Whole Genome and Phenotypic Approaches to Study *Vibrio cholerae* Pathogenicity and Survival Mechanisms

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Department of Microbiology

Noakhali Science and Technology University

Noakhali 3814, Bangladesh.

Certification

It is hereby certified that the student bearing Roll No. 2305002F has carried out the thesis entitled, **" Whole Genome and Phenotypic Approaches to Study *Vibrio cholerae* Pathogenesis and Survival Mechanisms"**, for the partial fulfilment of his Masters of Science Degree in Microbiology from Noakhali Science and Technology University, Noakhali, Bangladesh, under my academic supervision in the Molecular Ecology and Metagenomics Laboratory, Infectious Disease Division (IDD), International Center for Diarrheal Disease Research, Bangladesh (icddr,b). The thesis is the outcome of his original work and his worth submitting for the degree of Master of Science in Microbiology under the Faculty of Life Sciences of Noakhali Science and Technology University.

Dr. Munirul Alam
Emeritus Scientist & Head
Molecular Ecology and Metagenomics Laboratory,
Infectious Disease Division (IDD),
International Center for Diarrhoeal Disease Research (icddr,b)
Email: munirul@icddrb.org

Dr. Toslim Mahmud
Associate Professor,
Department of Microbiology,
Noakhali Science & Technology University,3814, Noakhali.
Mobile: +880 1725-706169
Email: toslim@nstu.edu.bd

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Author

Abstract

Despite *Vibrio cholerae* causing epidemic and pandemic cholera has been well-established in brackish water environments, the aquatic reservoir is often debated because the bacterium is rarely, if ever, isolated from surface water by culturing methods. Non-culturable state has been proposed as a survival strategy for *V. cholerae* wherein it can change shape from curved rod to coccoid, as detected by Direct Fluorescent Antibody (DFA) assay. Recent studies showed *V. cholerae* O1 cells in the aquatic habitat, persisting mostly as coccoid cells in clusters of biofilms comprising non-culturable cells that can regain culturalabity upon animal challenge to initiate seasonal cholera. In this study, we have attempted to isolate and detect the bacterium from water samples collected fortnightly from household ponds and nearby canals in the coastal area of Noakhali, Bangladesh. Serotyping and molecular approaches were employed to identify *V. cholerae* O1. Polymerase Chain Reaction (PCR) was employed to detect the virulence-specific genes and Multilocus Sequence Typing (MLST) of the bacterium. Whole Genome Sequencing (WGS) was performed for further characterization of the isolated *V. cholerae* strains. In addition, a laboratory microcosm was constructed to observe the impact of temperature on growth response of the bacterium. Of 11 *V. cholerae* isolated from water samples collected during May - October, 2024, nine proved positive for serogroup O1, as they carried specific genes: *omp*W, *rfb*O1, *tcp*A, *tox*R, *viu*B, but, all were non-toxigenic having no *ctx*A encoding cholera toxin subunit A. Two isolates were non-O1/non-O139 having *omp*W only. Furthermore, MLST analysis confirmed these nine *V. cholerae* O1 as sequence type 69 (ST-69), whereas the two non-O1/non-O139 as ST-982. WGS data showed the presence of pathogenicity and related genes, including *acfA, acfB, acfC, acfD, hlyA, makA, nanH, tagA, toxR, vasX, tcp* etc., but lacked the CTX prophage. Some of these isolates also had multiple antimicrobial resistance genes for ampicillin, ceftazidime, and sulfamethoxazole suggesting multidrug resistance. Biofilm assays revealed that *V. cholerae* O1 isolates having all biofilm genes (including *msh*ABCDEFGHIJKMN, *omp* U) failed to form a typical biofilm ring on a borosilicate glass tube, suggesting additional regulatory or structural factors that may influence the biofilm development. *V. cholerae* non-O1/non-O139 isolates lacked the key biofilm genes *msh*D and *omp*U and displayed no ring formation. Microcosm study revealed that at 37ºC, *V. cholerae* O1 grew actively isolates grew actively up to 49 days, whereas non O1/ non O139 strains became non-culturable after 35 days. In Microcosm at 4ºC, O1 cells failed to retain active growth state after 7 days, whereas the non-O1/non-O139 were active until 14 days. The overall results presented in this study might reflect the pathogenicity status, aquatic reservoir and possible transition from toxigenic to non-toxigenic strains, including the growth response and mechanism of the survival of *V. cholerae* O1 in the aquatic environment of the coastal areas of Noakhali, Bangladesh.

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Abbreviations

VNC – Viable but not culturable

ATCC - American Type Culture Collection

LPS- Lipopolysaccharide

eDNA - extracellular DNA

MSHA - Mannose-sensitive hemagglutinin

VPS - Vibrio Polysaccharide

TCBS - Thio-sulfate-citrate-bile salt

TTGA - Taurocholate Tellurite Gelatin Agar

GA - Glucose agar

PBS - Phosphate Buffer Solution

APW – Alkaline peptone water

LB broth – Luria Bertani broth

RPM - Revolutions Per Minute

ml – Mili liter

ALT – Ammonium-based Tissue Lysis buffer

AW1 – Wash buffer 1

AW2 – Wash buffer 2

PCR – Polymerase chain reaction

NFW – Nuclease-free water

TBE - Tris-Borate-EDTA

CLSI - Clinical and Laboratory Standards Institute

LA - Luria Agar

µl – Micro liter

AMR – Antimicrobial Resistance

WGS -Whole genome sequencing

MDR- Multi-drug resistance

NGS- Next-generation sequencing

tcpA- Toxin co-regulated pilus

MLST- Multi-locus Sequence typing

DNA – Deoxyribonucleic acid

1.1. Introduction

V. cholerae, the triggering factor of cholera, is a severe secretory diarrheal disease characterized by the sudden development of dehydration and hypovolemia [1]. The disease arises via the ingestion of food or water contaminated by bacteria, but typically *V. cholerae* spend a significant portion of their lives outside of human hosts [2, 3]. Floods and conflicts that allow for increased fecal pollution of water supplies are frequently linked to and exacerbate disease epidemics [3]. Only two of the more than 200 known serogroups of *V. cholerae*—serogroups O1 and O139—are known to be choleragenic, meaning they may cause cholera in humans [4]. A filamentous bacteriophage is present in *V. cholerae* strains that cause cholera. CTXΦ that produces the cholera toxin (CT) and the toxin coregulated pilus (TCP), a coordinately regulated virulence component that is both necessary for colonization and a CTXΦ receptor [1, 3]. It is concerning to note that many NOVC (non-O1 *V. cholerae*) isolates from various serogroups, including O37, O75, and O14, obtained from human illnesses, encode key virulence components (cholera toxin [CTX] and toxin co-regulated pili [TCP]) and can therefore cause cholera [5]. The *V. cholerae* pathogenicity island (VPI) or TCP island is a sizable cluster of genes necessary for TCP biosynthesis. The primary pilus component is encoded by *tcpA* within this cluster [6]. Environmental *V. cholerae* isolates are typically antigenically diverse and do not commonly possess cholera toxin genes (*ctx*A or *ctx*B) or toxin coregulated pilus (TcpA). However, environmental strains are epidemiologically and evolutionarily important as they may serve as a donor of genetic material through natural transformation. Similarly, they may acquire the O1 antigen and virulence genes through horizontal gene transfer [7].

At multiple stages of their life cycle, bacteria in a natural environment exhibit morphological changes [8]. These morphological alterations correspond with the two seasonal peaks of endemic cholera in Bangladesh in the spring and fall. The majority of *V. cholerae* O1 cells are coccoid within biofilms, although they can also be free-living, rod-shaped, and attached to plankton or particulate matter during the year's interepidemic periods [8, 9]. Multiple studies have shown that *Vibrios* are very adaptable in their adhesion to water surfaces, including those of plants, filamentous green algae, zooplankton, crustaceans, and insects. This behavior is defined as biofilm. Biofilm can shield its colonizers from the effects of heavy metals, contaminants, antibiotics, and temperature and pH fluctuations. Enhanced rates of conjugation in bacterial biofilms have been reported numerous times. It implies that fast evolution through horizontal genetic material transfer may take place in biofilm, creating the ideal environment

for the creation of novel diseases through the acquisition of virulence factors, antibiotic resistance, and environmental capacities for survival [9]. For instance, the introduction of a *Bacillus subtilis* strain carrying a conjugative transposon that imparts resistance to tetracycline in a microcosm containing dental plaque has been shown to acquire resistance genes in *Streptococcus* biofilm. The study illustrates the presence of a biofilm gene in some strains that really affects the creation of biofilms and how various environmental elements, such as temperature, may affect the ability to overcome stressful conditions in the production of biofilms [9]. Through the biofilm assay technique, the ability of biofilm formation of *V. cholera* may be assessed. High biofilm producer isolates may producer may form a ring in a borosilicate glass tube with 0.1% crystal violet. Besides, genotypic analysis demonstrates which genes are more frequently involved in this case. In this study, we showed the comparison of phenotypic and genotypic characteristics of *V. cholerae* isolates and *V. cholerae* biofilm and biofilm genes involved in this procedure.

1.2. Literature review

1.2.1. Cholera disease

Cholera is a severe and sometimes fatal diarrheal disease caused by the comma-shaped bacterium *V. cholerae*. Consuming food or water tainted by this microbe is how the illness is contracted. Cholera has virtually disappeared from developed countries due to improved hygiene standards and water quality; however, many underdeveloped countries that lack adequate infrastructure and have poor sanitation continue to endure the menace of the disease. Disease outbreaks are often associated with and accentuated by floods and conflict that allow increased fecal contamination of water supplies [3].

1.2.2. *V. cholerae* reservoirs in the aquatic environment

The aquatic environment provides many circumstances (temperature, salinity, and pH) and a variety of resources (carbon and energy needed for bacterial metabolism) that dictate the range of habitats that microorganisms can occupy. *V. cholerae* is a chemoheterotrophic bacterium capable of both respiratory and fermentative metabolism starting from a variety of organic substrates. It is a halotolerant microorganism mostly isolated from environmental locations where NaCl concentrations range from 0.2% and 3.0%. The optimal temperature for growth ranges from 30°C to 40°C [2]. To date, several environmental reservoirs have been

hypothesized for *V. cholerae,* like several marine and freshwater flora provide a survival advantage to associated *V. cholerae*. In addition to them, chironomids, protozoa, marine bivalves, aquatic birds, etc., also play a vital role in the survival of *V. cholerae* in the aquatic system [2]. According to very recent research, *V. cholerae* O47 could represent an emerging *Vibrio* pathogen with the potential to spread virulence and antimicrobial resistance features impacting the management of cholera-like diseases [5].

It has been postulated that under stress conditions, the *Vibrios* are converted to a viable but nonculturable (VBNC) form that is unable to be recovered by conventional culture techniques, and during inter-epidemic periods, the pathogen has to be in that state to persist primarily in the aquatic environment and reinfect humans under ideal circumstances [10, 11].

1.2.3. Epidemiology of cholerae

Cholera is recognized as one of the emerging and reemerging infections that pose a significant threat to several developing countries. An estimated 120,000 people perish from cholera outbreaks per year, and there are many more cases, the great majority of which entail children. The epidemiology of cholera is characterized by the following attributes: (i) an elevated level of clustering of cases by region and season; (ii) the highest rates of infection in children aged 1 to 5 in areas of endemic infection; (iii) patterns of antibiotic resistance that frequently vary from year to year; (iv) clonal diversity of epidemic strains; and (v) defense against the disease through enhanced sanitation and hygiene and prior immunity [10].

The annual number of cholera cases reported to the WHO per continent between 1989 and 2017 is displayed in Fig. Over the years, cholera outbreaks have fluctuated in magnitude and location; in 2017, a particularly high number of cases were documented. Significant cholera outbreaks were documented in Yemen, many African nations, and Haiti during 2017 and 2018 [12].

In 2021, 23 countries reported cholera outbreaks, mainly in the WHO Regions of Africa and the Eastern Mediterranean. This trend has continued into 2022, with over 29 countries (Figure 1.1) reporting cholera cases or outbreaks (Figure 1.2)

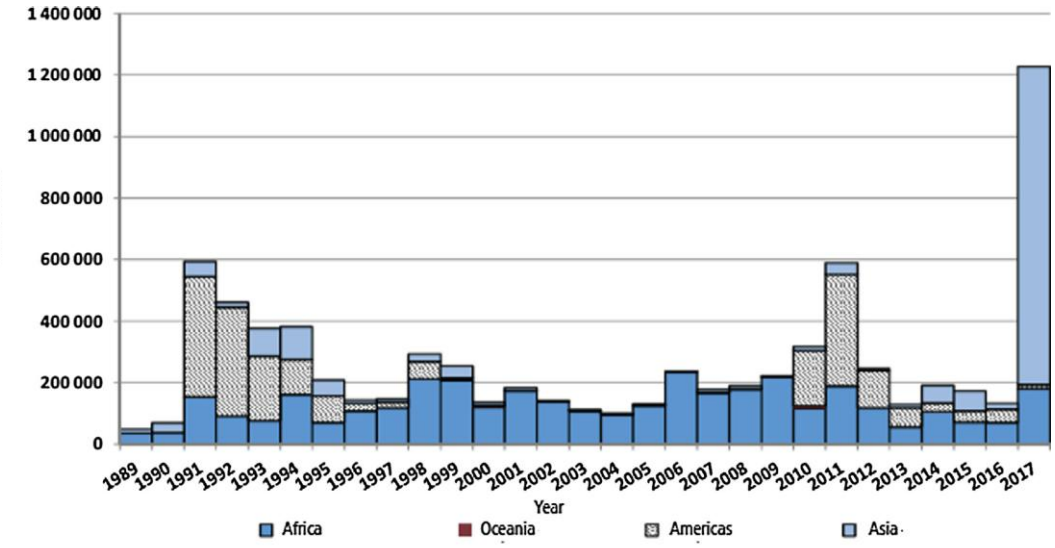


Figure 1.1: Number of cholera cases reported to WHO by year and by continent, 1989–2017.

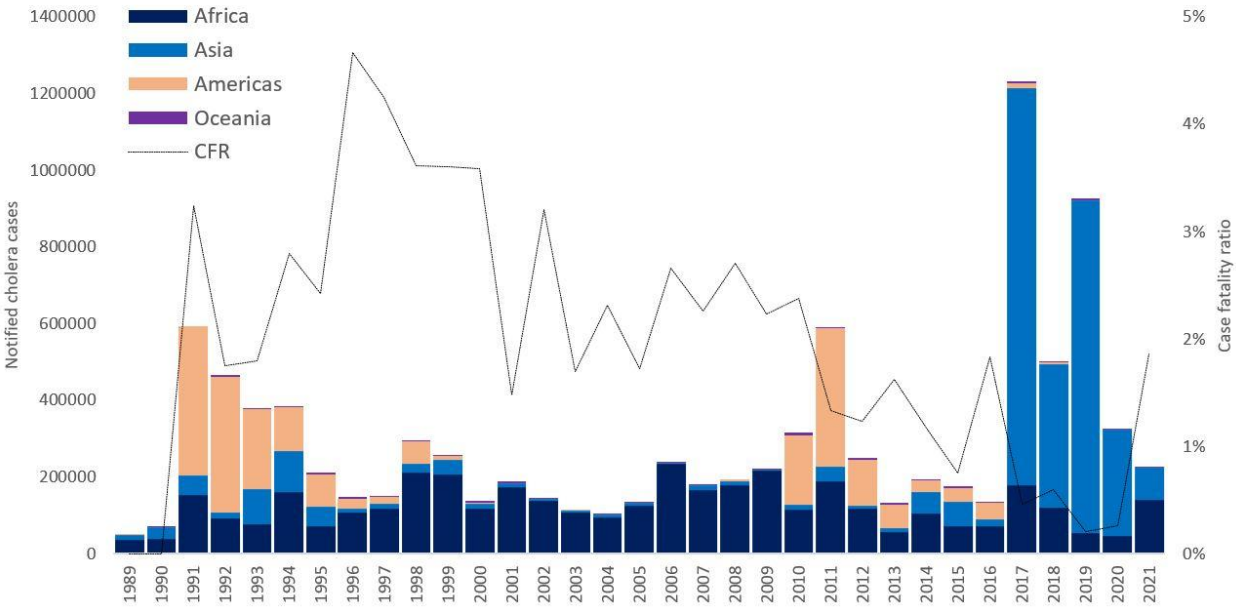


Figure 1.2: Cholera cases* reported to WHO by year and continent, global CFR, 1989-2021(WHO, 2024).

1.2.4. Early Pandemics

Since 1817, the world has recognized seven cholera pandemics. The first six pandemics are thought to have originated in the Ganges River delta. The seventh pandemic, still ongoing today, began in 1961 in Sulawesi, Indonesia (Shears, 1994). Pollitzer (1959) found that the

classical biotype caused the first five pandemics, whereas the classical strains were responsible for the sixth. Between the sixth and seventh pandemics, cholera was limited to small outbreaks generated by El Tor strains throughout a 38-year span (39).

1st Pandemic

Since the first cholera pandemic began in 1817, it has been established that there have been seven distinct pandemics. Except for the seventh pandemic, which started on the Indonesian island of Sulawesi, the pandemics arose on the Indian subcontinent, typically in the Ganges delta, then extended to other continents, impacting multiple countries over a long period of time [10]. According to multiple reports, the first cholera pandemic, also known as Asiatic cholera (1817–1823), started in Jessore, Bangladesh, and spread to Russia in the north; Myanmar, Thailand, Malacca, Malaysia, Singapore, Indonesia, the Philippines, China, and Japan in the east; Oman, Bahrain, and the Mediterranean region, as well as a portion of Iran, Iraq, Syria, Turkey, and Egypt in the west; and Sri Lanka, Mauritius, and Zanzibar in the south [11]. Although the precise number of deaths during this pandemic is unclear, Thailand documented 30,000 deaths, and there were speculations of roughly one million cases in India and Indonesia in 1820 [11].

2nd Pandemic

John Snow made significant epidemiological observations about the waterborne transmission of cholera in London between 1847 and 1854 during the late second and third pandemics [10]. The second cholera pandemic reached the British Isles in the early 1830s, expanded to the Ganges Delta in India, Indonesia, the Philippines, China, and Japan, attacked Afghanistan and Iran in 1829, and moved northward to affect Russia and northern and central Europe [11]. Ships from Ireland carrying sick immigrants carried the second pandemic to Canada [10]. Cholera struck Egypt in 1831, killing over 130,000 people [11].

3rd Pandemic

Over 100,000 cholera cases and 2000–3000 deaths were recorded by the World Health Organization (WHO) during the third cholera pandemic (1852–1859), which originated in

India and migrated to East Asia, Europe, and Africa. Cholera was pervasive in the US during the third epidemic [10, 11]. 10,739 people died from cholera outbreaks in London throughout the ensuing years (1853–1854). Over 150,000 people died in the United States during the two pandemics between 1832 and 1849 as a result of several outbreaks brought about by Irish immigrants arriving by ship from England. In 1854–1855, cholera made its way to South America (Brazil and Venezuela), where it killed 25,820 people in Puerto Rico [11]. Due to inadequate monitoring systems in many nations, the true number of cholera cases may be significantly higher due to underestimation of the illness [11].

4th Pandemic

About 30,000 people died during the fourth cholera pandemic (1863–1879), which erupted in the Ganges Delta and migrated to Mecca via pilgrims. In the same year, cholera killed 90,000 people in Russia [11], and toward the end of the fourth pandemic (during the 1870s), cities and towns along the Mississippi, Missouri, and Ohio rivers experienced cholera [10].

5th Pandemic

South America was severely impacted by the fifth pandemic, which resulted in widespread epidemics in numerous nations and was highlighted by high death rates in Argentina, Chile, and Peru [11]. The fifth pandemic wiped out 90,000 people in Japan in 1887–1889, approximately 200,000 in Russia in 1893–1894, 58,000 in Egypt, 250,000 in Europe, and 50,000 in the Americas. Robert Koch isolated the cholera-causing organism, known as "comma bacilli," from rice water stools of patients in Egypt in 1883 and India in 1884 [10].

6th Pandemic

More than 800,000 people died in India, 200,000 in the Philippines, and 500,000 in Russia during the sixth pandemic (1899–1923), which mostly affected people in the Near and Middle East as well as the Balkan Peninsula [10, 11]. From the middle of the 1920s until the start of the seventh pandemic in 1961, cholera was essentially limited to South and Southeast Asia, except for a significant outbreak in Egypt in 1947. In 1902, about 34,000 people perished in

Egypt. *V. cholerae* of the classical biotype was responsible for both the sixth and possibly the fifth pandemics [10].

