CHAPTER-I

INTRODUCTION

Aquatic environment is the natural lodgment for *Vibrio* species (Ortigosa et al., 1994, Farmer et al., 1992 and Heidelberg et al., 2002). They exist in association with a number of vertebrate species like fish, zooplanktons and in some instances plants (Islam et al., 1996). Of them, many species have acquired increasing importance because of the association of several of its members with human disease. The most feared of the *Vibrio* species is *V. cholerae*, the causative agent of cholera, a devastating disease of global significance. Other important vibrios of medical importance are *V. parahemolyticus, V. vulnificus, V. mimicus*, and to a lesser extent *V. fluvialis, V. furnissii, V. hollisae, and V. damsela*. Many studies have also implicated *V. alginolyticus* and *V. metschnikovii* in human disease, although their complete significance has not yet been established. The virulence of all medically important *Vibrios* is aided by a variety of traits that help breach human defenses (Farmer et al., 1992; Chakraborty et al., 1997). Among them, *V. cholerae* alone is responsible high morbidity and mortality (WHO, 2007 and CDC, 1993). Cholera (caused by toxigenic *V. cholerae* O1 and O139 Bengal strain) outbreak is common in most of the developing countries (Janda et. al., 1988). All these cases high morbidity and mortality as well as outbreaks are associated with poor environmental sanitation resulting to contamination of water and foods.

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 (Pokhrel and Viraraghavan., 2004). First bacteriologically confirmed epidemic cholera (subject of an international report) from Nepal was reported in 1958 (Abou-Gareeb et al, 1961). There are many reports of the isolation of *V. cholera*e from the

clinical samples including from cases of outbreaks (Ono *et al*., 2001, NPHL Annul Report; 2005).

In Nepal, outbreaks of cholera occur each year (Pokharel and Kubo et. al., 1996, Tamang et al.,2005, Bistha and Gautam, 1993) with the beginning of summer/rainy season (continues to post rainy season) and mainly associated with *V. cholera* 01 biotype El Tor Ogawa (Yamamoto *et al*., 1995, NPHL Annual Report 2004-2006). This is true even in the Katmandu Valley where the capital city is located (Phokharel and Kubo, 1996).

Bagmati River is the biggest river running across the Kathmandu Valley. This river also has religious importance especially for *Hindu* devotees. The devotees take holy bath in the river water and also drink the water particularly at *Pashupati Nath* temple (the famous god *Shiva* temple) area. The devotees also carry the river water with them and distribute to family member, relatives and also in the community. The river water is also being used to clean the green and leafy vegetables before taking to market. However, during recent years, this river has been heavily polluted and has become an urban drainage and site for waste dumping resulting into river of sewerage. These statements are supported by the reports of isolation of *V. cholerae* and other medically important *Vibrios* from the sewerage of Kathmandu Valley during peak rainy season (Rai et al., 2009). Environmental surveillance plays an important role in cholera control (Barrett et al., 1980). Cholera outbreaks can be predicted by detecting *V. cholerae* O1 and vibriophages in sewage water (Madico *et al.*, 1996). Besides the surveillance for cholera *Vibrios* from sewerage this study is attempted to isolate including *V. cholerae* O1, Non-O1 and other medically important *Vibrios* in sewage samples collected from different locations of Kathmandu Valley during winter season.

CHAPTER-II

OBJECTIVES

2.1 General objectives

To detect and characterize medically important *Vibrios* from the sewage of Kathmandu Valley during winter season

2.2 Specific objectives

i. To detect medically important *Vibrio* spp from the sewage of Kathmandu Valley.

ii. To detect the *V. cholerae* from the sewage of Kathmandu Valley.

iii. To characterize *V. cholerae* by biotyping and serotyping.

iv. To describe the antibiotics resistance pattern of isolates.

CHAPTER-III

LITERATURE REVIEW

3.1 Genus *Vibrio*

The genus *Vibrio* consists of Gram-negative straight or curved rods, motile by means of a single polar flagellum. *Vibrios* are capable of both respiratory and fermentative metabolism. O_2 is a universal electron acceptor; they do not denitrify. Most species are oxidase-positive. In most ways *Vibrios* are related to enteric bacteria, but they share some properties with *Pseudomonads* as well. The Family Vibrionaceae is found in the "Facultatively Anaerobic Gram-negative Rods" in Bergey's Manual (1986), on the level with the family Enterobacteriaceae. In the revisionist taxonomy of 2001 (Bergey's Manual), based on phylogenetic analysis, Vibrionaceae, Pseudomonadaceae and Enterobacteriaceae are all landed in the Gammaproteobacteria. *Vibrios* are distinguished from enterics by being oxidase-positive and motile by means of polar flagella. *Vibrios* are distinguished from *Pseudomonads* by being fermentative as well as oxidative in their metabolism (Bergey's Manual., 1986).

3.2 Distribution of *Vibrios* in Environment

Vibrios are highly abundant in aquatic environments, including estuaries, marine coastal waters and sediments, and aquaculture settings worldwide. Several cultivation-dependent and independent studies have showed that *Vibrios* appear at particularly high densities in and/or on marine organisms, e.g., corals, fish, molluscs, seagrass, sponges, shrimp and zooplankton (Falzano et al., 1999, Diggles et al., 2000 and Vandenberghe et al., 1998, 1999, 2003). Large numbers of *Vibrio* and *Photobacterium* attached to the external surface of zooplankton have also been reported (Heidelberg, 2002). It has been suggested that a close partnership occurs between these bacteria and zooplankton.

The bio-film formation by *Vibrio* spp on the exoskeletons of these crustaceans and other marine organisms may in fact constitute a strategy to survive during starvation and/or other environmental stresses (Lipp et al., 2000). In biofilms these bacteria can use trapped and absorbed nutrients, resist antibiotics, and establish favorable partnerships with other bacteria or hosts. Copepods may, in turn, feed on these bacteria. *V. cholerae* moves along and attaches to surfaces with the aid of the flagellum and pili, this may act as adhesins. In as little as 15 min, *V. cholerae* forms microcolonies on surfaces; subsequently it produces exopolysacharides, which stabilize the pillars of the biofilm. A 15-m-thick biofilm, with pillars of cells and water channels, is formed within 72 h (Moorthy et al., 2004, Watnick et al., 1999, 2000, 2001). According to these authors, the strong ability of *V. cholerae* E1 Tor to form densely packed biofilms in the environment gives a survival advantage to this organism, which is the predominant cause of cholera. Because *V. cholerae* is closely associated with plankton, it is assumed that cholera outbreaks are linked with planktonic blooms and the sea surface temperature, and so such outbreaks may be predicted by monitoring these parameters by e.g., remote sensing (Lipp et al., 2002). The wide ecological relationships and ability to cope with global climate changes may be a reflection of the high genome plasticity of Vibrios (Lipp et al., 2002). Recently, a number of reports have highlighted the pathogenic potential of Vibrios toward humans and marine animals (e.g. corals, gorgonians, and shrimp), which may be coupled with rising of sea water temperature due to global warming (Kushmaro et al., 2001, Martin et al., 2002 and Rosenberg et al., 2002).

3.3 Medically important Vibrios

V. cholerae, *V. parahaemolyticus*, and *V. vulnificus* are serious human pathogens (Wachsmuth et al., 1994, CDC, 1999 and Finkelstein, 2002). *V.*

cholerae alone is responsible high morbidity and mortality (WHO, 1993, CDC, 1993). Cholera (caused by toxigenic *V. cholerae* O1 and O139 Bengal strain) outbreak is common in most of the developing countries (Janda et al., 1998 and Nair et al., 1994). Besides these, strains of the organism classified as *V. fluvialis* were first described by Furniss et al., in 1977.

Their organisms, designated group F, were isolated in 1975 from a patient with diarrhea in Bahrain, from patients with diarrhea in Bangladesh. In 1983 Brenner et al., confirmed the evidence *V. furnissii* which cause acute gastroenteritis.

The genus vibrio contains a number of species, of which 11 have a proven association with intestinal and extra intestinal diseases. The main species of importance with respect to foods are, *v. cholerae, V. parahaemolyticus* and *V. vulnificus.* Seafood is the most common vehicle for food borne infection, although water is historically associated with *V. cholerae* infection. *V. vulnificus* is also an important cause of wound infections. (Oliver et al., 1995).

3.4 Sewer system in Kathmandu Valley

Sewer is a drain which carries sewage. Bagmati River is the main drainage of the Kathmandu Valley, which originates from the Shivapuri Lekh (Baghdwar) situated to the north of the valley and it drains out all of the surface water of the valley through the only one exit along the southwestern edge of the valley at Chobhar gorge. The final outlet of the river is near the Katuwal Daha where the altitude of river bed is only 1220m. Major tributaries of the Bagmati River are the Bishnumati (flows North to South), the Manohara (flows Northeast to Southwest), the Dhobi Khola (flows N to S), the Hanumante (flows East to West), the Godawari (flows S to N), the Nakhu Khola (flows S to N), the Kodku Khola (flows S

to N), the Balkhu Khola (flows NW to SE) and the Bosan Khola (flows NW to SE).

The overall drainage pattern forms a typical example of the centripetal drainage system in the world. All the tributaries trending in different directions drain inwards to the center of the valley and join to the Bagmati River. The drainage basin has more or less indented circular shape and it is spread over an area of about 585 sq. Km (JICA, 1990).

3.5 Status of sewer system in Kathmandu Valley

In the Kathmandu Valley, around 70% of the households dispose their excreta directly into the sewer line while remaining 30% of the households still depend on onsite systems such as pit latrines and septic tanks. Onsite sanitation systems are prevalent mostly in the peri-urban areas of the Kathmandu Valley. Also, 30% of households in urban areas of Lalitpur, 8% in Bhaktapur and 18% in Kathmandu Districts still use septic tanks for disposal of excreta while in the peri-urban areas more than 50% of the households use such onsite systems (UN report).

3.6 Cholera

"Cholera is characterized by the sudden onset of effortless vomiting and profuse watery diarrhea with rapid dehydration and hypovolumic shock, which may cause death in 12-24 hours" (Greenwood 2008). Cholera is caused by toxigenic *V. cholerae*, a member of the family Vibrionaceae, is a facultatively anaerobic, Gram-negative, non-spore-forming curved rod, about 1.4–2.6μm long, capable of respiratory and fermentative metabolism; it is well defined on the basis of biochemical tests and DNA homology studies (Baumann et al., 1984). The bacterium is oxidase-positive, reduces nitrate, and is motile by means of a single, sheathed, polar flagellum. Growth of *V. cholerae* is stimulated by addition of 1%

sodium chloride (NaCl). However, an important distinction from other *Vibrio* spp is the ability of *V. cholerae* to grow in nutrient broth without added NaCl.

3.6.1 Cholera pathogenesis overview

The pathogenesis of *V. cholerae* infection serves as a paradigm for many classic features of bacterial virulence. *V. cholerae* is both an environmental organism and a human intestinal pathogen. The organism produces a prototypical enterotoxin. A pilus is required for colonization of the small intestine.Genes encoding virulence factors are clustered in two regions of the chromosome, on a pathogenicity island and on a filamentous bacteriophage; this suggests that horizontal gene transfer may lead to the development of new epidemic strains.Multiple virulence genes are coordinately regulated by environmental conditions. Biofilm formation is important in both infectivity and environmental persistence (Butterton et al., 1995).

3.6.2 Susceptibility

About one hundred million bacteria must typically be ingested to cause cholera in a normal healthy adult. This dose, however, is less in those with lower gastric acidity (for instance those using proton pump inhibitors. Children are also more susceptible, with two- to four-year-olds having the highest rates of infection. Individuals' susceptibility to cholera is also affected by their blood type, with those with type O blood being the most susceptible. Persons with lower immunity, such as persons with AIDS or children who are malnourished, are more likely to experience a severe case if they become infected. However, it should be noted that any individual, even a healthy adult in middle age, can experience a severe case, and each person's case should be measured by the loss of fluids,

preferably in consultation with a doctor or other health worker (WHO 2007).

3.6.3 Transmission

Cholera is typically transmitted by either contaminated food or water. In the developed world, seafood is the usual cause, while in the developing world it is more often water. Cholera has been found in only two other animal populations: shellfish and plankton.

People infected with cholera often have diarrhea, and if this highly liquid stool, colloquially referred to as "rice-water" or "faucet butt", contaminates water used by others, disease transmission may occur. The source of the contamination is typically other cholera sufferers when their untreated diarrheal discharge is allowed to get into waterways, groundwater or drinking water supplies. Drinking any infected water and eating any foods washed in the water, as well as shellfish living in the affected waterway, can cause a person to contract an infection. Cholera is rarely spread directly from person to person. Both toxic and nontoxic strains exist. Nontoxic strains can acquire toxicity through a temperate bacteriophage. Coastal cholera outbreaks typically follow zooplankton blooms, thus making cholera a zoonotic disease. Bacteria, when consumed, do not survive the acidic conditions of the human stomach.The few surviving bacteria conserve their energy and stored nutrients during the passage through the stomach by shutting down much protein production. When the surviving bacteria exit the stomach and reach the small intestine, they need to propel themselves through the thick mucus that lines the small intestine to get to the intestinal walls, where they can thrive. *V. cholerae* bacteria start up production of the hollow cylindrical protein flagellin to make flagella, the cork-screw helical fibers they rotate to propel themselves through the mucus of the small intestine.

