PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF *VIBRIO CHOLERAE* CAUSING 2012 DIARRHEAL OUTBREAK IN KATHMANDU

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Date: 9th June, 2013 **Surendra Raj Sharma**

LIST OF ABBREVIATIONS

UNICEF	:	United Nations Children's Fund
WHO	:	World Health Organization
NRCS	:	Nepal Red Cross Society
EDCD	:	Epidemiology Department and Disease Control Division
ET	:	Altered EI Tor
CL	:	Classical
PFGE	:	Pulsed Field Gel electrophoresis
SNP	:	Single Nucleotide Polymorphism
OmpW	:	Outer membrane protein
rfbO1	:	Somatic O antigen coding gene
rtx	:	Repeat in toxin
tcp	:	Toxin co-regulated Pilli
ctxB	:	Cholera Toxin B Subunit
tlc	:	Toxin linked cryptic plasmid
VSP	:	Vibrio Pathogenicity Island
NDDIC	:	National Digestive Diseases Information Clearinghouse
WGO	:	World Gastroenterology organization
HIV	:	Human Immuno Virus
CT	:	Cholera toxin
KDa	:	Kilo dalton
ADP	:	Adenosine Diphosphate
ADPR	:	ADP-Ribose
NAD	:	Nicotinamide Adinene Dinucleotide
AMP	:	Adenosine Mono Phosphate
cAMP	:	Cyclic Adenosine Mono Phosphate

PCR	:	Polymerase Chain Reaction
TCBS	:	Thiosulfate Citrate Bile Sucrose Agar
CCA	:	Chicken Cell Agglutination
PB	:	Polymyxin B
VP	:	Vogeus Praskeur Test
AFLP	:	Amplified Fragment Length polymorphism
DMAMA	:	Double Mismatch Amplification Mutation Assay
AGE	:	Acute Gastro Enteritis
ICDDRB	:	International Center for Diarrheal Disease Research
APW	:	Alkaline Peptone Water
µl	:	Micro liter
µg	:	Microgram
mg	:	Milligram
NA	:	Nutrient Agar
CIP	:	Ciprofloxacin
Rpm	:	Rate per minute
Min	:	Minute
d/w	:	Distilled water
PBS	:	Phosphate Buffer Saline
EDTA	:	Ethylene Diamine Tetracetic Acid

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ABSTRACT

Cholera is characterized by perfuse watery diarrhea which removes essential body fluid, nutrients leading to a condition of dehydration and malnutrition and often proved to be fatal if not treated. It is one of the common among diarrheal diseases and is emerging as a huge health burden in developing countries like Nepal. Etiological agent of Cholera namely *Vibrio cholerae*, in current scenario is transforming itself in to various forms and variants gaining novel pathogenic attribute and multidrug resistant property creating hurdles in clinical management. This research primarily aims to analyze genetic diversity, characterize *V. cholerae* strains prevalent in 2012 Kathmandu diarrheal outbreak according to various phenotypic, serological, genetic markers and pulsotyping. In this research probable Cholera samples were collected from diarrheal patients and *V. cholerae* strains were identified and screened using recent, precise microbiological, serological and molecular assays. Thus screened strains were further subjected to phenotypic tests, antibiotic susceptibility test, PCR based genotyping assays and Pulsed Field Gel Electrophoresis. Additionally each patient was directly interviewed for clinical history as well demographic information. Among selected highly probable Cholera samples (N=72) *V. cholerae* O1 (32%), *V. cholerae* non O1 (7%) and non specified pathogens (61%) were found. Similarly antibiotic susceptibility profile of selected strains identified that all isolated *V. cholerae O1* strains were 100 % resistant to Trimethoprim/Sulfamethoxazole (SXT) ,Nalidixic Acid (NA); Streptomycin and 100 % susceptible to Erythromycin; Gentamicin; Tetracycline, Ampicillin ,Azithromycin and Ciprofloxacin. Combined result from phenotypic and PCR based genotypic assays showed that *V. cholerae* O1 strain possessed Cholera toxin (ctxAB),rfbO1 O1 specific antigen, toxin coregulated pilli allele specific to ET Tor biotype $tcpA^{ET}$, Regulatory elements of Ctxϕ namely $rstR^{ET}$and rtxC, Toxin linked cryptic plasmid (tlc). Another genotyping test based on Double Mismatch Amplification Mutation Assay (DMAMA-PCR) assay also identified a variation pattern in ctxB gene ,which categorized these strains in to ctxB genotype 7 and revealed that these strain carried point mutation at 20^{th} amino acid position of ctxB sequence which caused change in code for amino acid (Histidine→Asparagine,(58^{th} nucleotide change C →A)). Further, PFGE pulsotype analysis of selected strains and control strain N19691 predicted 100% correlation among them and 90% correlation with N19691, hence suggesting all strains to be of seventh pendamic EI Tor ancestry and are of same clonal origin.

Keywords: Cholera, EI Tor, antibiotic susceptibility, ctxB genotype, PFGE, clonal

INTRODUCTION

1.1 Background

Every year there are about two billion cases of diarrheal disease estimated worldwide (WHO,2012). Diarrheal disease, which mostly results from contaminated food and water sources, is a leading cause of mortality and morbidity in the world. Among diseases causing death diarrheal disease holds fourth position in ranking (www.lifeexpectancy.com). Worldwide, around 1 billion people lack access to improved water and 2.5 billion have no access to basic sanitation (UNICEF, 2009). Diarrhea due to infection is widespread throughout the developing countries. In 2004, diarrheal disease was the third leading cause of death in low-income countries, causing 6.9% of deaths overall. In children under five years old, diarrheal disease is the second leading cause of death next to pneumonia. Out of the 1.5 million children killed by diarrheal disease in 2004, 80% were under two years old. (WHO and UNICEF, 2009).

In developing countries, children under three years old experience an average three episodes of diarrhea every year (UNICEF, 2009). Nepal, is one amongst country where infant and child mortality rate is high (Wang *et al*., 2011; Thapar *et al*., 2004). Diarrhea is the leading cause of mortality and morbidity in Nepal. Because of high incidence, diarrheal cases are increasing in Nepal by 20-50% (Water Aid, 2008; Dixit and Maskey, 2012). Although government and various organizations have been implementing diarrhea control program, it still remains a major public health problem (Karki *et al*., 2009 ; NRCS, 2012).

Globally, Cholera incidence has increased steadily since the beginning of the millennium with Cholera outbreaks persisting in Sub-Saharan Africa and Asia. Cholera continues to pose a serious public health problem among developing world (Thapar *et al.*, 2004), in populations which lack access to adequate water resources and sanitation (WHO, 2012; UNICEF, 2009; Pawlowski *et al.,* 2010). In 2011, 32% of cases were reported from Africa whereas between 2001 and 2009, 93% to 98% of total cases worldwide were reported from that continent; in that time Asia was also unremittingly affected from various outbreaks of diarrhea (WHO, 2012). Cholera is believed to be originated in South Asia specifically in Ganges River during 18^{th} century however its presence in very ancient period of Buddha and Hippocrates has also been documented in some historical books (Maheshwori *et al.*, 2011).

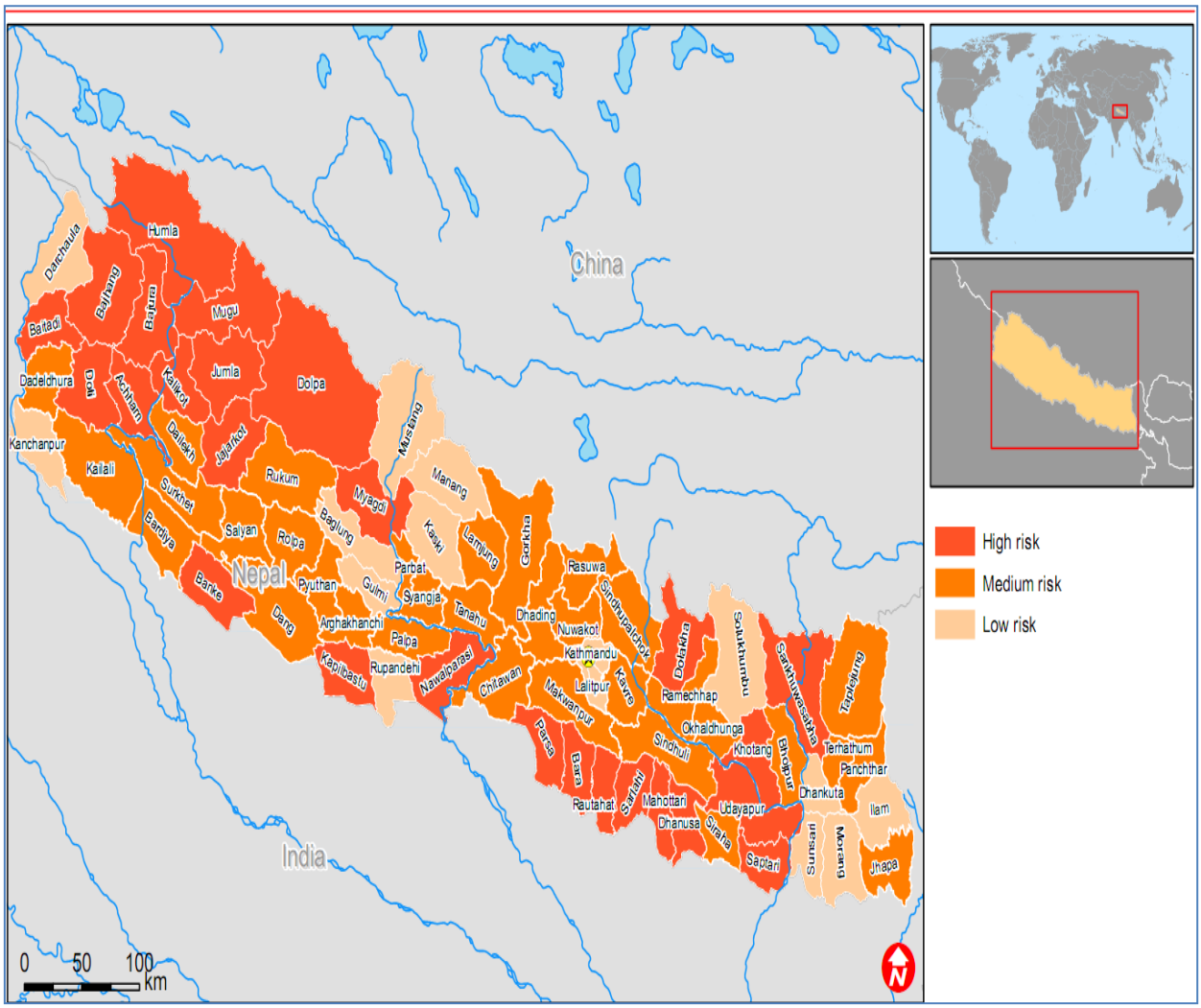


Figure 1: Map illustrating risk zones of diarrhea throughout Nepal (Adapted from: Nepal Red Cross Society appeal, 2010 "Potential diarrhea outbreak.")

Source of origin of Cholera in Nepal is virtually unknown however it is believed that its emergence and spreading occurred in Nepal from mid of 19th century. Few incidences of parallel outbreaks with similar nature in near region of India during that time supports the speculation which says, Cholera might probably introduced in Nepal via people travelling from India. After first emergence of diarrheal outbreak associated with Cholera in Nepal (Pillizer *et al.*, 1958), continuously many outbreaks were seen in various time periods of past 50 years. With the start of rainy season diarrheal outbreak becomes vicious problem and affects thousands of people in Nepal, in which Cholera holds prime position. Since 2007, Nepal is continuously facing diarrheal outbreaks for the past 5 years in many places, affecting high number of population. Immense focus has been made because of massive threat created by death as a consequence of diarrheal disease. Various unpublished reports from Epidemiology and Disease Control Division (EDCD),

Nepal; depicts that in past years 2009-2011 about more than 74767 diarrheal cases were reported along with death of 441 people. In those diarrheal burdens number of districts affected by diarrhea was 18 in average each year. In 2009, sudden emergence of diarrheal epidemic in most of the districts of mid-western and far-western regions of Nepal affected more than 5,000 peoples. Among them 340 people lost their life. Calculated attack rate and Case fatality ratios were found to be 8.2% and 1% respectively for Jajarkot district during that time. Various studies conducted in that time concluded Cholera as prime cause for mortality in Jajarkot, diarrheal outbreak (Bhandari *et al.*, 2009). This fact threatened Nepal via its possible emergence of Cholera as outbreak in other areas as well with the start of rainy season. Talking about 2009 epidemic of Jajarkot district alone, more than 100 people died in just four months, affecting over 400 people (EDCD/N, 2009 unpublished report; Dixit and Maskey, 2012).

Cholera in Kathmandu was first seen in 1958 (Pillizer *et al.*, 1958). Over the past 50 years many diarrheal outbreaks were reported in Kathmandu and among the various forms of diarrhea Cholera holds major position. Every year various Cholera cases in the form of sporadic cases and diarrheal outbreak are reported in Kathmandu. Review of past research works carried on diarrhea in Kathmandu depicted that there is high incidence of *Vibrio cholerae* (Karki *et al.*, 2010; Shrestha *et al*., 2010). Kathmandu a highly populated city carries a huge potential of diarrheal outbreak and sporadic cases in rainy season because of contamination in food and water supply system. Beside these during diarrheal outbreaks emergence of multidrug resistance pathogens is also in increasing trend (Shrestha *et al.*, 2010). Hence there is necessity of continuous monitoring and research in this area. Particularly talking about *V. cholerae*, various studies conducted in antibiotic surveillance all over Nepal in past years identified strains possessing resistant property towards highly recommendable drugs specifically Tetracycline, Nalidixic acid, Ciprofloxacin, Cotrimoxazole (Shyakya *et al.*, 2012).

In the present context because of constantly changing genetic behavior and nature of Cholera associated pathogen namely *V. cholerae* diarrhea management is problematic (Safa *et al,* 2008) .This research in the current time aims to apply various methodology to best identify, characterize genotype based on molecular markers as well to predict and compare genetic diversity among the strain isolated and reference strain. Similarly analysis of pulsotype, phenotypic and genotypic feature of the isolated strain will depict current position of prevalent strain in evolutionary lineage as well as diversity among strain isolated. Conclusively various results and outcomes of this research are expected to provide valuable information to scientific community and policymakers to facilitate Cholera management.

1.2 Justification of study

Nepal, in spite of being a small country carries huge health burden. Diarrheal outbreak is considered as a persistent health problem in developing countries. Nepal being one among the developing countries is no longer safe from these health consequences. Every year in Nepal diarrheal outbreak occurs and appropriate management of such situation is quite problematic due to various limitations, among them one of prime reason remains to be lack detection and identification of exact etiological agents causing diarrheal outbreak (Dixit and Maskey, 2012).

As far as current seventh epidemic in Asia and Africa is concerned, previous *Vibrio cholerae* ET have been replaced by a new hybrid strain belonging to ET possessing Cholera toxin of classical biotype. It has been remarked that Asian strains of Cholera posses novel phenotypic and genotypic attributes in recent outbreaks (Nair *et al*., 2002). Since 2001, these hybrids strains of Cholera of both CL and ET biotypes are continuously seen and isolated in various outbreaks from clinical cases in Asia and Africa (Safa *et al.*, 2008).These reported strains are found to be new pathogen capable of causing fatal as well as serve diarrhea (Siddique *et al*., 2010). Recent Cholera outbreak in Haiti 2010 blamed Nepal for its origin moreover its perfect justification of its connection has not been confirmed but strains seen in that outbreak were quite similar to strains prevalent during that time in Nepal, according to some phenotypic, genotypic attributes and Pulsed field gel electrophoresis pattern (Rene. *et al.*, 2011; Hasan *et al.*, 2012). Globally, in context of increasing drug ineffectiveness of antimicrobial towards the *V. cholerae* because of gene transfer and other intrinsic mechanism (Kikota *et al*., 2011) Cholera management is quite problematic. This fact in current time depicts that Nepal is no longer far from this consequences. Hence it is worth to study antibiotic susceptibility trends of currently prevalent strains. On the other hand trend of change in *V. cholerae* genetic elements over the past year (Alam *et al.;* 2012) also stress out the fact that it is necessary to study genetic diversity among strains prevalent in outbreak and with reference strains of origin to identify genetic differences and relatedness.

For perfect management of any diarrheal case in-depth lab analysis and identification of etiological agent is essential. Nepal is still depending on classical methods of diagnosis and identification. Review of past studies regarding diarrheal investigation conducted in Nepal gives us glimpses that all efforts of studies are solely focused on either culture or study of epidemiological factors. To overcome every possible limitation here we are combining all these methods with highly optimized molecular diagnosis and genotyping methods which are expected to provide details of etiological agents of diarrheal outbreak focusing on *V. cholerae*.

1.3 Objectives

1.3.1 General objective

To characterize *V. cholerae* according to genotype, genetic elements and genetic diversity causing 2012 diarrheal outbreak in Kathmandu.

1.3.2 Specific Objective

- To select a newer, fast, cheaper, reliable, rapid, specific, sensitive and optimized method for isolation, detection and genotyping of *V. cholerae* from clinical samples.
- To select best optimized method for isolation of *V. cholerae* DNA from diarrheal samples.
- To characterize *V. cholerae* according to their serotype from samples isolated using highly specific serological test.
- To perform molecular characterization and genotyping based on Polymerase chain reaction assays.
- To perform Pulsed Field Gel Electrophoresis to retrieve information about diversity and clonal attributes among isolated strains.
- To analyze possible route of origin and genetic similarity among sample isolated.
- To compare isolated strains with related strains from south Asia and beyond with reference to serological, genetic elements and features.
- To analyze symptomatic relation, epidemiological, demographic features associated with Cholera as well as its distribution and relation with genetic elements of isolated strains.

1.4. Rationale and scope of study

It is well known fact that constantly emerging threat of Cholera in the form of outbreak and sporadic fashion in Nepal is hot issue and major problem in health sector (Bhandari *et al.*, 2009). As far as progress towards introduction and implementation of the novel molecular diagnostic techniques suitable for Nepal is concerned, innovative molecular diagnostic methodologies in every applied and feasible aspect with high specificity, sensitivity, cost effectiveness, applicability in Nepal is far behind. Current practice of diagnosis are entirely focused on classical methods like culture and serological tests (Dixit and Maskey, 2012), which are proved to be less sensitive and effective and lack capability to identify exact genotype and genetic diversity in comparison to molecular diagnosis methods (Safa *et al.*, 2009). Here, this research aims to introduce molecular

diagnosis as well as genotyping methods which are suitable in Nepal's context. This research in the current time also extends the research platform generating further possibilities in development and improvement of molecular diagnostic technologies. Currently used methodologies in developed countries for diagnosis and genotyping such as Microarray, Single nucleotide polymorphism (SNP) analysis via sequencing are not so much affordable in comparison to the investment of our country towards the health sector and economic condition of people of Nepalese. On the other hand such facilities are not so well established in Nepal. Hence in the above scenario various methodologies used in this research carries huge significance and will add up few more steps in the field of scientific research. In other aspect this research also stresses on the study of genetic component as well as epidemiological factors playing governing role in the spread of Cholera outbreak. Hence it can reveal the hidden facts via generating many more answers towards the problematic burden rendered by Cholera especially in Nepal. In the premise of constantly changing genetic makeup of Cholera with changing frame of time and environment, is leading to the emergence of multidrug as well as novel mutated variant strains creating hurdles in clinical management (Glass *et al.,* 1980; Sack *et al.,* 2004; Saha *et al.*, 2006), which demands the constant research and monitoring in this area. This research implements all selected microbiological, serological and molecular methods to perform comparative analysis of various attributes such as multidrug resistance, serotype, genotype and genetic diversity. More specifically these methodologies include modified phenotypic tests, genotyping and identification tests based on polymerase chain reaction using marker genes which include genus specific Outer membrane protein gene (ompw), *V. Cholerae* specific O antigen gene (rfbO1), Cholera toxin gene coding "A" subunit (ctxA), Repeat in toxin (rtx), Toxin co-regulated pilli (tcp), Cholera toxin (ctxB), Toxin linked encryptic plasmind (tlc) and Pulsed Field Gel-electrophoresis. Combined result from all these approaches will depict the current scenario of multidrug resistance, genetic change, mutation or variation in Cholera toxin gene, genetic divergence as well as phylogenic relationship among the strains of Nepal Asia and beyond. This will further add a footstep to know probable lineage and position of Nepali strains in evolution of *V. cholerae*.

Literature review

2.1 Diarrhea

Diarrhea is defined as the passage of three or more loose or liquid stools per day or more frequent passage than in normal for the individual (WHO, 2012). However frequent passing of formed stools frequently is not always confirmed as diarrhea, nor is the passing of loose, "pasty" stools by breastfed babies. Diarrhea is basically a symptom developed as a consequence of an infection in the intestinal tract caused by a variety of etiological agents' i.e. bacterial, viral and parasitic organisms. The source of intestinal infection is fundamentally contaminated food or drinking-water, or from person-to-person as a result of poor hygiene and sanitary habit. There exist three clinical types of diarrhea which are, a)Acute watery diarrhea which lasts several hours or days, and includes Cholera; b)Acute bloody diarrhea also called dysentery; c)persistent diarrhea lasts 14 days or longer (NDDIC, 2012; WGO, 2008)

Clue of cause is depicted by clinical feature of diarrhea. To cause infectious diarrhea a person should ingest enough loads of microbes associated with diarrhea via various sources so that pathogen enters and colonize in our body overcoming various line of immune defense (Karper *et al.,* 1995). Typically routine passage of high volume, watery fluid associated with mal-absorption and is prominent feature of a diarrhea caused by small intestine disease. During diarrhea dehydrated condition is most frequently seen in large number of patients. Diarrhea caused by colonic involvement is more often associated with frequent small-volume stools, the presence of blood, and a sensation of urgency (NDDIC, 2012; WHO 2012).

Essential epidemiological factors and symptoms that must be appraised while conducting clinical study and treatment of acute diarrhea include travel history, ill people sharing same dormitory facility, recent antibiotic use, history of HIV or medically induced immune-suppression, sources of water (e.g., well water), recent food intake, history of profuse diarrheal episodes, dehydration, fever, hematochezia, nausea, vomiting, and abdominal pain (WGO, 2008). Important clinical features include abrupt versus gradual onset of symptoms, symptom duration, including bowel movement frequency, stool quantities, dysentery with fever, tenesmus, hematochezia or pus in the stool, signs of volume depletion, including thirst, tachycardia, orthostasis, decreased urine output, skin turgor, and lethargy or confusion, or both.(Pwalowski *et al*., 2010; WGO, 2008)

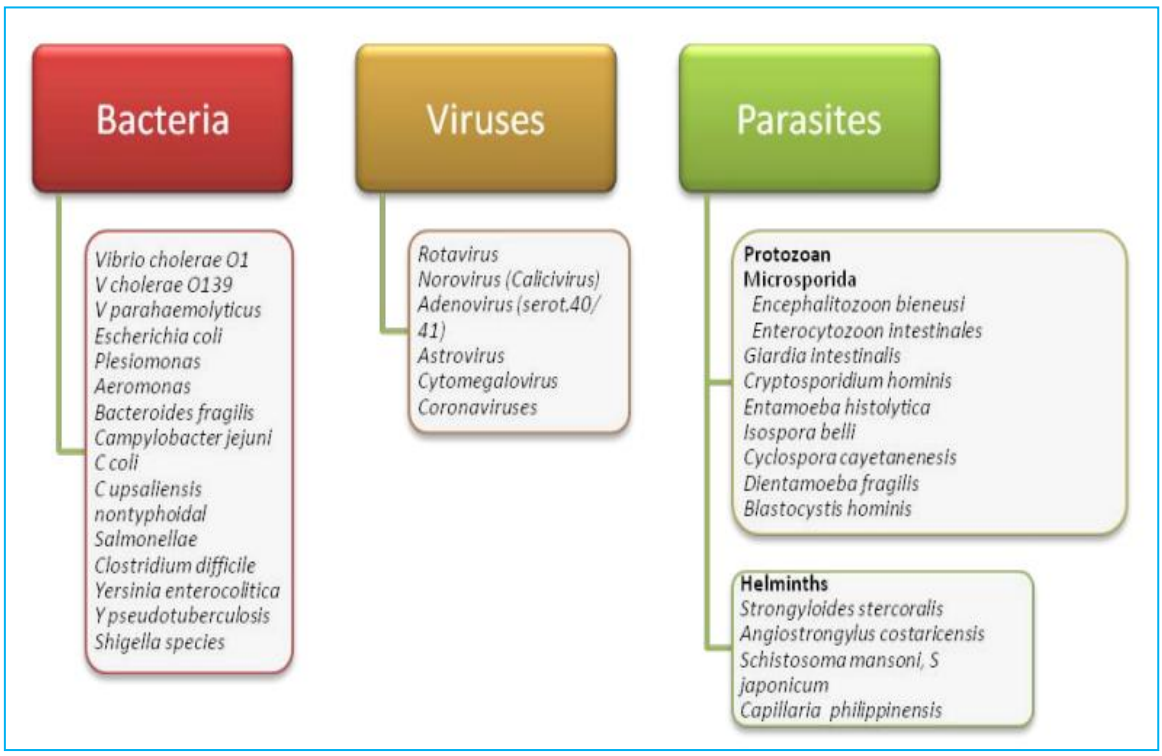


Table 1: Various causative agents of Diarrheal disease (Adapted from World Gastro-enterology Organization practice guideline: Acute diarrhea)

2.2 Mechanism of infectious diarrhea and associated symptomatic relation with their etiological agents

Because of large health burden and public health problem created by infectious diarrhea, extensive research is confined in this field from past few decades and has provided new insights in contrast to mechanism of diarrhea caused by diverse range of enteric pathogens. These pathogens are broadly classified as non-inflammatory, inflammatory or invasive according to their pathogenic behavior and effect. Broadly there exist three groups of organisms these basically cause two diarrheal syndromes which include non-inflammatory diarrhea and inflammatory diarrhea (Navananeethan and Giannella. 2008; Ou *et al.,* 2009). The non-inflammatory diarrheas are fundamentally caused by pathogens capable of producing enterotoxin which includes *Vibrio cholerae* and its variants or related species, enterotoxigenic *Escherichia coli* and also by some viruses possessing capability of adhering to the mucosa and disrupting the absorptive and or secretary processes of the enterocyte with no acute inflammation or mucosal destruction. On the other hand inflammatory diarrhea is caused by two groups of organisms' cytotoxin producing, noninvasive bacteria (e.g.entero-aggregative *Escherichia coli*, entero-hemorrhagic *Escherichia* coli and *Clostridium difficile*) or by invasive organisms (e.g. *Salmonella* spp., *Shigella* spp., *Campylobacter spp.*, *Entamoeba histolytica*). Adherence of cytotoxin producing organisms to the mucosa leads to

activation of cytokines and stimulation of the intestinal mucosa releasing inflammatory mediators. An invasive organism, which poses capability to produce cytotoxins, invades the intestinal mucosa and induces an acute inflammatory reaction via the activation of cytokines and inflammatory mediators. Irrespective of the underlying mechanism used by them, these various types of pathogen successfully evolved to evade and modulate the host defense systems (Abba *et al.,* 2009*; Flach et al.,* 2007*).*

Table 2: Comparison between inflammatory and non-inflammatory infectious diarrhea

Feature	Inflammatory diarrhea	Non –inflammatory diarrhea
Fecal leukocyte	Positive	Negative
Clinical presentation	Bloody ,mucoid small-volume diarrhea, tenesmus lower left quadrant abdominal cramps : may be febrile and toxic	Large volume ,watery diarrhea, no blood ,pus or tenesmus .May have nausea ,vomiting ,cramps, but no fever
Etiological Agents	*Shigella spp* ,*Salmonella spp*, amebic colitis, *Campylobactor spp*, EAEC, EHEC,EIEC, *Yersinia* spp ,*Clostiridium difficale*	Noro virus ,rota virus, *Vibrio cholerae* ,*Giardia lambella* ,ETEC ,Entero toxin producing bacteria ,*Staphylococcus aureus* ,*Cryptosporodium pravum*, *Clostridium perfrigens*
Site involvement	colon	Small intestine

(Adapted from Navaneethan, U. & Giannella, R. a, 2008. Mechanisms of infectious diarrhea. Nature clinical practice.)Abbreviations: EAEC ,enteroaggregative *Escherichia coli*, EHEC,enterohemorrhaghic *Escherichia coli* ; EIEC, enteroinvasive *Escherichia Coli,* ETEC ,enterotoxigenic ETEC ,enterotoxigenic *Escherichia Coli.*

Patients ingesting toxins or those with toxigenic infection typically have nausea and vomiting as prominent symptoms, along with watery diarrhea, but rarely have a high fever (WGO, 2008). Vomiting that begins within 6 hours of ingesting a food should suggests that it is caused by preformed toxin from bacteria such as *S. aureus* or *B. cereus.*If diarrheal disease begins within 8 to 14 hours of food ingestion in that case *C. perfringens* should be suspected (Navananeethan U and Giannella R. 2008). When the incubation period is longer than 14 hours and vomiting is also a significant symptom,

along with the diarrhea, viral agents should be considered. Parasites that do not invade the intestinal mucosa, such as *Giardia lamblia* and *Cryptosporidium* usually cause only mild abdominal discomfort. Giardiasis may be associated with mild steatorrhea, gaseousness and bloating (Pawloski *et al.*, 2010). Invasive bacterial infection such as *Campylobacter, Salmonella* and *Shigella* spp., and organisms producing cytotoxins, namely C*. difficile* and enterohemorrhagic *E. coli* (serotype O157:H7) etc., often exhibit symptoms like abdominal pain and low-grade fever. *Yersinia* and related organisms often infect the terminal ileum; caecum and manifest with right lower quadrant pain as well as tenderness suggesting acute appendicitis. Particularly in young children and older adults Hemolytic-uremic syndrome , thrombotic, thrombocytopenic purpura can occur in infections with enterohemorrhagic *E. coli* and *Shigella* organisms,*Yersinia* infection and other enteric bacterial infections may be accompanied by Reiter's syndrome (arthritis, urethritis, and conjunctivitis), thyroiditis, pericarditis, or glomerulonephritis. Enteric fever, caused by *Salmonella typhi* or *Salmonella paratyphi,* is a severe systemic illness manifested initially by prolonged high fevers, prostration, confusion and respiratory symptoms followed by abdominal tenderness, diarrhea, and rash (Farque and Albert, 1998).

