MEDICALLY IMPORTANT VIBRIOS FROM DIFFERENT ENVIRONMENTAL WATER SAMPLES FROM EASTERN NEPAL

A Dissertation Submitted to the Sunsari Technical College, Tribhuwan University, Dharan, Nepal, in Partial Fulfillment of the Requirements for the award of Degree of Master of Science in Microbiology (Medical)

By

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RECOMMENDATION

This is to certify that Mrs. JyotiDev has completed this dissertation work entitled **"MEDICALLY IMPORTANT VIBRIOS FROM DIFFERENT ENVIRONMENTALWATER SAMPLES FROM EASTERN NEPAL"** as a partial fulfillment of the requirements of M. Sc. Degree in Microbiology (medical) under our supervision. To our knowledge, this thesis work is an original research work of his and not been submitted for any other degree.

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CERTIFICATE OF APPROVAL

On the recommendation of Mr. **Hemanta Khanal** and Ms. **Saluna Bhandari,** this dissertation work of Mrs. **Jyoti Dev** entitled **"MEDICALLY IMPORTANT VIBRIOS FROM DIFFERENT ENVIRONMENTAL WATER SAMPLES FROM EASTERN NEPAL"**has been approved for the examination and is submitted to the Sunsari Technical College in partial fulfillment of the requirements for the degree of Master of Science in Microbiology (Medical).

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ABSTRACT

Safe drinking water, as defined by the guidelines, does not represent any significant risk to health over a lifetime of consumption. The most important bacterial diseases transmitted through water are cholera, typhoid fever and bacillary dysentery, focusing on the biology and ecology of the causal agents and on the diseases' characteristics and their life cycles in the environment. Diarrhoea, which is caused by poor sanitation, hygiene and water quality, is one of the most prevalent water borne disease in Nepal. This study was performed from June 2016 to November 2016. The aim of research was to evaluate significant of *vibrio* in environmental water in eastern regions of Nepal (especially around Dharan and Ithari). Altogether 60 water samples were collected and were incubated in alkaline peptone water and cultured in TCBS agar.Identification of the significant isolates was done by standard microbiological techniques and antibiotic susceptibility testing was done by Kirby Bauer disc diffusion method.The seven different species of *vibrios* isolated were *V. cholerae* (22.2%), *V. parahaemolyticus* (19.5%), *V. furnissii* (16.7%), *V. vulnificus* (16.7%), *V. fluvialis* (13.9%), *V.alginolyticus* (5.5%) and *V. metschnikovii* (5.5%). *Vibrios* isolates were 100% sensitive to Amikacin, Azithromycin, Ceftriaxone, Chloramphenicol, Doxycyclin, Imipenem, Levofloxacin, Norfloxacin and were varying degrees of resistance to Ampicillin, Ceftazidime, Cefotaxime, Ciprofloxacin, CoTrimoxazole, Erythromycin, Gentamicin, Nalidixicacid, Nitrofurantoin, Ofloxacin and Tetracycline. Among the isolates, *Vibrio Cholerae* was found to be higher percentage. Therefore, monitoring of *V. cholerae* in the environmental water samples in the interim period between cholera epidemics is of great significance for early warning of an outbreak, as well as prevention and control of cholera epidemics when they occur.

Keywords: Drinking water, TCBS agar, Alkaline peptone water, *V. Cholerae*, Cholera

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LIST OF ABBREVIATIONS

ATCC:	American Type Culture Collection
CDC:	Centers for Disease Control
CBS:	Central Bureau of Statistics
CPS:	Capsular Polysaccharide
CLSI:	Clinical and Laboratory Standards Institute
CAMP:	Cyclic Adenosine Monophosphatee
DoHS:	Department of Health Services
NPHL:	National Public Health Laboratory
NWSC:	Nepal Water Supply Corporation
OCHA:	United Nations Office for the Coordination of Humanitarian Affairs
SPSS:	Statistical Package for Social Sciences
TCBS:	Thiosulphate citrate bile salts sucrose
TCP:	Toxin Coregulating Pilus
TDH:	Thermostable Direct Hemolysin
TRH:	TDH Related Hemolysin
UNEP:	United Nations Environment Programme
UNICEF:	United Nations International Children's Emergency Fund.
WHO:	World Health Organization
WUSC:	Water Users and Sanitation Committee

CHAPTER I

1 INTRODUCTION AND OBJECTIVES

1.1 BACKGROUND

Water is essential to life. An adequate, safe and accessible supply must be available to all. Improving access to safe drinking-water can result in significant benefits to health. Every effort should be made to achieve a drinking water quality as safe as possible (WHO 2008). It forms 50% to 60% of our body weight and plays an active role in all vital processes of our body (Abed et al 2007). About 2 liters per day for a 60 kg person and 1 liter per day for a 10 kg child is essential (Bhandari et al 2009). On a global scale, there have been countless numbers of disease outbreaks and poisoning around the world resulting from the consumption of untreated or poorly treated drinking water (Mihdhdir 2009).

The world health organization (WHO) estimated that up to 80% of all sickness and diseases in the world are caused due to inadequate sanitation, polluted water or unavailability of water (Prasanna et al 2009). WHO has estimated 3–5 millions cases and 100,000–120,000 deaths are due to cholera. Gastroenteritis (associated with diarrhoea) is one of the major health problems in Nepal causing high morbidity (30,000 death/year) and mortality of 3.3 episodes per child. (WHO Fact Sheet 2014).

In Nepal, outbreaks of cholera occur each year with the beginning of summer rainy season continues to post rainy season (Pokhrel et al 1996; Bhandari et al 2009) and mainly associated with *V. cholerae* 01 biotype El Tor Ogawa. (Yamamoto et al 1995; NPHL 2004-2007). Diarrhoea, which is caused by poor sanitation, hygiene and water quality, is one of the most prevalent water borne disease in Nepal. During 1995/96, the incidence of diarrhoea among children below five years of age was 131 per 1,000 children. The mortality rate due to the diarrhoea was 0.34 per 1000 children under five years of age, while the case of fatality rate was 2.56 per 1,000 (CBS 2001).

In Jajarkot district of Nepal, large cholera outbreaks occurred in 2009 that took place for nearly three weeks, in which 3,000 people were affected and more than 80 were dead (WHO 2010). Similarly, in Nepalgunj, another outbreak affected more than 1500 people resulting deaths of eight people in late July and August of the year 2010 (OCHA 2010).

The most important pathogenic bacteria transmitted by the water route are *Salmonella Typhi*, the organism causing typhoid fever, and *Vibrio cholerae*, the organism causing cholera (Madigan et al 1997). Cholera caused by toxigenic *V. cholerae* O1 and O139 Bengal strain outbreak is common in most of the developing countries. (Janda et al 1988, Nair et al 1994). In Portugal, during the cholera epidemic of 1974, bottled mineral water was identified as one of the vehicles of transmission of *Vibrio Cholerae* (Blake et al 1977).

According to a recent United Nations International Children's Emergency Fund (UNICEF) report, about 800 million people in Asia and Africa are living without access to safe drinking water. Consequently, this has caused many people to suffer from various diseases. But protection of water sources and treatment of water supplies have greatly reduced the incidence of these diseases in developed countries (Sadeghi et al 2007). The *vibrios* was first named and described by Pacini in 1854 and 32 years later Koch isolated the organism (Koneman et al 2010).

Vibrios are Gram negative, curved shaped rods which are facultative anaerobes, oxidase positive (the only exception is *V. metschnikovii*). They are halophilic, mesophilic and chemoorganotrophic in nature and are widespread in marine and estuarine environments, very rarely found as freshwater inhabitants (Baumann et al 1984). They are actively motile by means of a single polar flagellum and the movement is of "darting type". In stained mucous flakes of cholera stool, the *vibrios* are arranged in parallel rows, which have been described by Koch as the 'fish- in-stream' appearance (Chakraborty 2003). *Vibrio* infections are generally acquired either through ingestion of foods and water contaminated with human faeces or sewage, raw fish and seafood, or they are associated with the exposure of skin lesions, such as cuts,

open wounds and abrasions, to aquatic environments and marine animals (Lee & Younger 2002).

Vibrios produce many extracellular enzymes including gelatinase, amylase, chitinase and Dnase (Joseph et al 1982). *Vibrios* are distinguished from enterics by being oxidase-positive and motile by means of polar flagella. *Vibrios* are distinguished from pseudomonas by being fermentative as well as oxidative in their metabolism. Among the *vibrios* that are clinically significant to humans, is *Vibrio cholerae,* the agent of cholera, is the most important *vibrio* (Bergey's manual of systematic bacteriology 2001). *Vibrios* are responsible for a number of clinical conditions such as cholera, gastroenteritis, septicaemia and wound infections (Doyle & Beuchat2007).

The species that are recovered from humans and potentially cause disease can be divided into two groups, namely *Vibrio cholerae* and the non- cholerae *vibrios*. Within the species of *V. cholerae*, there is much dissimilarity among the strains in both their pathogenic and epidemic potential. The strains can be divided according to differences in their cell wall composition, somatic O antigen, which forms the basis of the serotyping scheme that classifies the organisms into 139 different serogroup and flagellar H antigen which is common in all. Some of the non-cholera Vibrio species are *Vibrio vulnificus, Vibrio parahaemolyticus, Vibrio alginolyticus, Vibrio fluvialis, Vibrio damsela, Vibrio furnissii, Vibrio hollisae, Vibrio mimicus, Vibrio metschnikovii, Vibrio cincinnatiensis and Vibrio carchariae* (Koneman et al 2010)

Cholera, a life threatening disease whicht can spread rapidly as explosive epidemics from one region to another is characterized by profuse watery diarrhoea with flakes and mucus, dehydration and sometimes death, when adequate medical intervention is not instituted (Talkington et al 2011). Infections by *V. cholerae* can lead to epidemics, pandemics or may be endemic in specific areas. Since its discovery in 1817 there have been seven cholera pandemics. The most recent, seventh pandemic caused by *V. cholerae* 01, biotyoe EL Tor, which began in 1961 in Indonesia, rapidly spread to Asia,

Europe, Africa, and the South Pacific, and reached South and then Central America in 1991 (Koneman et al 2010).

V. cholerae alone is responsible for high morbidity and mortality (CDC 1993). This study was carried out in order to evaluate the presence of Vibrios in environmental water samples and bottled water samples and to compare the different water samples of eastern region of Nepal. Also to assess antibiotics patterns of *vibrios* and determine the prevalence of multidrug resistance patterns of the isolates. Hence, the outcome of the study will be helpful to manage the municipal water supply.

1.2 OBJECTIVES

1.2.1 General Objective

- To isolate and identify medically important *vibrios* from different environmental water samples from eastern Nepal.

1.2.2 Specific Objective

- To isolate and identify the medically important *vibrios* from different water samples.
- To study antibiotic sensitivity of the bacterial isolates.
- To compare the quality of tap water, bottled water and other sources.
- Serotyping of V. *cholerae* isolated from environmental water.

CHAPTER II

2 LITERATURE REVIEW

2.1 Water as life

Safe drinking water, as defined by the guidelines, does not represent any significant risk to health over a lifetime of consumption. Those who are generally at risk of waterborne illness may need to take additional steps to protect themselves against exposure to water borne pathogens, such as boiling their drinking water. Safe drinking water is required for all usual domestic purposes, including drinking, food preparation and personal hygiene. Improving access to safe drinking water can result in tangible benefits to health (WHO 2011). The World Health Organization in its "Guidelines for Drinking water Quality" publication highlighted at least seventeen different and major genera of bacteria that may be found in tap water which are capable of seriously affecting human health. According to the WHO, the mortality of water associated diseases exceeds 5 million people per year. From these, more that 50% are microbial intestinal infections, with cholera standing out in the first place (WHO 2008).

According to the most recent World Health Organization (WHO) estimates, 842000 diarrhoeal deaths in low and middle-income countries are caused by inadequate drinking-water, sanitation and hand washing practices (WHO 2014a). In addition, outbreaks of waterborne disease continue to occur in both developed and developing countries, leading to loss of life and significant disease and economic burden for individuals and communities. (Craun et al 2010). Diseases caused by contaminated water are among the ten most prevalent water borne diseases in Nepal (DoHS 1998). In Nepal, the incidence of diarrhoea is increasing in alarming rate. A report obtained from Teku Hospital in Kathmandu shows that 16.5 % of all deaths were due to water-borne disease (Metcalf & Eddy 2000). Probably the most important pathogenic bacteria transmitted by the water route are *Salmonella typhi*, the organism causing typhoid fever, and *Vibrio cholerae*, the organism causing cholera (Madigan et al 1997).