7th Pandemic

V. cholerae O1 of the El Tor biotype, which was initially discovered in the El Tor hamlet in Egypt in 1905, is the causative agent of the seventh pandemic, which is the largest of the pandemics in terms of both geographical distribution and duration [10, 11]. The first five pandemics may have been caused by *V. cholerae* O1 of the classical biotype. The seventh pandemic originated on the Indonesian island of Sulawesi in 1961 and quickly expanded to Java, Sarawak, and Borneo. The epidemic afflicted Malaysia, Thailand, Burma, Cambodia, Vietnam, India, Bangladesh, and Pakistan as it moved to the Asian mainland between 1963 and 1969 [10]. El Tor cholera outbreaks were reported in Afghanistan, Iran, Iraq, and neighboring Soviet countries shortly after it reached Pakistan. The seventh pandemic struck sub-Saharan West Africa in the early 1970s, causing explosive outbreaks that generated over 400,000 cases with a high death rate. These outbreaks were mostly driven by the population's lack of prior immunity and deficiencies in the health care system. Of the 36 nations that reported cholera in 1970, 16 were in Africa, and 28 were newly afflicted. Cholera reappeared in South America after more than a century when the seventh pandemic struck in the form of an explosive epidemic that started in Peru in January 1991. An epidemic of cholera was later recorded in Colombia and neighboring Ecuador. The most detrimentally affected groups in each of these nations were those with poor socioeconomic status who lacked access to clean water and sanitary facilities. According to estimates from the Pan American Health Organization, there were 750,000 cholera cases and 6,500 fatalities throughout the Americas between 1991 and 1992 [10]. El Tor cholera expanded to nearly every country where cholera is prevalent in around 25 years, primarily through widespread outbreaks [11]. Between 1982 and 1992, the classical biotype reappeared in some parts of Bangladesh and coexisted with Seasonal outbreaks of the seventh pandemic are still occurring in several developing nations, including Bangladesh and India. However, the eighth pandemic may have started in 1992 when V. cholerae from a non-O1 serogroup (now known as O139) caused widespread cholera outbreaks in Bangladesh and India and expanded to a few other nations [10].

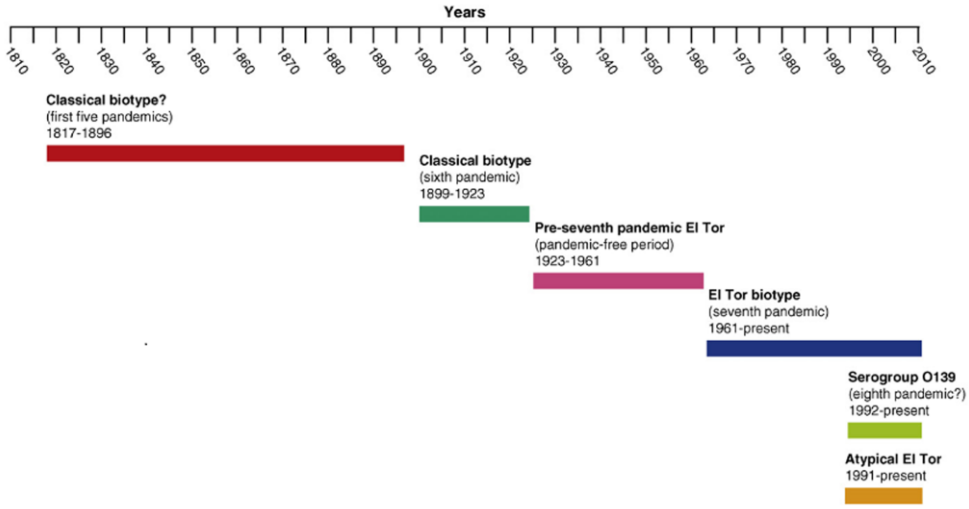


Figure 1.3: The Seven pandemic occurred worldwide from 1817-2010 [13]

El Tor strains [11]. The majority of these classical isolates were remarkably resistant to multiple drugs [11].

8th Pandemic

An epidemic of cholera was recorded in Madras and other locations in Bangladesh and India starting in late 1992 and lasting into 1993 [11, 14]. The causal agent was a *V. cholerae* non-O1 strain that was subsequently sero-grouped as O139, despite the clinical presentation being typical of cholera. *V. cholerae* O139 spread over Bangladesh, India, and other nations as the outbreak persisted throughout 1993 [11]. Since then, reports of *V. cholerae* O139 outbreaks or cases have come from Pakistan, Nepal, China, Thailand, Kazakhstan, Afghanistan, and Malaysia. According to recent surveillance conducted in 1996 and 1997, *V. cholerae* O139 coexists with El Tor vibrios and continues to generate cholera outbreaks in Bangladesh and India [11, 14]. Beginning in the early 1990s, cholera outbreaks continued in several Asian and African countries [11]. If outbreaks of cholera due to this new serogroup continue to occur and affect more countries, this may represent the eighth pandemic [14].

1.2.5. Classification

V. cholerae is classified serologically based on the variations in the O antigen composition. The O antigen specificity is derived from the O-specific polysaccharide, which is located on the outermost portion of the lipopolysaccharide (LPS). While more than 200 serogroups of *V. cholerae* have been detected, only serogroups O1 and O139 have been associated with epidemic cholera. *V. cholerae* strains that do not belong to serogroup O1 or O139 are often collectively referred to as non-O1/O139 *V. cholerae* or NOVC. Non-toxigenic, non-O1/O139 *V. cholerae* may cause sporadic cases of gastroenteritis and sepsis, but do not cause cholera-like sickness [1].

V. cholerae O1 is further classified by serotype. The three predominant serotypes are *V. cholerae* O1 Inaba, *V. cholerae* O1 Ogawa, and *V. cholerae* Hikojima. *V. cholerae* O1 Hikojima is an unstable form that is infrequent in the natural world [15]. Inaba and Ogawa serotypes are distinguished by the addition of a single 2-O-methyl group in the non-reducing terminal saccharide of the Ogawa O-specific polysaccharide [1].

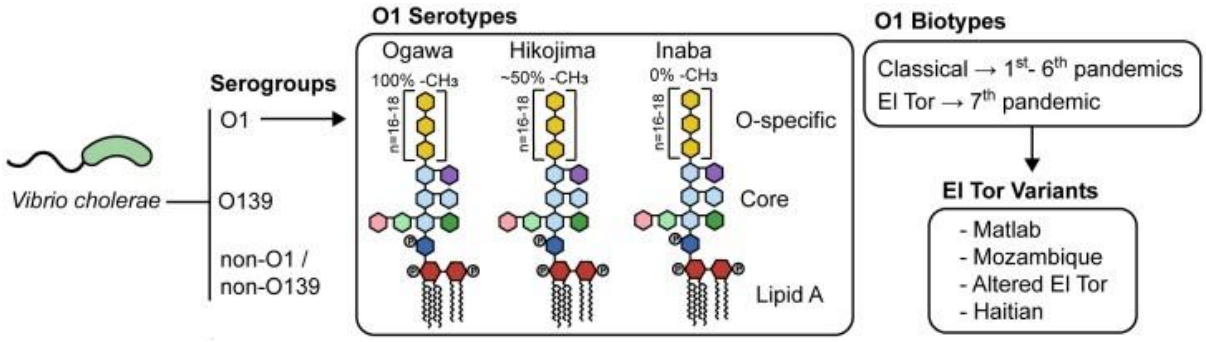


Figure 1.4: *Vibrio cholerae* classification [13]

Two biotypes are further subdivided into the *V. cholerae* O1 serogroup on the basis of phenotypic and genotypic characteristics [1, 12]. El Tor and classical biotypes are differentiated by biochemical distinctions and susceptibility to specific bacteriophages. The circulating classical biotype, which is currently believed to be extinct in nature, was totally supplanted by the El Tor biotype during the current cholera outbreak [1]. Variations in specific gene clusters, such as VSP-I and -II (Vibrio seventh pandemic island I and II) and RTX (repeat in toxin), have also been used to distinguish the two biotypes through genotypic analyses of particular genes

(*tcp*A (toxin co-regulated pilin A), *ctx*B (cholera toxin B), and *rst*R (repeat sequence transcriptional regulator) [16].

1.2.6. Genomic organizations of *Vibrio cholerae*

The genome of *V. cholerae* is unique among bacteria, consisting of two separate circular chromosomes. The complete genome sequence of reference strain *V. cholerae* N16961 comprises a total of 40,033,460 base pairs (bp), distributed between two chromosomes, designated as chromosome I (chr I) and chromosome II (chr II). Chromosome I, the larger of the two, contains 2,961,146 bp and primarily encodes genes essential for bacterial growth and viability. In contrast, chromosome II is smaller, consisting of 1,072,314 bp, and predominantly harbors genes of unknown function and hypothetical proteins [17].

1.2.7. Transmission of Cholerae

V. cholerae infection may be contracted by the ingestion of tainted food or water. Direct person-to-person transmission probably plays a significant role in transmission as well, even if exposure to environmental *V. cholerae* is believed to be a significant factor in infection in historically endemic areas [1]. While epidemic cholera frequently occurs close to waterways when weather conditions are favorable for the growth of the bacteria, endemic cholera has been linked to tidal seawater intrusions and seasonal climatic patterns. As a result, an interaction between the aquatic environment and fecal-oral spread of cholera has long been held [12]. Individuals with severe cholera can excrete as many as 10^{10}-10^{12} per liter of feces, and human-shed organisms appear to be transiently more contagious than organisms isolated from the aquatic environment. The fact that contaminated food and water are the means of transmission is reflected in individual risk factors for *V. cholerae* infection. Cholera risk factors include, for instance, drinking unfiltered or untreated water, although using soap is often linked to a decreased risk of infection. In foodborne outbreaks, risk factors may include consumption of specific contaminated foods, such as rice products, vegetables, or fruits [1].

Additional risk factors for *V. cholerae* infection are indicative of specific biological interactions between host and pathogen (virus). Hypochlorhydria lowers the infectious dose of *V. cholerae* that can cause infection, because the acidity of the stomach is a crucial barrier that prevents infection. Individuals with blood group O have a higher risk of developing severe cholera

compared to individuals of other blood groups. Nutritional factors also influence the risk of cholera; for example, retinol insufficiency is linked with an increased risk of cholera, while breastfeeding is protective [1].

Besides, mass gatherings, especially those with significant numbers and inadequate access to clean water and sanitary facilities, facilitate the spread of cholera and may be followed by additional transmission when participants return home [12].

1.2.8. Clinical manifestation

V. cholerae frequently causes asymptomatic infection or moderate diarrheal illness; however, in severe cases (asymptomatic infection), *V. cholerae* induces a profound secretory diarrhea that can be fatal in 6-24 hours in a healthy adult [1, 12]. The acidic environment of the stomach kills many organisms. After a typical incubation period of 12 days (but sometimes up to 5 days), patients experience a sudden onset of watery diarrhea. Vomiting is common and often precedes the onset of diarrhea. In cholera, the maximal rate of fluid loss often exceeds 1% of the body weight per hour, leading to rapid-onset profound dehydration that develops quickly. Over 24 hours, up to 20 liters of watery diarrhea can be excreted in adults. Stools are typically transparent with tiny white mucous flecks and referred to as rice-water stool because the fluid resembles water after rice is washed in it. The stool is extremely contagious, containing 10^9 *V. cholerae* organisms per ml of stool [1]. The cholera organisms found in stool are a combination of biofilm-like clumps and free-swimming *V. cholerae* cells [12].

Patients with severe dehydration from cholera may be apathetic or lethargic, have sunken eyes, dry mucous membranes, chilly, clammy skin, and reduced skin turgor. The peripheral pulse is weak or non-palpable. Acidosis can lead to irregular breathing. Cramping and weakness are commonly due to potassium deficiency. The majority of deaths from cholera are the result of low circulating blood volume caused by extreme dehydration. Hypoglycemia, hypokalemia, and hyper- or hyponatremia are other potentially life-threatening complications of cholera and are more common in young children [1].

1.2.9. Management of Epidemic Cholerae

Cholera prevention and control initiatives will be successful if transmission, for instance, can be effectively controlled at the home and/or community levels. Clean water, sanitation, and hygiene (WASH) initiatives and an oral cholera vaccination (OCV) campaign that primarily targets cholera hotspots are two cholera response approaches to eradicate cholera in at least 20 nations by 2030 and reduce mortality from the disease by 90% worldwide [11]. In regions where outbreaks are likely, OCV may be crucial for both long-term cholera control and outbreak prevention in conjunction with WASH. In regions where outbreaks are likely, OCV may be crucial for both long-term cholera control and outbreak prevention in conjunction with WASH. Through herd protection, OCV directly benefits both vaccine recipients and non-immunized people [11]. Through herd protection, OCV directly benefits both vaccine recipients and non-immunized people [15]. There are now four live oral cholera vaccines and roughly five killed oral vaccinations available [11]. Access to ORS/IV fluid treatment, epidemiological and laboratory surveillance, and antibiotics for severe cases are all part of the implementation of other integrated and comprehensive techniques for the prevention of cholera [11].

1.2.10. Stress response of *V. cholerae*

The nutrient-rich human small intestine and aquatic habitats are two distinct ecological niches that *V. cholerae* has successfully colonized. *Vibrios* must deal with a variety of physical, chemical, and biological challenges in the aquatic environment, such as nutrient scarcity, high temperatures, oxidative stress, bacteriophage predation, and protozoan grazing [8]. *Vibrios* endure exposure to low pH, bile acids, high osmolarity, iron restriction, antimicrobial peptides, and infrequent nutritional deprivation in the gastrointestinal system. *V. cholerae* must overcome as many harsh situations as it needs to survive and stay outside of the human host to achieve high titers in the gut. This is demonstrated by the fact that *V. cholerae's* pathogenicity and ability to colonize the small intestine are significantly reduced when genes producing the general stress response regulator RpoS (σS) or the RNA polymerase σE subunit (RpoE) that mediates the envelope stress response are disrupted [6].

1.2.11. Quorum Sensing

Quorum sensing is a cell-to-cell communication method in bacteria that involves the production, release, motility, conjugation, competence, virulence, and sensing of signaling molecules known as autoinducers [18]. Quorum sensing is used by *Vibrio species* to control a number of traits, including luminescence, pathogenicity, and biofilm development. This process involves several transcription regulators, such as AphA, HapR, and VpsT. The master quorum-sensing regulator HapR lowers the expression of virulence genes by blocking the transcription of aphA. Quorum sensing through the regulators AphA and HapR inhibits the formation of biofilms. At low cell density, AphA boosts the expression of the biofilm activator VpsT. HapR decreases the formation of biofilms at high cell densities by inhibiting vpsT and lowering the intracellular c-di-GMP pool [6, 18].

1.2.12. Biofilm formation

Biofilms are microbially derived sessile communities characterized by cells that are adhered to a substratum, an interface, or to each other; are embedded in a self-produced extracellular matrix; and present a distinctive phenotype with respect to growth rate and transcription profile [6, 9, 19]. For *V. cholerae*, biofilms are more resistant to bile salts and low pH [20]. The pathogenesis, transmission, and environmental survival of *V. cholerae* depend on biofilms, which makes it a perfect model system for biofilm research [6, 19]. Ingesting hyperinfectious biofilms has been proposed as a natural way to get cholera during epidemics and as a quick way to spread the illness [6]. Biofilm production is modulated by intricate networks of signal transduction channels in response to intracellular and external stimuli. Various cell-surface and cell-cell interactions are essential for *V. cholerae* biofilm attachment and stability. A variety of surface attachment molecules, such as adhesion proteins, pili, and flagella, are important in the creation of biofilms [9, 19].

mshA cluster

The 17 genes of the msh-cluster are arranged into two operons: *msh*HIJKLMNIJ, which codes for secretory proteins, and *msh*BACDOPQ, which produces the structural elements (subunits) of MSHA pili. In the intestine, the production of toxin-coregulated pili (TCP) increases, and the synthesis of MSHA is repressed; whereas the opposite process is observed in water [19].

1.2.12.1. Surface attachment

Pili

By inhibiting motility and increasing surface attachment and biofilm matrix synthesis, the prevalent signaling molecule bis-(3–5)-cyclic dimeric guanosine monophosphate (c-di-GMP) assists *V. cholerae* in evolving from a motile to a sessile biofilm state. Diguanylate cyclase enzymes (DGCs) produce the c-di-GMP molecule, which is then broken down by phosphodiesterase enzymes (PDEs). It can interact with various receptor proteins or RNA to modify cellular processes. Mannose-sensitive hemagglutinin (MSHA) pili, which are necessary for initial attachment, are conveniently produced at higher doses of c-di-GMP [19].

Flagellum

The gliding motility of *V. cholerae* is attributed to a single Na+-driven flagellum on one of the cell poles. Suppression of flagellar rotation stabilizes cell surface attachment and prevents separation during the early stages of biofilm development. In *V. cholerae*, c-di-GMP inversely controls motility and biofilm development. In comparison to the wild type, genetic disruption of *V. cholerae* flagellin, FlaA, or flagellum biosynthesis increases cellular c-di-GMP levels and promotes biofilm formation [19]. Analysis of c-di-GMP production in single cells suggests that an increase in cellular c-di-GMP levels after first surface attachment may be due to a decrease in flagellar activity and production [19].

Adhesion protein

Two such adhesins, FrhA and CraA, are found in *V. cholerae* and are expected to localize to the outer membrane through the type I secretion system. Initial adhesion and biofilm formation are dependent on strain and substrate for both FrhA and CraA. Loss of FrhA and CraA decreases initial attachment and biofilm formation in the classical strain of *V. cholerae* O1. GbpA, which is increased by c-di-GMP, also has a role in *V. cholerae* adherence to mucin and chitin surfaces, underscoring the various adhesion proteins and attachment mechanisms [19].

1.2.12.2. Formation of microcolonies

The biofilm-forming bacteria usually establish a brief relationship with the surface and other surface-attached microorganisms after initial attachment. It is likely able to search for a place to settle down and form a stable association as a member of a microcolony thanks to this temporary affiliation. In addition to moving the bacteria closer to the surface, flagellum-mediated motility is essential for parallel migration to the surface. In order for bacteria to spread across a surface and approach it, flagella are essential [19].

1.2.12.3. Extracellular Matrix Production

Vibrio Polysaccharide

The development of *V. cholerae* 3D biofilm structures and native cell packing in biofilms depends on the generation of VPS. Mature biofilms contain VPS, which is secreted upon surface adhesion [19, 21]. The vps genes are clustered in two regions, the vps-I cluster (vpsU, vpsA–K, VC0916–27) and the vps-II cluster (vpsL–Q, VC0934–39), separated by an intergenic region containing the rbm gene cluster that encodes biofilm matrix proteins. According to a number of studies, VPS may be generated during infection and aid in in vivo colonization and survival [21]. The VPS-dependent and independent biofilms differ in their environmental activators and in architecture [22].

Matrix Protein

The primary proteins that create the extracellular matrix needed for biofilm formation are RbmA, RbmC, and Bap1. These proteins are secreted into the matrix by the type II secretion system (T2SS); biofilm development is reduced when the T2SS is lost. RbmA is widely distributed throughout the mature biofilm and is one of the first proteins released into the biofilm matrix once a founder cell attaches to surfaces. The absence of cell-cell interaction in the loss of rbmA results in looser, less compact biofilms that are more susceptible to invasion and predation [19]. Cell clusters inside the biofilm are encased by RbmC [15], whereas Bap1, a homologue of RbmC that was first discovered by a transcriptome study of *V. cholerae* biofilm phases [23].

Other matrix proteins

Additionally, biofilms include outer membrane vesicles (OMVs) and extracellular DNA (eDNA). The biofilm matrix proteins RbmA, RbmC, and Bap are harbored by OMVs, which are composed of an outer leaflet of lipopolysaccharide, an inner leaflet of phospholipids, and outer membrane proteins (OMPs). Through horizontal gene transfer, eDNA contributes to the biofilm structure, interacts with VPS, and represents a potential source of nutrients and genetic material [19].

1.2.12.4. Dispersal mechanism

Cellular dispersal from biofilms can be controlled in two ways: passively, as shearing by fluid flow, and actively. Pilus retraction, downregulation of biofilm matrix synthesis, upregulation of matrix-degrading enzymes, and overexpression of motility—processes that are often initiated by intracellular levels of c-di-GMP—usually occur concurrently with active dispersal. The manipulation of MSHA pili interactions with a surface by cellular c-di-GMP levels is one such instance in *V. cholerae*. Low c-di-GMP levels cause MSHA pilus retraction, and increased retraction causes *V. cholerae* to spread from surfaces in the early phases of biofilm formation.

By altering the active state of important biofilm regulators, changes in cellular c-di-GMP levels brought on by the activation and inhibition of c-di-GMP metabolizing enzymes also affect flagellar motility, biofilm matrix formation, and ultimately biofilm dispersion. Reduced matrix component development may help biofilm dispersal, but for cells to be able to leave biofilms, they must also detach from the matrix, degrade the matrix, or do both [19].

