Production of Polyclonal Antisera of *Vibrio cholerae* O1 & O139 to Detect Pathogenic *Vibrio cholerae*

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfilment of the requirements for the degree of Bachelor of Science in Biotechnology

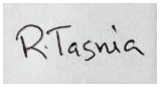
Department of Mathematics and Natural Sciences
Brac University
January 2023

Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I/We have acknowledged all main sources of help.

Sincerely Yours,



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Enrolling Semester- Spring 2018

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Approval

The thesis "Production of Polyclonal Antisera of *Vibrio cholerae* O1 & O139 to Detect Pathogenic *Vibrio cholerae*" submitted by Rifah Tasnia of Spring, 2018 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Biotechnology on 31st January 2023.
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Ethics Statement

I am Rifah Tasnia, student of B.Sc., Department of Mathematics and Natural Sciences, Brac University, do hereby declare that the thesis on "**Production of Polyclonal Antisera of *Vibrio cholerae* O1 & O139 to Detect Pathogenic *Vibrio cholerae***" is an original and authentic record of my research work carried out by me for the degree of Bachelor of Science in Biotechnology, under the joint supervision and guidance of Dr. Zahid Hayat Mahmud, Scientist and Head, Laboratory of Environmental Health , Laboratory Sciences and Services Division (LSSD), International Centre for Diarrheal Disease Research, Bangladesh (icddr,b) and Dr. Iftekhar Bin Naser, Assistant Professor, Biotechnology, Department of Mathematics and Natural Sciences, Brac University, Dhaka, Bangladesh.

It has not been submitted by me for any other degree.

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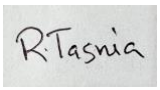

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Abstract

Cholera is endemic in Bangladesh due to serogroup O1, and people are still suffering from the disease seasonally in this geographic area. So, detecting the disease as soon as possible and making availability of materials to detect the disease is indispensable. Anti-sera are used to identify the serogroup of pathogenic *Vibrio cholerae*. Anti-sera are available commercially, but they are expensive, and getting the substance to the lab can take time. Thus, making antisera in the lab can be time and money efficient and can help local labs become self-sufficient. The purpose of this study and process was to show how to make the anti-sera on-site in the labs. The pathogenic strain of *V. cholerae* can be identified more quickly by utilizing *V. cholerae* antisera. The methods for recognizing *V. cholerae* serotype O1 and O139 are crucial as it is sensitive as well as repeatable because up to 60%-75% of all cholera cases are subclinical. During the study, the samples were taken from an area around ponds at a camp for Rohingya refugees where 42 suspected Vibrio were isolated using the common culture method. 26 of them had gelatinase activity and were oxidase positive. Based on colony morphology and gelatinase activity, isolates were obtained. On each isolate, common biochemical tests designed to identify Vibrio were conducted. 18 samples were determined to be *Vibrio cholerae* after conducting common biochemical tests. For molecular confirmation, multiplex PCR was then performed. *ToxR* genes were present in 18 of the isolates, but the lack of *ctxA* genes indicated that they were nonpathogenic O1/O139. To validate the findings and the prepared anti-sera the lab-prepared polyclonal antiserum was used where 3 samples agglutinated with the anti-sera confirming that they were nonpathogenic O139 *Vibrio cholerae*. The polyclonal antibodies were prepared by injecting dosage of dead cells of *V. cholerae* to New Zealand white rabbits and following all the procedures the polyclonal antisera were prepared which was further used to identify the pathogenic O1 and O139 serogroups of *V. cholerae* fast and cost effectively.

Keywords: *Vibrio cholerae*, Antisera, polyclonal, serotypes, environmental sample

This Thesis is Dedicated to my Family

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Firstly, I would like to express my gratitude to Almighty Allah for providing me the strength to complete the task on schedule.

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Sincerely,

Rifah Tasnia

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Abbreviations

WHO	World Health Organization
CFR	Case Fatality Rate
CFU	Colony Forming Unit
FEDSD	Field Epidemiology and Disease Surveillance Division
DNA	Deoxyribonucleic acid
PCR	Polymerase Chain Reaction
NIH	National Institute of Health
ECDC	European Center for Disease Control
TE	Tris EDTA
TBE	Tris Borate EDTA
TCBS	Thiosulfate Citrate Bile Salt
cAMP	Cyclic Adenosine Monophosphate
SEATO	Southeast Asia Treaty Organization
APW	Alkaline Peptone Water
TTGA	Tellurite-Taurocholate-Gelatin Agar
GA	Gelatine Agar
TSA	Trypticase Soy Agar
IgG	Immunoglobulin G
IgM	Immunoglobulin M
TCP	Toxin Coregulated Pilus
bp	Base Pair
spp.	Species (plural)
sp.	Species (singular)
VBNC	Viable but Non-Cultivable
ACE	Accessory Cholera Enterotoxin
AC	Adenylate Cyclase
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
ZOT	Zonula Occludens Toxin
dNTPs	Deoxynucleoside Triphosphates
RNA	Ribonucleic Acid
EPS	Extracellular Polymeric Substances
SXT	Self-Transmissible Transposon
pABs	Polyclonal Antibodies
CVA	CHROMagar Vibrio
icddr,b	International Center For Diarrheal Disease Research, Bangladesh

CHAPTER ONE: INTRODUCTION

1. Introduction

Millions of people have died from the terrible pandemic disease cholera, which is still a major health issue today. The word "cholera" comes from the Greek word "bilious," which means "terrible sickness." The illness is known as "Olauta" in Bangladesh. Since Hippocrates and Lord Buddha, it has been known to happen (Barua, 1992; Pollitzer et al., 1959). In nations with weak socioeconomic situations, where everyone cannot be guaranteed access to safe water and proper sanitation, this issue is still quite problematic. With the exception of the most recent pandemic, which started in celeb instead of its endemic center in the Ganges and Brahmaputra delta, mankind has endured seven pandemics (International Centre for Diarrhoeal Diseases Research et al., 1993; Pollitzer et al., 1959). With approximated cases which is around more than 5 million every year, the disease is endemic to the Indian subcontinent and continues to resurface elsewhere in Asia, Africa, and America (Glass & Black, 1992; Taylor et al., 1993). The condition is characterized by the loss of electrolytes and large amounts of fluid. The etiological agent of cholera is a toxigenic strain of the Gram-negative bacterium V. cholerae (Comstock et al., 1995). Typically, cholera is spread orally through feces, and a person only contracts the disease after ingesting the bacterium. A normal infectious dose of the pathogenic *V. cholerae* serotype O1 is between 10^5 and 10^6 live cells. If given with food, an infectious dose can be initiated by a much lower number (Levine et al., 1981). Cholera's infectious dose is high in healthy adult males. Environmental aquatic reservoirs are linked to cholera transmission and yearly seasonal outbreaks. By consuming contaminated food and beverages, one contracts the primary infection. During an epidemic, transmission from person to person happens through contaminated food or drink (S. Holmberg et al., 1984). Consuming contaminated fish, oysters, crabs, or other shellfish during an epidemic time is another way that it can spread (Feachem, 1981).

Only 40% of the villages in Bangladesh, where 80% of the population lives, have hygienic latrines. Common water bodies are contaminated by the typical methods of direct fecal discharge into ponds or rivers. People may come into touch with the cholera-causing agent when they bathe in, wash in, or drink from contaminated water. Primary transmission may occur when aquatic plants and animals from the marine environment are consumed as food (often without being fully cooked or through drinking water) (Miller et al., 1985). Cholera epidemics hit Bangladesh twice a year, with the first one peaking in the cooler months (September to December) and the second one being lesser and occurring in the summer (March-May). *V. cholerae* O1 cannot be isolated from surface

water when the inter-epidemic span is ongoing, but it is possible to isolate it from patients and surface-water during the epidemic season (Comstock et al., 1995; Siddique et al., 1992).

There are two main serogroups present for epidemic *Vibrio cholerae* known as O1 and O139. The two primary serotypes are called Inaba and Ogawa which falls under the *V. cholerae* O1 serogroup. They are known to replace each other throughout time, and the *V. cholerae* 0139 has recently developed (International Centre for Diarrhoeal Diseases Research et al., 1993). It is believed that the replacement of Ogawa and Inaba over time in an endemic area is a good indicator of the serotype's level of population immunity.

Vibrio cholerae, particularly the pathogenic strains of *V. cholerae* O1 and *V. cholerae* O139, cause the disease cholera which is a water-borne infectious disease, and *V. cholerae* non-O1 can also cause moderate-to-severe diarrhea that resembles cholera in Bangladesh as well as many different parts of world (Nair et al., 1994).

For the identification of the serotypes of *V. cholerae* O1 group-specific known as anti-A and type-specific marked as anti-B and anti-C antisera are used as they allow for the serological identification. Rabbits are immunized using cells of *V. cholerae* serotype Inaba and Ogawa that are heat-killed and are able to produce polyvalent antisera (Meeks et al., 2004). Antibodies are removed using group-specific antigen A against that antibody by cross-absorption which can create monospecific type sera (Mukerjee & Guha Roy, 1961). But the process can result in the concurrent deprivation of antibodies that are type-specific, which can make the production of such type antisera challenging. Sensitive as well as repeatable procedures are obtainable for the diagnosis of *V. cholerae* serotype O1, as up to 75% of the occurrence of cholera are subclinical (Merson, 1978). The objective of this work was to create an enzyme based on a highly specific polyclonal antibody for use in research laboratory and for epidemiological purposes.

1.1 Objectives of this study

The major purposes of this study are to prepare polyclonal antisera of *V. cholerae* O1 and O139 and validation of the prepared antisera by isolating *V. cholerae* from environmental samples.

- Preparing antisera with heat-killed vibrio strains in lab animals preferably New Zealand white rabbits.

- Isolation of *V. cholerae* from environmental samples
- Molecular detection of isolated samples of *V. cholerae*.
- Validation of prepared antisera by serotyping with the confirmed colonies.

CHAPTER TWO: LITERATURE REVIEW

2.0 Literature review

2.1 The Organism: *Vibrio Cholerae*

2.1.1 Taxonomy

Kingdom: Bacteria

Sub kingdom: Negibacteria

Phylum: Proteobacteria

Class: Gamma proteobacteria

Order: Vibrionales

Family: Vibrionaceae

Genus: *Vibrio*

Species: *Vibrio cholerae*

2.1.2 Historical Background of *Vibrio cholerae*

Cholera became more common in the 19th century. In 1817, India recorded the first pandemic, which later spread to other regions of the world. This disease gradually attracted greater attention as it spread beyond India and started to cause major concern on a global scale.

There have been seven pandemics so far (Table 2.1). Six pandemics struck the world after the initial one in 1816–1817, starting in 1826, 1852, 1863, 1881, 1889, and 1961. There is still a seventh pandemic. By Pollitzer, Kamal, and Barua (Islam), the causes and effects from the previous and ongoing pandemics and how they extended to different parts of the universe were thoroughly examined. The location of this pandemic's inception is crucial information. The epicenter of all previous pandemics and epidemics happened to be formed at the Ganges delta of Bengal but for the last which is the present pandemic known as the seventh one happened to originate from Indonesia. The name of the place is Sulawesi which is an Indonesian island. However, during this pandemic, the Bengal is still a hub for cholera as an endemic disease. Even

though it appeared that the disease had stopped spreading after the outbreaks, Bengal was always where it was still being reported. As a result, cholera has been an ongoing problem in Bengal since its inception. Bengal is regarded as the cholera's native land (International Centre for Diarrhoeal Diseases Research et al., 1993).

Cholera has almost certainly visited every nation on earth at some period, making it simpler to compile a list of those it didn't. The world's southernmost and northernmost regions normally did not become affected by this disease. In Asia, Chamchatka and Northern Siberia were unaffected. The most northern portions of North America, such as Newfoundland and Greenland, as well as Western Europe, including Iceland, the Faroe Islands, Shetland, and the Orkney Islands, remained unaffected. The southernmost areas of Chile, Argentina, and the Falkland Islands in South America were cholera-free (International Centre for Diarrhoeal Diseases Research et al., 1993).

After the sixth pandemic, forty years of stillness ensued. The last pandemic after this was caused by the *V. cholerae* O1 El Tor strain, which started in 1961 in and around Indonesia and spread rapidly throughout most of Asia and Eastern Europe. In 1970, this El Tor biotype was also introduced to West Africa, where it expanded very quickly. After a century-long hiatus, it was once more introduced to Peru in 1991. Cholera epidemics in the South Asian subcontinent were known to be generated by *V. cholerae* O139 in 1992, and as a result, it had spread to eleven more Southeast Asian countries. Aside from a few isolated occurrences, this serogroup has not been documented outside of these nations (International Centre for Diarrhoeal Diseases Research et al., 1993).

Table 2.1: Cholera pandemics since 1817 (Adopted from hunter 1997)

Pandemic	Causative Strain	Duration	Origin	Affected Regions
First	O1 (Classical)	1817-1823	Bangladesh	India, SE Asia, Middle East, East Africa
Second	O1 (Classical)	1826-1851	Bangladesh	India, SE Asia, Middle East, Africa, Europe, Americas

Third	O1 (Classical)	1852-1859	Bangladesh	India, SE Asia, Middle East, Africa, Europe, Americas
Fourth	O1 (Classical)	1863-1879	Bangladesh	India, SE Asia, Middle East, Africa, Europe, Americas
Fifth	O1 (Classical)	1881-1896	Bangladesh	India, SE Asia, Middle East, Africa, Europe, Americas
Sixth	O1 (Classical)	1899-1923	Bangladesh	India, SE Asia, Middle East, Africa, Europe, Americas
Seventh	O1 (El Tor)	1961-1975	Indonesia	India, SE Asia, Middle East, Africa, Europe, Americas
Eighth	O139	1991-	India	India, SE Asia

2.1.2.1 Global Update of cholera

In its 2013 The World Health Organization (WHO) reported that the number of cholera cases started to fall in 2012 following several years of continuous growth beginning in 2007. With a combined total of 24,53,931 cases, a case-fatality ratio (CFR) of 1.2%, and a death toll of 3,034, this study found. This report indicates a 58% decline in the number of incidents since 2011. A total of 48 countries across all regions registered cholera occurrences to WHO during this time period, which is a 17% decrease over 2011. Cholera occurrences related to *V. cholerae* were registered throughout the world. Similar to 2011, 27 nations on the African continent reported instances. The number of countries reporting cases reduced from 15 in Asia during 2011 to 12 by 2012, and from 9 in the Americas during 2011 to 6 for 2012. In 30 nations, the WHO has received a report of cholera cases out from Americans, Asia, Europe, and Oceania. 23 of the 30 countries that reported cholera deaths occurred on the African continent, making up 67% of the total worldwide. On the American continent, the Dominican Republic and Haiti were responsible for 962 fatalities, or 31% of the total. Throughout contrast, hundreds of thousands of cases of cholera were unrecorded in Asia due to the inadequate surveillance systems. More than two million episodes of severe watery diarrhea are reported annually in Bangladesh (WHO, 2011).

In 2010, during the month end of October a significant outbreak affecting the Dominican Republic and Haiti began. In 2012, 49 percent of all reported cases were associated with this outbreak. Around 7367 cases were reported from Asia, which represents 3% of the global total and an 81% drop from 2011 (38,298). Only important cases from Oceania were reported in Australia (Fig. 1.1). In 2012, WHO confirmed few outbreaks in different countries, where Africa accounted for 29 cases where America only confirmed 4 and 5 cases were shown in Asia. WHO received reports of 1,19,995 Hispaniola cases, or 49% of the global total. A visual representation of cholera cases reported to WHO by year and continent till 2016 is given below (Lonappan et al., 2020).

