Molecular Characterization, Antimicrobial Resistance, Biofilm Formation, and Whole-Genome Sequencing of *Vibrio* spp. Isolated from Shrimp in Noakhali, Bangladesh

M.S. Thesis

A dissertation submitted to the Department of Microbiology, Noakhali Science and Technology University in partial fulfillment of the requirement for the Degree of Master of Science in Microbiology

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CERTFICATE OF APPROVAL

This is to certify that the research work as thesis entitled "**Molecular Characterization, Antimicrobial Resistance, Biofilm Formation, and Whole-Genome Sequencing of *Vibrio* spp. Isolated from shrimp in Noakhali, Bangladesh**" submitted by Sajedul Islam bearing Roll: MUH2305MS103M, Session: 2022-2023 to the Department of Microbiology, Noakhali Science and Technology University, in the partial fulfilment of the requirement for the degree of Master of Science in Microbiology, is the original work and has been carried out under my supervision. I have gone through all the data and materials reported in this manuscript and certify their authenticity and correctness.

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DECLARATION

I hereby declare that the thesis entitled " **Molecular Characterization, Antimicrobial Resistance, Biofilm Formation, and Whole-Genome Sequencing of *Vibrio* spp. Isolated from shrimp in Noakhali, Bangladesh.**" has been submitted in partial fulfillment of the requirements for the Master of Science degree in Microbiology from Noakhali Science and Technology University. This research was conducted in the Microbiology laboratory at Noakhali Science and Technology University and has not been submitted for any other degree elsewhere.

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Abstract

Shrimp aquaculture is a vital economic sector in Bangladesh. *Vibrio* species are common foodborne pathogens that cause gastrointestinal tract inflammation. Multidrug resistance (MDR) in *Vibrio* spp. is a major health concern, especially in aquaculture and food chain. This research examined the prevalence, antimicrobial resistance (AMR), virulence characteristics, and biofilm-forming ability of *Vibrio* species in different retail shrimp samples from Noakhali, Bangladesh. A total of 241 shrimp samples were collected and studied through culture, molecular and whole-genome sequencing. *Vibrio* species were identified in 43.6% of samples, with 29.5% *V. parahaemolyticus* and 14.1% *V. cholerae*. Alarmingly, 70.5% of *Vibrio* isolates were multidrug-resistant (MDR) including significant resistance to ampicillin (88.6%), meropenem (81.9%), cefotaxime (71.4%), and ceftazidime (61.0%). Genotypic screening suggested the presence of ESBL genes bla-TEM (29.5%) and bla-SHV (10.6%). Biofilm production was positive in 62.9% of isolates, exhibiting interlink between biofilm strength and multidrug resistance. Virulence gene profiling demonstrated that all *V. cholerae* contain hlyA El Tor gene and tlh was prevalent in V. parahaemolyticus. Whole-genome sequencing of two severe MDR *V. parahaemolyticus* isolates identified a comprehensive array of antibiotic resistance genes, virulence factors, and genetic determinants associated with biofilm formation. These results underscore the intersection of pathogenicity, antibiotic resistance, and biofilm development in *Vibrio* species throughout the shrimp supply chain, constituting a significant public health issue. The study demonstrates the necessity for improved antimicrobial control, elevated hygiene standards, and ongoing surveillance to reduce public health hazards linked to contaminated seafood in Bangladesh.

Keywords: *Vibrio* species, *Vibrio parahaemolyticus*, *Vibrio cholerae*, shrimp, aquaculture, antimicrobial resistance (AMR), multidrug resistance (MDR), biofilm formation, virulence genes, extended-spectrum beta-lactamase (ESBL), whole-genome sequencing.

Chapter 1
Introduction

1. Introduction

The global shrimp aquaculture business is one of the fastest-growing areas in food production. It is an important source of animal protein, jobs, and export income for many developing countries, such as Bangladesh. Shrimp is a big part of the fishing industry, and it helps the economy a lot through both domestic consumption and commerce with other countries [1]. Bangladesh is the third best country in the world for capturing fish in open water and the fifth best for aquaculture productivity. The freshwater scampi (*Macrobrachium rosenbergii*) and the black tiger (*Penaeus monodon*) are the two types of shrimp that make the most money for our country when they are exported, showing how important shrimp are to our economy [2]. But bacterial contamination in prawns and other marine foods is still a concern that hurts both food safety and public health. A number of human foodborne illness cases have been linked to eating contaminated fresh-raw prawns, oysters, and other seafood [3]. When prawns are cultivated, processed, preserved, and stored under unsanitary conditions, they may become infected. Despite being a valuable food source with high nutritional benefits from proteins, vitamins, and minerals, eating seafood that contains human pathogenic bacteria may make one more susceptible to foodborne illness [4]. Seafood species, storage conditions, postharvest handling and processing during marketing, harvest location, environmental factors, and eating habits such as eating raw or minimally processed fish—all influence the likelihood of contracting a seafood-related illness. Metals, parasites, viruses, pathogenic bacteria, chemicals, and natural poisons are all present in seafood [5]. Among the bacterial diseases of concern, *Vibrio* species occupy a pivotal position because of their ubiquity in marine and estuarine habitats and pathogenic potential in humans and aquatic creatures [6].

Vibrio species are gram-negative, motile, facultatively anaerobic, curved rod-shaped bacteria that belong to the Vibrionaceae family that range in length from 1.4 to 2.6 μm and width from 0.5 to 0.8 μm. With a large terminal flagellum that propels them through the water, they are extremely mobile [7]. They are all oxidase positive and do not produce microcysts or endospores. They are facultative anaerobes that can metabolize both fermentatively and aerobically but they don't fix or nitrify molecular nitrogen. The majority of them can thrive on a mineral medium that contains ammonium chloride and D-glucose since they are all chemoorganotrophs. Ions of sodium promote the growth of all organisms. Glycerol, fructose, and maltose can all be used by them without

producing gas [8]. They are halophilic and frequently found in brackish and marine waters, where they coexist alongside plankton, fish, shellfish, and sediments. Some species are natural flora of aquatic animals, while others are harmful to humans and marine life. *Vibrio* infections in humans are frequently linked to eating raw or undercooked seafood, especially prawns, oysters, and other shellfish [9]. *Vibrio* species can be isolated using a variety of specialized selective bacteriological culture media such as Sucrose tellurite petal (STP) medium, taurocholate tellurite gelatin agar (TTGA), thiosulfate citrate bile salt sucrose agar (TCBS), and others are examples of these media. Certain non-selective plating media, like trypticase soy agar (TSA) and gelatin agar (GA), are also utilized for the development of *Vibrios* [10]. They do not ferment sucrose, *V. parahaemolyticus* and *V. mimicus* develop as green colonies on TCBS, but *V. cholerae* and *V. alginolyticus*, which ferment sucrose, form yellow colonies. This was unlikely because even *Vibrio* species had very diverse ideal growth circumstances (pH, salinity, and temperature). As an illustration, *V. vulnificus* can survive and thrive at temperatures ranging from 4 to 14°C, but it grows best at 25°C and low salinity (1-2%). Although *V. cholerae* could grow at 35°C and 10% salinity, it thrived at somewhat lower temperatures (30°C) and greater salinities (15%) [11]. *V. parahaemolyticus* requires a minimum temperature of 13°C to grow in broth, however other studies contend that a temperature as low as 5°C is enough [12]. *V. alginolyticus* strains showed broad temperature and salinity tolerance, resulting in growth at all measured temperatures (24°C–40°C) and salinities between 1% and 6% (wt/vol) NaCl with optimal growth between 30°C–36°C and 2%–4% NaCl [13].

A diverse array of products, such as hemolysins, proteases, biofilm formation factors, phospholipases, cytotoxins, quorum sensing molecules, siderophores, and phage presence, contribute to the pathogenicity of *Vibrio* species [14], [15], [16]. The swarming movement of *Vibrio* is associated with its virulence, while hemolysin is the most significant virulence factor identified in *Vibrio*, linked to both human and fish infections [17]. Ten Prior research identified several virulence genes, including Outer Membrane Protein (OMP), thermolabile hemolysin (TLH), toxR, collagenase, and cholera toxin, that enhance the pathogenicity of the organisms. Collagenase was extensively utilized in the molecular identification of *V. alginolyticus*, as it may degrade the basal epithelial membrane and connective tissue, resulting in extra-intestinal disease and diffusion into the bloodstream [18]**.** Eleven Minimum Twelve *Vibrio* species have been identified as pathogenic strains that cause foodborne illnesses. *V. parahaemolyticus*, in conjunction with other *Vibrio* species, accounts for 25.00% of all foodborne illnesses [19]. *V. parahaemolyticus*

generates hemolysin as an enterotoxin that causes lysis of blood cells in the infected species. The tlh gene encodes a thermolabile hemolysin, serving as a particular identifier for the identification of *V. parahaemolyticus* species [20]. *Vibrio parahaemolyticus* possesses numerous virulence factors, including thermostable direct hemolysin (tdh), TDH-related hemolysin (trh), and adhesin. The thermostable direct hemolysin, encoded by the tdh gene, is a significant virulence factor in *V. parahaemolyticus* [21]. Fourteen The etiology of cholera is intricate and involves multiple genes, including ctx, zot, tcp, and rfbO1. These genes serve as indicators of virulence. However, cholera toxin is the most critical pandemic marker [22]. Fifteen The identification of *Vibrio* species relies mostly on their physical and biochemical traits, which are validated by PCR.

The two main categories of human illnesses brought on by pathogenic bacteria of the *Vibrio* genus are cholera and non-cholera infections [23]. The causative agent of cholera is *V. cholerae*, a severe diarrheal sickness that is mostly brought on by consuming tainted food or water, but person to-person transmission is also a possible. Notably, fresh water also harbors *V. cholerae*. Not cholera-related *Vibrio* species, notably *Vibrio parahaemolyticus* and *Vibrio vulnificus*, cause vibriosis, a collection of disorders with varying clinical presentations according on the pathogen species, infection route, and host susceptibility [24]. While exposure to contaminated water can result in wound infection and subsequent septicemia, non-cholera bacterial ingestion can induce mild gastroenteritis or primary septicemia, which is caused by eating raw or undercooked infected food. Non-cholera *Vibrio* species are prevalent in seafood and seawater and inhabit environments with moderate to high salinity. The most significant environmental human infections that come from aquatic and marine environments are these microorganisms [23]. *Vibrio* spp. differ in their modes of transmission to humans; non-cholera *Vibrio* spp., including *Vibrio parahaemolyticus*, *Vibrio vulnificus,* and *Vibrio alginolyticus* are a major and expanding source of diseases transmitted by contaminated seafood and direct contact with water. *V. cholerae*, the cause of cholera, is unique in that it has both human and environmental phases in its life cycle [25]. *V. cholerae* can be found in fresh water as a free-living organism, in biofilms formed by numerous cells, or in conjunction with plankton. *V. cholerae* can infect humans directly through polluted water [26], [27]. Unlike other pathogenic non-cholera *Vibrio* spp., *V. cholerae* spreads from person to person via the feacal-oral pathway. *Vibrio cholerae* begins to express genes that encode virulence factors such as toxin-co-regulated pilus (Tcp) and cholera toxin once it enters the human host and reaches the small intestine. Cholera toxin is composed of two subunits, CtxA and CtxB, which bind to the

ganglioside (a sialylated glycosphingolipid) GM1 on enterocyte plasma membranes through the pentameric CtxB subunit. Bound cholera toxin is endocytosed and then transported retrograde to the endoplasmic reticulum (ER), where the subunits split. The release of the enzymatic CtxA subunit from the ER into the cytosol enables allosteric activation by ADP ribosylation factor 6 (ARF6). The ARF6-bound and active CtxA subunit then catalyses the ADP ribosylation of a G protein-coupled receptor, so activating adenylyl cyclase. Increased cellular cAMP levels activate protein kinase A (PKA)-dependent phosphorylation (P) of the cystic fibrosis transmembrane receptor (CFTR), which promotes ion and water efflux into the small intestine lumen, resulting in diarrhoea [23]. *Vibrio parahaemolyticus, Vibrio vulnificus,* and *Vibrio cholerae* are widespread pathogenic bacteria that can cause symptoms ranging from mild gastroenteritis to severe septicemia and cholera-like sickness. The *Vibrio* genus has over 100 species, many of which live freely in marine and estuarine habitats. Environmental factors including temperature, salinity, and nutrition availability affect their distribution and abundance [28]. Warmer temperatures, in particular, promote *Vibrio* growth and are frequently connected with seasonal peaks in *Vibrio*-related infections. In aquatic habitats, *Vibrio* species create symbiotic or commensal interactions with a variety of marine organisms such as prawns, molluscs, and plankton. These interactions facilitate their survival and transmission through the food web [29].

Shrimp are an extremely beneficial fish because they are high in omega-3 fatty acids, essential amino acids, and high-quality protein. However, it can also act as a reservoir or vector for harmful germs. Dangerous bacteria, particularly *Vibrio* species, can spread and get contaminated in local markets as a result of improper handling, unsanitary processing, and poor storage [30]. *Vibrio*-related infections are significantly more common in tropical and subtropical regions, where high temperatures promote bacterial development. The situation is particularly relevant in Bangladesh, where prawns are regularly picked from coastal aquaculture farms and sold in local markets without sufficient cold-chain facilities. Several investigations have detected *Vibrio* species in prawn samples from retail markets, raising concerns about seafood-related illnesses [31]. Additionally, the widespread and usually unregulated use of antibiotics in aquaculture techniques for disease prevention and growth promotion has contributed to the emergence of multidrug-resistant bacterial species, such as *Vibrio* spp., in the aquatic environment [32].

Vibrio species have the ability to colonise shrimp's gills, exoskeleton, and digestive tract in shrimp farming systems. Diseases like vibriosis, which is characterised by lethargy, decreased eating, and high mortality rates in prawn larvae and juveniles, can be brought on by pathogenic strains. A strain can still be detrimental to human health if it has virulence or antibiotic resistance genes. So, finding *Vibrio* in prawns that people eat is a very important evidence of public health threat [33]. Bacterial infections in aquatic animals are a major hazard to shrimp farming since they often lead to production losses. There are a variety of reasons why it is important to keep an eye on Vibrio species in prawns. First, it helps figure out how safe marine products are from bacteria and what the risks of becoming sick from food are. It also explains how pathogenic and antibiotic-resistant *Vibrio* strains are spread and how common they are in water. Third, it makes it easier to come up with effective plans for managing and reducing problems in the prawn supply chain, from farm to market [34]. So, finding and describing *Vibrio* species in prawns supplied in Bangladesh will assist the country improve its requirements for food safety and public health monitoring [35]. With the growing global concern over antibiotic resistance and environmental contamination, studies that focus on separating, identifying, and characterizing *Vibrio* species from seafood are becoming more and more important. This kind of research helps us learn more about how microbes interact in water and also supports the formulation of rules about how to use antibiotics and keep food safe [36].

Antibiotic resistance in *Vibrio* species is one of the most pressing concerns for aquatic microbiology and public health. Antibiotics have been used in aquaculture for a long time to help prawns and fish develop faster, cut death rates, and stop bacterial infections in production systems. However, using these compounds too often and carelessly has led to the growth of antibiotic-resistant bacteria in water habitats [37]. In aquaculture systems, antibiotics are often supplied through feed or added directly to the water. This means that the organisms that are supposed to take them in don't fully take them in. As a result, large amounts of these substances are discharged into the environment, where they selectively affect microbial ecosystems and encourage the horizontal spread of resistance genes [38]. Antibiotic resistance genes (ARGs), which can be dispersed throughout bacterial populations by mobile genetic elements like plasmids, transposons, and integrons, are thus stored in and transported by the aquatic environment [39]. Many *Vibrio* species have become multidrug resistant (MDR) to a variety of antibiotics, including as aminoglycosides, tetracyclines, fluoroquinolones, and β-lactams. For instance, *Vibrio vulnificus*

and *Vibrio cholerae* have shown resistance to several types of antimicrobial drugs, but *Vibrio parahaemolyticus* has showed resistance to ampicillin and streptomycin. It is concerning that these resistant strains of shellfish exist because they could reduce the effectiveness of widely used antibiotics in clinical settings [40]. Antibiotics like erythromycin, ciprofloxacin, chloramphenicol, and oxytetracycline are frequently used in prawn aquaculture in Bangladesh. The danger of antibiotic residues and resistant microorganisms getting into the food chain is increased when there are lax laws and surveillance systems in place. Research carried out in nearby prawn markets has shown that *Vibrio* strains resistant to several antibiotics are common, suggesting a new public health risk. The necessity of using antibiotics responsibly and keeping an eye out for resistance patterns in aquaculture settings are both highlighted by these findings [41].

Antibiotic resistance in *Vibrio* species may arise through intrinsic mechanisms or acquired genetic modifications. Intrinsic resistance involves inherent structural or functional traits that prevent antibiotic action, such as impermeable outer membranes or efflux pumps that expel antibiotics from bacterial cells. Acquired resistance, on the other hand, often results from mutations in chromosomal genes or the acquisition of resistance determinants through horizontal gene transfer. Limiting uptake of a drug due to permeability or impermeability of the outer membrane or cell wall can hinder the entry of antibiotics and also reduce permeability of antibiotics [42]. The efflux pump proteins use energy from ATP or transmembrane ion gradients to keep the antibiotic substances out of the bacterial cell before it can reach its target site and exert its effect [43]. Mutation and modification in the target of the antimicrobial agent, which reduces the binding of the antimicrobial agent [44]. *Vibrios* can also cause inactivation of drug either chemically modify the antibiotic by adding a chemical group to the scaffolds or hydrolyze the main drug structure with enzymes to eliminate antibiotic activity [45]. 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. Antibiotic resistance may also occur through one of several genetic mechanisms, including transformation, conjugation, or transduction [46], [47]. The emergence of antibiotic-resistant *Vibrio* species is dangerous for two reasons: it makes treating human infections caused by *Vibrio* more difficult and adds to the larger worldwide issue of antimicrobial resistance (AMR). AMR is one of the top ten global health challenges, according to the World Health Organization (WHO), which highlights

the value of integrated strategies like the One Health framework to combat the evolution of resistance across human, animal, and environmental interfaces (WHO 2021).

Biofilms are organized microbial communities covered in an extracellular polymeric material (EPS) that they produce on their own. Bacteria are protected by biofilm development against host immunological responses, environmental stressors, and antimicrobial treatments. *Vibrio* species that form biofilms are becoming more problematic due to the emergence of antibiotic resistance, which poses serious risks to aquaculture [48]. Antibiotics do not effectively treat bacterial infections that result in the production of biofilms. Bacterial cells aggregate on the host surface to create biofilms, which are made of a slime layer matrix and extracellular polysaccharides (EPS) that the bacteria produce on their own. The EPS matrix keeps external substances like antibiotic chemicals from entering the biofilm, which is more resilient than planktonic cells. As a result, using market antibiotics to eradicate biofilm is extremely difficult [49]. This form of development enables *Vibrio* populations to survive on a variety of biotic and abiotic surfaces, such as prawn shells, aquaculture equipment, and processing facilities. *Vibrio* spp. biofilm production occurs in multiple stages, including initial adhesion, microcolony formation, maturation, and dispersion. The process is controlled by quorum sensing (QS), a cell-to-cell communication system mediated by signaling molecules known as autoinducers. *Vibrio* cells use QS to coordinate gene expression in biofilm growth, virulence factor synthesis, and environmental adaption. Multiple quorum-sensing circuits work together to govern virulence and biofilm formation in *Vibrio cholerae*. *V. cholerae*, unlike other bacterial pathogens, inhibits virulence factor production and/or biofilm formation at high cell density in the presence of quorum-sensing autoinducers [50]. Numerous investigations have established a favourable relationship between *Vibrio* species' biofilm development and antibiotic resistance. This relationship is caused by intricate and multifaceted mechanisms [51]. Resistance cells within biofilms can act as genetic reservoirs, and the high cell density and close closeness of biofilm environments promote the horizontal transfer of resistance genes. Furthermore, biofilm development can be induced by exposure to sub-inhibitory antibiotic doses, generating a feedback loop that supports the evolution of resistance and bacterial survival [52].