2.3 Cholera overview

Cholera a ravaging disease, characterized as a profuse watery diarrhea accompanied by serve dehydration and death if untreated, is basically caused by highly diverse group of gram negative ,Gamma -proteobacteria that approximately contains 72 species (www. Bacterio.net) among them 12 species are clinically important and categorized in six different group. Fundamentally these groups include symbionts and commensals that are commonly found in or on marine animals as well as in many species with pathogenic significance (Farque and Albert, 1998; Tarr *et al*., 2011). In the wake of the most recent outbreaks, debate rekindled about the origin and transmission of the offending Cholera strain. It is well known fact that transmission of Cholera is primarily by fecal contamination of food and water and is rarely person to person. Interestingly, however, the bacterium lives naturally in both fresh and salt waters; fish and shellfish often are vehicles of transmission. To cause illness in a healthy adult, about 100 million bacteria typically must be ingested (Sack *et al.*, 2004). However, various host factors that influence the inoculums necessary for infection. The dose is less in persons with reduced gastric acid for instance, those using proton pump inhibitors. Younger children also are more susceptible to infection; with the highest rates occurring in 2 to 4 year olds. There is evidence that susceptibility to Cholera also is affected by blood type, with persons having type O blood being the most susceptible (Holmner *et al*., 2012). The cystic fibrosis genetic mutation in humans may confer a selective advantage over Cholera more

specifically Heterozygous carriers of the mutation who are unaffected by cystic fibrosis are more resistant to infection. In this model, the deficiency in the cystic fibrosis trans-membrane conductance regulator channel proteins causes interference with bacteria binding to the gastrointestinal epithelium, thus reducing the effects of an infection (Sigman *et al*., 2012). As prominent clinical feature of Cholera serve watery diarrhea, nausea, vomiting fever accompanied by serve dehydration are commonly seen as common symptoms in Cholera patients. Under absence of treatment Cholera can lead to hypovolemic shock, acidosis, and death (Karper *et al*., 1995). Fundamentally *Vibrio cholerae* is remarked as prime etiological agent of Cholera however various *Vibrios* are also found to be associated in Cholera and various infections (Bake, 1980). Of the *Vibrios* that are clinically significant to humans,*V. cholerae* is predominantly seen in various diarrheal outbreaks worldwide from last century.

In the developed world, Cholera associated bacterium usually is transmitted through consumption of contaminated seafood, whereas contaminated water is more often the source of the bacterium in developing countries. Cholera has been found in only two animal populations: Shellfish and plankton. Coastal Cholera outbreaks can follow zooplankton blooms, making Cholera a zoonotic disease. Patients infected with Cholera typically have profuse diarrhea and disease transmission occurs when this highly liquid stool, historically referred to as ‘‘rice water,’’ contaminates water used by others. Drinking any contaminated water, eating any foods washed in the water or consuming shellfish from the affected waterway can cause infection (Harpen *et al.*, 2003; Sigman *et al.*, 2012). In current scenario Cholera is gaining great attention and epidemiological importance because of its increasing trends of outbreak. In past two decade Cholera incidence is proceeding towards peak particularly in developing countries as consequence of poor hygiene, unsanitary behavior of people as well as poverty (Shakya *et al*., 2012; WHO and UNICEF, 2009).

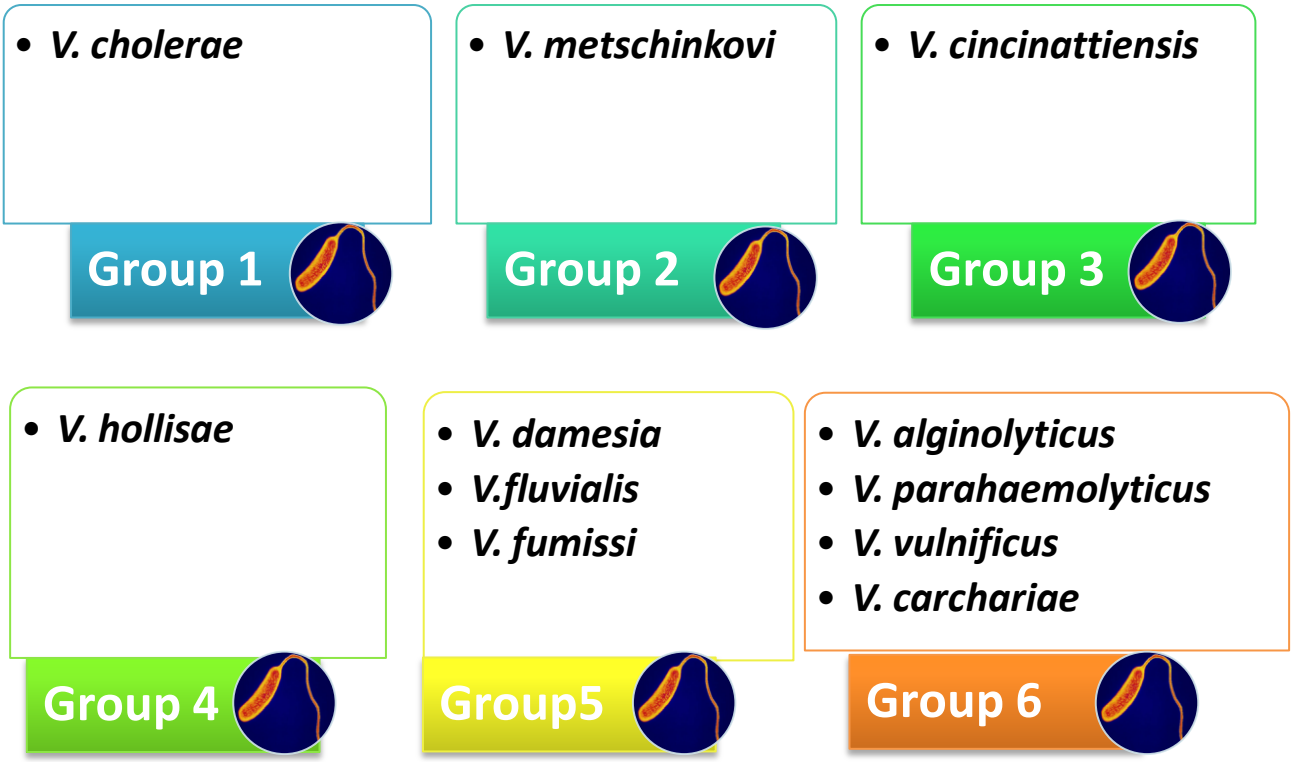


Figure 2: List of twelve clinically important vibrio species and their group (Partly adapted from " laboratory identification of Vibrio ",Center For Disease Control)

2.4 Cholera epidemiology and history

Cholera is vastly underreported, with an estimated 3–5 million cases and 100,000–130,000 deaths annually (WHO, 2010). Cholera is endemic in the developing countries of Asia and Africa, is responsible for epidemics in Asia, the Middle East, South and Central America. In 2009, the number of cases of Cholera reported to the World Health Organization (WHO) increased by 16% over the previous year (221,226 cases from 45 countries). Globally, the high incidence in the Americas in the early 1990s has shifted to a high incidence in Africa in the 2000s, with few cases in Asia. South Asia, especially Ganges delta region and its near territory is remarked as endemic region for Cholera since beginning of its recorded history. Epidemic attribute of Cholera was described in 1563 by Garcia del Huerto, a Portuguese physician at Goa, India (Todar, www. textbookofbacteriology.net; Sack *et al.,* 2004). The first pandemic occurred in 1817, with spread of the disease outside the Indian subcontinent along trade routes as far west as Southern Russia. The second pandemic began in 1826 and reached Europe by the early 1830s. When the infection reached the United Kingdom in 1831, the government's response led to the establishment of local Boards of Health and a ''Cholera Gazette,'' which served as a clearing house for tracking the epidemic. At that time, Cholera was believed to be spread by the 'miasma' (like a fog) coming from the river, but the classic 1854 epidemiologic study of John Snow, a surgeon from London, revealed the

association of the disease with contaminated drinking water before bacteria were known to exist. A New York Cholera epidemic led to the creation of the first Board of Health in the United States in 1866 and Cholera became the first reportable disease. In 1883, Robert Koch isolated the causative microorganism from the intestinal discharge of infected patients and proved conclusively that it was the agent of the disease. Three more pandemics ensued, the latest of which continued up to 1925 and involved Africa, Australia, Europe, and all the Americas (Koch, 1884; Karper *et al*., 1995). The current (seventh) pandemic began in Indonesia and expanded globally. The causative strain was a biotype of *Vibrio cholerae* serogroup O1 called El Tor, named for El Tor, Egypt, the site of a quarantine station where the bacterium was isolated in 1905 from Indonesian pilgrims traveling to Mecca (Pollitzer R ,1959). The El Tor serotype spread to India in 1964 to Africa in 1970, to Southern Europe in 1970 and to South America in 1991. In 1992, O139 Bengal, a newly described, non-O1 serogroup of *Vibrio cholerae*, caused unusual Cholera outbreaks in India and Bangladesh.In April 1997, a Cholera outbreak occurred among 90,000 Rwandan refugees living in temporary camps in the Democratic Republic of Congo. During the first 22 days of the outbreak, 1,521 deaths were recorded, most of which occurred outside healthcare facilities. The epidemic in Haiti in 2010 is the first recorded outbreak in that country in 100 years(Sigman *et al.*, 2012; Anna *et al.*, 2012).

In most of the outbreak worldwide before first sixth epidemic classical strains of *Vibrio Cholerae* almost disspperared whereas ET Tor strains and its variants emerged in outbreaks and sporadic cases in Africa, Asia. Beside these some different strains from different serogroup , O5 and O37 are also caused massive Cholera in Czechoslovakia and Sudan during period of 1960s and 1970s respectively (Safa *et al*., 2003). Since period of 1960s *Vibrio cholerae* is reported in Nepal with various diarrheal cases and outbreaks in rainy season. Cholerae incidence in Nepal is very fluctuating, sometime its proportion goes to high extent however some time it is reported as sporadiac cases indeed Cholera still account a top position among eiotological agent responsible for dirrhea. A recent study conducted in Kathmandu identified that in timeperiod of 2008 -2009, 27% *Vibrio cholerae* were found to be present in collected samples (Karki *et al*., 2012) which was the highest proportion among *Vibrio cholerae* and other pathogens identified in year 2002-2004 (Kansakar *et al*., 2011).

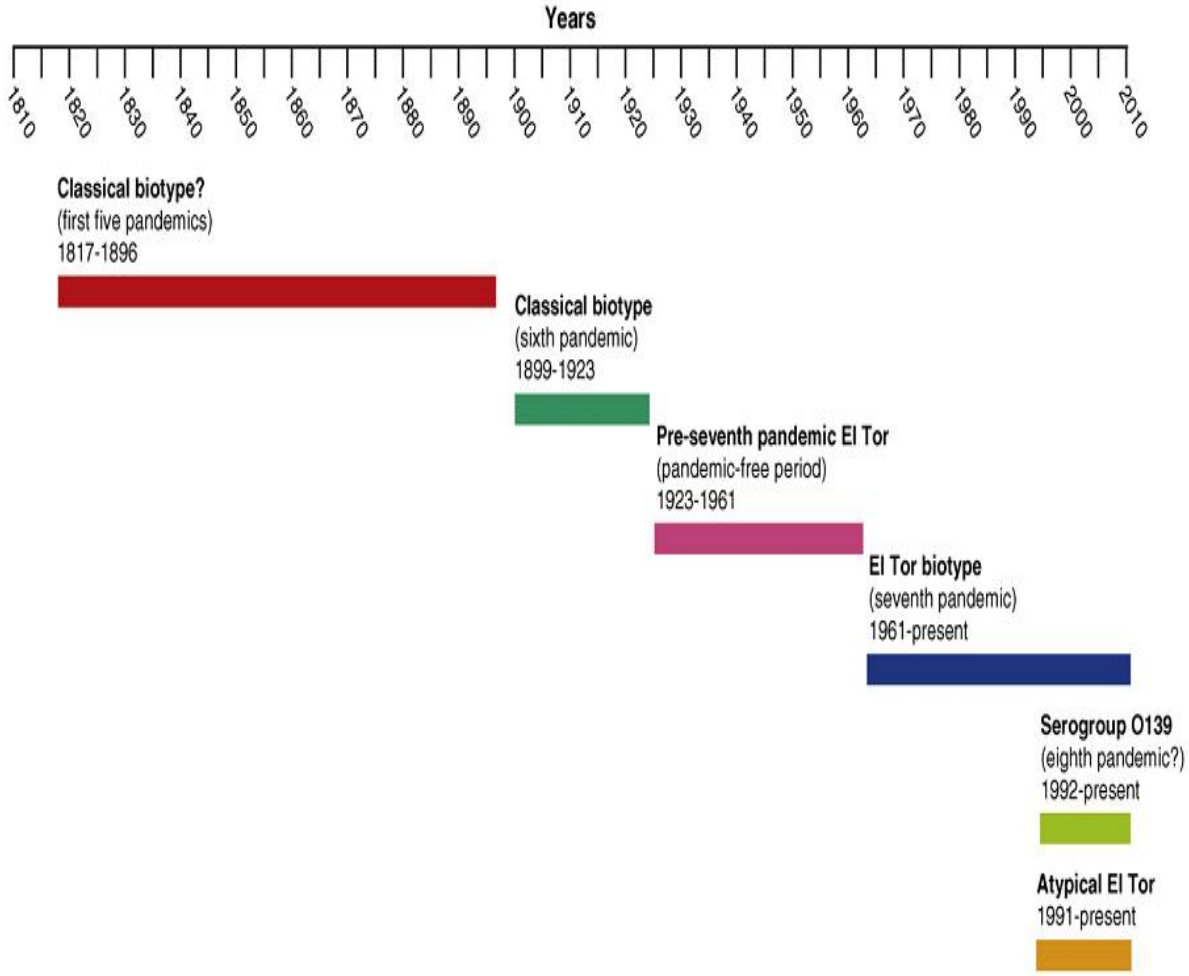


Figure 3: Historical overview of *Vibrio cholerae* outbreaks (Safa *et al*., 2003)

2.4 *Vibrio cholerae*

Vibrio cholerae is a comma-shaped, gram-negative aerobic or facultative anaerobic bacillus that ranges from 1 to 3 micrometers in length by 0.5 to 0.8 micrometers in diameter. It is identified easily by its unique, single, polar flagellum (Mandal *et al.*, 2011). Based on presence of lipopolysaccharide and capsule as structural component ie. Somatic antigen, *Vibrio cholere* are categorized as O1/O139 and non O1/O139 serogroups, among them serogroups O1 and O139 are epidemic and pandemic associated however non-O1 which are more than 206 type are not epidemic or pandemic associated. *Vibrio cholerae* O1 strains produce CT(Cholera toxin) and is often found to be associated with epidemic and pandemic Cholera however strains lacking ability to produce Cholera toxin (CT)is also found (Karper *et al.,*1981). Strains isolated from environment are usually CT negative and considered as non pathogenic (Levine *et al.,* 1983). Furthermore *Vibrio cholerae* of O1 serogroup are categorized into 3 serotype namely inaba ,ogaba and hikojima this type of biotyping is not applicable to non-O1 strains. An antigenic mutant or variants of O1 was also categorized as O139 which was

seen in Bangladesh in early 1993. This carried unique serological feature beside O1 and non O1 which it gained via mutation in genetic material encoding O1 antigen (Manning *et al*., 1994).

Scientific classification
Kingdom:Bacteria
Phylum:Proteobacteria
Class:Gamma Proteobacteria
Order:Vibrionales
Family:Vibrionaceae
Genus:*Vibrio*
Type species:*Vibrio Cholerae*

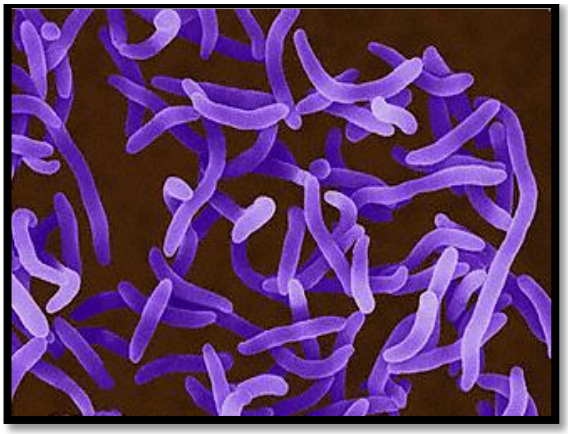


Fig 4: Electron micrograph of *Vibrio Cholerae,*
Source:www.microbewiki.kenyon.edu

Beside these very less no of strains belonging to *Vibrio cholerae* non O1/O139 are seen in Cholera outbreak. As these strains do not posses ability to aggulutinate O1 /O139 antigen hence categorized as O2-O138 on the basis of lipopolysaccharide, among them majority of strains are environmental and lacks ability to produce Cholera toxin and not associated with epidemics(Morris, 1990). Isolation of *V. cholerae* non-O1 from environmental sources in the absence of diarrheal cases is common. Another basis of classification based on phenotypic differences *V. cholerae* O1 is differentiated into two biotypes namely classical and EI Tor and differentiating phenotypic tests include sheep erythrocytes, agglutination of chicken erythrocytes, Voges Proskauer reaction, sensitivity to polymyxin B and specific phages (Karper *et al*., 1995; Safa *et al*., 2003; Sigman *et al*., 2012). Now days novel variants of *V. cholerae* O1 are seen in various outbreaks of India and Bangladesh (Alam *et al*., 2012). Most prominantly *V. cholerae* O1 is seen in various outbreaks since first outbreak of 18th century (Mekalanos ., 1997) moreover possible role of other *Vibrio* species and variants of *V. cholerae* is not controverted.

Classification Method	Epidemic Associated	Epidemic Non-Associated
Serogroups	O1 ,O139	Non O1(>200 exist)
Biotypes	Classcical ,E1 Tor	Biotype not applicable to non O1 type strains
Serotype	Ogaba(O factors : A&B) Inaba(O factors : A& C) Hikojima(Of actors : A,B&C)	These 3 Serotype not applicable to non O1 strains
Toxin	Produce Cholera toxin*	Usually do not produce Cholera toxin : sometimes produce other toxin

*Non toxigenic O1 strains, but are with epidemics

Table 2: Serological characteristics of *Vibrio cholerae*

2.6 Genome and pathogenic genetic element of *Vibrio cholerae*

Whole genome sequencing performed via random sequencing method (Fraser C.M *et al*., 1997) shows that *V. cholerae* posses two chromosome of variable sizes, 2,961,146 (chromosome 1) and 107, 2314 (Chromosome 2). Most of the genes required for the growth and viability are present on chromosome 1 but some gene are believed to be located on chromosome 2 and are believed to be essential for the normal cell growth and function, for example dsdA , thrS gene encoding ribosomal protein L20 and L35 . In addition to that many essential intermediaries of metabolic pathway are encoded by chromosome 2 (Hidelberg *et al.*, 2000). Comparative genomic analysis of two chromosomes clarified most of the suspense and it is found that there exist pronounced asymmetry between the distribution of the genes known to be essential for the growth and virulence between two chromosomes. More specifically more gene encoding DNA replication and repair, transcription, translation, cell wall biosynthesis and variety of central catabolic as well as biosynthetic pathways are carried in chromosome 2. Similarly most of gene known to be essential in bacterial pathogenicity those encoding the toxin

co-regulated pilus (TCP), Cholera toxin (CT), lipopolysaccharide and extracellular protein secretion machinery are also present on chromosome 1 while chromosome 2 also contains larger fraction (59%) of hypothetical protein and gene(42%) of unknown significance (Hidelberg *et.al.*, 2000). Most of the virulence factors harbored by *Vibrio cholerae* are encoded by accessory genetic elements. In various researches it is found that capability of *V. cholerae* to exhibit pathogenic activity is because of inherited genetic elements from various phages.

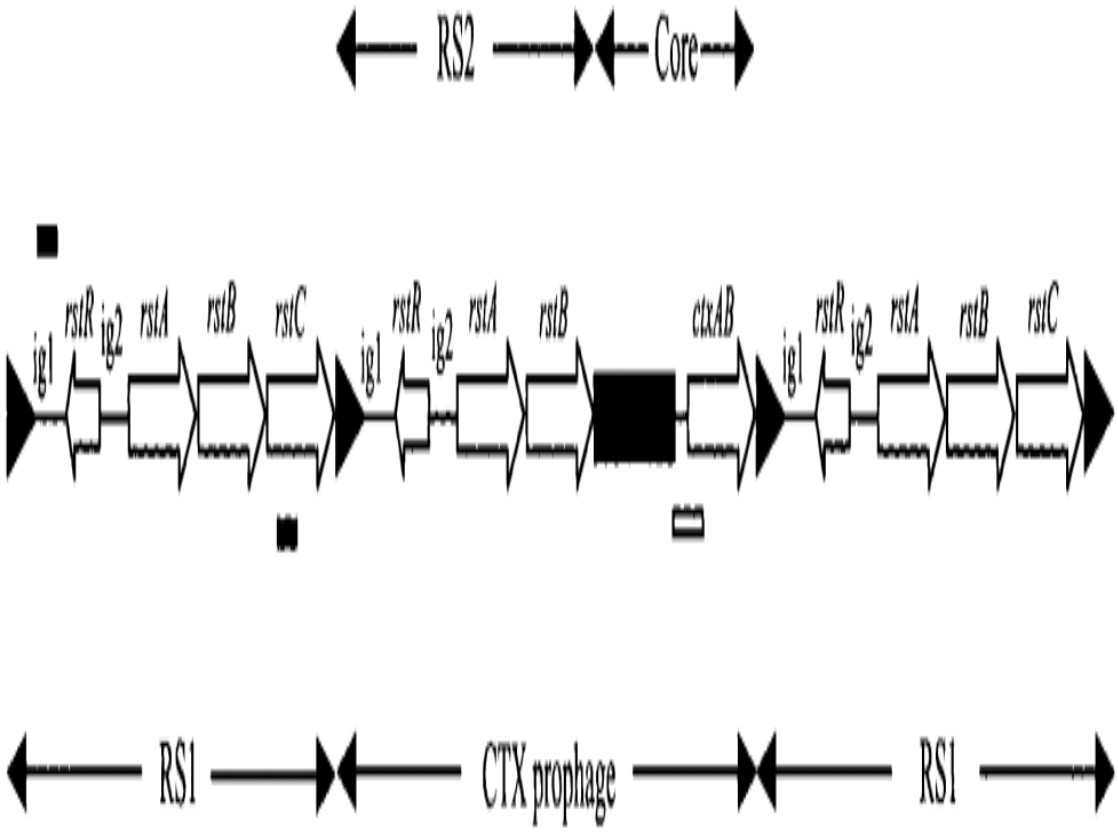


Figure 4: Schematic representation of the genetic organization of RS1 and a CTX prophage comprising an RS2 segment and a core region (not drawn to scale). The RS1 element is often flanked by the CTX prophage. Solid triangles represent end repeats and are the attachment sites of the prophage in the chromosomes of *V. Cholerae*. Open arrows represents genes present in the RS1, RS2 and core elements and their directions of transcription. The location of the ctxAB genes in the core is indicated. The solid rectangle is also part of the core and contains the genes cep, orfU, ace and zot. As shown, RS2 is identical to RS1 except for the presence of rstC in the latter element; ig1 and ig2 represent intergenic sequences. (Adapted from Nandi *et al.*, 2003)

The details of *Vibrio cholerae* genome revealed that there exist genome of filamentous phage namely CTXϕ in integrated form on chromosome 1. The 6.9 Kb CTXϕ genome is divided into 4.6 kb core region, which encodes Cholera toxin and psh, cep, orfU (gIII), ace, zot, all these functions are essential for virion morphogenesis and a 2.4 kb RS2 (repeat sequence 2) region, that encodes various component namely rstR, rstA and rstB, which are required for regulation, replication and integration of CTXϕ (Walder *et.al.*, 1997; Farque and Mekalanos, 2012). On the chromosome of *V. cholerae* CTXϕ is found to be flanked by 2.7kb RS1 (repeat sequence 1) elements. RS1 which have quite similar DNA sequence to RS2, another part of CTXϕ genome this also posses similar functional attributes however beside RS2, RS1 additionally encodes an open reading frame (ORF) namely rstC which lacks in RS2 (Walder *et.al.*, 1997). In EI tor biotype *V. cholerae* strains copies of both the CTX prophage and RS elements are frequently duplicated (Mekalanos, 1983). Toxin coregulated pilli (TCP) the receptor for entry of CTXϕ in to cell and its related gene clusters also located on large chromosome of *Vibrio* (Walder *et al*., 1996). The genes for TCP are part of the 'TCP–ACF element', alternatively referred to as Vibrio pathogenicity island (VPI) (Farque *et al*., 2003). All these pathogenic elements in *V. cholerae* are believed to be horizontally transferred genes from various sources. A region of nearly 40 kb, including the TCP–ACF gene clusters flanked on both sides by a putative 20-bp att-like attachment sequence, carrying a putative integrase gene and a transposase gene, constitutes the TCP–ACF element or the VPI. The structure, GC content and codon usage within the VPI suggests that it was recently acquired by *V. cholerae*.