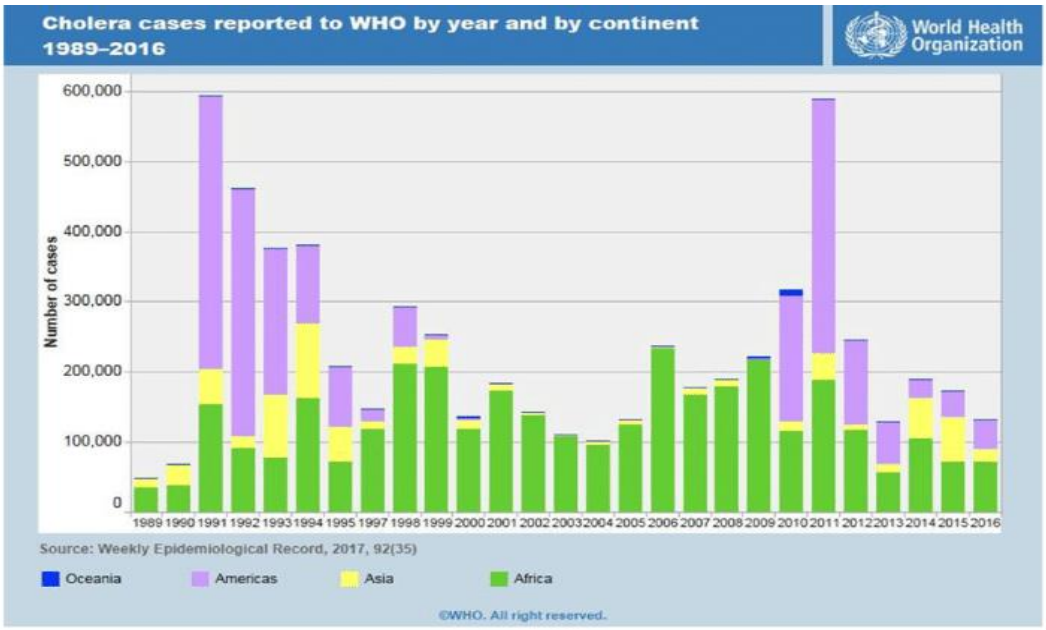


Fig. 2.1: Report of Cholera cases by WHO by year and continent from 1989 to 2016

2.1.2.2 Cholera: Bangladesh Perspective

Bangladesh is endemic for cholera. Additionally, it is a major cause of illness and mortality. Bangladesh, located within the delta of the Ganges, the place where all the cholera epidemic originated except the seventh one. According to studies, outbreaks of epidemics in Bangladesh typically happens two times a year, with the no. of cases being the highest between September and December, following the monsoon (Glass et al., 1982; Siddique et al., 1991, 1992). It was projected that the 1991 outbreak caused between 2.1 million and 2.3 million illnesses and over 8,000 deaths

(Siddique et al., 1992). From January to March 1993, a strain, that is new, of *V. cholerae* non-Ol, eventually named as *V. cholerae* 0139, which had never been known to trigger epidemics, started an outbreak of cholera in Bangladesh (International Centre for Diarrhoeal Diseases Research et al., 1993; Ramamurthy, 1993). This diarrheal illness killed more than 1,473 out of 1,07,297 patients in Bangladesh in 1993 (International Centre for Diarrhoeal Diseases Research et al., 1993).

2.1.2.3 Recent Outbreak Updates

Since the beginning of 2022, Pakistan and Bangladesh have witnessed an unprecedented increase in cholera, posing a growing health risk. As per the Field Epidemiology and Disease Surveillance Division (FEDSD) of National Institute of Health, Islamabad (NIH), the number of reported suspected cholera cases was 6,231 as of 17 April 2022. The statistical distribution of the cases varied throughout the nation, with Khyber Pakhtunkhwa, Sindh, Punjab, and Balochistan reporting, respectively, 2,596, 1,873, 956, and 697. As of 27 April 2022, the European Center for Disease Control (ECDC) reported 129 cases which has been confirmed by laboratory in Karachi, Pakistan. As per ECDC, the number of probable total cholera cases and deaths in Bangladesh is 495,433 and 29 respectively, inclusive of 33,832 cases from the Refugee Camps of Rohingyas in Cox's Bazar, Bangladesh. Till 13 March 2022, the number of laboratory-confirmed cases were 47. In addition to that, the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) reports that, in Dhaka, daily hospital admissions due to diarrhoeal diseases exceeded 1000 for the first time in the last 60 years, with 1,057 patients getting admitted, alone, on 16th March. The count reached an all-time high of 1,334 admissions on 28th March. Current reports indicate that Dhaka has been primarily affected by a probable cholera outbreak since the start of the year. However, it is also affecting other important cities, like Chittagong, Khulna, Barisal, Mymensingh, Rangpur, Sylhet, and Rajshahi. The government of Bangladesh, in collaboration with the World Health Organization, intends to provide oral vaccine of cholera to 2.3 million individuals (non-pregnant, older than 1 year) residing in diarrhea-prone districts.

2.1.3 General Characteristics

Vibrio cholerae, a member of the Vibrionaceae bacteria family, is a facultative anaerobe that moves by means of its flagellum. It's a Gram-negative bacterium that produces cytochrome C oxidase but does not create spores. Despite being facultative organisms, they develop considerably better in an aerobic environment. They thrive in alkaline conditions but are destroyed in conditions below pH 6. They are typically found in aquatic settings (freshwater, saltwater, wastewater, or brackish water) or in the intestine, vomit, and feces of human hosts (Harris, 2012). Its cells are bean-like rods measuring 1.3 m in length and 0.3 m in diameter. The optimal temperature for growth of *Vibrio Cholerae* is 37°C (98.6°F), but it can grow between 14°C and 40°C. Utilizing a single polar flagellum as a motility organelle, *Vibrio cholerae* is extremely mobile in liquid. Most *Vibrio Cholerae* can ferment glucose, maltose, sucrose, mannitol, dextrin, lactose, and starch; however, *V. cholerae* species identification among fecal isolates in TCBS media is done by applying only sucrose fermentation. These isolates typically produce extracellular DNase, lipase, -galactosidase, and Ornithine Decarboxylase, and use acetate. They do not synthesize Phenylalanine deaminase, urease, and they cannot grow on media containing inositol and KCN. This genus produces gas from glucose in a typical manner.

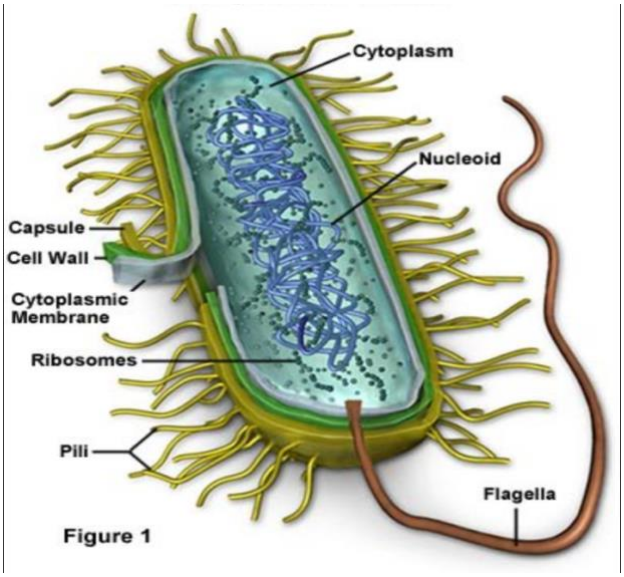


Figure 2.2: Basic structure of *Vibrio Cholerae*

V. cholerae has a vast array of strains and biotypes. Pathogenic along with nonpathogenic strains of *V. cholerae* differ in gene content related to their virulence. The O polysaccharides of *V. cholerae* have 140 serogroups and based on its O antigen, the bacteria have more than 200 serotypes; nonetheless, the serotypes O1 in addition to O139 have been identified as the culprits responsible for the pandemic and epidemic cholera outbreaks (Baron, 1996). These can be distinguished by their feature to manufacture toxin of cholera, for which encoding is done by the *ctx* gene. The *ctx* gene was a means to detect choleragenic *V. cholerae* in environmental samples. Based on morphological and genetic traits (Baron, 1996). there can be two biotypes of *Vibrio cholerae* O1, classical and El Tor.

2.1.3.1 Physicochemical properties

Vibrio cholerae is an anaerobe which is facultative. It can reduce nitrates to nitrites. It produces gas and acid (mainly H2 and CO2) at the time of growth on glucose and sucrose fermentation. By traditional biochemical tests for indole production and string test, which are conducted in clinical laboratories, *V. cholerae* is positive. They are also citrate, oxidase and gelatin hydrolysis positive but urease and hydrogen sulfide and methyl red test negative. Most *V. cholerae* strains can grow over a wide range of temperatures (approximately 14–40°C). Maximum growth rate is observed in the narrow range of 37–40°C. *V. cholerae* can grow best at alkaline condition within a pH range: 6.5–9.0 (approximate) (Hollenbeck et al., 2014).

2.1.3.2 Role as normal flora

The small intestine is colonized by *V. cholerae* where they secrete the potent cholera enterotoxin. In the bowel, the toxin is bound to the plasma membrane of intestinal epithelial cells and an enzymatically active subunit is released causing a rise in cyclic adenosine 51-monophosphate (cAMP) production. The high intracellular cAMP level is reason behind the cause of massive secretion of water and electrolytes into the intestinal lumen (Reidl & Klose, 2002).

2.1.3.3 Role as model organism

Research laboratories has been using *V. cholerae* for nearly a century because of the cholera pandemic of the Ganges Delta with an outbreak in Jessore, India, in 1817. It is studied because of its virulence factor, and fast-growth capacity. At 37°C this organism experiences ready growth on most of the laboratory media and upon overnight incubation, produces colonies.

2.1.3.4 Spectrum of Illness

The disease can be distinguished by a brief period of incubation (8 - 72 hours) followed by acute watery diarrhea, frequently along with vomiting, muscle cramps, in addition to consequences due to severe dehydration and metabolic acidosis. Rehydration is the primary treatment for cholera, although antibiotics have been demonstrated to be crucial and cost-effective adjuncts in severe patients and epidemic conditions. Antibiotics are not considered lifesaving under optimal treatment conditions, as patients can be treated with intravenous and oral rehydration fluids alone. Antibiotics are regarded as the standard treatment for cholera since they lower by approximately 50 percent the length of disease, the volume of diarrhea, and the rehydration requirements. When treating a high number of cases, duration reduction and symptom alleviation are particularly significant, and antibiotic treatment minimizes the expense and effort required to combat an outbreak (Comstock et al., 1995)

2.1.3.5 Seasonality of *V. cholerae*

Bangladesh and other South Asian nations are regarded as the cholera endemic zone. A regular seasonal pattern is maintained by cholera, in Bangladesh (Glass et al., 1982; Islam et al., 1993), with the biggest peak happening during the post monsoon season (Sept. to Jan). The second peak occurring during the pre-monsoon season (Mar. to May). The lower Ganges Delta retained a distinct yearly pattern of outbreaks as the birthplace of cholera. For instance, Dhaka and Matlab are researched as the endemic center of cholera in Bangladesh. In 1961, The epidemiologic research of this illness was initiated in Bangladesh (then East Pakistan) after the creation of the Pakistan Southeast Asia Treaty Organization (SEATO) Cholera Research Laboratory. From 1964

to 1966, (Martin et al., 1969) noticed in Dhaka that cholera outbreaks peaked annually in November, December, or January. During the epidemic of 1965-1966, they detected another peak in April-May (McCormack et al., 1969), in a Matlab study, evaluated the seasonality of cholera. The study was conducted between November of 1963 to June of 1966 and discovered that each year the cholera. outbreak was at its peak between November and January. Also, they detected a small secondary peak throughout April and June during the outbreak of 1966. (Merson et al., 1980) demonstrated in research conducted in Matlab, Bangladesh, between 1968 and 1977 that the occurrence of the classical biotype was highest between September and November. In March to April, a second, smaller peak was also detected. (Glass et al., 1982) analyzed the seasonal characteristics of both El Tor and conventional biotypes in Matlab, Bangladesh, where he accumulated 15 years of data. Similar seasonal patterns were identified for both El Tor and classical biotypes, as observed by (Merson et al., 1980). (Samadi et al., 1983) also investigated the seasonal characteristics of El Tor along with classic cholera. The forecasted peak of the El Tor outbreak was in October, while the classic pandemic peaked in December. The peak incidence of El Tor occurred before the onset of typical cholera, according to research. If the seasonality of cholera in Bangladesh (and the then East Pakistan) is analyzed based on available data within 1963 to 1980, it is clear that in endemic regions the incidence of cholera is typically seasonal (Figure 2.2).

During the interepidemic time, it is not possible to culture *V. cholerae* from water from the surface. However, at the season of the epidemic, isolation of *V. cholerae* can be done from both the patient's body and water from the surface (Khan et al., 1984). When not causing human intestinal problems, *V. cholerae* can be discovered in a variety of aquatic settings, including estuaries, rivers, ponds, etc (Huq et al., 1995; Turner et al., 2009). It may survive in the aquatic environment either as free-living plankton type organism in the water column or in association with zooplankton and phytoplankton (Islam et al., 1990; Samadi et al., 1983). In natural settings, *V. cholerae* remains connected to Anabaena spp., while in an artificial aquatic environment, *V. cholerae* Ol persists in the mucilaginous sheath of a blue-green alga, A. variabilis (Islam et al., 1990). Some researchers investigated its survivability of the O1 variant in various aquatic conditions. In artificial sea water, in the presence of copepods and chitin, Pruzzo and his colleagues discovered that, *V. cholerae* O1 remained viable for 14 days, without losing its adherent characteristics (Stauder et al., 2010).

Feachem demonstrated that, at 4°C and 30°C, it can survive in clean water for up to one month and 2-14 days respectively (Feachem, 1981).

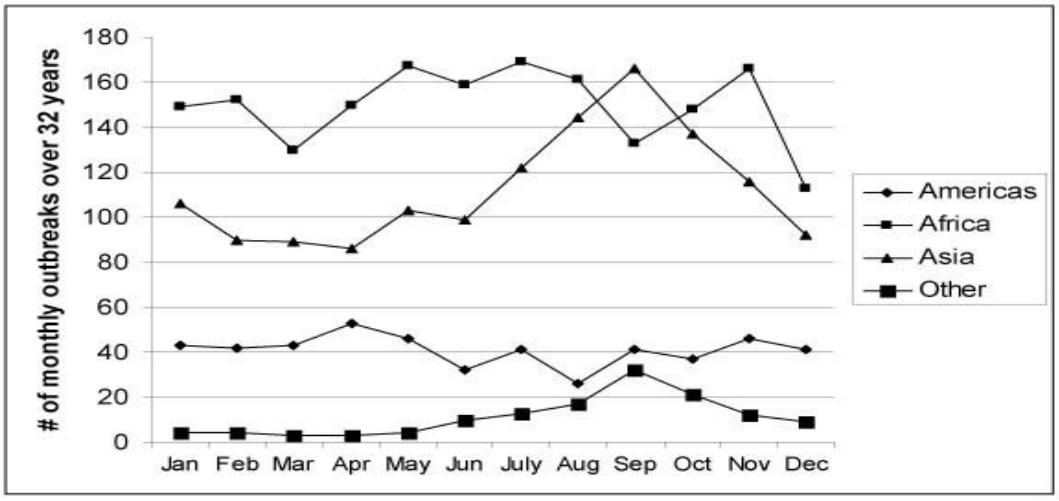


Fig 2.3: Monthly Cholera outbreaks by region (Emch et al. 2008)

Also, it is evident that, the epidemic in Bangladesh always reaches its peak during the winter season with a second small peak during the hot season.

2.1.3.6 Mode of Transmission of *V. cholerae*

Primarily, *Vibrio cholerae* transmission can happen by food and water that is contaminated with feces. For more than a century, the importance of water for transmitting cholera has been acknowledged. As was already established, this proof was shown in 1854, during the second cholera pandemic, by the London doctor and epidemiologist John Snow, who showed that illness was caused by consuming water from a system that pulled water from the Thames River below a sewage inflow. Several subsequent investigations have shown the importance of water in the dissemination of the disease (Glass & Black, 1992; Pollitzer et al., 1959). In a study conducted in Bangladesh it was portrayed that around 44% of shallow water which was sourced from communities with cholera, was positive in culture, for the organism, and not surprisingly, risk of infection associated with cooking, bathing, or washing with water from culture-positive sources

had a remarkable increase (Hughes et al., 1982). It has also been demonstrated that water contributed to the growth of diseases from South America. In a study conducted in Piura, Peru and Trujillo, it was determined that not boiling water was mainly linked with illness; contagion of urban water with feces was prevalent. In these and other research (Deb et al., 1986; Hughes et al., 1982; Ries et al., 1992; Taylor et al., 1993), the potential dangers provided by water storage containers, which are open, wide-mouthed and prevalent in houses in cholera-endemic regions, were also considered. Even though the supplied water to the family didn't contain *V. cholerae* initially, the containers were contaminated easily, which raised the potential of disease transmission within the household. Adding to this, research conducted in Calcutta, India, revealed that the adoption of containers that had narrow-mouth and was used for storage of water (i.e. into which utensils, hands, etc. cannot be placed) significantly reduced disease transmission (Deb et al., 1986). Transmission of cholera is possible by consuming food also. Seafood can be contaminated via sources from the environment and may act as a vector for both epidemic and endemic sickness, especially if it is raw or only slightly cooked (Baine et al., 1974; Blake et al., 1980). Additionally, there is proof that irrigating vegetables with unrefined sewage water can harbor and spread *V. cholerae* O1 (Cohen et al., 1971).