The ability of *Vibrio* species to establish strong biofilms on biotic and abiotic surfaces significantly increases their risk to public health and seafood safety: Biofilms easily form on storage containers, display trays, ice, cutting boards, and other food-contact surfaces in shrimp processing and retail

settings. These persistent reservoirs continuously seed products with pathogenic cells and are frequently resistant to regular cleaning and disinfection [53]. When biofilms reach maturity, they shield embedded *Vibrio* cells from physical removal and chemical sanitizers, allowing populations to persist in between processing cycles and raising the risk of spoiling and cross-contamination [54]. Crucially, biofilm communities have the ability to release cells or cell aggregates that spread into nearby water or food matrices, increasing human exposure and extending contamination chains. This spread is linked to an increased risk of infection when infected seafood is eaten raw or undercooked [55]. Biofilm-grown cells exhibit decreased susceptibility to antimicrobials and cleaning agents, which can result in persistent contamination, treatment failure, and larger or prolonged outbreaks of seafood-borne disease. This issue is made worse by the frequent co-occurrence of antibiotic resistance and biofilm formation in environmental and seafood-associated *Vibrio* isolates [56]. In Bangladesh, seafood including shrimp is frequently exposed to warm temperatures and unsanitary storage and marketing conditions, which might encourage the growth of biofilms and the spread of *Vibrio* spp. This emphasizes how crucial it is to put in place stringent hygiene protocols, efficient biofilm control techniques, and education initiatives for seafood handlers in order to lower the risk of contamination and protect the public's health.

The purpose of this study is to isolate and identify *Vibrio* species from shrimp samples taken from local markets in Noakhali, Bangladesh with a focus on antibiotic resistance patterns, biofilm building abilities and virulence profile in order to better understand the public health implications. Shrimp aquaculture is critical to Bangladesh's economy, yet antibiotic overuse and poor hygiene in production and retail areas contribute to the spread of multidrug-resistant *Vibrio* bacteria. These bacteria including *V. parahaemolyticus, V. cholerae, V. vulnificus,* and *V. alginolyticus*, can survive in aquatic habitats and processing facilities due to biofilm development, making them difficult to eradicate and raising the risk of foodborne transmission. The study aims to provide insight to seek resistance mechanisms and contamination pathways by connecting environmental factors, antibiotic use, and microbial behaviour. The findings are likely to help lead improved monitoring, encourage responsible antibiotic use in aquaculture, and reinforce Bangladesh's seafood safety and public health surveillance systems.

Aims and Objectives

The aims and objectives of this study were to determine the prevalence and elucidate antimicrobial resistance and virulence profiles of *Vibrio* spp. The purposes of this study are follows:

- To isolate and identify different *Vibrio* spp. such as *V. cholerae*, *V. parahaemolyticus, V. alginolyticus,* and *V. vulnificus* present in shrimp samples collected from different retail market of Noakhali.
- To determine the patterns of antibiotic resistance among *Vibrio* isolates.
- To detect the virulence gene and biofilm formation capabilities of the isolates.
- To analyze whole genome of potential MDR isolates.
- To evaluate the potential public health risks posed by antibiotic-resistant *Vibrio* species in shrimp.
- To provide recommendations for improving shrimp farming and handling practices to minimize the spread of antibiotic-resistant *Vibrio* species.

Chapter 2
Materials and Methods

2. Experimental Design

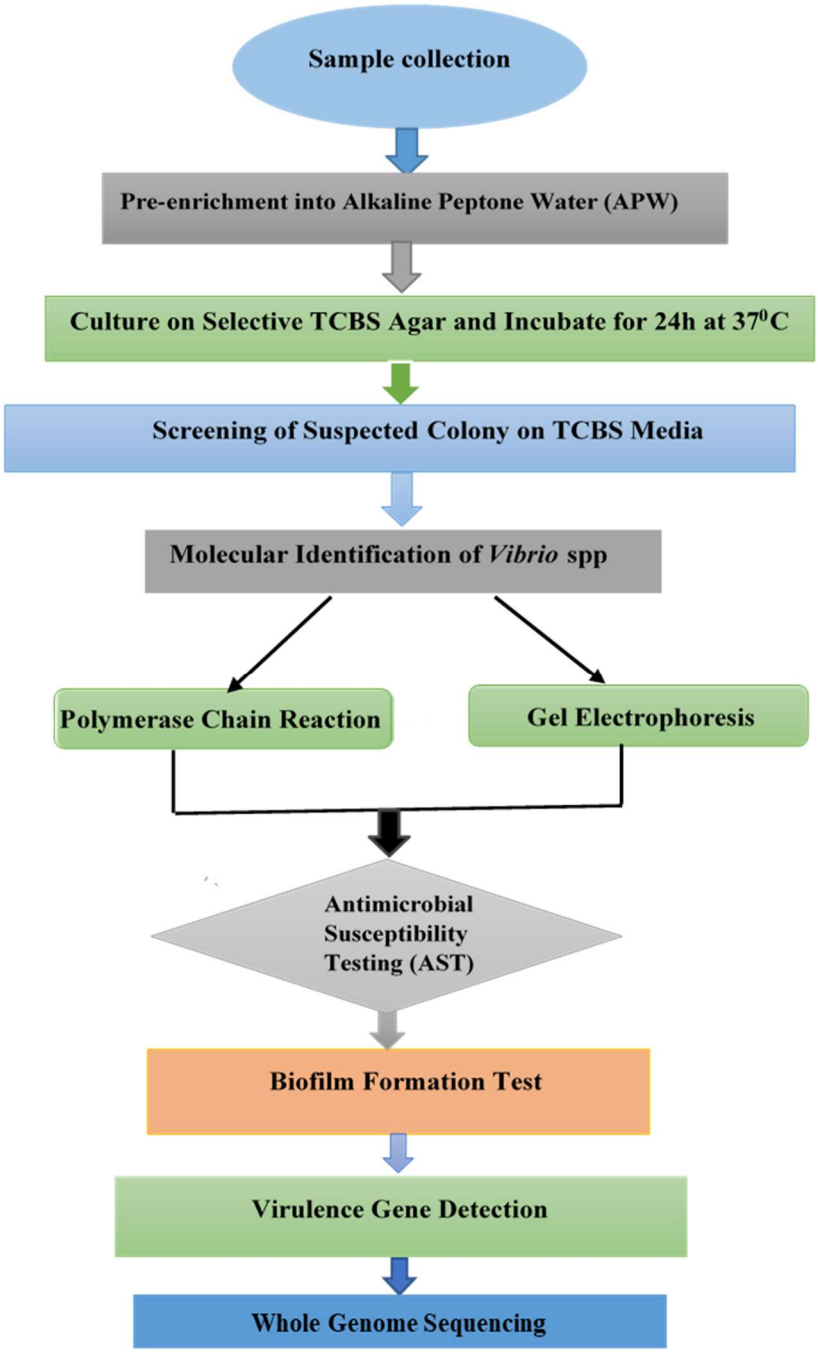


Figure 2.1: Overall procedure of the study

2.1 Ethical Statement

The study was carried out in accordance with the regulations of the institutional Animal Care, Health, and Safety Research Ethics Committee and was authorized by the research cell of Noakhali Science and Technology University (NSTU).

2.2 Sample Collection Site and Sample Type

A total of 241 shrimp samples were collected from Maijdee Bazar, Pouro Bazar, Sonapur Bazar, Chawmuhani Bazar, Bazra Bazar, Sonaimuri Bazar, Bangla Bazar and Chatkhil Bazar of Noakhali. Each of samples was brought using sterile plastic zipper bags. Then they were immediately transported to the Pasteur Laboratory of Noakhali Science and Technology University.

2.3 Sampling Time

The samples were collected in different time from February 2025 to September 2025.

2.4 Sample Collection and Transportation

240 different samples of shrimp were collected from different retail markets of Noakhali district. These samples were collected aseptically in sterile zipper bag. Each of the shrimp sample was placed in an individual sterile plastic zipper bag to avoid contamination. The samples were conveyed on ice in a cooler box from the sampling site to microbiology lab of Noakhali Science and Technology University (NSTU) as soon as possible for further processing and analysis.

Figure 2.2 : Sample collection and transportation

2.5 Sample Processing

The brain, leg, muscle, and intestine from each shrimp sample were blended and taken for enrichment in alkaline peptone water (APW) broth. After that, the broths were incubated overnight at 37°C for bacterial enrichment.

Figure 2.3: Sample processing

2.6 Isolation of Presumptive *Vibrio* spp.

Using a pre-made thiosulfate-citrate-bile salt-sucrose (TCBS) agar plate, a loop full of surface growth was streaked and incubated for 18 to 24 hours at 37°C, as previously reported [57]. Based on their morphology (size, shape, and color), bacterial colonies were inferred to be *Vibrio* spp. after incubation. For further purification, a single, distinct colony which was either yellow or green in color was subsequently streaked onto recently made TCBS agar plates which is believed to be a colony of *Vibrio* species.

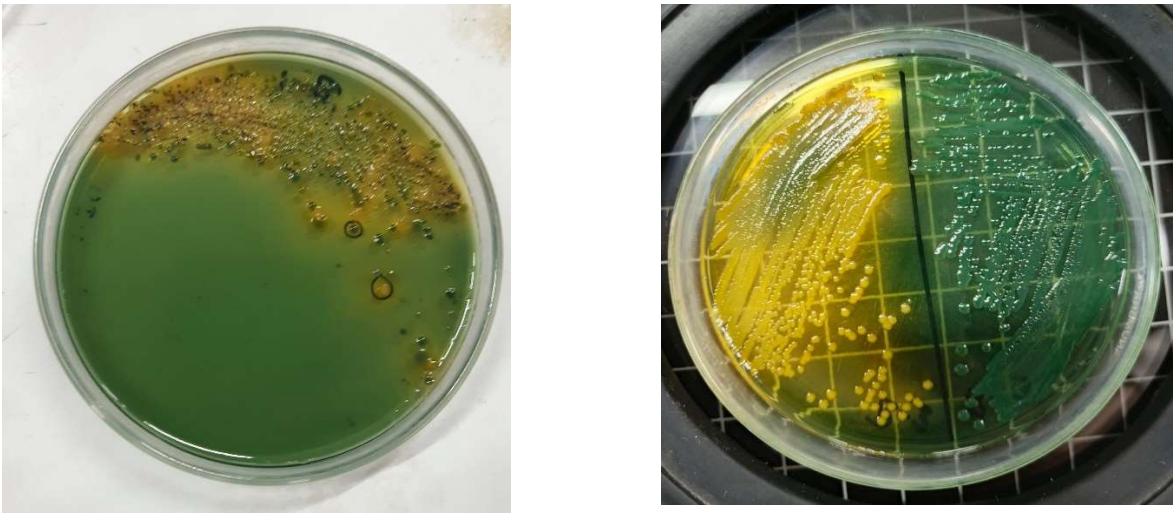


Figure 2.4: Presumptive *Vibrio* spp. on TCBS; Primary culture (Left), Sub-culture (Right)

2.7 Molecular Detection of Presumptive *V. cholerae, V. parahaemolyticus, V. alginolyticus, and V. vulnificus*

2.7.1 Preparation of genomic DNA

DNA extraction was carried out by boiled technique. The strains were grown in Tripticase Soy Broth (TSB) for 24 hours at 37°C. An estimated concentration of each microorganism after the incubation period was approximately 10^6 CFU ml^{-1} of vegetative forms. One mililiter culture from TSB was taken in eppendorf tube and centrifuged for 5 minutes at 15,000 rpm. The supernatant was discarded and the pellet was subjected to DNA extraction protocols. Then 500 µL distilled water was added in each tube and mixed by re-pipetting and centrifuged for another 5 minutes at 15,000 rpm. The supernatant was discarded followed by the addition of 350 µL nuclease free water (PCR water) into pellet. The tubes were boiled at 100°C for 15 minutes. After boiling, the mixtures were immediately heat shocked at -20°C for 5 minutes. Then the tubes were centrifuged for 15 minutes at 15,000 rpm. Finally, the supernatants were collected from each tube without disturbing pellets which contain DNA extract and preserved for PCR.

2.7.2 DNA concentration 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). Pure DNA preparation has an OD 260/OD 280 value of 1.8. The DNA was stored at -20 °C until further use.

2.7.3 Polymerase chain reaction

Different *Vibrio* spp. were finally confirmed using a polymerase chain reaction (PCR) assay that focused on the genus-specific groEL gene. Every Vibrio isolate was put through a multiplex PCR for the detection of *V. cholerae, V. parahaemolyticus, V. alginolyticus,* and *V. vulnificus* molecularly.

Table 2.1: List of primers used for specific detection of target *Vibrio* species.

Targeted gene	**Name of *Vibrio* spp.**	**Primer**	**Sequence**	**PCR condition**	**Product size(bp)**	**References**
groEL	*V. cholerae*	groVc1	GATCTTGACTGGCGGTGTTGTG	Initial denaturation of 5 min at 94°C followed by 30 cycles each having denaturation for 30 s at 94°C, annealing for 30 s at 69°C and extension for 30 s at	418	[58], [59], [60]
		groVc2	GTCACCCACCAGAGAAGAGAGT			
	V. parahaemolyticus	groVp1	GTCAGGCTAAGCGCGTAAGCA		644	
		groVp2	GCATGCCTGCGCTTTCTTTTTG			
	V. vulnificus	groVv1	GTTCGCGCTGGTGAAGGTTCA		192	

		gro Vv2	TGGCATACC AGAGTCTTT CTGTG	72°C, and final extension step for 5 min at 72°C		
	V. alginolyticus	groVa1	GATTCGGTG AAGAAGAGA TGATCTC		301	
		groVa2	TCTTCGTTG TCACCCGTT AGGTGA			

The reaction mixture for PCR was prepared by mixing the specific volume of the components in a proper sized tube.

Table 2.2: Volume of components for preparation of 15 µL PCR mixture

Composition	**Volume (µL)**
Master mix	7.5
Forward primer	0.5 x 4 = 2
Reverse primer	0.5 x 4 = 2
Template	3
Nuclease-free water	0.5
Total	15

All reagents of PCR master mix were thawed and gently vortexed before use. After the addition of appropriate ultrapure water and all reagents and extracted DNA as template was added. PCR tubes were gently vortexed and placed in thermal cycler to start PCR.

2.8 Gel Electrophoresis

2.8.1 Preparation of 1.5% agarose gel

- A 100 ml flask was used to prepare the gel solution. 0.75 gram Agarose was added to the flask containing 50 ml 1x TAE buffer, the mixture was heated at 100°C for 40 seconds and agarose solution was cooled. No detectable evaporation should be found.

- After that, 3 µL of Ethidium Bromide was added with cooled agarose solution.
- Agarose solution was poured into the bed and the level of the comb was adjusted so it rests evenly with a few millimeters (mm) of space between teeth and the tray.
- The gel was allowed to completely solidify and then the comb was removed and the electrophoresis apparatus chamber was filled with the required volume of diluted buffer and the gel was placed into the electrophoresis chamber, properly oriented, centered and leveled on the platform.

2.8.2 Sample loading procedure

3µL ladder was loaded into the first well and the other well were loaded with 5µL of each PCR product.

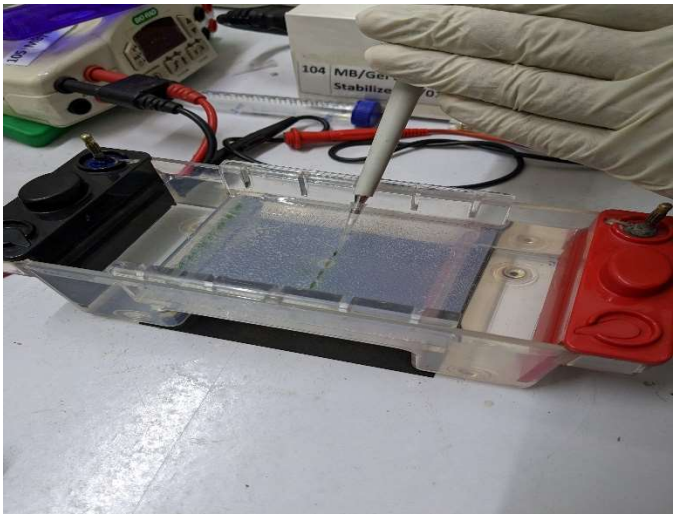


Figure 2.5: PCR product loading in the wells of electrophoresis gel

2.8.3 Running the gel

- After the samples were loaded, electrode terminals covered down carefully and apparatus chamber was properly oriented.
- The plug was inserted of the black wire into the black input of the power source (negative input) and red wire into the red input of the power source (positive input). The power source was set at the required voltage and conduct electrophoresis for the length of time.

- After approximately 40 minutes electrophoresis was completed.
- DNA fragments were visualized in Fluorochrome E (protein simple, USA) and the gel results were documented .

2.9 Antimicrobial Susceptibility Testing

The Kirbey Bauer method, a standardized agar-disc-diffusion technique, was used to assess the test isolates antimicrobial susceptibility in vitro [61]. It is an adaptation of Baur's approach. The antimicrobial test was conducted using Mueller-Hinton agar (Oxoid Limited, England) and commercially available discs. From 8 different antibiotic groups total 15 antibiotic discs were used in this study.

