I-CHAPTER

1. INTRODUCTION

Diarrhea and gastroenterititis still remain at the second position among the top ten diseases admitted to hospital. Every year it causes more than 2 billion cases and 3 million deaths resulting 4% of all death and 5% of health loss to disability (WHO, 2010). It is second major cause of death in children below 5 years of age resulting 1.5 million deaths every year and 1.5 million of death above this age group (CDC, 2010 and WHO, 2010). In Nepal, 30,000-40,000 people died annually due to diarrheal infection (Bista, 2001). It is endemic disease in Nepal constituting important cause of mortality and morbidity.

Acute diarrheal disease is a major health problem in many developing countries including Nepal. Acute diarrhea is characterized with the passage of three or more loose stools per day or more frequently than for normal individuals (CDC, 2010). It is normally associated with many viruses, bacteria and parasites. Commonly associated bacteria are *E. coli*, *Vibrio cholerae*, *Shigella* species, *Campylobacter* species and non typhoidal *Salmonella*. Cholera is one of the major acute diarrheal disease in the world and is caused by toxigenic strains of *Vibrio cholerae* O1 and O139 affecting more than 3-5 million people worldwide and 100,000-130,000 deaths a year as of 2010 (WHO, 2010).

Transmissions of acute diarrheal diseases along with cholera occur with the consumption of contaminated food and water. Water contaminated with human feces together with poor hygiene leads to rapid outbreak of diarrhea and cholera (WHO, 2010). World-wide around 1.1 billion people lack access to improved water sources and 2.4 billion have no basic sanitation. Diarrhea due to infection is widespread throughout the developing world. In Southeast Asia and Africa, diarrhea is responsible for as much as 8.5% and 7.7% of all deaths respectively (WHO, 2010).

Amongst the poor and especially in developing countries, cholera is major killer. Each year, different cholera outbreaks occur in many developing world especially in Africa, south East Asia and Central America associated with poor water quality and sanitation (Griffith et al., 2005). In Nepal it is endemic disease. There were numerous outbreaks of

cholera occurred in the past mostly during warm summer months when water quality become deteriorated (Tamang et al., 2005, Maharjan et al., 2007, Karki et al., 2010, EDCD Report, 2007). Most recent outbreaks in western part of Nepal also associated with the use of poor quality water and lack of sanitation (EDCD Report, 2010). Nepal is a developing country and majority of its people (approximately 80%) reside in rural areas with the lack of clear piped water and sanitation. Most of the people (55%) illiterate and proper toilet facility is available to 44% of household only. Such data are relevant with the concordance of cholera endemic status of Nepal (WHO, 2010).

Consumption of contaminated food and water lead to extensive rice watery diarrhea which could lead to the severe fluid loss and death within short time if left untreated (Sack et al., 2004). It is mainly treated by replacement of fluids and electrolytes through oral rehydration therapy. Antimicrobial therapy considerably reduces the severity of diarrhea and duration of illness and vibrios excretion. However emergence of multiple drug resistant (MDR) *Vibrio cholera* strains in recent years creates a problem during antibiotic therapy (Das et al., 2011, Pugliese et al., 2009). The drug resistance mechanism is mediated by many resistant genes and transferable. So increase in magnitude of drug resistance strains not only creates problem during cholera treatment, it also serves as a source of resistance to other bacteria (Burrus et al., 2004). Treatment of cholera with inappropriate drugs, excessive use of same drugs together with environmental factors are related to the emergence of drug resistance cases (Lipp et al., 2002).

Multiple drug resistance (MDR) is defined as resistance to $\geq$2 of the antimicrobial agents belonging different structural classesCDC, 2006). Multidrug resistance among common) pathogens has resulted into treatment failures and increased economic burden to bacterial .(pathogens, thus dictating their early and reliable detection (ASM, 2009 contain these

Vibrio cholera also lives naturally in aquatic environment. Their association with phytoplankton and zooplanktons in aquatic environment lead to their proliferation in water during inter epidemic period during which they frequently convert serotype and acquire resistances (Colwell, 1996 and Lipp et al., 2002). This indicates environmental roles for emergence of new cholera epidemics with new serotype and antibiotic resistances. Due to its erratic nature, it is difficult to predict when new toxigenic strains

arises with resistant properties thus creating epidemics and havoc to the people and health agencies throughout the world (WHO, 2010). So constant monitoring of this pathogen is of prime importance, whenever this new toxigenic and resistant strain arises it can be reported promptly and effective measures could be taken on time. Monitoring of the aquatic environment regularly also helps to predict about the epidemics.

Vibrio cholerae remain in viable but non culturable (VBNC) form in aquatic environment during the interepidemic period. It changes its serotype and acquires new resistant genes in environment which could lead to the emergence of next epidemic or outbreak with new characters. In Nepal number of outbreaks occurred in the past most notably during 2009 in Jajarkot and in Kavre during 2004. Emergence of toxigenic and resistant strains of *Vibrio cholerae* represents a clinical threat because of their resistance to different types of drugs and transfer of resistance to other types of organism as well as the impact to the people's health thus ensuing continual surveillance, rapid identification and determination of antibiotic susceptibility, to measure the prevalence and assess their impact to the health. Hence, this study was conducted with an aim to isolation and identification of the *Vibrio cholera* from the diarrheal stool samples and measuring the antibiotic resistances from both hospitals and outbreak areas.

II-CHAPTER

2. OBJECTIVES

General Objective 2.1

To describe serotype, biotype and antibiotic susceptibility of isolated *Vibrio cholerae* from the diarrheal stool samples of outbreak area and hospitals.

Specific Objectives 2.2

1. To determine the sample prevalence of cholera in diarrheal stool samples.
2. To describe the demographic factors related to cholera cases.
3. To identify the serotype and biotype of isolated *Vibrio cholerae*.
4. Toassess the antibiotic susceptibility of the isolated *Vibrio cholerae* by Kirby Bauer disc diffusion method and minimum inhibitory concentration (MIC) test.

CHAPTER-III

3. REVIEW OF LITERATURE

3.1 Cholera- Introduction

The word cholera is derived from Greek term χολέρα *kholera* that means flow of bile (Sack et al., 2004). It is also known as Asiatic or epidemic cholera is an infectious gastroenteritis caused by enterotoxin producing strains of bacterium *Vibrio cholerae* (Sack et al., 2004). It is the most feared epidemic diarrheal disease because of its severity. Dehydration and death can occur within hours of an infection (Smith and Angelo et al., 2008). Transmission to human is by water or food. It is a substantial health burden in Africa, Asia and South and Central America where it is endemic. The exact scale of problem is however uncertain because of weaknesses in the existing surveillance system, difficulties to clinically distinguish mild to moderate case of cholera from other causes of acute diarrhea and failures to report cases or even outbreaks to WHO, which acknowledge that only around 5-10% of cholera cases are actually reported (WHO, 2004).

3.2 Epidemiology of Cholera

3.2.1 Global Epidemiology of Cholera

It is estimated that cholera affects 3-5 million people worldwide and causes 100,000-130,000 death in a year as of 2010 (WHO, 2010). This occurs mainly in developing world (Reid et al., 2002). In the early 1980s death rates are believed to have been greater than 3 million a year (WHO, 2010). It is difficult to calculate exact no. of cases as many go unreported due to concerns that an outbreak may have negative impact on the tourism of the country (Sack et al., 2006). Cholera remains epidemic and endemic in many areas of the world. It is endemic disease in Nepal also (WHO, 2010).

Cholera is a disease that occurs in area of low economic income where sanitation, food and water hygiene are inadequate. Imported cases occasionally occur in traveller returning from epidemic areas (WHO Cholera Fact Sheet, 2010).

In areas without sewage disposal and clean water (as may occur after natural disaster or displaced population in areas of conflict) cholera can spread quickly and have a case fatality ratio of as high as 50%in vulnerable groups with limited medical care (WHO, 2010). An outbreak in Haiti, Zimbabwe and in Goma, Rwanda is excellent examples of this. In mid October 2010 cholera epidemic was broke in Haiti followed by catastrophic earthquake in January 12 which favors rapid progression of the cases (WHO Weekly Cholera Report, 2010).

Vibrios are highly abundant in aquatic environments including estuaries, marine waters, coastal water sediments and aquaculture setting worldwide. Several studies suggested that they are highly found in marine environments (Falzano et al., 1999, Diggeles et al., 2000, Vanderberge et al., 2003). The environmental and clinical strains of *Vibrio cholerae* are known to represent a single species (Lipp et al., 2002). A possibility of genetic exchange in the environment between the clinical and environmental strains lead to the emergence of new toxigenic strains which could serves as a new pathogenic strain and cause epidemics (Lipp et al., 2002).

Cholera is transmitted via the fecal oral route by consumption of water and food (Finkelstein, 2004). Although much is known about the mechanism behind the spread of cholera, this has not led to a full understanding of what makes cholera outbreaks happen in some places and not others. Lack of treatment of human feces and lack of treatment of drinking water greatly facilitate its spread but bodies of water can serve as reservoir and seafood shipped long distances can spread the disease. Cholera was not known in Americas for most of the 20^{th} century but it reappeared towards the end of the century and seems likely to persist (Blake, 1993).

Annual global figures reported to WHO in 2009 included 221226 cases and 4946 death from 45 countries (WHO, 2010). 98% cases were reported from Africa where outbreak that started at 2008 and lasted almost a year spread to South Africa and Zambia. Asia reported 82% decrease in cases in 2009 compared to 2008. In the Americas no cases were reported. However in 2010 explosive epidemic occurred in Haiti which matched the Asian type of strain (WHO, 2010).

Globally mortality and morbidity relating to cholera is likely to be grossly under reported due to the limitations of surveillance system (WHO, 2010). WHO estimates actual global burden of disease as 3-5 million cases and 100,000-130,000 death per year as of 2010.

3.2.2 Epidemiology of Cholera in Nepal

Cholera is endemic disease in Nepal (WHO, 2010). The first bacteriologically confirmed case of cholera was first reported in 1958 by Abou-Gareeb (Abou- Gareeb, 1961). Before 1992, cholera epidemic related to classical biotype but after 1992 the biotype El Tor was associated with the epidemic outbreak in Nepal (Yamamoto et al., 1995). *Vibrio cholerae* O1 serotype Ogawa biotype El Tor was major strain related to numerous outbreaks in Nepal.

Being the developing country, Nepal also faces massive problem of cholera and other associated acute diarrheal diseases during every monsoon season (Bista, 2001). The lack of treated water and poor sanitation is major cause of cholera (Pokharel et al., 2004). Every year 30,000-40,000 people died due to acute diarrheal diseases and cholera is major reason behind such large mortality (Bista, 2001).

Various outbreaks of cholera had been occurred in the past especially during warm summer months from June to November in Nepal (Pokhrel et al., 1996). In 1992 large outbreaks of cholera occurred in eastern Nepal largely affecting Bhutanese refugees and the agent was *Vibrio cholera* O1 biotype El Tor serotype Ogawa (Yamamoto et al., 1995). Kathmandu valley always suffer cholera outbreak in monsoon season due to deterioration of the water qualities (Pokharel et al., 2004). In 1996, *Vibrio cholera* O1 El Tor serotype Hikojima was major strain related to cholera cases in Kanti Hospital (Pokhrel et al., 1996). Till the year 2004 major strain isolated from Nepal related to *Vibrio cholera* O1 serotype Ogawa (Kansakar et al., 2011). In 2005 and 2006 *Vibrio cholera* O1 serotype Inaba was isolated in outbreaks of cholera from Kathmandu valley (NPHL Report, 2007 and Shrestha et al., 2008).

In recent year, large cholera outbreaks had occurred in western region of Nepal. In Jajarkot district alone more than 80 people died and 3,000 peoples were affected in three weeks period of July 2009 (EDCD Report, 2009). The outbreak was associated with the

poor hygiene and use of contaminated water. The lack of quick medical facility after the patient contracted with the cholera agent and consequences of acute diarrhea has further increases the morbidity and mortality in such places (WHO, 2010). In Nepalgunj outbreak of cholera was occurred in late July and August 2010, which was also related with the use contaminated water affecting more than 2500 people just in a week time (Republica News August 26, 2010). Eight people were died including at least five children during that outbreak (WHO, 2010).

3.3 History

During the cholera epidemic in 1854 in Florence Italy, Paccini first described the comma shaped gram negative Vibrio the comma bacillus responsible for cholera (Lipp et al., 2002), which was subsequently named as *Vibrio cholerae* by Robert Koch. However cholera likely has its origin in the Indian subcontinent, it has been prevalent in the Ganges delta since ancient times (Sack et al., 2004). The disease is first spread by trade routes to Russia in 1817, then to Eastern Europe and from Europe to North America (Sack et al., 2004). Even though its isolation and nomenclature was done late, the cholera related disease was prevalent since ancient times. There have been seven cholera pandemics in the past 200 years with the last originating in Indonesia in 1961 (Cholera CBS News, 2010).

Except for the seventh pandemics, all the pandemics were arose from Indian subcontinent and spread to other continents affecting many countries and extending over many years (Faruque et al., 1998). That's how from the local disease, cholera becomes one of the most wide spread and deadly disease of the nineteenth century, killing an estimated tens of millions of people (Lee et al., 2003). In Russia alone between 1847 and 1851 more than one million people had perished of the disease (Hosking, 2001). It killed 150,000 Americans during the second pandemic (Byrne et al., 2008). Between 1900 and 1920, perhaps 8 million people died of cholera in India (Hays et al., 2005).

Prior to the seventh pandemic which started at 1961 all the outbreaks were traced to the classical biotypes of *Vibrio cholerae* O1 (Lipp et al., 2002). The biotype O1 El Tor strain replaces the classical biotype by 1961 (Kaper et al., 1995). Toxigenic strain of *Vibrio*

cholerae O139 first emerged as a pandemic threat in south and eastern India and Bay of Bengal in 1992 and appears to have arisen by genetic exchange between *Vibrio cholerae* O1 El Tor (Rhyne et al., 1994, Walder et al., 1996). Recent evidence also suggest that O139 isolate may have arisen by genetic exchange with non O1 *Vibrio cholerae* from the environment as well as clinical strains of *Vibrio cholerae* O1 (Faruque et al., 1999).

Table 3.1: Cholera pandemics since 1817 (adapted from Tauxe 1998)

SN	Year	Origin	Organism
1	1817-1823	India	?
2	1829-1851	India	?
3	1852-1859	India	?
4	1863-1879	India	?
5	1881-1896	India	*Vibrio cholerae* O1 classical
6	1899-1923	India	*Vibrio cholerae* O1 classical
7	1961-present	Indonesia	*Vibrio cholerae* O1 El Tor
8	1992-present	India	*Vibrio cholerae* O139

The seventh pandemic which was originated in Indonesia, by various routes the causative agent *Vibrio cholerae* O1 El Tor spread across the Asia, the Middle East, Africa and parts of Europe. But in 1991 explosive O1 El Tor outbreak was occurred in America after absence of cholera there for 100 years and rapidly spread to central and south America with recurrent epidemics in 1992 and1993 (WHO, 2010). From the onset of epidemic in January 1991 to September 1, 1994 a total of 1,041,122 cases and 9,642 deaths (over all case fatality ratio 0.9%) were reported from the countries in the western hemisphere to the pan American health organization. In 1993 the number of reported cases and death were 204,543 and 2,362 respectively in that region (WHO Cholera Archive, 1995).

In 1982, in Bangladesh a classical biotype resurfaced with a new capacity to produce more severe illness and it rapidly replace the El Tor strain which was thought to be well entrenched. This classic strain has not yet produced a major outbreak in any other country (CDC, 2010).

In December 1992 large epidemic of cholera began in Bangladesh and large numbers of peoples have been involved, the organism has been characterized as *Vibrio cholerae* O139 Bengal. It is derived genetically from El Tor pandemic strain but changed its antigenic structure such that there is no existing immunity and all ages even in endemic are susceptible. The epidemic has continued to spread and it affects at least eleven countries of south and East Asia. The total number of cases is unknown because affected countries do not report infection caused by O1 and O139 separately (WHO, 2010).

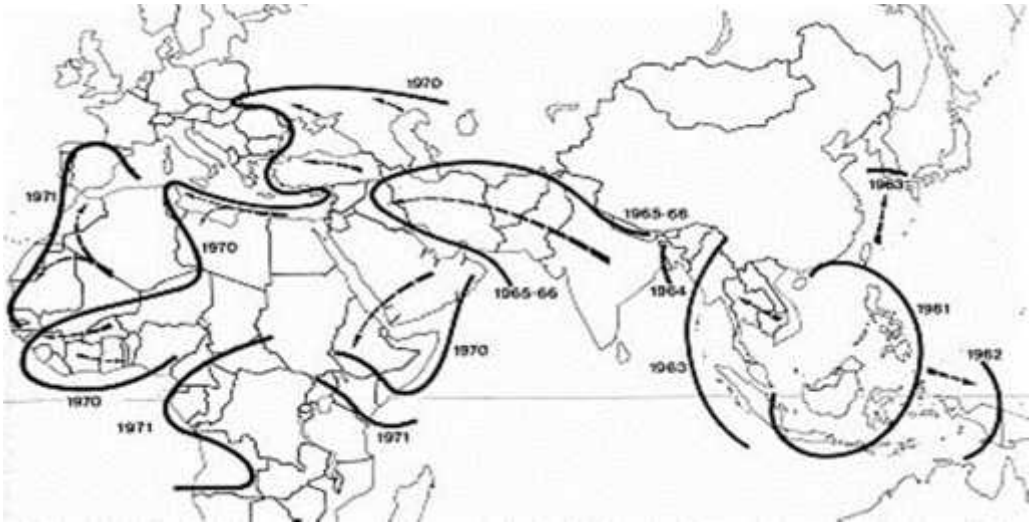


Figure 3.1: The global spread of cholera during the seventh pandemic, 1961-1971 (CDC, 2010)

In April 1997, a cholera outbreak occurred among 90,000 Rwandan refugees residing in temporary camps in Goma democratic republic of Congo (former Eastern Zaire). During the first 22 days of outbreak 1,521 deaths were recorded most of which was recorded in area lack of health care facilities (CDC and WHO Weekly report 1998). Outbreak claims more than 12,000 lives and 70,000 cases which is an excellent paradigm of how socioeconomic influences on the health status of the nation (Faruque et al., 1998).