Food within houses or other places can be affected (contaminated) by food-handlers (S. Holmberg et al., 1984; LOUIS et al., 1990), or organism may be contained in water used while preparing the food, if not boiled (Johnston et al., 1983). The source of a recent outbreak of cases in the US was tracked back to coconut milk that was frozen and was brought in from Thailand. Here, the floor on which the coconut meat was diced and later used to make coconut milk was washed with water from canals (Taylor et al., 1993). As previously said, food serves as an ideal medium for culture. For example, cooked rice, which has been shown to promote rapid growth of *V. cholerae* (Kolvin & Roberts, 1982), as well as neutral sauces like peanut sauce, serve this purpose. Additionally, it should be mentioned that acidic sauces with a pH of 5.0 or lower seem to protect against this bacterium (LOUIS et al., 1990). While food has repeatedly been blamed for spreading cholera to new areas (Glass & Black, 1992; Pollitzer et al., 1959), the organism's ability to thrive and the protection against gastric acidity suggest that food may have a bigger impact than previously thought on disease transmission in endemic areas. (Comstock et al., 1995).

Usually, cholera is transmitted by either contaminated food or water. In wealthy countries, the most common cause of death is seafood. In contrast, in underdeveloped nations, normal water is more prevalent. Most of the cholera cases in industrialized nations originate from contaminated food which occurs if the water is contaminated with sewage are used by people for harvesting oysters because *V. cholerae* accumulates in zooplankton and the oysters eat the zooplankton. Cholera-infected individuals' rice-water stools contaminate water, and if the contaminated water is consumed by others, disease transmission can occur. Drinking contaminated water and consumption of food washed with contaminated water might cause infection. Cholera is infrequently transmitted directly from person to person.

2.1.3.7 Morphology of *Vibrio Cholerae*

Vibrio cholerae, a comma shaped bacillus which possesses a single polar flagellum. The name *V. cholerae* is given because of their vibrating motility in fresh wet stool preparation. They are also called "comma bacilli" because of their unique comma shape. The bacteria are oxidase positive and do not form endospore or microcyst (Baumann, 1984b). The bacterium can grow in the media containing 0 to 6.5% salt but not in a media having 8% salt. *V. cholerae* grows at pH ranging 7.0 to 8.5 but a pH range of 7.5 to 8 is shown to be optimum and the ambient temperature is 37°C. Most used broth for the enrichment of *V. cholerae* is alkaline peptone water *(*APW). For isolation of the bacterial colonies many selective and semisolid culture media have been developed and among them Tellurite-Taurocholate-Gelatin Agar (TTGA) which is also known as Monsur's media (Monsur, 1961) and Thiosulfate-Citrate-Bile salt-Sucrose (TCBS) agar have gained wide popularity around researchers. On TCBS agar *V. cholerae* produces typical yellow colonies; while on TTGA plate colonies are rather semi-transparent and grayish in color with dark spot in the center and also surrounded by a zone of opacity due to gelatinase activity. There are some non-selective media such as Gelatin Agar (GA), Trypticase Soy Agar (TSA) etc. which are also used for *V. cholerae* cultivation (Comstock et al., 1995).

2.1.4. Classification and antigenic type of *V. Cholerae*

O antigen is the antigen used to characterize *V. cholerae*, which is primarily responsible for its pathogenicity. This antigen is stable at heated conditions and has a homopolymer containing the

amino-sugar D-perosamine (4-amino-4,6-dideoxy-D-mannose) in which the amino groups have been acetylated through 3-deoxy-L-glyco-tetronic acid. The variety of groups of this O antigen are known as serovars or serogroups. The rough (R) antigen of *Vibrio cholerae* is same across all species. It is difficult to differentiate between the R form and the S (smooth) form based on colony morphology alone, however identification of the R form can be done using the R antiserum. There is also a flagellar (H) antigen on the bacterium, although its utility for identification of species is restricted due to the existence of H epitopes shared by all species of Vibrio.

The O antigen of *V. cholerae* can be identified by a number of classification techniques. The most prevalent and frequently used system is Shimada and Sakazaki's (1973) typing technique, which employs antisera, that is raised against organisms which are heat-killed. This technique consists of about 200 distinct groups. Serogroups O1 and O139 of *V. cholerae* are toxigenic, while the remaining serogroups, designated non-O1/non-O139, are rarely toxigenic. *V. cholerae* Ol can be further characterized based on its morphological and antigenic features in two ways.

2.1.4.1 Serogroups of *V. cholerae*

Midway through the 1930s, a single antiserum was developed against the entire *V. cholerae* after discovery, where majority of vibrions isolated from cholera cases agglutinated with the raised antiserum. This test is characterized as an agglutination test because the bacteria in the elevated antiserum form clumps. The agglutination test became the primary criterion for detecting pathogenic *V. cholerae* over time. Based on O antigen, *V. cholerae* was split into six serologically identified groups (I to VI) (Gardner & Venkatraman, 1935). The majority of O antigens are thermostable polysaccharides. *V. cholerae* O1 or agglutinable *V. cholerae* is pathogenic *V. cholerae* that may agglutinate with O antisera. Non-agglutinating vibrios (NAGs) or non-cholera vibrios are the colloquial terms for organisms that resemble vibrios morphologically but do not agglutinate in this serum (Group II to VI). According to current terminology, they are known as *V. cholerae* non-O1 or other Vibrio species. Non-O1 vibrios and other types of vibrios are prevalent in pond, lake, river, and ocean environments (Farmer et al., 1984).

From the action of cholera toxin, pathogenic *V. cholerae* O1 is the serogroup that causes severe watery diarrhea. In contrast, *V. cholerae* non-O1 is known to cause cholera to a considerably lesser

extent than *V. cholerae* O1 (Spira et al., 1981). A new serogroup of *V. cholerae* was discovered and designated as *V. cholerae* O139 in 1992, which is popularly known as Bengal (Islam et al., 1993; Ramamurthy et al., 1993). *V. cholerae* 0139 does not express O1 antigen due to the lack of at least two genes from the O1 biosynthetic gene cluster. On the basis of O antigen, more than 200 serogroups have been found to date (Colwell, 2002). *V. cholerae* Ol and non-Ol have a common heat-labile flagellar H antigen, although distinct species of vibrios possess a range of additional H antigens (Tassin et al., 1983). A common R antigen identification was done, and it was similar across all *V. cholerae* strains (Shimada & Sakazaki, 1973). The classification stages are shown below-

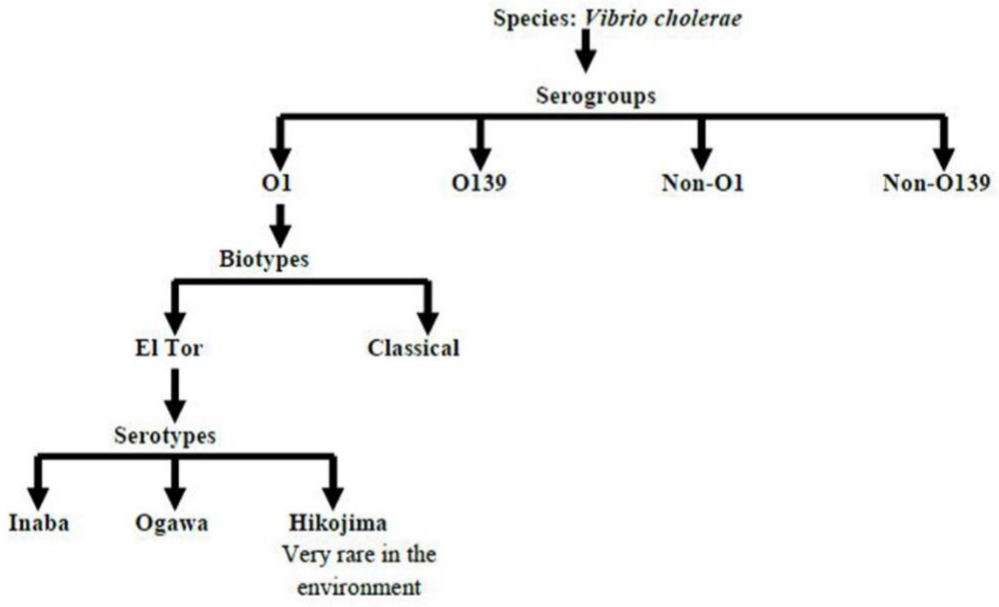


Fig 2.4 Classification stages of *V. cholerae*

2.1.4.2 Serovar and Biovar of *V. cholerae* Ol

All *Vibrio cholerae* strain has lipopolysaccharide in their structure. The lipopolysaccharides of *V. cholerae* can produce powerful inflammatory reaction. The lipopolysaccharide consists of lipid A,

an endotoxin, core polysaccharide and O polysaccharide sidechain. The O polysaccharide can subdivide the *V. cholerae* into many serogroups. Cholera toxin (CT) is produced by *V. cholerae* O1 and it is linked to cholera pandemics and epidemics. There are further strains of *V. cholerae* O1 that don't produce Cholera toxin because the gene for cholera toxin is absent (Comstock et al., 1995). The most important confirmation for identifying *V. cholerae* O1 is agglutination in polyvalent hyperimmune antisera produced against entire bacteria including all O1 antigenic components. Agglutination is really the cross-linking of many homogenous bacteria with immunoglobulin G (IgG) and immunoglobulin M (IgM) found in animal serum inoculated against that specific bacterial species (Baumann, 1984a).

2.1.4.2.1 Biotypes

Bio-typing is an important method of classification, epidemiologically for O1 strains of *V. cholerae*. Classification of the two biotypes of serogroup O1 of *V. Cholerae* are done on the basis of various genotypic and phenotypic markers, and those are named as El Tor and classical. The serotypes of El Tor are then namely as Hikojima and Ogawa, Inaba (Comstock et al., 1995). The most dominant serotype is Ogawa, on the other hand Hikojima serotype is found to be very unstable and rare in the environment as this is the transitional state. It expresses both Inaba and Ogawa properties. There are some distinctness between the El Tor and classical biotypes of *V. cholerae* O1. The differences are as follows:

- The adaptability of El Tor is finer than the classical biotype as it can subsist in the environment as well as in the host (human) as the intestinal epithelium can be colonized better by them (Finkelstein, 1996).
- However, in the comparison of toxigenicity, the *V. cholerae* O1 classical biotype is lead to belief to be more virulent and toxigenic (Koelle et al., 2005).
- Phenotypically the El Tor biotype is responsive to both Mukerjee El Tor phage 5 and 50 IU of polymycin B, on the other hand the classical biotype is responsive to Classical phage IV. Besides, agglutination of biotype, El Tor, can be seen through erythrocytes of chickens, it also gives a positive Voges-Proskauer test reaction. The classical biotype does not give a positive reaction for the same test (Mandal et al., 2011).

V. cholerae O1 classical biotype, as a causative agent, is thought to have caused the first six cholera pandemics, resulting in both asymptomatic and symptomatic cases. After the 6th pandemic it is assumed to be extinct and then the seventh and present cholera pandemic is thought to be associated with the El Tor biotype. The appearance of this pandemic was first noticed in 1905 in the village of El Tor, Sinai, Egypt and therefore it is named as El Tor. More asymptomatic infections were caused by it compared to any other biotype. With help of current advancement, it was possible to alter *V. cholerae* O1 El Tor isolates to produce cholera toxin of the classical biotype. The O139 serogroup, also known as Bengal, is contained on different strains which are genetically diverse and both non-toxigenic and toxigenic; genetically, El Tor *V. cholerae* is closer to this strain (Ansaruzzaman et al., 2004).

From O1 serogroup the three Hikojima Ogawa and Inaba serotypes are different, depending on the structures of their antigens. They are differentiated in terms of their somatic (O) antigen. The Ogawa serotype contains A and B antigens, the Inaba serotype contains A and C antigens, while Hikojima contains unstable antigenic types having all three antigens (A, B and C) which makes this serotype a transitional state. The Ogawa and Inaba serotypes are found to be positive for Ogawa and Inaba antisera, respectively. Hikojima serotype is found to be positive for both Ogawa and Inaba antisera (Shimada et al., 1994).

The two biovar or biotypes based on their phenotypic characterization is shown below. (Table 2.2)

Table 2.2: Biotypes of *V. cholerae* O1

Biotypes	**Reactions**					
	VP Test	Resistance to polymyxin B (50U)	Agglutination of chicken erythrocytes	Hemolysis of sheep red blood cells	Lysis by Classical IV phage	Lysis by El Tor V phage
Classical	-	-	-	-	+	-
El Tor	+	+	+	+	-	+

To examine biotypes for characterizing *V. cholerae*, the Voges-Proskaeur reaction, agglutination with chicken erythrocytes, and other tests are considered the most useful. Variations in the DNA sequences of the genes that encodes the toxin coregulated pilus (TCP) from the different type of strains are used in the second approach of bio typing (Keasler & Hall, 1993). In isolated colonies connected to the fifth and sixth pandemics, V. cholerae O1's characteristic biotype was discovered. The El Tor biotype, however, predominated during the seventh pandemic and is now largely to blame for cholera outbreaks globally.

2.1.4.2.2 Serotypes

Inaba, Ogawa, and Hikojima serotypes are further subdivided under the serogroup O1 of *V. cholera*e (Table 2.3). Hikojima is an uncommon condition, and this entire classification is founded on the three O antigen components A, B, and C, with A being either common or group specific. The B and C components are assumed to be unknown in nature, however the factor A is thought to be the homopolymer of D-perosamine. The serotype differences are mainly quantitative.

- The Ogawa- strain produces the antigens that are A and B. They also produce a small amount of C.
- The Inaba- strain produces only the A and C antigens. Specific Antisera from Inaba and Ogawa are made by absorbing with the other serotype.
- The Hikojima serotype contains all three factors (Sakazaki, 1992), and so, they react with both antisera of Inaba and Ogawa.

The subtype known as Hikojima, which is uncommon and unstable, is often referred to as a stage between Ogawa and Inaba. Some authority frequently fails to recognize this subtype and instead describe cultures as Inaba or Ogawa, and that depends on which serum elicits the strongest response (Mooi & Bik, 1997).

Table 2.3: Serotype determination of *V. cholerae* O1

Serotypes	Major O antigen determinants	Agglutination in absorbed antiserum	
		Ogawa	Inaba
Ogawa	A, B	+	–
Inaba	A, C	–	+
Hikojima	A, B, C	+	+

2.1.4.2.3 Evolution of atypical El Tor strains

Classical strains have been steadily replaced by El Tor strains as the cholera source since the beginning of the seventh pandemic. The traditional biotype reportedly disappeared in Bangladesh in 1973 but reappeared in 1982. The classical biotype and El Tor biotype co-circulated for a decade (the latest isolation which was documented was in 1992) (Samadi et al., 1983; Siddique et al., 1991). Only in Bangladesh was the brief reappearance of Ol classical strains reported. Classical strains are assumed to be extinct; thus, the origin of the classical *rst* R and classical *ctx* alleles and their mechanism of transmission to El Tor strains in Bangladesh remain a mystery. Some classical strains may still exist in Bangladesh's aquatic ecosystems and may have served as gene donors. Due to their low incidence or existence in a viable but non-cultivable (VBNC) condition, these strains may have evaded discovery by traditional culture procedures, but they still possess pathogenic potential. In the early 1990s, the existence of classical strains in Bangladesh's aquatic ecosystems was recorded, lending credence to the notion that they had not been totally eradicated in Bangladesh (Siddique et al., 1991).