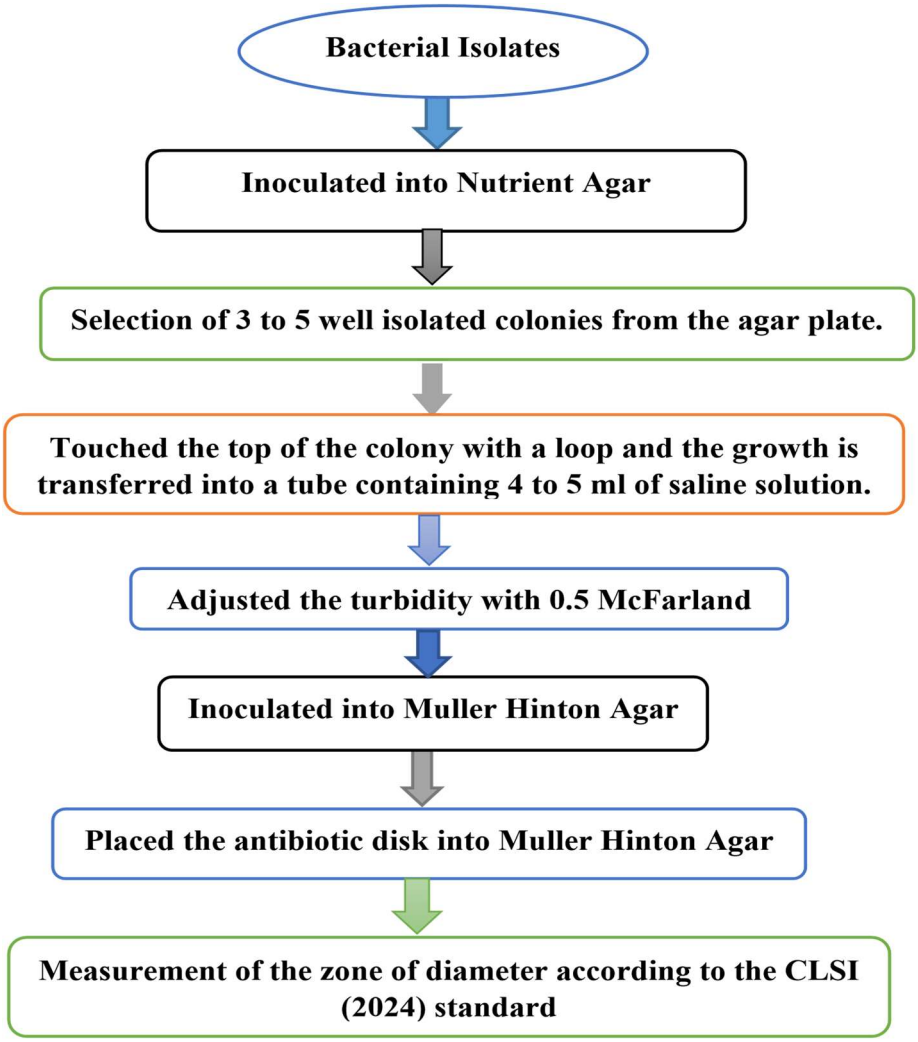


Figure 2.6: Methodology for Antimicrobial Susceptibility Test

Table 2.3: Antimicrobial agents, their disc concentrations and zone interpretative reference according to CLSI M100-Ed34 published in February, 2024.

Antimicrobial Groups	Antimicrobial agents	Disc conc. (µg)	Zone interpretation (diameter in mm)		
			S	I	R
Penicillin	Ampicillin (AMP)	10	≥ 17	14-16	≤ 13
Cephalosporins	Cefotaxime (CTX)	30	≥ 26	23-25	≤ 22
	Ceftazidime (CAZ)	30	≥ 21	18-20	≤ 17
Carbapenems	Imipenem (IPM)	10	≥ 23	20-22	≤ 19
	Meropenem (MR)	10	≥ 23	20-22	≤ 19
Aminoglycoside	Amikacin (AK)	30	≥ 17	15-16	≤ 14
	Gentamycin (GEN)	10	≥ 15	13-14	≤ 12
Quinolones	Ciprofloxacin (CIP)	5	≥ 26	22-25	≤ 21
	Levofloxacin (LE)	5	≥ 21	17-20	≤ 16
	Nalidixic acid (NA)	30	≥ 19	14-18	≤ 13
Phenicol	Chloramphenicol (C)	30	≥ 18	13-17	≤ 12
Macrolides	Erythromycin (E)	15	≥ 18	14-17	≤ 13
	Azithromycin (AT)	15	≥ 18	14-17	≤ 13
Nitrofurantoin	Nitrofurantoin (NIT)	300	≥ 17	15-16	≤ 14

2.9.1 Preparation of inoculums

For each isolate the inoculums was prepared as described here.

- To obtain a fresh culture, each of the isolates was resurrected on a Nutrient Agar (NA) plate from a stock culture and then subcultured on a different MHA plate. Then, using a sterile inoculating loop, isolated 1-2 pure colonies were chosen from the agar plate and moved into a test tube with 5 mL of Mueller Hinton Broth (MHB) (Oxoid Limited, UK) (Appendix I).

- The inoculated broth was adjusted to the McFarland standards by incubation at 37°C until it achieved or exceeded the turbidity of the 0.5 McFarland standards (usually 2-6 hours). McFarland 0.5 turbidity standard was prepared by mixing 0.05 mL of 1.175% barium chloride dihydrate ($BaCl_2.2H_20$), with 9.95 mL of 1% sulfuric acid (H_2SO_4).
- The turbidity of the actively growing broth culture was adjusted with sterile broth to obtain turbidity optically comparable to the point of the 0.5 McFarland standards.

2.9.2 Inoculation of test plates

Ideally, a sterile cotton swab was dipped into the modified suspension of the inoculums within 15 minutes of changing the suspension's turbidity. The swab was firmly pressed against the tube's inner wall above the fluid level after being rotated multiple times. This cleared the swab of extra inoculum. A Mueller Hinton agar plate's dry surface was inoculated by streaking the swab over the whole surface of the sterile agar. This process was performed twice more, rotating the plate by about 60° each time to guarantee that the inoculum was distributed evenly. As a final step, the edge of the agar was swabbed. The lid was left slightly open for 3 to 5 minutes, but no longer than 15 minutes, to allow any excess surface moisture to be absorbed before placing the drug-impregnated discs.

2.9.3 Application of discs to inoculated agar plates

Using sterile forceps, the predetermined battery of antimicrobial discs was dispensed onto the infected agar plate's surface. Each disc was squeezed down to guarantee full contact with the agar surface. Whether the discs were dispensed with a dispenser or individually device, they were equally spaced with a maximum distance of 24 mm between centers. Within fifteen minutes of the discs being applied, the plates were inverted and put in an incubator that was heated to 37°C.

2.9.4 Reading plates and interpretation

Following 16-18 hours of incubation, each plate was observed for the zone of inhibition, which appeared as a uniformly circular area with a continuous lawn of bacterial growth. The diameters of these inhibition zones (determined by the naked eye) were measured, including the diameter of the disc itself, and recorded to the nearest millimeter. Any faint growth or tiny colonies visible only with a magnifying lens at the edge of the inhibition zone were disregarded. Zone sizes were evaluated according to performance standards for antimicrobial susceptibility testing and the

organisms were classified as susceptible, intermediate, or resistant to various antibiotics based on Clinical Laboratory Standard Testing (CLST), 2024.

2.10 Molecular Detection of Extended Spectrum Beta-lactam (ESBL) Gene

Antibiotic resistance genes include Extended Spectrum Beta lactam (ESBL) gene were detected among the all *Vibrio* isolates that shows resistance pattern in disc diffusion assays. Three ESBL (blaCTX-M, blaTEM and blaSHV) gene were identified using all extracted DNA. For PCR amplification, gene specific primers were used describe in table 2.4. The PCR reaction was conducted by using 10 pmol of forward and reverse primers in a 25µl volume with 5µl (5ng) of DNA template, 12.5µl of 2X Emerald Amp® MAX PCR Master Mix (code no. RR320A, Takara) and 5.5µl of nuclease free water. PCR program was run by Bio-Rad T100 thermal cycler (Model: T100, Bio-Rad Laboratories) following primer specific conditions described in Table 2.4. Finally, PCR products were then visualized on a 1.5 % agarose gel stained with ethidium bromide under UV light to confirm the presence of specific gene with reference product band.

Table 2.4: List of primers used for genotypic characterization of ESBL resistant isolates.

Targeted gene	Primer	Sequence	PCR condition	Product size	Reference
ESBL	*bla*-CTX-M	Forward 5′-CGGGAGGCAGATGGGTGT-3′	Initial denaturation at 95°C for 5 minutes, 35 cycles of 30 seconds' denaturation at 95°C, annealing at 58°C for 30 seconds per extension at 72°C for 30 seconds and a final extension for 5 minutes at 72°C	381	[119]
		Reverse 5′-TCGGCTCGGTACGGTCGA3′			
	bla-TEM	Forward 5′-GTCGCCGCATACACTATTCTCA-3′		258	
		Reverse 5′-CGCTCGTCGTTTGGTATGG-3′			

	bla-SHV	Forward 5′-GCCTTGACCGCTGGGAAAC-3′		319	
		Reverse 5′-GGCGTATCCCGCAGATAAAT-3′			

2.11 Biofilm Formation Test

Tube method

Biofilm formation was detected by in vitro methods: Tube method (TM). Described by Christensen et al., this is a qualitative method for biofilm detection. A loopful of test organisms was inoculated in 3 mL of trypticase soy broth with 1% glucose in test tubes. The tubes were incubated at 37ºC for 24 h. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were then stained with crystal violet (0.1%) for 5 min. Excess stain was washed with deionized water. Tubes were dried in inverted position. The scoring for tube method was done according to the results of the control strains. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as weak/none, moderate and high/strong [62].

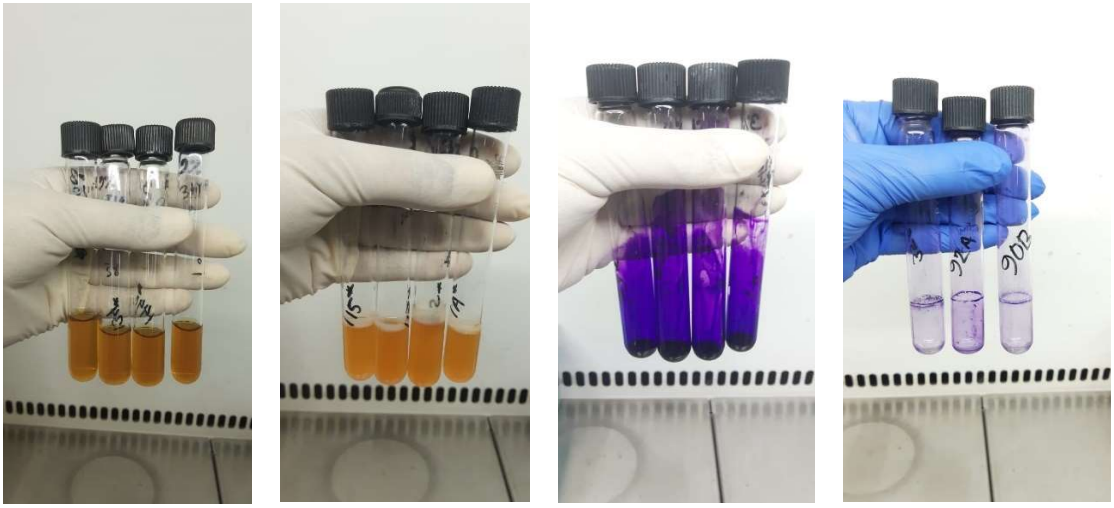


Figure 2.7: Biofilm formation test (Tube Method)

2.12 Molecular Detection of Virulence Gene in *Vibrio* spp.

A total of seven virulence gene were tested by polymerase chain reaction among the all *Vibrio* isolates that shows virulence pattern of those isolates. From them four gene for *Vibrio cholerae* including hlyA EI Tor, rtx, ompU, TcpA and the other three gene for *Vibrio parahaemolyticus* which are Tdh, Tlh, Stn. PCR was conducted by using the previously extracted DNA of all isolates and gene specific primers describe in table 2.5. The PCR reaction was conducted by using 10 pmol of forward and reverse primers in a 13µl volume with 6.5 µl of 2X EmeraldAmp® MAX PCR Master Mix (code no. RR320A, Takara), 2 µl (2ng) of DNA template, 1 µl of both forward and reverse primer and 2.5µl of nuclease free water. PCR program was run by Bio-Rad T100 thermal cycler (Model: T100, Bio-Rad Laboratories) following primer specific conditions described in **Table 2.5**. Finally, PCR products were then visualized on a 1.5 % agarose gel stained with ethidium bromide under UV light to confirm the presence of specific gene with reference product band.

Table 2.5: List of primers and PCR condition for specific detection of virulence gene in *Vibrio* species

Name of *Vibrio* spp.	**Target Gene**	**Primer Sequence**	**PCR Condition**	**Product Size (bp)**	**References**
Vibrio cholerae	***hly*A El Tor**	F: GAGCCGG CATTCAT CTGAAT	Initial denaturation at 94°C for 2 minutes, 30 cycles of 30 seconds denaturation at 94°C, annealing at (as mentioned below) for 30 seconds, extension at 72°C for 30 seconds in each cycle and a final extension for 2 minutes at 72°C Annealing temperature: *hlyA* El Tor - 55°C *TcpA* - 53°C *ompU* - 56°C *rtx* - 52°C	481	[63], [64]
		R: CTCAGCG GGCTAAT ACGGTTT A			
	***Tcp*A**	F: GAAGAAG TTTRTAA AAGAAGA ACA		451	
		R: GAAAGGA CCTTCTTT CACGTTG			

	ompU	F: ACGCTGACGGAATCAACCAAAG		869	
		R: GCGGAAGTTTGGCTTGAAGTAG			
	rtx	F: GCGATTCTCAAAGAGATG		1366	
		R: CACTCATTCCGATAACCAC			
Vibrio parahaemolyticus	***Tdh***	F: GTAAAGCTCTCTGACTTTTGGAC	Initial denaturation at 94°C for 2 minutes, 30 cycles of 30 seconds denaturation at 94°C, annealing at (as mentioned below) for 30 seconds, extension at 72°C for 30 seconds in each cycle and a final extension for 2 minutes at 72°C Annealing temperature: *Tdh* - 55°C *Tlh* - 55°C *Stn* - 56°C	269	[65], [66]
		R: TGGAATAGAACCTTCATCTTCACC			
	Tlh	F: AAAGCGGATTATGCAGAAGCACTG		450	
		R: GCTACTTTCTAGCATTTTCTCTGC			
	Stn	F: GGTGCAACATAATA		375	

		AACAGTC AACAA			
		R: TAGTGGT ATGCGTT GCCAGC			

2.13 Whole Genome Analysis

2.13.1 Whole genome sequencing, assembly and strain characterization

The total DNA from the isolates was extracted utilizing the QIAamp® DNA Mini Kit (QIAGEN, Germany) and then quantified with a Qubit 4 fluorometer (Thermo Fisher Scientific, USA). Prior to DNA sequencing, a native barcoding kit (SQK.NBD.114.24) was utilized for library preparation, and the sequencing was conducted on the Oxford Nanopore MinION™ Mk1C platform using the FLO-MIN114 (R10.4.1) flow cell. The MINKNOW program (version 1.11.5) was employed for data collection. The Guppy (v.6.3.2), in high accuracy mode, was utilized for basecalling the MinION™ Mk1C sequencing data (FAST5 files) to generate pass reads (FASTQ format) with a mean quality score > 9. The adaptor and barcode sequences were removed via Porechop (v.0.2.4) [67], and low-quality bases (q<10) were removed with Nanofilt [68]. The de novo assembly workflow was employed to construct the quality-trimmed reads into contigs using Flye (v.2.9.4) [69]. Further, genome polishing was done by Racon (v.1.5.0) [70].

The Type (Strain) Genome Server (TYGS) was employed to evaluate the species [71]. The potential contamination within the genome and its completeness was evaluated using ConEst16S (https://www.ezbiocloud.net/tools/contest16s) [72], CheckM (v1.2.4) [73], QUAST (v.5.2.0), and BUSCO (https://busco.ezlab.org/). The average nucleotide identity (ANI) was assessed using the OrthoANI tool available on the Ezbiocloud server (https://www.ezbiocloud.net/tools/orthoaniu) [74].

2.13.2 Genome annotation, mapping, subsystem and metabolic pathway analysis

The assembled sequence undergone annotation through Prokka [75], RAST [76], and Prokaryotic genome annotation pipeline (PGAP) of NCBI [77]. The circular genome map was constructed using Proksee server [78]. Analysis of different subsystems and functional metabolic pathways

were carried out through Patric server [79] and RAST [76]. The biosynthetic gene clusters were determined through antiSMASH7.0 tool [80].

2.13.3 Assessment of antimicrobial resistance genes, virulence genes, mobile genetic elements, and prophase analysis

Antimicrobial resistance genes were investigated through CARD server [81]. Following that, mobile genetic elements and virulence genes were explored through mobileOG-db [82] and Victors [83], respectively. Phage integration within the bacterial genome was examined using the PHAge Search Tool Enhanced Release (PHASTER) [84], [85] and the PHAge Search Tool with Enhanced Sequence Translation (PHATEST) [86].

2.13.4 Pan genome analysis, pathogenicity and MLST profiling

A thorough assessment of the pangenome, along with a comparative genome study, was conducted to gain insight into the intrinsic diversity exhibited by the target strain. This initiative aimed to identify unique differences in the presence or absence of specific genes or gene families, known as Presence-Absence differences (PAVs), across strains from diverse geographical locations and sources. 10 whole genome sequences of *V. parahaemolyticus* strains obtained from human sources from different countries and 13 whole genome sequences of the same species from non-human sources were downloaded from Bacterial and Viral Bioinformatics Resource Center (BV-BRC). Pangenome and comparative genome analysis was carried out with total 27 genomes V. parahaemolyticus including the genome of our test isolate *V. parahaemolyticus* SU37A, and *V. parahaemolyticus* SU91A using Roary [87], a tool that rapidly builds large scale pan genomes identifying the core and accessory genes. Pathogenicity profiling and prediction of the pathogenicity of all the isolates towards human hosts was carried out through PathogenFinder (v2.0) [88] webtool of Center for Genomic Epidemiology and analyzed as well as visualized using Tidyverse package of RStudio.

Chapter 3
Results

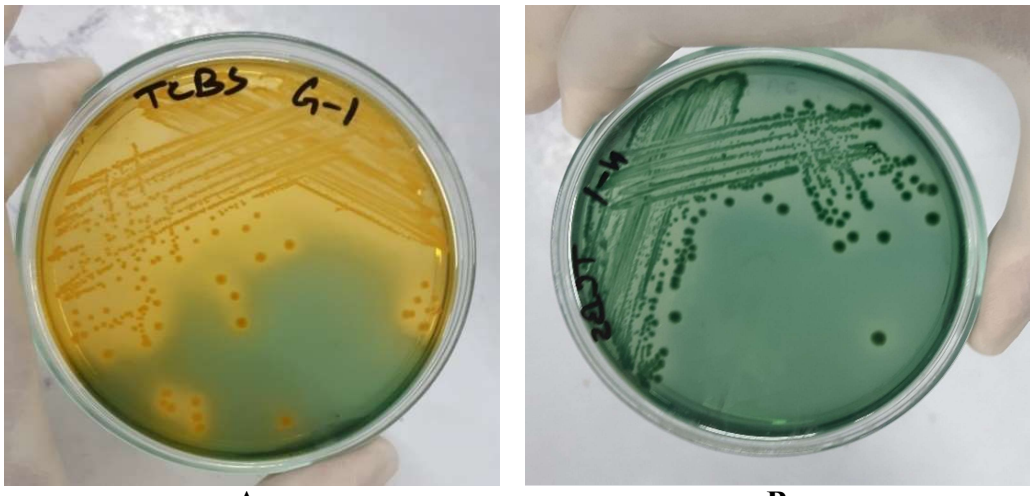
3.1 Growth on Selective Enrichment Media

The number of *Vibrio* spp. was increased in sample and the growth of other organisms was inhibited when the samples were pre enriched in Alkaline Peptone Water (APW). The turbidity indicate that the bacteria have been enriched properly.

Figure 3.1: Pre enrichment of Vibrio in APW

3.2 Detection of *Vibrio* spp. on TCBS

Through conventional culture approaches, we determined a total number of 241 different bacterial species in all shrimp samples. we found 2 different *Vibrio* species including *V. cholerae* and *V. parahaemolyticus*. After 18 to 24 hours incubation suspected *V. cholerae* and *V. parahaemolyticus* were found to appear as yellow and green colonies respectively on TCBS agar plates.



