13 µL was used for PCR amplifications, which contained specific primer pairs, PCR mastermix, nuclease-free water, and template DNA (**Table 2.2**). The PCR condition was set as **Table 2.7** for the selected isolates and after the amplification of the PCR product, gel electrophoresis was performed as described in Method 2.4.4.

Table 2.6: The list of common VAGs of *Salmonella* with their annealing temperature and product size.

Antibiotic resistance gene	Primer sequence (5′–3′)	Annealing temp. (ºC)	Size (bp)
blaTEM-1	F: CCGTGTCGCCCTTATTCC	54.5	445
	R: AGGCACCTATCTCAGCGA		
blaCTX-M-1	F: TTAGGAAGTGTGCCGCTGTA	54.4	499
	R: CCGTTTCCGCTATTA CAAACC		
tetA	F: GGTTCACTCGAACGACGTCA	50.7	577
	R: CTGTCCGACAAGTTGCATGA		
sul1	F: TTCGGCATTCTGAATCTCAC	47.6	822
	R: ATGATCTAACCCTCGGTCTC		

Table 2.7: PCR conditions at different stages for antimicrobial resistance genes.

Primer	Denaturation	Denaturation after the first cycle	Annealing	Extension	No. of cycles	Final extension
blaTEM-1	94ºC (2 min)	94ºC (30sec)	54.5ºC (30sec)	72ºC (1min)	30 X	72ºC (5min)
blaCTX-M-1	94ºC (2 min)	94ºC (30sec)	54.4ºC (30sec)	72ºC (1min)	30 X	72ºC (5min)
tetA	94ºC (2 min)	94ºC (30sec)	50.7ºC (30sec)	72ºC (1min)	30 X	72ºC (5min)
sul1	94ºC (2 min)	94ºC (30sec)	47.6ºC (30sec)	72ºC (1min)	30 X	72ºC (5min)

2.6 Biofilm Forming Test

Biofilm formation was assessed to detect the organism's capacity to survive, persist, and remain protected under harsh or stressful environmental conditions [155]. The biofilm formation was quantified by the tube-based crystal violet assay. Overnight cultures were diluted 1:100 in tryptic soy broth (TSB) supplemented with 1% glucose, and 5 mL aliquots were incubated in sterile glass test-tubes at room temperature for 48 h. After incubation, planktonic cells were

removed, and tubes were gently washed with distilled water. Biofilms were air-dried and stained with 0.1% (w/v) crystal violet for 15 min. Excess stain was removed by rinsing with distilled water, and the bound dye was solubilized with 3 mL of 30% (v/v) acetic acid. Solubilized dye was mixed, and optical density was measured at 570 nm in a spectrophotometer. Biofilm strength was classified using the OD cut-off (ODc = mean OD of negative control + 3×SD). This ODc value was used as the threshold to classify biofilm production. OD values of all test strains were then recorded, and biofilm intensity was interpreted according to Stepanović's criteria (non-adherent, weak, moderate, or strong) [156].

Chapter 3

Results

3. Results

3.1 Isolation and Identification of Bacterial Isolates

A total of 100 fish samples were collected for the study. Among all samples, 224 pure colonies were picked from HEA culture media (**Figure 3.1**). Among all of them, 145 were selected as presumptive *Salmonella* spp. based on cultural, Gram staining (**Figure 3.2**), and other biochemical tests, as shown in **Table 3.1**.

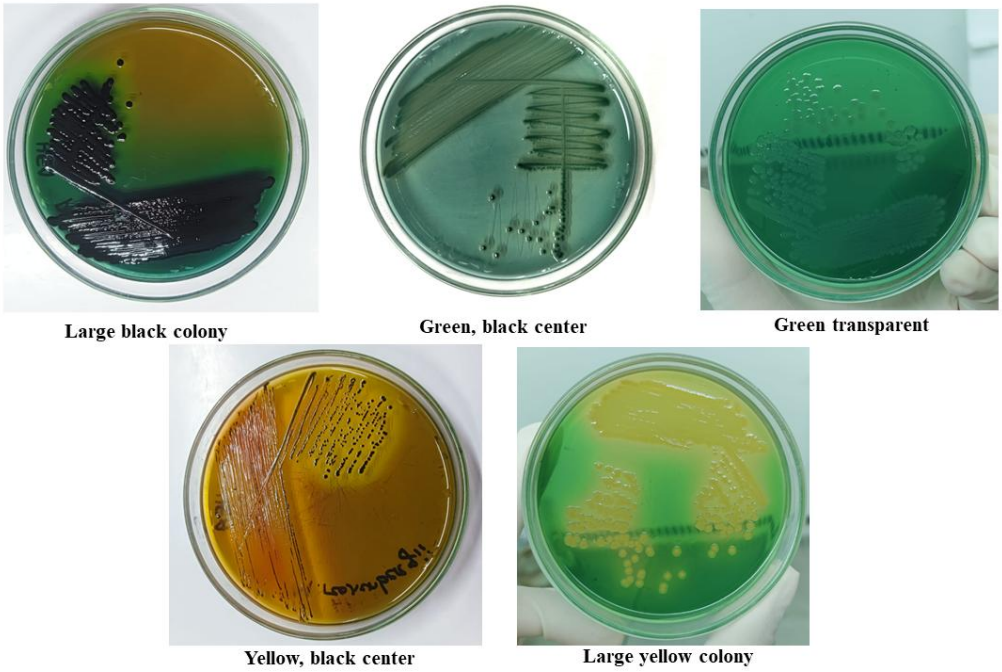

Figure 3.1: Presumptive *Salmonella* colonies on Hecton Enteric Agar Media (HEA).

Among the six distinct retail markets from which the samples were obtained, samples from Dotter Haat exhibited the highest prevalence of *Salmonella* at 6.2%, followed by Sonapur Bazar in second place with 4.12%, Pouro Bazar with a prevalence of 2.8%, 1.4% in Maijdee Bazar, and 2.07% in Choumuhuni Bazar indicating the varying severity of *Salmonella* across different retail markets. In contrast, Kalitara Bazar showed no evidence of pathogenic *Salmonella* spp. in their fish samples (**Figure 3.3**).

According to the sample types, the prevalence of positive samples was 20.8% in *P. monodon,* 4.2% in *M. rosenbergii*, 4.2% in *P. merguiensis,* and 8.33% in *H. nehereus from* Dotter Haat. On the other hand, 12.5% in *O. pabda*, 4.2% in *P. monodon*, 4.2% in *M. rosenbergii*, and 4.2% in *H. nehereus* from Sonapur Bazar. In contrast, from Pouro Bazar, 8.33% of prevalence was

observed in *H. nehereus*, 4.2% in *P. monodon*, and 4.2% in *P. merguiensis*. Conversely, from the samples of Maijdee Bazar, both *P. monodon* and *M. rosenbergii* showed 4.2% prevalence. In the case of Choumuhuni Bazar, the highest prevalence was observed in P. monodon and M. rosenbergii, revealing a prevalence of 4.2% (**Figure 3.4**).

During the isolation, identification of bacterial colonies having typical cultural characteristics was selected as presumptive for *Salmonella* serovars. For this, general-purpose and differential selective media such as HEA and SS agar were used to culture the organism, although not all of them are equally suitable for all the serovars of *Salmonella.* In the present study, specific enriched media and biochemical tests mentioned above were also used. The colony characteristics of *Salmonella* spp. such as green and with or without a black center on HEA plates, were similar to the findings of other authors (**Figure 3.1**).

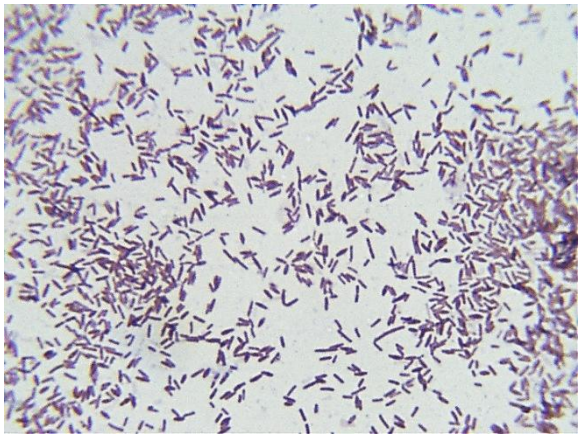

Figure 3.2: Gram staining result of a presumptive *Salmonella* isolate.

During the gram staining procedure, all presumptive *Salmonella* isolates appeared as Gram-negative rods under light microscopy (100× magnification) (**Figure 3.2**). The cells were observed as short, straight, rod-shaped bacilli, occurring singly or in short chains, consistent with the typical morphology of *Salmonella* spp. No Gram-positive contaminants were detected in pure cultures.

Table 3.1: The group of all positive isolates according to the colony characteristics.

Sample ID	Media	Colony characteristics	TSI				MIU			Citrate	Oxidase	Catalase	MR	VP	Decarboxylase
			Slat	Butt	H2S	GAS	Motility	Indole	Urease						
PM1	HEA	Large black colony	A	A	+	-	+	+	+	+	+	+	+	-	+
PM2	HEA	Green, black center	K	A	+	-	+	-	-	+	-	+	+	-	+
PM3	HEA	Green, black center	K	A	+	-	+	+	+	-	-	+	+	-	+
MR1	HEA	Green, black center	K	A	+	-	-	-	-	-	-	+	+	-	-
MR2	HEA	Yellow, black center	K	A	+	+	+	-	-	+	+	+	+	-	+
MR3	HEA	Yellow, black center	K	A	+	+	+	-	-	+	-	+	+	-	-
MR4	HEA	Green, black center	K	A	+	-	-	+	+	-	-	+	-	-	-
MR5	HEA	Yellow, black center	K	A	+	-	+	+	+	-	-	+	-	-	-
MR6	HEA	Medium black colony	K	A	+	+	+	-	-	+	-	+	+	-	-
MR7	HEA	Green transparent, green center	K	A	-	-	-	-	-	-	-	+	+	-	+
MR8	HEA	Green transparent, green center	A	A	-	-	-	+	+	-	-	+	-	-	+
PM4	HEA	Green, no black center	K	A	+	-	-	-	-	-	-	+	-	-	+
PM5	HEA	Yellow, black center	K	A	+	-	+	+	+	-	-	+	-	+	+
PM6	HEA	Green, black center	K	A	-	-	-	-	-	-	-	+	-	+	+
PM7	HEA	Green, black center	A	A	-	-	-	+	+	-	-	+	+	-	+
MR10	HEA	Large black colony	K	A	+	+	+	-	-	+	+	+	-	-	-
MR11	HEA	Green, black center	A	A	+	-	+	+	+	+	+	+	+	-	+
MR12	HEA	Green, black center	K	A	+	-	+	-	-	+	-	+	-	-	-
MR13	HEA	Green, black center	K	A	+	-	+	+	+	-	-	+	+	+	-
MR14	HEA	Yellow, black center	K	A	+	-	-	-	-	-	-	+	-	-	+
PM8	HEA	Yellow, black center	K	A	+	+	+	-	-	+	+	+	+	+	+
PM9	HEA	Green, black center	K	A	+	+	+	-	-	+	-	+	+	-	+
PM10	HEA	Yellow, black center	K	A	+	-	-	+	+	-	-	+	+	-	-
MR15	HEA	Medium black colony	K	A	+	-	+	+	+	-	-	+	-	-	+
MR16	HEA	Green transparent, green center	K	A	+	+	+	-	-	+	-	+	-	-	-
P1	HEA	Green transparent, green center	K	A	-	-	-	-	-	-	-	+	-	-	+
P2	HEA	Green, no black center	A	A	-	-	-	+	+	-	-	+	+	-	+
P3	HEA	Yellow, black center	K	A	+	-	-	-	-	-	-	+	+	-	+

