A Project Report

On

"IDENTIFICATION OF TOXIGENIC VIBRIO CHOLERAE IN FISH, TANK WATER AND SWAB FROM FISH CUTTING BOARD"

Submitted by

Suzon Ahamed

ID: 182-34-803

Department of Nutrition & Food Engineering

Daffodil International University

Supervised By

Ms. Nasima Akter Mukta
Assistant Professor,
Department of Nutrition & Food Engineering
Faculty of Allied Health Sciences
Daffodil International University

Dr. Mohammed Badrul Amin
Associate Scientist,
Laboratory of Food Safety & One Health
International Centre for Diarrhoeal Diseases Research, Bangladesh

Date of Submission: 05th October, 2022

Certificate of Approval

This is to certify that the research work presented in the report titled **"Identification of Toxigenic Vibrio Cholerae in Fish, Tank water and Swab from fish cutting board"** is being submitted by Suzon Ahamed, bearing ID: 182- 34-803 is an authentic work which is carried out in the laboratory of Food Safety and One Health, International Centre for Diarrhoeal Research, Bangladesh. The project report is approved for the partial fulfillment of the Bachelor degree of Science in Nutrition and Food Engineering.

Dr. Nizam Uddin
Associate Professor and Head
Department of Nutrition & Food Engineering
Daffodil International University

Ms. Nasima Akter Mukta
Assistant Professor,
Department of Nutrition & Food Engineering
Daffodil International University

Dr. Mohammed Badrul Amin
Associate Scientist,
Laboratory of Food Safety & One Health
International Centre for Diarrhoeal Diseases Research, Bangladesh

Letter of Transmittal

Date: 05th, October 2022

Dr. Nizam Uddin

Associate Professor & Head

Department of Nutrition & Food Engineering

Daffodil International University.

Subject: **Submission of Project Report**

Dear Sir,

This is my pleasure to submit the project report titled "Identification of Toxigenic Vibrio Cholerae in Fish, Tank water and Swab from fish cutting board" as partial fulfillment for the requirement of the BSc. in Nutrition & Food Engineering (NFE) program. I would like to thank you for your support and guidance.

I made my best effort in collecting and studying necessary data to make the report as analytical as possible. The practical and analytical knowledge gathered during the thesis work will be fruitful for my career.

I therefore would like to submit this report to you for your kind suggestion and consideration.

Sincerely yours,

Suzon Ahamed

ID: 182-34-803

Dept. of Nutrition and Food Engineering

Daffodil International University.

Declaration

This project report entitled “Identification of Toxigenic Vibrio Cholerae in Fish, Tank water and Swab from fish cutting board” is being submitted to the Department of Nutrition and Food Engineering, Faculty of Allied Health Sciences, Daffodil International University, Dhaka, Bangladesh as a part of partial fulfillment of the requirements for the degree of Bachelor of Science in Nutrition & Food Engineering. This project report is unique and carried out by Suzon Ahamed’s authentic work.

Submitted By

Suzon Ahamed

ID: 182-34-803

Department of Nutrition & Food Engineering

Daffodil International University

Supervised By

Ms. Nasima Akter Mukta
Assistant Professor,
Department of Nutrition & Food Engineering
Faculty of Allied Health Sciences
Daffodil International University

Dr. Mohammed Badrul Amin
Associate Scientist,
Laboratory of Food Safety & One Health
International Centre for Diarrhoeal Diseases Research, Bangladesh

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ABSTRACT

Bangladesh continues to be plagued with cholera, one of the deadliest infectious illnesses in the world. In many nations, the toxic *Vibrio cholerae* that causes cholera continues to be a serious public health issue. The main causes of this illness are poor hygiene, inadequate sanitation, and drinking tainted water. Recent cholera outbreaks demonstrated the illness's escalating intensity, duration, and severity. The current study was conducted to assess the prevalence of toxic *Vibrio cholerae* in fish, tank water and swab from fish cutting board in retail and wholesale market during the non-outbreak period. 45 fish samples, 16 tank water samples and 16 swabs from fish cutting board samples were obtained between May 2022 and September 2022. In order to identify *V. Cholerae* in the samples, bacteriological tests were performed and the results were verified for the outer membrane protein (OmpW) by the polymerase chain reaction (PCR). Following that, the isolates were tested for the cholera enterotoxin gene (ctx) and the toxin co-regulated pilus gene (tcpA). Fish, tank water and swab from fish cutting board contained 53%, 87% and 56% of *V. Cholerae* contamination, respectively. We have found that *V. Cholerae* was present in 16 Tilapia isolates and 8 Pangas isolates. 14 isolates from tank water and 9 from swab from fish cutting board samples tested positive for *V. Cholerae*. Furthermore, out of 77 isolates 5 were found positive for tcpA and 4 isolates for ctxA were detected. In conclusion, pathogenic, toxic *V. Cholerae* species are found in aquatic habitats, survive there, and are still isolable even when there aren't any outbreaks. This is one of the biggest obstacles to cholera control programs and a major public health concern.

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LIST OF ABBREVIATIONS AND ACRONYMS

ASPW	**Alkaline Saline Peptone Water**
Bp	**Base pair**
LB	**Luria broth**
PCR	**Polymerase Chain Reaction**
rpm	**Revolution Per Minute**
TCBS	**Thiosulfate Citrate Bile Sucrose**
TSA	**Trypton Salt Agar**

1.Introduction

1.1 Background Information

There are quite a few incidents of human infections caused by microbes transmitted by fish or aquatic environments, depending on the season, the patient's contact with fish, dietary habits, and the state of the patient's immune system (Novotny et al., 2004). When contaminated effluent is introduced to aquatic environments due to storms, floods, or sewage spills, infection rates frequently reach their peak (Altekruse et al., 1997). Vibrios are common in aquatic habitats, where they can be found coexisting with plankton or existing free in the water. As a result of the bacteria's preference for warm water, breakouts are more common in the summer (Iwamoto et al., 2010; Paz, 2009). Cholera is one of the most harmful gastroenteric illnesses. Enterotoxin-producing V. cholerae of serogroups O1 and O139 are the main culprits behind epidemic cholera. Most cases of cholera occur in poorer nations with poor sanitation because the disease is frequently spread to people by consuming tainted food or water (Traoré et al., 2014).

A lot of nations throughout the world rely on fish and fisheries products as a source of foreign exchange in addition to being vital nutritionally. Providing around 3.74% of the country's GDP, 2.7% of export profits, and 22.23% of the country's agricultural output, Bangladesh's fisheries and aquaculture industries have risen to become the second-largest contributors to export earnings. Fish exports from Bangladesh, including frozen fish, dry fish, salted fish, and dehydrated fish, are rising daily due to the large number of international markets, which include the USA, UK, Japan, Belgium, Netherlands, Thailand, Germany, China, France, Canada, Spain, and Italy. Only 62 of Bangladesh's 129 fish processing facilities have received EU accreditation. Therefore, it is crucial to preserve the frozen fish's quality to ensure its acceptance in global trade and to prevent consumer health issues (Sanjee & Karim, 2016).

Due to their increased nutritional value, which includes a high protein content and minimal to no carbohydrate and fat value, fish are a major concern for export revenues. But throughout different stages of handling, processing, and transportation, fish may become contaminated in retail market.This contamination may be caused by tank/ice/wash water, leaks, insect and pest harborage, employees, and processing equipment like forklifts. Additionally, processing and

storage of seafood might lead to contamination (Bryan, 1980; Gangarosa et al., n.d.). Foodborne pathogens like Vibrio spp. that are naturally present in aquatic areas or Salmonella spp. that are produced from sewage-contaminated water can also cause contamination.