Remarkably, the CTXϕ that encodes CT uses TCP as its receptor for infecting new strains and these two horizontally moving elements are linked evolutionarily (Boyd *et al*., 2002; Farque *et al.*, 2003).Some other clusters of genes that having putative additional roles in pathogenesis are also reported and most of these have features which suggests that they were recently acquired. These include the *V. cholerae* RTX (repeat in toxin) gene cluster and also genes for a new type IV pilus. The RTX family includes a group of related exotoxins produced by a variety of pathogenic Gram-negative bacteria (Cotte *et al., 1992).* The RTX toxin gene cluster *in V. cholerae* encodes the presumptive cytotoxin (rtxA), an acyltransferase (rtxC) and an associated ATP-binding cassette transporter system (RtxB and RtxD, two proteins for toxin transportation). It is physically linked to the core element in the *V. cholerae* genome, although its activity is independent of the core element. Phenotypically, these genes are proven to be associated with cytotoxicity in HEp-2 cells (Chow *et al*., 2001).

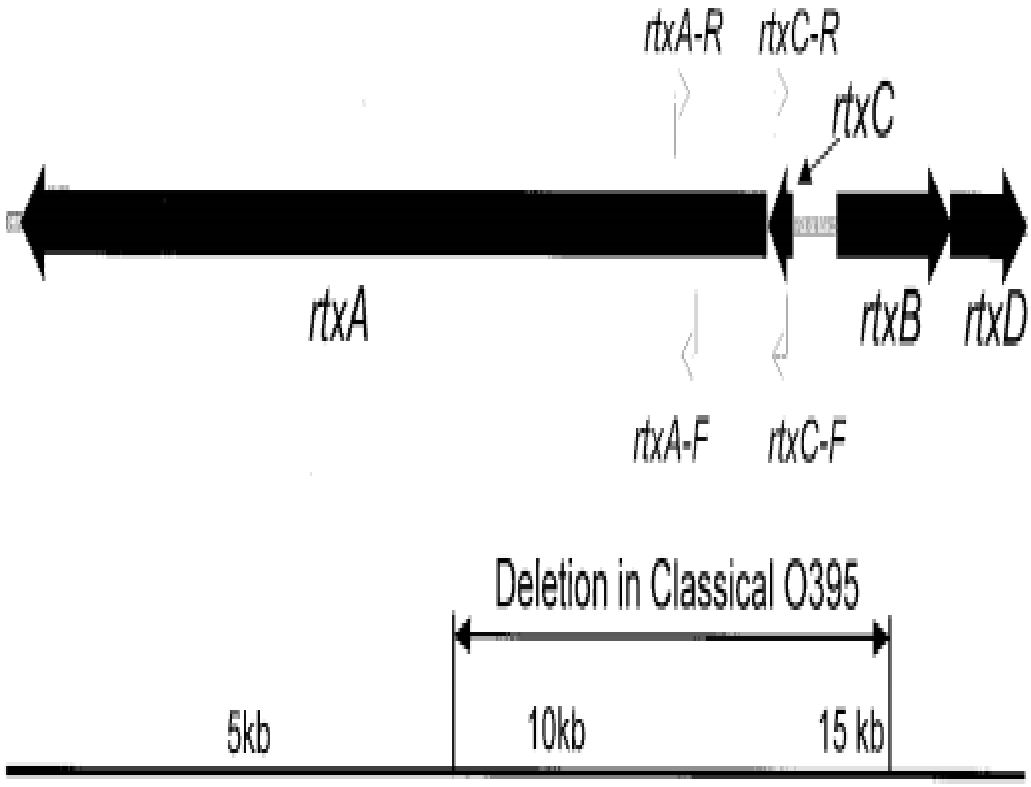


Figure 6: Genomic structure of RTX element (Adapted from Chow *et al.,* 2001*)*

Recently, DNA microarrays have been used to perform a comparative genomic analysis of *Vibrio* cholerae isolates (Dejiman *et al.,* 2002*).* These studies have identified seventh pandemic-specific genes which are grouped into two chromosomal islands, the VSP-1 and VSP-2 (Vibrio seventh pandemic island-1 and -2). The GC content of these islands suggests that they were recently acquired. A study determined that VSP-1 and VSP-2 appear to be absent in over 100 diverse non-O1, non-O139 and TCP-negative O1 strains, suggesting that the acquisition of VSP-1 and VSP-2 coincided with the emergence of the seventh pandemic clone of *V. cholerae* (Jermyn WS and Boyd EF, 2002). A novel 75 Kb chromosomal insert that encoding genes for neuraminidase (nanH) and amino sugar metabolism, was proposed to correspond to a pathogenicity island designated VPI-2 (Jermyn WS and Boyd EF, 2002) .On the other hand beyond structural genes of CT and TCP, other regulatory genes, Tox R that controls their expression in vivo (Lee *et al*., 1999), is also present on chromosome 1. Similarly numerous potential toxin genes including several haemolysis, proteases and lipases for example haemaglutinin protease (hap) protein which affects the protein involved in maintaining of integrity of epithelial cell in intestine (Wu . *et al*., 1996) and hlyA encoding haemolysin having enterotoxic activity are also present in chromosome 1 (Amam *et al*., 1998).

Gene Cluster	Putative function and Features
CTX prophage	Chromosomally integrated CTXϕ genome , originally perceived as a transposon – like genetic element (CTX genetic element) that carries the cholera toxin gene (ctxAB) .Other genes including zot ,ace and cep originally proposed as virulence ,have now been shown to encode functions necessary for morphogenesis of CTXϕ
TCP island or VPI	A 40 kb chromosomal region flanked on both sides by a 20- bp att like attachment sequence and comprised of tcp and acf gene clusters as well as genes encoding a putative integrase and a transposase .The major colonization factor of *V. Cholerae* is TCP , also receptor for CTXϕ consists of tcpA subunits encoded by the tcpA gene located in tcp gene cluster.
VSP1-VSP2	*Vibrio* seventh pandemic islands consists of clusters of open reading frames ,found in strains of seventh pandemic .The genes encode hypothetical functions presumed to be necessary for evolutionary fitness and epidemic spread of seventh pandemic clone
VPI -2	A 57.3 kb gene cluster encoding genes for neuraminidase (nanH) and amino sugar metabolism ,which has the characteristic features of a pathogenicity island.
RTX gene cluster	RTX gene encode cytotoxic activity and is physically linked to the CTX prophage in the *V. cholerae* might be associated with residual adverse properties displayed by certain live ,attenuated cholera vaccines
Integron island	A large 125.3 kb gene cluster that constitutes an efficient gene capture system .Presumably ,the intregons permits *V. Cholerae* to entrap genes from microorganisms and thus constitute mechanism for the clustering and spread of the pathogens as well genes of other biochemical functions.
New type IV-A pilus gene cluster	A 5.4 kb pil gene cluster in *V. cholerae* that resembles the *Aeromonas hydrophila* tap gene cluster and other type IV-A pilus assembly operons.Proteins encoded by this gene cluster include a prepillin peptides that is important for toxin secretion,mannose sensitive hemaglutinin(MSHA) and TCP production.

Table 4: Summary of major gene cluster of *Vibrio cholerae* and their features (Adapted from: S.M. Farque and J.J Mekalanos, 2003)

2.7 *Vibrio cholerae*, pathogenesis

Pathogenesis of Cholera is complex phenomenon and governed via numerous factors that help bacteria to colonize in the epithelium of the small intestine and to produce toxins. The massive dehydrating diarrheal condition, which is peculiar characteristics of Cholera, is because of action or Cholera toxin (CT) or Choleragen. Serve dehydrating action is assisted by synergistic action of various other regulatory gene products (Kaper, *et al*., 1995). Structurally CT is categorized in A-B subunit group of toxin. It contains 5

identical B subunits and single A unit of molecular weight 11.6 KDa and 27.2 KDa respectively (Hrist, *et al.*, 1991).

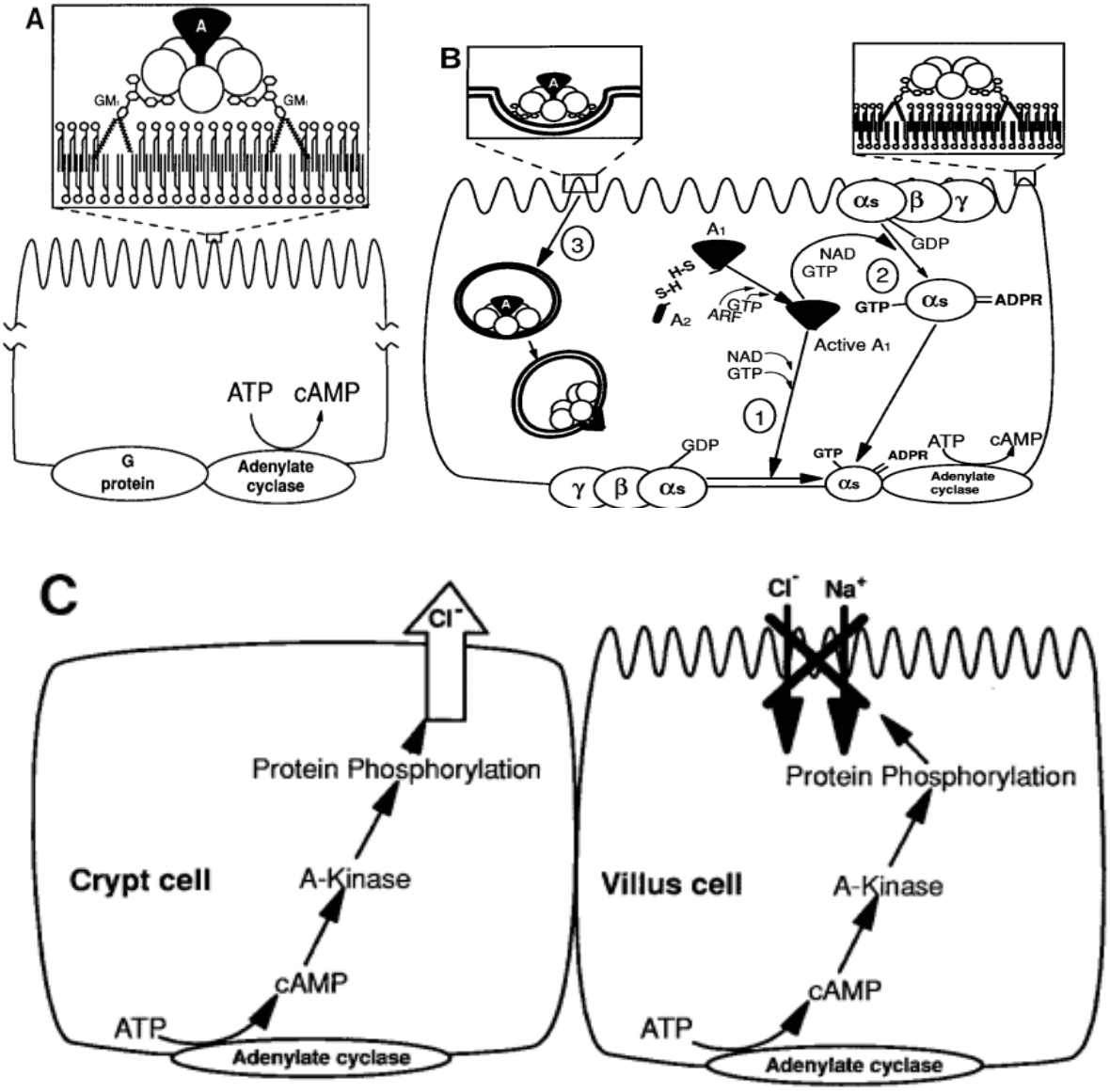


Figure 7: Mode of action of CT A) Adenylate cyclase located in intestinal epithelial cell is regulated via G protein.CT finds interaction with its G_{M1} ganglioside receptor via B – subunit pentamer B) A subunit enters probably via endosome and gets cleaved proteolytically in its disulfide bond yielding two peptides A_1 and A_2 . Thus activated peptide A_1 transfers ADP- ribose moiety (ADPR) from NAD to α subunit of G protein. ADP ribosylated α subunit thus dissociates from other subunit of G protein and activates adenylate cyclease (AMP), thereby raising intercellular concentration. c) Increased cyclic AMP (cAMP) activates protein kinase A, causing protein phosphorylation which consequently leads to increased CL^- secretion by intestinal crypt cells and decreased NaCl coupled absorption by villi cells (Kaper, *et al.*, 1995).

Beside CT other various toxins are produced by *Vibrio cholerae* which include a)Zot toxin that increases permeability of small intestinal mucosa by affecting intracellular tight junction or zonula occuludens (Fasano *et al*., 1991). b) Ace toxin, it is another toxin identified by Trucksis *et al.,* 1993 which shows structural similarity to virulence protein Spv of *Salmonella dublin* (Krause *et al*., *1991)* and it is found that it has possible role in fluid accumulation as noticed in rabbit ileal loops. Similarly another study in pig supported and showed new fact that it increases vascular permeability and cAMP concentration in the ileum thus similar role is believed to be possible in human (Kapral *et al*., 1976; Kenne et *al*., 1982). As far as gene location is concerned above mentioned toxins genes are found to be located on the same open reading frame of CTXϕ (Kaper *et al*., 1994). c) Haemolysin /cytolysin, this is a protein encoded by hlyA gene that may posses lytic ability on human with reference to lytic behavior shown by *E. Coli* strains containing cloned hlyA gene in chicken and rabbit (Richardson *et al*., 1986). In comparison to CT induced diarrheal condition, watery fluid in response to haemolysin was invariably bloody with mucous (Ichinose *et al*., 1987), on the other hand cytolysin showing fluid accumulating response was seen in rabbit and this was proposed to be identical to El Tor haemolysin (Mc Cardell *et al*., 1985; Spira *et al*., 1986). Beside these additional miscellaneous toxin namely Shiga like toxin, ST toxin new Cholera toxin, Sodium channel inhibitor, Thermo stable direct haemolysin are also present and they exhibit various role in diarrhea (Kaper *et al*., 1995).

2.8 *Vibrio cholerae* O1 genomic evolution and its variants

Review of the sixth pandemic showed that there was prevalence almost exclusively of the *Vibrio cholerae* O1 classical biotype whereas the current seventh pandemic has been caused by the O1 El Tor biotypes, which was first isolated in the Indonesian island of Sulawesi in 1961. Since then, several novel genetic variants of *V. cholerae* O1 El Tor have emerged and re-emerged. During past outbreaks most noticeable newly emerged strains were variants of *V. cholerae O1* serogroup, named as O139 or Bengal (Ramamurty *et al.,* 1993). This serogroup was first recognized in 1992 in India and Bangladesh, which initially displaced the local existing O1 El Tor strains indeed it, is restricted to Asia. The current pandemic in Asia and Africa is currently largely attributed to new variants of *V. cholerae* showing traits of both the classical and El Tor biotypes and El Tor biotype strains producing the classical Cholera toxin. A new term 'Atypical El Tor' is coined for these new variants of *V. cholerae* and which include the Matlab types I, II, and III, altered El Tor, Mozambique El Tor and hybrid El Tor strains (Safa *et al*., 2003).

- **The Matlab variants**

These are naturally occurring atypical El Tor variants that were first observed by Nair and colleagues among hospital strains of *Vibrio cholerae* O1 isolated between 1991 and 1994 in Matlab, Bangladesh (Nair *et al*., 2002). In that time certain strains showed conflicting phenotypic results hence could not be biotyped as either classical or El Tor and were designated as Matlab (MT) variants. Ribotyping (Nair *et al*., 2002) and PCR-based identification of the El Tor-specific gene clusters VSP-I, VSP-II and RTX (Safa *et al*., 2006) suggested that the source of origin of MT variants is El Tor biotype. However, Pulsed-field Gel Electrophoresis analysis showed the patterns that these strains were mixture of typical strains namely classical and El Tor biotypes (Safa *et al., 2006*). Further studies showed that many of the MT variants showed the presence of rstR ^{Cla} and ctxB1 alleles, which are characteristic of CTXϕ Cla of classical strains (Safa *et al., 2006;* Nair *et al*., 2002). Based on the range of the above mentioned phenotypic and genotypic variations, MT strains were grouped into three types namely Matlab variant I, II and III. More specifically fundamental tests for phenotypic differentiation include chicken cell agglutination (CCA), polymyxin B test, Vogeus Proskeur (VP), phageIV and phage V test in which all these three strains exhibit negative result to CCA and VP test. In polymyxin B test Matlab variant shows sensitivity while other variants ie I and III exhibit resistivity. In case of phagetyping phage IV is resistant to all variants however phage V is resistant to matlab variant I and sensitive to variants II and III (Safa *et al., 2006).*

- **The Mozambique variants**

Mozambique is a Cholera-endemic country in East Africa, where a large and extended Cholera outbreak was reported in 2004. In that outbreak there was involvement of *Vibrio. cholerae* strains which displayed phenotypic characteristics of the El Tor biotype. However, the result of rstR and ctxB genotyping was consistent with the classical biotype as they showed presence of classical rstR and ctxB1 genotype (Ansaruzzaman *et al.,* 2004; Das B *et al*., 2007). In the small chromosome the Mozambique variants carried, two tandem copies of the prophage whose sequence was almost identical to that of the typical CTXϕ Cla (Ansaruzzaman *et al.,* 2004; Das B *et al*., 2007). Particularly, this was the first report about atypical El Tor strains harbouring CTXϕ Cla in Africa. Recently, similar strains carrying tandem copies of CTXϕ Cla in the small chromosome have also been isolated in samples from Vietnam collected in time period between 1995 and 2004 (Nguyen *et al*., 2009). Unlike the Mozambique variants, some of these strains have an additional CTXϕ Cla in the large chromosome (Nguyen *et al*., 2009).

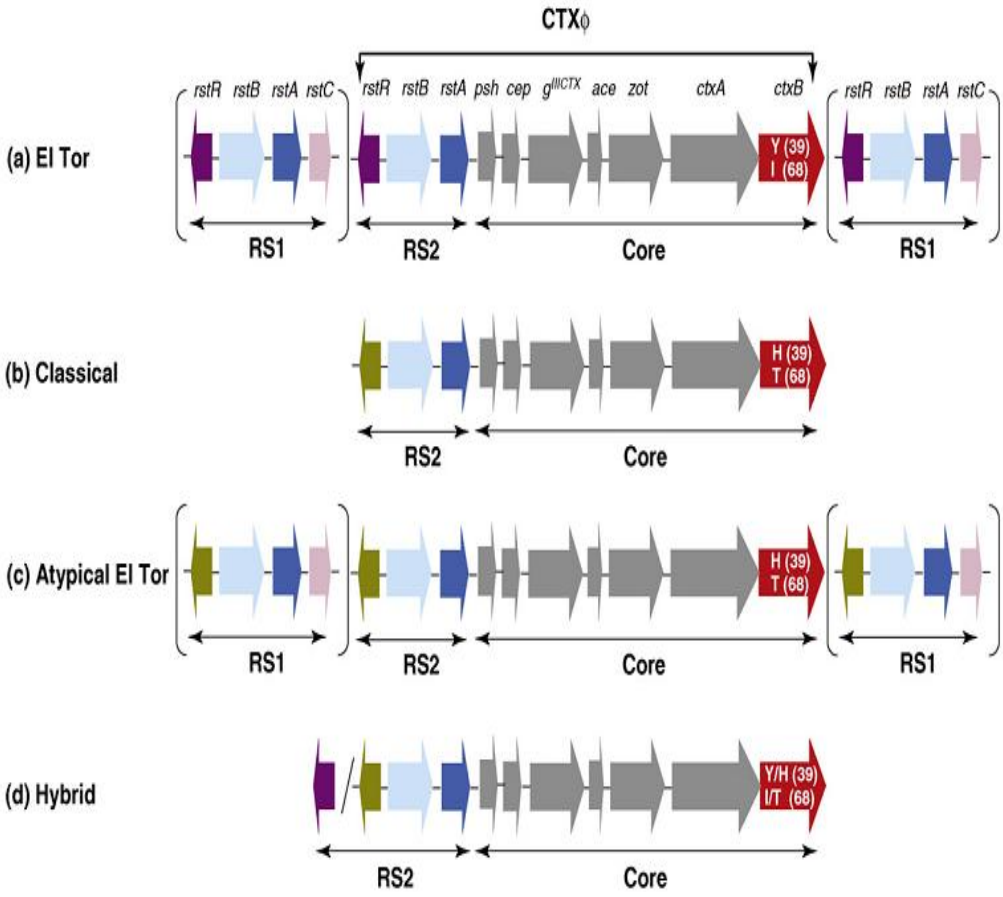


Figure 8: Comparison of structure of CTXϕ and the RS1 element in different *Vibrio Cholerae* O1 variants. In the prototype El Tor (a) and atypical El Tor (c) strains, excursuses define the RS1 elements that flank the integrated CTXϕ genome. (b) CTXϕ in prototype classical strains, RS1 element is absent. (d) A hypothetical hybrid CTXϕ. In ctxB gene letter H (histidine), T (threonine), Y (tyrosine) or I (isoleucine) indicate polymorphic positions within that gene segment (Adapted from, Safa A *et al*., 2003).

- **Altered El Tor**

These strains were isolated before 2001 in Bangladesh which carried the ctxB3 allele and possessed ability to produce CT of the typical El Tor biotype. However, all El Tor strains isolated after that date in this country harbor ctxB1, producing CT of the classical bio- type (Nair *et al*., 2006). These atypical El Tor strains have been designated 'altered El Tor' in the recent literature (Nair *et al*., 2006). Interestingly, CTXϕ from atypical El Tor strains isolated from Vietnam in 2007–2008 contains both ctxB1 and rstREl (Nguyen *et al*., 2009). Recent review by Safa A, 2009 suggested the term 'hybrid CTXϕ' which refers to prophages displaying combinations of classical and El Tor features; for example, rstREl/ctxB1 (as in the mentioned Vietnamese variants) or rstRCla/ctxB3 (Safa *et al.,* 2009*)*.

- **Hybrid El Tor / Several El Tor strains**

All these strains were isolated between 1991 and 2004 from Asian and African countries other than India, Bangladesh and Mozambique and these strains also harbor the classical CT allele ctxB1 hence these variants were designated as 'hybrid El Tor' (Safa *et al.,*2008). Many more investigation has revealed that several of these strains carry both rstR Cla and rstR El, indicating the presence of two different copies of CTXϕ, either as a tandem array or located on different chromosomes.

2.9 *Vibrio cholerae* and phylogenetic relation with other related *Vibrio* strains

Up to now more than 60 complete or draft whole genome sequences are available in the public domain for various *Vibrio* species, which include over 26 *V. cholerae* strains. Among them *Vibrio spp*., *Vibrio mimicus* was thought to be genetically related to *V. cholerae* (Thompson *et al.,* 2009) as it was originally reported to be a biochemically atypical group of *V. cholerae* strains (Dabis *et al.,* 1981). A clearer estimate of the relationship emerged via genome-based phylogenetic analysis is presented in literature (Choo *et al., 2010)* where both *V. cholerae* and *V. mimicus* were concluded to represent two genomically distinctive groups of bacteria among 37 various strains considered.

