Molecular evaluation of *Vibrio cholerae* O1 strains in India to decipher the mechanism of higher virulence and antimicrobial resistance

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BY

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CERTIFICATE FROM THE SUPERVISOR(S)

This is to certify that the thesis entitled "**Molecular evaluation of *Vibrio cholerae* O1 strains in India to decipher the mechanism of higher virulence and antimicrobial resistance**" submitted by Smt. **Sreeja Shaw** who got her name registered on **2nd November, 2020** for the award of Ph. D. (Science) degree of Jadavpur University, is absolutely based upon her own work under the supervision of **Dr. Asish Kumar Mukhopadhyay** and that neither this thesis nor any part of it has been submitted for either any degree / diploma or any other academic award anywhere before.

AKMukhopadhyay 10/10/2023

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Declaration

I do, hereby, declare that the work embodied in this thesis entitled "**Molecular evaluation of *Vibrio cholerae* O1 strains in India to decipher the mechanism of higher virulence and antimicrobial resistance**" submitted for the award of Doctorate of Philosophy (Science) in Life Science and Bio-technology, is the completion of work carried out under the supervision of **Dr. Asish Kumar Mukhopadhyay**, Scientist-F (Sr. Deputy Director), at the Division of Bacteriology, ICMR-National Institute of Cholera and Enteric Diseases, Kolkata. Neither this thesis nor any part of it has been submitted for either any equivalent degree/diploma or any other academic award elsewhere.

Date: 10.10.2023

Place: **Kolkata, India**

Sreeja Shaw

(Sreeja Shaw)

Dedicated to

my

Beloved Parents

and

Husband

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Sreeja Shaw
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Abbreviations

TCBS	*Thiosulfate citrate bile salt sucrose*
V. cholerae	*Vibrio cholerae*
APW	*Alkaline peptone water*
TSI	*Triple sugar iron agar*
TSB	*Triple sugar iron broth*
MHA	*Muller Hinton agar*
VP	*Voges Proskauer*
MIC	*Minimal inhibitory concentration*
AMP	*Ampicillin*
CHL	*Chloramphenicol*
MEM	*Meropenem*
CTX	*Cefotaxime*
CRO	*Ceftriaxone*
CAZ	*Ceftazidime*
SX	*Sulfamethoxazole*
TM	*Trimethoprim*
GM	*Gentamicin*
STR	*Streptomycin*
NA	*Nalidixic acid*
AZM	*Azithromycin*
°C	*Degree Celsius*
A_{260}	*Absorbance at 260 nm*
A_{280}	*Absorbance at 280 nm*
DNA	*Deoxy ribonucleic acid*
RNA	*Ribonucleic acid*
BSA	*Bovine serum albumin*
CFU	*Colony forming unit*
cDNA	*Complementary DNA*
CTAB	*Cetyltrimethylammonium bromide*

DMEM	*Dulbecco's modified eagle's medium*
DMSO	*Dimethylsulfoxide*
EDTA	*Ethylenediaminetetraacetic acid*
FA	*Fluid accumulation*
FBS	*Fetal bovine serum*
LB	*Luria Bertani*
M	*Molar*
ml	*Millilitre*
mM	*Milli molar*
mm	*Millimeter*
NaCl	*Sodium Chloride*
NaHCO3	*Sodium bicarbonate*
NaAz	*Sodium azide*
ctxA	*Cholera toxin A*
ctxB	*Cholera toxin B*
rtxA	*repeats in toxin*
tcpA	*Toxin coregulated pilus*
VPI	*V. cholerae Pathogenicity Island*
VSP	*Vibrio seventh pandemic islands*
ng	*Nanogram*
nm	*Nanometer*
OD	*Optical density*
PBS	*Phosphate buffered saline*
PCR	*Polymerase chain reaction*
qRT-PCR	*Real-Time Quantitative Reverse Transcription PCR*
TAE	*Tris acetate EDTA*
TE	*Tris EDTA*
µg	*Microgram*
µl	*Microlitre*
kbp	*Kilo base pair*
CRVC	*Carbapenem-resistant Vibrio cholerae*

MDR	*Multidrug resistant*
PFGE	*Pulse field gel electrophoresis*
ICEs	*Integrating conjugative elements*
MGEs	*Mobile genetic elements*
NAD	*Nicotinamide adenine dinucleotide*
ADP	*Adenosine diphosphate*
TMB	*Tetramethylbenzidine*
PolB	*Polymyxin B*
WHO	*World Health Organization*
TSS	*Transformation & storage solution*
AMR	*Antimicrobial resistance*
CAMP	*Cationic antimicrobial peptide*

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Molecular evaluation of *Vibrio cholerae* O1 strains in India to decipher the mechanism of higher virulence and antimicrobial resistance

Abstract

Vibrio cholerae, the causative agent of the diarrheal disease cholera can frequently modify their genotypic and phenotypic attributes to adapt well in the environment and cause severe outbreaks in the community. The present study aimed to characterize the changing genetic features of recently circulating *V. cholerae* O1 strains in India with special emphasis on alterations in the resistance profile towards third generation cephalosporin antibiotics.

In a study period of 4 years (2018-2021), a total of 474 *V. cholerae* O1 strains isolated from different cholera endemic states which were further classified into two groups, West Bengal (n=116) and other regions of India (n=358) (Maharashtra, Gujarat, Delhi, Tamil Nadu, and Karnataka). All the isolates from other regions of India belonged to El Tor biotype and carried the Haitian alleles of virulence-associated genes, *ctxB, tcpA,* and *rtxA* with polymyxin B-sensitive phenotype. Moreover, a deletion in the VSP II region, a remarkable trait of Yemen cholera outbreak strains has been checked by a newly developed PCR which demonstrated the presence of this deletion (VSP-IIC) within all the isolates from other regions of India. However, only 6% (7/116) of the West Bengal strains exhibited classical *ctxB* with El Tor *rtxA* along with polymyxin B-resistant, non-hemolytic trait and harboured VSP-II rather than VSP-IIC type. These strains were found to contain a new variant of *hlyA* with a 6 bp deletion in addition to the 11 bp deletion which is a characteristic feature of non-hemolytic classical *hlyA*. We have also evaluated various roles of polymyxin B-sensitive allele ($carR^S$) in comparison to the resistant allele ($carR^R$) in terms of pathogenicity and observed that polymyxin B sensitivity has been associated with higher IL-8 production in INT 407 cell line which is linked to further higher motility and adherence compared to the resistant strains. The mRNA expression study of flagellar genes *flhDC* is significantly higher in the case of polymyxin B-sensitive Haitian variant strains. However, the contribution of $carR^S$ allele in suckling mice colonization and fluid accumulation in rabbit illeal loop model has given similar results to the $carR^R$ allele.

All of the studied strains followed the antibiogram pattern similar to that exhibited by multidrug resistant El Tor vibrios. Interestingly six of the isolates from Gujarat (2019) revealed resistance against carbapenem and hence were further characterized. The whole genome sequencing of these isolates indicated the presence of a 142kb mega-sized plasmid belonging to IncA/C1 which harbours genes such as bla_{NDM-1}, bla_{CMY-6}, bla_{DHA-7}; *aph(3')-VI, aph(6)-Id, aac(6')-Ib10, aac(3)-IId, rmtC* conferring resistance against the antibiotics beta-lactam and aminoglycosides, respectively. The plasmid was conjugally transferred into *Escherichia coli*, *V. parahaemolyticus, Shigella flexneri* 4a, *Salmonella enterica*, and *V. cholerae* O1 Ogawa and depicted similar resistance patterns which may pose a high risk in the management of enteric diseases. The analysis by pulse field gel electrophoresis also showed that all of the six strains were clonally similar to each other.

This study indicated the prevalence of Haitian variant *V. cholerae* O1 strains linked to various hypervirulent features as well as the developing resistance against carbapenem antibiotics, which may aid in the spreading of this catastrophic disease. Therefore, continuous monitoring is important for better management and the control of antibiotic resistant *V. cholerae* O1 strains.

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Chapter 1

Introduction

1. Introduction

Among the various infectious diseases, diarrhea is one of the most common and important health hazards in the world, particularly in developing countries. Infectious diseases with the potential to become pandemics have consistently emerged and spread throughout the world. It continues to be the world's greatest cause of death, particularly for children under five (Feleke et al., 2022). Many of the well-known illnesses thought to be under control are reemerging and becoming causes of serious global concern. *Vibrio cholerae* is an organism contributing to this global fear of the reemerging infectious disease, cholera. An approximate 1.3-4 million cases with 21,000 to 143,000 deaths have been found to occur globally every year (Ali et al., 2015; Saha et al., 2021). The disease is manifested by voluminous watery stools and extreme loss of fluid and electrolytes that may lead to death in the absence of prompt and appropriate treatment (Kaper et al., 1995; Finkelstein RA., 1996).

V. cholerae is a Gram negative, non-spore forming, halophilic, highly motile, curved rod with a single polar flagellum, belonging to the subdivision Gamma proteobacteria and family Vibrionaceae (Baumann et al., 1984). *V. cholerae* infection begins with the ingestion of the organism, usually through contaminated food and water resulting from the poor sanitary system and inadequate water supply (Taylor et al., 2015). The incubation periods for these organisms vary depending on the inoculum and the susceptibility of the person who has been exposed and it varies between 12 to 72 hrs (Levine et al., 1981). Following oral consumption, it colonizes the small intestine by overcoming the acidic barriers of the stomach with the help of major colonization factor called toxin coregulated pilin (TcpA) associated with other factors such as mannose sensitive hemagglutinins (MSHA), core-encoded pilus (*cep*), and accessory colonization factor (*ace*) (Kaper et al., 1995; Taylor et al., 1987). A number of signaling cascades become activated and secrete the potent ADP-ribosylating exotoxin known as cholera toxin (CT) (Taylor et al., 1987; Kaper et al., 1995). Thereafter, cholera toxin binds to the GM_1 receptors localized on the intestinal epithelial cell surface and successively results in loss of electrolytes and water from the cell to the intestinal lumen and causes watery diarrhea, characteristics of the onset of the disease cholera. CT is encoded in the genome of a temperate bacteriophage (CTXΦ) (Waldor et al., 1997). CTXΦ infects the bacterium, causes lysogenic conversion, and helps in the production of cholera toxin. Non-toxigenic strains of *V. cholerae* become toxic after acquiring genes encoding TCP and CT during evolution.

More than 200 serogroups of *V. cholerae* have been identified based on their lipopolysaccharide structure (LPS), but only serogroups O1 and O139 are capable of causing epidemic and pandemic cholera. Serogroup O1 is comprised of two biotypes, classical and El Tor based on some phenotypic attributes (sheep blood hemolysis, chicken erythrocyte agglutination, Voges Proskauer test, polymyxin B susceptibility and phage sensitivity assays) and three serotypes, Ogawa, Inaba and Hikojima, based on the variations in antigenic determinants. *V. cholerae* strains often undergo shifts between the two serotypes, possibly due to immune pressure within the population. Historic events suggest that the isolates, which prevailed in earlier decades, were of the classical biotype being responsible for the first six pandemics. The potential of cholera to manifest as explosive outbreaks and its ability to cause major pandemics that progressively affect numerous countries across many continents over a prolonged time course are two distinguishing epidemiologic characteristics of the disease. Since 1817, the world has witnessed seven pandemics of cholera. The Indian subcontinent was the origin of almost every cholera pandemic phase. The persistence of cholera in Asia has been long-standing. The current ongoing seventh pandemic of cholera first originated in Indonesia in 1961 with the introduction of a newer biotype, El Tor. After that, it continuously underwent different genetic modifications and thus evolving as new variants, which have disseminated in different geographical locations and finally they incorporated in the continents of Asia, Africa, Australia, Europe, and Americas (Mutreja et al., 2011; Weill et al., 2017; Domman et al., 2017; Mashe et al., 2020). Until 1992, epidemic cholera was believed to be caused by only the O1 serogroup. In 1992, a large cholera outbreak occurred in Madras that subsequently spread to Bangladesh, Pakistan, Thailand, and Indonesia. The causative agent of this outbreak has been recognized to belong to a new serogroup and have been demarcated as O139 and synonym Bengal, after its place of origin. This new organism has appeared to be hybrid of O1 and non O1 with El Tor characteristics like the production of CT and possession of TCP (Rhine and Taylor, 1994) and typical non-O1 characteristics of polysaccharide capsule production. However, this organism lacks O1 LPS along with some genetic components important for O1 antigen (Manning, et al., 1994). However, it was restricted to South Asia and has not spread so far (Siddique et al., 2010).

Several recent events marked the epidemiological importance of this disease. During 2010, a catastrophic outbreak of cholera hit the Caribbean country Haiti which affected more than 500,000 people and among them, around 8000 died (Chin et al., 2011; Ghosh et al., 2014a). Cholera endemic had not been reported in Haiti for over a century. Many published reports

foreground Asian countries as the origin of this outbreak and promptly origin of this epidemic became a matter of dissension. It is one of the remarkable examples of the present global cholera pandemic, which was caused by a new El Tor variant strain with hypervirulent genetic traits.

During 2017, an Asian country, Yemen, experienced the world's worst and largest cholera outbreak, which affected more than 1 million people in this country (Weill et al., 2019). The higher incidence rate (5,000 new cases a day) has presented this affliction at the forefront of the public health agenda (Kupferschmidt et al., 2017; WHO, 2017). Furthermore, this situation persisted in Yemen also in the subsequent years. A report on 17th June 2019 announced that heavy rainfall and extensive flood in the country had deteriorated the humanitarian catastrophe with more than 3 million suspected cases and 639 deaths during six months (Deen et al., 2020). The inability to predict the perceptible pattern of outbreaks and the emergence of the newer variant *V. cholerae* isolates with severe clinical symptoms and increased antimicrobial resistance was allied to limitations of laboratory markers to a large extent. However, recent studies indicated the failure in the treatment of cholera patients infected by recently emerging strains of *V. cholerae* O1 (Das et al., 2020). Nowadays, *V. cholerae* shows resistance against all commonly used antibiotics, tetracycline, quinolones, cotrimoxazole, ampicillin, macrolides, ciprofloxacin, and even carbapenem. These are no longer effective in the treatment of cholera. This resistance is achieved by genetic mutation, frequent acquisition of extrachromosomal mobile genetic elements (MGEs) through conjugation, exporting drugs via efflux pumps, etc (Ghosh et al., 2011; Spagnoletti et al., 2014; Carraro et al., 2016).

In recent times, *V. cholerae* has become an uncontrolled ferocious bacterial pathogen causing major outbreaks worldwide. It predominates in the environment during inter-epidemic periods undetectably by outpacing our efforts to prevent the disease after natural calamities like floods and earthquakes that cause an unsanitary environment and overcrowding. Thus, the present study enlightens the path to understanding the changing nature of evolving strains of *V. cholerae* O1 with intense antibiotic resistance properties through genetic modifications, which may cause severe disease outcomes in the upcoming period.

Chapter 2

Importance of the Study

2. Importance of the Study

The displacement of classical biotype by the ongoing El Tor occurred in 1961, marking the beginning of the seventh cholera pandemic. According to studies, the seventh pandemic El Tor strains were less virulent than classical biotype strains in terms of the virulence-related genes they contained. However, El Tor strains are more environmentally fit than classical biotype strains, so they have largely taken the position of the former. A few decades after its appearance, El Tor strains were well adapted to their surroundings and evolved into a more virulent form by acquiring classical determinants. The former acquired the classical type of cholera toxin subunit B (*ctxB*) and replaced the El Tor *ctxB* in the El Tor backbone during the 1990s. Following that, they gradually gathered the hypervirulent variants of the virulence genes, which suddenly led to a huge epidemic of cholera in Haiti in 2010 where cholera was absent for about a century. Comparative genomic studies showed the presence of point mutations in the genome of strains causing Haitian outbreak in the virulence genes such as *ctxB* and *tcpA* to make the strains more virulent. Due to a point mutation in the Haitian *rtxA* gene, it became non-functional. Studies suggested that the contribution of *rtxA* in virulence might be compensated by toxin genes, the *hlyA*-encoded hemolysin/cytolysin (HlyA), and *ctxB*. Retrospective studies with the archived strains from Kolkata, West Bengal showed the presence of Haitian genetic traits of these virulence genes were present in these isolates before the outbreak of cholera occurred in Haiti. Haitian *ctxB* was found to be present in Kolkata, West Bengal strains since 2006, Haitian *rtxA* from 2004, and Haitian *tcpA* from 2003. Other studies indicated Kolkata, India as the origin of these disastrous Haitian outbreak strains that shared a common lineage.

The main difference between biotypes lies in certain biochemical tests such as polymyxin B susceptibility, chicken blood cell agglutination, sheep blood hemolysis, Voges Prokauer test, and phage susceptibility assay. Generally, El Tor biotype strains which emerged in 1961, and prevailing also in the current years, has consistently been found with their typical El Tor biotype characteristics including the hemolytic with polymyxin B resistance phenotype. However, this pattern has been changed with the emergence of polymyxin B-sensitive El Tor variant strains in 2012 in Kolkata, West Bengal, and then spread around the world (Samanta et al., 2015). Surprisingly, in 2016, a large cholera outbreak occurred in an Asian country, Yemen that has been marked as the worst cholera outbreak in cholera history, and the isolated strains had the same polymyxin B-sensitive phenotype as the Kolkata strains. Other studies

have shown that the Yemeni strains shared a common lineage with the polymyxin B-sensitive El Tor strains from Kolkata, India.

Also, the emergence of non-hemolytic El Tor *V. cholerae* has been reported with several mutations in the gene encoding hemolysin (*hlyA*) which leads to truncated protein (Fan et al., 2019). *hlyA* is a major virulence factor found in El Tor *V. cholerae* in chromosome II. This pore-forming toxin has the ability to cause cell death by acting on targeting membrane (Khilwani and Chattopadhyay, 2015).

Moreover, *V. cholerae* has a special genetic makeup and extraordinary competence, which together enable the cholera bacterium to adapt quickly to challenging environmental conditions and withstand the harmful effects of antimicrobial agents. This enteric pathogen is well-known for being multidrug resistant (MDR). By hydrolyzing the central structure or adding a chemical group to the scaffolds, *V. cholerae* can, like other bacteria, degrade or alter antibiotic scaffolds. The most typical method of drug resistance in *V. cholerae*, involves chemical modification of antibiotics using an enzymatic function of acquired genetic characteristics which can occur via horizontal gene transfer and develops resistance to a variety of antibiotics, including tetracycline, fluoroquinolones, aminoglycosides, chloramphenicol, and even carbapenem.

Hence, the present study provides insights into the epidemiology of recently evolving variants of *V. cholerae* O1 strains with different attributes associated with higher virulence and disease pathogenesis. In addition to this, it would also allow us to understand the molecular mechanism of emerging carbapenem resistant strains, which may help us to restrict the spread and raise awareness against this growing threat.

Chapter 3

Review of Literature

3. Review of Literature

3.1. Cholera: the dreadful diarrheal disease

Cholera is a severe dehydrating diarrheal disease that affects millions of people worldwide. It remains a global public threat in many developing countries associated with a lack of basic infrastructure concerning access to safe drinking water and proper sanitation, especially in Africa and Asia. The disease's unique epidemiologic feature is its propensity to emerge as outbreaks that, if uncontrolled, could turn into epidemics. In terms of severity and explosive nature to appear as an outbreak, there are very few diseases that can compete with cholera. Each year, it has been found that the number, size, and case fatalities of cholera outbreaks are gradually rising and therefore it continues to inflict high rates of morbidity, especially in the countries of Asia, Africa, and Latin America.

3.2. Historical Perspective

Although the origin of the term cholera is debatable, it may have originated from the Greek words chole (bile) and rein (flow), which describe bile flow in that language. Although cholera as a disease is likely not as old as others like smallpox or tuberculosis, it has unquestionably existed for at least a thousand years. Others have proposed that the term derives from the Greek word cholera, which means roof gutter. It is referred to as Visuchika in Ayurveda and is specifically defined in Susruta from the 7th century B.C. According to Pollitzer, Schmidt's translation of a Sanskrit text whose origin is thought to be Tibetin or around 802 AD strongly implies the name “cholera” (Mekalanos et al, 1997). In Hebrew, cholera means “bad disease”. All these diverse forms imply that cholera may have been known to ancient civilizations in the Middle East, the Mediterranean, and the Indian subcontinent (Barua and Greenough, 1992).

3.3. Discovery of *Vibrio cholerae*: the causative agent of cholera

The Gram negative proteobacteria *V. cholerae* is the aetiologic agent of the cataclysmic diarrheal disease cholera which continues to be a public threat. When toxigenic *V. cholerae* is consumed, typically through contaminated food or water, cholera infection occurs. The incubation period of cholera ranges from several hours to a few days and is dependent in part on inoculum size (Levine et al., 1981). In 1854, Filippo Pacini, an Italian anatomist first described the etiological agent responsible for cholera. He performed an autopsy of cholera

patients right away and examined histological samples of the intestinal mucosa. In the course of these investigations, Pacini made the initial discovery of a bacillus with a comma shape that he called a "Vibrio." In 1854, he published a paper named, "Microscopical observations and pathological deductions on cholera" where he explained the organism and its contribution to the disease. His microscopic slides had labeled clearly with the date and nature of his examination. He discovered several curved bacteria that he named "Vibrio cholera" reside in the intestines of cholera victims (Pollitzer, 1959). This discovery by Pacini was overshadowed by the work of Robert Koch in 1883, who worked with cholera in Egypt and proved that a comma-shaped organism was responsible for cholera (Pollitzer, 1959). The organism was given the name Kommabazillen by Koch, and the name *Vibrio comma* was used for several decades before Pacini's groundbreaking work was acknowledged and the name was replaced by *Vibrio cholerae*.

3.4. Taxonomy & serological classification

V. cholerae, member of the genus *Vibrio* are Gram negative, non-sporing curved rods. They can move through monotrichous flagella that are encased in a sheath with oxidase positive (Ryan and Ray 2004; Bronze and Greenfield 2005) trait and able to ferment glucose, producing acid but no gas. The growth is stimulated by sodium, and is halophilic in nature.

Scientific position of *Vibrio cholerae*

Domain – Bacteria

Phylum – Proteobacteria

Class – Gamma proteobacteria

Order – Vibrionales

Family – Vibrionaceae

Genus – *Vibrio*

Species – *cholerae*

The ‘O’ antigen is the major surface antigen used for serotyping of *V. cholerae*. Apart from this somatic ‘O’ antigen, another antigen, a flagellar (‘H’) antigen is also present. But, for serotyping of *V. cholerae,* the H antigen is of little value as all the strains in a given species possess identical ‘H’ antigen (Shinoda et al., 1976). Serological studies have depicted that *V.*

cholerae strains possess identical rough (R) antigens irrespective of their 'O' antigen type (Shimada et al., 1973). Each needs to be adsorbed in the preparation of O somatic antiserum. Organism expressing only the rough antigen is usually indistinguishable from the corresponding smooth (S) form by colony morphology and biochemical tests but may be differentiated by agglutinating with rough antiserum (Sakazaki, 1992).

The heat stable 'O' antigen is composed of a homopolymer containing the amino acid sugar D- persamine (4-amino, 4, 6-dideoxy-D-mannose) in which the amino groups are acylated by 3-deoxy-L-glycero-tetronic acid (Redmond, 1975; Kenne et al., 1982; Manning et al., 1994). There are >200 serogroups in *V. cholerae* designated with 'O' followed by the sequential numbers of detection. Among them, only O1 and O139 serogroups can cause epidemic and pandemic cholera. Others are commonly grouped as non-O1/non-O139. These organisms have been identified by the agglutination with O1 group-specific antiserum directed against the O1 specific LPS component of the cell wall and by expression of their enterotoxigenicity. In 1992, cholera was also caused by the serogroup O139. This serovar was identified by the non-agglutination with O1 specific antiserum but agglutination with the unique O139 antigenic group-specific antiserum.

3.4.1. Serogroup O1

The O1 serogroup of *V. cholerae* has long been linked to cholera epidemics and pandemics. The genes involved in 'O1' antigen biosynthesis are present on the chromosomally located wbe operon which is ~20kb in length and comprises 21 complete open reading frames (ORFs) (Manning et al., 1994).

Based on some phenotypic features, the O1 serogroup is classified into two biotypes, classical and El Tor. These phenotypic traits are susceptibility to polymixin B, haemagglutination of chicken erythrocytes, haemolysis of sheep erythrocytes, the Voges–Proskauer test, which measures the production of acetylmethylcarbinol, and phage susceptibilities (Kaper et al., 1995; Faruque et al., 1998). Apart from the phenotypic differences based on bacteriological traits, there are differences in the pattern of infection caused by the two biotypes.

Each of the O1 biotype can further be classified into the serotypes Ogawa, Inaba, and Hikojima. Ogawa strains can produce the antigenic factors A and B with a small amount of C antigen, while the strains with the Inaba serotype are found to produce the A and C antigens only (Sakazaki, 1992). The third serotype, Hikojima is found to contain all three antigenic

factors but is unstable and rarely found in nature (Kelly et al., 1991). The conversion or switching between the serotypes, Ogawa and Inaba has also been reported at a frequency of approximately 10^{-5} but the converse is further less (Manning et al., 1994).

Bacteriophage typing allows for additional differentiation of *V. cholerae* O1 strains that have the same biotype and serotype. A recent typing method that used 10 typing phages could be able to distribute a thousand *V. cholerae* strains into 146 phage types (Chattopadhyay et al., 1993). Cholera toxin is present in 95% of all *V. cholerae* strains belonging to the O1 serogroup. Only a few episodes of diarrhoea or extraintestinal infections have been associated with *V. cholerae* O1 strains that lack the cholera toxin (Johnston et al., 1983; Morris et al., 1984).

3.4.2. Serogroup O139

Early in 1993, the first reports of a new epidemic of a severe, cholera-like disease originating from India and Bangladesh, making obsolete the straightforward distinction between *V. cholerae* O1 and *V. cholerae* non-O1 (Albert et al., 1993; Ramamurthy et al., 1993). The etiology of this outbreak was initially thought to be a non-O1 *V. cholerae* because it did not agglutinate with O1 antiserum. However, additional research indicated that this organism belonged to a new serogroup, which was given the name O139 and the synonym "Bengal" to recognize the origin of this strain. This strain did not belong to any of the O serogroups previously discovered for *V. cholerae* (Shimada et al., 1993).

Earlier molecular studies revealed that the O139 has originated from the O1 El Tor serogroup with a large foreign genomic DNA fragment insertion that encodes genes specific to O139 and concomitant deletion of most of the O1 antigen–specific gene cluster (Calia et al., 1994; Manning et al., 1994; Stroeher et al., 1995). Recent studies suggested that both O1 and non-O1 serogroups are forefathers of the O139 vibrios (Faruque et al., 2000).

Salient virulence characteristics, such as cholera enterotoxin (CT) and toxin coregulated pilus (TCP) in O139 serogroup are identical with the typical El Tor *V. cholerae* O1 strains (Hall et al., 1993; Rhine et al., 1994). It was suggested that the El Tor vibrios were the backbone of the emergence of the O139 serogroup (Waldor and Mekalanos, 1994). However, this organism lacks the O1 LPS and is devoid of some of the genetic elements obligatory for O1 antigen production (Manning et al., 1994).

3.4.3. Serogroups non O1 & non O139

The "non-O1, non-O139 *V. cholerae*" strains are those that are recognized as *V. cholerae* based on biochemical assays but do not agglutinate with either O1 or O139 antisera. Names such as 'non-cholera vibrios' (NCVs) or non-agglutinable vibrios (NAGs) were used for these *V. cholerae*. Therefore, all the serogroups fall under the group of non-O1, non-O139 *V. cholerae* except serogroups O1 and O139. Most of these serogroups do not produce CT and have never been linked to cholera epidemics, but they have been linked to rare outbreaks of diarrheal illnesses (Cheasty et al., 1999; Morris and Black, 1985). Because some non-O1, non-O139 *V. cholerae* cause septicemia, it's possible that they have an invading nature (Sanyal, 1992). They are frequently found in estuarine areas, and the infections caused by these strains usually have environmental origins (Morris, 1990). Besides being a human pathogen and free-living organism, these non-O1, non-O139 strains are typically isolated from shellfish, particularly from filter feeders such as oysters.

3.5. Biotypes

Vibrio cholerae O1 has been classified into two biotypes based on Voges Proskauer test, chicken blood cell agglutination, haemolysis of sheep erythrocytes, sensitivity to polymyxin B, and susceptibility to biotype-specific phages (Table 3.1) (Kaper et al., 1995). Biotypes share a very close relationship with the epidemiology of *V. cholerae*. Due to exchanging, gaining, and losing factors they keep their circulatory motion flawless in the environment. According to the study, the genome of *V. cholerae* strains is experiencing subtle modifications that can affect strains' virulence, quick transmission, and spread. The emergence of El Tor variant strains containing classical phenotypes has already been reported. In 2012, Polymyxin B-resistant El Tor was completely replaced by Polymyxin B-sensitive strains of *V. cholerae* in Kolkata (Samanta et al., 2015). There are also many reports regarding the emergence of non-hemolytic El Tor strains of *V. cholerae* (Fan et al., 2019). These cryptic changes in *V. cholerae* genome show its versatile nature to adapt well to the environment. Thus, these characteristics are no longer used to identify the two biotypes.

Table 3.1. *Phenotypic features to differentiate classical and El Tor biotypes.*

Phenotypic assays		**Result displayed by biotypes**	
		Classical	**El Tor**
Chicken blood cell agglutination		-	+
Hemolysis of sheep erythrocytes		-	+
Voges Proskauer		-	+
Polymyxin B Sensitivity (50U disk)		+	-
Lysis by	Classical IV bacteriophage	+	-
	El Tor V bacteriophage	-	+

3.6. Seven pandemics of cholera

Cholera continues to pose difficulties and amaze us despite more than a century of research. Cholera now has a worldwide presence with many people dying each year. Since 1817, there have been seven major cholera pandemics (Faruque et al., 2003).

The year 1817, first disclosed the well-documented cholera pandemic in India; which spread through China, East Africa, and much of Oceania. Historical records unequivocally show cholera epidemics over the Indian subcontinent, even though it is unknown how widely it spread in antiquity (Pollitzer, 1959). It has been endemic in South Asia, especially the Ganga delta region. Early in the 1830s, the second cholera pandemic spread to the British Isles. It then invaded North America through Canada in 1832 and moved south across the Mississippi Valley. A period stretching from 1852 to 1859 is described as the period of the third pandemic and during this pandemic in 1854, the physician John Snow demonstrated that all the cases of cholera in the localized London epidemic could be traced to a single contaminated water pump (McNeil, 1976). It was during this time stretch when Pacini found a large number of curved bacteria in the stools of cholera victims and termed them “Vibrio cholera”. Cholera was widespread in the United States during the third pandemic, and by the conclusion of the fourth pandemic, it had spread to cities and villages along the Mississippi,

Missouri, and Ohio rivers (Billings et al, 1975). The fifth pandemic extensively affected South America; it caused large epidemics in many countries and was characterized by high motility in Argentina, Chile, and Peru (Laval et al, 1989). Robert Koch discovered the cholera-causing "comma bacilli" from patients' rice water feces in Egypt in 1883 and in India in 1884, during the fifth epidemic (Koch, 1884). The stretch of the sixth pandemic is from 1899 to 1923 and this one mostly involved the population of the Middle East and its surroundings and Balkan Peninsula (Pollitzer, 1959).

The 7th pandemic was introduced in 1961 in Indonesia and run through the entire Southeast Asia by the end of 1962. The pandemic during the year 1963 to 1969 was spread in India, Bangladesh, Pakistan, Iraq, Afghanistan, Iran, and the nearby republics of Soviet Union (Kamal, 1974). After then, cholera spread quickly, causing outbreaks in West Africa in the early 1970s and the Arabian Peninsula and its neighboring countries in the 1970s (Goodgame et al., 1975).

3.7. Journey of *V. cholerae*

3.7.1. Global replacement of classical biotype by El Tor biotype

The bacteria responsible for cholera was first discovered in 1884 at the start of the fifth pandemic and considered as the serogroup O1 based on their serological antigenic response (Pollitzer, 1959; Sakazaki 1992). The biotyping of *V. cholerae* O1 was further categorized into classical and El Tor based on certain biological assays (Kelly, 1991). Among the recorded seven pandemics of cholera, the 5th and the 6th were caused by the *V. cholerae* serogroup O1 of the classical biotype. Though, the distinction among the six pandemics is not clear. According to some reports, the first six pandemics were a single continued pandemic that was governed by the classical biotype for almost 80 years. At the El Tor quarantine station in 1905, Gotschlich discovered a novel strain of *V. cholerae* O1 among Indonesian pilgrims who had passed away from cholera-like symptoms. The first identification revealed that the organisms resembled *V. cholerae* and they differ from the existing strains in that they can lyse blood cells (Mosley, 1969). After that, for about 30 years they caused sporadic outbreaks in Indonesia affecting Sarawak, Java, and Borneo. Despite causing a severe outbreak in Celebes, Indonesia in 1936, this strain was not given the name El Tor until it attained international recognition. In the early 1960s, the public authority recognized the El Tor strain as a newer variant of *V. cholerae* that are responsible for causing outbreaks in numerous areas around the world. This dissemination ultimately resulted in the seventh

pandemic of cholera (Barua and Greenough, 1992). The intensity of terrestrial dissemination and length of this pandemic were noticeably different from those of past periods. The *V. cholerae* O1 El Tor epidemic spread throughout practically the entire world over a period of time of around 50 years. The preexisting classical biotype of *V. cholerae* O1 was entirely supplanted by the El Tor biotype as the primary epidemic strain in the areas where it was endemic for a year after its initial appearance.

3.7.2. Emergence of *V. cholerae* O139

A huge increase in the global incidence of cholera in the 1990s represents a reversal in the global cholera scenario. Mainly two important but unrelated events were responsible for this reversal. The reemergence of cholera in America (in 1991) after 100 years (Tauxe and Blake, 1992) and the genesis of a new epidemic causing cholera strain in Southern India (Ramamurthy et al., 1993) were considered as a root cause. The appearance of a novel toxigenic strain in the Indian subcontinent (in October 1992), which did not agglutinate with O1 antiserum was an unrivaled change in the history of cholera (Nair et al., 1994). The two biotypes of the organism *V. cholerae*, either classical or El Tor was accountable for all the pandemics till 1991, (till the seventh pandemic), but in October 1992, the sudden emergence of a non-O1 serogroup (O139 synonym Bengal) in Southern India with epidemic potential was a mysterious event in the history of cholera. After the discovery of *V. cholerae* O139 Bengal as the second etiologic agent of epidemic cholera (in October 1992) in the south Indian coastal city of Madras, the long-held belief that only *V. cholerae* belonging to serogroup O1 is capable of causing epidemic and pandemic cholera has been refuted. Within months of emerging in Madras, *V. cholerae* O139 caused a series of cholera outbreaks that spread across the whole Indian subcontinent (Albert, 1996). It has also spread to several neighboring countries in Asia. The infection caused by O139 was indistinguishable from typical cholera (Bhattacharya et al., 1993) and it spread swiftly to all cholera endemic areas of India (Nair et al., 1994) and other parts of Asia. Cholera outbreaks due to O139 serogroup was also recorded in several different countries of the world (WHO, 1995; Dalsgaard et al., 1995; Nair et al., 1996). The eagerness and ample spread of this new serogroup causing cholera led the researchers to think that the genesis of O139 was the beginning of the eighth pandemic (Faruque et al., 1998) which was not happen as isolation of *V. cholerae* O139 gradually diminished.

3.7.3. Hybrid variants of *V. cholerae* O1

In Matlab, Bangladesh, Nair and colleagues discovered a novel strain of *V. cholerae* O1 that combined characteristics of the El Tor and traditional biotypes. These strains were known as the *V. cholerae* O1 Matlab variants and were thought to be a cross between the two biotypes. The significance of these hybrids was highlighted by the discovery that *V. cholerae* O1, which was isolated from a significant and prolonged cholera outbreak in Mozambique, an endemic cholera country in East Africa, in 2004, had phenotypic traits of the El Tor biotype. Interestingly, the results of *rstR* and *ctxB* genotyping were similar to the traditional classical biotype (Ansaruzzaman et al., 2004). The importance of these hybrids was further spotlighted while *V. cholerae* O1 Mozambique isolates were found to carry the CTX prophage of classical type (during early 2004) unless otherwise resembled the El Tor biotype. Later, similar El Tor variant strains were recovered from other cholera epidemics in Africa and Asia's endemic regions (Safa et al., 2008). According to a retrospective investigation of *V. cholerae* strains from Kolkata, India, the classical type *ctxB* first appeared in 1990, despite El Tor type *ctxB* still existing in nearly equal numbers at the time. An unusual event occurred in 1991 when strains with both the classical and El Tor type *ctxB* became more prevalent (Mukhopadhyay et al., 2014). In 1994, isolation of strains with El Tor *ctxB* became rare, and the major *ctxB* allele was of the classical type. Previous reports from our lab showed that *V. cholerae* O1 El Tor variant strains were isolated in 1995 onward and in 1995 it replaced the prototype El tor biotype strains in Kolkata (Raychoudhuri et al., 2009).

3.7.4. Appearance of Haitian variant *V. cholerae* O1

With 7,490 deaths and 586,625 illnesses, the disastrous cholera outbreak in Haiti in late October 2010 brought this ancient scourge to the top of the global public health agenda for the first time in almost a century (Piarroux et al., 2011). Many published reports foreground Asian countries as the origin of this outbreak and/or due to indigenous strains (Reimer et al., 2011; Chin et al., 2011; Hendriksen et al., 2011). Analysis of the entire genomes of Haitian isolates of *V. cholerae* found some unusual alterations in various chromosomal regions (Chin et al., 2011). These include SNPs in the *ctxB*, *tcpA*, *rtxA*, and *rstB2* area as well as structural variations in the superintegron, VSP-2, and SXT genes. The likely source of the cholera outbreak strain in Haiti was discovered using a third-generation, single-molecule DNA sequencing technique (Chin et al., 2011). They resolved the genome sequences of two Haitian cholera samples and three cholera samples from elsewhere around the world. The Haitian

samples and the seventh pandemic variant strains isolated in Bangladesh in 2002 and 2008 shared a close relationship, according to a thorough analysis and comparison of crucial DNA features among the various cholera samples using genome sequencing, which included single nucleotide variations, insertions, and deletions of specific regions of the genome, and structural variations. Thus, Haitian outbreak introduced a newer variant of 7^{th} pandemic El Tor biotype with unique mutations in different segments of their genome.

Later on, these Haitian variant strains were found to circulate all over the world and causing outbreaks that are more serious than the former outbreaks. Even, the Haitian variant strains are more virulent (Ghosh et al., 2019).

3.7.5. Emergence of Haitian variant polymyxin B-sensitive *V. cholerae*

V. cholerae is generally classified into two biotypes, classical and El Tor, depending on several phenotypic tests. Among them, Polymyxin B susceptibility assay is used as a major marker in determining biotypes and remains reliable for decades. The classical biotype of the first six pandemics was sensitive and the seventh pandemic El Tor biotype strains are relatively resistant to this antibiotic.

The earlier pandemic strains were classical biotype and polymyxin B-sensitive, but they were hypervirulent and which might be a reason for their instability to nature. Subsequently, the classical strains have been replaced by the less virulent seventh pandemic El Tor biotype strains which are thought to be more stable to the environment along with the property of polymyxin B resistance. Since the first emergence of El Tor biotype strains, in 1961, several El Tor variants have been found to emerge in different parts of the world with the variation and modification of their virulence genetic attributes. However, all of them were polymyxin B resistance like the prototype El Tor strains. Suddenly, in 1993 a new type of serogroup was found to cause cholera like the O1 strains and was named O139. These O139 serogroup strains were also polymyxin B-resistant. After that in 2010, a new variant of El Tor biotype has been evolved which caused a disastrous outbreak in Haiti but with the feature of polymyxin B- sensitivity. But, this pattern has changed with the emergence of polymyxin B-sensitive Haitian variant strains in 2012 in the strains of Kolkata, West Bengal (Samanta et al., 2015). From India, this variant strain has traveled through many countries to cause outbreaks. In 2016-2018, Yemen has been faced the worst outbreak of cholera caused by the polymyxin B-sensitive Haitian variant strains, where millions of people were affected and it is known to be the world's largest cholera outbreak in history (Weill et al., 2019). This

variant now continuously causes major outbreaks in different parts of the world like recently in Algeria, Somalia, Niger, Nigeria, Mozambique, etc, and replaced the polymyxin B-resistant allele completely (ECDC, 2022). In this study, we have tried to evaluate the importance of polymyxin B sensitivity in virulence.

(a) Mechanism of *carR* gene associated with polymyxin B susceptibility

V. cholerae is continuously evolving by changing different genetic factors for better adaptation to the environment, which becomes expressed phenotypically. Polymyxin B resistance has been used as a useful marker to differentiate between the classical and El Tor biotypes. El Tor vibrios can modify lipid A with glycine or diglycine residues to increase the net positive charge to inhibit the binding of the cationic antimicrobial peptide (CAMP) polymyxin B to cause resistance to the drug and the CarR-CarS two-component system has proven to contribute significantly to this modification process by regulating the promoter region of *almEFG* (Bilecen et al., 2015). CarS (VC1319) is a membrane-bound histidine kinase that phosphorylates the soluble CarR (VC1320) at the D55 amino acid residue and helps to bind to the *almEFG* promoter. CarR acts as a positive regulator for the operon. After the translation of *almEFG*, glycine moieties are added to AlmF (VC1578) with the help of AlmE (VC1579). Then, AlmG (VC1577), which is a membrane-bound protein, transfers the glycines from AlmF to the negatively charged lipid A molecule of the inner membrane. Subsequently, the glycinylated lipid molecules are transported to the outer surface of the outer membrane and reduce the net negative charge on the outer surface. This protection causes the bacterial surface to reduce its net negative charge and removes the site for CAMP binding (Figure 3.1) (Bilecen et al., 2015).

Recently, a point mutation at the 265th nucleotide position (G265A) in *carR* gene was known for the emergence of polymyxin B-sensitive Haitian variant strains and the sequence analysis study of the *carR* gene has also found that these strains differ from polymyxin B-sensitive classical strain O395. Due to the point mutation, the CarR^{S} strains are unable to activate the expression of the alm operon and thus induces polymyxin B-sensitivity (Figure 3.1) (Samanta et al., 2020).

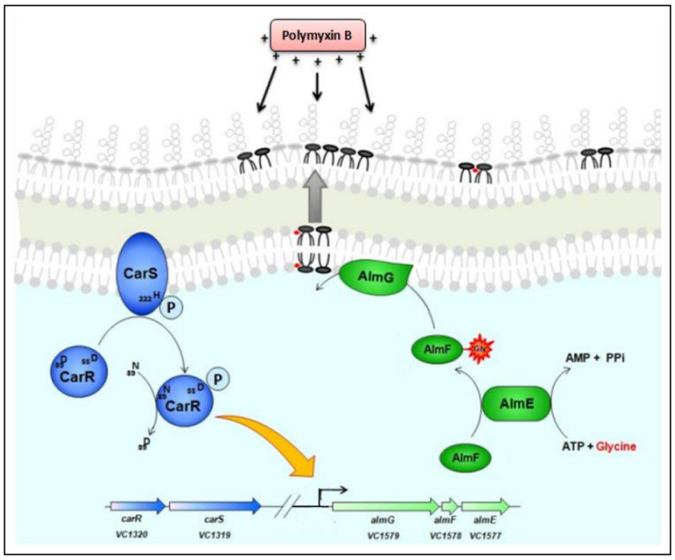

Figure 3.1. *Mechanism of polymyxin B resistance in prototype V. cholerae O1 El Tor. OM, Outer membrane; P, periplasmic space; IM, inner membrane; C, cytosol.*

3.8. Present global scenario of cholera

Transmission of cholera is strongly correlated with the lack of access to sanitary facilities and clean water. Peri-urban slums and camps for internally displaced people or refugees are examples of typical at risk places where the necessary measures for clean water and sanitation are not being maintained. An increase in the risk of cholera transmission might result from the effects of a humanitarian crisis, such as the breakdown of water and sanitation systems or the relocation of populations to insufficient and crowded camps.

The WHO reported in May 2011 that there were 7816 deaths (CFR, 1.3%) in 2011 compared to 7543 deaths (CFR, 3.5%) in 2010 (WHO 2011). Worldwide, the number of reported cholera cases that died in 2010 increased by 52% compared to 2009, with a case-fatality rate (CFR) of 2.38% (WHO 2010). Overall, it is estimated that approximately 2.83 million cases and 95,000 deaths are caused by cholera per year (Ali et al., 2015; Deen et al., 2020).

However, the dynamics of cholera in recent years, along with the advent of newer variant strains causing more severe clinical symptoms and rising antimicrobial resistance, as well as climate change, are helping to push the burden of cholera to the top of the global public health agenda.

The Asian countries mainly include India and then Bangladesh where the highest number of cases and deaths were reported annually during the years 2008-2012 (Ali et al., 2015). Moreover, the Integrated Disease Surveillance Program of India has identified 13 out of 36 states as having an endemic cholera outbreak, according to data from the years 2010 to 2015.

(Ali et al., 2017). According to reports, India has the greatest cholera burden (Figure 3.2) (Ali et al., 2015).

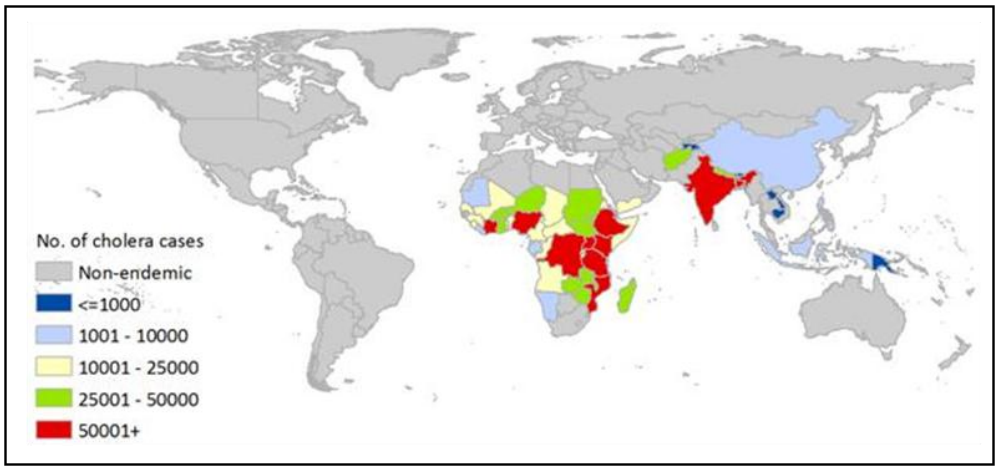

Figure 3.2. *Cholera cases from endemic countries were reported to WHO as of 2015.*
Source: Ali et al., 2015

For years, many cholera cases have been reported in African nations, including the Democratic Republic of the Congo, Somalia, Zambia, Ethiopia, Nigeria, South Sudan, and Cameroon, all of which experienced catastrophic outbreaks in 2017. Since 1999, there hasn't been a cholera outbreak in the Americas, but the cholera outbreak in Haiti in 2010 is still a global public health concern. In Oceania, Australia has been found to report cholera cases also in the year 2019 (Figure 3.3).