Humans typically contract cholera by consuming contaminated food and drink. On the basis of human volunteer experiments, it has been determined that, depending on the health of a specific person, ingesting roughly 10^4–10^6 V. cholerae O1 organisms is likely to result in clinical cholera (Cash et al., 2015). Contaminated potable water is the main way that toxigenic *V. cholerae* spreads in developing nations. The quality of the water and ice used to process the fish is also crucial for producing high-quality fish because contaminated water and ice can infect the entire processing facility (Sanjee & Karim, 2016). In this article, we propose that fish serve as significant reservoirs and carriers of V. cholerae. In fact, in several parts of the world, eating salt fish, sardines, dried fish, and other fish was linked to cholera (Senderovich et al., 2010).

In many developing nations with low socioeconomic conditions, subpar sanitary infrastructure and public hygiene, and a lack of safe drinking water, cholera, a highly epidemic diarrheal illness, continues to wreak havoc. Over 8000 cases of cholera that resulted in death were reported by 21 nations in the Western Hemisphere in 1992–1993 and totaled 800 000 cases. In Bangladesh, the cholera pandemic is severe. Over 8,000 fatalities and between 210,000 and 235,000 illnesses were attributed to the outbreak of 1991 (Faruque et al., 1998). Cholera deaths of 14,000 were reported in Rwandan refugee camps in July 1994.While a cholera outbreak in April 1997 resulted in a total of 1521 deaths among 90 000 Rwandan refugees living in temporary camps in the Democratic Republic of the Congo (Siddique et al., 1995).

Cholera enterotoxin which is produced in large quantities by V. cholerae is a key virulence factor (CT). The cholera toxin is an enterotoxin that has two subunits (Spanglert, 1992). Adenylate cyclase is activated by subunit A, which results in a significant loss of cellular function when unwell. The small intestine's epithelial cell surface receptors are contacted by the toxin through the B subunit (Guidolint & Manning, 1987). The chromosomal genes ctxA and ctxB, which code for the A and B subunits, are expressed as a single transcriptional unit (Guidolint & Manning, 1987;

Mekalanos et al., 1983). However, toxin production does not correspond with serotype since some V. cholerae O1 strains may not produce CT and V. cholerae non-O1 may be enterotoxigenic (Kaper et al., 1981). Recently, it was revealed that another serotype (O141) contains ctx operon. The prophage V. cholerae strains receives the ctx operon from the filamentous bacteriophage CTX, which also carries the ctxA and ctxB genes. The cholera toxin (CT), which causes the potentially fatal diarrheal disease cholera gravis brought on by an outbreak of cholera, is encoded by the ctxAB genes. While some *V. cholerae* strains that lack ctx can still infect humans, these infections are much less serious and rarely life-threatening. Toxigenic cholera can be distinguished from the majority of non-toxigenic *V. cholerae* strains by the presence of ctxAB (Dalsgaard et al., 2001).

Antibiotics are frequently given as part of the treatment plan, however in numerous nations, the number of resistant forms of V. cholerae has increased. Since 1991, more than 45% of V. cholerae strains have been ampicillin-resistant, and this resistance rate remained consistently high until 2006, when it reached 91.3%. Cotrimoxazole resistance spiked in 1995 with 56.3% and did so again in 2006 (Kacou-N et al., 2012). Streptomycin and chloramphenicol were two of the first antibiotics that were successfully used to treat cholera between the 1940s and the 1960s (Das et al., 2020). In 1962, Calcutta saw a demonstration of the use of tetracycline in the treatment of cholera. In the 1970s, sulphamethoxazole-trimethoprim (SXT) was first used to treat cholera. Tetracycline, chloramphenicol, and SXT all worked just as well at getting rid of V. cholerae in cholera patients. To lessen the intensity and length of the sickness, ciprofloxacin and azithromycin are also prescribed to cholera patients (Das et al., 2020).

In order to raise awareness about food safety, the present study was conducted to examine the microbiological quality of the raw fish. Due to their close relationships with fish processing and preservation, this study also looked into the microbiological condition of water and ice.

1.2 Specific objectives

i. To determine the magnitude of *V. cholerae* in fish, water and swab from fish cutting board.

ii. To establish the virulence characteristics of the isolated *V. cholera*e.

2. Literature Review

2.1 Description of *Vibrio cholerae*

Vibrio cholerae is a Gram-negative, facultative anaerobic, curved rod-shaped bacterium that belongs to the family of Vibrionaceae. The bacterium is oxidase-positive, reduces nitrate, and moves by means of a single, sheathed, polar flagellum. It is between 1.4 and 2.6 millimeters in length. The organism is currently divided into more than 206 serogroups according to the "O"somatic antigens. But only the O1 serogroup and the just-identified O139 are linked to pandemic and epidemic cholera. *V. Cholerae* O1 serogroup isolates have been further classified into three serotypes: Inaba, Ogawa, and Hikojima, and two biotypes: Classical and El Tor. The non-O1 and non-O139 strains can occasionally be found in diarrhoeal patients, as well as in a range of extra-intestinal illnesses, wounds, ear, sputum, urine, and cerebrospinal fluid. All ready-to-eat foods should be free of the non-O1 and non-O139 *V. Cholerae*, despite the fact that these are not of significant epidemiological concern (Lemaistre et al., 1990).