1.2.13. Microcosm

Microcosms are controllable biological models that simplify and replicate intricate, diverse natural ecosystems. The core principle is that all units in nature, regardless of their size, exhibit similarities in structure and function. As an experimental tool for simulating specific ecosystems, microcosms can be applied in laboratories or natural environments [24]. In the laboratory, a small boundary of an ecosystem with a specific microbial community like *V. cholerae* O1, O139, or non-O1/O139 may be observed with different environmental conditions

(temperature, salinity, pH, ion concentration, etc.). The phenotypic and genotypic changes due to environmental parameter change may be visualized and compared with the large ecosystem.

1.2.14. Advantages of *V. cholerae* biofilm formation

The capacity to pick up transmissible genetic elements more quickly is one benefit of living in biofilms. This implies that fast evolution through horizontal gene transfer may take place in a biofilm, creating the ideal habitat for the creation of novel diseases through the acquisition of virulence components, antibiotic resistance, and environmental survival skills [25].

It has been shown that *V. cholerae* biofilms are more resistant to acid inactivation [6]. Besides, compared to planktonic cells, biofilm-derived cells may be more successful in competing for scarce resources in the small intestine due to their increased expression of their phosphate absorption mechanism [20].

Living in a city has additional benefits. Living together is beneficial during difficult times. In a similar vein, biofilm-associated cells have greater resistance to a variety of harmful chemicals, including detergents, chlorine, and antibiotics [25].

1.3 Aims & Objectives

1. To understand the fitness dynamics of pathogenic and non-pathogenic *V. cholerae* in the coastal region of Bangladesh
2. To track the lineage of isolated pathogenic strains through different molecular techniques including WGS.
3. To identify the mechanism of biofilm formation in water microcosm.

Materials &Method

2.1. Sample collection:

Samples of water and plankton were collected every two weeks from nine separate water reservoirs in the Noakhali region. These reservoirs are natural bodies of water where local people wash dishes, take baths, and sometimes drinking also. All samples were collected by using aseptic techniques in sterile dark Nalgene bottles, placed in an insulated plastic box, and transported at ambient air temperature from the site of collection to the central laboratory of the International Centre for Diarrheal Disease Research, Bangladesh (icddr,b), in Dhaka. All samples were processed the following day, with approximately 20 h elapsing between sample collections in the field and processing in the laboratory. Samples were collected at 15-day intervals. Their names have been listed here.

Table 1: Sample area list

Serial No	Area of Sample	Nature of the water reservoir
1	Noakhali Police Training Center	Pond
2	Primary training institute	Pond
3	Housing pond	Pond
4	Circuit house pond	Canal
5	Housing pond 2	Pond
6	Nil dighi	Dighi
7	Lawyer colony lake	Lake
8	Pouro Park Lake	Lake
9	Noakhali General Hospital Dighi	Dighi

Sample collection Period:

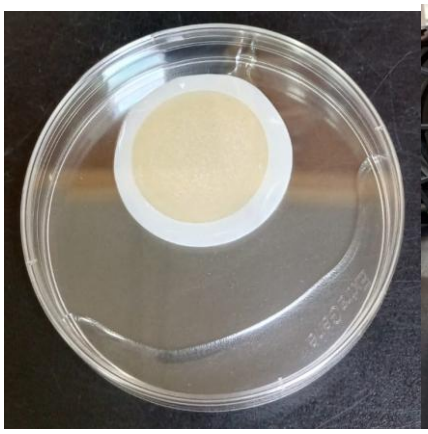
Samples were collected from May 2024- October 2024.

Processing

Following selective medium culture, typical *V. cholerae* colonies were isolated and identified using standard procedures (34).

- About 200ml of each sample was filtered through a 0.22µm membrane filter.
- The filter was then suspended in 5 mL of Phosphate buffer saline (PBS).
- From this suspension, 2 ml was inoculated into 18 ml of APW and kept for 6 hours at 37ºC, and incubated on TTGA plates and incubated for 18-24 hours.

(a)



(b) **(c)**

Figure 2.1: Sample processing; (a) Filtration, (b) PBS with filter paper, (c) APW enrichment

2.2. Isolation

V. species are selectively isolated using Thiosulfate-citrate-bile-salt-Sucrose agar (TCBS), Taurocholate Tellurite Gelatin Agar (TTGA), and Glucose agar (GA) based on their ability to ferment sucrose or utilize glucose. These media provide an optimal environment for *Vibrio spp*. Growth while inhibiting the development of other microorganisms.

2.3. Preparation of TCBS agar

TCBS agar, namely Thio-sulfate-citrate-bile salt agar, is a selective and differential medium for isolating *V. cholerae* and other *Vibrio* species. Mainly, high pH rates (8.5-9.0) inhibit the growth of most intestinal flora and allow selective growth of *Vibrio* species. The presence of sucrose and bile salts makes the medium both selective and differential, distinguishing *Vibrio sp* based on their ability to ferment sucrose. Bromothymol blue and thymol blue are incorporated as pH indicators, with yellow colonies indicating sucrose fermentation by *Vibrio spp*.

Agar Preparation

2.4. Preparation of TTGA agar

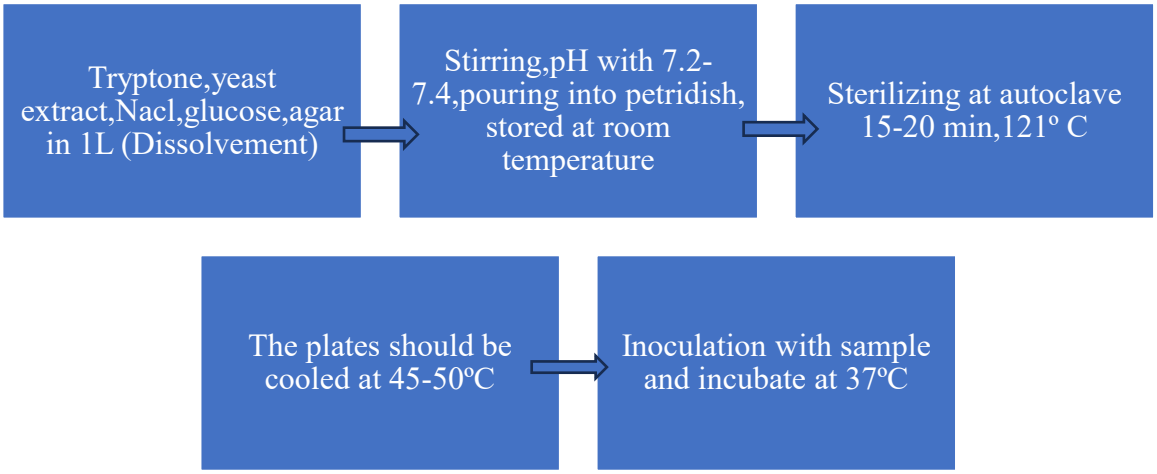
Taurocholate Tellurite Gelatin Agar is a selective medium used for isolating and identifying *V. cholerae* and other *Vibrio* species. This medium inhibits the growth of intestinal flora and promotes the growth of *Vibrio* species due to the presence of taurocholate and tellurite. Besides, 4-methylumbrlliferyl-β- D-galactoside allows for the detection of β-galactosidase activity, enhancing the detection of *V. cholerae.*

Agar Preparation

2.5. Preparation of GA agar

Glucose agar (GA) is used for isolating *Vibrio* species from environmental samples. This medium is mainly based on the principle that *Vibrio* species can use glucose as their carbon and energy source and may produce acid and reduce pH. The low pH and reduced redox potential create an anaerobic condition that helps *Vibrio species* grow.

Agar preparation



2.6. Serotyping

The glass slide agglutination procedure has been used for detecting serotypes of *V. cholerae* O1 or O139.

Materials

- ✓ Pure culture of *V. cholerae* (Grown in gelatin agar)
- ✓ Clean glass slide
- ✓ Inoculating loops
- ✓ *V. cholerae* O1 polyvalent antiserum
- ✓ *V. cholerae* O139 antiserum
- ✓ Inaba antiserum
- ✓ Ogawa antiserum

Procedure

1. 5µl lines of antisera should be placed using a micropipette on the glass slide for each serogroup (O1 and O139). Lines should be paralleled and spaced sufficiently to avoid cross-contamination.
2. Single colony from culture plate using a sterile loop. Mix the bacteria into each line to create a smooth suspension.
3. Gently rocking the slide back and forth for 30s-40s and waiting for visible clumping (agglutination)

Figure 2.2: Serological Test

Interpretation

- Agglutination in the O1 line ➞ *V. cholerae* O1 positive
- Agglutination in the O139 line ➞ *V. cholerae* O139 positive
- Agglutination in the Inaba line ➞ *V. cholerae* O1 Inaba positive
- Agglutination in the Ogawa line ➞ *V. cholerae* O1 Ogawa positive

2.7. DNA extraction

2.7.1. Boil DNA extraction from bacterial isolate

- A single colony should be picked from a fresh culture medium and mixed with 700µl PBS (Phosphate Buffer Solution) in a microcentrifuge tube.
- Then the mixture should be vortexed for about few minutes
- Then the mixture should be kept in a heat block at 100ºC for 10 minutes at 1600 rpm to lyse the cells.
- After boiling, the tube was immediately cooled on ice for 5 minutes.
- The samples were centrifuged at 13000 rpm for 10 minutes
- Finally, the supernatant should be transferred into another microcentrifuge tube.
- The DNA extract was stored at -20ºC

2.7.2. Boiled DNA extraction from enriched water sample

- 1.5ml of the 6-hour APW-enriched water sample was transferred into a sterile 1.5ml microcentrifuge tube.
- The sample was centrifuged at 13000 rpm for 10 minutes to pellet the bacterial cells.
- The supernatant was carefully discarded, and the pellet was retained.
- The pellet was resuspended in 700µl of PBS by vortex or pipetting.
- The tube was placed in a heat block at 100ºC and boiled for 10 minutes at 1600 rpm to lyse the cells.
- After boiling, the tube was immediately placed on ice for 5 minutes to cool.
- The sample was centrifuged again at 13000 rpm for 10 minutes to remove cell debris.
- The supernatant containing crude genomic DNA was carefully transferred to a sterile microcentrifuge tube.

- The extracted DNA was stored at -20ºC for further procedure.

2.7.3. Genomic DNA extraction

DNA extraction from the bacterial isolate was done by using the Qiagen DNeasy Blood and Tissue Kit.

- A single colony was inoculated into 3 ml of LB broth.
- The culture was incubated for 18 hours at 37ºC with shaking at 150 rpm.
- On the following day, 1.5 ml of the liquid culture was transferred into a 1.5 ml Eppendorf tube.
- The sample was centrifuged at 13000 rpm for 10 minutes.
- The supernatant was gently discarded, and the pellet was retained.
- The pellet was resuspended in 20µl of PBS and mixed thoroughly by vortexing or pipetting.
- A volume of 180 µl of ATL buffer was added and mixed well by pipetting.
- Subsequently, 20µl of Proteinase K solution(800U/ml) was added and mixed well by pipetting.
- The mixture was incubated at 56ºC for 1 hour.
- The solution was then incubated at 95ºC for 5 minutes to inactivate the Proteinase K.
- Afterward, 2 µl of RNase A solution (10mg/ml) was added.
- The mixture was incubated at 37ºC for 30 minutes.
- A volume of 200 µl of AL solution was added.
- Then, 200 µl of 99% ethanol was added.
- The entire solution was transferred to a QiAamp spin column and centrifuged at 8000 rpm for 1 minute.
- The spin column was placed into a clean 2 ml collection tube, and the old tube containing the filtrate was discarded.
- A volume of 500 µl of AW1 solution was added to the column, followed by centrifugation at 8000 rpm for 1 minute.
- The spin column was transferred to a new clean 2 ml collection tube, and the previous one was discarded.
- Then, 500 µl of AW2 solution was added and centrifuged at 14000rpm for 3 minutes.

- The spin column was again placed into a new 2 ml collection tube, and the old tube was discarded.
- The empty column was centrifuged at full speed (1560 rpm) for 1 minute to remove residual wash buffer.
- The spin column was placed into a new 1.5 ml Eppendorf tube, and the collection tube was discarded.
- A volume of 50 µl of RNase-free water was added directly to the membrane.
- The column was incubated at room temperature for 5 minutes.
- Genomic DNA was eluted by centrifugation at 14800 rpm for 1 minute, and the column was discarded.
- DNA concentration was measured using a Nano Drop spectrophotometer.
- The DNA-containing Eppendorf tube was stored at -20ºC for future use.

2.8. Molecular detection and identification of *Vibrio cholerae*

PCR technique

2.8.1. Initial identification by *ompW* PCR (Species specification PCR)

DNA extracted from APW-enriched water or samples was subjected to PCR amplification targeting the outer membrane protein W(*omp*W) gene, which is conserved in *V. cholerae.* This gene was used as a species-specific marker for the primary identification of *V. cholerae.*

Figure 2.3: *omp*W PCR program

PCR reactions were prepared using species-specific primers for *ompW*, and amplification was carried out under standard thermocycling conditions. The presence of an amplicon of the expected size (approximately 588bp) was considered indicative of *V. cholerae.*

Table 2: Master Mix composition for *omp*W PCR

Name of the reagent	Volume(µl)
Go-taq	10
Nuclease-free water	6
Forward primer	1
Reverse primer	1
Total	=18

Table 3: Primer sequence for *omp*W PCR

Primer	Sequence	Position	Amplicon size
Forward primer (TS) ompW-F	CACCAAGAAGGTGACTTTATTGTG	589-611	588bp
Reverse primer (TAS) ompW-R	GAACTTATAACCACCACCCGCG	1156-1174	

Table 4: PCR conditions for *omp*W PCR

Segment	Condition	Cycle No
Pre denaturation	94ºC for 5 minutes	1
Denaturation	94ºC for 30 seconds	35
Annealing	64º C for 30 seconds	
Extension	72º C for 30 seconds	
Final extension	72ºC for 7 minutes	
Infinite hold	12ºC	1

2.8.2. Multiplex PCR (Serogroup confirmation)

The samples that are *ompW* positive were further analyzed using multiplex PCR to determine the serogroup and toxigenic potential. This assay simultaneously targeted the following genes-

- *rfb*O1: marker for O1 serogroup
- *rfb*O139: marker for O139 serogroup
- *ctx*A: cholera toxin subunit A gene, indicating toxigenic strains.

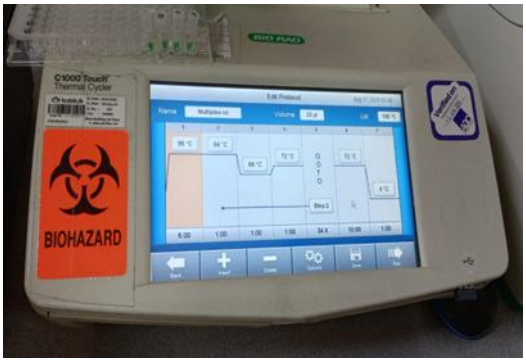


Figure 2.4: Multiplex PCR program

Table 5: Multiplex PCR conditions and master mix recipe

Name of reagent	Concentration(µl)
Gotaq	10 µl
NFW	6 µl
rfbO1 F	1.2 µl
rfbO1 R	1.2 µl
rfbO139 F	0.8 µl
rfbO139R	0.8 µl
ctxA F	0.5 µl
ctxA R	0.5 µl
Total	= 21 µl

Table 6: Primer sequences for Multiplex PCR

Name	Sequence	Amplicon size	Specificity
rfbO1 F	GGTTCACTGAACAGATGGG	192bp	O1 serogroup
rfbO1 R	GGTCATCTGTAAGTACAAC		
rfbO139 F	AGCCTCTCTTTATTACGGGTGG	449bp	O139 serogroup
rfbO139R	GTCAAACCCGATCGTAAAGG		
ctxA F	CGGGCAGATTCTAGACCTCCTG	302bp	Toxigenic strain
ctxA R	CGATGATCTTGGAGCATTCCCAC		

Table 7: PCR conditions for Multiplex PCR

Segment	Condition	Cycle No
Pre denaturation	95ºC for 5 minutes	1
Denaturation	94ºC for 1 minute	35
Annealing	55º C for 1 minute	
Extension	72º C for 1 minute	
Final extension	72ºC for 10 minutes	
Infinite hold	12ºC for 18 hours	1

Multiplex PCR was performed using specific primer sets in a single reaction tube. Amplicons were resolved by agarose gel electrophoresis, and the presence or absence of specific bands was used to classify the isolate as follows:

- ✓ *rfbO1*(+), *ctxA*(+) ⟶ *V. cholerae* O1, toxigenic
- ✓ *rfbO139*(+), *ctx*A(+) ⟶ *V. cholerae* O139, toxigenic
- ✓ *ompW* (+) only ⟶ Non O1 /O139 or nontoxigenic strain

2.8.3. *Viu*B PCR

This PCR method targets the *viuB* gene of *V. cholerae,* which confirms the presence or absence of *V. cholerae* in any sample.

Figure 2.5: *viu*B PCR program

Table 8: Master mix composition for *viu*B PCR

Reagent	**Concentration**
Go-taq	10
NFW	6
viuB F	1
viuB R	1
Total	=18

Table 9: Primer sequence for *viu*B PCR

*viu*B F	5'- GGTTGGGCACTAAAGGCAGAT-3'
*viu*B R	5'-TCGCTTTCTCCGGGGCGG-3'

Table 10: PCR conditions for *viu*B PCR

Segment	**Condition**	**Cycle No**
Pre denaturation	95ºC for 3 minutes	1
Denaturation	95ºC for 30 seconds	35
Annealing	64º C for 30 seconds	
Extension	72º C for 1 minute	
Final extension	72ºC for 5 minutes	
Infinite hold	4ºC for 18 hours	1

2.8.5. *tcp*A PCR

This method is used to identify *tcpA* gene which is the genetic determinant of virulence *of V. cholerae.*

Table 11: Master mix composition for *tcp*A

Reagent	**Concentration**
Go-taq	10
NFW	6
*tcp*A . Forward primer	0.8
*tcp*A. Reverse primer-cl	0.6
*tcp*A. Reverse primer-El tor	0.6
Total	=18µl

Table 12: Primer sequences for *tcp*A

***tcp*A. Forward primer**	5'-CACGATAAGAAAACCGGTCAAGAG-3'
***tcp*A Reverse primer-Cl**	5'-TTACCAAATGCAACGCCGAATG-3'
***tcp*A. Reverse primer-El tor**	5'-CGAAAGCACCTTCTTTCACACGTTG-3'

Table 13: PCR condition for *tcp*A

Segment	**Condition**	Cycle No
Pre denaturation	95ºC for 3 minutes	1
Denaturation	95ºC for 30 seconds	35
Annealing	56º C for 25 seconds	
Extension	72º C for 45 seconds	
Final extension	72ºC for 5 minutes	
Infinite hold	4ºC for 18 hours	1

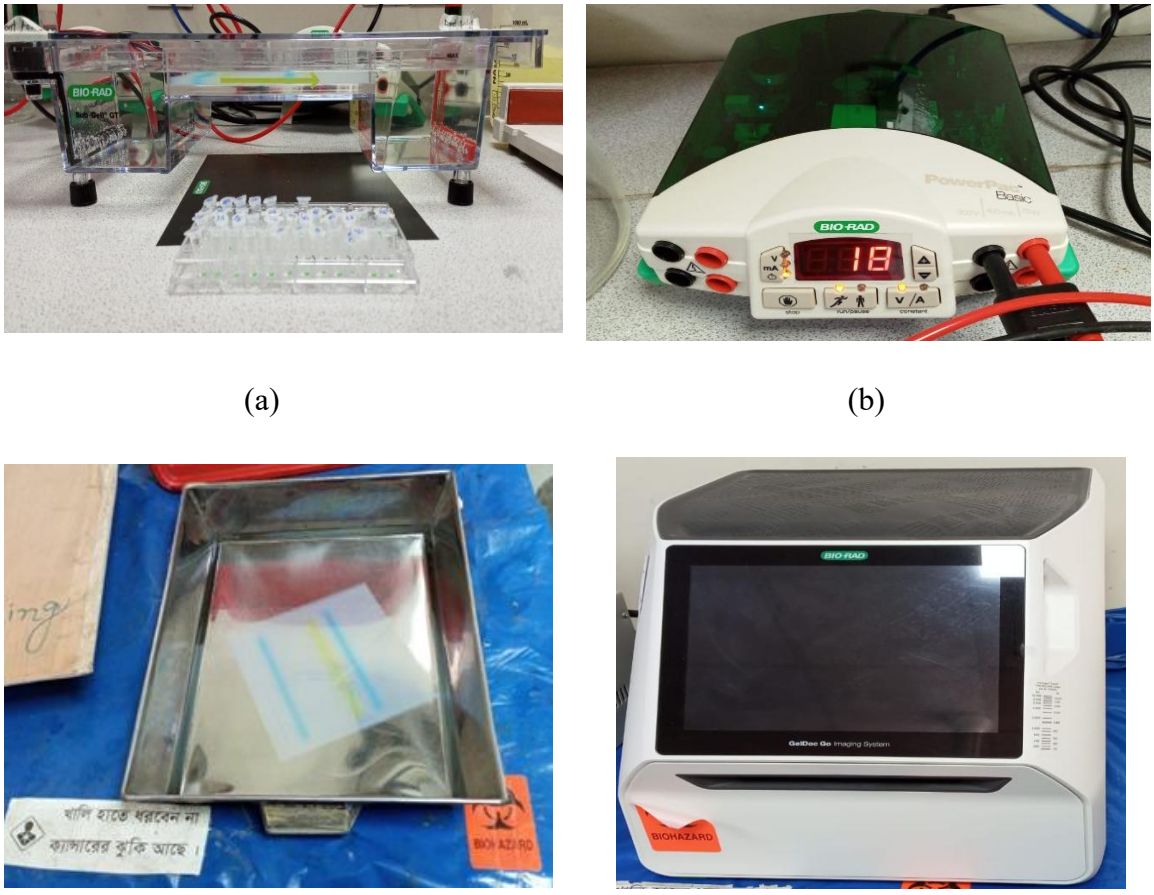
2.9.0.5x Agarose Gel Preparation

Requirements

1. Agarose gel powder
2. 0.5x TBE buffer
3. Microwave oven
4. Gel cassette
5. Gel chamber
6. Ethidium bromide
7. Gel doc

Procedure

Following the completion of polymerase chain reaction (PCR), a 1% agarose gel is prepared using 0.5× Tris-Borate EDTA (TBE) buffer. The PCR products are loaded into the gel wells and subjected to electrophoresis at 110V for 60 minutes. After the run, the gel is immersed in a 0.01% ethidium bromide (EtBr) solution for 60 minutes to allow staining. The stained gel is then visualized using a gel documentation (Gel Doc) system.