Once the cholera bacteria reach the intestinal wall, they no longer need the flagella to move. The bacteria stop producing the protein flagellin, thus again conserving energy and nutrients by changing the mix of proteins which they manufacture in response to the changed chemical surroundings. On reaching the intestinal wall, *V. cholerae* start producing the toxic proteins that give the infected person a watery diarrhea. This carries the multiplying new generations of *V. cholerae* bacteria out into the drinking water of the next host if proper sanitation measures are not in place.

The cholera toxin (CTX or CT) is an oligomeric complex made up of six protein subunits: a single copy of the A subunit (part A), and five copies of the B subunit (part B), connected by a disulfide bond. The five B subunits form a five-membered ring that binds to GM1 gangliosides on the surface of the intestinal epithelium cells. The A1 portion of the A subunit is an enzyme that ADP-ribosylates G proteins, while the A2 chain fits into the central pore of the B subunit ring. Upon binding, the complex is taken into the cell via receptor-mediated endocytosis. Once inside the cell, the disulfide bond is reduced, and the A1 subunit is freed to bind with a human partner protein called ADP-ribosylation factor 6 (Arf6). Binding exposes its active site, allowing it to permanently ribosylate the Gs alpha subunit of the heterotrimeric G protein. This results in constitutive cAMP production, which in turn leads to secretion of H_2O, Na^+, K^+, Cl^-, and HCO_3^- into the lumen of the small intestine and rapid dehydration. The gene encoding the cholera toxin is introduced into *V. cholerae* by horizontal gene transfer. Virulent strains of *V. cholerae* carry a variant of temperate bacteriophage called CTXf or CTXφ (Lan R and Reeves, 2002).

Microbiologists have studied the genetic mechanisms by which the *V. cholerae* bacteria turn off the production of some proteins and turn on the production of other proteins as they respond to the series of chemical

environments they encounter, passing through the stomach, through the mucous layer of the small intestine, and on to the intestinal wall. Of particular interest have been the genetic mechanisms by which cholera bacteria turn on the protein production of the toxins that interact with host cell mechanisms to pump chloride ions into the small intestine, creating an ionic pressure which prevents sodium ions from entering the cell. The chloride and sodium ions create a salt-water environment in the small intestines, which through osmosis can pull up to six litres of water per day through the intestinal cells, creating the massive amounts of diarrhea. The host can become rapidly dehydrated if an appropriate mixture of dilute salt water and sugar is not taken to replace the blood's water and salts lost in the diarrhea (Lan R and Reeves, 2002)

By inserting separate, successive sections of *V. cholerae* DNA into the DNA of other bacteria, such as *E. coli* that would not naturally produce the protein toxins, researchers have investigated the mechanisms by which *V. cholerae* responds to the changing chemical environments of the stomach, mucous layers, and intestinal wall. Researchers have discovered a complex cascade of regulatory proteins controls expression of *V. cholerae* virulence determinants. In responding to the chemical environment at the intestinal wall, the *V. cholerae* bacteria produce the TcpP/TcpH proteins, which, together with the ToxR/ToxS proteins, activate the expression of the ToxT regulatory protein. ToxT then directly activates expression of virulence genes that produce the toxins, causing diarrhea in the infected person and allowing the bacteria to colonize the intestine. Current research aims at discovering "the signal that makes the cholera bacteria stop swimming and start to colonize (that is, adhere to the cells of) the small intestine (Lan R and Reeves, 2002)

3.6.4 Diagnosis

A rapid dip-stick test is available to determine the presence of *V. cholerae*. In those samples that test positive, further testing should be done to determine antibiotic resistance. In epidemic situations, a clinical diagnosis may be made by taking a patient history and doing a brief examination. Treatment is usually started without or before confirmation by laboratory analysis.

Stool and swab samples collected in the acute stage of the disease, before antibiotics have been administered, are the most useful specimens for laboratory diagnosis. If an epidemic of cholera is suspected, the most common causative agent is *V. cholerae* O1. If *V. cholerae* serogroup O1 is not isolated, the laboratory should test for *V. cholerae* O139. However, if neither of these organisms is isolated, it is necessary to send stool specimens to a reference laboratory. Infection with *V. cholerae* O139 should be reported and handled in the same manner as that caused by *V. cholerae* O1. The associated diarrheal illness should be referred to as cholera and must be reported in the United States (WHO 2007).

3.6.5 Cholera toxin

Cholera toxin (CTX or Ctx, or CT) is a protein complex secreted by the bacterium *V. cholerae*. CTX is responsible for the massive, watery diarrhea characteristic of cholera infection. Structurally, the cholera toxin is an oligomeric complex made up of six protein subunits: a single copy of the A subunit (part A, enzymatic), and five copies of the B subunit (part B, receptor binding). The three-dimensional structure of the toxin was determined using X-ray crystallography by Zhang et al. in 1995. The five B subunits—each weighing 12 kDa, and all coloured blue in the accompanying figure—form a five-membered ring. The A subunit has two important segments. The A1 portion of the chain (CTA1) is a

globular enzyme payload that ADP-ribosylates G proteins, while the A2 chain (CTA2) forms an extended alpha helix which sits snugly in the central pore of the B subunit ring(DeHan et al., 2004).

3.6.6 Epidemiology

There are references to the deaths due to the dehydrating diarrhoea dating back to the Hippocrats (460-377 BC) and Galen (129-216 AD). The mode of transmission of the cholerae through the water was first proven by a London physician, John Snow in 1849. In 1883, Robert Koch successfully isolated *V. cholerae* from the intestinal discharges of the cholerae patient and proved conclusively it was etiological agent of the cholera (WHO, 1993). Modern knowledge about cholera, however, dates only from the beginning of the 19th century when researchers began to make progress towards a better understanding of the causes of the disease and its appropriate treatment. The 1st pandemic, or global epidemic, started in 1817 from its endemic area in South-East Asia and subsequently spread to other parts of the world. The 1st and subsequent pandemics inflicted a heavy toll, spreading all over the world before receding (WHO, 2009).

Cholera is both an epidemic and endemic disease. The epidemicity and endemicity of a disease depend on the Characteristics of the agent, and those of the system (environment).Characteristics of the agent which influence its distribution include its ability to survive in a given environment, its virulence, the average number of organisms required to cause infection, etc. Characteristics of the system which affect the distribution of the agent include the number of susceptible, and the opportunities it provides for transmission of the infection. Global experience has shown that the introduction of cholera into any country cannot be prevented, but cholera can create only in the areas where sanitation is defective. Epidemiological determinants include causative

agent, host resistance, toxin production and reservoirs of infection, carriers, infective dose and period of communicability of the organism (WHO, 2007).

The first long-distance spread of cholera to Europe and the Americas began in 1817, such that by the early 20th century, six waves of cholera had spread across the world in devastating epidemic fashion. Since then, until the 1960s, the disease contracted, remaining present only in southern Asia. In 1961, the "El Tor" biotype (distinguished from classic biotypes by the production of hemolysins) reemerged and produced a major epidemic in the Philippines to initiate a seventh global pandemic. Since then, this biotype has spread across Asia, the Middle East, Africa, and parts of Europe (WHO, 2007).

There are several characteristics of the El Tor strain that confer upon it a high degree of "epidemic virulence" allowing it to spread across the world as previous strains have done. First, the ratio of cases to carriers is much less than in cholera due to classic biotypes (1: 30-100 for El Tor vs. 1: 2 - 4 for "Classic" biotypes).

Second, the duration of carriage after infection is longer for the El Tor strain than the classic strains. Third, the El Tor strain survives for longer periods in the extraintestinal environment. Between 1969 and 1974, El Tor replaced the classic strains in the heartland of endemic cholera, the Ganges River Delta of India (WHO 2007).

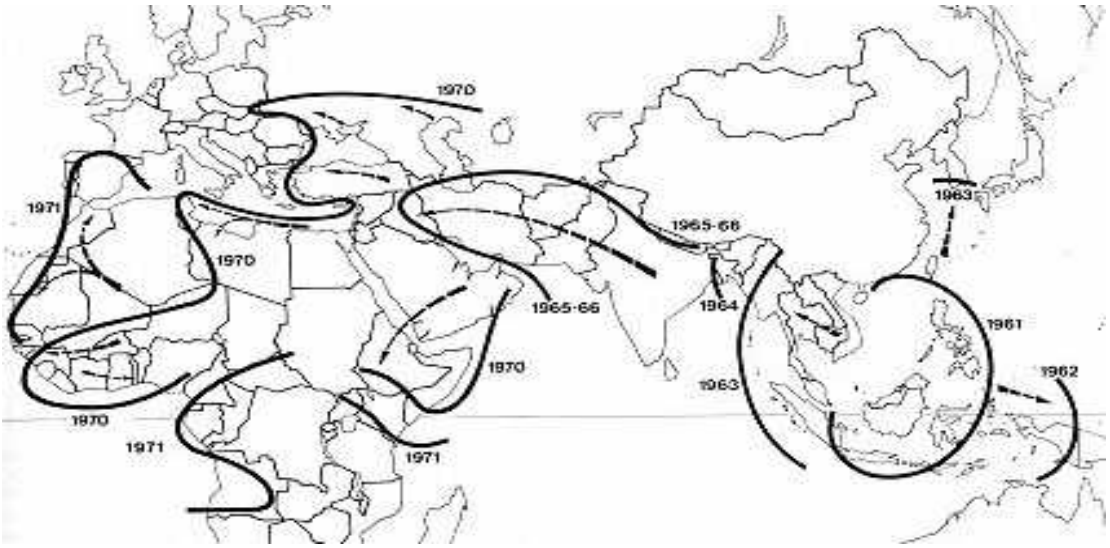


Fig. 3.1: The global spread of cholera during the seventh pandemic, 1961-1971(CDC)

El Tor broke out explosively in Peru in 1991 (after an absence of cholera there for 100 years), and spread rapidly in Central and South America, with recurrent epidemics in 1992 and 1993. From the onset of the epidemic in January 1991 through September 1, 1994, a total of 1,041,422 cases and 9,642 deaths (overall case-fatality rate 0.9%) were reported from countries in the Western Hemisphere to the Pan American Health Organization. In 1993, the numbers of reported cases and deaths were 204,543 and 2362, respectively.

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

In December, 1992, a large epidemic of cholera began in Bangladesh, and large numbers of people have been involved. The organism has been characterized as *V. cholerae* O139 "Bengal". It is derived genetically from

the El Tor pandemic strain but it has changed its antigenic structure such that there is no existing immunity and all ages, even in endemic areas, are susceptible.

The epidemic has continued to spread. and *V. cholerae*O139 has affected at least 11 countries in southern Asia. Specific totals for numbers of *V. cholerae* O139 cases are unknown because affected countries do not report infections caused by O1 and O139 separately (WHO, 2009).

In April 1997, a cholera outbreak occurred among 90,000 Rwandan refugees residing in temporary camps in the Democratic Republic of Congo. During the first 22 days of the outbreak, 1521 deaths were recorded, most of which occurred outside of health-care facilities.

In the United States, cholera was prevalent in the 1800s but has been virtually eliminated by modern sewage and water treatment systems. However, as a result of improved transportation, more persons from the United States travel to parts of Latin America, Africa, or Asia where epidemic cholera is occurring. U.S. travelers to areas with epidemic cholera may be exposed to the bacterium. In addition, travelers may bring contaminated seafood back to the United States. A few food borne outbreaks have been caused by contaminated seafood brought into this country by travelers. Greater than 90 percent of the cases of cholera in the U.S. have been associated with foreign travel (WHO, 2009).

In 1961, the 7th cholera pandemic wave began in Indonesia and spread rapidly to other countries in Asia, Europe, Africa and finally in 1991 to Latin America, which had been free of cholera for more than one century.

The disease spread rapidly in Latin America, causing nearly 400,000 reported cases and over 4000 deaths in 16 countries of the Americas that year (WHO, 2009).

In 1992, a new serogroup – a genetic derivative of the EI Tor biotype – emerged in Bangladesh and caused an extensive epidemic. Designated *V. cholerae* 0139 Bengal, the new serogroup has now been detected in 11 countries and likewise warrants close surveillance.

While no evidence is available to gauge the significance of these developments, the possibility of a new pandemic cannot be excluded. EI Tor, for example, was originally isolated as an avirulent strain in 1905 and subsequently acquired sufficient virulence to cause the current pandemic (WHO, 2007).

30 January 2009, Zimbabwe's cholera outbreak, one of the worlds largest ever recorded, is far from being brought under control. An enhanced response is needed to urgently reverse an epidemic that has so far infected more than 60,000 people and killed more than 3,100 since August 2008 (WHO, 2009).

3.6.7 Emergence of the toxigenic cholera Vibrios from environment

Environmental conditions affect both overall abundance and, potentially, the serogroup of *V. cholerae* in the environment. Recombination and acquisition of foreign DNA appear to be common features among the vibrios. Ctx gene production are rarely found in serogroups other than O1 and O139 isolates may have arisen from genetic exchange with non-O1 *V. cholerae* strains as well as clinical strains osf O1. The possibility of genetic exchange in the environment allows the potential emergence of the new toxigenic clones. In *V. cholerae* and other vibrios, dormant state, in which metabolically active cells cannot be cultured on microbiological media, is induced by changes in environmental condition, including temperature and salinity (Karaolis et al., 1995).