Multiple microbial species and significant amounts of phage and free DNA are found in aquatic settings. It is known that natural cell lysis of *V. cholerae* and lytic phages play an essential role in the release of bacterial and phage DNA into the environment (Dziejman et al., 2005). The enhanced fitness of the El Tor strains may have contributed to the elimination of classical strains. Consequently, a population of "homeless" free classical CTX prophage has evolved.

Typically, the free classical CTX prophages cannot produce functional virions. To secure their existence, they may infect additional hosts. By lateral gene transfer and recombination, the free classical CTX prophage may have entered the genetic background of a range of *V. cholerae* Ol and non-01/non-0139 serogroups or similar Vibrio species, such as *V. mimicus* (Bag et al., 2008). Unknown is the selection pressure that causes aberrant El Tor strains to harbor classical CTX prophage. The classical biotype is associated with more severe diarrhea than El Tor strains, which may facilitate the formation of classical CTX prophage and, consequently, the capacity to create classical type CT in El Tor strains. This occurrence may have contributed to the spread of more toxic strains in the environment (Comstock et al., 1995).

2.1.5. Pathogenicity and clinical significance of *V. Cholerae*

The human small intestine becomes infected with the bacterium *V. cholerae* O1 and O139, which results in cholera (International Centre for Diarrhoeal Diseases Research et al., 1993). According to Chowdhury (1988), the following compounds are produced by cholera Vibrios:

Enterotoxin

Accessory Cholera Enterotoxin (Ace)

RDE

Mucinade

Desquamating factor

Sodium pump inhibitor

Soluble haemolysin

The severity of infection and disease results from a series of interactions between the pathogenicity features of the pathogen and human host defensive mechanisms. The size of the inoculum, motility, chemotaxis, synthesis of key enzymes (including mucinase, protease, chitinase, and neuraminidase), presence of adhesions, and development of heat-labile cholera enterotoxin (CT) are determinants of the infection. CT generating strains are responsible for severe diarrhea, hypotension, and death within 12 hours if left untreated (Jesudason et al., 1993).

There are also some non O1 *V. cholerae* serotype can produce cholera toxin (CT), which is normally only produced by the epidemic type of *V. cholerae* O1 and O139. (Rahim & Aziz, 1992). When sufficient organisms are swallowed and some cells survive the stomach's acidic environment, infection occurs. The organisms colonize the small intestine, grow rapidly, and produce cholera toxin in adequate quantities. In severe cases, there is significant diarrhea, and a considerable volume of rice-water stool that are clear fluids with mucus flecks and they pass without pain. One liter of fluid can be expelled per hour. Usually, there is vomiting and minimal appetite to preserve nutrients. If the patient is not treated, prostration will develop along with indicators of extreme dehydration, loss of skin elasticity, and no urine excretion. Extreme dehydration causes death to happen very quickly once symptoms appear (S. D. Holmberg & Farmer III, 1984).

2.1.5.1 Cholera enterotoxin

Cholera enterotoxin, often known as CT or choleragen, is the cause of the severe dehydration diarrhea. This heat-labile protein could be generated by toxigenic strains of *V. cholerae*. Cholera enterotoxin *V. cholerae* generates several compounds that are extracellular and also toxic to eukaryotic cells. Although Koch (1884) suggested the existence of a chemical secreted by a comma-shaped bacillus in the human gut that causes cholera, the theory did not acquire traction until the 1970s. An Indian scientist, Sambhunath De, did not discover and define the Vibrio toxin until 1959, more than 140 years after the first epidemic began. Although not all Vibrio species produce toxins, the majority do. Twelve of the thirty identified species of this bacterium genus are pathogenic to humans (Choopun et al., 2002). Several scientists, including Finkelstein (1963), Richardson (1969), and Field (1979), took the effort to purify and determine the configuration of

the toxin of cholera. Nevertheless, vast majority of environmental isolates of *V.cholerae ctx* sequence (Comstock et al., 1995) are non-toxigenic *V. cholerae*. Strains or mutants of *V. cholerae* who are incapable of generating cholera toxin are also incapable of causing the disease, then again, presence of other toxins may induce a lesser type of diarrhea. Cholera toxin is the best researched biochemical and genetic virulence component of *V. cholerae* (Baudry et al., 1992).

2.1.5.1.1 The cholera toxin- Structure and action

An ADP-ribosylating toxin of the A-B type is cholera toxin. This toxin is a member of the enterotoxin family and is composed of two functional polypeptides, A and B. (Field, 1979). CT's structure is characteristic of the group of toxins with A-B subunits. One A subunit, five identical B subunits, make up the molecule of CT. In animal or intact cell systems, none of the components alone have substantial secret genic activity. While the A subunit performs a specific intracellular enzymatic function, the B subunit aids in the binding of holotoxin to the receptor of eukaryotic cell.

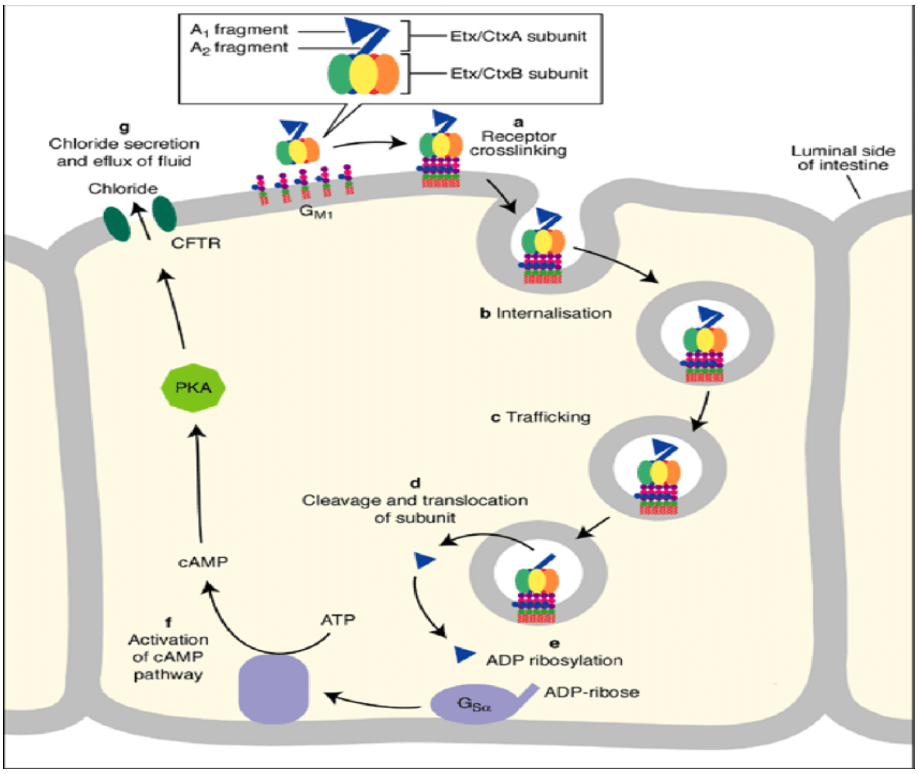


Fig 2.5: The actions of cholera toxin

After being released by bacteria in the infected gut, cholera toxin binds to enterocytes, which are intestinal cells (epithelial cell). Interaction between the B subunit of toxin that are pentameric and GM1 ganglioside which is a receptor of intestinal cell results in binding and endocytosis of the toxin. By separating the Al domain from the A2 domain, the active A1 enzyme is generated. Through an ADP ribosylation process, the active A1 portion of A subunit that has toxin reaches the cytosol and initiates protein G. Continuously, G protein in its GTP-bound state stimulates to create cAMP from adenylate cyclase (AC). The elevated cAMP levels stimulate the transmembrane conductance regulator for cystic fibrosis (CFTR). When the CFTR is activated, infected enterocytes release a substantial number of ions and water, which causes diarrhea with a lot of fluid (Zhang, 2006).

2.1.5.1.2 Additional accessory *V. cholerae* toxins

Besides cholera toxin, *V. cholerae* yields other more toxins. These include zot (zonula occludens toxin), accessory cholera enterotoxin (ace), neuraminidase, disulfide isomerase, protease, and hemolysin-cytolysin toxin, among others (Kurazono et al., 1995).

2.1.5.1.3 Zonula occludens (zot) toxin

By altering the intracellular structure of the mucosa of the small intestine, the zot toxin produced by *V. cholerae* makes it more absorbent. Hydrostatic pressure may cause Zot to leak water and electrolytes into the lumen, causing diarrhea (Uzzau et al., 1999).

2.1.5.1.4 Accessory cholera enterotoxin (ace)

Trucksis and his colleagues found the gene encoding *V. cholerae's* accessory cholera enterotoxin (Trucksis et al., 1993). The gene, according to the researchers, results from an open reading frame that comes right before the zot gene.

2.1.6 Reservoir of infectious *V. cholerae*

The reservoir of any contagious agents is animal, any person, plant soil, arthropod, or blend of these, where this infective agent ordinarily exists and proliferates, where it hinges primarily on for

subsistence, and from which it can be transmitted to a susceptible host. Koch (1884) first hypothesized that aquatic environments may serve as reservoirs for vibrios when during the epidemic of cholera in 1883, he isolated bacillus that had comma like shape, from a reservoir in Calcutta. After Koch, numerous studies have been conducted to examine the possibility of aquatic environments as vibrio reservoirs.

(Hood & Winter, 1997) ensured that existence of *V. cholerae* in water is conditional on adjourned particles. They noticed that the survival duration of viable cells in filtered and centrifuged saltwater decreased in proportion to the degree of filtration and centrifugation. As the process of filtering as well as centrifugation diminish the impurity, the organism's feasibility may be influenced by the occurrence of particles. Also, the serotype *V. cholerae* 01 favors an epibiotic environment. These can be found from estuarial and marine water. Also, water that are particle-free and so they concluded that this *V. cholerae* 01 can exist in planktonic form for a period.

Although cholera is prevalent in many tropical regions, the marine environment serves as a reservoir for the vibrios, as the majority of cholera outbreaks began in coastal locations (Lowenhaupt et al., 1998).

2.1.7 Molecular method for detection of specific genes

The general evaluation criteria for describing systems include Reproducibility, typeability, discriminatory Power, and interpretability. Currently, the proximity of bacterial isolates is determined by one or more phenotyping techniques, such as serotyping, bio typing, phage typing, and antibiotic resistance pattern. Phenotyping characteristics are not constant under all environmental or cultural conditions. The greatest advantage of molecular method is its greater precision. Additional benefit of molecular technique:

(i) DNA can always be retrieved from bacteria, making every strain capable of being typed.

(ii) Similar DNA-analytical methodologies are used for the genotypic method of any origin.

(iii) Genomic DNA is a stable feature whose composition is not affected by

cultural condition or preparation method.

(iv) It permits statistical data analysis and is easy to automate (Bingen et al., 1993).

PCR and RAPD is the most prevalent molecular technique utilized worldwide now.

2.1.7.1 Polymerase chain reaction for detection of specific gene

PCR is known to be in vitro approach where a specific section of DNA gets amplified. When Kary Mullis and his colleagues established PCR as a technology, it was possible to use it to produce vast quantities of genes that were single copy based on genetic DNA (Mullis et al., 1986). The polymerase chain reaction makes use of several amplifications via cycles of template denaturation, primer annealing, and primer elongation of DNA sequences (Saiki et al., 1988). The primers are designed such that DNA polymerase can move through the area between them by hybridizing to the target sequence's complementary strands.

The additional items are complementary as well as their ability to bind primers over multiple cycles of amplification effectively double the volume of the DNA that is initially targeted and that was produced in prior cycles. The outcome is an approximately up to 2n exponential buildup of a given target DNA segment, while n is the number of amplification cycles done (Saiki et al., 1988).

Typically, after 20 to 30 cycles of PCR, enough amplified product is created to permit detection on a gel stained with ethidium bromide. The response consists of components, that are template, reaction buffer, primers, and magnesium chloride, combination of dNTPs and polymerase that are thermostable.

The template must be sufficient for ethidium bromide-based visualization of PCR products. The presence of impurities in the DNA preparation can reduce the efficacy of PCR. The examples can be the detergent, urea, SDS sodium acetate, also, occasionally residues from DNA purification from agarose gels (Saiki et al., 1988).

A set of appropriate primers should efficiently hybridize to the sequence of concern while hybridizing little to other sequences that are also present in the sample. Up to 10 kb, the space

between the primers is quite variable. However, there is a significant decrease in synthesis efficiency for distances greater than 3 kb (Jeffreys et al., 1988). A primer should be between 20 and 30 bases long. It is highly unlikely that primers that are longer will considerably boost precision.

Taq DNA polymerase used the most frequently is polymerase that are thermostable. These polymerases enabled the usage of temperatures that are higher for annealing as well as extension, that increased the rigor of the template of primer hybridization, also consequently some specificity of yields. The mistake rate of Taq DNA polymerase, which was first expected to be 2x10-4 nucleotides/cycle, is a crucial feature (Saiki et al., 1988). Comparing with other enzymes the absence of 3'=5'-exonuclease activity in the purified enzyme reduces mistake rates.

Ion concentration of magnesium influences enzyme the activity, melting temperature of the template, primer annealing, and product of the PCR, fidelity, also the production of primer dimers (Innis & Gelfand, 1999). The reaction condition is supported by the reaction buffer.

2.1.8 Biofilms

Microbes necessarily do not exist in the form pure type cultures of discrete single cells; rather, they aggregate at surfaces to create polymicrobial masses of poly microbes such as mats, films, flocks, or "biofilms." A biofilm is a surface-accompanying population of microbes developing and encased in their extracellular polymeric substances (EPS) which is their own matrix (Kolari, 2003). Extracellular polymeric substances are primarily comprised of proteins, polysaccharides, lipids, and nucleic acids; not only do they deliver the mechanical solidity of biofilms, but they also create a consistent, three-dimensional network po polymers. This network oversees the interconnection and temporary immobilization of biofilm cells. Flagella, pili, and fimbriae are bacterial extracellular structures that can also maintain the matrix (Flemming et al., 2002). Keeping extracellular enzymes close to their cells, the matrix formed by biofilm also works like a peripheral digestive method. These type of enzymes allow biofilm cells to digest dissolved solid and colloidal biopolymers. The matrix also serves as a repository of genes for horizontal gene transfer obtained from DNA and other lysed cell components. The sorption of xenobiotics by EPS, which can also

serve as a source of nutrients, contributes to environmental detoxification. Some of the EPS properties are merely biodegradable, and the total destruction of all properties needs a diverse array of this type enzymes. The organisms are protected by the matrix from aridness by maintaining a microenvironment that is highly hydrated. In addition, it protects against charged biocides, also oxidizing, certain medicines and the cations that are metallic, UV radiation, some types of (but not all) protozoan grazers, as well as host immunological defenses (Donlan & Costerton, 2002).

2.1.8.1 Molecular assembly principles of *V. cholerae* biofilms

On solid surfaces, *V. cholerae* can form biofilms, which are huge multicellular structures. The biofilm functions as the organism's microenvironment. It promotes the survival and longevity of microorganisms by increasing their resilience to diverse stressors (e.g., chlorine, antibiotics).

On abiotic surfaces, *V. cholerae* 01 El Tor and 0139 are capable of forming a three-dimensional biofilm. Biofilm development is essential to the life cycle of *Vibrio cholerae* because, during interepidemic times, it promotes the organism's environmental persistence within natural aquatic habitats. Exopolysaccharide from *V. cholerae* serves as the biofilm matrix and is necessary for the development of matured biofilm formations (Yildiz & Schoolnik, 1999). It needs matrix proteins, mainly RbmA, RbmC, and Bapl, to keep the wild-type biofilm's structural reliability (Fong & Yildiz, 2007).