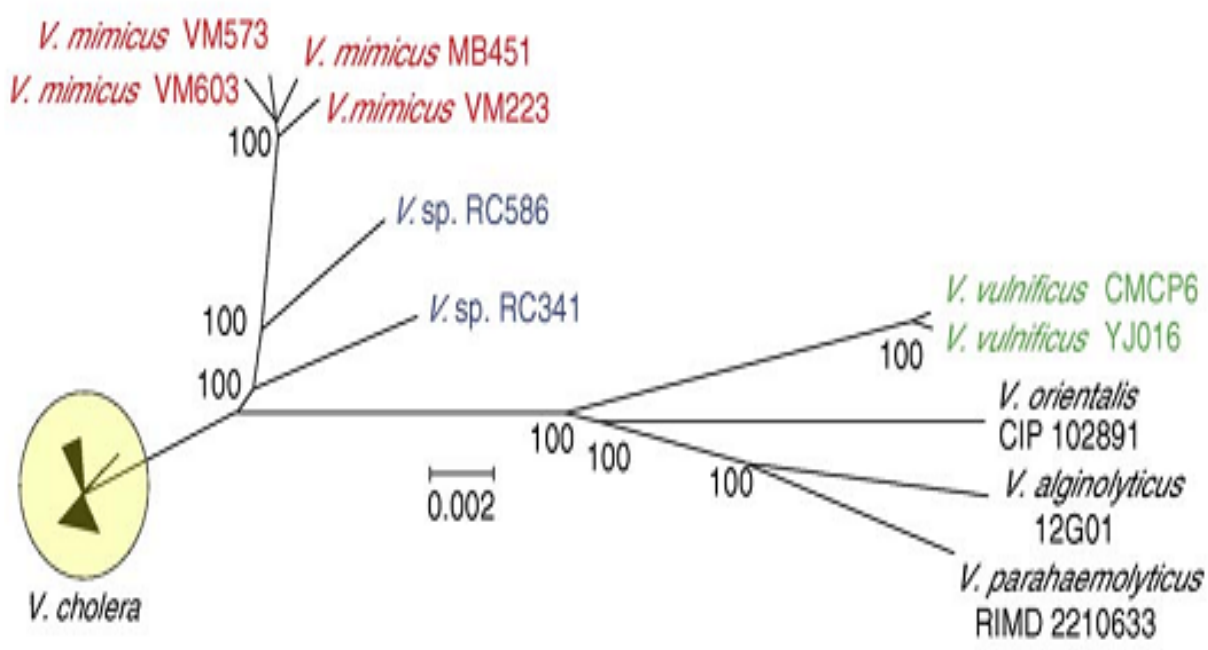


Figure 9: Neighbor-joining tree showing evolutionary relationships of various *Vibrio* strains. The calculation was based on 663 orthologous ORFs (820,348 bp). Bar represents 0.002 substitutions per site. (Adapted from Choo YJ *et al., 2010*)

2.10 Summary of genesis of the seventh pandemic *V. cholerae*

Till date or in current situation there still exists continuation of ever known longest seventh pandemic in the world. There exist huge genetic change and variations going in strains prevalent in various areas of Asia and Africa and beyond, primarily strains are emerging and re-emerging from preexisted strains however possibility of emergence of novel strains in from different lineage is not rejected. Among various approaches applied so far for study of genomic behavior, recent whole genome based comparisons of representative *Vibrio cholerae* strains belonging to various serogroups and genomically distinctive lineages indicated that isolates responsible for the seventh pandemic are clonal, despite they belong to different serotype namely O1 or O139 (Chun *et al.*, 2009). The entire seventh pandemic *V. cholerae* strains sequenced so far shares an almost identical genome backbone, while variation is particularly confined in their gene contents. Within the phylocore genome 1(PG-1) group, a major difference between O1 El Tor strains belonging to the seven and non-seventh pandemic lineages lies in two genomic islands known as *V. cholerae* seventh pandemic islands VSP-1 and VSP-2, which were initially identified from microarray-based (Dziejman *et al.,* 2002) and whole genome sequencing-based (Chun *et al.,* 2009) studies.

Several lines of evidence suggest that genomic islands might have introduced into an organism ancestral to all present seventh pandemic strains which is likely a major initiation for the prevalence of current seventh pandemic clones (Chun *et al.,* 2009). In seventh pandemic strains presence of VSP is fluctuating, VSP-1 with 13 ORFs was found in all of the sequenced seventh pandemic strains whereas VSP-2 was found to be completely deleted in the strain in an altered El Tor (CIRS101) isolate from Bangladesh. Presence of VSP-2 like genomic islands was also seen in non-O1/ non-O139 strains as well as other species, such as *V. vulnificus* (Chun *et al.,* 2009). Similarly, VSP-1 or VSP-1-like genomic islands were found in non-O1/non-O139 strains (Chun *et al.,* 2009; O'shea *et al., 2004)*. In recent, study by Grim *et al.* survey over 300 *Vibrio* strains using multiplex PCR and found that a small fraction of non-O1/non-O139, including *V. mimicus*, contained VSP-1-like elements, which indicated the prevalence of this genomic island across both *V. cholerae* and *V. mimicus*. However, their relatedness to pathogenicity is still unknown.

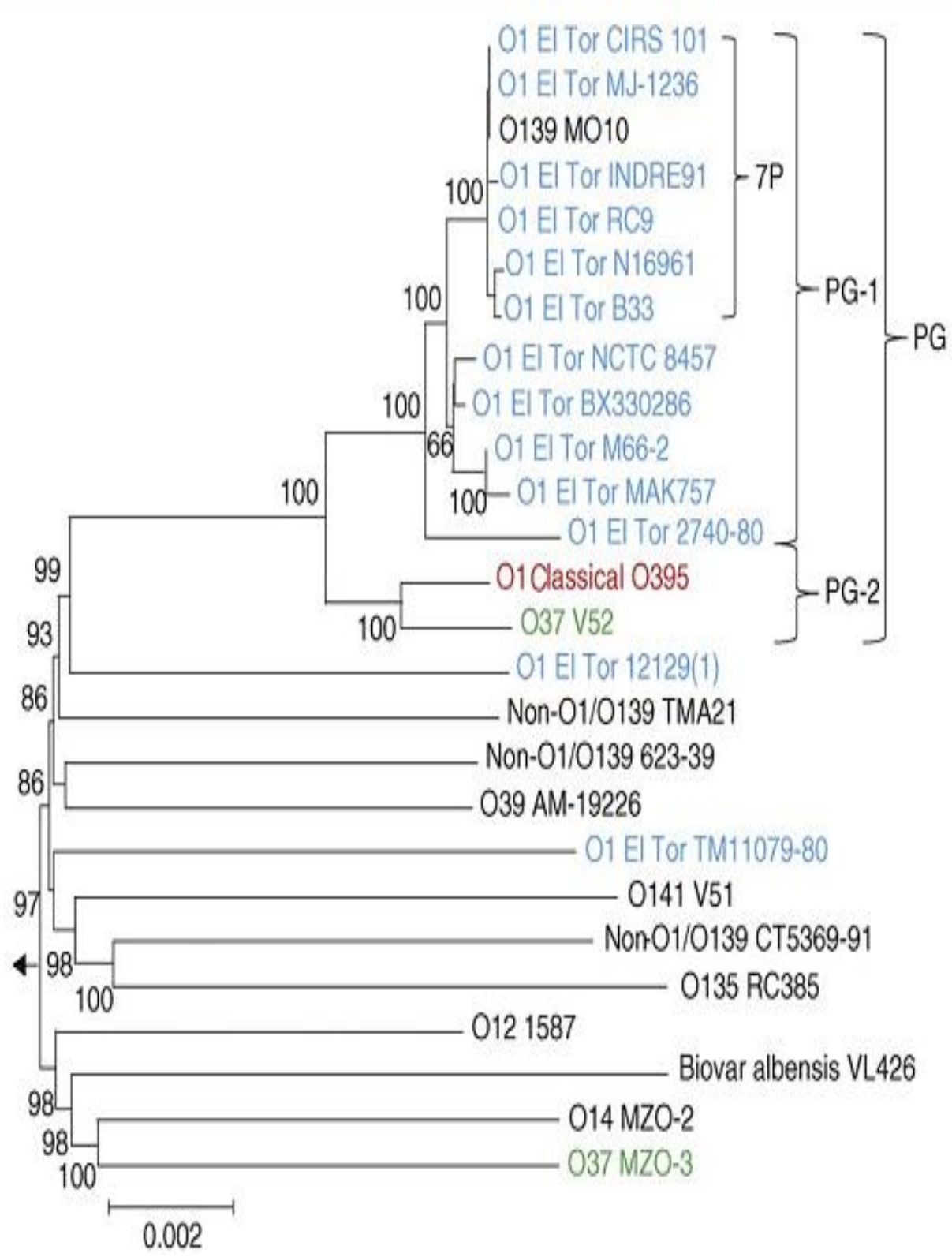


Figure 9: Neighbor-joining tree showing evolutionary relationships of 26 *V. cholerae* strains. The calculation was based on 1712 orthologous ORFs (1,709,262 bp). Only orthologous genes showing >95% nucleotide sequence similarity to those of V. Cholerae N16961 were selected. Bar represents 0.002 substitution per site, Abberberation 7P : seventh pendamic, PG: Phylocore group (Adapted from Choo YJ *et al., 2010*).

Detailed comparative genomics of currently predominant 'atypical clones' of the seventh pandemic suggested that insertion of a hybrid type CTX-phage and an integrative conjugative element (ICE) may have lead to their establishment of novel successful clinical clones over other seventh pandemic groups. There exist many hypothesis about how different genomic islands are inserted or deleted along the phylogenetic lineages, however further functional studies on these mobile elements are required to elucidate how seventh pandemic clones were established and shifted geographically across time.

2.11 Phenotypic, serological, molecular characterization and geno-typing of *Vibrio cholerae*

There exist various methods for identification and characterization of clinically important *Vibrio* species, namely old microbiological, biochemical and recent advanced as well as highly specific molecular identification and genotyping methods. The minimum identification of *Vibrio* requires only serologic conformation of the O serotype antigens with suspect isolates moreover absolute characterization may include deep analysis of biochemical property as well as genomic comparison using recent molecular tool Polymerase chain reaction (PCR) assay for specific marker gene detection (ompw, ctx, tcp etc); sequencing, microarray (Tarr *et al.*, 2011; Pinto *et al., 2005*).

2.11.1 Phenotypic, serological test and methods for characterization of *Vibrio cholerae*

Since first successful culture of *V. Cholerae* by Robert coach various methods are derived and adopted to differentiate *V. Cholerae* from other enteric pathogen which includes optimized culture and biochemical differentiation methods. Beside classical culture methods using Thiosulphate Citrate bile salt sucrose (TCBS) agar, Gelatin Agar (GA) etc. (Kaper *et al.,* 1995) various phenotypic test are derived among them commonly adapted methods include Chicken cell agglutination (CCA), Polymyxin B(PB), Sheep erythrocyte haemolysis, Modified Voges Proskauer (VP), Succeptibility identification test to IV and V phages. Brief description about some commonly used phenotypic tests are summarized below.

Table: 5 Common phenotypic methods used for identification and characterization of *Vibrio cholerae*

Phenotypic Tests	Description
Chicken Cell Agglutination(CCA)	Chicken erythrocytes are mixed with a *Vibrio cholerae suspension* on a glass slide. In case of positive result agglutination will occur within 30-60 sec. Classical biotype is differentiated from E1 tor strains, as it does not induce agglutination where as E1 tor strains induce agglutination (Kay, AB *et al.*, 1994).
Polymyxin B(PB)	It is a antibiotic susceptibility test which is determined by agar disc diffusion method. Classical strain shows sensitivity(inhibition zone ≥12 mm) ,where as EI Tor strain are generally resistant (inhibition zone ≤1 - 2mm) However sensitivity towards PB is also seen in previously reported cases (Safa *et al.*, 2008; WHO Lab Manual ; 1987)
Sheep erythrocyte Haemolysis	This test is used to detect β -haemolytic property of *Vibrio cholerae* on sheep blood agar (Kay, A.B *et al.*, 1994). EI Tor strains are commonly haemolytic positive where as classical strain show negative result. It is limited in use because some EI Tor variant (US Gulf coast isolates) show non haemolytic pattern (Kaper, J B *et al.*, 1995).
Modified Voges-Proskauer(VP)	In modified VP test bacteria are grown in VP containing 1% NaCl broth, on addition of colored reagents if dark pink red color with in 5 min is observed it is interpreted as positive, whereas no color or yellow orange color development is interpreted as negative test. (Kay, A.B *et al.*, 1994). Classical Strains are negative just opposite to EI tor strains which are positive.
Susceptibility test towards IV and V phages	This test predicts the susceptibility of *Vibrio cholerae* against phage IV and V (Kay, A.B *et al.*, 1994). Classical strains are sensitive to phage IV and resistant to V and conversely EI tor strains are resistant to IV and sensitive to V. Moreover some O1 strains showing unpredictable pattern either sensitive or resistant to both phages have been reported (Safa *et al.*, 2008).

3.11.2 Molecular characterization and methods for genotyping of *Vibrio cholerae*

Recent advancement in the field of genotyping is constantly putting forward novel techniques focusing on microbial genotype or DNA sequence .These tools are highly applicable in biological research and in the establishment of large databases of characterized organisms. Genotyping methods have various advantages beyond classical tools and highly used to retrieve information on subtypes of the organism, their source of origin of infection and to recognize particular virulent strains of the organism. These methods include Pulsed-field gel electrophoresis, ribotyping, CTX typing, amplified fragment length polymorphism (AFLP), enterobacterial intergenic consensus sequence-PCR, multilocus sequence typing and microarray methods etc.

In another aspect these tools are more often used for the determination of genetic changes of toxigenic and non toxigenic *V. cholerae* strains, origin of infection and relationship between clinical and environmental strains, with the simultaneous detection of the number of copies and types of CTX prophages and genes required for persistence in diverse aquatic environments. (Singh and.Mohapatra, 2008). Among various molecular characterization and genotyping techniques, this study basically utilizes PCR based molecular characterization and genotyping using specific marker gene such as ctxA, rtxC ,rstC, tlc,tcpA,rstR and ctxB.Similarly non PCR based approaches used in this study was pulsotyping to analyze genome polymorphism using Pulsed Gel Electrophoresis method .

- **ctxB typing :**

Based upon complete sequence analysis of ctxB gene coding CT B subunit several non random point mutation patterns are identified. Among various different biotype and serogroups of *V. cholerae* different alleles are categorized based on nucleotide polymorphism created by mutaton, as listed in table 6. Targeting specific primer against these position genotyping for various can be performed. Recently developed PCR method namely Mismatch amplification mutation PCR assay (Masatomo, *et al.*, 2008) and Double Mismatch Amplification Mutation Assay (DMAMA) (Naha *et al.*, 2012) can discriminate three ctxB allelic subtype of *V. cholerae* O1.

- **rstR typing:**

Repeat sequence transcriptional regulator (rstR) is often used for genotyping. Various alleles are categorized based upon variation of nucleotide sequence among classical and EI tor and these are represented as $rstR^{classical}$ or rstR1or $rstR^{cla}$ and $rstR^{EITor}$ or rstR2 or $rstR^{EI}$ respectively. In addition to this for new variants O_{139} and environmental

strains with various alleles are defined. Specific PCR based methodology using specific primer for genotyping of *V. cholerae* O1 strains are well illustrated by Mwansa *et al*., (2006) and Kimsey *et al*., (2003).

Subtypes	*ctxB* genotype	Nucleotide positions (amino acid positions)							
		58 (20)	72 (24)	83 (28)	101 (34)	115 (39)	138 (46)	165 (55)	203 (68)
O1	1	C (His)	A (Gln)	A (Asp)	A (His)	C (His)	T (Phe)	A (Lys)	C (Thr)
	2	C (His)	A (Gln)	A (Asp)	A (His)	C (His)	G (Leu)	A (Lys)	C (Thr)
	3	C (His)	A (Gln)	A (Asp)	A (His)	T (Tyr)	T (Phe)	A (Lys)	T (Ile)
	7	A (Asn)	A (Gln)	A (Asp)	A (His)	C (His)	T (Phe)	A (Lys)	C (Thr)
O139	3	C (His)	A (Gln)	A (Asp)	A (His)	T (Tyr)	T (Phe)	A (Lys)	T (Ile)
	4	C (His)	A (Gln)	A (Asp)	A (His)	T (Tyr)	T (Phe)	A (Lys)	C (Thr)
	5	C (His)	A (Gln)	C (Ala)	A (His)	C (His)	T (Phe)	A (Lys)	C (Thr)
	6	C (His)	A (Gln)	A (Asp)	C (Pro)	T (Tyr)	T (Phe)	A (Lys)	C (Thr)
O27	8	C (His)	C (His)	C (Ala)	A (His)	C (His)	T (Phe)	A (Lys)	C (Thr)
O37	9	C (His)	A (Gln)	A (Asp)	A (His)	C (Leu)	G (Leu)	C (Asn)	C (Thr)

[a]Nucleotide position +1 corresponds to the A of the ATG start codon in ctxB

Table 6: Different genotypes of Vibrio based on difference in point mutation pattern in ctxB nucleotide of *V . Cholerae*[a] (Ashrafus S, *et al.*, 2009)

- **tcpA typing:**

Toxin coregulated pilli (TCP) fundamentally a IV type of pilli that servres as receptor for CTXϕ virions plays essential role in colonization of *V. cholerae* in colon (Kaper *et al.*, 1995). The gene tcpA that codes for major pillus protein A, is based upon variation of nucleotide of which at C-terminal domain between classical and EI tor variants.

- **Pulsotyping by Pulsed Field Gel Electrophoresis (PFGE):**

  It is one of the typing methods and is also referred as “gold standard” for molecular typing. Principally this method utilizes advantage of use of infrequently cutting restriction enzyme in order to generate large molecular weight genetic fragments and digested products are subjected to pulsating polarized electric current to generate electrophoretic banding pattern which is then analyzed via commercially available software to obtain phylogenetic tree (Singh and Mohapatra H, 2008).

Material and Methods

4.1 Collection of samples and survey data

The study was performed in Kathmandu and samples were collected from patients visiting Sahid Sukraj Tropical Disease Hospital, Kathmandu during outbreak season of year 2012 from June to September. From diarrheal registered cases during study period, 72 highly probable rectal swab and stool samples with particular characteristic feature of Cholera were collected using Cary Blair media (Himedia, Mumbai India). Fundamental criteria for selection of sample and collection of survey data was (1) Rice watery stool (2) Watery stool (3) Patients admitted with Acute Gastro Enteritis (AGE) developing probable Cholera suspected symptoms.

4.2 Isolation and Culture of Bacterial Strains

A loopful sample was enriched in alkaline peptone water broth (Himedia) with 3% NaCl and incubated for 18 hrs and then streaking was done on Thiosulphate Citrate Bile Salt Agar Plates (Himedia) and incubated at 37 for 24 hrs. Positive colonies were selected on the basis of phenotypic feature, more specifically yellowish colonies.

4.3 Serological identification, serotyping and Rapid Test for Sero-grouping

Serogrouping and serotyping were performed by using Rapid kits (Rapid Kit, ICDDRB) and Slide Agglutination methods. For serogrouping by Rapid test approximately 800µl of Alkaline peptone water (APW) cultured broth was taken and rapid stripe kits were dipped and respective band pattern were compared to identify respective serogroup. For Slide Aggluntination test respective antibody with amount of approx 5 µl of specific serotype biotype viz. Inaba, Ogawa and Hikojima was dispensed in glass slide and a loopful of culture was mixed, based on agglutination serotype was noted.

4.4 Antibiotic susceptibility test and Biotyping using phenotypic tests

4.4.1 Antibiotic susceptibility test:

Susceptibility to antibiotics was performed by disk diffusion, as described both by Bauer *et al.* (Bauer *et. al*., 1966) and the Clinical and Laboratory Standards Institute (CLSI manual,2010), using commercial antibiotic discs. A total of nine antibiotics (Oxoid, United Kingdom): Erythromycin (E, 15µg); Gentamicin (CN, 10µg); Trimethoprim/Sulfamethoxazole (SXT, 30µg), Tetracycline (TE, 30µg), Ampicillin (AMP,

30µg), Streptomycin (S, 10µg), Azithromycin (AZM, 15µg), Nalidixic acid (NA, 30µg) and Ciprofloxacin (CIP, 5µg) were employed. Characterizations of the resistance or susceptibility profiles of the isolates were determined by measuring the inhibitory zone and comparing it with an interpretative chart to determine sensitivity to the antibiotics. Biotyping was done by number of phenotypic tests: Chicken erythrocyte agglutination (CCA), sensitivity to Polymyxin B and Mukerjee CL phage IV and ET phage V tests. Brief description about procedure of tests is mentioned below.

4.4.2 Chicken Cell Agglutination Test:

Fresh chicken red blood cell was used for this assay. A 2.5% (vol/vol) suspension of washed (3 times) and packed (by centrifugation) cells was made in normal saline after the final wash. A large loopful of the red cell suspension was placed on a glass slide. A small portion of the bacterial colony or growth from a nutrient agar (Himedia) was added to the red cells with a needle or loop, and was mixed well and agglutination was observed. Results were interpreted as positive for strain which showed agglutination till 30 to 60 seconds.

4.4.3 Sensitivity test against Polymyxin B:

A 50-unit Polymyxin B disk (Mast Diagnostics, Merseyside, U.K.) was used for this test. In this test known strains of classical and El Tor biotypes were used as controls and zone of inhibition was measured. Strain showing inhibition zone ≥12mm was interpreted as sensitive and vice versa.

4.4.4 Mukerjee Cl phage IV and ET Phage V tests:

Phage typing of isolated strains was done following previously mentioned procedure (Mukerjee *et. al.,* 1963). In this test selected strains isolated from diarrheal samples were streaked and grown in Nutrient Agar media. After complete growth of selected strain in agar media reference phage (Phage IV and Phage V) suspension was inoculated in culture plate using micropipette (approx 10 µl) and incubated at 37 °c for 24 hrs. Plaque or clear zone was observed. Plaque or clear lysis in culture infected with reference phages was interpreted as sensitive and infected culture with phage suspension with no clear zone is interpreted as resistant.

4.5 Extraction of crude DNA from culture

The DNA was extracted via boiling method (Alam *et. al.*, 2012). In brief, 2 ml overnight Alkaline peptone water (APW) culture broth was taken and centrifuged at maximum speed (13000 rpm/min) to harvest cell pellet. Once after harvesting of cell pellet, approximately 500 -1000 µl of sterile PBS (phosphate buffer saline) was added, mixed

properly and boiled at 100 ◦C for 10 min in water bath and immediately transferred to -20◦c for 30 minute and centrifuged at maximum speed (13000rpm/min) for 10 min and crude DNA extract was collected and stored .

Primer	Sequence (5'-3')	Targeted gene	Annealing temperature	Amplicon size(bp)	Reference
ompW F ompW R	CAC CAA GAA GGT GAC TTT ATT GTG GGT TTG TCG AAT TAG CTT CAC C	*ompW*	64 °C	304	Nandi *et al.* (2000)
rfbO1 F rfbO1 R	GTT TCA CTG AAC AGA TGG G GGT CAT CTG TAA GTA CAA C	*rfbO1*	55 °C	192	Hoshino *et al.* (1998)
rfbO139 F rfbO139 R	AGC CTC TTT ATT ACG GGT GG GTC AAA CCC GAT CGT AAA GG	*rfbO139*	55 °C	449	Hoshino *et al.* (1998)
ctxA F ctxA R	ACA GAG TGA GTA CTT TGA CC ATA CCA TCC ATA TAT TTG GGA G	*ctxA*	55 °C	308	Hoshino *et al.* (1998)
tcpA El Tor R tcpA F	CGA AAG CAC CTT CTT TCA CAC GTT G CAC GAT AAG AAA ACC GGT CAA GAG	*tcpA* El Tor	60 °C	453	Rivera *et al.* (2001)
tcpA Class R tcpA F	TTA CCA AAT GCA ACG CCG AAT G CAC GAT AAG AAA ACC GGT CAA GAG	*tcpA* Classical	60 °C	620	Rivera *et al.* (2001)
Rv-cla	CCTGGTACTTCTACTTGAAACG	*ctxB*			Morita *et al.* (2008)
ctxB-F3	GTTTTACTATCTTCAGCATATGCGA	*ctxB*	55 °C	191	Naha et al. (2012)
ctxB-F4	GTTTTACTATCTTCAGCATATGCGC	*ctxB*	60 °C	191	Naha et al. (2012)
rstR1 F rstR3A R	CTT CTC ATC AGC AAA GCC TCC ATC TCG AGT TGT AAT TCA TCA AGA GTG	*rstR* Classical	50 °C	500	Mwansa *et al.* (2006)
rstR2 F rstR3A R	GCA CCA TGA TTT AAG ATG CTC TCG AGT TGT AAT TCA TCA AGA GTG	*rstR* El Tor	50 °C	500	Mwansa *et al.* (2006)
rtxC F rtxC R	CGA CGA AGA TCA TTG ACG AC CAT CGT CGT TAT GTG GTT GC	*rtxC*	55/56 °C	265	Chow *et al.* (2001)
rstC1 rstC2	AAC AGC TAC GGG CTT ATT C TGA GTT GCG GAT TTA GGC	*rstC*	52.4 °C	238	O'Shea *et al.* (2004)
tlc3 tlc4	GGG AAT GTT GAG TTC TCA GTG GTT GCG AAG TGG ATT TTG TG	*tlc*	55.5 °C	1,548	O'Shea *et al.* (2004)

Table 7: List of primers used in study

4.6 Genomic identification of *Vibrio cholerae* from samples using PCR based assays

Genomic identification and characterization of the *Vibro* from culture was done through polymerase chain reaction (PCR) amplification of specific genes at specific condition as described below.

4.7.1 Singlex PCR for identification of *Vibrio cholerae*

Outer membrane protein coding gene namely OmpW gene specific to *Vibrio* species was amplified for confirmation of identification of *Vibrio* from other enteric pathogen using specific set of primers (listed in table 7) under the optimized condition as described in table 8 and 9 below.

Table 8: Master Mix Components for ompW Singlex PCR

Ingredients	Volume Per reaction	Remarks
d/w	10.8µl	MilliQ
10x Pfu buffer	2.0µl	Thermo scientific
$MgCl_2$(50mm)	1.0µl	Sigma
dNTPs (12.5µm)	2.0µl	Thermo scientific
Ompw primer F(10 pmol)	1.0µl	-
Ompw reverse R(10 pmol)	1.0µl	
Pfu polymerse	0.2µl	Thermo scientific
DNA template	2.0µl	-
Total	20 µl	-

Table 9: Thermocycling condition for ompW Singlex PCR

Step	Temperature	Time	Cycle
1	94◦C	5 min	1
2	94◦C	30 sec	35
	64◦C	30 sec	
	72◦C	1 min	
3	72◦C	7 min	1
4	4◦C	Hold	-

4.7.2 Multiplex PCR for serogrouping of *Vibrio cholerae* O1 /O139 and ctxA gene detection

Once after singlex PCR, to identify and confirm specific serotype (O1, O139 or non O1/O139) with Cholera toxin gene Cholera toxin A (CtxA), Somatic antigen gene specific for O1 and O139 strain, rfbO1 and rfbO139 respectively were amplified using optimized multiplex PCR protocol and parameters listed in table 10 and 11 .