3.6.8 Cholera in Nepal

First bacteriologically confirmed epidemic cholera (subject of an international report) was reported in 1958 (Abou-Gareeb et al., 1961). Many investigators reported the isolation of *V. cholerae* from the diarrheal fecal samples in Nepal (Pokhrel and Gautam, 1993; Pokhrel et. al.,, 2004; Tamang *et al.,* 2005). Ono *et al*., 2001, reported isolation of *Vibrio* species ranking second position among the enteric pathogens detected from the fecal samples of Kathmandu valley. Karki *et al*., 2008, reported the isolation of *V. cholerae* at the rate of 27.1% (57 positive samples out of 210 stool samples detected). Outbreaks of cholera have occurred annually in Nepal (Pokharel et al., 1996, Bistha et al., 2000 and Tamang et al., 2005). *V. cholera*e 01 biotype El Tor Ogawa is the major cause of epidemic in Nepal (Yamamoto et al., 1995 and NPHL Annual Report 2004-2007). Non aggluntinable (NAG) *Vibrios* are included in the species of *V. cholera*e. Some, strains elaborate cholera toxin but most do not. Prior 1992, non- O1 strain were recognized to cause sporadic cases and rare out breaks of diarrheal disease, but were not associated with large epidemics (NPHL Annul Report, 2005).

The retrospective study was conducted during January to September in the year 1997 by Karki and Tiwari. Total three hundred and forty nine stool samples were collected from diarrhoea patients from different places of Kathmandu valley and examined at National Public Health Laboratory (NPHL), Teku, Kathmandu. Acute diarrhoea becomes epidemic in rainy season and is a major public health problem of the city. In this study, people with poor hygiene practice and poor education were infected more than other people. Among the 349 patients with the gastrointestinal disease, 26.0% were found to have bacterial infection. Out of which, 88 (25.1%), one (0.28%), one (0.28%), and one (0.28%) were found to be *V. cholerae* 01, *V. cholerae* 0139, *Shigella dysenteriae* and *Escherichia coli* respectively. Cholera cases were found almost throughout the year in the

city though the numbers increased during the rainy season. It was highest during July (34.6%) followed by August (32.35%), September 32% and June (6.89%). Higher prevalence was found in urban areas (83.52%) than in rural areas (16.48%).

3.7 Distinguishing features of *Vibrios* from other genera

Environment (sewage) contains mixed population microorganism. Members of the genera *Vibrio, Aeromonas, Plesiomonas, Campylobacter* and *Helicobacter* share a few common features. *Vibrios* are differentiated from related genera and by certain features (Table-3.1).

Table-3.1: Distinguishing characteristics Vibrios from related genera (Janda., 1998)

+, positive; -, negative; +/-, variable

Properties	*V. cholerae* *V. mimicus*	Other *Vibrio* spp.	*Aeromonas*	*Plesiomonas*
Growth on Nutrientbroth or on nutrient agar	+	-	+	+
0% NaCl	+	+	-	-
6% NaCl				
Susceptibility to O/129				
10µg				
150µg	+	+/-	-	+/-
	+	+	-	+/-
Ampicillin susceptibility 10µg	+/-	+	-	-
Gas from glucose	-	-	+/-	-
Fermentation of				
m-inositol	-	-	-	+
L-arabinose	-	-/+	+/-	-
Utilization of aminoacids				+
Lysine	+	+	-	+
Arginine	-	-	+	+
Ornithine	+	+	-	
String test	+	+	-	-

3.8 Properties of medically important *Vibrios*

Table-3.2: Some important biochemical tests of medically important Vibrios

Properties	*V. cholerae*	*V. parahaemolyticus*	*V. vulnificus*	*V. alginolyticus*
Glucose fermentation	+	+	+	+
Gas from glucose	-	-	-	-
Oxidase	+	+	+	+
Indole	+	+	+	+/-
VP	+/-	-	-	+
Citrate	+/-	+	+	+/-
Sucrose fermentation	**+**	**-**	**-**	**+**
Lactose fermentation	+	-	+	-
Growth in 0% NaCl	+	-	-	-
Growth in 6% NaCl	-	+	+/-	+
Growth in 10% NaCl	-	+	-	-

3.9 Antigenic characteristics of Vibrios

All motile *Vibrios* contain somatic O and H flagellar antigens was described by Gardner and Venkatraman in 1935. Also, they classified cholerae *Vibrios* and biochemically similar Vibrios based on antigenic structure into two groups: i) Group A *Vibrios* possessing a common H antigen but distinct 'O' component, ii) and rest group B *Vibrios* comprising of a heterogeneous collection of Vibrios distinct from group A biochemically and antigenically.

Group A vibrios were originally divided on the basis of O- antigen into six serological sub- groups (now called O serogroups or serovars from I toVI. Now more than 139, O serogroups, all sharing the common H antigen are recognized, referred to as O serogroups or serovars.

***V. cholerae* O1:-** both classical and El Tor cholerae biotypes belongs to the same O subgroup 1 and are antigenically indistinguishable and referred to as *V. cholerae* O1. On the basis of the differences in minor (subsidiary) O antigen (A, B, C), *V. cholera*e O1 are subdivided into three subtypes Ogawa, Inawa and Hikojima (Nobechi, 1993).

***V. cholerae* non O1:** *V. cholerae* serovares O2 - O139 with rather similar biochemical characters and same antigens as *V. cholerae* O1 but different O antigens are called non - O1 *V. cholerae.*

El Tor Vibrios: Gotschlich isolated a *Vibrio* at the El Tor Quarantine station on the Sinai Peninsula in Arebia who had died of dysentery or gangrene of the colon and the organism was called El Tor Vibrio. The El Tor variant is distinguished from the classical biotype by the ability to express a haemolysin, gives positive result of the Voges-Prokaur test and resistance to polymyxin B. The two biotypes can also be recognized by their differential susceptibility to specific phages (Table3.2).

Table-3.3: Biochemical differences between classical and El Tor biotype

Characteristics	Classical	El tor
1. haemolysis on sheep RBC	-	+
2. VP reaction	usually weak or -	strongly or +
3. Susceptibility to polymyxin B	+	-
4. Sensitivity to phage V (Mukhejee & Basu)	-	+
5. Sensitivity to phage IV	+	-

3.10 Molecular and epidemiological methods of typing Vibrios

3.10.1 Phage typing

The strain of the classical biotype of *V. cholerae* O1 can be divided into five (I-IV) types (Mukerjee and Basu, 1968). A forth phage (IV) lysed all classical but not El tor strain. All El Tor strains were lysed by IV phage. Later, Basu and Mukharjee establied a scheme for El Tor strain of VI types on the basis of lysis pattern of the four phages. A different scheme for all *V. cholerae* was introduced in which 14 phages were used, and 24 patterns were prominent of lysis among El Tor strain (Glass et al., 1982).

Lysogenity is widespread in the *V. cholerae* O1 and has been used in typing. The lysogenity of El tor strain explains apparent instability of some types (Nicole et al., 1971).

3.10.2 Ribo typing and PCR based methods

Amplified fragment length polymorphism (AFLP), florescence in situ hybridization (FISH), amplified polymorphic DNA (RAPD), repetitive extragenic palindromes(rep) and restriction fragment length polymorphism (RFLP), along with multilocus enzyme electrophoresis (MLEE) and multilocus sequence typing (MSLT) have yielded the most valuable information about the new insights into the population structure of some species of the Vibrinaceae and have also provided a means of identifying these organism (Dikshoorn et al., 2001, Gurtler & Mayall, 2001 and Rademaker et al., 1998)

3.11 Vibrios and the environment

Climate change and weather variability pose threats for water-borne diseases, some food-borne diseases and marine and coastal issues, including harmful algal blooms and ecological disruption. It has been demonstrated that changes in precipitation, temperature, humidity, salinity, and wind have a measurable effect on the quality of water used for drinking, recreation, and commerce, while heavy rainfall has been associated with water-borne disease outbreaks throughout the world. Temperature also influences the occurrence of bacterial agents, toxic algal blooms (red tides), and survival of viral pathogens that cause shellfish poisoning.

In general, pathogenic vibrio species are normally present in low numbers when compared with the more abundant saprophytes, but their presence in a certain environment always means a risk of transmission to higher organisms including man, especially if they become concentrated by filter-feeding organisms living in the same habitat. Climate-related increases in sea surface temperature can lead to higher incidence of water-borne cholera and shellfish poisoning. The incidence of Vibrios species depends

on the contents of salts and nutrients and on the water temperature. The highest incidence is thus found in those areas where the water temperature has been above 20° C for a long period.

Cholera is well recognized as a "water-borne" disease, and the population dynamics of the causative agent, *Vibrio cholerae*, demonstrates a close linkage with selected physical, chemical, and biological parameters of natural water.

Climatic factors have also been shown to be significant in cholera epidemics: a linkage has been proposed between the most recent epidemic of cholera in Peru and the El Niño-derived climatic and environmental changes. *Vibrio cholerae* has been found to be associated with marine zooplankton, and blooms from warmer sea surface temperatures could expand this important reservoir from which cholera epidemics may arise. Environmental factors, such as warm water and moderate salinity, can increase the number of *V. parahaemolyticus* and V. *vulnificus* organisms in shellfish.

Environmental reservoirs for *V. cholerae* make eradication of this disease almost impossible because the bacterium has been associated with blue crabs, shellfish, copepods, and aquatic vegetation. There is compelling evidence that *V. cholerae* may proliferate in copepod egg sacks, which can then be ingested in untreated drinking water. These associations mean that spread of these aquatic organisms, as in a copepod bloom, could potentially cause spread of the disease. Researchers have implicated seasonal outbreaks of cholera in Bangladesh with blooms of aquatic organisms and plankton blooms may also have accelerated spread of cholera in Peru in 1991, although direct evidence is lacking. The association of pathogens such as cholera with plankton has important implications for increased risk of disease through stimulation of plankton

blooms. It has been suggested that nutrient enrichment through anthropogenic activity and even climate change (in particular, warming trends) may be important factors in the spread of waterborne disease.

3.12 Epidemiology of vibrio diseases

Vibrio infections are becoming increasingly common in Europe and United States. The CDC estimates that 8028 Vibrio infections and 57 deaths occur annually in the United States.

Vibrio infections were reported among bathers from several European countries in the summer of 2006, apparently related to an increase in water temperature, which is favorable to the growth of Vibrio species.

Three people in the Netherlands developed infections caused by Vibrio alginolyticus in July 2006 after swimming in the Oosterschelde, a large inlet on the North Sea, at separate but nearby locations. Health authorities in the state of Mecklenburg-Vorpommern, Germany have reported three cases of wound infections with the bacterium Vibrio vulnificus so far last summer. Three people in Blekinge County in southeast Sweden were reported to have developed mild to severe wound infections caused by non-agglutinating (not O1 or O139) and non-toxin-producing Vibrio cholerae bacteria after outdoor water contact (Baltic Sea and possibly an irrigating pond) ; all 3 people had skin breakages, and two had other underlying diseases.

Two cases of septicemia caused by Vibrio cholerae non-O1, non-O139 were reported in different regions of Poland during one month in summer 2006.

3.13 Ecology of vibrios and the environmental parameters influencing their presence in the aquatic environment

Cholera behaves ecologically like a three-factor complex. There is growing evidence that a reservoir for this disease exists in bays and estuaries. Once an epidemic starts, transmission is by fecal-oral spread from carriers recovered from the disease and from asymptomatic, infected persons. So far, no aquatic animal reservoir has been found, although persistence in shellfish for several weeks has been demonstrated. A better understanding of the ecology would help us predict the effect of global climate change and prepare us to react.

Although environmental parameters have routinely been measured by using water samples collected aboard research ships, the available data sets are sparse and infrequent. Furthermore, shipboard data acquisition is both expensive and time-consuming. Interpolation to regional scales can also be problematic. Moreover, available data are mainly referred to *V. cholerae* but few have been obtained from the other pathogenic vibrios. In some studies conducted in cholera-endemic areas in the world, satellite data were used to monitor the timing and spread of cholera.

For example, public domain remote sensing data for the Bay of Bengal were compared directly with cholera case data collected in Bangladesh from 1992-1995. The remote sensing data included sea surface temperature and sea surface height. It was discovered that sea surface temperature shows an annual cycle similar to the cholera case data. Sea surface height may be an indicator of incursion of plankton-laden water inland, e.g., tidal rivers, because it was also found to be correlated with cholera outbreaks. (Sechi et al; 2000.)