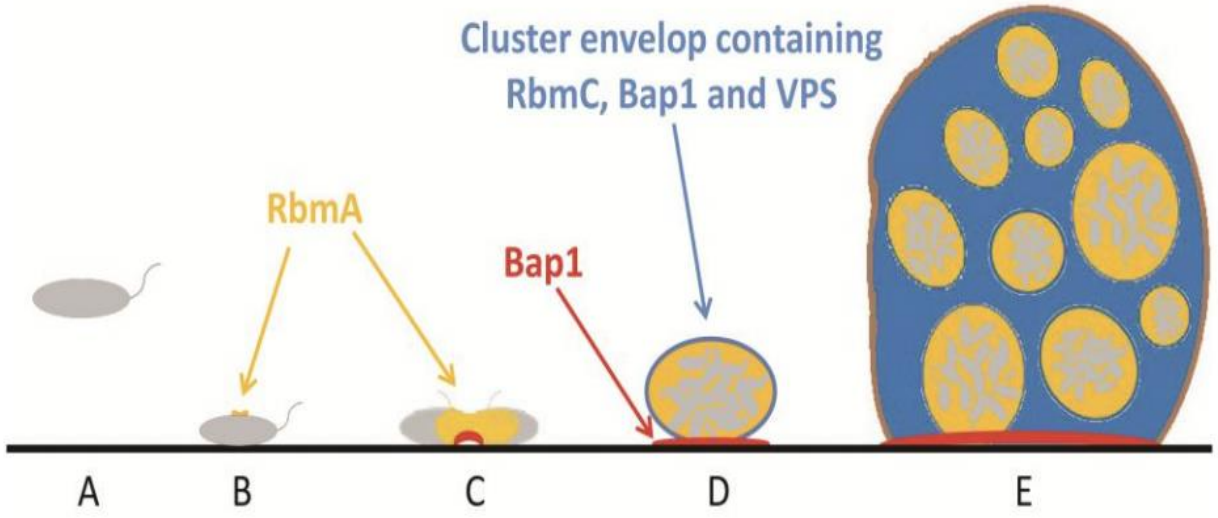


Fig 2.6: Involvement of VPS, RbmA, RbmC, and bap1 in the biofilm formation of *Vibrio cholerae*

(A) When a single planktonic cell comes into contact with the surface, (B) first attachment takes place, and RbmA (blue) builds up all over the cell surface. (C) Bapl (green), which ensures that the daughter cell adheres to the surface, arises at the interface of cell-surface at the division site that is initial as the founder cell divides. (D) A clustered cell including VPS, RbmC, and Bapl, that is encased in a envelope (red) that is flexible develops as the cells continue to divide. RbmA and Bapl production and deposition are both boosted as clusters form on the surfaces of individual cells. (E) When distinct cell clusters grow plus come into touch with additional clusters the biofilm happens to mature.

2.1.9 Antibiotics against *V. cholerae*

V. cholerae typically does not induce systemic illness. Antibiotics cannot be the only therapy option for the condition. They are not necessary to eliminate cholera symptoms. Nonetheless, there happens to be benefits in combining antibiotics with treatments like oral rehydration. Antibiotics should be utilized to shorten the length of disease by roughly fifty percent and to lower the amount

of *V. cholerae* excreted through diarrhea (Greenough et al., 1964; Lindenbaum et al., 1967; Pierce et al., 1968). It is possible to minimize certain severity of symptoms through lowering the volume of diarrhea, antibiotics (Greenough et al., 1964; Lindenbaum et al., 1967; Pierce et al., 1968). The process is critical in underdeveloped nations due to the limited availability of drinking water that are regarded as safe besides oral rehydration treatments.

A patient can be saved by a solution from dehydration if that contains sodium chloride, glucose, trisodium citrate and potassium chloride. It should be delivered orally or intravenously (WHO, 2002). Antibiotics named quinolones and tetracycline were extensively used to treat cholera (Mhalu et al., 1979; Towner et al., 1980). However, various strains showed resistant to some antibiotics which then confined their usage to individuals had acute dehydration (Garg et al., 2001). In most acute instances, one dosage of doxycycline and fluid replacement therapy are adequate to stabilize the patient. Alternately, tetracycline can be taken in multiple doses. For young toddlers, erythromycin in liquid form is recommended. In a clinical trial performed randomly, the antibiotic erythromycin had the highest recovery rates among children (Roy et al., 1998).

Despite of being beneficial, WHO has strict policy against widespread usage of antibiotic due to the rapid development of antimicrobic resistance. Besides other bacterial illnesses, Cholera became also challenging to cure due to microbial antibiotic resistance (Hedges & Jacob, 1975). Antibiotic treatment should only be administered to patients with severe dehydration.

2.1.9.1 The resistance of antibiotic among toxic *V. cholerae* strains

Multiple-drug-resistant *V. cholerae* 01 caused cholera outbreak in rural subdistrict called Matlab, in Bangladesh, in 1979 (Glass et al., 1983). Ampicillin, Tetracycline, streptomycin, kanamycin, and sulfamethoxazole trimethoprim resistance was found in 16.7% of the samples that were isolated from the outbreak, and among the isolates that were 10%, it was found that they were also resistant to atleast one of these four antibiotics, which includes tetracycline. These isolates had an antibiotic resistance plasmid that could be conjugated to *E. coli* K-12, was also found. A single multidrug-resistant strain of *V. cholerae* O1 was found to be the cause of the outbreak, according to epidemiological investigation of the cases (Glass et al., 1983).

The pattern of drug resistance had changed by 1986. Streptomycin, tetracycline, chloramphenicol, nalidixic or amoxicillin acid resistance was not present in any of the strains that were isolated from patients affected by cholera in Dhaka in January 1986 (Ichinose et al., 1987).

The 0139 *V. cholerae* serogroup that arose between 1992 and 1993 was susceptible to tetracycline (International Centre for Diarrhoeal Diseases Research et al., 1993). Waldor and his team discovered that *V. cholerae* 0139 had a 62-kb transposon-like self-transmissible element that are also called SXT element, that made it resistant to sulfamethoxazole-trimethoprime and streptomycin (Burrus et al., 2006). Efflux pumps, chromosomal mutations, conjugative transposons, conjugative plasmids, self-transmissible and integrons are chromosomally combining SXT essentials are all involved in *V. cholerae's* drug resistance.

2.1.9.2 Relationship between biofilm formation and antimicrobial resistance

Due to their protective impact against a variety of conditions, microorganism-produced biofilm has been defined as a barrier. There are considerable changes in physiology, morphology, and gene expression pattern between biofilm and planktonic cells. Biofilm-formed bacteria are far more resistant to antimicrobials (biocides and antibiotics) than free-swimming bacteria. Various multifactorial processes have been postulated to explain this enhanced resistance (Costerton et al., 1999; Donlan & Costerton, 2002; Watnick & Kolter, 1999). EPS may build permeability barriers or form compounds with antimicrobials, impeding their antimicrobial effect.

- The outer layers of EPS may neutralize reactive oxidants faster than they diffuse.
- Extracellular enzymatic activity within the biofilm could be sufficient to degrade antimicrobials.
- At the deepest biofilm layers, changed pH, CO2, O2 and cation concentrations may influence the antibacterial action of antimicrobial agents.
- Biofilm-associated bacterial cells may have decreased sensitivity due to altered permeability, metabolism, or growth rate.

2.1.10 Production of Antibodies

Serum immunoglobulins with a specific binding affinity for a certain antigen are known as antibodies. The conventional procedures for inducing antibodies entail immunization with filtered or moderately filtered antigen preparations, even it is possible to detect antibodies in the blood serum of people or patients who were exposed to specific infections. Although carbohydrates, nucleic acids, tiny chemical compounds (haptens) coupled to suitable carriers of proteins, and cells but cell extracts could also be utilized as antigens, proteins and peptides are the most often used antigens.

Typically, the first to know is if monoclonal or polyclonal antibodies are needed. While antibodies that are monoclonal could have exceptional specificity or could be produced for practical application but antibodies that are polyclonal are especially useful for the immunoprecipitation besides immunoblotting selection of animal species that are to be used for monoclonal antibodies. The effectiveness of vaccination depends in part on whether highly specific antibodies are mandatory, if so then genetically determined strains could be beneficial, also antibodies with high cross-reactivity.

It's also important to consider how much antibody is required. Clones of hybridomas, which are myeloma tumors that are tolerant for the formation of immunoglobulins that are monoclonal and somatic cell crossbreeds of the B cells from spleen of immunized animal. A monoclonal antibody, however, requires a significant upfront investment, whereas a polyclonal antiserum can be produced in relatively large quantities from numerous genetically similar rats or mice or a single rabbit.

The approach mentioned is applicable to larger animals like sheep or goats as well as smaller ones like rabbits, rats, mice, and hamsters. For polyclonal and monoclonal antiserum, the appropriate animals are rabbits, mice, rats, and hamsters with a few minor changes. Once a stable hybridoma line has been developed, the issue of how to produce a lot of monoclonal antibodies must be addressed (Cooper & Patterson, 2008).

2.1.10.1 Polyclonal antibodies

The term "polyclonal antibodies" (pAbs) refers to a diverse group of antibodies that are normally produced by various B-cell lymphocytes of a mammal and are targeted against various epitope of the microbial protein. A bacterium's antigenically active component, and serological testing is depended on coat protein's epitopes. As they are able to recognize and bind to various autoantigens of a particular bacterial antigen allows them to form lattices with the antigens. The most typical laboratory species used to make pAbs include rabbit, mouse, guinea pig, chicken, goat, and sheep. In comparison to other animals, rabbits are preferred because of their size and comparatively long lifespan. In order to produce polyclonal antiserum, pure bacterial preparation or bacterial protein is injected intramuscularly or intravenously weekly into animals (Hu et al., 2014). Animals are bled to collect antiserum carrying antibodies for use in serological assays. pAbs are commonly employed for certain bacteria identification including the serotype of *Vibrio cholerae*.

2.1.10.2 Selection of Animal to Prepare Polyclonal Antisera

Antibody formation is a process that requires desired antiserum. It’s also important to have evolutionary space between species from where the required protein needs to be produced. Also, the species of that said animal should immunize as well as previous exposure to the immunizing agent also play a role in this regard. Due to their genetic differences from the sources of the proteins most frequently examined in humans are mice and rabbits. Rabbits can bleed up to 25 cc of serum per time without suffering any discernible negative consequences. Mouses that are inbred are possibly the ideal method for research that are smaller in scale. These also rely on carefully determined antibody requirements. Because mice are smaller than humans, far less antigen suspension is required for immunization, as well as the maximum volume of serum can be extracted from a sole bleed is 0.5 ml. When more serum is required or when the increased evolutionary distance is favorable, rats and hamsters may be employed. These species can yield as much as 5 ml of serum with repeated bleeding.

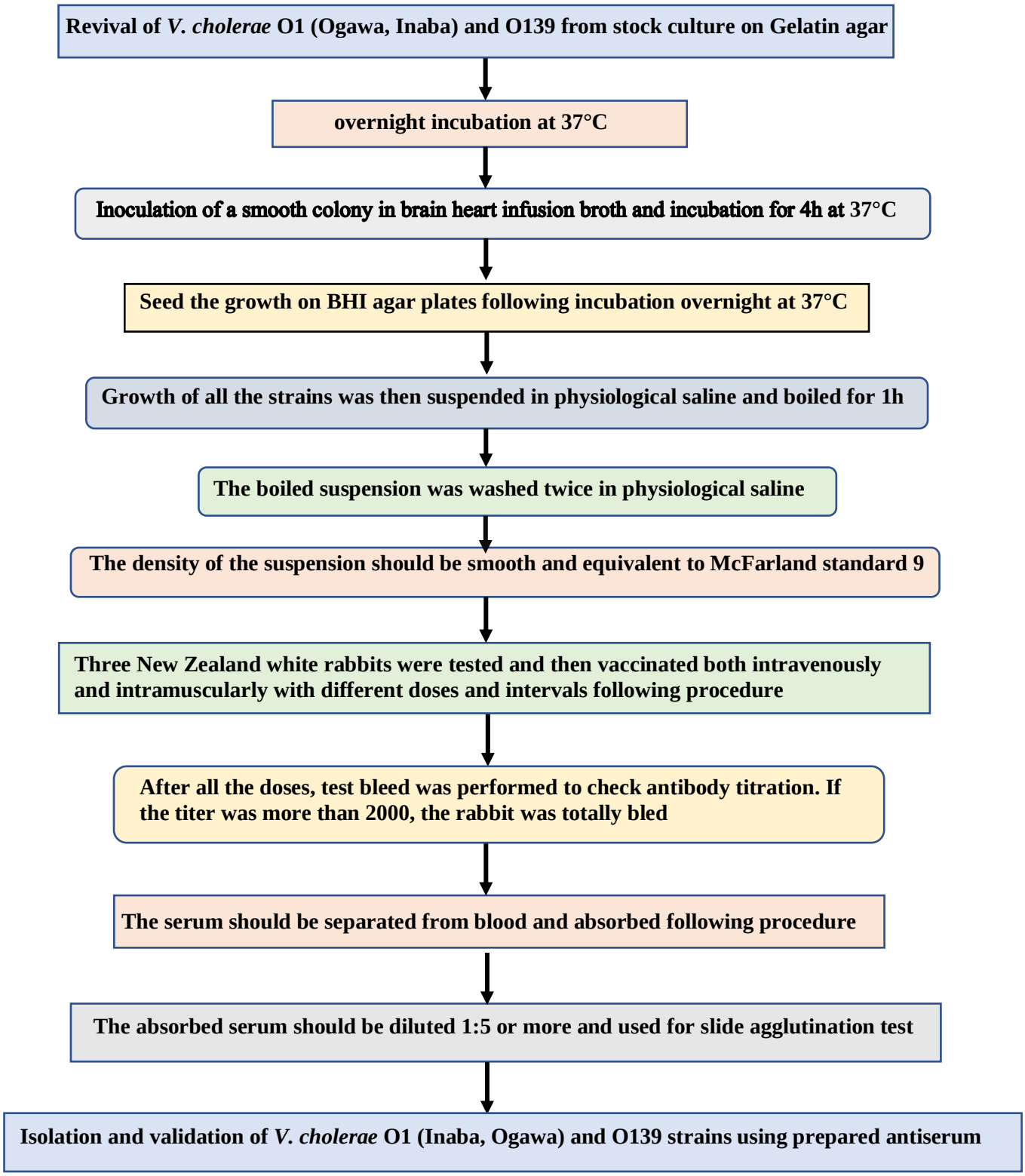
In general, the best sources of specific antisera are produced from New Zealand rabbits that can be red or white in color, since each bleed can provide 30 to 50 ml of whole blood. One rabbit can give a consistent source of a particular antiserum over time with booster treatments because they can live for 5 to 6 years. In this sense, the prescribed intervals between booster shots are not crucial;

following the initial and secondary booster injections, the animal may rest for a few months before receiving a further booster. However, in order to guarantee a high titer, blood collection must be done 7 to 14 days following each booster (Cooper & Patterson, 2008).

CHAPTER THREE: MATERIALS AND METHODS

3.0 Materials and methods

The entire study methodology is briefly given in the flowchart below



This experiment was carried out at the Laboratory of Environmental Health icddr, b. This investigation was aimed to prepare polyclonal antisera against pathogenic *Vibrio cholerae* in order to make the detection fast and cost effective amidst an ongoing epidemic. All equipment, media composition and reagents used in this study are given in the appendix I, II and III respectively.

3.1 Collection and revival of *V. cholerae* strain

The stock cultures of pathogenic *vibrio cholerae* strains were maintained at room temperature in T1N1 agar. There were stock cultures available in the clinical microbiology lab of icddr, b, so it was collected from there. The strains were directly taken from there with a sterile loop and streaked on three different Gelatin Agar plates followed by incubation overnight at 37°C. Then the plates were observed for the appearance of colonies of *V. cholerae*. That's how the pathogenic *V. cholerae* strains were revived from storage.

3.2 Preparation of vaccines from the strains

The *Vibrio cholerae* strains that were used for antiserum production are as follows:

1. For antiserum against *V. cholerae* O1 Ogawa (Classical) clinical isolated strain "E.16434" was used.
2. For antiserum against *V. cholerae* O1 Inaba (Classical) clinical isolated strain "G-146" was used.
3. For antiserum against *V. cholerae* O139 clinical isolated strain "AV-71044" was used.

To prepare the vaccines, at first, the clinical strains were revived, and a smooth colony was streaked on Brain Heart Infusion (BHI) agar followed by incubation at 37°C for better yield. The cells were then scraped out with a sterile loop and suspended in normal saline. After accumulating enough cells, the test tubes were boiled for an hour in a heat block in 110°C. The boiled suspension was mixed well in a vortex machine and washed twice in normal saline. Then the density of the normal saline was measured to be equivalent to McFarland standard 9. The vaccine doses were injected on day 1, day 3, day 6, day 9 and day 12 respectively. A booster dose was given on day 19^{th}.