A **B**

Figure 3.2: Presumptive colonies of *Vibrio* spp. on TCBS; *V. cholerae* (A), *V. parahaemolyticus* (B)

3.3 Genotypic Detection of *Vibrio* spp.

Among 105 *Vibrio* positive isolates, gro Vp was positive for 71 isolates with the percentage of 67.62% and groVc was positive for 34 isolates with the percentage of 32.38%. No isolate was positive for groVa and groVv.

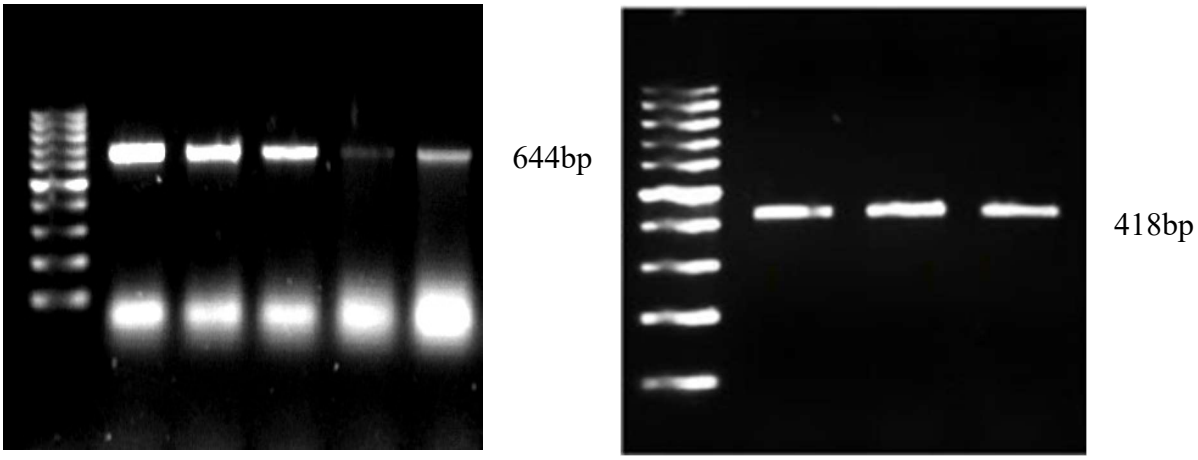


Figure 3.3: Genotypic detection of *Vibrio parahaemolyticus* (Left) and *Vibrio cholerae* (Right)

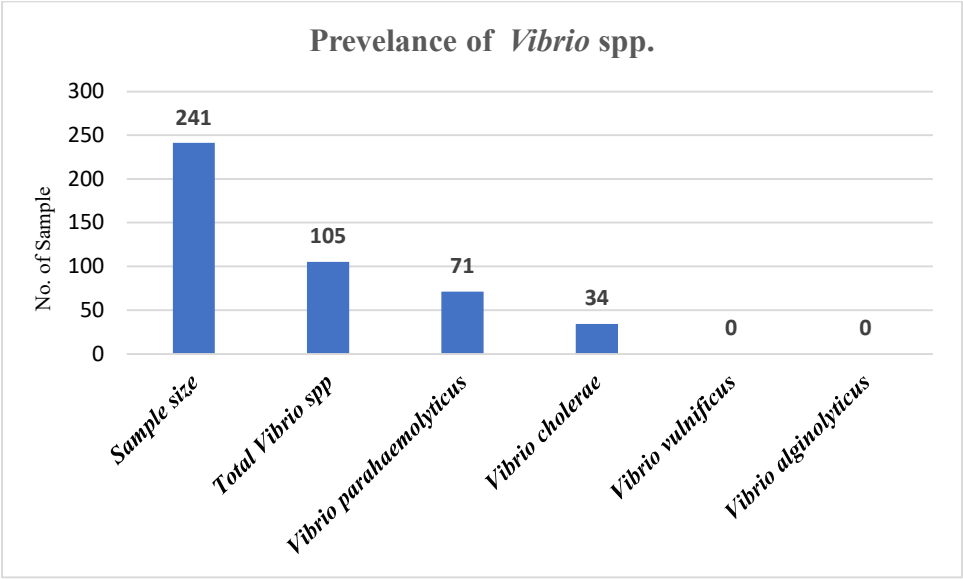


Figure 3.4: Prevelance of *Vibrio* spp. in shrimp sample

Occurrence of *V. cholerae*, *V. parahaemolyticus, V. Vulnificus,* and *V. alginolyticus*

Among 241 samples thirty-four (14.12%) contained *Vibrio cholerae* and seventy-one (29.46%) were positive for *V. parahaemolyticus* . The percentage of *V. cholerae* found in *Macrobrachiumn rosenbergii* (22%) was higher than that of *Penaeus monodon* (17%) and *Litopenaeus vannamei* (12%). The presence of *V. parahaemolyticus* was more in *Penaeus monodon* (36%) compared to samples of *Macrobrachium rosenbergii* (19%) and *Litopenaeus vannamei* (26%). No isolate was positive for *V. vulnificus* and *V. alginolyticus*.

3.4 Result of Antibiotic Susceptibility Test

The antimicrobial susceptibility and resistance characteristics of all chosen isolates were evaluated against 8 different antibiotic groups and a total of 15 antibiotic discs. The resistance among the selected isolates was observed predominantly in the antibiotic: ampicillin, ceftazidime, meropenem, cefotaxime.

Table 3.1: Antimicrobial susceptibility test result

ID	E	GEN	CTX	CIP	NA	C	NIT	MR	AK	AZM	AMP	IPM	CAZ	LE
1A	S	S	R	S	S	S	S	R	S	S	R	S	R	S
3D	S	S	R	S	S	S	S	I	S	S	R	S	R	S
5A	S	S	I	S	S	S	I	R	S	S	R	S	S	S
13A	S	S	R	S	S	S	S	R	S	S	R	S	R	S
14A	R	S	R	R	I	S	R	R	R	R	R	S	R	S
17	I	S	R	S	S	S	R	R	S	S	R	S	R	S
19	S	S	R	S	S	S	S	R	I	S	R	S	I	S
21A	S	S	R	S	S	S	R	I	S	S	R	S	R	S
22	S	S	R	S	S	S	S	R	S	S	R	I	I	S
23	I	S	R	I	S	S	I	R	R	S	R	S	R	S
24A	S	S	R	I	S	S	S	R	S	S	R	S	R	S
25A	S	S	R	S	S	S	S	I	I	S	R	S	I	S
26A	S	S	R	S	S	S	R	R	I	S	R	S	R	S
28A	S	S	R	S	S	S	R	R	I	S	R	S	R	S
29B	R	S	I	S	S	I	R	R	S	I	R	S	R	S
31A	S	S	R	S	S	S	R	I	S	S	R	S	R	S
34A	R	S	R	S	I	S	R	R	S	R	R	S	I	S
35A	I	S	I	I	S	S	I	R	S	S	R	S	I	S

36A	R	R	R	S	S	I	I	R	S	R	R	S	R	S
37A	R	S	R	S	I	S	R	R	S	R	R	S	I	S
38A	I	S	R	S	S	S	R	R	I	S	R	S	R	S
38C	S	S	R	S	S	I	S	R	S	S	R	S	R	S
39	S	S	R	I	S	I	R	R	I	S	R	S	I	S
40	S	S	R	S	I	S	S	R	S	S	R	S	R	S
42A	S	S	R	S	S	S	R	R	I	I	R	I	R	S
43A	I	S	R	S	S	S	S	I	S	S	R	S	I	S
45	R	S	R	S	S	S	R	R	S	R	R	I	R	S
49	I	S	R	S	S	S	R	R	I	S	R	I	R	S
61	R	S	R	S	I	I	S	R	S	R	R	S	R	S
64	R	S	R	S	I	R	R	R	I	R	R	I	R	S
65	I	S	R	S	S	S	R	R	I	I	R	S	R	S
75A	S	S	R	S	S	S	I	R	S	S	I	S	R	S
76	S	S	R	S	S	S	R	R	S	S	R	S	R	S
77A	S	S	R	S	S	S	S	R	S	S	R	S	R	S
81C	S	S	R	S	S	S	R	R	I	S	R	S	R	S
87C	I	S	R	S	S	S	R	R	R	S	R	S	R	S
88A	S	S	R	S	S	S	S	I	I	S	R	S	S	S
89A	S	S	R	S	S	S	I	I	S	S	R	S	S	S
90B	I	S	R	I	S	I	R	R	I	R	R	S	R	S
91A	R	S	R	I	I	S	R	R	S	R	R	S	R	S
92A	S	S	R	S	S	S	S	R	S	S	R	S	S	S
93A	S	S	R	S	S	S	S	R	S	S	R	S	I	S
94	S	S	R	I	R	S	R	R	S	I	R	S	R	S
104	S	S	R	S	S	S	S	R	S	S	R	S	S	S
105	S	S	I	S	S	S	S	R	S	S	R	S	S	S
106	I	S	R	S	I	S	R	R	S	S	R	S	S	S
107	S	S	R	S	S	S	S	R	S	S	R	S	S	S
108	I	S	R	S	S	S	R	R	I	I	R	R	R	S
109	S	S	R	S	S	S	R	R	I	S	R	I	R	S
110	I	S	R	S	I	S	S	R	S	S	R	S	R	S
111	I	I	R	I	S	S	R	R	S	S	R	S	R	S
112	S	S	R	S	S	S	I	R	S	S	R	S	S	S
114	I	S	R	S	S	S	R	R	I	S	R	I	R	S
115	S	S	R	S	S	S	R	R	I	S	R	I	R	S
116	S	S	R	S	S	S	S	R	S	S	R	S	R	S

117	S	S	R	S	S	S	I	R	S	S	R	I	I	S
118	S	S	R	S	S	S	S	R	S	S	R	S	R	S
119	S	S	R	S	S	S	S	R	S	S	R	S	S	S
120	S	S	R	S	S	S	I	R	I	S	R	S	I	S
124	S	S	R	I	S	S	S	R	S	S	R	S	R	S
129	R	S	R	S	S	R	I	R	S	R	R	S	R	S
130	S	S	R	S	S	S	R	R	S	S	R	S	R	S
132	S	I	R	S	S	S	R	R	S	S	R	S	R	S
134	S	S	R	S	S	S	S	R	S	S	R	S	R	S
143	S	S	R	S	S	S	S	R	S	S	R	S	R	S
144B	S	S	R	S	S	S	S	R	S	S	R	R	R	S

151B	S	S	I	S	S	S	S	R	S	S	R	S	R	S
152	R	S	I	S	S	S	S	R	R	S	R	R	R	S
153A	S	S	S	R	I	S	S	R	S	S	R	R	I	S
157C	S	S	I	S	S	S	R	R	I	S	I	S	R	S
158A	S	S	R	S	S	S	I	R	S	S	R	I	R	S
167	S	S	R	S	S	S	S	S	S	S	S	S	S	S
168A	S	S	S	S	S	S	S	I	S	S	R	I	I	S
168B	S	S	I	S	S	S	S	S	S	S	S	S	S	S
169A	S	S	S	S	S	S	S	I	I	S	I	S	I	S
169B	I	S	I	S	S	S	S	R	R	S	R	S	R	S
172	S	S	R	S	S	S	S	R	S	S	R	I	I	S
174A	S	S	S	S	S	S	S	R	S	S	R	S	R	S
176A	I	S	R	S	S	S	S	R	S	S	S	R	I	S
177B	S	S	I	S	S	S	S	R	S	S	R	S	R	S
180A	S	S	S	S	S	S	S	R	S	S	R	S	I	S
181B	S	S	S	I	S	S	S	R	S	S	R	S	R	S
182	S	S	R	S	S	S	R	R	S	S	R	S	R	S
185A	I	S	R	S	S	S	S	I	I	S	R	S	I	S
186	S	S	I	S	S	S	S	R	S	S	R	S	R	S
187	S	S	R	S	S	S	I	R	S	S	R	S	R	S
188	S	S	S	S	S	S	S	S	S	S	I	S	S	S
189	I	S	S	S	S	S	I	R	S	I	R	I	R	S

191	S	S	I	S	S	S	S	R	S	S	R	S	R	S
193	I	S	I	S	S	S	S	I	I	S	R	S	I	S
195	I	S	I	S	S	S	S	R	S	S	S	R	I	S
196B	S	S	S	S	S	S	S	S	S	S	I	S	S	S
197	I	S	R	S	S	S	I	R	S	S	R	I	R	S
200	R	S	S	S	S	S	S	S	S	R	R	S	I	S
203	I	S	S	S	S	S	S	S	S	I	R	I	I	S
205B	I	S	I	S	I	S	S	I	S	S	R	R	S	S
208	R	S	S	I	S	R	S	I	S	R	S	R	S	S
211	S	I	I	S	S	S	R	R	R	S	S	I	S	S
213B	S	S	R	S	S	S	I	R	S	S	I	S	R	S
215	S	S	I	S	S	S	R	R	S	S	R	S	R	S
217A	S	S	R	S	S	S	S	R	S	S	R	S	R	S
222	S	S	R	I	S	I	R	R	I	S	R	S	I	S
224B	S	S	S	S	I	S	S	R	S	S	R	S	R	S
229	S	S	R	S	S	S	R	R	I	I	R	I	R	S
236A	I	S	R	S	S	S	S	I	S	S	R	S	I	S

*S= Sensitive, I=Intermediate, R= Resistant

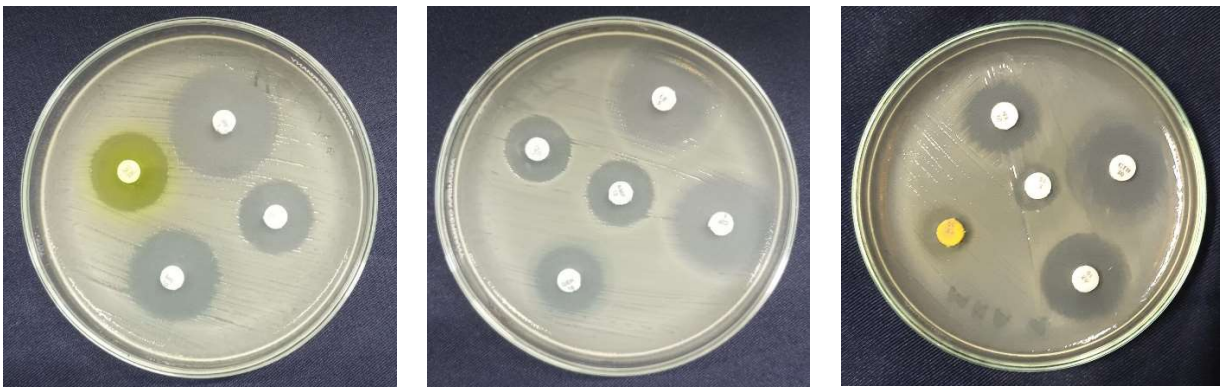


Figure 3.5: Antimicrobial susceptibility test by disc diffusion

By antimicrobial susceptibility test, the highest percentage of the *Vibrio* isolates were phenotypically resistant to ampicillin (88.57%), followed by meropenem (81.90%), cefotaxime (71.43%), ceftazidime (60.95%), nitrofurantoin (34.29%), erythromycin (12.38%), azithromycin (11.42%), amikacin (9.52%), imipenem (7.6%) and chloramphenicol, gentamicin, nalidixic acid, ciprofloxacin (1-3%). No isolates were resistant to levofloxacin.

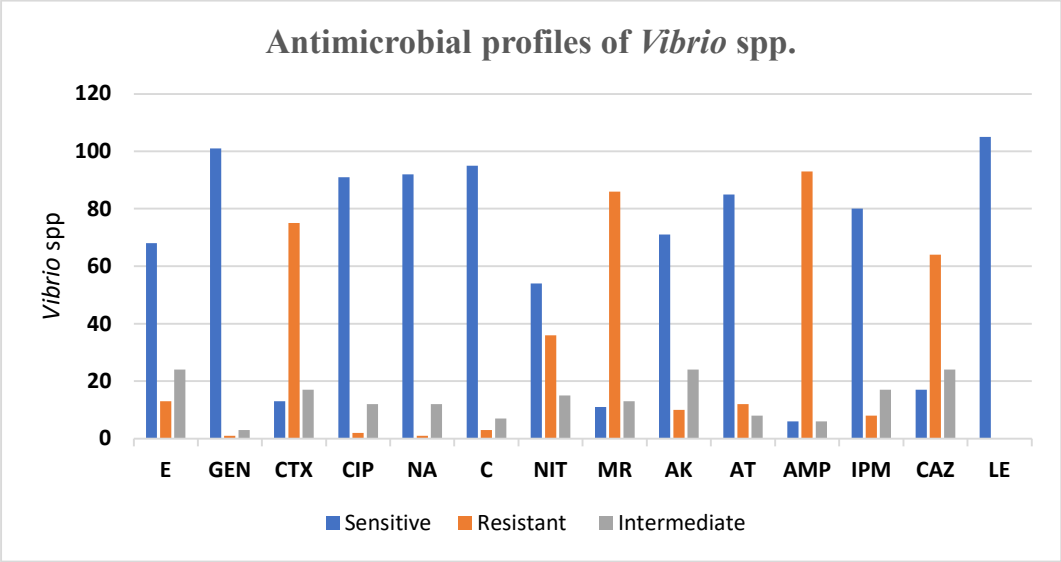


Figure 3.6: Antimicrobial profiles of *Vibrio* spp.

Prevalence of Multi-drug Resistant (MDR) and MAR index of *Vibrio parahaemolyticus* and *Vibrio cholerae*

The MDR isolates were identified by assessing their resistance phenotype against antibiotics from a minimum of three distinct groups. There were 74 *Vibrio* isolates that were MDR in nature (70.48%,). Among them 69 % (49/71) of the *Vibrio parahaemolyticus* and 88.24% (30/34) of the *Vibrio cholerae* were found to be MDR. A significant proportion of isolates had a MAR index greater than 0.2 (85/105). The range of the MAR index was between 0.0 and 1.0. Twenty-nine distinct resistance patterns were also found in the isolates of *Vibrio* spp.