Vibrio cholerae serogroup O1, serotype Inaba was reported on January 6, 2015 in Kenyatta National Hospital in Nairobi, Kenya (WHO & CDC 2014). In 2010 *Vibrio parahaemolyticus* caused an outbreak of food poisoning in Maryland (Julie et al 2016). According to International Medical Corps organization People living without access to safe water and sanitation are most at risk of contracting cholera. But 80 % of cases can be treated successfully with the right treatment. The quality of water for drinking has deteriorated because of the inadequacy of treatment plants, direct discharge of untreated sewage into rivers and inefficient management of the piped water distribution system (UNEP 2001).

2.2 Access of safe drinking water

2.2.1 In Nepal

As per government estimates, as of now, 85% of the total population has access to basic water supply services and 62% has access to basic sanitation facilities (access to toilets). Approximately 40,000 water supply schemes of various types are estimated such as rural, semi-urban and urban; gravity flow, pumping and combination of both. Most of the rural and semi–urban water schemes are operated and managed by Water Users and Sanitation Committee (WUSC), a kind of community based organization formed by the consumers themselves, whereas the urban schemes are operated by a water utility such as Nepal Water Supply Corporation (NWSC), a semi-government agency (WHO 2014).

2.2.2 In Globe

According to the WHO, 71% of the global population (5.2 billion people) used a safely managed drinking-water service according to 2015 data, 89% of the global population (6.5 billion people) used at least a basic service and 884 million people lack even a basic drinking-water service, including 159 million people who are dependent on surface water. Globally, at least 2 billion people use a drinking water source contaminated with faeces. In low and middle income countries, 38% of health care facilities lack an

improved water source, 19% do not have improved sanitation, and 35% lack water and soap for hand washing (WHO 2017 fact sheet).

2.3 Water borne infection

Waterborne diseases are caused by contaminated water, which is any water source that contains pathogenic microorganisms. Among the many types of microorganisms that may cause waterborne diseases are followings (Ashley 2016).

2.3.1 Viral infection

The most important waterborn viral disease is Hepatitis caused by hepatitis virus and symptoms include fever, chills, jaundice, dark urine and abdominal discomfort. Other includes Poliomyelitis caused by polio virus, Conjunctivitis caused by adenovirus virus and Gastroenteritis caused by rota virus (Ashley 2016).

2.3.2 Protozoal infection

Amoebiasis is a common water-borne diseasecaused by protozoa*Entamoeba histolytica*.Symptoms include fatigue, diarrhoea, flatulence, abdominal discomfort and weight loss. Other protozoal disease is Cryptosporidiosis Symptoms include diarrhoea and abdominal discomfort. Most important protozoal disease is Giardiasis caused by protozoa*Giardia*.Symptoms includes diarrhoea and abdominal discomfort (Ashley 2016).

2.3.3 Bacterial infection

Most important family of bacteria that causes waterborn disease includes:

2.3.3.1 *Enterobacteriaceae*

The Enterobacteriaceae are a large family of Gram-negative bacteria such as *Salmonella, Escherichia coli, Citrobacter, Enterobacter, Serratia, Morganella, Proteus, Providencia, Yersinia pestis, Klebsiella*, and *Shigella*. Some of the diseases caused by enterobacteriaceae are shigellosis caused by bacteria *Shigella*. Symptoms include bloody stool, diarrhoea and fever and typhoid fever caused by bacteria *Salmonella*. Symptoms include fever,

headache, constipation, diarrhea, nausea, vomiting, loss of appetite and an abdominal rash (Ashley 2016).

2.3.3.2 *Campylobacteriaceae*

Campylobacter is a bacteria that causes diarrheal illness, gastroenteritis and food poisoining. *Campylobacter* is primarily associated with poultry, animals, and humans (Ashley 2016).

2.3.3.3 *Vibrionaceae*

The family *Vibrionaceae*, first described by Veron (1965), with the intent of grouping a number of non enteric, fermentative, gram negatives rods that were oxidase positive and motile by means of polar flagella (Koneman et al 2010). The families Vibrionaceae currently includes eight validly published genera and are Aliivibrio, Catenococcus, Enterovibrio, Grimontia, Listonella, Photobacterium, Salinivibrio, and Vibrio (Krieg & Holt 1984).

2.3.3.3.1 The Genus *Vibrio*

The first description of a *Vibrio* species took place in 1854, when the Italian physician Pacini discovered *V. cholerae*. They are gram-negative, oxidase-positive, rod- or curved rod shaped facultative anaerobes. Generally, the organisms are halophilic, mesophilic and chemoorganotrophic in nature (Thompson et al 2004). There are 12 species that are routinely isolated from human clinical samples, and the diseases in which they are concerned include diarrhoeal disease, gastroenteritis, septicemia and wound infections (Daniels et al 2000). *Vibrio* species are abundant in aquatic environment worldwide (Ortigosa et al 1989; Barbieri et al 1999). Among the more than 200 known *Vibrio cholera* serogroups, only the O1 and O139 strains are pathogenic, causing epidemics and pandemics throughout history (Sack et al 2004).

2.3.3.3.2 Vibrios as Human Pathogens

The genus *Vibrio* presently contains twelve species pathogenic to humans. Those of prime medical concern are *V. cholerae, V. parahaemolyticus and V. vulnificus.* Other organisms implicated as opportunistic pathogens are *V. alginolyticus, V. damsela, V. fluvialis, V. furnissii, V. hollisae, V. mimicus, V. metschnikovii, V. cincinnatiensis and V. carchariae* (Koneman et al 2010).

2.3.3.3.3 *Vibrio cholerae*

Cholera is a highly infectious disease, caused by infection of the small intestine with *V. cholerae* O1 and O139 serotypes and is characterized by massive acute diarrhoea, vomiting, and dehydration. Death occurs in severe and untreated cases. *Vibrio cholerae,* the etiological agent of cholera is wide spread in estuarine and marine waters around the world, where salinity is between 4 to 17% (Huq et al 2005). *V. cholerae* is often transmitted by water but fish or fish products in contact with contaminated water or faeces from infected persons also frequently serve as a source of infection (Colwell 1996).

Infections by *V. cholerae* can lead to epidemics, pandemics or may be endemic in specific areas.Since its discovery in 1817 there have been seven cholera pandemics. The most recent, seventh pandemic caused by *V. cholerae* 01, biotyoe EL Tor, which began in 1961 in Indonesia, rapidly spread to Asia, Europe, Africa, and the South Pacific, and reached South and then Central America in 1991 (Koneman et al 2010).

The emergence and rapid spread of cholera caused by a new serotype designated 0139 Bengal in October 1992 in nine countries (Nepal, India, Bangladesh, Pakistan, Thailand, Malaysia, Burma, Saudi Arabia and China) suggests the possibility for the beginning of the eighth pandemic (Koneman et al 2010). From 1996 to 2002, most cases of cholera in Bangladesh were caused by *V. cholera* 01 biotype EL Tor, but in March 2002, *V.cholera* 0139 re-emerged as the predominant pathogen. (Farugque et al 2003). The bacteria are carried around the world mostly by asymptomatic carriers and the most recent outbreak occurred in Haiti and it was linked to a clone in Nepal (Talkington et al 2011).

2.3.3.3.4 *Vibrio parahaemolyticus*

V. parahaemolyticus is associated with gastroenteritis manifested by profuse, watery diarrhoea free from blood and mucus, abdominal cramps, nausea, and vomiting. Outbreaks of *V. parahaemolyticus* food poisoning are associated with consumption of raw molluscs and cooked crustaceans in America and Europe, but in Japan, South East Asia, India and Africa raw fish is implicated as a vehicle for transmission (Jay et al 2005). Hospitalization is rare, unless severe fluid loss has occurred, and the illness is generally self-limiting after a few days (Huq et al 1979). A few extra intestinal infections have been reported, in particular, from wounds (Blake et al 1980) and a rare pneumonic form of *V. parahaemolyticus* infection has also been reported (Yu & Uy-Yu 1984).

V. parahaemolyticus is usually a member inhabiting coastal waters (Adams & Moss 2008), but has been recently isolated from fresh water fish (Noorlis et al 2011). Seafood which is contaminated with *V. parahaemolyticus* and not cooked sufficiently may cause diarrhoeal disease (Mihajlovic et al 1982). Since 1996, pandemic spread of *V. parahaemolyticus* infections due to single clone has been reported in several Asian countries. (Tuyet et al 2002)

2.3.3.3.5 *Vibrio vulnificus*

V. vulnificus is regarded as an emerging pathogen and infection in humans was first reported in 1964 in the USA and in 1987 in Taiwan (Harwood et al 2004; Hsueh et al 2004). It is an opportunistic pathogen in the elderly or in immune-compromised individuals with impaired liver function, or underlying disease such as cirrhosis, diabetes mellitus or those on steroid therapy (Harwood et al 2004; Drake et al 2007).

V vulnificus can also cause an infection of the skin when open wounds are exposed to warm seawater. It causes three important disease syndromes; septicaemia, necrotising wound infections and gastroenteritis with a mortality rate of 40 to 50% occurring one to two days after onset of the symptoms (Harwood et al 2004; Hsueh et al 2004; Cazorla et al 2011).

2.3.3.3.6 *Vibrio alginolyticus*

Vibrio alginolyticus is an opportunistic pathogen occasionally associated with cases of gastroenteritis and diarrhoea and causing systemic infections in immune-compromised individuals with severe burns, cancers or with a history of alcohol abuse (Oliver & Kaper 1997). In healthy individuals *V.alginolyticus* is associated with extra intestinal infections such as wound or ear infections (Novotny et al 2004). *V. alginolyticus* often occurs as an opportunistic pathogen in mixed bacterial infections of extra-intestinal wounds in man (Bonner et al 1983).

2.3.3.3.7 *Vibrio damsela* (photobacterium damsela)

Vibrio damsel was formerly called CDC Group EF-5. It has been reported as the cause of human wound infections, primarily after exposure of salt water (Koneman et al 2010) This organism is a marine pathogen associated with traumatic wound infections acquired in warm tropical and semi-tropical coastal areas (Kreger 1984).

2.3.3.3.8 *Vibrio fluvialis*

Formerly designated as CDC Group EF-6, *V. fluvialis* has been isolated from humans with diarrhoea. This species was isolated from fecal culture of more than 500 patients with diarrhoea at the Cholera Research Laboratory in Bangladesh during a 9 month period from 1976-1977 (Huq et al 1980). *V. fluvialis* is an important cause of cholera like bloody diarrhoea and causes wound infection with primary septicaemia in immune compromised individuals (Huq et al 1985).

2.3.3.3.9 *Vibrio furnissii*

Vibrio furnissii is considered to be a causative agent of acute gastroenteritis with symptoms including diarrhoea, abdominal cramps, nausea, and vomiting (Dalsgard et al 1997). The pathology of *V. furnissii* in gastroenteritis is potentially related to cytolysin and hemolysin production (Maghalhaes et al 1993). *V. furnissii* has been reported as an unusual cause of bacteraemia and skin lesions after ingestion of seafood (Derber et al 2011).

2.3.3.3.10 *Vibrio hollisae*

V. hollisae has been associated with blood infections and cases of diarrhoea (Hickman et al 1982). This bacterium has been isolated from cases of gastroenteritis and septicaemia (Abbott & Janda 1994) and was also reported to be a cause of bacteraemia developed by seafood consumption (Rank et al 1988).

2.3.3.3.11 *Vibrio mimicus*

V. mimicus has been isolated from a variety of clinical disorders associated with exposure to aquatic environments. Formerly classified as sucrose negative *V. cholerae, Vibrio mimicus* has been isolated from shellfish and water as well as from human diarrhoeal stools and ear infections (Koneman et al 2010).

2.3.3.3.12 *Vibrio metschnikovii*

Vibrio metschnikovii is often isolated from the environment but rarely isolated from human clinical specimens. The first documented case of human infection was from the blood of a patient with cholecystitis at Cook Country Hospital in Chicago (Jean et al 1981). Hansen and colleagues reported two cases of *Vibrio metschnikovii* septicemia: one fatal case involving a patient with liver cirrhosis, renal insufficiency, and diabetes, and another in 82 year old women with respiratory problems and an infected leg lesion, who was treated successfully (Hansen et al 1993).

2.3.3.3.13 *Vibrio cincinnatiensis*

V. cincinnatiensis was ascribed to the pathogenic group of *Vibrios* (Brayton & co-workers 1986). In the single case reported, the organism was isolated from a case of septicaemia and meningitis in an elderly immune-competent man who had apparently not been recently exposed to seafood or saltwater (Bode et al 1986). Meningitis caused by pathogenic *Vibrios* is rare but often fatal (Hughes et al 1978).

2.3.3.3.14 *Vibrio carchariae*

The pathogenic *V. carchariae* was first isolated from a brown shark (Grimes et al 1984). This organism was later isolated and characterised from the wound of a girl who was attacked by a shark and had developed a series of deep parallel lacerations on her left leg with extensive damage to the gastronomies and soleus muscles (Pavia et al 1989).