Since the worst cholera outbreak occurred in Yemen, Asia has experienced a sharp increase in the number of cholera cases worldwide. In 2019, 923 037 cholera cases and 1911 cholera deaths were reported globally, for a CFR of 0.2%, including 61 941 cholera cases and 886 cholera deaths outside Yemen (CFR, 1.4%).

Due to the appearance of the SARS-CoV-2 (COVID-19) pandemic in 2020, cholera cases significantly decreased globally (WHO, 2021) (Figure 3.3). The preventive measures of COVID-19, such as more handwashing and hygiene, social distancing, and prohibition of large gatherings, developed general hygiene in health care facilities, and even household lockdowns reduced the transmission of cholera. The WHO received case reports from 80 countries in total, of which 27 reported 323 320 cholera cases and 857 fatalities, with a case-fatality rate (CFR) of 0.27% (WHO, 2021), whereas 53 nations reported no cases for the year. Following Yemen's exclusion, which was responsible for 85% of the cases reported to the WHO on a worldwide scale in 2020, 47 608 cases and 742 deaths (CFR, 1.5%) were reported from other countries. The number of cholera cases reported worldwide decreased by 65% in

2020 compared to 2019, with Yemen reporting 68% fewer cases and all other countries reporting 23% fewer cases. In 2020, there were 55% fewer recorded cholera deaths worldwide.

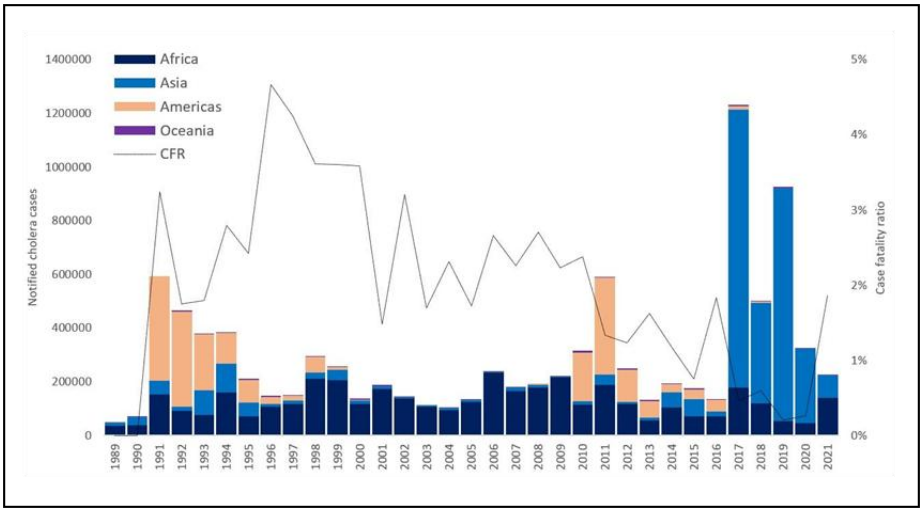

Figure 3.3.*Year and continent-wise cholera cases reported to WHO (1989-2021).*
Source: World Health Organization (16 December 2022). Disease Outbreak News; Cholera – Global situation. Available at: https://www.who.int/emergencies/disease-outbreak-news/item/2022-DON426)

The number of cholera cases and the geographic distribution of those cases have both increased since 2021. Cholera outbreaks were reported in 23 countries in 2021, primarily in the WHO Regions of Africa and the Eastern Mediterranean. This trend has been continuing till 2022 with cholera cases reported from over 29 countries (Figure 3.4). 13 of them, including Lebanon, which reported its final cases in 1993, and Syria, which reported its last cases in 2000, had no records of cholera in 2021 (Larkin et al., 2023).

However, a number of the afflicted countries recorded higher case-fatality ratios than in past years. The global average for cholera case fatality ratio in 2021 was 1.9%, while the African average was 2.9%. Yet, because so many cases go unreported, it is unknown how widespread cholera is. Study shows that cholera is a global problem that is present on every continent and has been spreading more frequently in recent years, causing outbreaks in countries associated with poor water, sanitation, and hygiene (WaSH) (Cholera, 2020).

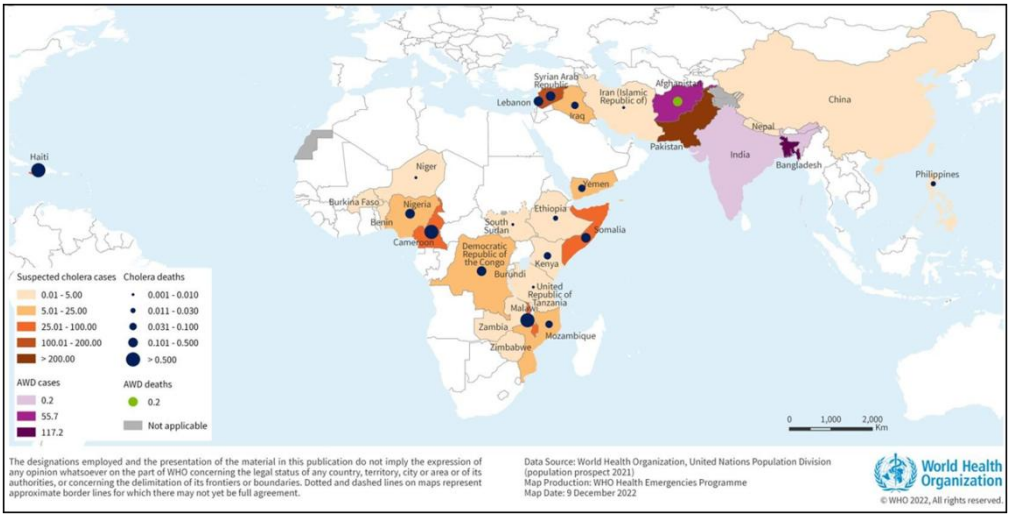

Figure 3.4. *Incidence of cholera cases reported to WHO from different countries in 2022.*
Source: World Health Organization (16 December 2022). Disease Outbreak News; Cholera – Global situation. Available at: https://www.who.int/emergencies/disease-outbreak-news/item/2022-DON426

3.9. Pathogenesis of *V. cholerae*

The pathogenesis of cholera is closely linked to the synthesis and activity of the enterotoxin known as cholera toxin (CT). CT, a heterohexameric protein was first discovered in India (De and Chatteijee, 1953). A protein released by *Vibrio* was identified and purified by Finkelstein et al. (1964, 1969, 1970), who also demonstrated that the potent enterotoxin caused fluid accumulation in guinea pigs' ileal loops. CT is the prototype of A-B subunit toxins and an ADP ribosylating toxin which contain one A (MW 27 kDa) and five identical B (MW 11.6 kDa) subunits (Gill, 1976, 1981; Lai, 1976; Ohmoto, 1976). The A subunit has an active enzymatic function that functions intracellularly, while the B subunit is responsible for binding holotoxin to the eukaryotic cell receptor. Neither of the subunits possesses secretogenic activity alone. The stochiometric analysis of AB_5 suggests that the A subunit lies on a central axis of a ring of five B subunits. For enzymic activity, A subunit must be nicked proteolytically to fragments A1 and A2, which are linked by a disulfide bond. A1 fragment (22 kDa) possesses catalytic activity and A2 fragment (5kDa) connects A1 to B ring through a single disulfide bond, which must be cleaved for the expression of enzymatic activity.

CT binds with the receptor GM_1 ganglioside of host mucosal cells which are sialic acid containing oligosaccharide covalently attached to a ceremide lipid. A neuraminidase secreted by *V. cholerae* removes the sialic acid residue from GM_1, gangliosides. Following the release of the A1 subunit from the toxin, which is most likely accomplished by reducing the disulfide bond connecting it to the A2 subunit and entering the host cell by an unknown translocation mechanism. Released A1 fragment binds to NAD (Cassel and Pfeuffer 1978; Galloway and

Van Heyningen, 1987) and catalyzes the ADP ribosylation of a GTP binding regulatory G protein, G_{Sa}, associated with adenylate cyclase (Cassel and Pfeuffer, 1978; Gallowayand Van Heyningen, 1987). The G protein that participates in transmembrane signaling consists of three protein chains or subunits known as alpha, beta, and gamma. As soon as it is activated, the GTP-bound alpha subunit separates from the beta and gamma subunits and diffuses along the plasma membrane's inner surface, where it eventually connects with adenylate cyclase to catalyse the synthesis of cAMP. Increased cAMP causes fluid and electrolyte imbalances that, in turn, cause the body to secrete water and electrolytes into the gut lumen, which can result in severe diarrhoea, fluid loss, and, in certain circumstances, dehydration and metabolic acidosis (Kaper et al., 1995).

3.10. Control & prevention of cholera

Effective cholera preventive and control initiatives are well known and rely on the implementation of integrated and comprehensive approaches that involve activities outside of the health sector. High coverage with oral cholera vaccines (OCV) leads to a significant reduction in cholera transmission in a variety of settings, despite the fact that cholera is frequently predictable, preventable, and can ultimately be eliminated by providing access to clean water, proper sanitation facilities, and satisfactory hygiene conditions. The use of OCV as a cholera control method was reaffirmed in the 64th World Health Assembly Resolution WHA 64 (2011), "Cholera: Mechanism for Control and Prevention." A WHO initiative to control and prevention of cholera was found with the undertaking of the global task force on cholera control (GTFCC) in 2017, which targeted 47 cholera endemic countries including India to end cholera, by 90% by the year 2030.

3.10.1. Oral cholera vaccines

Cholera is generally treatable with ORS, but it is difficult to overcome the risk in sudden outbreak situations. There are three WHO qualified oral cholera vaccines- Shanchol™, Euvichol®, and Dukoral®. Dukoral® is administered with a buffer solution and involves two doses. It is found to give protection against *V. cholerae* serogroup O1 and to some extent against Enterotoxigenic *Escherichia coli*. It is administered to people with the age of >2 years which gives protection against the organism for at least two years with a prompt immune response (7-10 days after the 2nd dose) and has a good safety profile. However, the limited production of Dukoral and the marketing, which is restricted to travelers, is a drawback of

this vaccine in treating cholera effectively (WHO, 2017). Instead, the vaccines Shanchol™ and Euvichol® are not required to administer with buffer solution and both are essentially the same vaccine derived from the same transmission of technology. Both vaccines protect from *V. cholerae* serogroup O1 and O139. The vaccines Shanchol™ and Euvichol® can be administered to children aged between 1-2 years with an interval of 2 weeks between the two doses. They are reported to give protection for at least three years with >60% efficacy. However, levels of protection are lower among children under five. Shanchol™ and Euvichol® have well safety profiles. Both vaccines constitute the OCV stockpile. In 2010, World Health Organization (WHO) published a guideline to use the vaccines in conjugation with other preventive strategies in the cholera-endemic regions and outbreak situations.

3.11. Virulence factors of *V. cholerae*

As far as we are aware, the pathogenesis of cholera involves a complex, multifactorial process that includes *V. cholerae* colonization, coordinated virulence factor expression, and toxin activity. There are still many unanswered problems regarding the pathophysiology of *V. cholerae*, although its main characteristics are widely understood. The unexpected complexity of the regulation of *V. cholerae*'s virulence factors suggests that the organism undergoes cryptic changes as it adapts to the human body.

3.11.1. Toxins of *V. cholerae*

(a) Cholera Toxin (CT)

Cholera enterotoxin, commonly known as CT or choleragen, causes severe, dehydrating diarrhoea that is a hallmark of the disease. The idea that the culprit causing cholera generates "a particular poison" that acts on the intestinal epithelium and that the symptoms of cholera could be "considered essentially as a poisoning" was first put out by Robert Koch in 1884. The presence of such a cholera toxin (CT) was, however, not clearly shown until 1959. De then in his now-classical one-page Nature study could report that cell-free culture filtrates from *V. cholerae* (of the classical biotype) when administered directly into ligated loops of the small intestine of rabbits may produce intestinal fluid accumulation (De, 1959). Initially, it was believed that the toxin was made up of a single subunit that could aggregate into different sizes with varying degrees of toxicity. Then, Lönnroth and Holmgren, and others showed that CT is composed of two types of subunits: a 56 kDa oligomer made up of numerous identical "light" subunits that are responsible for receptor binding and a single

"heavy" subunit that is toxic-active and 28 kDa in size. These subunits were later given the names B (for binding) and A (for toxic-active) (Holmgren et al, 1973). In the assembled, the circular B-subunit homopentamer (CTB pentamer), which is in charge of toxin binding to cells, contains the toxic-active A subunit (CTA). The 11.6 kDa B subunit monomers have 103 amino acids, while the 28 kDa CTA has 240 amino acids. Despite being created as a single polypeptide chain, CTA undergoes post-translational modification by *V. cholerae* protease, which results in the formation of two fragments, CTA1 and CTA2, which are nevertheless still connected by a disulfide bond. CTA1 has the hazardous (enzymatic ADP-ribosylating) action of CTA, whereas CTA2 is used to introduce CTA into the CTB pentamer.

The complex is then taken up by endocytosis after the B-subunit homopentamer of the AB5 enterotoxin binds to the GM1 ganglioside receptors of the host intestinal epithelial cells. The A-subunit consists of the A1 and A2 domains, which are connected by a single disulfide link and a serine protease-sensitive cleavage site. (Gill and Rappaport, 1979). The alpha subunit of trimeric G-protein (Gs), a guanine nucleotide (GTP) binding regulatory protein, is ADP-ribosylated when the A1 subunit separates from the A2 subunit through proteolysis and reduction. This results in the stabilization of the GTP-bound form, constitutive activation of adenylate cyclase, and uncontrolled cyclic AMP synthesis. The cystic fibrosis transmembrane conductance regulator (CFTR) chloride ion channels are phosphorylated by protein kinase A (PKA), which opens them and results in elevated sodium conductance (Figure 3.5) (Goodman and Percy, 2005). The outflow of fluids and electrolytes into the lumen of the small intestines, which is the result of greater sodium conductance, is what leads to the dreadful watery diarrhoea, dehydration, and collapse of the circulatory system.

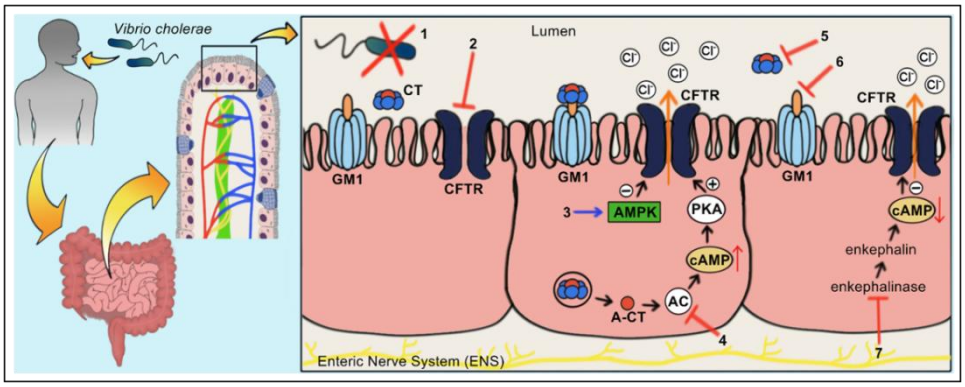

Figure 3.5. *Pathogenesis of cholera & mechanism of cholera enterotoxin.*
Source: Sousa et al., 2020

CT1 is possessed by all the classical biotype isolates and the isolates of the US gulf coast. Whereas CT2 is confined by the El Tor biotype strains (Finkelstein et al., 1987). Later on, the serogroup O1 was also found to contain the CT2. Another classification is based on the *ctxB* nucleotide sequences and consists of three types. Genotype 1 was carried by the classical biotype along with the US gulf coast strains. Genotype 2 was found to harbor solely with the El Tor strains Australian Peru isolates and genotype 3 is found in the seventh pandemic El Tor strains and the strains of Latin America (Olsvik et al., 1993). Therefore, the *V. cholerae* found in the earlier seventh pandemic contained the CT2 epitype containing cholera enterotoxin with nucleotide sequence similarity of genotype 3 while the classical biotype strains contained CT1 epitype and belonged to the genotype 1. Raychaudhuri et al. in 2008 amended the pre-existed biotypic scheme of classifying CT and *ctxB*. Further, a study in Bangladesh, which includes the strains isolated during the last five decades, examined the monoclonal classical and El Tor specific antibodies and to deduce the amino acid sequences the *ctxB* nucleotide sequences were compared. Their study revealed the production of CT of the classical biotype strain since 2001 which indicated a major change in the seventh pandemic El Tor strains (Nair et al., 2006). Therefore, the CT epitype and genotype linked with the El Tor seventh pandemic strains in Bangladesh have changed to the CT1 epitype with genotype 1 from the prototype CT3 epitype and genotype 3. The appearance of CT1 in El Tor strains have also been reported from other parts of the world (Ansaruzzaman et al., 2004; Nair et al., 2002; Tamplin et al., 1990). The clones from the US Gulf Coast were noticed to possess classical CT (Olsvik et al., 1993). Moreover, a recent study in Kolkata, India has reported the existence of classical CT in El Tor biotype strains and replaced completely the prototype El Tor CT since 1995 along with the presence of El Tor *rstR* carried by the hybrid CTX prophage (Raychaudhuri et al., 2009). Further, the Haitian isolates those are found in Haiti, Kolkata and other places recently carried a unique mutation at the signal sequence of the premature classical cholera toxin subunit B (Talkington et al., 2011; Goel et al., 2008; Son et al., 2011). This study together with the appearance and dissemination of Haitian kind of *ctxB* allele in Kolkata and Delhi, India (Naha et al., 2012; Ghosh et al., 2017) represented an evolutionary genetic background of the currently circulating *V. cholerae* O1 strains.

(b) Zonula occludens toxin (Zot)

Enterotoxin zonula occludens toxin (Zot) alters the intercellular tight junction, causing the small intestinal mucosa to become more permeable. Because of the increased intestinal permeability brought on by Zot, it is theorized that diarrhoea is caused by the loss of water and electrolytes into the lumen under the influence of hydrostatic pressure. Instead, a weakening of the tight junctions could allow the absorption of bacterial endotoxins or other substances, which would then encourage symptoms like nausea, vomiting, and headaches. It is currently unknown how Zot affects tight junctions. The 1.3 kb open reading frame of the *zot* gene, which is situated just upstream of the *ctx* locus (Baudry et al., 1992), has the potential to generate a 44.8 kDa polypeptide. Both *V. cholerae* O1 and non-O1 strains have *zot* gene sequences, and the strains with *ctx* sequence almost usually have *zot* sequence as well (Johnson et al., 1993; Kaper et al., 1995; Karasawa et al., 1993).

(c) Accessory cholera enterotoxin (Ace)

Accessory cholera enterotoxin (Ace), which can boost short circuit current in Ussing chambers, is another enterotoxin (Trucksis et al., 1993). It also raises the potential difference, just like CT. The ace gene, which is related to coliphage M13 gene VI, is positioned right upstream of the *zot* gene. Ace most likely works by accumulating and inserting itself into eukaryotic membranes to create an ion channel (Trucksis et al., 1993). The projected amino acid sequence of Ace is remarkably similar to a family of eukaryotic ion transporting ATPases, including the calcium-transporting ATPase from rat brain, the human plasma membrane calcium pump, and the cystic fibrosis (CF) gene's product, the CF transmembrane regulator (CFTR). The *V. cholerae* isolates of the classical and El Tor biotypes have been shown to share the same nucleotide sequence for the Ace protein.

(d) Repeats in toxin (RtxA)

Multifunctional autoprocessing repeats in toxin (MARTX) is a RTX family toxin and the largest open reading frame (ORF) consisting of 13,677 bp in *V. cholerae* genome (Heidelberg et al., 2000). In El Tor biotype *rtxA* has been found to reside in a gene cluster along with the other three genes *rtxB, rtxC* and *rtxD,* and contribute to the cytotoxicity of the Hep-2 cells (Lin et al., 1999). The Rtx exhibits a GD-rich repeated motif. RtxC, an activator, and the RtxB and RtxD ABC transporter systems are necessary for its function. According to

the study, RTX toxins from *V. cholerae* contribute to actin cross-linking and depolymerization in HEp-2 cells (Fullner and Mekalanos, 2000). A domain of the *V. cholerae* RTX toxin (RtxA) that covalently cross-links actin has been identified by Sheahan et al. (2004). RtxA consists of four domains, an actin cross-linking domain (ACD), alpha/beta hydrolase (ABH) domain, Rho inactivation domain (RID), and a cysteine-protease domain (CPD) (Sheahan et al., 2004). The *rtxA* gene has been found to be linked with *ctx* genetic element.

Both gaining and losing factors help in pathogen evolution that influence disease progression. Bacteria in the environment mature quickly and acquire nucleotide polymorphism through genetic change. This is the case with *V. cholerae*, which is exemplified by the wide diversity and distinctive variants of the RTX toxin produced by the *rtxA* gene found in environment-associated strains that lead to localized outbreaks of diarrhoea and food-borne illness. Contrarily, El Tor *V. cholerae* strains linked to severe diarrhoea during the seventh pandemic have undergone only minor changes, until the modified El Tor became the most common cause of cholera, including the epidemic in Haiti in 2010. These strains from the Haitian pandemic are more virulent and have been linked to a novel variation of the main virulence factor, cholera toxin. It was also noted that these strains of the Haitian epidemic have an inactivated RTX toxin gene, which also happened during the classical cholera pandemics. This circumstance emphasizes that El Tor cholera evolution is proceeding along a similar route of growing dependence on cholera toxin while eliminating other secretory elements (Dolores et al., 2013).

(e) Hemolysin (HlyA)

Another biotyping marker to distinguish classical and El Tor is the hemolysis of sheep blood erythrocytes. The hemolysin/cytolysin is initially made as an 82 kDa protein and organized in two steps to a 65 kDa active cytolysin (Yamamoto et al., 1990). *hlyA* gene which encode this hemolysin, are present in classical, El Tor, and non-O1 strains of *V. cholerae* (Brown and Manning, 1985). The absence of hemolytic activity in classical strains was expressed by the finding that the *hlyA* gene of classical strains has a deletion of 11 bp, leading to the synthesis of a truncated 27 kDa product (Alm and Manning, 1990). *hlyA* is a major virulence factor found in El Tor *V. cholerae* in chromosome II and the product is secreted through the Type-I Secretory system (T1SS). It was found that the purified hemolysin (Honda and Finkelstein, 1979) is cytolytic for different mammalian cells and erythrocytes in culture and severely

lethal for mice. It can cause accumulation of intestinal fluids in the ligated ileal loops in rabbits and the appearance of the fluid is invariably bloody and with mucous (Ichinose et al., 1987). The haemolysin in El Tor biotype damages eukaryotic cells by forming selective anion-channels of 1 to 2nm in diameter to the target membranes (Zitzer et al., 1995). The Haitian outbreak strains have also been found to contain the *hlyA* of the El Tor type and have the characteristic functional similarity with the prototype El Tor strains (Chin et al., 2011). Like polymyxin B, this biomarker has been no longer useful to distinguish between two biotypes due to the emergence of non-hemolytic El Tor variants. This is a result of genetic alteration, such as the *hlyA* gene's existence or absence, *hlyA* mutations, or variations in how this gene expresses itself (Fan et al., 2019). In this study, we have also observed non-hemolytic phenotypes among the El Tor variant strains.

(f) Hemagglutinin/Protease (HapA)

Hemagglutinin/protease (HapA) is produced by the soluble Zn-metalloprotease in *V. cholerae* and is encoded by the *hapA* gene (Hase and Finkelstein, 1991). Several physiologically significant host proteins, including mucin, can be proteolytically degraded by HapA (Finkelstein et al., 1983). By interfering with tight junction-associated proteins, HapA alters the paracellular barrier of intestinal epithelial cells in culture (Mel et al., 2000; Wu et al., 1996). In vitro penetration of a mucin-containing gel by *V. cholerae* was dependent on the expression of *hapA*, and *hapA* inactivation improved HT29-18N2 cells' adherence to mucin (Benitez et al., 1997; Silva et al., 2003). Finkelstein et al. (1992); Hase and Finkelstein (1991) found that although the infant rabbits and suckling mice infected with *hapA* mutants did not provide evidence that *hapA* is a critical virulence determinant, it has been found to contribute to reactogenicity against the live attenuated cholera vaccine strains in humans (Garcia et al., 2005).

3.11.2. Factors related to attachment & colonization

(a) Motility & flagella

Vibrio cholerae can swim by using its single polar-sheathed flagellum and motility has been identified as an important virulence property (Follet and Gordon, 1963). The swimming pattern of *V. cholerae* can be used for rapid identification of them in the infected patients stool through dark field microscopy and is known as “shooting star” (Benenson et al., 1964). The flagellar core is constituted of two proteins of 47 kDa and 49 kDa. A membrane sheath

that appears to be continuous with the outer membrane covers the flagellar core. These proteins are not agglutinated by or obstruct *V. cholerae*'s mobility by antibodies (Richardson and Parker, 1985). Flagellum also serves as adhesin irrespective of its role in motility (Attridge and Rowley, 1983; Eubanks et al., 1977). Researchers have developed antibodies recently, which can inhibit the motility of *V. cholerae* by interacting with O-specific polysaccharide, a component of lipopolysaccharide (Charles et al., 2020). It has been shown that the non-motile mutants have reduced virulence in the rabbit model of cholera (Richardson, 1991) but they do not show decreased colonization in the infant mouse model (Klose and Mekalanos, 1998).

(b) Toxin coregulated pilus (TCP)

Long filamentous pili-making bundles on the surface of *V cholerae* aid in the colonization of the small intestine and subsequent secretion of the cholera toxin, known as TCP due to the coordinated activation of the cholera toxin genes (*ctxA* and *ctxB*) and the *tcp* gene cluster by the ToxT transcriptional activator. It is one of the major key determinants, which can contribute to the pathogenesis of cholera. TCP belongs to the class of type IV b pilus. The *tcp* operon is found on the Vibrio Pathogenicity Island (VPI) of chromosome 1, where the genes for the TCP assembly components are located. Numerous pilin subunits produced by the *tcpA* gene combine to form TCP oligomers. TCP filaments have a diameter of about 80 Å and a length of several microns. The *V. cholerae* cells have these exposed filamentous extensions on their surfaces, which are used to communicate the creation of microcolonies and colonization of the small intestine (Kirn et al., 2000). The cholera toxin released by the bacteria is contained by interaction with TCP, which also causes cells to agglutinate in microcolonies on the small intestinal epithelial cells, protecting the bacteria from host defense. These pili also serve as a receptor for the cholera toxin phage (CTXΦ), which can infect non-pathogenic *V. cholerae* and transform it into a pathogenic form by transferring the genes producing cholera toxin (Waldor and Mekalanos, 1996). TCP in recipient bacteria must therefore be functioning for CTX transduction to occur.

The secretion of TcpF, a soluble colonization factor, depends on TCP (Kirn et al., 2003; Kirn and Taylor, 2005; Megli et al., 2011). In the absence of TCP, *V. cholerae* strains cannot colonize in the neonatal mouse infection model and human volunteers (Taylor et al., 1987; Herrington et al., 1988). Though they are fundamental to the *V. cholerae* life cycle and

infection process, the mechanisms by which TCP cohere among them and to CTXΦ are poorly understood.

All the El Tor and classical strains possess Tcp with 82.83% homology in the predicted protein sequences (Iredell and Manning, 1994; Rhine and Taylor, 1994). The synthesis and regulation of Tcp is complex and controlled by ToxR system (Ogierman et al., 1993; Kaufman et al., 1993). *V. cholerae* O139 also expressed Tcp when they are grown under conditions that approve expression of the Tcp in El Tor O1 and share homogeny in the *tcpA* gene sequence with *V. cholerae* El Tor biotype (Hall et al., 1993; Rhine and Taylor, 1994). The nucleotide sequence of the *tcpA* gene of two non-toxic vibrios from clinical and environmental sources was characterized by Novais et al., 1999, as being similar with only one change at the 559th nucleotide locations. In the same region of the classical and El Tor biotypes, the nucleotide sequence analysis revealed 76% and 71% nucleotide identity (Novais et al., 1999). A new variant of *tcpA* was isolated in a CT-positive clinical non-Ol, non-O139 strain by Nandi et al., 2000. Nucleotide sequence analysis revealed that the new variant had 74% and 72% homology with the *tcpA* of O1 classical and El Tor biotype strains, respectively (Nandi et al., 2000). Another new *tcpA* allele was identified in the environmental non-O1, non-O139 strains (Mukhopadhyay et al., 2001). In 2010, Haitian cholera outbreak further introduces another variant of *tcpA* allele. This Haitian *tcpA* sequence differs from the El Tor *tcpA* sequence only with a single base substitution at 266th nucleotide position (Talkington et al., 2011; Son et al., 2011). These entirely new variants suggest that there are still different *tcpA* alleles present in *V. cholerae*. The TcpA protein sequence has both highly conserved and hypervariable areas, which raises the possibility that strong selection pressure is driving this evolution (Nandi et al., 2000). Due to their critical involvement in pathogenesis, these surface-displayed virulence factors make attractive research topics for vaccines and antibacterial treatments.

(c) Mannose-sensitive hemagglutinin (MSHA)

A thin, flexible pilus, Mannose-sensitive Hemagglutinin was identified by Jonson et al. It is composed of 17 kDa subunits. MSHA is found to be expressed by the El Tor biotype strains and rarely found in strains of classical biotype (Jonson et al., 1991). MSHA amino acid sequence shows homology with the type IV pilus (Jonson et al., 1994). It is found to be associated with the chicken blood cell agglutination of *V. cholerae* O1 El Tor biotype and the

receptor binding site may be found in the actual structural subunit rather than in a minor pilus protein (Jonson et al., 1991).

Challenge with *mshA* null mutants in animal models and adult volunteers suggested that there is no obvious role of MSHA in the colonization of *V. cholerae* in the human digestive tract (Attridge et al., 1996; Tacket et al., 1998). It has also been reported that MSHA, and not the TCP is involved in colonization to the abiotic surfaces (Watnick and Kolter, 1999; Watnick et al., 1999). Thus, MSHA might have a definite role in the environmental survival of *V. cholerae*.

(d) Core-encoded pilus (Cep)

The 4.5kb core elements contain a gene encoding a component that promotes colonization in suckling mice in addition to the toxin genes *ctx*, *zot*, and *ace*. It has been shown that the *cep* (core-encoded pilus) locus encoded an 82-amino acid protein sequence (Pearson et al., 1993). The flexible pilus of *Aeromonas hydrophila* shows similarities with the protein's projected amino acid sequence. However, the *cep* gene product has not yet been associated with a pilus structure. The colonization capability of *V. cholerae* strains with deleted *cep* loci was shown to be reduced by 13 to 21-fold in an infant mouse model (Pearson et al., 1993).

(e) Other pili

In addition to TCP, at least two additional fimbrial kinds were described, and it was hypothesized that a single strain may express a variety of fimbrial types (Hall et al., 1988). Pili of *V. cholerae* O1 were purified, and they exhibited mannose- and fructose-sensitive hemaglutination activity (Ehara et al., 1987). Nakasone and Iwanaga, 1990 isolated pili from a *V. cholerae* non-O1 strain with a subunit size of 16 kDa. A pilus that is common to numerous *V. cholerae* strains of different serogroups was identified when an antibody produced against this pilus reacted with 12 of 49 *V. cholerae* non-O1 strains and 25 of 99 *V. cholerae* O1 strains.

3.12. Virulence gene regulators in *V. cholerae*

In *V. cholerae*, virulence gene regulation is a multifactorial process. In cholera pathogenesis, the two most important virulence factors are CT and TCP, which are controlled by a regulatory cascade that consists of three different transcriptional activators, ToxT and ToxR (Figure 3.6). Among them, TcpP and ToxT are found in the VPI while ToxR is the third one

that is found in the ancestor *V. cholerae* chromosome (Karaolis et al., 1998; Osorio and Klose, 2000).

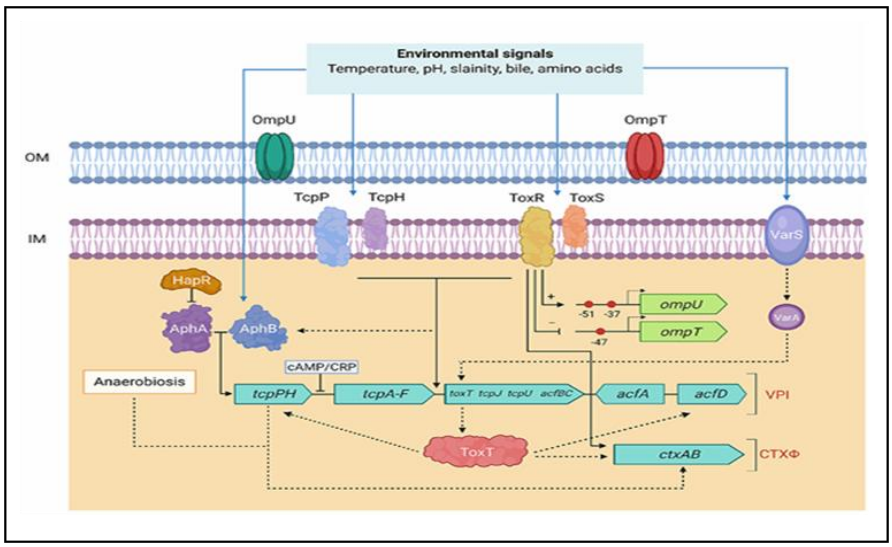

Figure 3.6. *Tox-mediated virulence genes regulatory cascade.*
Source: Ramamurthy et al., 2020

3.12.1. ToxR

A 32 kDa transmembrane protein belonging to the outer membrane protein regulator-R (Omp-R) family, ToxR is produced by the *toxR* gene (Miller et al., 1987). The regulation of virulence genes in response to environmental cues is greatly influenced by ToxR. Upstream of the *ctxAB* structural genes, a tandemly repeated 7 bp DNA sequence is where it binds. This binding contributes to an increase in *ctxAB* transcription, which leads to greater levels of CT expression (Miller and Mekalanos, 1984; Miller et al., 1987). ToxR not only regulates the expression of cholera enterotoxin but also the TCP colonization factor, the accessory colonization factor, the outer-membrane proteins OmpT and OmpU, three lipoproteins, and the accessory colonization factor (Taylor et al., 1987; Peterson and Mekalanos, 1988; Miller and Mekalanos, 1988; Parsot et al., 1991). The ToxR increases the expression of most of these factors, however, OmpT expression is lowered by ToxR (Miller and Mekalanos, 1988). This bitopic membrane protein consists of a periplasmic domain that helps in sensing the signal, a cytoplasmic DNA binding domain that can regulate the virulence genes transcription, and a transmembrane domain (Hsiao and Zhu, 2020). The activity of ToxR is enhanced by two gene products, *toxT* and *toxS*. As ToxR directly controls the expression of genes in addition to *toxT*, the ToxR regulon can be supposed to interact in two different

modes, the ToxT-dependent branch, and the ToxT-independent branch (Champion et al., 1997).

3.12.2. ToxT

The second transcriptional regulator ToxT is a 32kDa protein encoded by the gene *toxT* and activated by ToxR. It can act as both a monomer or a dimer. ToxT immediately starts the expression of virulence genes, particularly the CT and Tcp, after being triggered by ToxR and TcpP. *aldA* (aldehyde dehydrogenase), *acfA* (accessory colonisation factor), *tagA* (ToxR-activated gene-A), and *tarAB* (ToxT-activated RNA) are some examples of other accessory virulence genes that are activated by ToxT (Ramamurthy et al., 2020). ToxT is also reported to self-regulate its expression through an auto-regulatory loop (Yu and DiRita, 1999).

3.12.3. TcpH & TcpP

TcpP/TcpH consists of a pair of regulatory proteins, which are necessary for *toxT* transcription. These *tcpP* and *tcpH* genes are found in the TCP cluster of Vibrio Pathogenicity Island (Karaolis et al., 1998). *tcpP* deficient strains are not able to induce the *toxT* transcription (Hase and Mekalanos, 1998). Additionally, strains with *tcpH* mutations showed irregular toxT transcription and could not activate the virulence components like CT and TCP that are downstream of *toxT* (Carroll et al., 1997; Yu and DiRita, 1999). C58, one of the four cysteine residues found in the cytoplasmic domain of TcpP, is essential for the protein's functionality as well as for intestinal colonization in suckling mouse model and the activation of virulence genes (Shi et al., 2020). Although, there is no such data regarding the binding of TcpP to intestinal cells.

3.12.4. VieA

The genome of *V. cholerae* contains 52 response regulators and one of them is ***vieA*****,** a cyclic diguanylate phosphodiesterase and member of an operon encoding the VieSAB signal transduction pathway that is differently expressed in *V. cholerae* biotypes (Wang et al., 2015). In the El Tor biotype, this operon is almost completely silent, whereas it is expressed in the classical biotype to govern around 10% of the genome (Ayala et al., 2018).

The response regulator VieA, the auxiliary protein VieB, and the hybrid sensor kinase VieS constitute the VieSAB signal transduction system. The VieA protein has three domains: a receiver, a helix-turn-helix (HTH), and an EAL domain (Tamayo et al., 2005). By modifying

the c-di-GMP pool, VieS regulates gene expression through VieA, while VieB collaborates with VieS to prevent VieA phosphorylation (Martinez-Wilson et al., 2008; Mitchell et al., 2015). Thus, phosphorylated VieA transmits the host signal to regulate two adversely related cellular processes: promoting disease by *toxT* activation and impeding biofilm maintenance through decreased c-di-GMP levels. Although VieB lacks a DNA binding domain, it does have the conserved aspartate residue for response regulator phosphorylation. Instead, it has a structural motif that promotes protein-protein interactions and enables it to bind to VieS and prevent autophosphorylation of that protein. Because VieB accumulates at high transcriptional levels of the *vieSAB* operon, a negative feedback loop forms that prevents further stimulation of virulence from this source (Zhou et al., 2020).

H-NS and the master quorum sensing regulator HapR prevent the transcription of *vieSAB* in *V. cholerae* of the El Tor biotype (Wang et al., 2015).

Comparative genomic sequencing reveals a point mutation in Haitian *V. cholerae* strains with the El Tor strains at the position 235th nucleotide (C to T) which might replace the amino acid leucine (L) to phenylalanine (F) (Carignan et al., 2016). It has been reported recently that this point mutation does not play any significant role in the case of CT or ToxT production whereas, when the nonsynonymous point mutation in *vieA* (C235T, residue L79F) was introduced into N16961, the expression of TcpA decreased, indicating that the point mutation in Haitian *vieA* modifies VieA activity toward the *tcp* operon (Carignan et al., 2016).

In this study, we have studied a retrospective analysis to know the prevalence of Haitian *vieA* in Kolkata, West Bengal isolates.

3.12.5. Other virulence gene regulators

Some additional regulators are directly involved in various aspects of ToxR/TcpP/ToxT regulatory network within the cell.

tcpPH transcription necessitates two cytoplasmic proteins, AphA and AphB, which assist the connection of the ToxT regulon to quorum sensing (Skorupski and Taylor, 1999).

Recent studies indicated the importance of nucleotide derived small molecules in virulence and biofilm formation (Hsiao and Zhu, 2020). These secondary messengers are also important for bacterial environmental adaptation. One such small molecule is the well-studied cyclic AMP-cyclic AMP receptor protein (CRP) complex, that can negatively influence the gene expression of *toxR* virulence regulon in the *V. cholerae*. CRP negatively regulates the genes in the *toxR* regulon as evidenced by the transcription of the *ctx* and *tcp* genes in *crp*

mutant strains of *V. cholerae* even under non-inducing circumstances (Skorupski and Taylor, 1997). The mechanism of such regulation is unknown but might either by activating the ToxR/TcpP/ToxT cascade or by affecting the respective *tcp* and *ctx* promoters directly (Figure 3.6).

In *V. cholerae*, H-NS, also thought to be another negative regulator of several genes of ToxR virulence regulon. Members of the Enterobacteriaceae have a high prevalence of H-NS, which influences how virulence genes are expressed on plasmids or pathogenicity islands. In the absence of ToxR, TcpP, or ToxT, high levels of *toxT*, *tcpA*, and *ctx* transcription occur in *hns* mutants, indicating that H-NS functions as a "silencer" that only permits these virulence regulators to influence virulence gene transcription in the presence of the suitable environmental stimuli (Nye et al., 2000).

3.13. The CTXΦ genome

By infecting and integrating the lysogenic cholera toxin phage (CTX phage) into the bacterial chromosomes, toxic *V cholerae* strains are produced. It is a filamentous phage with a single-stranded DNA gene for cholera toxin measuring 7 kb in size (Waldor and Mekalanos, 1996). CTXΦ genome has two regions, a 4.6 kb "core" region contains the *ctxAB* and a 2.4 kb region termed as repeat sequence2 (RS2) which includes the genes important for DNA synthesis, regulation, and integration to the host cells (Waldor et al., 1997). RS was named so due to repeat sequence as this part is often duplicated directly in the flanking region of CTX element in *V. cholerae* (Mekalanos, 1983). The integrated CTXΦ is frequently flanked by another RS sequence called RS1 which is similar to RS2. Both the RS elements contain three open reading frames (ORFs), RstR, RstA2, and RstB2 (in RS2) and RstR, RstA1, RstB1 (in RS1) with identical *rstR* sequences and contain intergenic regions (*ig1* and *ig2*). RS1 contains an additional ORF designated *rstC* (Waldor et al., 1997). The *rstA2* gene is essential for phage replication; the *rstB2* gene is required for CTXΦ integration and the *rstR* gene contains the repressor protein, RstR, which help to repress the transcription of *rstA2*. To influence the transcription of *rstA2* and maybe other downstream genes, the amino terminus of RstR attaches to an operator site in the intergenic region between the divergently transcribed *rstR* and *rstA2* genes (Waldor et al., 1997). While RstR is a phage repressor, RstC is an antirepressor protein that inhibits its function. It also increases the transcription of genes necessary for phage development, which aids in the transmission of both RS1 and CTXΦ. Additionally, RstC's antirepression aids in the induction of CT genes like *ctxAB*, which may

increase the virulence of *V. cholerae* (Davis et al., 2002). The *ctxAB* genes are found in the core area of the CTX element alongside the genes *zot*, *ace*, *cep*, and *orfU* (Richardson and Wozniak, 1996). The CTX phage's morphogenesis also requires the coordinated expression of a number of genes, such as those encoding the phage coat proteins Cep and OrfU as well as the protein needed for CTX assembly (Zot) (Waldor and Mekalanos, 1996).

After the CTXΦ element is excised from the donor chromosome, it undergoes a site-specific recombination event between the *attRS* sites that are located at both ends of the CTX element before circularizing into a replicative form (RF). When inserted into other *V. cholerae* strains, this RF either replicates as a plasmid or integrates into the bacterial chromosome (Richardson and Wozniak, 1996).

CTXΦ is integrated into both the chromosomes of *V. cholerae* classical biotype strains, whereas the El Tor biotypes contain only one copy of CTXΦ in the first chromosome. Classical CTXΦ is unable to replicate because of the array of the prophage structure. But the El Tor strains are able to produce infected phage particles. It has also been reported that the recently circulating *V. cholerae* O1 strains from Kolkata have an altered orientation of CTXΦ and corresponding RS1 element which disables the excision of phage genome and production of phage particles (Ochi et al., 2021).

3.14. Comparative genome analysis of *V. cholerae*

Two chromosomes were found in *V. cholerae*, according to Trucksis et al. (1998). When undigested genomic DNA was electrophoresed, two megabase-sized fragments were visible, which led to the discovery of this. The lower fragment (replicon II) shows as a more clear band on the gel, but the top fragment (replicon I) appears as a diffuse smear on the gel, indicating that it was chromosomal DNA.

The two *V. cholerae* replicons correspond to two chromosomes for the following reasons. First, the size of the second, smaller replicon is equivalent to 40% of the whole genome. Second, both replicons are present in all three of the *V. cholerae* strains that were investigated, which include the classical, El Tor, and O139 serotypes. Third, the stoichiometry of both replicons with one another is fixed. The CTX element is present in both replicons of the classical biotype, which is the only instance of duplication that has been identified thus far among the genes found in each replicon. The inability to create a complete genetic map of *V. cholerae* using conventional chromosomal mobilizing techniques may be

explained by the discovery that the *V. cholerae* chromosome consists of two distinct and circular replicons.

3.15. *V. cholerae* pathogenicity island (VPI)

The *V. cholerae* pathogenicity island (VPI), first found by Karaolis et al. in 1998, is a chromosomal pathogenicity island (PAI) that is present in epidemic and pandemic strains but absent in nontoxigenic strains. A few environmental strains of *V. cholerae* were also reported to contain the VPI gene (Chakraborty et al., 2000). The PAI is 39.5 kb in size and has a lower G+C concentration than the rest of the *V. cholerae* genome, at 35%. The VPI consists of genes like *tcpA*, which encodes a crucial colonization factor and functions as the receptor for CTX, *toxT*, *tcpP*, and *tcpH*, which encode virulence gene regulators, as well as genes like ORFI and *int* that may be necessary for the transfer and integration of the VPI. In 1998, Karaolis and colleagues hypothesized that every gene on the VPI is likely to have a crucial role in virulence, either directly or indirectly through the transfer and motility of the VPI. This hypothesis created the possibility of the formation of new epidemic and pandemic strains. At a location close to a gene that looks like tRNA, the VPI is interpolated. It is flanked by *att* sites, which are used as specialized attachment sites between this element and the host bacterial chromosome. It has both a putative integrase and transposase gene. Three functional zones can also be used to separate the VPI (Karaolis et al., 2001). The three regions are as follows: (i) the VPI "left region," which contains a gene encoding a potential transposase as well as several ORFs whose functions are unknown and still being researched; (ii) the VPI "central region", which contains many genes of the *tcp* gene cluster encoding proteins for the TCP structure as well as the *tcpP* and *tcpH* genes involved in the coordinate regulation of TCP and CT expression; and (iii) the remaining *tcp* genes for transport and assembly, the *toxT* regulator of CT and TCP production, the *tcpJ* (gene essential for processing), *int* (high homology to phage like integrases), and the *acf* gene cluster (appears to play a role in colonization) are all included in the VPI's "right area" (Karaolis et al., 2001). Specific environmental strains of *V. cholerae* that can successfully colonize the human digestive tract may be changed by VPI. Additionally, after being infected by CTX, the VPI permitted these strains to develop toxigenicity. Therefore, the VPI may be the fundamental genetic component needed for epidemic and pandemic disease. If non-O1, non-O139 strains of *V. cholerae* acquire VPI and CTX, it is entirely feasible that they will be the source of future cholera epidemics. The VPI of the sixth pandemic (classical biotype) and the seventh

pandemic (El Tor biotype) strains have been extensively sequenced and compared by Karaolis et al., 2001. There were several proteins whose estimated sizes varied substantially between the strains as a result of frameshift mutations, in addition to different nucleotide and amino acid polymorphisms (Karaolis et al., 2001). Accordingly, it appears that *V. cholerae*, as a species, has a pool of diversity in the central region of the VPI, which has helped to promote the formation and evolution of the pathogenic forms through horizontal gene transfer (HGT) and genetic recombination.