P4	HEA	Green, black center	K	A	+	-	+	-	-	+	-	+	+	-	-
P5	HEA	Green, black center	K	A	+	-	+	+	+	-	-	+	+	-	-
PM13	HEA	Large black colony	K	A	+	-	-	-	-	-	-	+	+	+	-
PM14	HEA	Green, black center	K	A	+	+	+	-	-	+	+	+	+	+	+
PM15	HEA	Green, black center	K	A	+	+	+	-	-	+	-	+	-	-	-
MR19	HEA	Green, black center	K	A	+	-	-	+	+	-	-	+	-	-	+
MR20	HEA	Yellow, black center	K	A	+	-	+	+	+	-	-	+	+	-	-
MR21	HEA	Yellow, black center	K	A	+	+	+	-	-	+	-	+	+	+	+
MR22	HEA	Green, black center	K	A	-	-	-	-	-	-	-	+	-	-	+
MR23	HEA	Yellow, black center	A	A	-	-	-	+	+	-	-	+	-	+	+
MR24	HEA	Medium black colony	K	A	+	-	-	-	-	-	-	+	-	-	-
P10	HEA	Green transparent, green center	A	A	+	-	+	+	+	+	+	+	-	+	-
P111	HEA	Green transparent, green center	K	A	+	-	+	-	-	+	-	+	+	-	-
P12	HEA	Green, no black center	K	A	+	-	+	+	+	-	-	+	-	+	-
P15	HEA	Yellow, black center	K	A	+	-	-	-	-	-	-	+	+	+	-
PM18	HEA	Green, black center	K	A	+	+	+	-	-	+	+	+	-	+	+
PM19	HEA	Green, black center	K	A	+	+	+	-	-	+	-	+	+	+	-
PM20	HEA	Large black colony	K	A	+	-	-	+	+	-	-	+	-	+	+
PM21	HEA	Green, black center	K	A	+	-	+	+	+	-	-	+	+	+	-
PM22	HEA	Green, black center	K	A	+	+	+	-	-	+	-	+	+	+	-
PM23	HEA	Green, black center	K	A	-	-	-	-	-	-	-	+	+	-	-
PM24	HEA	Green transparent, green center	A	A	-	-	-	+	+	-	-	+	-	-	+
PM25	HEA	Green transparent, green center	K	A	+	-	-	-	-	-	-	+	-	-	+
PM26	HEA	Green, no black center	K	A	+	-	+	+	+	-	-	+	-	-	-
PM27	HEA	Yellow, black center	K	A	-	-	-	-	-	-	-	+	+	-	-
PM28	HEA	Medium black colony	A	A	-	-	-	+	+	-	-	+	+	-	+
PM29	HEA	Green transparent, green center	K	A	+	+	+	-	-	+	+	+	+	-	+
MR26	HEA	Green transparent, green center	A	A	+	-	+	+	+	+	+	+	+	+	+
MR27	HEA	Green, no black center	A	A	+	-	+	+	+	+	+	+	+	-	+
MR28	HEA	Yellow, black center	K	A	+	-	+	-	-	+	-	+	+	-	-
MR29	HEA	Green, black center	K	A	+	-	+	+	+	-	-	+	-	-	-
MR30	HEA	Green, black center	K	A	+	-	-	-	-	-	-	+	-	-	-
MR31	HEA	Large black colony	K	A	+	+	+	-	-	+	+	+	+	-	-

MR32	HEA	Green, black center	K	A	+	+	+	-	-	+	-	+	+	-	-
MR33	HEA	Green, black center	K	A	+	-	-	+	+	-	-	+	-	-	-
MR34	HEA	Green, black center	K	A	+	-	+	+	+	-	-	+	-	-	-
GS1	HEA	Yellow, black center	K	A	+	+	+	-	-	+	-	+	-	-	-
GS2	HEA	Yellow, black center	K	A	-	-	-	-	-	-	-	+	-	-	+
GS3	HEA	Green, black center	A	A	-	-	-	+	+	-	-	+	+	-	+
PM30	HEA	Yellow, black center	K	A	+	-	-	-	-	-	-	+	-	-	+
PM31	HEA	Medium black colony	K	A	+	-	+	+	+	-	-	+	+	-	+
PM33	HEA	Green transparent, green center	K	A	-	-	-	-	-	-	-	+	-	-	+
PM34	HEA	Green transparent, green center	A	A	-	-	-	+	+	-	-	+	+	-	+
MR36	HEA	Green, no black center	K	A	+	+	+	-	-	+	+	+	-	-	-
MR37	HEA	Yellow, black center	A	A	+	-	+	+	+	+	+	+	+	-	-
PM35	HEA	Green, black center	A	A	+	-	+	+	+	+	+	+	+	-	+
PM36	HEA	Green, black center	K	A	+	-	+	-	-	+	-	+	+	+	-
GS4	HEA	Large black colony	K	A	+	-	+	+	+	-	-	+	-	+	+
GS5	HEA	Green, black center	K	A	+	-	-	-	-	-	-	+	-	-	-
MR38	HEA	Green, black center	K	A	+	+	+	-	-	+	+	+	-	-	+
MR39	HEA	Green, black center	K	A	+	+	+	-	-	+	-	+	+	-	-
MR40	HEA	Green transparent, green center	K	A	+	-	-	+	+	-	-	+	+	-	+
P17	HEA	Green transparent, green center	K	A	+	-	+	+	+	-	-	+	+	+	-
P18	HEA	Green, no black center	K	A	+	+	+	-	-	+	-	+	+	-	+
PM39	HEA	Yellow, black center	K	A	-	-	-	-	-	-	-	+	+	+	-
PM40	HEA	Medium black colony	A	A	-	-	-	+	+	-	-	+	+	-	+
PM41	HEA	Green transparent, green center	K	A	+	-	-	-	-	-	-	+	-	-	-
PM42	HEA	Green transparent, green center	K	A	+	-	+	+	+	-	-	+	-	-	+
P21	HEA	Green, no black center	K	A	-	-	-	-	-	-	-	+	+	-	+
P22	HEA	Yellow, black center	A	A	-	-	-	+	+	-	-	+	+	-	+
P23	HEA	Green, black center	K	A	+	+	+	-	-	+	+	+	-	-	+
P24	HEA	Green, black center	A	A	+	-	+	+	+	+	+	+	-	-	+
GS8	HEA	Large black colony	A	A	+	-	+	+	+	+	+	+	-	-	-
PM45	HEA	Green, black center	K	A	+	-	+	-	-	+	-	+	-	-	-
PM46	HEA	Green, black center	K	A	+	-	+	+	+	-	-	+	+	+	+
PM47	HEA	Green, black center	K	A	+	-	-	-	-	-	-	+	-	+	-

MR43	HEA	Yellow, black center	K	A	+	+	+	-	-	+	+	+	+	-	+
MR44	HEA	Yellow, black center	K	A	+	+	+	-	-	+	-	+	-	-	-
GS11	HEA	Green, black center	K	A	+	-	-	+	+	-	-	+	+	-	+
BD1	HEA	Yellow, black center	K	A	+	-	+	+	+	-	-	+	-	+	-
BD2	HEA	Medium black colony	K	A	+	+	+	-	-	+	-	+	+	-	+
BD3	HEA	Green transparent, green center	K	A	-	-	-	-	-	-	-	+	+	+	-
BD4	HEA	Green transparent, green center	A	A	-	-	-	+	+	-	-	+	+	-	+
BD5	HEA	Green, no black center	K	A	+	-	-	-	-	-	-	+	-	+	-
BD6	HEA	Green transparent, green center	K	A	+	-	+	+	+	-	-	+	-	-	+
BD7	HEA	Green, black center	K	A	-	-	-	-	-	-	-	+	-	+	-
BD12	HEA	Green, black center	A	A	-	-	-	+	+	-	-	+	+	+	+
BD13	HEA	Large black colony	K	A	+	+	+	-	-	+	+	+	+	+	-
BD14	HEA	Green transparent, green center	A	A	+	-	+	+	+	+	+	+	+	+	-
BD15	HEA	Green, black center	A	A	+	-	+	+	+	+	+	+	+	+	+
BD16	HEA	Green, black center	K	A	+	-	+	-	-	+	-	+	+	+	-
BD17	HEA	Green transparent, green center	K	A	+	-	+	+	+	-	-	+	+	+	+
BD18	HEA	Green, black center	K	A	+	-	-	-	-	-	-	+	-	-	-
BD24	HEA	Green, no black center	K	A	+	+	+	-	-	+	+	+	-	-	+
BD25	HEA	Yellow, black center	K	A	+	+	+	-	-	+	-	+	+	-	-
BD26	HEA	Medium black colony	K	A	+	-	-	+	+	-	-	+	+	-	+
BD27	HEA	Green transparent, green center	K	A	+	-	+	+	+	-	-	+	-	-	-
BD28	HEA	Green transparent, green center	K	A	+	+	+	-	-	+	-	+	-	-	+
BD29	HEA	Green, no black center	K	A	-	-	-	-	-	-	-	+	-	-	-
BD30	HEA	Yellow, black center	A	A	-	-	-	+	+	-	-	+	-	+	+
BD39	HEA	Green, black center	K	A	+	-	-	-	-	-	-	+	+	-	-
BD40	HEA	Green, black center	K	A	+	-	+	+	+	-	-	+	-	-	+
BD41	HEA	Large black colony	K	A	-	-	-	-	-	-	-	+	+	-	+
BD42	HEA	Green, black center	A	A	-	-	-	+	+	-	-	+	-	-	+
BD43	HEA	Green, black center	K	A	+	+	+	-	-	+	+	+	+	-	+
BD44	HEA	Green, black center	A	A	+	-	+	+	+	+	+	+	-	-	+
BD45	HEA	Green, black center	A	A	+	-	+	+	+	+	+	+	+	-	+
BD60	HEA	Yellow, black center	K	A	+	-	+	-	-	+	-	+	+	-	+
BD61	HEA	Green, black center	K	A	+	-	+	+	+	-	-	+	+	-	+