In United States cholera was prevalent in 1800s, but has been virtually eliminated by modern sewage and water treatment system (Finkelstein, 2004). However as a result of transport and travel to epidemic areas, US travellers bring the cholera back to the country

from the epidemic areas and also by the contaminated shell food from the coastal areas. Greater than 90% of cholera cases of US have been associated with foreign travel (FDA Report, 2009).

In Nepal first bacteriologically confirmed epidemic cholera cases was reported in 1958 (Abou-Gareeb, 1961). Outbreaks of cholera have been occurred annually in Nepal (Pokhrel et al., 1996, Bista, 2000 and Tamang et al., 2005). *Vibrio cholerae* Ogawa El Tor strain is major cause of epidemic in Nepal (Yamamoto et al., 1995, NPHL Annual Cholera Report, 2007 and Tamang et al., 2005). Recent outbreaks in western part of Nepal in 2009 were also associated with O1 El Tor Ogawa strain (NPHL Unpublished Report, 2010).

3.4 *Vibrio cholerae*

3.4.1 Taxonomy

According to the Bergey's manual 2004, the organism *Vibrio cholerae* is classified under the class Gammaproteobacteria which include five orders. They are Oceanospirillales, Alteromonadales, Vibrionales, Aeromonadales and Enterobacteriales. *Vibrio cholerae* lies in order Vibrionales under the family Vibrionaceae which include genus *Vibrio* (44 species), *Photobacterium* (6 species) and *Salinivibrio* (one species). The classification scheme is based upon examination of structure, function and regulation of proteins, comparison of molecular % of G+C content, DNA-DNA hybridization, 5S rRNA cataloging and sequence comparisons and 16S rRNA sequencing.

3.4.2 Morphology

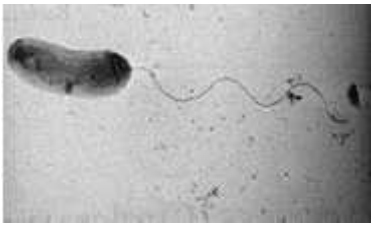


Fig 3.2: Vibrio cholerae

Vibrio cholerae is gram negative small and slightly curved rod or comma shaped rod, 0.5-0.8×1.4-2.8 µm in size (Bergey's Manual, 2004). The organism is motile with single polar flagellum and shows typical darting motility.

In liquid medium *Vibrio cholerae* produce single sheathed polar flagellum which is 24-30 nm in diameter and 14-16 nm in thickness with wavelength 1.4-1.8 µm (Baumann et al., 1984, Janda, 1998). However, they produce unsheathed flagellum in solid medium (Bergey's Manual, 2004). They produce lateral flagellum in solid media (Baumann et al., 1984) which permits swarmer migration across solid surface and results progressive spreading of bacterial colony (Maccarter and Silverman, 1990). Fimbriae are also produced by a no. of pathogenic vibrios such as *Vibrio cholerae* O1 and non O1 vibrios, *Vibrio parahemolyticus* and *Vibrio vulnificus* (Hales et al., 1998, Honda et al., 1988). Capsules have been detected in surrounding cells of strains of *Vibrio cholerae* O139 and *Vibrio vulnificus* strains (Janda, 1998).

3.4.3 Cultural Characteristics

Vibrio cholerae is facultative anaerobic organism capable of both respiratory and fermentative mechanism (Bergey's Manual, 2004). On most non selective media containing 0.5-1% NaCl, they give smooth, convex colonies with 2-3mm in diameter after overnight incubation at 37ºC. Of the many selective media developed, Thio Sulphate Citrate Bile Sucrose (TCBS) agar medium is most convenient and widely used selective media for isolation of vibrios developed by Kobayashi et al. in 1963. On TCBS agar vibrios can be separated in to two groups on the basis of results of sucrose fermentation. The sucrose fermenting strains give yellow colonies while non fermenting strains give green colonies. *Vibrio cholerae* being sucrose fermenter gives yellow colony on TCBS agar medium while *Vibrio parahemolyticus*, *Vibrio mimicus* and *Vibrio vulnificus* give green colonies.

Selective media designed for isolation of members of this genus have revolved around their resistance to toxic levels of certain organic and inorganic compounds (KI), antimicrobial agents (Polymyxin B) and tolerance to highly alkaline conditions of pH 8.6 (Janda, 1998).

On taurocholate tellurite gelatin agar medium *Vibrio cholerae* produce grayish colonies (1-2mm) with dark centers (due to the reduction of tellurite to tellurium) surrounded most often by opaque zone due to gelatin production (Albert, 1994). TCBS medium is superior to Monsur's TTGA medium as colonies of *Vibrio cholerae* on TTGA are difficult to differentiate, TTGA is comparatively difficult to prepare and the level of potassium tellurite added can inhibit the bacterial colony if excess slightly (Cheesbrough, 1984).

Alkaline peptone water (pH 8.6) containing 0.86% of NaCl is good enrichment medium for *Vibrio cholerae*. The broth should be subcultured to TCBS medium after 5-6 hour of enrichment at 37ºC. Longer incubation allows the overgrowth of unwanted organism which could override the vibrios. It can be also incubated for longer period by lowering the temperature to 18-22ºC (Janda, 1998). APW can be used as a transport medium if the time required is less than 6 hours, for longer time requirement, Cary Blair transport medium is used (Cheesbrough, 1984). Buffer glycerol saline is not preferred as glycerol is inhibitory to vibrios (Forbes et al., 2007).

Beside TCBS and GATT medium, alkaline meat extract medium (MEA) and alkaline bile salt agar can be used to isolate vibrios (Vandepitte et al., 2004).

3.4.4 Biochemical characters

Vibrios can be distinguished from other members of Enterobacteriaceae, *Pseudomonas*, *Aeromonas* and *Plesiomonas* spp. on the basis of biochemical characters. The positive oxidase test is character of *Vibrio* which separates from the Enterobacteriaceae. It is fermentative organism hence differs from *Pseudomonas* spp., however *Aeromonas* and *Plesiomonas* spp. possess little bit difficulty but they can't grow in high salt concentration while vibrios can grow so, they can be distinguished.

The various species within the genus *Vibrio* can be separated by various biochemical tests, more importantly by amino acid decarboxylation test. *Vibrio cholera* O1 group always fall in to the Heiberg 1 fermentation pattern; that is they ferment sucrose and mannose but not arabinose and they produce acid but not gas. It also possesses lysine and ornithine decarboxylase but not arginine dihydrolase.

TABLE3.2: Biochemical characteristics of human pathogenic Vibrionaceae (Adapted from Elliot et al.

		V. algi-nolyticus	*V. cholerae*	*V. fluvialis*	*V. furnissii*	*V. hollisae*	*V. metschni-kovii*	*V. mimicus*	*V. parahae-molyticus*	*V. vulnificus*
TCBS agar		Y	Y	Y	Y	NG	Y	G	G	G
Oxidase		+	+	+	+	+	−	+	+	+
Arginine dihydrolase		−	−	+	+	−	+	−	−	−
Ornithine decarboxylase		+	+	−	−	−	−	+	+	+
Lysine decarboxylase		+	+	−	−	−	+	+	+	+
Growth in (w/v):	0% NaCl	−	+	−	−	−	−	+	−	−
	3% NaCl	+	+	+	+	+	+	+	+	+
	6% NaCl	+	−	+	+	+	+	−	+	+
	8% NaCl	+	−	V	+	−	V	-	+	-
	10% NaCl	+	−	−	−	−	−	−	−	−
Growth at 42°C		+	+	V	−	nd	V	+	+	+
Acid from:	Sucrose	+	+	+	+	−	+	−	−	−
	D-Cellobiose	−	−	+	−	−	−	−	V	+
	Lactose	−	−	−	−	−	−	−	−	+
	Arabinose	−	−	+	+	+	−	−	+	−
	D-Mannose	+	+	+	+	+	+	+	+	+
	D-Mannitol	+	+	+	+	−	+	+	+	V
	ONPG	−	+	+	+	−	+	+	−	+
	Voges-Proskauer	+	V	−	−	−	+	−	−	−
Sensi-tivity to:	10 µg O/129	R	S	R	R	nd	S	S	R	S
	150 µg O/129	S	S	S	S	nd	S	S	S	S

	Gelatinase	+	+	+	+	−	+	+	+	+
	Urease	−	−	−	−	−	−	−	V	−

Abbreviations: TCBS, thiosulfate-citrate-bile salts-sucrose; Y = yellow NG = no or poor growth S = susceptible nd = not done
G = green V = variable among strains R = resistant V = variable

Sucrose fermenting character differentiate *Vibrio cholerae* from other members of *Vibrio*, which can grow in media without addition of salt while others require salt for their growth in agar media. *Vibrio mimicus* can also grow in salt free medium but its inability to ferment sucrose differentiates from cholera vibrios (Bauman et al., 1988 and Janda, 1998).

There is difference on biochemical character in different strains of *Vibrio cholera* O1 referred as biotypes and they are classical and El Tor. They can be separated from each other by Voges- Proskauer reaction, hemolysis on sheep blood agar, susceptibility to Polymyxin B, agglutination of chick erythrocytes and lyses by bacteriophages (Bauman et al., 1998 and Kelly et al., 1991).

Table 3.3: Phenotypic differences between classical and El Tor strains

Test	Classical	El Tor
Haemolysis of sheep erythrocytes	−	+
Agglutination of chicken erythrocytes	−	+
Voges–Proskauer reaction	−	+
Polymyxin B resistance	s	r
Phage IV	s	r
Phage 5	r	s

Present cholera pandemic is associated with El Tor strain however classical strains have caused many outbreaks in recent years (Lee et al., 1992). The classical strain seems better survivor at aquatic environment than El Tor strain (Matson et al., 2007).

Like El Tor biotype, *Vibrio cholerae* O139 is also positive for Voges-Proskauer reaction, show variable hemolysis pattern on sheep erythrocytes, agglutinates chick erythrocytes, resistant to Polymyxin B, Mukharjee phage IV and El Tor phage 5. They are sensitive to

specific kappa phages. Like El Tor strain it is also resistant to vibriostatic agent O129 (Albert, 1994); giving strong evidence that the organism arises due to genetic exchange in environment between O1 strain and non O1 strains (Lipp et al., 2002).

3.4.5 Antigenic structures

Vibrio cholerae possess following type of antigenic properties.

Flagellar H- antigens: All strains of *Vibrio cholerae* possess same type of flagellar antigens determined by immunodiffusion. H-antigens of *Vibrio cholerae* may be difficult to detect by slide or tube agglutination because the sheath prevents the antigenic exposure which can be removed by phenol or formalin pretreatment or by aging the cultures.

Capsular antigens: Capsules have been detected surrounding cells of *Vibrio cholerae* O139 and other non cholera vibrios, which can be detected by variety of staining technique such as uranyl acetate, poly cationic ferritin and ruthenium red (Janda, 1998). The polysaccharide capsule found to be interfering with O antigens in some instance (Hayat et al., 1993).

Fimbrial antigens: Fimbriae are produced by number of pathogenic vibrios such as *Vibrio cholerae* O1 and non O1 vibrios and other non cholera vibrios (Hales et al., 1998). Several different morphological types of fimbriae have been described in *Vibrio cholerae* O1. These include wavy pilli (3 nm), rigid filaments which is 5-6 nm wide and 180-800 nm long (Hales et al., 1998). These pilli composed of protein TcpA and TcpA formation is co regulated with cholera toxin expression and is key determinant of *in vivo* colonization.

Somatic or surface O- antigens: The outer membrane of cell wall consists of many layers but lipopolysaccharide layer is predominant one. The lipopolysaccharide layer consists of lipid A which forms a part of lipid bilayer, a core region and serotype specific O-polysaccharide antigenic side chain (Simanda et al., 1994). The core of LPS composed of single unit of 2-keto 3 deoxy-D-mannose octulosonic acid and a fructose unit (Guidolin and Manning 1987). The core attaches to lipidA portion by 2 keto 3 deoxy-D-

mannose octulosonic acid and to the O side chain by fructo-furanoside linkage (Fallarino et al., 1997).

The O-antigens play major role in antigenic variation and pathogenesis of cholera. O-antigens are heat stable cell wall polysaccharide which plays major role in differentiating *Vibrio cholerae* in to different serogroup and serotype (Finkelstein, 2004). Over 200 serogroup of *Vibrio cholerae* have been classified on the basis of O-antigens (Lipp et al., 2002) of which only two serogroup O1 and O139 are responsible for epidemic cholera cases (Lipp et al., 2002, Finkelstein 2004, Janda 1998, Bauman 1988 and Faruque et al., 1998).

The serogroup O1 has been distinguished in to 3 different serotypes namely Ogawa, Inaba and Hikojima and each serotype may display classical or El Tor biotype. The serotype Ogawa and Inaba express common antigen A and differentiated by the type specific antigens B (Ogawa) and C (Inaba). An additional serotype Hikojima contains both type specific antigens and is rare (Finkelstein, 2004).

Table 3.4: Antigenic Determinants of *Vibrio cholerae*

Serotype	O-antigens
Ogawa	A, B
Inaba	A, C
Hikojima	A, B, C

Vibrio cholerae O139 is new type of strain arose from homologous recombination event. So it expresses part of O1 El Tor O-antigens and remaining of non O1 strain. The recombination event also lead to capsular antigen production, a feature often associated with non O1 strains (Bik et al., 1995 and Dumontier et al., 1998). So O139 strain expresses typical O-antigens partly resembles O1 El Tor strain and partly with non O1 cholera vibrios.

There are various unusual sugars found in surface lipopolysaccharide which determines the antigenicity. The serogroup related sugar include fucose, arabinose, D-glycero-D-

mannohepstose, D-glucosamine, D-galactosamine, 3-amino-3 6 dideoxyglucose, 3 amino-3 dideoxygalactose, 2 keto-3 deoxyoctonic acid (Iguchi et al., 1995). The O-polysaccharide side chains of *Vibrio cholerae* O1 is homopolymer of D-perosamine approximately 17-18 unit in length (Manning et al., 1994 and Knirel et al., 1997). The amino group of perosamine unit is commonly acetylated with 3-deoxy-L-glycerotetronoic acid. Quinovosamine is thought to be capping sugar (Manning et al., 1994). 4-amino 6 dideoxy-2-O methyl mannose is only found in serotype Ogawa (Eton et al., 1994).

The genes responsible for O1 antigen biosynthesis has been designated *wbeT* and are localized on a 21.6 kb *sacI* fragment of DNA, this is highly conserved sequence. The only changes observed between Ogawa and Inaba serotypes are related to mutation in the *wbe* T region, which causes serotype conversion in *Vibrio cholera* O1 serogroup (Garg et al., 2002). However only partial genes of *wbeT* is found in O139 serogroup which gives strong evidence that O139 arises after genetic modification of O1 strains and non O1 strains of *Vibrio cholerae* (Albert, 1994)

3.4.6 Pathogenesis of cholera

The pathogenic action of *Vibrio cholerae* is exerted on the intestinal epithelium. The function of intestine is to absorb the nutrients and other salts and also maintaining the fluidity of the intestinal lumen. The membrane fluidity in the intestinal lumen depends upon absorption of nutrients and active absorption and secretion of electrolytes. The disease stage can arise if there is any imbalance in the membrane fluidity i.e. less fluid or more fluid in intestine (Finkelstein, 2004).

Infection due to *Vibrio cholerae* begins with consumption of contaminated food and water. In the developed world, seafood is usual cause while in developing world it is more often water (Sack et al., 2003). Cholera has been found only in two other animal populations beside human: shellfish and plankton (Sack et al., 2003). It has been shown that more than one million *Vibrio cholerae* cells are required to induce infection and diarrhea. However the dose is about 10,000 cells in those with lower gastric acidity (for instance those using proton pump inhibitors). Children are also susceptible with two to four years of age having the highest rate of infection. Individual's susceptibility is also

affected by the blood type, those with type O blood being the most susceptible (Sack et al. 2003 and Harris et al., 2005).

Perhaps, the higher dose of bacteria is required to overcome the stomach acidity as vibrios are very sensitive to the stomach acidity (pH 1-2). After passing through the stomach's acid barrier, the organism colonizes on the epithelial wall of small intestine (Finkelstein, 1988).

Colonization of the Small Intestine: There are several characteristics of pathogenic *V. cholerae* that are important determinants of the colonization process. These include adhesins, neuraminidase, motility, chemotaxis and toxin production. If the bacteria are able to survive the gastric secretions and low pH of the stomach, they are well adapted to survival in the small intestine. *Vibrio cholerae* is resistant to bile salts and can penetrate the mucus layer of the small intestine, possibly aided by secretion of neuraminidase and proteases (mucinases). They withstand propulsive gut motility by their own swimming ability and chemotaxis directed against the gut mucosa (Finkelstein, 2004).

Specific adherence of *V. cholerae* to the intestinal mucosa is probably mediated by long filamentous fimbriae that form bundles at the poles of the cells. These fimbriae have been termed Tcp pili (for toxin co regulated pili); because expression of these pili genes is co regulated with expression of the cholera toxin genes (Tayler et al., 1987). Not much is known about the interaction of Tcp pili with host cells, and the host cell receptor for these fimbriae has not been identified. Tcp pili share amino acid sequence similarity with N-methylphenylalanine pili of *Pseudomonas* and *Neisseria* (DiRita et al., 1991).

Two other possible adhesins in *V. cholerae* are a surface protein that agglutinates red blood cells (hemagglutinin) and a group of outer membrane proteins which are products of the *acf* (accessory colonization factor) genes (Karaolis et al., 1998). *acf* mutants have been shown to have reduced ability to colonize the intestinal tract. It has been suggested that *V. cholerae* might use these nonfimbrial adhesins to mediate a tighter binding to host cells than is attainable with fimbriae alone (Levin et al., 1998 and Karaolis et al., 1998).

V. cholerae produces a protease originally called mucinase that degrades different types of protein including fibronectin, lactoferrin and cholera toxin itself. Its role in virulence is not known but it probably is not involved in colonization since mutations in the mucinase gene (designated hap for hemagglutinin protease) do not exhibit reduced virulence. It has been suggested that the mucinase might contribute to detachment rather than attachment. Possibly the vibrios would need to detach from cells that are being sloughed off of the mucosa in order to reattach to newly formed mucosal cells (Levin et al., 1998).