Table 3.2: Multidrug resistance and multiple antibiotic resistance index of *Vibrio* isolates from shrimp

Pattern No	Antibiotic resistance patterns	No. of antibiotics (classes)	No. of isolates	Overall no. of MDR isolates (%)	MAR index
1	CAZ, MR, AMP, CTX	4 (3)	13	70.48% (74/105)	0.3
2	CAZ, NIT, MR, AMP, CTX	5 (4)	15		0.4
3	CAZ, MR, AMP	3 (3)	14		0.2
4	CAZ, NIT, AMP, CTX	4 (3)	3		0.3
5	E, CAZ, MR, C, AT, AMP, CTX	7 (5)	1		0.5

6	E, CAZ, CIP, NIT, MR, AK, AT, AMP, CTX	9 (7)	1		0.6
7	CAZ, MR, AK, AMP, CTX	5 (4)	3		0.4
8	E, NIT, MR, AMP, CTX	5 (5)	1		0.4
9	E, GEN, CAZ, MR, AZM, AMP, CTX	7 (5)	1		0.5
10	E, CAZ, NIT, MR, AT, AMP	6 (5)	2		0.4
11	CAZ, C, MR, AMP	4 (4)	3		0.3
12	E, CAZ, NIT, MEM, AZM, AMP, CTX	7 (5)	2		0.5
13	CAZ, NIT, MR, AMP	4 (4)	2		0.3
14	CAZ, MR, AMP, IPM, CTX	5 (3)	1		0.4
15	CAZ, MR, AT, AMP, CTX	5 (4)	2		0.4
16	CAZ, NIT, AMP, MR, CTX, IPM	6 (4)	1		0.4
17	CAZ, NA, NIT, MER, CTX, AMP	6 (5)	1		0.4
18	CAZ, AT, NIT, MR, CTX, AMP	6 (5)	1		0.4
19	E, CAZ, C, NIT, MER, AT, AMP, CTX	8 (6)	1		0.6
20	E, CAZ, CTX, AMP, AT, MR	6 (4)	2		0.4
21	CAZ, AMP, CTX	3 (2)	3		0.2
22	MR, AMP, IPM	3 (2)	3		0.2
23	CAZ, CTX, MR	3 (2)	2		0.2
24	CTX, MR, AMP	3 (3)	4		0.2
25	CTX, MR, IPM	3 (2)	3		0.2
26	MR, AMP	2 (2)	5		0.1
27	CAZ, AMP	2 (2)	7		0.1
28	AMP	1(1)	3		0.07
29	CTX	1(1)	1		0.07

***MDR = Multidrug- resistant, MAR = Multiple Antibiotic Resistance

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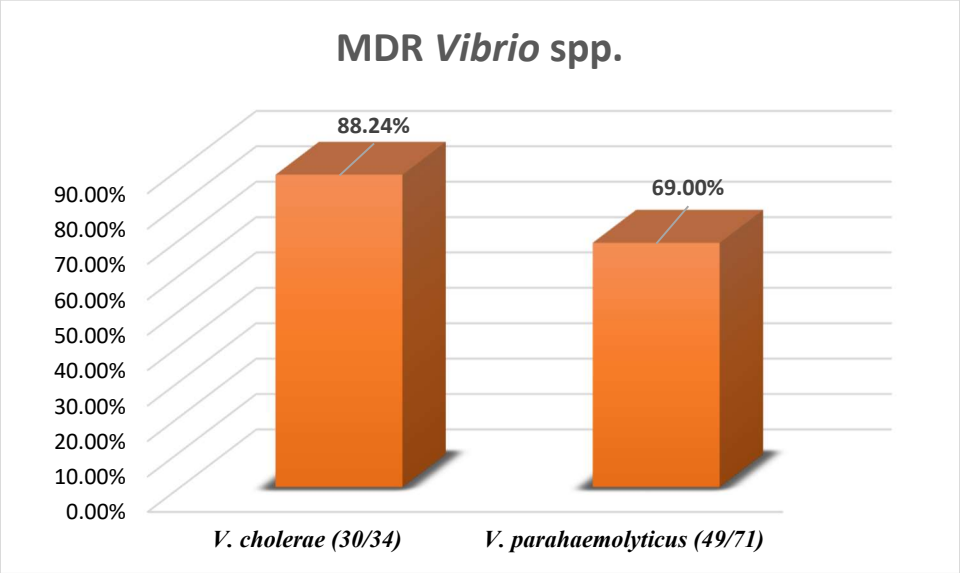

Figure 3.7: Prevalence of MDR *Vibrio cholerae* and *Vibrio parahaemolyticus*

3.5 Genotypic Detection of Extended Spectrum Beta-lactam (ESBL)

All *Vibrio* isolates were screened for certain antibiotic resistant gene belonging to the distinct group; ESBL. The presence of ESBL was determined by screening for resistant genes, including bla-TEM, bla-CTX-M and bla-SHV. 29.52% (31/105) isolates were positive for blaTEM and 10.61% (7/105) isolates were positive for bla- SHV. All the isolates were negative for bla-CTX-M.

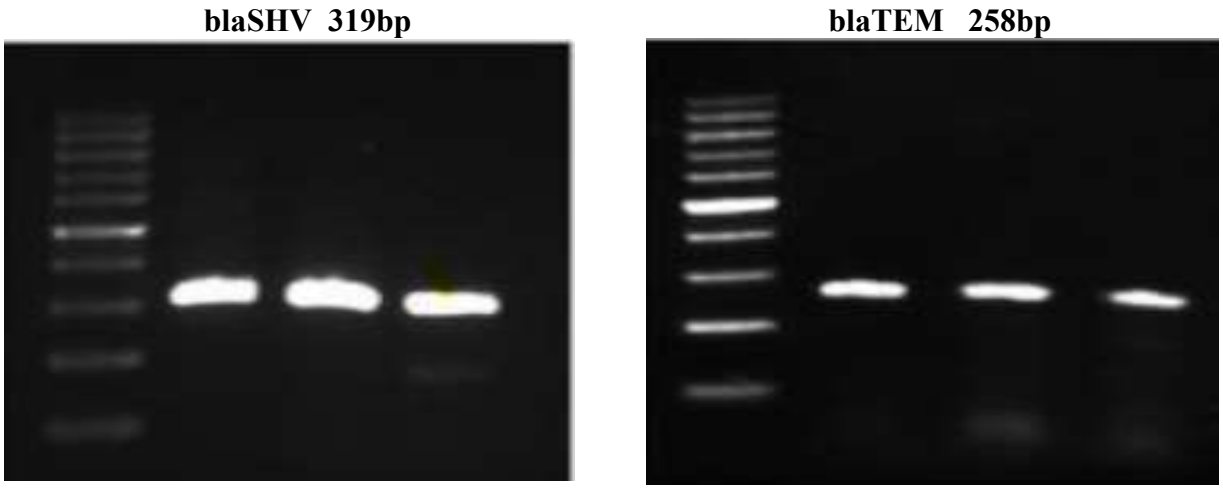

Figure 3.8: Genotypic detection of ESBL (bla- SHV and bla-TEM)

3.6 Biofilm Test Result

Among 105 positive isolates 41 were strong biofilm former, 21 were moderate biofilm former and 24 were weak biofilm former as like Table 3.3 and Table 3.4. The rest didn't form any biofilm.

Table 3.3: Biofilm formation by *Vibrio cholerae*

Isolate	**Biofilm Formation**	**Isolate**	**Biofilm Formation**
1A	Strong	120	Strong
22	Strong	129	Strong
38C	Weak	130	Moderate
104	Strong	132	Weak
105	Strong	134	Moderate
106	Moderate	143	Moderate
107	Strong	144B	Weak
108	Moderate	151B	Moderate
109	Strong	153A	Moderate
110	Moderate	172	No
112	Strong	176A	Strong
114	Weak	191	No
115	Strong	195	Strong
116	No	215	Moderate
117	Strong	222	Weak
118	Strong	229B	Weak
119	Strong	236A	No

Table 3.4: Biofilm formation by *Vibrio parahaemolyticus*

Isolate	**Biofilm Formation**	**Isolate**	**Biofilm Formation**
3D	Weak	91A	Strong
5A	Strong	92A	Strong
13A	Strong	93A	Strong
14A	Weak	94	Strong
17	Weak	111	Moderate

19	Strong	124	Weak
21A	Moderate	152	Strong
23	Strong	157C	No
24A	Weak	158A	Weak
25A	Strong	167	No
26A	No	168A	Moderate
28A	Moderate	168B	Strong
29B	Strong	169A	Weak
31A	Strong	169B	No
34A	Moderate	174A	Strong
35A	Strong	177B	No
36A	Strong	180A	Strong
37A	Moderate	181B	No
38A	Strong	182	Strong
39	Weak	185A	Weak
40	Strong	186	No
42A	Strong	187	Moderate
43A	Strong	188	No
45	Moderate	189	Strong
49	Weak	193	Weak
61	Strong	196B	Moderate
64	No	197	No
65	Weak	200	Weak
75A	Weak	203	Weak
76	No	205B	No
77A	Weak	208	Moderate
81C	No	211	No
87A	Strong	213B	Moderate
88A	Moderate	217	Weak
89A	No	224	Weak
90B	Strong		

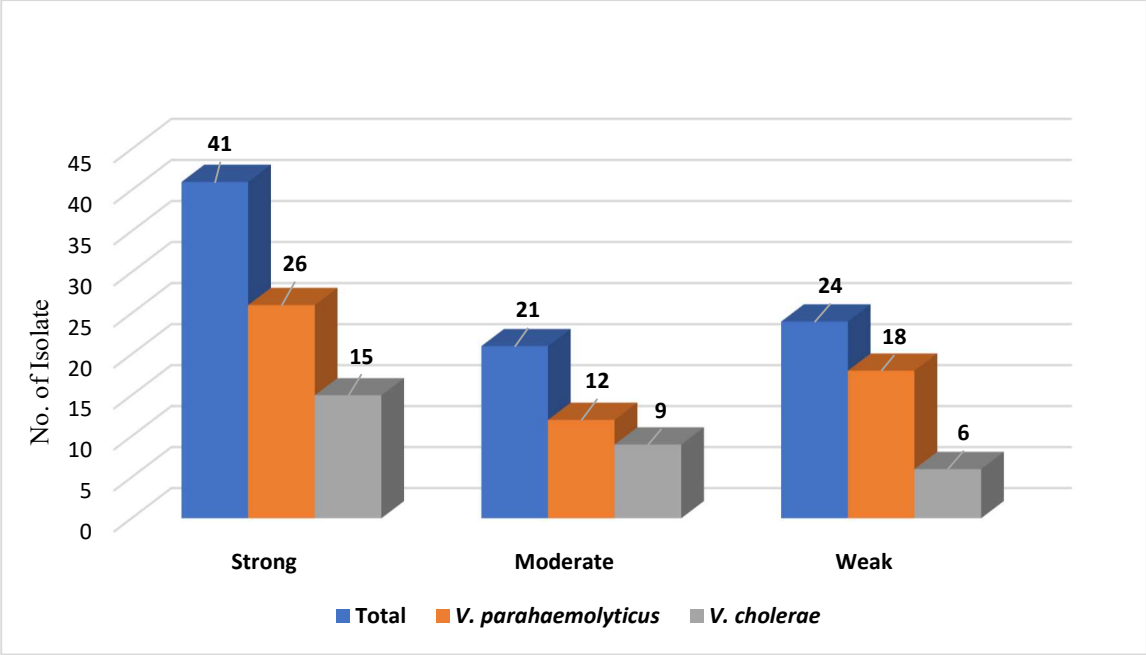

Figure 3.9: Biofilm formation of *Vibrio* spp.

3.7 Molecular detection of virulence genes

Seven virulence gene were tested by polymerase chain reaction among the *Vibrio* isolates. From them four gene for *Vibrio cholerae* include ng hlyA EI Tor, rtx, ompU, TcpA and the other three gene for *Vibrio parahaemolyticus* which are Tdh, Tlh, Stn. It shows virulence pattern of those isolates as Table 5 and 6.

Table 3.5: Virulence gene in *Vibrio cholerae* from shrimp sample

Isolate	hlyA EI Tor	rtx	ompU	TcpA
1A	+	−	−	−
22	+	−	−	−
38C	+	−	−	−
104	+	−	−	−
105	+	−	−	−
106	+	−	−	−
107	+	−	−	−
108	+	+	+	−

109	+	+	+	−
110	+	+	+	−
112	+	+	+	−
114	+	−	−	−
115	+	+	−	−
116	+	−	+	−
117	+	+	−	−
118	+	+	+	−
119	+	+	−	−
120	+	−	−	−
129	+	+	−	−
130	+	+	−	−
132	+	+	−	−
134	+	−	−	−
143	+	+	−	−
144B	+	−	−	−
151B	+	−	−	−
153A	+	+	+	−
172	+	−	+	−
176A	+	−	+	−
191	+	−	−	−
195	+	−	+	−
215	+	+	+	−
222	+	−	+	−
229B	+	−	+	−
236A	+	−	+	−

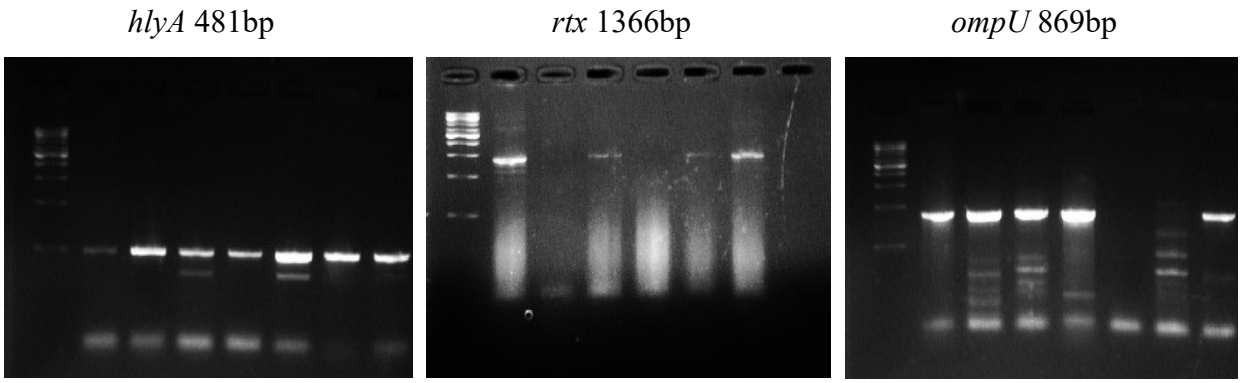

Figure 3.10: Detection of virulence gene in *Vibrio cholerae*

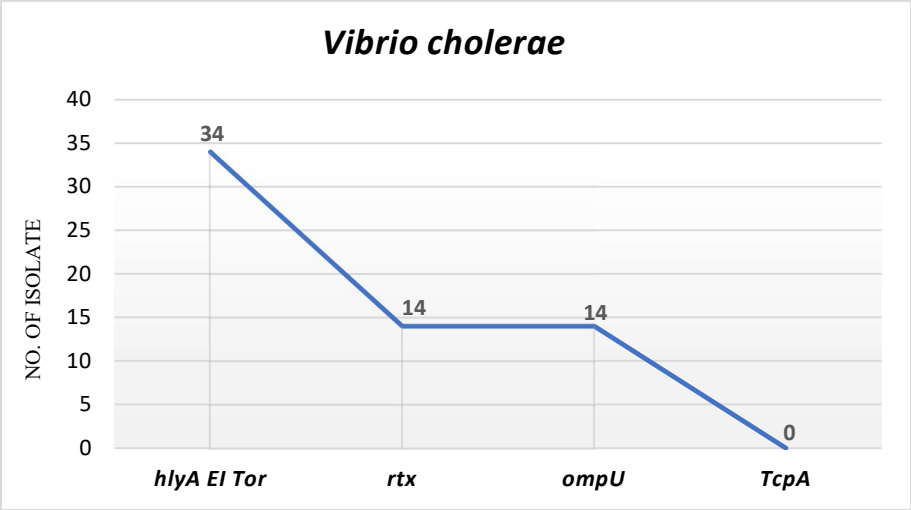

Figure 3.11: Virulence pattern in *Vibrio cholerae*

Table 3.6: Virulence gene in *Vibrio parahaemolyticus* from shrimp sample

Isolate	Tdh	Tlh	Stn
3D	−	+	+
5A	−	+	+
13A	−	−	−
14A	−	+	+
17	+	−	−
19	+	−	−

21A	−	−	−
23	+	−	−
24A	+	−	−
25A	+	−	+
26A	+	−	−
28A	−	+	−
29B	−	+	−
31A	+	−	−
34A	−	+	−
35A	−	+	−
36A	−	+	−
37A	−	+	−
38A	−	+	−
39	−	+	+
40	−	+	+
42A	−	+	−
43A	−	+	−
45	−	+	+
49	−	+	−
61	−	+	−
64	−	+	+
65	−	+	−
75A	−	+	−
76	−	+	−
77A	−	+	−
81C	−	+	−
87A	−	+	−
88A	−	+	−
89A	−	+	−
90B	−	+	−
91A	−	+	+

92A	−	+	+
93A	−	+	−
94	−	+	−
111	−	+	−
124	−	+	−
152	−	+	−
157C	−	+	−
158A	−	+	−
167	−	+	−
168A	−	+	−
168B	−	+	−
169A	−	+	−
169B	−	+	−
174A	−	+	−
177B	−	+	−
180A	−	+	−
181B	−	+	−
182	−	+	−
185A	−	+	−
186	−	+	−
187	−	+	−
188	−	+	−
189	−	+	+
193	−	+	−
196B	−	+	−
197	−	+	−
200	−	+	−
203	−	+	−
205B	−	+	−
208	−	+	−
211	−	+	−

213B	−	+	−
217	−	+	−
224	−	+	−

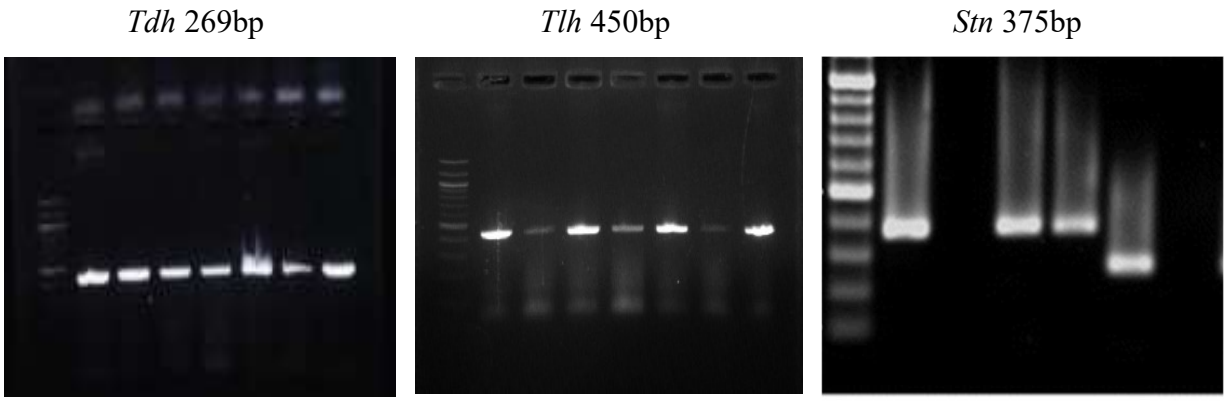

Figure 3.12: Detection of virulence gene in *Vibrio parahaemolyticus*

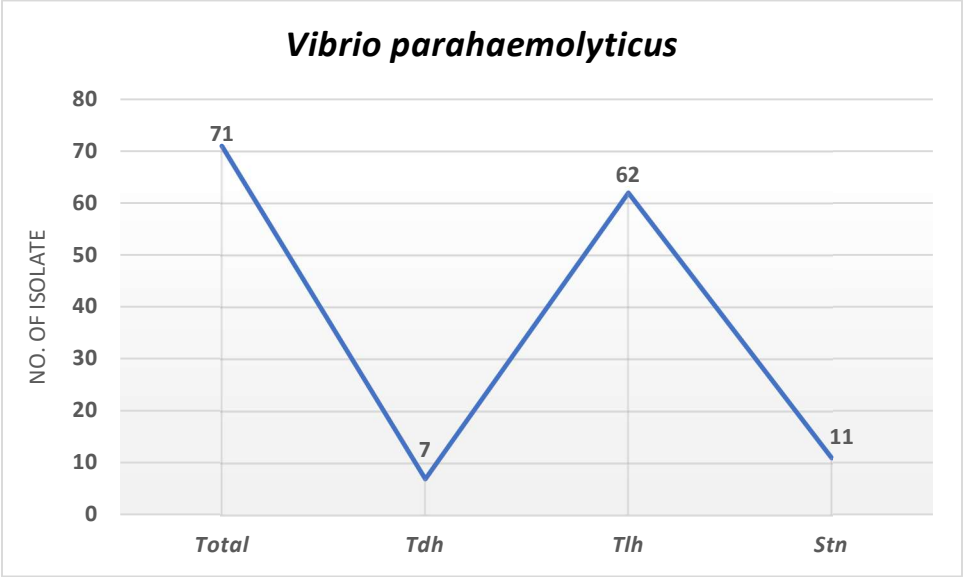

Figure 3.13: Virulence pattern in *Vibrio parahaemolyticus*

3.8 Whole Genome Analysis

Whole genome sequence analysis and functional annotation

The identification based on whole genome sequencing confirmed that both isolates are *Vibrio parahaemolyticus*. Additionally, the validation through the ANI score reinforced the identification process, revealing that the examined isolates were indeed belong to the member of *V. parahaemolyticus*. This finding was emphasized by the significant nucleotide identity surpassing 96% in comparison to the reference strains. Subsequently, isolates 37A and 91A were classified as "*Vibrio parahaemolyticus* SU37A" and "*Vibrio parahaemolyticus* SU91A," respectively. The complete genome sequences of both isolates have been submitted to GenBank at NCBI, assigned the accession numbers JBQRUG000000000.1 and JBQWLY000000000.1. The genome sequence analysis revealed high quality, characterized by completeness and minimal contamination. The genome's completeness, together with both coarse and fine consistency, also suggested its high quality. The genome size and GC content of the sequenced isolates corresponded closely with the known genomes of *V. parahaemolyticus*.