BD62	HEA	Yellow, black center	K	A	+	-	-	-	-	-	-	+	-	-	+
BD63	HEA	Medium black colony	K	A	+	+	+	-	-	+	+	+	-	-	+
PB9	HEA	Green transparent, green center	K	A	+	+	+	-	-	+	-	+	-	-	+
PB10	HEA	Green transparent, green center	K	A	+	-	-	+	+	-	-	+	+	-	+
PB11	HEA	Green, no black center	K	A	+	-	+	+	+	-	-	+	+	-	+
PB12	HEA	Yellow, black center	K	A	+	+	+	-	-	+	-	+	+	-	+
PB13	HEA	Green, black center	K	A	-	-	-	-	-	-	-	+	+	-	+
PB14	HEA	Green, black center	A	A	-	-	-	+	+	-	-	+	+	-	+
PB15	HEA	Large black colony	K	A	+	-	-	-	-	-	-	+	+	-	+
PB16	HEA	Green, black center	K	A	+	-	+	+	+	-	-	+	-	+	+
PB17	HEA	Green, black center	K	A	-	-	-	-	-	-	-	+	-	+	+
PB18	HEA	Green, black center	A	A	-	-	-	+	+	-	-	+	+	-	+
PB19	HEA	Yellow, black center	K	A	+	+	+	-	-	+	+	+	+	-	+
PB20	HEA	Green, black center	A	A	+	-	+	+	+	+	+	+	-	-	+
PB21	HEA	Green, black center	A	A	+	-	+	+	+	+	+	+	-	-	+
PB22	HEA	Yellow, black center	K	A	+	-	+	-	-	+	-	+	-	+	+
PB23	HEA	Medium black colony	K	A	+	-	+	+	+	-	-	+	-	-	+
PB24	HEA	Green transparent, green center	K	A	+	-	-	-	-	-	-	+	+	+	+

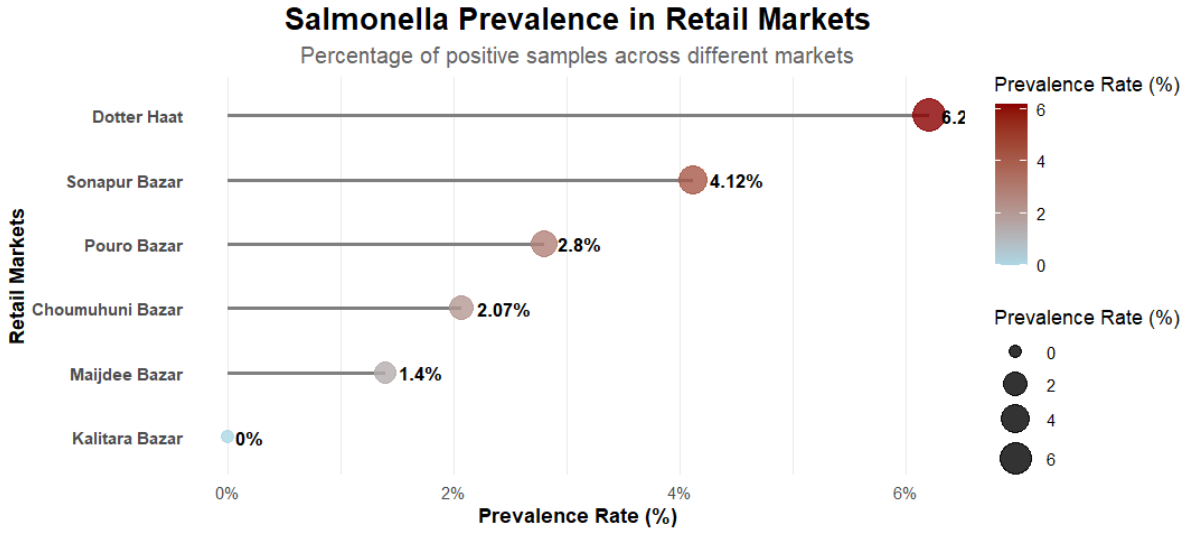

Figure 3.3: Percentage of *Salmonella*-positive samples detected in various retail markets.

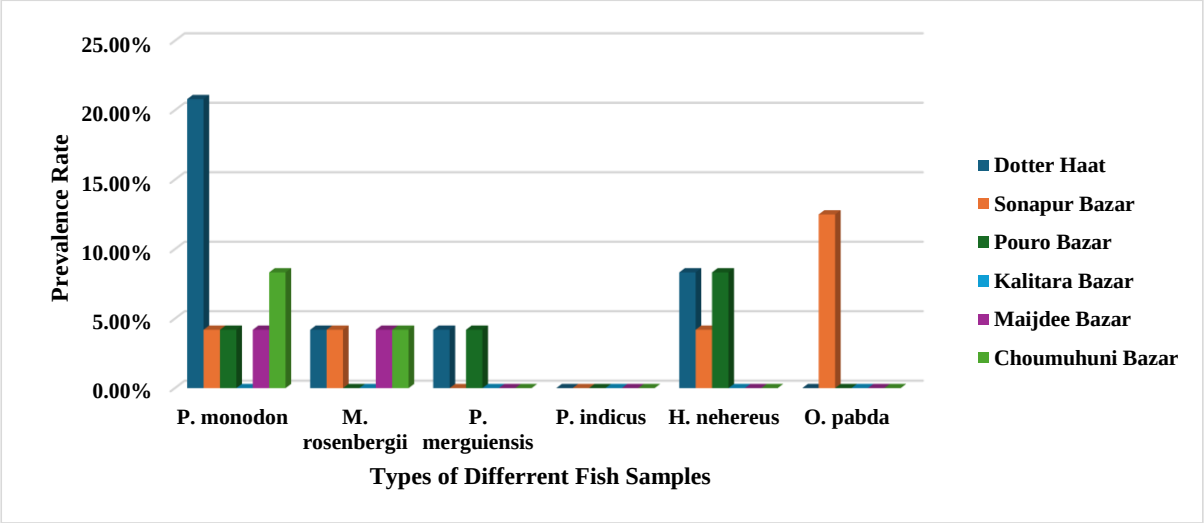

Figure 3.4: Prevalence of *Salmonella* spp. from various samples collected from six different retail markets of Noakhali, Bangladesh.

3.1.1 Biochemical Pattern of the Isolates

Various biochemical tests were performed (**Figure 3.5**) on the selected 145 isolates, and they were presumptively identified according to the standard guideline (Bergey's manual of determinative bacteriology, 2012) and were grouped together (**Table 3.1**). According to the guideline, the biochemical test results of *Salmonella* were observed in **Figure 3.5**.

The isolates, which were tentatively identified as *Salmonella* spp. demonstrated characteristic biochemical reactions indicative of the genus. In Triple Sugar Iron (TSI) agar, the isolates exhibited an alkaline slant and acid butt (K/A) with hydrogen sulfide (H_2S) production, indicative of glucose fermentation and sulfur reduction. Motility Indole Urea (MIU) tests indicated motility, indole negativity, and urease negativity. The organisms tested negative for citrate utilisation. In the MR-VP assay, the isolates exhibited a positive methyl red (MR) result and a negative Voges–Proskauer (VP) result, indicating the occurrence of mixed-acid fermentation. Lysine Iron Agar (LIA) results indicated lysine decarboxylation accompanied by hydrogen sulfide (H_2S) production, characteristic of *Salmonella* spp. Furthermore, all isolates tested positive for catalase activity and negative for oxidase activity, thereby providing additional confirmation of their classification within the Enterobacteriaceae family. Overall, these biochemical features align with *Salmonella* spp. and substantiate their identification.

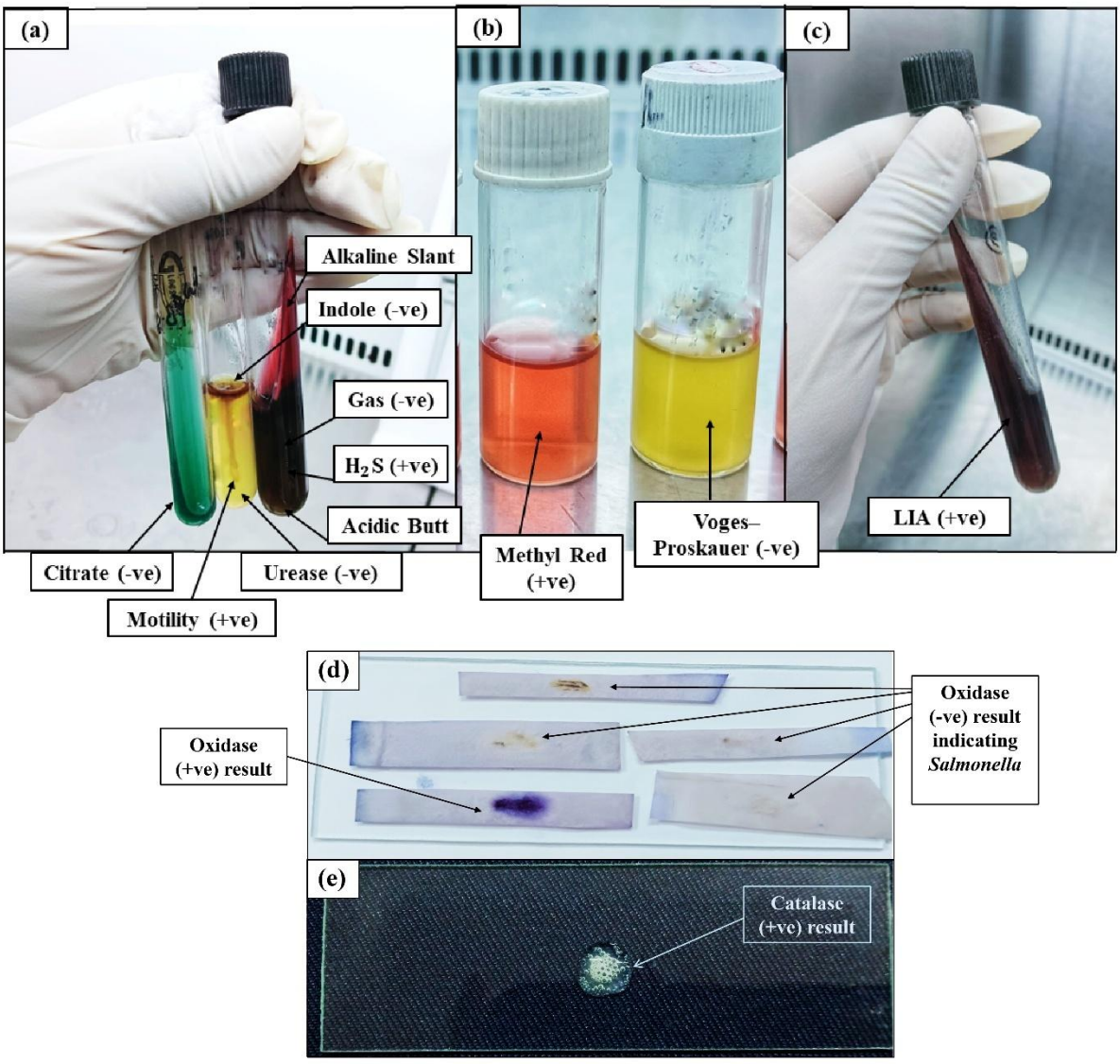

Figure 3.5: Biochemical test results for *Salmonella* spp. (a) Citrate, MIU, TSI test, (b) MR-VP test, (c) LIA test, (d) Oxidase test, (e) Catalase test.