2.4 Pathogenesis of *Vibrios sps.*

V. cholera usually enters the body orally through contaminated water and food. On reaching the small intestine by using its own mechanism, such as motality, chemokines, and production of enzymes (haemagglutinin and protease), *V. cholera* reaches the mucous layer of the small intestine. Haemagglutinin and protease break mucin and fibronectin of the mucosa. Subsequently, bacteria adhere to the intestinal wall facilitated by Toxin-co regulating pilus (TCP). Vibrios, once adhered to the intestinal wall, produce cholera toxin. The toxin activates cAMP, which inhibits the absorption of sodium transport and activates the excretory chloride transport in the intestinal epithelial cells. This leads to an accumulation of sodium chloride in the lumen of intestine. The high osmolarity of the intestinal fluid is balanced by large secretion of water, which overcomes the absorptive capacity of the lumen, eventually causing diarrhea (Parija 2012).

Vibrio parahaemolyticus strains have a number of different virulence factors including adhesins, thermostable direct hemolysin (TDH*)* and TDH related hemolysin (TRH) as well as two type secretion systems, T3SS1 and T3SS2 (Makino et al 2003).

The important virulence factors associated with the pathogenesis of *V. vulnificus* are its capsular polysaccharide (CPS), iron acquisition protein, flagellar hook protein, haemolysin, and metalloprotease (Gulig et al 2005). V. vulnificus reach the bloodstream by invading the intestinal mucosa. Patients with hepatic disease are particularly susceptible to septicemia (Tacket et al1984).

V. fluvialis produces several toxins that may be important in pathogenesis including an enterotoxin-like substance, lipase, protease, cytotoxin, and hemolysin (Kothary et al 2003).

2.5 Clinical complication of *Vibrios sps.*

Profuse watery diarrhea and severe abdominal cramp is the most important manifestation of cholera. The cholera stool is profusely watery that is colorless and odorless, free of proteins and speckled with mucous and often is described as rice water stool. It contains few leukocytes, but no erythrocytes. Vomiting is another important manifestation of cholera and occurs in early stage of the disease. Cholera if left untreated can lead to severe loss of fluid and electrolytes due to diarrhea and vomiting. This could lead to isotonic dehydration, metabolic acidosis, hypokalemia, and hypovolemic shock. In untreated patients, case fatality rate has been estimated to vary from 25% to 50% (Parija 2012).

Symptoms of *Vibrio parahaemolyticus* infection may include watery diarrhea, abdominal cramps, nausea, vomiting, fever, headache and bloody diarrhea (CDC 2013). Medical conditions that predispose to *V. vulnificus* bacteremia include liver dysfunction and syndromes leading to increase iron deposition, chronic cirrhosis, hepatitis, thalassemia major, hemochromatosis, and a history of heavy alcohol consumption (Janda et al 1988). *V. fluvialis* gastroenteritis are similar to those caused by *Vibrio cholera* with the additional finding of bloody stools, which is suggestive of an invasive pathogen (Oliver and Kaper 1997).

2.6 Diagnosis of *Vibrios sps*

Among 46 isolates obtained from the various samples, 36 were identify as the genus *Vibrio* on the basis of phenotypic characterization like Gram staining, oxidase test, catalase test and salinity tolerance and other biochemical tests namely string test, voges-proskauers's (VP test), arginine dihydrolase, utilization of citrate, ornithine, lysine, mannitol, arabinose, sucrose, glucose, triple sugar iron agar (TSI), sulphite indole motality (SIM)

and urease test. As well as sero-typing for *Vibrio cholera* is done by using antisera (poly O1, Ogawa and Inaba) (Denka Seiken Co. Ltd, Japan).

The most commonly used enrichment medium is alkaline peptone water (Rennels et al1980). Thiosulphate-citrate-bile salts-sucrose (TCBS) agar is a selective medium commonly used to isolate members of the genus *Vibrio* (Pfeffer & Oliver 2003). Sucrose fermenters produce yellow coloured colonies and non-fermenters produce green to blue-green colonies. Both yellow and green colonies measuring from 2 to 9 mm in diameter on TCBS agar were subjected for gram staining, biochemical testing as well as sero-typing using antisera (poly O1, Ogawa and Inaba) (Denka Seiken Co. Ltd, Japan).

Antimicrobial susceptibility test (AST) for the isolates was determined using various antimicrobial discs (HiMedia) like Amikacin, Ampicillin, Azithromycin, Cefotaxime, Ceftazidime, Ceftriaxone, Chloramphenicol, Ciprofloxacin, CoTrimoxazole, Doxycycline, Erythromycin, Gentamicin, Imipenem, Levofloxacin, Nalidixic Acid, Nitrofurantoin, Norfloxacin, Ofloxacin, and Tetracycline. AST was performed using the modified Kirby-Bauer disk diffusion method as recommended by Clinical and Laboratory Standards Institute guidelines (CLSI guidelines).

2.7 Treatment of Vibrios infection

Antibiotic Treatment: Tetracycline or doxycycline is the drug of choice for adults (Parija 2012). Erythromycin or azithromycin recommended as first-line drugs for pregnant women and children. Ciprofloxacin and doxycycline recommended as second-line drugs for children (CDC 2015).
Zinc Treatment: Zinc is recommended for the treatment of pediatric diarrhoea more generally when available, supplementation (10-20 mg zinc per day) should be started immediately (CDC 2015).

Rehydration Therapy: Rehydration is the cornerstone of treatment for cholera. Oral rehydration salts and, when necessary, intravenous fluids and electrolytes, if administered in a timely manner and in adequate volumes, will reduce fatalities (CDC 2015).

Patients with noncholera *Vibrio* wound infection or septicemia are much more ill and frequently have other medical conditions. Medical therapy consists of Prompting, initiation of effective antibiotic therapy, intensive medical therapy with aggressive fluid replacement and vasopressors for hypotension and septic shock to correct acid-base and electrolytes abnormalities that may be associated with severe (CDC 2015).

2.8 Prevention and Control of Vibrios infection

The preventive measures against cholera include general preventive measures and cholera vaccination. General preventive measures include early identification and case management, improved water supply and sanitation, improved personal hygiene, and health education.Vaccines include Killed cholera vaccines, Nonliving oral B subunit whole cell vaccine, and live oral cholera vaccine (Parija 2012).

CDC recommends for prevention of water borne diseases by: the drink and use of safe water, while brushing, cooking, making ice etc. always one should use safe water. To make water safe to drink and use it should be boiled or treat with a chlorine product or household bleach and should stored in a clean, covered container. Before eating, preparing food, after using latrine or toilet, and after taking care of someone ill with diarrhea should always washed hands with soap and safe water. Latrines and surfaces contaminated with feces should clean using a solution of 1 part household bleach to 9 parts water. Food is cooked well, should be covered. Fruits and vegetables should be peeled before eating. Kitchen and bathroom should be cleaned regularly. Consider getting vaccinated before travel, a single-dose live oral cholera vaccine called Vaxchora. Vaxchora is recommended for adults who are 18 to 64 years old and are traveling to an area of active cholera transmission with toxigenic *Vibrio cholera* O1 (the bacteria strain that most commonly causes cholera) to prevent infection (CDC 2015).

CHAPTER-III

3 MATERIALS AND METHODOLOGY

3.1 Materials and equipments

The materials, equipments and reagents required for this work have been included in the Appendix A, C and D.

3.2 Methodology

3.2.1 Study site and study period

The study site was mainly focused on eastern region of Nepal, particularly in Dharan and Ithari and its vicinity. The study was performed in the laboratory of Sunsari Technical College from June 2016 to November 2016 during peak rainy season. The aim of research was to evaluate significant of *vibrios* in environmental water samples.

3.2.2 Study population

The study was conducted in various water samples (tap water, bottled water, drainage, river water, pond and sewage) of eastern Nepal.

3.2.3 Sample size

A total 60 sample was taken.

Number of sample = $4PQ/\varepsilon 2$

Where ε = Error

3.2.3.1 Inclusion criteria

Samples which are collected aseptically with proper labeling, sufficient volume were accepted.

3.2.3.2 Exclusion criteria

Samples with improper labeling, insufficient volume and inappropriately collected and transported were rejected.

3.2.4 Sample collection and Transportation:

3.2.4.1 Collection of Tap Water

For tap water one sterile bottle, of 200 ml capacity containing sodium thiosulfate (to neutralize chlorine if present) was used. The mouth of the tap was cleaned by using clean cloth to remove any dirt. Then the sterilization of mouth of tap was done with the help of flame for one minute. The tap was turned on and allowed the water to run for 1-2 minutes at a medium flow. Sterilized bottle was opened and filled with water by leaving a small air space tomake shaking before analysis easier. Finally stopper was placed on the bottle and brown paper protective cover was fixed with the string. The water samples were transported to the laboratory of Sunsari college in ice box within 2 hours of collection, because *V. cholerae* survives better in specimens held at 4˚C than in frozen samples (WHO 1997).

3.2.4.2 Collection of Bottle Water

For bottle water commonly available brands of bottled water were purchased from local market and samples were transported to the laboratory of Sunsari College in ice box.

3.2.4.3 Collection of Water from other environmental sources.

For river, sewage and other flowing water samples, the Moore's swab method was used for sampling water. The samples were transported to the laboratory in an ice chest to prevent possible overheating on the same day of collection.

3.2.5 Physiochemical analysis of sample.

pH: pH was measured by using pH meter.

Temperature: Temperature was measured by using thermometer.

3.2.6 Analytical technique for isolation of vibrios.

3.2.6.1 Membrane filteration technique

This technique was done for tap water and bottled water. In the membrane filtration method, 100 ml of the sample was transfer aseptically into a properly

disinfected filteration assembly containing a sterile membrane filter (pore size 0.45 µm). A vacuum was applied and the sample was drawn through the membrane filter. Filter was placed in 100 ml of (alkaline peptone water) APW in a flask and incubated at 37˚C for 6 to 8 hours and further plated to TCBS (Thiosulfate-citrate-bile salts-sucrose agar) and incubated at 37˚C for 18 to 24 hour (Senior 2007).

3.2.6.2 Moore's technique

This method was applied for sewage and other flowing water samples. Moore's swabs were made by cutting pieces of cotton gauze 2 to 4 feet long by 6 inches wide (60 to 120 cm by 15 cm), folding or rolling the gauze lengthwise several times to form a tight cylindrical roll, and tying the center with a strong wire. The swabs were wrapped in heavy paper and sterilized by autoclaving. The ends of the wires holding the swabs were tied to nylon fish line, and the swabs were suspended by the lines in the water or sewage to be tested and left in place for 24 to 48 (usually 24) hour. The swabs were then removed, the wires holding the swabs were cut aseptically, and the swabs were submerged in alkaline peptone broth with a pH of 8.6. The jars were transported to the laboratory in an ice chest to prevent possible overheating on the same day of collection.300 to 500 ml of APW should be added to the specimens immediately after arrival of the samples at the laboratory. Specimens were incubated at 37˚C for 6 to 8 hours and streaked to TCBS agar using one large or two smaller loopfuls from the surface and topmost portion of the broth, since vibrios preferentially migrates to this area. The tube should not be shaked or mixed before subculturing (Timothy et al 1980).

3.2.7 Identification of Vibrios

The identification of bacterial isolates was done by using standard microbiological techniques as describe in Bergey's Manual of systematic bacteriological which included the study of colony morphology, staining reactions and biochemical tests like catalase, oxidase, triple sugar iron, sulphite indole motality, citrate utilization, urea hydrolysis and other tests required for the identification of bacteria (Cheesbrough 2006). Suspected colonies were sub-cultured on nutrient agar (NA) and the colonies on NA were

subjected for biochemical tests and serotyping using polyvalent V. cholerae O1 sera and also with Inaba and Ogawa antisera (Denka Seiken Co. Ltd, Japan) for V. cholera O1 (Feeley & Balows 1974).

For the characterization of other medically important species of Vibrios, colonial characters and other biochemical tests, such as string test, voges-proskauers, nitrate, lysine, arginine dihydrolase, ornithine, mannitol, arabinose, sucrose, lactose, glucose and salt tolerance test were employed. Related details are included in the Appendix C and E.

3.2.8 Antibiotic susceptibility test

Modified Kirby-Bauer disc diffusion technique was used as recommended by Clinical and Laboratory Standards Institute guidelines (CLSI) and World Health Organization by using Mueller Hinton Agar (MHA) for antibiotic susceptibility tests (Banfi et al 2013). Using this test, antimicrobial resistant is detected by allowing the antibiotics to diffuse into an agar medium with the test organism. Visible growth of bacteria occurs on the surface of the agar showing clear zone of inhibition. Following incubation, the diameter of the zone of inhibition around each disc is measured in millimeters and compared with standard chart (Sharma et al 2011). To differentiate the classical from El Tor *V.cholerae* species poymixin B disc test was done (Han & Khie1963).