Table 10: Optimized Master Mix Components for rfBO1/O139 and ctxA Multiplex PCR

Ingredients	Volume Per reaction	Remarks
d/w	14.7µl	MilliQ
10x Pfu buffer	3.0µl	Thermo scientific
$MgCl_2$(50mm)	1.0µl	Sigma
dNTPs (2.5mm)	2.5µl	Thermo scientific
rbfO1 primer F(10 pmol)	1.5µl	-
rbfO1 primer R(10 pmol)	1.5µl	
O139 primer F(10 pmol)	0.8µl	
rfbO139 primer R (10 pmol)	0.8µl	
CtxA primer F(10 pm)	0.5µl	
CtxA primer R(10 pm)	0.5µl	
Pfu polymerse	0.2µl	Thermo scientific
DNA template	3.0µl	-
Total	30 µl	-

Table 11: Thermocycling conditions for rfBO1/O139 and ctxA Multiplex PCR

Step	Temperature	Time	Cycle
1	95◦C	5 min	1
2	94◦C	30 sec	35
	58◦C	30 sec	
	72◦C	1 min	
3	72◦C	7 min	1
4	4◦C	Hold	-

Table 12: Master Mix Components for tcpA multiplex PCR

Ingredients	Volume Per reaction	Remarks
d/w	14.5 µl	MilliQ
10x Pfu buffer	0.2 µl	Thermo scientific
$MgCl_2$(50mm)	1 µl	Sigma
dNTPs (12.5µm)	2 µl	Thermo scientific
tcpA primer F(10 pmol)	1 µl	-
tcpA Cl reverse primerR(10 pmol)	1 µl	
tcpA El Tor reverse primer R(10 pmol)	1 µl	
Pfu polymerse	0.2 µl	Thermo scientific
DNA template	3 µl	-
Total	20 µl	-

Table 13: PCR Parameters for tcpA multiplex PCR

Step	Temperature	Time	Cycle
1	94◦C	3 min	1
2	94◦C	1 min	29
	62◦C	1.30 min	
	72◦C	7 min	
3	72◦C	7 min	1
4	4◦C	Hold	-

4.7.3 Genomic characterization, genotyping using PCR based assays

To complement the biotype characterization by phenotypic tests, various PCR based assays using various biotype specific marker genes were carried out under modified condition, using previously mentioned primers and procedures. These assay include amplification of specific marker gene viz. toxin co-regulated pilli tcpA allele (CL and ET) (Rivera *et. al*., 2001); rstR gene encoding the phage transcriptional regulator (Kimsey *et.*

al., 1998); rtxC gene of RTX (repeat in toxin) (Chow *et. al*., 2003), Cholera toxin ctxB variation identification via double mismatch mutation amplification PCR assay (Naha *et. al.,* 2012). Beside this presence of toxin linked cryptic plasmid was also analyzed via PCR assay through method and condition described previously (O'Shea *et. al.,* 2004).

Table 14: Optimized PCR parameter for DMAMA PCR assay

Ingredients	Volume Per reaction	Remarks
d/w	10.8µl	MilliQ
10x Pfu buffer	2 µl	Thermo scientific
$MgCl_2$(50mm)	1 µl	Sigma
dNTPs (2.5mm)	2 µl	Thermo scientific
primer R(10 pmol) (ctxB F3)	1 µl	-
primer R(10 pmol) (ctxB F4)	1 µl	-
Pfu polymerse	0.2 µl	Thermo scientific
DNA template	2 µl	-
Total	20 µl	-

Table 15: Optimized PCR condition for DMAMA PCR assays

Step	Temperature	Time	Cycle
1	96◦C	2 min	1
2	96◦C	0.10min	24
	56◦C	0.10min	
	72◦C	0.30min	
3	72◦C	2min	1
4	4◦C	Hold	-

Table 16: Optimized PCR parameter for rtxC,rstC and tlc PCR assay

Ingredients	Volume Per reaction			Remarks
	rstC	rtxC	tlc	
d/w	10.8 µl	10.8 µl	10.8 µl	MilliQ
10x Pfu buffer	2 µl	2 µl	2 µl	Thermo scientific
$MgCl_2$(50mm)	1 µl	1 µl	1 µl	Sigma
dNTPs (2.5mm)	2 µl	2 µl	2 µl	Thermo scientific
primer R(10 pmol)	1 µl	1 µl	1 µl	-
primer R(10 pmol)	1µl	1µl	1µl	-
Pfu polymerse	.2 µl	.2 µl	.2 µl	Thermo scientific
DNA template	2 µl	2 µl	2 µl	-
Total	20 µl	20 µl	20 µl	-

Table 17: Optimized PCR condition for rtxC, rstC and tlc PCR assays

Step	Temperature(^{0}C)			Time(min)			Cycle		
	rtxC	rstC	tlc	rtxC	rstC	tlc	rtxC	rstC	tlc
1	94	94	94	5	5	3	1	1	1
2	94	94	94	1	0.40	1	29	34	24
	56	56	56	1	0.40	1			
	72	72	72	1	1.30	1			
3	72	72	72	7	2	1	1	1	1
4	4	4	4	Hold			-		

Table 18: Optimized PCR parameter for rstR1 and rstR2 PCR assays

Ingredients	Volume Per reaction		Remarks
	rstR1	rstR2	
d/w	14.5 µl	14.5 µl	MilliQ
10x Pfu buffer	2.5 µl	2.5 µl	Thermo scientific
$MgCl_2$(50mm)	2.5 µl	2.5 µl	Sigma
dNTPs (2.5mm)	0.8µl	0.8µl	Thermo scientific
primer R(10 pmol)	0.75 µl	0.75 µl	-
primer R(10 pmol)	0.75 µl	0.75 µl	-
Pfu polymerse	0.2 µl	0.2 µl	Thermo scientific
DNA template	3 µl	3 µl	-
Total	25 µl	25 µl	-

Table 19: Optimized PCR condition for rst R1 and rstR2 PCR assays

Step	Temperature(^{0}C)		Time(min)		Cycle	
	rstR1	rstR2	rstR1	rstR2	rstR1	rstR2
1	96	96	2	2	1	1
2	96	96	0.10	0.10	24	24
	56	56	0.10	0.10		
	72	72	0.30	0.30		
3	72	72	2	2	1	1
4	4	4	Hold		-	

4.7.4 Amplified Product Detection

2% Agarose gel (100 ml) was prepared in 0.5X TBE buffer and 1.5µl (0.15µg/ml) of Ethidium bromide was added to it .Gel cassette was prepared and amplified PCR product were loaded along with the 6X gel loading dye. Voltage (94 volts) was supplied for approx 2hours and the gel was visualized under Biorad GELDOC System. Two different gene Ruler (ladder) 100 bp (New England Biolab, United Kingdom), consisting of DNA fragments ranging from1, 517 to 100 base pair ,and 1Kb ruler consisting of were used as a molecular marker.

4.7.5 Pulsed Field Gel Electrophoresis and Image analysis

The whole agarose-embedded genomic DNA for *Vibrio cholerae* was prepared. Pulsed field gel electrophoresis (PFGE) was carried out with a contour-clamped homogeneous electrical field (CHEF-DRII) apparatus (Bio-Rad), according to procedures described elsewhere (Cooper *et*. *al.*, 2006). The conditions used for separation were as follows: 2 to 10 s for 13 h, followed by 20 to 25 s for 6 h. An electrical field of 6V/cm was applied at an included field angle of 120°. Genomic DNA's of the test strains were digested by the NotI restriction enzyme (Gibco-BRL, Gaithersburg, MD) and *Salmonella enterica* serovar Braenderup was digested by XbaI, with the fragments being used as molecular-size markers. The restriction fragments were separated in 1 pulsed-field-certified agarose in 0.5X TBE (Tris-borate-EDTA) buffer. In the post electrophoresis gel treatment step, the gel was stained and de-stained. The DNA was visualized using a UV transilluminator and images were digitized by a one-dimensional gel documentation system (Bio-Rad).The fingerprint pattern in the gel was analyzed using a computer software package, Bionumeric (Applied Maths, Belgium). After background subtraction and gel normalization, the fingerprint patterns were subjected to typing on the basis of banding similarity and dissimilarity using Dice similarity coefficient and unweighted-pair group method (UPGMA) using average linkage clustering methods, as recommended by the manufacturer; these were graphically represented as dendrograms.

RESULTS

5.1 Isolation and culture of *Vibrio cholerae* from samples and serological test

Among collected samples (n=72), 28 *Vibrio* related bacteria were isolated. Among them 23 strains typically shown *V. cholerae* O1 positive result in rapid serological test and produced characteristics yellow colonies specific to *V. cholerae* when they were grown on Thiosulphate Citrate Bile Salt (TCBS) Agar. In another slide agglutination test, among collected samples 23 strains shown agglutination with monovalent Ogawa antisera specific to *V. cholerae* O1 Ogawa and remaining five strains were found to be non O1 strains as they were unable to show positive agglutination like *V. cholerae* O1.

5.2 Singlex and Multiplex PCR assay for identification of *Vibrio cholerae* and Cholera toxin gene (ctxA)

Among 72 samples 28 samples amplified *Vibrio* species specific outer membrane protein gene (OmpW) of amplicon size 304 bp in Singlex PCR assay but other 44 strains failed to amplify primer specific for *Vibrio* species. Hence 28 Singlex PCR positive samples were identified as *Vibrio* species. Similarly in Multiplex PCR assay of collected samples, 23 strains amplified *V. cholerae* O1 specific somatic antigen rfbO1 of amplicon size 192 bp and showed the presence of Cholera toxin gene ctxA (amplicon size 308 bp). Among screened 28 *Vibrio* strains 5 strains were identified only as *Vibrio* species by Singlex PCR as they failed to amplify gene targeted against detection of rfbO1 and ctxA, suggesting that these strains are not *V. cholerae* O1 or O139 and do not posses Cholera toxin gene, which is hallmark of pathogenic *V. cholerae.* Hence these strains are non O1 or O139 strains.

	Microbiological ,Serologial identification and serogrouping			**PCR Identification and serogrouping**				**Result**
				Singlex	Multiplex			
ID	TCBS Culture	Rapid test /serogroup	Agglutination test/ serotyping	ompW	rfbO1	rfbO139	ctxA	
PC1	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*
PC2	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*
PC3	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*
PC4	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*
PC5	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*
PC6	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*
PC7	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*
PC8	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*
PC9	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*
PC10	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*
PC11	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*
PC12	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*
PC13	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*
PC14	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*

Table 20: Summary of microbiological, serological and serotyping assays of *Vibrio cholerae* isolated from clinical samples.

	Microbiological ,serologial identification and serogrouping			**PCR Identification and serogrouping**				
				Singlex	Multiplex			
ID	TCBS Culture	Rapid test /serogroup	Agglutination test/serotyping	ompW	rfbO1	rfbO139	ctxA	Result
PC15	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*
PC16	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*
PC17	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*
PC18	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*
PC19	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*
PC20	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*
PC21	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*
PC22	Shiny Yellow colonies	O1	Ogawa	+	+	-	+	*V. cholerae O1 Ogawa*
TK12	Pale Yellow colonies	ND	NA	+	-	-	-	*V. cholerae nonO1*
TK25	Pale Yellow colonies	ND	NA	+	-	-	-	*V. cholerae nonO1*
TK30	Pale Yellow colonies	ND	NA	+	-	-	-	*V. cholerae nonO1*
TK31	Pale Yellow colonies	ND	NA	+	-	-	-	*V. cholerae nonO1*
TK44	Shiny Yellow colonies	O1	Ogawa	+	-	-	-	*V. cholerae nonO1*
TK50	Pale Yellow colonies	ND	NA	+	-	-	-	*V. cholerae nonO1*

ND: Not Determined, NA: Not applicable

Table 21: Summary of microbiological, serological and serotyping assays of *Vibrio cholerae* isolated from clinical samples.

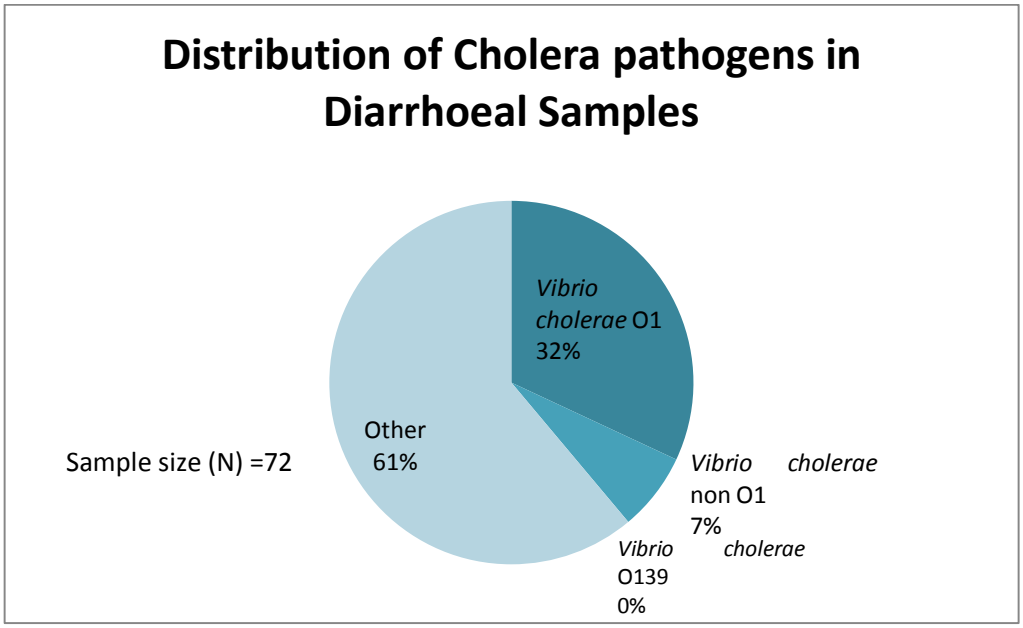
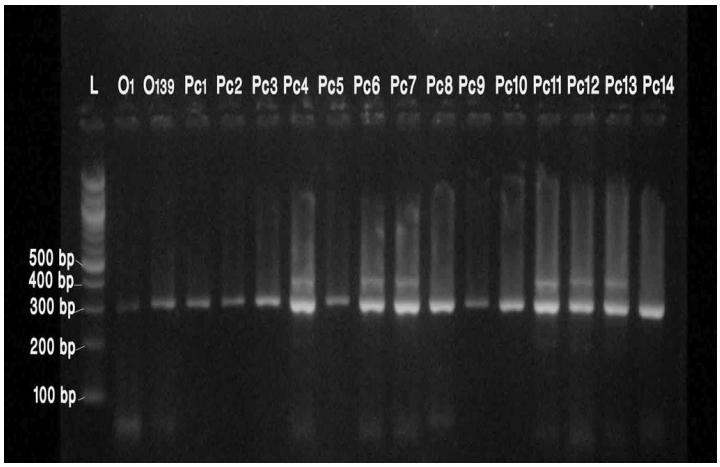


Figure 11: Distribution of Cholera pathogens in selected clinical samples identified via Singlex, Multiplex PCR and serological assay

List of Gel-electrophoresis photographs of Singlex and Multiplex PCR optimized for rapid identification of *V. cholerae* O1 & O139 and Cholera toxin gene (ctxA).



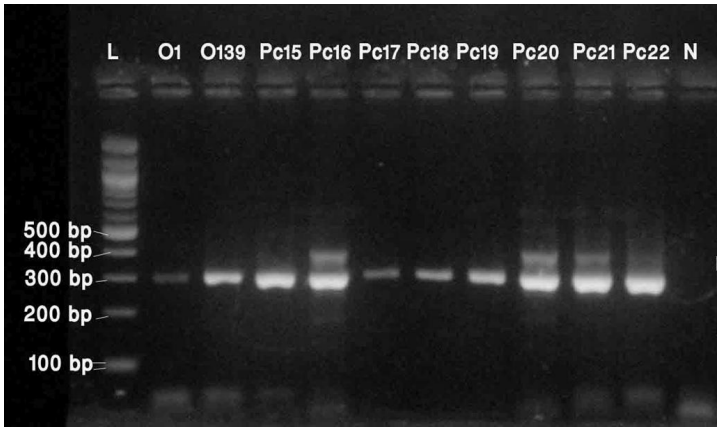
L: 100 bp ladder

O1& O139: Positive Control

PC1-PC14: Samples

Amplicon Size: 304 bp

Figure 12: Gel Photograph, Singlex PCR of ompW gene encoding outer membrane protein specific to *Vibrio cholerae* (PC1-PC14)



L: 100 bp ladder

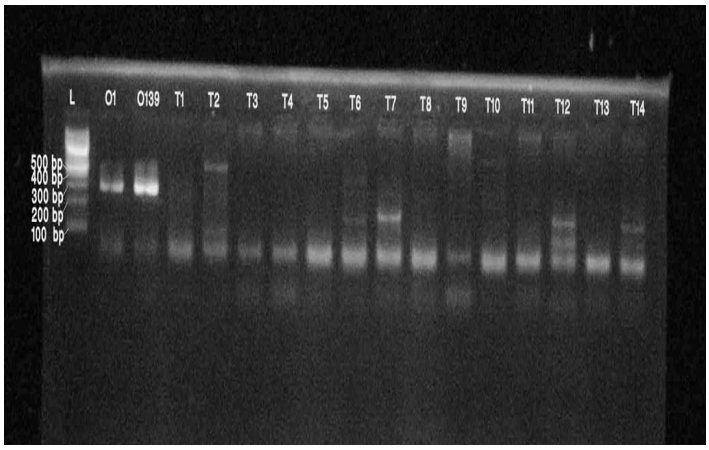
O1& O139: Positive Control

N: Negative Control

PC15-PC22: Samples

Amplicon Size: 304 bp

Figure 13: Gel Photograph, Singlex PCR of ompW gene encoding outer membrane protein specific to *Vibrio cholerae* (PC15-PC22)



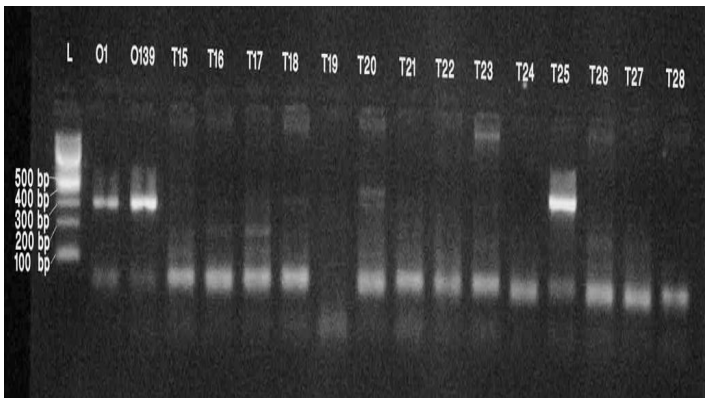
L: 100 bp ladder

O1& O139: Positive Control

T1-T14: Samples

Amplicon Size: 304 bp

Figure 14: Gel Photograph, Singlex PCR of ompW gene encoding outer membrane protein specific to *Vibrio cholerae*(T1-T14)



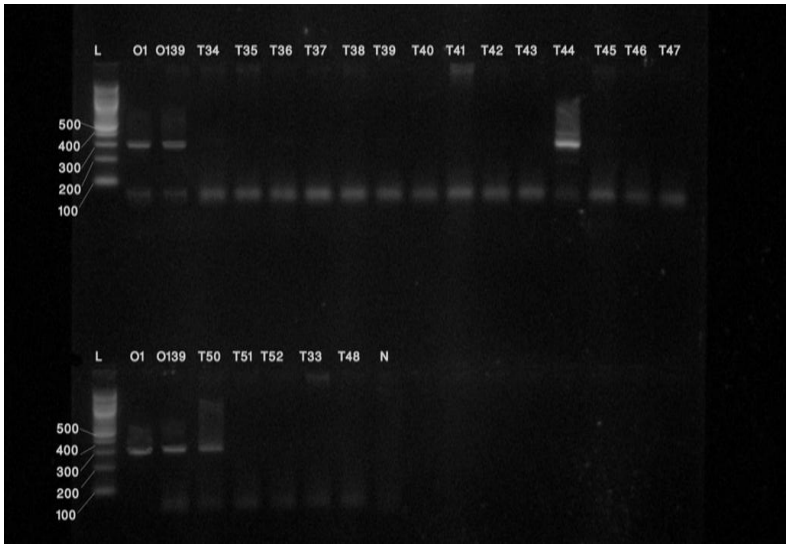
L: 100 bp ladder

O1& O139: Positive Control

T15-T23: Samples

Amplicon Size: 304 bp

Figure 15: Gel Photograph, Singlex PCR of ompW gene encoding outer membrane protein specific to *Vibrio cholerae* (T15-T23)



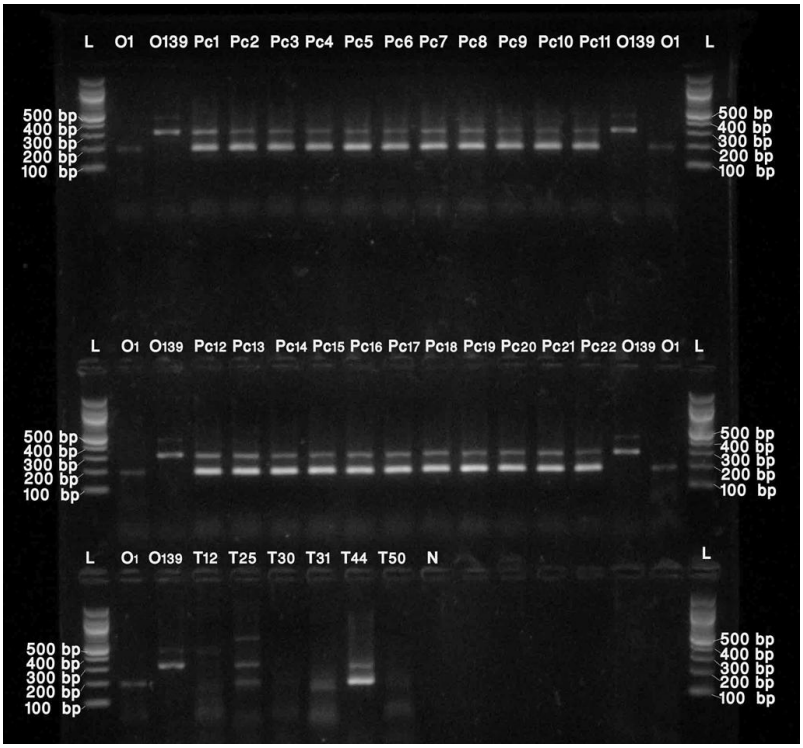
L: 100 bp ladder

O1& O139: Positive Control

T34-T51: Samples

Amplicon Size: 304 bp

Figure 16: Gel Photograph, Singlex PCR of ompW gene encoding outer membrane protein specific to *Vibrio cholerae* (T34-T51)



O1 & O139: Positive control

L: 100 bp Ladder,

N: Negative control,

PC1-22, T12, T30, T31, T50: samples

Amplicon Size:

rfbO1: 192bp

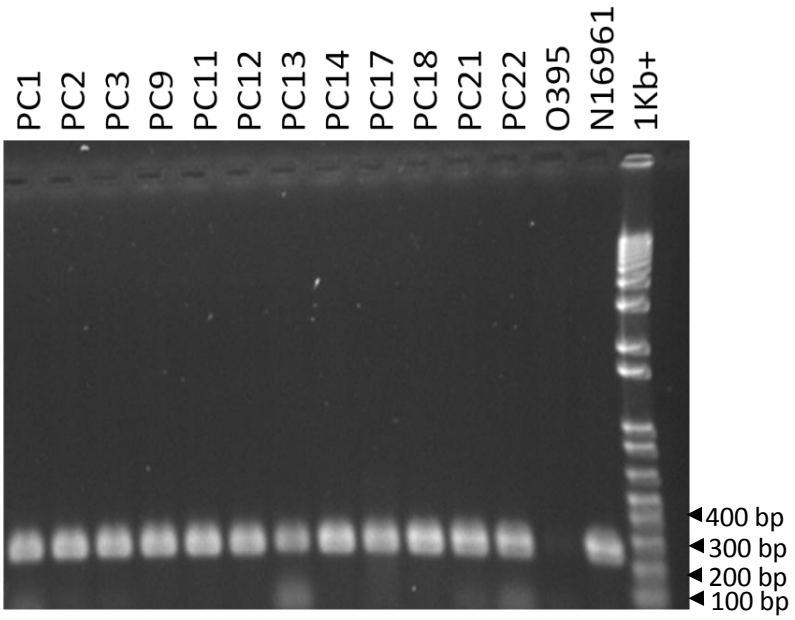
rfBO139: 449bp

CtxA: 208 bp

Figure 17: Gel Photograph, Multiplex PCR assay for simultaneous detection of rfbO1, rfbO139 CtxA gene of *Vibrio cholerae* (PC1-22, T12, T30, T31 and T50)

5.3 Biotyping by Phenotypic and PCR based assay

Result of phenotypic and genotypic characteristics of 12 selected *Vibrio cholerae* O1 strains is presented in table 31 below. All of the selected *V. cholerae* O1 strains were primarily identified as ET biotype based on specific phenotypic characteristics. In which all strains exhibited agglutination with Chicken erythrocytes (CCA), sensitivity towards ET-specific phage V, resistance to both Polymyxin B (50U) and CL specific phage. All the phenotypically confirmed ET stains were again subjected to PCR based biotyping tests and amplified primers for the rtxC gene unique to ET biotype thus by confirming their ET type traits. Furthermore all ET strains amplified primers for toxin linked cryptic plasmid (tlc) showing presence of tlc gene. Similarly using another PCR based rst typing method considering rstC gene, a unique genetic component of EI Tor present in satellite phage known as RS1 (Safa A. *et al*., 2009); rstR1 gene of RS2 element regulating CTXϕ specific to EI tor genotype; rstR2 regulating CTXϕ specific to classical genotype further genotypic screening was done. In this genotyping test all the selected strains amplified primer of two genes namely rstC,rstR2 excluding primer of rstR1 hence this further confirmed that all these strains are typical EI Tor variants of *V. cholerae* O1. Additionally another genetic component responsible for coding toxin co-regulated pilli gene (tcpA) which is receptor for entry of CTXϕ that specific to ET Tor denoted as $tcpA^{ET}$ was amplified by all selected strains which depicted that all these strains possesed ET Tor type tcpA allele.