3.2 Molecular Characterisation and Genotyping of the *Salmonella* spp.

3.2.1 Virulence-associated Gene-specific PCR for Detection of *Salmonella*

A total of 145 presumptive *Salmonella* isolates from fish samples were subjected to the *Salmonella*-specific gene (invA) and were confirmed as *Salmonella* positive by the predicted product size of 321-bp DNA fragments (**Figure 3.6a**). The electrophoresed DNA isolates revealed a list of 24 positive isolates (**Table 3.2**), indicating the presence of the invA gene. Two other virulence genes (hilA and fimA) were also detected through PCR in the 24 confirmed *Salmonella* isolates. The amplicon size for hilA and fimA was 499 and 85 bp, respectively. Among 24 positive isolates confirmed by the invA gene, 19 were positive for fimA (**Figure**

3.6b) and 23 for hilA (**Figure 3.6c**). The presence and prevalence of specific virulence genes across all isolates and retail markets are illustrated in **Figures 3.7 and 3.8**.

Table 3.2: Selected 24 isolates' sample ID and their updated indicator profiles.

Serial No.	Sample ID	New Indicator	Serial No.	Sample ID	New Indicator
1	PM-2	Sal-1	13	MR-36	Sal-13
2	PM-10	Sal-2	14	MR-25	Sal-14
3	PM-18	Sal-3	15	P-5	Sal-15
4	PM-15	Sal-4	16	P-16	Sal-16
5	PM-30	Sal-5	17	BD-12	Sal-17
6	PM-22	Sal-6	18	BD-16	Sal-18
7	PM-25	Sal-7	19	BD-22	Sal-19
8	PM-28	Sal-8	20	BD-25	Sal-20
9	PM-32	Sal-9	21	BD-28	Sal-21
10	PM-45	Sal-10	22	PB-12	Sal-22
11	MR-28	Sal-11	23	PB-27	Sal-23
12	MR-29	Sal-12	24	PB-30	Sal-24

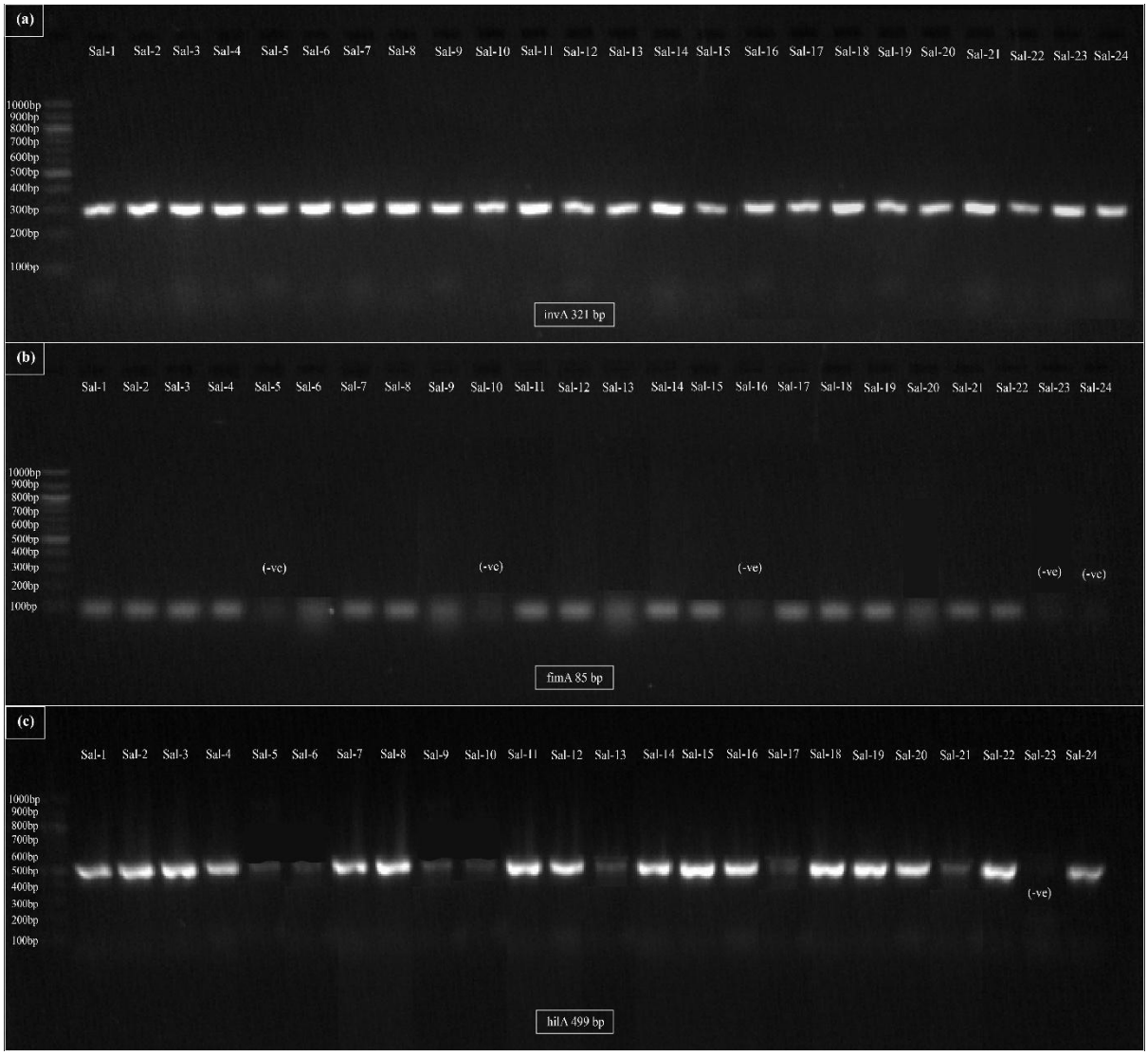

Figure 3.6: Visualisation of virulence-associated genes from different isolates, including (a) invA, (b) fimA, and (c) hilA; (-ve) = absence of virulence genes.

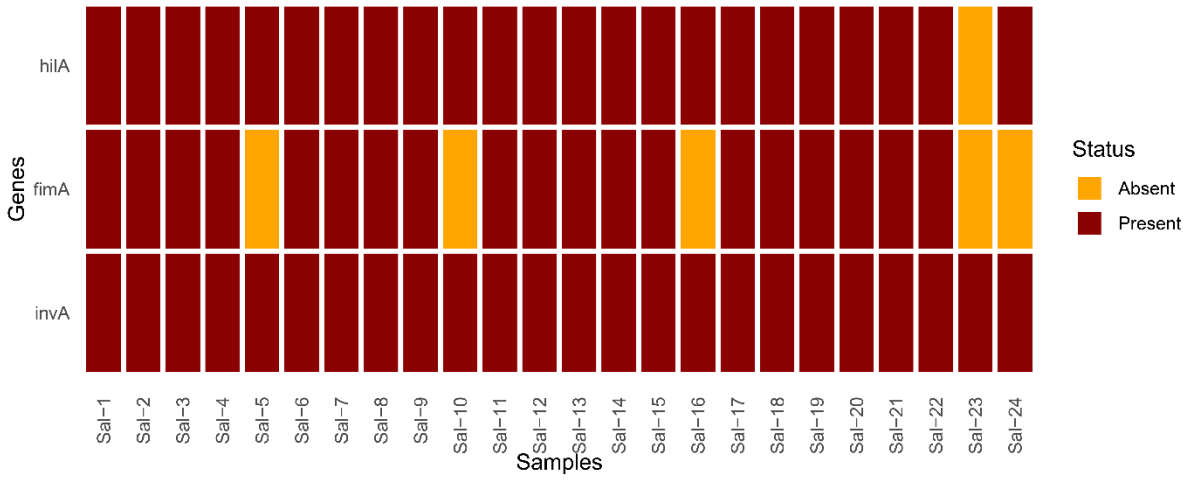

Figure 3.7: A heatmap illustrating the number of positive isolates among all the presumptive *Salmonella* spp., identified with specific virulence-associated genes.

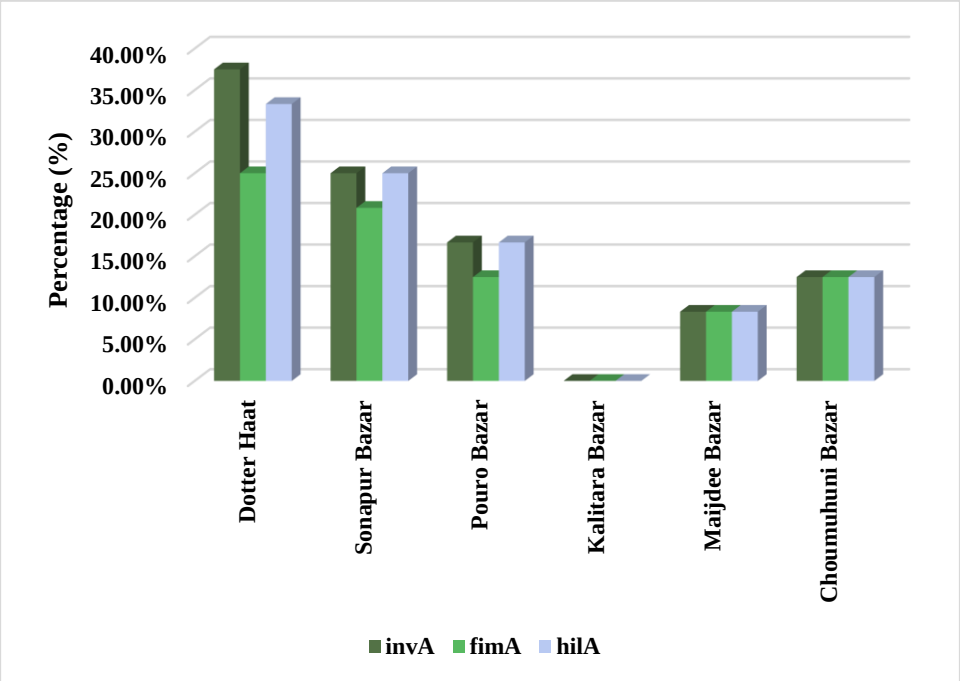

Figure 3.8: Prevalence of the virulence genes among different retail markets of Noakhali.

3.3 Antibiogram of Confirmed *Salmonella* Isolates

3.3.1 Antibiotic Susceptibility

A total of 24 isolates were selected as presumptive *Salmonella* according to their morphological, biochemical, and molecular characteristics. These isolates were tested against 14 commonly used antibiotics (Oxoid, UK) belonging to 6 different groups of antibiotics (**Figures 3.9** and **3.10**). The results were interpreted following the CSLI 2025 guidelines (**Table 3.3**). It revealed that among the 24 isolates, 100%, 75%, 50%, 33.33%, 29.17%, 29.17%, and 25% were resistant to Ceftazidime, Ampicillin, Levofloxacin, Azithromycin, Tetracycline, Ofloxacin, and Ciprofloxacin, respectively. On the other hand, higher sensitivity was recorded against Cefepime, Meropenem, Trimethoprim-sulfamethoxazole, and Chloramphenicol. The MAR index of the selected isolates is shown in **Table 3.4**. The 24 isolates displayed variable antibiotic resistance patterns, with the number of resistant antibiotics ranging from 1 to 6 per isolate. The MAR indices ranged from 0.07 to 0.43, indicating that several isolates originated from environments with high antibiotic exposure. Notably, isolates Sal-3, Sal-5, Sal-9, and Sal-20 exhibited the highest MAR index (0.36), reflecting resistance to six antibiotics. In contrast, Sal-15 showed the lowest MAR index (0.07), suggesting minimal exposure to antimicrobial selective pressure. Based on resistance across antibiotic classes, a majority of isolates (over 70%) qualified as MDR, exhibiting resistance to three or more antibiotic classes. This

demonstrates a substantial burden of multidrug resistance within the isolate population. Resistance was most frequently observed in β-lactams (particularly CAZ-30) and fluoroquinolones in many isolates. Overall, the analysis reflects a high prevalence of multidrug resistance and elevated MAR indices, underscoring the potential public health risk and the need for improved antimicrobial stewardship.