3.16. Vibrio seventh pandemic island-I and II (VSP-I and VSP-II)

Two genomic areas are only found in the seventh pandemic El Tor and O139 strains by the genomic microarray analysis, according to the research of Dziejman et al (2002). They are known as VSP-1 and VSP-II, the Vibrio seventh pandemic island I and II, respectively. The 16-kb region known as VSP-I includes ORFs VC0175 through VC0185. 40% of this region's G+C content is unusual (compared to 47% for the overall genome) (Dziejman et al., 2002). A P4-like integrase is encoded by the 27-kb region known as VSP-II, which is integrated at a tRNA methionine locus (VCO516.1) (O'Shea et al., 2004). VSP-II was first recognized as a 7.5 kb region containing the ORFs VC0490-VC0497 (Dziejman et al., 2002).

Later, it was discovered that the VSP-II area in *V. cholerae* biotype El Tor and O139 serogroup isolates truly comprised a 26.9 kb region (VC0490-VCO516) (O'Shea et al., 2004). In addition to two transcriptional regulators, a type IV pilus, a DNA repair protein, two methyl accepting chemotaxis proteins, and a P4-like integrase (VCO516) next to the tRNA-methionine locus (VCO517.1), VSP-II encodes these proteins (O'Shea et al., 2004).

Studies suggested that the presence of VSP-II might benefit bacterial fitness and survival. A new bacteriocin and a pyocin protein were found in the VSP-II after comparative genomic investigations of the *V. cholerae* El Tor reference strain N16961 and a set of strains responsible for an outbreak of the disease in Florida (Haley et al., 2014).

However, it is unclear how the genes found in the VSP-II element function. Numerous reports have been made regarding the VSP-II variants in El Tor strains from many continents, including Asia, Africa, and Latin America (Imamura et al., 2017). Imamura and his colleagues detected four main types of heterogeneity in the VSP-II region of *V. cholerae* strains isolated from Kolkata, West Bengal, between 2007 and 2014 (Figure 3.7) (Imamura et al., 2017). In 2016-2018, the Asian country Yemen was the witnessed of the worst cholera

outbreak caused by the hypervirulent El Tor variant strains contains VSP-IIC which is a significant feature of these strains (Weill et al., 2019).

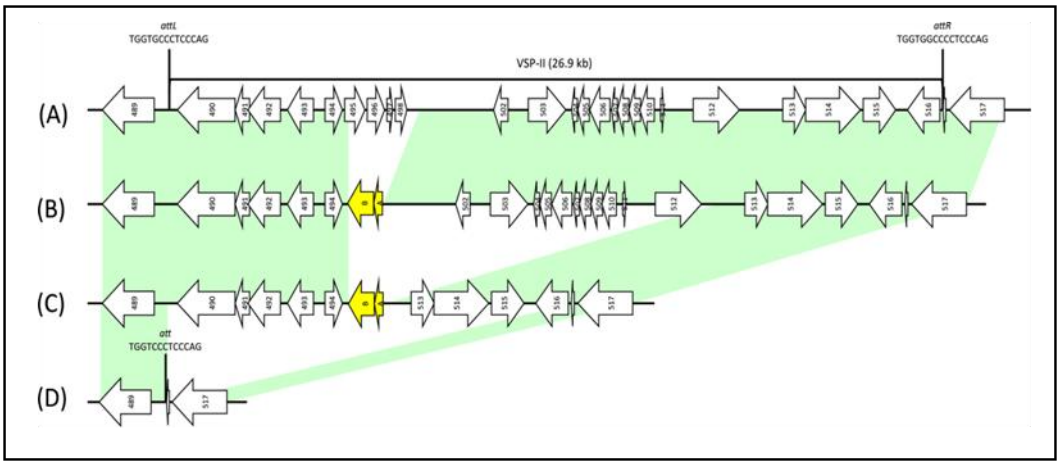

Figure 3.7*. Genomic organization of VSP-II in Kolkata isolates (A) Intact VSP-II of reference strain N16961, (B) VSP-IIB, (C) VSP-IIC, (D) VSP-IID of VSP-II negative strain.*
Source: Imamura et al., 2017

3.17. Drug resistance in *V. cholerae*

Antibiotics are chemical compounds that can inhibit or kill bacterial species, synthesized from microbes as a weapon to kill other microbes. Antibiotics are generally perform its work by inhibiting cell wall synthesis, cell membrane integrity, inhibiting DNA, RNA, and protein synthesis, or intruding essential cellular metabolic pathways (Figure 3.8).

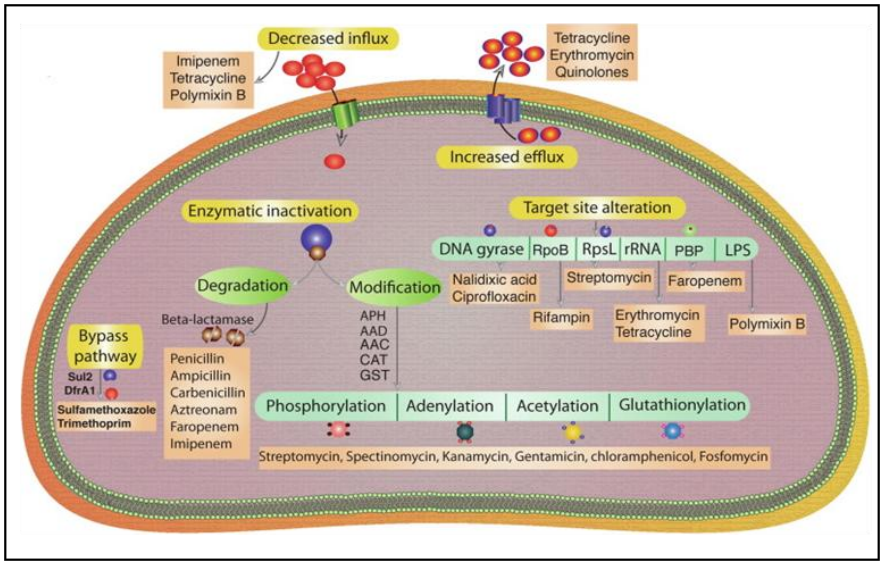

Figure 3.8*.Mechanism of antibiotic resistance in Vibrio cholerae.*
Source: Das et al., 2020

Antibiotic treatment helps to reduce the duration and transmission of cholera, especially during an outbreak. Antibiotics are not generally used for cholera and thus *V. cholerae* O1

strains were shown to be sensitive up to the 1970s. But this pattern of sensitivity has changed significantly with the emergence of drug resistance *V. cholerae* strains (Ghosh et al., 2011; Mandal et al., 2012; Mohanraj et al., 2020; Quilici et al., 2010) and showed altered AMR profile. However, for several decades *V. cholerae* has emerged as a notorious multidrug resistant (MDR) enteric pathogen (Das et al., 2020). The dissemination of the genetic determinants of antibiotic resistance has been facilitated by acquisition of plasmids, integrating conjugative elements (ICEs), superintegron, transposable elements, and insertion sequences (Das et al., 2020; Hochhut et al., 2001). Harboring such elements led the strains more prone to cause fatal disease.

The first report of *V. cholerae* O1 El Tor strains showed reduced susceptibility towards third-generation cephalosporin has appeared in Argentinian cholera outbreak (Rossi et al., 1993). After that, several reports have been published stating the resistance against third-generation cephalosporins and most of them were from Indian subcontinent (Ceccarelli et al., 2016; Krishna et al., 2021). There are very few reports indicating carbapenem resistant *V. cholerae* O1 strains which consider this drug as last resort of antibiotic. The unique carbapenem structure comprises the β-lactam ring renders this class of antibiotics remarkably stable (Goh et al., 2022). Due to the steric hindrance provided by the 6-α-1R-hydroxyethyl moiety at the β-lactamase binding site, carbapenems are much more stable and efficient than other β-lactams (Zhanel, et al., 2005).

According to WHO, carbapenem is considered “critically important antimicrobials”. However, the carbapenem resistance is continuously skyrocketing clinical and economic costs (Goh et al., 2022).

In the present study, we have characterized recently emerging carbapenem resistant *V. cholerae* O1 strains carrying plasmid indicating an alarming concern towards public health.

Chapter 4

Objectives

4. Objectives of the Study

Cholera, the devastating diarrheal disease continues to be an important health concern in developing countries that lack safe drinking water and proper hygiene. *V. cholerae* has the potential to adapt and thrive in unfavorable environments by changing their phenotypic or genotypic characteristics (Daboul et al., 2020). This ability also helps them cause more severe outbreaks in different regions of the world. South Asia, particularly the Ganges-Brahmaputra delta, has long been considered the ‘‘homeland of cholera”. This Ganges delta in India, which encompasses mostly West Bengal, was thought to be the reservoir of the organism *V. cholerae* and the place of origin of the disease cholera. Reports suggested that the strains that caused the large outbreaks in other continents have accumulated the toxic genetic components from the Indigenous strains and caused outbreaks in other parts of the world. However, the recently emerging El Tor variant strains with polymyxin B-sensitive phenotypic traits cause disastrous outbreaks worldwide. Pathogenesis of *V. cholerae* is continuously becoming mysterious with the emergence of new variant strains. Although the pathogenesis of *V. cholerae* is a complex, multifactorial process, consisting of *V. cholerae* colonization, co-ordinate expression of virulence factors and toxin action play a key role in producing profuse watery diarrhea.

Moreover, the unique genetic makeup and remarkable competency of *V. cholerae* are the key factors that help the cholera pathogen adapt rapidly to adverse environmental conditions and resist the detrimental effects of antimicrobial agents. Harboring chromosomal mutations, and extrachromosomal mobile genetic elements (MGEs) from closely or distantly related bacterial species through horizontal gene transfer contributes to major roles in *V. cholerae* drug resistance, which leads to fatal disease outcomes.

Therefore, the present study was designed to highlight the changing genetic features that contribute to the higher virulence of the currently circulating *V. cholerae* O1 strains in different endemic regions of India, as well as to understand the natural mode of adaptation of these strains along with the antibiotic resistance pattern during the course of time.

Keeping the above views in mind, the present study was designed with the following objectives:-

1. To analyze the changing genetic features of currently circulating *V. cholerae* O1 isolates in India.

2. To know the antimicrobial resistance pattern in recently circulating *V. cholerae* strains along with its mechanism of resistance.
3. To understand the inflammatory response of El Tor strain and recently emerged polymyxin B-sensitive El Tor variant on intestinal epithelial cell line.
4. To compare the pathogenicity like colonization ability and fluid accumulation in animal models when infected with polymyxin B-resistant El Tor and polymyxin B-sensitive El Tor variant strains.

Chapter 5

Materials & Methods

5. Materials & Methods

5.1. Collection of clinical *V. cholerae* O1 strains

An active epidemiological surveillance program is generally conducted by the ICMR-National Institute of Cholera and Enteric Diseases (ICMR-NICED) with the stool samples of the patients suffering from diarrhea and admitted to the ID&BG hospital (IDH) and B. C. Roy hospital (BCH) in Kolkata, India aiming to the study of the diarrheal diseases. IDH and BCH are the two main referral centres for diarrhea in West Bengal for years and people from the city and other neighboring districts also received treatment from these hospitals around the year. Another group of clinical isolates of *V. cholerae* O1 was received from different hospitals from five states of India including, Shri Vasnatrao Naik Govt. Medical College in Yavatmal (Maharashtra); State Public Health Laboratory in Baroda, SMIMER Medical College in Surat, AMC MET Medical College in Ahmedabad and P.D.U Govt Medical College in Rajkot (Gujarat); Tirunelveli Medical College in Tirunelveli (Tamil Nadu); Manipal Hospital in Bangalore, Kodagu Institute of Medical Sciences in Madikeri (Karnataka) and Rajan Babu Institute of Pulmonary Medicine & Tuberculosis in (Delhi), India (Table 5.1).

Hence, this study mainly focused on the cholera of different diarrheal endemic states located in India with duration of 4 (2018-2021) years.

Table 5.1. *Isolation details of V. cholerae strains in this study.*

Sl No.	Studied Regions	State of Isolation	Place of Isolation	Year of Isolation	No. of Isolates (n)
1	West Bengal	West Bengal	Kolkata	2018-2021	116
2	Other regions of India except West Bengal	Maharashtra	Yavatmal	2018	22
3		Gujarat	Baroda	2018-2020	79
4			Surat	2018-2019	56
5			Ahmedabad	2021	81
6			Rajkot	2019, 2021	4
7		Tamil Nadu	Tirunelveli	2020	4
8		Karnataka	Bangalore	2020	38
9			Madikeri	2020	2
10		Delhi	Delhi	2019, 2021	72
Total				**2018-2021**	**474**

5.2. Bacteriology

5.2.1. Collection and transportation of stool samples

Clinical stool samples were collected from patients of any age group suffering from diarrhea and admitted to the ID&BG and B.C. Roy hospitals in Kolkata, West Bengal. The stool samples were collected in the McCartney bottles using a sterile catheter and the specimens were processed within 1 hour of collection. In some cases, rectal swabs were taken from the patients, from whom stool could not be obtained, using sterile cotton-tipped swab sticks. Swab sticks were placed in Cary Blair (Difco, USA) transport media and transferred to the laboratory for analysis.

5.2.2. Isolation and selective identification of the strains

Stool samples were directly streaked onto thiosulfate citrate bile salt sucrose (TCBS) agar (Eiken, Japan) plate for 18-24 h with the help of cotton swab and incubated at 37°C overnight. The TCBS media served as the selective and differential media for *V. cholerae* as this organism produced yellow colored colonies due to its ability of sucrose fermentation. Alkaline peptone water (APW) is used for the enrichment of bacteria present in stool as well as in rectal swab samples. For this, swab sticks or a small portion of the diarrheal stool was inoculated into APW allowed the growth for 4-6h at 37°C into a shaker, and then streaked onto TCBS plates. After incubation, a typical, large yellow colour colony was picked and sub-cultured on Luria Bertani agar containing 1% NaCl (1% LA) in the form of a lawn and further incubated at 37°C for 18-24 hours. *V. cholerae* colonies grow as 2-4 mm in diameter, round, slightly flattened, sucrose fermenting, yellow with opaque center and translucent peripheries.

5.2.3. Presumptive identification of *V. cholerae* strains

(a) Oxidase test

This test is used to identify bacteria containing the respiratory enzyme, cytochrome oxidase. The cytochrome oxidase enzyme catalyses the transport of electrons from a donor compound to the final electron acceptor-oxygen. In this test, an artificial electron donor, phenylenediamine, a redox dye in its reduced form, is used to reduce the cytochrome oxidase. If the enzyme is present, the colourless dye will turn immediately into purple colour. No colour change is a negative test.

A small amount of overnight culture from the 1% LA plate was taken with the help of a sterile toothpick and rubbed on blotting paper soaked with the oxidase reagent (N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride). The presence of a deep purple colour indicated a positive reaction.

(b) String test

This test was performed on a slide by suspending 18 to 24h growth from culture on LA plate. A drop of 0.8% aqueous solution of sodium deoxycholate was then added. If the result is positive, the bacterial cells will be lysed by the sodium deoxycholate, the suspension will lose turbidity, and DNA will be released from the lysed cells causing the mixture to become viscous. A mucoid "string" is formed when an inoculating loop is drawn slowly away from the suspension.

(c) Triple sugar iron agar

This test works on the principle that a microorganism's ability to ferment sugars and produce hydrogen sulfide. TSI agar contains three carbohydrates, glucose, sucrose and lactose with a pH indicator dye phenol red. TSI slants (Becton Dickinson and Company, USA) were inoculated by stabbing the butt with the small amount of culture taken from 1% LA plate by using straight wire and also streaking on the surface of the medium by using loop. The inoculated slants were incubated at 35°C for 18 to 24 hours. *V. cholerae* strains produced alkaline slants over acid butts as recorded by the appearance of purple and yellow color (K/A reaction) and did not produce any gas bubbles (Kaper, 1979).

5.2.4. Serological confirmation

Based on the presence of O-antigens on the cell surface of *Vibrio cholerae*, over 200 serogroups are identified. Among them only serogroups, O1 and O139 cause cholera. Detection of a particular serogroup is depending on the agglutination reaction with the antiserum that is specific to that serogroup. Agglutination tests were performed by taking a small amount of the culture and rubbed on a drop of polyvalent O1 specific antisera on a glass slide. The strains that agglutinated with the O1 were tested further with Ogawa and Inaba antisera to determine the serotype of O1 serogroup.

5.2.5. Preservation of *V. cholerae* strains

All the confirmed strains of *V. cholerae* O1 were preserved in 0.8% nutrient agar (NA) stab and 16% glycerol stock and stored at room temperature and -80°C respectively.

Nutrient agar stab contained 0.8% of agar in a test tube plugged with cotton, autoclaved and solidified. The confirmed culture was picked up with a sterile straight wire from the 1% LA plate and stabbed into the NA stab. The stab was then incubated at 37°C for 18h and after incubation; the cotton plug was replaced with a rubber cork, sealed with parafilm and stored at room temperature. Glycerol stock was prepared by growing the confirmed culture in Luria-Bertani broth (LB) for 4-6hrs at 37°C. Then 540µl of the culture was mixed with 260µl of sterile 50% glycerol to make a total volume of 800µl. The stock was mixed by pipetting and kept at -80°C for further use. The microorganism stored in the form of stabs and stocks remained viable for years. To use further, the stab and stocks were revived by streaking on the TCBS plate.

5.3. Biotyping of *V. cholerae* O1

V. cholerae O1 is further categorized into biotypes, classical and El Tor, based on a few phenotypic tests such as polymyxin B susceptibility, Voges-Proskauer (VP) test, sheep blood haemolysis, chicken blood cell agglutination and susceptibility to biotype specific phages. These two biotypes differ in their genetic constituents and pathogenicity.

5.3.1. Polymyxin B susceptibility test

Polymyxin B susceptibility assay is a very reliable phenotypic test generally used for the classification of two biotypes of *V. cholerae* O1. Classical strains were polymyxin B sensitive and El Tor strains were polymyxin B resistant.

(a) Polymyxin B (50U) susceptibility checked by Kirby-Bauer disc diffusion method

A small amount of culture was taken to inoculate into Tryptone Soya Broth (TSB) and grown for the 0.08-0.13 at OD_{600} (0.5 McFarland standard). Then the culture was spread over Mueller-Hinton agar (MHA) plates with the help of a sterile cotton swab stick. After 5 minutes, a polymyxin B (50U) disc was placed on the center of the plate and incubated overnight at 37°C. The diameter of the zone of inhibition (in mm) was measured according to the guidelines of European Committee on Antimicrobial Susceptibility Testing (EUCAST)

(EUCAST, 2019). The breakpoint followed was: Resistant ≤8mm, intermediate 9-11mm and sensitive ≥12mm. Escherichia coli ATCC 25922 was used to check disc accuracy.

(b) Polymyxin B (50µg/ml) plate susceptibility assay

A single colony was picked from the TCBS plate and inoculated in LB broth media for 4-6 hours. One loopful of this culture was streaked on LB Agar plates containing polymyxin B at a final concentration of 50µg/ml. A corresponding set of plates without polymyxin B were prepared to check whether the sensitive strains were viable and still capable of growing on LB Agar plates. Resistant strains were grown on the LA-PolB50 plate whereas; sensitive strains were unable to grow (Samanta et al., 2018).

5.3.2. Voges-Proskauer (VP) test

This test was performed to detect the ability of a microorganism to produce acetylmethycarbinol from glucose fermentation. In this method, the test organism was inoculated and grown in VP broth for 24-48 hrs at 37°C. VP broth or glucose peptone broth contains glucose as a carbon source. Bacteria ferment glucose, and produced acetoin or acetylmethyl carbinol which is converted to diacetyl in the presence of alpha-napthol and 40% KOH with atmospheric oxygen. El Tor strains yield a positive reaction in VP test by the production of pink coloration. Such coloration was due to presence of 2, 3- butanediol. El Tor strains have the capacity to produce 2, 3- butanediol by carbohydrate fermentation while classical strains produce organic acids as their fermentation end product (Chatterjee et al., 2009).

5.3.3. Sheep blood hemolysis (SBH)

Tryptic soy agar (TSA) was prepared, with 5% sheep blood. One drop (~10µl) of an overnight culture of test strains in TSB was placed over the blood agar plate, soaked and incubated at 37°C for 24 hrs. The appearance of a greenish transparent zone indicated a positive reaction. Non-hemolytic strains are found to show opaque zones around the drops on the blood agar plate. Classical strains were non-hemolytic whereas, the El Tor strains are found positive for this test (Liu, 1959).

5.3.4. Chicken blood cell agglutination (CCA)

Chicken cell agglutination is another test to differentiate between the classical and the El tor biotypes. Chicken whole blood was collected and mixed with an equal volume of Alsevers'

solution (anti-coagulating agent). 1ml of blood was centrifuged at 2500 rpm for 2 mins and the cell pellet was washed 4-6 times with 1ml phosphate buffered saline (PBS). Then the pellet was re-suspended in 100µl PBS, mixed by pipetting and one drop was placed over a clean glass slide. A small amount of bacterial culture was taken with a toothpick and rubbed on the drop of the blood for agglutination reaction. El Tor strains are able to agglutinate chicken red blood cells (RBCs) as they possess cell-surface-associated mannose-sensitive hemagglutinin (MSHA) and are active on chicken erythrocytes (https://www.ncbi.nlm.nih.gov/books/NBK8407/). Classical strains do not harbor MSHA and thus, cannot agglutinate chicken RBCs (Finkelstein, 1964).

5.4. Determination of antibiotic susceptibility profile

One-third of the total isolated *V. cholerae* O1 strains were taken to note the antimicrobial susceptibility pattern. A number of recent generation antibiotics were chosen and the susceptibility was analyzed with the following methods:

5.4.1. Kirby-Bauer disc diffusion test

Representative *V. cholerae* O1 isolates were taken to examine their susceptibility to fifteen antibiotics viz. ampicillin (10µg), tetracycline (30µg), chloramphenicol (30µg), sulfamethoxazole/trimethoprim (23.75/1.25µg), ciprofloxacin (5µg), nalidixic acid (30µg), norfloxacin (10µg), ofloxacin (5µg), gentamycin (10µg), streptomycin (10µg), ceftazidime (30µg), ceftriaxone (30µg), cefotaxime (30µg), meropenem (10µg), azithromycin (10µg) and doxycycline (30µg) by Kirby-Bauer disk diffusion method using commercially available antibiotic disks (Difco, B.D, USA and Himedia, India) under the criteria recommended by European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2019). The *E. coli* strain ATCC 25922 was used as the control to check disc accuracy for this assay (Bauer et al., 1966). Breakpoints were interpreted following the guideline mentioned by EUCAST.

5.4.2. Minimal inhibitory concentration (MIC) test with E-strips

MIC is necessary to determine the extent of antimicrobial resistance property of a particular organism. In this study, MICs have been performed with the strains that showed resistance against latest generation antibiotics. The tested strain was grown and spread as the same technique used for the disc diffusion method. E-test gradient strip (HiMedia, India) was placed on the agar surface and the plate was incubated at 37°C overnight with an inverted position. On the next day, a zone of inhibition was found and the MIC value was taken at the

point where the zone interacted with the strip. Breakpoints were interpreted following the guideline mentioned by EUCAST (EUCAST, 2019).

5.5. Molecular characterization of isolated *V. cholerae* O1 strains

5.5.1. Polymerase chain reaction (PCR)

This technique was developed by Kary Mulis during 1980 based on the principle of enzymatic amplification of DNA. It requires a DNA template, two oligonucleotide primers of length from 15-25 bases, deoxyribonucleotides (dNTPs) and the enzyme Taq polymerase. A thermal cycler that can shuffle the temperature of its blocks shortly (ramp rate) was used for the amplification (making copies) of the DNA fragment of our interest. This PCR product was run in an agarose gel for staining and viewing of the amplified product (Mullis et al., 1986).

(a) Isolation of genomic DNA

Several methods have been used to isolate DNA from the bacterial cell.

i) Boiled template method

The principle of this method is the heating and subsequent chilling which causes lyses of the bacterial cell and release the DNA content that remains in the suspension pelleting down other cell components. A small amount of bacterial culture was taken from the LA plate and suspended in a 1.5ml micro-centrifuge tube containing 200µl of autoclaved water. Vortexed the tubes and placed them in boiling water bath for 10 mins followed by snap chilling on ice for 5 mins and centrifugation at 10000 rpm for 10 mins. The supernatant was collected and directly used as a template for PCR.

ii) Phenol-chloroform method

This method is also used to separate nucleic acids from other cellular components by using a mixture of phenol, chloroform, and isoamyl alcohol, which can help in the partitioning of lipids and debris into an organic phase, leaving the DNA in the aqueous phase. One loopful of an overnight culture from LA plate was suspended in 200µl of Tris-EDTA buffer (pH 8.0) and then lysed by vigorous vortexing with a mixture of phenol: chloroform: isoamyl alcohol (25:24:1) saturated with 10 mM Tris and 1mM EDTA (Sigma-Aldrich, St Louis, MO, USA). The supernatant was collected carefully after centrifugation at 12,000 rpm for 15 min and

was extracted once by adding an equal volume of a mixture of chloroform: isoamyl alcohol (24:1) and centrifuged for 15 min at 12,000 rpm. The supernatant containing the DNA was diluted 30 times in autoclaved water and used as template for PCR analysis. All the centrifugation steps were carried out at room temperature.

iii) Cetyltrimethylammonium Bromide (CTAB) method

A modification of the method of Murray and Thompson, 1980 was used for DNA extraction. Cells were harvested from 16-18 h grown culture in LA by centrifugation at 3000 rpm for 1 min at 4°C suspended in 1ml PBS. Bacterial pellet was resuspended in 540µl of TE buffer (pH-8), followed by sequential addition of 60µl 10% SDS solution and 9µl freshly prepared proteinase K solution (20 mg/ml). The suspension was then incubated at 50°C for 1 h for the digestion of proteinaceous material. After incubation, 6µl of RNase A (final 100 µg/ml) was added and incubated at 37°C for ½ h. Then 100ul of 5M NaCl solution was added followed by the addition of 80ul CTAB/NaCl and incubated at 65°C for 10 minutes. The DNA was extracted once with equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1) by collecting the aqueous phase at each of the extraction steps followed by the centrifugation at 12,000 rpm for 10 min, into fresh microfuge tubes. The aqueous phase was transferred to a fresh micro-centrifuge tube and the DNA was precipitated with 0.6 volume of isopropanol and recovered as an insoluble pellet by centrifugation at 10,000 rpm at 4°C for 10 min. The DNA pellet was washed with 70% ethanol, dried and then suspended in 80µl of TE buffer (pH 8.0). The concentration of the DNA was determined spectrophotometrically. Then the DNA was diluted to 20-100ng/µl and used as template for PCR (Murray and Thompson, 1980).

(b) Quantification and quality of the nucleic acid

Quantification of both DNA and RNA was carried out by spectrophotometric measurement of the samples. For this 1µl portion of the nucleic acid solution was used to measure the optical density at 600nm wavelength (OD_{260}) value in a NanodropTM spectrophotometer. It was assumed that a DNA (double stranded) solution has a concentration of 50µg/ml would give an OD value of 1 (Sambrook et al., 2001). The OD_{260} values of the samples were used to estimate DNA concentration by unitary method. Similarly for RNA samples, it was understood that a solution having a concentration of 33µg/ml would give unit OD value (Sambrook et al., 2001). Here again, the OD_{260} values of the samples were used to estimate

the total RNA concentration by the unitary method. The purity of the sample was checked by determining the ratios of OD values separately measured at 260 and 280 nm respectively. The purity of RNA is close to 2.0.

DNA preparation with an OD_{260} to OD_{280} close to 1.8 was always used for digestion with restriction enzymes and other molecular biological applications. Agarose gel electrophoresis of the DNA preparation confirmed that it did not contain any contaminating materials.

(c) Preparation of PCR reaction mixture and cycling condition

To run a PCR amplification assay, it is important to constitute the PCR master mix with the appropriate proportion. Commercially available PCR kit was used (GeNei, India and TaKaRa, Japan). For a single PCR 20µl of total reaction volume was set up. The components were added as follows: 10X reaction buffer-2.0 µl, 200 µM of each dNTPs, 100-300 pmoles of each forward and reverse primer, 2Units of Taq DNA polymerase and 20-100ng of DNA template.

The complete PCR mixture was then placed in a thermal cycler machine with the following steps: initial denaturation at 96°C for 2mins; 25-35cycles of denaturation at 94°C for 30secs, annealing at 54-60°C for 25-30secs and extension at 72°C for 25-60secs; and final extension at 72°C for 4mins. PCR cycle, annealing temperature and time, and extension time are varied for different amplifications details of which are given in Table 5.2.

(d) Agarose gel electrophoresis and documentation

Gel electrophoresis has been used to separate DNA molecules (PCR amplified) and to estimate the size of nucleic acid molecules of known length by comparison with the migration of molecules of known length (molecular size marker or DNA ladder) in agarose gels. The agarose gel was stained in a solution containing the fluorescent dye ethidium bromide (EtBr) (Sigma). By principle, this planar molecule binds to DNA by intercalating between the base pairs. Binding concentrates ethidium in the DNA and also increases its intrinsic fluorescence. As a result, when the gel is illuminated with ultraviolet light, the regions of the gel containing DNA fluoresce much more brightly than the regions of the gel without DNA. For the PCR amplified products, 4µl of the product was mixed with 1µl of bromophenol blue loading dye (Appendix) and loaded on agarose (SRL, India) gels (concentration of agarose is varied according to the size of the DNA molecule). Electrophoresis was carried out in 1X Tris-acetate EDTA (TAE) buffer (Appendix) in a

horizontal gel electrophoresis apparatus (BioRad, USA) at 100 volts for 1h using a power supply (BioRad). For molecular weight determination, a 100 bp or 1 kb ladder (New England Biolabs, NEB, UK) was run along with the PCR products. After electrophoresis, the gels were stained in a freshly prepared EtBr solution (0.5mg/ml) for 15 min. The gels were then viewed and documented with Gel-Doc 2000 system (BioRad).

Table 5.2. *Primers used in this study.*

Primer	Sequence (5'-3')	Target	Amplicon (bp)	Annealing (°C)	Reference
rtxAR1	TGTGAACCACGTCTGCC	*rtxA*	187	54	Ghosh et al., 2014a, 2014b
rtxAR2	TGTGAACCACGTCTGCT				
rtxAF	ATCGGAATGAGTGAGAAAGACC				
ctxBF3	GTTTTACTATCTTCAGCATATGCGA	*ctxB*	191	58	Naha et al., 2012
ctxBF4	GTTTTACTATCTTCAGCATATGCGC				
ctxBRv-cla	CCTGGTACTTCTACTTGAAACG				Morita et al., 2008
tcpA-F1	CCAGCTACCGCAAACGCAGA	*tcpA*	167	56	Ghosh et al., 2014a, 2014b
tcpA-F'2	CCAGCTACCGCAAACGCAGG				
tcpA-El-Rev	CCGACTGTAATTGCGAATGC				
gyrAF	TGCTCTTCCTGATGTGCGTGATG	*gyrA*	177	60	Ghosh et al., 2014a, 2014b
gyrAR1	GATGGTGTCGTAAACCGCTA				
gyrAR2	GATGGTGTCGTAAACCGCTC				
rstBF1	ATTCTGAAGGGGTGAGTCGTA	*rstB*	160	58	Ghosh et al., 2014a, 2014b
rstBR2	CTGGTCATCGCGTCACTGGAT				
rtxC-F	CGACGAAGATCATTGACGAC	*rtxC*	263	55	Chow et al., 2001
rtxC-R	CATCGTCGTTATGTGGTTGC				
rstC1	AACAGCTACGGGCTTATT	*rstC*	238	52.4	Zaw et al., 2019
rstC2	TGAGTTGCGGATTTAGGC				

CIIF	CTCACGCTGAACAG CAAGTC	cII	766	55	Maiti et al., 2006
CIIR	TTGCTTGAATCGAA AGGACA				
rstR1(F)	CTTCTCATCAGCAA AGCCTCCATC	*rstR*	500	50	Nusrin et al., 2004
rstR2(F)	GCACCATGATTTAA GATGCTC				
rstA3(R)	TCGAGTTGTAATTC ATCAAGAGTG				
VC0502F	TCATCAGTTAGCAC ACGAAC	VC0502	476	52	O'shea et al., 2004
VC0502R	GCTATCGTTATACT TGGCG				
VC0494F	GGGGAGGTGTTTCA ATCTGTC	VC0495-VC0512 deletion	2700	56	This study
VC0513R	CTCCACTGCTATTC CGGT				
VC0506_Fp	GCGTATCGTGTGTT GACCG	VC0506	323	56	
VC0506_Rp	CGCTAGTTCCTGAT GCACTG				
VC0510_Fp	CGATGCCCTAACCA ATCCTG	VC0510	246	56	
VC0510_Rp	GGTGTTGAGTCTCC AGATGG				
vprA_SacI_F	GGTCGAGAGCTCGT CCCCATGTCTAACA ACCC	*carR*	714	58	Samanta et al., 2020
vprA_SacI_R	CCATCGGAGCTCGG TTTACCAAGTGTCA GGCAC				
Pcv_Ins_F2	CTGCAGGTCGACGG ATC	Variable	Variable (depends on the size of insert)	56	Samanta et al., 2020
Pcv_Ins_R	ACATGTGGAATTGT GAGCGG				
Bla_{NDM-1}-F	GGGCAGTCGCTTCC AACGGT	bla_{NDM-1}	475	60	Shanthi et al., 2014
Bla_{NDM-1}-R	GTAGTGCTCAGTGT CGGCAT				
VieA_F1	CCTAAGCTAGGTGT GGTGATCC	*vieA*	181	62	This study
VieA_F2	CCTAAGCTAGGTGT GGTGATCT				
VieA_R	GTGACTGGTTTCGG GCTC				
hlyA_489F	GGCAAACAGCGAA ACAAATACC	*hlyA*	481(El Tor)	60	Rivera et al., 2001
hlyA744F	GAGCCGGCATTCAT CTGAAT		727/738 (El		

			Tor/classical)		
hlyA_1184R	CTCAGCGGGCTAATACGGTTTA				
recA_RT_Fp	GTCGCAAGCAATGCGTAAAC	*recA*	158	55	This study
recA_RT_Rp	CCAAACGAACAGAAGCGTAGA				
GAPDH Fp	CATGTTCGTCATGGGTGT GAACCA	*GapDH*	160	60	
GAPDH Rp	AGTGATGGCATGGACTG TGGTCAT				
IL-8_F	GCCAAGGGCCAAGAGAA TATC	*Il-8*	176	60	
IL-8_R2	GCTAGCAGACTAGGGTTGC				
flhC RT_F	GCCTTGGTTGAAGGCTCC	*flhC*	153	55	
flhC RT_R	CCAGGGGATTTCAGCTGG				
flhD RT_F	GAATTGGCGCGCATTGATC	*flhD*	156	55	
flhD RT_R	CAGTTGGCTCTGGTTTCCC				

5.5.2. Different types of PCR

PCR can be of multiple types based on the purpose and requirement of the amplified product. Depending on the purpose of this study a number of PCR types have been used.

a) Simplex PCR (Singleplex PCR)

Simplex PCR is the amplification of a single product within a PCR reaction using only one set of primers (one forward and one reverse). This is the simplest method of optimization, amplification, and detection of a gene from an unknown species. The primers designed require a little optimization and there is no competition for the targets between the primers.

b) Mismatch amplification mutation assay (MAMA) PCR

This is generally a variant of simplex PCR, which amplifies a single gene product within a reaction tube. MAMA PCR is used to distinguish between two known genetic alleles differing only at a single nucleotide position. Two reactions set up were prepared to amplify two different genetic alleles and involves three primers. One is common to both the allelic

variants and used for both PCR sets. Whereas, the other two variable primers contain the desired mutation differing only at a single base and are used in two different reaction tubes. These two variable primers have been designed in such a way that extreme 3' end of a forward or reverse (not both) primer includes the mismatched base (Morita et al., 2008). Another primer-template mismatch at the 3' proximity of oligonucleotide primer just before the last nucleotide that has little or no effect on the amplification of genes were incorporated to overcome the effect of mispairing of the other primer which differs only at a single base. Utilizing this PCR, the allelic variants of the genes *ctxB, tcpA, rtxA, vieA, gyrA, rstR* were analyzed for the presence of genetic alleles in the currently circulating *V. cholerae* strains (Table 5.2).

c) Double-mismatch amplification mutation assay (D-MAMA) PCR

This method is a type of MAMA PCR that distinguishes three alleles of a gene that differ in two distant positions with single point mutations (Naha et al., 2012; Morita et al., 2008). Three reaction tubes were set up for three specific alleles. The detection of three recently circulating *ctxB* alleles i.e., classical, El Tor, and Haitian were performed with D-MAMA PCR assay in this study. These three alleles differ in two different nucleotide positions. Classical *ctxB* and El Tor *ctxB* differ at the 203rd nucleotide position whereas; Haitian *ctxB* varies at the 58th nucleotide position from the classical *ctxB* (Naha et al., 2012; Morita et al., 2008).

d) Development of a new PCR assay for the detection of deletion in the VSP-II

To know whether the current Indian strains of *V. cholerae* have a deletion in the region (VC0495–VC0512) of the VSP-II (Weill et al., 2019); a new PCR assay has been developed. For this PCR, primers (Table 5.2) have been designed from the flanking regions of VC0495 and VC0512 (VC0494F/ VC0513R) which would give an amplicon of 2.7 kb if the region VC0495–VC0512 is absent. The temperature cycling condition was optimized to two-step amplification for long-length product formation with the enzyme XT Polymerase following the manufacturer's protocol (XT-20 PCR system, GeNei, India). The thermal cycling was set up for initial denaturation at 95°C for 2 mins, then 5 cycles of denaturation for 30s at 95°C, annealing for 30s at 55°C and extension for 6 mins at 72°C and another 10 cycles of denaturation for 30s at 94°C, annealing for 30 s a 56°C, and extension for 6 mins at 72°C (+5

s/cycle for each successive cycle) with a 10 min final extension at 72°C. An isolate of the Yemen cholera outbreak (YMN-170240) was taken as the positive control in this PCR.

e) PCR for gene cloning (allelic substitution)

In this study, a number of allelic substitutions comprised of single base mutations have been performed. Forward and reverse primers were designed in such a way that the single mutated base lies in the middle of the amplified product and the product is supposed to be about 800bp long. Moreover, the restriction site for SacI restriction enzyme (GAGCTC) was added to the 5' ends of both the primers followed by the addition of 4 to 6 random bases for strong and appropriate binding of the cutting enzyme (Table 5.2). The desired allele was amplified from a strain and used for the allele replacement within another strain that contains a different allele. The PCR amplified product was then run in a 1% agarose gel and purified with a kit using the manufacturers' protocol (QIAquick gel extraction kit, Qiagen, USA).

5.5.3. Nucleotide sequencing

(a) Primer sequences, PCR reaction mixture, and condition

The primers (hlyA744F/ hlyA_1184R) used to amplify *hlyA* gene of *V. cholerae* and PCR condition are given in the table 5.2. PCR amplification was carried out in 20µl reaction mixture containing 10X PCR buffer (Bangalore Genei, Bangalore, India) 10 ng of template DNA, 200 nM of each forward and reverse primer (10 pmol/µl), 200µM of each dNTP (Roche Diagnostics, Mannheim, Germany) with 1 unit of Taq DNA Polymerase (Bangalore Genei, Bangalore, India) and 1.5mM $MgCl_2$ (Bangalore Genei, Bangalore, India).

(b) Purification of the PCR product

The PCR product to be sequenced was purified using the Qiaquick PCR purification kit (Qiagen, GmBH, Germany). The buffers used for purification were provided with the kit. The PCR product was diluted by adding 5 volumes of buffer PB and mixed thoroughly by pipetting. The diluted product was loaded on a Qiaquick spin column placed in a 2 ml collection tube and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and the column was washed with 750 µl of PE buffer. The tube was centrifuged twice for 1 min each at 13,000 rpm to remove residual ethanol, and the flow-through was discarded each time. The DNA was eluted by adding 30 µl of sterilized water or elution buffer to the center of the Qiaquick membrane and allowing the column to stand for 10 min at room temperature.

The eluted DNA was collected in a sterile 1.5 ml microfuge tube by centrifugation at 13,000 rpm for 1 min. 2 µl of the purified DNA was loaded on a 2% agarose gel to estimate the amount of DNA present in the sample. This PCR product purification method was used while sequencing.

(c) Cycle sequencing

For the cycle sequencing reaction, a specific amount of DNA present in purified PCR product was added as template depending on the size of the product to be sequenced. The PCR primer pair used to generate the amplicon was generally used for sequencing both strands of the amplicon. The sequencing mix was obtained from Big Dye Terminator v3.1 kit (Applied Biosystems, USA) and consisted of Big Dye® Terminator 3.1 Sequencing Buffer (5X) and ready-to-use reaction mixture (2.5X). The terminators used were di-deoxynucleotides labeled with the fluorescent dyes. For cycle sequencing, the following components were added in a 0.2 ml thin-walled PCR tube: Sample DNA amounts to 100ng/kb of template to be sequenced, Ready reaction mixture (2.5X)- 2µl, Big Dye Sequencing Buffer (5X)-1µl, Primer (forward or reverse)- 1µl (from 3.3 pmoles/ul) and Sterile triple-distilled water to a final volume of 10µl. Cycle sequencing was carried out in a thermal cycler (Applied Biosystems, USA). The tubes were allowed to stand at 96ºC for 2 min followed by 25 complete cycles comprising of denaturation at 96ºC for 10 seconds, annealing at 50ºC for 5 seconds and extension at 60ºC for 4 minutes and finally allowed to reach 4°C before the tubes were taken out from the thermal cycler. The cycle sequencing products were kept on ice for further purification.

(d) Cycle sequencing product purification

After cycle sequencing, the products of each reaction (10µl) were pippetted into a 1.5 ml tube and diluted by adding 30ul sterile deionized water. In the same tube 5µl of 3M sodium acetate (pH 4.8) and 50ul of 95% ethanol (Sigma) was added. The contents were thoroughly mixed by vortexing and placed on ice for 15 min. Tubes were centrifuged at 13,000 rpm for 15 min at room temperature and the supernatant were carefully aspirated with a separate finetipped pipette tip for each sample and discarded. Next, the pellets were rinsed in 70% ethanol and centrifuged at 13,000 rpm for 10 min. The residual fluid was removed by aspiration and the pellets were dried completely in a vacuum centrifuge or air. The dried pellets were re-suspended in 20µl of Hi-Di™ Formamide (Applied Biosystems, USA) by

vortexing vigorously for 1 min and heating at 95°C for 2 min in a thermal cycler. Samples were snap chilled on ice for 5 min and vortexed thoroughly. Tubes were now stored at -20°C until they were loaded in the automated DNA sequencer (ABI PRISM 3100, Applied Biosystems).

(e) Capillary electrophoresis

The purified extension products from cycle sequencing reactions were loaded onto sample tubes, sealed with septa, and arranged on a 96-well tray. The tray was loaded to an automated sequencer (PE Applied Biosystems) and samples were subjected to electrophoresis through 47cm x 50µm capillary (PE Applied Biosystems) coated with POP-6 (Performance Optimized Polymer-6), (PE Applied Biosystems). Based on the nature of end-labeled bases four different types of fluorescence could be obtained. The machine recorded the different types of fluorescence obtained from end labeled molecules and analyzed the data to generate the output file with nucleotide sequence for the sample. The raw sequencing data was analyzed using the ABI PRISM DNA Sequencing Analysis Software (PE Applied Biosystems).

(f) Assembly and analysis of sequencing data

The nucleotide sequence data were assembled to generate sequence contig for each gene from respective forward and reverse sequence read using software (DNASTAR, USA). Then searches for identical sequences were performed using the Basic Local Alignment Search Tool (BLAST) programme available on the National Centre for Biotechnology Information (NCBI) network server.

5.5.4. Molecular studies of carbapenem-resistant *V. cholerae* O1 strains

In this study, we have found carbapenem-resistant *V. cholerae* O1 strains (AKM-1 to AKM-6) containing plasmid. Thus, to characterize those strains in depth, the following studies have been done.

(a) Transfer of plasmid through conjugation assay

To determine the mobility of the plasmid, a conjugation assay was performed by using carbapenem resistant *V. cholerae* O1 wild type strains carrying plasmid as a donor with the different enteric pathogens, such as *Escherichia coli* J53 (sodium azide resistant), *Shigella*

flexnerii 4a (IDH 6498), *Salmonella enterica* (BCH 0645), *V. parahaemolyticus* (K12929), and a NDM-1 negative polymyxin B-resistant *V. cholerae* O1 (IDH 3291) as recipients. Overnight grown cultures of donor (wild type bla_{NDM-1} containing *V. cholerae* O1 strain) and recipient strains (*E. coli* J53, *S. flexneri*, *S. enterica* and *V. parahaemolyticus*, *V. cholerae* O1) in LB broth containing selective antibiotics were mixed in 1:1 ratio in different tubes containing LB broth with no antibiotics and incubate all the tubes overnight at 37˚C in static condition. After incubation, serial dilutions were prepared of each conjugated culture and take 100µl of the diluted culture was to spread over the plates containing ceftriaxone (5µg/ml) plus 100 µg/ml of sodium azide supplemented MacConkey agar for *E. coli* J53, XLD, and TCBS agar were used for the screening *of S. flexneri, S. enterica* and *V. parahaemolyticus,* respectively. 50µg/ml polymyxin B plus ceftriaxone (5µg/ml) supplemented TCBS plates were used for the screening of polymyxin B-resistant *V. cholerae* O1 transconjugants. In all cases, the donor and recipient suspensions were also diluted in phosphate buffer saline (PBS) and plated on selective plates for each donor and recipient to confirm the purity and count the colonies. Plates were incubated overnight at 37˚C in an incubator to grow (Figure 5.1). The frequency of transfer was calculated for each recipient strain by the formula of no. of transconjugants that appeared on selective media (CFU/ml)/no. of donor bacteria present when mating was initiated (CFU/ml) (Sarkar et al., 2015).