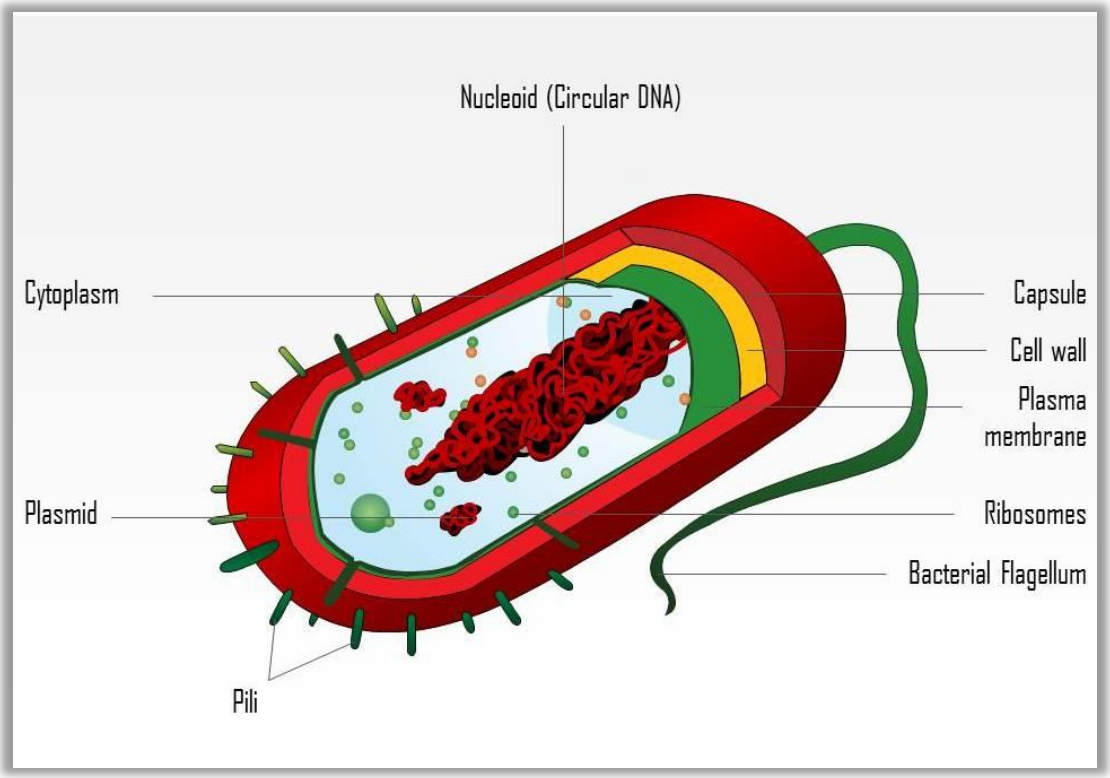

Figure 1: Vibrio Cholerae Cell Structure

2.2 Epidemiology of Cholera in Bangladesh

Cholera surveillance in Bangladesh has uncovered crucial details regarding the disease's epidemiology as well as changes in the characteristics of V. cholerae strains isolated from several epidemics over time. The Ganges delta, which was the origin of all cholera pandemics but the sixth, is where Bangladesh is located. The water levels in ponds and rivers in Bangladesh vary according to the dry and wet seasons because of the low-lying deltaic environment. The monsoons also flush sewage from the villages into the rivers, which raises river levels. In rural areas, surface water is used directly by residents for drinking, bathing, cooking, and irrigation. The population is now growing faster than there are available homes and sanitary facilities can accommodate. In Bangladesh, where cholera is endemic, outbreaks follow a predictable seasonal pattern. For more than 35 years, the International Centre for Diarrhoeal Disease Research (ICDDR,B) and the old Pakistan Seato Cholera Research Laboratory have conducted systematic cholera surveillance in Bangladesh (Martin et al., 1869; Samadi et al., 1983).

According to several studies, epidemic outbreaks in Bangladesh typically happen twice a year, with the highest number of infections occurring from September to December, right after the monsoon season. In the spring, between March and May, cholera cases also reach a slightly lower peak. More than 90% of cases of cholera in Bangladesh up to 1970 were brought on by the traditional Inaba serotype; by 1972, the traditional Ogawa serotype was responsible for 85% of cases. Since its appearance in Bangladesh in 1969/1973, the El Tor biotype of Vibrio cholerae O1 has fully displaced the conventional biotype. But starting in 1982, the classical biotype returned as the main epidemic biotype in Bangladesh, where it coexisted with the El Tor vibrios until 1992 (Samadi et al., 1983).

2.3 Pathogenesis and Ecology

Occasional outbreaks are caused by contaminated drinking water and/or faulty food preparation in densely populated areas that are home to endemic toxigenic strains. The primary cause of outbreaks is contaminated water with free-living V. cholerae cells, followed by contaminated food, particularly water-produced goods like fish and vegetables. Consuming tainted food or water is

the first step in contracting V. cholerae infection. The organism colonizes the epithelium of the small intestine after passing past the acid barrier of the stomach via the toxin-coregulated pili and other colonization factors like the various haemagglutinins, auxiliary colonization factor, and core-encoded pilus. After that, the adhering vibrios exude cholera enterotoxin, which interferes with the transport of ions by intestinal epithelial cells, across the bacterial outer membrane into the extracellular environment. Cholera's acute diarrhea is caused by the cells' subsequent loss of water and electrolytes (Reidl & Klose, 2002). In addition, V. cholerae exists naturally in aquatic habitats, primarily in warm climates (water temperature). Contrary to popular belief, most environmental O1 strains are not toxic, and non-O1 and non-O139 strains are more frequently isolated from rivers and estuaries than O1 and O139 strains (Reidl & Klose, 2002).

According to a theory, under stressful circumstances, vibrios change into a viable but non-culturable (VNC) form that cannot be retrieved using traditional culture techniques. Such VNC forms are capable of infecting other organisms, which causes them to change back into the culturable form. Additional research has shown that V. cholerae O1 can associate with a wide range of phytoplankton, blue-green algae, and other aquatic species. These relationships (symbiosis) aid in survival, and it is likely that the vibrios obtain nutrients from the host as a result (Faruque et al., 1998). Cholera epidemics also follow a predictable seasonal pattern in endemic locations. However, according to Mukhopadhyay et al. (1998), there is clonal variation among epidemic strains of V. cholerae O1 and O139 as evidenced by the frequent observation of variances in genetic characteristics across these strains. These occurrences have sparked debate over whether seasonal outbreaks of V. cholerae are brought on by recurrent occurrences of the same strains or by the ongoing development of new toxigenic clones from non-toxigenic progenitors.

2.4 Molecular Mechanism of Virulence in *Vibrio cholera*e

The cholera toxin, produced by Vibrio cholerae enterotoxin (ctx genes), is a pathogen. Both ctx-A and ctx-B encode the toxin's A and B subunits, respectively (Sánchez & Holmgren, 2011). Each gene is a component of the same operon. The expression of a regulon, which includes the ctxoperon and the tcp operon (toxin co-regulator pilus involved in fimbriae synthesis), is regulated by environmental cues like as temperature, pH, osmolarity, and certain amino acids (Faruque et al., 1998). Tox-R, Tox-S, and Tox-T were discovered to be the proteins in charge of regulating the

expression of this regulon. Approximately two-thirds of the amino terminal portion of the transmembranous protein tox-R is exposed to the cytoplasm. In addition, Tox-R dimers bind to the ctx-AB operon's operator region and trigger the transcription of that gene (Lemaistre et al., 1990). Tox-S serves as a sensor protein that phosphorylates Tox-R, causing it to change from its inactive DNA binding form to its active form, and Tox-R and Tox-S appear to comprise a typical two component regulatory system. For the purposes of this instance, Tox-T is a cytoplasmic protein that activates transcription within the tcp operon (Sánchez & Holmgren, 2011). Additionally, Tox-R stimulates the expression of Tox-T, and Tox-T in turn stimulates the transcription of tcp genes to produce pili. According to Faruque et al. (1998), Tox-R and Tox-S interact to detect environmental changes and send a molecular signal to the chromosome that triggers the transcription of genes for attachment (pili formation) and toxin production. For this reason, it is beneficial to target Tox-R when conducting environmental research. Furthermore, it is logical to assume that the environmental factors present in the human gastrointestinal tract (temperature, low pH, high osmolarity, etc.), as opposed to those present in the extra-intestinal (aquatic) environment of the vibrios, are those that are required to induce the formation of the virulence factors required to infect (Akoachere et al., 2013). This explains why environmental isolates discovered during non-epidemic periods are typically not toxic.

2.5 Role of Vibrio cholerae bacteriophage CTXΦ in the Survival and Persistence of Toxigenic Strains of Vibrio cholerae

According to a study done in Bangladesh, toxigenic V. cholerae strains can be made to create extracellular CTX phage particles under the right circumstances (Faruque et al., 1998). The naturally existing V. cholerae strains O1 and O139 are hence inducible lysogens of CTX. Furthermore, it is conceivable that unexplained environmental conditions may cause toxigenic V. cholerae to become lysogenic CTX-positive, releasing extracellular CTX-positive particles into the aquatic environment in natural ecological situations. Through interactions with non-toxigenic strains present in the environment and in the human population that consumes the ambient waters, the cell-free phage particles contribute to the formation of novel toxic strains of V. cholerae. Only V. cholerae cells that express TCP can be infected by the phage (CTX), as it uses TCP as its receptor (Faruque & Mekalanos, 2012). This provides more evidence to support the idea that CTX

likely contributes significantly to the evolution of new toxigenic strains of V. cholerae in natural environments. Additionally, it has been shown that the intestinal environment, where virulence factors like TCP are fully produced, is where CTX more effectively infects recipient V. cholerae strains (Faruque et al., 2002). While the conversion of non-toxigenic V. cholerae is favored within the gastrointestinal tract of the mammalian host, the natural selection and persistence of the novel toxigenic strains may involve both intestinal and environmental factors, the immune status of the host population, and the antigenic characteristics of the new pathogenic strain. Environmental cues such optimal temperature, sunshine, and osmotic conditions are likely what regulate the induction of CTX lysogens (Faruque & Mekalanos, 2012).