CholeraToxin: Cholera toxin activates the adenylate cyclase enzyme in cells of the intestinal mucosa leading to increased levels of intracellular cAMP, and the secretion of H_2O, Na^+, K^+, Cl^-, and HCO_3^- into the lumen of the small intestine. The effect is dependent on a specific receptor, monosialosyl ganglioside (GM1 ganglioside) present on the surface of intestinal mucosal cells. The bacterium produces an invasin, neuraminidase, during the colonization stage which has the interesting property of degrading gangliosides to the monosialosyl form, which is the specific receptor for the toxin (O'Neal et al., 2005 and Finkelstein, 2004).

The toxin has been characterized and contains 5 binding (B) subunits of 11,500 Daltons, an active (A1) subunit of 23,500 Daltons, and a bridging piece (A2) of 5,500 Daltons that links A1 to the 5B subunits. Once it has entered the cell, the A1 subunit enzymatically transfers ADP ribose from NAD to a protein (called Gs or Ns), that regulates the adenylate cyclase system which is located on the inside of the plasma membrane of mammalian cells (Finkelstein, 2004).

Enzymatically, fragment A1 catalyzes the transfer of the ADP-ribosyl moiety of NAD to a component of the adenylate cyclase system. The process is complex. Adenylate cyclase (AC) is activated normally by a regulatory protein (GS) and GTP; however activation is normally brief because another regulatory protein (Gi) hydrolyzes GTP. The normal situation is described as follows (Finkelstein, 2004).

The A1 fragment catalyzes the attachment of ADP-Ribose (ADPR) to the regulatory protein forming Gs-ADPR from which GTP cannot be hydrolyzed. Since GTP hydrolysis

is the event that inactivates the adenylate cyclase, the enzyme remains continually activated. This situation can be illustrated (Finkelstein, 2004).

Thus, the net effect of the toxin is to cause cAMP to be produced at an abnormally high rate which stimulates mucosal cells to pump large amounts of Cl^- into the intestinal contents. H_2O, Na^+ and other electrolytes follow due to the osmotic and electrical gradients caused by the loss of Cl^-. The lost H_2O and electrolytes in mucosal cells are replaced from the blood. Thus, the toxin-damaged cells become pumps for water and electrolytes causing the diarrhea, loss of electrolytes, and dehydration that are characteristic of cholera (Faruque et al., 1998 and Finkelstein, 2004).

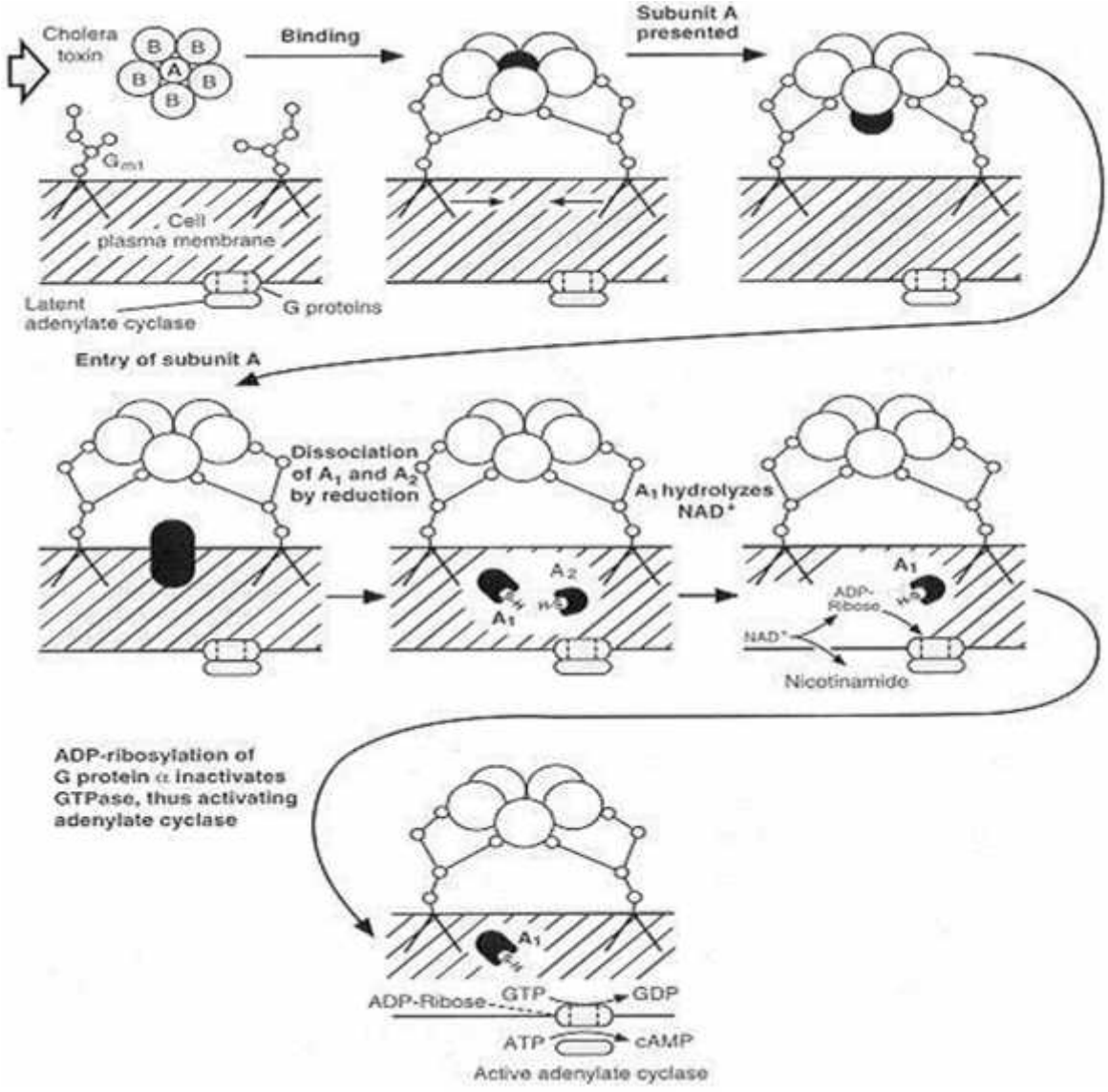


Figure 3.3: Mechanism of action of cholera enterotoxin according to Finkelstein in Baron, Chapter 24. Cholera toxin approaches target cell surface. B subunits bind to oligosaccharide of GM1 ganglioside. Conformational alteration of holotoxin occurs, allowing the presentation of the A subunit to cell surface. The A subunit enters the cell. The disulfide bond of the A subunit is reduced by intracellular glutathione, freeing A1 and A2. NAD is hydrolyzed by A1, yielding ADP-ribose and nicotinamide. One of the G proteins of adenylate cyclase is ADP-ribosylated, inhibiting the action of GTPase and locking adenylate cyclase in the "on" mode.

Genetic Organization and Regulation of Virulence Factors in *Vibrio cholerae*: In *Vibrio cholerae*, the production of virulence factors is regulated at several levels. Regulation of genes at the transcriptional level, especially the genes for toxin production and fimbrial synthesis, has been studied in the greatest detail.

V. cholerae enterotoxin is a product of *ctx* genes. *ctx*A encodes the A subunit of the toxin, and *ctx*B encodes the B subunit. The genes are part of the same operon. The transcript (mRNA) of the *ctx* operon has two ribosome binding sites (rbs), one upstream of the A coding region and another upstream of the B coding region. The rbs upstream of the B coding region is at least seven-times stronger than the rbs of the A coding region. In this way the organism is able to translate more B proteins than A proteins, which is required to assemble the toxin in the appropriate 1A: 5B proportion. The components are assembled in the periplasm after translation. Any extra B subunits can be excreted by the cell, but A must be attached to 5B in order to exit the cell. Intact A subunit is not enzymatically active, but must be nicked to produce fragments A1 and A2 which are linked by a disulfide bond. Once the cholera toxin has bound to the GM1 receptor on host cells, the A1 subunit is released from the toxin by reduction of the disulfide bond that links it to A2, and enters the cell by an unknown translocation mechanism. One hypothesis is that the 5 B subunits form a pore in the host cell membrane through which the A1 unit passes (Waldor et al., 1996, Mekalanos et al., 1997 and Finkelstein, 2004).

Transcription of the *ctx*AB operon is regulated by a number of environmental signals, including temperature, pH, osmolarity, and certain amino acids. Several others *V. cholerae* genes are coregulated in the same manner including the *tcp* operon, which is concerned with fimbrial synthesis and assembly. Thus the *ctx* operon and the *tcp* operon

are part of a regulon, the expression of which is controlled by the same environmental signals (Lipp et al., 2002).

The proteins involved in control of this regulon expression have been identified as ToxR, ToxS and ToxT (Skorupski et al., 1997). ToxR is a transmembranous protein with about two-thirds of its amino terminal part exposed to the cytoplasm. ToxR dimers, but not ToxR monomers, will bind to the operator region of *ctx*AB operon and activate its transcription. ToxS is a periplasmic protein. It is thought that ToxS can respond to environmental signals, change conformation, and somehow influence dimerization of ToxR which activities transcription of the operon (DiRita et al., 1997). ToxR and ToxS appear to form a standard two-component regulatory system with ToxS functioning as a sensor protein that phosphorylates and thus converts ToxR to its active DNA binding form. ToxT is a cytoplasmic protein that is a transcriptional activator of the *tcp* operon. Expression of ToxT is activated by ToxR, while ToxT, in turn, activates transcription of *tcp* genes for synthesis of *tcp* pili (Higgins et al., 1996).

Thus, the ToxR protein is a regulatory protein which functions as an inducer in a system of positive control. Tox R is thought to interact with ToxS in order to sense some change in the environment and transmit a molecular signal to the chromosome which induces the transcription of genes for attachment (pili formation) and toxin production (Skorupski et al., 1997). It is reasonable to expect that the environmental conditions that exist in the GI tract (i.e. 37^{o} temperature, low pH, high osmolarity etc.), as opposed to conditions in the extra intestinal (aquatic) environment of the vibrios; are those that are necessary to induce formation of the virulence factors necessary to infect (Parsot and Mekalanos et al., 1990). However, there is conflicting experimental evidence in this regard, which leads to speculation of the ecological function of the toxin during human infection (Waldor et al., 1998).

3.4.7 Host defense and immunity against cholera

Infection with *V. cholerae* results in a spectrum of responses ranging from life-threatening secretory diarrhea to mild or unapparent infections of no manifestation except

a serologic response. The reasons for these differences are not known. One idea is that individuals differ in the availability of intestinal receptors for cholera vibrios or for their toxin, but this has not been proven. Prior immunologic experience is certainly a major factor. For example, in heavily endemic regions such as Bangladesh, the attack rate is relatively low among adults in comparison with children.

After natural infection by *V. cholerae*, circulating antibodies can be detected against several cholera antigens including the toxin, somatic (O) antigens, and flagellar (H) antigens. These antibodies are also raised by parenteral injection of antigens as vaccine components. Antibodies directed against *Vibrio* O antigens are considered vibriocidal antibodies because they will lyse *V. cholerae* cells in the presence of complement and serum components. Vibriocidal antibodies reach a peak 8-10 days after the onset of clinical illness, and then decrease, returning to the baseline 2 - 7 months later. Their presence correlates with resistance to infection, but they may not be the mediators of this protection, and the role of circulating antibodies in natural infection is unclear.

After natural infection, people also develop toxin-neutralizing antibodies but there is no correlation between antitoxic antibody levels and the incidence of disease in cholera zones.

Since cholera is essentially a topical disease of the small intestine, it would seem that topical defense might be a main determinant of protection against infection by *V. cholerae*. Recurrent infections of cholera are in fact, rare, and this is probably due to local immune defense mediated by antibodies secreted onto the surfaces of the intestinal mucosa. Moreover, in children who are nursing cholera is less likely to occur, presumably due to protection afforded by secretory antibody in mother's milk.

Secretory IgA, as well as IgG and IgM in serum exudate, can be detected in the intestinal mucosa of immune individuals. Although these antibodies presumably have to function in the absence of complement they still bring about protective immunity. Motility is important in pathogenesis, and antibodies against flagella could immobilize the vibrios. Antibodies against flagella or somatic O antigens could cause clumping and arrested

motion of cells. Antitoxic antibodies could react with toxin at the epithelial cell surface and block binding or activity of the toxin. Since the process by which the vibrios attach to the intestinal epithelium is highly specific, antibodies against *Vibrio* fimbriae or other surface components (LPS) could block attachment.

3.4.8 Cholera Vaccines

The observation that natural infection confers effective and long-lasting immunity against cholera has led to efforts to develop a vaccine which will elicit protective immunity. The first attempts at a vaccine in 1960s were directed at whole cell preparations injected parenterally (Ryan et al., 2000). At best, 90% protection was achieved and this immunity waned rapidly to the baseline within one year (Clemens et al., 1990). Purified LPS fractions from different biotypes have also been given as vaccines with variable success. The cholera toxin can be converted to toxoid in the presence of formalin and glutaraldehyde. The toxoid is a poor antigen, however, and it elicits a very low level of protection (Lon Roth et al., 1973, Ryan et al., 2000).

At present time two type of cholera vaccine is available, the killed oral vaccine and live attenuated oral vaccine.Both have been shown to be safe, immunogenic and efficacious (Ryan et al., 2000, Ivanoff et al., 2002). Killed oral cholera vaccine Ducoral™ (licensed by SBL vaccine AB Biotech, Sweden) is recommended by WHO in 1999 contains formalin killed whole cell cholera O1 (both Ogawa and Inaba serotype and biotype classical and El Tor) added with purified recombinant cholera toxin B subunit (Peltola et al., 1991 and Lopez-Gigosos et al., 2007). The vaccine also advertises protection against entero toxigenic *E. coli* (ETEC) which produces a toxin (LT) identical to cholera toxin, and which is an important cause of traveller's diarrhea (Peltola et al., 1991 and Lopez-Gigosos et al., 2007). This is given orally with buffer to neutralize stomach acidity which provides 90% immunity during 6 month on all ages of people after administration of 2 doses in1-2 weeks apart. Now a day it is administered in a 3 doses to 2-6 year children followed by booster dose in 6 months and 2 doses in old children and adults followed by booster dose in 2 years (Clemens et al., 1991 and van Loon et al., 1996).

Attenuated live vaccine consist of *Vibrio cholerae* O1 Inaba strain (CVD 103-HgR) which has been engineered to produce B subunit (CTB) but not A of cholera toxin (CT). The vaccine is designed by Berna Biotech Switzerland as Orochol™. This is also given orally with buffer to neutralize stomach acidity and protection was found to be 80% in diarrheal cases (Tacket et al., 1999). The ideal properties of such a "vaccine strain" of the bacterium would be to possess all the pathogenicity factors required for colonization of the small intestine (e.g. motility, fimbriae, neuraminidase, etc.) but not to produce a complete toxin molecule. Ideally it should produce only the B subunit of the toxin which would stimulate formation of antibodies that could neutralize the binding of the native toxin molecule to epithelial cells (Tacket et al., 1999).

A new vaccine has been developed to combat the *Vibrio cholerae* Bengal strain that has started spreading in epidemic fashion in the Indian subcontinent and Southeast Asia. The Bengal strain differs from previously isolated epidemic strains in that it is serogroup O139 rather than O1, and it expresses a distinct polysaccharide capsule. Since previous exposure to O1 *Vibrio cholerae* does not provide protective immunity against O139, there is no residual immunity in the indigenous population to the Bengal form of cholera (Ledon et al., 2003).

The noncellular vaccine is relatively nontoxic and contains little or no LPS and other impurities. The vaccine will be used for active immunization against *Vibrio cholerae* O139 and other bacterial species expressing similar surface polysaccharides. In addition, human or other antibodies induced by this vaccine could be used to identify *Vibrio cholerae* Bengal for the diagnosis of the infection and for environmental monitoring of the bacterium (Ryan et al., 2006).

3.4.9 Treatment

Successful treatment of cholera patient depends on rapid replacement of fluid and electrolyte losses. With proper treatment, mortality is less than 1% of reported cases. Fluid and electrolytes can be replaced rapidly through either oral or intravenous routes, intravenous therapy is required for patients who are in profound shock or cannot drink.

The administration of isotonic maintenance solution should be continued until the diarrhea ceases.

Antibiotic therapy is not generally recommended for treatment of cholera. However in severe cases, antibiotics are given which reduces the duration of the illness, the volume of stool and the duration of shedding of vibrios in the feces (Greenough et al., 1964, Lindenbaum et al., 1967 and Pierce et al., 1968).

When antibiotics are to be used, it is essential to choose one to which the organism is susceptible. For the treatment of cholera, an oral or intravenously administered solution containing glucose, sodium chloride, potassium chloride and tri sodium citrate can save a patient from dehydration (WHO, 2002). The antibiotic tetracycline and quinolones have been widely used but the emergence of *Vibrio cholerae* strains resistance to antibiotics has restricted their use to patient with severe dehydration (Garg et al., 2001). In severe cases, single dose of doxycycline (a member of class Tetracycline) co-administered with fluid replacement therapy is usually sufficient to stabilize the patient. Alternatively a multidose treatment of tetracycline can be administered; in the case of young children liquid erythromycin is preferred (WHO, 2004).

Though there are obvious benefits to individuals who are treated with antibiotics, the World Health Organization (WHO) does not recommend their general use because antibiotics contribute to increasing antimicrobial resistance making cholera and other bacterial infections more difficult to treat (Glass et al., 1980 and Sack et al., 2003). Use of antibiotics to treat cholera should be strictly regulated to patient suffering from severe dehydration (WHO, 2010).

3.4.10 Mechanism of antimicrobial resistance in *Vibrio cholerae*

The strategies adopted by *Vibrio cholerae* to combat antimicrobials are as follows.

Resistance for quinolone and fluoroquinolone: Resistance to this group of drugs has been recorded from early 2000s in India and other countries after the extensive use of this drug. The resistance mechanism is associated with the mutation in *gyrA* and *parC* gene

which codes for DNA gyrase and DNA topoisomerase respectively (Ramamurthy and Ghosh, 2011), both collectively referred as quinolone resistance determining region. In addition to this, proton motive force dependent efflux is also involved in quinolone resistance in clinical isolate of *Vibrio cholerae* (Baranwal et al., 2002).