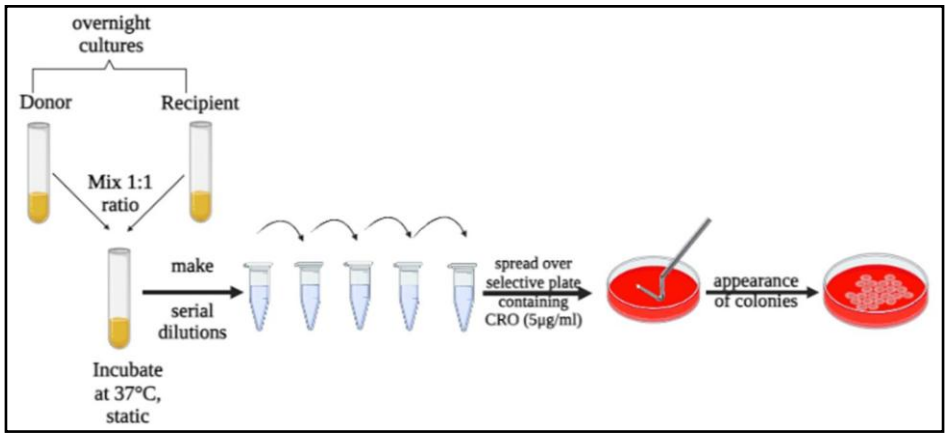

Figure 5.1. *A schematic representation of conjugation assay to transfer plasmid containing NDM-1.*

(b) Plasmid extraction

Carbapenem resistant *V. cholerae* O1 isolates were cultured on TCBS plates. Colonies from selective media were then inoculated into 5ml LB broth containing ceftriaxone (10µg/ml) antibiotic and incubated at 37°C overnight in a shaker. After incubation, the liquid culture

was transferred into 1.5ml eppendrof tubes and centrifuged to harvest the cells at the bottom of the tube. Plasmid was then extracted from the cell pellet by using Kado and Liu method from donors, recipients, and transconjugants according to the protocol stated in Chowdhury et al (2016) and analyzed by gel electrophoresis using 0.8% agarose.

(c) Whole genome sequencing (WGS)

Total genomic DNA from all the eight isolates (AKM-1 to AKM-6; IDH 3291 and TC 3291) were extracted from overnight grown culture containing ceftriaxone (5µg/ml) antibiotic by using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The quality and quantity of extracted DNA samples were measured by using the NanoDrop lite spectrophotometer (Thermo Fisher Scientific, Delaware, United States) and further visualized through electrophoresis by running the DNA sample on 0.8% agarose gel.

All the eight isolates were sequenced by using the Illumina NovaSeq 6000 to obtain short reads. The extracted genomic DNA was re-quantified by nanodrop-based spectrophotometric and Qubit based fluorometric method. Whole genome DNA sequencing library preparation was performed by using Nextera XT DNA Library Prep Kit (illumina) as per manufacturer's instructions. Paired end (2 x 250 bp) sequencing of these libraries was performed in Novaseq 6000 (Illumina). Quality of the final libraries was checked using high sensitivity D1000 screen tape in 2200 TapeStation (Agilent) and final library quantification was performed in Qubit Fluorometer. Raw reads were further trimmed with the help of Trim-galore. Unicycler was used to assemble the Illumina reads (Wick et al., 2017) and annotated by Prokka (Prokka, 2014).

Additionally, and two isolates (AKM-3 & AKM-4) were further subjected to Oxford Nanopore Technologies (MinION Sequencer, SQK-RBK110.96, New York, NY, USA) to obtain long reads.

Library preparation was done using library construction kits according to the manufacturer's instructions. Libraries were sequenced with R9 flow cells (active pores number $\geq$ 800) on a MinION sequencer. Real-time basecalling was performed using Guppy version 6.4.6 with the corresponding basecalling model as indicated in each workflow, which was integrated in the MinKNOW software 22.12.5. Fastq data were obtained for further analysis. For hybrid assembly, both the short reads (from Illumina) and the long reads were combined using

Unicycler to obtain the complete genome. The quality of the assemblies was checked using CheckM software (Parks et al., 2015).

WGS analysis is described in section of 5.8 (5.8.1 and 5.8.2).

5.5.5. Allele replacement using suicide vector

For the allele replacement of a wild type strain within the genome the suicide vector, pCVD442 (Donnenberg et al., 1991) has been used which is 6345 bp (Table 5.3). The complete plasmid map is available in Addgene server. In brief, this plasmid vector carried ampicillin resistant marker *bla-Tem9, sacB* marker for counter selection and multiple cloning sites from which SacI site was selected for this study. The plasmid that has been stored in a DH5α strain of *E. coli* was used. The complete procedure is as follows: vector isolation, vector digestion with SacI, removal of 5' phosphate group of the vector to prevent self ligation, PCR amplification of insert DNA, digestion of insert with the same enzyme SacI, ligation of vector and insert (1:3 molar ratio), transformation of the ligated product into *E. coli* strain SM10λpir, conjugation with wild type *V. cholerae* strain and proper selection and confirmation of the exconjugants and mutant.

(a) Isolation of plasmid

The plasmid pCVD442 was isolated from the strain *E. coli* DH5α using the QIAGEN plasmid Midi kit following manufacturers' instruction. In brief, 100ml culture with 100µg/ml ampicillin was grown overnight, harvested by centrifugation at 6000 rpm for 15 mins at 4°C in a centrifuge (Biofugestratos, Heraeus), pellet was resuspended and lysed followed by precipitation. Then the sample was centrifuged at 20000xg for 30mins at 4°C. Supernatant was collected and loaded onto a Qiagen tip 100 column for DNA binding. The column was then washed with 2x10ml wash solution and eluted with 5ml of elution buffer. The eluted fraction was further mixed with 3.5ml (0.7 vol) of room-temperature iso-propanol and centrifuged at 20000xg for 15mins. The supernatant was discarded and the pellet was washed with 2ml of room-temperature 70% ethanol and again centrifuged at 20000xg for 10mins. Supernatant was discarded, and the pellet was air-dried and suspended with 200-400µl of TE buffer (pH 8.0). The isolated plasmid was then run in a 55 0.8% agarose gel. The band which comes in the ~3.7kb region of the molecular marker was recognized as the supercoiled form of the 6345bp pCVD442 and propagated with the sample in downstream processes.

Table 5.3. *Strains and plasmids used for cloning.*

Strain or plasmid	Description	Reference or Source
Strains		
E. coli		Lab collection
SM10λpir	*thi thr leu tonAlacYsupErecA* (RP4-2-Tc::Mu) λpir R6K Km^r π	
V. cholerae		Samanta et al., 2020
IDH 7230	*V. cholerae* O1 El Tor variant, Amp^S, Str^R, $PolB^S$	
IDH 7230MT	IDH 7230, $PolB^R$	
N16961	*V. cholerae* O1 El Tor, Amp^s, Str^R, $PolB^R$	
N16961MT	N16961, $PolB^S$	
Plasmids		
pCVD442	oriR6K mobRP4 sacBAmp^R	Donnenberg et al., 1991
pVA1	pCVD442, VC1320a†	Samanta et al., 2020
pVA2	pCVD442, VC1320b††	

†VC1320a, carR with a G at 265th nucleotide position; ††VC1320b, carR with an A at 265th nucleotide position; Amp, Ampicillin; Str, Streptomycin; PolB, Polymyxin B.

(b) Restriction digestion of plasmid and insert

The isolated plasmid is quantified and 500ng was subjected to SacI restriction digestion using the following reaction set up for 50µl: pCVD442 500ng, 5X buffer 4 (NEB) 10µl, BSA (100X, NEB) 0.5µl, SacI-HF enzyme 1µl (20U) and autoclaved water upto 50µl. The mixture was incubated at 37°C water bath overnight and heat-inactivated at 65°C for 10 mins followed by snap chilling in ice. For the insert digestion, the PCR amplified product was purified and concentration was measured. 200ng of the purified product was subjected to SacI restriction digestion and the above-mentioned reaction setup was followed. After overnight incubation and enzyme deactivation both the vector and insert (50µl) were run in 0.8% agarose gel, stained with EtBr, and desirable bands were cut (6345 bp for pCVD442 and

insert for the respective size) followed by gel extraction with QIAGEN gel extraction kit. To prevent the self-ligation of the vector in the vector-insert ligation process, SacI digested linear pCVD442 was subjected to phosphatase treatment which removes the 5' phosphate groups of the linear DNA. The reaction was set up to a volume of 50µl which consists of linear pCVD442 ~300ng, 5X buffer 4 (NEB) 10µl, BSA (100X, NEB) 0.5µl, Calf intestinal alkaline phosphatase (CIP, NEB) 1µl (5U) and autoclaved water upto 50µl. The mixture was incubated at 37°C water bath for at least 4 hrs. No heat inactivation is required. The mixture was then directly subjected to gel run and gel extraction before further use.

(c) Ligation of vector and insert

A 20µl ligation reaction was set up using vector 100ng and insert 300ng (1:3 molar ratio), 10X T4 ligation buffer (Thermo scientific, USA) and 2µl of T4 ligase (40U). A negative control was set up using all the components mentioned above except the insert to check whether the phosphatase treatment has been worked. The reactions were allowed to occur in a water bath set at 22°C and kept overnight.

(d) Preparation of competent cells

For the transformation experiment, it is necessary to make the bacterial cell competent. SM10λpir has been used for this purpose as it contains pir protein which is necessary for conjugal tube formation during conjugation (Table 5.3). 5ml of bacterial culture was grown overnight in LB broth at 37°C. A fresh subculture in 50ml LB broth (1:100) was made and grown up to an $O.D_{600}$ ~0.5. Cells were kept in ice for 45-60mins and centrifuged at 1500xg for 10mins at 4°C. Supernatant was discarded and the pellet was suspended with 5ml of TSS (Appendix). The suspension was again centrifuged at 1500xg for 10mins at 4°C and the pellet was suspended in 1ml of TSS. The suspension was kept in ice 56°C for 30mins and 50ul aliquots were made one of them was used for transformation experiment and others were kept at -80°C for further use up to 2 months.

(e) Chemical transformation of the ligated product

The ligated reaction mix was mixed with 50µl TCM (Appendix) and kept in ice for 15-20mins. The ligation negative control setup was also mixed with the same volume of TCM and incubated in ice. Aliquots of SM10λpir competent cells were also kept in ice. A third setup was prepared using the intact undigested pCVD442 and TCM to check the efficiency of

the competent cells. After incubation, all the three tubes were mixed with 50µl of competent cells and kept in ice for another 15-20 mins. The tubes were then subjected to heat shock in a water bath maintained at the temperature of 42°C for 90 secs and immediately kept in ice for 3 mins. An aliquot of 50µl competent cell was also heat treated and preceded with the other three setups to check the viability of the cells after heat shock treatment. 200µl of pre-warmed LB broth was added to all the tubes and incubated at 37°C in shaking condition for 1hr. After 1 hr, the tubes were taken and spread over the LA with 100 µg/ml ampicillin (LA-AMP) plate (positive selection marker for pCVD442). Only the tube for a viability checkup was spread over the LA plate without ampicillin since SM10λpir does not possess ampicillin resistance marker. The plates were incubated overnight at 37°C. Typical positive colonies were selected and verified with the respective PCR of the insert and with the primers Pcv_Ins_F /R2 that have been designed in this study from the flanking regions of the multiple cloning site of pCVD442. This PCR would yield a 101 bp product if there is no insertion and an additional length of the insert if it contains the insert (Table 5.2).

(f) Conjugation

Construction of the *carR* allelic substitution and deletion mutant of *V. cholerae* was performed with the strains that are streptomycin (100µg/ml) resistant and ampicillin (100µg/ml) sensitive. The strain SM10λpir with the plasmid construct was ampicillin resistant but streptomycin sensitive and was used as the donor strain. Both the strains (donor and recipient) were streaked on the LA with 100µg/ml streptomycin (LA-STR), LA-AMP, and LA-AMP-STR plates and incubated overnight at 37°C to check their susceptibility. On the next day, a small amount of culture from the LA-AMP plate (donor) was taken and spread with an inoculation loop on a LA plate without any selection and incubated for 1hr. The recipient strain from the LA-STR plate was taken and spread on the 1hr incubated LA plate over the donor strain in the same way and incubated 37°C overnight. Next day, with the help of an inoculation loop small amounts of culture were picked up from the mating plate by touching different areas over the plate and streaked on a LA-AMP-STR plate for the selection of exconjugants and incubated overnight.

(g) Counter selection with sucrose

After incubation, a single exconjugant colony was picked and streaked on a NaCl deficient nutrient agar plate with 5% sucrose (NA-SUC) for the selection of strains that lose the vector

plasmid after recombination. Recombination would yield three types of colonies on the LA-SUC plate, the strains that reverted to their original genotype with reversal recombination, the strains with double crossovers and yield the desired mutant genotype, and third one is the strain with the integrated plasmid (a result of a single crossover event) with the presence of both alleles of the gene of our interest that overcame the counter selection.

(h) Confirmation by PCR and nucleotide sequencing

The colonies on the sucrose selection plate have been screened with the allele specific PCR and desired colonies (mutant) were confirmed with nucleotide sequencing. Finally, the mutant strains were stored in the form of 15% glycerol stock and kept at -80°C for further use.

5.5.6. Pulse field gel electrophoresis (PFGE)

In normal electrophoresis, all linear double-stranded DNA molecules that are larger than a certain size migrate through agarose gels at an equal time and cannot be separated as distinct bands. To solve this issue, Schwartz and Cantor, 1984 first reported the development of PFGE capable of resolving DNA of up to 10 megabase (Mb) pairs in length. Large DNA molecules, ranging from $2x10^4$ to 10^7 base pairs (20 kb to 10 Mb) in length, can be separated by size with PFGE. This technique depends on the unique behavior of large DNAs in an electric field that is turned on and off (pulsed) sequentially with the alternating, orthogonal electric fields. When an electric field is applied in the gel, the DNA molecules migrate in the direction of the field and also stretch out lengthwise. If the current is stopped, the molecules begin to "relax" into random coils. The time required for relaxation is directly proportional to the length of a molecule. The electric field then is reapplied at 90° or 180° in the first direction. Longer molecules relax less than shorter ones during the time the current is turned off. Since the molecules must relax into a random coil before moving off in a new direction, longer molecules start moving in the direction imposed by the new field more slowly than the shorter ones. Repeated alternation of the field direction gradually forces large DNA molecules of different sizes further and further apart.

(a) Media, buffers and other reagents

The media required are LB broth and agar plate and listed in the Appendix. The composition of the buffers i.e., cell suspension buffer, washing buffers (with and without phenyl methyl

sulphonyl fluoride, PMSF), 10X restriction digestion buffer (buffer H), 0.5X Tris-borate EDTA (TBE) and other reagents such as PMSF solution, lysozyme solution, proteinase K solution are listed in the Appendix.

(b) Preparation of DNA plugs

To perform PFGE, the genomic DNA was brought out from the bacterial cells in the Agarose embedded form following the methodology described by Kurazono et al., 1994 with certain modifications. The strains of *V. cholerae* were revived on LA plates and a single colony was inoculated into 3ml of LB. Following incubation at 37°C for 16-18 h with shaking (150rpm), 30ul of the culture was used as seed inoculum to generate fresh 3ml culture, which was allowed to grow at room temperature for 1 hr till it reached unit OD measured at 600 nm. 1 ml of normalized bacterial culture was taken and harvested by centrifugation at 1,000xg for 5 minutes at 4°C, washed and resuspended in 500µl of cell suspension buffer and incubated at 50°C for 10 minutes. A 2% low melting molecular grade agarose (Bio-Rad) in water was also equilibrated at 50°C. The cell suspension was then mixed with an equal volume of the low melting molecular grade agarose (Bio-Rad) at 50°C and the mixture was poured into plug moulds (Bio-Rad). The solidified gel plugs were cut into two small sizes and then transferred into 2 ml tubes and soaked in 1 ml of lysozyme solution at 37°C overnight. Following incubation, the gel plugs were rinsed twice in distilled water and were then again put in proteinase K solution and incubated at 50°C overnight. The solution containing proteinase K was discarded from the tubes containing the plugs. The gel plugs were then first washed with the 1X washing buffer for an hour at room temperature with gentle agitation, followed by washing for 1 h with the wash buffer containing PMSF. Two more washing at room temperature for 1 h followed this washing step with the 0.1X wash buffer without PMSF. After removing the washing buffer the gel plugs were equilibrated in 1 ml of 1X restriction enzyme buffer (Buffer H, TaKaRa) for the desired enzyme (Not I) for 1h at room temperature. The 1X restriction enzyme buffer was then replaced with 0.6 ml of the fresh 1X restriction enzyme buffer and 50U of the Not I restriction enzyme was added and mixed gently. The plugs were then incubated at 37°C overnight. Following the incubation, gel plugs were washed with 1ml of 1X "washing buffer" for 30 min at room temperature. The "washing buffer" was then removed and the gel plugs were equilibrated with gel running buffer (0.5 X TBE) for 30 minutes at room temperature. A similar set was prepared with the

strain *Salmonella* serotype Braenderup (H9812) which has been used as the universal control for PFGE and digested with the restriction enzyme XbaI (50U).

(c) Electrophoresis

A 100 ml 1% gel was made using pulse field certified agarose (Bio-Rad) in 0.5 X TBE. PFGE chamber of the CHEF Mapper (Bio-Rad) was filled with 2 litre of 0.5 X TBE and cooled to 14°C using the cooler model 1000 minichiller (Bio-Rad). The gel plugs were kept on the comb heads and the comb was placed on the casting tray and then 1% low melting Agarose was slowly poured. After 1hr of solidification, the gel was carefully placed into the electrophoresis chamber. PFGE of bacterial strains were performed by using contourclamped homogeneous electric field method on a CHEF-mapper (Bio-Rad) using 0.5X TBE buffer for 40.24 hr. The temperature of buffer during the run was maintained at 14°C. Run conditions were generated by auto-algorithm mode of CHEF Mapper Pulsed Field Gel Electrophoresis system using a size range of 20-300 kb. The calibration factor was kept fixed at 1.5 and all other parameters including initial and final switch times were generated by default [electrophoresis buffer: 0.5X TBE pH 8.0; run temperature 14°C; agarose PFC grade and concentration 1%; electrical parameters: 6 V/cm; run time 40.24 hr; included angle 120°C; initial switch time 2.98s and final switch time 26.95s with linear ramping factor].

(d) Documentation of the result

After the completion of the electrophoresis the gel was stained with EtBr solution (0.5mg/ml; Sigma), and visualized and documented using Gel Doc 2000 system (Bio-Rad). The banding patterns were then analyzed and the dendrogram was constructed with Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium).

5.5.7. Quantitative real-time (qRT) PCR for gene expression

Relative mRNA expression of the genes *flhC* and *flhD* were studied among the wild type and mutant *V. cholerae* strains to find out the role of *carR*-sensitive allele in motility. For this, conditional growth was subjected to the strains and used for the extraction of total cellular RNA for qRT PCR. For the IL-8 expression analysis of eukaryotic cell line (INT 407), a similar method was followed unless otherwise mentioned.

(a) RNA isolation

For bacterial RNA isolation, overnight LB cultures were diluted 1:100 in fresh LB with selective antibiotic and grown for and OD 600nm of 0.6-0.8. Cells were normalized to 1.0 OD and 1.5ml of the normalized culture was harvested and washed with PBS. The pellet was suspended in 1ml of TRIzol reagent (Ambion, Life Technologies), homogenized by pipetting vigorously and kept at room temperature for 5mins. 200µl of chloroform (Merck) was added and mixed by inverting the capped tube several times and kept again at room temperature for 10mins. After 10mins, the tube was centrifuged at 13,000 rpm for 15 mins at 4°C. Clear supernatant (~300µl) was collected carefully without disturbing the bottom layer and equal volume of isopropanol was added and mixed by inverting the tube. The tube was then incubated at room temperature for 10 mins and centrifuged at 13,000rpm for 10 mins at 4°C. The supernatant was discarded carefully and the nucleic acid material was seen to precipitate as white to transparent mass in the bottom of the tube. The tube was then soaked by keeping the tube inverted over a tissue and 75% chilled ethanol solution was added. The tube was vortexed and centrifuged at 13,000 rpm for 8 mins at 4°C. Supernatant was decanted carefully, the pellet was air dried for 15-20 mins and dissolved with 30µl RNase free autoclaved water by pipetting well. The extracted RNA can be stored at -80°C for 2 weeks. The RNA extraction from the eukaryotic cell line (INT 407) was carried out using the same protocol.

(b) Removal of contaminant DNA from RNA

The concentration of the extracted RNA was measured using a NanoDrop spectrophotometer and normalized to 400ng/µl. A 30µl of 400ng/µl concentration of RNA was made in such a way that it can accommodate the 3µl 10X DNaseI buffer and 2µl (40U) within the 30ul total volume. For example, if the RNA concentration comes to 800 ng/µl, then the RNA needed to make 30 µl of 400 ng/µl concentration is 15 µl, 10X buffer 3 µl, DNaseI enzyme 2 µl, and 10µl RNase free water. The reaction setup was subjected to incubation in a thermal cycler using the condition as follows: 37°C for 3.5 hrs, 75°C for 10 mins and 4°C for 10 mins. After the reaction was over, a PCR for *recA* gene was run using 5µl of the DNaseI treated RNA as template for checking for any remaining traces of contaminated DNA. If no bands were found with the DNaseI treated sample in the PCR, then it was considered as DNA

contamination-free RNA and were used for the next step of cDNA preparation. For the eukaryotic cell line (INT 407), GAPDH gene was used for PCR checking instead of *recA*.

(c) cDNA synthesis

A total of 2-3 µg of RNA was used for the synthesis of total cDNA according to the manufacturers' protocol (Revert Aid first strand cDNA synthesis kit, Thermo Scientific, USA). A 20µl reaction volume was prepared. First, 5 µl of RNA, 1µl of random hexamer (for eukaryotic sample 1 µl of oligodT primer was used instead of random hexamer) and 6 µl of autoclaved double distilled water were added and the reaction mixture was heated at 65°C for 5 mins and snap chilling on ice. Then, 4 µl of 5X reaction buffer, 2 µl of dNTP mix, 1µl of ribolock (20 U/µl) and 1µl of reverse transcriptase (200 U/µl) enzyme were added into the snap chilled mix. The final reaction was subjected to thermal cycling using the condition 42°C for 90 mins, 70°C for 10 mins and 4°C on hold. After the process was done, the concentration of the DNA was measured using a NanoDrop spectrophotometer and finally normalized to 1000 ng/µl (for eukaryotic cDNA 1500 ng/µl) and used for qRT PCR.

(d) Reaction mixture and temperature cycling for qRT PCR

qRT PCR was performed using the fluorescent SyBr green dye (Thermo Scientific, USA) which fluoresces when intercalated in double stranded DNA. To quantify the relative mRNA expression for a particular gene, three types of reaction were set up (each wells contain 20µl). The reaction mix for standard was set up using 10 µl of 2X SyBr green mix, 0.6 µl of each forward and reverse primers (usually *recA* for bacterial and GAPDH for eukaryotic samples were used), 7.8 µl of water and 1 µl of template of known concentration (prepared previously using PCR amplification of the standard gene such as *recA* followed by gel run, gel purification and concentration determination). Usually, six reactions for standard were prepared using different known template concentrations. Four of the concentrations of 1 ng/µl, 0.2 ng/µl (diluting 1ng/µl 5-times), 0.04 ng/µl, 0.08 ng/µl, positive and negative controls. The reaction mix for unknown was prepared for two genes, one is the calibrator gene *recA* (or GAPDH) and the other is the gene of our interest. The reaction mixture for each was prepared as follows: 2X SyBr green mix 10 µl, 0.6 µl of each forward and reverse primer, 4.8ul of water, and 4µl of total cDNA (~4000ng/µl). All the primers have been designed in such a way that the GC% was 50-55 and the tm was about 55°C (Table 5.2). The reaction was prepared in a 96-well plate, sealed, centrifuged (2000 rpm- 2 mins) and run in

LightCycler 480 Instrument II (Roche diagnostics, UK) using the following program: pre-incubation at 95°C for 3 mins, 40 cycles of denaturation at 94°C for 20secs, annealing 55°C for 20 secs and extension 72°C for 20 secs (as the product length was 150-200 bp) with a single acquisition at the extension step (band width channel 465-510) followed by a cooling step at 40°C for 30secs.

(e) Analysis of result

After the run has ended the standard curve and the Ct values were generated using the Abs Quant/2nd Derivative Max program. The Ct values were then compared between the calibrator and the experimental gene using the $2^{-\Delta\Delta Ct}$ method to know the relative expression of a particular gene among different strains (Pfaffl et al. 2002). Fold increase and error bars were then calculated using the Graphpad prism software (Ver. 6.0).

5.5.8. Motility assay

Motility assay was performed in this study with the polymyxin B-sensitive and resistant strains to find out whether there is any difference in motility between them. Overnight grown LB broth cultures were diluted in fresh LB broth (1:100) and incubated at 37°C until it reached an OD_{600} nm of 0.5 (in triplicate). 5 µl of the culture was taken and placed like a drop at the centre of a 0.3% LB agar plate and incubated carefully at 30°C for 16-18 hrs. The diameter of growth (in mm) was measured after the incubation period and the bar diagram was plotted with error bars using mean ± standard deviation.

5.6. Tissue culture assay

Tissue culture assay generally represents the experiments involved in the infection of bacteria to the mammalian cultured cell-lines. In this study, the INT 407 (intestine 407, Human intestinal epithelial cell line) cell line was used to analyze the inflammatory response induced by the strains of *V. cholerae* carrying various polymyxin B phenotypic trait (Table 5.4).

5.6.1. Culturing methods

Revival of the stored cell line includes the following steps. Stored cell line was taken out from liquid nitrogen (-196°C) and placed in a floater kept in a beaker containing pre warmed (37°C) water. 1ml of 20% fetal bovine serum (FBS, Gibco) containing Dulbecco's modified eagles medium (DMEM, Gibco) and pen-strep-glutamine (Gibco) was added to the vial,

mixed, and emptied into a T-25 flask (Tarsons, India) containing 5ml of 20% DMEM (20%FBS + DMEM + pen-strep-glutamine). The flask was then shaken briefly and incubated at 37°C with 5% CO_2 till it reached confluence. Sub-culturing was performed in T-75 flask containing the same media composition except the FBS concentration (10% in this case). After the cells of the T-25 flask reached confluence, the media was discarded, washed the cells with 1X PBS and 1ml of 1X Trypsin-EDTA solution (0.025% trypsin and 0.01% EDTA in PBS, Gibco) was added. The flask was then incubated at 37°C for 3-5mins. After the cells were dislocated from the flask bottom surface, 2 ml of 10% DMEM was added, mixed and taken in a 15 ml centrifuge tube. The tube was centrifuged to 1500g for 2 mins. Supernatant was discarded and 1ml of fresh 10% DMEM was added and mixed thoroughly by pipetting. A T-75 flask previously filled with 14ml of 10% DMEM was used to inoculate 250µl of the above cell suspension for sub-culturing and incubated at 37°C with 5% CO_2 till it reached confluence.

Table 5.4. *V. cholerae strains used in tissue culture and animal study.*

Strains	Polymyxin B phenotype	Reference or Source
IDH 7230MT	Resistant	Samanta et al., 2020
N16961, IDH 3439, IDH 3672, IDH 3291		Lab collection
IDH 7230, N16961MT	Sensitive	Samanta et al., 2020
IDH 12699, IDH 12694, IDH 12460		This study

5.6.2. Induction of IL-8 after infection with *V. cholerae* strains

(a) Cell culture infection and stimulation

The human intestinal epithelial cell line INT 407 was grown and maintained in Dalbecco's Modified Eagle's Medium (DMEM, GIBCO-BRL, Gaithersburg, MD) at pH 7.4, supplemented with 10% fetal bovine serum (GIBCO-BRL, Gaithersburg, MD) containing penicillin/streptomycin in the presence of 5% CO_2 at 37°C. Cells were seeded in canted neck T-75 tissue culture flasks (Falcon, USA). Two days later when the cells were confluent, the medium was put off, detached the cells by the treatment of Trypsin- EDTA and washed the cells with PBS. Cells were again seeded in 6 wells plate containing DMEM with 10% FBS with antibiotics. After confluency, the medium was replaced by DMEM with no FBS or antibiotics. Simultaneously, bacteria from the overnight culture were suspended and the

OD_{600} was normalized to 1.0 in PBS were added to each well at an MOI 40. An uninfected control well was also prepared. After 2 hrs of infection, media was decanted and total RNA was extracted by adding TRIzol™ Reagent (Ambion) into the wells.

The qRT PCR has been done for the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of the cell line and the gene of our interest interlukin-8 (IL-8) to analyze the level of induction of the pro-inflammatory cytokines according to the protocol mentioned in the section 5.5.7. The Ct values were converted to relative expression difference using the formula $2^{-\Delta\Delta Ct}$ mentioned by pffafl. The experiment was performed in triplicate for a single strain (Woida et al., 2020).

5.6.3. Adherence assay to intestinal cell line INT 407

For quantitative adherence assays, the intestinal epithelial cells INT407 cell line was seeded in a six-well culture plate at a density of about 5 x 10^5 cells per well. Two days later, the medium was taken off and washed with PBS and about 1x10^7 bacterial culture suspended in fresh DMEM medium without antibiotic were added to each well. The infected plates were incubated for 2 h at 37°C under 5% CO_2. Cells were then washed vigorously three times with PBS to remove the non-adherent bacteria. The number of cell-associated CFUs was determined after lifting off the monolayer by scraping and vortexing the cells to dissociate the bacteria. The percentage of adhesion was calculated as 100x cell-associated CFU/ (cell-associated CFU+CFU present in the supernatant).

5.6.4. Cell line preservation

For the preservation of cell line, the confluent cell of a T-75 flask was processed. The media of the flask was discarded and washed with PBS twice. 2ml of trypsin-EDTA was added and incubated 3mins at 37°C with 5% CO_2. 6ml 10% DMEM was added and collected the cells in a 15ml centrifuge tube. Centrifuge the cells at 1500g for 2mins, supernatant was discarded and resuspend the cells in 6ml of PBS. Centrifuged again and the supernatant was discarded. 2ml of chilled Cell banker solution (FBS:DMSO = 9:1) was added and mixed thoroughly by pipetting. 1ml of aliquots were made into cryovials and placed into an 1^o cooler (Tarsons, India). The cooler was then kept at 4°C for 1hr, at 0°C for 1hr, -20°C for 2hrs and -80°C for overnight. On the next day, the cryovials from the cooler was shifted to the liquid nitrogen (-196°C) for long term preservation.

5.7. Biological Tests

5.7.1. Ethics statement

All animal experiments were conducted following the standard operating procedure as outlined by the committee for supervision and control experiments on animals (CPCSEA), Government of India. The animal experiment protocol was approved by the Institutional Animal Ethics Committee of ICMR-National Institute of Cholera and Enteric Diseases (68/GO/ReBi/S/99/CPCSEA, 18/07/2019-17/07/2024).

5.7.2. Bacterial strains used in animal experiments

Strains having *carR*^{S} (IDH 7230, N16961MT, IDH 12699, IDH 12695, IDH 12460) and *carR*^{R} (IDH 7230MT, N16961, IDH 3291, IDH 3672, IDH 3439) alleles were used in animal experiments to understand their roles in virulence and pathogenicity (Table 5.4).

5.7.3. Infant mouse colonization assay

Colonization assay was performed in a suckling mice model with the modified protocol described by Angelichio et al., 1999. Briefly, 4 to 5-day-old suckling BALB/c mice were separated from their mothers 1 h before inoculation with *V. cholerae*. The cells for the inocula were grown at 30°C in LB broth (30 ml) supplemented with streptomycin (100 µg/ml). The overnight culture was diluted 1:1,000 in LB. Each mouse was then inoculated with 10 µl of 10^6 bacterial cells. The bacterial titters in each inoculum were determined by plating serial dilutions of the inocula on the appropriate plates. Infected mice were kept at 26°C in the absence of their mother. They were then sacrificed at the designated time points. Small intestines of infected individuals were cut into segments and mechanically homogenized in PBS (100 µl) with a Tissue homogenizer (POLYTRON PT1600E, Kinematica AG). Then serial dilutions were plated onto LB agar supplemented with streptomycin (100µg/ml) to enumerate viable *V. cholerae* cells expressed in log of CFU/gm of intestine.

5.7.4. Rabbit illeal loop assay for detection of enterotoxigenecity

V. cholerae is generally found to adhere to the intestinal surfaces and secretes the potent cholera toxin which binds to the GM_1 receptor of the intestinal cell and through downstream processes it constitutively opens the ion channels and as a result fluid accumulation is observed in the intestinal lumen. Pathogenicity is directly proportional to the amount of fluid accumulation.

Experimental strains including a positive control strain (*V. cholerae* N16961) were grown in LB broth overnight at 37°C. Subculture (1:100) was done in 8ml fresh LB broth with 100µg/ml streptomycin and grown for an OD_{600} nm of 1.0 at 37°C. 1 ml of the normalized culture was harvested and washed with 1 ml of PBS. The cells ($1x10^8$) were then suspended in 1ml PBS which was then injected to the ligated ileum.

New Zealand white rabbits were used in this study with either sex weighing 2.0–2.5 kg. All animals were maintained under specific pathogen free condition in ventilated cages in the Animal House facility of NICED and provided with sterilized food and water. New Zealand white rabbits were fasted for 48 h before the surgery and only fed water ad libitum. Rabbits were anesthetized by intramuscular injection of ketamine (35 mg/kg body weight) and xylazine (5 mg/kg body weight). A laparotomy was performed, and the ileum was washed and ligated into discrete loops of about 10 cm. Each segment was separated by uninoculated segments of 1 to 2 cm. The first loop was filled with the strain N16961 as positive control. Test loops were inoculated with 10^9 CFU of the challenge strains in PBS. Negative-control loops were infused with PBS. The intestine was put back into the peritoneal cavity; animals were stitched up and kept to their cages at 25°C feeding only water for 14-16hrs. After overnight incubation, the rabbit was sacrificed with CO_2 exposure and the abdomen was reopened to take out the loops. The total intestinal looped segment (~70cm) was dissected out and washed with PBS. Cleaned intact segment was placed on a white paper for imaging. The intact first loop (N16961 inoculated) and then the other loops were cut out and the accumulated fluid was taken into 50ml falcon tubes by placing the fluid filled segment in the tube and puncturing with a scissor. Accumulated fluid volume was measured with steri-pipette and fluid accumulation ratio (ml/cm of intestine) was calculated. This experiment was performed in triplicate to determine the significance of the result derived (Ghosh-Banerjee et al., 2010).

5.7.5. Histopathology

(a) Sample preparation

Samples were collected from the intestine of mice and rabbit model. Tissue samples were dipped in 10% formalin buffer (Sigma) for fixation. Then the samples were put into 70%, 90% and 100% alcohol each for 30 mins to dehydrate the tissue. After that, samples were processed in xylene I and xylene II solution each for 30mins to clear the sample. Finally, after treatment with chloroform (30mins) samples were put in paraffin bath to prepare the tissue

block for microtome. Then tissues were cut into 3µm section and placed on a glass slide with the help of egg albumin (Sigma).

(b) Haematoxylin and eosin (HE) staining

Deparafinization was done to remove the wax from the samples. Tissue containing slides were then treated with xylene solutions following hydration of the tissue samples with down gradation of alcohol treatment each for 5mins. After that slides were dipped into Haematoxylin (Sigma) solution (5mins). After washing and dehydration slides were dipped in Eosin stain (Sigma). Slides were further subjected to upgradation of alcohol treatment. Super saturated lithium–carbonate was used for blueing. Finally, samples were washed in xylene solutions and mounted in DPX (Sigma).

After the completion of the staining procedure, visualization and documentation was done with the help of a light microscope.

5.8. Bioinformatics

The tools in bioinformatics were used in this study for whole genome sequencing analysis of *V. cholerae* O1 strains and in silico analysis of the DNA, plasmid, and gene sequences.

5.8.1. Genome analysis, annotation & antimicrobial resistance gene identification

Raw reads were assembled *de novo* using AssemblerSPAdes software v4.4.0.1. The genome was annotated using the RAST server (Rapid Annotation using Subsystem Technology- http://rast.nmpdr.org) available from the PATRIC database (Pathosystems Resource Integration Centre -https://www.patricbrc.org). The Centre of Genomic Research's database was utilised to perform Multi Locus Sequence Typing (MLST) to determine the sequence type of *V. cholerae* strains based on the seven housekeeping genes *adk, gyrB, mdh, metE, pntA, purM,* and *pyrC* (https://cge.cbs.dtu.dk/services/MLST/). Genome assemblies were screened for the presence of antimicrobial resistance genes using the ResFinder web server v2.1 (https://cge.cbs.dtu.dk/services/ResFinder/).

5.8.2. Plasmid analysis and molecular typing by MLST

The presence of plasmid was analyzed using Plasmid Finder 1.3 available from the CGE server (https://cge.cbs.dtu.dk/services/) from the whole genome sequencing of the study isolates. The incompatibility (Inc) type of the plasmid was analyzed using pMLST (https://pubmlst.org/plasmid/). The mobile genetic element (MGE) finder was screened using

the MGE finder available at CGE server (https://cge.food.dtu.dk/services/MobileElementFinder/). Comparative plasmid analysis was performed using CGview server (https://proksee.ca/).

5.8.3. Statistical analysis

Statistical significance for the quantitative methods was analyzed using Kruskal-Wallis oneway ANOVA or two-way ANOVA in GraphPad Prism ver. 6.0 for the animal experiments. For the qRT PCR analysis, statistical significance was carried out using one-way ANOVA and Dunnett's multiple-comparison tests. Apart from these experiments, others were calculated using mean ± standard deviation. P-values of <0.05 were marked as statistically significant for all the tests.

Chapter 6

Results

Objective I

To analyze the changing genetic features of currently circulating *V. cholerae* O1 isolates in India.

6.1. Isolation and identification of *V. cholerae* strains

In this study, 474 *V. cholerae* strains were obtained from different cholera-endemic regions of India during the period of 2018-2021. Stool samples were received from IDH and BCH hospitals in Kolkata, West Bengal. Whereas, stab samples were obtained from Shri Vasnatrao Naik Govt. Medical College in Yavatmal (Maharashtra); State Public Health Laboratory in Baroda, SMIMER Medical College in Surat, AMC MET Medical College in Ahmedabad and P.D.U Govt Medical College in Rajkot (Gujarat); Tirunelveli Medical College in Tirunelveli (Tamil Nadu); Manipal Hospital in Bangalore, Kodagu Institute of Medical Sciences in Madikeri (Karnataka) and Rajan Babu Institute of Pulmonary Medicine & Tuberculosis in (Delhi), India. All the strains were grouped into two categories: a) West Bengal, and b) other regions of India, to compare the changing features of strains between the cholera-prone region, West Bengal with other states of India where cholera is frequent but not so much focused on earlier.

All the *V. cholerae* strains were identified by their colony morphology as they appear like round, yellow, sucrose fermenting ability with an elevated centered colony on Thiosulphate-Citrate Bile salt Sucrose agar (TCBS) plates after incubating for 18 h at 37˚C. The large yellow colour colony was selected for further sub culturing on LB agar plates. This culture was used for presumptive tests such as oxidase and string to distinguish *V. cholerae* strains from *Aeromonas sp*. Strains were found to give a purple color when mixed with Oxidase reagent and the formation of thread-like structures with sodium deoxycholate solution and thus showed typical Oxidase and string positive features specific for *V. cholerae.*

6.2. Predominance of Ogawa serotype

The LB agar containing cultures of *V. cholerae* isolates were tested for agglutination with polyvalent O1 serogroup specific antisera to confirm their serogroup. All the strains were found to agglutinate with the polyvalent O1 antisera and hence were confirmed as *V. cholerae* O1. A further serological classification was also performed for monovalent O1-Ogawa and O1-Inaba specific antisera to determine the serotype. Out of 116 strains from West Bengal and 358 from outside of West Bengal, 88% (102/116) and 96% (344/358) strains have been shown to agglutinate with O1-Ogawa- specific antisera respectively. Whereas rest of the strains belonged to the O1-Inaba serotype (Table 6.1).

Table 6.1. *Phenotypic characteristics of V. cholerae O1 strains isolated during 2018-2021.*

Sl No.	Place of Isolation	Serotype (%)	Biotyping attributes			
			Voges Proskauer Test	Chicken Blood Cell Agglutination	Sheep Blood Hemolysis (%)	Polymyxin B Susceptibility (%)
1	West Bengal	Ogawa (88)/ Inaba (12)	+	+	+ (94)/ - (6)	Sensitive (94)/ Resistant (6)
2	Other regions of India	Ogawa (96)/ Inaba (4)	+	+	+	Sensitive

6.3. Emergence of *V. cholerae* O1 strains with altering biotyping features

V. cholerae is a versatile organism and this versatility has been exhibited by many types of changes in their genome that help them better adapt to the environment. Thus, to reveal the changing features of recently circulating strains, we have compared the different attributes of the isolates from West Bengal to the other regions of India during the period 2018-2021.

6.3.1. Strains were able to produce acetoin from glucose fermentation

V. cholerae O1 El Tor strains have the ability to produce acetylmethyl carbinol or acetoin from glucose fermentation and as a result, they are considered VP positive. Conversely, classical strains were unable to ferment glucose and thus were negative for this reaction. In this study, all the strains were considered positive in the VP test similar to the prototype El Tor (Table 6.1).

6.3.2. Strains showed agglutination of chicken blood cell

Another test to differentiate the El Tor biotype from the classical biotype is chicken blood cell agglutination. The El Tor biotype shows visible agglutination when mixed with chicken blood cells, whereas the classical biotype cannot. All the strains included in this study were classified into El Tor as they have shown typical clumping of blood cells (Table 6.1).

6.3.3. Appearance of nonhemolytic strains in West Bengal

An important test for identifying the *V. cholerae* biotype is sheep blood hemolysis. Typically, the classical biotype is unable to lyses sheep blood erythrocytes, whereas the El Tor biotype is able to do so and, after 24-48 hours of incubation, produces a clear zone at the site of inoculation on the blood agar plate. 6% (7/116) of the total strains from West Bengal were

unable to lyse sheep blood erythrocytes similar to the classical biotype strains (Figure 6.1). Therefore, these strains were considered non-hemolytic. Whereas, all the isolates from other regions except West Bengal showed hemolytic traits similar to the El Tor strains (Table 6.1).

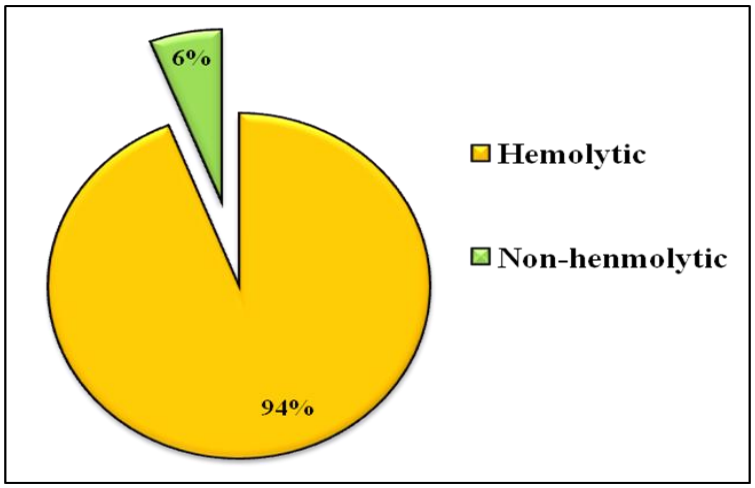

Figure 6.1. *Prevalence of hemolytic and non-hemolytic phenotypes among the West Bengal isolates in 2018-2021.*

6.3.4. Co-existence of polymyxin B-sensitive and resistant strains

Another key marker for the differentiation of the biotypes of *V. cholerae* O1 is polymyxin B susceptibility. Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guideline for the family Enterobacteriaceae as no interpretive guideline was mentioned there for *V. cholerae.* To verify the results, all the strains that showed resistance and sensitivity were streaked on the LB agar plate containing 50µg/ml polymyxin B. Strains showing resistance in the disc diffusion test were found to grow on the LB agar with polymyxin B plate whereas, those found sensitive in the disc diffusion method were growth deficient in that medium. The classical biotype was sensitive to this antibiotic whereas El Tor was relatively resistant. However, this pattern of sensitivity has been changed with the emergence of polymyxin B sensitive El Tor variant strains in 2012 in West Bengal (Samanta et al., 2015). Both the phenotypes are continuously engaged in competition to adapt well in nature and polymyxin B-resistant strains are again found to dominate in 2015 followed by a complete absence. In 2017, one isolate was found to be resistant to polymyxin B indicating the re-emergence of polymyxin B-resistant strains (Figure 6.2).

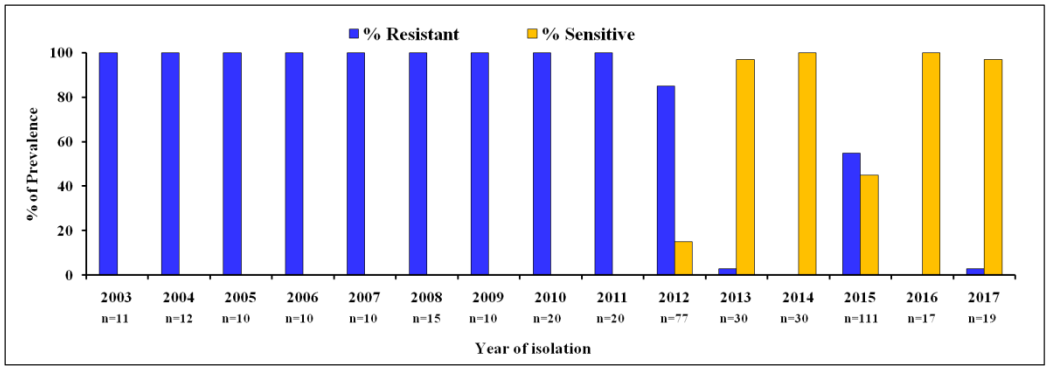

Figure 6.2. *Yearly distribution of polymyxin B-resistant and sensitive V. cholerae O1 isolates (n=402) in West Bengal during 2003-2017.*

In this study, 6% (7/116) strains from West Bengal gave a zone diameter of ≤10mm in Kirby-Bauer disc diffusion test similar to the polymyxin B-resistant El Tor reference strain N16961 (Figure 6.3). These strains were isolated in the year of 2018 in West Bengal. Interestingly, polymyxin B-resistant El Tor variant strains were again completely disappeared after 2018 from West Bengal. The remaining isolates (94%) from West Bengal and all the strains from other regions of India showed a zone of inhibition of ≥12mm and were unable to grow on LB agar plate with 50µg/ml polymyxin B similar to the polymyxin B-sensitive reference strain O395 in the period of 2018-2021.