3.3 Model animal selection

Animals are crucial in research studies to develop medical treatments, produce pharmaceuticals and vaccines, assess the toxicity or poisonousness of the medicine, and confirm the value and welfare of medicinal products that are intended to benefit humans. Research studies on animals and living creatures have been carried out since 500 BC (Ch, 2016). Animal models are essential not just for people but also for treating animals because many human diseases can occasionally pose a harm to them (Barré-Sinoussi & Montagutelli, 2015). Depending on the type of studies, different types of animals are used.

The study aimed to produce polyclonal antisera to *Vibrio cholerae* O1 and O139 by vaccinating three New Zealand white rabbits both intravenously and intramuscularly at the same time with different doses and intervals to perform identification of pathogenic *Vibrio cholerae* fast and cost effectively from recent cholera outbreak in Bangladesh. The three New Zealand white rabbits were sourced from Animal Resources Facilities, icddr, b. The rabbits were named according to the serotypes of the vaccines.

Table 3.1: Selected rabbits and their weight

Names	Weight
Inaba	2.20 kgs
Ogawa	2.25 kgs
O139	2.46 kgs

The rabbit that was injected Inaba (O1) serotype weighed 2.20 kgs, Ogawa (O1) serotype weighed 2.25 kgs and O139 serotype weighed 2.46 kgs. Prior to vaccination, the rabbits were weighed, and on the day before the vaccination, all the sampled animals were observed and found to be in good clinical health. Their blood samples were taken to confirm no presence of the vaccine.

In order to maintain their sterility, each rabbit was housed in a three-tiered cage alone, away from any other rabbits or animals. The temperature and humidity inside the compartment were continuously recorded. The ambient temperature was 24.21°C and there were no noticeable

variations in temperature between the measurement days. The room's air currents were moving at 0.01 m/s.

Fig 3.1: Three New Zealand White rabbits chosen and marked

3.4 Blood sample test for pre-immunization

After choosing the suitable animal to produce antisera, bloods were collected to ensure that the rabbits were not pre-immunized to *Vibrio cholerae*.

Fig 3.2: Collecting blood sample to confirm no presence of immunity against cholera

The blood samples were then taken to the lab ensuring sterility. The blood serums were collected by centrifuging them at 3000 rpms for 15 minutes and keeping them still for a few hours in room temperature allowing the blood to clot underneath. Then the serums were tested against the *Vibrio cholerae* O1 (Ogawa, Inaba) and O139 by serology.

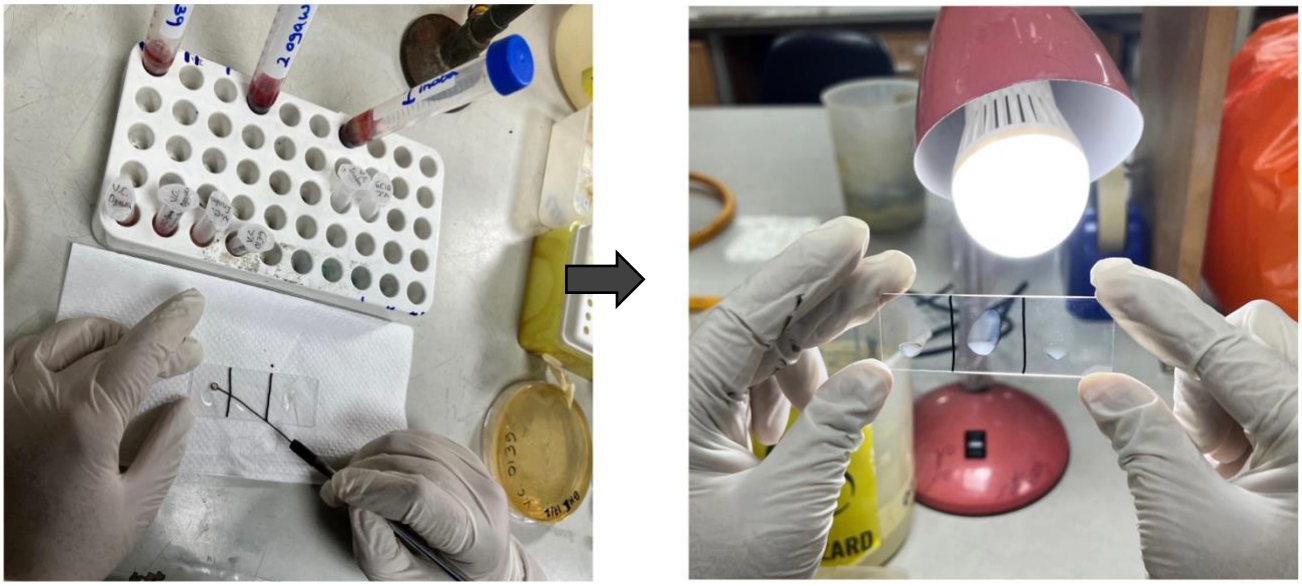


Fig 3.3: Serology testing of *V. cholerae* with pre-immunized serum shows no clotting

Here, the serum was collected in 1.5 ml tube and marked accordingly. 5 µl of serum was taken in each part of glass sides. Then, with a sterile loop, cultured O1 (Ogawa, Inaba) and O139 were mixed in each part of the serum taken on a glass slide allowing it some time to clot. In front of the lamp, we can see that no clots were formed meaning that the blood serum of the rabbits were not immune to *V. cholerae*.

3.5 Vaccine Doses and Intervals

After ensuring that the rabbits were not immune to *V. cholerae*, they were vaccinated both intravenously and intramuscularly at the same time. While vaccinating the rabbits, the sterility of all the equipment was ensured.

Fig 3.4: Rabbits are vaccinated intravenously and intramuscularly in Animal Resources Facilities, icddr, b

The dosage and intervals were maintained properly following protocols. Single-use only Tuberculin Syringes were used every time to vaccinate the rabbits. Two vets from Animal Resources Facilities, icddr,b assisted the whole procedure. The following doses and intervals were maintained throughout the study (Table 3.2):

Table 3.2: Doses and intervals of the heat-killed *V. cholerae* vaccines

Days	**1**	**3**	**6**	**9**	**12**
Dose for intravenous injection (ml)	**0.1**	**0.3**	**0.5**	**0.7**	**1.0**
Dose for intramuscular injection (ml)	**1.0**	**1.5**	**2.0**	**2.5**	**2.5**

After day 12, a booster dose was given on day 19 with 1.5 ml of antigen intravenously and 3.0 ml of antigen intramuscularly. Then after 7 days 1 ml blood sample was taken to check the antibody titration. All the antisera were diluted 400 times.

To ensure high antibody titer, another booster was given on day 26 with 1.5 ml of antigen intravenously and 3.0 ml of antigen intramuscularly. Again, after 7 days, 1 ml blood sample was taken to check the antibody titration. All the antisera were diluted upto 400 times. After checking the titration, the rabbit was totally bled the next day. The serum was then separated and absorbed following the procedure.

3.6 Absorption of serum

The serum was absorbed with a rough strain of *V. cholerae* to remove cross reacting antibodies. For the absorption of Inaba serum, the rough strains of Ogawa bacteria and vice versa should be used. For the absorption of O139 either Inaba or Ogawa strains can be used but, in our procedure, we used Inaba strains. The protocol for absorption is given below-

- 1.0g of wet weight of live bacteria should be added to 1.0 ml of serum
- Incubation for 1hr at 37°C, followed by incubation overnight at 4°C.
- Centrifugation at 8000 rpm for 5 minutes followed by removing the pellet.
- The first, second and third steps should be repeated with boiled cells.
- Lastly, membrane filtration (with 0.45 μm pore size membrane) should be done to absorb the serum.

3.6.1 Preparation of Cells

To absorb produced serum, a huge number of cells needed to be cultured. Hence, we used BHI broth for enrichment of cells. We used 500 ml sterile plastic bottle. BHI broth media was prepared following procedure. The broth was poured in 10-12 plastic bottles inside biosafety cabinet and then they were sent to a shaking incubator for 18h-24h at 37°C maintaining 120 rpm. We kept the cap of the bottle slightly loose as *V. cholerae* is facultative anaerobe.

Fig 3.5: Enriched *V. cholerae* in BHI broth after overnight incubation

Inaba, Ogawa cells were enriched this way. After that, we poured the enriched medium in 6 falcon tubes of 50 ml each time for centrifugation at 4000 rpm for 15 minutes to acquire cell pellet. The supernatants were discarded with bleach to kill the remaining cells in the supernatant.

Fig 3.6: 50ml falcon tubes in centrifuge machine

3.6.2 Absorption in live cells

The serum was mixed and vortexed with the live cells and then incubated at 37°C for 1 hr. After that it was kept overnight at 4°C. The mixture was then moved to 1.5 ml microcentrifuge tubes following centrifugation at 8000 rpm for 5 minutes to remove the pellet.

Fig 3.7: Eppendorf tubes in centrifuge machine to remove the pellet

3.6.3 Absorption in dead cells

To prepare dead cells, we mixed the live cells with distilled water. Mixed and vortexed them well and transferred them in glass test tubes. Then they were placed in heat block at 110°C for 1hr to heat kill the cells. Then, following centrifugation, we discarded the supernatant and mixed the serum with the pellet (dead cells). Then they were incubated at 37°C for 1hr. After 1hr it was held overnight at 4°C. Then the mixture was transferred in 1.5ml microcentrifuge tubes following centrifugation at 8000 rpm for 5 minutes to remove the pellet.

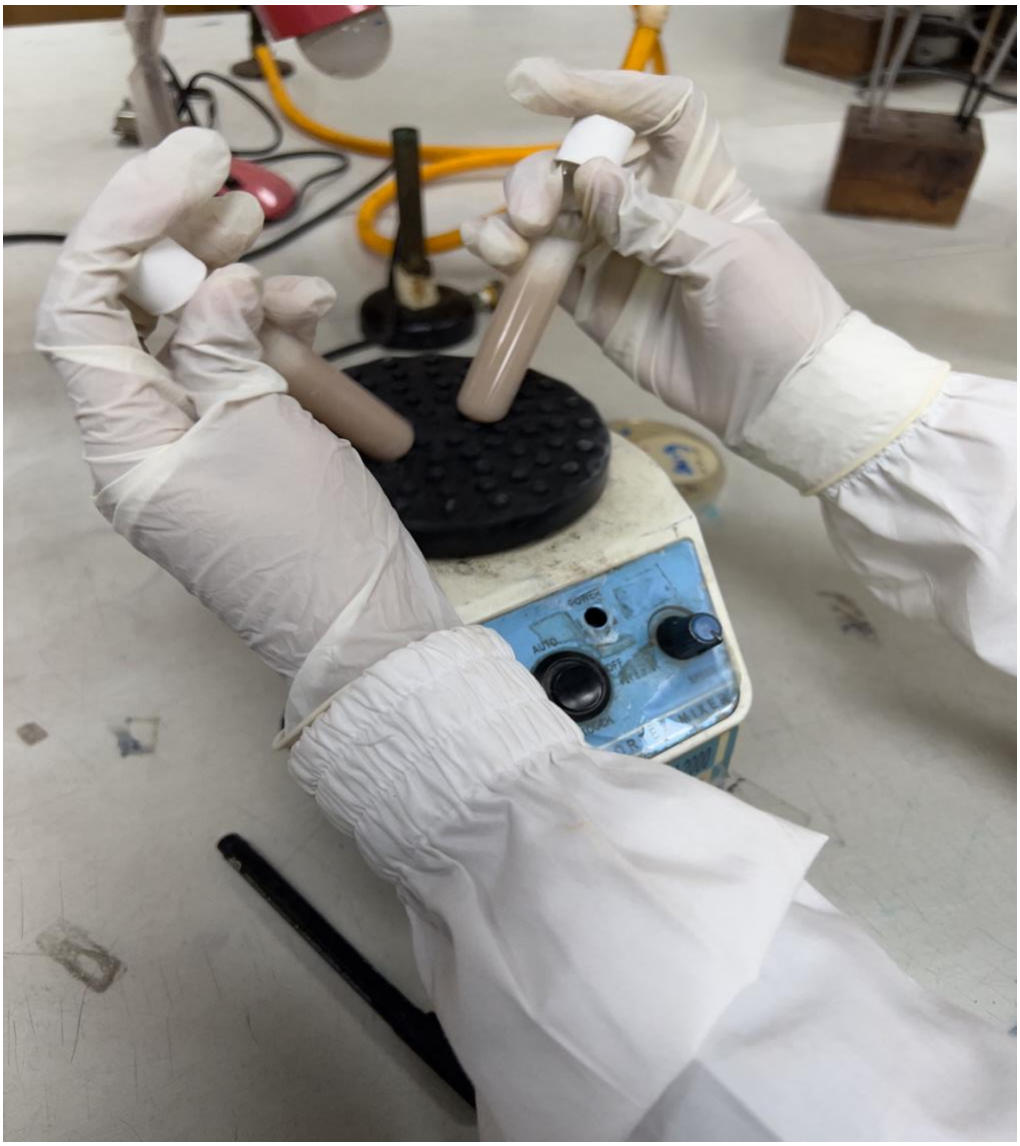


Fig 3.8: Mixing live cells and distilled water in a vortex machine

3.6.4 Filtration of the serum

After absorbing the serum in live cells and boiled cells, the serum was further absorbed in the membrane filter. For this, a membrane filter of mdi syringe filter with 0.45µm pore size and 25mm in diameter was used. The membrane filter was joined with disposable syringe and pushed through the filter to get the absorbed serum. The resulting filtrate contained the desired anti-sera.

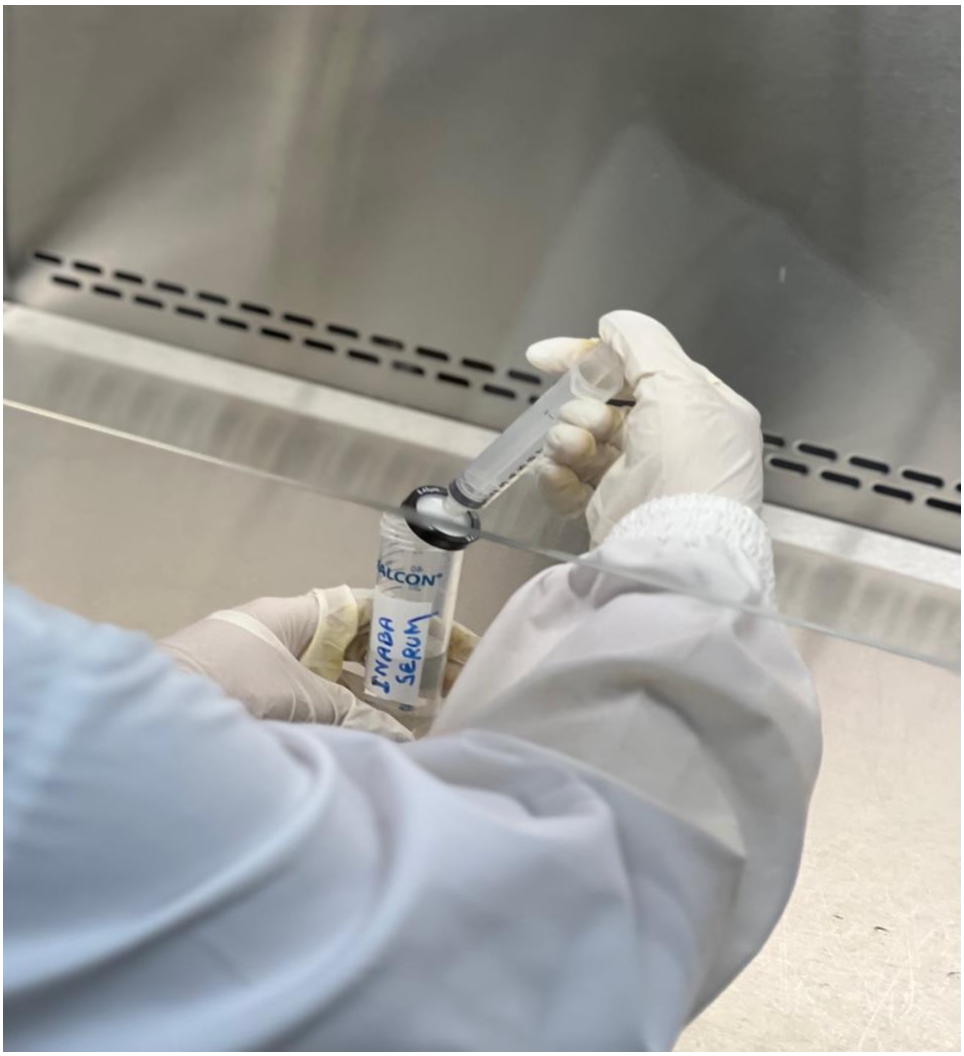


Fig 3.9: Absorption of serum with filter membrane

3.7 Isolation and validation of *V. cholerae* strain using prepared Antiserum

3.7.1 Media Preparation

3.7.1.1 Alkaline Peptone Water (APW): The water samples were initially enriched in 3X APW (Alkaline Peptone Water) media in order to isolate *V. cholerae*. This media is composed of 1L of distilled water, 30g of peptone, and 30g of NaCl. Using NaOH, the pH of this medium was brought down to 8.8. The media was then autoclaved at 121°C for 15 minutes at 15 lbs of pressure to sterilize it.