Figure 3.9: Antibiotic sensitivity test of a presumed *Salmonella* isolate (Sal-2) with 14 different types of antibiotic discs.

Table 3.3: Results of disc diffusion susceptibility testing following CLSI guidelines 2025.

Sample ID	β-lactam						Macrolide	Tetracycline		Fluoroquinolone			Folate pathway antagonist	Phenicol
	AMP-10	CTX-30	CAZ-30	FEP-30	IMP-10	MRP-10	AZM-15	TE-30	DO-30	CIP-5	LE-5	OFX-5	COT-25	C-30
Sal-1	23 (S)	34.5 (S)	8 (R)	30 (S)	25.5 (S)	29 (S)	0 (R)	0 (R)	11 (I)	30 (I)	19 (R)	29 (I)	26 (S)	20 (S)
Sal-2	0 (R)	28 (S)	0 (R)	28 (S)	22.5 (I)	27 (S)	15.5 (S)	12 (I)	13 (I)	27 (I)	25 (I)	26.5 (I)	23.5 (S)	20 (S)
Sal-3	0 (R)	24 (I)	14 (R)	35 (S)	27 (S)	30.5 (S)	19.5 (S)	15 (S)	17 (S)	14 (R)	13.5 (R)	15 (R)	23 (S)	28 (S)
Sal-4	0 (R)	30.5 (S)	6 (R)	28 (S)	22 (I)	29 (S)	15 (S)	13 (I)	13.5 (I)	27 (I)	26 (I)	28 (I)	24.5 (S)	24 (S)
Sal-5	20 (S)	26 (S)	15 (R)	28 (S)	20 (I)	23 (S)	12 (R)	20 (S)	13 (I)	20 (R)	18 (R)	17 (R)	16 (S)	18 (S)
Sal-6	13 (R)	28 (S)	15 (R)	32 (S)	21.5 (I)	25 (S)	13 (S)	0 (R)	11 (I)	23.5 (I)	16 (R)	23 (I)	18 (S)	20 (S)
Sal-7	8 (R)	30 (S)	8 (R)	30 (S)	28 (S)	28 (S)	14 (S)	14 (I)	14 (S)	18 (R)	28 (I)	18 (R)	20 (S)	25 (S)
Sal-8	12.5 (R)	33.5 (S)	0 (R)	28 (S)	22 (I)	26 (S)	10 (R)	13 (I)	13 (I)	25 (I)	23 (I)	25 (I)	26 (s)	24 (S)
Sal-9	22 (S)	23 (I)	14 (R)	35 (S)	21.5 (I)	31.5 (S)	8 (R)	19.5 (S)	11.5 (I)	16.5 (R)	16.5 (R)	27 (I)	17.5 (S)	28 (S)
Sal-10	0 (R)	30.5 (S)	6 (R)	28 (S)	20.5 (I)	26 (S)	15 (S)	12.5 (I)	15 (S)	27 (I)	21 (I)	19.5 (R)	23.5 (S)	20 (S)
Sal-11	0 (R)	28 (S)	15 (R)	28 (S)	25 (S)	29 (S)	0 (R)	16 (S)	12 (I)	23 (I)	20 (R)	15 (R)	24 (S)	23 (S)
Sal-12	0 (R)	27.5 (S)	15 (R)	32 (S)	22 (I)	24.5 (S)	0 (R)	12 (I)	12.5 (I)	25 (I)	23 (I)	24 (I)	24.5 (S)	26 (S)
Sal-13	0 (R)	25 (I)	8 (R)	30 (S)	27 (S)	26.5 (S)	0 (R)	17 (S)	13 (I)	26.5 (I)	18.5 (R)	26 (I)	25 (S)	28 (S)
Sal-14	0 (R)	32 (S)	0 (R)	28 (S)	27.5 (S)	24 (S)	18 (S)	0 (R)	11 (I)	28.5 (I)	25 (I)	28.5 (I)	26 (S)	25 (S)
Sal-15	20.5 (S)	33 (S)	14 (R)	35 (S)	25 (S)	30 (S)	17 (S)	13 (I)	13 (I)	30 (I)	27 (I)	29.5 (I)	18 (S)	24 (S)
Sal-16	18 (S)	26 (S)	6 (R)	28 (S)	26 (S)	25 (S)	19.5 (S)	12.5 (I)	16 (S)	20 (R)	26 (I)	16 (R)	20 (S)	21.5 (S)
Sal-17	0 (R)	28.5 (S)	15 (R)	28 (S)	20.5 (I)	25 (S)	18 (S)	0 (R)	12.5 (I)	28 (I)	19 (R)	30 (I)	22.5 (S)	20.5 (S)
Sal-18	0 (R)	24 (I)	15 (R)	32 (S)	22 (I)	23 (S)	0 (R)	12 (I)	17 (S)	26 (I)	16 (R)	25 (I)	21.5 (S)	19.5 (S)
Sal-19	0 (R)	29.5 (S)	8 (R)	30 (S)	28.5 (S)	28 (S)	15 (S)	14 (I)	12 (I)	27 (I)	25 (I)	24 (I)	18 (S)	22.5 (S)
Sal-20	13 (R)	23.5 (I)	0 (R)	28 (S)	26 (S)	25 (S)	0 (R)	0 (R)	20 (S)	14 (R)	24 (I)	19 (R)	28 (S)	25 (S)
Sal-21	17 (S)	30.5 (S)	14 (R)	35 (S)	21.5 (I)	26 (S)	13 (S)	12.5 (I)	11.5 (I)	23 (I)	18 (R)	25 (I)	26 (S)	27 (S)
Sal-22	0 (R)	26.5 (S)	6 (R)	28 (S)	25 (S)	24 (S)	15 (S)	0 (R)	13 (I)	22.5 (I)	20 (R)	23 (I)	20 (S)	28 (S)
Sal-23	0 (R)	30 (S)	15 (R)	28 (S)	20 (I)	25 (S)	14 (S)	13.5 (I)	12 (I)	24.5 (I)	25 (I)	27 (I)	19.5 (S)	18 (S)
Sal-24	0 (R)	25 (I)	15 (R)	32 (S)	23 (S)	24 (S)	17.5 (S)	0 (R)	13 (I)	25 (I)	14 (R)	26 (I)	25 (S)	20 (S)

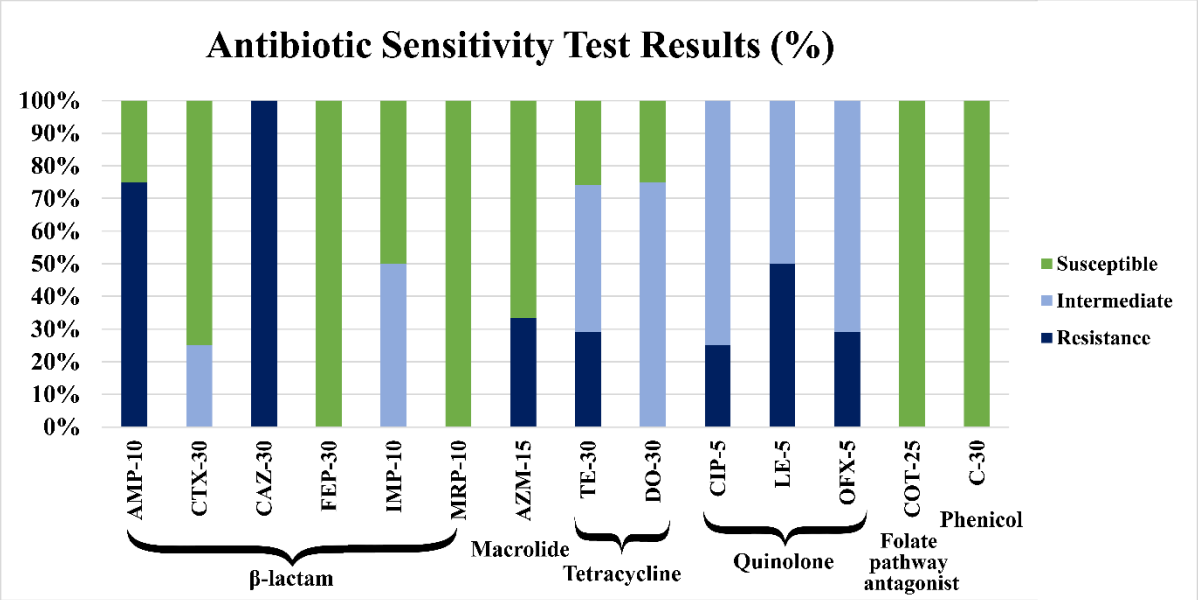

Figure 3.10: Diagrammatic representation of the antibiotic resistance pattern of the isolates.

Table 3.4: Overview of antibiotic resistance, MAR index distribution, and MDR status in the isolate collection.

Sample ID	Resistance type	Resistant pattern	No. of Antibiotic Class	MAR Index
Sal-1	MDR	CAZ, AZM, TE, LE	4	0.29
Sal-2	-	AMP, CAZ	1	0.14
Sal-3	MDR	AMP, CAZ, CIP, LE, OFX	2	0.36
Sal-4	-	AMP, CAZ	1	0.14
Sal-5	MDR	CAZ, AZM, CIP, LE, OFX	3	0.36
Sal-6	MDR	AMP, CAZ, TE, LE	3	0.28
Sal-7	MDR	AMP, CAZ, CIP, OFX	2	0.29
Sal-8	MDR	AMP, CAZ, AZM	2	0.21
Sal-9	MDR	CAZ, AZM, CIP, LE	3	0.28
Sal-10	MDR	AMP, CAZ, OFX	2	0.21
Sal-11	MDR	AMP, CAZ, AZM, LE, OFX	3	0.36
Sal-12	MDR	AMP, CAZ, AZM	2	0.21
Sal-13	MDR	AMP, CAZ, AZM, LE	3	0.29
Sal-14	-	AMP, CAZ	1	0.14
Sal-15	-	CAZ	1	0.07
Sal-16	MDR	CAZ, CIP, OFX	2	0.21
Sal-17	MDR	AMP, CAZ, TE, LE	3	0.28
Sal-18	MDR	AMP, CAZ, LE	2	0.21
Sal-19	-	AMP, CAZ	1	0.14
Sal-20	MDR	AMP, CAZ, AZM, CIP, OFX	3	0.36
Sal-21	-	CAZ, LE	2	0.14
Sal-22	-	AMP, CAZ	1	0.14
Sal-23	-	AMP, CAZ	1	0.14
Sal-24	MDR	AMP, CAZ, LE	2	0.21

3.3.2 Antimicrobial Resistance Gene Identification

A total of 24 confirmed *Salmonella* isolates from fish samples were subjected to different antibiotic resistance genes (blaTEM-1, blaCTX-M-1, tetA, and sul1) to confirm their antibiotic resistance pattern (**Figure 3.11**). Depending on the antibiotic susceptibility test results, the selected 24 isolates showed antibiotic resistance against four groups of antibiotics (β-lactam, macrolide, tetracycline, and quinolone). However, the genes blaTEM-1, blaCTX-M-1, tetA, and sul1 were selected because they represent major antibiotic classes commonly associated with resistance in Enterobacteriaceae, particularly in diarrheal pathogens (e.g., *Salmonella* spp.). Based on the analysis, all 24 isolates exhibited the presence of the blaTEM-1 gene; 12 isolates contained blaCTX-M-1; 7 isolates demonstrated the presence of the tetA gene; and another 7 isolates exhibited the presence of the sul1 gene (**Figure 3.12**). A chord diagram illustrating all selected isolates in relation to the specific primers (invA, fimA, hilA, blaTEM-1, blaCTX-M-1, tetA, and sul1) is shown in **Figure 3.13**.