The turbidity of broth culture of test organism was matched to that of Mc Farland 0.5 solution and was swabbed uniformly on the surface of Mueller Hinton Agar (MHA). The antibiotics discs were then placed aseptically over the lawn of test organisms. After incubation at 37 ◦c for 18-24 hours, the diameter of the zone of inhibition around each disc was measured. Control strains were used in parallel as a part of quality control test systems. The resistance and susceptibility were interpreted according to the National committee for clinical laboratory standards.

3.2.9 Screening for multidrug resistant (MDR) isolates

The bacterial isolates showing resistance to three or more than three classes of antibiotics during antibiotic susceptibility testing on MHA was reported as MDR isolates.

3.3 Quality control

Laboratory equipment like an incubator, autoclave, hot air oven, refrigerator, weighing balance etc. was regularly monitored for their performance and immediately corrected if any deviation occurred. The temperature of the incubator and refrigerator were monitored every day. Reagents and biochemical media were checked for manufacture and expiry date and proper storage. After preparation, each reagent were labeled with preparation date, expiry date and stored in proper conditions. Sterility testing, performance testing and quality control of antibiotic disc were done with *E. coli* ATCC 25922 and Staphylococcus aureus ATCC 25923. For antimicrobial susceptibility testing, inoculums broth was matched with 0.5 Mc Farland solutions.

3.4 Data analysis

Data was analyzed by using SPSS software. A value of $p \leq 0.5$ was assumed where even applicable and 95% confidence intervals along with exact p- value were presented.

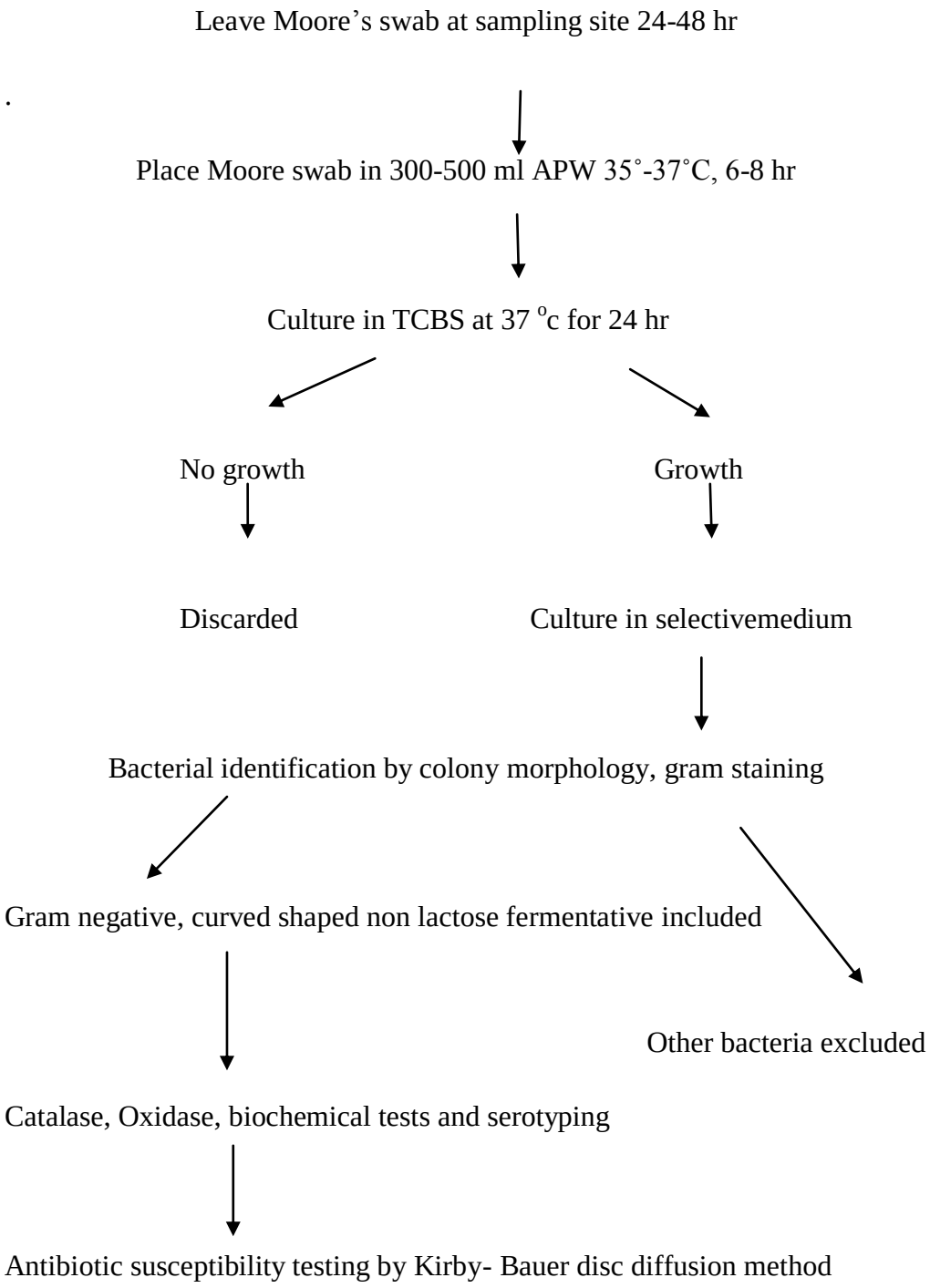


Figure 1. A Flow chart for detection of *vibrios* from Moore's Swab Technique

CHAPTER –IV

4 RESULTS

4.1 Study Population

This study was community based comparative cross sectional study conducted in the laboratory of Sunsari Technical College, Dharan from June 2016 to November 2016. 30 tap water samples, 10 bottled water samples and 20 surface water samples (river, sewage, pond,& drainage) were taken from Dharan municipality and nearby place and were tested for *Vibrios* contamination, pH and temperature. Further, identification of microorganisms and antibiotic susceptibility pattern was performed by standard methods. Twenty surface water samples were tested to find out the route of contamination in tap water. For collection of sample simple random sampling technique was used. From the obtained data the mean, standard deviation were calculated and according to the nature of data p-value was determined by applying Chi square test by using Statistical Package for Social Sciences (SPSS).

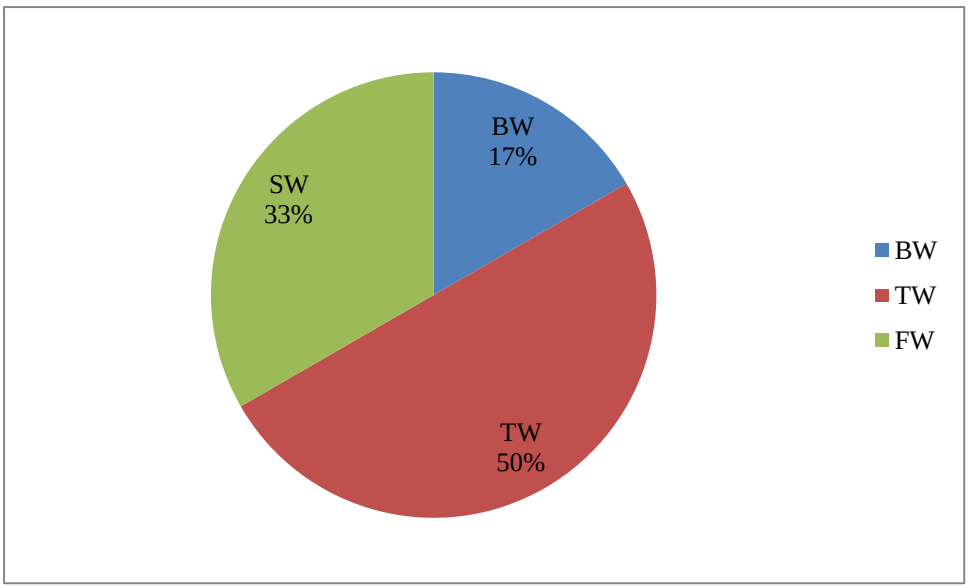


Figure 2. Percentage of water samples taken for study population.

4.2 Different characteristic of water samples.

The minimum temperature of tap water was 11◦C and maximum temperature was 26◦C with mean temperature 15.87◦C and standard deviation (SD) 4.0. For bottle water minimum temperature was 16◦C and maximum temperature was 18 ◦C with mean temperature 17.2◦C and standard deviation 0.79. The minimum temperature of surface water was 11 ◦C and maximum temperature was 27 ◦C with mean temperature 20.75 ◦C and standard deviation (SD) 4.08. The minimum pH of tap water was 6.7 and maximum pH was 8.3 with mean pH 7.22 and standard deviation 0.40. The minimum pH of bottled water was 6.9 and maximum pH was 7.4 with mean pH 7.05 and standard deviation 0.21. For surface water minimum pH was 6.7 and maximum pH was 8.3 with mean pH 7.75 and standard deviation (SD) 0.46. There was significant difference among the three groups of water samples in temperature and pH. After further analysis it was found that Temperature and pH between tap water and surface water was not significant. But temperature and pH of bottled water were significantly different between tap and surface water. The reasons behind the different values of temperature and pH among the selected sources may be due to different level of purification and treatment of water.

Variables		Type of water			Total Mean ±SD	p -value
		Tap (n=30)	Bottle (n=10)	Surface water (n=20)		
Temp(◦C)	Minimum	11	16	11	17.72 ± 4.27	<0.001
	Maximum	26	18	27		
	Mean ±SD	15.87 ± 4.0	17.2 ± 0.79	20.75 ± 4.08		
PH	Minimum	6.7	6.9	6.7	7.37 ± 0.48	<0.001
	Maximum	8.3	7.4	8.3		
	Mean± SD	7.22 ± 0.40	7.05 ± 0.21	7.75 ± 0.46		

Table 1. Different characteristic of water samples

4.3 Number of water samples contaminated with *vibrios*

Out of 30 tap water only 4 (13.3%) was contaminated with *vibrios*. But there were no any contamination in bottled water. 19 (95.0%) surface water samples were contaminated with *vibrios*. There was significant difference in contaminates rates of *vibrios* between all the three types of water samples. Bottled water was founded to be safer than tap water and surface water samples. Tap water was less contaminated than surface water. Surface water was highly contaminated.

Types of water	Growth		Total	P -value
	Present	Absent		
Tap	4 (13.3%)	26 (86.7%)	30 (100%)	<0.001
Bottle	-	10 (100%)	10 (100%)	
Surface	19 (95%)	1 (5%)	20 (100%)	
Total	23 (38.3%)	37 (61.7%)	60 (100%)	
P -value	<0.001	<0.001		

Table 2. Number of water samples contaminated with *vibrios*

4.4 Percentage of water samples contaminated with different species of *vibrios.*

Out 60 samples included in this study, 4 tap water (13.3%) and 19 surface water (95%) were contaminated with *Vibrios*. Among 19 surface water 32 medically important *vibrios* (total different 7 species) were identified. In tap water samples 4 *vibrios* (total 3 species) were detected. Altogether 36 isolates were identified. Among them *V. cholerae* was present in 3.3% of tap water and 35% of surface water samples. 6.7% of tap water and 20% of surface water samples were contaminated with *V. furnissii*. *V. fluvialis* was present in 3.3% of tap water and 20% of surface water samples. 30% *V.vulnificus*, 35% *V. parahaemolyticus*, 10% *V. alginolyticus* and 10% of *V. metschnikovii* were present only in surface water samples, these bacteria were not found in tap water samples. All bottled waters were free from contamination with *vibrios.*

Bacteria		Tap water n=30	Bottle water n=10	Surface water n=20
V. cholerae	Present	1 (3.3%)	-	7 (35%)
	Absent	29 (96.7%)	10 (100%)	13 (65%)
	Total	30 (100%)	10 (100%)	20 (100%)
V. vulnificus	Present	-	-	6 (30%)
	Absent	30 (100%)	10 (100%)	14 (70%)
	Total	30 (100%)	10 (100%)	20 (100%)
V. parahaemolyticus	Present	-	-	7 (35%)
	Absent	30 (100%)	10 (100%)	13 (65%)
	Total	30 (100%)	10 (100%)	20 (100%)
V. furnissii	Present	2 (6.7%)	-	4 (20%)
	Absent	28 (93.3%)	10 (100%)	16 (80%)
	Total	30 (100%)	10 (100%)	20 (100%)
V. fluvialis	Present	1 (3.3%)	-	4 (20%)
	Absent	29 (96.7%)	10 (100%)	16 (80%)
	Total	30 (100%)	10 (100%)	20 (100%)
V. alginolyticus	Present	-	-	2 (10%)
	Absent	30 (100%)	10 (100%)	18 (90%)
	Total	30 (100%)	10 (100%)	20 (100%)
V. metschnikovii	Present	-	-	2 (10%)
	Absent	30 (100%)	10 (100%)	18 (90%)
	Total	30 (100%)	10 (100%)	20 (100%)

Table 3. Percentage of water samples contaminated with vibrios

4.5 Frequency of medically important Vibrios in water samples.

Among the total 60 samples collected for the study 36 medically important *Vibrios* were identified. From which *V. Cholerae* was found more than others (22.2%) which is medically important. Next to it was *V. Parahaemolyticus* (19.5%), than *V. vulnificus* and *V. furnissii* (16.7%), followed by *V. fluvialis* (13.9%), *V. alginolyticus* (5.5%) and *V. metschnikovii* (5.5%).