Efflux pumps: Bacteria may acquire efflux pumps that export antibacterial agents before it can reach its target site and exert its effect. *Vibrio cholerae* use multiple efflux pump system to export broad range of antibiotics, dyes and detergents (Paulsen et al., 1996).

The chromosome of *Vibrio cholerae* contains many putative genes of multidrug resistance and toxic compounds extrusion family. These include *vcmM, vcmB, vcmD, vcmH and vcmN*. Elevated MICs of multiple drugs such as fluoroquinolone and aminoglycosides were reported in susceptible *E. coli* when these genes are transformed. It has been also shown that *vcmB, vcmD* and *vcmH* genes were Na+ dependent.

Integrons: These are naturally occurring gene acquisition systems which help bacteria capture exogenous genes and incorporate them in to their genomes (Dubois et al., 2007). It has *intI* gene facilitating the site of recombination at *att* point. It play prominent role in dissemination of drug resistance because they frequently carry drug resistance genes. Till this date over 100s of such genes have been discovered, out of these few have been found in *Vibrio cholerae* even though integrons have been detected in large no of isolates around the world (Ghosh et al., 2010). An integron carrying *qnr* gene is responsible for resistance to Ciprofloxacin have been detected (Fonseca et al., 2008 and Kim et al., 2010).

Integrative and conjugative elements (ICE): These are linear DNA molecules which can integrate in to bacterial genome and move through conjugation. These have the capacity to incorporate genes of drug resistance to DNA repair pathways (Burrus et al., 2006). SXT is an ICE in *Vibrio cholerae* which carry resistance genes to Sulfomethoxazole-Trimethoprim, Streptomycin and Chloramphenicol. The SXT gene contains tandem sequences which can mediate gene transfer to form novel ICEs (Burrus et al., 2004).

Plasmid: One of the common modes of the dissemination of drug resistance is through the plasmid. *Vibrio cholerae* O1 strains isolated from clinical specimens before 1970s are sensitive to Tetracycline but due to extensive use of the drug, resistance occurred. A multidrug resistant *Vibrio cholera* harbours 200 Kb self transmissible plasmid that mediates resistance to Tetracycline, Ampicillin, Chloramphenicol, Kanamycin, Gentamycin and Sulfomethoxazole-Trimethoprim (Yamamoto et al., 1995).

Extended spectrum beta lactamases (ESBLs): Beta lactamase production is demonstrated in Ampicillin resistant strain of *Vibrio cholerae* (Sundaram et al., 1994). CTX-Mase which hydrolyzes cefotaxime is mostly coded by trans-genes and found in *Vibrio cholerae* (Rasmussen et al., 2004). Which belongs to the class A beta lactamase which functions as ESBL and confers resistance to penicillin, extended spectrum cephalosporin and monobactam.

CHAPTER IV

4. MATERIALS AND METHODS

The laboratory works were carried out in National Public Health Laboratory (NPHL) and in the laboratory of Central Department of Microbiology (CDM), Tribhuvan University (T.U.) for MIC test. The study was conducted from April to December of 2010. The diarrheal stool specimen admitted to 10 different hospitals of Kathmandu, 2 hospitals of Butwal, at laboratory of NPHL and specimens from Nepalgunj outbreak area were taken under the investigation. Altogether 240 samples were processed for the isolation of *Vibrio cholerae*. The materials used during the study period are given in the appendix II.

4.1 Specimen collection and transport

The samples were collected in clean sterile wide mouthed container and processed immediately after its arrival in the laboratory. Rectal swabs were the specimen of choice from the outbreak area and brought in Cary Blair Transport media at 4ºC to the laboratory as soon as possible. Rectal swab was taken by inserting and rotating the swab 2-3 cm deep through the rectal sphincter noticing that some fecal materials were also brought in the swab. Those who can pass stool at a time, it was collected in a wide mouthed container and swab was dipped in to the stool and brought in a Cary Blair medium. The patient's clinical history was taken during the time of sample collection. The samples were collected only from the patient who did not have any treatment during that time. The data collection form is given in appendix I.

4.2 Processing of the Specimens

Culture: The specimen was placed on alkaline peptone water (APW) (in a volume of 1:10) and incubated at 37ºC for 6 hours. In case of swab it was dipped in to the alkaline peptone water directly. The stool sample was also inoculated in Thio Sulfate Citrate Bile Salt Sucrose agar (TCBS) medium and MacConkey agar (MA) and incubated at 37ºC for 24 hours. Similarly the APW inoculated sample was inoculated on TCBS and MA and incubated at 37ºC for 24 hours.

After incubation, the plates were observed for the presence of yellow button shaped sucrose fermenting colonies in TCBS agar medium and late lactose fermenting colonies in MA. Then the colonies were further identified by biochemical tests.

Isolation and identification: After culture, the visual growth of the organism was observed on the agar medium. Then the organism was isolated and identified on the basis of Gram staining reactions and biochemical properties such as Oxidase Test, Catalase Test, Methyl Red Test, Voges Proskauer Test, Citrate Utilization Test, Triple Sugar Iron Agar Test, Sulfide-Motility and Indole Test, Urea Hydrolysis Test, Amino acid Decarboxylation Test, Salt Concentration Tolerance Test and Acid Production Tests. The details of these biochemical processes are given in the appendix III.

4.3 Serotyping of the *Vibrio cholerae*

For the serotyping of *Vibrio*, the suspected colonies from TCBS and MA were sub-cultured on NA as the direct testing from these medium is not recommended. Hence serological tests were performed only from NA after 24 hours of incubation.

Slide agglutination technique was used for this purpose. A uniform milky smear was prepared of the isolate with normal saline on the clean glass slide. Then specific polyvalent O1 antiserum was added in to the smear and the slide was tilted in to and fro motion for some time. The visible agglutination was observed for the positive test which was further classified into Ogawa, Inaba and Hikojima serotypes by using respective serotype specific antisera by Denka Siken Co. Ltd., Japan.

4.4 Biotyping of *Vibrio cholerae*

Epidemic strain of *Vibrio cholerae* can be further differentiated into Classical and El Tor biotype on the basis of following test:

Haemolysis of sheep RBC: El Tor strain hemolysed the sheep RBC which was clearly visible during inoculation in 5% sheep blood agar medium.

VP reaction: The test organism was inoculated in MR/VP medium and incubated at 37ºC. After overnight incubation, the Barritt's reagent A and B were put in the medium at a ratio of 3:1. A positive reaction was indicated by the appearance of dark red color.

Susceptibility to Polymyxin B: Polymyxin B was placed on the swabbed surface of Muller Hinton agar with the test organism. After overnight incubation the El Tor strain shows resistance while classical strain was sensitive.

4.5 Antibiotic sensitivity pattern of the *Vibrio cholerae*

After identification of the organism, the sensitivity test was performed *in vitro* for the isolates. The test was performed by Kirby Bauer disc diffusion method and minimum inhibitory concentration method.

Primary susceptibility of the isolates was performed by Kirby Bauer disc diffusion method; a standard dilution of the test isolate was prepared by matching it with 0.5 McFarland turbidity standards and was uniformly swabbed over the Muller Hinton agar medium. Then various antibiotic discs were placed on the medium and incubated at 37ºC for 24 hours. After incubation the zone of inhibition were measured and results were interpretated as per the guideline given by the Clinical Laboratory Standard Institute (CLSI).

The minimum inhibitory concentration test was performed for only five antibiotics, Ampicillin, Chloramphenicol, Ciprofloxacin, Nalidixic acid and Tetracycline by microtitre broth dilution method. The test performed according to the guideline given by the CLSI (performance standard for antimicrobial susceptibility test M100-S20). The procedures are listed in appendix IV.

4.6 Purity plate culture

Purity plate indicates whether the experiment was performed under aseptic condition or not. For the purpose it was done during each biochemical test and MIC test. Before the test, half NA plate was inoculated with the test organism and half plate inoculated after

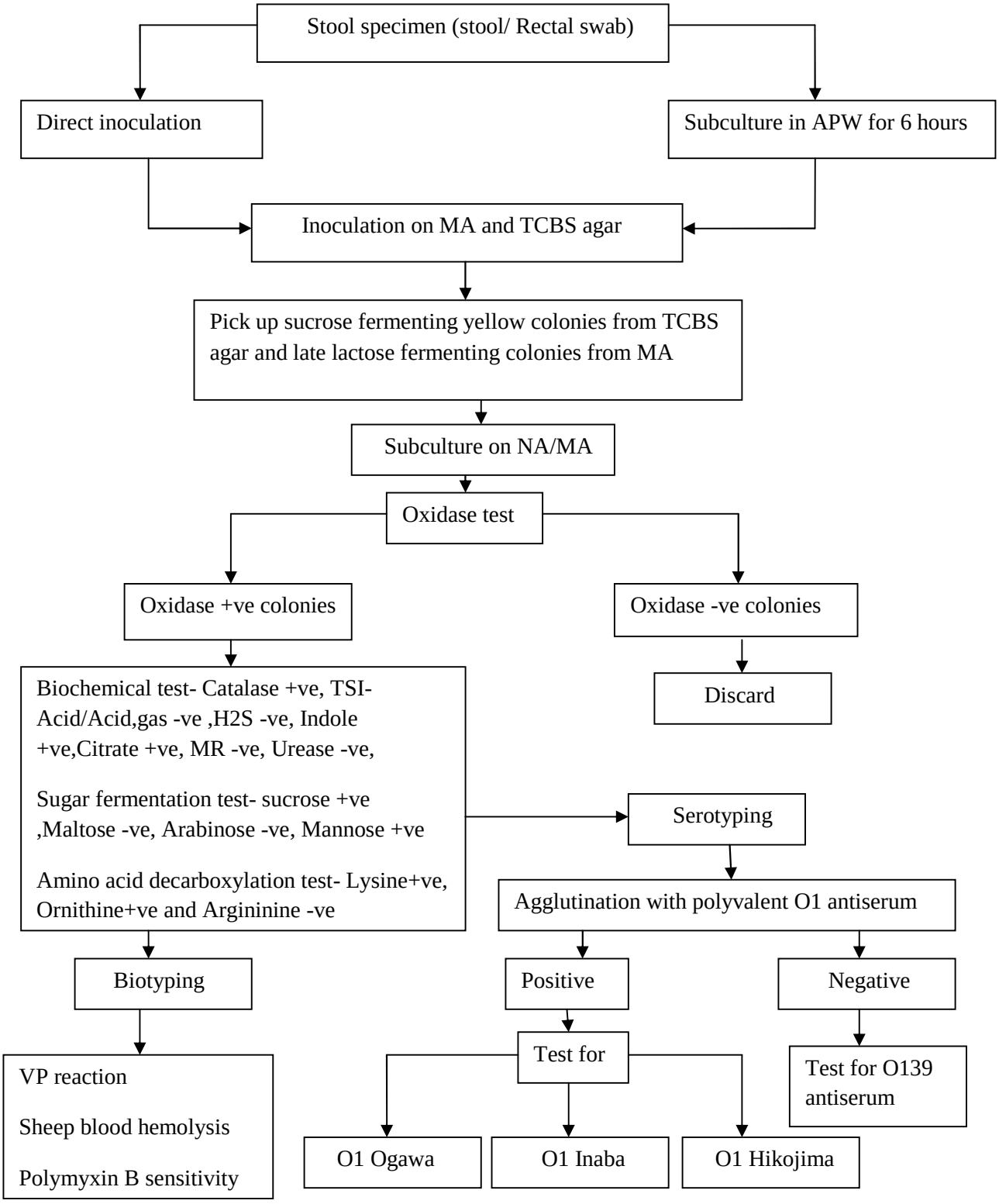
performing the test. The growth of the organism in pure form in both pre and post inoculation area indicates that the experiment was performed aseptically.

4.7 Quality control

For the confirmation of accuracy of the test results, quality control was maintained throughout the period of the study. Strict aseptic condition and techniques were followed by the batch test of the culture medium and monitoring the antibiotic disc and powder by using standard ATCC culture as recommended by CLSI.

All the necessary quality controls of media and culture plates were performed during the experiment. The media lot no. and expiry date were also checked. TCBS medium and other biochemical medium were also tested with standard reference control strains for the performance of the medium. During AST by disc diffusion test, the *E. coli* ATCC 25922 was taken as control throughout the test procedure. It was also taken as reference strain during MIC test. *Proteus vulgaris* ATCC 13315 and *Klebsiella pneumoniae* ATCC 31388 were taken as control strain for amino acid decarboxylation and VP reaction test apart from *E. coli* ATCC 25922. All of the instruments used were checked for their efficacy and temperature and pH control. All antibiotics disc and powder were checked for their expiry date.

Flow chart for identification of *Vibrio cholerae* (CDC, 2010)



CHAPTER-V

5. RESULTS

During the study period, a total of 240 samples were processed for the isolation of *Vibrio cholerae*. Out of total samples, 44 were taken from outbreak area and 196 were the hospital samples admitted to the different hospitals. Out of total samples 110 were from male and 130 were from female.

5.1: Samples processed and positive cases

During the study period, 240 diarrheal samples were investigated. Out of those samples, 44 (18.3%) were taken from diarrheal outbreak areas. Remaining 196 samples (81.7%) were taken from different hospitals where patient admitted their diarrheal samples. From 240 samples 48 cases were found to be positive for *Vibrio cholerae*. 20 (8.7%) strains were isolated from outbreak samples and 28 (11.3%) strains were isolated from the hospital samples resulting total sample prevalence was 20%.

Table 5.1: Distribution of the positive cases in total samples

Samples taken from	Sample processed		Positive cases		Prevalence
	No.	%	No.	%	
Hospitals	196	81.7	28	11.3	20%
Outbreak area	44	18.3	20	8.7	
Total	240	100	48	20	

Among 48 isolated strains of *Vibrio cholerae*, 18 strains (37.5%) were isolated from male and 28 strains (62.5%) were isolated from female patients.

Table 5.2: Gender wise distribution of the cholera cases in outbreak and hospital samples

Cases	Male		female		total	
	No.	%	No.	%	No.	%
Outbreak	9	18.75	11	22.9	20	41.7
Hospital	9	18.75	19	39.6	28	58.3
Total	18	37.5	30	62.5	48	100

9 strains (18.75%) were isolated in outbreak samples and 9 (18.75%) from hospital samples in case of male patients and 11 (22.9%) strains were isolated from outbreak samples and 19 strains (39.6%) were isolated from hospital sample taken from female patients. There was no significant difference in occurrence between cholera cases and gender of the patients (P>0.05).

On the basis of age wise distribution of the *Vibrio cholerae* cases, 50% strains were isolated from the age group 0-20 and 41.7% strains were isolated from age group 20-50. There was no significant difference in occurrence between cholera cases and the age group of the patients (P>0.05).

Table 5.3: Age and gender wise distribution of the *Vibrio cholerae* isolates

Age group	Male		Female		Total	
	Number	%	Number	%	Number	%
0-20	9	18.7	15	31.3	24	50.0
20-50	8	16.7	12	25.0	20	41.7
50- above	1	2.1	3	6.2	4	8.3
Total	18	37.5	30	62.5	48	100

5.2 Results of Serotyping

Serotyping results showed that all the strains agglutinate polyvalent O1 antisera hence all *Vibrio cholerae* belonged to serogroup O1. Reaction with specific antisera showed that all strains agglutinate Ogawa antisera and hence all isolated *Vibrio cholerae* strains were belonged to serogroup O1 and serotype Ogawa.

Table 5.4: Results of Serotyping of isolated *Vibrio cholerae* strains

Vibrio cholerae serogroup	*Vibrio cholerae* serotype	Number of case	Percentage of case
Serogroup O1	Ogawa	48	100
	Inaba	0	0
	Hikojima	0	0

5.3 Results of Biotyping of the isolated *Vibrio cholerae*

All of the isolated strains of *Vibrio cholerae* were subjected to biotyping by Voges Proskauer reaction, Sheep blood hemolysis and Polymyxin B sensitivity test. All 48 isolated *Vibrio cholerae* strains gave positive VP reaction. All strains were resistant to 300U of Polymyxin B and all strains hemolysed sheep RBC on sheep blood agar medium.

Table 5.5: Results of biotyping for *Vibrio cholerae*

S.N.	Test performed	Results		Remarks
		No. of strain positive	No. of strain negative	
1	Voges Proskauer test	48	0	All the isolated strains of *Vibrio cholerae* were of biotype El Tor.
2	Sheep blood hemolysis	48	0	
3	Polymyxin B sensitivity	0	48	

On the basis of results of serotyping and biotyping all the strains of isolated *Vibrio cholerae* were belong to serogroup O1 serotype Ogawa and biotype El Tor.

5.4 Month wise distribution of cholera cases

According to the month wise isolation of *Vibrio cholerae* strains 72% were isolated during August.

Table 5.6: Distribution of cholera cases in different months

Month	Frequency	Sample percentage
April	0	0.0
May	0	0.0
June	0	0.0
July	5	10.4
August	35	72.0
September	7	14.6
October	0	0.0
November	1	2.1
December	0	0.0

5.5 Antibiotic susceptibility test (AST)

5.5.1 Kirby Bauer disc diffusion method

All the isolated strains of *Vibrio cholerae* O1 were completely sensitive to Amikacin, Gentamicin, Ceftriaxone and Cefotaxime. All the strains were completely resistant to Nalidixic acid, Cotrimoxazole and Furazolidone. 89.6% strains were resistant to Ampicillin while 8.3% were intermediate and 2.1% were sensitive. 41.7% strains were sensitive to Chloramphenicol and 56.2% intermediate and 2.1% was sensitive. For Erythromycin, 41.7% strains were sensitive, 56.2% intermediate and 2.1% were resistant. For Ciprofloxacin, 8.3% strains were sensitive, 35.4% intermediate and 56.3% were resistant. For Ofloxacin, 4.2% strains were sensitive, 35.4% intermediate and 60.4% were resistant. In case of tetracycline, 35.4% were resistant while 64.6% were sensitive. All the strains resistant to Tetracycline were isolated from the outbreak sample (17 out of 20), all strains of *Vibrio cholerae* isolated from hospital sample were sensitive to Tetracycline.