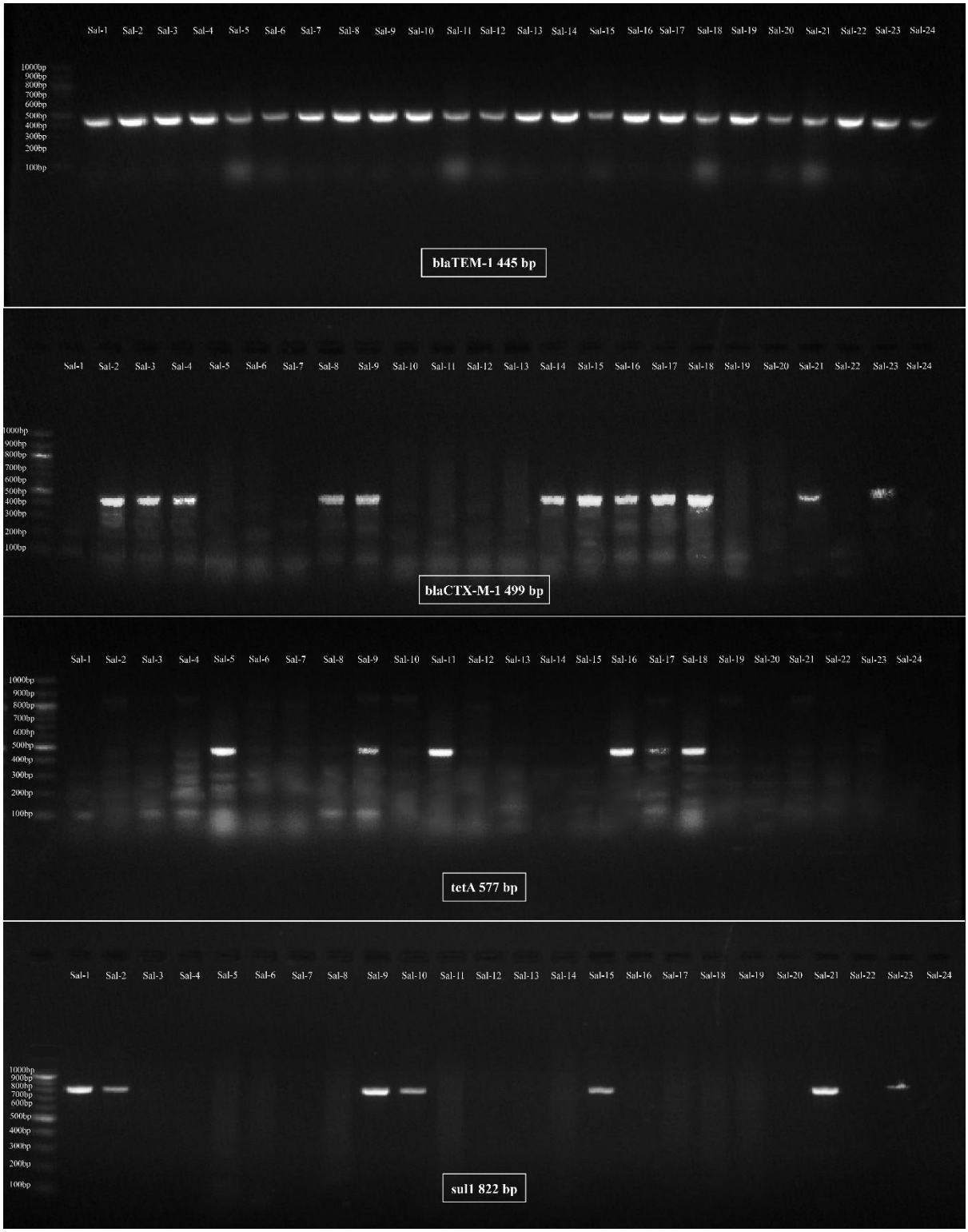

Figure 3.11: PCR detection of antimicrobial resistance genes (blaTEM-1, blaCTX-M-1, tetA, sul1) in the selected bacterial isolates.

Figure 3.12: A heatmap to identify the presence of different antibiotic resistance genes in the selected 24 isolates.

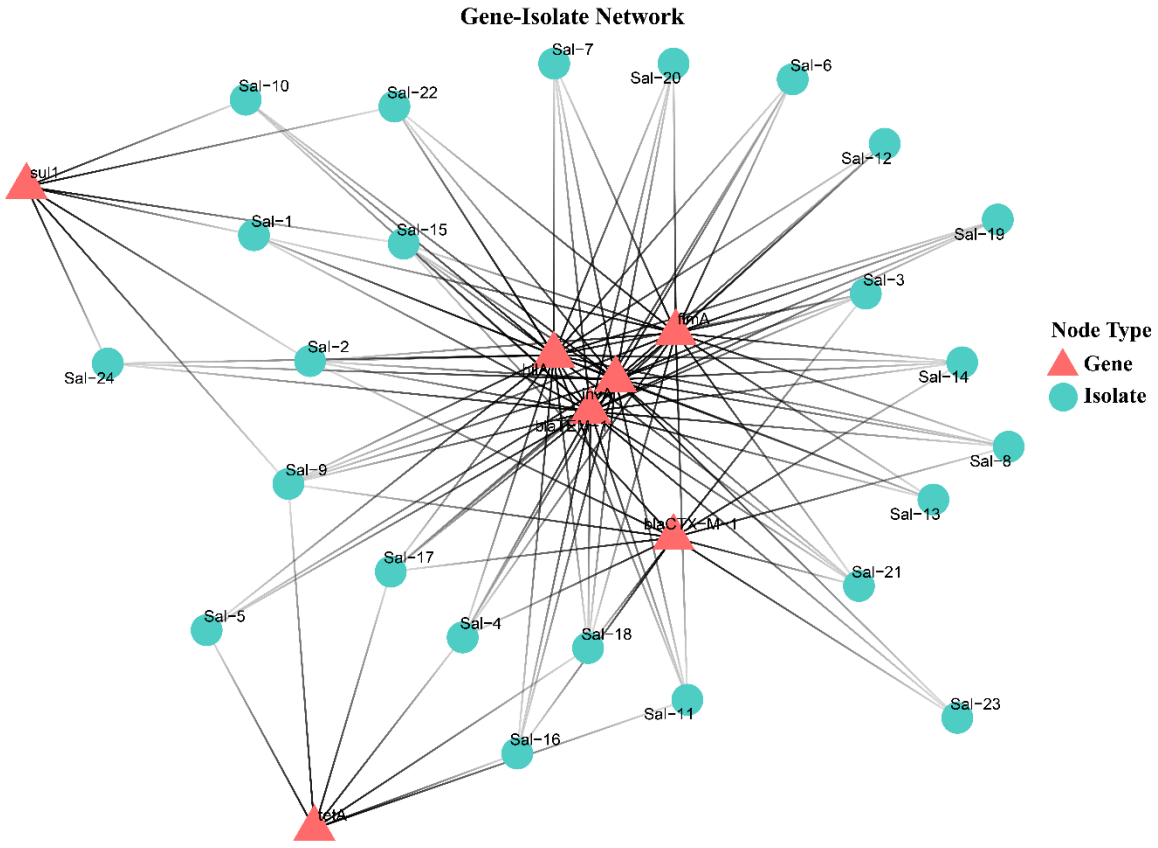

Figure 3.13: A gene-isolate network illustrating the presence of the specific genes among all the selected isolates.

3.4 Biofilm Forming Test

The quantitative assessment of biofilm formation among the 24 Salmonella isolates (**Figures 3.14** and **3.15**) revealed clear variability in their adherence capacity, as reflected by their measured OD values. According to the analysis, isolates exhibiting low OD values (Sal-2, Sal-

10, Sal-16, Sal-19) correspond to weak or minimal biofilm formation, suggesting limited ability to persist under stressful or nutrient-restricted conditions. Those with moderate OD values (Sal-20, Sal−11, Sal−17, Sal−9, Sal−22, Sal−13, Sal−14, Sal−7, Sal−24, Sal−1, Sal−12, Sal−21, Sal−23, Sal−6, Sal−18) indicate a moderate biofilm-forming capability, representing an intermediate level of environmental resilience. In contrast, isolates showing high or extreme OD values (Sal−4, Sal−5, Sal−8, Sal−15, Sal−3) demonstrate strong biofilm-forming potential, highlighting their enhanced capacity to survive, colonise surfaces, and withstand harsh conditions. The distribution of OD values for all isolates is summarised in **Figure 3.15.**

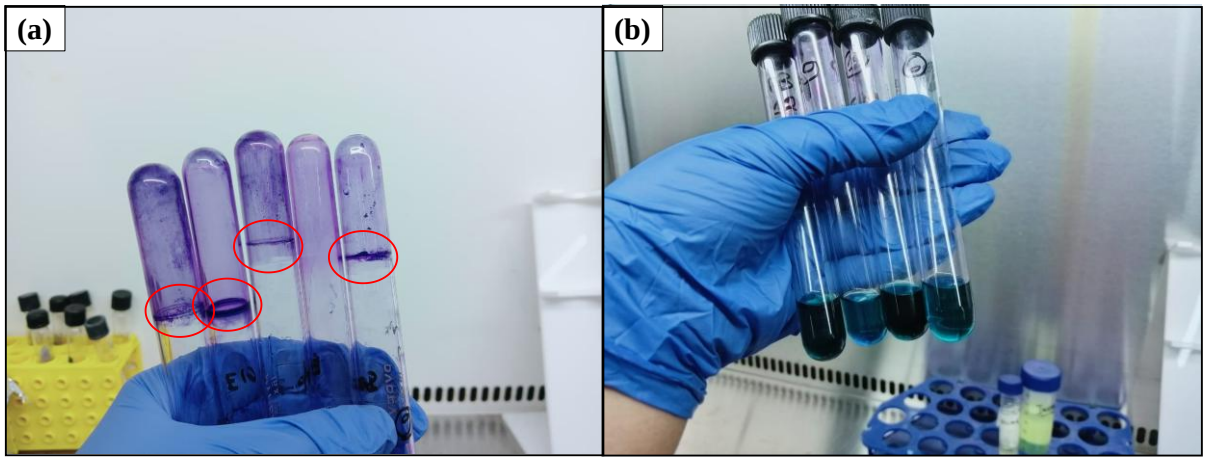

Figure 3.14: Biofilm-forming test. (a) Biofilm ring formation, (b) Different conc. of biofilm after adding glacial acetic acid.