The members of the genus Vibrio include harmless aquatic strains as well as strains capable of causing epidemics of cholera. Diarrhoea caused by

Vibrio cholerae is attributed to cholerae enterotoxin (CT) codified by the ctx operon and regulated by a number of virulence genes such as toxT, toxR and toxS. Fifty-two Vibrio strains were isolated from different aquatic environments in and around Sardinia and searched by PCR for the presence of ctxA, zot, ace, toxR, toxS, toxT, tcpA and vpi virulence genes in the genomes of the isolates. The toxR operon was found in 27 Vibrio alginolyticus strains out of 42 analysed, in three out of four V. cholerae non-O1 strains and in three Vibrio parahaemolyticus isolates. A positive amplification for the virulence pathogenic island (vpi) was produced by five V. alginolyticus strains. Finally, the ace expected amplification fragment was found in two V. alginolyticus isolates whereas the amplification with zot primers produced the expected fragment in one V. alginolyticus isolate. Differentiation of these strains with a PCR fingerprinting technique revealed no association between the presence of virulence genes and a particular fingerprinting pattern. Although most Vibrio species are considered non-pathogenic or only potentially harmful to humans, the finding of V. cholerae virulence genes in other members of the genus Vibrio, and the recent reports of the creation and evolution of pandemic strains of V. cholerae, may give a new perspective to the significance of these results. (Deriu et al; 2000)

3.14 Clinical manifestations

The clinical manifestation of cholera begins an average of 2 to 3 days after ingestion of the bacilli. Cholera characterized by the sudden onset of effortless vomiting and profuse watery, non-bloody diarrhea is known as rice water stool because of its appearance and can result in the loss of average of one litre of fluid every hour. The resulting severe loss of fluid and electrolyte may lead to dehydration, metabolic acidosis, hypokalemia with cardiac arrhythemia and renal failure. Anuria develops, muscle cramps occur and the patient quickly becomes weak and lethargic with loss of skin turgor, low blood pressure and absent or thread pulse. In untreated cases, the mortality from cholera is 40-60%. Rapidly instituted fluid and electrolyte replacement reduce the mortality to less than 1%. (Bhatt C.P., 2006)

CAPTER-IV

MATERIALS AND METHODS

4.1 Materials

List of materials is given in appendix I

4.2 Methods

A total of 60 sewage samples were collected from the different sites of sewer system of Kathmandu Valley (Fig 4.1) between 10th November 2008 to 6th February 2009 using principle based on Moore's technique. Briefly, this technique involves the use of cotton gauge (swab) wrapped on one end of a piece of six inch diameter pipe (five inch long) placed horizontally into the sewerage in opposite to sewerage flow for overnight. Samples [Moore's technique based swabs were submersed in alkaline peptone water (broth) pH of 8.6] were transported to research laboratory of CDM, TU in cold condition (ice chest).

The samples were incubated at 37^{0}C for 8 hours followed by culture on thiosulfate-citrate-bile salts-sucrose (TCBS) agar and incubated at 37^{0}C for overnight. The TCBS plates showing colonies resembling *V. cholerae* and other *Vibrios* were subjected for identification following standard bacteriological procedures. The organisms were inoculated into triple sugar iron (TSI) agar. The TSI reaction as alkaline or acid slant, acid butt with no gas and H_2S was suspected to be *V. cholera*e. Based on the TSI reaction, suspected colonies were sub-cultured on nutrient agar (NA) and the colonies on NA were subjected for serotyping using polyvalent *V. cholerae* 01 sera and also with Inaba and Ogawa anti-sera as described by Feeley *et al* (1974). For the characterization of other species, colonial characters and biochemical tests (such as sucrose fermentation, lysine

utilization, motility, indole test, oxidase positive, swarming, growth on 0, 2, 3, 6% NaCl and others) were employed.

4.1 Study area

Duplicate (day and night) sewage sample was collected from the different sewer sites of KTM Valley (Fig-4.1) as described below.

a) Balaju: Five samples were collected from the sewage discharging into the Bisnumati River.
b) Kirtipur: Samples includes sewage of Panga drains, Nagau drains and sewerage of Tribhuwan University discharging into Bagmati River.
c) Lalitpur: Samples were collected from Satdobato, Gwarko, Lagankhel drains.
d) Bhaktapur: Sampling sites included Jadibuti, Kausaltar, Gatthaghar and Sanothimi drains.
e) Others: Some sewerages of Chakrapath, Chabahil, Pashupati area and Baneshwor were also included to collect the sewage samples.

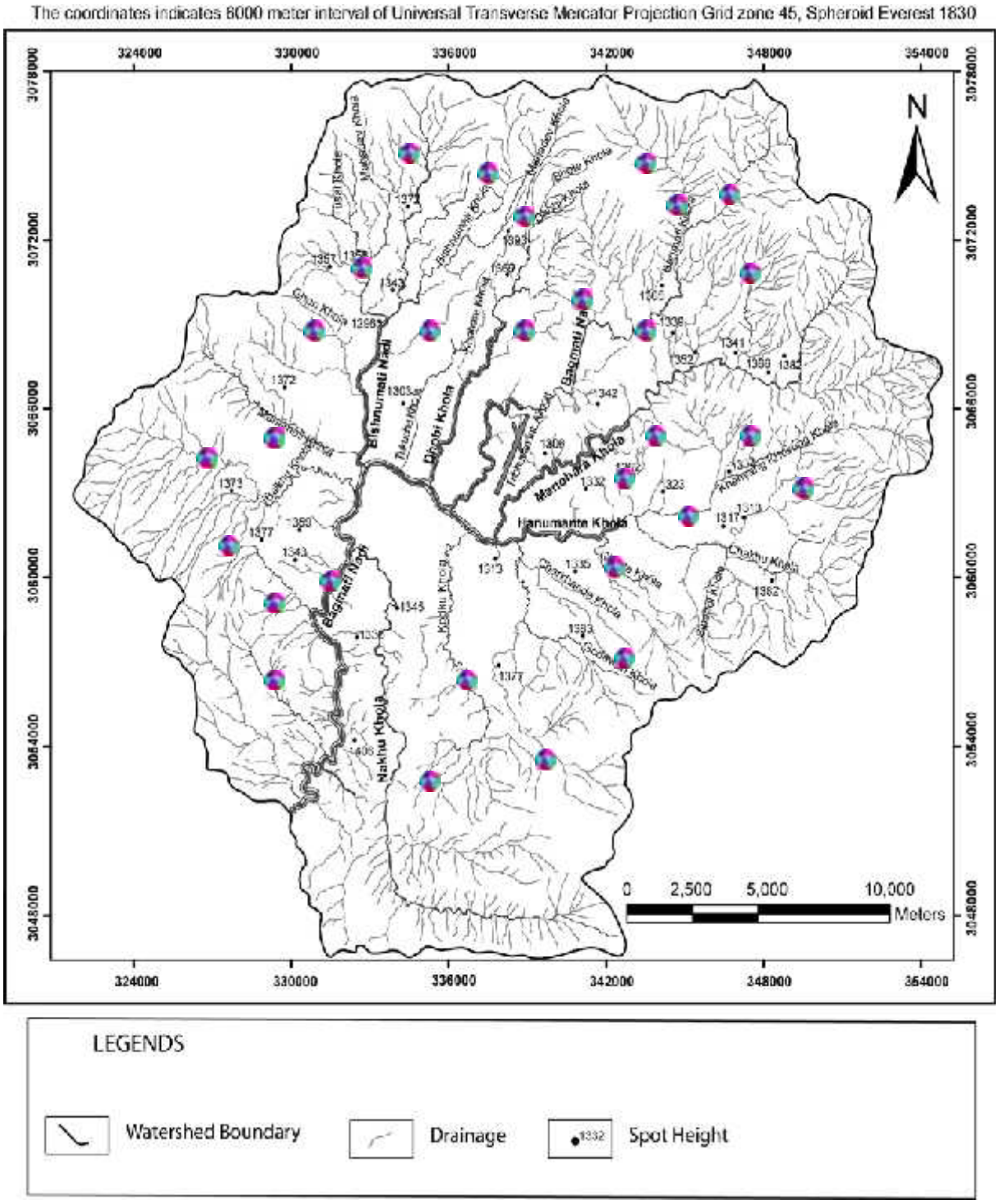


Fig. 4.1: Drainage pattern of the Bagmati River (Source: Nepal Survey Division)

- Indicates the Sampling Sites

4.2 Identification

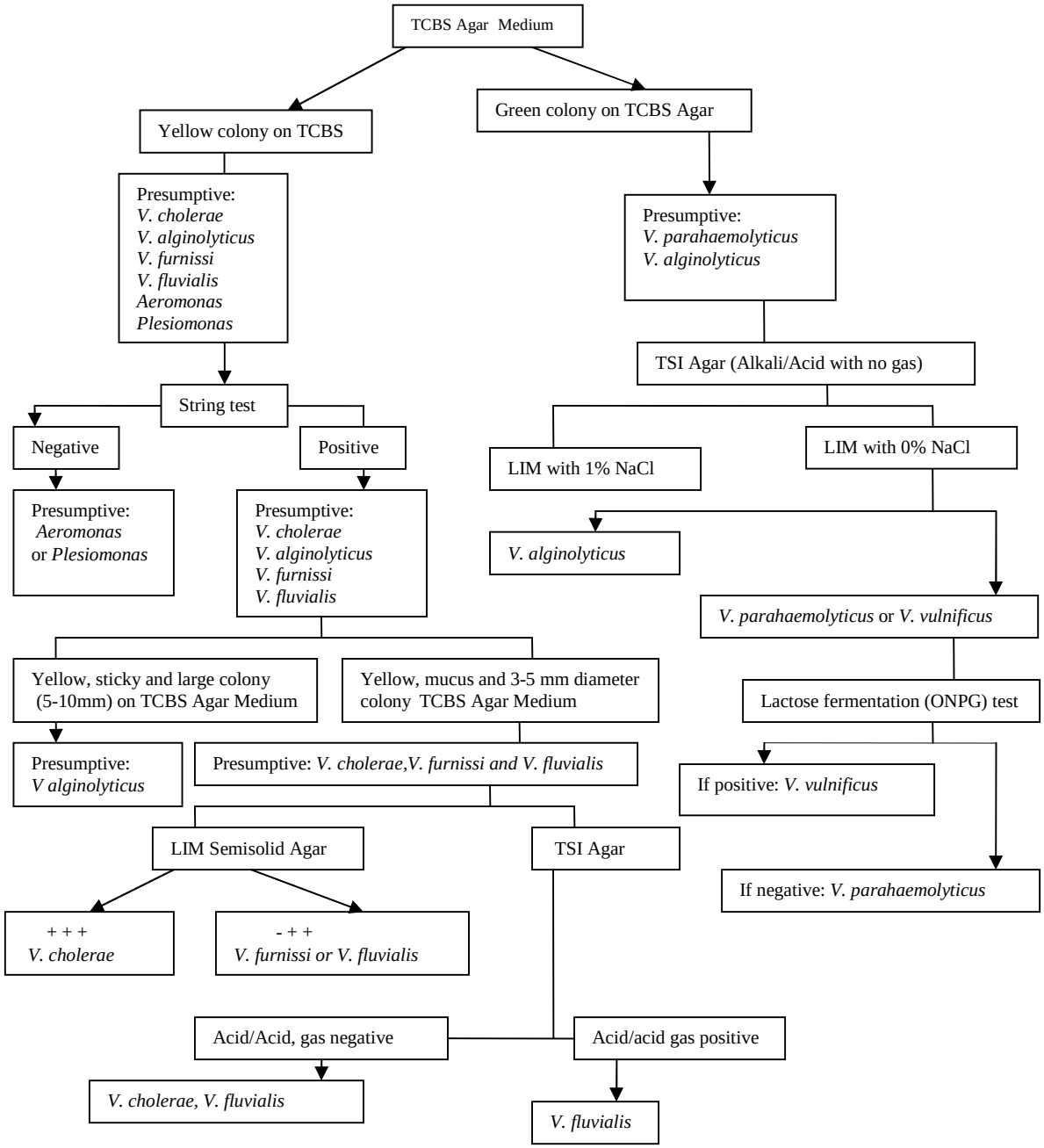
4.2.1 Colonical characterization on TCBS Agar

V. cholerae, V. furnissi and V. fluvialis produce yellow, mucus and 3-5 mm diameter colony on TCBS agar medium. On other hand, *V. alginolyticus* produces yellow, sticky and large colony (5-10 mm). *V. parahaemolyticus* being sucrose non fomenter produce green colonies on TCBS. Some strains of *V. vulnificus* and *V. alginolyticus* also produce green colonies on TCBS agar medium (Flow chart: 4.1).

4.2.2 Biochemical Identification

On Triple Sugar Iron Agar, most of *V. cholerae* typically ferments sucrose and yields an acid slant on TSI and few yield alkaline slant. Therefore, all isolates showing a TSI reaction of alkaline or acid slant, acid butt, no gas, and no H_2S were considered suspect *V. cholerae*. Subsequent biochemical tests like indole, lactose fermentation, lysine decarboxylation or deamination, gas production along with motility were performed to characterize medically important *Vibrio* isolates (Flow chart- 4.1).

Flow Chart 4.1: Characterization of medically important Vibrios



(Cheesbrough M, 1993)

4.3 Serotyping of the *V. cholerae*

Rapid slide agglutination method was employed for serotyping using *V. cholera* O1 antisera (Denka Seiken Co. Ltd. 12-1, Nihonbashikabuto- Cho Chuo-Ku, Tokyo, Japan).