2.6 Characteristics of Vibrio cholera

The gram-negative rod v. cholerae is very motile and comma shaped. Because "vibrio" in Latin means "to quiver," the name of the genus was inspired by the vigorous movement of V. cholerae. All other vibrio species are halophilic with the exception of v.cholerae and v.mimicus. Initial isolates are slightly bent, but when cultured in a lab, they can take on the appearance of straight rods. The bacterium possesses pili and a flagellum at one of its cell poles. The majority of intestinal commensals are killed by alkaline medium; however, they are sensitive to acid. It can engage in both respiratory and fermentative metabolism since it is a facultative anaerobe. It has a length ofμ1.3 μm, a diameter of 0.3 μm, and an average swimming speed of about 75.4 μm/sec (Shigematsu et al., 1995).

2.7 Diversity and evolution

Cholera epidemics are brought on by the O1 and O139 serogroups of V. cholerae. The bulk of outbreaks are caused by O1, while O139, which was initially discovered in Bangladesh in 1992, is only found in Southeast Asia. A cholera-like sickness can be brought on by numerous additional V. cholerae serogroups, both with and without the cholera toxin gene (including the nontoxigenic strains of the O1 and O139 serogroups). Serogroups O1 and O139 toxigenic strains are the only ones to have produced widespread outbreaks. Both the classical and El Tor biotypes of V. cholerae O1 have two different serotypes, Inaba and Ogawa. Although there is no difference in the signs

and symptoms of infection, most El Tor biotype infections are asymptomatic or cause only moderate disease. Classical V. cholerae O1 infections have become infrequent in recent years and are only found in some regions of Bangladesh and India. New mutant strains have recently been discovered in numerous regions of Asia and Africa. According to observations, these strains of cholera had greater case fatality rates and more severe symptoms (Siddique et al., 1995).

2.8 Fish as Possible Reservoirs of *V. Cholerae*

More than 50 years ago, pathogenic *V. Cholerae* O1 strains were used to infect sardines (Stolephorus) and mullets in a laboratory experiment. The amount of Vibrio in the water was 10^2 cells/m. After the fish were exposed to the bacterium, the strains were found in their intestines. In a different laboratory test, the gut of zebrafish was colonized with V. cholerae O1 strains (Runft et al., 2014). They discovered that the bacteria developed tiny colonies after adhering to the fish intestinal epithelium. They proposed that pathogenic V. cholerae strains may use zebrafish as a host model (Rowe et al., 2014; Runft et al., 2014). Large quantities of V. cholerae serogroups O1 and O139 were found in fish scale samples collected in Mozambique, which was published as proof of the occurrence of pathogenic serogroups of V. cholerae in fish (M du Preez, 2010). These researchers used a direct fluorescent antibody approach to gather their proof. Tanzanian scientists discovered V. cholerae O1 in the gills of Tilapia after testing it for the cholera toxin gene (Hounmanou, 2015). Two marine fish in Cochin, India were found to contain V. cholerae O1 isolates that were positive for the ctxA and tcpA genes (the type of the fish wasn't mentioned). Additionally, 141 non-O1/O139 isolates from unknown marine fish species were found in the same investigation (Kumar & Lalitha, 2013).

3. Materials and Methods

3.1 Overview of The Whole Study Design

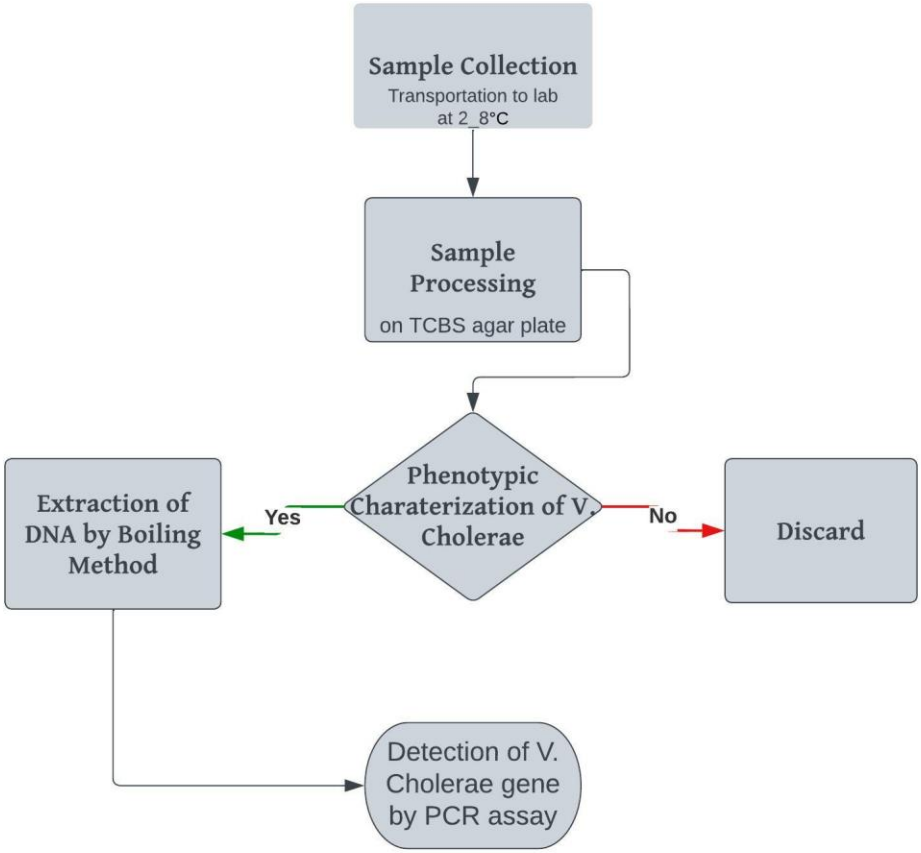

Figure 2: Overview of This Study Design

3.1 Study Area

This study was carried out in Dhaka city, Norshingdi and Cumilla. There were two Retail Market and one wholesale market in Dhaka city and two ponds from Norsingdi and Cumilla . 13 samples from Mirpur, 6 Retail Market and 24 samples from Uttara Retail market were collected. 10 samples were collected from Abdullahpur Wholesale Market. And also 20 samples from the pond of Norshingdi and 10 samples from the pond of Cumilla were collected.

3.2 Study Design

The study was across-sectional study conducted on water, fish and swab collected from the study area.

3.3 Study Materials

During this study, Fish, tank water and swab from fish cutting board were collected from the Retail, wholesale market and the ponds. There were two kinds of fish, one is Fresh Tilapia and another one is Fresh Pangas. During processing fish samples were taken from fish skin. This is because fish filet (the main edible part) is regarded as sterile and bacteria that occur in it are due to cross-contaminants from the environment (water)/tank water and handlers. Fish skin is thus representative parts of fish to give inferences on possible microorganisms present in fish.

3.4 Sample Collection

A total of 77 samples from fish, tank water and swab from fish cutting board were collected where 13 were from Retail market of Mirpur 6, 24 were from Uttara Retail Market, 10 were from Abdullahpur wholesale market, 20 were from the pond of Norshingdi and 10 were from the pond of Cumilla The samples were characterized and analyzed for the detection of toxigenic *Vibrio Cholera* isolates.