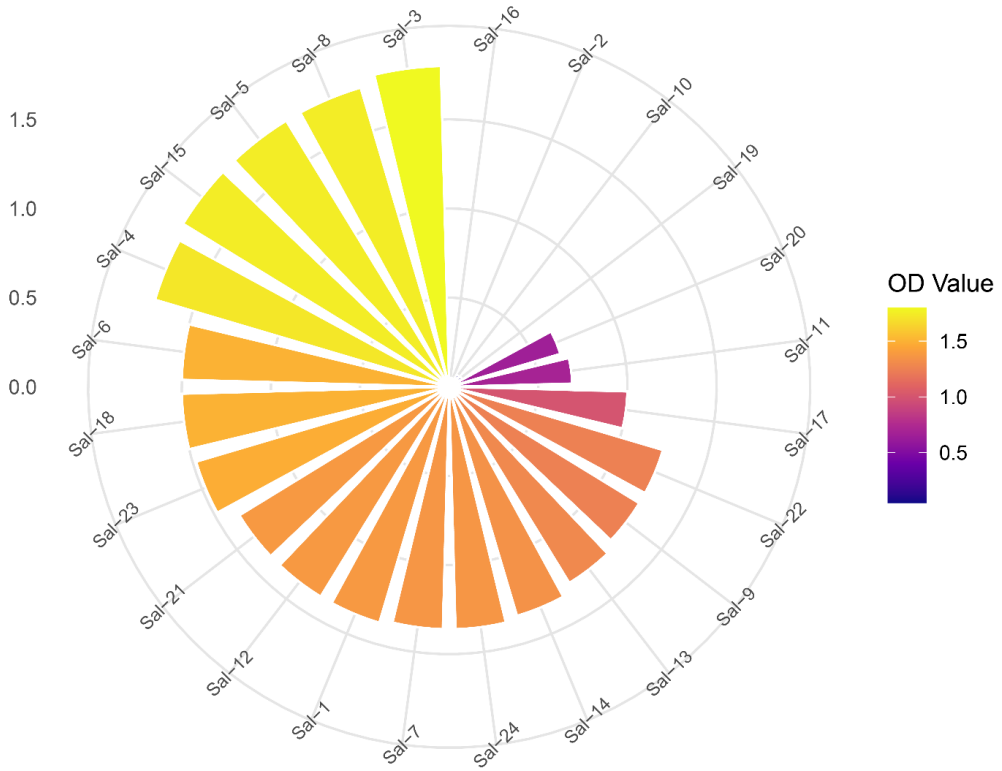

Figure 3.15: Radial visualisation showing optical density distribution of 24 selected isolates.

Chapter 4

Discussion

4. Discussion

This study provides important baseline data on the occurrence, pathogenic potential, and AMR profiles of *Salmonella* spp. isolated from commonly consumed fish sold in retail markets of Noakhali, Bangladesh. Fish constitutes a major source of animal protein for the Bangladeshi population, yet microbiological surveillance at the retail level remains limited. By integrating phenotypic identification, virulence gene profiling, antibiotic susceptibility testing, detection of resistance genes, and biofilm assessment, this work offers a comprehensive picture of the public health risks posed by foodborne *Salmonella*. The findings are particularly significant in the context of increasing antimicrobial use in aquaculture, weak enforcement of hygiene practices in wet markets, and the growing global concern over MDR foodborne pathogens. To our knowledge, this is among the first studies to report detailed associations between *Salmonella* virulence, AMR, and biofilm in retail fish markets in the Noakhali region, thereby filling a critical knowledge gap and supporting evidence-based food safety interventions.

The detection of *Salmonella* in 24% of analysed fish samples demonstrates a substantial level of contamination across multiple retail markets in Noakhali. This prevalence aligns with previous studies in Bangladesh and globally, which reported *Salmonella* occurrence in fish and shrimp at varying rates depending on sampling methods, environmental conditions, and market hygiene. For instance, Khan et al. (2024) documented pathogenic bacteria, including *Salmonella*, in retailed shrimp in Bangladesh [1], while Novotny et al. (2004) highlighted fish as an important vehicle for bacterial pathogens in several countries [13].

The presence of *Salmonella* across both freshwater and marine species in this study further supports earlier observations that fish often act as passive carriers due to faecal contamination of ponds, runoff from poultry farms, and poor sanitation in handling environments [61], [63]. Similarly, Ehetasum Hossain et al. (2018) reported widespread bacterial contamination in fish from cultured and natural ponds in the Noakhali region, reinforcing the role of environmental exposure in facilitating pathogen persistence [26].

Compared to studies in other regions, the prevalence observed here is higher than the 1.2–8.5% contamination rates reported in international surveys [62], suggesting regional factors such as inadequate cold-chain maintenance, poor infrastructure, and unregulated handling practices may contribute significantly. The findings highlight the urgent need for stricter hygiene practices at retail markets, as inadequate sanitation during processing has been repeatedly identified as a major route of *Salmonella* transmission in food supply systems [106], [110].

The study identified high frequencies of key virulence genes: invA (100%), hilA (95.8%), and fimA (79.2%), among 24 confirmed isolates. The universal presence of invA, a highly conserved marker associated with epithelial invasion, corroborates its well-established role as a reliable diagnostic indicator for *Salmonella* and its widespread occurrence in foodborne isolates [112].

The predominance of hilA, a transcriptional regulator in SPI-1 responsible for invasion and induction of host inflammatory responses, is comparable to findings from other environmental and foodborne studies where SPI-1 genes were consistently preserved, enabling rapid pathogenicity [113], [115]. High hilA prevalence suggests that retail fish isolates possess substantial invasive capability, underscoring a noteworthy pathogenic threat.

Similarly, the detection of fimA, involved in fimbrial adhesion, aligns with studies demonstrating its importance in attachment to host tissues and surfaces, enhancing environmental persistence [114]. Previous research has shown that foodborne *Salmonella* often retain fimbrial adhesion genes to survive processing and storage conditions [112].

The variability of virulence gene distribution among markets observed in this study may reflect differences in contamination sources, market sanitation practices, or environmental conditions. Such heterogeneity has been reported in other developing regions where fish markets differ significantly in hygiene infrastructure [4], [106].

Antibiotic susceptibility testing revealed worrying resistance trends among the *Salmonella* isolates. High resistance rates were observed against commonly used antibiotics, including ampicillin (75%), ceftazidime (100%), azithromycin (33.33%), tetracycline (29%), ciprofloxacin (25%), levofloxacin (50%), and ofloxacin (29%). In contrast, comparatively higher susceptibility was noted to cefepime, meropenem, cotrimoxazole, and ciprofloxacin, although reduced susceptibility to cefotaxime, imipenem, and doxycycline was also evident in some isolates.

The PCR analysis of antibiotic resistance genes revealed resistance to multiple antibiotic classes, including β-lactams, macrolides, tetracyclines, and quinolones. The universal detection of blaTEM-1 (100%) indicates widespread β-lactam resistance, a common issue in aquaculture environments where penicillin and early-generation cephalosporins are frequently used [36], [37].

Moreover, the presence of blaCTX-M-1 in 50% of isolates is particularly concerning, as extended-spectrum β-lactamase (ESBL)-producing *Salmonella* strains have been increasingly associated with treatment failures and outbreaks worldwide. Similar findings were reported by Rahman et al. (2018), who observed multidrug-resistant (MDR) *Salmonella* in milk and meat in Bangladesh [23]. The detection of tetA and sul1, both at 29.2%, is consistent with earlier reports showing that tetracycline and sulfonamide resistance genes are commonly selected for in aquaculture due to unregulated antibiotic use [36], [38], [41]. These results correspond with global patterns, where MDR *Salmonella* frequently emerge in food-producing animals exposed to prolonged antibiotic pressure [133], [135].

Comparative studies from other countries reveal similar challenges: for example, cephalosporin- and fluoroquinolone-resistant *Salmonella choleraesuis* strains were identified in swine in Taiwan and Thailand [125], while North Indian poultry farms reported widespread resistance to bacitracin and polymyxins [126]. The patterns observed in the present study are therefore reflective of a global AMR crisis in the food production sector.

The co-occurrence of virulence and resistance genes, as observed here, is particularly alarming because such combinations may facilitate more severe infections and increase the likelihood of horizontal AMR gene transfer within food supply chains [130].

All isolates in this study exhibited measurable biofilm-forming ability, with several classified as strong producers. Biofilms are known to enhance microbial survival by providing protection against disinfectants, desiccation, and antibiotic exposure. Previous studies have reported that biofilm-forming *Salmonella* strains can persist on fish surfaces, equipment, and market environments for extended periods, facilitating repeated contamination events [3], [4].

The identification of strong biofilm producers such as Sal-3, Sal-4, Sal-5, Sal-8, and Sal-15 mirrors findings from other studies where biofilm-forming capacity was associated with increased resistance to environmental stressors and greater difficulty in eliminating pathogens from retail surfaces [60], [110].

Furthermore, the coexistence of biofilm ability with virulence genes (invA, hilA, fimA) and AMR genes (blaTEM-1, blaCTX-M-1, tetA, sul1) presents a severe public health concern. Biofilms can act as reservoirs for ARG dissemination and provide ideal conditions for horizontal gene transfer, as noted in multiple reviews on bacterial persistence in aquatic environments [42], [112].

The detection of pathogenic, multidrug-resistant, and biofilm-forming *Salmonella* spp. in retail fish markets underscores significant risks for consumers. Fish is a staple protein source in Bangladesh, and contaminated seafood has been linked to outbreaks and international trade rejections. For example, seafood exports from Bangladesh, India, and Indonesia were rejected by the United States due to *Salmonella* contamination in 2018–2019 [47], [48].

However, domestic markets lack routine microbial screening, placing resource-limited consumers at disproportionate risk. This gap contrasts with strict international standards requiring zero *Salmonella* in 25 g of seafood [47]. Previous studies similarly reported high microbial loads in locally consumed fish due to inadequate hygiene practices, reflecting broader challenges in Bangladesh's food safety management system [26], [106]. Additionally, unregulated antibiotic use in aquaculture contributes to AMR development, which can spread to human pathogens through consumption or environmental pathways. Given that retail markets often lack sufficient sanitation, the persistence of MDR *Salmonella* in these environments poses a continuous threat to community health.

Although the study achieved comprehensive phenotypic and PCR-based characterisation, further genomic resolution is necessary to fully elucidate the epidemiology of circulating *Salmonella* strains. Serotyping and whole genome sequencing (WGS) could not be conducted due to time and funding limitations, similar to constraints noted in other resource-limited studies. WGS would enable detailed analysis of ARG mobility, plasmid profiles, virulence evolution, and phylogenetic relatedness, which are the key elements for effective source attribution and integration with global surveillance initiatives [117], [118].