Three drops of normal saline were placed on a clean, grease free glass slide. The colony to be tested (*V. cholerae* suspect) was picked with the help of sterile wire loop and emulsified on the drops. One drop of *V. cholerae* O1 polyvalent antiserum was added on either one emulsified drop to detect the presence of *V. cholerae* O1. *V. cholerae* O1Ogawa, Inawa and antisera were added on polyvalent positive slides to detect the specific strains of the *V. cholerae*. For the observation, the slide after the addition of the antisera, was tilted to and fro to see the distinct agglutination.

4.4 Antibiotics susceptibility test

4.4.1 Media

Muller Hinton agar medium was used for antimicrobial susceptibility test, which is recommended by CLSI (Clinical Laboratory Standardization Institution) [formerly NCCLS 2002 (National Committee for Clinical Laboratory Standard)

4.4.2 Test plate

Ideally a test plate with internal diameter of 90 mm uniform depth of 4 mm used and containing a standard volume of 25 ml media was used (CLSI, 2002)

4.4.3 Inocula preparation

A single pure isolated colony of *V. cholerae* O1 was inoculated from an overnight growth into a Muller Hinton broth tube and incubated at 35-37^{o}c for 3-4 hours.

The growth of test organism was standardized by adjusting the turbidity equivalent to 0.5 Mac Farland opacity standards (prepared by adding 0.95 ml of $BaCl_2$ solution to 0.5 ml of 1% H_2SO_4) which is equivalent to the 1-2x10^6-1-2x10^8 CFU/ml (CLSI, 2002). The comparison was conveniently made by observing the tubes against a background of white paper having print of alphabets. Turbidity was adjusted by diluting the broth with sterile saline if necessary.

4.4.4 Selection of the antimicrobial agent

Only limited numbers of selected antibiotics were tested for the susceptibility. The panel of antimicrobial agents depends on appropriate selection by a therapeutic committee comprising physicians, microbiologist and infection control body. In this study antibiotics used were Amikacin, Nalidixic Acid, Nor-floxacin, Ciprofloxacin, Tetracycline, Ofloxacin and Polymyxin B.

4.4.5 Inoculation and incubation

A sterile swab was dipped into the suspension and the swab was rotated several times, pressing it firmly on the inside of the tube wall above the fluid level to remove the excess fluid from the swab. The test plate was inoculated evenly with swab by rotating the plate each time to ensure an even distribution of the inoculums. The inoculated plate was left to dry for 3-5 minutes at room temperature with the lid closed. The antibiotics discs were placed onto the surface of the inoculated plates using a disc dispenser or a sterile needle and the disc are slightly pressed onto the surface. Not

more than 6 discs were placed on a plate (one in centre six in circle 15 mm apart from the edge of the plate). The inoculated plates were incubated at 35^0C-37^0Cfor 18-24 hours.

4.4.6 Measurement of the inhibition zone

After overnight incubation, the inhibition zone was measured in mm with a rule scale on under surface of the plate without opening the lid. The end point of inhibition was judged by naked eye at the edge where growth started. The antibiotic disc manufacturer's (HiMedia Laboratories Pvt. Limited, India) chart was followed for the interpretation of zone of inhibition with control strain.

4.4.7 Quality control of Disc

Quality control of disc was performed by using purity culture of E. coli ATCC 25922 and S. aureus ATCC 25923.

4.5 Data analysis

Data analysis for isolation of medically important *Vibrio* spp frequency employed the comparative and descriptive study among other investigators.

CHAPTER-V

RESULTS

5.1 Distribution of *Vibrios*

Out of 60 sewer samples, 57 medically important *Vibrios* were isolated where *V. parahemolyticus* was dominating isolates followed by *V. cholerae* (Table-5.1).

Table-5.1: Frequency of isolates

Vibrios	n	%
V. parahaemolyticus	**26**	**45.6**
V. cholerae	12	21.0
V. alginolyticus	11	19.3
V. furnissi	4	7.0
V. fluvialis	2	3.5
V. vulnificus	2	3.5
Total	57	100

5.2 Serotypes of *Vibrio* spp

All biochemically resembling *V. cholerae* (n=12) were subjected to serotyping using polyvalent *V. cholerae* O1 anti-sera, where all isolates were found to be non agglutinable (NAG).

5.3 Antibiogram of biochemically related *V. cholerae*

Only biochemically resembling cholera Vibrios (n=12) were subjected to antibiotic susceptibility test (Table-5.4.1-3), where two isolates were found to be multi antibiotic resistant.

Multi antibiotic resistant isolates are defined as the isolates which are resistant to two or more antibiotics of different chemical classes in this study.

Table 5.4.1: Antibiogram *V. cholerae* isolates

N	Antibiotics	Sensitive isolates		Resistance isolates	
		Number	% sensitive	Number	% resistance
12	Ak	12	100	----	0
	Na	10	83.3	2	16.7
	Nx10	12	100	----	0
	C	10	83.3	2	16.7
	Cfx	11	91.7	1	8.3
	T	10	83.3	2	16.7
	Of	12	100	----	0
	Pb	9	75	3	25

Note: Ak, Amikacin; Na, Nalidixic Acid; Nx 10, Norfloxacin; C, Chloramphenicol: Cfx, Ciprofloxacin; T, Tetracycline Of, Ofloxacin and Pb, Polymixin B.

Table 5.4.2: Antibiogram *V. cholerae* isolates

Isolates	Ak	Na	Nx10	C	Cfx	T	Of	Pb
SN^{3}	S	S	S	S	R	S	S	S
SN^{5}	S	S	S	S	S	S	S	S
SN^{24}	S	R	S	R	S	R	S	S
SN^{20}	S	S	S	S	S	S	S	S
SN^{32}	S	S	S	S	S	S	S	R
SN^{18}	S	S	S	S	S	S	S	S
SN^{13}	S	S	S	S	S	S	S	S
SN^{27}	S	S	S	S	S	S	S	S
SN^{9}	S	S	S	S	S	S	S	R
SN^{37}	S	S	S	S	S	S	S	S
SN^{12}	S	R	S	R	S	R	S	S
SN^{46}	S	S	S	S	S	S	S	R

Note: S, sensitive; R, resistance.

From antibiotic susceptibility test, 50% isolates were susceptible to all tested antibiotics. Where some isolates were resistant to Ciprofloxacin, Nalidixic acid, Chloramphenicol, Tetracycline and Polymyxin B (Table-4.1.3).

Table 5.4.3: Antibiogram *V. cholerae* isolates

Antibiogram	Frequency	%
All tested antibiotics	6	50
Cfr^R	1	8.3
Na^R, C^R, T^R	2	16.6
Pb^R	3	25

CHAPTER-VI
DISCUSSION AND CONCLUSION

6.1 Discussion

Vibrios are natural inhabitants of aquatic environment where water plays a central role for the transmission human pathogenic *Vibrio* Spp. *Bagmati River* is ultimate drainage of KTM Valley residents and the water of the river is used for many purposes such as irrigation, washing clothes, cleaning green vegetable even for drinking (*Hindu* devotees around *Pashupatinath* temple) which can attribute the outbreak and as well as the source of *Vibrio* infection. For this study we collected samples from the sewerages using principle based on Moore technique because sewer surveillance by using the Moore swab offers an effective way to determine whether *V. cholerae* 01 infection are occurring in an area with sewage systems. It can also lead to detection of asymptomatic infections and mild disease which would not lead infected persons to seek medical assistance or have stool cultures performed. Sewer surveillance using the Moore swab had also found evidence of infections occurring in areas where surveillance of diarrheal illness failed to detect cholera. Culturing community sewage by the Moore swab method proved to be an economical and effective way of determining areas where *V. cholerae* infections were occurring (Barrett et al., 1980).

Out of all isolates, nearly half of isolates were characterized as *V. parahemolyticus* (45.6%). To the knowledge, in developed countries infection due to *V. parahaemolyticus* is considered as serious health problem (Farmer et al., 1992).

V. cholerae in this study was isolated at the rate of 21%. This finding was lower than those reported from elsewhere during peak rainy days. In Argentina Emiliani et al., (1997) found *V. cholerae* in 69.4% of samples studied. Similarly, in Bangladesh Huq et al., (1990) reported isolation of *V. cholerae* at the rate of 64.27% from the aquatic environment. In Japan and India, however, V. cholerae was positive in all river water and drinking water samples studied (Yamai et al., 1987; Thomson et al., 1998). Rai et al (2007) isolated *V. cholerae* (43.5%) from the sewerages to Bagmati River during peak rainy days. So it is revealed that the prevalence of *V. cholerae* in rainy days is higher than winter. This lower prevalence rate of could be applied to justify the outbreak of cholera in KTM Valley where outbreaks of cholera occur each year (Pokharel et al., 1996, Tamang et al., 2005, Bistha et al., 2000) with the beginning of summer/rainy season (continues to post rainy season). Many investigators reported the isolation of *V. cholerae* from the diarrheal fecal samples in Nepal (Pokhrel et al., 1993; Pokhrel et al., 2004; Tamang et al., 2005). Ono et al., 2001, reported isolation of *Vibrio* species ranking second position among the enteric pathogens detected from the fecal samples of Kathmandu valley. Karki et al., 2008, reported the isolation of *V. cholerae* at the rate of 27.1% (57 positive samples out of 210 stool samples detected).

This study was unable to detect the *V. cholerae* O1 using poly O1 anti-sera. However, epidemiologically, all biochemically resembling cholera *Vibrios* are important for possible cholera outbreak because according to the Finkestein (1973), *Vibrios* resembling *V. cholerae* but failing to agglutination in cholera antisera, had been strongly implicated as causative agents of both sporadically occurring and focal cholera like diarrheal disease.

This can also be compared with the clinical isolates responsible for cholera outbreak in Nepal where outbreak of cholera has been mainly due to the *V. cholerae* O1 Ogawa sero-group (NPHL annual report 2004-2006).

In this study other clinically important *Vibrio* isolates were identified as *V. alginolyticus* (19.3%), *V. furnissi* (7%), *V. fluvialis* (3.5%) and *V. vulnificus* (3.5%). This result can be compared with the research conducted on clinical isolates by Singh (2000), who analysed total of 236 diarrheal clinical samples at Kanti Children Hospital, Maharajgung and Sukraraj Tropical Hospital, Teku. Of total sample analyzed, 50 (21.18%), 3(1.275%) and (0.435%) were *V cholera*e, *V. parahaemolyticus* and NAG respectively.

Pant and Mittal (2007) recovered *Salmonella, Shigella and Vibrio* from an Up flow Anaerobic Sludge Blanket Reactor based sewage treatment plant located at a suburb of Delhi, India and they investigated pathogenicity of the treated wastewater with possible risk factors. In their study, frequencies of occurrence of all three were 100% at all the stages of sewage treatment. Furthermore, recovery of *Vibrio* spp was the highest among all the pathogens.

The survivable of the *Vibrios* in sewage during rainy days may be correlated with fluctuation in the quality of Bagmati River with higher pollution in dry summer and lower in rainy season. In rainy season sewage may get diluted. Polluted water dilution may give the favorable physiochemical parameters like pH, temperature, BOD, DO, COD, toxic metals etc support the longevity of Vibrios in sewage in rainy days. According to the Singleton et al., (1982), *V. cholerae* O1 survives poorly in water at temperatures below 10^0C and actively proliferates in nutrient-

rich waters at temperatures above 20^0C. However, Madico et al., (1996), found that ambient temperatures greater than 20^OC were associated with increased numbers of *V. cholerae* O1 bacteria in sewage samples and documented cholera cases and also claimed that in tropical countries, where ambient temperatures are nearly always above 20^0C, factors other than temperature may influence the seasonality of cholera.

But, Sunny et al., (2001) examined the abundance and distribution of *V. cholerae* in recreational beach waters and tributaries of Southern California. Total bacterial direct counts were also determined to understand the relationships between the presence of *V. cholerae* and environmental conditions and he found the presence of *V. cholerae* is independent of sewage pollution. *V. cholerae* were detected in all samples taken from rivers, creeks and wetlands of coastal Southern California where salinities were between 1 to 34 parts per thousand (ppt), but was not found at a freshwater sampling site in upper San Juan Creek. The highest density of *V. cholerae* was found in San Diego Creek with a concentration of 4.25×105 CFU/L. The geographical distribution of *V. cholerae* was inversely correlated with salinity. High concentrations of *V. cholerae* were more frequently detected in waters with lower (but above 0) salinity. The results of this study provide insight into the ecology of this aquatic species and are potentially important to the understanding of the epidemiology of cholera on a global scale.

This study was limited to characterize *V. cholerae* O1. So, NAG isolates during the study may include *V. cholerae* O139 which was reported in many cholera cases in Nepal. The clinical cases of *V. cholerae* serogroup O139 has been reported in 1993 (24 cases), 1994(5 cases),1997(1 cases

imported from India), and year 2000 out of 175 laboratories confirmed *V. cholerae* cases 42 isolates were O139 (NPHL Annual Report 2004-2006).

In this study, only *V. cholerae* were subjected for antibiogram against the common drugs. All of the isolates were sensitive to the Amikacin, Ofloxacin and Norfloxacin. Only two isolates were multi antibiotics resistant. Similar type of result was carried out by the Rajkarnikar (2000).