(a) (b)

(c) (d)

Figure 2.6: Agarose gel electrophoresis; a) Gel Chamber; b) Gel machine; c) Ethidium bromide staining; d) Gel doc

2.10. Library Preparation

A DNA library was prepared using the Illumina Nextera XT kit. The purpose of this procedure is to describe a standardized laboratory protocol for DNA library preparation of enteric bacterial organisms using the Illumina® DNA Prep kit.

Materials

Reagents:

- ✓ TB1 (Tagmentation Buffer 1)
- ✓ BLT (Bead-Linked Transposon)
- ✓ TSB (Tagment Stop Buffer)

- ✓ TWB (Tagment Wash Buffer)
- ✓ EPM (Enhanced PCR Mix)
- ✓ NFW (Nuclease Free Water)
- ✓ UD Index Adapters (Illumina)
- ✓ IPB: Illumina Purification Beads
- ✓ RSB: Resuspension Buffer
- ✓ WGS: Whole Genome Sequencing
- ✓ Ethanol
- ✓ Nuclease-free water

Equipment:

- ✓ Qubit 4.0 Fluorometer (Thermo Fisher Scientific)
- ✓ Magnetic stand (e.g. DynaMag- 96)
- ✓ Thermal cycler with Heated Lid
- ✓ Microcentrifuge(280×g)
- ✓ Vortex mixer

Protocol

1. DNA quantification and Normalization

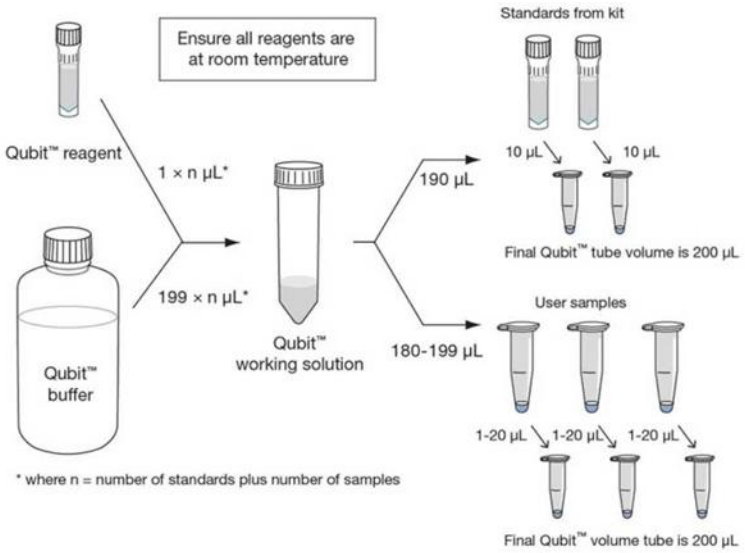


Figure 2.7: Quantifying DNA using Qubit

- Genomic DNA is quantified using the Qubit dsDNA HS Assay kit. (Fig)
- The DNA concentration is adjusted to 10 ng/µl using nuclease-free water.

2. Dilution and Tagmentation: DNA is fragmented and tagged with the adapter sequences and bound to the BLT during these steps.

2.1.DNA dilution

- 10µl of genomic DNA (10ng/ µl) is transferred into a 96-well PCR plate.
- 20 µl of nuclease-free water is added to reach a final volume of 30 µl.

2.2. Tagmentation Master Mix Preparation

- First of all, BLT (from the refrigerator) and TB1 (from the freezer) should be allowed to come to room temperature.
- BLT should be vortexed for 10s for resuspension.
- TB1 should be vortexed to mix and perform a quick spin.
- For each sample, 11 µl of TB1 and 11 µl of BLT are mixed in an Eppendorf tube and vortexed to resuspend the beads well.

2.3. Tagmentation Reaction

- 20 µl of the tagmentation master mix is added to each DNA sample (Final volume:50 µl).
- Pipet gently to mix well, resuspending the beads, and inspect the wells to ensure that samples and master mix are homogeneous before incubation.

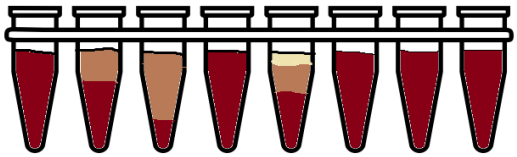


Figure 2.8: Example of homogeneous and non-homogeneous samples.

- The reaction is incubated in a thermal cycler with the lid at 100ºC
    - 55ºC for 15 minutes
    - Hold at 10ºC indefinitely

N.B.: 1st PCR is done

3. Post-Tagmentation Clean-up: DNA (now tagged with adapters and bound to the BLT) will be washed before amplification.

3.1. Stop Reaction

- At first, TSB should be kept at room temperature for 30 min, and no precipitation should be present.
- TSB (Tagmentation Stop Buffer) was kept in a heat block (37°C, 200 rpm, 10 min).
- 10 µl of TSB is added to each well.
- Slowly pipetting 10 times for resuspension.
- Seal the PCR tube and run 2nd PCR. (Post- tag program; Incubation at 37ºC for 15 minutes, lid pre-heated at 105ºC.
- While samples are incubating, EPM is on ice and thawed at room temperature.

3.2. Magnetic Bead Binding

- After samples reach 10ºC, the Eppendorf should be removed from the thermal cycler, and a quick spin.
- The plate is placed on a magnetic stand for 3 minutes until the supernatant clears.
- The supernatant is carefully discarded.

3.3. TWB washes

- 100µl of TWB is added, and the beads are resuspended by gentle pipetting
- The wash step is repeated three times.

4. Amplifying Tagmented DNA: The indexes are added to the tagmented DNA, and amplification occurs. DNA will be released from the BLT beads during PCR.

4.1.EPM Master Mix Preparation

- EPM should be vortexed and quickly spun.
- **Master Mix**: 22 µl of EPM and 22 µl of NFW are mixed per sample, vortexed, spun, and centrifuged at 280g for 10s.

4.2. Master Mix Addition

- Residual TWB is removed from the beads.
- 40 µl of the EPM master mix is added and gently pipetting to mix well, ensuring resuspension of the pellet.

4.3. Adding an index

- 10 µl of unique UD index adapters are added to each well.
- Pipetting a minimum of 10 times to mix.
- It should be ensured that all samples are homogeneous. This step is crucial for effective indexing and subsequent product recovery.
- The plate is centrifuged at 280×g for 30s.

4.4.PCR amplification

- Thermal cycling is carried out as follows
    - 68ºC for 3 minutes
    - 98ºC for 3 minutes
    - 5 cycles of:98ºC(45s),62ºC (30s),68ºC (2 min)
    - Hold at 10ºC
- Centrifuging at 280 × g for 1 minute

4.5. Safe Stopping Point

- Libraries can be stored at 4ºC for up to 3 days before final cleanup

5. Cleanup libraries: This dual bead clean-up procedure is for purification and size selection of libraries. Target fragment sizes are 800-1000 bp.

5.1. Preparation of reagents:

1. If using SPB, it should be brought to room temperature (at least 30 minutes) from the refrigerator.

2. RSB should be brought to room temperature (from frozen) and vortexed to mix**.**

3. Enough fresh 80% ethanol should be diluted for all samples.

- 100% Ethanol- 0.4ml × n
- NFW- 0.1ml × n

n Number of samples

4. SPB/IPB should be vortexed and inverted for full resuspension.

5. IPB master mix;

- IPB- 40.8× n
- NFW- 44.2×n

n Number of samples

6. If the PCR tube was retrieved from cold storage, centrifuge it at 280 x g for 1 minute.

7. The PCR tube should be placed on the magnet for 5 minutes.

8. 45 µl of supernatant should be transferred (now containing the DNA) to new wells.

9. Remove the sample tube from the magnet.

10. SPB/IPB master mix should be vortexed thoroughly, and 85µl to each sample

11. Pipet to mix a minimum of 10 times

12. Incubation at room temperature for 5 minutes.

13. PCR tubes should be placed on the magnet for 3-5 minutes.

14. During incubation, the stock SPB/IPB should be vortexed.

15. After incubation, with the tubes still on the magnet, 125 µl of supernatant (containing DNA) should be transferred to new wells (longest DNA fragments are being left behind).

16. The PCR tube should be removed from the magnet, and 15 µl of well-suspended stock SPB/IPB to the supernatant.

17. Gently pipette a minimum of 10 times to mix.

18. Incubation at room temperature for 5 minutes.

19. PCR tubes should be placed on the magnet for 3-5 minutes.

20. Supernatant should be removed and discarded (desired libraries are bound to the beads; shortest libraries in the supernatant are being discarded).

21. The steps below should be performed (9.5.17.1. through 9.5.17.3.) twice, for a total of two washes:

- While the tubes are on the magnet, 170 µl of fresh 80% ethanol should be added.
- Incubation for 30 seconds.
- Supernatant should be removed and discarded.

22. Beads should be allowed to air dry for 3-5 minutes.

23. PCR tubes should be removed from the magnet and 32 µl of RSB. It is recommended to add RSB quickly to each sample.

23. Pipetting gently and thoroughly to mix.

24. Incubate at room temperature for 2-5 minutes.

25. Placed on a magnet for 3 minutes.

26. 30 µl of the supernatant should be transferred into a new well.

Note: This is the final cleaned product. The safe stopping point is at -20ºC.

6. Pool libraries

1. Amplified DNA libraries are pooled into a new tube.

2. The final concentration (e.g.8.62 ng/ µl) is measured using the Qubit fluorometer.

3. Molarity Calculation

The molarity(nM) is calculated using the formula:

(Qubit reading ng/µl/ (660g/mol x 800bp)) x 10^6

- The average fragment size is assumed to be 1000bp.

7. Dilution and Neutralization

- **Dilution**: To achieve 4nM, 21.32µl of pooled DNA is diluted with 28.68µl of RSB.
- **Denaturation**: 5µl of the diluted sample is mixed with 5µl of 0.2M NaOH and incubated at room temperature for 5 minutes.

- **Neutralization**: 5 µl of Tris-HCl (200nM) and 985µl HT1 buffer is added to achieve a final 8 pM solution.

8. Library loading

- 62.5µL of the 20pM library is diluted with 39.5µl of HT1 buffer to reach 12.5pM
- The final solution is loaded onto the sequencing cartridge

9. Final Preparation for Sequencing

- A final dilution is prepared by mixing
  - 10µl of 10 nM primer mix
  - 490 µl HT1 buffer
  - 8 pM adapter mix

Note: All steps are performed under sterile conditions with precise incubation control to maintain library quality.

2.11. Sequencing

WGS was done by using the Illumina NextSeq platform.

Figure 2.9: Illumina Next-seq Sequencer

2.12. Bioinformatic analysis

Genome annotation was carried out using Prokka and Bakta; CARD Resistance Gene Identifier was used to identify antimicrobial resistance genes, and CDS for biofilm gene, VirulenceFinder for virulence gene [26].

Multi-locus Sequencing Typing

Multi-locus sequencing typing (MLST) was performed using the PubMLST, cgMLST (Core genome MLST) database, based on the analysis of seven housekeeping genes.

Sequences of *V. cholerae* O1 and non-O1/non-O139 and reference strains of El Tor (N16961) were downloaded from the Pathogenwatch (https://pathogen.watch/genomes) database and NCBI GenBank.

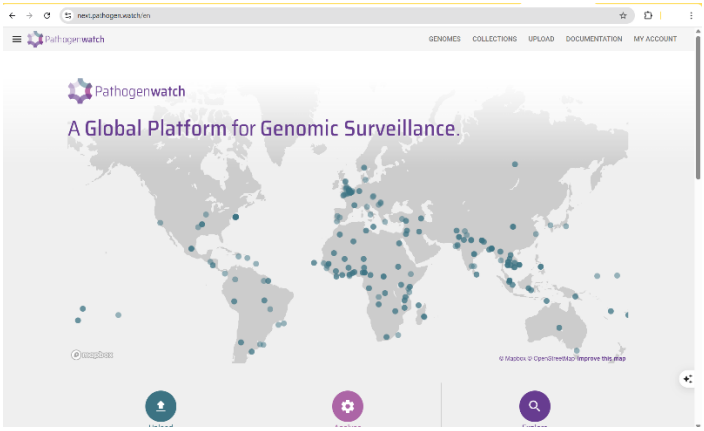


Figure 2.10: Pathogenwatch database

2.13. Construction of a Phylogenetic Tree

The phylogenetic tree was constructed on the basis of the presence of biofilm gene *mshA* and *hap* that are present at nine isolates of *V. cholerae* O1 and absence at two strains identified as *Vibrio* non O1/non O139. The analysis was performed using CSI phylogeny (https://cge.cbs.dtu.dk/services/snpTree/)pipeline integrating a suite of widely used

bioinformatics tools and annotated and visualized using Interactive Tree of Life (iTOL) [26]. The *V. choleare* O1 El Tor N16961 has been used as a reference group (NCBI GeneBank).

2.14. Antimicrobial Resistance Test

Antimicrobial resistance (AMR) testing, used to determine the effectiveness of antibiotics against bacterial isolates. It helps identify whether bacteria are susceptible, intermediate, or resistant to specific antimicrobial agents. One commonly used laboratory method is the Disk Diffusion Assay. During incubation, antibiotics permeate into the agar, creating a concentration gradient. As bacteria proliferate on the agar surface, they form a visible lawn of growth. The sensitivity of bacteria to a given antibiotic is determined by measuring the zone of inhibition, which is the clear area surrounding each antibiotic disc where bacterial growth is suppressed. This data is valuable for monitoring antibiotic resistance and guiding public health strategies to mitigate the impact. The antibiotics that are used to determine the resistance characteristics of *V. cholerae*-

According to the **CLSI** guideline [27]-

Table 14: Antibiotic List

Classification	Antibiotics	Inhibit	Zone of inhibition		
			S	I	R
Penicillin	Ampicillin (AMP)	Cell wall synthesis	≥17	14-16	≤13
Tetracycline	Tetracycline (TE)	Protein synthesis	≥15	12-14	≤11
	Doxycycline (DO)		≥14	11	≤10
Macrolid	Erythromycin (E)	Protein synthesis	≥23	14-22	≤13
	Azithromycin (AZM)		≥13	NA	12
Fluoro-quinolones	Ciprofloxacin (CIP)	Nucleic acid synthesis	≥21	16-20	≤15
Sulfonamides	Sulfamethoxazole (SXT)	Nucleic acid synthesis	≥16	11-15	≤10

Materials

- ✓ Muller Hinton Broth
- ✓ Muller Hinton Agar plate
- ✓ Antibiotic Disc
- ✓ Cotton swab
- ✓ Syringe needle, etc.

Procedure

1. **Preparation of bacterial inoculum:**

- One isolated colony is collected from a pure culture plate using a sterile loop and transferred into a vial containing Muller-Hinton Broth.
- Incubate the vial at 37ºC, 150rpm for 4 hours

2. **Muller Hinton Agar plate preparation**

- The bacterial suspension is evenly streaked onto Muller-Hinton Agar Plates using a sterile swab, ensuring a uniform bacterial lawn without gaps.

3. **Application of Antibiotic Disc**

- Antibiotic discs containing a known concentration of various antibiotics are placed onto the inoculated agar surface using sterile forceps.
- The discs are gently pressed down to ensure proper contact with the agar and evenly spaced on the plate.

4. **Incubation**

- The plates are incubated at 37ºC for 18 to 24 hours.

5. **Measurement of Zone**

- After incubation, the diameter of the zone of inhibition around each antibiotic disc is measured using a ruler.
- This zone represents the area where bacterial growth is inhibited due to the presence of an antibiotic.

6. **Analysis of results**

- The measured zone diameters are compared against established breakpoints set by the Clinical and Laboratory Standards Institute (CLSI) or other regulatory authorities.

- Based on the Minimum Inhibitory Concentration (MIC) required to prevent bacterial growth, bacteria are classified as susceptible, intermediate, or resistant to each antibiotic.

7. **Results reporting**

- The results are documented, specifying the antibiotic name, concentration, and classification (susceptible, intermediate, or resistant).

Adherence to proper laboratory procedures is crucial when performing antibiotic susceptibility tests. Safety precautions must be implemented to prevent contamination and ensure the reliable handling of potentially infectious agents. Additionally, high-quality antibiotic discs, culture media, and other test materials should meet regulatory standards to maintain accuracy.

2.15. Biofilm Assay

Equipment

1. 1x LB broth
2. Borosilicate glass tube
3. 0.1% crystal violet
4. Distilled water
5. Dimethyl sulfoxide (DMSO)

Procedure

1. Borosilicate glass tubes were filled with 1ml LB broth and inoculated with *V. cholerae* O1 and *Vibrio* non O1/non O139 grown in LB broth at 37ºC for 2 hours [22].
2. After inoculation, tubes were incubated at 4ºC and 37ºC for 24 hours.

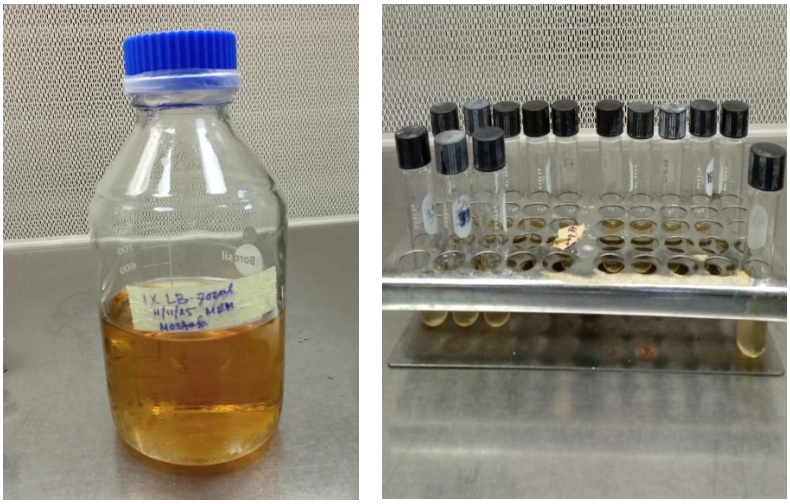


Figure 2.11: a) 1x LB broth; b) Bacterial inoculation

3. Next day, broth cultures from all tubes were poured off, tubes were then rinsed vigorously with distilled water to remove non-adherent cells.
4. Then the tubes were filled with 1.2 ml of a 0.1% crystal violet solution, allowed to incubate for 30 minutes, and again rinsed vigorously with distilled water.
5. Biofilm formation was quantified by measuring the optical density at 600 nm of a solution produced by extracting cell-associated dye with 1.5ml of dimethyl sulfoxide.