Table 5.7: Results of AST by Disc diffusion method for isolated *Vibrio cholerae*

S.N.	Sample code	A	FR	NA	TS	T	AM	GM	E	CIP	OFX	C	CRO	CTX
1	OVc18	R	R	R	R	R	S	S	S	IMD	R	S	S	S
2	OVc19	R	R	R	R	R	S	S	S	R	R	S	S	S
3	OVc20	R	R	R	R	R	S	S	S	R	R	S	S	S
4	OVc21	R	R	R	R	R	S	S	S	R	R	S	S	S
5	OVc22	R	R	R	R	R	S	S	S	R	R	S	S	S
6	OVc37	R	R	R	R	R	S	S	S	IMD	R	S	S	S
7	OVc23	R	R	R	R	R	S	S	S	R	R	S	S	S
8	OVc24	IMD	R	R	R	R	S	S	S	IMD	R	S	S	S
9	OVc25	R	R	R	R	R	S	S	S	R	R	S	S	S
10	OVc26	R	R	R	R	R	S	S	S	R	R	S	S	S
11	OVc27	R	R	R	R	R	S	S	S	IMD	IMD	S	S	S
12	OVc28	R	R	R	R	R	S	S	S	R	R	S	S	S
13	OVc29	R	R	R	R	R	S	S	S	R	R	S	S	S
14	OVc30	R	R	R	R	R	S	S	S	R	R	S	S	S
15	OVc31	R	R	R	R	R	S	S	S	R	R	S	S	S
16	OVc32	R	R	R	R	S	S	S	S	R	R	S	S	S
17	OVc33	R	R	R	R	S	S	S	S	R	R	S	S	S
18	OVc34	R	R	R	R	R	S	S	S	R	R	S	S	S
19	OVc35	R	R	R	R	S	S	S	S	R	R	S	S	S
20	OVc36	IMD	R	R	R	R	S	S	S	R	R	S	S	S
21	HVc1	IMD	R	R	R	S	S	S	IMD	R	R	IMD	S	S
22	HVc2	R	R	R	R	S	S	S	IMD	R	R	IMD	S	S
23	HVc3	R	R	R	R	S	S	S	IMD	R	R	IMD	S	S
24	HVc4	R	R	R	R	S	S	S	IMD	IMD	R	IMD	S	S
25	HVc5	R	R	R	R	S	S	S	IMD	IMD	IMD	IMD	S	S
26	HVc6	R	R	R	R	S	S	S	IMD	IMD	S	IMD	S	S
27	HVc7	R	R	R	R	S	S	S	IMD	IMD	IMD	IMD	S	S
28	HVc8	R	R	R	R	S	S	S	IMD	IMD	IMD	IMD	S	S
29	HVc9	S	R	R	R	S	S	S	IMD	IMD	IMD	IMD	S	S
30	HVc10	R	R	R	R	S	S	S	IMD	IMD	IMD	IMD	S	S
31	HVc11	R	R	R	R	S	S	S	IMD	S	IMD	IMD	S	S
32	HVc12	R	R	R	R	S	S	S	IMD	IMD	R	IMD	S	S
33	HVc13	IMD	R	R	R	S	S	S	IMD	R	R	IMD	S	S
34	HVc14	R	R	R	R	S	S	S	IMD	R	R	R	S	S
35	HVc15	R	R	R	R	S	S	S	IMD	IMD	S	IMD	S	S
36	HVc16	R	R	R	R	S	S	S	IMD	IMD	IMD	IMD	S	S
37	HVc39	R	R	R	R	S	S	S	IMD	IMD	IMD	IMD	S	S
38	HVc40	R	R	R	R	S	S	S	IMD	R	IMD	IMD	S	S
39	HVc41	R	R	R	R	S	S	S	IMD	R	IMD	IMD	S	S
40	HVc42	R	R	R	R	S	S	S	IMD	R	R	IMD	S	S
41	HVc43	R	R	R	R	S	S	S	IMD	IMD	IMD	IMD	S	S
42	HVcA	R	R	R	R	S	S	S	IMD	S	IMD	IMD	S	S
43	HVcB	R	R	R	R	S	S	S	IMD	S	IMD	IMD	S	S
44	HVcC	R	R	R	R	S	S	S	IMD	IMD	IMD	IMD	S	S
45	HVc44	R	R	R	R	S	S	S	IMD	S	IMD	IMD	S	S
46	HVcD	R	R	R	R	S	S	S	IMD	R	IMD	IMD	S	S
47	HVc38	R	R	R	R	S	S	S	R	R	R	IMD	S	S
48	HVc17	R	R	R	R	S	S	S	IMD	R	R	IMD	S	S

Table 5.8: Overall distribution of AST results

Antibiotics used	Resistant		Intermediate		Sensitive		CLSI zone size guideline(mm)
	No.	%	No.	%	No.	%	
Ampicillin	43	89.6	4	8.3	1	2.1	R-<13, S- ≥17
Furazolidone	48	100	0	0.0	0	0.0	R- ≤18, S- ≥18
Nalidixic acid	48	100	0	0.0	0	0.0	R- ≤13, S- ≥19
Cotrimoxazole	48	100	0	0.0	0	0.0	R- ≤10, S- ≥16
Tetracycline	17	35.4	0	0.0	31	64.6	R- ≤14, S- ≥19
Amikacin	0	0.0	0	0.0	48	100	R-≤13, S- ≥ 16
Gentamicin	0	0.0	0	0.0	48	100	R-≤14, S- ≥ 17
Erythromycin	1	2.1	27	56.2	20	41.7	R-≤ 13, S≥ 23
Ciprofloxacin	27	56.3	17	35.4	4	8.3	R- ≤17, S ≥21
Ofloxacin	29	60.4	17	35.4	2	4.2	R- ≤17, S ≥21
Chloramphenicol	1	2.1	27	56.2	20	41.7	R- ≤12, S- ≥18
Ceftriaxone	0	0.0	0	0.0	48	100	R- ≤14, S-≥23
Cefotaxime	0	0.0	0	0.0	48	100	R- ≤13, S- ≥21

5.5.2 Minimum inhibitory concentration (MIC) test

MIC test was performed for five different antibiotics, Nalidixic acid, Ampicillin, Tetracycline, Chloramphenicol and Ciprofloxacin.

Results of MIC test showed that 100% strains were resistant to Nalidixic acid. 89.6% strains were resistant to Ampicillin. 35.4% strains were resistant to Tetracycline while 41.7% intermediate and 22.9% were sensitive. All Tetracycline resistant strains were isolated in outbreak samples. 97.9% strains were sensitive to Chloramphenicol and 2.1% intermediate. 35.4% strains were sensitive to Ciprofloxacin, 60.4% were intermediate and 4.2 % were resistant. 4.2% *Vibrio cholerae* strains isolated from outbreak samples were resistant to Ciprofloxacin and remaining were intermediate while 11(22.9%) strains were intermediate and remaining (17 strains) were sensitive in case of *Vibrio cholerae* isolated from the hospital sample.

Table 5.9: MIC results for all isolated strains of *Vibrio cholerae*

S.N.	Sample code	Nalidixic acid(mg/L)	Ampicillin (mg/L)	Tetracycline (mg/L)	Chloramphenicol (mg/L)	Ciprofloxacin (mg/L)
1	OVc18	64	16	8	0.5	0.25
2	OVc19	64	16	8	0.5	0.25
3	OVc20	32	16	8	0.5	0.25
4	OVc21	32	16	8	0.5	1
5	OVc22	32	16	8	0.5	0.5
6	OVc37	32	16	8	0.5	0.25
7	OVc23	32	16	8	0.5	0.5
8	OVc24	32	8	8	0.5	0.25
9	OVc25	32	16	8	0.5	0.25
10	OVc26	32	16	8	0.5	0.25
11	OVc27	32	16	8	0.5	0.25
12	OVc28	32	16	8	0.5	0.25
13	OVc29	32	16	8	0.5	0.25
14	OVc30	32	16	8	0.5	0.25
15	OVc31	32	16	8	0.5	0.25
16	OVc32	64	16	2	0.5	0.25
17	OVc33	64	16	2	0.5	0.25
18	OVc34	64	16	8	0.5	2
19	OVc35	64	16	2	0.5	0.5
20	OVc36	64	8	8	0.5	0.5
21	HVc1	64	8	2	2	0.25
22	HVc2	64	16	2	2	0.25
23	HVc3	64	16	2	2	0.25
24	HVc4	64	16	2	2	0.125
25	HVc5	64	16	2	2	0.125
26	HVc6	32	16	2	2	0.125
27	HVc7	32	16	2	2	0.125
28	HVc8	32	16	2	2	0.125
29	HVc9	32	8	2	2	0.125
30	HVc10	32	16	2	2	0.125
31	HVc11	32	16	2	2	0.125
32	HVc12	32	16	1	2	0.125
33	HVc13	32	8	1	2	0.25
34	HVc14	32	16	1	4	0.25
35	HVc15	32	16	1	2	0.125
36	HVc16	32	16	1	2	0.125
37	HVc39	64	16	1	2	0.125
38	HVc40	64	16	1	2	0.25
39	HVc41	32	16	4	2	0.25
40	HVc42	32	16	4	2	0.25
41	HVc43	32	16	1	2	0.125
42	HVcA	32	16	1	2	0.125
43	HVcB	32	16	4	2	0.125
44	HVcC	32	16	4	2	0.125
45	HVc44	32	16	4	2	0.125
46	HVcD	32	16	4	2	0.25
47	HVc38	64	16	1	2	0.25
48	HVc17	64	16	1	2	0.25

Table 5.10: Overall MIC results of the isolates

Antibiotics	Resistant		Intermediate		Sensitive		CLSI guideline (mg/L)
	No.	%	No.	%	No.	%	
Nalidixic acid	48	100	0	0.0	0	0.0	S- ≤ 2, R- ≥ 16
Ampicillin	43	89.6	5	10.4	0	0.0	S- ≤ 2, R- ≥ 16
Tetracycline	17	35.4	20	41.7	11	22.9	S- ≤ 1, R- ≥ 8
Chloramphenicol	0	0.0	1	2.1	47	97.9	S- ≤ 1, R- ≥8
Ciprofloxacin	2	4.1	29	60.4	17	35.4	S- ≤ 0.125, R- ≥ 1

5.5.3: Comparison between AST by Kirby Bauer disc diffusion method and MIC test

From MIC test and AST by Kirby Bauer disc diffusion method 100% resistance observed against Nalidixic acid. 89.6% strains were resistance against Ampicillin as result observed in both methods (AST by Kirby Bauer disc diffusion method and MIC test). One strain which was sensitive during AST by Kirby Bauer disc diffusion method was found intermediate in MIC test for Ampicillin. From both methods, 35.4% strains were found resistant against Tetracycline. But 21 strains (43.8%) which were sensitive during AST by Kirby Bauer disc diffusion method was found intermediate during MIC test and only 10 strains (20.8%) were sensitive against Tetracycline. In case of Chloramphenicol, 1(2.1%) strain which was resistant during AST by Kirby Bauer disc diffusion method was found intermediate and all remaining strains were found sensitive during MIC test. For Ciprofloxacin, 27(56.2%) strains were found resistant during AST by Kirby Bauer disc diffusion method. Out of this only 2(4.2%) strains were found sensitive during MIC test. 17 (35.4%) strains were intermediate and 4(8.4%) strains were sensitive during AST by Kirby Bauer disc diffusion method but 17 (35.4%) strains were found sensitive and 29 (60.4%) strains were found intermediate in MIC test. All the strains isolated from outbreak samples were either resistant or intermediate.

Table 5.11: Comparison of results obtained between AST by Kirby Bauer disc diffusion method and MIC test by microtitre broth dilution method

Antibiotics used	Results	Resistant		Intermediate		Sensitive	
		No.	%	No.	%	No.	%
Nalidixic acid	AST	48	100	0	0.0	0	0
	MIC	48	100	0	0.0	0	0
Ampicillin	AST	43	89.6	4	8.3	1	2.1
	MIC	43	89.6	5	10.4	0	0
Tetracycline	AST	17	35.4	0	0.0	31	64.6
	MIC	17	35.4	20	41.7	11	22.9
Chloramphenicol	AST	1	2.1	27	56.2	20	41.7
	MIC	0	0.0	1	2.1	47	97.9
Ciprofloxacin	AST	27	56.2	17	35.4	4	8.4
	MIC	2	4.1	29	60.4	17	35.4

Table 5.12: Comparison between results of AST and MIC test for resistance strains

Antibiotics used	R strains from AST	R strains from MIC	Matching percentage
Nalidixic acid	48	48	100
Ampicillin	43	43	100
Tetracycline	17	17	100
Chloramphenicol	1	0	0.0
Ciprofloxacin	27	2	7.4

5.5.4 Mean MIC value for the isolated strains

The mean MIC value for Nalidixic acid was 40.3 mg/L, Ampicillin was 14.9 mg/L, Tetracycline was 3.2 mg/L, Chloramphenicol was 1.2 mg/L and Ciprofloxacin was 0.23 mg/L.

Table 5.13: MIC range of the isolates in percentage and mean MIC value of total isolated strains of *Vibrio cholerae* O1 Ogawa El Tor

Antibiotic used	Concentrations of antibiotics (mg/L) and percentage of strains											Geometric mean MIC value
	128	64	32	16	8	4	2	1	0.5	0.25	0.125	
NA	0.0	33.3	66.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	42.67
AMP	0.0	0.0	0.0	89.6	10.4	0.0	0.0	0.0	0.0	0.0	0.0	15.17
TET	0.0	0.0	0.0	0.0	35.4	12.5	29.2	22.9	0.0	0.0	0.0	4.16
CHLO	0.0	0.0	0.0	0.0	0.0	2.1	56.2	0.0	41.7	0.0	0.0	1.42
CIP	0.0	0.0	0.0	0.0	0.0	0.0	2.1	2.1	8.3	52.1	35.4	0.28

(NA-Nalidixic acid, AMP- Ampicillin, TET- Tetracycline, CHLO- Chloramphenicol, CIP- Ciprofloxacin)

16 (33.3%) strains had given MIC value of 64 mg/L and 32 (66.6%) strains had given 32 mg/L for Nalidixic acid. For Ampicillin, 43 (89.6%) strains had given MIC value of 16 mg/L and remaining 5 (10.4%) strains had given 8 mg/L. Against Tetracycline, 17 (35.4%) strains had given MIC value of 8 mg/L, 6 (12.5%) strains given 4 mg/L, 14 (29.2%) given 2 mg/L and 11 (22.9%) given 1 mg/L. In case of chloramphenicol, only one strain (2.1%) had given MIC value of 4 mg/L, 27 (56.2%) given 2 mg/L and remaining 20 (41.7%) strains given MIC value of 0.5 mg/L. Against Ciprofloxacin, 1 strain (2.1%) had given MIC value of 2 mg/L, 1 strain (2.1%) given 1 mg/L, 4 strain (8.3%) given 0.5 mg/L, 25 strain (52.1%) given MIC value of 0.25 mg/L and 17 (35.4%) strains given the MIC value 0.125 mg/L.

5.5.5 Multiple drug resistant strains

All 48 strains isolated were multiple drugs resistant. All strains were resistant against at least 3 different types of drugs. 97.9% strains were resistant to 4 different types of drugs, 68.8% were resistant against 5 different types of drugs, 52.1% strains were resistant against 6 different types of drugs and 33.3 % drugs were resistant to 7 different types of drugs.

Table 5.14: Distribution of Resistant strains against the drugs

No. of antibiotics	No. of R strains	% of R strains
Two Drugs	48	100
Three drugs	48	100
Four drugs	47	97.9
Five drugs	33	68.8
Six drugs	25	52.1
Seven drugs	16	33.3

5.5.6 Types of antibiotic resistant patterns: R-type distribution

On the basis of resistant pattern observed on antibiogram test by Kirby Bauer disc diffusion method, 13 different resistant patterns were observed. These R-types were classified on the basis of sensitivity pattern of Ampicillin, Tetracycline, Ciprofloxacin, Ofloxacin, Erythromycin and Chloramphenicol. Beside these antibiotics, all of the isolates were resistant to Nalidixic acid, Furazolidone, Cotrimoxazole and sensitive to Amikacin, Gentamicin, Ceftriaxone and Cefotaxime. All the partial sensitive strain also considered sensitive during classification of R-type strains. On the basis of R-type distribution, 12 outbreak strains were RRRRSS type and 14 hospital strains were RSSSSS type. One strain was totally sensitive to all of six drugs considered during R-type classification.

Table 5.15: No. of different R-Types isolated (On the basis of AST by disc diffusion test)

S.N.	A	T	Cip	Ofx	E	C	Sample code	Total	%
1	R	R	R	R	S	S	OVc 19,20,21,22,23,25,26,28,29,30,31,34	12	25
2	R	S	R	R	R	S	HVc 38	1	2.1
3	R	S	R	R	S	S	OVc 32,33,35 HVc 2,3,17,42	7	14.5
4	R	R	S	S	S	S	OVc 27	1	2.1
5	R	R	S	R	S	S	OVc 18,37	2	4.2
6	R	S	S	R	S	S	HVc 4,12	2	4.2
7	R	S	S	S	S	S	HVc 5,6,7,8,10,11,15,16,39,43,A,B,C,44	14	29.1
8	R	S	R	R	S	R	HVc 14	1	2.1
9	R	S	R	S	S	S	HVc 40,42,D	3	6.2
10	S	R	S	R	S	S	OVc 24	1	2.1
11	S	R	R	R	S	S	OVc 36	1	2.1
12	S	S	R	R	S	S	HVc 1,13	2	4.2
13	S	S	S	S	S	S	HVc9	1	2.1
							Total	48	100

CHAPTER-VI

6. DISCUSSION AND CONCLUSION

6.1 DISCUSSION

died every year due to acute diarrheal diseases people 40,000-In Nepal annually 30,000 and cholera is major problem among them. The burden is high in mid and far western region of Nepal. In 1994, mortality rate was higher in far eastern region (29.2/100000). .(2005 ,.ses to 2.79% in 2001 (Maharjan et alHowever case fatality rate is decrea ,Worldwide It is estimated that 3.5 million people affected from cholera and causes 100,000-130,000 deaths in a year as of 2010 (WHO, 2010).