O395: Negative control (Classical Strain)

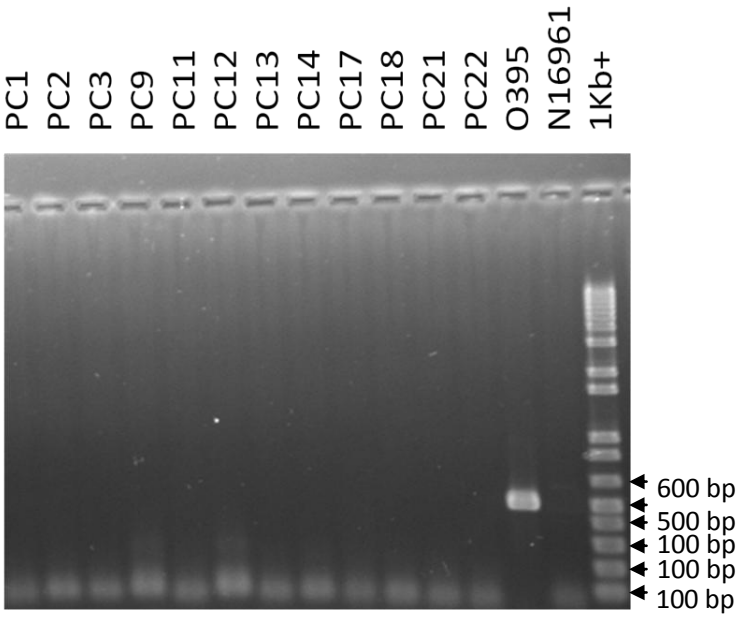
N16961: Positive Control (EI Tor)

PC denoted by numbers: Selected samples

Ladder: 1Kb

Amplicon size: 238 bp (rstC)

Figure 18: PCR gel image of rstC gene component of satellite phage RS1 specific to EI Tor



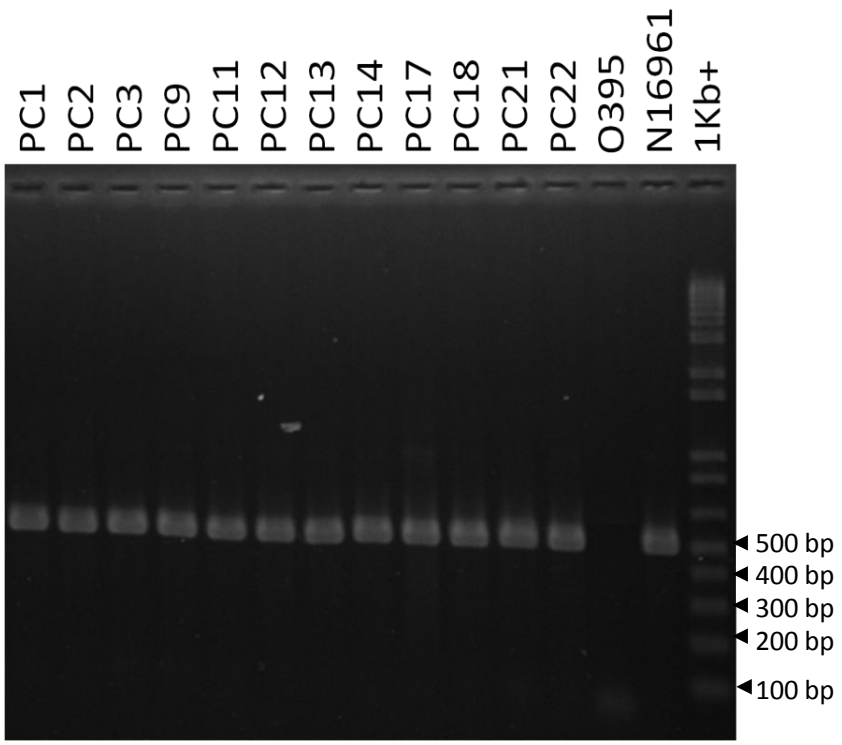
O395: Positive control (Classical Strain)

N16961: Negative Control (EI Tor)

PC denoted by numbers: Selected samples

Ladder: 1Kb ,Amplicon size: 500 bp(rstR1,classical)

Figure 19: PCR gel image of rstR1 gene of RS2 element regulating CTXϕ.



O395: Negative control (Classical Strain)

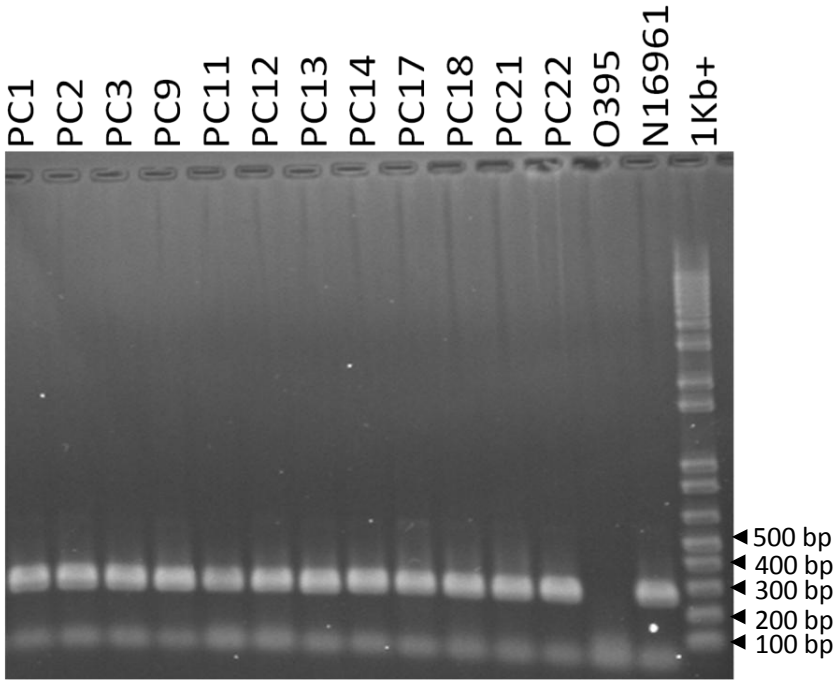
N16961: Positive Control (EI Tor)

PC denoted by numbers: Selected samples

Ladder: 1Kb

Amplicon size: 500 bp(rstR2,EI Tor)

Figure 20: PCR gel image of rstR2 gene of RS2 element regulating CTXϕ.



O395: Negative control (Classical Strain)

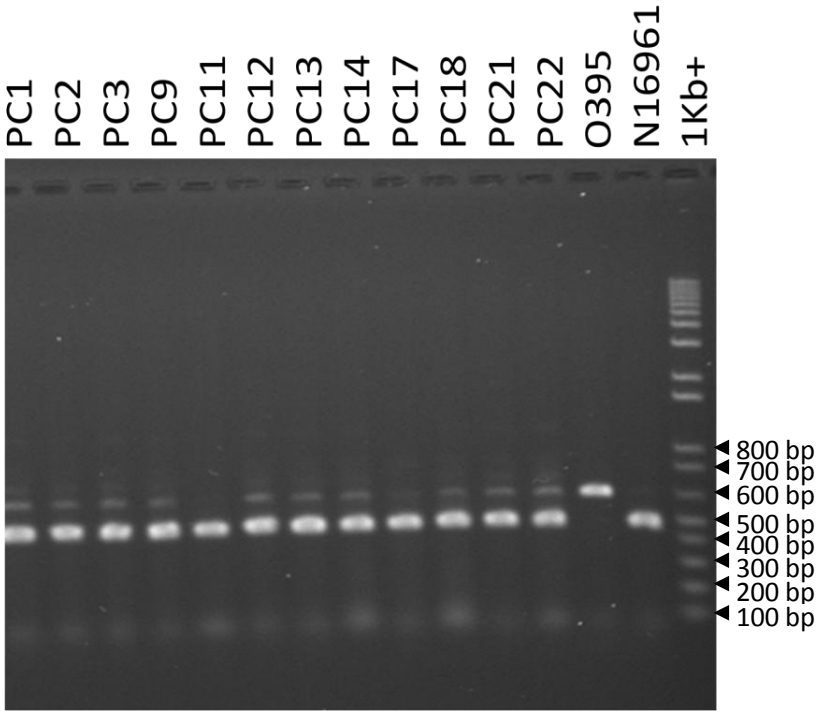
N16961: Positive Control (EI Tor)

PC denoted by numbers: Selected samples

Ladder: 1Kb

Amplicon size: 238 bp(rstC,EI Tor)

Figure 21: PCR gel image of rtxC gene component present in Repeat in toxin (RTX) gene.



O395: Positive control (Classical Strain)

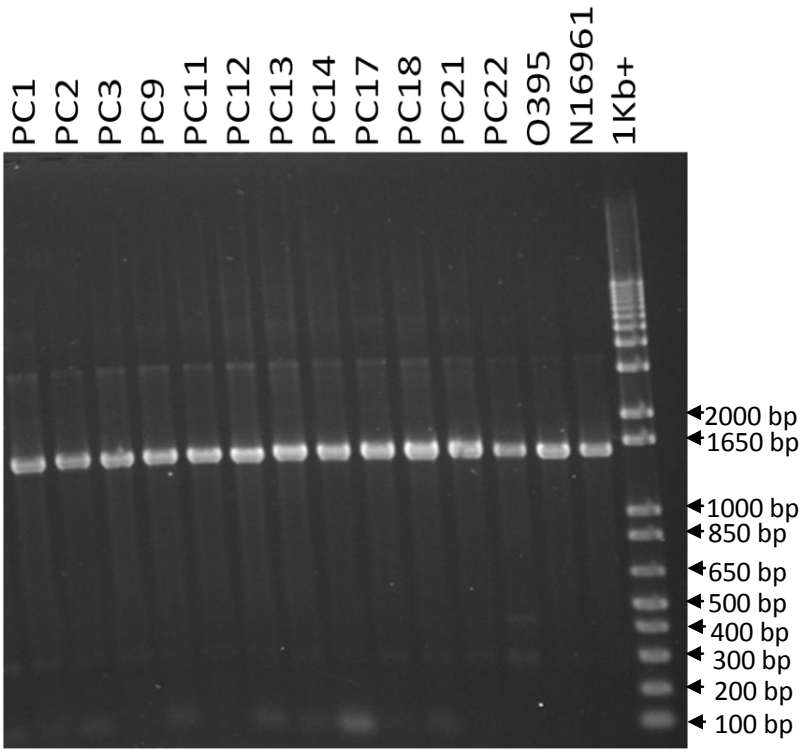
N16961: Positive Control (EI Tor)

PC denoted by numbers: Selected samples

Ladder: 1Kb

Amplicon size: tcpA EI Tor: 453bp,tcpA Classical: 620 bp

Figure 22: PCR gel image of simultaneous detection of tcpA allele specific to classical and EI Tor



O395: Positive control (Classical Strain)

N16961: Positive Control (EI Tor)

PC denoted by numbers: Selected samples

Ladder: 1Kb

Amplicon size:1,548 bp(tlc)

Figure 23: PCR gel image of tlc (toxin linked cryptic plasmid)

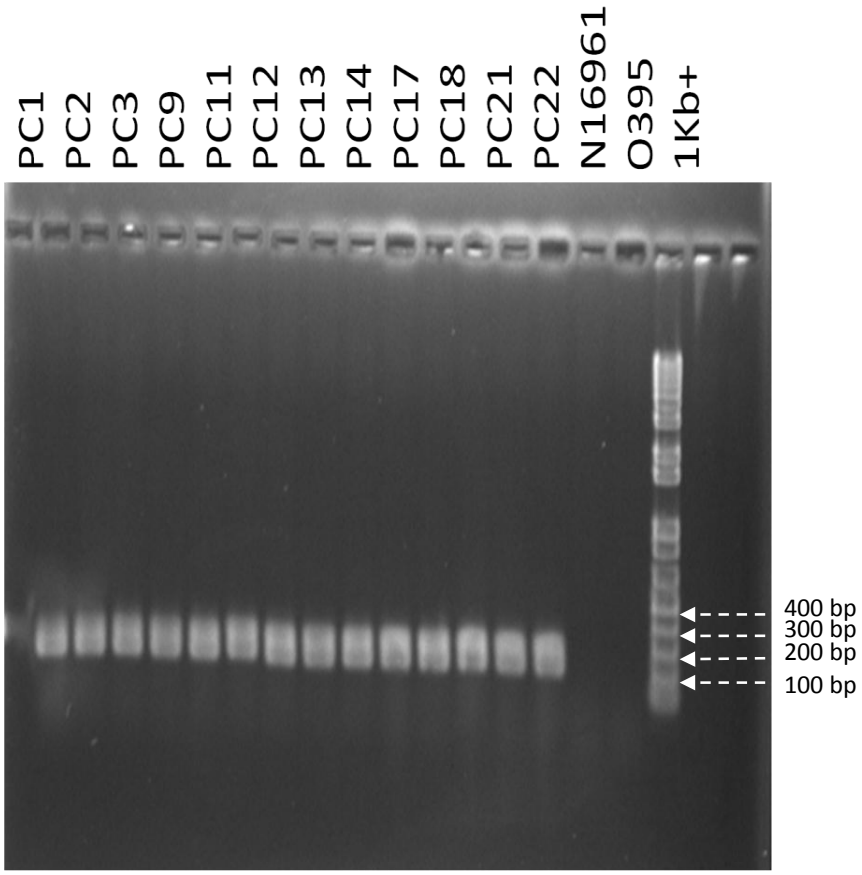
5.4 Antibiotic Susceptibility Testing

Result of response of *V. cholerae* O1 strains towards six different antibiotics disclosed that all the selected strains were 100% resistance to antibiotics namely Trimethoprim/Sulfamethoxazole (SXT), Nalidixic Acid (NA); Streptomycin while response towards other antibiotics among total of nine antibiotics Erythromycin; Gentamicin; Tetracycline, Ampicillin, Azithromycin and Ciprofloxacin was susceptible. Cumulative analysis of antibiotic response suggested that there was presence of multidrug resistance in *V. cholerae* strain prevalent in 2010 Kathmandu diarrheal outbreak.

5.5 CtxB genotyping by Double Mismatch Amplification Mutation Assay (DMAMA)-PCR

Selected *V. cholerae* O1 strains, including the O395 (CL), N16961 (ET) were analyzed by Double Mismatch Amplification Mutation Assay (DMAMA)-PCR technique to determine the ctxB genotype. As shown in table 22, all *V. cholerae* O1 ET strains isolated amplified double mismatch amplification primer designed for Haitian genotype and depicted that these strains belong to ctxB genotype 7. Similarly DMAMA-PCR also revealed these strains

comprise point mutation at 58th nucleotide position of the ctxB gene which was also reported recently from *V. cholerae* O1 strains in India (Kolkata) and Haiti in 2006 and 2010-2012 respectively (Naha *et al*., 2012).



O395: Control (Classical Strain)

N16961: Control (EI Tor)

PC denoted by numbers: Selected samples

Ladder: 1Kb

Amplicon size: ctxB classical 191 bp

.

Figure 24: PCR gel image of Double-Mismatch-Amplification-Mutation Assay (DMAMA) for ctxB genotyping

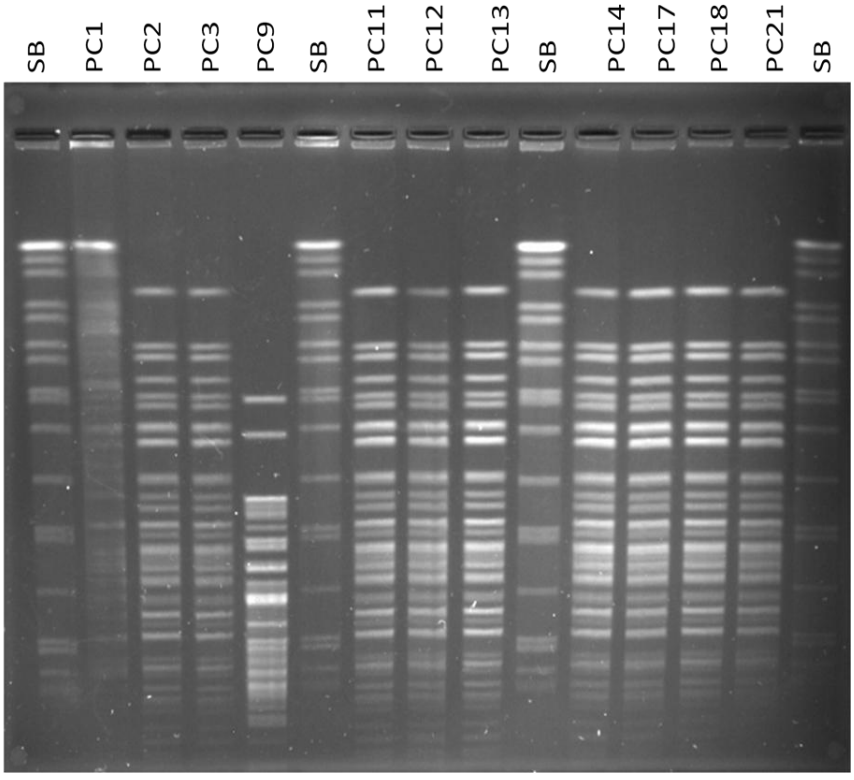
Table 22. Phenotypic , genotypic and drug resistance properties of *12 Vibrio cholerae* O1 causing Cholera in Kathmandu, Nepal

Country	Strain ID	Year of Isolation	Source	Serotype	*rfbO1*	Phenotypic Properties				Genetic screening by PCR							Resistance Profile
							Sensitivity										
						CCA	PMB (50U)	CL Specific phage IV	El Tor-Specific phage v	*ctxA*	*tcpA type*	*ctxB genotype*	[a] *rstR type*	*rtxC*	*rstC*	*tlc*	
Nepal	PC1	2012	Clin	Ogawa	+	+	R	R	S	+	ET	B7	ET	+	+	+	SXT, NA, S
	PC2	2012	Clin	Ogawa	+	+	R	R	S	+	ET	B7	ET	+	+	+	SXT, NA, S
	PC3	2012	Clin	Ogawa	+	+	R	R	S	+	ET	B7	ET	+	+	+	SXT, NA, S
	PC9	2012	Clin	Ogawa	+	+	R	R	S	+	ET	B7	ET	+	+	+	SXT, NA, S
	PC11	2012	Clin	Ogawa	+	+	R	R	S	+	ET	B7	ET	+	+	+	SXT, NA, S
	PC12	2012	Clin	Ogawa	+	+	R	R	S	+	ET	B7	ET	+	+	+	SXT, NA, S
	PC13	2012	Clin	Ogawa	+	+	R	R	S	+	ET	B7	ET	+	+	+	SXT, NA, S
	PC14	2012	Clin	Ogawa	+	+	R	R	S	+	ET	B7	ET	+	+	+	SXT, NA, S
	PC17	2012	Clin	Ogawa	+	+	R	R	S	+	ET	B7	ET	+	+	+	SXT, NA, S
	PC18	2012	Clin	Ogawa	+	+	R	R	S	+	ET	B7	ET	+	+	+	SXT, NA, S
	PC21	2012	Clin	Ogawa	+	+	R	R	S	+	ET	B7	ET	+	+	+	SXT, NA, S
	PC22	2012	Clin	Ogawa	+	+	R	R	S	+	ET	B7	ET	+	+	+	SXT, NA, S
India	O395	1965	Clin	Ogawa	+	-	S	S	R	+	CL	B1	CL	-	-	+	-
Bangladesh	N16961	1971	Clin	Inaba	+	+	R	R	S	+	ET	B3	ET	+	+	+	-

[a] Determine by double mismatch amplification mutation assay (DMAMA) PCR (Naha et al., 2012); Clin, clinical; PMB, polymyxin B; R, resistant; s, sensitive; ET, El Tor; CL, classical; CCA, chicken cell agglutination SXT, trimethoprim/sulfamethoxazole; NA, Nalidixic Acid; S, Streptomycin

5.6 Pulsed Field Gel Electrophoresis Analysis

A total of 11 representative strains out of 23 confirmed *V. cholerae* O1 strains isolated in 2012 Kathmandu diarrheal outbreak were analyzed by Pulsed-field Gel Electrophoresis (PFGE) to reveal the genetic relatedness of strains that were circulating in Kathmandu along with primitive *V. cholerae* O1 strain from Bangladesh 1971. The *NotI* restriction enzyme digested the genomic the genomic DNA into variable fragments and the fragment sizes ranged from 20 to 350 kb. All of the tested *V. cholerae* strains had very closely related PFGE banding patterns, suggesting that they belong to the same clonal lineage as depicted by the preliminary analysis bands gel electrophoresis. Among selected 11 strains two strains PC1 and PC9 showed bit different band pattern because of inappropriate enzyme digestion however analysis and comparison of genetic elements from PCR confirmed that these strains are also of similar genotype as other isolated strains from Kathmandu.



PC Denoted by numbers: Selected samples

SB: *Salmonell enterica* serovar Braenderup DNA digested by XbaI

Figure 25: PFGE gel image of selected *Vibrio. cholerae* O1 strains

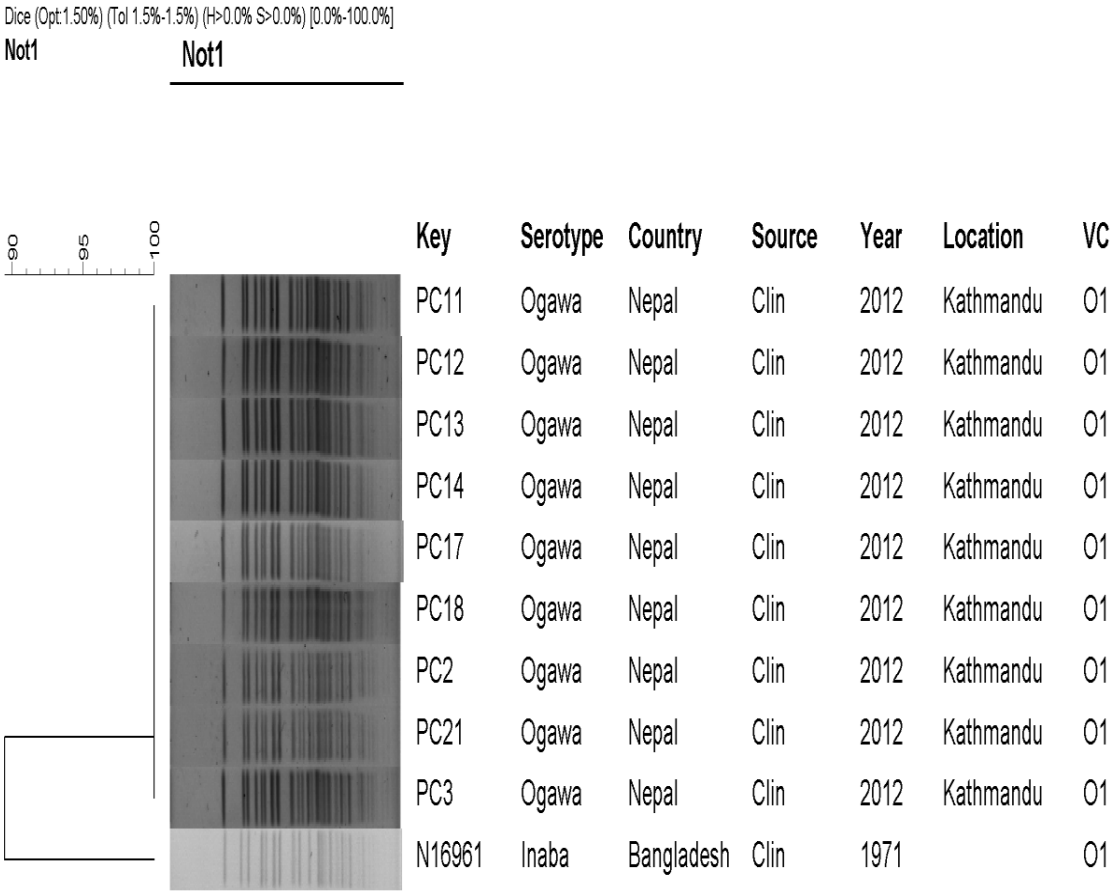


Figure 26: Phylogenetic relationship among strain isolated in 2012 Kathmandu diarrheal outbreak and N16961 (Determined via PFGE band pattern using Bionumerics Software)

5.7 Analysis of survey data

Result from survey in this study illustrated that there was no significant relation between prevalence of Cholera in male and female. Highest no of Cholera cases were seen in 20-40 (67 %) year age groups and were predominant among young patients. Basically children are considered as main victim of diarrhea but majorly people of young and working age of 20-40 years were more affected in this outbreak. Incidence of Cholera was gradually increased from June, reached at peak in August and gradually decreased with start of September. This trend was quite similar with the trend observed in another study in 2010 diarrheal outbreak (Shrestha *et al.*, 2010). This pictured the fact that Cholera burst in form of diarrheal

outbreak in period of August and September. As major clinical feature or manifestation vomiting/nausea, dehydration, watery diarrhea with high frequency (9 times per day in average) and duration (3 days in average) was observed in patients. In comparison to *Vibrio cholerae* O1 , Non O1/O139 associated Cholera cases were only 7 % in selected samples and clinical manifestation was quite less. In patients associated with *V. cholerae* non O1/O139 dehydration, vomiting and low diarrheal duration (2 days in average) and frequency was observed. With reference to first place of confirmed Cholera (PC1, Tahachal) case identified in 2012 Kathmandu diarrheal outbreak almost all other Cholera associated patients were from Tahachal and its near territories hence this fact illustrates that *V. cholerae* is colonized majorly in Tahachal and is spreading its near areas . Use of untreated water for drinking proposes was common in patients associated with Cholera. Based on this evidence it can be presumed that spreading of Cholera around Kathmandu valley was possibly due to use of unsafe, contaminated water, poor hygiene and sanitation.