According to the study carried out by Rajkarnikar, of 50 stool samples were taken from the Sukraraj Tropical Hospital and Kanti Children Hospital, 48 % of isolates were resistance to Nalidixic Acid, Ciprofloxacin and Chloramphenicol. And 4% isolates were resistant to Ofloxacin, Nalidixic Acid, Chloramphenicol and 8 % were resistant to the tetracycline. Only two isolate in this study were multidrug resistant strains.

Since the Tetracycline is the drug of choice for the cholera because of its effectiveness, non toxic and ease of availability in cheap. However, in this study, among *V. cholerae* O1 isolates 7.69 % were resistant to the Tetracycline. This can be compared with the results given by Jain et al., (1991 and 1993), according to them, El Tor strains Vibrios isolated from the rural areas of Loni, Showed resistance to one or more antibiotics particularly Tetracycline. Similarly, Giono (1995) reported *V. choleare* resistance to Tetracycline and Doxycycline. Moolasart et al., (1998) studied the efficacy of Tetracycline and Norfloxacin in the treatment of the acute watery diarrhea in Thailand. He found that tetracycline therapy was good as Norfloxacin therapy for quick recovery and for bacterial eradication in the patient.

This study did not perform characterization of toxin production by the isolates. Isolates could comprise both toxigenic and non-toxigenic.

However, both types are equally important. Comparative genomics study has demonstrated that environmental isolates of *Vibrio* species lack toxin encoding genes in their bacterial chromosomes (Dziejman et al., 2002). But, due to the high similarity of genetic makeup of the environmental isolates and their pathogenic counterparts, the environmental isolates may have a high possibility to take up genes responsible for pathogenesis through plasmid, bacteriophage and integron and then carry diverse combinations of virulence genes (Chiang and Mekalanos, 1999). As a result, environmental isolates of *Vibrio* species may act as a reservoir for the potential spreading of virulence genes in the natural environment.

6.2 Conclusion

Medically significant Vibrios were detected from the sewage of Kathmandu valley in winter. In Kathmandu valley, Bagmati River is the only drain for sewage disposal. The liquid waste generated from residents carry the sewage with faecal excreta. Cholera patients and cholera convalescents may excrete toxigenic *V. cholerae* and are shed back to the sewage. Water flood during peak rainy days pollute the surface water but all surface water in Kathmandu Valley has been tapped for drinking purpose. So, consumption of surface water without disinfection might be the cause of cholera outbreak. In this study, almost all medically important *Vibrios* were isolated including *V. cholerae* which indicates that the potential source of *Vibrio* infection could be sewage. The result suggests that the proper sanitation and consumption of safe drinking water (disinfect water) can prevent the possible infection.

CHAPTER-VII

SUMMARY AND RECOMMENDATION

7.1 Summary

1. Total 60 random samples (in early and mid winter) were taken from the sewage of Kathmandu valley by Moore technique.
2. 57 medically important Vibrios were isolated.
3. Out of 57 isolates, *V. cholerae, V. parahaemolyticus, V. alginolyticus, V. furnissi, V. fluvialis* and *V. vulnificus* were characterized by biotyping and found to be 12 (21%), 26 (45.6%), 11(19.3%), 4(7%), 2(3.5%) and 2(3.5%), respectively.
4. All *V. cholerae* isolates were NAG using poly O1 anti-sera.
5. *V. parahaemolyticus* was dominant isolate which is followed by *V. cholerae* which indicates that the potential source of *Vibrios* infection and cholera outbreak could be sewage. The result suggests that the proper sanitation and consumption of safe drinking water can prevent the possible *Vibrios* infection and cholera outbreak.
6. On anti-biogram, out of 12 isolates (*V. cholerae*), two isolates were multi-antibiotic resistant isolates.

7.2 Recommendation

1. This study detected medically important multi-antibiotic resistant strains. So, further study on toxigenicity of the *Vibrio* spp is recommended.

2. Sustainable sewer surveillance system and community awareness programs should be launched to prevent and trace the possible *Vibrio* infection.

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Picture 1: Sucrose fermerter and non fermeter (primary isolates)

Picutre 2: Sucrose fermenter isolate

Appendix I

Culture Media

Different types of cultural media such as enrichment media, selective media, and differential media were used. Composition and preparation of different types of culture media are given below.

1. Buffered peptone water

Ingredients	gm/litre
Peptone	10.0
Sodium chloride	5.0
Disodium phosphate	3.5
Potassium dihydrogen phosphate	1.5
Final pH (at 25^{o}C)	7.2 +- 0.2

Twenty grams of the ingredients was suspended in 1000 ml distilled water and dissolved completely. The medium was distributed in 20 ml or desired in culture bottles and autoclaved at 121^{o}C for 15 minutes.

2. Peptone water (CM9)

Ingredients	gm/litre
Peptone	10.0
Sodium chloride	5.0
Water	1000 ml

pH before adjustment to alkaline was peptone water 7.2 +- 0.2,

Fifteen grams of the medium was dissolved in 1000 ml of distilled water. It was then adjusted the pH to 8.6. This was then distributed in to the test tubes and autoclaved at 121^{o}C for 15 minutes.

3. Alkaline peptone water

Ingredients	gm/litre
Peptone	10.0
NaCl	10.0
Distilled water	1000
Final pH (at 25^{o}C)	8.5 +- 0.2

Fifteen grams of the medium powder was suspended in 1000 ml distilled water and dissolved completely. Then the medium was dispensed in 20ml amount in test tubes and autoclaved at 121^{o} for 15 minutes.

4. MacConkey Agar

Ingredients	gm/litre
Peptone	20.0
Lactose	10.0
Sodium Taurocholate	5.0
Sodium Chloride	5.0
Neutral Red	0.04
Agar	20.0
Final pH (at 25^{o} C)	7.4 +- 0.2

Fifty-five grams of the medium was suspended in 1000 ml of distilled water and then boiled completely. Then the medium was sterilized by autoclaving at 121^{o}C (151bs pressure) for 15 minutes.

5. Mueller Hinton Agar

Ingredients	gm/litre
Beef, Infusion form	300.0
Casein Acid Hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH (at 25^{o} C)	8.6 +- .02

38 grams of the medium was suspended in 1000 ml of distilled water and the medium was warmed to dissolve. 10 ml was distributed in test tubes and sterilized by boiling in water bath for 10 minutes.

6. Nutrient Agar

Ingredients	gm/litre
Peptone	10.0

Sodium Chloride	5
Beef Extract	10.0
Yeast Extract	1.5
Agar	12.0
Final pH (at 25^{o} C)	7.4 +- .02

Thirty-seven grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^{o} C (151bs pressure) for 15 minutes.

7. Nutrient Broth

Ingredients	gm/litre
Peptone	5.0
Sodium Chloride	5.0
Beef Extract	1.5
Yeast Extract	1.5
Final pH (at 25^{o} C)	7.4 +- .02

Thirteen grams of the medium was dissolved in 1000ml distilled water and autoclaved at 121^{o}C for 15.

8. Thiosulphate Citrate Bile Sucrose (TCBS) Agar

Ingredients	gm/litre
Yeast Extract	5.0
Peptone	10.0
Sodium Thiosulphate	10.0
Sodium Citrate	10.0
Ox bile	8.0
Sucrose	20.0
Sodium Chloride	10.0
Ferric Citrate	1.0
Bromothymol Blue	0.04
Thymol Blue	0.04
Agar	15.0

Final pH (at 25^{o} C) 8.6 +- .02

Eighty-nine grams of the medium was dissolved in 1000 ml water and heated to boiling to dissolve the medium completely (DO NOT AUTOCLAVE) then cooled to 50^{0}C and poured into the sterile petri plates.

Appendix II

Biochemical Tests Media

1. MR-VP medium

Ingredients	gm/litre
Buffered Peptone	7.0
Dextrose	5.0
Dipotassium Phosphate	5.0
Final pH (at 25^{o} C)	6.9 +- .02

Seventeen grams of ingredient was dissolved in 1000 ml distilled water and 3ml of medium was distributed in each test tube and autoclaved at 121^{o}C for 15 minutes.

2. Triple Sugar Iron Agar (TSI)

Ingredients	gm/litre
Peptone	10.0
Tryptone	10.0
Yeast Extract	3.0
Beef Extract	10.0
Lactose	10.0
Saccharose	1.0
Dextrose	0.2
Ferrous sulphate	0.2
Sodium Chloride	5.0
Sodium Thiosulphate	0.3
Phenol Red	0.024
Agar	12.0
Final pH (at 25^{o} C)	7.4 +-.02

Sixty-five grams of the medium was dissolved in 1000 ml of distilled water and sterilized autoclaving at 151bs (121^{o}C) pressure for 15 minutes. The medium was allowed to set in sloped form with a butt about 1 inch of thickness.

3. Urea Broth Base

Ingredients	gm/litre
Monopotassium phosphate	9.1
Disodium Phosphate	9.5
Yeast Extract	0.1
Phenol Red	0.001
Final pH (at 25^{o} C)	6.8 +- 0.2

1.87 grams of ingredients were dissolved in 95 ml distilled water . This was sterilized by autoclaving at 15 lbs pressure (121^{o}C) for 15 minutes . This was cooled to 55^{o}C and aseptically added 5 ml of sterile 40% urea solution , mixed well and dispensed 10 ml amounts into sterile tubes.

4. Lysine Indole Motility Agar

In gridients	gm/litre
Pancreatic digest gelatin	10
Pancreatic digest casein	10
Yeast extract	3
l- lysine hydrochloride	10
Dextrose	1
Ferric ammonium citrate	0.5
Agar	2
Bromocresol purple	20

Final pH 6.6 +_ 0.2 at 25^{o}C

56.5 grams of ingredient was dissolved in 1000 ml distilled water and 3ml of medium was distributed in each test tube and autoclaved at 121^{o}C for 15 minutes.

Appendix III

Staining and test reagents

1. For Gram's Stain

(a) Crystal Violet solution

Crystal Violet	20g
Ammonium Oxalate	9.0g
Ethanol or Methanol	195ml
Distilled Water to	1 litre

(b) Lugol's Iodine

Potassium Iodine	20.0g
Iodine	0.0g
Distilled water	1000ml

(c) Declouriser

70% acetone

(d) Safranin (counter stain)

Safranin	5.0g
Distilled water	1000ml

2. Normal saline

Sodium chloride	0.85g
Distilled water	100ml

3. Test Reagents

a. For Voges –Proskauer Test

Solution A

Alfa Napthol	15.0g
Ethyl alcohol	100ml

Solution B

Potassium Hydroxide 40.0g

Distilled water 000ml

b. For Catalase test

Catalase Reagent (3%H202)

Hydrogen peroxide 3 ml

Distilled water 97 ml

c. For Methyl Red Test

Methyl Red solution

Methyl red 0.05ml

Ethyl alcohol (absolute) 28ml

Distilled water 22ml

d. For indole test

Kovac's indole reagent

Isoamyl alcohol 30ml

p-diethyl amino

Bezeldehyde 2.0g

Hydrochloric acid 10ml

e. For Oxidase Test

Oxidase reagent (impregnanted in what man's No. 1 filter paper)

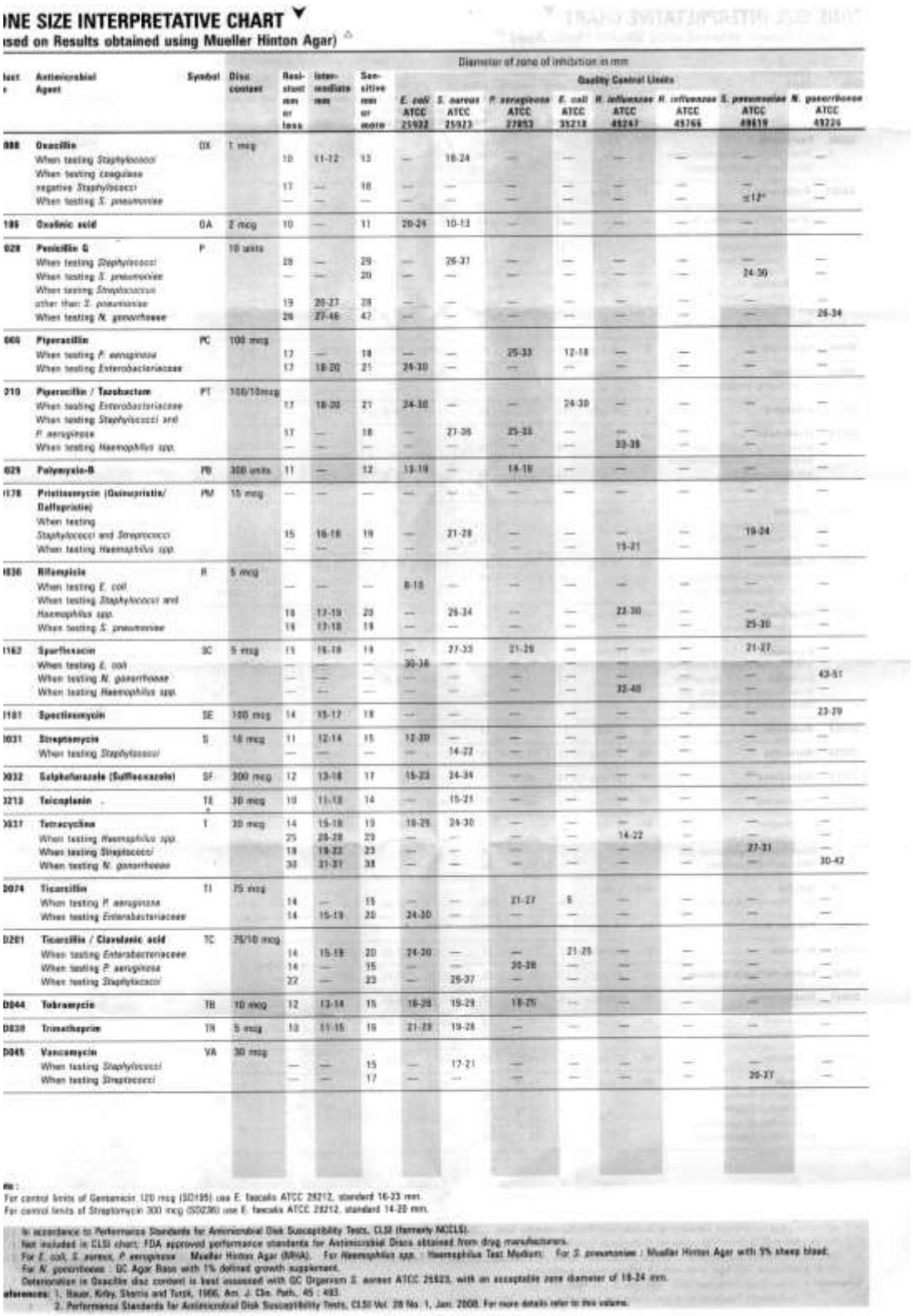
Tetramethyl p-phenylene 1gm

Diamine dihydrocholoride

Distilled water 100ml

Appendix IV

Zone Size Interpretative Chart (Antibiotic Susceptibility Test Discs)