3.7.1.2 Gelatin Agar (GA) Medium: To prepare this medium 30g gelatin, 10g peptone, 10g NaCl and 20g agar was mixed in 1L distilled water. After mixing the components, the pH was adjusted to 7.4 followed by boiling. Then the media was sterilized by autoclaving at 121°C for 15minutes at 15 lbs pressure. GA is a nonselective medium for *V. cholerae* which is indicated by the production of an opaque zone around colonies which resembles a halo (Kay et al., 1994).

3.7.1.3 Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar Medium: A selective differential media to isolate and grow Vibrio organisms from samples is the TCBS agar medium plate. Here, we can confirm if the sample belongs to Vibrio species by preparing the media with BD Difco TCBS agar. *Vibrio cholerae* usually appears medium and yellow in TCBS agar as they can ferment sucrose. *Vibrio vulnificus* appear yellow or translucent colonies and *Vibrio parahaemolyticus* appear colorless colonies with a center that is green.

3.7.1.4 CHROMagar Vibrio Agar (CVA): CHROMagar Vibrio is another ready-made medium mixture that we prepared to isolate and detect *V. cholerae, V. parahaemolyticus* and *V. vulnificus* after mixing and autoclaving at 121°C for 15minutes at 15 lbs pressure.

3.7.2 Sample Processing and Isolation

To isolate *V. cholerae*, we took 42 environmental samples from ponds near Rohingya Refugee Camp that were suspected to cause cholera disease. For analysis, the samples were put and allowed to come to room temperature before being homogenized by a vortex machine. The samples were then enriched in APW media. The ratio of APW to sample here is 1:2. In a 50ml falcon we took 10 ml APW and 20 ml sample. The falcon tubes were incubated at 37°C for 18h-24h. Following the incubation period, the falcon tubes that had opaque APW consistency were taken as *V. cholerae* suspected samples. They were further streaked on TCBS agar plate.

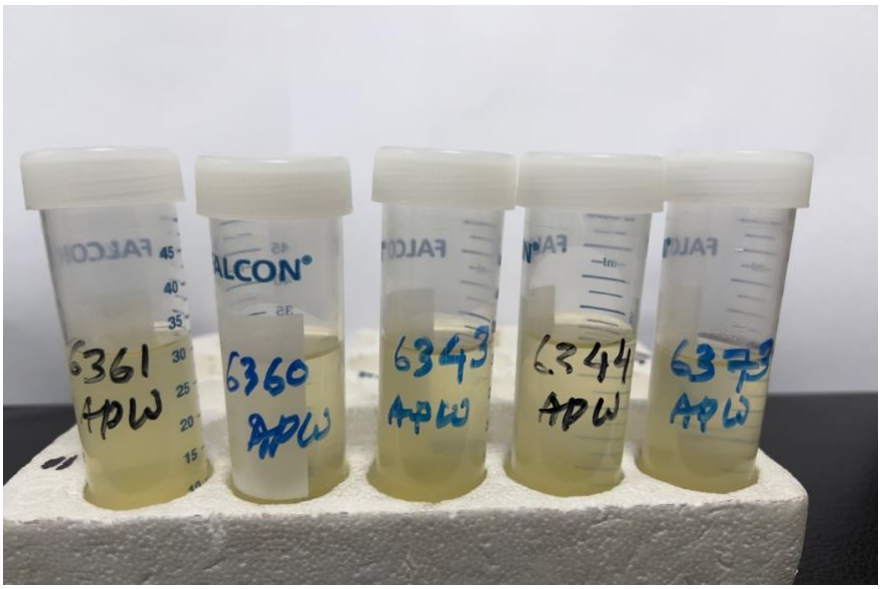


Fig 3.10: Samples in Alkaline Peptone Water for enrichment

The suspected *V. cholerae* samples were further streaked on TCBS agar plate. After incubation, medium and yellow colonies were considered as putative *V. cholerae.* Following incubation, *V. cholerae* colonies were counted. Further, five single colonies from each plate were picked and patched on CHROMagar Vibrio, TCBS agar media and GA media, one colony in one slot at a time, and incubation temperature was at 37°C for the period of 18 h-24 h. Growth and distinctive colony morphology on these media confirmed the presence of *Vibrio cholerae.* Medium and yellow colonies on TCBS, Green blue to turquoise blue in CVA and white colonies with opaque zones around due to gelatinase activity in GA media were considered as *V. cholerae*. Then these confirmed colonies were chosen for molecular characterization.

3.8 Molecular Confirmation

A single or a few copies of DNA can be amplified across multiple orders of magnitude using the biochemical technique known as polymerase chain reaction (PCR), which was created in 1983 by Kary Mullis. This technique can produce copies of thousands to millions of a specific DNA sequence. The process depends on thermal cycling, which involves repeatedly heating and cooling the reaction to cause DNA melting and enzymatic DNA replication. The DNA polymerase (after which the technique is named) and primers, which are short DNA fragments with complementary

sequences to the target region, are essential elements to permit selective and recurrent amplification. The DNA produced during PCR is employed as a template for replication as the process moves on, starting a chain reaction in which, the DNA template is exponentially amplified. Usually, it takes 20 to 35 cycles of PCR to yield enough amplified product for it to be seen on stained gel comprised of ethidium bromide. To identify pathogenic and non-pathogenic O1 and O139 serotypes PCR technique is performed.

3.8.1 Requirements for PCR

Reagents essential for performing PCR are listed in Table 3.4

Table 3.3 Reagents Used in PCR

No	Reagents
i.	Target DNA
ii.	PCR buffer
iii.	MgCl2
iv.	dNTPs (dATP, dTTP, dCTP and dGTP)
v.	Primers
vi.	Taq DNA polymerase
vii.	Nuclease Free Water

3.8.2 Boiled DNA template preparation for PCR

The DNA extraction by boiled method is mentioned below (Hossain et al., 2021)-

- Label the Eppendorf tube accordingly
- Take 600µl of autoclaved DI in the Eppendorf tube
- Take a loopful of colony from the culture plate into 600µl of autoclaved DI and mix well
- Subject to heat treatment for 10 mins
- Subject to chilling for 10 mins
- Centrifuge at 13000 rpm for 7-8 mins
- Take 100µl of supernatant from the top and put into fresh 1.5ml Eppendorf tube

3.8.3 Preparation of Reaction Mixture for PCR

The components of the PCR reaction mixture were combined at the specified quantities listed in Table 3.4. Separate primer sets were utilized for each test. The reaction mixture with the template DNA was placed in a sealed PCR tube, and the entire thing was briefly spun down in a centrifuge. The heat cycler was then used to put the PCR tubes (BIO RAD). Tubes were kept at -20°C until further analysis after PCR was completed.

Table 3.4: Components of PCR reaction mixture

Component	Volume
Template DNA	**2µl**
2X Green Master mix	**12.5µl**
***toxR* F**	**1µl**
***toxR* R**	**1µl**
***ctxA* F**	**1µl**
***ctxA* R**	**1µl**
Nuclease Free Water	**6.5µl**
Total Volume	**25µl**

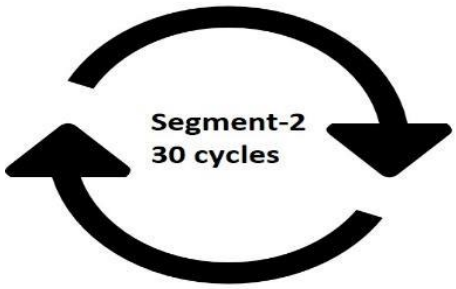
3.8.4 Multiplex PCR for detection of *toxR* and *ctxA* genes to identify pathogenic/non-pathogenic O1 and O139

With the help of two sets of primers, a multiplex PCR was carried out to find out the existence of the virulence gene *ctxA* and the species-specific *toxR* gene of *V. cholerae* in our samples. The quantities listed in Table 3.4 were used to create a master mixture that was used for both test strains at once. A PCR tube sized 0.2 ml was used to run PCR. The following PCR process specification, which is listed in Table 3.5, was applied to the reaction mixture, resulting in 35 cycles of amplification. DI was used as the negative control, and *V. cholerae* O1, E.16434 was utilized as the positive control.

Table 3.5: Primers used to determine the presence of *V. cholerae* O1 and O139 serotype

Primer name	Primer Sequence	Band Size
***toxR* F**	**CCTTCGATCCCCTAAGCAATAC**	**779bp**
***toxR* R**	**AGGGTTAGCAACGATGCGTAAG**	
***ctxA* F**	**CGGGCAGATTCTAGACCTCCTG**	**564bp**
***ctxA* R**	**CGATGATCTTGGAGCATTCCCAC**	

Table 3.6 PCR conditions for *toxR* and *ctxA* multiplex reactions (Mehrabadi et al., 2012)

Process	Temperature (°C)	Time	Segment
Initial denaturation	94°C	5min	Segment 1
Denaturation	94°C	1 min	
Annealing	57°C	1 min	
Extension	72°C	2 min	
Final extension	72°C	10 minutes	Segment 3

3.8.5 Post-amplification finding: Agarose gel electrophoresis

The method for agarose gel electrophoresis was used for the post-amplification detection of PCR. By resolving the PCR result in 1% agarose gel produced with 0.5X TBE buffer, the successful amplification was evaluated. Ethidium bromide (EtBr) was used to stain the gel and imaged in a UV-transilluminator following migration for approximately 2.5 hours at 60 volts (Appendix I). GeneRuler 100bp plus DNA ladder (Thermo scientific) was always run concurrently with the test samples during migration in order to determine the size of the DNA band(s) in the PCR product (Ansaruzzaman et al., 2007). After migration, DNA band(s) with the amplicon size(s) stated above

were regarded as successful amplification of the particular genes. The positive results were further subjected to serotyping.

3.9 Serological assay of the molecular confirmed *V. cholerae*

After molecular confirmation the confirmed colonies were tested with the prepared antisera. Serological analysis was carried out in a microscope glass slide in the size of 25X75mm and 1mm thick (Thermo Scientific). The glass slide was divided in 5 sections consisting of Ogawa, Inaba and O139 antisera and positive and negative control.

CHAPTER FOUR: RESULTS

4.0 Results

The present study was designed to produce polyclonal antisera against *V. cholerae* and isolate *V. cholerae* to check the efficacy and validate the prepared antisera. 42 environmental samples were collected from ponds near Rohingya Refugee Camp that were suspected of having *V. cholerae* contamination and their molecular confirmation was done by PCR.

4.1 Sample Processing isolation, identification, and determination of *V. cholerae*

Samples were first enriched in APW. The samples having growth in APW media showed opaque concentration following incubation. Those samples were then taken and sub-cultured on TCBS agar media.

Fig 4.1: Medium sized yellow colonies on TCBS agar plate is indicative of *V. cholerae*

Following streaking on TCBS agar plate, 5 colonies were taken from each plate and patched on GA, CVA and TCBS media to further confirm the existence of *V. cholerae*. In GA media *V. cholerae* is indicative of a zone that is opaque around colonies which resembles a halo. In TCBS, it's indicative of medium yellow colonies and in CVA the green blue to turquoise blue colonies is considered as *V. cholerae.*

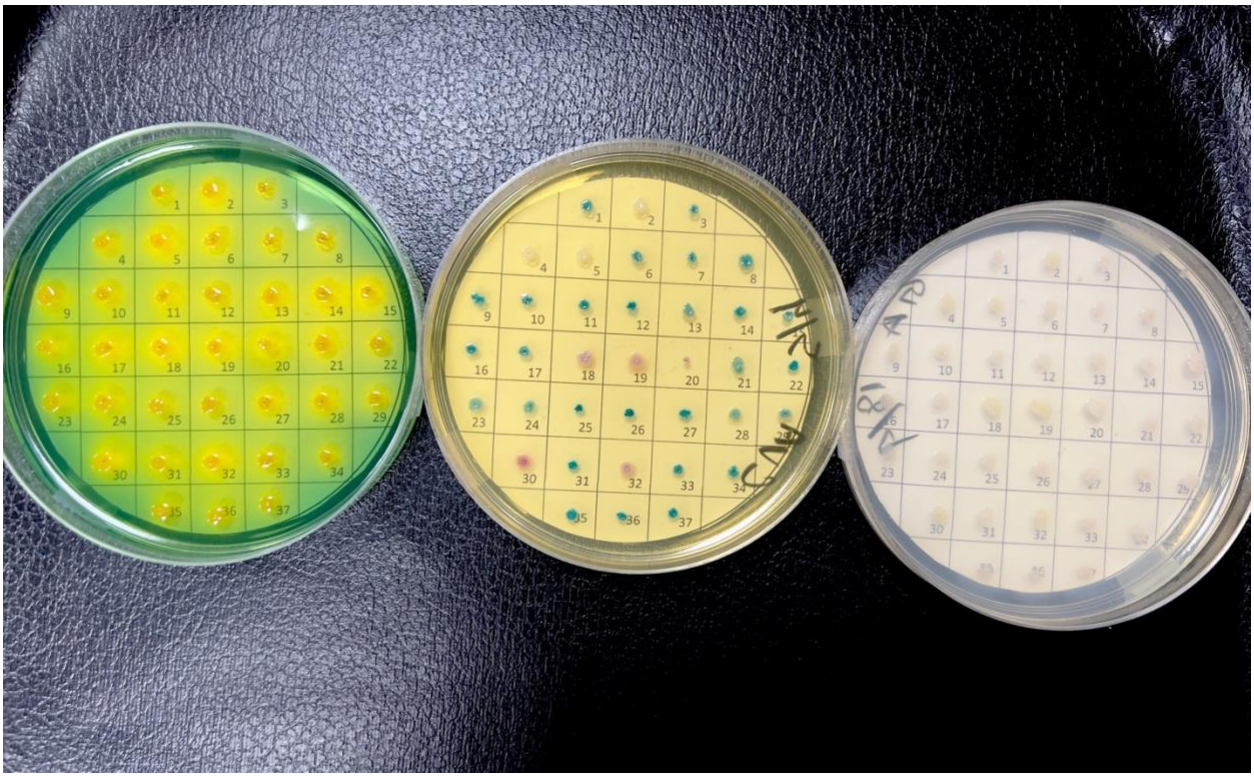


Fig 4.2: *V. cholerae* on TCBS, CVA and GA plate. Here, the slot 13 is the positive control and the slot 15, 23, 24, 28, 29 indicates putative *V. cholerae* due to their growth and distinctive color

4.2 Detection of *toxR and ctxA* genes by PCR

The test strains were subjected to multiplex PCR targeting two genes named *toxR* and *ctxA. toxR* is indicative of *V. cholerae* and *ctxA* gene is responsible for Cholera toxin. After performing the multiplex PCR, it was found that the strains contained *toxR* gene meaning that the strains were

positive for *V. cholerae*. As they didn't have the disease-causing gene means they were non-pathogenic.