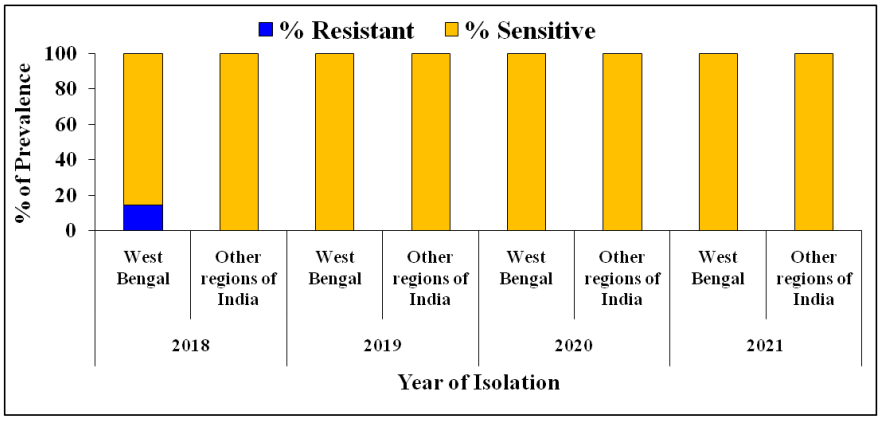

Figure 6.3. *Prevalence of polymyxin B-sensitive and resistant strains in West Bengal and other regions of India (2018-2021).*

6.4. Analysis of Haitian genetic alleles of virulence genes

After the occurrence of the disastrous Haitian cholera outbreak in 2010, cholera has become a global public health burden for the first time in almost a century with its severity and propensity. The isolated strains possessed virulence genetic characteristics that were different from the existing ones. The strains that carried point mutations in the major virulence-associated genes in *V. cholerae* O1 are the cholera toxin subunit B (*ctxB*), toxin co-regulated

pilus (*tcpA*), and repeats-in-toxin (*rtxA*) which have been proved for their hypervirulent properties (Naha et al., 2012; Ghosh et al., 2019). Hence, in this study, we were interested to find out whether our studied isolates harbor these hypervirulent alleles of the major virulence-associated genes.

6.4.1. Prevalence of Haitian *ctxB*

V. cholerae O1 strains have been found to exhibit different variants of *ctxB* alleles, mostly as a result of non-random point mutation. Almost 14 genotypes of *ctxB* have existed. Among them, genotypes 1, 3, and 7 are associated with the pandemic strains. Classical biotype strains contained genotype 1, or classical *ctxB* (*ctxB1*), which predominated earlier. El Tor biotype with *ctxB* genotype 3 (*ctxB3*) appeared in 1961. The preceding El Tor *ctxB* was gradually displaced in 1990 by the re-appearance of classical *ctxB*. Haiti experienced a cholera pandemic in 2010 as a result of the emergence of a novel variant of the *ctxB* allele (*ctxB7*) of *V. cholerae* O1. These strains generate classical *ctxB* except for an additional mutation in the signal sequence region where an asparagine (N) residue replaces a histidine (H) at the 20th amino acid position (H20N) of cholera toxin subunit B-precursor (pre-CTB). Our previous study mentioned the distribution pattern of predominating classical and Haitian *ctxB* in Kolkata during 2004-2011 and reported the existence of Haitian *ctxB* (*ctxB*7) in Kolkata, West Bengal since 2006, long before the Haitian cholera outbreak (Naha et al., 2012) (Figure 6.4). At present, two *ctxB* genotypes of *V. cholerae*, classical *ctxB* and Haitian *ctxB* were existed in nature. Our earlier retrospective study with the *V. cholerae* O1 strains isolated during 2012-2017 from Kolkata, West Bengal was analyzed for the presence of different *ctxB* alleles and compared with that of the previous isolates. It has been found that Haitian *ctxB* replaced the classical *ctxB* for the first time in 2012 and dominated the classical *ctxB* throughout the study period except for the years 2015 and 2017 (Figure 6.4).

In this study, a total of 116 strains were isolated from West Bengal and 358 strains from other regions of India during 2018-2021. Genotyping screening for the detection of different variants of *ctxB* gene has been performed with the help of MAMA PCR and the result indicated the prevalence of Haitian *ctxB* allele among the total isolates. Only 6% (7/116) of the strains from West Bengal carried classical *ctxB* genotype (Figures 6.5 and 6.6A1).

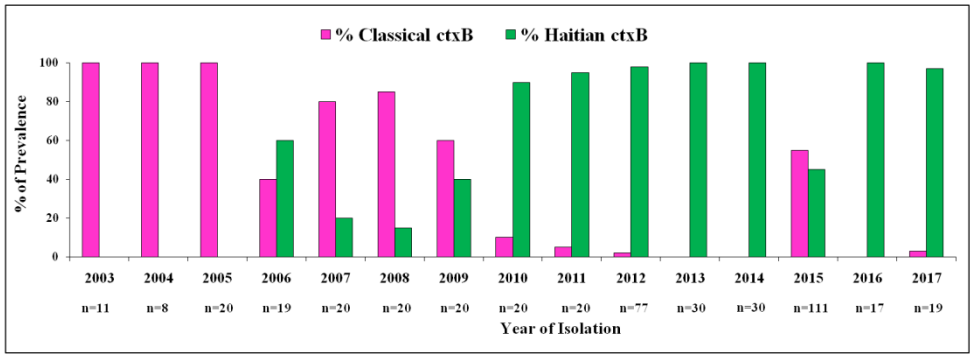

Figure 6.4. *Distribution of different ctxB alleles among V. cholerae O1 isolates in Kolkata, West Bengal during 2003-2017 (n=442).*

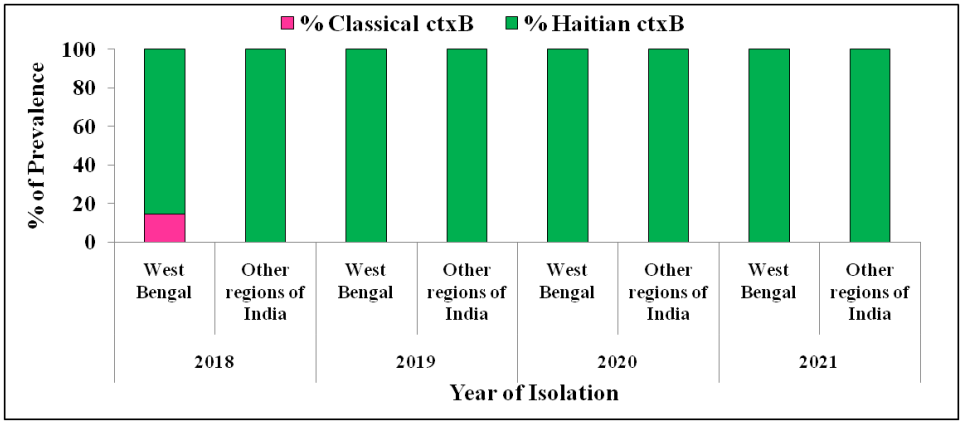

Figure 6.5. *Prevalence of Haitian alleles of ctxB among the strains of West Bengal and other regions of India except West Bengal (2018-2021).*

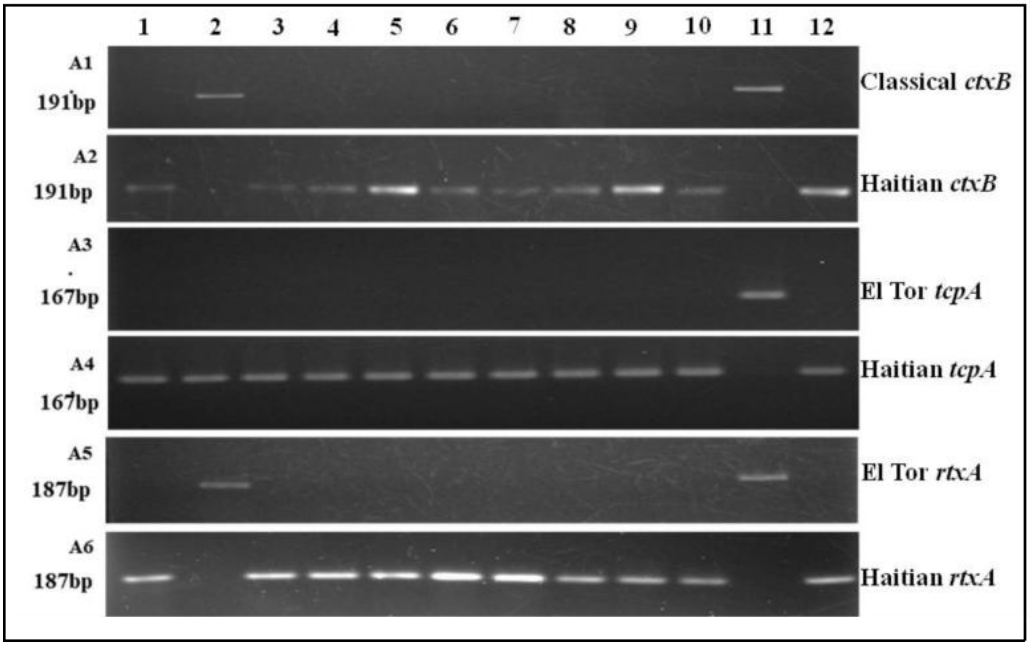

Figure 6.6. *PCR assay for the presence of ctxB, tcpA and rtxA alleles in Vibrio cholerae O1 isolates of different states in India. Lanes 1-10, representative isolates; Lane 11 and 12, respective positive controls.*

6.4.2. Existence of only Haitian allele of *tcpA* among the isolates

TcpA protein is a type IVb pilus required for *V. cholerae* colonization. Two biotypes of *V. cholerae* are associated with pandemic disease, classical and El Tor. The TcpA of the two biotypes is 82% identical, with variability being found in the C terminus of the protein. A single point mutation was responsible for the emergence of Haitian *tcpA* allele that was found to link with higher virulence and pathogenicity (Ghosh et al., 2019). Our earlier reports from Kolkata, West Bengal suggested that Haitian *tcpA* first appeared in Kolkata, West Bengal in the year 2003 and completely replaced the El Tor *tcpA* in 2004 (Figure 6.7). The retrospective study with the *V. cholerae* isolates from Kolkata during 2012-2017 was found to give bands with Haitian *tcpA* specific PCR and no single strain was found positive for El Tor *tcpA* (Figures 6.6A3 and 6.6A4). It is also noted that El Tor *tcpA* has not appeared in a single isolate since 2004 (Figure 6.7). Thus, to know whether recently circulating strains in India also harbor the hypervirulent Haitian allele, we have been interested in screening the Haitian *tcpA* among the recently isolated *V. cholerae* O1 strains. Our results showed that all the isolates (n=474) contained Haitian *tcpA* as the major colonization factor irrespective of the genotypes of *ctxB* and *rtxA* (Figures 6.8, 6.6A3, and 6.6A4).

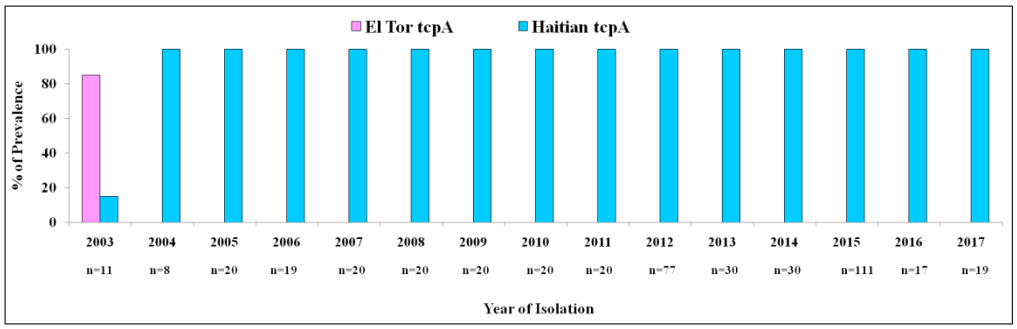

Figure 6.7. *Distribution of different tcpA alleles among V. cholerae O1 isolates in Kolkata, West Bengal during 2003-2017.*

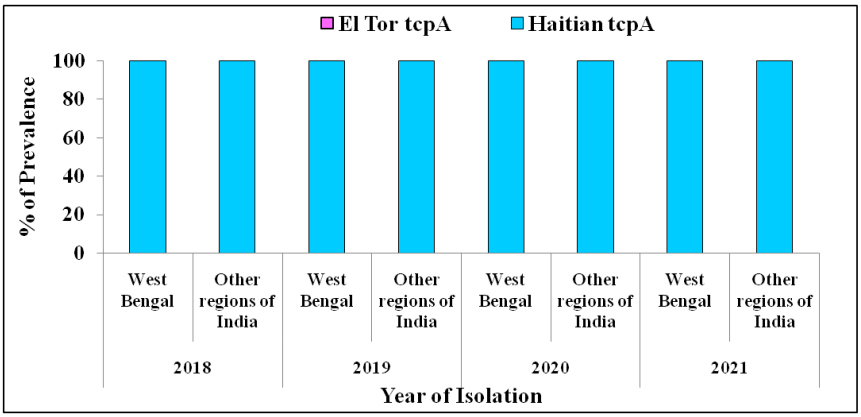

Figure 6.8. *Prevalence of Haitian alleles of tcpA among the strains of West Bengal and other regions of India (2018-2021).*

6.4.3. Predominance of non-functional Haitian *rtxA*

The *rtxA* gene is the largest open reading frame (ORF) of the *V. cholerae* genome. Classical strains have a deletion of 7,869 bp and thus remove the 5′ end of the *rtxA* gene as well as the RTX promoter region and genes for toxin maturation and secretion. Whereas El Tor *rtxA* which appeared with the emergence of El Tor biotype has a complete reading frame and functions as a potent cytotoxin. However, the Haitian strains of *V. cholerae* have a point mutation in the reading frame of functional *rtxA* that introduced a premature stop codon, which disables its function. It is believed that the inactivation of *rtxA* in Haitian isolates may contribute as a genetic background for the formation of a highly pathogenic Haitian *ctxB* genotype, even though this Haitian *rtxA* is less powerful than El Tor *rtxA*. Two genotypes of *rtxA* were found to be present in nature currently, El Tor and Haitian *rtxA*.

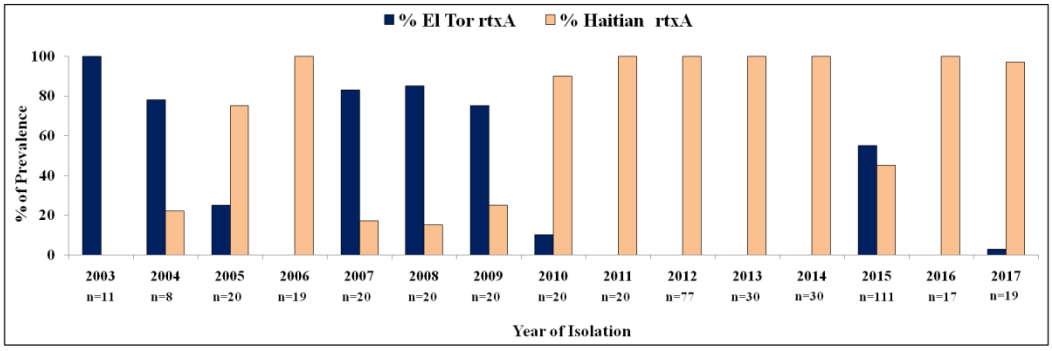

Figure 6.9. *Distribution of different rtxA alleles among V. cholerae O1 isolates in Kolkata, West Bengal during 2003-2017.*

Based on the sequence polymorphism, a PCR-based detection of only El Tor and Haitian *rtxA* has performed. Our earlier studies showed that the Haitian *rtxA* was present in West Bengal since 2004 before the occurrence of Haitian cholera outbreak (Figure 6.9). However, after the complete replacement of El Tor *rtxA* in 2006, it again found to dominate during the years

2007, 2008, 2009, and 2015. After that, Haitian *rtxA* remained as the major dominating genotype except in 2017.

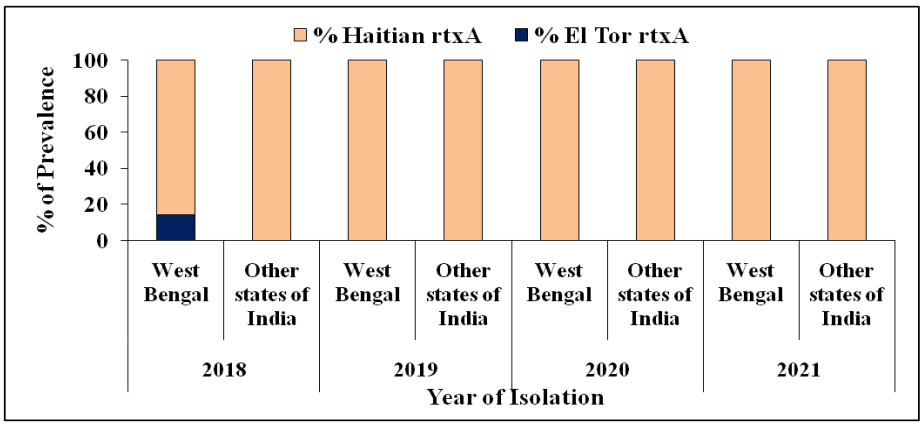

Figure 6.10. *Prevalence of Haitian and El Tor rtxA alleles among V. cholerae O1 isolates in India during 2018-2021.*

In this study, we have found the re-emergence of El Tor *rtxA* in 6% of the isolates in West Bengal associated with the classical *ctxB* genotype (*ctxB1*) (Figures 6.10, 6.6A5, 6.6A1, and 6.5) whereas Haitian *rtxA* was present in the rest of the strains received in the period of 2018-2021 (Figure 6.10).

6.4.4. Prevalence of Haitian *gyrA* among the isolates

DNA gyrase is a heterotetramer composed of two A and two B subunits, encoded by the gyrase A and gyrase B gene respectively. It is the target of quinolones. Haitian strains have a point mutation in *gyrA* which was responsible for the change in amino acid serine to isoleucine at the position of the 83rd nucleotide. This mutation further plays a role in quinolone resistance phenotype (Hasan et al., 2012).

Our earlier report revealed the existence of Haitian *gyrA* allele in Kolkata from 1994 before the occurrence of Haitian outbreak. Thus, to determine the prevalence of Haitian allele among the isolates, we have performed a MAMA PCR by using the primers for El Tor and Haitian *gyrA* alleles (Table 5.2) and found that all the *V. cholerae* O1 isolates given band similar to Haitian control strain EL-1786 (Figure 6.11). This study indicates the existence of only Haitian allele of *gyrA* from 1994-2021 in West Bengal.

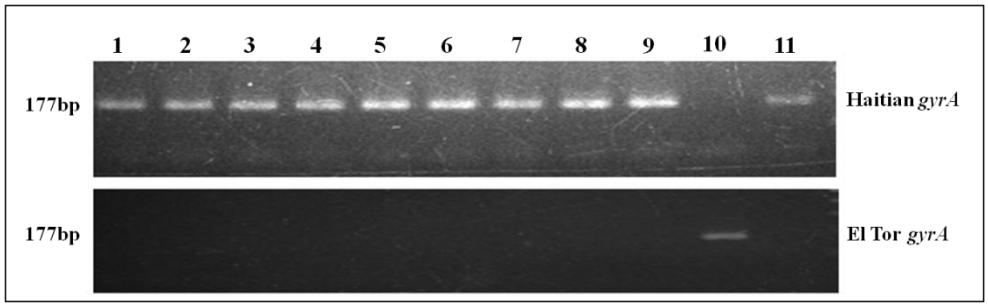

Figure 6.11. *A MAMA PCR based assay for gyrA allele in representative strains of V. cholerae O1 isolates in different states of India by using primers (gyrAF/gyrAR1) and (gyrAF/gyrAR2) for Haitian and El Tor strains. Lanes 1-9 contains representative isolates, Lane 10 represents N16961 (El Tor) and Lane 11 represents EL-1786 (Haitian).*

6.4.5. Existence of Haitian *vieA* allele in West Bengal before the Haitian cholera outbreak

VieA is a part of VieSAB three component signal transduction pathway and plays various roles in biofilm, motility, and the pathogenesis of cholera. Studies showed that El Tor *vieA* is almost silent. But a point mutation in *vieA* at the position 235th nucleotide (C235T) was responsible for the emergence of Haitian *vieA*. As the Haitian strains were characterized as hypervirulent strains of *V. cholerae*, we were interested to perform a retrospective study of the prevalence of Haitian *vieA* allele in West Bengal strains. As a result, like *ctxB*, *tcpA*, *rtxA* and *gyrA*, we found that Haitian *vieA* has been present in West Bengal since 2004 before the occurrence of Haitian outbreak (Figure 6.12).

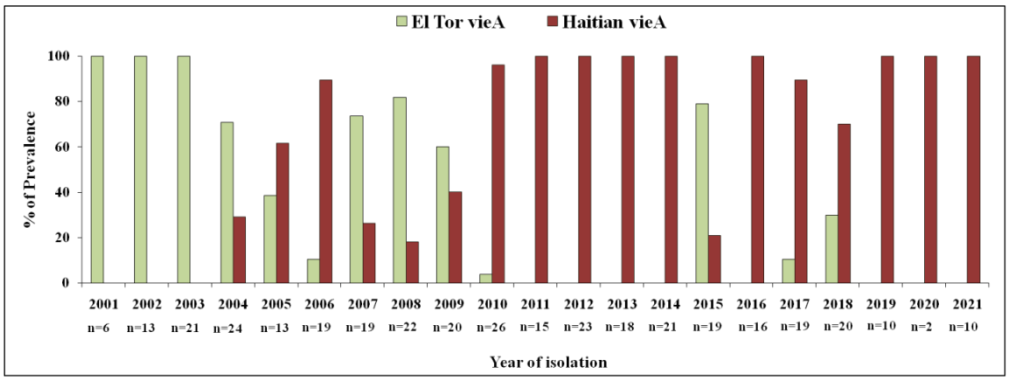

Figure 6.12. *Yearwise distribution of El Tor and Haitian vieA among V. cholerae O1 isolates (n= 356) in Kolkata, West Bengal during 2001-2021.*

6.4.6. Non hemolytic phenotype results from truncated *hlyA*

Like polymyxin B, the emergence of non-hemolytic El Tor *V. cholerae* has also been reported with several mutations in the gene encoding hemolysin (*hlyA)* which leads to truncated protein (Fan et al., 2019). *hlyA* is a major virulence factor found in El Tor *V.*

cholerae in chromosome II. This pore forming toxin has the ability to cause cell death by act on targeting membrane (Huntley et al., 1997). Classical strains are non-hemolytic due to the 11bp deletion in their nucleotide sequence which introduces a premature stop codon. Thus, the resulting protein is non-functional. Mechanisms that were responsible for non-hemolysis of the epidemic O1 El Tor strains are complex and not only confined to gene mutation but also deficiencies of transcription and extracellular transport of HlyA (Fan et al., 2019). Thus, to evaluate whether this non-hemolytic phenotype found in our strains was also linked to genetic mutation or not, we have performed a multiplex PCR assay by using primers (hlyA_489F-1184R/hlyA_744F-1184R) specific for El Tor and both El Tor & classical respectively (Table 5.2). Strains having non-hemolytic phenotype were taken to perform the PCR assay and all strains were found to be given bands similar to classical strain O395 (Figure 6.13). This observation indicates that they are trying to emerge as stable variants in nature by acquiring classical traits like in the case of polymyxin B. To confirm this observation, we have performed nucleotide sequencing of two of those 7 non-hemolytic strains and two hemolytic strains isolated during 2018 from West Bengal. Nucleotide sequencing data of non-hemolytic strains revealed a different nucleotide sequence of *hlyA* compared to the classical *hlyA*. A comparative nucleotide sequence analysis study has been done with the El Tor reference strain N16961 (accession no. AE003852.1) by using BLASTn and found a 17 bp deletion at the position of 733-749 nucleotide (Figure 6.14). This study revealed a new *hlyA* variant, which contains an additional 6 bp deletion compared to the 11 bp deletion found in classical *hlyA* (Fig 6.14). This deletion also leads to the introduction of a premature stop codon which results in a truncated protein like classical HlyA. Strains with hemolytic activity have nucleotide sequences similar to El Tor *hlyA*.

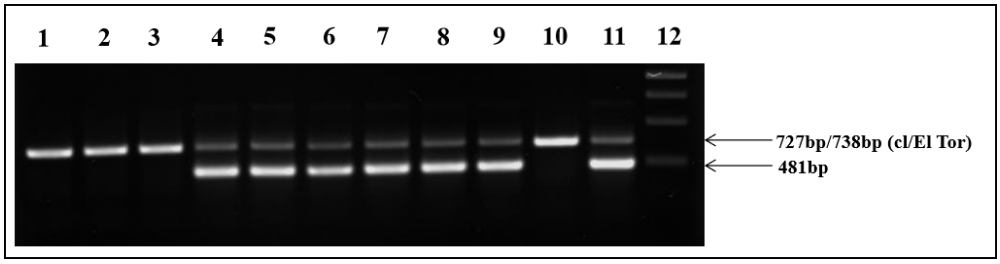

Figure 6.13. *A multiplex PCR based assay for classical and El Tor hlyA gene of V. cholerae O1 strains. Lanes 1-3 contain strains with non-hemolytic phenotype. Lanes 4-9 contain strains with hemolytic trait. Lanes 10 and 11 represent classical (O395) and El Tor (N16961) control strains respectively. Lane 12 represents 100 bp ladder (New England Biolab Inc., Beverly, MA, USA). cl, classical; El, El Tor.*

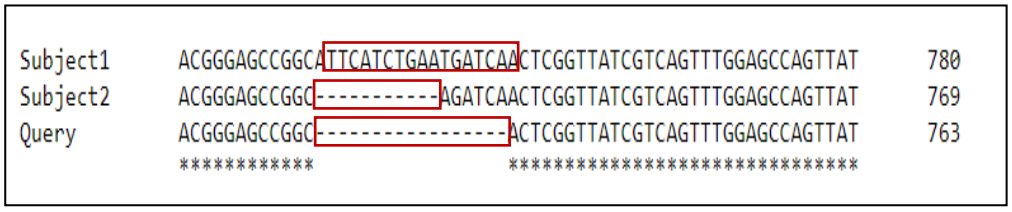

Figure 6.14. *Alignment of hlyA sequence among N16961 (subject1), O395 (subject2) and the non-hemolytic West Bengal isolate (query). Deleted regions are marked within red boxes.*

6.5. Presence of El Tor- specific genetic markers

In addition to the conventional phenotypic characteristics, the classical and El Tor biotypes are also distinguished by several genetic characteristics such as the CTXΦ integration in chromosome II, the presence of *rtxC*, *rstC*, *ctxB* and *rstR* alleles. The seventh pandemic El Tor strains were comprised of a unique feature with the absence of CTX prophage in chromosome II (Heidelberg et al., 2000; Maiti et al., 2006), whereas the classical biotype has CTX prophages in both chromosomes. The absence of CTX phage integration will be marked by the appearance of band of 766 bp in size and our result showed that no strains harboured CTX prophage in their chromosome II (Figure 6.15). Further, El Tor strains comprise the genes *rtxC*, *rstC*, and El Tor- specific *rstR* allele (Nusrin et al., 2004). Conversely, the classical biotype does not have *rtxC*, *rstC* genes, but contained classical *rstR*. All the strains from this study harboured the genes *rtxC*, *rstC* and El Tor- specific *rstR* like the typical El Tor biotype (Table 6.2).

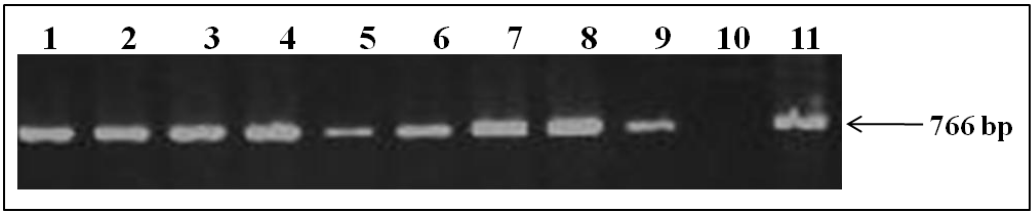

Figure 6.15. *A PCR based assay to check any integration of CTX-phage in chromosome II by using (cIIF/cIIR) primers among the studied strains. Lanes 1-9 contains representative isolates, Lane 10 represents O395 (Classical) and Lane 11 represents N16961 (El Tor).*

Table 6.2. *Characteristics of El Tor specific genetic markers among the V. cholerae O1 isolates under this study.*

Place of Isolation	El Tor specific genetic markers		
	***rtxC* (%)**	***rstC* (%)**	***rstR* (%)**
West Bengal	Present (100)	Present (100)	El-100 Cla-0
Other regions of India	Present (100)	Present (100)	El-100 Cla-0

**Cla-classical, El-El Tor.*

6.6. Deletion of a large fragment in the VSP- II region revealed similarity with Yemeni strain

The 2016-2018 Yemen cholera outbreak has been marked as the world's worst outbreak caused by *V. cholerae* O1 El Tor typified by several genetic changes, including a deletion of a large genomic fragment (including genes VC0495– VC0512) in the VSP-II region (Weill et al., 2019). A newly developed PCR assay in the present study confirmed that all the hemolytic isolates of *V. cholerae* contained a deletion in the VSP-II with the amplicon of 2.7 kb in size (Figure 6.16a). The Yemeni strain used as a positive control also produced a 2.7 kb product. Based on the similarity in amplicon size with the VSP-II of the strains characterized by Imamura and colleagues it can be proposed that the strains of this study have partially deleted VSP-II and possessed VSP-IIC type (Imamura et al., 2017). The standard strain N16961 which has the ~27 kb intact VSP- II region (VSP- IIA) gave no band as the PCR condition applied was inadequate to amplify this large fragment. Similar to strain N16961, the 6% (7/116) strains from West Bengal containing non-hemolytic trait were also gave no band in this PCR (Figure 6.16a). This might have been due to the presence of VSP-II regions other than the VSP- IIC type (VSP-IIA or B). The strains with negative results were further confirmed for the presence of the representative genes VC0502, VC0506, and VC0510 of the VSP-II region located between the genomic region VC0494 to VC0512 and found to yield bands in the PCR assays (Figures 6.16b–6.16d). The presence of the internal control genes, VC0502, VC0506 and VC0510 confirmed that 6% of the West Bengal strains harboured either VSP-IIA or B type.

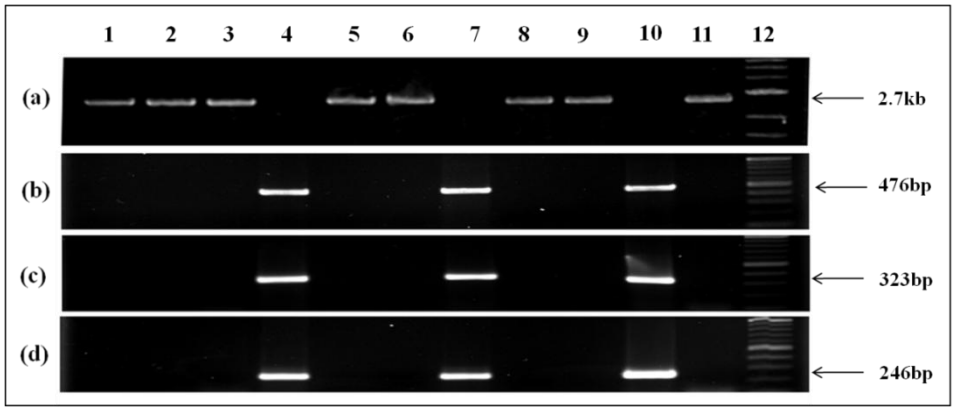

Figure 6.16. *Polymerase chain reaction (PCR) assay using primer pair VC0494F/VC0513R to check the deletion of genes VC0495–VC0512 in the VSP-II region. This deletion is a remarkable feature of Yemen cholera outbreak strains (a). PCR for the presence of loci VC0502 (b), VC0506 (c) and VC0510 (d) of the VSP-II region. Lanes 1–9 represent the clinical isolates of different regions of India; lane 10, N16961; lane 11, YMN-170240 (Yemen outbreak strain) and lane 12, 1kb size ladder (for panel a) or 100bp size marker (for panels b, c and d) (New England Biolab Inc., Beverly, MA, USA).*

6.7. PFGE analysis showed a different pulsotypic pattern among the nonhemolytic isolates

To determine the genetic relatedness of the El Tor nonhemolytic strains with the hemolytic strains, a total of 21 representative strains from 2018 were analysed. Dendrogram analysis showed five different pulsotypes with an overall similarity of 95% (Figure 6.17). Hemolytic strains clustered in the pulsotype P2, whereas nonhemolytic strains showed four different pulsotypic patterns, P1, P3, P7, and P8. Hemolytic El Tor strains (P2) belonged to Ogawa serotype, sensitive to polymyxin B and harboured Haitian *ctxB* (*ctxB7*) allele. In contrast, nonhemolytic strains of this study were identified as Inaba serotype, polymyxin B-resistant, and possessed classical *ctxB* (*ctxB1*) genotype. Almost 96% similarity was observed between the pulsotypes P2 and P1, whereas P3, P7 and P8 pulsotypes, which consist of nonhemolytic strains shared 93% genomic identity with P1 and P2. Among the pulsotypes, P2 is predominated by 15 Ogawa serotype strains with hemolytic trait. Interestingly, the other four pulsotypic variants consisted of the 6 strains of West Bengal among which genomic differences exist and belong to the Inaba serotype (Figure 6.17). To determine the possible genomic relatedness of the strains in this study with the polymyxin B-sensitive Yemeni isolate responsible for the cholera outbreak in Yemen, we also included one Yemeni strain in the analysis and found ~94% similarity. In addition, we have also taken four polymyxin B-sensitive Haitian variant strains isolated during 2013-2014 from Kolkata, West Bengal to understand the evolutionary relationship between them and found that overall 93% similarity

to the Yemeni strain as well as to the hemolytic strains indicating a close genetic relatedness (Figure 6.17).

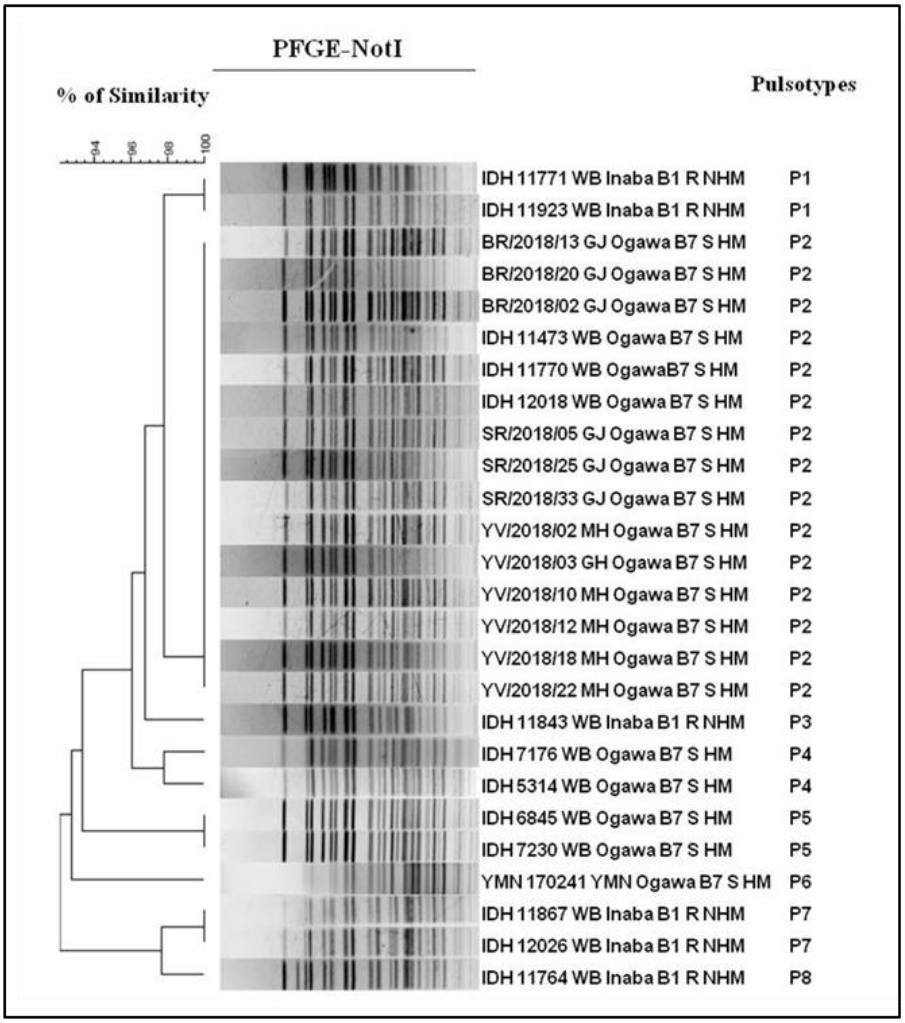

Figure 6.17. *Pulse-field gel electrophoresis patterns of NotI-digested genomic DNA of Vibrio cholerae O1 strains isolated from Gujarat, Maharashtra, West Bengal, and Yemen. The gel image was analysed using BioNumerics version 4.0 software (Applied Maths) based on the single-linkage method to generate the dendrogram. Percent similarity is shown on the upper left side. Polymyxin B-sensitive, hemolytic, Haitian ctxB-containing Ogawa strains formed a single cluster, whereas polymyxin B-resistant, nonhemolytic, classical ctxB-carrying Inaba strains were found to form different banding patterns. Strain IDs were mentioned at the end of each lane followed by the place of isolation, serotype, ctxB genotype, polymyxin B susceptibility and hemolysis pattern. WB, West Bengal; GJ, Gujarat; MH, Maharashtra; YMN, Yemen; B1, classical ctxB; B7, Haitian ctxB; R, polymyxin B-resistant; S, polymyxin B-sensitive; NHM, nonhemolytic; HM, hemolytic.*

Objective II

To know the antimicrobial resistance pattern in recently circulating *V. cholerae* strains along with its mechanism of resistance.

6.8. Changing drug resistance pattern in recent *V. cholerae* O1 isolates in India

Antibiotic resistance is not common for *V. cholerae* but there have been many reports published recently that state the emergence of antibiotic resistant *V. cholerae* O1 (Mandal et al., 2012; Mohanraj et al., 2020; Quilici et al., 2010). However, for several decades *V. cholerae* O1 has emerged as a notorious multidrug-resistant (MDR) enteric pathogen (Das et al., 2020). The dissemination of the genetic determinants of antibiotic resistance has been facilitated by the acquisition of plasmids, integrating conjugative elements (ICEs), superintegron, transposable elements, and insertion sequences (Das et al., 2020; Hochhut et al., 2001). Harboring such elements led the strains more prone to cause fatal diseases. Therefore, it is important to know the current status of antibiotic susceptibility of the recent strains.

6.8.1. Emergence of carbapenem resistant *V. cholerae* O1 in Gujarat, India raises an alarming risk

In this study, a total of 57 representative *V. cholerae* O1 isolates from the 116 strains from West Bengal and 182 from 358 strains from other regions of India isolated during 2018-2021 have been taken to test their antimicrobial susceptibility pattern by the Kirby-Bauer disc diffusion method of the commonly used antibiotics namely ampicillin (10µg), tetracycline (30µg), chloramphenicol (30µg), sulfamethoxazole/trimethoprim (23.75/1.25µg), ciprofloxacin (5µg), nalidixic acid (30µg), norfloxacin (10µg), ofloxacin (5µg), gentamicin (10µg), streptomycin (10µg), ceftazidime (30µg), ceftriaxone (30µg), cefotaxime (30µg), azithromycin (10µg), doxycycline (30µg) and meropenem (10µg) using the commercially available discs (BD, Difco, USA). Recent reports suggest that the resistance phenotype against different drugs in *V. cholerae* is becoming complex. Over 92% of the strains from West Bengal and other regions showed resistance against streptomycin and sulfamethoxazole/trimethoprim (Figures 6.18A and 6.18B). All the West Bengal strains were resistant to nalidixic acid. Beginning in early 1990, there was a sharp rise in resistance to tetracycline, chloramphenicol, ampicillin, and nalidixic acid. Ampicillin resistant strains were mostly prevalent in West Bengal (18/57), Maharashtra (4/182), Gujarat (46/182), and Delhi (20/182). The first report of MDR *V. cholerae* isolates from serogroup O1 that were resistant to tetracycline, streptomycin, and chloramphenicol were reported in 1970. About 8% of the isolates from West Bengal were found to be resistant to tetracycline (Figure 6.18A). Only 3 isolates (1.6%) from Gujarat showed resistance against tetracycline. Chloramphenicol

resistance was also seen in a few of the isolates, which were also reported in Africa and southern India. Reduced susceptibility to quinolones was also reported in Western Africa and India. However, the strains in this study did not show resistance to the latest generation of quinolones. Moreover, reduced susceptibility has been observed in a few strains of West Bengal, Gujarat, Karnataka, and Delhi. *V. cholerae* O1 isolates from Gujarat showed a vast range of drug resistance patterns compared to the strains from other states. 3% (6/182) of isolates from Gujarat isolated during 2019 showed resistance against the third generation of beta lactam antibiotics (cefotaxime, ceftriaxone, and ceftazidime). These 3% strains also showed a resistant phenotype against the carbapenem group of antibiotics such as meropenem, which is known as the last resort drug along with resistance against gentamicin (Figure 6.18B). The emergence of extensive drug resistance (XDR) and MDR strains of *V. cholerae* has occurred due to the acquisition of plasmids, integrating conjugating elements (ICEs), superintegrons, transposable elements, and insertion sequences. Although carbapenem-resistant *Vibrio cholerae* (CRVC) strains have been occasionally observed.

6.8.2. Major focus on carbapenem-resistant *V. cholerae* O1 (CRVC) isolates

Globally, the prevalence of antimicrobial resistance (AMR) in *V. cholerae* strains is constantly growing. Carbapenems are considered "critically important antimicrobials" by the World Health Organization (WHO) because they are one of the few accessible options to treat bacteria that are multi-drug resistant. The genomic plasticity of *V. cholerae* strains allows them to respond to a wide range of environmental stimuli, including antimicrobials.

The bacterium is naturally competent at obtaining DNA from the environment through all three of the main HGT pathways, namely transformation, conjugation, and transduction. Moreover, carbapenem resistance results in enormous clinical and financial expenses. The unusual carbapenem structure, which includes the β -lactam ring, distinguishes carbapenems as a distinct class of antibiotics and contributes to their outstanding stability in the presence of β -lactamases. The most prevalent method of drug resistance in *V. cholerae* is the chemical modification of antibiotics using an enzymatic function of acquired genetic features.

In this study, we found 6 isolates were resistant to meropenem out of 63 strains from Gujarat in 2019. As the carbapenem-resistance phenotype for *V. cholerae* O1 strains is rare and a global alarming health concern, this observation caught our attention to characterizing these strains in detail to understand the mechanism of carbapenem-resistance by phenotypic and genotypic methods.

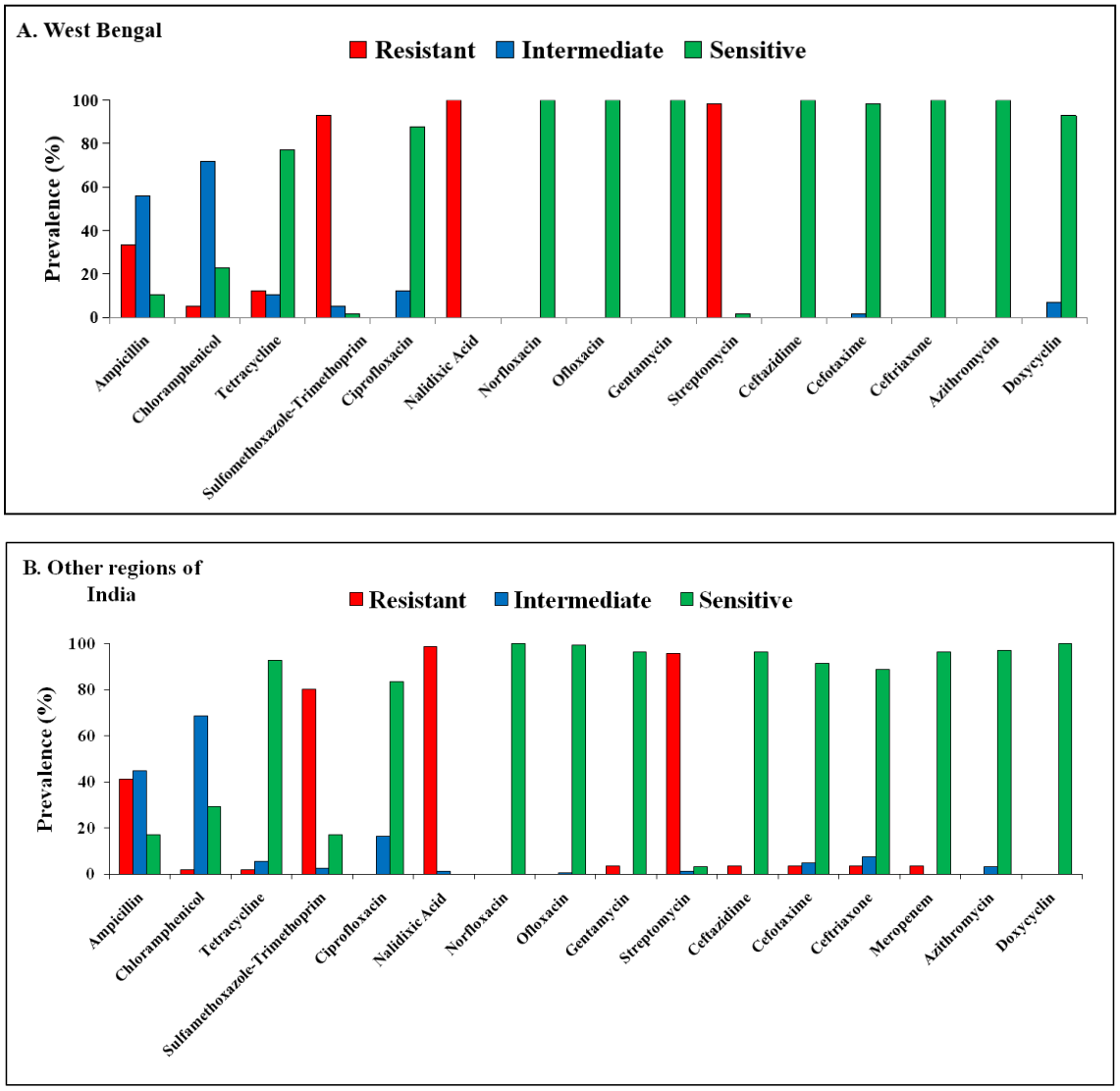

Figure 6.18. *Antibiotic susceptibility patterns of V. cholerae O1 strains from West Bengal (A) and other regions of India except West Bengal (B) in 2018-2021 by Kirby-Bauer disc diffusion method using the commercially available discs of the commonly used antibiotics.*

6.8.3. Determination of MIC phenotype of CRVC strains

Apart from the disc diffusion, the six strains were also checked with meropenem, ampicillin, ceftriaxone, and gentamicin E-strips to determine the minimal inhibitory concentration (MIC) values. All six strains were found to give MIC values of 16-4µg/mL for meropenem (Figure 6.19, Table 6.3). The concentration has further been measured by following the CLSI interpretive criteria for *Vibrio* spp, which is sensitive ≤1.0µg/ml and resistant ≥4.0µg/ml (CLSI, 2015). All six CRVC strains displayed a higher MIC value for the antibiotics ampicillin, ceftriaxone, and gentamicin (Table 6.3).