Following de novo genome assembly and annotation, the coding sequence (CDS) ratios of *Vibrio parahaemolyticus* isolates SU37A and SU91A were predicted to be 0.929 and 1.012, respectively. These values indicate a high-quality assembly, as they fall within the standard CDS ratio range (0.8–1.2) generally accepted by the NCBI for well-assembled bacterial genomes. A notable proportion of hypothetical proteins (≥30.0) was identified in both isolates, suggesting substantial functional and evolutionary diversity. The presence of these proteins suggests the possible existence of unidentified genes or uncharacterized functional elements in the genome. Hypothetical proteins are inferred from open reading frames (ORFs) whose translated products remain unknown and lack functional annotation. The comprehensive investigation of these proteins in *V. parahaemolyticus* may result in the discovery of biological roles that were not previously recognized, as well as the identification of novel diagnostic biomarkers or therapeutic targets for the purpose of managing infections that are caused by this bacterium.

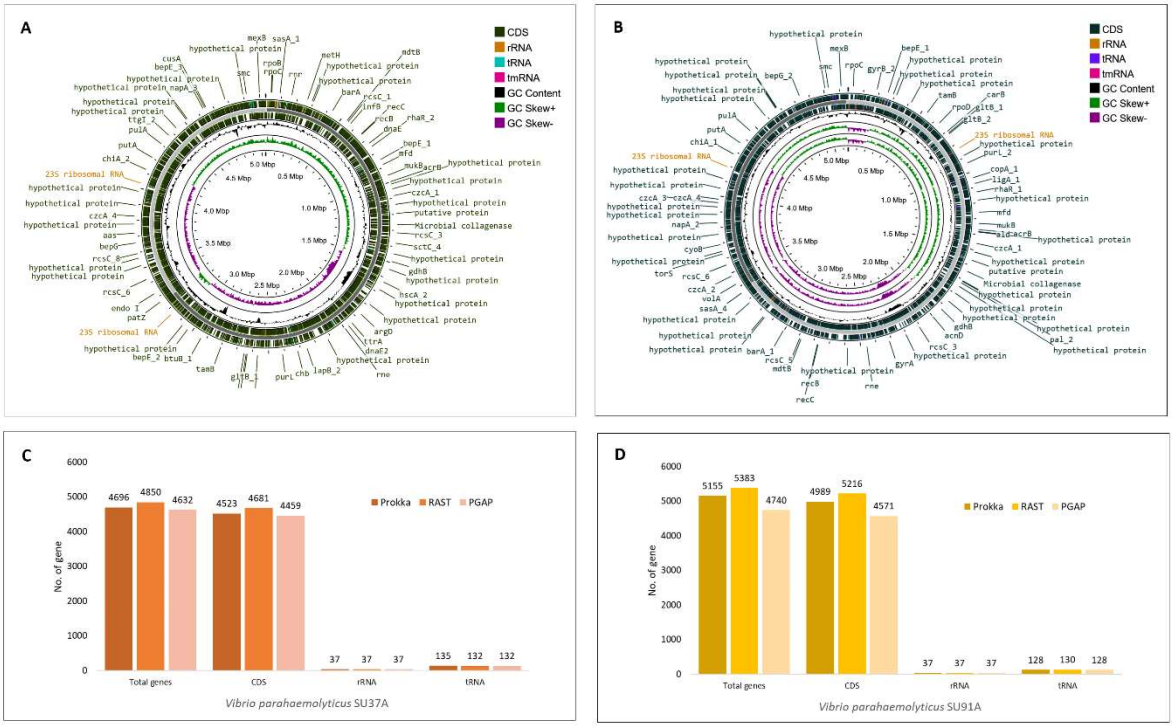

Figure 3.14: Whole genome sequence analysis of *Vibrio parahaemolyticus*

Antimicrobial resistance genes, and mobile genetic elements analysis

The analysis of antimicrobial resistance genes in the *V. parahaemolyticus* SU37A and SU91A strains uncovered an array of resistance factors across several antibiotic categories. Both strains contained tet(35) and TxR, which provide resistance to tetracycline antibiotics through ATP-binding cassette (ABC) efflux pumps, enabling the active expulsion of the antibiotic. In a similar vein, the CRP and adeF were recognized as elements of resistance-nodulation-cell division (RND) efflux systems, which confer resistance to various classes of antibiotics, such as macrolides, fluoroquinolones, penams, and tetracyclines.

In both strains, resistance to beta-lactam antibiotics was also identified due to the presence of CARB-18, a type of beta-lactamase, along with homologs of PBP3 from *Haemophilus influenzae*. These factors contribute to resistance against cephalosporins, cephamycins, and penams through mutations in penicillin-binding proteins (PBPs). The S385T substitution in the PBP3, may decrease the protein's affinity for beta-lactams, hence imparting resistance via modification of the antibiotic

target. The above strategy enables the SU37A and SU91A strains to persist in building its cell wall despite antibiotic exposure, constituting a major route for beta-lactam resistance [89].

The presence of vanY and vanT genes within the vanG cluster suggests a potential for resistance through alterations in cell wall targets.

Further multidrug resistance of the strains was observed with rsmA, a gene linked to the RND efflux pump that imparts resistance to fluoroquinolones, diaminopyrimidines, and phenicols. The findings collectively emphasize the strains' ability to resist antimicrobial agents, highlighting their potential implications in clinical settings and the difficulties encountered in managing infections caused by these strains.

However, the SU37A isolate was identified to possess a variant of the parE gene, which is homologous to the parE gene found in *Escherichia coli*, and is linked to resistance against fluoroquinolone antibiotics. A D476N point mutation in the ParE protein confers this resistance, leading to structural changes in the DNA gyrase subunit, which is the target of fluoroquinolones. As a result, the mutation may halt the antibiotic's ability to bind to its target, allowing the bacterium to endure exposure to fluoroquinolones. This mechanism illustrates the alteration of antibiotic targets, and the identification of the mutated parE indicates that the SU37A has a genotype resistant to fluoroquinolones.

Furthermore, an analysis of the *Vibrio parahaemolyticus* SU37A and SU91A genomes revealed the existence of several mobile genomic elements (MGEs), which include activities associated with replication, recombination and repair, phage integration, excision, horizontal transfer, and stability/defense mechanisms. Several of these MGEs clustered close major antibiotic resistance gene, PBP3 and ParE, indicating a possible involvement in the acquisition and spread of resistance genes among these isolates.

Virulence associated gene analysis

Over 60 virulence associated genes were identified in the *Vibrio* strains, specifically in *V. parahaemolyticus* strain SU37A and *V. parahaemolyticus* strain SU91A. The regulatory and stress response genes — rpoS, rpoE, crp, hfq, and arcA — were found to be common in both strains. These genes are crucial for modifying virulence and facilitating the adaptation of *Vibrio* species to diverse and often stressful conditions, including nutritional scarcity and host immunological responses. The rpoS and rpoE genes encode sigma factors (σ^S and σ^E) that modulate stress

responses and pathogenicity by enhancing bacterial survival under nutritional scarcity, oxidative damage, and envelope stress [90]. The crp gene functions as a universal regulator that connects carbon metabolism to virulence, modulating pathogenic variables based on nutritional availability [91]. The small RNA–mediated regulation of stress, motility, and quorum sensing is modulated by the hfq gene, which encodes an RNA chaperone [92].

Energy metabolism genes, including frdC, atpA, and sucA, promote anaerobic respiration and energy conservation in low-oxygen habitats. The strains also reported to have redox homeostasis genes, such as gshB and pare, which protect against oxidative stress by producing glutathione and activating peroxiredoxins [93]. Moreover, other membrane stability genes, tolB and VC2206, were also found to be common within the strains, maintaining cell membrane integrity and defending against immunological assault [94]. Finally, signal transduction regulators (cyaA and rpoB) within the strains may control the expression of global virulence genes in response to environmental signals [95].

Biosynthetic gene cluster and metabolic pathways investigation

The genomes of *V. parahaemolyticus* strain SU37A and strain 91A featured several major biosynthetic gene clusters, including four core biosynthetic gene clusters and various additional biosynthetic genes, transport-related genes, regulatory genes, and other genes (Fig. 5a). Genes for NI-siderophore biosynthesis, betalactone, ectoine, and arylpolyene comprised the four primary biosynthetic gene clusters. The presence of several conserved biosynthetic gene clusters associated with the production of secondary metabolites, such as arylpolyene, ectoine, beta-(β-)lactone, and NI-siderophore biosynthetic pathways, was revealed through genome-wide analysis of *Vibrio parahaemolyticus* isolates. These clusters, which are primarily recognized for their ability to adapt to environmental conditions, are now being perceived as indirect or direct contributors to the pathogenicity and ecological viability of *V. parahaemolyticus*. The production of carotenoid-like polyene pigments, which are structurally similar to bacterial lipids found in Gram-negative outer membranes, is encoded by arylpolyene biosynthetic gene clusters. These compounds contribute to the structural integrity of biofilms and provide oxidative stress protection, which contributes to bacterial survival in conditions of environmental stress and reactive oxygen species (ROS) generated by the host [96], [97]. Consequently, the presence of arylpolyene clusters in the genomes of both the 37A and 91A V. parahaemolyticus strains suggests that they may be involved in

oxidative resilience and persistence, which are crucial factors in the establishment of infections and the long-term colonisation of host environments.

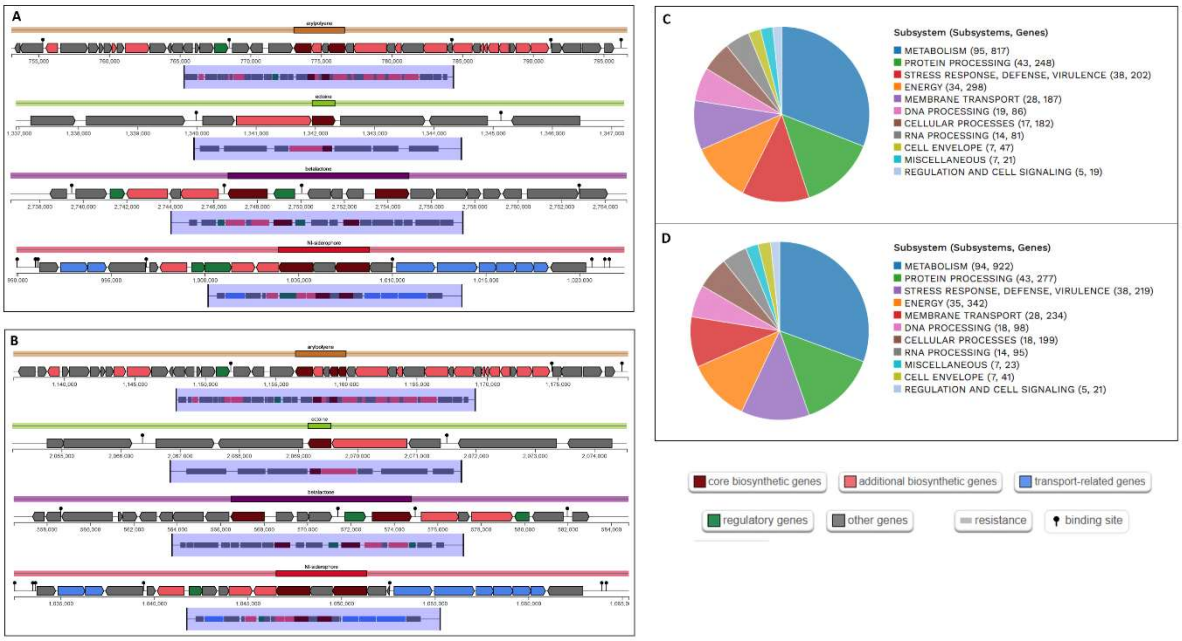

Figure 3.15: Biosynthetic gene cluster and metabolic pathways investigation

Subsystem analysis showed that the isolates had genes involved in metabolism, protein processing, stress response and defense, virulence, respiration, membrane transport, cellular processes, regulation, and cell signaling. The metabolic pathway study also found pathways involved in important processes. These included glucose metabolism, amino acid metabolism, glycan biosynthesis and metabolism, and signal transduction. Additional routes including lipid metabolism, energy metabolism, polyketide biosynthesis, secondary metabolite biosynthesis, and the biodegradation and metabolism of xenobiotics. The analysis of metabolic pathways in the isolates uncovered multiple routes for secondary metabolite biosynthesis, such as those for carotenoid, zeatin, phenylpropanoid, flavonoid, and novobiocin production. Furthermore, various pathways for the degradation of xenobiotics have been identified, including those for 2,4-dichlorobenzoate, toluene, xylene, geraniol, ethylbenzene, trinitrotoluene, styrene, atrazine, and caprolactam. The existence of these pathways highlights the isolates' ability to endure and adjust to hostile surroundings, showcasing their metabolic flexibility and strength. The combined metabolic capabilities of SU37A and SU91A seem to play a crucial role in their survival, metabolic functions, virulence, pathogenicity, and biochemical interactions associated with diabetic foot infections.

Chapter 4
Discussion

4. Discussion

This study offers an in-depth investigation of the prevalence, antimicrobial resistance (AMR), pathogenicity potential, and biofilm-forming ability of Vibrio species isolated from shrimp samples in Noakhali, Bangladesh. The findings indicate significant public health hazards linked to shrimp eating in this region, emphasizing a high incidence of multidrug-resistant (MDR) and aggressive Vibrio strains, which are supported by strong biofilm development. This discourse examines these critical findings within the broader context of regional aquaculture practices, global antibiotic resistance challenges, and food safety concerns.

Vibrio species were found in 43.57% (105 out of 241) of the shrimp samples that shows how badly this food item is contaminated. The accurate identification of *V. parahaemolyticus* (29.46%) as more prevalent than *V. cholerae* (14.12%) aligns with their recognized ecology, given that *V. parahaemolyticus* is a primary disease associated with seafood. The absence of *V. vulnificus* and *V. alginolyticus* suggests either their genuinely diminished prevalence in the examined markets or the influence of specific environmental and host factors, such as shrimp species. The elevated prevalence of *V. cholerae* in *Macrobrachium rosenbergii* (freshwater scampi) relative to the marine Penaeus monodon is notably compelling. Although *V. cholerae* is commonly linked to freshwater, its occurrence in both species suggests a multifaceted contamination route, likely arising during post-harvest processing, transit, or cross-contamination in retail environments where freshwater and marine species are sold in close proximity under unsanitary conditions. A recent study showed that *Vibrio* spp. was found in 60.2% of 216 shrimp and environmental samples from a different investigation done in Bangladesh's southwestern coastal region. While shrimp and water samples from Gher farms showed less contamination with *Vibrio* spp., a comparable study in southern Bangladesh found that only 27% of shrimp samples were contaminated with *V. parahaemolyticus* [98]. Another study found that 60.25% (241 out of 400) of retail shrimp meat samples from different production methods and geographic areas in northern California had *Vibrio* in them [99]. This work is the first in Bangladesh, to our knowledge, to use a molecular method to find all four *Vibrio* species—*V. parahaemolyticus*, *V. alginolyticus*, *V. vulnificus*, and *V. cholerae*—in different types of retail shrimp. Comparisons (similar, higher, and lower) between the current results and global research may differ from our findings due to variances in testing procedures, sample sizes and kinds, settings, and geographical distributions, among other factors.

This finding is a stark warning that the risk goes beyond farming and affects the entire supply chain.

The most concerning discovery of this investigation is the extensive and significant antibiotic resistance present among the *Vibrio* isolates. The exceedingly elevated resistance rates to ampicillin (88.57%), meropenem (81.90%), cefotaxime (71.43%), and ceftazidime (60.95%) constitute a significant public health emergency. Environmental *Vibrio* strains commonly exhibit great resistance to ampicillin, often attributable to intrinsic causes. The elevated quantity of ampicillin-resistant Vibrio isolates in this investigation aligns with prior research conducted in Bangladesh [98], [100]. The resistance to third-generation cephalosporins (cefotaxime, ceftazidime) and carbapenems (meropenem) is profoundly concerning, aligning with similar research outcomes from other countries, as these antibiotics are essential for managing severe human infections. The significant incidence of multidrug-resistant (MDR) *Vibrio* isolates (70.48%), with *V. cholerae* (88.24%) exhibiting a higher tendency for multidrug resistance than *V. parahaemolyticus* (69%), suggests a substantially contaminated reservoir of resistance genes within the aquaculture environment. The presence of resistance to antibiotics from the cephalosporin, aminoglycoside, fluoroquinolone, and folate pathway inhibitor families, which are recommended for treating *Vibrio* spp. infections, represents a significant issue by diminishing available treatment options [101].

The high Multiple Antibiotic Resistance (MAR) index (>0.2 for 80.95% of isolates) shows that these habitats are under a lot of antibiotic pressure, which is probably because antibiotics are used too much and for no good reason in shrimp aquaculture. The previous investigation by Lee et al. [102] found that 70% of the *Vibrio* isolates had a MAR index higher than 0.2, which is lower than what we found. This strategy, meant to stop sickness in ponds with a lot of people, has led to the growth of resistant bacteria, which subsequently go up the food chain and make frontline drugs useless against vibriosis or cholera. The genotypic identification of Extended-Spectrum Beta-Lactamase (ESBL) genes provided a genetic basis for the observed phenotypic resistance. The presence of bla-TEM (29.52%) and bla-SHV (10.61%) genes signifies a unique genetic resistance profile. The high number of these genes shows that mobile genetic elements with resistance determinants have been acquired.