2.16. Microcosm Preparation`

Estuarine Microcosm [9]

Materials:

1. 1x LB broth (250 ml)
2. Autoclaved Conical flask
3. Autoclaved water
4. Inoculating loop
5. Burner
6. Shaking incubator
7. Filter paper
8. Water Filter

Method

1. The water sample should be filtered using filter paper and a filter machine.

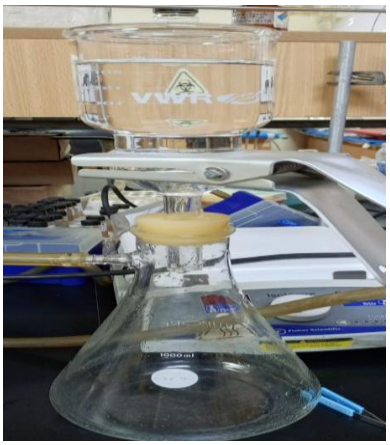




Figure 2.12: Water filtration process for Microcosm

2. About 125ml (3 times) of filtered water should be collected into a conical flask and autoclaved.
3. Strains are grown on LA agar medium.
4. After that, about 50 ml of 1x LB broth should be taken in a 4-ounce bottle.
5. From the LA medium, 1/2 colonies are inoculated into 1x LB broth, mixed properly, and kept for overnight incubation at a shaking incubator with 100 rpm, 37ºC.
6. After incubation, 1.8 mL broth solution was inoculated into 2 mL Eppendorf.
7. Then centrifuge at 8000 rpm for 5 min.

8. The supernatant should be removed without disturbing the pellet, and the pellet should be washed with 1 ml PBS (Phosphate Buffer Saline), mixed by pipetting, and centrifuged at 8000 rpm for 5 minutes (Repeated two times)
9. Suspended with 1 ml PBS and mixed by pipetting.
10. About 250µl solution should be added to each conical flask with autoclaved water, and mixed gently.
11. A conical flask should be set up at two temperatures (37ºC, 4ºC).
12. Weekly microbial survivability should be kept under observation.

2.17. Microcosm Plate Counting

Every week, bacterial growth has been observed by culturing on TTGA and TCBS agar plates. For this,

- Neat concentration, 10^{-1}, 10^{-2}, 10^{-3} dilution should be made into Eppendorf (100µl microcosm water + 900µl PBS)
- Mixed by gently pipetting
- About 50µl of neat and diluted water should be dropped into an agar plate and kept for incubation overnight.
- Next day, the bacterial growth number should be counted.
- CFU count and logarithmic count should be displayed on a graph.

2.18. Simple staining

To visualize biofilm formation, simple staining mechanism should be applied according to [9].

- Water samples from the microcosms were aseptically collected and placed on clean glass slides.
- Air dried
- Stained with 0.1% crystal violet for 1 minute
- washed, air dried, and visualized using a light microscope

Figure 2.13: Simple staining for microscopic examination

Results

3.1. Phenotypic characteristics.

3.1.1. *V. cholerae* growth on TCBS Plate

V. cholerae formed large, smooth, round, and yellow colonies due to its ability to ferment sucrose, producing acid that lowers the pH and turns the bromothymol blue indicator yellow.

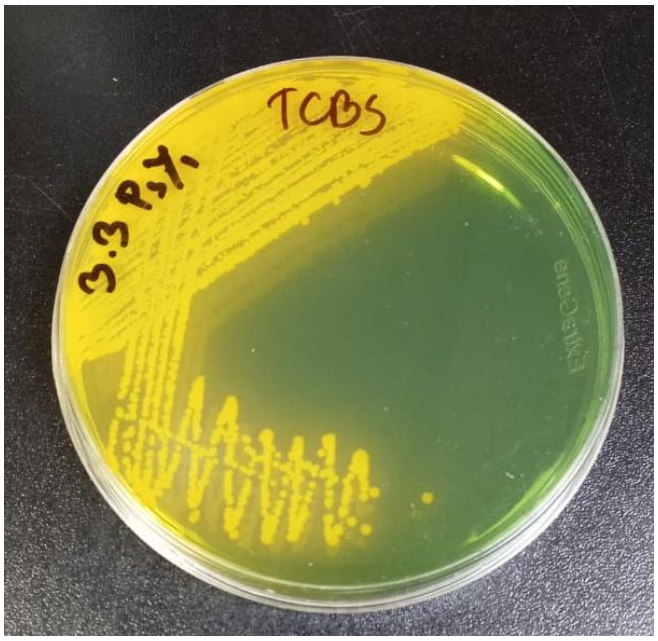


Figure 3.1.1: Colony morphology on TCBS agar

3.1.2. *V. cholerae* growth on TTGA plate

All *V. cholerae* O1 isolates produced small to medium-sized, round colonies that are gray to black in color. The black appearance is due to tellurite reduction by the bacteria. These colonies often have a shiny or glistening surface and display gelatinase activity, causing liquefaction of the medium around the colony.

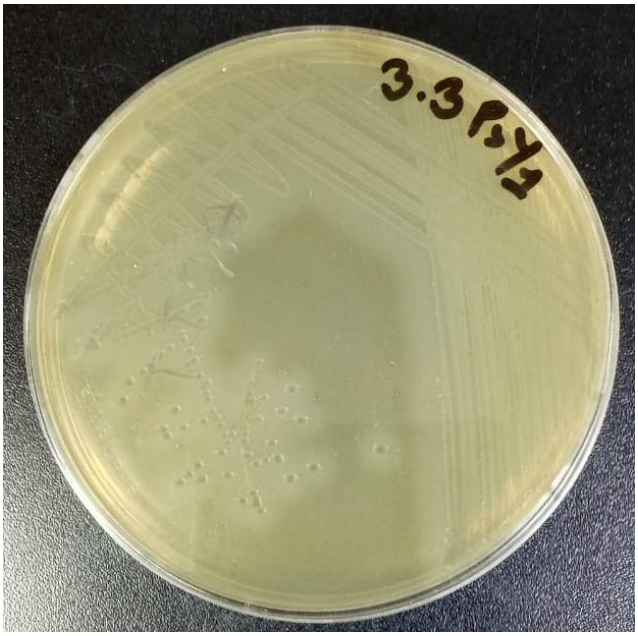


Figure 3.1.2: Colony morphology on TTGA agar

3.1.3. *V. cholerae* growth on Gelatin Agar plate

Colonies of *V. cholerae* O1 may be translucent to opaque with a smooth and moist appearance. Zones of gelatinase activity by the strain, and this hydrolytic activity is indicated by clear halos around the colonies.

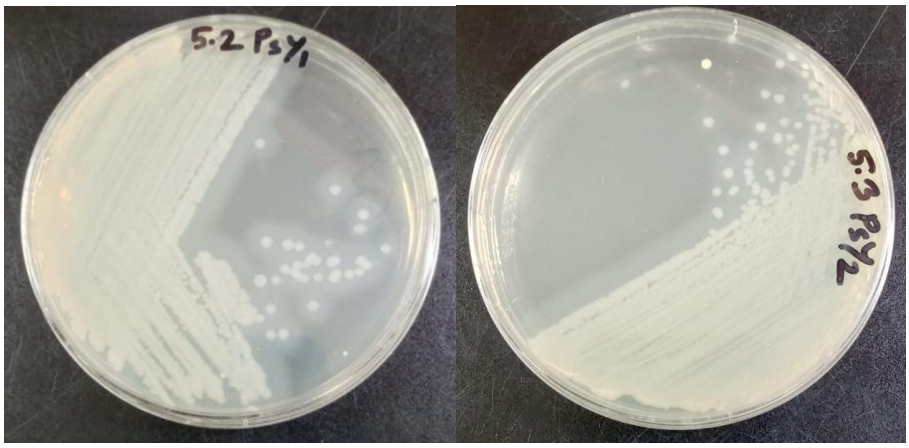


Figure 3.1.3: Colony morphology on GA plate

3.2. Serological Test

All of the isolated colonies used in this study showed agglutination with O1 antisera, but two did not defined as *V. cholerae* non O1/non O139. As the O1 serogroup is classified by Inaba and Ogawa, all *V. cholerae* O1 isolates were again examined by these two serotypes. Mostly are identified with Inaba antisera, and three strains are neither Inaba nor Ogawa**.** The following table 15 shows the result:

Table 15: Serological Test result

Sample ID	Serogroup	Serotype
2.2 WsY1	VC Poly O1	Inaba
3.3 PsY1	VC non O1/non O139	
5.2 PsY1	VC Poly O1	
5.3 WsY2	VC Poly O1	
5.3 PsY2	VC Poly O1	Inaba
5.4 PsY2	VC non O1/non O139	
6.1 WsY3	VC Poly O1	Inaba
8.1 WsY1	VC Poly O1	Inaba
8.2 WsY2	VC Poly O1	Inaba
9.3 WsY1	VC Poly O1	Inaba
9.3 WsY4	VC Poly O1	Inaba
ATCC 14033	VC Poly O1	Inaba

3.3. Concentration of DNA

Genomic DNA was extracted from enriched *V. cholerae* cultures grown in LB broth using the Qiagen DNeasy Blood & Tissue Kit. The quality and quantity of the extracted DNA were assessed by measuring absorbance at 260/230 nm and 260/280 nm wavelengths. DNA concentration and purity values are summarized in the following table.

Table 16: Sample DNA Concentration and purity Measurements

Sample ID	ng/µl	A260/280	A260/230
2.2 WsY1	73.7	1.82	2.04
3.3 PsY1	680.3	1.9	2.33
5.2 PsY1	99.2	1.85	1.78
5.3 WsY2	97.7	1.84	1.99
5.3 PsY2	105	1.86	1.79
5.4 PsY2	427	1.85	1.79
6.1 WsY3	147.8	1.85	1.76
8.1 WsY1	111.1	1.85	2
8.2 WsY2	159.5	1.82	1.42
9.3 WsY1	110.6	1.86	1.94

9.3 WsY4	76.7	1.83	1.2
ATCC 14033	228.7	1.85	2.01

3.4. Molecular characterization

The isolates that are primarily identified as *Vibrio* species are then subjected to PCR-based detection to confirm the isolates as *Vibrio cholerae.* DNA was extracted from all of the isolates using Qiagen DNeasy Blood & Tissue Kit, and PCR was done for genes that are specific to *Vibrio cholerae* species-*omp*W gene, *rfb*O1 gene for serogroup O1, *ctx*A gene for cholerae toxin, *tcp*A for toxin co-regulated pilus. All PCR results are summarized in table 20 and described subsequently.

Table 17: Molecular characterization of isolated bacterial strains

Strains	**Multi-VC**			***omp*W**	***viu*B**	***tcp*A**
	***rfb*O1**	***rfb*O139**	***ctx*A**			
2.2 WsY1	+	-	-	+	+	El Tor
3.3 PsY1	+	-	-	+	+	-
5.2 PsY1	+	-	-	+	+	El Tor
5.3 WsY2	+	-	-	+	-	El Tor
5.3 PsY2	+	-	-	+	+	El Tor
5.4 PsY2	+	-	-	+	+	-
6.1 WsY3	+	-	-	+	+	El Tor
8.1 WsY1	+	-	-	+	+	El Tor
8.2 WsY2	+	-	-	+	+	El Tor
9.3 WsY1	+	-	-	+	-	-
9.3 WsY4	+	-	-	+	+	El Tor
ATCC 14033	+	-	-	+	+	-

3.4.1. Multi-VC

In the Multiplex PCR, three sets of primers were used: one targeting the *rfb*O1 gene specific to the *V. cholerae* O1 serogroup, another targeting *ctx*A, the gene encoding cholera toxin. Among the 11 isolates, 11 showed positive amplification for *rfb* O1 and but negative for *ctx*A*, rfb*O139; indicating that they are non-toxigenic *V. cholerae* O1. Lane 13 contains negative control, lane 12,14 contain O1 positive control and lane 15 contain O139 positive control.

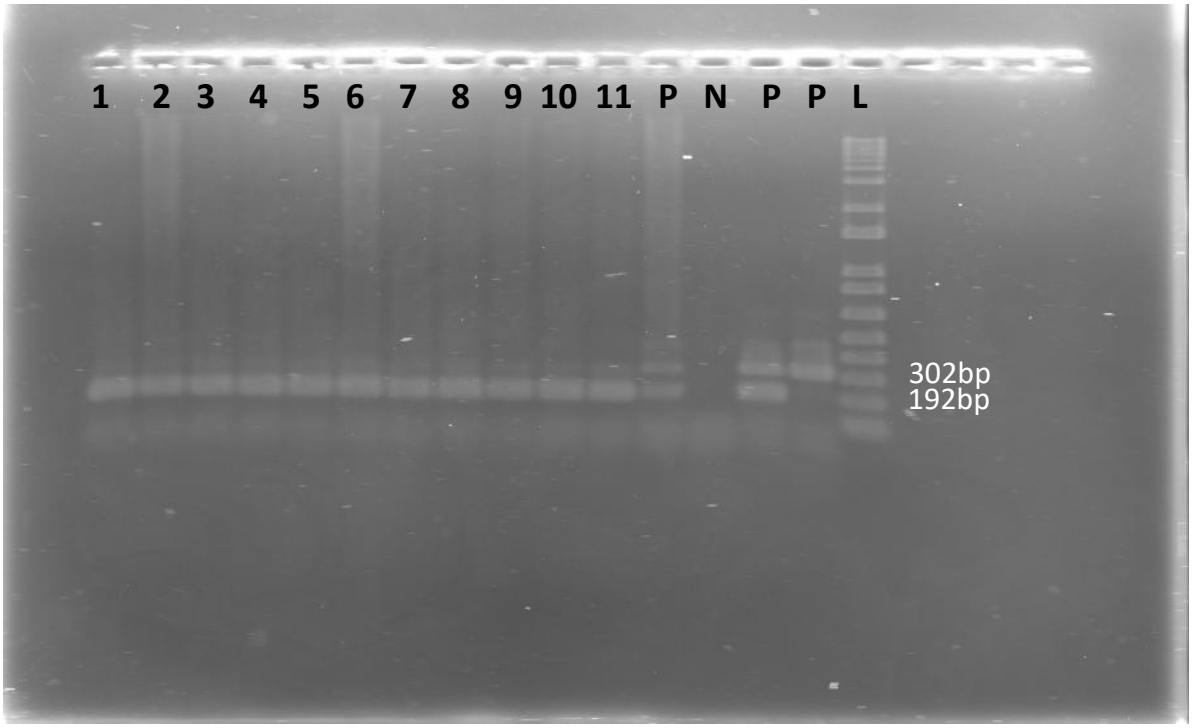


Figure 3.2.1: Representative PCR amplification results for *rfb*O1 and *ctx*A genes.

3.4.2. *omp*W

All of the isolates tested positive for the *omp*W gene, which produces 304bp amplicons, confirming the isolates as *V. cholerae.* Lane 17 contains a 1Kb plus ladder, while lanes 15 and 16 contain positive and negative controls. This result indicates that all isolates belong to *V. cholerae* species containing *omp*W gene.

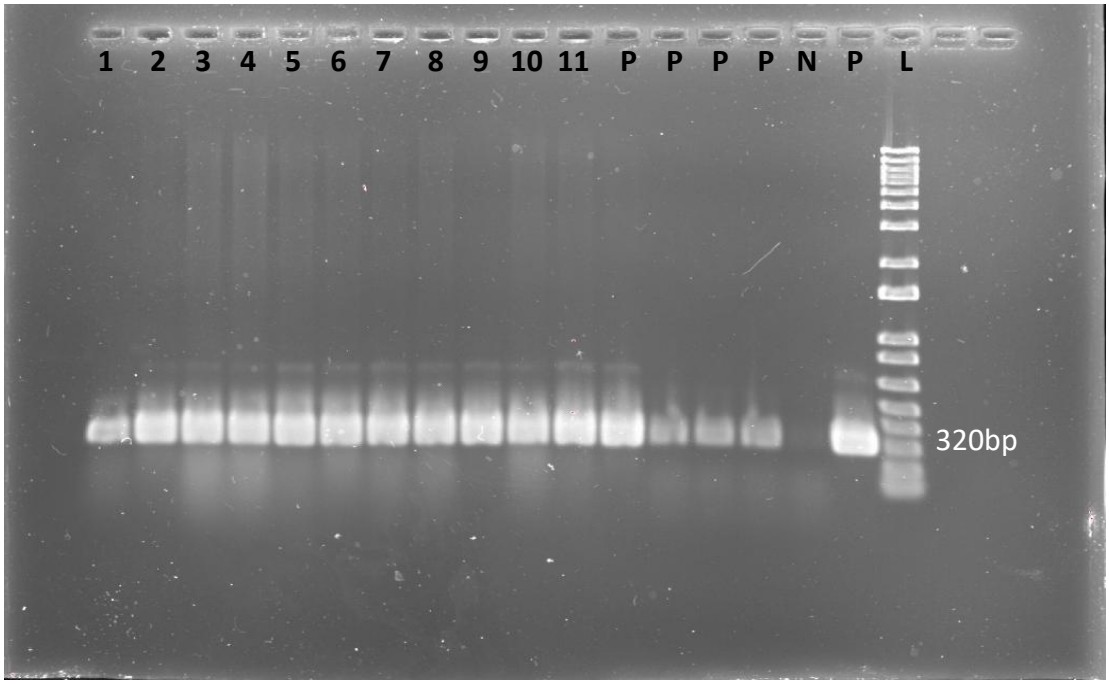


Figure 3.2.2: Representative PCR amplification result for the *omp*W gene

3.4.3. *tcp*A

Polymerase chain reaction (PCR) was utilized to distinguish the *tcp*A gene, which encodes the toxin co-regulated pilus, between the El Tor and classical biotypes of *V. cholerae.* The expected amplicon size was 471 bp for the El Tor biotype and 617bp for the classical biotype. Among 11 isolates, 9 were positive, and 2 were negative, indicating the absence of the *tcp*A gene. Lanes 13 and 14 contain positive and negative controls, and Lane 15 contains a 1Kb plus ladder. The absence of the *tcp*A gene was in 3.3 PsY2, 5.4 PsY2, and 9.3WsY4 isolates.

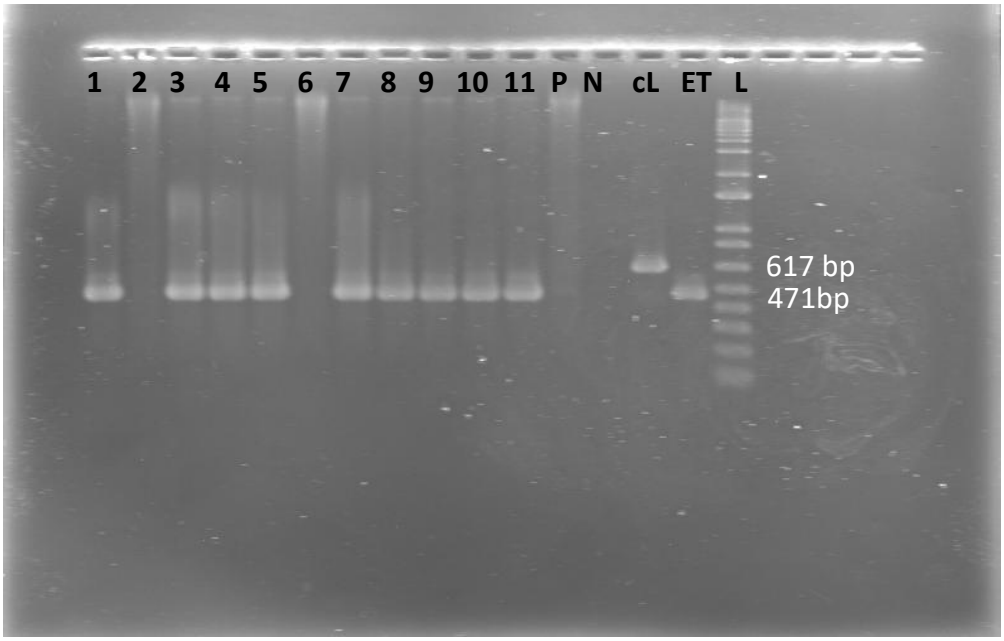


Figure 3.2.3.: Representative PCR amplification of the *tcp*A gene.

3.4.4. *viu*B

Polymerase chain reaction was utilized for the *viu*B gene. Among 11 isolates, 9 were positive, and 2 were negative for the *viu*B gene amplification. Lane 13 was for the positive control, and Lane 14 was for the negative control.