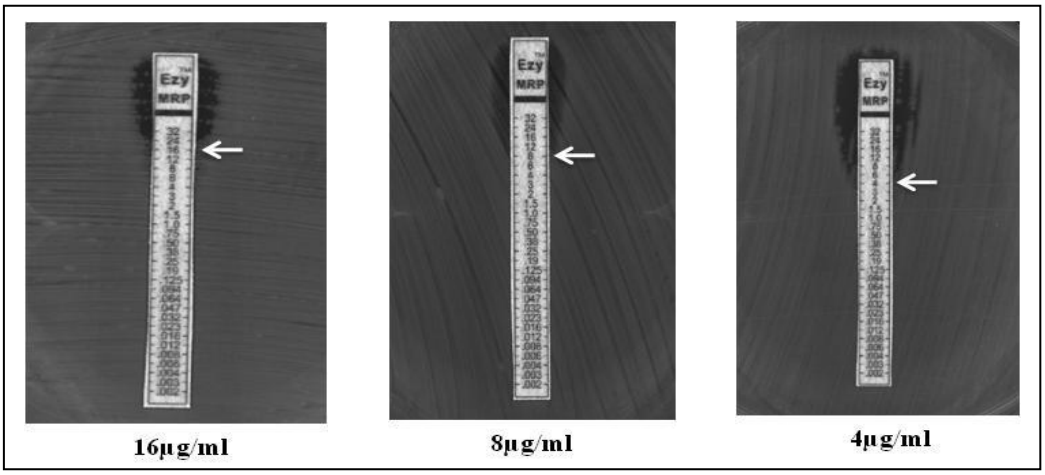

Figure 6.19. *MIC values of meropenem for the wildtype CRVC strains isolated from Gujarat in 2019 indicated by white arrows.*

Table 6.3. *Antibiotic resistance profile and respective MIC values of wildtype carbapenem resistant V. cholerae O1 strains.*

Sl. No.	Wild Type CRVC Strain Id	Resistant Profile	MIC (µg/ml)			
			MEM	AMP	CRO	GM
1	AKM-1	AMP, CTX, CRO, CAZ, GM, MEM, NA, SXT, STR	16	>256	>256	>256
2	AKM-2		4	>256	>256	>256
3	AKM-3		8	>256	>256	>256
4	AKM-4		16	>256	>256	>256
5	AKM-5		16	>256	>256	>256
6	AKM-6		4	>256	>256	>256

6.8.4. Presence of *bla*$_{NDM-1}$ among the CRVC strains

Resistance against this group of antibiotics is associated with the production of the enzyme β-lactamase able to bind and hydrolyze the β-lactam ring. However, carbapenemase-producing *V. cholerae* are mainly associated with *bla*$_{NDM-1}$ emergence and successful plasmid dissemination. Genotypic screening has been done for the *bla*$_{NDM-1}$ gene among the six isolates and all strains were found to be positive (Figure 6.20). The dissemination of mobile carbapenemases among bacterial pathogens is of great concern, not only because these

enzymes confer resistance to carbapenems and other β-lactam group of antibiotics, but also shows resistance against other group of antibiotics (Chowdhury et al., 2016), leaving very few medications options available. Pathogenic bacteria can transfer the carbapenemase gene, *bla*$_{NDM-1}$ to other bacteria and thus multidrug-resistant or extreme drug-resistant phenotypes results.

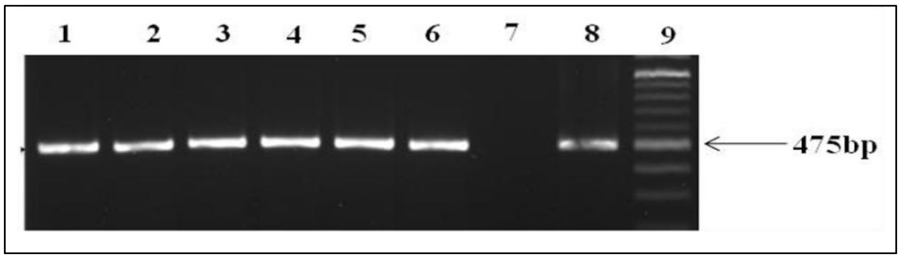

Figure 6.20. *A PCR based detection of bla*$_{NDM-1}$ *gene among the carbapenem resistant V. cholerae isolates. Lanes 1-6, carbapenem resistant V. cholerae strains; Lanes 7 and 8, negative and positive controls respectively; Lane 9, the 100bp size ladder (New England Biolab Inc., Beverly, MA, USA).*

6.8.5. Detection of a plasmid carrying *bla*$_{NDM1}$ gene and transfer of resistance in different enteric pathogens

Studies showed that the *bla*$_{NDM1}$ gene is associated with plasmids with different incompatibility groups. Thus, we have performed conjugation experiments to determine the presence of plasmids. For this study, one of the wild-type *bla*$_{NDM1}$ positive *V. cholerae* O1 strains has been taken as a donor. On the other hand, *Escherichia coli J53*, and other enteric pathogens such as *Shigella flexneri, Salmonella enterica, Vibrio parahaemolyticus* and *V. cholerae* O1 were used as recipient strains. The growth of transconjugants on selective media containing ceftriaxone antibiotic indicates positive results, which were further confirmed by plasmid extraction from donor, recipients, and transconjugants by Kado & Liu method & analyzed by 0.8% gel electrophoresis. In addition, PCR assay was used to detect the presence of *bla*$_{NDM1}$ along with the determination of the antibiogram profile by disc diffusion assay and MIC. Plasmid profiles of donor and transconjugants showed a single band at similar positions, indicating the transfer of the plasmid to all enteric pathogens taken as recipients for this study (Figure 6.21). MIC of meropenem of the enteric pathogens used as recipients has been reduced after acquiring the plasmid compared to their respective wild type (Figure 6.22). For meropenem, the MIC value of transconjugants was 16-3 µg/ml, but higher MIC values were observed for other antibiotics such as, ampicillin, ceftriaxone, and gentamicin (Table 6.4). Moreover, PCR results showed that all the transconjugants contained the gene *bla*$_{NDM1}$. In addition, the antibiogram profile obtained from the Kirby Bauer disc diffusion

assay of all transconjugants showed resistance phenotypes against the following antibiotics, ampicillin, ceftriaxone, cefotaxime, ceftazidime, gentamicin, and meropenem indicates that all the transconjugates may contain similar plasmid with uniform resistance genes through successful conjugation with different enteric pathogens (Table 6.4).

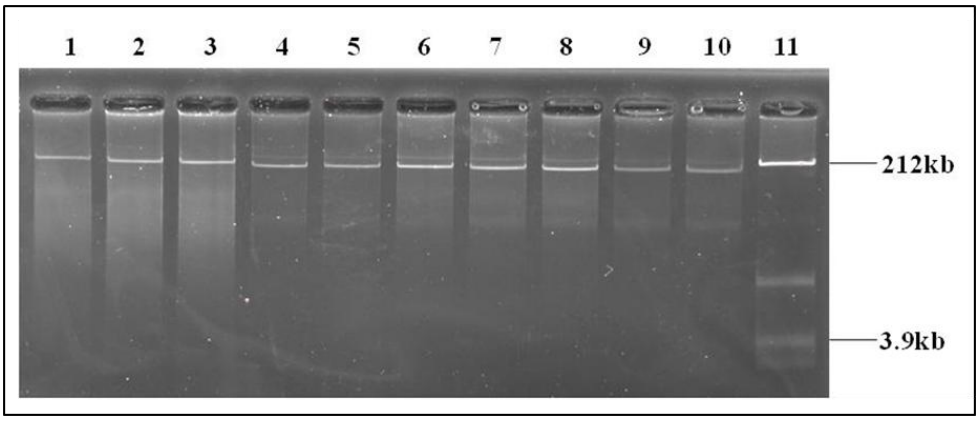

Figure 6.21. *Plasmid profile of donor and transconjugants. Lanes 1-5, plasmid from donor strains; lanes 6-10, plasmid from transconjugants; lanes 11, Shigella flexneri YSH 6000.*

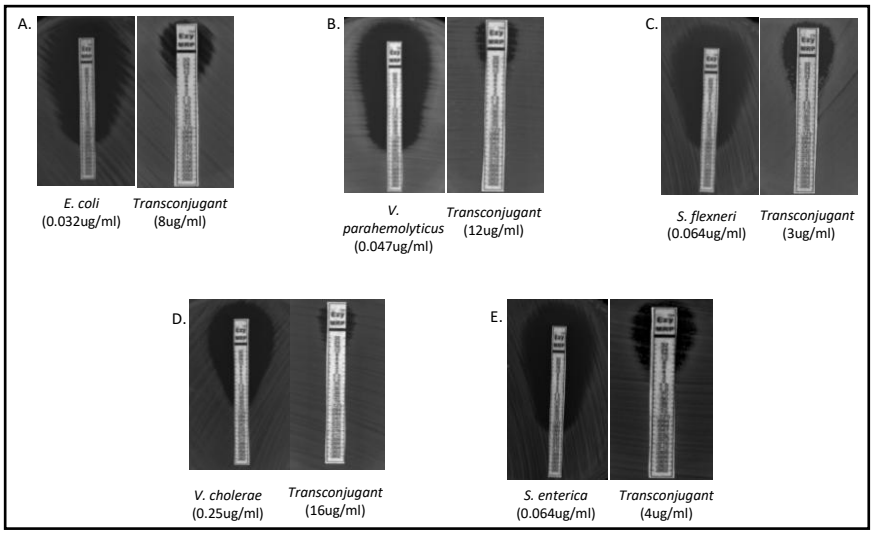

Figure 6.22. *MIC of meropenem of wild type recipients and their respective transconjugants.*

Table 6.4. *Antibiotic resistance profiles of recipients and their transconjugants*

Strain Id	Organism	Resistant Profile	MIC (µg/ml)				*Frequency of Transfer
			MEM	AMP	CRO	GM	
J53	*E. coli* (Recipient)	-	0.032	0.125	0.075	0.19	-
TC-J53	*E. coli* (transconjugant)	AMP, CRO, MEM, GM	8	>256	>256	>256	$2.9x10^{-2}$
K12929	*V. parahaemolyticus* (Recipient)	AMP	0.047	24	0.50	0.125	-
TC-K12929	*V. parahaemolyticus* (Transconjugant)	AMP, CRO, MEM, GM	12	>256	>256	>256	$1.22x10^{-3}$
IDH 6498	*Shigella flexneri 4* (Recipient)	AMP, SXT, STR	0.064	>32	0.38	0.125	-
TC-6498	*Shigella flexneri 4* (Transconjugant)	AMP, SXT, STR, CRO, MEM, GM	3	>256	>256	>256	$2.35x10^{-4}$
IDH 3291	*V. cholerae* O1 (Recipient)	AMP, SXT, STR, PolB	0.25	4	0.47	0.38	-
TC-3291	*V. cholerae* O1 (Transconjugant)	AMP, SXT, STR, PolB, CRO, MEM, GM	16	>256	>256	>256	$1.4x10^{-5}$
BCH 0645	*Salmonella enterica* (Recipient)	AMP, C, CIP, NOR, OFX, NA, AZM	0.064	>256	0.25	0.25	-
TC-0645	*Salmonella enterica* (Transconjugant)	AMP, C, CIP, NOR, OFX, NA, AZM, CRO, MEM, GM	4	>256	>256	>256	$1.42x10^{-6}$

Notes: *Frequency of transfer was calculated as number of transconjugants per donor cell.

6.8.6. Conjugation efficiency determination

The transfer frequency has been calculated and it ranged from $1.42*10^{-6}$ to $2.9*10^{-2}$ (Table 6.4). The highest frequency has been observed in *E. coli* strain J53 ($2.9x10^{-2}$) followed by *V. parahaemolyticus, S. flexneri, V. cholerae* O1, and *S. enterica.*

6.8.7. PFGE shows a similar clonality pattern among the CRVC strains

PFGE analysis was conducted for six carbapenem-resistant wild type isolates of *V. cholerae* O1 isolated from Gujarat in 2019 and all of them (AKM-1 to AKM-6) showed banding patterns with ~98% similarity, indicating that they are clonally similar to each other (Figure 6.23). In addition to six CRVC strains, three non-NDM strains (BR-26, BR-32, and BR-34) isolated from the same region during 2019 have also been included to determine the genetic relatedness among them. The analysis displayed an overall 89% similarity between NDM positive and negative strains.

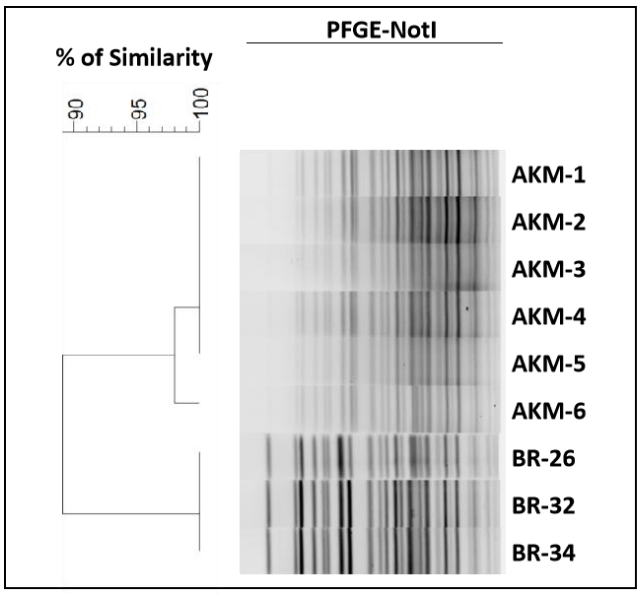

Figure 6.23. *Pulse-field gel electrophoresis patterns of NotI-digested genomic DNA of* bla_{NDM-1} *harbouring Vibrio cholerae O1 stains (AKM-1 to AKM-6) isolated from Gujarat 2019. The dendrogram was generated using the software BioNumerics version 4.0 (Applied Maths) based on the single linkage method. Percentage similarity is shown at the left-hand side.*

6.8.8. Genome analysis and AMR profile

A total of 8 whole genome sequencing has been carried out by using Illumina NovaSeq 6000. Among them, six isolates were CRVC strains carrying plasmid and another two were *V. cholerae* recipient (IDH 3291) and transconjugant (TC-3291). Oxford Nanopore Technologies was performed for two CRVC strains (AKM-3 and AKM-4), the complete genome sequence of AKM-3-2 and AKM-4-2 was obtained by combining both the short and long reads. The genomes were of ~ 4Mb, with a GC content of 47.6%, which was similar to the genome sequences of the other *V. cholerae* isolates. WGS analysis found that all the CRVC strains were identified as belonging to ST-69.

Table 6.5. *Whole genome sequencing statistics of V. cholerae O1 strains.*

Features	**AKM-1#**	**AKM-2#**	**AKM-3#**	**AKM-4#**	**AKM-5#**	**AKM-6#**	**IDH 3291#**	**TC-3291#**	**AKM-3-2***	**AKM-4-2***
Sequence size	4091857	4158710	4167372	4161688	4166479	4164351	4025389	4162212	4244181	4244181
Number of contigs	110	94	118	94	94	97	92	107	3	3
GC content (%)	47.7	47.7	47.6	47.6	47.6	47.6	47.5	47.6	47.6	47.6
Shortest contig size	104	104	102	101	104	104	104	105	142367	142367
Median sequence size	1418	1269	1174	1135	1134	1325	1188	1197	1048983	1048983
Mean sequence size	37198.7	44241.6	35316.7	44273.3	44324.2	42931.5	43754.2	38899.2	1414727.0	1414727.0
Longest contig size	555061	663448	560108	564993	594918	594998	652682	405575	3052831	3052831
N50 value	197719	246154	197656	197719	197719	187476	235586	227635	3052831	3052831
L50 value	7	5	8	6	6	7	5	8	1	1

*#Draft genome, *Complete genome.*

WGS analysis revealed the presence of multiple AMR genes in the studied isolates. This includes resistance to beta-lactams such as AmpC-β-lactamases (*bla*$_{\text{CMY-6}}$, *bla*$_{\text{DHA-7}}$), carbapenemases-metallo-β-lactamases (*bla*$_{\text{NDM-1}}$); aminoglycosides such as aminoglycosides modifying enzymes (*aac(3)-IId*, *aph(3')-VI*, *aac(6')-Ib10*, *aph(3")-Ib*, *aph(6)-Id*, *strA*, *strB)*, *16S rRNA methyl transferases (rmtC);* sulfonamide (*sul2*); trimethoprim (*dfrA1*); chloramphenicol (*florR*, *catB9*); and bleomycin (*ble*$_{\text{MBL}}$) (Table 6.5). Correlation between the phenotypic and genotypic resistant pattern confirms the presence of respective genes for the phenotypic resistance shown in antibiotic susceptibility tests. Analysis of the genetic arrangement revealed the linkage of ARGs with multiple mobile genetic elements (MGEs), including plasmids, insertion sequences, transposons and mobile resistance cassettes linked with *IntI*.

Table 6.6. *Correlation between phenotypic and genetic resistance profiles of CRVC strains.*

Phenotypic Resistant Profile	Antibiotic Class	Resistant Gene Profile
AMP, CTX, CAZ, CRO, MEM	Beta-lactam	*bla*$_{CMY-6}$, *bla*$_{NDM-1}$, *bla*$_{DHA-7}$
STR, GM	Aminoglycosides	*strA, strB, rmtC, aph(3')-VI, aph(3")-Ib, aph(6)-Id, aac(6')-Ib10, aac(3)-IId*
NA	Fluoroquinolones	*gyrA, parC, parE*
SX	Sulfonamide	*sul1, sul2*
TM	Trimethoprim	*dfrA1*
Reduced susceptibility towards CHL	Phenicols	*catB9, floR*

6.8.9. Sequence analysis of *bla*$_{NDM-1}$ plasmid

The assembled genome showed a single plasmid which we refer to as pNDM-VC, consisting of ~142 kb with an average GC content of 51.4% with 174 predicted coding sequences (CDSs). The plasmid belongs to sequence type 1 (ST-1) of IncA/C plasmid group with typical IncA/C replicon and putative genes such as *parA*, *parB*, *repA*, and *A053*. The IncA/C encodes for multiple acquired AMR genes against several antimicrobial classes such as *bla*$_{NDM-1}$, *bla*$_{CMY-6}$, *bla*$_{DHA-7}$, *aac(3)-IId*, *aph(3')-VI*, *aac(6')-Ib10*, *aph(3")-Ib*, *aph(6)-Id*, *rmtC*, *sul1* and *ble*$_{MBL}$ genes (Figure 6.24). The plasmid sequence obtained from WGS had high similarity to the *E. coli* (KC999035) and *V. cholerae* (CP007636) plasmids and thus these plasmids were used as a reference to reconstruct the circular pNDM-VC plasmid. Both the plasmid of *E. coli* and *V. cholerae* shows 99% identity with 98% query coverage and 99% identity with 78% query coverage, respectively. The presence of conjugal transfer genes and integrons flanking the resistance genes suggests the likely acquisition from other species of Enterobacteriaceae. The resistance genes are located in two regions in which the first region contains *sul1*, *aac(3)-IId*, *aph(3')-VI*, *aac(6')-Ib10*, *aph(3")-Ib*, *aph(6)-Id*, *rmtC*, *ble*$_{MBL}$, *bla*$_{NDM-1}$, *qacE*. These antimicrobial resistance (AMR) genes are situated between the integrons (3' CS of class I integron) and the IS26 sequence along with the conjugal transfer genes *traGH* that are present in upstream of this region and play an important role in the dissemination of plasmid encoding AMR genes. The second region, including *bla*$_{CMY-6}$ was

flanked by *tnpA* transposase, and the T4SS cluster which contains the conjugal transfer (*tra*) genes.

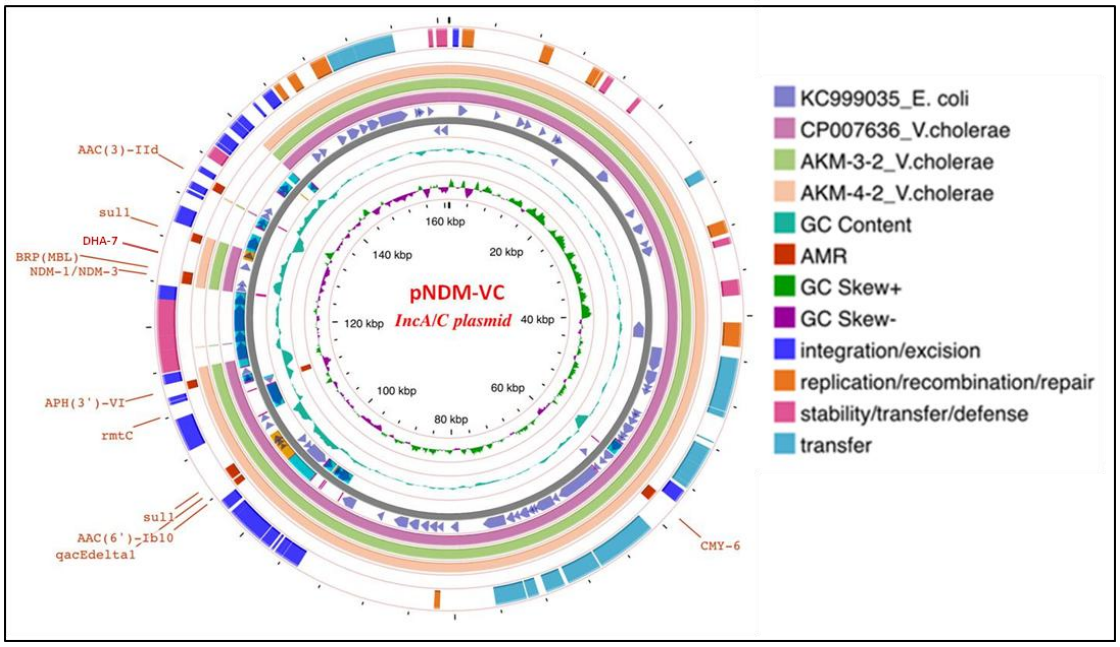

Figure 6.24. *Salient features of bla$_{NDM-1}$ encoding plasmid pNDM-VC. Starting from inside the first circle indicates the GC content and the second circle represents the GC skew (dark green, GC +, purple, GC -), the third circle represents the arrangement of contigs. Fourth and fifth circles indicate the reference genomes of E. coli (KC999035) and V. cholerae (CP007636) plasmids respectively. The sixth and seventh circles represent the complete plasmids with CDS regions being the outermost circle. The AMR genes are denoted by red colour, and transfer apparatus using blue colour.*

6.8.10. NDM plasmid carrying NotI restriction sites

Another PFGE was performed to further confirm the transfer of the plasmid in the *V. cholerae* recipient strain, and we found that the NDM negative recipient and its transconjugant only differed in a single band with ~132 kb size, which is present in the donor strain. This observation indicates the presence of NotI restriction sites in the plasmid, as we have obtained a 142 kbp plasmid from WGS. By using BLASTn software to compare the plasmid sequence with the NotI restriction sequence, we have found three NotI sites in the plasmid at the positions of 105952, 109225, and 115848th nucleotides, respectively. Results of this digestion produced fragment sizes of 3273, 6623, and 132470 bp (Figure 6.25B). Among them, the largest fragment produced by NotI digestion is the 132 kbp indicated by a red box in the Figure 6.25A, which was present in both donor and transconjugant but not in the recipient strain. Whereas, the other two fragments are unable to be visualized due to their small sizes. However, both the donor and the NDM-negative *V. cholerae* O1 recipient (IDH 3291) strains showed ~97% similarity before conjugation but this pattern has been shifted to

98% similarity after the transfer of the plasmid to the recipient strain indicating a successful transfer of the complete 142kb plasmid (Figure 6.26).

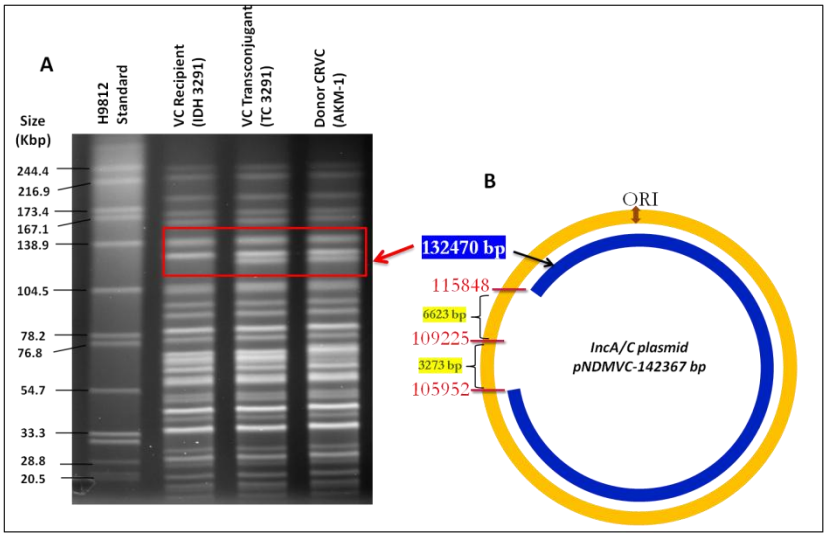

Figure 6.25. *(A) Pulse-field gel electrophoresis pattern of NotI-digested genomic DNA of V. cholerae O1 strains, From left to right, lane 1 represents the Salmonella serotype Braenderup (H9812) as control strain digested by XbaI; 2-4 represents* bla_{NDM-1} *negative recipient (IDH 3291), transconjugant (TC 3291) and* bla_{NDM-1} *harbouring donor (AKM-1) strains respectively; (B) Schematic representation of the NotI restriction sites in the plasmid, Yellow colour indicates the circular 142kb plasmid, Blue colour semi-circle indicates the largest fragment with ~132kb size produced by the digestion of Not I.*
ORI, origin of replication.

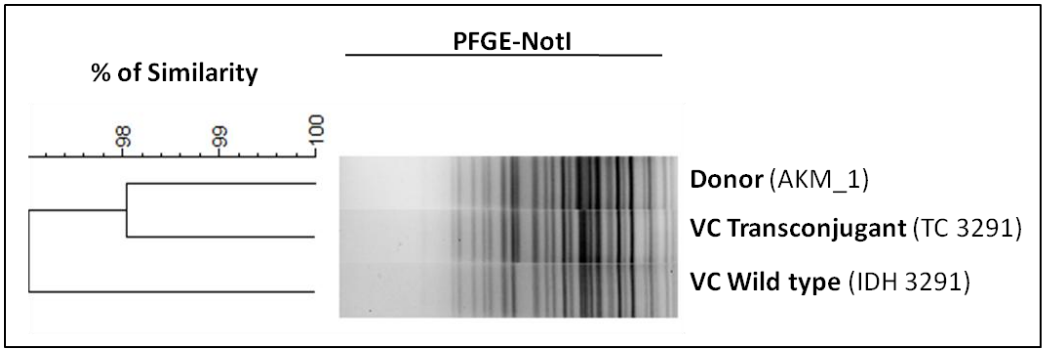

Figure 6.26. *Dendrogram analysis of* bla_{NDM-1} *harboring donor, transconjugant and its wild type NDM - negative strains using BioNumerics version 4.0 software (Applied Maths) based on the single-linkage method to generate the dendrogram. Percent similarity is shown on the upper left side.*

Objective III

To understand the inflammatory response of El Tor strain and recently emerged polymyxin B-sensitive El Tor variant on intestinal epithelial cell line.

6.9. Importance to study the role of polymyxin B-sensitive phenotype in the pathogenesis of cholera

Polymyxin B is known to be a major marker to distinguish classical strains from El Tor. But the emergence of polymyxin B-sensitive Haitian variant strains has changed this phenomenon and it disseminated all over the world to cause catastrophic outbreaks like recently in Yemen, Algeria (Weill et al., 2018; Benamrouche et al., 2022). Our earlier reports stated that, the newly emerged sensitive strains had a single mismatched base at the 265th position of the *carR* gene and the difference was also reflected in their amino acid sequences when the nucleotide sequences were translated. Thus, the *carR* gene is responsible for the different polymyxin-B phenotypes.

Sequencing analysis of *carR* gene revealed that the sensitive strains contained a single point mutation at the 265th nucleotide position (G265A) compared to the polymyxin B-resistant El Tor reference strain N16961. It has also been noted that the 265th nucleotide position of the classical biotype strains was conserved for the base G as found in the El Tor polymyxin B resistant strain N16961. Thus, to confirm the role of G-to-A change in polymyxin B susceptibility, the A residue of a polymyxin B sensitive clinical isolate of Kolkata, West Bengal IDH 7230 was replaced with 'G' to construct the isogenic resistant mutant strain IDH 7230MT. In addition to this, we also made a G265A substitution in *carR* of the polymyxin B resistant El Tor standard strain N16961 and constructed its isogenic mutant N16961MT with the polymyxin B- sensitive trait (Samanta et al., 2020). This result indicated that G265A point mutation in *carR* was responsible for the phenotypic shift of currently circulating polymyxin B-sensitive *V. cholerae* O1 El Tor strains (Samanta et al., 2020). However, there is no such report, yet which stated the role of the sensitive allele in the pathogenesis of cholera. Thus, we have been interested to study the various roles of the polymyxin B sensitive allele in comparison to the resistant allele in terms of pathogenicity.

However, very little is understood about how the host and bacteria interact (Bandyopadhaya et al., 2007). The primary site of entry for enteric infections is epithelial cells, which may send out early cues for the acute mucosal inflammatory response by releasing proinflammatory cytokines and inflammatory mediators. When pathogens enter the colon, a complicated interaction between nonspecific inflammatory mechanisms and adaptive immune activities takes place. The disease cholera was regarded as the typical non-inflammatory disease. However, lactoferrin is a significant indicator of intestinal inflammation when it is observed in stool samples. Additionally, acute *V. cholerae* O1

infection triggers mucosal innate immune responses that include neutrophil infiltration, mast cell and eosinophil degranulation, and the production of proinflammatory cytokines (Qadri et al., 2004).

The pathogenesis of cholera is multifactorial at the molecular level and involves a number of virulence genes that promote the recruitment of diverse immune cells to the mucosal surface and the development of the disease by upregulating the cytokines that cause an inflammatory response (Chatterjee et al., 2013). According to studies, INT407 cells were selected as a well-recognized model for the host-*V. cholerae* interaction primarily for research on the inflammatory response. Epithelial cells are considered to represent an integral component of the mucosal immune system, as they provide the underlying mucosa with the first signals of infection. Thus, to understand the pathogenesis pattern induced by polymyxin B sensitive strains in comparison with resistant strains, a study of mRNA expression of inflammatory cytokine has been performed against the mammalian intestinal epithelial cell line INT407.

The evidence from studies clearly indicates that both adherence and motility of *V. cholerae* to intestinal epithelial cells may induce the IL-8 mRNA expression. Thus, for better understanding, we have also performed motility and adherence assays with the same strains (Table 5.4).

6.9.1. Sensitive allele containing strains contributes to more Il-8 mRNA expression

Infected cells were analyzed for the interleukin 8 (IL-8) inductions with the help of quantitative RT PCR and normalizing with glyceraldehydes 3-phosphate dehydrogenase (GAPDH) taken as housekeeping control with the primer sets IL-8_RT_F/R2 and GAPDH Fp/Rp (Table 6.1). The IL-8 mRNA expression was found to be significantly higher ($p=<0.05$) in the strains containing *carR*-sensitive allele (IDH 7230 and N16961MT) compared to the strains containing resistant alleles (IDH 7230MT and N16961). Similar study has also been carried out with the Haitian variant clinical strains with naturally containing *carR*-sensitive allele isolated from West Bengal (IDH 12699, IDH 12695, and IDH 12460) in 2019 and resistant allele (IDH 3672, IDH 3291, and IDH 3439) during 2010-2011. This result also exhibited that polymyxin B-sensitive strains have the ability to induce more IL-8 expression compared to the polymyxin B-resistant strains (Figure 6.27).

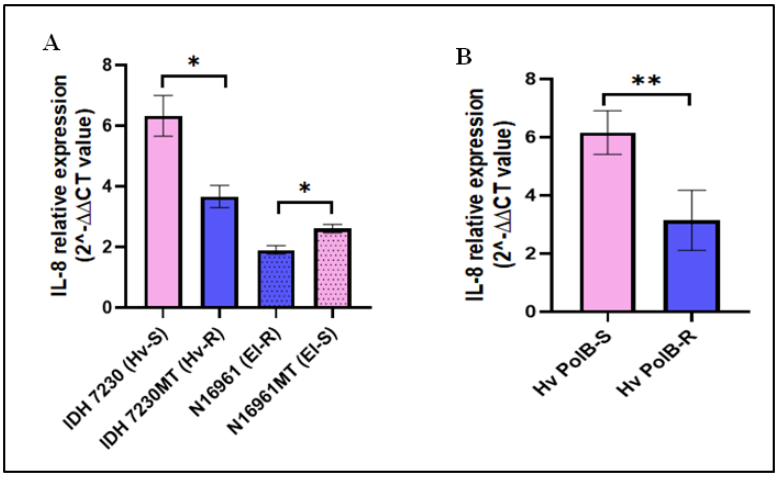

Figure 6.27. *Graphical representation of IL-8 mRNA expression induced by (A) different carR alleles containing strains and their isogenic mutants, (B) group of clinical isolates with naturally occurring different polB phenotypes.*
Asterisk indicates statistically significant p-value <0.05; Hv, Haitian variant; S, sensitive; El, El Tor; R, resistant; PolB, polymyxin B.

6.9.2. Sensitive strains found to be adhered more compared to resistant strains to intestinal epithelial cell

Adherence to intestinal epithelial cells is essential for colonization of *V. cholerae* in the intestinal context. Thus, adherence assay has been studied to know whether the polymyxin B-sensitive strains have the ability to adhere more to human epithelial intestinal cell line and help to induce higher IL-8 mRNA expression compared to the resistant strains. In this experiment, we have taken strains with *carR*-sensitive (IDH 7230) and *carR*-resistant (N16961) alleles with their isogenic mutants (IDH 7230MT and N16961MT) respectively to know the role of the $carR^S$ allele in adherence study. In addition, we have also taken six clinical Haitian variant strains with polymyxin B-sensitive (IDH 12699, IDH 12695, and IDH 12460) and resistant (IDH 3672, IDH 3291, and IDH 3439) phenotype for better understanding. As a result, both the sensitive allele containing strains (IDH 7230 and N16961MT) were found to be adhere more (p= <0.05) with the INT 407 compared to their isogenic counterparts (IDH 7230MT and N16961) (Figure 6.28A). However, a little change has been observed between the groups of clinical isolates with naturally occurring *carR* alleles (Figure 6.28B).

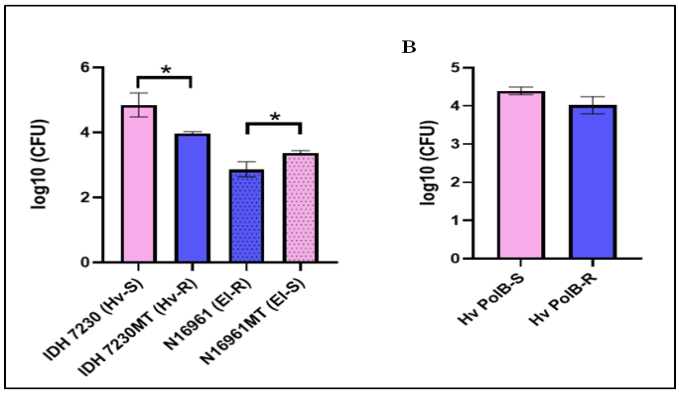

Figure 6.28. *Graphical representation of adherence assay demonstrated by (A) different carR alleles containing strains and their isogenic mutants, (B) group of clinical isolates with naturally occurring different polB phenotypes.*
Asterisk indicates statistically significant p-value <0.05; Hv, Haitian variant; S, sensitive; El, El Tor; R, resistant; PolB, polymyxin B.

6.9.3. Sensitive strains have higher motility compared to resistant strains

As the IL-8 induction is associated with both the adherence and motility, we have performed the motility assay between the *carR*-sensitive and resistant alleles along with their isogenic mutants. In addition to this, similar study with the Haitian variant polymyxin B-resistant and sensitive clinical strains has also been performed to understand the role of *carR*-sensitive allele in motility. All the strains with *carR*-sensitive allele showed higher motility on 0.3% agar plate compared to the strains with *carR*-resistant allele irrespective of the genetic background (Figure 6.29). It is evident from the result that *carR* contributes significantly to motility when tested in vitro and $CarR^S$ promotes motility which might further play a role in the induction of IL-8.

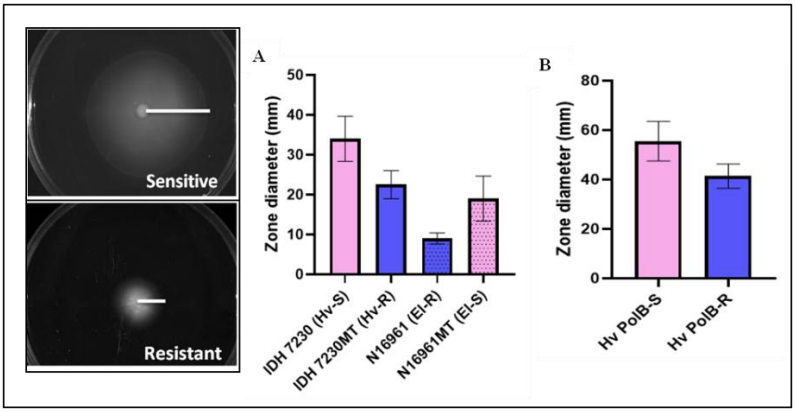

Figure 6.29. *Motility assay of (A) different carR alleles containing strains and their isogenic mutants, (B) group of clinical isolates with naturally occurring different polB phenotypes.*
Hv, Haitian variant; S, sensitive; El, El Tor; R, resistant; PolB, polymyxin B.

6.9.4. Varying expression of flagellar genes *flhDC* in different *carR* genetic background

To understand the difference in motility, we did comparative sequence analysis of various flagellar genes between the El Tor (N16961) and Haitian (EL-1786) strains by using BLASTn software and found that two genes *flhD* and *flhC* are absent in El Tor strains but present in Haitian strains. Several studies suggested that motility is linked to the virulence of *V. cholerae* (Correa et al., 2005), but it has been difficult to evaluate the role of flagellar genes in the pathogenesis of cholera. Our earlier reports have also indicated that the Haitian variant strains manifest higher virulence compared to the El Tor strains (Ghosh et al., 2019). Thus, we have studied the expression of *flhDC* in both natural isolates containing $carR^S$ and $carR^R$ alleles and their isogenic mutants. Higher mRNA expression of *flhDC* has been observed in the strains harboring $carR^S$ allele such as IDH 7230 and other sensitive isolates (IDH 12695, IDH 12699, and IDH 12460) compared to the strains with $carR^R$ allele (IDH 7230MT, IDH 3291, IDH 3672, and IDH 3439) (Figure 6.30). This result indicates the possible role of $carR^S$ allele in motility by inducing the master regulator *flhDC*.

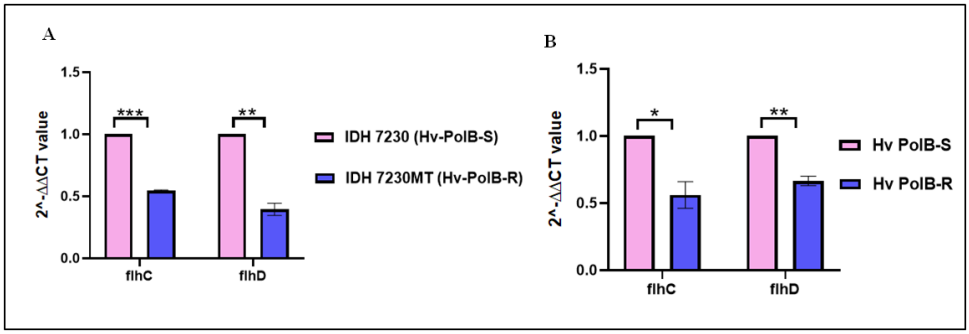

Figure 6.30. *Relative mRNA expression of flagellar genes flhDC in (A) different carR alleles containing strains and their isogenic mutants, (B) group of clinical isolates with naturally occurring different polB phenotypes.*
Asterisk indicates statistically significant p-value <0.05; Hv, Haitian variant; S, sensitive; El, El Tor; R, resistant; PolB, polymyxin B.

Objective IV

To compare the pathogenicity like colonization ability and fluid accumulation in animal models when infected with polymyxin B-resistant El Tor and polymyxin B sensitive El Tor variant strains.

6.10. Significance of Polymyxin B-sensitive Haitian variant strains in higher virulence

It has been noted from the previous studies that the *V. cholerae* O1 strains have emerged as a more pathogenic form causing large outbreaks. Since the beginning of the first pandemic, the organism is emerging with new genetic features that have made them more virulent. Our earlier studies showed that how polymyxin B-resistant Haitian variant strains linked with higher virulence (Ghosh et al., 2019). *V. cholerae* is continuously evolving to get better advantage and stability in nature. The emergence of Haitian variant strains with polymyxin B-sensitive phenotype has been first observed in 2012 in Kolkata, West Bengal and the gene, which is responsible for this changing phenotype is *carR* as mentioned in 6.9 section. Therefore, the difference in IL-8 expression between polymyxin B-sensitive and resistant strains has driven us to study the role of $carR^S$ allele in vivo system.

For in vivo experiments, we have taken $carR^S$ and $carR^R$ allele containing El Tor (N16961) and Haitian variant (IDH 7230) strains and their respective isogenic mutants. In addition, we have also taken Haitian variant clinical isolates with different polymyxin B phenotypes for the better understanding (Table 5.4).

6.10.1. No difference in fluid accumulation between polymyxin B-resistant and sensitive strains

The rabbit ileal loop assay has been performed with $carR^S$ (IDH 7230, N16961MT, IDH 12695, IDH 12699, and IDH 12460) and $carR^R$ (IDH 7230MT, N16961, IDH 3291, IDH 3672, and IDH 3439) alleles containing strains. As a result, no significant difference has been obtained in fluid accumulation between polymyxin B-sensitive and resistant strains (Figure 6.31). But, it is clear from the result that recently circulating Haitian variant polymyxin B-sensitive strain and its isogenic polymyxin B-resistant mutant showed higher fluid accumulation than those of the El Tor polymyxin B-resistant strain N16961 and its isogenic polymyxin B-sensitive mutant (N16961MT) (Figure 6.31). Therefore, this result suggests that CarR plays a role in regulating pathogenicity of *V. cholerae* O1 in a strain-specific manner when infected with the rabbit intestine.

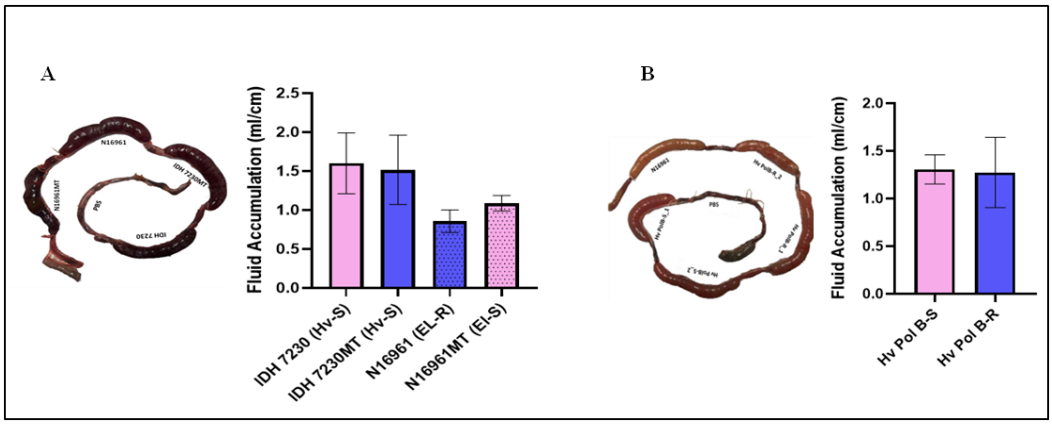

Figure 6.31. *Fluid accumulation (FA) ratio in rabbit ileum infected with (A) different carR alleles containing strains and their isogenic mutants, (B) group of clinical isolates with naturally occurring different polB phenotypes.*
Hv, Haitian variant; S, sensitive; El, El Tor; R, resistant; PolB, polymyxin B.

6.10.2. Similar colonization potential shown by the strains with polymyxin B-resistant and sensitive phenotype in infant mice

Pathogenicity mainly depends on colonization, which is required for disease manifestation. Thus, in vivo colonization in infant mice model was performed to predict the colonization ability of polymyxin B-sensitive and resistant strains. No significant difference has been found between different *carR* alleles (Figure 6.32). However, like rabbit illeal loop assay, the recently circulating Haitian variant strains are more efficient in colonization in vivo compared to the El Tor strains.

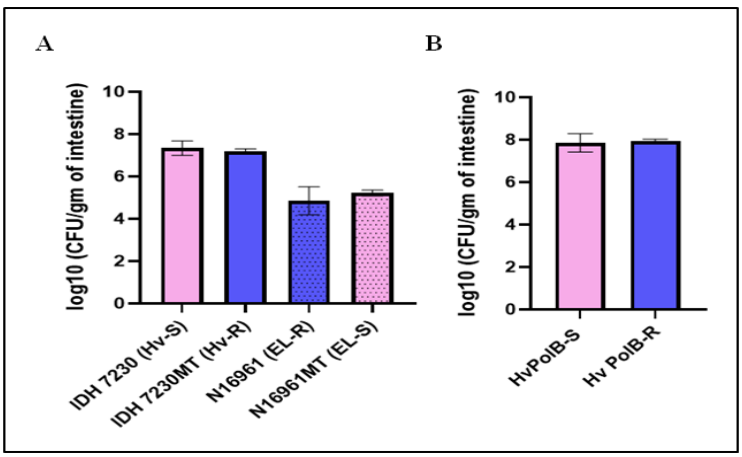

Figure 6.32. *Colonization potential in suckling mice model infected by (A) different carR alleles containing strains and their isogenic mutants, (B) group of clinical isolates with naturally occurring different polB phenotypes.*
Hv, Haitian variant; S, sensitive; El, El Tor; R, resistant; PolB, polymyxin B.

6.10.3. Histology of rabbit intestinal tissue challenged with polymyxin B-sensitive and resistant strains

Histopathological analysis was done with the rabbit tissue challenged with Haitian variant polymyxin B-sensitive and resistant strains. Both the strains showed acute inflammatory cell infiltrates within mucosa and sub-mucosa (Figure 6.33). Sections treated with sensitive strains showed a complete loss of surface and glandular epithelium whereas, the loss of goblet cells and crypts has been found in the section treated with the resistant strains. Therefore, no such significant difference has been found in case of in vivo system which again indicates that *carR*^{S} might play similar role with the *carR*^{R} allele containing strains in terms of pathogenicity.