3.5 Isolation and identification of *Vibrio Cholerae* isolates

10 g of Fish sample weighed and dispensed into 90 mL of sterile Alkaline Saline Peptone Water (ASPW) in a sterile Poly Bag. And 10 ml of tank water filtrated and deep it into 45 mL of sterile Alkaline Saline Peptone Water (ASPW) in a sterile Falcon Tube. And also 1 mL of swab measured and dispensed into 9 mL of sterile Alkaline Saline Peptone Water (ASPW) in a sterile Test Tube.

After vortexing on high speed for 1 min, the suspension tank water and swab were incubated at 42°C for 18-24 hours and the suspension of the fish samples were incubated for 6 hours at 42°C. after 6 hours later 1 ml of suspension of fish samples were transferred into 9 mL of sterile Alkaline Saline Peptone Water (ASPW) in a sterile test tube and incubated it for 18-24 hours at 42°C. After incubation, a loopful sample was inoculated on Thiosulfate Citrate Bile Sucrose agar, TCBS (Oxoid, UK). All plates were incubated at 37°C for 18–24 hours. After incubation, yellow colonies which is typical for *V. Cholerae* on TCBS agar (sucrose fermenting, ≥ 2 mm) suspected as *Vibrio cholerae* (Oxoid, UK) and if the colony doesn't show yellowish colony which is atypical was discarded. From the TCBS plates, one typical colony was extracted and sub cultured on TSA agar for pure colony. After overnight incubation of the plates at 37°C, one or two pure colony taken from each plate and suspended in a test tube containing LB broth which was used later for the DNA extraction.

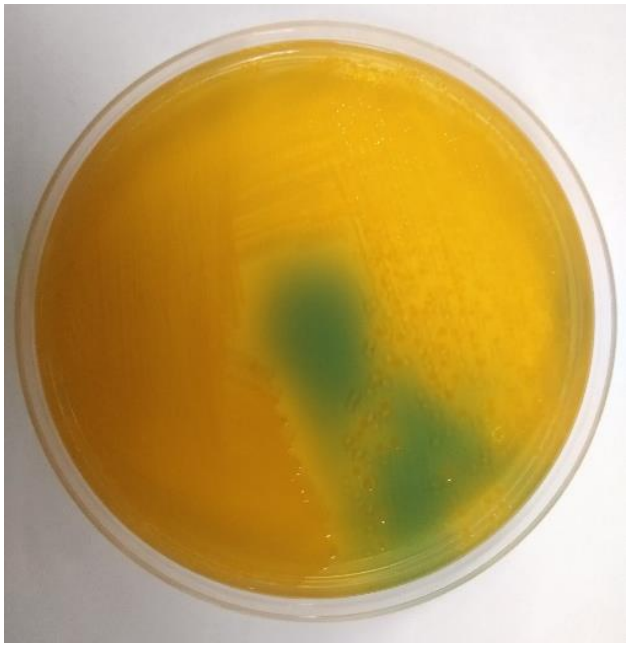

Figure 3: Culture of Vibrio Cholerae (Yellowish colony) on TCBS Plate

3.6 Molecular confirmation of *Vibrio cholerae* and identification of virulence genes

3.6.1 Template DNA Extraction

Bacterial DNA was extracted by Boiling DNA extraction method. Bacterial isolates were grown overnight on LB Broth at 37°C. Around 20 µl of suspension were transferred in 180 µl Auto calved distilled water into a microcentrifuge tube. After mixing the suspension by vortex mixture, suspension was boiled at 100°C for 10 minutes and it was rapidly cooled in ice for 5-10 minutes. This process is called the heat shock treatment. The cell lysate was then centrifuged at 6200 rpm for 6 minutes to precipitate the cell debris. 100 µl of supernatant was transferred to another set of microcentrifuge tube and the DNA was stored at -20°C. The DNA was used as a template DNA for PCR.

3.6.2 Polymerase Chain Reaction (PCR)

Polymerase chain reaction was performed for the detection of the presence of different genes by using different sets of primers for each gene.

Basic PCR reagents

1. Filtered Deionized Water (FDW)
2. 10X PCR Buffer (with MgCl2) (New England Biolabs, UK)
3. dNTP (10 mM per dNTP) (New England Biolabs, UK)
4. Taq DNA polymerase (5U/ µl) (New England Biolabs, UK)
5. Agarose (SIGMA, USA)
6. Ethidium bromide (10 mg/ml)
7. TBE (Tris-Borate EDTA) buffer
8. 100 bp DNA size standard (New England Biolabs, UK)
9. Primers

3.6.3 Detection of Toxigenic *Vibrio Cholerae* genes by PCR assays

Primer sequences **(Table 1)** and PCR parameters **(Table 2)** for detection of the *Vibrio Cholerae* are mentioned in the following table.

Table 1: Primers sequences for *V. Cholerae* genes

Targeted genes	Primer sequence	Expected size (bp)
CtxA *Cholera toxin gene*	F-CAGTCAGGTGGTCTTATGCCAAGAGG R-CCCACTAAGTGGGCACTTCTCAAACT	219
OmpW Outer membrane protein	F-CACCAAGAAGGTGACTTTATTGTG R-GAACTTATAACCACCCGCG	588
tcpA Toxin coregulated pilus	F-CAC GAT AAG AAA ACC GGT CAA GAG R-CGA AAG CAC CTT CTT TCA CGT TG	295

Table 2: PCR programs used in this study to determine ESBL types among V. Cholerae isolates are presented in this table

Genes	No. of cycles	Denaturation tem and time	Annealig tem and time	Elognation temp and time	Final extention time and temp
OmpW	35	94°C for 1 min	1 min at 50°C	72°C for 1 min	72°C for 5 min
Ctx A	35	94°C for 1 min	1 min at 50°C	72°C for 1 min	72°C for 5 min
tcpA	35	94°C for 1 min	1 min at 50°C	72°C for 1 min	72°C for 5 min

3.6.4 Agarose Gel Electrophoresis

The amplification products were horizontally electrophoresed in 1.5% agarose gel (1.5 g) in 100 ml of 0.5 TBE (Tris- borate EDTA) buffer at ambient temperature for 1 hour at a current of 90 volts (50 mA). For each sample, 1 µl of amplified DNA was combined with 1 µl of tracking dye (bromophenol blue), and then placed into a separate well of the gel (5 mm thick). 100 bp DNA standard size (The New England Biolabs) was employed as a marker to gauge the molecular size of the amplified products. After staining the gel for 30 minutes at room temperature with Ethidium bromide (0.5 g/ml), DNA bands were visible and photographed as instructed.

4. Results

4.1 **Identification of *Vibrio cholerae by* Using colony Morphology**

All the sample types were plated on TCBS agar media and incubated at 37°C for Overnight. They showed yellowish colonies on TCBS agar plate **(Figure 4, left**). From each sample a single well isolated presumed typical *Vibrio Cholerae* colony was selected and sub-cultured on TSA agar plate to obtain pure culture **(Figure 4, right**).