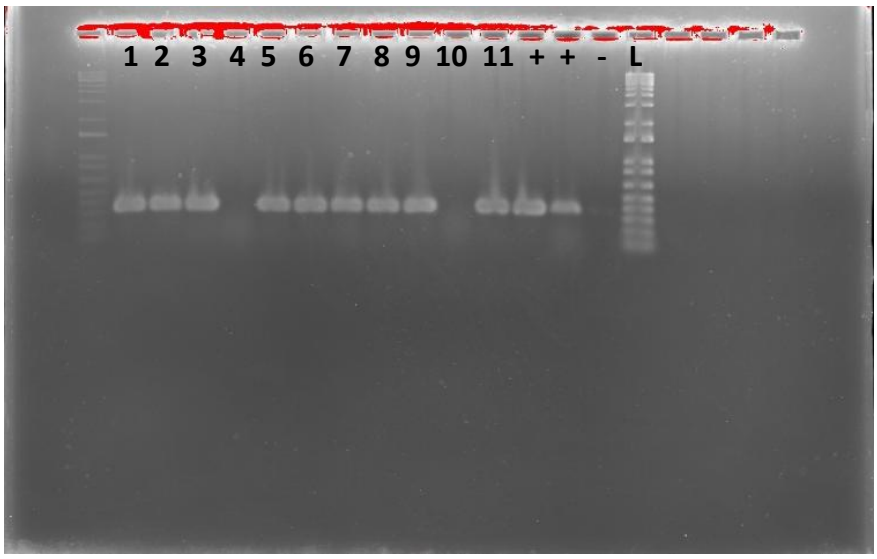


Figure 3.2.4: Representative PCR amplification of *viu*B gene.

3.5. Post sequencing analysis

3.5.1. Quality scores of the sequenced genome

Eleven isolates were subjected to whole-genome sequencing using the Illumina platform. Following sequencing, raw reads were quality trimmed and assembled into contigs using BaseSpace and SPAdes. The quality of the assembled contigs was assessed using Staramr and the result has been shown in table 28 which consists of Number of contigs, genome length and N50 (length of contigs compared to 50% of total assembly length).

Table 18: Quality scores of assembled contigs, sequence type, and plasmid

Isolate Name	No. Contigs	Genome Length	N50
8.1WsY1_S41_contigs	71	4000406	246323
9.3WsY1_S43_contigs	67	4000544	246323
3.3PsY1_S35_contigs	36	3978474	282180
5.2PsY1_S36_contigs	68	3980584	279094
5.4PsY2_S39_contigs	40	3978374	325637
6.1WsY3_S40_contigs	66	3998551	246323
2.2WsY1_S34_contigs	68	3998863	205408
5.3PsY2_S38_contigs	70	3999234	246323
8.2WsY2_S42_contigs	69	4000834	246323
5.3WsY2_S37_contigs	63	4001577	279097
9.3WsY4_S44_contigs	69	4002511	279097

3.5.2. MLST typing

All (n=11) *V. cholerae* O1 isolates exhibited identical multilocus sequence typing (MLST) profiles across all seven housekeeping gene loci analyzed. About nine isolates were assigned to sequence type ST 69, and two were ST 984.

Table 19: MLST profile of eleven *Vibrio cholerae* isolates

Genome Name	ST	adk	gyrB	mdh	metE	pntA	purM	pyrC
8.1WsY1_S41_contigs	69	7	11	4	37	12	1	20
9.3WsY1_S43_contigs	69	7	11	4	37	12	1	20
3.3PsY1_S35_contigs	984	2	38	161	230	69	1	259
5.2PsY1_S36_contigs	69	7	11	4	37	12	1	20

5.4PsY2_S39_contigs	984	2	38	161	230	69	1	259
6.1WsY3_S40_contigs	69	7	11	4	37	12	1	20
2.2WsY1_S34_contigs	69	7	11	4	37	12	1	20
5.3PsY2_S38_contigs	69	7	11	4	37	12	1	20
8.2WsY2_S42_contigs	69	7	11	4	37	12	1	20
5.3WsY2_S37_contigs	69	7	11	4	37	12	1	20
9.3WsY4_S44_contigs	69	7	11	4	37	12	1	20

3.5.3. Virulence gene profile

Whole genome sequencing analysis shows that among 11 isolates, 9 isolates were positive to *acfA, acfB, acfC,acfD, hylA, makA, nanH, tagA, vasX and tcp* whereas two isolates were negative to them as they are *V. cholerae* non O1/non O139.

Table 20: Virulence gene profile of *V. cholerae* isolates

Genome Name	***acfA***	***acfB***	***acfC***	***acfD***	***ctxA***	***hlyA***	***makA***	***nanH***	***tagA***	***toxR***	***vasX***	***tcp***	***ctxB***
2.2WsY1	+	+	I	+	-	+	+	+	+	-	+	+	-
3.3PsY1	-	-	-	-	-	+	-	+	-	+	I	-	-
5.2PsY1	+	+	I	+	-	+	+	+	+	+	+	+	-
5.3PsY2	+	+	I	+	-	+	+	+	+	+	+	+	-
5.3WsY2	+	+	I	+	-	+	+	+	+	+	+	+	-
5.4PsY2	-	-	-	-	-	+	-	+	-	+	I	-	-
6.1WsY3	+	+	I	+	-	+	+	+	+	+	+	+	-
8.1WsY1	+	+	I	+	-	+	+	+	+	+	+	+	-
8.2WsY2	+	+	I	+	-	+	+	+	+	+	+	+	-
9.3WsY1	+	+	I	+	-	+	+	+	+	+	+	+	-
9.3WsY4	+	+	I	+	-	+	+	+	+	+	+	+	-

3.5.4. AMR gene profiling

Among all isolates, 9 isolates were resistant to *varG, catB9, floR, gyrA, sul2, strA, strB, strB, dfrA* and 2 isolates 3.3PsY1 and 5.4PsY2 were sensitive to all.

Table 21: AMR gene profile of eleven *V. cholerae* isolates

Genome Name	*varG*	*catB9*	*floR*	*gyrA*	*sul2*	*strA*	*strB*	*dfrA1*
2.2WsY1	+	+	+	+	+	+	+	+
3.3PsY1	-	-	-	-	-	-	-	-
5.2PsY1	+	+	+	+	+	+	+	+
5.3PsY2	+	+	+	+	+	+	+	+
5.3WsY2	+	+	+	+	+	+	+	+
5.4PsY2	-	-	-	-	-	-	-	-
6.1WsY3	+	+	+	+	+	+	+	+
8.1WsY1	+	+	+	+	+	+	+	+
8.2WsY2	+	+	+	+	+	+	+	+
9.3WsY1	+	+	+	+	+	+	+	+
9.3WsY4	+	+	+	+	+	+	+	+

3.5.5. Msh cluster profiling

Whole genome sequencing analysis revealed that *V. cholerae* biofilm forming master gene cluster, msh gene clusters (*mshA-mshN*) are present in nine isolates but only *mshD* gene is incomplete in two strains 2.2 WsY1 and 5.4 PsY2.

Table 22: MSH clusters profile of eleven *V. cholerae* isolates

Genome Name	*msh A*	*msh B*	*msh C*	*msh D*	*msh E*	*msh F*	*msh G*	*msh H*	*msh I*	*msh J*	*msh K*	*msh M*	*msh N*
2.2WsY1	+	+	+	+	+	+	+	+	+	+	+	+	+
3.3PsY1	+	+	+	-	+	+	+	+	+	+	+	+	+
5.2PsY1	+	+	+	+	+	+	+	+	+	+	+	+	+
5.3PsY2	+	+	+	+	+	+	+	+	+	+	+	+	+
5.3WsY2	+	+	+	+	+	+	+	+	+	+	+	+	+
5.4PsY2	+	+	+	-	+	+	+	+	+	+	+	+	+
6.1WsY3	+	+	+	+	+	+	+	+	+	+	+	+	+
8.1WsY1	+	+	+	+	+	+	+	+	+	+	+	+	+
8.2WsY2	+	+	+	+	+	+	+	+	+	+	+	+	+
9.3WsY1	+	+	+	+	+	+	+	+	+	+	+	+	+
9.3WsY4	+	+	+	+	+	+	+	+	+	+	+	+	+

3.5.6. Biofilm gene profiling

Among 11 isolates, 9 isolates showed the presence of all biofilm gene including *hap*A, *omp*U, *rpo*S, *lux*, *msh*A but 2 isolates showed incomplete presence to *omp*U and *msh* cluster. The table 23 shows the brief of result.

Table 23: Biofilm gene profiling

Strain ID	*hapA*	*ompU*	*rpoS*	*lux*	*Msh cluster*
2.2 WsY1	Present	Present	Present	Present	Present
3.3 PsY1	Present	Incomplete	Present	Present	Incomplete
5.2 PsY1	Present	Present	Present	Incomplete	Present
5.3 WsY2	Present	Present	Present	Present	Present
5.3 PsY2	Present	Present	Present	Present	Present
5.4 PsY2	Present	Incomplete	Present	Present	Incomplete
6.1 WsY3	Present	Present	Present	Incomplete	Present
8.1 WsY1	Present	Present	Present	Present	Present
8.2 WsY2	Present	Present	Present	Present	Present
9.3 WsY1	Present	Present	Present	Present	Present
9.3 WsY4	Present	Present	Present	Present	Present

Findings of incomplete gene of *omp*U leads to the nucleotide blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with a reference Genome ID- 003779 [28] and found the result -

Table 24: Nucleotide blast with *omp*U gene of Genome ID- 003779.

Srain ID	Length	Identities	Gap
2.2WsY1_S34_contigs	1052	1052/1053(99%)	0/1053(0%)
3.3PsY1_S35_contigs	1079	884/1122(79%)	111/1122(9%)
5.2PsY1_S36_contigs	1052	1052/1053(99%)	0/1053(0%)
5.3PsY2_S38_contigs	1052	1052/1053(99%)	0/1053(0%)
5.3WsY2_S37_contigs	1052	1052/1053(99%)	0/1053(0%)
5.4PsY2_S39_contigs	1079	884/1122(79%)	111/1122(9%)
6.1WsY3_S40_contigs	1052	1052/1053(99%)	0/1053(0%)
8.1WsY1_S41_contigs	1052	1052/1053(99%)	0/1053(0%)
8.2WsY2_S42_contigs	1052	1052/1053(99%)	0/1053(0%)
9.3WsY1_S43_contigs	1052	1052/1053(99%)	0/1053(0%)
9.3WsY4_S44_contigs	1052	1052/1053(99%)	0/1053(0%)

Table 24 shows the genome length, similarity with reference genome and genome gap in assembly of incomplete gene *ompU.* This result gives an idea why the presence of incomplete gene differs biofilm formation.

3.6. Genomic Annotation

Whole genome sequences were annotated by using Bakta and visualized by Proksee. Here, genes responsible for antimicrobial resistance, biofilm formation, CDS, and virulence are shown in figure 3.13. In the left side, presence of *msh* cluster, *hap A, luxU* indicates the presence of biofilm gene and *hlyA* gene indicates the presence of the virulence gene.

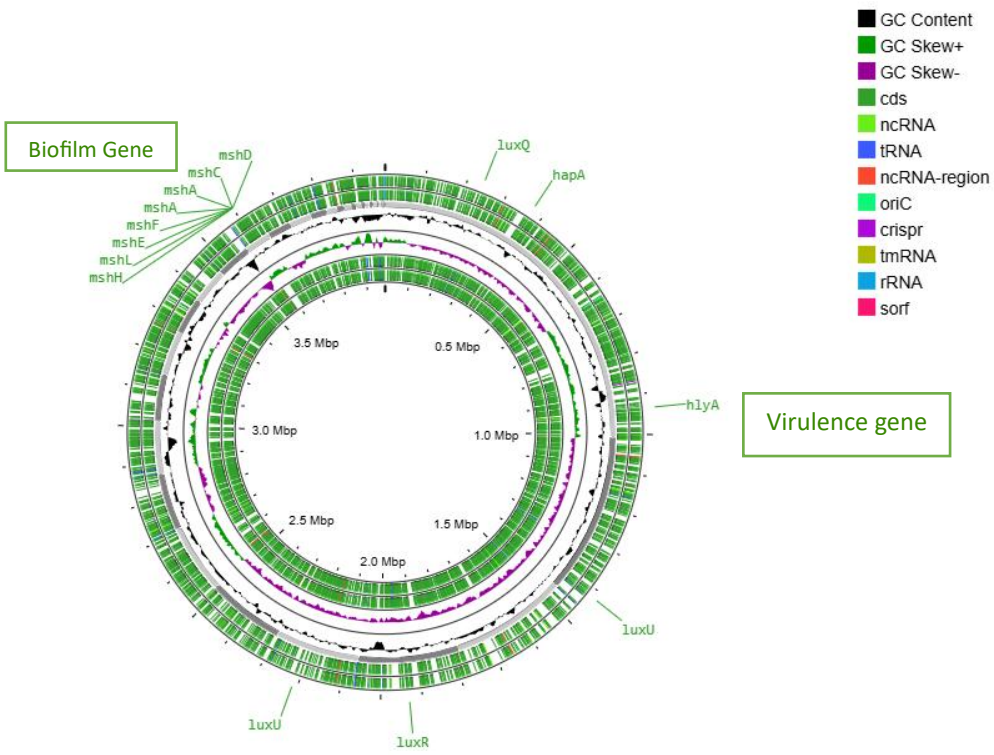


Figure 3.3.1: Bakta-based genome annotation of 3.3 PsY1 isolate

Genome annotation figure of 5.2 PsY1 isolate shows the presence of *mshA, luxU, ompU, hap* genes are responsible for biofilm gene; *tcpA, Rpos, toxS* genes for virulence characteristics and *varG, dfrA1, floR, sul2, cat B9, gyrA etc.* indicating the presence of AMR genes.

Genome annotation figure of 8.2 WsY2 isolate shows the presence of *mshA, luxU, ompU, hap* genes are responsible for biofilm gene; *tcpA, Rpos, toxS*, *nanH* genes for virulence characteristics and *varG, dfrA1, floR, sul2, cat B9, gyrA etc.* indicating the presence of AMR genes.

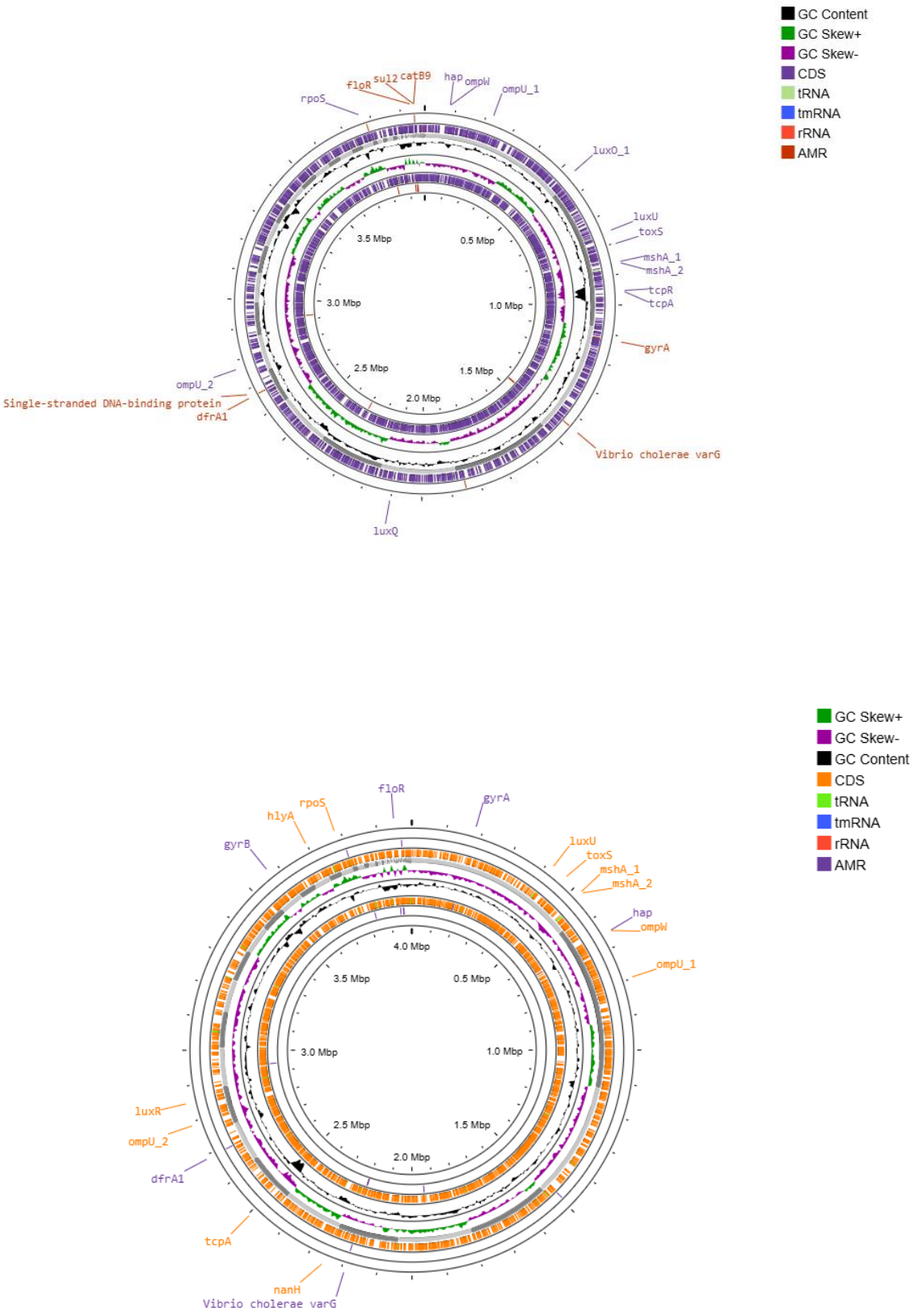


Figure 3.3.2: Bakta-based genome annotation of 5.2 PsY1 isolate and 8.2WsY2 isolate

3.7. Dataset

There are so many whole genome sequences (WGS) of *V. cholerae* O1 and non-O1/non-O139 available in the Pathogenwatch database from different countries. For this study, 39 sequences were selected and downloaded from different countries. Combined with 11 newly sequenced isolates and reference strain- El Tor strain N16961, a total of WGS were included in the final dataset for analysis.

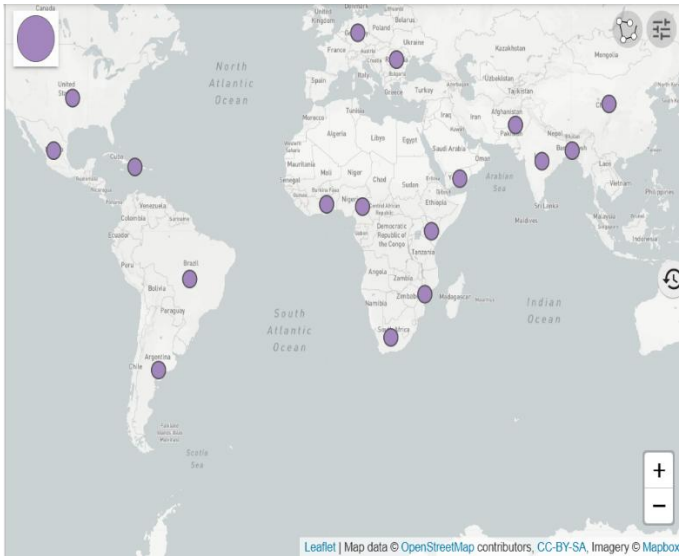


Figure 3.3.3: Geographic distribution of *V. cholerae* isolates downloaded from PathogenWatch

3.8. Phylogenetic analysis

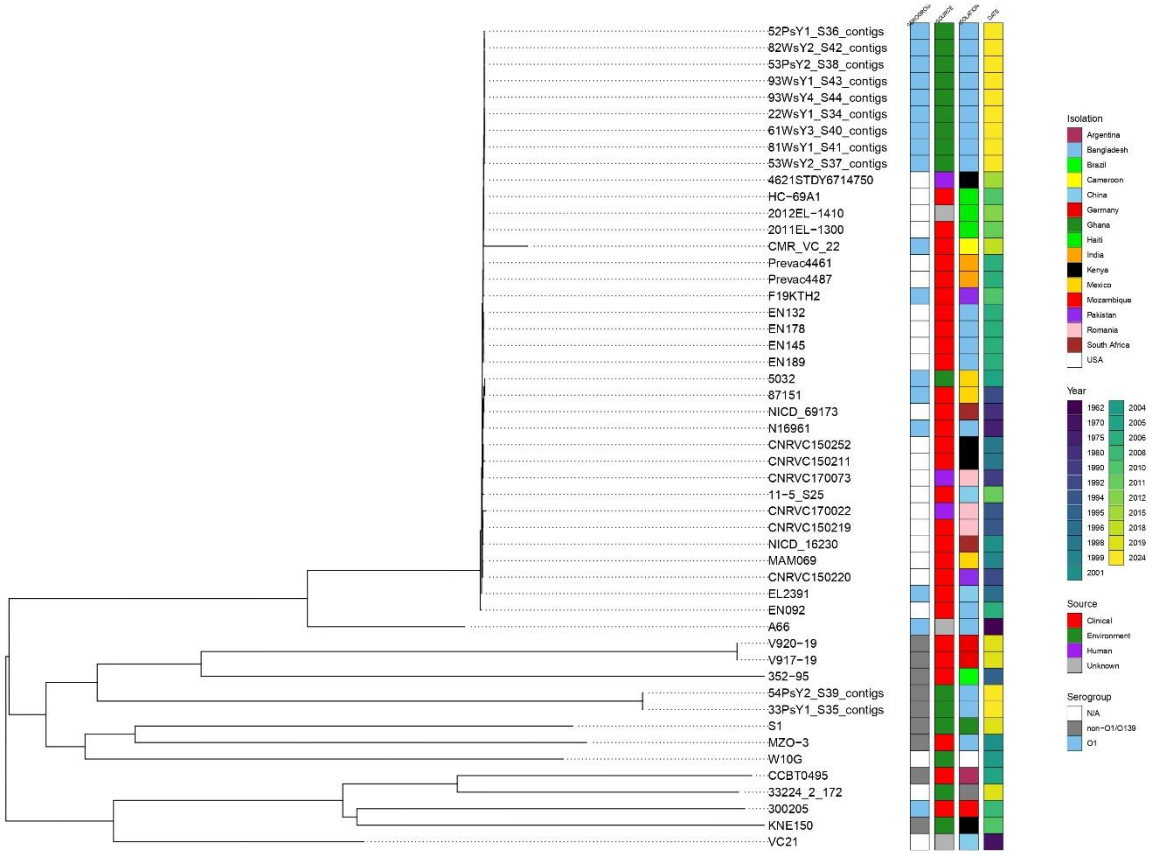


Figure 3.3.4. A maximum likelihood phylogenetic tree was constructed from whole genome sequences of 40 *V. cholerae* isolates with reference genome El Tor N16961 based on the presence or absence of biofilm gene (*msh*A, *hap*A). The isolates are marked in different colours, highlighting their position. The accompanying color bar represents the year, source, country, and serogroup (32).