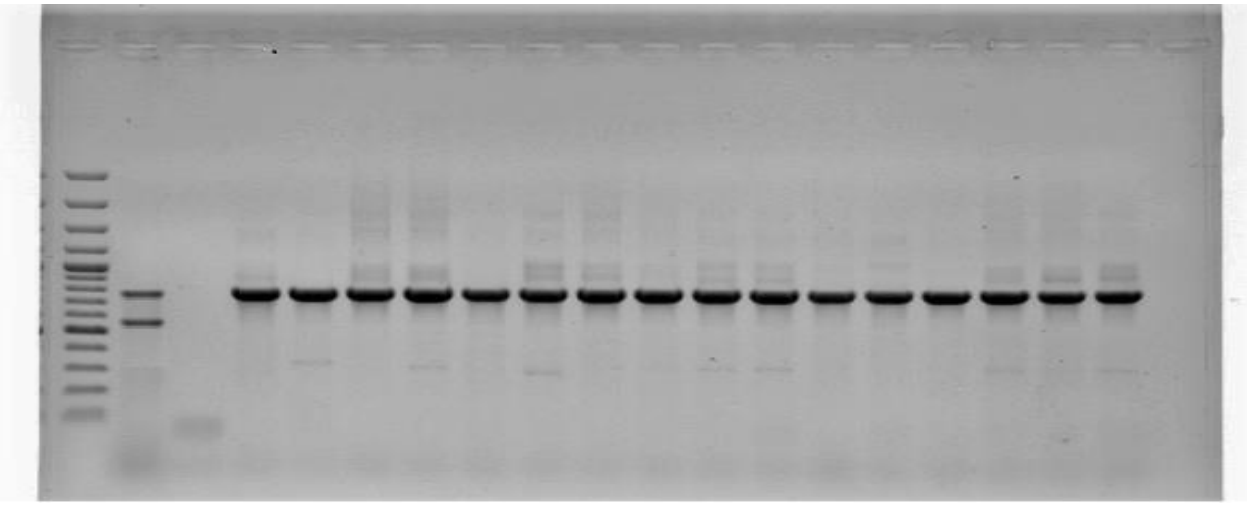


Fig 4.3: *toxR* and *ctxA* genes showed after running agarose gel electrophoresis of multiplex PCR. Lane 1 shows 100 bp plus DNA ladder and lane 2 shows 779 bp and 564 bp amplicons of these genes of *V. cholerae* O1, E.16434 (classical) control strain. Lane 3 represents no template control. Lane 4 to 19 show 779bp *toxR* genes for our samples.

4.3 Antibody titration

7 days after the first booster dose injected in the rabbits, 1 ml blood sample was drawn from each rabbit to check the antibody titration. All the antisera were diluted 400 times. The results are as followed (Table 3.2)-

Table 4.1: Antibody titration checking up to 400 times diluted antiserum (After 1st booster)

Antisera	Dilution factor- 100 times	Dilution factor- 200 times	Dilution factor- 400 times
Inaba	✓ **(Weak Ogawa serotype clotting)**	✓ **(No Ogawa serotype clotting)**	✓
Ogawa	✓	✓ **(Very weak)**	✗
O139	✓	✗	✗

To get higher titration another booster dose was given after the first one. Then after 7 days, 1 ml blood sample was taken to check the antibody titration. All the antisera were diluted upto 400 times. The results are mentioned in table 3.2-

Table 4.2: Antibody activity checking up to 400 times diluted antiserum (After 2nd booster)

Antisera	Dilution factor- 100 times	Dilution factor- 200 times	Dilution factor- 400 times
Inaba	✓	✓	✓
Ogawa	✓	✓ **(weak)**	✗
O139	✓	✗	✗

4.4 Serum collection

After confirming a high titer, the rabbit was totally bled to collect the blood serum with our desired antibody. From each rabbit different amount of blood serum was collected and the results are given below-

Table 4.3 Total count of blood and serum from the rabbits

Vaccines Injected	Blood Collected	Serum Obtained
Inaba	**49 ml**	**26 ml**
Ogawa	**53 ml**	**27 ml**
O139	**57 ml**	**28 ml**

4.3 Serotyping of confirmed isolates

After confirming the presence of *toxR* gene in agarose gel electrophoresis, we tested our prepared *V. cholerae* anti-sera on those isolates obtained from the environment.

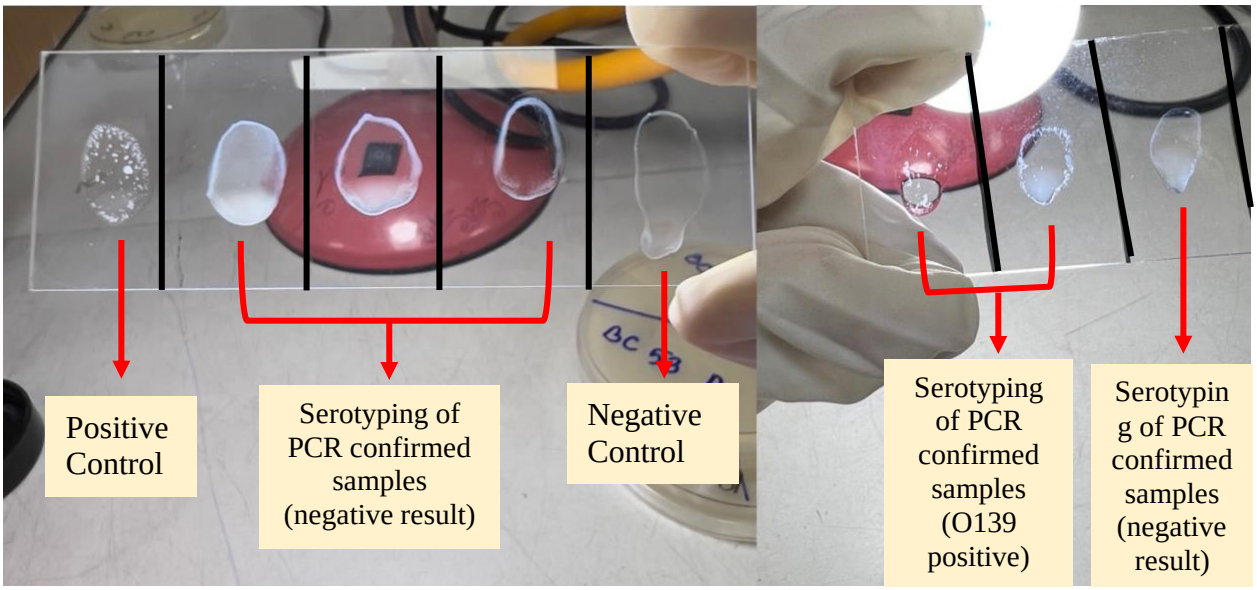


Fig 4.4: *V. cholerae* polyclonal anti-sera is taken in eight slots. The first one has positive control, and the fifth slot has negative control. 2nd, 3rd, 4th and 8th slots did not agglutinate with the anti-sera. 6th and 7th slots agglutinated with O139 antisera.

CHAPTER FIVE: DISCUSSION

Discussion

Since cholera originated on the Indian subcontinent and expanded to other nations in Asia, Africa, and Hispaniola, it continues to have an impact on a number of nations worldwide. Acute watery diarrhea is a defining symptom of the illness, which is brought on by intestinal infection with *Vibrio cholerae*, serogroup O1 or O139. If left untreated, severe cases of this can result in fast increasing severe dehydration and, in some circumstances, death within a few hours (Sack et al., 2021). Besides pathogenic serotype of *V. cholerae* O1 and O139, there are also nonpathogenic O1 and O139 serotype of *V. cholerae* that participate showing syndromes of cholera disease. The mechanism of these kind of virulent cholera also plays an important role as they have the capacity to flare into a localized outbreak (International Centre for Diarrhoeal Diseases Research et al., 1993) and they can also be identified by the anti-sera. Research on cholera is still going on and anti-sera of the bacteria *V. cholerae* happened to be an important component regarding this factor.

During the study, using conventional culture technique 42 suspected *Vibrio cholerae* were isolated. Among them, 36 isolates showed gelatinase activity and were oxidase positive. Depending on the colony morphology and gelatinase activity isolates were collected. All the isolates were subjected to standard biochemical tests designed to identify *Vibrio cholerae*. By conventional biochemical tests, 26 were found positive as *Vibrio cholerae*. Then for molecular confirmation, multiplex PCR was performed. Among them 18 isolates had *toxR* genes present but *ctxA* gene was absent confirming that they were nonpathogenic O1/O139.

In our study, all the 18 isolates were subjected to serological analysis by slide agglutination test, using polyclonal and specific antisera *V. cholerae* O1 and O139. Among them 3 isolates were found to agglutinate with *V. cholerae* O139 specific polyclonal antisera. If a filamentous bacteriophage infects *V. cholerae* and also exist on chromosome of toxic strains but absent in strains that are non-toxic (Boyd et al., 2000; Faruque et al., 1998), *ctx* genetic elements are detected (Mekalanos et al., 1983). In our isolates the *ctxA* genes were not found hence the isolates were confirmed as non-O139. Most *V. cholerae* serotype, particularly those from environmental samples, have been discovered to not have the genes needed to make cholera toxin (CT), and the potential for exchanging the genetic in the ecosystem makes the creation of new clones, that are toxigenic, possible.

After administration of heat-killed *V. cholerae* vaccines, the B cells that are antigen-stimulated proliferate to produce a significant memory B cell in the rabbit, which rapidly became activated as the booster injection was administered. As a result, after receiving a booster shot, there is a shorter delay before the appearance of the particular antibody than there was after receiving the initial immunization. Additionally, a noticeably greater titer of a particular antibody is produced and maintained for a longer time. 7 to 14 days after booster, antibody production reaches its peak. Because memory B cells exist, a less amount of antigen is needed to elicit a potent secondary reaction. A specific antibody response can be evoked up to six months or a year after the final booster since memory B cells have a long lifespan (Cooper & Patterson, 2008).

In general, the rabbits known as New Zealand rabbits that can be either red or white in color make the finest sources of particular antisera since each bleed can yield 30 to 50 ml of entire blood. One rabbit can give a consistent source of a particular antiserum over time with booster treatments because they can live for 5 to 6 years. In this sense, the prescribed intervals between booster shots are not crucial; following the initial and secondary booster injections, the animal may rest for a few months before receiving a further booster. However, in order to guarantee a high titer, blood collection must be done 7 to 14 days following each booster. The most preferable negative control is preimmunize serum from the same animal. If more control sera are necessary, pooled serum from naive animals or immune serum from animals inoculated with completely unrelated antigens will be sufficient. (Cooper & Patterson, 2008).

Adjuvant research has focused on developing potent adjuvants that lessen animal trauma throughout the past ten years. Less-noxious adjuvants should be utilized whenever possible to lessen animal pain, as stated in the alternate protocol. Additionally, Complete Freund's adjuvant (CFA) usage in lab animals is restricted in some nations. The results vary greatly as of issued comparisons of profitable adjuvants with fundamental Complete Freund's adjuvant (CFA) / Indirect Fluorescent Antibody (IFA) procedure. Contrarily, CFA/IFA typically results in larger titers of more potent antibodies in less period (Chen et al., 1992).

By using *V. cholerae* antisera, it is possible to identify the pathogenic strain of *V. cholerae* faster. As around 75% of cholera cases were thought to be subclinical (Merson, 1978), it became critical to ensure procedures for identifying *V. cholerae* serotype O1 and O139 as they are sensitive and repeatable which can be achieved by the anti-sera.

CHAPTER SIX: CONCLUSION

6.0 Conclusion

This study and procedure aimed to demonstrate how to prepare and validate the anti-sera locally in the labs. The samples we used to validate the produced antisera were environmental collected from a pond from the Rohingya Refugee camp. After confirming the molecular characterization and serology tests, non-pathogenic *V. cholerae* O139 was discovered among all the isolates of *V. cholerae*. Serology testing was done by making the use of the polyclonal antisera prepared by injecting vaccines in the New Zealand white rabbits. Anti-sera are available commercially which is expensive and the whole process of bringing the product to the lab can be time consuming. Hence preparing antisera in the lab can be cost effective, save time, and can make local labs self-sufficient. The countries or local labs with limited resources for research can prepare their own anti-sera in order to continue their research.

By using *V. cholerae* antisera, it is possible to identify the pathogenic strain of *V. cholerae* faster.

Since the subclinical cholera cases are up to 75% (Merson, 1978), it's critical to ensure having procedures for identifying *V. cholerae* O1 and O139 as it can be sensitive and repeatable which is possible to achieve by the anti-sera.

CHAPTER SEVEN: REFERENCES

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APPENDICES

Appendix I

Unless otherwise mentioned, all media were sterilized by autoclaving at 121°C for 15 minutes at 15 lbs pressure. Distilled water was used for preparation of all media. The media used in this study has given below:

Media Composition

TCBS (Thiosulfate Citrate Bile Salt Sucrose Agar) Media

Ingredients	Amount (g/L)
TCBS Agar	89
Distilled Water	1 L
Agar	3

GA (Gelatine Agar)

Ingredients	Amount (g/L)
Gelatine	30
Peptone	10
NaCl	10
Distilled Water	1L
Agar	20
pH	7.4

ChromAgar Vibrio (CVA)

Ingredients	Amount (g/L)
Agar	15.0
Peptone and yeast extract	8.0

Salts	51.4
Chromogenic mix	0.3
pH	9.0+/-0.2

APW (Alkaline Peptone Water)

Ingredients	Amount (g/L)
Peptone	10
NaCl	10
Distilled Water	1L
pH	8.8

Tryptone Salt Agar (T1N1)

Ingredients	Amount
Tryptone	10g
NaCl	10g
Distilled Water	800ml
Agar	20g

Bacto Brain Heart Infusion (BHI) Broth

Ingredients	Amount (g/L)
Calf brain	7.7
Beef heart	9.8
Proteose peptone	10
Dextrose	2

NaCl	5
Disodium Phosphate	2.5
pH	7.4+/-0.2

Appendix II

Solutions and Reagents

1M Tris-HCl

121.1g tris base was dissolves in 800ml of distilled water. The pH was adjusted to the desired value by adding concentrated HCl and the final volume was made up to 1L with distilled water. The solution was sterilized by autoclaving and stored at room temperature.

TE buffer (pH 8.0)

10mM tris HCl (pH 8.0), 1mM EDTA was prepared by diluting concentrated stocks of 1M tris-HCl and 0.5 M EDTA. The buffer was stored at 4°C.

0.5 M EDTA

186.1 g Na2EDTA·2H2O and 20.0 g of NaOH pellets were added and dissolved by stirring in 800 ml distilled water on a magnetic stirrer. The pH was adjusted to 8.0 with a few drops of 10M NaOH and the final volume was made up to 1L with distilled water. The solution was sterilized by autoclaving and stored at room temperature.

MacFarland 9 Unit

1% v/v solution of sulfuric acid is prepared by adding 1 mL of concentrated sulfuric acid to 99 mL of water.

Ethidium Bromide Solution

5 µl of ethidium bromide was dissolved in per 100 mL deionized water.

Appendix III
Apparatus Used

Autoclave (Model HL-42E)	Tokyo, Japan
Centrifuge machine	Sigma, USA
Class-11 Al biological safety cabinet	Thermo Forma, USA.
Duran bottle	Scott Germany.
Falcon Tubes	Thomas Scientific
Electric balance model no. 2 1 OS	Sartorius, Germany.
Eppendrof tubes (1, 5m)	Eppendrof, Germany
Freezer (-30°C)	Thermo Forma, USA.

Fridge (4°C)	West frost.
Fridge 8°C model no. M1R-253	Japan.
Gel Documentation	Bio-Rad, USA.
Incubator	Memmert, Germany.
Incubator, WTB binder, model no. D-78502	Germany.
Glassware	Pyrex brand, USA.
Microscope Glass Slide	Thermo Scientific
Microcentrifuge, Eppendrof centrifuge	Germany.
Micropipettes	Eppendrof, Germany.
Micropipette tips	Labsystems, Finland.
Microwave oven, model no. CE2933N	Samsung, Korea
PCR machine	MJ Research.
Power supply	BIO-RAD, USA.
pH meter, model no. MP220.	Toledo, Germany,
Shaker incubator	Thermo Forma, USA.
Sterilizer	Memmert, Germany
Vortex Mixer	Fisher Brand, England.

THE END