Vibrio species	N	%
V. cholerae	8	22.2
V. parahaemolyticus	7	19.5
V. furnissii	6	16.7
V. vulnificus	6	16.7
V. fluvialis	5	13.9
V. alginolyticus	2	5.5
V. metschnikovii	2	5.5
Total	**36**	**100**

Table 4. Frequency of medically important Vibrios in water samples. N=60

4.6 Antibiotic susceptibility pattern of the *Vibrio cholerae*

All the 8 isolates of *V. Cholerae* were 100% sensitive to Ampicillin, Amikacin, Azithromycin, Ceftriaxone, Chloramphenicol, Doxycycline, Erythromycin, Imipenem, Levofloxacin, and Norfloxacin. This was followed by Tetracycline, 87.5%, Ciprofloxacin, 87.5%, Cefotaxime, 75%, Ofloxacin, 75%, Gentamicin, 62.5%. While all isolates were, 100% resistant to Nalidixic Acid, Nitrofurantoin, Ceftazidime, and Co-Trimoxazole.

Antibiotics used	Sensitive		Resistant	
	No.	%	No.	%
Amikacin	8/8	100	-	-
Ampicillin	8/8	100	-	-
Azithromycin	8/8	100	-	-
Cefotaxime	6/8	75	2/8	25
Ceftazidime	-	-	8/8	100
Ceftriaxone	8/8	100	-	-
Chloramphenicol	8/8	100	-	-
Ciprofloxacin	7/8	87.5	1/8	12.5
Co-Trimoxazole	-	-	8/8	100
Doxycycline	8/8	100	-	-
Erythromycin	8/8	100	-	-
Gentamicin	5/8	62.5	3/8	37.5
Imipenem	8/8	100	-	-
Levofloxacin	8/8	100	-	-
Nalidixic Acid	-	-	8/8	100
Nitrofurantoin	-	-	8/8	100
Norfloxacin	8/8	100	-	-
Ofloxacin	6/8	75	2/8	25
Tetracycline	7/8	87.5	1/8	12.5

Table 5. Antibiotic susceptibility pattern of the *V. cholera* 01 (ogawa, biotype eltor) isolates (n=8)

4.7 Antibiotic susceptibility pattern of the *V. Parahaemolyticus*

All the 7 isolates of *V. Parahaemolyticus* were 100% sensitive to Amikacin, Azithromycin, Ceftriaxone, Chloramphenicol, Ciprofloxacin, CoTrimoxazole, Doxycycline, Imipenem, Levofloxacin, Nalidixic Acid, Nitrofurantoin, Norfloxacin, Ofloxacin, and Tetracycline. This was followed by Cefotaxime 85.7%, Gentamicin 85.7%. While all strains were 100% resistanceto Ampicillin followed by Ceftazidime 71.4% and Erythromycin 71.4%.

Antibiotics used	Sensitive		Resistant	
	No.	%	No.	%
Amikacin	7/7	100	-	-
Ampicillin	-	-	7/7	100
Azithromycin	7/7	100	-	-
Cefotaxime	6/7	85.7	1/7	14.3
Ceftazidime	2/7	28.6	5/7	71.4
Ceftriaxone	7/7	100	-	-
Chloramphenicol	7/7	100	-	-
Ciprofloxacin	7/7	100	-	-
Co-Trimoxazole	7/7	100	-	-
Doxycycline	7/7	100	-	-
Erythromycin	2/7	28.6	5/7	71.4
Gentamicin	6/7	85.7	1/7	14.3
Imipenem	7/7	100	-	-
Levofloxacin	7/7	100	-	-
Nalidixic Acid	7/7	100	-	-
Nitrofurantoin	7/7	100	-	-
Norfloxacin	7/7	100	-	-
Ofloxacin	7/7	100	-	-
Tetracycline	7/7	100	-	-

Table 6. Antibiotic susceptibility pattern of *V. Parahaemolyticus* isolates (n=7).

4.8 Antibiotic susceptibility pattern of the *Vibrio furnissii*

All the 6 isolates of *V. furnissii* were 100% sensitive to Amikacin, Azithromycin, Cefotaxime, Ceftriaxone, Chloramphenicol, Ciprofloxacin, CoTrimoxazole, Doxycycline, Erythromycin, Gentamicin, Imipenem, Levofloxacin, Nalidixic Acid, Nitrofurantoin, Norfloxacin, Ofloxacin and Tetracycline. While all isolates were 100% resistanceto Ampicillin and Ceftazidime.

Antibiotics used	Sensitive		Resistant	
	No.	%	No.	%
Amikacin	6/6	100	-	-
Ampicillin	-	-	6/6	100
Azithromycin	6/6	100	-	-
Cefotaxime	6/6	100	-	-
Ceftazidime	-	-	6	100
Ceftriaxone	6/6	100	-	-
Chloramphenicol	6/6	100	-	-
Ciprofloxacin	6/6	100	-	-
Co-Trimoxazole	6/6	100	-	-
Doxycycline	6/6	100	-	-
Erythromycin	6/6	100	-	-
Gentamicin	6/6	100	-	-
Imipenem	6/6	100	-	-
Levofloxacin	6/6	100	-	-
Nalidixic Acid	6/6	100	-	-
Nitrofurantoin	6/6	100	-	-
Norfloxacin	6/6	100	-	-
Ofloxacin	6/6	100	-	-
Tetracycline	6/6	100	-	-

Table 7. Antibiotic susceptibility pattern of the *V. furnissii* isolates (n=6).

4.9 Antibiotic susceptibility pattern of the *Vibrio vulnificus*

The 6 isolates of *V. vulnificus* were 100% sensitive to Amikacin, Ampicillin, Azithromycin, Cefotaxime, Ceftriaxone, Chloramphenicol, Ciprofloxacin, CoTrimoxazole, Doxycycline, Gentamicin, Imipenem, Levofloxacin, Nitrofurantoin, Norfloxacin, Ofloxacin, and Tetracycline and 100% resistance to Ceftazidime followed by Erythromycin 66.7%. Nalidixic Acid was 50% sensitive.

Antibiotics used	Sensitive		Resistant	
	No.	%	No.	%
Amikacin	6/6	100	-	-
Ampicillin	6/6	100	-	-
Azithromycin	6/6	100	-	-
Cefotaxime	6/6	100	-	-
Ceftazidime	-	-	6/6	100
Ceftriaxone	6/6	100	-	-
Chloramphenicol	6/6	100	-	-
Ciprofloxacin	6/6	100	-	-
Co-Trimoxazole	6/6	100	-	-
Doxycycline	6/6	100	-	-
Erythromycin	2/6	33.3	4/6	66.7
Gentamicin	6/6	100	-	-
Imipenem	6/6	100	-	-
Levofloxacin	6/6	100	-	-
Nalidixic Acid	3/6	50	3/6	50
Nitrofurantoin	6/6	100	-	-
Norfloxacin	6/6	100	-	-
Ofloxacin	6/6	100	-	-
Tetracycline	6/6	100	-	-

Table 8. Antibiotic susceptibility pattern of the *V. vulnificus* isolates (n=6).

4.10 Antibiotic susceptibility pattern of the *Vibrio fluvialis*

All the 5 isolates of *V. fluvialis* were 100% sensitive to Amikacin, Azithromycin, Cefotaxime, Ceftriaxone, Chloramphenicol, Ciprofloxacin, CoTrimoxazole, Doxycycline, Gentamicin, Imipenem, Levofloxacin, Nitrofurantoin, Norfloxacin, Ofloxacin and Tetracycline and 100% resistance to Ampicillin, Ceftazidime. Nalidixic Acid and followed by 60% resistant to Erythromycin.

Antibiotics used	Sensitive		Resistant	
	No.	%	No.	%
Amikacin	5/5	100	-	-
Ampicillin	-	-	5/5	100
Azithromycin	5/5	100	-	-
Cefotaxime	5/5	100	-	-
Ceftazidime	-	-	5/5	100
Ceftriaxone	5/5	100	-	-
Chloramphenicol	5/5	100	-	-
Ciprofloxacin	5/5	100	-	-
Co-Trimoxazole	5/5	100	-	-
Doxycycline	5/5	100	-	-
Erythromycin	2/5	40	3/5	60
Gentamicin	5/5	100	-	-
Imipenem	5/5	100	-	-
Levofloxacin	5/5	100	-	-
Nalidixic Acid	2/5	40	3/5	60
Nitrofurantoin	5/5	100	-	-
Norfloxacin	5/5	100	-	-
Ofloxacin	5/5	100	-	-
Tetracycline	5/5	100	-	-

Table 9. Antibiotic susceptibility pattern of the *V. fluvialis* isolates (n=5)

4.11 Antibiotic susceptibility pattern of the *V. alginolyticus*

All the 2 isolates of *V. alginolyticus* were 100% sensitive to Amikacin, Azithromycin, Cefotaxime, Ceftriaxone, Chloramphenicol, Ciprofloxacin, CoTrimoxazole, Doxycycline, Gentamicin, Imipenem, Levofloxacin, Norfloxacin, Nitrofurantoin, Ofloxacin and Tetracycline. 100% resistance to Ampicillin, Ceftazidime, Nalidixic Acid and Erythromycin was 50 sensitive as described in table 10.

Antibiotics used	Sensitive		Resistant	
	No.	%	No.	%
Amikacin	2/2	100	-	-
Ampicillin	-	-	2/2	100
Azithromycin	2/2	100	-	-
Cefotaxime	2/2	100	-	-
Ceftazidime	-	-	2/2	100
Ceftriaxone	2/2	100	-	-
Chloramphenicol	2/2	100	-	-
Ciprofloxacin	2/2	100	-	-
Co-Trimoxazole	2/2	100	-	-
Doxycycline	2/2	100	-	-
Erythromycin	1/2	50	1/2	50
Gentamicin	2/2	100	-	-
Imipenem	2/2	100	-	-
Levofloxacin	2/2	100	-	-
Nalidixic Acid	-	100	2/2	100
Nitrofurantoin	2/2	100	-	-
Norfloxacin	2/2	100	-	-
Ofloxacin	2/2	100	-	-
Tetracycline	2/2	100	-	-

Table 10. Antibiotic susceptibility pattern of the *V. alginolyticus* isolates (n=2)

4.12 Antibiotic susceptibility pattern of the *Vibrio metschnikovii*

All the 2 isolates of *V. metschnikovii* were 100% sensitive to Amikacin, Azithromycin, Ceftriaxone, Chloramphenicol, Ciprofloxacin, CoTrimoxazole, Doxycycline, Gentamicin, Imipenem, Levofloxacin, Nitrofurantoin, Norfloxacin, and Tetracycline. While all strains were 100% resistance to Ampicillin, Cefotaxime, Ceftazidime, Erythromycin and Nalidixic Acid. Ofloxacin was 50% sensitive.

Antibiotics used	Sensitive		Resistant	
	No.	%	No.	%
Amikacin	2/2	100	-	-
Ampicillin	-	-	2/2	100
Azithromycin	2/2	100	-	-
Cefotaxime	-	-	2/2	100
Ceftazidime	-	-	2/2	100
Ceftriaxone	2/2	100	-	-
Chloramphenicol	2/2	100	-	-
Ciprofloxacin	2/2	100	-	-
Co-Trimoxazole	2/2	100	-	-
Doxycycline	2/2	100	-	-
Erythromycin	-	-	2/2	100
Gentamicin	2/2	100	-	-
Imipenem	2/2	100	-	-
Levofloxacin	2/2	100	-	-
Nalidixic Acid	-	-	2/2	100
Nitrofurantoin	2/2	100	-	-
Norfloxacin	2/2	100	-	-
Ofloxacin	1/2	50	1/2	50
Tetracycline	2/2	100	-	-

Table 11. Antibiotic susceptibility pattern of the *V. metschnikovii* isolates (n=2).