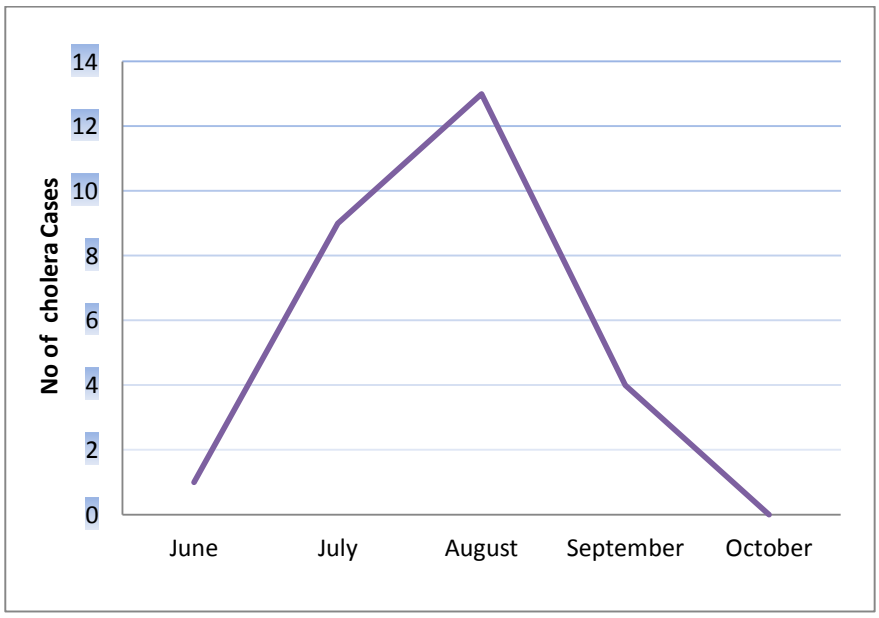


Figure 27: Trend of Cholera cases (both O1 and Non O1 associated) in Kathmandu Diarrheal outbreak, 2012

ID	Sex	Age	Duration days	Diarrheal Frequency	Fever	abdominal pain	Vomiting/ Nausea	Dehydration	Drug Use*	Associated Strain
PC1	M	19	2	7	Yes	No	yes	No	NS	*Vibrio cholerae O1 Ogawa*
PC2	F	40	2	30	No	Yes	yes	No	Ab	*Vibrio cholerae O1 Ogawa*
PC3	F	23	3	10	No	Yes	yes	Yes	NS	*Vibrio cholerae O1 Ogawa*
PC4	M	11	3	8	No	Yes	yes	No	AD	*Vibrio cholerae O1 Ogawa*
PC5	F	20	3	5	No	Yes	yes	Yes	AD	*Vibrio cholerae O1 Ogawa*
PC6	F	35	3	14	No	Yes	yes	Yes	NO	*Vibrio cholerae O1 Ogawa*
PC7	F	27	2	5	No	Yes	no	Yes	AD	*Vibrio cholerae O1 Ogawa*
PC8	F	22	2	11	No	No	yes	Yes	AD	*Vibrio cholerae O1 Ogawa*
PC9	F	78	4	20	Yes	Yes	yes	No	AD	*Vibrio cholerae O1 Ogawa*
PC10	F	41	3	11	No	Yes	yes	No	AD	*Vibrio cholerae O1 Ogawa*
PC11	F	23	6	30	No	Yes	yes	yes.	AD	*Vibrio cholerae O1 Ogawa*
PC12	M	23	2	5	No	Yes	no	Yes	AD	*Vibrio cholerae O1 Ogawa*
PC13	F	40	2	15	No	Yes	yes	No	AD	*Vibrio cholerae O1 Ogawa*
PC14	F	35	3	11	Yes	Yes	yes	Yes	No	*Vibrio cholerae O1 Ogawa*
PC15	M	28	3	8	No	Yes	yes	Yes	No	*Vibrio cholerae O1 Ogawa*
PC16	F	28	3	5	No	Yes	yes	Yes	No	*Vibrio cholerae O1 Ogawa*
PC17	M	25	2	8	No	Yes	yes	Yes	No	*Vibrio cholerae O1 Ogawa*
PC18	M	14	3	7	No	Yes	yes	Yes	No	*Vibrio cholerae O1 Ogawa*
PC19	M	17	2	8	Yes	Yes	yes	Yes	No	*Vibrio cholerae O1 Ogawa*
PC20	M	20	1	6	No	No	yes	Yes	AD	*Vibrio cholerae O1 Ogawa*
PC21	M	20	2	10	No	No	yes	Yes	AD	*Vibrio cholerae O1 Ogawa*
PC22	M	35	4	18	No	No	yes	Yes	AB	*Vibrio cholerae O1 Ogawa*
TK12	M	25	3	2	No	Yes	No	Yes	No	*Vibrio cholerae O1 Ogawa*
TK25	M	72	2	2	No	No	No	No	No	*Vibrio cholerae Non O1*
TK30	M	31	2	2	No	Yes	No	No	No	*Vibrio cholerae Non O1*
TK31	F	24	4	3	No	Yes	No	No	No	*Vibrio cholerae Non O1*
TK44	F	17	7	6	Yes	Yes	yes	Yes	No	*Vibrio cholerae O1 Ogawa*
TK50	M	27	4	3	No	No	No	No	No	*Vibrio cholerae Non O1*

*Drug used before hospital admission (without consulting medical personal) NS: Non specific drugs (Aciloc, Analgesic, Antipyretic), AD: Antidiarrhoeal drugs (metronidazole), AB: antibiotics, No: Drug not used, M: Male, F: Female

Table 23: Summary of epidemiological data associated with confirmed Cholera patients

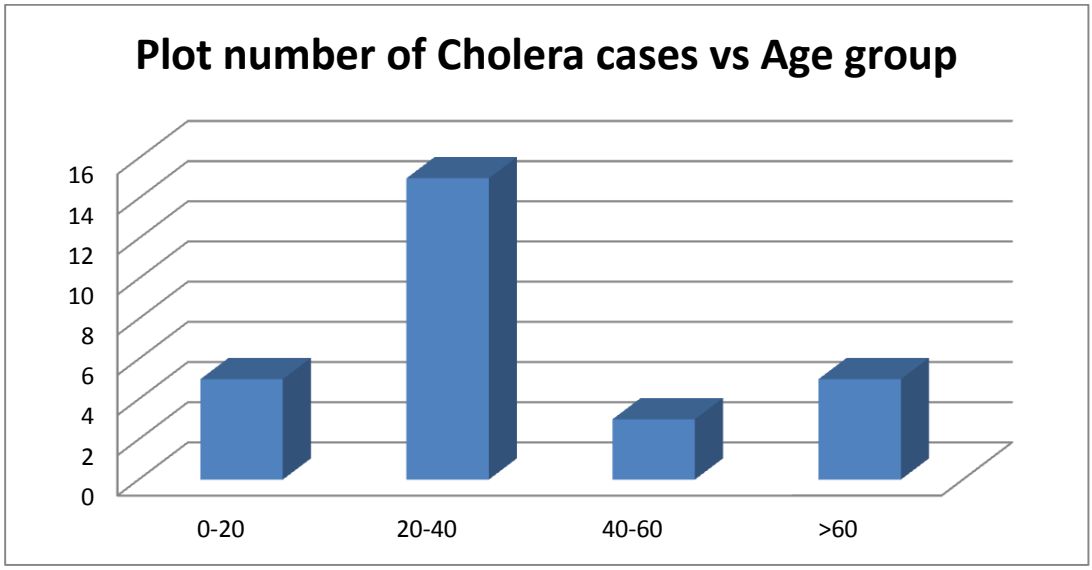


Figure 28: Distribution of *Vibrio cholerae* associated diarrhea in different age group

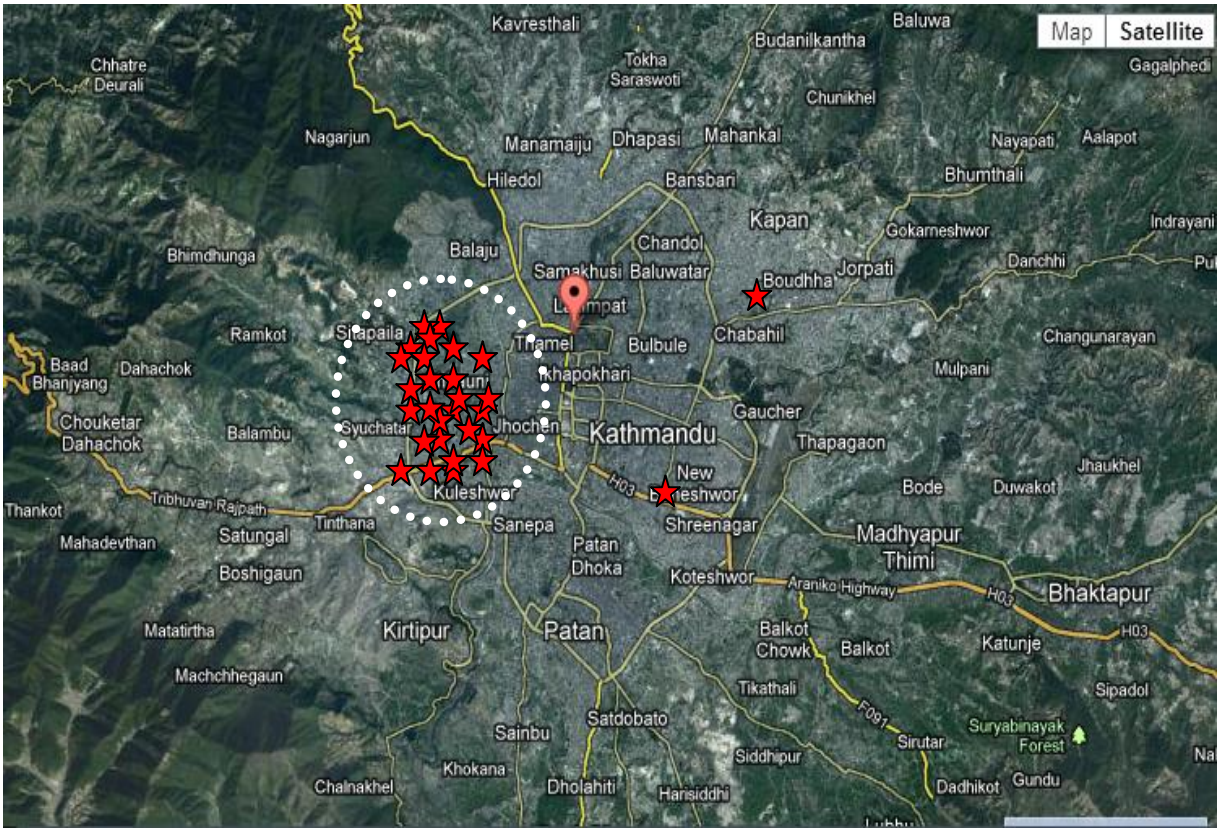


Figure 29: Geographical distribution of Cholera in 2012 Kathmandu outbreak

Table 24: Summary of demographic information of patients with confirmed *Vibrio cholerae O1* associated Cholera in 2012 Kathmandu diarrheal outbreak

ID	Age	Sex	Location	Drinking water	Travel History
PC1	19	M	Tahachal	Jar, Tap(U)	No
PC2	40	F	Teku bhansar	Tap ,(U)	No
PC3	23	F	Kalimati	Tap(U)	No
PC4	11	M	Swoyambhu	Tap(U)	No
PC5	20	F	Bhimsensthan	Tap(U)	No
PC6	35	F	Dallu	Tap(U)	No
PC7	27	F	Ravi bhawan	Jar, Tap(U)	No
PC8	22	F	Ravi bhawan	Jar ,Tank (U)	No
PC9	78	F	Swoyambhu	Tap(U)	No
PC10	41	F	Sankhamul	Tap, Tank(U)	No
PC11	23	F	Tahachal	Tap, Tank(U)	No
PC12	23	M	Kalanki	Tap (U)	No
PC13	40	F	Dallu	Tap(U)	No
PC14	35	F	Swoyambhu	Tank (U)	No
PC15	28	M	Swoyambhu	Tank(U)	No
PC16	28	F	Naya Bazar	Tap tank(U)	No
PC17	25	M	Mahabouddha	Tap ,Well (U)	No
PC18	14	M	Swoyambhu	Jar,Tank(U)	No
PC19	17	M	Bagdol, chobahal	Tank (U)	No
PC20	20	M	Buddha Nagar	Jar,Tap(U)	No
PC21	20	M	Sitapaila	Tank,Tap(U)	No
PC22	35	M	Teku	Tank water(U)	No
TK44	19	M	Tahachal	Jar , Tank(U)	No

M: Male, F: Female indicates: Untreated water not treated with disinfectant or boiled prior to use for drinking purpose

DISCUSSIONS

Summarizing information obtained from various researches conduced in Nepal, especially in Cholera, illustrated that classical approaches of identification and characterization are extensively used. Methodologies and approaches used so far in the identification and characterization of *Vibrio cholerae* in Nepal primarily includes microbiological culture, biochemical, serological and antibiotic susceptibility tests. Moreover these researches have contributed in various ways but it is not always reliable to depend upon these methods in the current scenario of changing genetic and phenotypic nature of *V. cholerae*. This research is mainly aimed to adopt modified microbiological, serological, molecular genotyping method to well identify genotype, genetic diversity and characterize the *V. cholerae* strains causing 2012 diarrhoeal outbreak in Kathmandu.

6.1 Selection of Phenotyping and Genotyping methods for identification of *Vibrio cholerae*

Traditional methods of identification using culture, biochemical and serological phenotypic tests are not able to fully identify and characterize the exact genotype and serotype because with the emergence of new variants of *V. cholerae,* some phenotypic tests are showing contradictory results (Shah *et. al.*, 2009). For instance shifting and transformation of one strains to another via unique mutation, as in shifting of O1 EI Tor to O139 by deletion and replacement of gene cluster encoding enzyme involved in the lipopolysaccharide (LPS) O-side chain synthesis, generated novel variants with unique serotype like *V. cholerae* O139. This raised the possibility of generation of problematic situation in identification and characterization due to the genetic change (Farque and Mekalanos, 2003).

Various phenotypic tests, for example Polymyxin B, shows fluctuating result and are not so reproductive as well as effective sometime. Similarly in case of phage typing some O1 strains either sensitive or resistant are also reported which generated trouble in result analysis. Hence, it is not always appropriate to totally depend upon phenotyping for differentiation and characterization of isolated strains as these tests cannot explain all attributes that clearly defines, identifies *Vibrio* and its biotype (Safa *et. al*., 2009). Thus above fact further delineates the essentiality of continuous development, improvement, adaptation of novel techniques in phenotypic, genotypic identification and characterization. Here in this research microbiological and serological tests were further complemented by singlex and multiplex PCR using markers viz. *V. cholerae* species specific outer membrane protein coding gene (ompW), virulence associated gene ctxA

that encoding the "A" subunit of Cholera toxin (CT) and gene encoding O1 or O139 specific antigen rbfO1 or rfbO139 (Hosino *et. al.*, 1998) for identification and detection of pathogenic potential via identification of presence of genes. As far as PCR based identification of Cholera and Cholera toxin detection by Singlex PCR using ompW and Multiplex PCR using rfbO1 or rfBO139 as well as ctxA as marker gene is concerned, it is found to be quite reproductive and specific. Thus these approaches not only identified *Vibrio cholerae* species but also differentiated serogroups O1/O139 or non O1 and simultaneously detected major pathogenic marker gene (ctxA) responsible for major pathogenic behavior shown by Cholera toxin. Hence this method can easily discriminate *Vibrio* belonging to non O1, O1, O139 groups. In the aspect of molecular diagnosis this may be one of the reliable methods for rapid identification of *V. cholerae* from clinical samples.

5.2 Selection of genetic markers for characterization of *Vibrio cholerae*

A large number of genetic markers are used for genetic characterization of *V. cholerae* among them genetic marker used in this study majorly includes genes associated with virulence namely ctxB, rxtC, rstC, rstR, rcpA and tlc. Use of single marker for characterization is not authentic because single marker cannot represent all genotype hence different markers are necessary for complete characterization. Characterization via use of virulence determinant genes is quite helpful and specific because it gives various clues about genetic change, distribution and potential pathogenic attributes. Each marker has different attributes such as distinct variation pattern, extra or unique genetic region, repeated sequences etc. Hence study of all these components can provide valuable information about diversity of strains and their genetic development (Rivera *et. al.,* 2001). Cholera toxin ctxB gene, encoding Cholera toxin subunit B is one of the highly used marker. In recent days continuous genetic changes and variation in ctxB are identified (Safa *et al.*, 2009) based on which strains are categorized. A genetic component of repeat in toxin (rxtC) which is deleted or truncated in classical strains is another potent marker for identification of ET Tor biotype.

Similarly selection of genes of the RS1- element (rstR and rstC) can also discriminate strains as different genotype, these gene segment are remarked as genetic component playing major role in the functioning of RS1 gene which overall is linked with CTXϕ functioning and associated pathogenesis (Walder *et. al.*, 1997). More specifically presence of rstC, rstR genes coding activator protein and repressor respectively signifies that strains are virulent as well as ET Tor type (Safa *et. al.,* 2006, Nair *et al.*, 2002). Similarly another gene prerequisite for the infection which is denoted as tcpA, encoding

subunit A of toxin co-regulated pilli of TCP, possessing important role in colonization is another potent marker (Karper *et. al.*, 1995; Farque *et. al.*, 2003). Toxin linked cryptic plasmid, whose role is believed to be pathogenic is another highly recommended marker for genetic characterization. Hence presence of these genes also depicts the pathogenic potential of strain as well as their genotype (Oshea *et. al.*, 2004, Safa *et. al.*, 2009).

6.3 Identification of ctxB genotype and sequence variation of cholera toxin B subunit encoding gene (ctxB genotyping)

Cholera toxin (CT), encoded by gene named ctxAB gene is hallmark of *Vibrio cholerae* which *is* responsible for serve Cholera associated diarrhea. After the first identification of the divergence pattern within corresponding amino acid sequence of the ctxB gene encoding B subunit of Cholera toxin in beginning of late 1990's. Considering sequence variation generated via substitution at specific amino acid position denoted as 20^{th} ,24^{th},28^{th},34^{th},39^{th},46^{th},$55^{th}$and 68^{th} nucleotide, various genotypes for *V. cholerae* are defined which includes various genotype 1 to genotype 11 (Safa *et. al.*, 2009, Marin *et. al.*, 2011). *V. cholerae* O1 with genotype 1,2,3,7,10,and 11 and *V. cholerae* with O139 genotype 3,4,5 and 6 are identified worldwide till now (Marin *et. al.*, 2011). Genotype 1 is considered as strain possessing primitive ctxB sequence belonging to classical biotype of worldwide and various territories of US gulf coast, with reference to which other variants are compared and categorized. This primitive strain in its nucleotide sequence contains message for coding His, Gln, Asp, His, His, Phe, Lys, Thr amino acids in position 20, 24, 28, 34, 46, 55 and 68 respectively (Safa *et al.*, 2009). Genotype 2 which shows altered sequence variation in specific position 46 {Phe→leu(T→G)} was reported in pre-seventh pandemic period from Australia . Genotype 3 is remarkable feature of ET biotype strains isolated from seventh pandemic and various outbreaks of Latin America (Safa *et. al.*, 2009; Olsvik *et. al.,* 1993).

In this study strains isolated in 2012, Kathmandu Diarrheal Outbreak were found to be majorly *V. cholerae* O1 ogawa consisting ctxB genotype 7 containing sequence variation in 20th amino acid position {His→Asn specific nucleotide variation (C→A)}, which was determined by double mismatch amplification mutation assay (DMAMA–PCR).This genotype was also reported in recent *V. cholerae O1* strains from India (Naha *et al*., 2012). Genotype 7 was first identified in Kolkata in 2007 (Naha *et. al.*, 2012) and similar genotype was also found in Bangladesh in 2008 -2010 with transition of genotype from 1 to 7 (Alam *et. al.*, 2012). *Vibrio cholerae* O1 with ctxB 7 genotype was also responsible for serve Cholera epidemic in Haiti 2010 (Alam *et. al.*, 2012). Significance of this mutation is not fully understood. Moreover presumption that points and suspects that there may be some effect on functional property of Cholera toxin is also not refused.

Analysis of clinical history of patients in this study also depicted that these strains have high pathogenic potential. In most of Cholera confirmed patients in this study, high level of dehydration was observed which may be due to elevated activity of Cholera toxin as well as other virulent factors. Review of various studies conducted based on volunteer study accounted the fact that Cholera toxin is key factor for diarrheal condition (Karper *et. al.*, 1995). High level of diarrheal duration and dehydration may be consequence of genetic variation in Cholera toxin gene indeed, its perfect link with scientific evidence is still not verified.

6.4 Serological variants and antibiotic susceptibility profile of isolated strains in this study and strains of past outbreaks in Nepal

After first emergence of Cholera in Nepal various studies were conducted in Cholera and primarily concentrated on drug sensitivity profiling. Various microbiological and serological studies done in past identified that strain predominantly seen in Nepal was *V. cholerae O1* Ogawa, however Hikojima was seen or less often isolated than inaba (Pokharel *et. al.,* 1997). Additionally all these strains were found to be possessing toxin producing ability (Maharjan *et. al.*, 2007). There is no strong evidence that proves first emerged serotype in Nepal was either Ogawa or Inaba but various studies identified that there was shifting of Ogaba strain towards inaba in period of 2004-2007. Later there was again re-emergence and persistent existence of Ogawa strains from 2008 (Shyakya *et. al.*, 2012) to till now.Current study also showed that strains isolated in 2012 Kathmandu Diarrheal Outbreak were all ogawa type. Sero-conversion is common phenomenon and often conversion of Ogaba to inaba with high proportion is seen in various places around the world. Prime reason for sero-conversion is more frequently due to the single base change in the rfbT gene which creates premature stop codon in gene and these mutated strains are called inaba. The factor that causes sero-conversion might probably due to selective pressure from anti-Ogawa immune response generated during infection process (Kaper *et. al.*, 1995; Sakhya *et. al.*, 2012).

Emergence of multidrug resistance *V. cholerae* in global scenario is in rapidly increasing trend and is creating hurdles in clinical management (Alam *et. al.*, 2012). Especially in Asian countries this problem is vicious and rapid with remarkable change in genetic component leading to multiple drug resistance (Nair *et. al.,* 2002; Jesudason *et. al.*, 1990). During past two decades of Cholera outbreak in Nepal *V. cholerae* O1 strains showing resistance to Tetracycline (TE), Furozolidine (Fr), Nalidixic acid (NA), Cortimoxazole (CM), Ciprofloxacin (CIP), Erythromycin (E), Trimethoprim/Sulfamethoxazole (SXT) (Karki *et. al.*, 2010; Shyakya *et. al.*, 2012;

Shrestha *et. al.*, 2010) were emerged. In this research antibiotic susceptibility pattern of selected strains showed that all selected strains were 100% resistant to Trimethoprim/Sulfamethoxazole (SXT), Nalidixic Acid; Streptomycin and 100% sensitive towards Azithromycin, Gentamycin, Erythromycin Ampicillin, Tetracycline, Ciprofloxacin. Thus above result depicted that there exist multidrug resistance as all isolates were resistant to least three different classes of antimicrobial drugs indeed Tetracycline is found to be quite effective in isolated strains of 2012 Kathmandu diarrheal outbreak. Nalidixic acid, which is recommended as first line of treatment of drug for children was seen insensitive to currently prevalent strains hence resistance towards this antibiotic, may be problematic.

In comparison to previous studies of antibiotic surveillance there is temporal variation in the susceptibility pattern and shows that there is circulation of strains having resistance with least three different antimicrobials (Karki *et. al.*, 2010; Shakya *et. al.*, 2012). Interestingly all selected strains in this study showed sensitivity towards Tetracycline indeed in past some strains with Tetracycline resistance were also reported (Shakya *et. al.*, 2012). There exists no such remarkable trend of resistance pattern observed in past and current study however comparative study well illustrated that antibiotic sensitivity behavior is fluctuating and increasing in each year. Some common and highly recommended antibiotics like Nalidixic Acid, Sulfomethoxazole/Trimethoprim are almost insensitive hence this may lead to complication in clinical management.

Emergence and increment in antibiotic resistance may account for many reason, fundamentally antibiotic resistance in Cholera is consequence as well as function of various mechanism and genetic element, which makes it feasible by exporting drugs through efflux pumps, drug insensitivity by chromosomal mutations or developing genetic resistance via the exchange of conjugative plasmids, conjugative transposons, integrons or self- transmissible chromosomally integrating SXT elements (Kitakoa *et. al.*, 2011). Specifically talking about probable reason of drug resistance in the strains prevalent in recent outbreak and past may be presence of SXT element or class 1 integrons possessing multidrug resistance gene cluster which was recently identified in various related strains from Bangladesh and India (Alam *et. al.,* 2012). Another research reported presence of point mutation in gyrA (ser83 →ile) and parC ser81→leu) gene in which *V. cholerae* considered in that study showed resistance attribute towards Trimethoprim, Sulfomethoxazole, Furazolidone, Streptomycin and Nalidixic acid, which were isolated from Haiti, 2012 (Hasan *et. al.*, 2012).

Year of isolation	*V. cholerae* Serotype	No of isolates	Percentage of resistant isolates[a]						Reference
			Tet	NA	Sxt	Cip	Ery	Fur	
2007	Inaba	72^{N}	13%	100%	63%	6%	7%	100%	Shakya *et al.*, 2012
	Ogawa	132^{N}	54%	100%	84%	3%	31%	100%	
2008	Ogawa	148^{N}	10%	100%	100%	7%	19%	100%	
2009	Ogawa	109^{N}	7%	100%	97%	1%	2%	100%	
2010	Ogawa	45	15%	100%	100%	7%	11%	100%	
	Ogawa	16^{w}	0%	100%	100%	6%	0%	100%	
2012	Ogawa	24^{K}	0%	100%	100%	0%	0%	ND	This study

[a]Partially resistant isolates included, Tet:Tetracyclin, NA:Nalidixic acid, Sxt: Trimethoprim/sulfamethoxazole, Fur: Furazoolidine , W : isolated from West Nepal ND:Not Done ,N: represents strains isolated from various region of Nepal, K : represents Strains isolated from Kathmandu,2012

Table 25: Comparative analysis of antimicrobial susceptibility profile of *V. cholerae O1* strains isolated in previous study and this study

6.5 Comparative analysis of genotypic and phenotypic feature of Nepali strains with various strains of the world

Genotypic and phenotypic basis of characterization broadly illustrated that there exist various variants of *V. cholerae* strains all around the world. A common scheme of categorization according to phenotypic and genotypic feature is listed below in table 26. Fundamentally two types of strains are seen till seventh outbreaks and categorized as typical biotypes, which are classical and EI Tor. Among these two typical strains with various phenotypes can clearly identify them from each other. EI tor which is believed to be emerged after emergence of classical strains in period between 19^{th} century replacing classical strains (Safa *et. al.,* 2009), after that various variants of E1 Tor specifically, primitive as well as hybrid variants are emerged in various outbreaks all

around the world. Various studies conducted based on phenotypic and genotypic assay in Cholera strains showed that later from beginning of seventh outbreak new variants were emerged (Alam *et. al.*, 2012; Safa *et. al.*, 2003) .These variant strains are termed as Atypical variants because these strains appear to have mix of both classical and EI Tor traits .

Table 26: Comparative analysis of genotype and phenotype of various variants of *V. cholerae* strains

Biotype or variant		Phenotypes[b]					Genotypes		
		CCA	PB	VP	Phage IV	Phage 5	tcpA	rstR	ctxB
Typical biotypes	Classical	-	s	-	s	r	Cla	Cla	B1
	El Tor	+	r	+	r	s	El	El	B3
Atypical variants	Matlab (type I)	-	r	-	r	r	Cla	Cla	B1
	Matlab (type II)	-	s	-	r	s	Cla	El	B3
	Matlab (type III)	-	r	-	r	s	Cla	Cla/El	B1/B3
	Altered El Tor	+	r	+	r	s	El	Cla/El	B1
	Mozambique	+	r	+	r	s	El	Cla	B1
	Hybrid El Tor	+	r	+	r	s	El	Cla/El	B1

Abbreviations: CCA, Chicken cell agglutination; PB, Polymyxin B; VP, Voges-Proskauer.Key: +, positive; negative; r, resistant; s, sensitive. A slash (/) indicates variable results (Adapted from Safa *et. al.*, 2009)

Based on results shown by this study, strains causing 2012 Kathmandu diarrheal outbreak are majorly *V. cholerae* O1 Ogawa EI Tor with CtxB genotype 7. All these strains possessed various marker gene that are identifying feature of EI Tor *V. cholerae* O1, which include EI Tor specific gene segment rtxC of repeat in toxin RTX gene, $tcpA^{ET}$, $rstR^{ET}$. Additionally all these strain showed presence of tlc (toxin linked cryptic plasmid),

rstC gene which is a unique gene segment present in CTX gene in EI Tor variants and absent in classical biotype.

6.6 Analysis of genetic diversity, phylogenetic relationship among isolated strains and reference strain N16961

Pulsed Field Gel Electrophoresis (PFGE), an authentic molecular tool, is routinely used for typing, comparison and genetic diversity analysis of various enteric pathogens including *Vibrio cholerae*. Overview of past studies done so far in *V. cholerae* typing and genomic analysis coincide with PFGE analysis derived conclusions, which suggests that there exist circulation of *V. cholerae* and its clonal variants in global scenario (Chin *et. al.*, 2011; Murtaja *et. al.*, 2011). Analysis of pulsotype banding pattern of isolated strains in this study with Bangladesh N16961 strains via Bionumerics software generated a phylogenetic tree which depicted that all the isolated strains from Kathmandu lies in same cluster, hence suggesting that these strains are from same clonal linage. However, they were found in various distant place or geographical regions of Kathmandu and its territories. As far as relatedness with N16961 strain is concerned these strains were slightly diverse in pulsotype pattern.