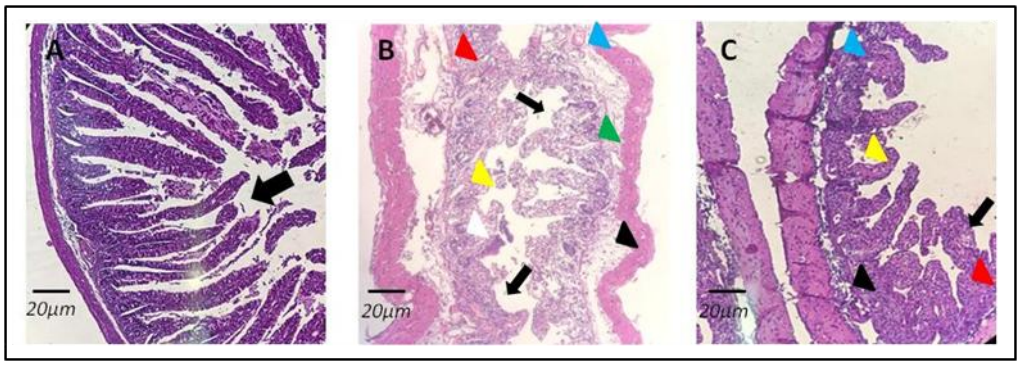

Figure 6.33. *Haematoxylin and Eosin stained sections of rabbit intestine challenged with V. cholerae O1 polymyxin B-sensitive and resistant strains. (A) Control loop showing intact villi with simple columnar epithelium, intact deeper crypts, submucosa, and muscle layers. (B) Loop infected by resistant strain showing moderate damage; inflammatory cell infiltrates within mucosa and sub-mucosa accompanied by crypt abscesses and loss of goblet cells and crypts. Surface epithelium, deeper glands, and muscle layers are damaged; focal ulceration and hemorrhage. (C) Sensitive strain infected loop indicates transmural necrosis. There is complete loss of surface and glandular epithelium with collapse of mucosa and severe acute inflammatory cell infiltration accompanied by areas of congestion accompanied by crypt abscesses. Original magnification ×40; scale bars 20 µm; arrows-inflammatory cell infiltrates within mucosa (solid) and submucosa (arrowheads); crypt abscess (blue arrowhead), goblet cell loss (green arrowhead); crypt loss (yellow arrowhead); hemorrhage (red arrowhead); focal ulceration (white arrowheads).*

Chapter 7

Discussions

7. Discussions

The cholera disease has used to be quite rampant in India and even now, there have been fluctuating frequencies of the disease in different parts of the country in the past few years. The battle against cholera is still ongoing after seven pandemics and widespread outbreaks around the world. Every year, it affects almost 4 million people and 21,000 deaths worldwide (Ganesan et al., 2020). As a result, from 2017 to 2019, there was an increase in the number of cholera cases, reaching 1.2 million cases in 2017 and 0.9 million in 2019 (Cholera, 2019, Weekly Epidemiological Record, World Health Organization. 2020). Outbreaks of cholera are often reported in South Asia and many African countries (Griffith et al., 2006). Since the worst cholera outbreak occurred in Yemen, Asia has experienced a sharp increase in the number of cholera cases worldwide. Yemen accounted for 84% and 41%, respectively, of all cases and deaths linked to cholera in 2017, with 5654 fatalities reported to the WHO from a total of 1.2 million cases worldwide (Deen et al., 2020). However, the appearance of SARS-CoV-2 (COVID-19) pandemic in 2020 has significantly decreased the cholera cases globally due to the maintaining proper sanitation and hygiene (WHO, 2021). Again, in 2021, both the number of cholera cases and their geographic dispersion have risen, and this trend has been continuing till 2022 with cholera cases being reported from over 29 countries. Cholera has long been an endemic disease in most of Asia, but it has only lately spread to Africa and Haiti. Since its resurgence in 2010, cholera outbreaks have been observed in Haiti for the first time in more than a century. The higher cases of cholera have been reported mainly from Asia and Africa due to improper hygiene and sanitation which results in the poor quality of the drinking water those have been contaminated with fecal wastes. Thus, World Health Organization (WHO) employed the global task force on cholera control (GTFCC) in 2017 which targets mostly the improvement of water, sanitation, and hygiene (WaSH) in the 47 cholera endemic countries in Asia and Africa including India to reduce the number of cases by 90% and to end cholera by 2030.

The causative agent of cholera, *Vibrio cholerae* frequently modifies its genetic structure, which may enable the bacterium to become more pathogenic and spread to new cholera endemic areas (Lekshmi et al., 2018). The epidemiology and the biotypes of *V. cholerae* are closely related. Due to aspects like exchanging, gaining, and losing, they maintain immaculate circulatory motion in the environment. The first six of the seven recognized cholera pandemics are believed to have been caused by the classical biotype of *V.*

cholerae O1, while the current seventh pandemic is thought to be caused by the El Tor biotype. With each pandemic, the frequency, length, and intensity increased, and the current pandemic is the longest and shows no signs of abating. The main difference between biotypes lies upon certain biochemical tests such as polymyxin B susceptibility, chicken blood cell agglutination, sheep blood hemolysis, Voges Prokauer test and phage susceptibility assay.

V. cholerae historically persisted effectively through successive changes with classical, El Tor and O139 strains and also by the El Tor biotype harbouring the classical *ctxB*. The acquisition of classical biotype markers in the El Tor biotype, which are thought to be epidemiologically significant, is one of several modifications in the *V. cholerae* O1 genomes that have been demonstrated by a number of genetic investigations (Bhandari et al., 2021). Nevertheless, cholera is an under-recognized problem in India; its endemicity in the country has been evidenced since ancient times, and the Kolkata city of the West Bengal state located in the Gangetic delta has been hailed as the "homeland of cholera," with regular outbreaks and pronounced seasonality.

In order to understand the changing genetic makeup of recently circulating *V. cholerae* O1 strains in India and their role in higher virulence, the present study was conducted to document the bacteriological and epidemiological analysis of clinical samples isolated from different cholera endemic states (West Bengal, Maharashtra, Gujarat, Delhi, Tamil Nadu, and Karnataka) in India during 2018-2021 and classified into two groups, West Bengal and other regions of India.

This study showed that *V cholerae* O1 Ogawa was the predominant etiological agent. Recent *V. cholerae* O1 strains were found to have increased their pathogenic ability with a sequential accumulation of traits towards a hypervirulent classical biotype with the El Tor biotype genetic backbone. In the late 90s, it was found that a few amino acid sequences of the cholera toxin B (CT-B) subunit of El Tor strains have been replaced by the classical type of CT-B in India and other countries. Further, in 2012, another classical biotype marker, polymyxin B susceptibility was acquired by El Tor biotype strains (Samanta et al., 2015).

All the strains in our study were found to follow El Tor characteristics by producing acetoin in VP test and showed visible agglutination with chicken blood cells. However, a different pattern has been found in West Bengal strains for polymyxin B-sensitivity and sheep blood hemolysis tests.

Generally, El Tor biotype strains which emerged in 1961 and prevailed also in the current years, have consistently been found with their typical El Tor biotype characteristics including

the polymyxin B resistance phenotype. However, during 2012, this pattern changed due to the emergence of polymyxin B-sensitive trait in El Tor variant strains and was first found in Kolkata, West Bengal, and then spread around the world (Samanta et al., 2015). Suddenly, the previously dominating polymyxin B-resistant El Tor strains of West Bengal were found to be predominated in 2015 over the sensitive strains and again reemerge in 2018 associated with both classical and El Tor genetic features (Shaw et al., 2022). This phenomenon perhaps indicates the similar environmental fitness of both types and is engaged in an inter-competitive event of survival for environmental fitness.

Polymyxin B was known to be a major marker from ancient times to distinguish the biotypes. However, due to the emergence of polymyxin B-sensitive Haitian variant strains, it is no longer a reliable marker. This phenotypic difference may result from the genetic change. Herrera et al. (2014) characterized the CarR ($CarR^R$) protein found in a polymyxin B-resistant strain and showed that the $CarR^R$ protein is phosphorylated at the D55 residue and becomes activated by CarS (Histidine kinase), i.e., the D55 residue in $CarR^R$ is an important site of phosphorylation for the activation of the protein function (Herrera et al., 2014). Earlier study from our lab revealed the factor associated with the sensitivity of polymyxin B which is the D89N (A265G) substitution of the response regulator CarR by comparing with the polymyxin B-resistant El Tor strain N16961 (Samanta et al., 2020). As a result of the point mutation, the DNA binding cleft of CarR has become constricted and it cannot bind efficiently to the promoter of AlmEFG, the proteins responsible for lipid A modification of El Tor *V. cholerae* and involved in polymyxin B resistance. The inefficient binding of CarR to the AlmEFG promoter reduces the expression of almEFG and also lipid A modification which makes the strain susceptible to polymyxin B. The sensitivity is the leading cause of the recently occurring outbreaks in different regions like in Yemen and Algeria.

As the role of the sensitive allele of *carR* has not yet been elucidated, we have focused on revealing the biological significance of the $carR^S$ allele. The epithelial cells are considered an integral component of the mucosal immune system, as they provide the underlying mucosa with the first signals of infection. *V. cholerae* O1 infection triggers mucosal innate immune responses that include neutrophil infiltration, mast cell and eosinophil degranulation, and the production of proinflammatory cytokines. (Qadri et al., 2004). Our study shows that the IL-8 mRNA expression was found to be significantly higher ($p=<0.05$) in case of polymyxin B-sensitive Haitian variant strains compared to polymyxin B-resistant strains. Moreover, the results from adherence and motility assays support the prior studies, which suggest both

adherence, and motility of *V. cholerae* to intestinal epithelial cells may induce the IL-8 mRNA expression (Chaudhuri et al., 2012). Therefore, we hypothesized that the increased adherence to INT 407 cell line by the sensitive strains induce more IL-8 secretion compared to the resistant strains. Our earlier studies with the *in-silico* analysis suggested that the G265A mutation is beneficial for the environmental survival over other naturally existing *carR* alleles, and this might be a cause for the disastrous outbreaks of cholera that occurred in Yemen and Algeria recently.

Bilecen et al. mentioned that $CarR^R$ regulates glycine and diglycine modification of lipid A, confers polymyxin B resistance, and is required for intestinal colonization, although this phenotype is strain dependent (Bilecen et al., 2015; Cheng et al., 2015). They performed the colonization assay in infant mouse model only with the $carR^R$ allele. However, there are no such reports regarding the role of $CarR^S$ in colonization and pathogenicity in comparison with $CarR^R$ in animal models. Our in vivo study in suckling mice colonization suggested that polymyxin B-sensitive strains containing $carR^R$ allele exhibited comparable level of colonization with the $CarR^R$ allele. A similar result has also been obtained from fluid accumulation study in rabbit illeal loop model. Due to the fact that CarR is a response regulator in a two component system, it is possible for it to perform a variety of roles in *V. cholerae* and to contribute to virulence through many pathways. However, the pathogenesis of cholera is a multifactorial process, involving many genes at different stages which then aid in colonization and disease progression.

Another biotyping marker in *V. cholerae* is HlyA, responsible for the hemolytic activity and also has the ability to cause cell death by acting on targeting membrane. Classical strains are non-hemolytic due to the 11 bp deletion in their nucleotide sequence, which introduces a premature stop codon. Thus, the resulting protein is non-functional. Mechanisms responsible for non-hemolytic phenotype of the epidemic O1 El Tor strains are complex and not only confined to gene mutation but also deficiencies of transcription and extracellular transport of HlyA (Fan et al., 2019). In this study, the difference in the phenotypic properties of hemolysin was observed for 6% West Bengal strains. Nucleotide sequencing data of non-hemolytic strains isolated from West Bengal revealed a new *hlyA* variant which contains an extra 6bp deletion in addition to the nucleotide deletion (11bp) found in classical *hlyA* (Figure 7.1). The presence of this 17bp deletion also introduces a premature stop codon similar to the classical *hlyA* and thus the resulted HlyA protein is truncated and devoid of hemolysis feature. The presence of the type I secretion system has also been reported to be associated

with the hemolytic trait of *V. cholerae* (Boardman & Fullner Satchell, 2004). However, strains carrying polymyxin B-resistant trait was associated with non-hemolytic phenotype carrying the new variant *hlyA*. While, all the polymyxin B-sensitive strains contained the *hlyA* nucleotide sequence similar to the El Tor *hlyA* and thus showed visible hemolysis on blood agar plates.

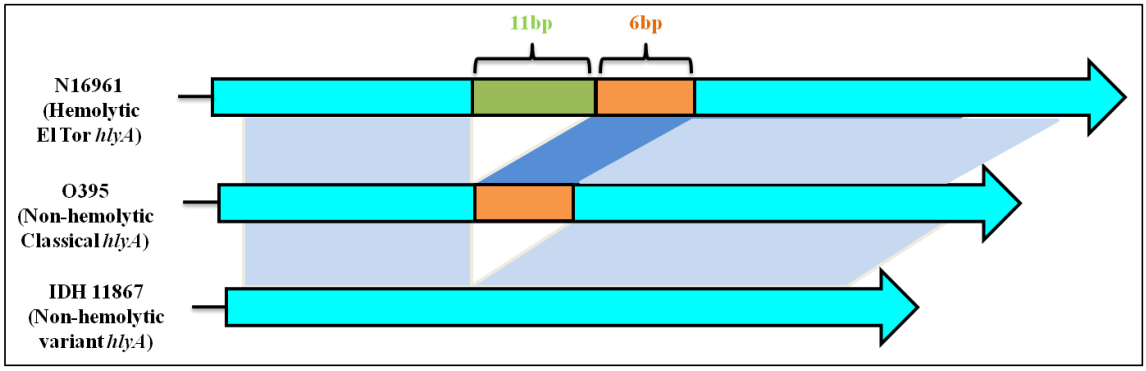

Figure 7.1. *Schematic representation of new variant hlyA found in V. cholerae O1 Inaba isolates from West Bengal during 2018.*

Several pathogenic variants of *V. cholerae* O1 emerged in the past and disseminated all over the world, causing large cholera outbreaks (Nair et al., 2002; Raychoudhuri et al., 2009). Since the beginning of the first pandemic the organism is emerging with new genetic features making them more virulent. In the past, it was thought that cholera began in Asia and spread in three waves from there. All the three waves began with Asiatic cholera. The wave-1 spanned 1961-1999 and the isolates in this wave contains CTX-1 that harbors *rstR*$^{\text{El Tor}}$ on chromosome 1, CT genotype-3 (*ctxB3*), but not the ICE of the SXT/R391 family. Wave-2 isolates were dominant between 1978 and 1984, and first identified in the Indian subcontinent. These isolates have *ctxB1* and tandem repeat of CTX-2 on chromosome 2. Some of them may contain CTX-1 and/or RS1 on chromosome 1. CT genotypes-4 to 6 (*ctxB4-ctxB6*) were detected in *V. cholerae* O139 isolated from Bangladesh during 1999–2005. The wave-3 isolates harbor CT-genotype 1 (1991–2010). The cholera outbreak in Haiti during 2010 introduces a novel variant of *ctx*B genotype (*ctxB7*) of *V. cholerae* O1, which is caused by the wave 3 strains (Piarroux et al., 2011; Reimer et al., 2011). The wave-3 isolates mainly have *rstR*$^{\text{El Tor}}$:TLC:RS1:CTX3 to CTX6. Our studies with *V. cholerae* O1 strains isolated from West Bengal, India during 2003-2017 revealed the prevalence of two *ctxB* genotypes, 1 or 7 and suggested the presence of wave-3 El Tor strains which are associated with the characteristics such as polymyxin B susceptibility and the *rtxA* genotype. Strains that

were resistant to polymyxin B had either CT-genotype 1 or 7. However, the wave-3 polymyxin B-sensitive strains were solely linked to CT-genotype 7 (Samanta et al., 2015).

Genetic analysis of Haitian strains revealed mutations in the major virulence-associated genes such as *ctxB*, *tcpA* and *rtxA* and are linked to higher pathogenicity (Talkington et al., 2011). Our earlier observations suggested that a similar variant genotype existed in strains isolated from cholera cases in Kolkata, West Bengal before the occurrence of Haitian cholera outbreak (Ghosh et al., 2014a; 201b; Naha et al., 2012) and was found to prevail in India and other countries (Bhattacharya et al., 2016; Bhuyan et al., 2016; Bwire et al., 2018; Ghosh et al., 2017; Kumar et al., 2014; Pal et al., 2017; Weill et al., 2017). All the hemolytic strains contained Haitian alleles of *ctxB* (*ctxB*7), *tcpA* and *rtxA* (inactivated). The non-hemolytic strains, however, carried the functional El Tor *rtxA* with the hypervirulent classical allele of *ctxB* (*ctxB1*).

The historical cholera pandemic caused by classical strains is also known for a naturally occurring deletion that eliminates more than 7 kb from the rtx locus, deactivating the MARTX toxin. Due to its size and potential for harming growth, the toxin may slow down the rapid growth that is necessary for greater dispersion. However, studies showed that the RTXA toxin is fully redundant in function when it associated with the functional *hlyA* and also with the Haitian *ctxB* which is linked to higher pathogenicity (Dolores et al., 2015). In the present study, we have also found that the non-hemolytic El Tor variant strains contained non-functional Haitian *rtxA* which was associated with classical *ctxB*.

In addition to major virulence genes, the devastating outbreak in Haiti also causes mutation in other virulence genes such as, *vieA*, *gyrA*, etc. VieA is a cyclic diguanylate phosphodiesterase and a member of an operon encoding the VieSAB signal transduction pathway that is differently expressed in *V. cholerae* biotypes (Wang et al., 2015). This operon is almost silent in the El Tor biotype, whereas it is expressed in the classical biotype (Ayala et al., 2018). Comparative genomic sequencing reveals a point mutation in Haitian *V. cholerae* strains with the El Tor strains at the position 235th nucleotide (C to T) which might replace the amino acid leucine (L) to phenylalanine (F) (Carignan et al., 2016). To track the existence of Haitian allele in West Bengal strains, we have performed a retrospective study and found Haitian *vieA* has been present in West Bengal since 2004 before the occurrence of Haitian outbreak as like the occurrence of other Haitian alleles of virulence genes. During our study, we also observed that the existence of Haitian *vieA* allele only in Haitian *rtxA* background. It has been recently reported that this point mutation does not play any significant role in the case of CT

or ToxT production. Whereas, when the nonsynonymous point mutation in *vieA* (C235T, residue L79F) was introduced into N16961, the expression of TcpA decreased, indicating that the point mutation in Haitian *vieA* modifies VieA activity toward the tcp operon (Carignan et al., 2016).

A serine to isoleucine substitution at the 83rd amino acid position was responsible for the emergence of Haitian *gyrase A* gene, which is associated with quinolone resistance in clinical *V. cholerae* strains (Hasan et al., 2012). This mutation was also reported from India, Nigeria and Cameroon (Baranwal et al., 2002; Garg et al., 2000; Islam et al., 2009; Quilici et al., 2010). Our earlier studies showed the presence of this genetic event was first detected in Kolkata, West Bengal strains since 1994 before the Haitian outbreak (Ghosh et al., 2014a) and present till now as stable trait in the recent isolates. The pathogenicity of the bacterium *V. cholerae* is mainly dependent on a discrete set of genetic determinants and their coregulated expression. It was also believed that *V. cholerae* El Tor isolates have an improved environmental fitness based on the observation that they have displaced the classical strains. On the other hand, classical strains of *V. cholerae* are known to produce more severe cholera (Kaper et al., 1995).

Comparative genomic analysis of classical and El Tor strains led to the discovery of Vibrio Seventh Pandemic Islands (VSP) (Dziejman et al., 2002). The seventh pandemic El Tor strains were found to have two distinct genomic islands, VSP-I and VSP-II acquired by lateral gene transfer (Dziejman et al., 2002; Nguyen et al., 2018). In El Tor reference strain N16961, VSP-II encodes genes for type IV pilin, chemotaxis proteins, an AraC-like transcriptional regulator, a DNA repair protein, and a P4-like integrase (O'Shea et al., 2004). Although it is still unknown how these regions contributes to the pathogenicity of the organism. However, it was hypothesized that these regions may increase the environmental fitness to the El Tor strains.

Several variants of VSP-II have been reported in El Tor strains isolated from different continents, including Asia, Africa, and Latin America (Taviani et al., 2010; Bhuiyan et al., 2012; Imamura et al., 2017). Therefore, the characterization of VSP-II types is important to understand the genetic lineages involved in the global transmission of cholera. Imamura and his colleagues analyzed the genomic organization of VSP II in the Kolkata strains and found heterogeneity in VSP II. Based on the genomic arrangements, they classified this region into four types, intact VSP II which is present in the El Tor reference strain N16961; VSP IIB contain a short VSP II with 1257 bp of genes for transposases A and B by replacing a

segment from 119th nucleotide of VC0495 to 1320 bp downstream of the VC0498. VSP IIC also contains transposases A and B by replacing the region from VC0495-VC0512 and the fourth is the VSP IID which is devoid of VSP II region.

V. cholerae O1 El Tor that has caused a huge cholera outbreak in Yemen during 2016–18 with a high case-infection ratio possessed several unique genetic features as mentioned before along with a deletion in the VSP-II region (Weill et al., 2019). Since the deletion of this VSP-II region in the seventh pandemic El Tor vibrios is an uncommon feature and thus we analyzed this feature in all the recent El Tor vibrios in this study. Interestingly, all of the hemolytic strains have the similar deletion in VSP-II region with the Yemeni *V. cholerae* strains. Whereas, the non-hemolytic strains from West Bengal displayed the possible presence of the intact VSP-II similar to El Tor reference strain N16961. This supports the observation reported by Imamura and colleagues (Imamura et al., 2017) and later found in Yemen cholera outbreak strains, indicating the presence of variability in VSP-II region. Our PCR results signifies VSP-II fragment deletion that corresponds to the VSP-IIC type associated with *ctxB7* genotype (Shaw et al., 2022) and it is believed that the non-hemolytic strains with classical biotype have been found for the first time in Indian El Tor strains.

Recent reports suggested that the strains that caused Yemen cholera outbreak originated in South Asia and mostly from the Indian subcontinent and then spread through Africa from 2014 to Yemen in 2016 (Benamrouche et al., 2022; Mutreja & Dougan, 2020; Weill et al., 2019). The genotypic and phenotypic features of these Indian isolates were similar to the isolates of this study. Moreover, we found that the Polymyxin B-sensitive El Tor variant Kolkata strains (isolated during 2013-2014) associated with *ctxB7* genotype have a similar PFGE pattern (94% similarity) with the current isolates and also with the Yemen outbreak strain suggesting a close relationship among them. This study also indicates that the Yemeni strains might have originated from Southeast Asia. Benamrouche et al. (2022) also suggested that the polymyxin B-sensitive El Tor variant strains were identified in South Asia, travelled to Eastern Africa and Yemen, and then spread to Algeria.

In *V. cholerae* O1, antibiotic resistance is one of the important phenotypic traits in differentiating the biotypes and also in effectively managing the cholera infection. Resistance to streptomycin, nalidixic acid and sulfamethoxazole-trimethoprim is the common feature of almost all seventh pandemic El Tor vibrios and can be classified as multidrug-resistant (MDR) strains (Dey et al., 2014).

Antibiotic resistance in *V. cholerae* O1 is a crucial phenotypic characteristic to distinguish the biotypes and also effective in the management of cholera infection. Rehydration with oral or intravenous fluids is the mainstay of cholera treatment. Antimicrobial agents may lessen the severity and the duration of diarrhea in extent cases (Sjölund-Karlsson et al., 2011). Cholera has commonly been treated with sulfamethoxazole/trimethoprim, fluoroquinolones (e.g., ciprofloxacin), Tetracyclines (e.g., doxycycline), macrolides (e.g., erythromycin). Beginning in early 1990, there was a sharp rise in the resistance to tetracycline, chloramphenicol, ampicillin, and nalidixic acid. According to a recent trend, practically all clinical isolates of *V. cholerae* are resistant to commonly used drugs (Verma et al., 2019). The current isolates of *V. cholerae* showed an increasingly complicated resistance profile to different antibiotics. Over 92% of the studied isolates showed resistance to streptomycin, nalidixic acid, and sulfamethoxazole-trimethoprim similar to the seventh pandemic MDR El Tor vibrios (Dey et al., 2014). This is probably due to the presence of mobile genetic elements such as SXT gene clusters and/or the integrative/conjugative elements acquired from the other species (Mohammed et al., 2018). Among the fluoroquinolone antibiotics, ciprofloxacin is recommended as the drug of choice to treat cholera (Rashed et al., 2017; Narendrakumar & Thomas, 2018). In Bangladesh, *V. cholerae* is increasingly becoming resistant to this antibiotic (Khan et al., 2015; Rashed et al., 2017). The resistance against commonly used drugs limits very few options available to treat cholera. The antimicrobial resistance of current *V. cholerae* O1 strains has also diverged from the patterns reported in the past. But, the isolates in this study did not exhibit resistance to the latest generation quinolones but reduced susceptibility towards ciprofloxacin/doxycycline has been found in the strains from West Bengal, Gujarat, Karnataka and Delhi similar to the recent study reported in 2018 (Narendrakumar & Thomas, 2018). It was also reported that the susceptibility of *V. cholerae* to tetracycline is decreasing in many countries (Das et al., 2011; Garg et al., 2000; Kuma et al., 2014; Mandal et al., 2012; Sjölund-Karlsson et al., 2011). Resistance against this antibiotic has been observed in a few isolates from West Bengal and Gujarat. Chloramphenicol resistance has also been detected in a few isolates. Among all the antibiotics, strains were exhibited their sensitivity towards azithromycin. Although, a decreased sensitivity towards this antibiotic has also been found recently (Mohanraj et al., 2020). AMR is currently one of the most urgent health issues facing the entire world. It causes more complicated therapies, extended hospital stays, greater rates of morbidity and mortality, and significant social and economic consequences (Goh et al., 2022). The advent

of multi-drug resistance superbugs that are resistant to carbapenems further makes this situation worse.

Present study indicates the carbapenem resistant phenotype among the six strains from Gujarat isolated during 2019 and this is a rare scenario for the *V. cholerae* O1 strains as these antibiotics are not generally prescribed to treat cholera. Therefore, this observation caught our attention to investigate the molecular insights into the emergence of carbapenem resistance among the recent isolates of *V. cholerae* O1 strains under this study.

In addition to nalidixic acid, streptomycin, and sulphamethoxazole/trimethoprim resistance, these CRVC strains exhibited resistance phenotype against carbapenems (meropenem) and third generation cephalosporins (ceftazidime, cefotaxime, and ceftriaxone) along with the aminoglycosides (streptomycin and gentamicin) associated with $bla_{\text{NDM-1}}$ gene.

The initial documentation regarding reduced susceptibility of *V. cholerae* O1 El Tor strains to third generation cephalosporins emerged during an outbreak of cholera in Argentina (Rossi et al.,1993). Subsequently, numerous published reports have highlighted resistance to third generation cephalosporins, with a majority originating from the Indian subcontinent (Mandal et al., 2012; Ceccarelli et al., 2016; Krishna et al., 2021). Nevertheless, only a handful of reports have indicated resistance to meropenem in *V. cholerae* O1 strains, which falls within the category of last resort of antibiotics under the carbapenem (Goh et al., 2022).

Based on the sequences obtained from the whole genome of CRVC isolates, the phenotypic susceptibility for each antibiotic used has been compared with the absence/presence of specific AMR genes relevant to each antimicrobial type. In addition to $bla_{\text{NDM-1}}$, the presence of AmpC β-lactamase genes $bla_{\text{CMY-6}}$, $bla_{\text{DHA-7}}$ also confers resistance against β-lactam antibiotics. Similarly, aminoglycoside resistance was associated with *strA, strB, rmtC, aac(3)-IId, aph(3')-VI, aac(6')-Ib10, aph(3")-Ib, aph(6)-Id* genes; sulfonamide resistance with *sul1*, *sul2*; trimethoprim resistance with *dfrA1* and chloramphenicol resistance with *florR* and *catB9*. Among the six CRVC strains, four (AKM-1, AKM-2, AKM-5 and AKM-6) of them harboured the mobile genetic elements such as pVCR94DeltaX (encodes sulfamethoxazole resistance), a new IncA/C plasmid, pVC1307, and *intI1*. Whereas, other two (AKM-3 and AKM-4) contained only pVC1307, *intI1*. The pVC1307 generally displayed resistance against ampicillin, nalidixic acid, chloramphenicol, azithromycin, gentamicin, and amikacin (Wang et al., 2016). However, all the six isolates showed sensitivity to azithromycin and a reduced sensitivity phenotype for chloramphenicol. PFGE analysis also showed that all the CRVC strains were almost similar (98%) indicating that they

were genetically similar clone. MLST analysis showed that they belonged to ST-69. Recent research from Asia and Africa also shown that most of the seventh pandemic El Tor (7PET) strains identified as ST-69 (Ramamurthy et al., 2019; Jubyda et al., 2023).

The ability to modify drug is one of the common mechanism for carbapenem resistance, and a group of metallo-β-lactamases serving as an example as found in this study. Due to their wide spectrum of substrates, these enzymes have a significant impact on public health today and are becoming more prevalent in clinically significant Gram-negative bacteria (Palzkill 2013). The recently discovered New Delhi metallo-β-lactamase (NDM-1) is a new member of Ambler class of β-lactamase belonging to class B, conferring resistance to carbapenems which is emerging worldwide and mainly found in pathogenic Enterobacteriaceae (Dortet et al., 2014; Chowdhury et al., 2016; Sarkar et al., 2015).

The spread of mobile carbapenemases among bacterial pathogens is a major cause of concern, because these enzymes impart resistance to carbapenems, but also because they exhibit resistance to other antibiotic groups, leaving very few treatment options (Palzkill 2013; Chowdhury et al., 2012). Pathogenic bacteria can spread the carbapenemase gene, $bla_{\text{NDM-1}}$, to other bacteria, leading to phenotypes that are very or multiple-drug resistant.

Thus, we have performed conjugation study with *E. coli* J53 and other enteric pathogens such as *S. enterica*, *S. flexnerii*, *V. parahaemolyticus* and also *V. cholerae* O1 to demonstrate the presence of plasmid carrying $bla_{\text{NDM-1}}$ and interestingly the resistance profiles of ampicillin, ceftriaxone, cefotaxime, ceftazidime, meropenem, and gentamicin have been successfully transferred to all the transconjugants. This indicates that the $bla_{\text{NDM-1}}$ positive isolates may have similar plasmids with the uniform resistance genes and hence, confer the same resistance phenotype which poses a high risk. Mobile genetic elements, including plasmids, conjugative transposons, integrons, and SXT elements are essential for the dissemination of resistance genes among different species (Wang et al., 2016). However, the degree of carbapenem resistance caused by the NDM plasmid differed between hosts. In this study, we found that *E. coli* J53 showed the highest transfer frequency compared to the other bacterial species used as recipients.

Studies also indicated that the bla_{NDM} is typically located on large plasmids that can be easily transferred between different bacterial species (Kumarasamy et al., 2010; Rolain et al., 2010). These plasmids can vary in type with examples including IncA/C, IncL/M, and IncR, and they can range in size from 30 to 300 kb (Carattoli et al., 2015; Gamal et al., 2016). From the WGS analysis, we found that all the CRVC isolates contained a 142 kb mega sized plasmid

belonging to IncA/C1 type. This group of plasmid has attracted increased attention partly because of their ability to contribute resistance to a number of antimicrobial drugs and easily transfer to different bacterial communities (Johnson et al., 2012; Fernandez-Alarcon et al., 2011) as we have seen in the present study.

Analysis showed that the plasmid containing $bla_{\text{NDM-1}}$ revealed high homology with the *E. coli* plasmid (KC999035.4) and *V. cholerae* plasmid (CP007636), and carrying resistance genes against the following antibiotic classes, beta lactam ($bla_{\text{NDM-1,}}$ $bla_{\text{CMY-6,}}$ $bla_{\text{DHA-7.}}$), sulfonamide (*sul2*), aminoglycosides (*rmtC, aph(3')-VI, aph(3'')-Ib, aph(6)-Id, aac(6')-Ib3*), bleomycin (ble_{MBL}) and quaternary ammonium compound (*qacE*). Studies also indicated that the AmpC beta lactamases, which are transferred through IncA/C type plasmids are found in the *E. coli* and *Salmonella sp*. isolated from both human and animal in the Canada, Europe, and United states (Carattoli et al., 2012). The majority of the bla_{NDM} sequences found in GenBank are surrounded by the ble_{MBL} gene downstream and an intact or truncated copy of ISAba125 upstream. In addition, these genes have a variety of MGEs detected in their vicinity, along with the genes for replication (*repA*), transfer (*tra*) and partition system (*parA*, *parB*, *A053*), that may potentially help to mobilize them. (Partridge and Iredell, 2012). It is possible that the IncA/C bla_{CMY} -carrying plasmid acquired the $bla_{\text{NDM-1}}$ within its scaffold as a result of a secondary event given the evolutionary link between NDM-1-bearing plasmids and the IncA/C plasmids carrying bla_{CMY} (Carattoli et al., 2012).

In conclusion, this study highlighted the epidemiological importance of cholera with the significant events in the evolution of recently circulating *V. cholerae* O1 variants that are more clinically relevant with their higher pathogenic potentials. This study also provide valuable information regarding the emerging threat of carbapenem resistant *V. cholerae* O1 strains which may increase serious underlying medical conditions. The altered pattern of AMR forecasts future resistant strains to these useful antimicrobials. Therefore, there is an urgent need for the development of effective strategies for controlling the spread of carbapenemase associated with *V. cholerae* O1.

Chapter 8

Highlights

8. Highlights of the Study

The salient features of the study have been furnished below:

I. To analyze the changing genetic features of currently circulating *V. cholerae* O1 isolates in India.

- The versatile nature of *V. cholerae* O1 strains make them important in the field of epidemiology. Thus, the present study has focused on the characterization of the recently circulating strains isolated from different cholera endemic regions of India in the period of 2018-2021 and found that 6% of the West Bengal strains belonged to the Inaba serotype and contained polymyxin B-resistant phenotype with the genetic backbone of classical *ctxB* and El Tor *rtxA*.
- We have found a new *hlyA* variant among the Inaba strains isolated from West Bengal in 2018 with non-hemolytic feature like classical strains but contained a 6 bp deletion in addition to 11 bp deletion which present in classical *hlyA*.
- The strains from other states of India were found to contain the hypervirulent Haitian virulence genes with polymyxin B-sensitive phenotype.
- All the hemolytic strains contained a deletion in VSP-II region (ΔVC0495-VC0512), a significant feature of the Yemen cholera outbreak strains, detected by our newly developed PCR.
- All the recent isolates of *V. cholerae* O1 in this study shared a close relationship with the strains that caused the devastating cholera outbreak in Yemen during 2016-2017.
- Our study also indicates that the polymyxin B-sensitive El Tor variant strains may have originated from Southeast Asia.

II. To know the antimicrobial resistance pattern in recently circulating *V. cholerae* strains along with its mechanism of resistance.

- Antibiogram profile showed that over 92% of the strains from West Bengal and other regions displayed resistance against streptomycin, sulfamethoxazole/trimethoprim and nalidixic acid.
- About 8% (5/57) of the isolates from West Bengal and 1.6% (3/182) from Gujarat showed resistance against tetracycline.

- Interestingly, 3% strains (6/182) from Gujarat isolated in 2019 showed resistance against meropenem (MIC values of 16-4μg/mL) which is used as the last line of treatment against MDR pathogens.
- All six strains (3%) contain bla_{NDM-1}, bla_{CMY-6}, bla_{DHA-7} beta lactamase genes which confer resistance to carbapenems and other β-lactam group of antibiotics.
- Whole genome sequencing of the NDM positive strains showed a 142 kbp mega plasmid which belonged to IncA/C1 incompatibility group and had a high similarity with the plasmids of *E. coli* (KC999035) and *V. cholerae* (CP007636).
- The plasmid carrying AMR genes confers resistance against β- lactam (bla_{CMY-6}, bla_{NDM-1}, bla_{DHA-7}), aminoglycosides (*aph(3')-VI, aph(3")-Ib, aph(6)-Id, aac(6')-Ib3, rmtC)* and sulfonamide (*sul1*) antibiotics.
- WGS analysis showed that the CRVC strains carrying mobile genetic elements such as pVCR94DeltaX, pVC1307, and *intI1*.
- Conjugation study indicates that the plasmid is easy to transfer into *E. coli* J53, and different enteric pathogens including, *Salmonella enterica, Shigella flexneri, V. parahaemolyticus* and even *V. cholerae* O1 strains.
- The resistance profiles against ampicillin, ceftriaxone, ceftazidime, cefotaxime, meropenem, and gentamicin of transconjugants indicates that the plasmid has successfully transferred to all the transconjugants and may carry similar plasmids with the uniform resistance genes.
- The highest transfer frequency has been found for *E. coli* (J53) followed by *V. parahaemolyticus*, *Shigella flexneri, V. cholerae* O1 and *Salmonella enterica.*
- PFGE analysis showed that all the six CRVC strains were clonally similar to each other.
- Plasmid sequence and PFGE analysis revealed the presence of three NotI restriction sites in the plasmid.
- MLST analysis showed that all the CRVC strains belonged to ST69.

III. To understand the inflammatory response of El Tor strain and recently emerged polymyxin B-sensitive El Tor variant on intestinal epithelial cell line.

- Polymyxin B-sensitive Haitian variant strains induce more inflammatory cytokine such as IL-8 mRNA expression compared to the resistant strains.

- The result of higher motility and adherence to the INT 407 cell line was found for the strains containing the *carR*-sensitive allele compared to the resistant allele.
- Polymyxin B-sensitive Haitian variant strains showed higher expression of flagellar genes *flhDC* compared to the resistant strains indicating the contribution of the $carR^S$ allele in the regulation of *flhDC* expression.

IV. To compare the pathogenicity like colonization ability and fluid accumulation in animal models when infected with polymyxin B-resistant El Tor and polymyxin B sensitive El Tor variant strains.

- The in vivo colonization study in the suckling mice model suggested the colonization potential of polymyxin B-resistant Haitian variant strains is similar to the resistant strains. But it is clear that Haitian variant strains colonize more compared to the canonical El Tor.
- There were no significant differences obtained in the virulence of the resistant and sensitive strains in the rabbit model and it varied in a strain specific manner.

Overall, this study helps in understanding the virulence and emerging drug resistance properties of recently circulating *V. cholerae* O1 strains in India and also to aware people of the hypervirulent nature of the strains and to track them easily during the pandemic situations worldwide.

Chapter 9

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9. References

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Chapter 10

Publications & Conferences

10. Publications & Conferences

Publications in peer-reviewed journals:

- **Shaw S**, Samanta P, Chowdhury G, **Ghosh D**, Dey TK, Deb AK, Ramamurthy T, Miyoshi S, Ghosh A, Dutta S, Mukhopadhyay AK. Altered Molecular Attributes and Antimicrobial Resistance Patterns of *Vibrio cholerae* O1 El Tor Strains Isolated from the Cholera Endemic Regions of India. **Journal of Applied Microbiology**. **2022**. 133(6):3605-3616.

- Ghosh D, Choudhury G, Samanta P, **Shaw S**, Deb AK, Bardhan M, Manna A, Miyoshi S, Ramamurthy T, Dutta S, Mukhopadhyay AK. Characterization of diarrhoeagenic *Escherichia coli* with special reference to antimicrobial resistance isolated from hospitalized diarrhoeal patients in Kolkata (2012-2019), India. **Journal of Applied Microbiology**. **2022**. 132(6), 4544-4554.

- Samanta P, Mandal RS, Saha RN, **Shaw S**, Ghosh P, Dutta S, Ghosh A, Imamura D, Morita M, Ohnishi M, Ramamurthy T, Mukhopadhyay AK. A Point Mutation in *carR* Is Involved in the Emergence of Polymyxin B-Sensitive *Vibrio cholerae* O1 El Tor Biotype by Influencing Gene Transcription. **Infection and Immunity**. **2020** Apr 20;88(5).

- Naha A, Mandal RS, Samanta P, Saha RN, **Shaw S**, Ghosh A, Chatterjee NS, Dutta P, Okamoto K, Dutta S, Mukhopadhyay AK. Deciphering the possible role of ctxB7 allele on higher production of cholera toxin by Haitian variant *Vibrio cholerae* O1. **PLoS Neglected Tropical Diseases. 2020** Apr 1;14(4):e0008128.

Manuscript in communication:

- Sharma D, Chowdhury G, , **Shaw S**, Anand S, Gupta S, Singhal S, Mukhopadhyay AK. Molecular characterization of *Vibrio cholerae* O195 strain isolated from sputum sample of a patient of Bronchiectasis in Delhi, India. Submitted in Indian Journal of Medical Microbiology on 13th July, 2023.

Manuscript under Preparation:

- **Shaw S**, Mukhopadhyay AK. Genomic analysis of IncA/C plasmid mediated *bla*$_{NDM}$ producing 7PET *V. cholerae* isolated from India.

Published abstract of papers presented at different conferences:

- **Sreeja Shaw**, Prosenjit Samanta, Goutam Chowdhury, Debjani Ghosh, T. Ramamurthy, Shanta Dutta, Asish Kumar Mukhopadhyay. Molecular attributes of *Vibrio cholerae* O1 strains isolated from different cholera endemic regions of India during 2018: A comparative study (Poster presented at 16th Asian Conference of Diarrheal Disease and Nutrition (ASCODD), Kolkata, India on 11th-13th Nov 2022).

- **Sreeja Shaw**, Prosenjit Samanta, Asish Kumar Mukhopadhyay. Genetic and phenotypic variability among the recent *Vibrio cholerae* O1 isolates of India [Presented poster virtually in the International Conference (Biosangam 2022) held at Department of Biotechnology, MNNIT Allahabad, Prayagraj, India on March 10-12, 2022].

Workshops:

- Participated in the Hands on Workshop on "Next Generation Sequencing, AMR Screening and Metagenomics" during the 16th Asian Conference of Diarrheal Disease & Nutrition (ASCODD) in Kolkata, India on the 12th November, 2022.

- Attended the Workshop on "Ethics in Human Research and Good Clinical Practice" organized by ICMR-National Institute of Cholera & Enteric Diseases (NICED) during 7th May, 2019.

Appendix

Reagents and Solutions:

- **Bromophenol Blue Loading Dye (10ml)**

Reagents	**Amount**
1M Tris-HCl (pH-7.5)	100µl
Glycerol	6ml
Bromophenol blue	0.003gm
500mM EDTA	1.2ml
Distilled Water	2.7ml

- **50X Tris-acetate EDTA (TAE) (1lt)**

Reagents	**Amount (g/L)**
Tris Base	242gm
Glacial acetic acid	55ml
0.5M EDTA(pH-8.0)	18.6gm
Adjust the volume with sterile reagent grade water to 1lt	

- **Transformation & storage solution (TSS) (50ml)**

Reagents	**Amount**
PEG8000	5gm
1M MgCl2	1.5ml
DMSO	2.5ml
Luria Bertani broth	46ml
Adjust the volume with sterile reagent grade water to 50ml	

- **TCM**

Reagents
11mM Tris Cl
11mM CaCl2, 2H2O
11mM MgCl2

- **Luria Bertani Broth (1lt)**

Reagents	**Amount (g/L)**
Tryptone	10g
Yeast Extract	5gm
NaCl	10gm
Distilled water	1000ml

- **Luria Bertani Agar (1lt)**

Reagents	**Amount (g/L)**
Tryptone	10g
Yeast Extract	5gm
NaCl	10gm
Bacto Agar	20gm
Distilled water	1000ml

- **Nutrient agar Stab (1lt)**

Reagents	**Amount (g/L)**
Nutrient Broth	8.00gm
BactoAgar	8gm
NaCl	10gm
Distilled water	1000ml

- **Cell Suspension Buffer (10ml)**

Reagents	**Amount**
1.0M Tris-HCl, pH 7.2	0.1ml
5.0M NaCl	0.04ml
0.5M EDTA, pH 8.0	1.0ml
Adjust the volume with sterile reagent grade water to 10ml	

- **10X restriction digestion buffer (buffer H)**

Reagents	**Amount**
500mM Tris HCl (pH 7.2)	2.5ml
100mM $MgCl_2$	0.5ml
10mM Dithiothreitol	0.5ml
1000mM NaCl	1ml
Distilled water	0.5ml

- **1X Washing Buffer (20ml)**

Reagents	**Amount**
1M Tris-HCl,pH 7.2	400µl
0.5M EDTA,pH 8.0	2.0ml
100mM PMSF	200µl
Adjust the volume with sterile reagent grade water to 20ml	

- **Phenylmethylsulfonyl fluoride (PMSF) (1lt)**

Reagents	**Amount**
PMSF	174.19gm
Sterile Distilled Water	1000ml

- **Phosphate Citrate Buffer**

Reagents	**Amount (g /L)**
Di-sodium hydrogen phosphate	2.67
Potassium dihydrogen phosphate	1.088
Potassium chloride	0.1998
Sodium chloride	7.946
Volume adjusted to 1L with double distilled water Final pH 7.4	

- **Lysozyme Solution (50ml)**

Reagents	**Amount**
Lysozyme	50mg
1M Tris-HCl, pH 7.2	0.5ml
5M NaCl	0.5ml
Na-deoxycholate	0.1gm
Na-laurylsarcosine	0.25gm
Adjust the volume with sterile reagent grade water to 50ml	

- **Proteinase K Solution (50ml)**

Reagents	**Amount**
Proteinase K	50mg
0.5M EDTA,pH 8.0	10ml
Na-deoxycholate	0.1gm
Na-laurylsarcosine	0.5gm
Adjust the volume with sterile reagent grade water to 50ml	

- **AKI medium (100ml):**

Reagents	**Amount**
Bactopeptone	1.5gm
Yeast Extract	0.4gm
NaCl	0.5gm
$NaHCO_3$	0.3gm
Adjust the volume with sterile reagent grade water to 100ml	

- **Bicarbonate Buffer (800ml):**

Reagents	**Amount**
Sodium Bicarbonate	1.05gm
Sodium Carbonate (anhydrous)	9.275gm
Distilled Water	800ml

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ORIGINAL ARTICLE

Altered molecular attributes and antimicrobial resistance patterns of *Vibrio cholerae* O1 El Tor strains isolated from the cholera endemic regions of India

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Abstract

Aims: The present study aimed to document the comparative analysis of differential hypervirulent features of *Vibrio cholerae* O1 strains isolated during 2018 from cholera endemic regions in Gujarat and Maharashtra (Western India) and West Bengal (Eastern India).