NE SIZE INTERPRETATIVE CHART

sed on Results obtained using Mueller Hinton Agar)

Antimicrobial Agent	Symbol	Disc content	Resistant mm or less	Intermediate mm	Sensitive mm or more	Diameter of zone of inhibition in mm — Quality Control Limits: *E. coli* ATCC 25922	*S. aureus* ATCC 25923	*P. aeruginosa* ATCC 27853	*E. coli* ATCC 35218	*H. influenzae* ATCC 49247	*H. influenzae* ATCC 49766	*S. pneumoniae* ATCC 49619	*N. gonorrhoeae* ATCC 49226
Oxacillin	OX	1 mcg											
When testing *Staphylococci*			10	11-12	13	—	18-24	—	—	—	—	—	—
When testing coagulase negative *Staphylococci*			17	—	18	—	—	—	—	—	—	—	—
When testing *S. pneumoniae*			—	—	—	—	—	—	—	—	—	≤12*	—
Oxolinic acid	OA	2 mcg	10	—	11	20-24	10-13	—	—	—	—	—	—
Penicillin G	P	10 units											
When testing *Staphylococci*			28	—	29	—	26-37	—	—	—	—	—	—
When testing *S. pneumoniae*			—	—	20	—	—	—	—	—	—	24-30	—
When testing *Streptococcus* other than *S. pneumoniae*			19	20-27	28	—	—	—	—	—	—	—	—
When testing *N. gonorrhoeae*			26	27-46	47	—	—	—	—	—	—	—	26-34
Piperacillin	PC	100 mcg											
When testing *P. aeruginosa*			17	—	18	—	—	25-33	12-18	—	—	—	—
When testing *Enterobacteriaceae*			17	18-20	21	24-30	—	—	—	—	—	—	—
Piperacillin / Tazobactam	PT	100/10mcg											
When testing *Enterobacteriaceae*			17	18-20	21	24-30	—	—	24-30	—	—	—	—
When testing *Staphylococci* and *P. aeruginosa*			17	—	18	—	27-36	25-33	—	—	—	—	—
When testing *Haemophilus spp.*			—	—	—	—	—	—	—	33-38	—	—	—
Polymyxin-B	PB	300 units	11	—	12	13-19	—	18-19	—	—	—	—	—
Prototsomycin (Quinupristin/ Dalfopristin)	PM	15 mcg	—	—	—	—	—	—	—	—	—	—	—
When testing *Staphylococci* and *Streptococci*			15	16-18	19	—	21-28	—	—	—	—	19-24	—
When testing *Haemophilus spp.*			—	—	—	—	—	—	—	19-21	—	—	—
Rifampicin	R	5 mcg											
When testing *E. coli*			—	—	—	8-15	—	—	—	—	—	—	—
When testing *Staphylococci* and *Haemophilus spp.*			16	17-19	20	—	26-34	—	—	23-30	—	—	—
When testing *S. pneumoniae*			19	17-18	19	—	—	—	—	—	—	25-30	—
Sparfloxacin	SC	5 mcg	15	16-19	19	—	27-33	21-26	—	—	—	21-27	—
When testing *E. coli*			—	—	—	30-36	—	—	—	—	—	—	—
When testing *N. gonorrhoeae*			—	—	—	—	—	—	—	—	—	—	43-51
When testing *Haemophilus spp.*			—	—	—	—	—	—	—	32-40	—	—	—
Spectinomycin	SE	100 mcg	14	15-17	18	—	—	—	—	—	—	—	23-29
Streptomycin	S	10 mcg	11	12-14	15	12-20	—	—	—	—	—	—	—
When testing *Staphylococci*			—	—	—	—	14-22	—	—	—	—	—	—
Sulphafurazole (Sulfisoxazole)	SF	300 mcg	12	13-16	17	15-23	24-34	—	—	—	—	—	—
Teicoplanin	TE	30 mcg	10	11-13	14	—	15-21	—	—	—	—	—	—
Tetracycline	T	30 mcg	14	15-18	19	18-25	24-30	—	—	—	—	—	—
When testing *Haemophilus spp.*			25	26-28	29	—	—	—	—	14-22	—	—	—
When testing *Streptococci*			18	19-22	23	—	—	—	—	—	—	27-31	—
When testing *N. gonorrhoeae*			30	31-37	38	—	—	—	—	—	—	—	30-42
Ticarcillin	TI	75 mcg	14	—	15	—	—	21-27	6	—	—	—	—
When testing *P. aeruginosa*			14	15-19	20	24-30	—	—	—	—	—	—	—
When testing *Enterobacteriaceae*													
Ticarcillin / Clavulanic acid	TC	75/10 mcg	14	15-19	20	24-30	—	—	21-25	—	—	—	—
When testing *Enterobacteriaceae*			14	—	15	—	—	30-38	—	—	—	—	—
When testing *P. aeruginosa*			22	—	23	—	29-37	—	—	—	—	—	—
When testing *Staphylococci*													
Tobramycin	TB	10 mcg	12	13-14	15	18-26	19-28	19-25	—	—	—	—	—
Trimethoprim	TR	5 mcg	10	11-15	16	21-23	19-28	—	—	—	—	—	—
Vancomycin	VA	30 mcg											
When testing *Staphylococci*			—	—	15	—	17-21	—	—	—	—	—	—
When testing *Streptococci*			—	—	17	—	—	—	—	—	—	20-27	—

For control limits of Gentamicin 120 mcg (SD195) use *E. faecalis* ATCC 29212, standard 16-23 mm.
For control limits of Streptomycin 300 mcg (SD236) use *E. faecalis* ATCC 29212, standard 14-20 mm.

In accordance to Performance Standards for Antimicrobial Disk Susceptibility Tests, CLSI (formerly NCCLS).
Not included in CLSI chart; FDA approved performance standards for Antimicrobial Discs obtained from drug manufacturers.
For *E. coli*, *S. aureus*, *P. aeruginosa*: Mueller Hinton Agar (MHA). For *Haemophilus spp.*: Haemophilus Test Medium. For *S. pneumoniae*: Mueller Hinton Agar with 5% sheep blood.
For *N. gonorrhoeae*: GC Agar Base with 1% defined growth supplement.
Deterioration in Oxacillin disc content is best assessed with QC Organism *S. aureus* ATCC 25923, with an acceptable zone diameter of 18-24 mm.

References: 1. Bauer, Kirby, Sherris and Turck, 1966, Am. J. Clin. Path., 45 : 493.
2. Performance Standards for Antimicrobial Disk Susceptibility Tests, CLSI Vol. 28 No. 1, Jan. 2008. For more details refer to this volume.

ZONE SIZE INTERPRETATIVE CHART ▼

(Based on Results obtained using Mueller Hinton Agar) △

Product Code	Antimicrobial Agent	Symbol	Disc content	Diameter of zone of inhibition in mm									
				Resistant mm or less	Intermediate mm	Sensitive mm or more	Quality Control Limits						
							E. coli ATCC 25922	*S. aureus* ATCC 25923	*P. aeruginosa* ATCC 27853	*E. coli* ATCC 35218	*H. influenzae* ATCC 49247	*H. influenzae* ATCC 49766	*S. pneumoniae* ATCC 49619	*N. gonorrhoeae* ATCC 49226
SD725	**Cefpodoxime**	CEP	10 mcg										
	When testing *Enterobacteriaceae* and *Staphylococci*			17	18-20	21	23-28	19-25	—	—	—	—	—	—
	When testing *Haemophilus spp.*			—	—	21	—	—	—	—	25-31	—	—	—
	When testing *N. gonorrhoeae*			—	—	29	—	—	—	—	—	—	—	35-43
	When testing *S. pneumoniae*			—	—	—	—	—	—	—	—	—	28-34	—
SD209	**Cefprozil**	CFZ	30 mcg										
	When testing *Enterobacteriaceae* and *Staphylococci*			14	15-17	18	21-27	27-33	—	—	—	20-27	—	—
	When testing *S. pneumoniae*			—	—	—	—	—	—	—	—	—	25-33	—
SD062	**Ceftazidime**	CA	30 mcg	14	15-17	18	25-32	16-20	22-29	—	—	—	—	—
	When testing *Haemophilus spp.*			—	—	26	—	—	—	—	27-38	—	—	—
	When testing *N. gonorrhoeae*			—	—	31	—	—	—	—	—	—	—	35-43
SD110	**Ceftizoxime**	CX	30 mcg	14	15-19	20	30-36	27-35	12-17	—	—	—	28-34	—
	When testing *N. gonorrhoeae*			—	—	30	—	—	—	—	—	—	—	42-51
	When testing *Haemophilus spp.*			—	—	25	—	—	—	—	29-38	—	—	—
SD065	**Ceftriaxone**	CI	30 mcg	13	14-20	21	29-35	22-28	17-22	—	—	—	—	—
	When testing *Haemophilus spp.*			—	—	26	—	—	—	—	31-38	—	—	—
	When testing *S. pneumoniae*			—	—	—	—	—	—	—	—	—	30-39	—
	When testing *Streptococcus* other than *S. pneumoniae*			24	25-26	27	—	—	—	—	—	—	—	—
	When testing *N. gonorrhoeae*			—	—	35	—	—	—	—	—	—	—	39-51
SD061	**Cefuroxime Sodium (Parenteral)**	CU	30 mcg	14	15-17	18	20-26	27-35	—	—	—	—	—	—
	When testing *N. gonorrhoeae*			25	26-30	31	—	—	—	—	—	—	—	33-41
	When testing *Haemophilus spp.*			16	17-19	20	—	—	—	—	—	26-36	—	—
SD050	**Cephalothin**	CH	30 mcg	14	15-17	18	15-21	29-37	—	—	—	—	26-33	—
SD040	**Cephotaxime**	CE	30 mcg	14	15-22	23	29-35	25-31	19-22	—	—	—	—	—
	When testing *Haemophilus spp.*			—	—	26	—	—	—	—	31-39	—	—	—
	When testing *S. pneumoniae*			—	—	—	—	—	—	—	—	—	31-38	—
	When testing *Streptococcus* other than *S. pneumoniae*			25	26-27	28	—	—	—	—	—	—	—	—
	When testing *N. gonorrhoeae*			—	—	31	—	—	—	—	—	—	—	39-48
SD041	**Cephoxitin**	CN	30 mcg	14	15-17	18	22-29	23-23	—	—	—	—	—	—
	When testing *N. gonorrhoeae*			23	24-27	29	—	—	—	—	—	—	—	33-41
SD011	**Chloramphenicol**	C	30 mcg	12	13-17	18	21-27	19-26	—	—	—	—	—	—
	When testing *Haemophilus spp.*			25	26-28	29	—	—	—	—	31-40	—	—	—
	When testing *S. pneumoniae*			20	—	21	—	—	—	—	—	—	23-27	—
	When testing *Streptococcus* other than *S. pneumoniae*			17	18-20	21	—	—	—	—	—	—	—	—
SD241	**Ciexacin**	CIN	100 mcg	14	15-18	19	26-32	—	—	—	—	—	—	—
SD058	**Ciprofloxacin**	CF	5 mcg	15	16-20	21	30-40	22-30	25-33	—	—	—	—	—
	When testing *Haemophilus spp.*			—	—	21	—	—	—	—	34-42	—	—	—
	When testing *N. gonorrhoeae*			27	28-40	41	—	—	—	—	—	—	—	48-58
SD192	**Clarithromycin**	CW	15 mcg											
	When testing *Streptococci*			16	17-20	21	—	—	—	—	—	—	25-31	—
	When testing *Staphylococci*			13	14-17	18	—	26-32	—	—	—	—	—	—
	When testing *Haemophilus spp.*			10	11-12	13	—	—	—	—	11-17	—	—	—
SD051	**Clindamycin**	CD	2 mcg											
	When testing *Staphylococci*			14	15-20	21	—	24-30	—	—	—	—	—	—
	When testing *Streptococci*			15	16-18	19	—	—	—	—	—	—	19-25	—
SD009	**Colistin**	CL	10 mcg	10	—	11	11-17	—	11-17	—	—	—	—	—
SD010	**Co-Trimoxazole (Trimethoprim/ Sulphamethoxazole)**	CO	1.25/ 23.75 mcg	10	11-15	16	23-29	24-32	—	—	24-32	—	—	—
	When testing *S. pneumoniae*			15	16-18	19	—	—	—	—	—	—	21-28	—
SD012	**Doxycycline Hydrochloride**	DO	30 mcg	12	13-15	16	18-24	23-29	—	—	—	—	—	—
SD237	**Enoxacin**	EN	10mcg											
	When testing *Enterobacteriaceae* and *Staphylococci*			14	15-17	18	20-26	22-28	22-29	—	—	—	—	—
	When testing *N. gonorrhoeae*			31	32-35	36	—	—	—	—	—	—	—	43-51
SD013	**Erythromycin**	E	15 mcg											
	When testing *Staphylococci*			13	14-22	23	—	22-30	—	—	—	—	—	—
	When testing *Streptococci*			15	16-20	21	—	—	—	—	—	—	25-30	—

▼ : In accordance to Performance Standards for Antimicrobial Disk Susceptibility Tests, CLSI (formerly NCCLS).