More specifically talking about interspecies correlation among strains isolated from Kathmandu 2012 Diarrheal Outbreak, 100% correlation was predicted by band pattern analysis through Bionumerics software on the basis of banding similarity and dissimilarity using Diece similarity coefficient and unweighted-pair group method (UPGMA) using average linkage clustering methods. Hence it is depicted that these strains are circulated within the valley from same clonal linage. Similarly comparison of these strains with standard strain N16961 these strains again showed 90% correlation hence suggesting their evolutionary link towards N16961 strain. This supports the fact that progenitor of currently prevalent Cholera strains are those strains seen in Bangladesh and Indian territories since 7th outbreak.

6.7 Position of Nepali *Vibrio cholerae* strains in evolutionary lineage

Various researches focused on evolutionary phenomenon associated with *V. cholerae* depicted that these strains were originated from various environmental strains through gene acquisition from diverse sources most importantly from filamentous phages. These entire exogenous genetic elements contributed *V. cholerae* for its pathogenic behavior and its better adaptability in environment (Safa *et. al.,* 2009; Faruque and Mekalanos, 2003).

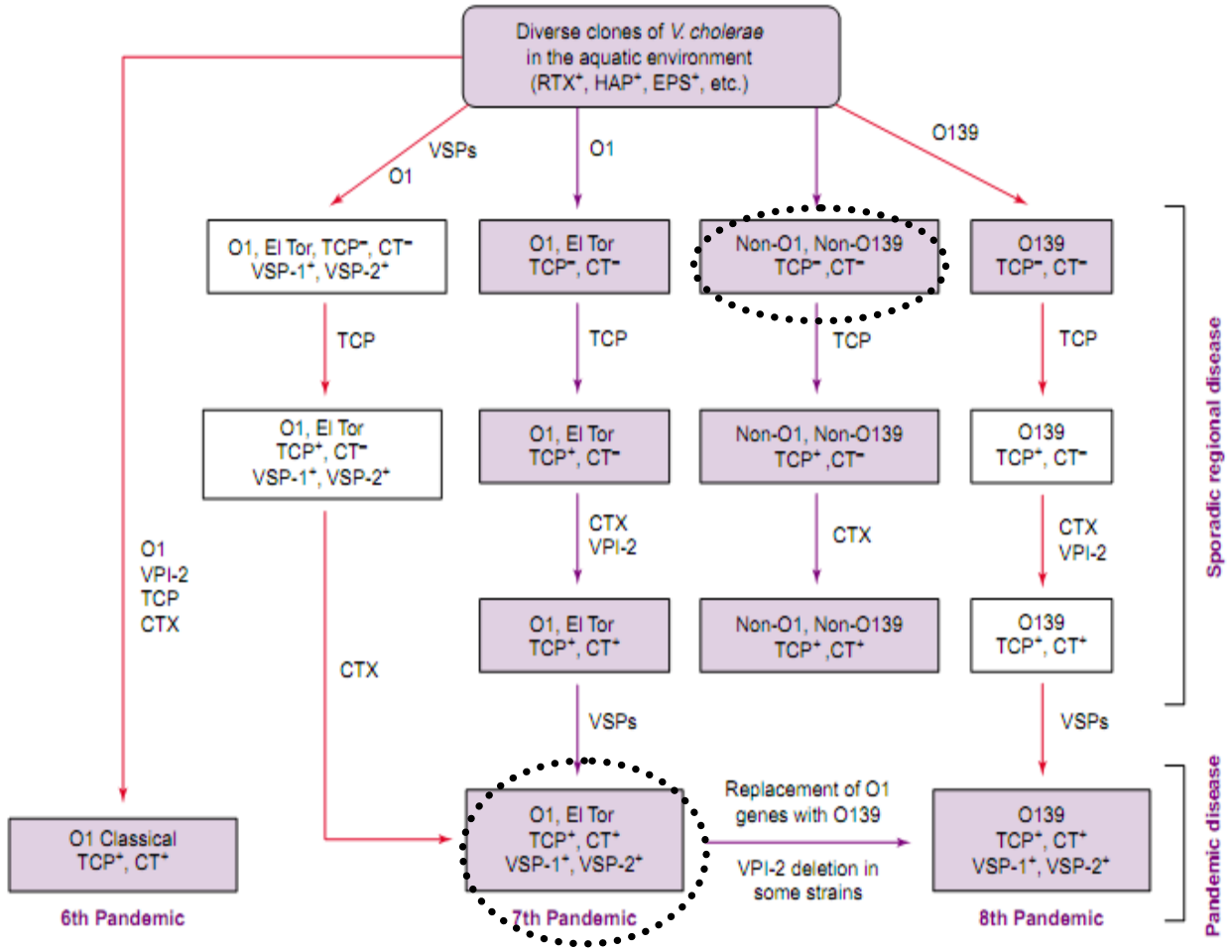


Figure 28: Overview of possible evolutionary lineages for emergence of pandemic strains of *Vibrio cholerae* globally. Lineages are based on the existence of intermediate strains carrying combinations of horizontally acquired gene clusters and other considerations. Shaded boxes describe strains that exist, whereas, non-shaded boxes designate strains that could hypothetically exist but have not yet been isolated. Arrows represent horizontal gene transfer events that are the most likely to have occurred or possible and hypothesized based on the existence of intermediate strains that have similar polymorphic markers (for example, ribotypes and allelic types of variable genes such as tcpA, rstR, toxT, among others).Similarly marked part indicates the probable stage of Nepalese *V cholerae* strains (considered in this study) in evolutionary path. Abbreviations: CTX, cholera toxin phage; EPS, extracellular protein secretion gene cluster; HAP, hemagglutinin protease gene; O1 and O139, various O antigen gene clusters; RTX, repeat in toxin gene cluster; TCP, toxin co-regulated pilus gene cluster also referred to as VPI; VPI, Vibrio pathogenicity island; VPI-2, the island that includes the nanH gene cluster; VSP, Vibrio seventh pandemic island; VSP-1 and VSP-2, the islands found in seventh pandemic El Tor strains and eighth pandemic O139 strains(Adapted from : Shah M. Faruque and John J. Mekalanos;2003)

Presence of genetic component of major pathogenic islands of seventh pandemic strains namely Cholera toxin (ctx), Toxin co-regulated pilli (tcp), Toxin linked cryptic plasmid (tlc) and Repeat in toxin RTX in *Vibrio cholerae* O1 ET Tor strains isolated in recent diarrheal outbreak,2012 indicates that these strains are probably originated from ancestor of seven pandemic family. Recent study conducted via Whole genome sequence typing (WGST) and phylogenetic approaches identified that all *V. cholerae* isolates from Nepal belong to a single well-supported monophyletic group which also contains isolates from Bangladesh and Haiti. Thus it is evident that strains prevalent in Nepal are quite similar with strain from Bangladesh and Haiti, which are identified as seventh pandemic clones (Murteza *et. al., 2011;* Hendriksen *et. al., 2011).* In another study, analysis of variation in ctxB gene and MLVA pattern showed that all strains prevalent in Nepal are similar. Strains with only subtle difference in ctxB variation reported in time period of 2007-2010 (Shakya *et. al., 2011).* This indicates that there is no huge difference in strains of Nepal and they might have emerged from single source. Similarly ctxB genotyping of isolated strains in recent diarrheal outbreak of 2012 showed that they belongs to ctxB 7 genotype, which resembles to genotype of Haiti 2010 and Calcutta, India in 2006-2007 (Naha *et. al.,* 2012). Identification of presence of CT^- , non O1 and O139 *V. Cholerae or* related strains in this study via PCR based assay also suspect that there might be presence of another closely related *V. cholerae* strain whose further characterization is essential.

Another approach in our study via PFGE depicted that currently prevalent strain belongs to same phylogenetic cluster hence it is discernible that all strains were emerged from same ancestor. Close relatedness of these strains with N16961 thus evinced, closest ancestor of currently prevalent *V. cholerae* strains in Nepal .Therefore these strains might have evolved from N16961 and its related clonal strains which probably migrated to Nepal via people travelling from Indian territories. Even though there is possibility of long evolution to achieve current stage. However it is quite difficult to come about all events in environment of Nepal because emergence of novel strains of *V. cholerae* is well supported by marine environment. Indeed current strain seems to be well adapting in all sort of circumstances of environment in Nepal, which is depicted by occurrence of outbreaks and sporadic cases every year. Till now it became well clear that all strain prevalent in Nepal are definitely from seventh pandemic lineage and there exist no such phenotypic and serological *V. cholerae* O1 variants. Further more intensive characterization for better understanding of *V. cholerae* evolution and adaptation in Nepal is essential to uncover hidden facts.

CONCLUSIONS

6.1 Overall conclusion

Cholera, an acute diarrheal illness caused by the bacterium *Vibrio cholerae*, remains a major public health concern for millions of poor people of the developing world where pure drinking water is scarce. Recent studies suggest that *V. cholerae* outbreaks can be highly local, occurring simultaneously in various locations where favorable environmental conditions prevail (Stine *et al*., 2008; Alam *et a*l. 2011). Also, *V. cholerae*, a pandemic pathogen having the coastal and estuarine link has the unique ability to rapidly spread and kill people at any corner of the world. Recent outbreak of *V. cholerae* altered El Tor in Haiti, which linked and blamed South Asian countries especially Nepal for its source attribution, although a very little work is done on the phenotypic and genetic characteristics of *V. cholerae* causing endemic Cholera in Nepal, a high altitude country located at the Himalayan valley (Hendriksen *et. al*., 2011). On stand point of these fact there was an essence of detail study on genetic and phenotypic attributes of *V. cholerae* associated with diarrhea outbreak. This research is mainly focused to identify genotype, changing genetic component and to predict genetic diversity among some selected reference *V. cholerae* O1 strain prevalent in recent diarrheal outbreak of Kathmandu, 2012. Methodologies adapted here includes phenotypic assays namely Chicken cell agglutination, Phage typing, Polymyxin B sensitivity test, PCR-based identification and genotyping tests using markers gene viz., *ompU*, *rfB*O1/139, *ctxA*, *ctxB*, *rstR*, *rtxC*, *rstC*, *tcpA*, and *tlc*.

Results from all these phenotypic and genotypic tests depicted that in recent Kathmandu diarrheal outbreak prominently *V. cholerae* O1 Ogawa EI Tor strains were prevalent however interestingly some non O1 *V. cholerae* were also found to be associated with diarrhea. Prevalence of the *V. cholerae* O1 Ogawa and *V. cholerae* non O1 was 32% and 7% respectively in selected Cholera suspected samples (N=72) moreover there was also presence of other non categorized diarrheal pathogens in high percentage i.e. 61% . Molecular characterization on other side illustrated that *V. cholerae* O1 strains causing 2012 diarrheal outbreak in Kathmandu contain high number of virulent gene and comport high pathogenic potential.

More specifically, genotypic test implemented here disclosed presence of genetic component Cholera toxin (ctxAB), rfbO1 O1 specific antigen, toxin co-regulated pilli allele specific to ET Tor biotype $tcpA^{ET}$, regulatory elements of Ctxϕ namely $rstR^{ET}$and rtxC, Toxin linked cryptic plasmid (tlc) in strains causing 2012 Kathmandu diarrheal outbreak. CtxB genotyping test based on Double Mismatch Amplification Mutation Assay (DMAMA-PCR) identified a variation pattern in ctxB gene, based on which these strains are categorized as genotype 7. This genotypic test also revealed the fact that these strains carried point mutation at 20^{th} amino acid position of ctxB sequence. This mutation is found to be responsible for changing genetic code of amino acid

(Histidine→Asparagine, (58th nucleotide change C →A))(Safa *et. al.,* 2009*)*. In comparison to Haitian strains of 2012 outbreak (Hendriksen *et al*., 2011) and some strains from Calcutta, India (Nandi *et. al.*,2012) this variation pattern is similar with currently prevalent strains of Kathmandu. Based on various phenotypic and genotypic marker tests and assays these strains are identified as *Vibrio cholerae* O1 Ogawa ET tor moreover some non O1 strains with unknown pathogenic significance were also identified in selected clinical samples.There exist close relationship among 2012 Kathmandu strains and all selected *V. cholerae O1* strains showed similar genotype i.e. ctxB genotype 7. As far as genetic components are concerned all genetic elements possessed by currently prevalent strains of Kathmandu were similar to Haitian strains of 2012 outbreaks (Nandi *et al.*, 2012). All these harbored genetic components encoding Cholera toxin, toxin encryptic plasmid (tlc), toxin coregulated pilli (tcp) are believed to be important in increased epidemic potential, high pathogenic attribute and better adaptability in changing environment during adverse condition (Farque and Mekalanos, 2003). In comparison to the classical strains seen in previous outbreaks First to Fifth, primitive, environmental and progenitor strains (Farque and Mekalanos, 2003) all these genetic components are novel and gradually harbored from various sources during evolutionary process. Transfer of genetic component from exogenous sources is suspected to be prime reason for elevated pathogenic and epidemic potential of currently prevalent strains (Farque and Mekalanos, 2003; Alam *et al.*, 2012). Interestingly, Cholera associated pathogens seem to be well adapting in Nepal indeed there lacks coastal and estuarine links. Hence various schemes and approaches used so far in the categorization of these strains via PCR based genotyping clearly depicted importance of this study.

In another prospect, this study also plays a pivotal role in the study of changing genetic element of *V. cholerae* strains in current time. In order to address facts this research endorsed novel approaches for Cholera identification particularly stressing on PCR based molecular diagnostic tools along with modified phenotyping methodologies which are quite effective in context of Nepal and synchronizes advantage of rapid output, high sensitivity, specificity, reproducibility as well as cost effectiveness. Increasing trend of multidrug resistance is another part of headache in clinical management of Cholera, comparative study of antimicrobial sensitivity profile in this study produced very valuable results and illustrated that there exists multidrug resistance in Cholera strains prevalent in current as well as past outbreaks of Nepal. Analysis of governing factor in molecular aspects links that acquired resistance may probably due to transfer of SXT genetic element more specifically class 1 integron (Alam *et al.*, 2012), mutation in gyrA and parC gene which hinders sensitivity of quinolone drugs (Hasan *et al.*, 2012). Another approach of study for genetic diversity analysis via PFGE in this study identified that there is 100 % correlation among strains isolated in 2012 Kathmandu diarrheal outbreak and 96 % correlation with ET Tor N16961. This renders that all isolated strains are from same clonal lineage and they are closely related with old ancestor ET Tor N16961.

Cholera is remarked as climate driven disease and carries potent threat all around Nepal. Particularly talking about Kathmandu probable burden and complication raised by Cholera is incontestable. High density of population and urbanization unmanaged water supply chain and sewage system in Kathmandu makes Cholera outbreak feasible in every rainy season. Evidence delineated by this study shows that in 2012 Kathmandu diarrheal outbreak there was prevalence of multidrug resistant *V. cholerae* O1 Ogawa, ET Tor variants with genotype or genetic marker ompW, rfbO1,CtxB-7, $rstR^{ET}$, rstC, $tcpA^{ET}$, rtxC, tlc. Finally, the data presented here underscores the need of close monitoring of *V. cholerae* emerging in diarrheal outbreak and sporadic cases in Nepal as well as across the globe.

6.2 Future prospective

- The evolution and continuing emergence of new pathogenic variants of *V. cholerae* over the past 100 years have posed major challenges to governments that attempt to control and manage this highly infectious disease. Recent investigations of the new variants have provided clues that are helping researchers to understand the probable origin of pathogenic variants from non-pathogenic ancestors and the natural events that might have contributed to their improved understanding in evolutionary fitness indeed there lacks many more questions to uncover like how these variants are generated, What are the factors that play role in such process?
- Cholera caused by the classical biotype is more severe than that produced by El Tor strains; however, the El Tor biotype is considered better able to survive in the environment. Nevertheless, it is unknown that how the classical CT affects the pathogenicity of atypical El Tor strains. Given that CT is directly responsible for the major clinical signs of the disease; genetic changes in the CT genes could alter the clinical manifestation of Cholera. Additionally, such a subtle genetic change might influence the efficacy of current Cholera vaccines, which could stimulate both antitoxic and antibacterial immunity hence this area also directs many more research platforms.
- The properties that make one pathogenic clone of *V. cholerae* more evolutionarily fit than another are not clearly understood. Recent studies have identified diverse environmental strains that show pathogenic potential, but these strains do not cause epidemics of Cholera thus many more reason that needs to be explored in this sector.
- It is also not clear what determines the seasonal appearance of epidemic strains and outbreaks of cholera in regions where it is endemic. It has been suggested that during inter-epidemic periods, epidemic *V. cholerae* exist in an unexplained ecological association with aquatic organisms, and studies have illustrated their ability to associate with a variety of such organisms including crustaceans,

zooplankton, phytoplankton and algae. The associations prolong survival and presumably the bacteria gain nutrients from their aquatic hosts. However, it is not clear whether such association is specific for strains carrying one or more virulence-associated genes or a general phenomenon for all *V. cholerae*. This also raises questions as to whether factors that have been described as virulence factors in the context of human infection with *V. cholerae* have other roles when the bacterium resides in its environmental habitat.

- Many studies have focused on gross genetic differences among *V. cholerae* strains, such as the presence or absence of individual genes or gene clusters. Further research is also required in characterizing the role of finer genetic variations in the biology of *V. cholerae*. The existence of different tcpA alleles in various non-epidemic serogroups, including toxigenic and non-toxigenic strains from clinical and environmental sources have been documented recently. The different tcpA alleles might have evolved in response to selective pressures that vary between the environment and the host. Similarly, considerable genetic mosaics in both the CTXϕ and the tcp gene cluster have been detected in non-epidemic environmental *V. cholerae* isolates. Some of these strains possess new alleles of the tcpA, toxT and tcpF genes. Different alleles of the CTXϕ prophage repressor, rstR, have also been found. Interestingly, some of the non-O1 and non-O139 environmental strains possessing novel tcpA, toxT and tcpF alleles were able to colonize mice, indicating that non-canonical virulence alleles could still have virulence function hence there is need of further follow up via new research.
- In contrast to Nepal, there is no such a favorable environment for easy growth and establishment of *V. cholerae* indeed there is always a constant emergence of *V. cholerae* in diarrheal outbreaks in every season hence it unanswered that how these strains are adapting in this environment instead of adverse scenario? This gives scope for further research.
- In this study we identified that there was association of non O1 *V. cholerae* with unknown pathogenic significance in Cholera like diarrhea which are not well characterized till date hence there is a need of research in this topic.
- In Nepal, from most of cases of Cholera suspected samples it is difficult to isolate organism. Recent studies demonstrated that there may be presence of non culturable form of *Vibrios* having pathogenic significance hence there is need of further research for characterization and identification.
- Multidrug drug resistance strains are constantly emerging in Nepal every year, which is becoming a major problem in clinical management hence it is necessary to focus more research in sorting out reasons for increasing such multidrug resistance pattern in Nepal.

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APPENDICES

APPENDIX1

8.1 LIST OF MATERIALS

1. Culture tubes & media plates
2. Biosaftey cabinet II
3. Water bath
4. Centrifuge
5. Sample transport kit
6. Thermo cycler
7. Autoclave

8.2 LIST OF CHEMICALS AND MEDIA

8.2.1 Carry Blair transport media (Himedia)

Composition**

Ingredients	Gms / Litre
Disodium phosphate	1.100
Sodium thioglycollate	1.500
Sodium chloride	5.000
Agar	5.000
Final pH (at 25°C)	8.4±0.2

**Formula adjusted, standardized to suit performance parameters

8.2.2 TCBS media (Himedia)

Composition**

Ingredients	Gms / Litre
Proteose peptone	10.000
Yeast extract	5.000
Sodium thiosulphate	10.000
Sodium citrate	10.000
Oxgall	8.000
Sucrose	20.000
Sodium chloride	10.000
Ferric citrate	1.000
Bromo thymol blue	0.040
Thymol blue	0.040
Agar	15.000

Final pH (at 25°C) 8.6±0.2

**Formula adjusted, standardized to suit performance parameters

Directions

Suspend 89.08 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Do not autoclave. Cool to 50°C and pour into sterile Petri plates.

8.2.3 Alkaline peptone water (APW)

Composition**

Ingredients	Gms / Litre
Peptic digest of animal tissue	10.000
Sodium chloride	10.000
Final pH	(at 25°C) 8.4±0.2

**Formula adjusted, standardized to suit performance parameters

Directions for use:

Suspend 20 grams in 1000 ml distilled water. Heat if necessary to dissolve the medium completely. Dispense as desired and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

8.2.4 Muller Hilton Agar (Himedia)

Ingredients**	Gms / Litre
Beef, infusion from	300.000
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.000
Final pH (at 25°C)	7.3±0.1

**Formula adjusted, standardized to suit performance parameters

Directions for use:

Suspend 38 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well before pouring.

8.2.5 Nutrient Agar (Himedia)

Ingredients**	Gms / Litre
Peptic digest of animal tissue	300.000
Sodium chloride	5.000
Beef extract	1.500
Yeast extract	1.500
Final pH (at 25°C)	7.4±0.2

**Formula adjusted, standardized to suit performance parameters

Directions for use:

Suspend 28 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well before pouring.

8.2.6 Preparation of Phosphate Buffer Saline

PBS (Phosphate Buffer Saline):	(1 Liter)
NaCl	8 gm
Na2HPO4.2H2O	1.44 gm
KCl	0.2 gm
KH2PO4	0.2 gm

pH was maintained to 7.3 to 7.4 with HCl

8.2.7 Preparation of Tris EDTA buffer

10 mM Tris HCl (pH-8.0)

10 mM EDTA (pH-8.0)

8.2.8 Preparation of Agarose gel (2%)

Agaose : 2gm

TAE buffer : 100 ml

Direction for use:

Agarose is gently mixed and boiled to dissolve it completely once it gradually cools down to 30 degree add approx 2µl EtBr,mix gently and poured in gel casting frame.

8.2.9 Preparation of TBE buffer

Tris Base	54 g
Boric acid	27.5 g
0.5 M EDTA (pH-8.0)	20 ml

Make final volume upto 1 liter (final pH-8.0)

8.2.10 Preparation of 6X Gel loading dye

10 mM Tris (pH-8.0)

0.03% Bromophenol blue

60 % Glycerol

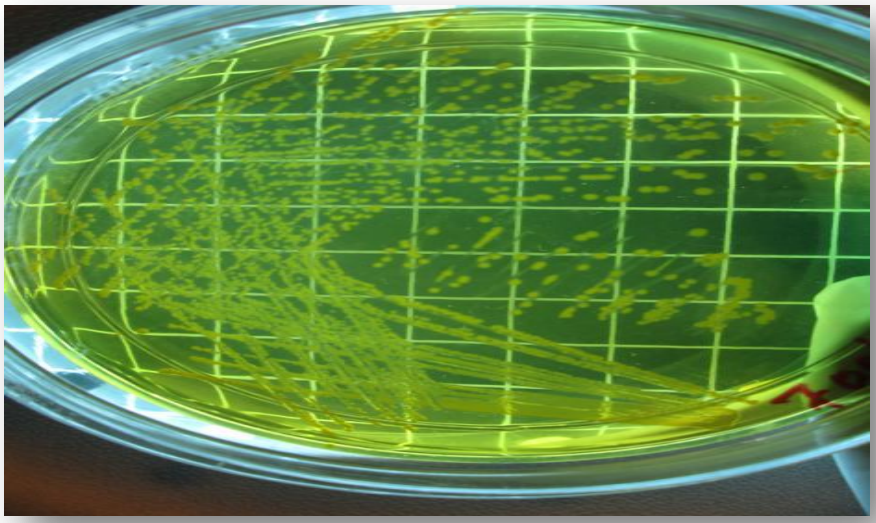
60 mM EDTA

APPENDIX2

Photos Plates

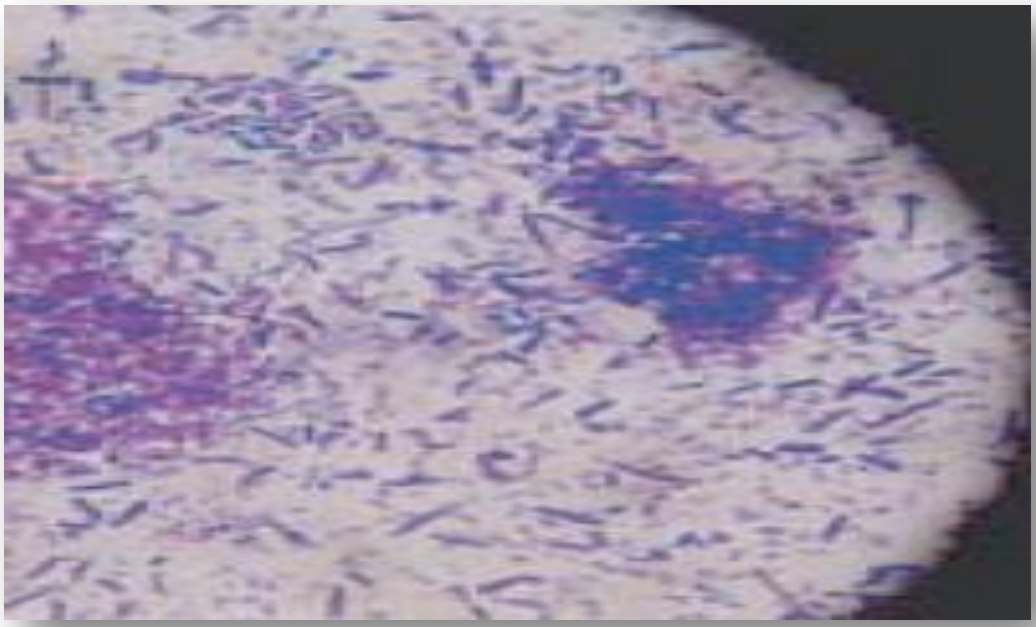
Picture: Researcher collecting probable Cholera sample

Picture: Sampling site Sukraj Tropical and Communicable Disease Hospital



Picture: Typical colony of *Vibrio cholerae* on TCBS media

Picture: Typical Cholera sample (rice watery stool)



Picture: Microscopic view of stained *Vibrio cholerae*

Picture: Serological screening of *Vibrio cholerae*

CHAPTER 1: INTRODUCTION

CHAPTER 2: LITERATURE REVIEW

CHAPTER 3: METHODS AND MATERIALS

CHAPTER 4: RESULTS

CHAPTER 5: DISCUSSIONS

CHAPTER 6: CONCLUSIONS

CHAPTER 7: REFERENCES

CHAPTER 8: APPENDICES