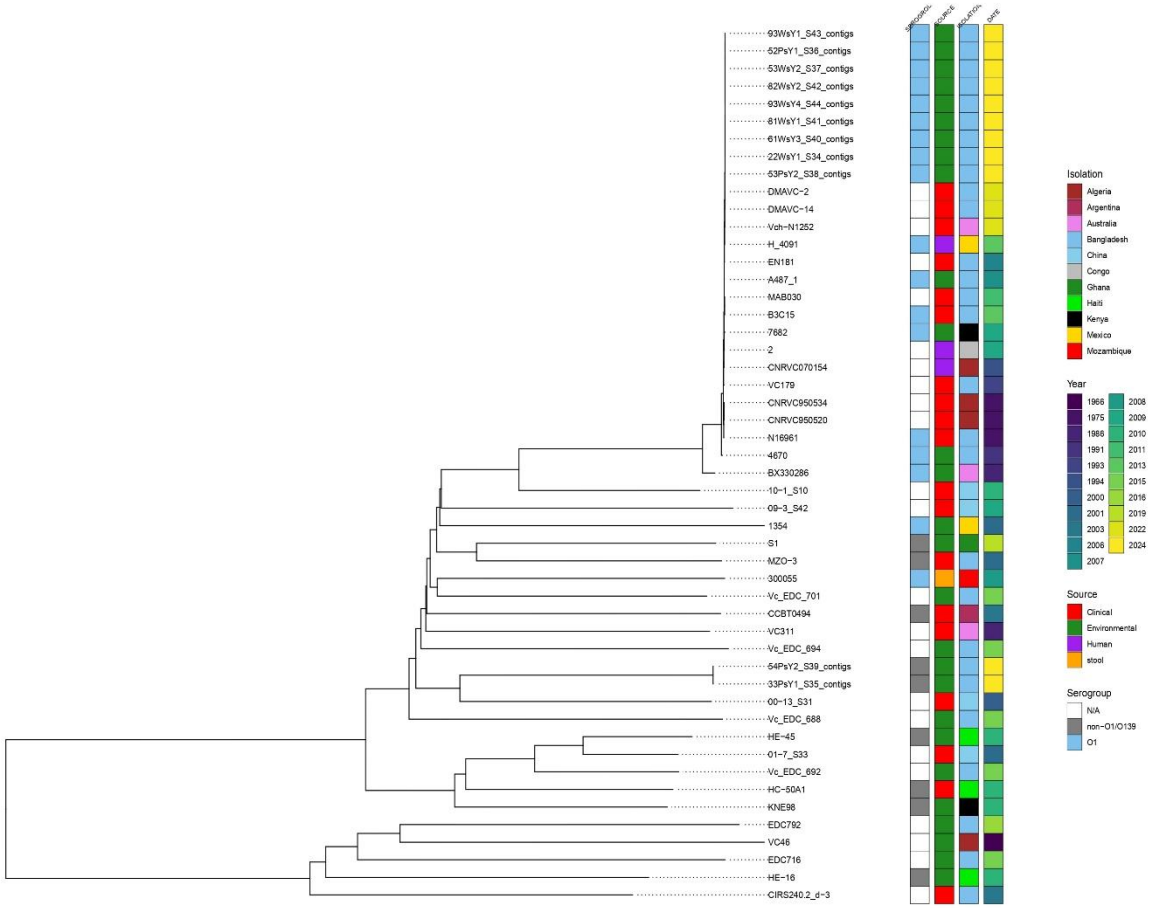


Figure 3.3.5: Maximum likelihood phylogenetic tree constructed from whole genome sequences of 50 *Vibrio cholerae* isolates with reference genome El Tor N16961 based on the presence or absence of AMR gene (*var*G, *flo*R, *gyr*A). The isolates are marked in a different color, highlighting their position. The accompanying color bar represents the year, source, country, and serogroup (32).

3.9.AMR test result

All of the isolates were tested for their response to commonly prescribed 7 different antibiotics (Ampicillin, Ciprofloxacin, Sulfamethoxazole, Erythromycin, Tetracycline, Azithromycin, Doxycycline) according to Clinical and Laboratory Standard Institute (CLSI) guidelines.

All *V. cholerae* O1 isolates were sensitive to Tetracycline (TE), Azithromycin (AZM), and Doxycycline (DO). About 75% strains are resistant to Sulfamethoxazole. Only one strain is resistant to Ciprofloxacin

The results of the antimicrobial susceptibility test are presented in the following Table. The antibiotics tested include Ampicillin 10µg (AMP), Ciprofloxacin 5µl (CIP), Erythromycin 15µl (E), Sulfamethoxazole 25 µg (SXT), Tetracycline 30 µg (TE), Azithromycin 15 µg (AZM), and Doxycycline 30 µg (DO). The interpretation of results is denoted as follows-

S- Sensitive

I-Intermediate

R-Resistant

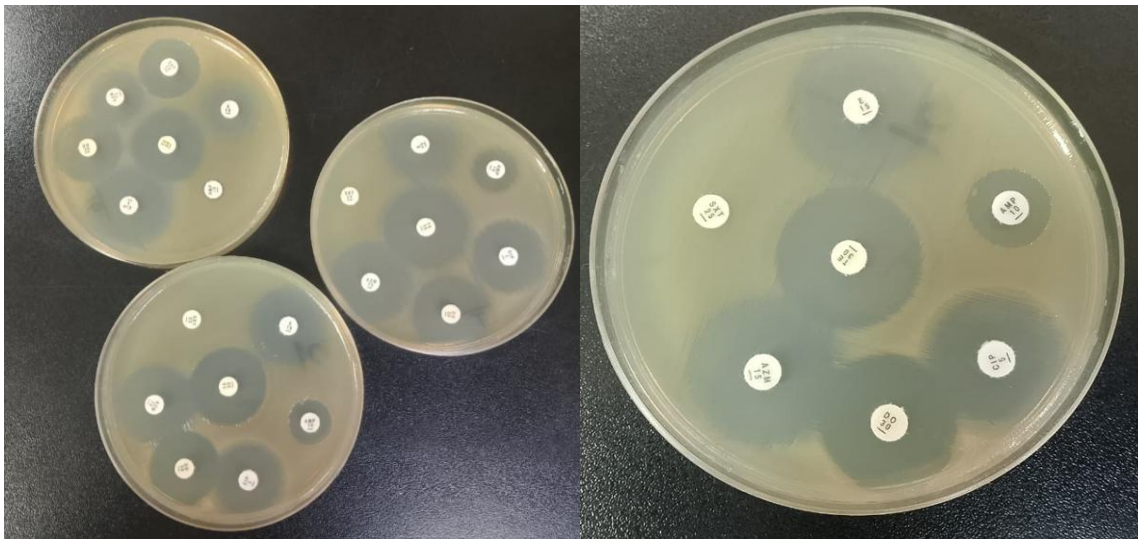


Figure 3.4: Zone of inhibition at AMR test

According to CLSR guidelines, the zone of inhibition has been measured at mm (25)

Table 25: Zone of inhibition

Sample ID	Ampicillin (AMP)		Ciprofloxacin (CIP)		Sulfamethoxazole (SXT)		Erythromycin (E)		Tetracycline (TE)		Azithromycin (AZM)		Doxycycline (DO)	
2.2 WsY1	15	I	25	S	0	R	25	S	30	S	28	S	27	S
3.3 PsY1	22	S	33	S	27	S	22	I	29	S	28	S	25	S
5.2 PsY1	15	I	27	S	0	R	30	S	30	S	30	S	25	S
5.3 WsY2	20	S	25	S	0	R	25	S	30	S	28	S	27	S
5.3 PsY2	15	I	25	S	0	R	25	S	30	S	29	S	26	S
5.4 PsY2	20	S	30	S	27	S	24	S	29	S	26	S	25	S
6.1 WsY3	16	I	21	S	0	R	29	S	31	S	30	S	30	S
8.1 WsY1	15	I	24	R	0	R	29	S	31	S	30	S	30	S
8.2 WsY2	10	R	25	S	0	R	26	S	30	S	28	S	25	S
9.3 WsY1	12	R	28	S	0	R	20	I	26	S	25	S	25	S
9.3 WsY4	18	S	28	S	0	R	20	I	28	S	27	S	25	S
ATCC-14033 (Control)	20	S	30	S	25	S	20	I	25	S	25	S	22	S

Table 26: Keynote highlighting resistance

Sample ID	AMR Test
2.2 WsY1	Resistant to Sulfamethoxazole
3.3 PsY1	Sensitive to all antibiotics
5.2 PsY1	Resistant to Sulfamethoxazole
5.3 WsY2	Resistant to Sulfamethoxazole
5.3 PsY2	Resistant to Sulfamethoxazole
5.4 PsY2	Sensitive to all antibiotics
6.1 WsY3	Resistant to Sulfamethoxazole
8.1 WsY1	Resistant to Sulfamethoxazole
8.2 WsY2	Resistant to Ampicillin and Sulfamethoxazole
9.3 WsY1	Resistant to Ampicillin and Sulfamethoxazole
9.3 WsY4	Resistant to Sulfamethoxazole
ATCC-14033 (Control)	Sensitive to all antibiotics

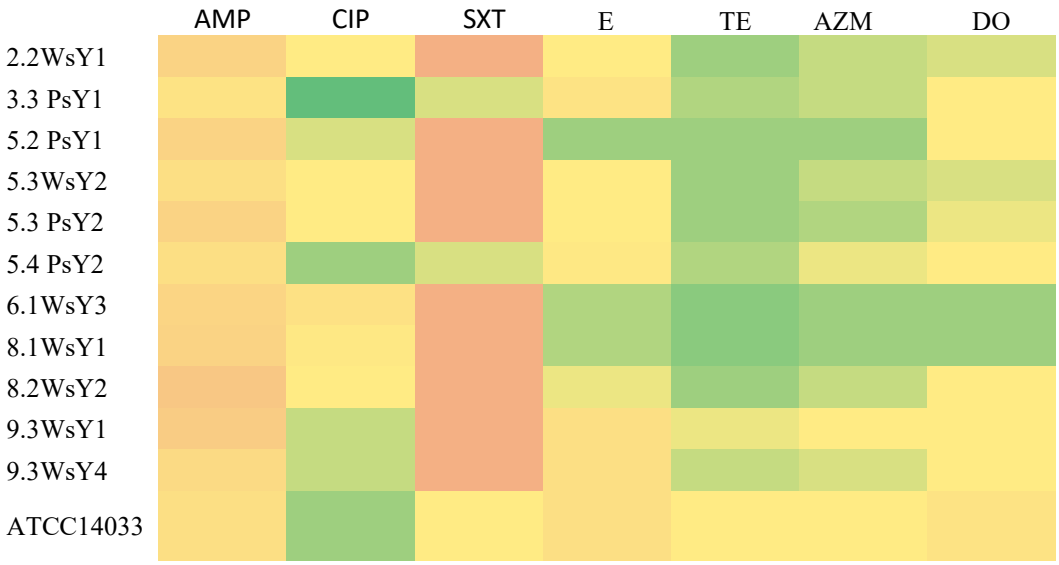


Figure 3.5: AMR heatmap

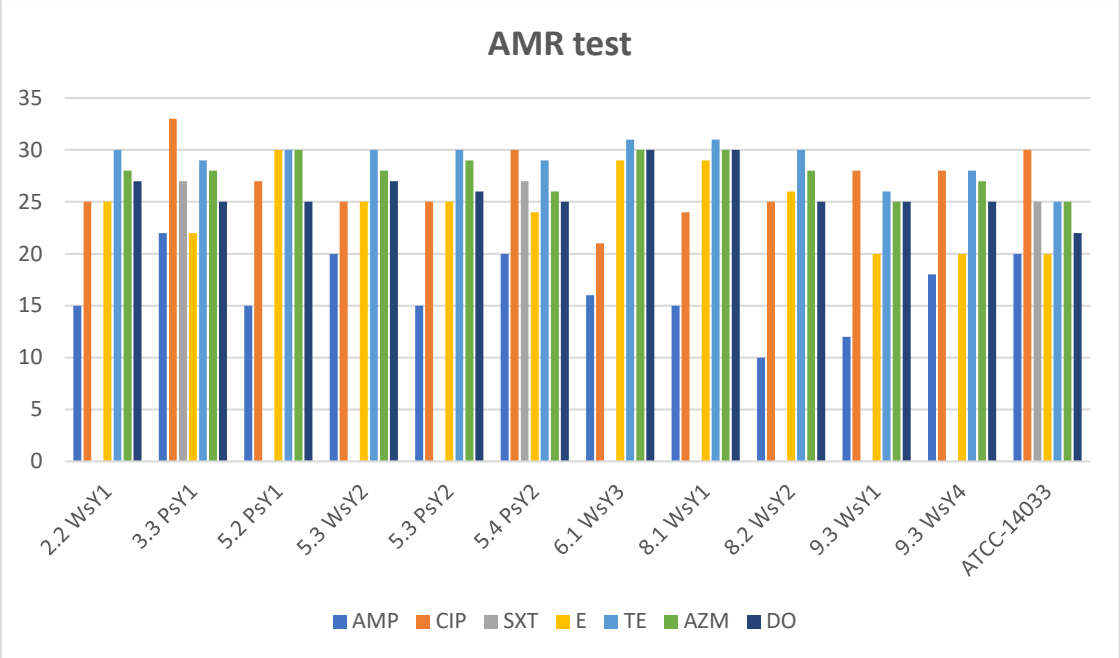


Figure 3.6: A column graph with zone of inhibition of antimicrobial agents

3.10. Biofilm assay result

Crystal violet-stained biofilms produced a purple-colored ring on a borosilicate glass tube, as this strain has the capacity to bind biofilms. The biofilm produced by experimental strains on borosilicate glass tubes was stained with crystal violet (5, 31).

Figure 3.7: Ring formation on borosilicate glass tube compared with N16961 control

3.11. Comparative results of biofilm phenotypic and genotypic characteristics

By comparing the phenotypic and genotypic result of biofilm formation, we found that the presence of following genes reveal high to low crystal violet ring at borosilicate glass wall. Presence of incomplete gene, slow biofilm formation may be occurred or not. But in the case of 9.3 PsY1, no ring formation was visible instead of presence of all biofilm gene.

Table 27: Presence of ring due to biofilm formation

Strain ID	Ring formation	*hap*A	*omp*U	*rpo*S	*lux*	*msh*
2.2 WsY1	++	Present	Present	Present	Present	Present
3.3 PsY1	+	Present	Incomplete	Present	Present	Incomplete
5.2 PsY1	++	Present	Present	Present	Incomplete	Present
5.3 WsY2	+++	Present	Present	Present	Present	Present
5.3 PsY2	+++	Present	Present	Present	Present	Present
5.4 PsY2	-	Present	Incomplete	Present	Present	Incomplete
6.1 WsY3	+	Present	Present	Present	Incomplete	Present
8.1 WsY1	+++	Present	Present	Present	Present	Present
8.2 WsY2	++	Present	Present	Present	Present	Present
9.3 WsY1	+++	Present	Present	Present	Present	Present
9.3 WsY4	-	Present	Present	Present	Present	Present

3.12. Bacterial growth counting

With 7 days interval, we have counted bacterial growth from the microcosm water by culturing onto TCBS and TTGA agar. We have made Neat, 10^-1, 10^-2 and 10^-3 dilutions from microcosm water and 50 µl from all dilution was cultured onto agar plate and the number was converted into CFU/ml and log value. The table displays the log value with days of count.

At 4ºC, microbial count TTGA displays that *V. cholerae* non O1/non O139 strain was culturable till 14 days. Strain 8.2 WsY2 indicating *V. cholerae* O1 became nonculturable after 7 days which explain high biofilm forming activity (Table:28)

Table 28: Bacterial growth count at TTGA plate (Microcosm water from 4ºC)

Sample Name	Log value							
	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49
3.3 PsY1	5.688	4.845	3.81	0	0	0	0	0
8.2WsY2	4	0	0	0	0	0	0	0

At 37ºC, microbial count at TTGA- 8.2 WsY2 was culturable till 49 days and 3.3 PsY1 was non-culturable state at day 35 (Table 29)

Table 29: Bacterial growth count at TTGA plate (Microcosm water from 37ºC)

Sample Name	Log value							
	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49
3.3 PsY1	5.732	4.301	4.279	4.246	4.146128	3.301	0	0
8.2 WsY2	5.903	5.02	4.716	3.73	3.892095	3.792	3.84	3.84

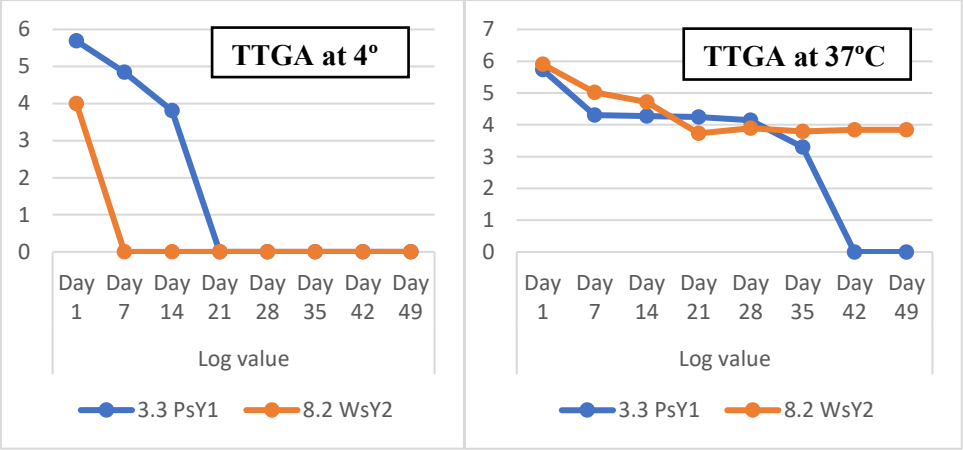


Figure 3.8.1: Microbial growth at 4ºC and 37ºC on TTGA agar plate

Bacterial growth counting at TCBS

At 4ºC, Table 30 indicates that there is no growth of any isolates till day 49 on TCBS agar. 8.2 WsY2 was at non culturable state at next counting whether 3.3 PsY1 continued growth till 14.

Table 30: Bacterial growth count at TCBS plate (Microcosm water from 4ºC)

Sample Name	Log value							
	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49
3.3 PsY1	5.845	4.643	2.505	0	0	0	0	0
8.2 WsY2	3.301	0	0	0	0	0	0	0

At 37ºC, microbial growth count, Table 31 indicates that there is no growth of any isolates till day 49 on TCBS agar. 3.3 PsY1 and 8.2 WsY2 were non culturable after 14 days and two were non culturable after 7 days only indicating enhanced biofilm-forming ability.