The Whole Genome Sequencing (WGS) of *V. parahaemolyticus* SU37A and SU91A provided clear insight into the genetic composition of these isolates. The detection of a mutant parE gene,

which confers fluoroquinolone resistance, and genes responsible for the alteration of penicillin-binding proteins (PBPs) in SU37A elucidates the resistance mechanisms that may not have been entirely evident from disc diffusion testing alone. The identification of a collection of efflux pump genes (e.g., tet(35), adeF, CRP, rsmA) offers a convincing rationale for the extensive multidrug resistance (MDR) phenotype, as these pumps may extrude many, structurally diverse classes of antibiotics. The co-localization of resistance genes with mobile genetic elements (MGEs) is a significant discovery, as it illustrates the possibility for horizontal gene transfer to other bacteria, including human pathogens, in environmental or gastrointestinal contexts, hence exacerbating the AMR dilemma. In addition to their resistance, the pathogenic potential of these isolates is a significant risk. Virulence gene profiling indicated that all *V. cholerae* isolates included the hlyA El Tor gene, which encodes hemolysin, a pore-forming toxin capable of inducing tissue damage and promoting intestinal invasion. Although the prominent pandemic marker tcpA was lacking, the detection of rtx (repeats-in-toxin) and ompU (outer membrane protein) in a considerable percentage of isolates suggests that these strains maintain high pathogenicity. The rtx toxin cluster contributes to cytotoxicity and inflammation, whereas ompU increases adhesion to host cells and provides resistance to host antimicrobial peptides. The presence of the tlh (thermolabile hemolysin) gene in almost all *V. parahaemolyticus* helps species identification; nevertheless, the infrequent occurrence of the tdh and stn genes, established indicators of acute gastroenteritis, implies that these retail strains may be less prone to induce conventional food poisoning. The WGS data identified more than 60 virulence-associated genes in SU37A and SU91A, including regulators such as rpoS and crp, which govern stress response and virulence in adverse environments like the human host. This comprehensive virulence repertoire, albeit lacking traditional toxins, highlights that these environmental strains have a "stealth" pathogenicity, capable of inducing opportunistic infections, particularly in immunocompromised individuals.

The capacity of these robust and pathogenic strains to generate biofilms exacerbates the risk. *Vibrio* spp. can endure on shrimp surfaces, processing equipment, and market settings, with 62.86% of isolates exhibiting strong or moderate biofilm formation capabilities. According to a latest study most *Vibrio* isolates showed a moderate ability (48.61%) to high ability (37.50%) for biofilm formation [103] which is consistent to our study. Importantly, most MDR *Vibrio* isolates showed a higher rate of moderate-to-strong biofilm-forming ability (92.86%; 95%CI: 66.13–99.82%). Biofilms function as a protective barrier, increasing resistance to disinfectants and

antibiotics, while also acting as a persistent source of contamination. This study illustrates the strong relationship between biofilm production and antimicrobial resistance (AMR), establishing a detrimental cycle: biofilms safeguard resistant cells and promote the horizontal transfer of resistance genes, while sub-lethal quantities of antibiotics can subsequently stimulate more biofilm formation. This renders eradication from the food processing chain exceedingly challenging and facilitates the persistence and dissemination of MDR Vibrio within the ecosystem.

The ability of these strong and harmful strains to make biofilms makes the risk much higher. *Vibrio* spp. can survive on shrimp surfaces, processing equipment, and in market environments, with 62.86% of isolates demonstrating robust or moderate biofilm forming skills. A recent study found that most *Vibrio* isolates had a moderate (48.61%) to high (37.50%) ability to produce biofilms [103], which is in line with our study. Most MDR *Vibrio* isolates had a higher rate of moderate-to-strong biofilm-forming capacity (92.86%; 95%CI: 66.13–99.82%). Biofilms serve as a protective barrier, enhancing resistance to disinfectants and antibiotics, while simultaneously operating as a continual source of contamination. This study demonstrates the robust correlation between biofilm formation and antimicrobial resistance (AMR), creating a harmful cycle: biofilms protect resistant cells and facilitate the horizontal transfer of resistance genes, while sub-lethal concentrations of antibiotics can subsequently enhance biofilm development. This makes it very hard to get rid of MDR *Vibrio* from the food processing chain, which helps it stay in the ecosystem and spread.

Chapter 5
Conclusion

5. Conclusion

The findings of this study provide evidence that shrimp that are for sale in retail markets in the Noakhali district are a major reservoir for species of the genus *Vibrio* that develop biofilms, are pathogenic resistant to many drugs. These three high-risk characteristics all coming together in a well-liked food product is a significant threat to the safety of food and the health of the general public. The issue was most likely caused by the over use of antibiotics in aquaculture, which leads to the selection of resistant strains that are able to survive even the most powerful antibiotics. It is necessary to act immediately in response to the findings that have been discovered. An integrated strategy is necessary, which will encompass the following elements: the stringent enforcement of regulations pertaining to the use of antibiotics in aquaculture, along with the promotion of alternatives such as probiotics and vaccines; the implementation of improved hygiene and cold-chain management throughout the shrimp supply chain, beginning at the farm and ending at the market; continuous surveillance of AMR in seafood and the environment using both phenotypic and genotypic methods; and the provision of education for farmers, handlers, and consumers regarding safe practices. This research offers a scientifically grounded basis that serves as a vital alert for policymakers, who must act to safeguard both public health and the economic sustainability of the shrimp industry, which is vital to Bangladesh.

References

[1] S. M. N. Alam, 'Portraying the Bangladesh Shrimp Industry: A SWOT Analysis', *Sustainability*, vol. 16, no. 3, p. 1290, Feb. 2024, doi: 10.3390/su16031290.

[2] Z. F. Haque *et al.*, 'Molecular Detection and Antibiotic Resistance of Vibrio cholerae, Vibrio parahaemolyticus, and Vibrio alginolyticus from Shrimp (Penaeus monodon) and Shrimp Environments in Bangladesh', *Aquac Res*, vol. 2023, pp. 1–11, Feb. 2023, doi: 10.1155/2023/5436552.

[3] P. R. Yaashikaa, A. Saravanan, and P. S. Kumar, 'Isolation and identification of Vibrio cholerae and Vibrio parahaemolyticus from prawn (Penaeus monodon) seafood: Preservation strategies', *Microb Pathog*, vol. 99, pp. 5–13, Oct. 2016, doi: 10.1016/j.micpath.2016.07.014.

[4] T. T. T. Vu, T. Alter, and S. Huehn, 'Prevalence of Vibrio spp. in Retail Seafood in Berlin, Germany', *J Food Prot*, vol. 81, no. 4, pp. 593–597, Apr. 2018, doi: 10.4315/0362-028X.JFP-17-366.

[5] J. Oehlenschläger, 'Seafood: Nutritional Benefits and Risk Aspects', *International Journal for Vitamin and Nutrition Research*, vol. 82, no. 3, pp. 168–176, Jun. 2012, doi: 10.1024/0300-9831/a000108.

[6] M. Khan, S. I. Paul, Md. M. Rahman, and J. A. Lively, 'Antimicrobial Resistant Bacteria in Shrimp and Shrimp Farms of Bangladesh', *Water (Basel)*, vol. 14, no. 19, p. 3172, Oct. 2022, doi: 10.3390/w14193172.

[7] E. Haque, J. Hussain, S. Shankar, S. Haldar, and S. Chatterjee, 'Implication of Vibrio biofilms in human and seafood sector', in *Understanding Microbial Biofilms*, Elsevier, 2023, pp. 247–260. doi: 10.1016/B978-0-323-99977-9.00038-7.

[8] J. J. Farmer, 'The Family Vibrionaceae', in *The Prokaryotes*, New York, NY: Springer New York, 2006, pp. 495–507. doi: 10.1007/0-387-30746-X_17.

[9] J. D. Oliver, 'The Biology of *Vibrio vulnificus*', *Microbiol Spectr*, vol. 3, no. 3, Jun. 2015, doi: 10.1128/microbiolspec.VE-0001-2014.

[10] A. Huq, B. J. Haley, E. Taviani, A. Chen, N. A. Hasan, and R. R. Colwell, 'Detection, Isolation, and Identification of *Vibrio cholerae* from the Environment', *Curr Protoc Microbiol*, vol. 26, no. 1, Aug. 2012, doi: 10.1002/9780471729259.mc06a05s26.

[11] R. Sultana *et al.*, 'Isolation and Identification of Vibrio Species from Different Types of Water Sources Along with Their Drug Susceptible Pattern', *Biomedical and Biotechnology Research Journal*, vol. 8, no. 2, pp. 207–212, Apr. 2024, doi: 10.4103/bbrj.bbrj_138_24.

[12] D. W. Miles, T. Ross, J. Olley, and T. A. McMeekin, 'Development and evaluation of a predictive model for the effect of temperature and water activity on the growth rate of Vibrio parahaemolyticus', *Int J Food Microbiol*, vol. 38, no. 2–3, pp. 133–142, Sep. 1997, doi: 10.1016/S0168-1605(97)00100-1.

[13] W. A. Norfolk, C. Shue, W. M. Henderson, D. A. Glinski, and E. K. Lipp, '*Vibrio alginolyticus* growth kinetics and the metabolic effects of iron', *Microbiol Spectr*, vol. 11, no. 6, Dec. 2023, doi: 10.1128/spectrum.02680-23.

[14] G. Aguirre-Guzman, H. Mejia Ruiz, and F. Ascencio, 'A review of extracellular virulence product of Vibrio species important in diseases of cultivated shrimp', *Aquac Res*, vol. 35, no. 15, pp. 1395–1404, Sep. 2004, doi: 10.1111/j.1365-2109.2004.01165.x.

[15] A. Nakhamchik, C. Wilde, and D. A. Rowe-Magnus, 'Cyclic-di-GMP Regulates Extracellular Polysaccharide Production, Biofilm Formation, and Rugose Colony Development by *Vibrio vulnificus*', *Appl Environ Microbiol*, vol. 74, no. 13, pp. 4199–4209, Jul. 2008, doi: 10.1128/AEM.00176-08.

[16] A. Rønneseth *et al.*, 'Comparative assessment of *Vibrio* virulence in marine fish larvae', *J Fish Dis*, vol. 40, no. 10, pp. 1373–1385, Oct. 2017, doi: 10.1111/jfd.12612.

[17] B. Austin and X.-H. Zhang, 'Vibrio harveyi: a significant pathogen of marine vertebrates and invertebrates', *Lett Appl Microbiol*, vol. 43, no. 2, pp. 119–124, Aug. 2006, doi: 10.1111/j.1472-765X.2006.01989.x.

[18] I. Frans, C. W. Michiels, P. Bossier, K. A. Willems, B. Lievens, and H. Rediers, '*Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention', *J Fish Dis*, vol. 34, no. 9, pp. 643–661, Sep. 2011, doi: 10.1111/j.1365-2761.2011.01279.x.

[19] F. Feldhusen, 'The role of seafood in bacterialfoodborne diseases', *Microbes Infect*, vol. 2, no. 13, pp. 1651–1660, Nov. 2000, doi: 10.1016/S1286-4579(00)01321-6.

[20] J. L. Nordstrom, M. C. L. Vickery, G. M. Blackstone, S. L. Murray, and A. DePaola, 'Development of a Multiplex Real-Time PCR Assay with an Internal Amplification Control for the Detection of Total and Pathogenic *Vibrio parahaemolyticus* Bacteria in Oysters', *Appl Environ Microbiol*, vol. 73, no. 18, pp. 5840–5847, Sep. 2007, doi: 10.1128/AEM.00460-07.

[21] A. DePaola, J. L. Nordstrom, J. C. Bowers, J. G. Wells, and D. W. Cook, 'Seasonal Abundance of Total and Pathogenic *Vibrio parahaemolyticus* in Alabama Oysters', *Appl Environ Microbiol*, vol. 69, no. 3, pp. 1521–1526, Mar. 2003, doi: 10.1128/AEM.69.3.1521-1526.2003.

[22] F. G. R. de Menezes *et al.*, 'DETECTION OF VIRULENCE GENES IN ENVIRONMENTAL STRAINS OF Vibrio cholerae FROM ESTUARIES IN NORTHEASTERN BRAZIL', *Rev Inst Med Trop Sao Paulo*, vol. 56, no. 5, pp. 427–432, Sep. 2014, doi: 10.1590/S0036-46652014000500010.

[23] C. Baker-Austin *et al.*, 'Vibrio spp. infections', *Nat Rev Dis Primers*, vol. 4, no. 1, pp. 1–19, Jun. 2018, doi: 10.1038/s41572-018-0005-8.

[24] N. Howard-Jones, 'Robert Koch and the cholera vibrio: a centenary.', *BMJ*, vol. 288, no. 6414, pp. 379–381, Feb. 1984, doi: 10.1136/bmj.288.6414.379.

[25] E. J. Nelson, J. B. Harris, J. Glenn Morris, S. B. Calderwood, and A. Camilli, 'Cholera transmission: the host, pathogen and bacteriophage dynamic', *Nat Rev Microbiol*, vol. 7, no. 10, pp. 693–702, Oct. 2009, doi: 10.1038/nrmicro2204.

[26] M. Alam *et al.*, 'Viable but nonculturable *Vibrio cholerae* O1 in biofilms in the aquatic environment and their role in cholera transmission', *Proceedings of the National Academy of Sciences*, vol. 104, no. 45, pp. 17801–17806, Nov. 2007, doi: 10.1073/pnas.0705599104.

[27] M. Sultana *et al.*, 'Biofilms Comprise a Component of the Annual Cycle of Vibrio cholerae in the Bay of Bengal Estuary', *mBio*, vol. 9, no. 2, May 2018, doi: 10.1128/mBio.00483-18.

[28] F. L. Thompson, T. Iida, and J. Swings, 'Biodiversity of Vibrios', *Microbiology and Molecular Biology Reviews*, vol. 68, no. 3, pp. 403–431, Sep. 2004, doi: 10.1128/MMBR.68.3.403-431.2004.

[29] M. T. Islam, M. Alam, and Y. Boucher, 'Emergence, ecology and dispersal of the pandemic generating Vibrio cholerae lineage.', *Int Microbiol*, vol. 20, no. 3, pp. 106–115, Sep. 2017, doi: 10.2436/20.1501.01.291.

[30] M. Tanveer, E. Ntakiyisumba, and G. Won, 'Prevalence and risk factors of seafood-borne Vibrio vulnificus in Asia: a systematic review with meta-analysis and meta-regression', *Front Microbiol*, vol. 15, Mar. 2024, doi: 10.3389/fmicb.2024.1363560.

[31] P. Sudan *et al.*, 'Prevalence and antimicrobial resistance of food safety related Vibrio species in inland saline water shrimp culture farms', *International Microbiology*, vol. 26, no. 3, pp. 591–600, Jan. 2023, doi: 10.1007/s10123-023-00323-7.

[32] M. Khan, S. I. Paul, Md. M. Rahman, and J. A. Lively, 'Antimicrobial Resistant Bacteria in Shrimp and Shrimp Farms of Bangladesh', *Water (Basel)*, vol. 14, no. 19, p. 3172, Oct. 2022, doi: 10.3390/w14193172.

[33] D. V. Lightner, 'Virus diseases of farmed shrimp in the Western Hemisphere (the Americas): A review', *J Invertebr Pathol*, vol. 106, no. 1, pp. 110–130, Jan. 2011, doi: 10.1016/j.jip.2010.09.012.

[34] T. Herawati *et al.*, 'Report on Vibrio Species Contamination in Shrimp From the Coast of Pangandaran, West Java, Indonesia', *Environ Microbiol Rep*, vol. 17, no. 5, Oct. 2025, doi: 10.1111/1758-2229.70210.

[35] M. Sohidullah *et al.*, 'Exploration of Shrimp and Their Environments for the Detection of Antibiotic Resistance Genes of Vibrio parahaemolyticus and Spectrophotometry of Shrimp Muscles for Heavy Metals and Their Human Health Risk Assessment in Bangladesh', *J Food Prot*, vol. 88, no. 4, p. 100475, Mar. 2025, doi: 10.1016/j.jfp.2025.100475.

[36] T. Herawati *et al.*, 'Report on Vibrio Species Contamination in Shrimp From the Coast of Pangandaran, West Java, Indonesia', *Environ Microbiol Rep*, vol. 17, no. 5, Oct. 2025, doi: 10.1111/1758-2229.70210.

[37] J. S. Mok *et al.*, 'Distribution and antimicrobial resistance of Vibrio parahaemolyticus isolated from fish and shrimp aquaculture farms along the Korean coast', *Mar Pollut Bull*, vol. 171, p. 112785, Oct. 2021, doi: 10.1016/j.marpolbul.2021.112785.

[38] A. Sapkota *et al.*, 'Aquaculture practices and potential human health risks: Current knowledge and future priorities', *Environ Int*, vol. 34, no. 8, pp. 1215–1226, Nov. 2008, doi: 10.1016/j.envint.2008.04.009.

[39] A. Di Cesare *et al.*, 'Aquaculture Can Promote the Presence and Spread of Antibiotic-Resistant Enterococci in Marine Sediments', *PLoS One*, vol. 8, no. 4, p. e62838, Apr. 2013, doi: 10.1371/journal.pone.0062838.

[40] L.-H. Lee and P. Raghunath, 'Editorial: Vibrionaceae Diversity, Multidrug Resistance and Management', *Front Microbiol*, vol. 9, Mar. 2018, doi: 10.3389/fmicb.2018.00563.

[41] S. Chowdhury *et al.*, 'Antibiotics usage practices in aquaculture in Bangladesh and their associated factors', *One Health*, vol. 15, p. 100445, Dec. 2022, doi: 10.1016/j.onehlt.2022.100445.

[42] A. H. Delcour, 'Outer membrane permeability and antibiotic resistance', *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, vol. 1794, no. 5, pp. 808–816, May 2009, doi: 10.1016/j.bbapap.2008.11.005.

[43] J. Sun, Z. Deng, and A. Yan, 'Bacterial multidrug efflux pumps: Mechanisms, physiology and pharmacological exploitations', *Biochem Biophys Res Commun*, vol. 453, no. 2, pp. 254–267, Oct. 2014, doi: 10.1016/j.bbrc.2014.05.090.

[44] D. I. Andersson and D. Hughes, 'Antibiotic resistance and its cost: is it possible to reverse resistance?', *Nat Rev Microbiol*, vol. 8, no. 4, pp. 260–271, Apr. 2010, doi: 10.1038/nrmicro2319.

[45] G. De Pascale and G. D. Wright, 'Antibiotic Resistance by Enzyme Inactivation: From Mechanisms to Solutions', *ChemBioChem*, vol. 11, no. 10, pp. 1325–1334, Jul. 2010, doi: 10.1002/cbic.201000067.

[46] S. Bag *et al.*, 'Molecular Insights into Antimicrobial Resistance Traits of Commensal Human Gut Microbiota', *Microb Ecol*, vol. 77, no. 2, pp. 546–557, Feb. 2019, doi: 10.1007/s00248-018-1228-7.

[47] S. M. Soucy, J. Huang, and J. P. Gogarten, 'Horizontal gene transfer: building the web of life', *Nat Rev Genet*, vol. 16, no. 8, pp. 472–482, Aug. 2015, doi: 10.1038/nrg3962.

[48] M. Arunkumar, F. LewisOscar, N. Thajuddin, A. Pugazhendhi, and C. Nithya, 'In vitro and in vivo biofilm forming Vibrio spp: A significant threat in aquaculture', *Process Biochemistry*, vol. 94, pp. 213–223, Jul. 2020, doi: 10.1016/j.procbio.2020.04.029.

[49] H.-S. Joo and M. Otto, 'Molecular Basis of In Vivo Biofilm Formation by Bacterial Pathogens', *Chem Biol*, vol. 19, no. 12, pp. 1503–1513, Dec. 2012, doi: 10.1016/j.chembiol.2012.10.022.

[50] B. K. Hammer and B. L. Bassler, 'Quorum sensing controls biofilm formation in *Vibrio cholerae*', *Mol Microbiol*, vol. 50, no. 1, pp. 101–104, Oct. 2003, doi: 10.1046/j.1365-2958.2003.03688.x.