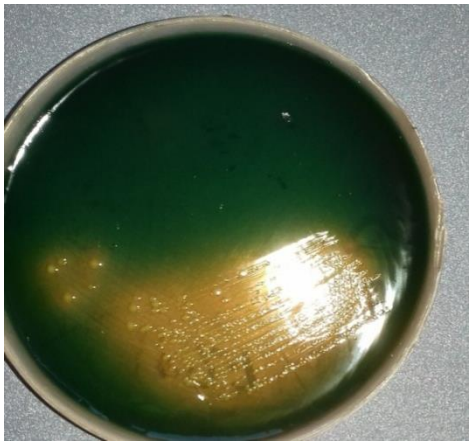
4.13 Multidrug resistance among different bacterial isolates

Among total bacterial isolates, 11 (44.4%) out of 36 showed multidrug resistance (MDR) towards antibiotics. Out of 8 *V. cholerae*, all were MDR strain. Out of 7 *V. parahaemolyticus* 1 were MDR strain. There were no any MDR in case of V. furnissi. Out of 5 *V. Vulnificus* 2 were MDR. Similarly out of 5*V. Fluvialis* 3 were found to be MDR. In case of *V. Alginolyticus* there were no any MDR and all isolates of *V. Metschnikovii* were found to be MDR.

Organisms	MDR	Total isolates
V. cholerae	8	8
V. parahaemolyticus	1	7
V. furnissi	-	6
V. vulnificus	2	5
V. fluvialis	3	5
V. alginolyticus	-	3
V. metschnikovii	2	2
Total	**16**	**36**

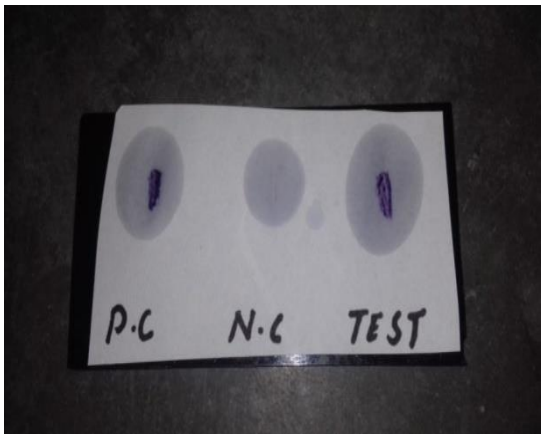
Table 12. Multidrug resistance among different bacterial isolates

PHOTOGRAPHS

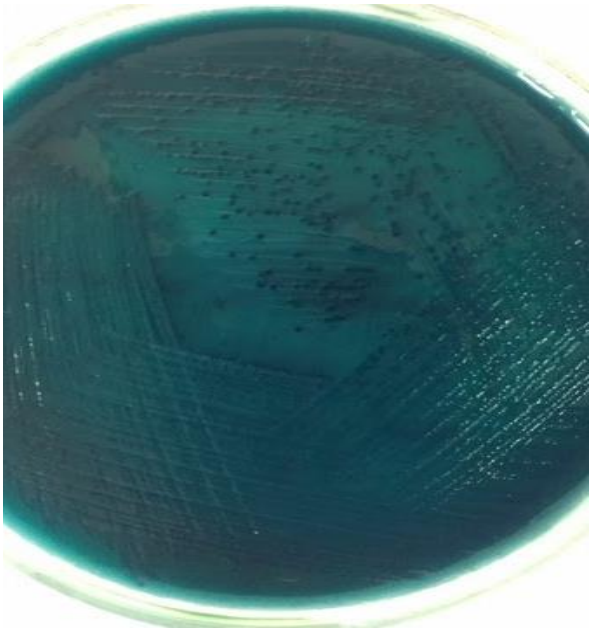


Photograph 1. Colony of *Vibrio cholerae* inTCBS

Sample No: 25

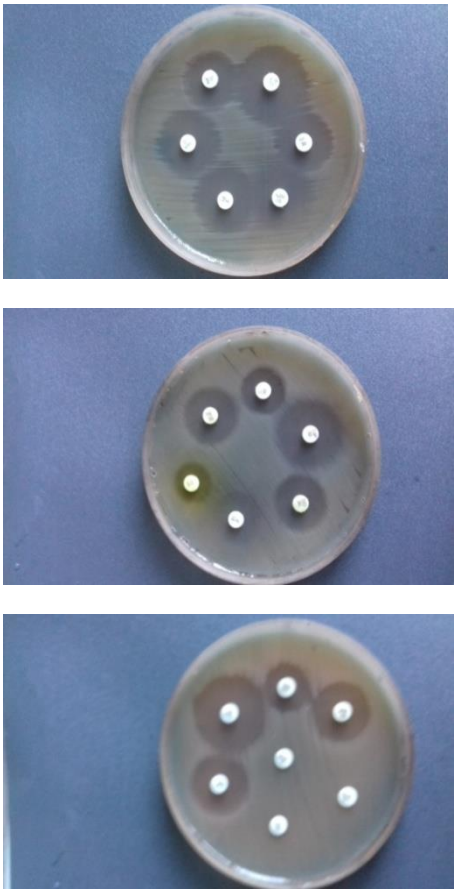


Photograph 2. Oxidase test



Photograph 3. Colony of *V. parahaemolyticus* in TCBS, Sample No: 28

Photograph 4. Biochemical tests of *Vibrio cholerae*



Photograph 5. Antibiotics sensitivity test of *V. Cholerae*

CHAPTER V

5 DISCUSSION

Water is essential to life, but many people do not have access to clean and safe drinking water and many die of waterborne bacterial infections (Joao 2010). Environmental waters are an important reservoir for *Vibrio cholerae*, and effective surveillance of the pathogen can help to warn of and prevent infection with this potentially fatal pathogen. Among more than 200 known *Vibrio cholerae* serogroups, only the O1 and O139 strains are pathogenic, causing epidemics and pandemics throughout history (Sack et al 2004). Therefore environmental surveillance for the presence of *Vibrio cholerae* is of utmost importance for the effective public health protection of cholera.

Cholera is one of the most predominant diarrhoeal diseases in Nepal. Recently there have been reports of increased drug resistance toward commonly used antibiotics among the strains of *Vibrio cholerae*, causing serious problem in management of the cholera cases (Vila & Pal 2010). Therefore in the present study antibiotic sensitivity of the bacterial isolates was also done to find out the multi drug resistant *Vibrios.*

In this study, drinking water samples from tap and bottled water, water samples from surface water (river, drainage, sewage and pond) from eastern region of Nepal (Dharan and Ithari) were screened for detection of *vibrios*, and to evaluate and compare the quality of water and its suitability for human consumption. Present study, therefore, was done to find out the existence of *V. cholerae* and other pathogenic species of *Vibrios* in water samples as well as to study antibiotic sensitivity of the bacterial isolates.

This study was conducted from June 2016 to November 2016, during the peak rainy seasons. The tap water samples were collected from the wards of Dharan and Ithari municipality following aseptic technique. Commonly available brands of bottled water were taken from local market and water samples from other sources (river, pond, sewage) were collected by the Moore's technique as it is considered a practical and effective technique for *V. cholerae* detection

in sewage, river and other flowing water sources.Many investigators have used the same method for the isolation of medically important *Vibrios* (both cholera and non cholera *Vibrios)* from sewerage/environment and for tracing the source of infection (Barrett et al1980; Madico et al 1996).

Altogether 60 water samples (30 tap water, 20 other sources of water from river, pond, sewage and 10 bottled water) were collected, temperature and pH were taken in a aseptic way and then sample were incubated in alkaline peptone water and cultured in TCBS agar. After sub-culturing in Nutrient agar, identification of the significant isolates was done by standard microbiological techniques and antibiotic susceptibility testing was done by Kirby Bauer disc diffusion method.

Though twelve Vibrio species *V. cholerae, V. parahaemolyticus, V. vulnificus, V.alginolyticus, V.furnissii, V.fluvialis, V.damsela, V.mimicus, V.hollisae, V.cincinnatiencis,V.harveyi and V.metchnikovii* have been documented as potential food-borne disease agents in humans (Thompson & Swings 2006; Adams & Moss 2008) but in my study only seven species *V. cholerae, V. parahaemolyticus, V. vulnificus, V.alginolyticus, V.furnissii, V.fluvialis* and *V.metchnikovii* were found.

36 medically important Vibrios were identified in 23 water samples out of total 60. Among the 20 other sources of water samples showed growth of 32 medically important *Vibrio* species where as Out of 30 tap water samples only 4 *Vibrios* were isolated. Such a large scale of isolation could be due to heavy contamination with untreated sewerage outlets of the city. None of any isolation of *Vibrios* was found in bottled water samples. Which is an assurance of safe drinking.Temperature and pH between tap water and bottled water did not vary significantly. But there were differences in pH and temperature among the water samples from other sources with drinking water samples.

In this study quality of bottled water was found to be better than of tap and other sources of water which was in agreement with the findings of Yasin et al 2012 and Islam et al 2010. But there are various reports regarding cholera outbreaks due to drinking of contaminated bottled water. In 1994 the centre

for disease control and prevention reported an outbreak of bottled water associated cholera in united state (EI-Salam et al 2008). Similarly in Portugal, during the cholera epidemic of 1974, bottled mineral water was identified as one of the vehicles of transmission of *Vibrio cholerae* (Blake et al 1977).

Our study showed that, 95% of natural sources of water along with sewage were found to be contaminated with *Vibrios*. The contamination of sources may be due to activity of humans and animals in the watershed area, however only 13.3% of tap water samples were found to be contaminated with *Vibrios.* This may be due to ineffective treatment of water before distribution or due to distribution from contaminated sources without water treatment or the contamination of tap water in the leakage parts of pipes.

In this study, evidence of *V. cholerae* was found in 22.2% of the total number of water samples A study by Karki & Tiwari in Kathmandu reported 25.1% cholera cases in 2007 and study by Tamang *et al* in Kavre reported 31% of positive cases for *V. cholera* in 2005. In the study done by Pappu et al incidence of *vibrio cholera* was found to be 26.7% in 2016. This was approximately similar to our result. But study done by (Rai K R et al 2012) 43.5 % of *Vibrio cholerae* were isolated among total samples. Similarly 64.2% of *Vibrio cholerae* has been reported in Argentina and in Bangladesh (Emiliani et al 1997; Huq et al1990). Yamai et al (1996) in Japan and Thomson et al (1998) in India however reported *Vibrio cholerae* were positive in all river water and drinking water samples. In this study finding of *Vibrio cholerae* was lower than those reported from elsewhere during peak rainy days.

All *Vibrio cholerae* strains isolated from water samples belonged to serogroup O1, Ogawa serotype, biotype El Tor. Similar types of result were shown by study done in other parts of Nepal. Sameer et al 2014 ; Pappu et al 2016 ; Bhandari et al 2009 ; Bhandari & Bhusal 2013 ; Gautam et al 2012 ; Karki et al 2010; Dixit et al 2014 reported all *V. cholerae* strains isolated from surface water were confirmed to be serogroup O1, Ogawa serotype, biotype El Tor. However in contrast to our study, all three serotypes Ogawa (64 %), Inaba (35 %) and Hikojima (1 %) were isolated by Mall & Dumre 2007. In the study

done by (Rai K R et al 2012) both Hikojima (all classical biotypes) and Ogawa (El Tor biotypes and Classical) serotypes were isolated. Nepal Public Health Laboratory (NPHL) reported *V. cholerae* O1 sero-types, Ogawa, Hikojima from the clinical cases of Nepal and few cases had been associated with Inaba sero-type in 2007. Such serotype shifting is a common phenomenon in *V. cholerae* (Garg et al 2000; Narang et al 2008; Sarkar et al 1999).

Three strains; *V. cholerae* O1 biotype El Tor, *V. cholerae* O1 biotype Classical and *V. cholerae* O139 have been frequently isolated in cholera outbreaks in Asian countries (Faruque et al 1998). Although classical *V. cholerae* O1 caused the fifth and sixth pandemics, and presumably the earlier pandemics, the seventh pandemic was attributed to the El Tor. Infections with classical strains are generally more severe than those with El Tor strains (Kaper et al 1995) However; investigators have reported *V. cholerae,* O1 biotype El Tor Ogawa as the major cause of cholera outbreak in Nepal (Ise et al 1996, Tamang et al 2005, Bhandari 2009, Karki et al 2010)

All *V. cholerae* O1 isolated from water samples were multi drug resistant (MDR). Similar type of result was reported by Shrestha et al 2015. Sameer et al 2014 reported the rate of multidrug resistance to be 100 % which is similar to our findings but Pappu et al 2016 had reported only 6.45 % of the strains of *Vibrio cholera* to be multidrug resistant. Multidrug resistant *Vibrio cholerae* has been reported from all around the world including Pakistan, Bangladesh, India and Nepal (Shrestha et al 2015). Indiscriminate use of antibiotics in the treatment of cholera and other enteric diseases has led to the emergence of antibiotic resistance among *V. cholerae*. Epidemics of MDR cholera (both classical and El Tor biotypes) have been reported worldwide (Mandal et al 2011)

In present study all isolates of *V. cholerae* were 100% sensitive to Ampicillin, Amikacin, Azithromycin, Ceftriaxone, Chloramphenicol, Doxycycline, Erythromycin, Imipenem, Levofloxacin and Norfloxacin while most of the isolates were sensitive to Tetracycline (87.5%), Ciprofloxacin (87.5%), Cefotaxime (75%), Ofloxacin (75%), and Gentamicin (62.5%). All strains were 100% resistant to Nalidixic Acid, Nitrofurantoin, Ceftazidime and Co-

Trimoxazole. In a study by Karki et al 2010, 100% sensitivity was observed for Ampicillin and 100% resistivity was observed for cotrimoxazole which supports my findings but in contrast Pappu et al 2016 ; Shrestha et al 2015 ; Das et al 2011 had reported the isolates to be 100% resistant to Ampicillin. Karki and Tiwari 2007 reported 100% resistant to Ampicillin contrast to our findings and 97.8% isolates were susceptible to ciprofloxacin which was similar with our findings. Sameer et al 2014 and Bhandari et al 2013 reported all strains were resistant to nalidixic acid and Co-Trimoxazole which support our findings.