Table 31: Bacterial growth count at TCBS plate (Microcosm water from 37ºC)

Sample Name	Log value							
	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49
3.3 PsY1	5.903	4.643	3.94	0	0	0	0	0
8.2 WsY2	5.778	3.84	2.3	0	0	0	0	0

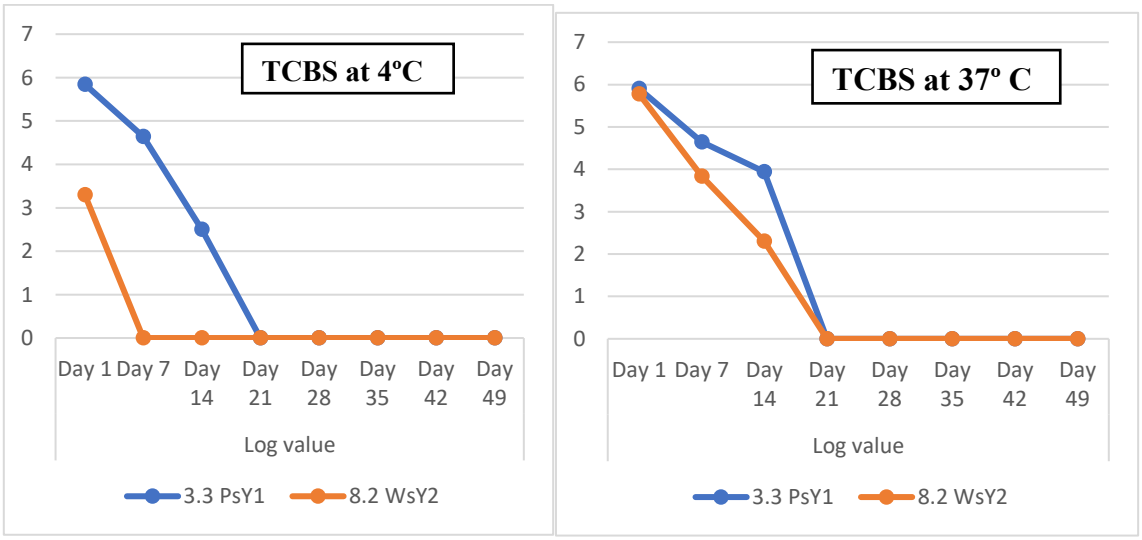


Figure 3.8.2: Microbial growth at 4ºC and 37ºC on TCBS agar plate

Figure 3.8.1 and 3.8.2 exhibit the real picture of bacterial growth in the microcosm water cultured onto TCBS and TTGA agar.

3.13. Microscopic examination

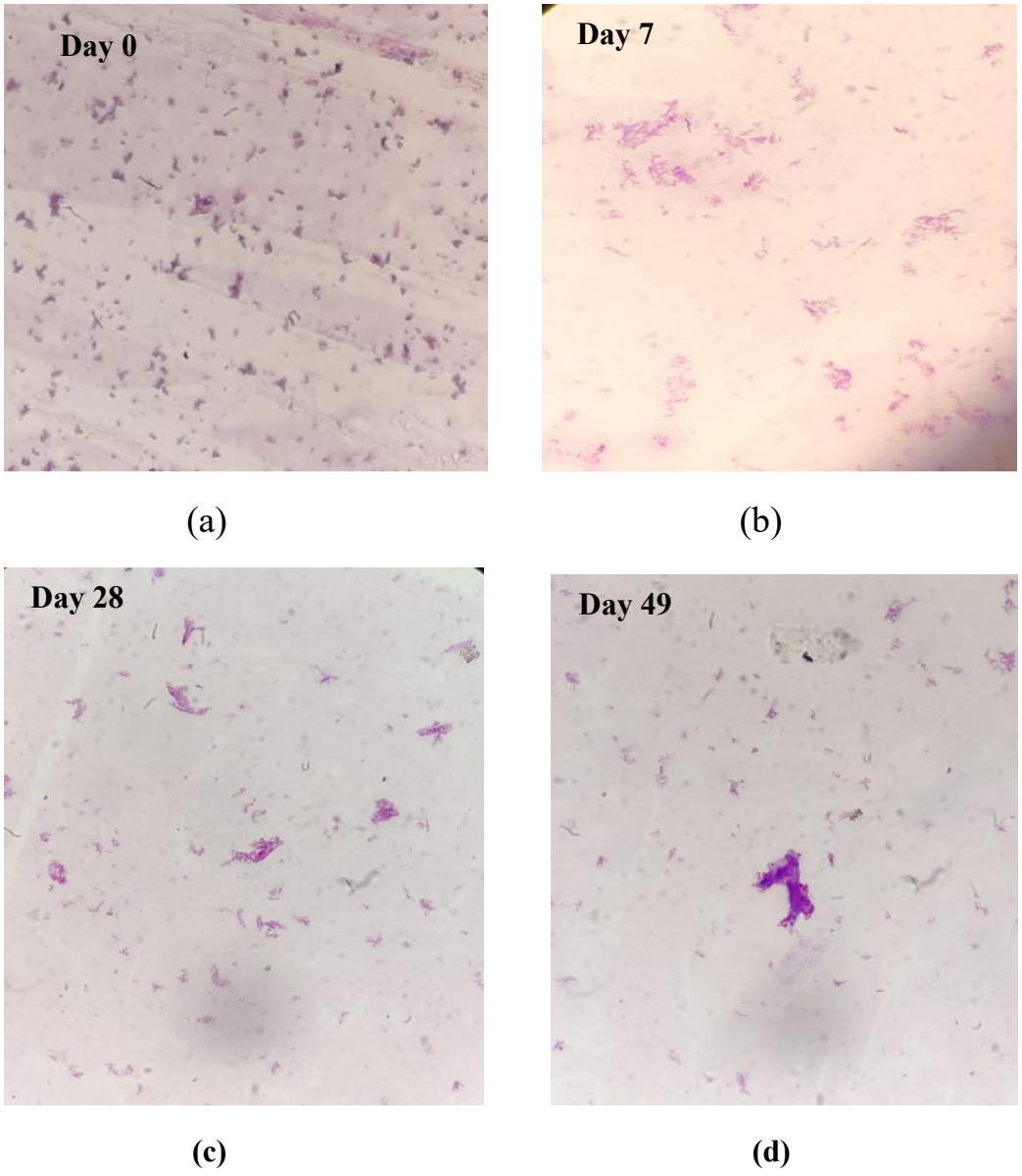


Figure 3.8.3: Micrographs showing *V. cholerae* O1 (8.2 WsY1) in MW microcosms at different stages; (a) Day 0; (b) Day 7; (c) Day 28; (d) Day 49 at 37º C

Using a light microscope, *V. cholerae* O1 (5.4 PsY1) from microcosm (37°) was visualized as rod shaped free floating bacteria Fig 3.8.3(a). After 7 days, bacteria were started to transform into coccoid shape Fig 3.8.4 (b) and after 28 days it started to form cluster like structure Fig 3.8.5 (c). Finally, after 49 days, more aggregated form with large biofilm structure had been found under phase contrast microscope.

Discussion

V. cholerae O1 is a highly contagious disease that has regularly surfaced since 1817. There have been around seven pandemics so far, and the 1992 appearance of *V. cholerae* O139 has sparked worries of an eighth pandemic. We have collected environmental samples from various sites in the Noakhali area of Bangladesh in order to investigate the epidemiology of *V. cholerae* and their ability to form biofilms that represent an annual life cycle of *V. cholerae* and could cause an unanticipated epidemic during a seasonal period [8].

In this study, 11 *V. cholerae* isolates collected over a seasonal period were examined. Samples were collected five times at fifteen-day intervals. All isolates produced yellow colonies on TCBS agar and translucent colonies with clear halo zones on GA medium, confirming their ability to hydrolyze gelatin. Serological testing showed that two isolates did not agglutinate with O1 or O139 antisera and were therefore classified as non-O1/non-O139. About nine isolates agglutinated with *V. cholerae* O1 antisera, which indicates that these carry the O1 serogroup.

PCR-based examination displays the presence of different genes (*ompW, viuB, tcpA*, etc.) in different isolates. The ability of pathogenic *V. cholerae* to cause diseases depends primarily on the expression of two virulence factors: encoding cholera toxin (CT), a potent enterotoxin, and a pilus colonization factor known as toxin coregulated pilus (TCP). The *ctxA* and *ctxB* genes that encode cholera toxin (CT) subunits A and B, respectively [29]. In this study, Multi-VC PCR displays the presence of *rfb*O1 and the absence of *ctxA* in all isolates indicating the absence of CT toxin. Targeting the *tcp*A gene identified 10 out of 11 isolates as positive for the El Tor type *tcp*A, suggesting that the majority of *V. cholerae* isolates likely evolved from the O1 El Tor biotype. The remaining two isolates were negative for *tcpA*. According to WGS (Whole Genome Sequencing), our isolates' virulence gene profiles showed that the *acf*A*, acf*B*, acf*C*, acf*D*, hly*A*, nan*H*, tag*A*, toxR, vasX*, and *tcp* gene cluster were present. Additionally, they lack the toxigenic genes *ctxA* and *ctxB*. These genes are present in nine isolates, but not in two that differ from the reference genome *Vibrio cholerae* El Tor N16961.

The quality scores of constructed contigs have been added to the table following genome sequencing analysis. MLST analysis revealed that about nine whole genome sequences belonged to sequence type ST69, identical to El Tor N16961, and two belonged to 984 based on seven housekeeping genes, which indicates that 9 isolates belong to O1 El Tor and the remaining two belong to non-O1/non-O139.

Bakta-based genome annotation revealed the presence of biofilm gene, virulence gene, AMR genes with clear visualization, and these properties were visualized by Proksee, a Genome Annotation Bioinformatic Tool.

According to the studies constructing Phylogenetic tree, the eleven investigated isolates of *V. cholerae* were positioned in two different phylogenetic positions. The first was represented by the nine isolates of investigated sample and *V. cholerae* O1 sequences collected from NCBI clustered based on *V. cholerae* O1 and the other position was represented by the two isolates of investigated sample and *V. cholerae* non O1/non O139 sequences collected from NCBI gene bank based on *V. cholerae* non O1 or O139 (37). The maximum likelihood phylogenetic tree Figure 3.11, constructed from whole genome sequences of 50 *V. cholerae* isolates with reference genome El Tor N16961, shows that isolates positioned near to each other on the tree carry a similar virulence gene, biofilm gene profile. In contrast, isolates placed on distant branches suggest different gene patterns, indicating variation in biofilm-forming ability.

The other maximum likelihood phylogenetic tree, Figure 3.12, constructed from whole genome sequences of 50 *V. cholerae* isolates with reference genome El Tor N16961, shows the nine isolates that cluster closely share a similar combination of AMR genes, such as *var*G*, flo*R*, gyr*A*, dfr*A*, sul2, and cat*B9, indicating comparable resistance characteristics. On the other hand, isolates positioned distant branches like the other two isolates (3.3 PsY2, 5.4 PsY2) exhibit distinct AMR profiles.

In addition to being sensitive to tetracycline, azithromycin, and doxycycline, all isolates (100%) were resistant to ampicillin, ceftazidime, furazolidone, and streptomycin and intermediately resistant to ciprofloxacin according to CLSI [27]. A few isolates also displayed intermediate resistance to other medications. Based on antimicrobial resistance, a potential heatmap has been developed. The resistance pattern with common AMR genes, such as *var*G*, flo*R*, sul2, gyr*A*, str*A*, str*B*, cat*B*9, and dfr*A, was shown by whole genome sequence-based analysis [30]. By examining this pattern, we found that nearly all *Vibrio cholerae* isolates possess these genes; however, two strains (3.3PsY1 and 5.4 PsY2) lacked any AMR gene and so did not exhibit any antibiotic resistance traits.

We conducted a biofilm assay analysis to see phenotypic traits such as biofilm development [22]. Two of the nine *V. cholerae* O1 isolates were unable to produce ring-like structures on the borosilicate glass tube wall. Strong crystal violet rings were generated by certain stains,

whereas moderate rings were formed by others. In order to create biofilm, *hap*A*, omp*U, and *tox*R are present. Two strains, indicating *Vibrio* non O1/non O139 (3.3PsY1 and 5.4 PsY2) were unable to form biofilm due to the incomplete genome of the *mshD* and *ompU* genes, according to comparative analysis with genetic and phenotypic characteristics. We also found that weak biofilm formation had occurred in the absence of any gene. A variety of virulence adaptive genes (VAP) and alleles are circulating in the environment. Some key virulence factors are acquired by a genome encoding a crucial mix of VAPs (Virulence Adaptive polymorphism), enabling it to develop into a virulent, potentially pandemic clone. In the conventional O395 context, it was discovered that *ompU* alleles provided effective host colonization, which is consistent with an *ompU,* VAP having contributed to the establishment of pandemic *V. cholerae* [28]. So this is the alarming issue with these strains. However, the strain 9.3WsY4 is confusing. It demonstrated no biofilm ring development rather than being present in every gene. There may be other genetic or environmental factors that are responsible in this regard.

The *msh* cluster profiling showed that all about genes (*mshA-mshN*) were found in nine isolates but *msh*D was incomplete in two isolates that are not identical to El Tor N16961. The presence of this cluster ensures that they all have biofilm-forming ability, low or high and an incomplete gene may cause low biofilm-forming ability.

Environmental signals including temperature, pH. osmolarity, iron availability, oxygen tension have been proposed to influence the initial bacterial attachment during the process of biofilm formation [9]. Results of the present laboratory-based microcosm study suggest that biofilm formation exhibits a powerful mechanism for the persistence of *Vibrios* in the natural environment. The genome contains a core area that encodes the CT gene, which affects the severity of cholera and other genes which code for virion morphogenesis. The *ctxA* and *ctxB* genes, which encode CT subunits A and B, comprise component of the lysogenic bacteriophage CTXφ [29]. *V. cholerae* strains found in natural aquatic environments can be toxigenic or non-toxigenic and *V. cholerae* O1 in estuarine microcosms loses CTX φ and becomes non-toxigenic [29, 31]

V. cholerae O1 cell in microcosm became nonculturable, as measured by TCBS agar, within 10-15 days, as has been reported by other investigators [29]. We tested the ability of two strains—one *V. cholerae* O1 isolates and one *V. cholerae* non-O1/non-O139—to investigate survival under stressful environments, such as 4ºC and 37ºC, over a 49-day period. Comparatively, *V. cholerae* O1 at 37ºC were culturable for more days than 4ºC. A possible

explanation of the extended active growth of *V. cholerae* at lower temperature (4º) is that the bacterium has lower biofilm forming capacity at lower temperature, as the biofilm formation is negatively linked with the culturability of *V. cholerae* in the environment [29]. Figures 3.11 and 3.12 display their growth rate as a log number. However, incubating cultures at 4°C resulted in slower biofilm formation and longer culturability compared to ~25°C, indicating that biofilm generation is temperature-dependent and associated with culturability loss [29]. In this study, incubating cultures at 37°C resulted in slower biofilm formation and longer culturability compared to 4°C has added a new insight about their survivability. Using microscopic examination, *V. cholerae* O1 strain was visualized from microcosm (day 0, day 7, day 28, day 49) and found the transformation from free-floating cells into coccoid shape clustered form. The growth of strain 8.2WsY2 was invisible at cultural form at TCBS agar plate count at 7 days that was related to the biofilm like aggregates existing at VBNC form [29]. Previous study shows that *V. cholerae* O1 in estuarine microcosms loses CTX φ and becomes non-toxigenic observing through molecular identification using PCR [31]. The isolates of this study are lack of toxigenic gene *ctx*A*, rtx*R *etc.* but other toxigenic gene like R*pos, nan*H etc. whether may be lost or not should be studied for further investigation.

4.1 Concluding remarks

This study provides a complete genomic and phenotypic characterization of *V. cholerae* O1 and *V. cholerae* non O1/non O139 from Noakhali, Bangladesh, offering important information about their evolutionary history, antimicrobial resistance, and virulence profiles. Phylogenetic analysis based on whole-genome sequencing revealed the relatedness with other strains found in other countries. Biofilm-associated genes are present in all isolates that showed phenotypic visualization in the crystal violet biofilm assay. It has been found that the incomplete presence of a single biofilm gene may dramatically reduce the ability of biofilm formation, whereas the presence of all biofilm genes did not show any biofilm-forming activities of the *V. cholerae* O1 isolate. Which another factor may influence that dissimilarities should be further analyzed. The incomplete presence of *omp*U concerns us to arise a pandemic situation, existing as VAP (Virulence Adaptive Polymorphism) that occurred with *V. cholerae O395* Classical biotype. Through microcosm analysis, we have found that *V. cholerae* O1and *V. cholerae* non O1/non O139 isolate continued actively growing after 49 days at 37ºC, and became non culturable at 4ºC only at 7 days. Result shows the ability of biofilm formation of *V. cholerae* O1 and non

O1/non O139 at 4ºC is more frequent than 37º C. This result represents a major public health concern as biofilm-associated cells show high persistence in a stressed environment, and this long-term survival strategy may facilitate them in transmission during outbreak situations with altered gene expression, enhanced virulence potential, and so on.

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APPENDIX-I

APPARATUS

Autoclave
Bag sealer
Centrifuge, Eppendorff
Deionizer, E-Pure, Branstead
Distilled water plant, Autosi!
Disposable micropipette tips
Disposable syringe
Electronic balance
Eppendorff tube
Incubator
Gel chamber and power supply, Life Technologies
Glass wares, Pyrex brand, USA
Inverted Microscope
Fluorescence Microscope, Axioskop 40, Carl Zeiss, Germany
Digital Camera, AxioCam MRc, Carl Zeiss, Germany
Laminar airflow
Magnetic stirrer
Micropipette, Eppendorff
Microoven, Sharp
Petridishes, Sterilin
pH meter
Refrigerated superspeed-centrifuge. Model RC-5B, Sorval
Spectrophotometer
Sterilizer
Slide, Gold seal
Sheff Mapper, Bio Rad
UV- transilluminator. Bio Rad
Vortex, Fisher
Wafer bath

APPENDIX-II

Microbiological media:

Media used were prepared by standard methods using appropriate compositions. Components used were high grade and were produced either by Sigma or Difco, USA. All media were sterilized by autoclaving for 20 minutes. The composition used for different media have been shown below:

1. Luria-Bertani (LB) Broth

Ingredients	Amount
Bacto-tryptone	10.0 g
Bacto-yeast extract	5.0 g
NaCl	10.0 g
Distilled water	1.0 L
pH adjusted to 7.4	

2. Taurocholate Tellurite Gelatin Agar (TTGA)

Ingredients	Amount
Tryptone	10.0 g
Sodium-uiurocholate	5.0 g
NaCl	10.0 g
Gelatin	30.0 g
Agar	16.0 g
Distilled water	1.0 L
pH adjusted to 8.5–9.0	

3. Gelatin Agar

Ingredients	Amount (g/L)
Tryptone	10.0 g
Trypticase (BBL)	10.0 g
Gelatin	30.0 g
Agar	16.0 g

Distilled water	1.0 L
pH adjusted to 7.4	

4. T1N1 Broth

Ingredients	**Amount**
Tryptone	10.0 g
NaCl	10.0 g
Distilled water	1.0 L
pH adjusted to 7.5	

5. Thiosulphate Citrate Bile Sucrose (TCBS) Agar

Ingredients	**Amount**
Yeast extract	5.0 g
Peptone	10.0 g
Sodium thiosulphate	10.0 g
Sodium citrate	10.0 g
Bile	8.0 g
Sucrose	20.0 g
NaCl	10.0 g
Ferric citrate	1.0 g
Bromothymol blue	0.04 g
Thymol blue	0.04 g
Agar	14.0 g
Distilled water	1.0 L
pH adjusted to 8.6	

APPENDIX-III

LABORATORY REAGENTS

Reagents which were used in carrying out different methods together with their sources are mentioned below.

Normal saline

Ingredients	Amount
NaCl (sigma)	8.5 g
Distilled water	1.0 L
pH adjusted to 7.8	

Phosphate Buffer Saline (PBS)

Ingredients	Amount
NaCl (sigma)	8.5 g
KH_2PO_4	0.156 g
Na_2HPO_4	1.28 g
Distilled water	1.0 L
pH adjusted to 7.4	

Polymerase Chain Reaction (PCR) reagents

(Life technologies Tech-Links™, Gibco BRL, USA)

- 10X PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl)
- 50 mM $MgCl_2$
- 10 mM dNTP mix
- Taq DNA polymerase
- Control primer mix
- Sterile water
- Silicone oil
- Molecular weight marker

Gel loading buffer

Ingredients	Amount (g/L)
Sucrose	6.7
Bromophenol blue	0.04
Stored at 4°C	

Ethidium bromide solution

1.0 g of ethidium bromide was dissolved in distilled water to a final volume of 100 ml. The container was wrapped in aluminium foil and stored at 4°C.