In this study, 240 samples were processed for detection of *Vibrio cholerae*. Among them 44 samples were taken from Nepalgunj outbreak and 196 were the samples admitted to different hospitals. 48 cases were found to be positive (20 in outbreak samples and 28 in hospital samples) resulting sample prevalence 20%. This is similar to the findings of Ono et al., 2001 and Karki et al., 2010. All the strains isolated were *Vibrio cholerae* serogroup O1 serotype Ogawa and biotype El Tor. Ogawa is predominant serotype of *Vibrio cholerae* O1 associated with epidemics in Nepal in recent years. *Vibrio cholerae* O1 serotype Ogawa biotype El Tor was the only serotype isolated during the outbreak in Kavre in 2004 (Tamang et al., 2005), only Ogawa serotype was isolated during 2008 in Kathmandu valley (Karki et al., 2010) and unpublished reports of NPHL confirmed that only serotype Ogawa of *Vibrio cholera* O1 biotype El Tor was responsible in epidemics in mid and far western Nepal in 2009.

In Nepal incidence of diarrheal diseases rises sharply each year during warm summer months. The lack of proper water or use of contaminated water is a major cause of diarrhea among inhabitants of Kathmandu valley (Ono et al., 2001). Due to lack of proper resources and treated water, people in rural area suffer similar fate (Kelly, 1990). In every monsoon season, Nepal suffers outbreak of diarrheal diseases and cholera is important cause of acute diarrheal disease (Pokhrel et al., 1996 and Rajkarnikar, 2004). In this study, all the strains were isolated during the monsoon period. 72% of cholera agent was

isolated in August in this study. In Nepal the cholera cases appear at peak during the June, July and August which is related with extensive contamination of water resources with sewages and fecal materials. In 2007, from June first to July 25, 305 cases of cholera had been reported with 5 deaths which were coincided with 72% deterioration of the water quality in Kathmandu valley at that time. The quality of water was 28% unsatisfactory during the month of May (EHA Letter WHO, 2007). The outbreak of cholera in Nepalgunj was also related to the contamination of water sources (EDCD Report, 2010).

During the year 2005 and 2006 *Vibrio cholerae* O1 serotype Inaba was the predominant strain responsible for cholera epidemics in the Kathmandu valley and surrounding places (NPHL Report, 2008). All the strains of *Vibrio cholerae* O1 isolated at Kathmandu were belonging to serotype Inaba in 2005 (Shrestha et al., 2008). Hikojima strain was not frequently isolated in recent epidemics. It was reported that Hikojima was major strain isolated from cholera cases during the year 1996 in Kathmandu (Pokhrel et al., 1996).1% Hikojima strains were isolated during the year 2007 in Kathmandu (NPHL Report, 2010). Classical strains were responsible for epidemic of cholera before the evolution of El Tor variety. Since 1992, only El Tor biotypes were isolated from the epidemic of Nepal (Yamamoto et al., 1995, Pokharel et al., 1996, Kubo et al., 1997, Bista 2001, Rajkarnikar 2004, Shrestha et al., 2008 and Karki et al., 2010).

Outside Nepal, cholera outbreak in east Delhi during the year 2007 to 2009 was predominated by the *Vibrio cholerae* O1 serotype Ogawa (Das et al., 2011). Cholera outbreaks in late 2007 to early 2008 in northern Vietnam were associated with serotype Ogawa (Nguyen et al., 2009). Cholera outbreak in Kelantan Malaysia in late 2009 was also associated with serotype Ogawa (Ang et al., 2010). During this study period in October 2010 cholera outbreak was occurred in Haiti which was also related to the serotype Ogawa biotype El Tor. According to CDC the strains isolated in Haiti outbreak was matched with the south Asian cholera O1 strains and belong to serotype Ogawa. This showed that frequent outbreak often associated to Ogawa serotype in recent years in Nepal as well as other countries.

Serotype conversion is a frequent mechanism between *Vibrio cholera* O1 Ogawa and Inaba serotypes (Stroeher et al., 1992, Lipp et al., 2002, Ramamurthy et al., 2004 and Dutta et al., 2006), which determines the dominant serotype in different outbreaks of cholera. In Nepal serotype Ogawa was predominant until 2004 and overridden by Inaba serotype in 2005-06 and again Ogawa was predominant (NPHL Report, 2007). In Bangladesh during the year 2005 and 2006 the major strains isolated from the cholera outbreaks was predominantly of Inaba serotype; however Ogawa strain predominates in later years. In northern India major strain isolated during the year 2002 to 2004 was serotype Ogawa which was overridden by the Inaba serotype in July 2004 onwards (Taneja et al., 2005). The *rbfT* gene (now *wbeT* gene) was responsible for serotype conversion. The gene *wbeT* which was found in Inaba strain after experiment in *E. coli* K-12 strain, some mutation leads to the change in the character of the organism and serotype changes. *rbfT* gene not involve in the O antigen synthesis but is required for determining the Ogawa serotype specificity. It is not the B-antigenic determinant itself but presumably is an O antigen modifying enzyme. Inaba strains are effectively *wbeT* mutants and presumably have arisen as a result of selection due to immune response during a cholera infection. Thus serotype conversion during the epidemics is simply a mutant enrichment procedure with antibodies to the Ogawa specificity providing the selection. Since Inaba strains are *wbeT* mutants, the reciprocal serotype conversion from Inaba to Ogawa requires the reversion of original mutation which cannot be detected in vitro. Moreover G+C content showed that *wbeT* had been acquired via horizontal gene transfer (Stroeher et al., 1992). The frequency of conversion of Ogawa to Inaba is approximately 10^{-5} where as from Inaba to Ogawa was rare and may be strain dependent (Garg et al., 2000).

In this study, out of 48 *Vibrio cholerae* strain isolated, 18 were isolated from male and 30 were from female patients. There was no significance difference between cholera cases in between the two genders ($P>0.05$). The finding was similar to Ono et al., 2001 and Karki et al., 2010.

Age wise distribution of cholera cases showed that the cases were high in the age group 0-20 (50.0%) and followed by 20-50 (41.7%). High number of cases in the age group 0-

20 may be due to productive age group as these group are involved in numerous daily activities so have greater chances of exposing to the disease distribution sources. The high number of cases in 20-50 age group also suggested that other persons were also exposed to the disease agent indicating that the outbreak started from the common sources and all were equally exposed. However, low incidence at the age group 50-above also suggested that these group are not active than other groups and hence exposed to the agent in less number than others. There was no significant difference in occurrence of cholerae cases and the age group ($P>0.05$). During this study, extensive data about patient's socioeconomic condition couldn't be taken, which could have further explore the cause of this result. Although in the outbreak areas, there wasn't good water supply and sanitation was also bad which were the major reason behind the outbreak as in the case of other places.

In Nepal rainy season starts from early June and last for the September. The arrival of monsoon brings the no. of acute diarrheal diseases and cholera is a major diarrheal disease among them. It is because of the poor quality of drinking water due to contamination with sewage and floods etc. the majority of cholera cases appear from May to October. No cholera cases detected from December to May in Nepal (Pokhrel et al., 1996). In this study, cholera cases were detected in June, July, August, September and November. 72% cholera cases were detected in August, 14.6% in September, 10.4% in July and 2.1% in November. No cases were detected in October. The highest cholera cases were detected in August during 2009 in outbreaks of cholera in western part of the country (NPHL Report, 2009). Shrestha et al., 2008 also reported that majority of cholera cases appeared in August during the year 2005. However the frequency of cholera cases fluctuates during the monsoon months. During three year study of cholera cases in Nepal from 1958 to 1960, peak cholera cases were detected during the month August followed by October and June in 1958, in July during the year 1959 and 1960 (Abou-Gareeb, 1961). The frequency ranges from months to months as suggested by Maharjan et al., 2007, Pokharel et al., 2005 and Karki et al., 2010).

In other countries, cholera cases also appear during the hot summer months due to the deterioration of the water quality. In Zimbabwe, between August 2008 to February 2009;

70640 cholera cases were detected with 3467 deaths (Bhattacharya et al., 2009). In Vietnam 3 cholera outbreaks were occurred from November 2007 to February 2008 (Nguyen et al., 2009). Das et al., 2011 also reported that peak cholera cases appeared during the monsoon period in Delhi. The high no. of cholera cases were associated with the poor water quality due to contamination of water sources, rainfall and flooding in central America, Africa and south east Asia from 1995-2005 (Griffith et al., 2006).

In Bangladesh, outbreak usually occurs twice in a year with highest no. of cases arises just after monsoon and some cases during spring. It has been known that occurrence of epidemics coincide with the increased number of cholera agent in aquatic environment. Phage predation of *Vibrio cholerae* has been reported to be a factor that influences the temporal dynamics of cholera epidemics in Bangladesh (Faruque et al., 2005).

In aquatic environment, *Vibrio cholerae* proliferates rapidly at the temperature above 20º C but survives poorly below 10º C (Madicoel et al., 1996). *Vibrio cholerae* undergo viable but non culturable form (VBNC) and exists in association with other aquatic organism during inter epidemic period. The no. of *Vibrio cholerae* increases in aquatic system with the increased population of phytoplankton and zooplanktons. The peak population appears in two cycles, one cycle during February to April and next during August to September. The timing correlates with very well incidence of diarrhea and cholera cases (Kiorbe et al., 1994). So the environ mental factors also play the major role in cholera epidemics (Colwell, 1996). Now cholera epidemic can be predicted in coastal area by monitoring the parameters in aquatic environments (Colwell, 1996). In endemic areas, the cholera cases tends to show seasonal trends which is related to the ecology of *Vibrio cholerae* in the environment where high no. is observed during the times of warmer water temperatures and phytoplankton and zooplankton blooms (Colwell, 1996, Lipp et al., 2002).

In this study, antibiotic susceptibility test (AST) was performed by Kirby Bauer disc diffusion method for 13 different antibiotics. All the different isolates were found sensitive to Amikacin, Gentamicin, Ceftraixone and Cefotaxime. 64.6% strains were sensitive to Tetracycline and remaining 35.4% were resistant and all were from

Nepalgunj outbreak, only 41.7% strains were sensitive, 54.1% were intermediate and 4.2% were resistant to Erythromycin. For Ciprofloxacin and Ofloxacin 56.3% and 60.4% strains were resistant respectively while 35.4% strains were intermediate for both and remarkably just 8.3% strains were sensitive to Ciprofloxacin and 4.2% strains were sensitive to Ofloxacin. Ofloxacin is thought to be more effective drug than Ciprofloxacin but it's not the case here. 41.7% strains were sensitive, 56.2% were intermediate and just one strain (2.1%) was resistant for Chloramphenicol. 89.6% strains were resistant, 8.3% were intermediate and only one strain (2.1%) was sensitive to Ampicillin. All the isolated strains were completely resistant to Nalidixic acid, Cotrimoxazole and Furazolidone.

During the outbreak in western part of the country in the summer of 2009, all the *Vibrio cholerae* O1 Ogawa strains were sensitive to Tetracycline, Ampicillin while total resistance against Nalidixic acid, Furazolidone and Cotrimoxazole (NPHL Report, 2009). Similar findings were reported during *Vibrio cholerae* O1 Ogawa strains isolated in 2008 in Kathmandu (Karki et al., 2010). All the *Vibrio cholerae* O1 Ogawa strains were sensitive to Tetracycline during outbreak in Kathmandu 2004 (Maharjan et al., 2007). In an outbreak of cholera in Kavre during 2004; all the isolated *Vibrio cholerae* O1 Ogawa strains were sensitive to Ampicillin, Chloramphenicol, Tetracycline, Doxycycline and Ciprofloxacin and total resistant against Cotrimoxazole (Tamang et al., 2005). All the isolated *Vibrio cholerae* O1 Inaba strains showed total resistance against Nalidixic acid and Cotrimoxazole, 84.9% strains were resistant to Furazolidone while total sensitivity towards Tetracycline, Ampicillin and Erythromycin (Shrestha et al., 2008)). In this study, resistance emerges against Tetracycline, Ciprofloxacin and Ofloxacin in large amount. Antimicrobial surveillance from 2002-2004 for isolated enteropathogens in Nepal suggested that all the isolated *Vibrio cholerae* O1 Ogawa strains were sensitive to Tetracycline, Ciprofloxacin during three year period, total resistance was seen against Furazolidone and Cotrimoxazole in 2004 and 2.24% resistance was reported against Erythromycin (Kansakar et al., 2011). Cotrimoxazole, Furazolidone and Nalidixic acid were totally resistant in this study. Nalidixic acid resistance was seen consistently throughout the years and it looks that *Vibrio cholerae* gains inherent resistance against it. However before 2002 it was effective drug and widely used for treatment of cholera but fluoroquinolones are effective and they are used now as recommended by WHO.

On the basis of all previous reports and findings, to my knowledge Tetracycline resistance is seen for the first time in Nepal during this study. Very high percentage of Ciprofloxacin and Ofloxacin resistance were seen during this study which was never reported during previous study in Nepal suggesting that Ciprofloxacin and Ofloxacin resistance begin to appear from the year 2010. Until 2008, all the isolated *Vibrio cholerae* O1 were sensitive to Ampicillin and only 17.54% resistance was reported in 2008 by Karki et al., 2010. In this study massive increase in resistance against Ampicillin was seen, about 89.6% resistance was observed. In 2009 outbreak in western Nepal about 83% isolates were sensitive (NPHL Report, 2009). Although only 2.1% and 4.2% resistance were observed for Chloramphenicol and Erythromycin majority of strains showed intermediate sensitivity (56.2% for Chloramphenicol and 54.1% for Erythromycin) suggesting that these drugs are also becoming less useful for the treatment purpose. Only Amikacin, Gentamicin, Ceftraixone and Cefotaxime showed good sensitivity results (100% sensitivity) and it can be said that these drugs are more effective for the treatment purpose.

The increasing amount of resistance against the commonly used drugs raised the concern. Even though antibiotic treatment is not a first choice for the treatment of cholera, the emergence of multiple drug resistant cases might create problem during antibiotic therapy. Further more such resistance mechanism is transferable and transfer of resistance gene from *Vibrio cholerae* to other enteric pathogens could lead to the unexpected havoc to the community and the health workers due to increasing resistance. In this study 16 (33.33%) strains of *Vibrio cholerae* O1 was found resistant against 7 drugs out of 13 drugs tested, all the isolates were resistant against at least 3 drugs, 97.9% strains were resistant against at least 4 drugs,68.4% strains were resistant against at least 5 drugs and 52% strains were resistant against at least 6 drugs.

The multidrug resistant strains detected by disc diffusion method were further checked for the minimum inhibitory concentration (MIC) level of some selected antibiotics. The MIC determination is gold standard method compared to disc diffusion method although it is not used routinely in lab (Collee et al., 1996). Five different antibiotics were selected and MIC level was tested by microtitre broth dilution method and results were

interpretated as per the guideline given by CLSI. Microtitre broth dilution method for MIC detection is very easy to perform, so it was used during this study. All resistant strains detected by disc diffusion test to Ampicillin were found resistant with mean MIC level 14.9 mg/L. Nalidixic acid MIC level was very high (40.3 mg/L) and all strains were resistant. All the resistant strains from disc diffusion test were found also resistant by MIC test for Tetracycline with MIC level ≥8 mg/L. However only one strain that was resistant during disc susceptibility test for Chloramphenicol was intermediate during MIC test. Out of 56.2% resistant strains for Ciprofloxacin from disc diffusion test, only two strains were resistant as their MIC value exceeded >0.5 mg/L and remaining were intermediate. The MIC test is sensitive method and hence some of the strains detected resistant during disc diffusion susceptibility method were detected intermediate during MIC determination for Ciprofloxacin. Furthermore, qualitative sensitivity method such as disc diffusion method can't detect the reduced susceptibility of the isolates as suggested by Islam et al., 2009. Although they appeared intermediate (during MIC test) against Ciprofloxacin, it still cannot be given to the patient in usual prescribed dose.

64.6% (31) strains were detected sensitive for Tetracycline by disc diffusion method but MIC determination showed that 20 strains (41.7%) were actually intermediate (mean MIC value was 3.2 mg/L) and it signified that the importance of MIC test because the strains were sensitive during disc diffusion test but they turn out to be intermediate after MIC test. Qualitative test like disc diffusion method failed to detect reduced susceptibility of the organism, also suggested by Islam et al., 2009. So the usual drug regimen prescribed for therapy may not work effectively for treatment purpose. This value was interpretated according to the CLSI guideline whose breakpoint value is ≥8 mg/L. But according to the European Committee of Antimicrobial Susceptibility Test (EUCAST), their breakpoint value for tetracycline is ≥2 mg/L and the mean MIC value here 3.2 mg/L which already passed EUCAST value and considered resistant. Out of 48 strains, only 11(22.9%) strains would be sensitive and remaining (77.1%) would be resistant according to EUCAST guideline. But EUCAST guideline was not used in this study.

In case of Ciprofloxacin, 56.2% strains were resistant detected from AST by disc diffusion method. MIC test detected that only 4.2% strain was resistant and remaining were intermediate. It suggests the sensitivity of MIC test but it still signify the drug amount required for therapeutic purpose because the strains were intermediate, either the dose should be increase or different drug should be prescribed for such cases. The qualitative sensitivity method such as disc diffusion method can't detect the reduced susceptibility of the isolates (Islam et al., 2009), which may be the reason that the isolates were detected intermediate (in MIC test) which was resistant during AST by disc diffusion method. The mean MIC value for total isolated strains was 0.23 mg /L which is high MIC level on average. Before this study, MIC test was not performed for *Vibrio cholerae* in Nepal and hence no value was available for comparison purpose. However mean MIC value for isolated *Vibrio cholerae* O1 strains in Bangladesh was 0.25 mg/L in 2006 which was more than the previous year suggesting the increasing resistance of Ciprofloxacin (Kareem et al., 2006). Islam et al., 2009 reported that Ciprofloxacin mean MIC value for the isolate *Vibrio cholerae* O1 strains was 0.5 mg/L in Zimbabwe.