Future work incorporating WGS, broader sampling, and environmental source tracking would strengthen conclusions and improve understanding of *Salmonella* transmission dynamics in Noakhali’s aquaculture supply chain.

Due to limited funding resources and the strict timeframe, it was not possible to perform serotyping or WGS within this project. However, these analyses are planned as part of the next phase of the research. Generating high-resolution genomic data will allow for more accurate source tracking, risk assessment, and integration with global *Salmonella* surveillance datasets. Such work will be crucial to understanding whether the isolates circulating in retail fish markets represent known epidemic clones or emerging strains carrying novel virulence or resistance determinants.

Chapter 5

Conclusion

5. Conclusion

This study revealed the presence of pathogenic, multidrug-resistant, and biofilm-forming *Salmonella* spp. in retail fish markets of Noakhali, Bangladesh, underscoring critical risks to food safety and public health. The detection of virulence genes (invA, hilA, fimA) together with AMR genes (blaTEM-1, blaCTX-M-1, tetA, sul1) highlights the widespread circulation of strains capable of causing severe infections and resisting multiple antibiotic classes. The ability of many isolates to form moderate to strong biofilms further enhances their environmental persistence and potential for cross-contamination within markets.

Although comprehensive characterisation was achieved through phenotypic and PCR-based methods, future work will include serotyping and whole genome sequencing (WGS) to more accurately elucidate the genetic relationships, ARG dissemination patterns, and epidemiological significance of these isolates. These advanced analyses were not feasible within the current study due to constraints in funding and the limited timeframe for thesis completion, but they remain a key priority for the continuation of this research.

Overall, the findings highlight the urgent need for improved hygiene in fish handling, stringent monitoring of antimicrobial use in aquaculture, and implementation of national surveillance programs to control the spread of antimicrobial-resistant *Salmonella*. Strengthening regulatory frameworks and raising consumer awareness will be essential steps toward ensuring safe fish consumption and protecting public health.

Chapter 6

References

6. References

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Chapter 7

Appendices

Appendix -1

Unless otherwise specified, all media, except Hektoen Enteric Agar and Salmonella-Shigella agar, were sterilised by autoclaving at 121 °C for 15 minutes at 15 lbs pressure. Double-distilled water was used to prepare all media. The media used in this thesis are given below:

Hektoen Enteric Agar (OXOID)

Ingredient Name	Amount (g/L)
Peptones	15.0
Lactose	14.0
Sucrose	14.0
Salicin	2.0
Bile salt mixture	2.0
Sodium chloride	5.0
Sodium thiosulphate	5.0
Ammonium iron (III) citrate (Ferric ammonium citrate)	1.5
Bromothymol blue	0.05
Acid fuchsin	0.08
Yeast extract	3.0
Agar-agar	13.5

***Salmonella-Shigella* Agar (HiMedia)**

Ingredient Name	Amount (g/L)
Beef Extract (or HM peptone B)	5.000
Peptic Digest of Animal Tissue / Peptone	5.000
Agar	13.5 – 15.0
Bile Salts Mixture	8.500
Sodium Citrate	10.000
Brilliant Green	0.00033
Lactose	10.000
Sodium Thiosulphate	8.500
Ferric Citrate	1.000
Neutral Red	0.025

Nutrient Agar (HiMedia)

Ingredient Name	Amount (g/L)
Peptone	5.0
HM Peptone B	1.5–3.0
Yeast Extract	1.5
Sodium Chloride (NaCl)	5.0
Agar	15.0

Buffered Peptone Water (HiMedia)

Ingredient Name	Amount (g/L)
Enzymatic digest of casein	10.0
Sodium chloride	5.0
Disodium hydrogen phosphate (dodecahydrate)	9.0
Potassium dihydrogen phosphate	1.5
Final pH (at 25°C)	7.0 ± 0.2

Rappaport-Vassiliadis Broth (OXOID)

Ingredient Name	Amount (g/L)
Soya Peptone	4.5 - 5.0
Sodium Chloride	7.2 - 8.0
Potassium Dihydrogen Phosphate	1.26 - 1.6
Di-potassium Hydrogen Phosphate	0.18
Magnesium Chloride	13.58
Malachite Green	0.036 - 0.04

Mueller Hinton Agar (OXOID)

Ingredient Name	Amount (g/L)
Beef Infusion	300
Casein Hydrolysate	17.5
Starch	1.5
Agar	17.0

Triple Sugar Iron Agar (OXOID)

Ingredient Name	Amount (g/L)
Peptone	20.0
Yeast extract	3.0
Meat extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Sodium chloride	5.0
Ferrous sulphate, heptahydrate	0.2
Sodium thiosulphate, pentahydrate	0.3
Phenol red	0.024
Agar	12.0

Motility Indole Urease Agar (HiMedia)

Ingredient Name	Amount (g/L)
Casein/Tryptone Hydrolysate	10
Sodium Chloride	5
Potassium Dihydrogen Phosphate	5

Dextrose	1
Agar	2–3
Phenol Red	0.01
Urea (supplement, added separately)	40% urea solution

Simmon-Citrate Agar (HiMedia)

Ingredient Name	Amount (g/L)
Sodium Citrate	2.0
Agar	15.0
Sodium Chloride (NaCl)	5.0
Ammonium Dihydrogen Phosphate	1.0
Dipotassium Phosphate	1.0
Magnesium Sulfate ($MgSO_4$)	0.2
Bromothymol Blue	0.08

Methyl Red-Voges Proskauer Media (HiMedia)

Ingredient Name	Amount (g/L)
Peptic digest of animal tissue	5
Dextrose	5
Dipotassium phosphate	5
Final pH (at 25°C)	7.5±0.1

Lysine Iron Agar (OXOID)

Ingredient Name	Amount (g/L)
Bacteriological peptone	5.0
Yeast extract	3.0
Glucose	1.0
L-lysine	10.0
Ferric ammonium citrate	0.5
Sodium thiosulphate	0.04
Bromocresol purple	0.02
Agar	14.5

Tryptone Soya Broth (HiMedia)

Ingredient Name	Amount (g/L)
Tryptone (pancreatic digest of casein)	17.0
Soya peptone (papaic digest of soybean meal)	3.0
Sodium chloride	5.0
Dextrose (Glucose)	2.5
Dipotassium hydrogen phosphate	2.5

Appendix -2 (Reagents)

TAE Buffer

242 g of tris-base, 57.1 ml of glacial acetic acid, and 100 ml of 0.5 M EDTA (pH 8.0) were combined, and distilled water was added to prepare 1 L. 1X concentrated TAE buffer was prepared by adding 10 ml of 10X TAE buffer to 450 ml of distilled water and stored at room temperature.

Ethidium Bromide (EtBr) Solution

10 µl of ethidium bromide was dissolved in 100 ml of TAE buffer to prepare a final concentration of 20 mg/ml and stored at 4 °C in the dark.

0.5M EDTA

186.1 g of $Na_2EDTA.2H_2O$ and 20.0 g of NaOH pellets were added and dissolved by stirring 800 mL of distilled water on a magnetic stirrer. The pH was adjusted to 8.0 with a few drops of 10 M NaOH, and the final volume was made up to 1 L with distilled water. The solution was sterilised by autoclaving and stored at room temperature.

Wizard® SV Gel and PCR Clean-Up System. Catalog No. A9282

Reagent	Purpose
Membrane Binding Solution	Helps in the binding of the PCR product
SV Minicolumn	For the binding of the PCR product
Collection Tube	For the collection of flow-through
Membrane Wash Solution	For washing purposes
Nuclease-Free Water	For the elution of purified DNA from the GD column
SYBR Green Master Mix	Used for real-time PCR amplification and fluorescence detection

Appendix -3

The important instruments and apparatus used in the study are listed below:

Instrument	Origin
ABI Prism 3130 Genetic Analyser	Applied Biosystems, USA
AlphaImager HP System (Versatile Gel Imaging)	Cell Bioscience, USA
Autoclave, Model HL-42AE	Hirayama Corp., Japan
Microcentrifuge (temperature-controlled)	Sigma, USA
Class II Microbiological Safety Cabinet	Nuaire, USA
Electric Balance, Scout SC4010	Shimadzu, Japan
Freezer (–30°C)	Liebherr, Germany
Horizontal Gel Electrophoresis Apparatus HI-SET	CBS Scientific, UK
Incubator	Japan
Microcentrifuge	Mikro 20, Germany
Microcentrifuge Tube	Eppendorf, Germany
Micropipettes	Eppendorf, Germany
Microwave Oven, Model D90N30 ATP	Butterfly, China
NanoDrop 2000	Thermo Scientific, USA
Thermal Cycler	Biometra, Germany
Thermal Cycler (Veriti 96 well)	USA
Thermal Cycler (ProFlex PCR System)	USA
pH Meter, Model MP220	Toledo, Germany
Power Pack	Vestfrost
Refrigerator (4°C)	Gerhardt, Germany
Room Temperature Horizontal Shaker	Japan
Steriliser, Model NDS-600D	England
Water Bath, Model SUM	Nuaire, USA
–80°C Freezer	Promega, USA
Maxwell® 16 Instrument	Promega, USA

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13	Mohammad Sharif Uddin, Md. Habib Ullah Masum, Md. Razib Hosen, Suhag Chandra Roy et al. " Multidrug Resistance and Virulence Gene Profiles of in Broiler Chickens: A Study From Noakhali, Bangladesh ", Veterinary Medicine International, 2025 Publication	<1%

*% detected as AI

AI detection includes the possibility of false positives. Although some text in this submission is likely AI generated, scores below the 20% threshold are not surfaced because they have a higher likelihood of false positives.

Caution: Review required.

It is essential to understand the limitations of AI detection before making decisions about a student's work. We encourage you to learn more about Turnitin's AI detection capabilities before using the tool.

Frequently Asked Questions

How should I interpret Turnitin's AI writing percentage and false positives?
The percentage shown in the AI writing report is the amount of qualifying text within the submission that Turnitin's AI writing detection model determines was either likely AI-generated text from a large-language model or likely AI-generated text that was likely revised using an AI paraphrase tool or word spinner.

False positives (incorrectly flagging human-written text as AI-generated) are a possibility in AI models.

AI detection scores under 20%, which we do not surface in new reports, have a higher likelihood of false positives. To reduce the likelihood of misinterpretation, no score or highlights are attributed and are indicated with an asterisk in the report (*%).

The AI writing percentage should not be the sole basis to determine whether misconduct has occurred. The reviewer/instructor should use the percentage as a means to start a formative conversation with their student and/or use it to examine the submitted assignment in accordance with their school's policies.

What does 'qualifying text' mean?
Our model only processes qualifying text in the form of long-form writing. Long-form writing means individual sentences contained in paragraphs that make up a longer piece of written work, such as an essay, a dissertation, or an article, etc. Qualifying text that has been determined to be likely AI-generated will be highlighted in cyan in the submission, and likely AI-generated and then likely AI-paraphrased will be highlighted purple.

Non-qualifying text, such as bullet points, annotated bibliographies, etc., will not be processed and can create disparity between the submission highlights and the percentage shown.