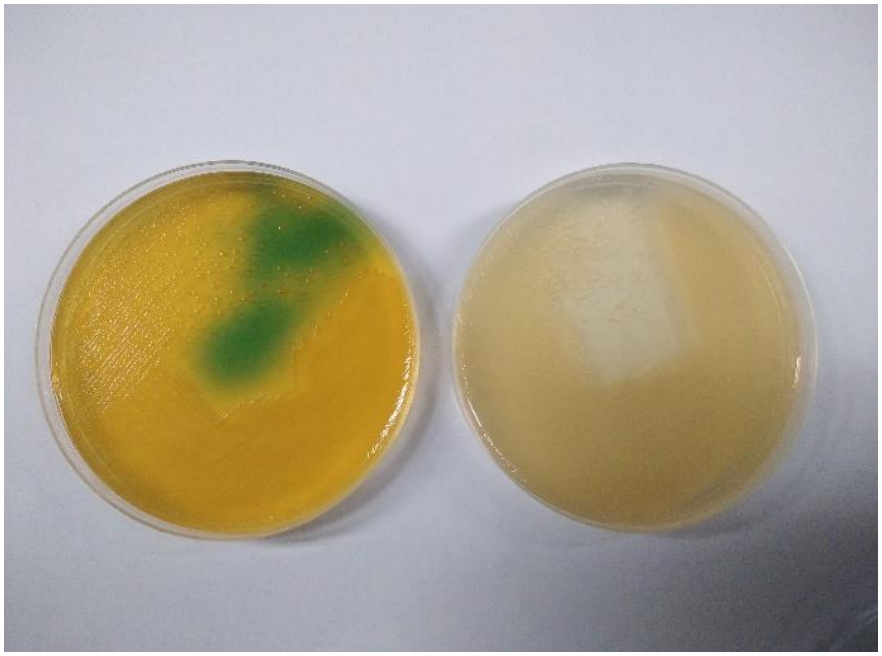

Figure 4: Representative image of Vibrio Cholerae colony on specific agar media. (Left) Yellowish colony of Vibrio Cholerae on TCBS agar plate and (Right) White colony on TSA agar plate.

Out of 77 samples, 60 samples were typical. A total 32(45) fish samples, 15(16) tank water samples and 13(16) swabs from fish cutting board samples were typical:

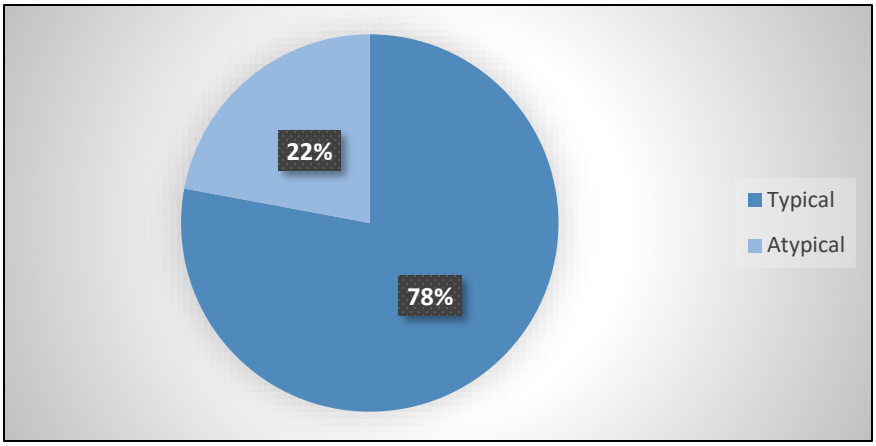

Figure 5: Overview of Colony Morphological Data

This figure shows that 78% (n=60) typical colony which means characteristic *Vibrio Cholerae* were found among the Fish, Tank water and Swab from fish cutting board samples. And, 22% (n=17) samples were atypical which means those samples did not have any characteristics *Vibrio Cholerae* colony.

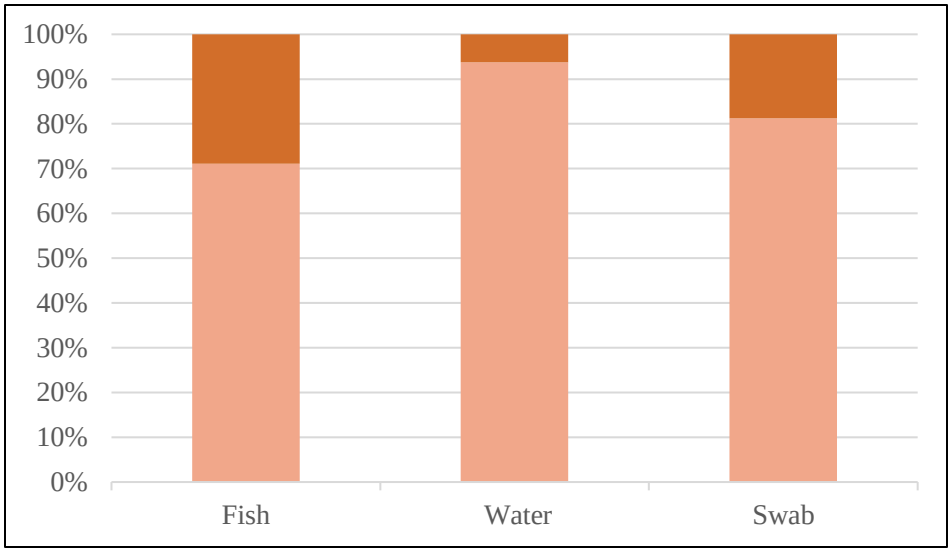

Figure 6: Percentage of V. Cholerae based on their colony morphology

This figure shows that 71% (n=32) fish samples, 93% (n=15) tank water samples and 81% (n=13) swab from fish cutting board samples were positive based on their characteristic's colony morphology on the TCBS agar plate.

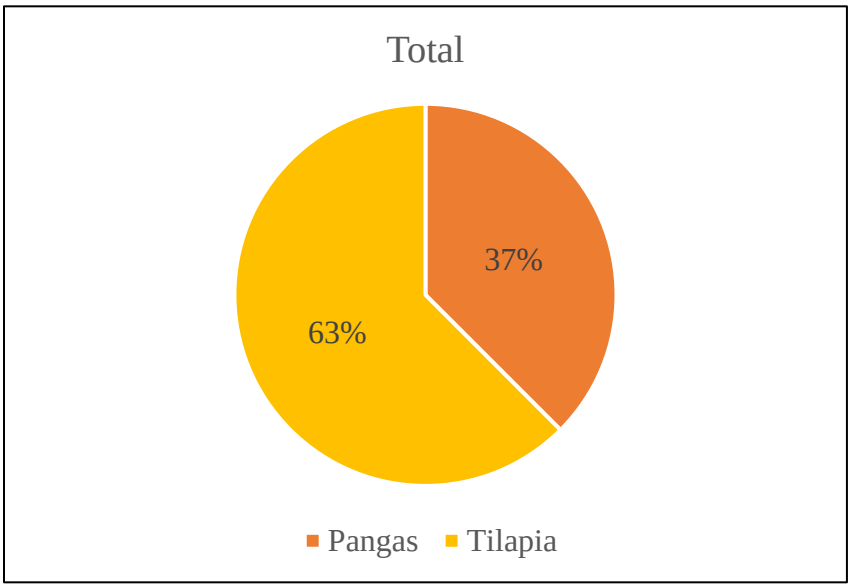

Figure 7: Percentage V. Cholerae positive fishes based on their colony morphology

This figure shows that 63% (n=20) Tilapia samples and 37% (n=12) Pangas samples were positive by their colony morphology.

4.2 Comparative prevalence of V. Cholerae genes among the fish, tank water and swab from fish cutting board Isolates

A total 77 samples were tested for the presence of different V. Cholerae genes. 45 were fish samples, 16 were tank water samples and 16 were swab from fish cutting board samples. Out of 45 fish samples, 24 fish were positive for ompW. 14 tank water samples were positive for ompW out of 16 water samples and 9 swabs from fish cutting board samples were positive for ompW out of 16 samples. A total 5 samples were positive for tcpA and 4 ctxA positive samples among 77 samples.

Presence of Vibrio Cholerae genes were visualized using agarose gel electrophoresis. Representative images of electrophoresis was shown in figure 3.7, 3.8 and 3.9 where amplification products of *ompW* gene is 588 bp**,** *tcpA* is 295bp) and *ctxA* is 219 bp.