[51] J. Kowalska, E. Maćkiw, M. Stasiak, K. Kucharek, and J. Postupolski, 'Biofilm-Forming Ability of Pathogenic Bacteria Isolated from Retail Food in Poland', *J Food Prot*, vol. 83, no. 12, pp. 2032–2040, Dec. 2020, doi: 10.4315/JFP-20-135.

[52] J. B. Kaplan, 'Antibiotic-Induced Biofilm Formation', *Int J Artif Organs*, vol. 34, no. 9, pp. 737–751, Sep. 2011, doi: 10.5301/ijao.5000027.

[53] T. Brauge, J. Mougin, T. Ells, and G. Midelet, 'Sources and contamination routes of seafood with human pathogenic *Vibrio* spp.: A *Farm-to-Fork* approach', *Compr Rev Food Sci Food Saf*, vol. 23, no. 1, Jan. 2024, doi: 10.1111/1541-4337.13283.

[54] I. Karunasagar, S. K. Otta, and I. Karunasagar, 'Biofilm formation by Vibrio harveyi on surfaces', *Aquaculture*, vol. 140, no. 3, pp. 241–245, Mar. 1996, doi: 10.1016/0044-8486(95)01180-3.

[55] R. E. Leighton *et al.*, 'Vibrio parahaemolyticus and Vibrio vulnificus in vitro biofilm dispersal from microplastics influenced by simulated human environment', *Front Microbiol*, vol. 14, Oct. 2023, doi: 10.3389/fmicb.2023.1236471.

[56] K. C. T. Nguyen, P. H. Truong, H. T. Thi, X. T. Ho, and P. Van Nguyen, 'Prevalence, multidrug resistance, and biofilm formation of Vibrio parahaemolyticus isolated from fish mariculture environments in Cat Ba Island, Vietnam', *Osong Public Health Res Perspect*, vol. 15, no. 1, pp. 56–67, Feb. 2024, doi: 10.24171/j.phrp.2023.0181.

[57] . WORLD HEALTH ORGANIZATION, *SELECTION AND APPLICATION OF METHODS FOR THE DETECTION AND ENUMERATION OF HUMAN-PATHOGENIC ... HALOPHILIC VIBRIO SPP. IN SEAFOOD : guidance*. WORLD HEALTH ORGANIZATION, 2017.

[58] M. T. Hossain, E.-Y. Kim, Y.-R. Kim, D.-G. Kim, and I.-S. Kong, 'Development of a *groEL* gene-based species-specific multiplex polymerase chain reaction assay for simultaneous detection of *Vibrio cholerae* , *Vibrio parahaemolyticus* and *Vibrio vulnificus*', *J Appl Microbiol*, vol. 114, no. 2, pp. 448–456, Feb. 2013, doi: 10.1111/jam.12056.

[59] R. Ahmed, S. M. Rafiquzaman, M. T. Hossain, J.-M. Lee, and I.-S. Kong, 'Species-specific detection of Vibrio alginolyticus in shellfish and shrimp by real-time PCR using the groEL gene', *Aquaculture International*, vol. 24, no. 1, pp. 157–170, Feb. 2016, doi: 10.1007/s10499-015-9916-5.

[60] Z. F. Haque *et al.*, 'Molecular Detection and Antibiotic Resistance of Vibrio cholerae, Vibrio parahaemolyticus, and Vibrio alginolyticus from Shrimp (Penaeus monodon) and Shrimp Environments in Bangladesh', *Aquac Res*, vol. 2023, pp. 1–11, Feb. 2023, doi: 10.1155/2023/5436552.

[61] G. M. Durán and D. L. Marshall, ‘Ready-to-Eat Shrimp as an International Vehicle of Antibiotic-Resistant Bacteria’, *J Food Prot*, vol. 68, no. 11, pp. 2395–2401, Nov. 2005, doi: 10.4315/0362-028X-68.11.2395.

[62] G. D. Christensen, W. A. Simpson, A. L. Bisno, and E. H. Beachey, ‘Adherence of slime-producing strains of Staphylococcus epidermidis to smooth surfaces’, *Infect Immun*, vol. 37, no. 1, pp. 318–326, Jul. 1982, doi: 10.1128/iai.37.1.318-326.1982.

[63] T. S. Bhowmick *et al.*, ‘Detection of virulence-associated and regulatory protein genes in association with phage typing of human Vibrio cholerae from several geographical regions of the world’, *J Med Microbiol*, vol. 58, no. 9, pp. 1160–1167, Sep. 2009, doi: 10.1099/jmm.0.008466-0.

[64] B. Meena *et al.*, ‘Studies on diversity of Vibrio sp. and the prevalence of hapA, tcpI, st, rtxA&C, acfB, hlyA, ctxA, ompU and toxR genes in environmental strains of Vibrio cholerae from Port Blair bays of South Andaman, India’, *Mar Pollut Bull*, vol. 144, pp. 105–116, Jul. 2019, doi: 10.1016/j.marpolbul.2019.05.011.

[65] P. Paria, B. K. Behera, P. K. Das Mohapatra, and P. K. Parida, ‘Virulence factor genes and comparative pathogenicity study of tdh, trh and tlh positive Vibrio parahaemolyticus strains isolated from Whiteleg shrimp, Litopenaeus vannamei (Boone, 1931) in India’, *Infection, Genetics and Evolution*, vol. 95, p. 105083, Nov. 2021, doi: 10.1016/j.meegid.2021.105083.

[66] A. V. Rizvi and A. K. Bej, ‘Multiplexed real-time PCR amplification of tlh, tdh and trh genes in Vibrio parahaemolyticus and its rapid detection in shellfish and Gulf of Mexico water’, *Antonie Van Leeuwenhoek*, vol. 98, no. 3, pp. 279–290, Oct. 2010, doi: 10.1007/s10482-010-9436-2.

[67] M. S. Uddin *et al.*, ‘First Insight into the Gut Microbiome of Rohu Fish from Halda River and Kaptai Lake Using 16S rRNA Sequencing’, Aug. 12, 2025. doi: 10.21203/rs.3.rs-7192852/v1.

[68] W. De Coster, S. D’Hert, D. T. Schultz, M. Cruts, and C. Van Broeckhoven, ‘NanoPack: visualizing and processing long-read sequencing data’, *Bioinformatics*, vol. 34, no. 15, pp. 2666–2669, Aug. 2018, doi: 10.1093/bioinformatics/bty149.

[69] M. Kolmogorov, J. Yuan, Y. Lin, and P. A. Pevzner, ‘Assembly of long, error-prone reads using repeat graphs’, *Nat Biotechnol*, vol. 37, no. 5, pp. 540–546, May 2019, doi: 10.1038/s41587-019-0072-8.

[70] R. Vaser, I. Sović, N. Nagarajan, and M. Šikić, ‘Fast and accurate de novo genome assembly from long uncorrected reads’, *Genome Res*, vol. 27, no. 5, pp. 737–746, May 2017, doi: 10.1101/gr.214270.116.

[71] J. P. Meier-Kolthoff and M. Göker, ‘TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy’, *Nat Commun*, vol. 10, no. 1, p. 2182, May 2019, doi: 10.1038/s41467-019-10210-3.

[72] I. Lee, M. Chalita, S.-M. Ha, S.-I. Na, S.-H. Yoon, and J. Chun, 'ContEst16S: an algorithm that identifies contaminated prokaryotic genomes using 16S RNA gene sequences', *Int J Syst Evol Microbiol*, vol. 67, no. 6, pp. 2053–2057, Jun. 2017, doi: 10.1099/ijsem.0.001872.

[73] D. H. Parks, M. Imelfort, C. T. Skennerton, P. Hugenholtz, and G. W. Tyson, 'CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes', *Genome Res*, vol. 25, no. 7, pp. 1043–1055, Jul. 2015, doi: 10.1101/gr.186072.114.

[74] I. Lee, Y. Ouk Kim, S.-C. Park, and J. Chun, 'OrthoANI: An improved algorithm and software for calculating average nucleotide identity', *Int J Syst Evol Microbiol*, vol. 66, no. 2, pp. 1100–1103, Feb. 2016, doi: 10.1099/ijsem.0.000760.

[75] T. Seemann, 'Prokka: rapid prokaryotic genome annotation', *Bioinformatics*, vol. 30, no. 14, pp. 2068–2069, Jul. 2014, doi: 10.1093/bioinformatics/btu153.

[76] R. K. Aziz *et al.*, 'The RAST Server: Rapid Annotations using Subsystems Technology', *BMC Genomics*, vol. 9, no. 1, p. 75, Dec. 2008, doi: 10.1186/1471-2164-9-75.

[77] T. Tatusova *et al.*, 'NCBI prokaryotic genome annotation pipeline', *Nucleic Acids Res*, vol. 44, no. 14, pp. 6614–6624, Aug. 2016, doi: 10.1093/nar/gkw569.

[78] J. R. Grant *et al.*, 'Proksee: in-depth characterization and visualization of bacterial genomes', *Nucleic Acids Res*, vol. 51, no. W1, pp. W484–W492, Jul. 2023, doi: 10.1093/nar/gkad326.

[79] J. J. Gillespie *et al.*, 'PATRIC: the Comprehensive Bacterial Bioinformatics Resource with a Focus on Human Pathogenic Species', *Infect Immun*, vol. 79, no. 11, pp. 4286–4298, Nov. 2011, doi: 10.1128/IAI.00207-11.

[80] K. Blin *et al.*, 'antiSMASH 7.0: new and improved predictions for detection, regulation, chemical structures and visualisation', *Nucleic Acids Res*, vol. 51, no. W1, pp. W46–W50, Jul. 2023, doi: 10.1093/nar/gkad344.

[81] A. G. McArthur *et al.*, 'The Comprehensive Antibiotic Resistance Database', *Antimicrob Agents Chemother*, vol. 57, no. 7, pp. 3348–3357, Jul. 2013, doi: 10.1128/AAC.00419-13.

[82] C. L. Brown *et al.*, 'mobileOG-db: a Manually Curated Database of Protein Families Mediating the Life Cycle of Bacterial Mobile Genetic Elements', *Appl Environ Microbiol*, vol. 88, no. 18, Sep. 2022, doi: 10.1128/aem.00991-22.

[83] S. Sayers *et al.*, 'Victors: a web-based knowledge base of virulence factors in human and animal pathogens', *Nucleic Acids Res*, vol. 47, no. D1, pp. D693–D700, Jan. 2019, doi: 10.1093/nar/gky999.

[84] D. Arndt *et al.*, 'PHASTER: a better, faster version of the PHAST phage search tool', *Nucleic Acids Res*, vol. 44, no. W1, pp. W16–W21, Jul. 2016, doi: 10.1093/nar/gkw387.

[85] Y. Zhou, Y. Liang, K. H. Lynch, J. J. Dennis, and D. S. Wishart, 'PHAST: A Fast Phage Search Tool', *Nucleic Acids Res*, vol. 39, no. suppl, pp. W347–W352, Jul. 2011, doi: 10.1093/nar/gkr485.

[86] D. S. Wishart *et al.*, 'PHASTEST: faster than PHASTER, better than PHAST', *Nucleic Acids Res*, vol. 51, no. W1, pp. W443–W450, Jul. 2023, doi: 10.1093/nar/gkad382.

[87] A. J. Page *et al.*, 'Roary: rapid large-scale prokaryote pan genome analysis', *Bioinformatics*, vol. 31, no. 22, pp. 3691–3693, Nov. 2015, doi: 10.1093/bioinformatics/btv421.

[88] A. Ferrer Florensa *et al.*, 'Whole-genome prediction of bacterial pathogenic capacity on novel bacteria using protein language models, with PathogenFinder2', Apr. 18, 2025. doi: 10.1101/2025.04.12.648497.

[89] V. Jakubu, M. Cechova, M. Musilek, L. Malisova, B. Zapletalova, and H. Zemlickova, 'Amino acid substitutions in PBP3 in *Haemophilus influenzae* strains, their phenotypic detection and impact on resistance to β-lactams', *Journal of Antimicrobial Chemotherapy*, vol. 80, no. 4, pp. 980–987, Apr. 2025, doi: 10.1093/jac/dkaf023.

[90] T. Dong and H. E. Schellhorn, 'Role of RpoS in Virulence of Pathogens', *Infect Immun*, vol. 78, no. 3, pp. 887–897, Mar. 2010, doi: 10.1128/IAI.00882-09.

[91] K. Skorupski and R. K. Taylor, 'Cyclic AMP and its receptor protein negatively regulate the coordinate expression of cholera toxin and toxin-coregulated pilus in *Vibrio cholerae*', *Proceedings of the National Academy of Sciences*, vol. 94, no. 1, pp. 265–270, Jan. 1997, doi: 10.1073/pnas.94.1.265.

[92] M. A. Faner and A. L. Feig, 'Identifying and characterizing Hfq–RNA interactions', *Methods*, vol. 63, no. 2, pp. 144–159, Sep. 2013, doi: 10.1016/j.ymeth.2013.04.023.

[93] W. Peng *et al.*, 'The role of glutathione for oxidative stress and pathogenicity of *Streptococcus suis*', *Virulence*, vol. 16, no. 1, Dec. 2025, doi: 10.1080/21505594.2025.2474866.

[94] R. Lloubès *et al.*, 'The Tol-Pal proteins of the Escherichia coli cell envelope: an energized system required for outer membrane integrity?', *Res Microbiol*, vol. 152, no. 6, pp. 523–529, Jul. 2001, doi: 10.1016/S0923-2508(01)01226-8.

[95] M. S. Thomas and S. Wigneshweraraj, 'Regulation of virulence gene expression', *Virulence*, vol. 5, no. 8, pp. 832–834, Nov. 2014, doi: 10.1080/21505594.2014.995573.

[96] T. A. Schöner *et al.*, 'Aryl Polyenes, a Highly Abundant Class of Bacterial Natural Products, Are Functionally Related to Antioxidative Carotenoids', *ChemBioChem*, vol. 17, no. 3, pp. 247–253, Feb. 2016, doi: 10.1002/cbic.201500474.

[97] I. Johnston *et al.*, 'Identification of essential genes for Escherichia coli aryl polyene biosynthesis and function in biofilm formation', *NPJ Biofilms Microbiomes*, vol. 7, no. 1, p. 56, Jul. 2021, doi: 10.1038/s41522-021-00226-3.

[98] A. B. Siddique *et al.*, ‘Characterization of Pathogenic Vibrio parahaemolyticus Isolated From Fish Aquaculture of the Southwest Coastal Area of Bangladesh’, *Front Microbiol*, vol. 12, Mar. 2021, doi: 10.3389/fmicb.2021.635539.

[99] B. Hirshfeld *et al.*, ‘Prevalence and antimicrobial resistance profiles of Vibrio spp. and Enterococcus spp. in retail shrimp in Northern California’, *Front Microbiol*, vol. 14, Jun. 2023, doi: 10.3389/fmicb.2023.1192769.

[100] M. S. RAHMAN, ‘Diversity of Vibrio Species’ and Their Antibiotic Resistance Patterns in Black Tiger Shrimp Penaeus monodon Fabricius, 1798 Cultured in South-West Region of Bangladesh’, *Asian Fish Sci*, vol. 33, no. 4, Dec. 2020, doi: 10.33997/j.afs.2020.33.4.004.

[101] V. Letchumanan, W.-F. Yin, L.-H. Lee, and K.-G. Chan, ‘Prevalence and antimicrobial susceptibility of Vibrio parahaemolyticus isolated from retail shrimps in Malaysia’, *Front Microbiol*, vol. 6, Jan. 2015, doi: 10.3389/fmicb.2015.00033.

[102] L.-H. Lee, N.-S. Ab Mutalib, J. W.-F. Law, S. H. Wong, and V. Letchumanan, ‘Discovery on Antibiotic Resistance Patterns of Vibrio parahaemolyticus in Selangor Reveals Carbapenemase Producing Vibrio parahaemolyticus in Marine and Freshwater Fish’, *Front Microbiol*, vol. 9, Oct. 2018, doi: 10.3389/fmicb.2018.02513.

[103] W. Mitsuwan *et al.*, ‘Multidrug Resistance, Biofilm-Forming Ability, and Molecular Characterization of Vibrio Species Isolated from Foods in Thailand’, *Antibiotics*, vol. 14, no. 3, p. 235, Feb. 2025, doi: 10.3390/antibiotics14030235.

APPENDIX-I

Media Composition

The composition of the media used in the present study has been given below. Mentioned all media were autoclaved at 121°C for 15 minutes.

1. Alkaline peptone water (APW)

Ingredients	Amount (gm/L)
Bacto-peptone	**10.0**
NaCl	10.0
Distilled water	1.0 liter
pH	9.0-9.2

2. Thiosulphate Citrate Bile salt Sucrose (TCBS) Agar

Ingredients	Amount (gm/L)
Bacto-yeast extract	5.0
Bacto-peptone	10.0
Sodium thiosulphate	10.0
Sodium citrate	10.0
Bile salts	8.0
NaCl	10.0
Ferric citrate	1.0
Bromothymol blue	0.04
Thymol blue	0.04
Agar	14.0
Distilled water	1.0 liter
pH	8.6

3. Muller Hinton Agar (Oxoid)

Ingredients	Amount (gm/L)
Beef extract	2.0
Agar	15.0
pH	7.3
Starch	1.5
Casein hydrolysate	17.5

Appendix-II

Reagents and buffers

Polymerase chain reaction (PCR) reagents

(Wako, Japan)

10XGene Taq Universal buffer (10 mM Tris-HCI, pH 8.3, 50 mM KCI, 1.5 mM $MgCl_2$, 0.01 mM EDTA, 0.1 mM dithiothreitol, 0.05% Tween 20, 0.05% Nonidet P-40, 5% 0.02 glycerol) dNTP mixture

Taq DNA polymerase

Control primer mix

Sterile water

Molecular weight marker

Normal saline

8.5g NaCl was dissolved in 800 ml deionized water. The pH was adjusted to 7.8 and the final volume was made up to 1 liter. The solution was sterilized by autoclaving and stored at room temperature.

Ethidium bromide solution

2.5 mg of ethidium bromide (Sigma, USA) was dissolved in 5 ml of distilled water at a concentration of 0.5 mg/ml. This solution was covered with aluminum foil and stored at room temperature.

TAE buffer

242 g of tris-base, 57.1 ml of glacial acetic acid, 100 ml of 0.5M EDTA (pH 8.0) was taken and distilled water was added to the mixture to make 1L. 1X concentrated TAE buffer was made by adding 10 ml 50X TAE buffer with 490 ml distilled water and stored at room temperature.

0.5 McFarland standard

A 0.5 McFarland turbidity standard was prepared by adding 0.5 ml of 1.175% (w/v) barium chloride ($BaCl_2.2H_2O$) solution to 99.5 ml of 1% sulphuric acid.

Appendix-III

The important instrument and apparatus used through the study are listed below:

Instruments	Origin
Autoclave, model no: 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 H1-SET	CBC Scientific, UK
Incubator	Japan
Microcentrifuge tube	Eppendorf, Germany

Micropipettes	Eppendorf, Germany
Microwave oven, Model: D90N30 ATP	Butterfly, China
Nanodrop 2000	Thermo Scientific, USA
pH meter, Model no: MP220	Eppendorf, Germany
Refrigerator (4°C)	Vest frost
Sterilizer, Model no: NDS-600D	Japan
Water bath, Model: SUM	England
-80°C Freezer	Nuaire, USA
Dry block Heating	Grant-bio, England
Automated thermo cycler, Model: 12137	Bio-Rad, Japan