Methods and Results: A total of 87 *V. cholerae* O1 clinical strains from Western India and 48 from Eastern India were analysed for a number of biotypic and genotypic features followed by antimicrobial resistance (AMR) profile. A novel polymerase chain reaction was designed to detect a large fragment deletion in the Vibrio seventh pandemic island II (VSP-II) genomic region, which is a significant genetic feature of the *V. cholerae* strains that have caused Yemen cholera outbreak. All the strains from Western India belong to the Ogawa serotype, polymyxin B-sensitive, hemolytic, had a deletion in VSP-II (VSP-IIC) region and carried Haitian genetic alleles of *ctxB*, *tcpA* and *rtxA*. Conversely, 14.6% (7/48) of the strains from Eastern India belonged to the Inaba serotype, polymyxin B-resistant, nonhemolytic, harboured VSP-II other than VSP-IIC type, classical *ctxB*, Haitian *tcpA* and El Tor *rtxA* alleles. Resistance to tetracycline and chloramphenicol has been observed in strains from both regions.

Conclusions: This study showed hypervirulent, polymyxin B-sensitive epidemic causing strains in India along with the strains with polymyxin B-resistant and nonhemolytic traits that may spread and cause serious disease outcomes in future.

Significance and impact of the study: The outcomes of this study can help to improve the understanding of the hyperpathogenic property of recently circulating pandemic *Vibrio cholerae* strains in India. Special attention is also needed for the monitoring of AMR surveillance because *V. cholerae* strains are losing susceptibility to many antibiotics used as a second line of defence in the treatment of cholera.

KEYWORDS

cholera, El Tor biotype, nonhemolytic, polymyxin B, *Vibrio cholerae*, vibrio seventh pandemic island II

INTRODUCTION

The global spread of cholera still remains a major public health concern in several countries. Several large outbreaks and epidemics have been reported recently in Haiti, Yemen, Congo, Niger and Nigeria (European Centre for Disease Prevention and Control (ECDC), 2022). Cholera prevails mainly in the developing countries of Asia and Africa, where people lack sanitation, proper hospital facilities and clean drinking water supply (Clemens et al., 2017; Kaper et al., 1995; Safa et al., 2008). This waterborne diarrheal infection is caused by the bacterium *Vibrio cholerae* with the O1 and O139 serogroups that possess cholera toxin (Albert, 1994; Kaper et al., 1995; Nair et al., 1994). Serogroup O1 is further subdivided into two biotypes, classical and El Tor, depending on their differing responses to phenotypic tests such as polymyxin B susceptibility, Voges-Proskauer test, chicken blood cell agglutination, sheep blood hemolysis and sensitivity to the respective biotype-specific phages. Overall, the pathogenic mechanisms of both the biotypes are almost identical. More severe clinical symptoms had been documented in cholera patients infected with the classical biotype, which was thought to cause the first six cholera pandemics, whereas the current seventh pandemic is caused by the El Tor biotype that expresses less virulence (Safa et al., 2010). Moreover, classical biotype strains were less stable in the environment, whereas the El Tor vibrios have a greater advantage to survive in the environment (Kaper et al., 1995; Nair et al., 2008; Sack et al., 2004; Samanta et al., 2018).

Recent *V. cholerae* O1 strains were found to have increased their pathogenic ability with a sequential accumulation of traits towards a hypervirulent classical biotype with the El Tor biotype genetic backbone. In the late 90s, it was seen that few amino acid sequences of cholera toxin B (CT-B) subunit of El Tor strains have been replaced by the classical type of CT-B in India and other countries. Further, in 2012, another classical biotype marker, polymyxin B susceptibility has been acquired in El Tor biotype strains.

Several pathogenic variants of *V. cholerae* O1 emerged in the past and disseminated all over the world, causing large cholera outbreaks (Nair et al., 2002; Raychoudhuri et al., 2009). In Haiti, a different type of El Tor strain caused the largest cholera outbreak in 2010 (Chin et al., 2011; Reimer et al., 2011). Genetic analysis revealed that the strains contain mutations in the major virulence-associated genes such as *ctxB*, *tcpA* (toxin co-regulated pilin) and *rtxA* (repeats-in-toxin) and are linked to their higher pathogenicity (Talkington et al., 2011). Our previous observations suggested that a similar variant genotype existed in strains isolated from cholera cases in Kolkata, West Bengal earlier to the Haitian cholera outbreak (Ghosh, Naha, Basak, et al., 2014; Naha et al., 2012) and was found to prevail in India and other countries (Bhattacharya et al., 2016; Bhuyan et al., 2016; Bwire et al., 2018; Ghosh et al., 2017; Kumar et al., 2014; Pal et al., 2017; Weill et al., 2017).

From 2016 to 2018, Yemen experienced a huge cholera outbreak with a high case-infection ratio, which affected more than 1 million people (Camacho et al., 2018; Weill et al., 2019). *Vibrio cholerae* O1 El Tor strains from this outbreak possessed a similar genetic trend as observed in Haiti along with new characteristics such as the polymyxin B susceptibility and a large deletion in the Vibrio Seventh Pandemic Island II (VSP-II) encoding gene (Weill et al., 2019). A recent study with the Kolkata strains isolated during 2007–2014 indicated the presence of four VSP-II types. VSP-IIA corresponds to the intact VSP-II region present in the El Tor reference strain N16961. Whereas VSP-IIB, C and D represent different types of VSP-II fragment deletion (Imamura et al., 2017). Considering these genetic changes, we targeted to determine the deletion in our studied strains. Moreover, our earlier studies correspondingly showed the presence of polymyxin B susceptibility of El Tor vibrios from Kolkata, West Bengal and other parts of India prior to the Yemen cholera outbreak (Samanta et al., 2015; Samanta et al., 2018).

In addition to the routine isolation and identification of *V. cholerae*, it is important to examine their genetic traits and relate to the epidemiology of cholera. In this study, we aimed to characterize *V. cholerae* O1 strains isolated from Gujarat and Maharashtra, located in the western parts of India in 2018 and compared them with the West Bengal strains to investigate their genetic attributes and other phenotypic markers.

MATERIALS AND METHODS

Bacterial strains

Vibrio cholerae strains ($n = 135$) included in this study were isolated from cholera patients in West Bengal, Gujarat and Maharashtra in 2018 (Table 1**)**. *V. cholerae* O1 control strains O395, N16961 and EL-1786 were used as a classical, El Tor and Haitian variant, respectively (Heidelberg et al., 2000; Samanta et al., 2018). One strain from the Yemen cholera outbreak has also been included as a positive control for the PCR to detect VSP-II deletion.

Media, reagents and antimicrobial susceptibility testing

Strains were isolated by plating them on a thiosulfate-citrate-bile salts-sucrose (TCBS) medium (Oxoid, UK) and then subcultured in Luria-Bertani (LB) broth and/or on LB agar (Difco, BD, USA) plates followed by oxidase and string tests (Neogy & Mukherji, 1970) for the confirmation of *V. cholerae*. To identify antibiotic susceptibility of the strains, a total of 83

TABLE 1 Isolation profile and phenotypic features of the *Vibrio cholerae* O1 strains used in this study

Sl no.	State of isolation	Place of isolation	No. of isolates (*n*)	Year of isolation	Serotype		Polymyxin B susceptibility		Hemolysis	
					Ogawa	Inaba	Sensitive	Resistant	Positive	Negative
1	Maharashtra	Shri Vasnatrao Naik Government Medical College, Yavatmal	22	2018	22 (100%)	0	22 (100%)	0	22 (100%)	0
2	Gujarat	State Public Health Laboratory, Baroda	22	2018	22 (100%)	0	22 (100%)	0	22 (100%)	0
		SMIMER Medical College, Surat	43	2018	43 (100%)	0	43 (100%)	0	43 (100%)	0
3	West Bengal	ID&BG Hospital	48	2018	41 (85.4%)	7 (14.6%)	41 (85.4%)	7 (14.6%)	41 (85.4%)	7 (14.6%)

representative strains from Gujarat ($n = 47$), Maharashtra ($n = 12$) and West Bengal ($n = 24$) were included in the testing. All these strains were tested for their susceptibility to ampicillin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), sulfamethoxazole-trimethoprim (23.75/1.25 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), norfloxacin (10 μg), ofloxacin (5 μg), gentamycin (10 μg), streptomycin (10 μg), ceftazidime (30 μg), ceftriaxone (30 μg), cefotaxime (30 μg), azithromycin (10 μg) and doxycycline (30 μg) by disk diffusion method using commercial disks (Difco, B.D, USA) in accordance with the protocol and breakpoints suggested by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2021). *Escherichia coli* strain ATCC 25922 was used as the control to check disc accuracy for this assay (Bauer, 1966).

To detect the polymyxin B susceptibility, a small volume of log-phase culture was spread over a Mueller Hinton Agar (MHA) plate and the disk with 50 U of polymyxin B (HiMedia, India) was placed over it. In addition, a loopful of culture was taken to streak on LB agar plates containing polymyxin B with a concentration of 50 μg/mL. Results were interpreted according to the criteria mentioned previously (Samanta et al., 2018).

Voges-Proskauer test

This test distinguishes the two *V. cholerae* biotypes which determine the production of acetylmethylcarbinol from glucose fermentation and is performed with the protocol mentioned by Yoon and Mekalanos (2006).

Hemolysis of sheep blood erythrocyte

Usually, classical biotype strains do not lyse sheep red blood cells and the El Tor biotype, being positive, forms a clear zone when grown on a 5% blood agar plate indicating lysis of sheep blood erythrocytes (Barrett & Blake, 1981).

Chicken blood cell agglutination test

When phosphate-buffered saline-washed chicken blood cells are mixed with the culture of El Tor strains on a glass slide, it aggregates and forms visible clumping. The classical strains are unable to agglutinate and hence classified as negative in this test (Barua & Mukherjee, 1965).

Serology

Denka-Seiken kit was used in the side agglutination test to identify the serotype of *V. cholerae* strains. Fresh cultures

grown overnight at 37°C in a nonselective medium were used in the test.

DNA extraction and PCR assays

DNA was extracted by phenol–chloroform extraction method and diluted to the desired concentration. Diluted DNA samples were used as templates in the allele-specific PCR assays to detect virulence-associated *ctxB*, *tcpA* and *rtxA* genes (Table 2; Ghosh, Naha, Pazhani, et al., 2014; Naha et al., 2012). The presence of El Tor-specific genetic markers was screened for the genes *rstR*, *rstC* and *rtxC* (Table 2; Chow et al., 2001; Nusrin et al., 2004; Zaw et al., 2019). The presence of CTX bacteriophage integration in chromosome II was also checked with the help of CIIF/CIIR primers (Table 2; Maiti et al., 2006).

Development of a new PCR assay for the detection of deletion in the VSP-II

To know whether the current Indian strains of *V. cholerae* have a deletion in the region (*VC0495–VC0512*) of the VSP-II (Weill et al., 2019), a new PCR assay has been developed. For this PCR, primers have been designed from the flanking regions of *VC0495* and *VC0512* (VC0494F/VC0513R) which would give an amplicon of 2.7 kb if the region *VC0495–VC0512* is absent. The temperature cycling condition was optimized to two-step amplification for long-length product formation with the enzyme XT-Polymerase following the manufacturer's protocol (XT-20 PCR system, GeNei, India). The thermal cycling was set up for initial denaturation at 95°C for 2 mins, then 5 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C and extension for 6 mins at 72°C and another 10 cycles of denaturation for 30 s at 94°C, annealing for 30 s at

TABLE 2 List of primers and polymerase chain reaction conditions used in this study

Primer	Sequence (5′-3′)	Annealing temperature (°C)	Amplicon size (bp)	References
ctxBF3	GTTTTACTATCTTCAGCATATGCGA	58	191	Naha et al. (2012)
ctxBF4	GTTTTACTATCTTCAGCATATGCGC	60		Naha et al. (2012)
ctxBRv-cla	CCTGGTACTTCTACTTGAAACG	60		Morita et al. (2008)
tcpA-F1	CCAGCTACCGCAAACGCAGA	56	167	Ghosh, Naha, Pazhani, et al. (2014)
tcpA-F'2	CCAGCTACCGCAAACGCAGG	56		Ghosh, Naha, Pazhani, et al. (2014)
tcpA-El-Rev	CCGACTGTAATTGCGAATGC	56		Ghosh, Naha, Pazhani, et al. (2014)
rtxAF	ATCGGAATGAGTGAGAAAGACC	54	187	Ghosh, Naha, Basak, et al. (2014)
rtxAR1	TGTGAACCACGTCTGCC	54		Ghosh, Naha, Basak, et al. (2014)
rtxAR2	TGTGAACCACGTCTGCT	54		Ghosh, Naha, Basak, et al. (2014)
rstR1(F)	CTTCTCATCAGCAAAGCCTCCATC	50	500	Nusrin et al. (2004)
rstR2(F)	GCACCATGATTTAAGATGCTC	50		Nusrin et al. (2004)
rstA3(R)	TCGAGTTGTAATTCATCAAGAGTG	50		Nusrin et al. (2004)
rstC1	AACAGCTACGGGCTTATTC	60	245	Zaw et al. (2019)
rstC2	TGAGTTGCGGATTTAGGC	60		Zaw et al. (2019)
rtxC-F	CGACGAAGATCATTGACGAC	55	263	Chow et al. (2001)
rtxC-R	CATCGTCGTTATGTGGTTGC	55		Chow et al. (2001)
CIIF	CTCACGCTGAACAGCAAGTC	55	766	Maiti et al. (2006)
CIIR	TTGCTTGAATCGAAAGGACA	55		Maiti et al. (2006)
VC0494F	GGGGAGGTGTTTCAATCTGTC	55	2700	This study
VC0513R	CTCCACTGCTATTCCGGT	55		This study
VC0502F	TCATCAGTTAGCACACGAAC	52	476	O'shea et al. (2004)
VC0502R	GCTATCGTTATACTTGGCG	52		O'shea et al. (2004)
VC0506_Fp	GCGTATCGTGTGTTGACCG	56	323	This study
VC0506_Rp	CGCTAGTTCCTGATGCACTG	56		This study
VC0510_Fp	CGATGCCCTAACCAATCCTG	56	246	This study
VC0510_Rp	GGTGTTGAGTCTCCAGATGG	56		This study

56°C, and extension for 6 mins at 72°C (+5 s/cycle for each successive cycle) with a 10 min final extension at 72°C. An isolate of the Yemen cholera outbreak (YMN-170240) was taken as the positive control in this PCR (Table 2). Further, to eliminate the possibility of false negative results of the PCR assay, the presence of internal genes between the loci *VC0494* and *VC0512* such as *VC0502*, *VC0506* and *VC0510* were checked with the primers and cycling conditions mentioned in Table 2.

Pulsed-field gel electrophoresis

A total of 26 *V. cholerae* strains were used to perform the pulsed-field gel electrophoresis (PFGE) according to the protocol standardized by PulseNet for *V. cholerae*. *Salmonella enterica* (serotype Braenderup H9812) was used as a molecular weight standard. The banding patterns were analysed by the software BioNumerics (Applied Maths, Kortrijk, Belgium), and DNA-banding profiles were compared using the procedure mentioned by Chowdhury et al. (2012).

RESULTS

Predominance of Ogawa serotype

One hundred and thirty-five *V. cholerae* O1 strains were studied from Maharashtra, Gujarat and West Bengal in 2018 (Table 1). All the strains were confirmed for their oxidase and string-positive characteristics and were found to agglutinate with polyvalent O1-specific antiserum. The strains from Gujarat and Maharashtra have shown to agglutinate with Ogawa-specific antisera but West Bengal strains showed a different serotyping pattern. About 85.4% (41/48) of them were Ogawa and 14.6% (7/48) were of Inaba serotype (Table 1).

Strains were able to produce acetoin from glucose and capable of chicken blood cell agglutination

VP test and chicken blood cell agglutination were performed to differentiate among classical and El Tor biotypes. All the strains from Gujarat, Maharashtra and West Bengal were found to produce acetoin from glucose fermentation and were able to agglutinate chicken blood erythrocytes. Hence, they were considered positive in the VP test and chicken blood cell agglutination assay similar to the prototype El Tor.

Appearance of nonhemolytic strains in West Bengal

Vibrio cholerae O1 strains from Gujarat and Maharashtra were found to lyse sheep blood erythrocytes when plated on 5% sheep blood agar plates and therefore classified as hemolytic like the typical El Tor strain. However, 14.6% (7/48) of the West Bengal strains were unable to lyse sheep blood erythrocytes similar to the classical biotype strains. Therefore, these strains were considered nonhemolytic. Other 85.4% (41/48) strains from the same region of West Bengal showed hemolysis resembling that of El Tor strains (Table 1).

Co-existence of polymyxin B-sensitive and resistant strains in West Bengal

Another key marker for the differentiation of the biotypes of *V. cholerae* O1 is polymyxin B susceptibility. All strains of Gujarat and Maharashtra and 85.4% (41/48) of the West Bengal strains were susceptible to polymyxin B, giving a zone diameter of 12–16 mm in the Kirby-Bauer disk diffusion method, similar to the classical control strain O395. The result was further confirmed with a polymyxin B (50 μg/ml) plate susceptibility assay. But, the strains from West Bengal have shown a different pattern with 14.6% (7/48) of the strains that were resistant to polymyxin B giving a similar result to that of the standard El Tor strain N16961 (Table 1).

Majority of the *V. cholerae* O1 strains contained Haitian genetic alleles of virulence gene markers

All the *V. cholerae* O1 strains from Western India, that is from Gujarat and Maharashtra were found to carry the Haitian alleles of *ctxB* (*ctxB7*), *tcpA* (*tcpA*^{CIRS}) and *rtxA* (Haitian type), which are associated with the hyperexpression of cholera toxin and higher colonization ability (Ghosh et al., 2019; Naha et al., 2020). But the strains of West Bengal showed a different result. Of 48 strains, 14.6% (7/48) of them showed nonhemolytic and polymyxin B resistance and were found to contain *ctxB* gene of classical type (*ctxB1*) along with the El Tor *rtxA*. Other 85.4% (41/48) strains that showed polymyxin B-sensitive characteristics, contained Haitian-type alleles of *ctxB* and *rtxA* similar to the strains from the Western region. It is noteworthy that all the Kolkata (West Bengal) strains carried the Haitian *tcpA* allele, irrespective of *ctxB* and *rtxA* allelic differences (Table 3).

TABLE 3 Major virulence genetic attributes of the *Vibrio cholerae* O1 strains in this study

			Genetic characteristics							
			ctxB (%)		tcpA (%)		rtxA (%)		VSP-II type (%)	
No.	Place of isolation	No. of isolates (*n*)	Classical	Haitian	El tor	Haitian	El tor	Haitian	VSP-IIC	Other VSP-II type
1	Maharashtra	22	0	100	0	100	0	100	100	0
2	Gujarat	65	0	100	0	100	0	100	100	0
3	Kolkata	48	14.6	85.4	0	100	14.6	85.4	85.4	14.6

Presence of El tor-specific genetic markers

In addition to the conventional phenotypic characteristics, the classical and El Tor biotypes are also distinguished by several genetic characteristics such as the CTXΦ integration in chromosome II, the presence of *rtxC*, *rstC* and *ctxB* and *rstR* alleles. The seventh pandemic El Tor strains were comprised of a unique feature of the absence of CTX prophage in chromosome II (Heidelberg et al., 2000; Maiti et al., 2006), whereas the classical biotype has CTX prophages in both chromosomes. Our result showed that all the strains did not harbour CTX prophage in their chromosome II. Further, El Tor strains comprise the genes *rtxC*, *rstC* and El Tor-specific *rstR* allele (Kimsey & Nair, 1998; Nusrin et al., 2004). Conversely, the classical biotype does not have *rtxC*, *rstC* genes, but contained classical *rstR*. All the strains from Gujarat, Maharashtra and West Bengal harboured the genes *rtxC*, *rstC* and El Tor-specific *rstR* like the typical El Tor biotype.

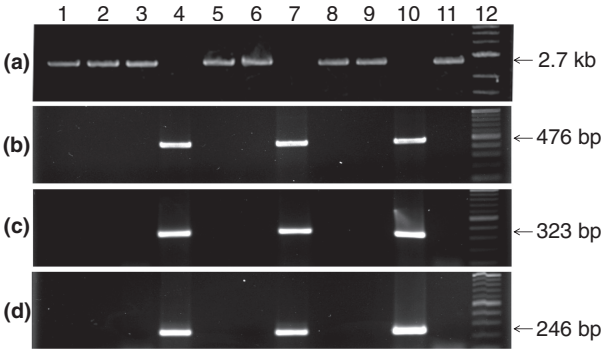

FIGURE 1 Polymerase chain reaction (PCR) assay using primer pair VC0494F/VC0513R to check the deletion of genes *VC0495–VC0512* in the VSP-II region. This deletion is a remarkable feature of Yemen cholera outbreak strains (a). PCR for the presence of loci *VC0502* (b), *VC0506* (c) and *VC0510* (d) of the VSP-II region. Lanes 1–9 represent the clinical isolates YV/2018/01, YV/2018/10, YV/2018/20, IDH11764, BR/2018/08, SR/2018/10, IDH12026, SR/2018/40 and IDH11473; lane 10, N16961; lane 11, YMN-170240 (Yemen outbreak strain) and lane 12, 1 kb size ladder (for panel a) or 100 bp size marker (for panels b, c and d) (New England Biolab Inc., Beverly, MA, USA).

Deletion of a large fragment in the VSP-II region revealed similarity with Yemeni strains

The 2016–2018 Yemen cholera outbreak has been marked as the world's worst outbreak caused by *V. cholerae* O1 El Tor typified by several genetic changes, including a deletion of a large genomic fragment (including genes *VC0495–VC0512*) in the VSP-II region (Weill et al., 2019). A newly developed PCR assay in the present study confirmed that all the *V. cholerae* O1 strains from Maharashtra and Gujarat and 85.4% (41/48) of the West Bengal strains contained a deletion in the VSP-II region as the PCR yielded an amplicon of 2.7 kb in size (Figure 1a). The Yemeni strain used as a positive control also produced a 2.7 kb product. Based on the similarity in amplicon size with the VSP-II of the strains characterized by Imamura and colleagues it can be proposed that the strains of this study have partially deleted VSP-II and possessed VSP-IIC type (Imamura et al., 2017). The standard strain N16961 which has the ~27 kb intact VSP-II region (VSP-IIA) gave no band as the PCR condition applied was inadequate to amplify this large fragment. Similar to strain N16961, 14.6% (7/48) of the strains from West Bengal gave no band in this PCR (Table 3). This might have been due to the presence of VSP-II regions other than the VSP-IIC type (VSP-IIA or B). The strains with negative results were further confirmed for the presence of the representative genes *VC0502*, *VC0506* and *VC0510* of the VSP-II region located between the genomic region *VC0494* and *VC0512* and found to yield bands in the PCR assays (Figure 1b–d). The presence of *VC0502*, *VC0506* and *VC0510* genes confirmed that 14.6% of the West Bengal strains harboured VSP-IIA or B type.

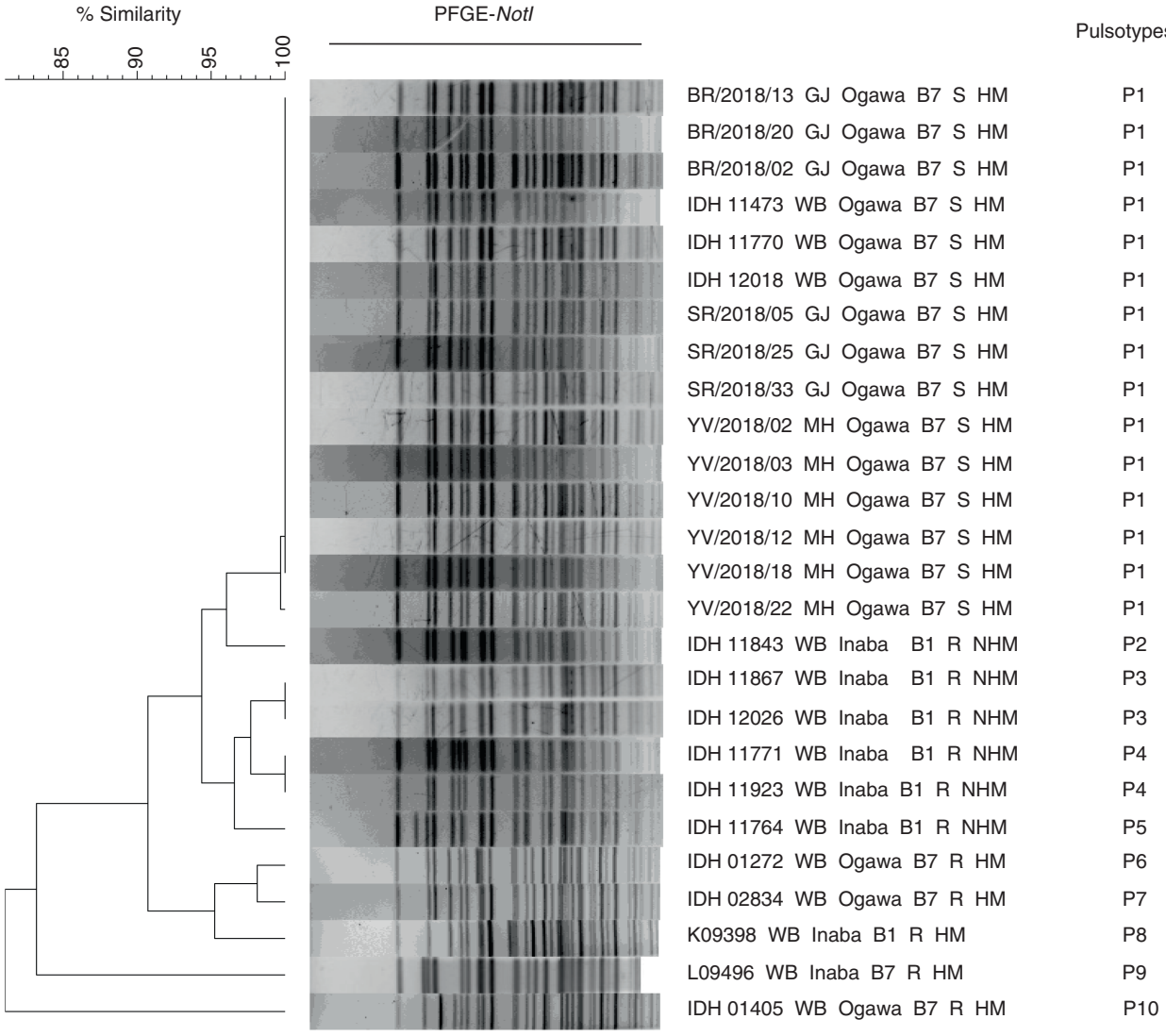

FIGURE 2 Pulse-field gel electrophoresis patterns of *Not*I-digested genomic DNA of *Vibrio cholerae* O1 strains isolated from Gujarat, Maharashtra and West Bengal during 2018. The gel image was analysed using BioNumerics version 4.0 software (Applied Maths) based on the single-linkage method to generate the dendrogram. Per cent similarity is shown on the upper left side. Polymyxin B-sensitive, hemolytic, Haitian *ctxB*-containing Ogawa strains formed a single cluster, whereas polymyxin B-resistant, nonhemolytic, classical *ctxB*-carrying Inaba strains were found to form different banding patterns. Strain IDs were mentioned at the end of each lane followed by the place of isolation, serotype, *ctxB* genotype, polymyxin B susceptibility and hemolysis pattern. WB, West Bengal; GJ, Gujarat; MH, Maharashtra; B1, classical *ctxB*; B7, Haitian *ctxB*; R, polymyxin B-resistant; S, polymyxin B-sensitive; NHM, nonhemolytic; HM, hemolytic.

PFGE analysis showed a different pulsotypic pattern among the nonhemolytic isolates

To determine the genetic relatedness of the El Tor non-hemolytic strains with the hemolytic strains, a total of 21 representative strains covering all three states were analysed. Dendrogram analysis showed five different pulsotypes with an overall similarity of 95% (Figure 2). Hemolytic strains clustered in the pulsotype P1, whereas, nonhemolytic strains showed four different pulsotypic patterns, P2, P3, P4 and P5. Hemolytic El Tor strains (P1) were belonged to Ogawa serotype, sensitive to polymyxin B and harboured Haitian *ctxB* (*ctxB7*) allele. In contrast, nonhemolytic strains of this study (P2–P5) were identified as Inaba serotype, polymyxin B-resistant and possessed classical *ctxB* (*ctxB1*) genotype. Almost 96% similarity was observed between the pulsotypes P1 and P2, whereas P3, P4 and P5 pulsotypes which consist of nonhemolytic strains shared 94% genomic identity with P1 and P2. Among the 5 major pulsotypes (P1–P5), P1 is predominated by 15 Ogawa serotype strains from Gujarat, Maharashtra and West Bengal. Interestingly, the other four pulsotypic variants (P2–P5) consisted of the 6 strains of West Bengal among which genomic differences exist and belong to the Inaba serotype (Figure 2). To determine the possible

genomic relatedness of the strains in this study with the Kolkata (West Bengal) isolates that are closely related to the Yemen cholera outbreak strains, another five strains (isolated during 2005–2010) were included in the PFGE analysis. These five strains from Kolkata were used by Weill et al. (2019) for the detection of phylogenic association with Yemeni strains. Our results showed that five strains belong to five different pulsotypes (P6-P10) which commonly shared almost 81% identity with the 21 representative isolates of the current study included in the PFGE analysis (Figure 2).

Antimicrobial resistance

All the strains showed resistance to nalidixic acid. About 99% (82/83) and 96% (80/83) of the strains were resistant to streptomycin and sulfamethoxazole-trimethoprim, respectively. Resistance to ampicillin has been found in 25% (21/83) of the strains, but is not predominant in any specific state. Tetracycline resistance (10.8%, 9/83) was seen in strains from Gujarat and West Bengal, whereas chloramphenicol resistance (3.6%, 3/83) was restricted in strains from Maharashtra and West Bengal only. Sensitivity and/or reduced susceptibility were found for the antibiotics ceftazidime, cefotaxime, ceftriaxone, ciprofloxacin, norfloxacin, ofloxacin, gentamycin, azithromycin and doxycycline in the isolates of all the states (Figure 3).

DISCUSSION

Cholera causes devastating outbreaks affecting almost 4 million people and 21,000 deaths worldwide in a year (Ganesan et al., 2020). Outbreaks of cholera are often reported in South Asia and in many African countries (Griffith et al., 2006). *Vibrio cholerae* frequently changes its genetic conformation that perhaps helping the organism to increase its pathogenicity and spread into other cholera endemic regions (Lekshmi et al., 2018). Historically, the disease-causing organism *V. cholerae* persisted effectively through successive changes with classical, El Tor and O139 strains and also by the El Tor biotype harbouring the classical *ctxB*. Several molecular studies have further shown the progression of several changes in the genomes of *V. cholerae* O1 characterized by the acquisition of classical biotype markers in the El Tor biotype, which are considered epidemiologically important (Bhandari et al., 2021).

Molecular studies with the recent *V. cholerae* O1 strains from different cholera endemic regions of India have been reported previously, but the regions of Western India have not been focused on in many earlier studies (Samanta et al., 2018). A large number of cholera cases emerged in western parts of India in 2018 and we have received a total of 87 strains for confirmation and further analysis. These typical El Tor strains remained susceptible to polymyxin B similar to that of our report (Samanta et al., 2015). Moreover, El Tor variant strains harbouring Haitian *ctxB* and *tcpA* alleles expressed higher virulence (Ghosh et al., 2019; Naha et al., 2020). *Vibrio cholerae* O1 El Tor that has caused a huge cholera outbreak in Yemen during 2016–18 with a high case-infection ratio and the strains possessed several unique genetic features as mentioned before along with a deletion in the VSP-II region (Weill et al., 2019). Since the deletion of this VSP-II region in the seventh pandemic El Tor vibrios is an uncommon feature, we analysed all the recent El Tor vibrios in this study. Interestingly, all of the strains from western India have the same deletion in VSP-II region indicating the similarity with the Yemeni *V. cholerae* strains. Whereas the West Bengal strains had both the forms of VSP-II. This supports the observation reported by Imamura and colleagues (Imamura et al., 2017) and later found in Yemen outbreak strains, indicating the presence of four different types of VSP-II region. Our PCR results signify VSP-II fragment deletion that corresponds to the VSP-IIC type.

As observed in this study, depending on the genetic and phenotypic characteristics, *V. cholerae* strains can be categorized into two groups. The first group includes the strains with Inaba serotype, which are nonhemolytic, polymyxin B resistance with VSP-II other than VSP-IIC type, classical *ctxB* and El Tor *rtxA*. These strains possibly emerged from the same lineage that prevailed in Kolkata during 2004–2010 (Ghosh, Naha, Basak, et al., 2014; Naha et al., 2012) and gained a classical biotype trait, that is nonhemolytic. The second group comprises strains with Ogawa serotype, which are hemolytic, polymyxin B-sensitive with partially deleted VSP-II region (VSP-IIC), and with Haitian *ctxB* and *rtxA*. Strains of this group share a close relationship with the lineage that has caused the devastating outbreak in Yemen (Morita et al., 2020). Remarkably, the strains of the first group emerged as nonhemolytic phenotypes like classical biotype, which has been found for the first time in Indian El Tor strains.

In *V. cholerae*, *hlyA* is a mainly responsible gene for the hemolytic activity. The difference in the phenotypic properties of hemolysin as observed in this study may be due to genetic modification such as the presence or absence of the *hlyA*, mutations in the *hlyA* and the differential expression pattern of this gene. The presence of the type I secretion system has also been reported to be associated with the hemolytic trait of *V. cholerae* (Boardman & Fullner Satchell, 2004). Currently, we are investigating the contributing factor(s) for this phenotypic shift and their role in the pathogenesis of recent *V. cholerae* O1 strains.

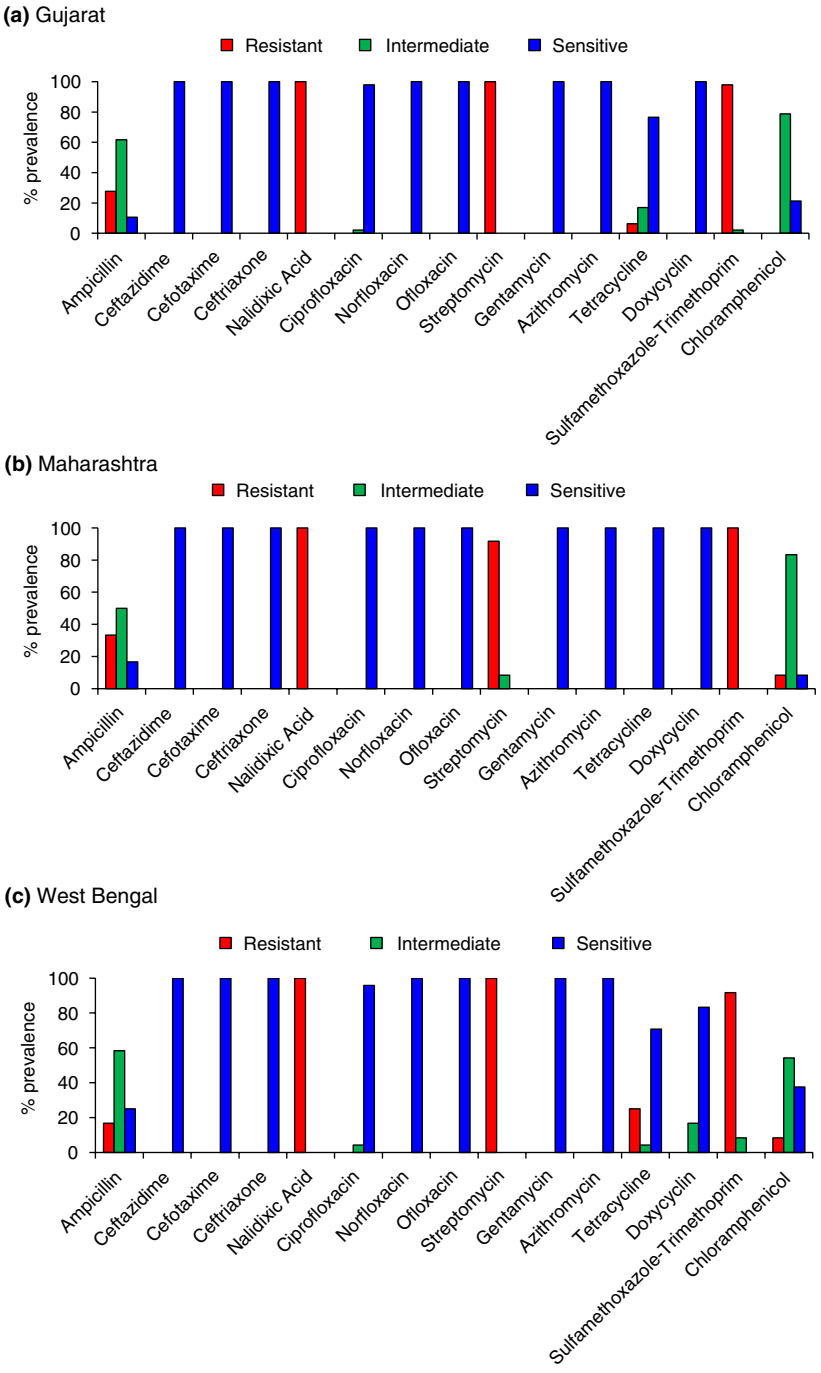

FIGURE 3 Antibiotic susceptibility patterns of *Vibrio cholerae* strains from Gujarat (a), Maharashtra (b) and West Bengal (c) by Kirby-Bauer disc diffusion method using the commercially available discs of the commonly used antibiotics.

Recent reports suggested that the strains that caused Yemen cholera outbreak during 2016–2018 originated in South Asia and mostly from the Indian subcontinent and then spread through Africa from 2014 to Yemen in 2016 (Benamrouche et al., 2022; Mutreja & Dougan, 2020; Weill et al., 2019). The genotypic and phenotypic features of these Indian isolates were similar to the isolates of this study. Moreover, we found that the Kolkata strains (isolated during 2005–2010) used to compare the phylogeny with the African, American, Asian and Middle East isolates (Weill et al., 2019) have a similar PFGE pattern (81% similarity) with the current isolates tested in this study (Figure 2). Therefore, it is evident from the reports that the isolates of this study might have shared a common ancestral origin with the South Asian isolates that caused the Yemen and African cholera outbreaks in recent years.

In *V. cholerae* O1, antibiotic resistance is one of the important phenotypic traits in differentiating the biotypes and also in effectively managing the cholera infection. Resistance to streptomycin, nalidixic acid and sulfamethoxazole-trimethoprim is the common feature of almost all seventh pandemic El Tor vibrios and can be classified as multidrug-resistant (MDR) strains (Dey et al., 2014). This is probably due to the presence of mobile

genetic elements from the other species such as SXT gene clusters and/or the integrative/conjugative elements (Mohammed et al., 2018). Among the fluoroquinolone antibiotics, ciprofloxacin is recommended as the drug of choice to treat cholera (Rashed et al., 2017). In Bangladesh, *V. cholerae* is increasingly becoming resistant to this antibiotic (Khan et al., 2015; Rashed et al., 2017). Generally, antibiotic susceptibility and bacterial virulence may have positive or negative or altogether no correlation depending upon several factors, including the type of bacterial species, specific virulence, unique/collective resistance mechanism(s), environment and the host (Beceiro et al., 2013). For example, antimicrobial resistance, either by mutation or acquisition of resistance determinants harboured by mobile genetic elements, may confer a biological cost for the bacteria. This fitness cost can affect the growth rate in vitro or the survival in the host or in the environment or the virulence function. However, bacteria can evolve and adapt to reduce this cost, by compensatory mutations or fine regulation of resistance expression (Brink, 2014). In contrast, the presence of resistance, nodulation and division (RND) genes in *V. cholerae* confers antimicrobial resistance by pumping out multiple antibiotics (Bina et al., 2018) and also enhances the virulence by increasing transcription of the virulence regulators TcpP and ToxT. On the other hand, the quinolone-modifying enzymes, which are responsible for quinolone resistance, seem no association with the virulence of the bacteria (Beceiro et al., 2013).

The antimicrobial resistance of current *V. cholerae* O1 strains has also diverged from the patterns reported in the past. As observed in the present study, reduced susceptibility towards ciprofloxacin/doxycycline has been reported in several states in India (Narendrakumar & Thomas, 2018). It was also reported that the susceptibility of *V. cholerae* to tetracycline is decreasing in many countries (Das et al., 2011; Garg et al., 2000; Kuma et al., 2014; Mandal et al., 2012; Sjölund-Karlsson et al., 2011). Changing AMR pattern forecasts future resistance strains towards these useful antimicrobials. Considering several genomic changes in *V. cholerae*, an active and holistic surveillance system is required in cholera endemic areas to adapt better disease management and to prevent the spread of any outbreaks.

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CONFLICT OF INTEREST

The authors declared that they have no conflict of interest.

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BACTERIAL INFECTIONS

A Point Mutation in *carR* Is Involved in the Emergence of Polymyxin B-Sensitive *Vibrio cholerae* O1 El Tor Biotype by Influencing Gene Transcription

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ABSTRACT Antimicrobial peptides play an important role in host defense against *Vibrio cholerae*. Generally, the *V. cholerae* O1 classical biotype is polymyxin B (PB) sensitive and El Tor is relatively resistant. Detection of classical biotype traits like the production of classical cholera toxin and PB sensitivity in El Tor strains has been reported in recent years, including in the devastating Yemen cholera outbreak during 2016-2018. To investigate the factor(s) responsible for the shift in the trend of sensitivity to PB, we studied the two-component system encoded by *carRS*, regulating the lipid A modification of El Tor vibrios, and found that only *carR* contains a single nucleotide polymorphism (SNP) in recently emerged PB-sensitive strains. We designated the two alleles present in PB-resistant and -sensitive strains *carR*^{r} and *carR*^{s} alleles, respectively, and replaced the *carR*^{s} allele of a sensitive strain with the *carR*^{r} allele, using an allelic-exchange approach. The sensitive strain then became resistant. The PB-resistant strain N16961 was made susceptible to PB in a similar fashion. Our *in silico* CarR protein models suggested that the D89N substitution in the more stable CarR^{s} protein brings the two structural domains of CarR closer, constricting the DNA binding cleft. This probably reduces the expression of the *carR*-regulated *almEFG* operon, inducing PB susceptibility. Expression of *almEFG* in PB-sensitive strains was found to be downregulated under natural culturing conditions. In addition, the expression of *carR* and *almEG* decreased in all strains with increased concentrations of extracellular Ca^{2+} but increased with a rise in pH. The downregulation of *almEFG* in CarR^{s} strains confirmed that the G265A mutation is responsible for the emergence of PB-sensitive El Tor strains.

KEYWORDS El Tor biotype, *Vibrio cholerae*, *almEFG*, *carR*, cholera, polymyxin B

Cholera, a life-threatening disease, is still present in developing countries, where people do not have a good sanitary system, good hygiene practices, and a safe drinking water supply (1). The Haitian outbreak during 2010 provided direct evidence of these health-related factors (2). Recently, Yemen, a country in Western Asia, experienced its worst cholera outbreak, which affected more than 1.1 million people. The high infection rate (69%) has placed the disease at the top of the public health agenda (3). The Gram-negative motile bacterium *Vibrio cholerae* O1 is the main cause of the disease and was responsible for the seven pandemics in the recorded history of cholera. The first six pandemics were presumed to be caused by the *V. cholerae* O1 classical biotype, and the current (seventh) pandemic is due to the El Tor biotype (1, 4, 5).

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PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Deciphering the possible role of *ctxB7* allele on higher production of cholera toxin by Haitian variant *Vibrio cholerae* O1

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Abstract

Cholera continues to be an important public health concern in developing countries where proper hygiene and sanitation are compromised. This severe diarrheal disease is caused by the Gram-negative pathogen *Vibrio cholerae* belonging to serogroups O1 and O139. Cholera toxin (CT) is the prime virulence factor and is directly responsible for the disease manifestation. The *ctxB* gene encodes cholera toxin B subunit (CTB) whereas the A subunit (CTA) is the product of *ctxA* gene. Enzymatic action of CT depends on binding of B pentamers to the lipid-based receptor ganglioside G_{M1}. In recent years, emergence of *V. cholerae* Haitian variant strains with *ctxB7* allele and their rapid spread throughout the globe has been linked to various cholera outbreaks in Africa and Asia. These strains produce classical type (WT) CTB except for an additional mutation in the signal sequence region where an asparagine (N) residue replaces a histidine (H) at the 20th amino acid position (H20N) of CTB precursor (pre-CTB). Here we report that Haitian variant *V. cholerae* O1 strains isolated in Kolkata produced higher amount of CT compared to contemporary O1 El Tor variant strains under *in vitro* virulence inducing conditions. We observed that the *ctxB7* allele, itself plays a pivotal role in higher CT production. Based on our *in silico* analysis, we hypothesized that higher accumulation of toxin subunits from *ctxB7* allele might be attributed to the structural alteration at the CTB signal peptide region of pre-H20N CTB. Overall, this study provides plausible explanation regarding the hypertoxigenic phenotype of the Haitian variant strains which have spread globally, possibly through positive selection for increased pathogenic traits.

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Journal of Applied Microbiology

ORIGINAL ARTICLE

Characterization of diarrhoeagenic *Escherichia coli* with special reference to antimicrobial resistance isolated from hospitalized diarrhoeal patients in Kolkata (2012–2019), India

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Abstract

Aims: This study analyses the prevalence and antimicrobial resistance (AMR) of major diarrhoeagenic *Escherichia coli* (DEC) pathotypes detected in hospitalized diarrhoeal patients in Kolkata, India, during 2012–2019.

Methods and Results: A total of 8891 stool samples were collected from the Infectious Diseases Hospital, Kolkata and screened for the presence of enteric pathogens. Multiplex PCR identified the presence of DEC in 7.8% of the samples, in which ETEC was most common (47.7%) followed by EAEC (38.4%) and EPEC (13.9%). About 54% cases were due to sole DEC infections. Majority of the mixed DEC infections were caused by the *Vibrio* spp. (19.1%) followed by Rotavirus (14.1%) and *Campylobacter* spp. (8.4%). ETEC and EAEC were associated significantly with diarrhoea in children <5 years of age, whereas EPEC and also ETEC were prevalent in patients aged between 5 and 14 years. AMR profile showed high prevalence of multidrug resistance (MDR) among DEC (56.9%) in which 9% were resistant to antibiotics of six different antimicrobial classes. Screening of the AMR conferring genes of DEC showed the presence of $bla_{\text{CTX-M3}}$ (30.2%) in highest number followed by bla_{TEM} (27.5%), *tetB* (18%), *sul2* (12.6%), *strA* (11.8%), *aadA1* (9.8%), $bla_{\text{OXA-1}}$ (9%), *dfrA1* (1.6%) and bla_{SHV} (1.2%).

Conclusions: These findings highlighted the high prevalence of MDR in major DEC pathotypes that could be considered as the leading aetiological bacterial agent responsible for diarrhoea and suggests a significant public health threat.

Significance and Impact of the Study: The results of this study can help to improve the understanding of the epidemiology of DEC infections in patients with diarrhoea. Monitoring of AMR surveillance needs special attention because the DEC isolates were highly resistant to commonly used antimicrobials in the treatment of diarrhoea.

KEYWORDS

antimicrobial resistance, diarrhoeagenic *Escherichia coli*, EAEC, EPEC, ETEC