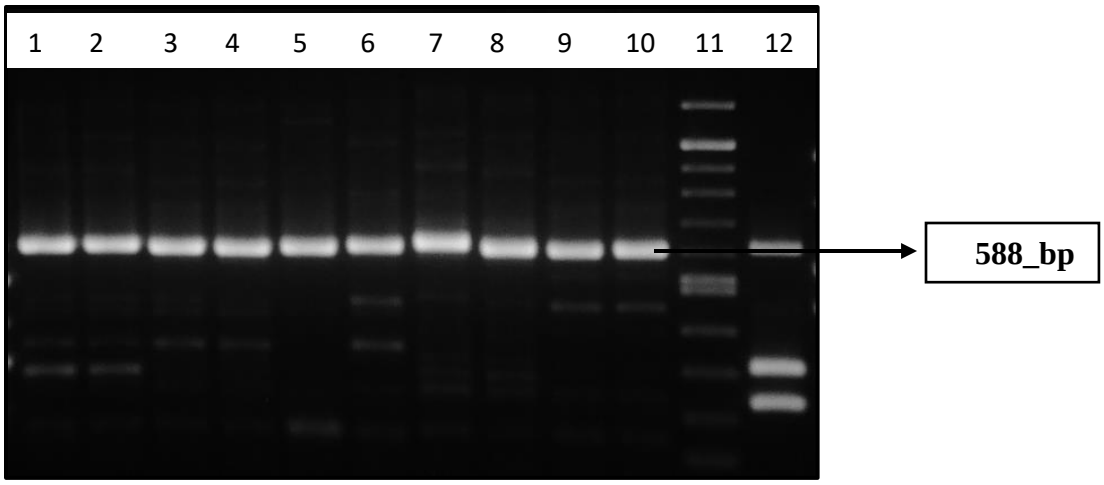

Figure 8: Agarose gel electrophoresis showing PCR amplification products of the ompW gene. The PCR positive samples showed band of 588 bp. Here, 100 bp ladder was used as marker. Lanes 1,2,3,4,5,6,7,8,9 and 10 represents test isolates. Lane 11 represents marker and lane 12 represents positive control.

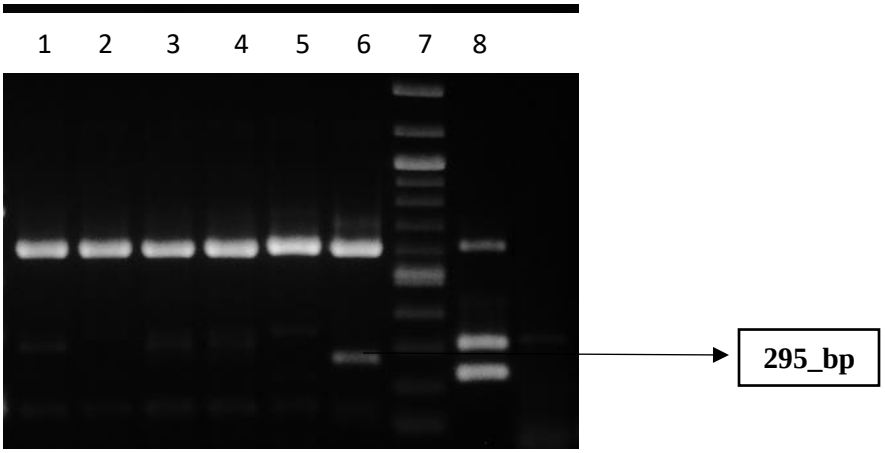

Figure 9: Agarose gel electrophoresis showing PCR amplification products of the tcpA gene. The PCR positive samples showed band of 295 bp. Here, 100 bp ladder was used as marker. Lanes 1,2,3,4,5 and 6 represents test isolates. Lane 7 represents marker and lane 8 represents positive control

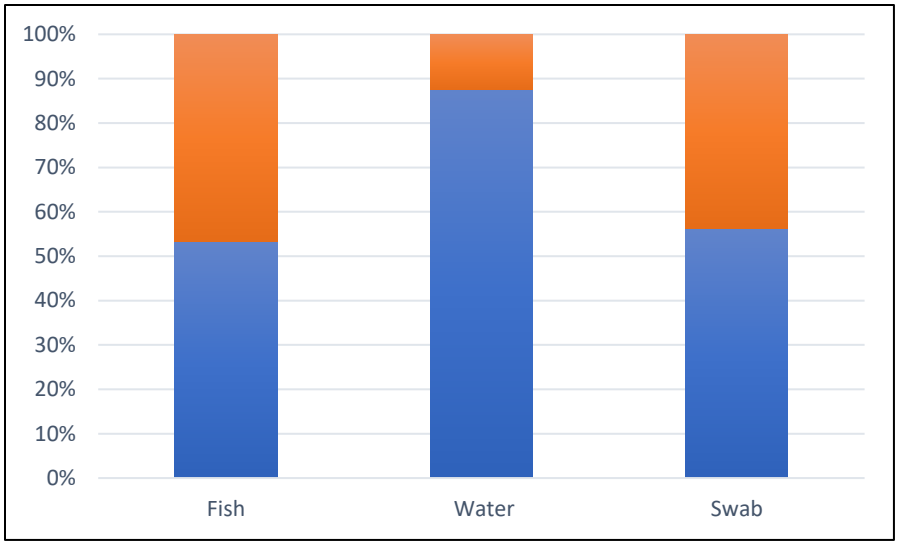

Figure 10: Percentage of positive *V. Cholerae* in fish, tank water and swab from fish cutting board

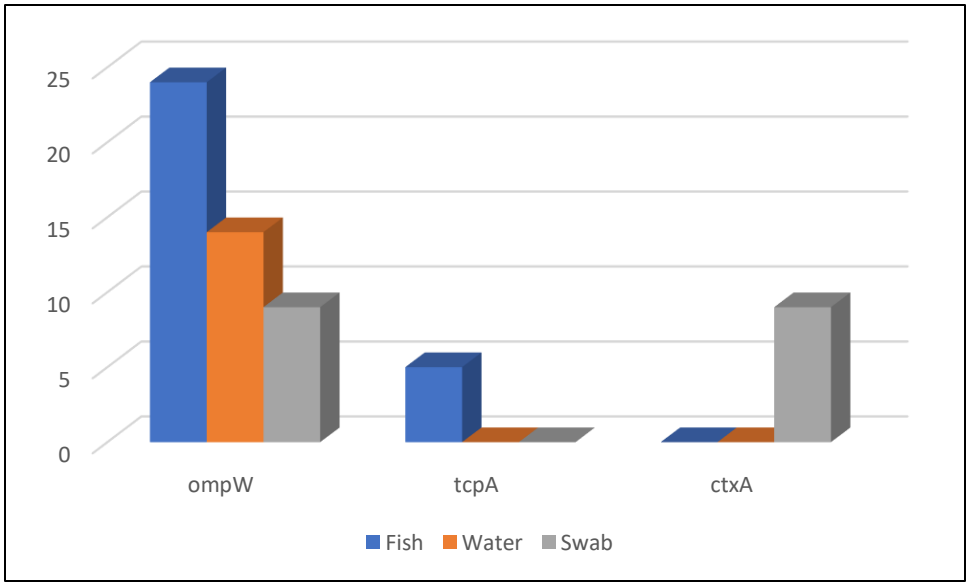

Figure 11: Comparative Prevalence of V. Cholerae genes (ompW, tcpA,ctxA) in fish, tank water and swab from fish cutting board