Although such amount of resistant cases were not reported from Nepal in previous year, multiple drug resistant cholera vibrios were reported vastly in neighbour India and Bangladesh. In Kolkata India, a study from 2004- 2009 showed that Tetracycline resistant cases were arise in the year 2007 onwards which was usually sensitive in previous years. The strains were also resistant to Nalidixic acid, Furazolidone and Cotrimoxazole. The MIC level was unusually high for isolate strain which was >16 mg/L (in our study it was ≥8mg/L) however the pattern was somewhat subsided (less than 50%) in the year 2009 (Bhattacharya et al., 2011). In Bangladesh, cholera agents isolated were resistant against Tetracycline, Ciprofloxacin, Erythromycin, Furazolidone and Cotrimoxazole in 2004-2005 (Faruque et al., 2007). The MIC level for Ciprofloxacin was also increases than the previous years signified that ciprofloxacin was in appropriate for cholera case management in Dhaka (Faruque et al., 2007). The gradual increase in MIC level for Ciprofloxacin was associated with resistant plasmid in Bangladesh (Kim et al., 2010).

There are wide array of mechanism that leads to the development of resistance to antibiotics in bacteria. Antibiotics used in agriculture and diverse prevention or growth promotion in animals have been ascribed to be one of the most underlying factors for development of resistance. The first clinically significant resistant cholerae epidemic was reported during 1964-1965 in Philippines where *Vibrio cholerae* strains were resistant against the no. of drugs (Kubahara et al., 1967 and Kobari et al., 1970). The resistance was mediated by R plasmid in these cases and later it was also confirmed in outbreak in Tanzania (Mhalu et al., 1979) and in Bangladesh (Glass et al., 1980). Even though the mechanism was same, the pattern of resistance was different leading to the strong hypothesis that it was dependent on the pattern of drug use and selective pressure for those multiple resistance.

With the increase use of extended group of antibiotics, resistance phenomenon began to increase in different places. The resistance pattern was largely depending on region, pattern of antibiotic used and point in time. Extensive use of drug at particular time results the quick resistance. In Nepal, Cotrimoxazole, Furazolidone and Nalidixic acid was used extensively during 1990-2000 which led to the emergence of resistance against these drugs. It was shown in Calcutta, resistance to Trimrthoprim-Sulfomethoxazole (TMP-SXT) emerged quickly over the course of year during which it was heavily used and this was added by explosion of resistance to Nalidixic acid when it became first line drug (Jesudasan and Saaya, 1997). In Bangladesh during 1979, the rate of resistance to certain antibiotics were rapidly increased concomitant with their use but then declined without any change in antibiotics use patterns (Glass et al., 1983). This leads to the finding that multiple genes were responsible for antibiotic resistances which were present in conjugative R plasmid. Beside these, a mobile genetic element SXT, integrones, beta lactamase, RND efflux systems and integrative and conjugative elements (ICEs) are also responsible for development of resistance in *Vibrio cholerae* (Ghosh and Ramamurthy, 2011).

The most common resistance mechanism against β-lactam antibiotics is the production of β-lactamase enzymes which hydrolyze the drug and render it inactive. Most of the plasmid encode resistance is manifested by one beta lactamase enzyme TEM-1 and there

are more than 20 other beta lactamases. TEM-ase has been reported in *Vibrio cholerae* and currently IncC plasmid that encode novel beta lactamase SAR-1 that are able to hydrolyze Carbenicillin and Penicillin G (Reid et al., 1986). CTX-M-2 type, PER-2 type and TEM-1 type ESBL enzymes were produced by *Vibrio cholerae* O1 isolated in Argentina (Petroni et al., 2002).

Resistance to erythromycin is conferred by modification of methylation of 23S rRNA by an erythromycin ribosyl transferase (Erm). These modifications reduce the affinity of macrolides such as erythromycin and Clindamycin class.Fluoroquinolone resistance is achieved by bacteria by mutation in genes encoding DNA gyrase and topoisomerase IV (coded *gyrA* and *parC* gene respectively). Nalidixic acid resistance is achieved by mutation in *gyrA* gene. *qnrVC3* gene which is responsible for protection of topoisomerase IV enzyme also confers low level of quinolone and fluoroquinolone resistance along the year, it was identified in mobile genetic SXT element of multiple drug resistance strain of *Vibrio cholerae* in Bangladesh (Kim et al., 2010).

The SXT element is 62 Kb self transmissible integrating and conjugative element (ICE) which carries the genes resistance for sulfomethoxazole, trimethoprim, streptomycin and Furazolidone as well as chloramphenicol (Burrus et al., 2006). Resistance against Furazolidone was carried out by SXT element in the MDR strain isolated during the year 1990-2004 in Zambia (Mwansa et al., 2006). SXT elements contain tandem SXT arrays and promote the formation of novel ICEs (Burrus et al., 2004). The ICEs formations depend upon conjugative transfer and independent of recA.

Many drug resistant genes are harboured by the plasmid called resistant (R) plasmid. A 200Kb self transmissible plasmid was detected in India which mediated resistance to Tetracycline, Ampicillin, Chloramphenicol, Kanamycin, Gentamicin, sulfomethoxazole and trimethoprim (Yamamoto et al., 1995). *Vibrio cholerae* O1 isolated from the patient in Uganda possessed 130 M-Da plasmid of incompatibility group 6-C that conferred resistance to Trimethoprim (by *dfr*I gene), Sulfonamides (*su*II gene), Tetracycline (*tet*C gene), Chloramphenicol (c*at*I gene), Ampicillin (mediated by other β-lactamase gene from bla^{TEM} and $bla^{SHV)}$ and Septinomycin (Krus et al., 1995). The plasmid pMRV150

increasingly found in the *Vibrio cholerae* O139 in Hangzhou china from 1994 onwards was found to be similar to the plasmid pIP1202, an IncC plasmid detected in an MDR *Yesinia pestis* isolated from bubonic plague patient in Madagascar (Pan et al., 2006).

Frequent drug resistance in bacteria is also mediated by integrones. Class-1 integrones are found widely among the clinical isolate of pathogenic bacteria. In Zambia, the MDR strain of *Vibrio cholerae* O1 isolated which was resistant to Tetracycline found to harbouring i*nt1I* gene in class I integron (Mwansa et al., 2006). Much of the SXT-TMP resistance was mediated by class 1 integron with the gene *ant 3"-1a* responsible for resistance in isolated *Vibrio cholerae* O1 strain in Vietnam (Dalsgraad et al., 1996).The MDR *Vibrio cholerae* O1 strains isolated in Accra Ghana during 2006, SXT-TMP resistance was mediated by both SXT element and class 2 integron bearing *dfrA1, sat* and *aadA1* gene cassettes. This was only one evidence that class2 integron responsible for drug resistance in African region (Opintan et al., 2008).

OmpU mediated efflux system of β-lactam antibiotic is also impervious to the presence of bile in the external solution where as permeability through ompT is reduced by bile in a concentration dependent manner (Simonet et al., 2003). Three genes out of six RND efflux system genes i.e. *vexAB, vexCD* or *vexEf* together with *tolC* of *Vibrio cholerae* NCTC 4716 elevated MIC level of various antibiotics when tested in susceptible *E. coli* (Borges-Walmsley et al., 2005).

It is hard fact that antibiotic resistance is growing phenomenon whereas rate of discovery of new antibiotic has been low (Varaldo et al., 2002). Fluoroquinolones are not commonly used to treat diarrheal disease because they are expensive. Tetracycline is the most effective drug for treatment of cholera and Cotrimoxazole also comes behind Tetracycline (Tjaniadi at al., 2003) which is widely used to treat other infection such as opportunistic infection in HIV patient as well. Cotrimoxazole is one of the widely used drugs in Nepal and it is best along with Furazolidone to treat children (Sabeena et al., 2001). The high rate of incidence of resistance to Cotrimoxazole and Furazolidone is also the alarming factor that these drugs cannot be used any more.

Although serotype conversion is frequent phenomenon, it was found that there was no difference in susceptibility patterns between two serotypes (Das et al., 2011). *Vibrio cholerae* has permanent habitat in aquatic environment and during the inter-epidemic period, their survival in aquatic environment and growth allow the dissipation of resistance to other serotype and other organisms and could bring another serious epidemics. Therefore there is constant need for the evaluation of such environments.

On the basis of antibiogram pattern, there was 13 different resistotypes were observed, which may be arises from different clones. Such resistotypes could contain different resistance genes responsible for drug resistance as mentioned above. The resistant pattern was different from place to places for e.g. Tetracycline, indicating their different source of emergence in outbreak area in Nepalgunj. This could be further analyzed by molecular study. Due to lack of resources, it couldn't be done in this study.

6.2 CONCLUSION

Cholera accounts for large proportion of acute diarrheal disease in Nepal. This study showed that strains isolated from samples of cholera outbreak in Nepalgunj and other hospital samples related to *Vibrio cholera* O1 serotype Ogawa and biotype El Tor. The isolated cholera strains are multidrug resistant and the strains are resistant to Nalidixic acid, Furazolidone, Cotrimoxazole, Ampicillin, Ciprofloxacin, Ofloxacin and Tetracycline in large amount. Only Gentamicin, Amikacin, Ceftraixone and Cefotaxime are the most effective drugs with Erythromycin and Chloramphenicol have moderate success. Emergence of Tetracycline resistance in such large amount is first time observed in Nepal and associated with outbreak strains. Emergence of resistance to such large amounts of antibiotics could create serious problem as the resistance phenomenon are transferable. The high MIC level for Ciprofloxacin and Tetracycline also suggests that drugs are increasingly become ineffective to combat with the pathogens or resistance phenomenon increasing with the time. So it should be noted in time and further strategy should be planned to avoid undesirable problem to the health sector.

VII-CHAPTER

7. SUMMARY AND RECOMMENDATION

7.1 SUMMARY

1. A total of 240 samples were processed, among them 44 samples were taken from Nepalgunj outbreak and 196 were taken from hospitals. 48 cases were found positive for cholera resulting prevalence of 20%.
2. Only *Vibrio cholera* O1 biotype El Tor serotype Ogawa was isolated during the study.
3. 37.5% cases of cholera was found in male and 62.5% cases were found in female patients and there was no association between cholera cases and gender (P>0.05).
4. Highest cases were found in age group 0-20 (50.0%) followed by age group 20-50 (41.7%). However there was no significant difference in occurrence between cholera cases and specific age group (P>0.05).
5. Cholera incidence was found higher in August (72%).
6. Antibiotic susceptibility test by Kirby Bauer disc diffusion method showed that 100% resistant to Nalidixic acid, Cotrimoxazole and Furazolidone, 86.9% strains were resistant to Ampicillin,56.4% resistant to Ciprofloxacin, 60.4% resistant to Ofloxacin and 35.4% resistant to Tetracycline. 100% strains were sensitive to Cefotaxime, Ceftriaxone, Amikacin and Gentamicin.
7. MIC result of the isolates showed that 100% resistant to the Nalidixic acid, which were also detected resistant by disc diffusion test.
8. 89.6% resistant strains (43 out of 48) tested by Kirby Bauer disc diffusion method for Ampicillin, all strains were also detected resistant during MIC test. Remaining 5 strains were found intermediate. 1 strain which was sensitive during AST by Kirby Bauer disc diffusion method was detected intermediate.
9. None of the strains were found resistant to Chloramphenicol as only one (2.1 %) strain was detect resistant during Kirby Bauer disc diffusion method. Only one strain was detected intermediate which was resistant during AST by Kirby Bauer disc diffusion method and remaining 47 strains were sensitive in MIC test.

10. For Tetracycline, all the resistant strains 35.4 % were isolated from outbreak samples. Out of remaining 64.6% sensitive strains, 41.7% strains (20 out of 31 strains) were actually turnout to be intermediate during MIC test.
11. For Ciprofloxacin, only 4.2% strains were detected resistant after MIC test out of 56.4% (27) resistant strains detected by disc diffusion test. The remaining 25 strains were detected intermediate during MIC test.
12. The mean MIC value for Nalidixic acid was 40.3 mg/L, Ampicillin was 14.9 mg/L, Tetracycline was 3.2 mg/L, Chloramphenicol was 1.2 mg/L and Ciprofloxacin was 0.23 mg/L.
13. 13 different R-type strains were detected on the basis of antibiogram pattern.

RECOMMENDATIONS 7.2

1. All of the strains were isolated in monsoon period with the contamination of water sources and lack of sanitation. So improvement of water quality and sanitation are suggested during this period.
2. Many drugs were found resistant towards the isolated strains of *Vibrio cholerae* and MIC value was also found high for most of the drugs. So determination of MIC value is also suggested during each epidemic to monitor the situation.
3. 13 different R-type of strains were found and were resistant to seven different drugs out of 13 tested. Further study of resistance mechanism is suggested in molecular level.
4. Widely used drugs were gradually become ineffective. So drug therapy system should be monitored to limit the emergence of resistance.
5. Since the *Vibrio cholerae* lives in aquatic environments and naturally interconvert serotype and acquire resistant genes while remaining in water. So study of aquatic environment for the presence of *Vibrio cholerae* and monitoring their situation in Nepal is strongly recommended.

CHAPTER VIII

8. REFERENCES

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APPENDIX I

Clinical and microbiological profile of the patient

Name of the patient: …… Date: …….

Age: …… Sex: ……

Specimen type: ……

Lab No.: …….

Clinical History: ……

Microbiological Investigation: ……..

Day One

1. Collection of the sample
2. Microscopy
3. Culture of specimen on MA, APW and TCBS agar and again culture on MA and TCBS agar from APW after 6 hour of incubation.

Day 2

1. Observation of the primary culture plate and enrichment media if left.
2. Gram staining of the desired colony
3. Subculture of the colony on the nutrient agar
4. Biochemical test

Test performed	Result
……	……..

Day 3

1. Observation of secondary plates
2. Results of biochemical test

Test performed	Result
…….	……..

3. Serotyping

Organism	antisera used
……	…….

4. Biotyping
5. Antibiotic susceptibility testing

Day 4

1. Results of Biotyping

Test performed	Result
⁾ VP test	……
⁾ Sheep Blood Hemolysis Test	…….
⁾ Polymyxin B sensitivity test	…….

2. Results of antibiotic susceptibility testing

S.N.	Antibiotic used	Result
1	Ampicillin	
2	Ciprofloxacin	
3	Ofloxacin	
4	Nalidixic acid	
5	Chloramphenicol	
6	Ceftriaxone	
7	Cefotaxime	
8	Erythromycin	
9	Amikacin	
10	Gentamicin	
11	Furazolidone	
12	Cotrimoxazole	
13	Tetracycline	
	Polymyxin B	

APPENDIX II

Equipments and Materials used during the study

Equipments

Autoclave: Sternite, Japan

Centrifuge: Heltich, Japan

Distillation plant: India

Refrigerator: Sanyo Japan, LG Korea

Hot Air Oven: Memmert, Germany

Incubator: Sakura, Japan

Water bath: Boekel 148003, Japan

Weighing balance: Choyo MP, Japan

Microscope: Olympus, Japan

Media used

Alkaline Peptone Water

Thio sulfate sucrose bile salt agar

MacConkey agar

Nutrient agar and nutrient broth

Nutrient broth

Mueller Hinton agar and Mueller Hinton broth

MR-VP medium

Simon citrate agar

Urea broth

Triple sugar iron agar

Sulfide indole motility agar

The entire medium used was from

1. Mast Company Limited UK
2. Oxoid Unipath Limited Hampshire UK

Reagents

NaCl	3% H_2O_2
Crystal violate	Paraffin oil
Gram's iodine	Normal saline
Absolute alcohol	Barritt's reagent
Safranin	Kovac's reagent

All of these reagents were brought from the local suppliers.

Antibiotic discs

Ampicillin (30 µg)

Chloramphenicol (30 µg)

Ciprofloxacin (5 µg)

Ofloxacin (5 µg)

Nalidixic acid (30 µg)

Ceftriaxone (30 µg)

Cefotaxime (30 µg)

Tetracycline (30 µg)

Furazolidone (100 µg)

Cotrimoxazole (25 µg)

Erythromycin (15 µg)

Amikacin (15 µg)

Gentamicin (15 µg)

Polymyxin B (300 U)

These entire antibiotic discs used were brought from Mast Co. Ltd. U.K.

Antibiotic powder

Ampicillin

Ciprofloxacin

Chloramphenicol

Naldixic acid

Tetracycline

All of these powder from High media private limited, India.

Microtitre broth plates: These were brought from Grinliner Company.

APPENDIX III

A. Composition and preparation of different culture media

All the culture media used were from the Mast co. ltd. U.K.

1. **Nutrient agar**

Composition	grams/litre
Peptic digest of animal tissue	5.00
Beef extract	1.50
Yeast extract	1.50
Sodium chloride	5.00
Agar	15.00
Final pH at 25ºC	7.4±0.2

28 gram of medium was suspended in 1000 ml of the distilled water and boiled to dissolve completely. Then medium was autoclaved at 121 ºC (15lbs pressure) for 15 min. the sterilized medium was then poured in to sterilized petridishes and then was allowed to cool.

2. **Blood agar base (Infusion agar)**

Composition	gm/ltr
Beef heart infusion form	500
Tryptose	10.00
Sodium chloride	5.00
Agar	15.00
Final pH at 25ºC	7.3±0.2

42.5 gram of the medium was suspended in 1000 ml of distilled water, dissolved by boiling and sterilized by autoclaved at 121ºC for 15 mins. After cooling to about 50-55 ºC, 5% v/v defibrinated sheep blood was added aseptically, then mixed with gentle rotation and poured in to sterilized petridishes and was allowed to cool.

3. MacConkey agar

Composition	gm/ltr
Peptic digest of animal tissue	17.00
Proteose peptone	3.00
Lactose	10.00
Bile salt	1.50
Sodium chloride	5.00
Neutral red	0.03
Agar	15.00
Final pH at 25ºC	7.1±0.2

51.3 gram of the medium was dissolved in 1000 ml of distilled water and then boiled to dissolve completely. The media was autoclaved at 121ºC for 15 mins. Sterilized medium was then poured in to sterile petridishes and was allowed to cool.