Pappu et al 2016 reported that all isolates were sensitive to tetracycline, doxycycline, levofloxacin and azithromycin, while most of the isolates were sensitive to chloramphenicol (96.77 %) and ciprofloxacin (93.55 %) which support our result. Shrestha et al 2015 reported that the majority of *V. cholerae* strains were identified as susceptible to tetracycline (100 %), ciprofloxacin (90.9 %), cefotaxime (81.8 %) and chloramphenicol (90.9 %) which is nearly similar with the results of our findings. However, in contrast to our study; Shah et al 2012 showed that 81.8 % of strains were resistant to tetracycline. *Garg* et al 2000 reported high level resistance to chloramphenicol in India. This result was contrast to our findings. Generally, fluoroquinolones are highly effective for treatment of cholera but recently fluoroquinolone resistant strains of *V. cholera* have been reported from India (Shrestha et al 2015*).*

Regarding other findings of *Vibrios* in our study after *V. cholerae* (22.2%), next finding were *V. parahaemolyticus* (19.5%), then *V. furnissii* and *V. vulnificus* (16.7%), *V. fluvialis* (13.9%) and lastly *V. alginolyticus* and *V. metschnikovii* were (5.5%). But in contrast to our result, Rai K R et al 2012 reported 43.5% of *V. cholerae*, next to which were *V. vulnificus* (23.9%) then *V. parahaemolyticus* (10.9%), *V. furnissii* (10.8%), *V. fluvialis* (6.5%) and *V. alginolyticus* (4.3%). In the study of Alphanso 2013 *V. cholerae* occurred in the highest frequency of 54% followed by V. vulnificus (41%) and V. alginolyticus (5%). This was in accordance with similar studies from India (Otta et al 1999; Vaseeharan & Ramasamy 2003).

All the isolates of *V. Parahaemolyticus* were 100% sensitive to Amikacin, Azithromycin, Ceftriaxone, Chloramphenicol, Ciprofloxacin, CoTrimoxazole, Doxycycline, Imipenem, Levofloxacin, Nalidixic Acid, Nitrofurantoin, Norfloxacin, Ofloxacin, and Tetracycline. This was followed by Cefotaxime 85.7%, Gentamicin 85.7%. While all strains were 100% resistance to Ampicillin followed by Ceftazidime 71.4% and Erythromycin 71.4%. Han et al 2007 reported isolates of *V. Parahaemolyticus* were resistant to ampicillin and susceptible to cefotaxime, ciprofloxacin and tetracycline which support our findings.Similarly, Chijioke et al 2014 reported isolates were sensitive to Ofloxacin (100%), Ciprofloxacin (100%) and Gentamicin (89.5%) which was similar to our results.

In this study *V. furnissii* were found to be 100% sensitive to Amikacin, Azithromycin, Cefotaxime, Ceftriaxone, Chloramphenicol, Ciprofloxacin, CoTrimoxazole, Doxycycline, Erythromycin, Gentamicin, Imipenem, Levofloxacin, Nalidixic Acid, Nitrofurantoin, Norfloxacin, Ofloxacin and Tetracycline. While all strains were 100% resistance to Ampicillin and Ceftazidime. K.Immaculate et al 2017 reported V. Furnissii were resistance against Ceftazidime which was similar to our findings.

Zanetti et al 2001; Manjusha et al 2005 and Elhadhi 2012 reported *V. vulnificus* isolates were resistant to Gentamicin, Amikacin, Ampicillin and isolates were susceptible to Norfloxacin, Cotrimoxazole and Tetracycline. On the other hand in this study *V. vulnificus* were 100% sensitive to Amikacin, Ampicillin, Azithromycin, Cefotaxime , Ceftriaxone, Chloramphenicol, Ciprofloxacin, CoTrimoxazole, Doxycycline, Gentamicin, Imipenem, Levofloxacin, Nitrofurantoin, Norfloxacin, Ofloxacin, and Tetracycline and 100% resistance to Ceftazidime followed by Erythromycin 66.7%. Nalidixic Acid was 50% sensitive. Similarly Chijioke et al 2014 reported isolates were 100% sensitive to Ciprofloxacin, Gentamicin and Ofloxacin.

In this study isolates of *V. fluvialis* were 100% sensitive to Amikacin, Azithromycin, Cefotaxime, Ceftriaxone, Chloramphenicol, Ciprofloxacin, CoTrimoxazole, Doxycycline, Gentamicin, Imipenem, Levofloxacin, Nitrofurantoin, Norfloxacin, Ofloxacin and Tetracycline. While all isolates

were 100% resistance to Ampicillin, Ceftazidime. Nalidixic Acid and Erythromycin were 60% resistant. Chijioke et al 2014 reported isolates of *V. fluvialis*to be 100%, 91.3%, 90% and 86.7% sensitive to Ciprofloxacin, Ofloxacin, Gentamicin and Ampicillin respectively.

V. alginolyticus were 100% sensitive to Amikacin, Azithromycin, Cefotaxime, Ceftriaxone, Chloramphenicol, Ciprofloxacin, CoTrimoxazole, Doxycycline, Gentamicin, Imipenem, Levofloxacin, Norfloxacin Nitrofurantoin, Ofloxacin and Tetracycline. While 100% resistanceto Ampicillin, Ceftazidime, Nalidixic Acid and Erythromycin was 50 sensitive. Alphanso 2013 reported all strains of V. alginolyticus to be 100% resistant to ampicillin which was similar with our findings.

V. metschnikovii were 100% sensitive to Amikacin, Azithromycin, Ceftriaxone, Chloramphenicol, Ciprofloxacin, CoTrimoxazole, Doxycycline, Gentamicin, Imipenem, Levofloxacin, Nitrofurantoin, Norfloxacin, and Tetracycline. While all isolates were 100% resistance to Ampicillin, Cefotaxime, Ceftazidime, Erythromycin and Nalidixic Acid. Ofloxacin was 50% sensitive. Chijioke et al 2014 reported isolates of *V. metschnikovii* were 100% sensitive to Gentamicin and Ciprofloxacin, 50% sensitive to Ampicillin and Ofloxacin.

From our study it can be concluded that contamination of vibrio sps. in drinking water is a significant problem not only in Nepal but also in other south Asian countries and other parts of the world. MDR is a crucial and dangerous health issue for human beings (Prescott et al 1999). It is also associated with epidemic outbreaks in various parts of the world (Levy 2001; Canton et al 2003). As environmental waters are important reservoirs for *Vibrios*. The effective surveillance of this pathogen can help in preventing further infections.

CHAPTER VI

6 CONCLUSIONS AND RECOMMENDATION

6.1 CONCLUSIONS

The present study was done in Sunsari technical college from June 2016 to November 2016 to isolate and identify the medically important *vibrios* from different water samples, to study antibiotic sensitivity of the bacterial isolates and to compare the quality of tap water, bottled water and other sources. Total 36 medically important Vibrios were identified. Among the 20 other sources of water samples collected by Moore's technique a total of 32 medically important *Vibrio* species were detected. Out of 30 tap water samples only 4 *Vibrios* were isolated. None any isolation of *Vibrios* was found in bottled water samples.

There were significant differences among the three groups of water samples in temperature and pH. Out of total samples included in our study, 13.3% of tap water and 95% of other sources of water were found to be contaminated with vibrios whereas the bottled water samples showed no contamination.

Altogether Seven different species of *Vibrios*; *V. cholerae, V. parahaemolyticus, V. vulnificus, V.alginolyticus, V.furnissii, V.fluvialis* and *V.metchnikovii* were identified. *V. Cholerae* was found to be the predominant species (22.2%). Next to it was *V. Parahaemolyticus* (19.5%), and followed *V. vulnificus* and *V.furnissii* (16.7%). *V. fluvialis* was found 13.9% and lastly *V.alginolyticus* and *V.metchnikovii* was found only 5.5%.

All *V. cholerae* strains isolated in this study were found Multi Drug Resistant. Azithromycin, Ciprofloxacin, Chloramphenicol, Doxycycline, Erythromycin, Levofloxacinand and Tetracycline were found still more potent antibiotics against *Vibrios* isolated during the study for preliminary treatment of cholera. The presence of medically important *Vibrios* in environmental water samples of eastern region of Nepal should not be ignored as it may cause outbreak of cholera and other Vibrio infections anytime in future.

6.2 RECOMMENDATIONS

1. Drinking water of tap due to finding of medically important *vibrio*sis majorpublic health problem in easten region of Nepal and should be addressed to avoid the possible water related outbreaks.

2. The antibiotics for the treatment of the cholera should be selected on the basis of the local antimicrobial susceptibility patterns of the *Vibrio cholerae*.

3. Azithromycin, Ciprofloxacin, Chloramphenicol, Doxycycline, Erythromycin, Levofloxacin and Tetracycline are suggested for preliminary treatment of cholera in Nepal.

4. For prevention of *Vibrios* infection proper sanitation, supply and consumption of safe drinking water and practice of personal hygiene is recommended.

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APPENDIX- A

List of equipment and materials used during the study

A. Equipments:

Autoclave	Oven
Incubator	Refrigerator
Microscope	Deep freeze
Digital balance	Distillation plant
Membrane filtration Jar	Water bath

B. Microbiological media (Hi-Media)

Alkaline peptone water	TCBS Agar
Nutrient Agar	Mueller- Hinton Agar
Simmon's citrate Agar	Urease Agar
SIM	TSI Agar
Nitrate broth	MRVP
Sugars	Aminoacids
NaCl solutions	

C. Chemicals/ Reagents

3% hydrogen peroxide	Oxidase reagent
Crystal violet	Gram's iodine
Acetone	Safranine
Kovac's reagent	Normal saline
Sulphalinic acid reagent	Alpha napthylamine reagent
zinc dust	

D. Miscellaneous

Glasswares	Forceps
Inoculating loops	Swab stick
Distilled water	Burner
Sticker	Cotton
Blotting paper	Dropper
Mineral oil	Immersion oil

APPENDIX- B

Model Sample Collection Form

Sampling Data:

Locality

Place

Source

Date of collection

Time of collection

Date of analysis

Time of analysis

Laboratoty sample No.:

Results:

PH

Temprature

Organism grown

Signature Date

APPENDIX- C

Microbiological Technique& Biochemical tests

A. Preparation of smear and Gram staining

Preparation of smear:

- A drop of sterile normal saline was put on a clean labeled glass slide.
- A portion ofcolony from culture plate was picked up with a sterile straight nichrome wire and emulsified with normal saline and spread evenly.
- The smear was air dried, and then heat fixed by passing the dried slide three times slowly through the flame and allowed to cool.

Gram stain:

- The heat fixed smear was covered with crystal violet stain for one minute.
- The slide was rinsed with clean water.
- All the water was tipped off and the smear was covered with iodine solution for one minute.
- Iodine was washed off with clean water.
- Decolorization was done rapidly with acetone and the smear was wash immediately with clean water.
- The counter stain was done with 0.1% carbol-fuchsin or safranin for 30 seconds.
- The stain was washed off with clean water.
- The smear was air dried.

Observation:

Gram stained smear was examined microscopicallyunder oil immersion at 100X. Findings were examined.

B. Catalase Test:

A small amount of a culture from nutrient agar plate was taken in a clean glass slide with a wooden applicator stick and about 2-3 drops of 3% hydrogen peroxide was put on the surface of the slide. The positive test was indicated by the formation of active bubbling of the oxygen gas. A false positive reaction may be obtained if the culture medium contains catalase (e.g. Blood Agar) or if an iron wire loop was used. The culture should not be more than 24 hours old.

C. Oxidase Test:

A stripe of filter paper is soaked with a little freshly prepared 1% solution of tetramethyle-p-phenylenediaminedihydrochloride and then at once used by rubbing a speck of culture on it with a loop. A positive reaction was indicated by an intense deep purple blue, appearing within 5-10 seconds

D. Preparation of inoculums for biochemical Tests:

With the help of a sterile straight wire, an isolated colony of organism to be tested from the culture plate was touched and inoculated in a sterile bottle containing alkaline peptone water and incubated at 37ºC for 4-6 hours.