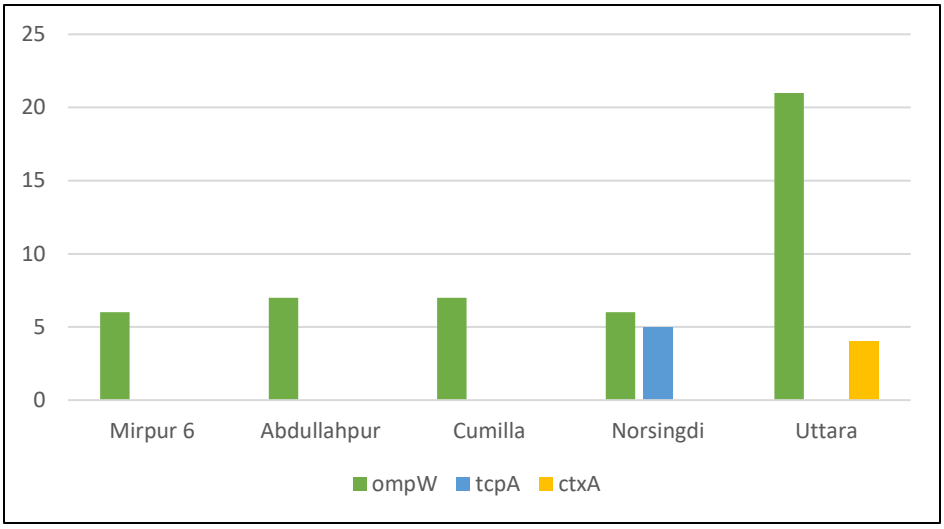

Figure 12: Comparative Prevalence of V. Cholerae genes (ompW, tcpA, ctxA) on different places.

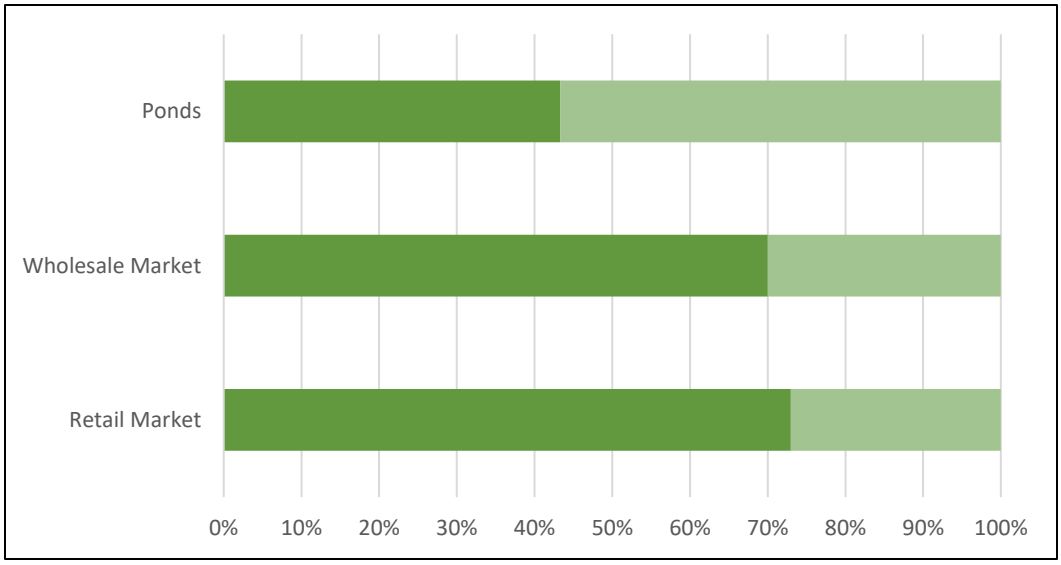

Figure 13: Percentage of positive vibrio cholerae in the supply chain of fish from ponds to Wholesale and then retail markets cutting board

5. Discussion

Fish is the most popular food item in Bangladesh which can be a potential source of *V. Cholerae* contamination as fish is cultured in water. Since *Vibrio Cholerae* is a naturally existing inhabitant of aquatic settings, water plays a crucial role in the spread of cholera. From the supply chain fish, ponds to wholesale market and finally retail market are the biggest threat for *V. Cholerae* contamination. According to the study's findings, the high levels of *V. Cholerae* found in the fish and tank water samples indicate that they may be environmentally normal, but they are still dangerous because people consume these fish and use water on a daily basis. 53% fish sample, 87% tank water samples were *V. Cholerae* positive. While 56% swab from fish cutting board were *V. Cholerae* positive. In fact, fish grown in water showed substantial contamination by *V. Cholerae* where fish harboring the pathogen. They induce non-choleric diarrhea in humans.

PCR verified the presence of pathogenic *V. Cholerae* in fish samples. Out of 77 samples, 47 were positively isolated. A total 57% (n=16) Tilapia and 47% (n=8) Pungas were *V. Cholerae* positive and also 87% (n=14) tank water samples were *V. Cholerae* Positive. Among 28 Tilapia samples 20 Tilapia samples were fresh fish which was collected from Cumilla and Norsingdi. From that fresh tilapia, 60% were *V. Cholerae* positive. And, remaining 8 Tilapia were obtained from the Retail market and wholesale market. This study found 50% tilapia were PCR positive for *V. Cholerae* from Retail and wholesale market. While 10 fresh Pangas were bought from Norsingdi out of 17 Pangas. Only 10% fresh pangas were *V. Cholerae* positive that is comparatively lesser than the fresh Tilapia samples. And, 100% (n=7) pangas were PCR Positive those were collected from Retail and wholesale market. This result says that Pangas which is collected from Retail and wholesale market is highly contaminated than fresh pangas from ponds. Tank water samples showed most *V. Cholerae* positive isolates. 87% (n=14) tank water samples and 56% (n=9) swab from fish cutting board samples were detected as *V. Cholerae* positive which indicates it can be the threat for the cross contamination of *V. Cholerae* during handling, cutting and processing of fish.

Comparative prevalence of tcpA and ctxA genes among 77 isolates, 5 isolates were positive for *tcpA from* the pond of Narsingdi. And 4 isolates were from Uttara retail market were ctxA positive. According to (Sánchez & Holmgren, 2011), the toxin co-regulated pilus (tcp-A) gene encodes for the creation of fimbriae, which enables the bacteria to be fastened to the host's intestinal epithelium, posing a major hazard to public health when present in isolates. The frequent occurrence of cholera outbreaks may be explained by the prevalence of toxic species of *V. Cholerae* from aquatic environments in non-outbreak periods (Akoachere et al., 2013).

Due to an inability to effectively eliminate these pathogens, human exposure to *V. Cholerae* through the consumption of contaminated fish can result in significant sickness in Dhaka city, Cumilla and Norsingdi. *V. Cholerae* was more prevalent in the fish and tank water. This circumstance means that either there will be additional human and animal contamination, including avian contamination or that the bacteria will continue to develop and spread throughout the system. The high incidence of *V. Cholerae* found in fish raised in ponds may be due to the high prevalence of the pathogen in the water. A total 18 samples 60% were *V. Cholerae* positive out of 30 ponds' samples which reveals that ponds are the great source of contamination of *V. Cholerae*. The level of bacterial contamination in fish will increase in direct proportion to the number of bacteria present in the pond water. Not only are fish from ponds consumed locally in the surrounding villages, but they are also exported to other city centers of the nation and other neighboring countries in the region. As a result, many people are exposed to *V. Cholerae*. Additionally, some non-pathogenic viable but unculturable species of *V. Cholerae* are found in foods and can withstand heat and other pressures to enter the human stomach where they can grow in the right conditions. Even with thorough cooking, such a condition can still result in the development of pathogenic organisms that cause sickness. Additionally, the risk still exists with regard to the dietary advice that advocates not overcooking fish

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