4. Nutrient broth (NB)

Composition	gm/ltr
Peptic digest of animal tissue	5.00
Sodium chloride	5.00
Beef extract	1.50
Yeast extract	1.50
Final pH at 25ºC	7.4±0.2

13 gram of the medium was dissolved in 1000ml of water and then boiled to dissolve completely. The medium was then dispensed in to the tubes about 3ml in each and autoclaved at 121ºC for 15 minutes. The sterilized medium was then cooled to room temperature.

5. Mueller Hinton agar (MHA)

Composition	gm/ltr
Beef Infusion	300.00
Casein Acid Hydrolysate	17.50
Starch	1.50
Agar	17.00
Final pH at 25ºC	7.3±0.2

38 gram of the medium was dissolved in 1000ml of distilled water and then boil to dissolve completely. The medium was autoclaved at 121ºC for 15 mins. The sterilized medium was then poured in sterilized petridishes and was allowed to cool.

6. Alkaline Peptone Water (APW)

Composition	gm/ltr
Peptone	10.00
Sodium chloride	10.00
Final pH at 25ºC	8.5±0.2

20 gram of alkaline peptone powder was dissolved in 1000 ml of distilled water and dispensed in to the tubes about 10-15 ml and the tubes were autoclaved at 121ºC for 15 minutes. Then the tubes were cooled to room temperature and stored.

7. Thio sulfate citrate bile salt sucrose agar (TCBS)

Composition	gm/ltr
Yeast extract	5.00
Peptone	10.00
Sodium thiosulfate	10.00
Sodium citrate	10.00
Ox gall	8.00

Sucrose	20.00
Sodium chloride	10.00
Ferric citrate	1.00
Bromothymol blue	0.04
Thymol blue	0.04
Agar	15.00
Final pH at 25ºC	8.6±0.2

89 gram of the medium was suspended in 1000 ml of distilled water and boiled to dissolve completely. The medium was not autoclaved and distributed in to sterile petridishes.

B. Composition and preparation of different biochemical test media

1. Simon citrate agar

Composition	gm/ltr
Magnesium sulfate	0.20
Mono ammonium dihydrogen phosphate	1.00
Dipotassium phosphate	1.00
Sodium citrate	2.00
Sodium chloride	5.00
Bromothymol blue	0.08
Agar	15.00
Final pH at 25ºC	6.8±0.5

24.2 gram of the medium was dissolved in 1000 ml of distilled water and boiled to dissolved completely.3 ml of medium was dispensed in each tube and autoclaved at 121ºC for 15 minutes. The sterilized mediums are allowed to settle at slant forming position.

2. Urea agar base (Christensen urea agar)

Composition	gm/ltr
Peptic digest of animal tissues	1.00
Dextrose	1.00
Monopotassium phosphate	0.8
Dipotassiu phosphate	1.20
Sodium chloride	5.00
Agar	15.00
Phenol red	0.012
Final pH at 25ºC	6.8±0.2

24 gram of the medium was suspended in 950 ml of water and dissolved by boiling and autoclaved at 121ºC for 15 minutes. After cooling to 50 ºC, 50 ml of sterile 40% urea solution was poured in to the medium and mixed with gentle rotation. Then 5 ml of the medium was dispensed in each tube and slant was prepared.

3. Sulfide indole motility (SIM) agar

Composition	gm/ltr
Peptic digest of animal	30.00
Beef extract	3.00
Peptonized iron	0.20
Sodium thiosulfate	0.025
Agar	3.00
Final pH at 25ºC	7.3±0.2

36.23 gram of the medium was dissolved in 1000 ml of distilled water and boiled to dissolve completely. Then it was dispensed in the test tube about 4 ml and autoclaved at 121 ºC for 15 minutes. Then it was cool down.

4. MR-VP medium

Composition	gm/ltr
Buffered peptone	7.00
Dextrose	5.00
Di- potassium phosphate	5.00
Final pH at 25ºC	6.9±0.2

17 gram of medium was dissolved in 1000 ml of distilled water and boiled to dissolve completely. 3 ml of medium was dispensed in each tube and autoclaved at 121ºC for 15 minutes.

5. Triple sugar iron (TSI) agar

Composition	gm/ltr
Peptic digest of animal tissue	10.00
Casein Enzymatic Hydrolysate	10.00
Yeast extract	3.00
Beef extract	3.00
Lactose	10.00
Sucrose	10.00
Dextrose	1.00
Sodium chloride	5.00
Ferrous sulphate	0.20
Sodium thiosulfate	0.30
Agar	12.00
Phenol red	0.024
Final pH at 25ºC	7.4±0.2

65 gram of the medium was dissolved in 1000ml of distilled water and dissolved completely. Then it was dispensed in to the tubes and autoclaved at 121ºC for 15 minutes. The sterilized medium in the test tube was then allowed to set in slant with a butt of 1inch thickness.

6. Preparation of decarboxylation broth

Composition	gm/liter
Yeast extract	5.00
Dextrose	1.00
Bromocresol purple	0.02
Final pH at 25ºC	6.8±0.2

Dissolve 14 gram of decarboxylation base powder in 1000 ml and 5 gram of required amino acid was added to it and boiled to dissolve. Then the broth was dispensed in each tube about 5 ml and autoclaved at 121 ºC for 15 minutes. Then it was allowed to cool down and stored at 2-8 ºC until use.

C. Composition and preparation of different staining reagent

1. Gram stain

a. Crystal violet solution

Crystal violet	20.00
Ammonium oxalate	9.00
Ethanol or Methanol	95.00ml
Distilled water	1000ml

Preparation: 20 grams of crystal violet was weighed in a clean piece of paper and transferred to a clean brown bottle. Then 95 ml of ethanol was added and mixed until the dye is completely dissolved. To the mixture, 9 grams of ammonium oxalate dissolved in 200ml of distilled water was added. Finally the volume was made 1000ml by addition of distilled water.

b. Lugol's Iodine

Potassium iodide	20 gm
Iodine	10gm
Distilled water	1000 ml

Preparation: to 250 ml of distilled water, 20 gm of potassium iodide was dissolved and 10 gm of iodine was mixed to it until it was dissolved completely. Finally the volume was made 1000mlby addition of distilled water.

c. Acetone alcohol decolorizer

Acetone	500ml
Ethanol (Absolute)	475ml
Distilled water	25ml

Preparation: 475 ml of ethanol was added to 25 ml of distilled water and mixed and kept in a clean bottle. Then immediately 500ml of acetone was added to the bottle and mixed well.

d. Safranin (Counter stain)

Safranin (2.5% in 95% ethanol)	10.00 ml
Distilled water	100 ml

Preparation: 2,5% of Safranin solution was prepared in 95% ethanol and 10 ml of prepared suspension was mixed in 100 ml of distilled water.

2. Normal saline

Sodium chloride	0.85gm
Distilled water	100ml

Preparation: o.85 gram of sodium chloride was weighed and added to a bottle containing 100ml of distilled water and mixed well to dissolve the salt completely and autoclaved. Then it was stored.

3. Biochemical Test Reagents

a. For catalase test

Catalase reagent (3% H_2O_2)

Hydrogen peroxide	1ml
Distilled water	9ml

Preparation: To the 9ml of distilled water, 1ml of hydrogen peroxide was added and mixed well so as to make 3% solution of hydrogen peroxide.

b. For oxidase test

Oxidase strip soaked in oxidase reagent

Tetra methyl para-phenylene diamine dihydrochloride(TPD)	1gm
Distilled water	100ml

Preparation: 1 gram of TPD was dissolved in 100 ml of distilled water and strips of Whatmann no. 1 paper was soaked and drained for anout 30seconds. Then the strip was freeze dried and stored in dark bottle tightly.

c. For indole test

Kovac's indole reagent

Para Dimethyl amino benzaldehyde	2.00gm
Isoamyl alcohol	30.00ml
Concentrated hydrochloric acid	10.00ml

Preparation: in 30 ml of isoamyl alcohol, 2 gram of para amino bemzaldehyde was dissolved and transferred to clean brown bottle. Then to this solution,10 ml of concentrated hydrochloric acid was added and mixed well.

d. For methyl red test

Methyl red solution

Methyl red	0.05gm
Ethyl alcohol	28.0ml
Distilled water	22.0ml

Preparation: 0.05 gm of methyl red was dissolved in 28 ml of ethanol and transferred to a clean brown bottle. To this, 22 ml of distilled water was added and mixed well.

e. For Voges Proskauer test

Barritt's reagent

Solution A

Alpha-Napthol	5.0gm
Ethyl alcohol	100ml

Preparation: 5gm of α-Napthol was dissolved in 25 ml ethanol and transferred in to clean bottle. Then final volume was made 100ml by adding ethanol.

Solution B

Potassium hydroxide (KOH)	40.0gm
Distilled water	100ml

Preparation: 40 gram of KOH was dissolved in 25 ml of distilled water and transferred in to the clean bottle and final volume was made 100ml by adding distilled water.

4. Turbidity standard equivalent to McFarland 0.5

1% V/V solution of sulphuric acid was prepared by adding 1ml of concentrated sulphuric acid to 99 ml of distilled water. 1% W/V solution of barium chloride was prepared by dissolving 0.5 gram of dehydrate barium chloride in 50 ml of distilled water. Then to the 99.5ml of 1% sulphuric acid solution, 0.5 ml of barium chloride solution was mixed and stirred continuously. Then the solution was transferred in to the clean screw capped tube and stored at dark place until use. The test tube for the broth preparation should be of same size as of McFarland tube. The tubes can be stored and used for six months.

APPENDIX IV

A. Procedure for gram staining (Forbes et al., 2007)

Gram staining is differential staining that differentiates all the bacterial species in to two large groups: gram positive and gram negative. Following steps are involved during gram staining.

1. A thin film of material to be examined was prepared and dried.
2. The material on the slide was heat fixed and allowed to cool before staining.
3. The slide was flooded with crystal violet stain and allowed to remain without drying for 10-30 second.
4. The slide was rinsed with tap water, shaking off excess.
5. Then the slide was flooded with iodine solution and allowed to remain on the surface without drying for twice as long as the crystal violet was in contact with the slide surface.
6. The slide was rinsed with tap water, shaking off excess.
7. The slide was flooded with acetone alcohol decolorizer for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolorizer. Thicker smear require more aggressive decolorization.
8. The slide was flooded with counter stain Safranin for 30 seconds and washed off with tap water.
9. The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 100X.

B. Procedure for Antibiotic Sensitivity Testing (AST) by Disc Diffusion Method.

In the treatment and control of infectious disease, AST is done to select effective antimicrobial drugs against suspected organisms. Disc diffusion method is widely used technique for susceptibility testing and done by Kirby Bauer disc diffusion method.

The following steps are involved in AST by Kirby Bauer disc diffusion method.

1. An isolated colony of organism was suspended in the nutrient broth and incubated at 37ºC for 4 hours. The turbidity was matched with 0.5 McFarland turbidity standards.
2. A sterile cotton swab was taken and introduced in to the tube taken out the organism and swabbed uniformly on the surface of Mueller Hinton agar medium.
3. The plate was allowed to dry and antibiotic disc were placed on the agar surface and incubated for 18-24 hours.

4. After incubation the zone size was measured and results were interpreted according to the standard guidelines.

C. Minimum inhibitory concentration (MIC) test

The stock solution of antibiotics was prepared by using following formula.

$$1000/ P \times V \times C = W$$

Where P= potency of given antibiotic.

V= volume required in ml.

C= final concentration of antibiotic.

W= weight of antibiotics to be dissolved in V ml.

Procedure of MIC test by microtitre broth dilution method

1. From the stock solution, the antibiotic solution was diluted to 2X strength required at a final concentration at column one of the plate. (For e.g. If final concentration is 128 mg/L then the dilution should be made up of 256 mg/L).
2. A sterile microtitre plate was taken. By using multipipette, 100µL of cation adjusted Mueller Hinton broth was placed in each column of the plate except column 12. Then the plate was labeled.
3. 100µL of 2X strength of antibiotic solution was placed in column 1 of the microtitre plate and was mixed by sucking up and down for 6-8 times. Care had taken, not to splash.
4. 100 µL of solution was withdrawn from column 1 and transferred to column two of the microtitre plate and mixed well. The antibiotic solution was diluted by two fold from column 1 to column 2.similar process was continued up to column 10.
5. From column 10, 100 µL of the solution was discarded rather than putting it to column 11.
6. A bacterial culture suspension was prepared by incubation of the culture in Mueller Hinton broth and the turbidity was matched wit0.5 McFarland turbidity standard.
7. 5 µL of the test solution of organism was placed from tube 11 to 1 with the help of micropipette.
8. The microtitre plate was covered with ELISA tape and incubated at 37ºC for 24-48 hours.
9. After incubation, the MIC was observed. The lowest dilution at which no growth was seen was regarded as MIC for the given antibiotic to that organism.
10. *E. coli* ATCC 25922 culture was taken as a standard reference strain during each experimental step.

APPENDIX V

ANTISERA

Vibrio cholerae immune sera SEIKEN

These are the antisera used for the serotyping of *Vibrio cholerae* O1 and each contains specific antibodies. The antisera are prepared by hyper immunizing rabbits with the standard Inaba type and Ogawa type strains. For immunization, the strains are pure cultured and suspended with saline and inactivated by heating at 100ºC for 2-3 hours. After bleeding, the antisera are separated, heated at 56 ºC for 30 minutes. Non specific agglutinins are removed and sterilized by ant bacterial filtration. As a preservative, sodium azide is added by 0.1 w/v %.

Products

Set: 4 vials (Polyvalent O1, Inaba, Ogawa and Hikojima type monovalent antisera)

Denka Seiken Co. Ltd.

3-4-2 Nihonbashikayaba-Cho, Chuo-Ku

Tokyo, Japan

Serotyping method

1. One drop of polyvalent O1 antisera was dropped on the clean glass slide. Then *Vibrio cholerae* colony was picked by inoculating loop and mixed with antisera. Then it was observed for the agglutination. If agglutination appears, then further testing with monovalent antisera was done.
2. Serotyping with monovalent antisera Ogawa, Inaba and Hikojima were done to screen out the serotype of the O1 Vibrio cholerae. The agglutination with the respective antiserum identify that the strain related to that serotype.
3. For non agglutinating Vibrio with polyvalent O1 antiserum, further serotyping with O139 antiserum should be done.

APPENDIX VI

Interpretation charts for antibiotics

A. Zone size interpretative chart (CLSI interpretation)

Antibiotic used	Concentration (µg)	Diameter of zone size(mm)			ATCC culture *E.coli* 25922 target zone size (mm)
		Resistant	intermediate	Sensitive	
Ampicillin	10	≤ 13	14-16	≥ 17	16-22
Ciprofloxacin	5	≤ 17	18-20	≥ 21	30-40
Ofloxacin	5	≤ 17	18-20	≥ 21	29-33
Nalidixic acid	30	≤ 13	14-18	≥ 19	22-28
Chloramphenicol	30	≤ 12	13-17	≥ 18	21-27
Ceftriaxone	30	≤ 13	14-20	≥ 21	29-35
Cefotaxime	30	≤ 14	15-22	≥ 23	29-35
Erythromycin	15	≤ 13	14-22	≥ 23	22-30
Amikacin	15	≤ 13	14-15	≥ 16	19-26
Gentamicin	15	≤ 14	15-16	≥ 17	19-26
Furazolidone	100	≤ 18		≥ 18	22-26
Cotrimoxazole	25	≤ 10	11-15	≥ 16	24-32
Tetracycline	30	≤ 14	13-18	≥ 19	18-25
Polymyxin B	300	≤ 11		≥ 11	13-19

B. Zone size interpretative chart (EUCAST interpretation)

Antibiotic used	Concentration (µg)	Diameter of zone size(mm)			ATCC culture *E.coli* 25922 target zone size (mm)
		Resistant	intermediate	Sensitive	
Ampicillin	10	≤ 14			16-22
Ciprofloxacin	5	≤ 19	20-21	≥ 22	30-40
Ofloxacin	5	≤ 19	20-21	≥ 22	29-33
Nalidixic acid	30				
Chloramphenicol	30	≤ 17		≥ 17	21-27
Ceftriaxone	30	≤ 20	21-22	≥ 23	29-35
Cefotaxime	30	≤ 18	19-20	≥ 21	29-35
Erythromycin	15				
Amikacin	15	≤ 13	14-15	≥ 16	19-26
Gentamicin	15	≤ 14	15-16	≥ 17	19-26
Furazolidone	100				22-26
Cotrimoxazole	25	≤ 13	11-15	≥ 16	24-32
Tetracycline	30				
Polymyxin B	300				

C. MIC value interpretation chart

Antibiotics	CLSI MIC value			EUCAST MIC value		
	Resistant	Intermediate	Sensitive	Resistant	Intermediate	Sensitive
Ampicillin	≥ 16	4-8	≤ 2	≥ 8	4	≤ 2
Ciprofloxacin	≥ 1	0.25-0.5	≤ 0.125	≥ 1		≤ 0.5
Chloramphenicol	≥ 8	2-4	≤ 1	≥ 8		≤ 8
Nalidixic acid	≥ 16	4-8	≤ 2			
Tetracycline	≥ 8	2-4	≤ 1	≥ 2		≤ 1

(Note: CLSI- Clinical Laboratory Standard Institute, EUCAST- European Committee on Antimicrobial Susceptibility Testing).

APPENDIX VII

STATISTICS

CHI- SQUARE TEST

Hypothesis

Null hypothesis H_o: There is no significant difference in occurrence between cholera cases and gender of the patients in outbreak and hospital samples.

Alternative hypothesis H_1: There is significant difference in occurrence between cholera cases and gender of the patients in outbreak and hospital samples.

Test statistics

Samples	Male	Female	Total
Outbreak	9	11	20
Hospital	9	19	28
Total	18	30	48

χ^2 calculated value = $N (ad-bc)^2 / (a+b) (a+c) (b+d) (c+d)$

= 0.822

Critical value

χ^2 tabulated value at 1 degree of freedom at 95% confidence level is 3.62.

Result

The χ^2 tabulated value is greater than calculate value, so H_o is accepted.

The P value when χ^2 calculated value is 0.822 is 0.3646 which is >0.05. Hence by conventional statistics, there is no significant difference in occurrence between cholera cases and gender of the patients.

Similarly P value is calculated for association between cholera cases and age group of the patients. The P value is 0.2563 which is > 0.05. Hence there is no significant difference in occurrence of cholera cases and age group of the patients.