E. Citrate utilization Test:

The sterile straight wire loop was dipped in the inoculums prepared for the biochemical test and then inoculated in a tube containing the Simmon's citrate media slope. This was incubated at 37 ºC overnight. The result was read as positive if there was streak of growth and colour of the medium changed to blue and negative if there was no change in the original green colour.

F. Triple Sugar Iron (TSI) Test:

The TSI agar is used to determine the ability of an organism to utilize specific carbohydrate incorporated in the medium (glucose, sucrose and lactose in concentrations of 0.1%, 1.0% and 1.0% respectively) with or without the production of gas along with determination of possible hydrogen sulphide production.

The sterile wire was dipped in the prepared broth and was inoculated in the test tube containing the slope of TSI. This was incubated at 37 ºC overnight. Colour changed to red indicated alkaline reaction and yellow indicated acidic reaction. Results interpreted as follows:

Red slant/ no change butt-glucose, lactose and sucrose non fermented.

Red slant/ Yellow butt- glucose fermented.

Yellow slant/ Yellow butt-glucose and sucrose or glucose and lactose or glucose, lactose and sucrose fermented.

A black precipitate in the butt indicates production of sulphide, bubbles or crack in the tube indicate the production of gas.

G. Urease Test:

The organism from the inoculums was inoculated in the Christensen's Urease media in the tube with the help of straight wire and incubated at 37 ºC overnight. If the organism produces urease the medium will turn into pink which was taken as a positive result. In negative result there was no change in colour.

H. SulphideIndole Motility (SIM) Test:

The sterile wire was dipped in the inoculum broth and inoculated into tube containing SIM medium and incubated at 37 ºC overnight. The haziness around the line of inoculums was taken as organism to be motile. To this overnight growth in SIM media a drop of Kovac's

reagent was added and shaken gently. Development of red coloured ring indicates presence of indole.

I. Nitrate test:

Nitrate broth was inoculated with broth inoculums of test organisms and incubated appropriate temperature for 24 to 48 hours. To this incubated broth one dropperfull of sulfanilic acid and one dropperfull of a α-naphthylamine was added. Interpretation of the result was as below

Nitrate Reduction Positive: (Redcolour after addingsulfanilic acid + alpha-naphthylamine; If no colour and even after adding zinc- No colour)

Nitrate Reduction Negative: (No colour after addingsulfanilic acid + alpha-naphthylamine followed by Redcolour after adding zinc)

J. Vogesproskauer (VP) Test:

The prepared inoculums broth of testorganism was inoculated into 2 ml of MRVP medium and was incubated at 37 ºC for 24 hours. After incubation, 0.6 ml of 5% alphanapthol was added and shaken vigorously. Again 0.2 ml of 40% of KOH was added and shaken vigorously and kept for 5 to 10 minutes. Development of pink to cherry red colour was taken as positive. No colour change was taken as negative.

K. Aminoacid Decarboxylase Test:

After inoculation into lysine, arginine and ornithine a 4 to 5 mm layer of sterile mineral oil was added to each tube and control tube of amino acid decarboxylase media. Positive decarboxylation is indicated by change from yellow to violet or reddish violet.

L. String Test:

A clean grease free slide was taken and a drop of 0.5% bile salt(Sodium deoxycholate or Sodium taurocholate) was dropped.An isolated colony of the bacterium was emulsified using an inoculating loop.Keeping on rubbing the loop vigorously for 2-3 mins until it appears viscous andgently the loop was pulled upwards from the slide. Formation of a thread like mucoid string indicates positive test.

APPENDIX- D

Composition and Preparation of Culture Medias & Reagents

The culture media was obtained from Hi-Media laboratories Pvt. limited, Mumbai. India.

1Alkaline peptone water (M618)

Ingredients	gms/litre
Peptic digest of animal tissue	10
Sodium chloride	10
Final pH (at 25ºC)	8.4±0.2

Preparation: 20 grams of the alkaline peptone water medium was suspended in 1000 ml of distilled water .pH was adjusted to 8.4±0.2 and was despense as desired and sterilized by autoclaving at 121ºc for 15 minutes at 15 lbs pressure.

2. Simmon's citrate agar (M099)

Ingredients	Gms/litre
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromothymol blue	0.08
Agar	15.0
Final pH (at 25°C)	6.8±0.2

Preparation: 24.28 grams of the medium wassuspended in 1000 ml distilled water. The medium was heated, to boiling, to dissolve completely. pH was adjusted and distributed in test tubes and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.After autoclaving tubes containing medium were tilted to form slant.

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3. TCBS Agar (M870)

Ingredients	Gms/litre
Peptone	10.0
Yeast extract	5.0
Sodium citrate	10.0
Sodium thiosulphate	10.0
Sodium cholate	3.0
Oxgall	5.0
Sucrose	20.0
Sodium chloride	10.0
Ferric citrate	1.0
Bromothymol blue	0.04
Thymol blue	0.04
Agar	15.0

Preparation: 89.08 grams of TCBS medium was suspended in 1000 ml of distilled water. The medium was dissolved completely by boiling. After cooling to 50°C medium was poured into sterile Petri plates. Autoclave was not required. Final pH (at 25°C) 8.8±0.2

4. Mueller Hinton Agar (M173)

Ingredients	Gms/litre
Beef, Infusion form	300.0
Casein acid hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH (at 25°C)	7.4±0.2

Preparation: 38 grams of medium was suspended in 1000 ml distilled water and the medium was warmed to dissolve.Then medium was sterilized by autoclaving at15 lbs pressure (121°C) for 15 minutes. After cooling it to 45-50°C medium was pourd into sterile Petri plates.

5. Triple Sugar Iron Agar (M021)

Ingredients	Gms/litre
Peptic digest of animal tissue	10.0
Casein enzymichydrolysate	10.0
Yeast extract	3.0
Beef extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Sodium chloride	5.0
Ferrous sulphate	0.2
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12.0
Final pH (at 25°C)	7.4±0.2

Preparation: 64.52 grams of powder was suspended in 1000 ml distilled water. The medium was completelydissolve by boiling .Mixture was distributed into test tubes and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The medium was allowed to set in sloped formwith a butt about 1 inch long.

6. SIM Medium (M181)

SIM Medium is recommended for determination of hydrogen sulphide production, indole formation and motility test.

Ingredients	Gms/litre
Beef extract	3.000
Peptic digest of animal tissue	30.000
Peptonized iron	0.200
Sodium thiosulphate	0.025
Agar	3.000
Final pH (at 25°C)	7.3±0.2

Preparation: 36.23 grams of the medium was suspended in 1000 ml distilled water and dissolved the medium completely by boiling. Then it was distributed in test tubes to a depth of about 3 inches and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Allow the tubes to cool in an upright position.

7. Christensen Urea Agar (M112)

Ingredients	Gms/litre
Peptone	1.0
Dextrose	1.0
Sodium chloride	5.0
Dipotassium phosphate	1.2
Mono-potassium phosphate	0.8
Phenol Red	0.012
Agar	15.0
Final pH (at 25°C)	7.4±0.2

Preparation: 24 grams of the medium was suspendedin 950 ml of distilled water and sterilized by autoclaving at 121ºC for 15 minutes. After cooling to about 45ºC, 50 ml of 40% urea was added and mixed well. Then 3 ml was dispensed in test tube and set at slant position.

8. Kovac's reagent for Indole

Composition:

P-dimethyleaminobenzaldehyde	5 g
Isoamyl alcohol	75 ml
Conc. Hydrochloric acid	25 ml

Preparation: Aldehyde was dissolved in alcohol by gently warming in water bath (50ºC-55ºC). It was then cooled and acid was added with care. It was kept protected from light and stored at 4ºC.

9. Oxidase reagents

Composition:

Tetramethyl p- phenylenediaminedihydrochloride (TPD)	1g
Distilled water	100ml

Preparation: 1 gm of TPD was dissolved in 100ml of distilled water. It was stored in dark coloured bottle and stored at 4ºC.

10. Crystal Violet:

Composition

Crystal violet	20.0 gm
Ethyl alcohol	95 ml
Ammonium oxalate	9.0 gm
Distilled water	1000 ml

Preparation: 20 gm of crystal violet was transferred to a clean brown bottle; Then 95 ml of ethyl alcohol was added and mixed until the dye was completely dissolved. To the mixture, 9 gm of ammonium oxalate dissolved in 200 ml of distilled water was added. Finally the volume was made 1 litre by adding distilled water. Before use it was stored for 24 hours.

11. Gram's Iodine

Composition:

Iodine	1.000 gm
Potassium iodide	2.000 gm
Distilled water	300.000 ml

Preparation: Potassium iodide was dissolved in distilled water. Then iodine was addedto it and mixed until it was dissolved completely.

12. Sugar media

To sterilized peptone water 1% of required sugar was added. Then 1% Andrade's indicator was added. It was distributed in sterile test tubes containing inverted Durham's tube and sterilized in water bath.

13. McFarland Standard

0.1 ml of 1% Bacl2 was added to 9.9 ml of 1% H2SO4 with constant stirring. The McFarland Standard was thoroughly mixed to ensure that it is evenly suspended.

APPENDIX- E

Table of Identifications of bacteria

Biochemical characteristics of vibrios

Gram stain: Characteristically curved or comma-shaped Gram negative rods

Test	V. cho	V. flu	V. alg	V. fur	V. Mets	V. para	V. vul
TCBS	Y	Y	Y	Y	Y	G	G
Oxidase	+	+	+	+	-	+	+
Catalase	+	+	+	+	+	+	+
Motile	+	+	+	+	+	+	+
Indole	+	+	+	-	-	+	+
Citrate	+	+	+	+	+	+	+
Urease	-	-	v	-	-	V	-
Gas	-	-	-	V	-	-	-
Nitrate	+	+	+	+	-	+	+
VP	v	-	+	-	+	-	-
string test	+	-	-	-	-	-	-
Arginine	-	+	-	+	-	-	-
Lysine	+	-	+	-	v	+	+
Ornithine	+	-	+	-	-	+	+
Sucros	+	+	+	+	+	-	-
Lactose	-	-	-	-	v	-	+
Glucose	+	+	+	+	+	+	+
Arabinose	-	+	-	+	-	V	-
Mannitol	+	+	+	+	+	+	v
Growth on 10% Nacl	-	-	+	-	-	-	-
Growth on 8% Nacl	-	v	+	-	v	+	-
Growth on 6% Nacl	-	+	+	+	+	+	+
Growth on 0% Nacl	+	v	-	V	v	-	-
Growth on ethanol	-	+	v	+	-	V	-
H2S	-	-	-	-	-	-	-

V: variable among strains, Y: yellow, G: green, +: positive test, -: negative test, S: susceptible, R: resistant.

Differentiation between Vibrio cholerae Biotypes

TEST	CLASSIAL	ELTOR
String test	+	+
B-haemolytic on sheep blood agar	-	+
VP test	-	+
Chicken RBC agglutination	-	+
Susceptibility to 50 U polymyxin B	S	R

+: positive test, -: negative test, S: susceptible, R: resistant.

APPENDIX- F

STATISTICAL PACKAGE FOR THE SOCIALN SCIENCES (SPSS) PRODUCTS

Variables	Type of water			Total	P value	Remarks
	Tap (n=30)	Bottle (n=10)	Others sources (n=20)			
Temp	15.87 ± 4.00	17.2 ± 0.79	20.75 ± 4.08	17.72 ± 4.27	<0.001	Sig
Ph	7.22 ± 0.40	7.05 ± 0.21	7.75 ± 0.46	7.37 ± 0.48	<0.001	Sig

grth * TYPE OF WATER Crosstabulation

			TYPE OF WATER			Total
			1	2	3	
grth	.00	Count	1	10	26	37
		% within TYPE OF WATER	5.0%	100.0%	86.7%	61.7%
	1.00	Count	19	0	4	23
		% within TYPE OF WATER	95.0%	.0%	13.3%	38.3%
Total		Count	20	10	30	60
		% within TYPE OF WATER	100.0%	100.0%	100.0%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	41.316[a]	2	.000
Likelihood Ratio	48.380	2	.000
Linear-by-Linear Association	30.527	1	.000
N of Valid Cases	60		

a. 1 cells (16.7%) have expected count less than 5. The minimum expected count is 3.83.

Descriptives

		Minimum	Maximum
temp	1	11	27
	2	16	18
	3	11	26
	Total	11	27
ph	1	6.7	8.3
	2	6.8	7.4
	3	6.7	8.3
	Total	6.7	8.3

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
temp	Between Groups	345.321	2	172.661	12.022	.000
	Within Groups	1005.309	70	14.362		
	Total	1350.630	72			
ph	Between Groups	4.526	2	2.263	14.582	.000
	Within Groups	10.863	70	.155		
	Total	15.389	72			