* : Not included in CLSI chart; FDA approved performance standards for Antimicrobial Discs obtained from drug manufacturers.

△ : For *E. coli*, *S. aureus*, *P. aeruginosa* : Mueller Hinton Agar (MHA). For *Haemophilus spp.* : Haemophilus Test Medium. For *S. pneumoniae* : Mueller Hinton Agar with 5% sheep blood. For *N. gonorrhoeae* : GC Agar Base with 1% defined growth supplement.

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(Based on Results obtained using Mueller Hinton Agar) △

Product Code	Antimicrobial Agent	Symbol	Disc content	Resistant mm or less	Intermediate mm	Sensitive mm or more	Diameter of zone of inhibition in mm — Quality Control Limits: *E. coli* ATCC 25922	*S. aureus* ATCC 25923	*P. aeruginosa* ATCC 27853	*E. coli* ATCC 35218	*H. influenzae* ATCC 49247	*H. influenzae* ATCC 49766	*S. pneumoniae* ATCC 49619	*N. gonorrhoeae* ATCC 49226
SD205	**Fosfomycin**	FO	200mcg											
	When testing *Enterobacteriaceae*			12	13-15	16	22-30	—	—	—	—	—	—	—
	When testing *Staphylococci*			—	—	—	—	25-33	—	—	—	—	—	—
SD737	**Gatifloxacin**	GF	5 mcg											
	When testing Enterobacteriaceae			14	15-17	18	30-37	—	20-28	—	—	—	—	—
	When testing *Staphylococci*			19	20-22	23	—	27-33	—	—	—	—	—	—
	When testing *Haemophilus spp.*			—	—	18	—	—	—	—	33-41	—	—	—
	When testing *Streptococci*			17	18-20	21	—	—	—	—	—	—	24-31	—
	When testing *N. gonorrhoeae*			33	34-37	38	—	—	—	—	—	—	—	45-56
SD256	**Gemifloxacin**	GM	5 mcg											
	When testing *Enterobacteriaceae* and *Staphylococci*			15	16-19	20	29-36	21-33	19-25	—	—	—	28-34	—
	When testing *Haemophilus spps*					18	—	—	—	—	30-37	—	—	—
SD016	**Gentamicin**	G	10 mcg	12	13-14	15	19-26	19-27	16-21	—	—	—	—	—
SD073	**Imipenem**	I	10 mcg	13	14-15	16	26-32	—	20-28	—	—	—	—	—
	When testing *Haemophilus spp.*			—	—	16	—	—	—	—	21-29	—	—	—
SD017	**Kanamycin**	K	30 mcg	13	14-17	18	17-25	19-26	—	—	—	—	—	—
SD216	**Levofloxacin**	LE	5 mcg											
	When testing *Staphylococci*			15	16-18	19								
	When testing *Enterobacteriaceae* and *Streptococci*			13	14-16	17	29-37	25-30	19-26	—	—	—	—	—
	When testing *Haemophilus spp.*			—	—	17	—	—	—	—	32-40	—	20-25	—
SD215	**Linezolid**	LZ	30 mcg				—	25-32	—	—	—	—	25-34	—
	When testing *Staphylococci*			—	—	21								
	When testing *Streptococci*			—	—	21								
SD296	**Lomefloxacin**	LO	10 mcg	18	19-21	22	27-33	23-29	22-28	—	—	—	—	—
	When testing *N. gonorrhoeae*			26	27-37	38	—	—	—	—	—	—	—	45-54
	When testing *Haemophilus spp.*			—	—	22	—	—	—	—	33-41	—	—	—
SD176	**Mecillinam**	MC	10 mcg											
	When testing *Enterobacteriaceae*			11	12-14	15	24-30	—	—	—	—	—	—	—
SD727	**Meropenem**	MR	10 mcg											
	When testing *Enterobacteriaceae*, *Staphylococci* and *P. aeruginosa*			13	14-15	16	28-34	29-37	27-33	—	—	—	—	—
	When testing *Haemophilus spp.*			—	—	20	—	—	—	—	20-28	—	—	—
	When testing *S. pneumoniae*			—	—	—	—	—	—	—	—	—	28-35	—
SD015	**Methicillin**	M	5 mcg	9	10-13	14	—	17-22	—	—	—	—	—	—
SD225	**Mezlocillin**	MZ	75 mcg	17	18-20	21	23-29	—	19-25	—	—	—	—	—
SD158	**Minocycline**	MI	30 mcg	14	15-18	19	19-25	25-30	—	—	—	—	—	—
SD220	**Moxalactam**	MX	30 mcg	14	15-22	23	28-35	18-24	17-25	—	—	—	—	—
SD217	**Moxifloxacin**	MO	5 mcg				28-35	28-35	17-25	—	31-39	—	25-31	—
	When testing *Staphylococci*			20	21-23	24								
	When testing *Haemophilus spp.*			—	—	18								
	When testing *Streptococci*			14	15-17	18								
SD246	**Nafcillin**	NAF	1 mcg											
	When testing *Staphylococci*			10	11-12	13	—	16-22	—	—	—	—	—	—
SD021	**Nalidixic Acid**	NA	30 mcg	13	14-18	19	22-28	—	—	—	—	—	—	—
***SD022**	**Neomycin**	N	30 mcg	12	13-16	17	17-23	18-26	—	—	—	—	—	—
SD046	**Netillin (Netilmicin Sulphate)**	NT	30 mcg	12	13-14	15	22-30	22-31	17-23	—	—	—	—	—
SD023	**Nitrofurantoin**	NF	300 mcg	14	15-16	17	20-25	18-22	—	—	—	—	23-29	—
SD057	**Norfloxacin**	NX	10 mcg	12	13-16	17	28-35	17-28	22-29	—	—	—	—	—
	When testing *S. pneumoniae*			—	—	—	—	—	—	—	—	—	15-21	—
***SD053**	**Novobiocin**	NV	30 mcg	17	18-21	22	—	22-31	—	—	—	—	—	—
SD087	**Ofloxacin**	OF	5 mcg	12	13-15	16	29-33	24-28	17-21	—	—	—	—	—
	When testing *Haemophilus spp.*			—	—	16	—	—	—	—	31-40	—	—	—
	When testing *Streptococci*			12	13-15	16	—	—	—	—	—	—	16-21	—
	When testing *N. gonorrhoeae*			24	25-30	31	—	—	—	—	—	—	—	43-51
	When testing *Staphylococci*			14	15-17	18								

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				Resistant mm or less	Intermediate mm	Sensitive mm or more	Quality Control Limits							
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SD088	**Oxacillin**	OX	1 mcg											
	When testing *Staphylococci*			10	11-12	13	—	18-24	—	—	—	—	—	—
	When testing coagulase negative *Staphylococci*			17	—	18	—	—	—	—	—	—	—	—
	When testing *S. pneumoniae*			—	—	—	—	—	—	—	—	—	≥12*	—
SD186	**Oxolinic acid**	OA	2 mcg	10	—	11	30-36	10-13	—	—	—	—	—	—
SD028	**Penicillin G**	P	10 units											
	When testing *Staphylococci*			28	—	29	—	26-37	—	—	—	—	—	—
	When testing *S. pneumoniae*			—	—	20	—	—	—	—	—	—	24-36	—
	When testing *Streptococci* other than *S. pneumoniae*			19	20-27	28	—	—	—	—	—	—	—	—
	When testing *N. gonorrhoeae*			26	27-46	47	—	—	—	—	—	—	—	28-36
SD066	**Piperacillin**	PC	100 mcg											
	When testing *P. aeruginosa*			13	—	18	—	—	25-32	12-18	—	—	—	—
	When testing *Enterobacteriaceae*			13	18-20	21	24-30	—	—	—	—	—	—	—
SD219	**Piperacillin / Tazobactam**	PT	100/10mcg											
	When testing *Enterobacteriaceae*			17	18-20	21	24-30	—	—	24-30	—	—	—	—
	When testing *Staphylococci* and *P. aeruginosa*			17	—	18	—	27-36	25-33	—	—	—	—	—
	When testing *Haemophilus* spp.			—	—	—	—	—	—	—	33-38	—	—	—
SD029	**Polymyxin-B**	PB	300 units	11	—	12	13-19	—	14-19	—	—	—	—	—
SD178	**Pristinamycin (Quinupristin/ Dalfopristin)**	PM	15 mcg	—	—	—	—	—	—	—	—	—	—	—
	When testing *Staphylococci* and *Streptococci*			15	16-18	19	—	21-28	—	—	—	—	18-24	—
	When testing *Haemophilus* spp.			—	—	—	—	—	—	—	16-21	—	—	—
SD030	**Rifampicin**	R	5 mcg											
	When testing *E. coli*			—	—	—	8-10	—	—	—	—	—	—	—
	When testing *Staphylococci* and *Haemophilus* spp.			16	17-19	20	—	26-34	—	—	33-38	—	—	—
	When testing *S. pneumoniae*			16	17-19	19	—	—	—	—	—	—	25-36	—
SD162	**Sparfloxacin**	SC	5 mcg	15	16-19	16	—	22-33	21-29	—	—	—	31-47	—
	When testing *E. coli*			—	—	—	30-36	—	—	—	—	—	—	—
	When testing *N. gonorrhoeae*			—	—	—	—	—	—	—	—	—	—	43-51
	When testing *Haemophilus* spp.			—	—	—	—	—	—	—	33-40	—	—	—
SD191	**Spectinomycin**	SS	100 mcg	14	15-17	18	—	—	—	—	—	—	—	23-29
SD031	**Streptomycin**	S	10 mcg	11	12-14	15	13-20	—	—	—	—	—	—	—
	When testing *Staphylococci*			—	—	—	—	14-22	—	—	—	—	—	—
SD032	**Sulphafurazole (Sulfisoxazole)**	SF	300 mcg	12	13-16	17	15-23	24-34	—	—	—	—	—	—
SD213	**Teicoplanin**	TE	30 mcg	10	11-13	14	—	15-21	—	—	—	—	—	—
SD037	**Tetracycline**	T	30 mcg	14	15-18	19	19-25	24-30	—	—	—	—	—	—
	When testing *Haemophilus* spp.			25	26-28	29	—	—	—	—	14-22	—	—	—
	When testing *Streptococci*			18	19-22	23	—	—	—	—	—	—	27-31	—
	When testing *N. gonorrhoeae*			30	31-37	38	—	—	—	—	—	—	—	30-42
SD074	**Ticarcillin**	TI	75 mcg											
	When testing *P. aeruginosa*			14	—	19	—	—	21-27	0	—	—	—	—
	When testing *Enterobacteriaceae*			14	15-19	20	24-30	—	—	—	—	—	—	—
SD281	**Ticarcillin / Clavulanic acid**	TC	75/10 mcg											
	When testing *Enterobacteriaceae*			19	15-19	20	24-30	—	—	21-29	—	—	—	—
	When testing *P. aeruginosa*			14	—	15	—	—	20-28	—	—	—	—	—
	When testing *Staphylococci*			22	—	23	—	29-37	—	—	—	—	—	—
SD044	**Tobramycin**	TB	10 mcg	12	13-14	15	18-28	19-29	19-35	—	—	—	—	—
SD039	**Trimethoprim**	TR	5 mcg	10	11-15	16	21-33	19-26	—	—	—	—	—	—
SD045	**Vancomycin**	VA	30 mcg											
	When testing *Staphylococci*			—	—	15	—	17-21	—	—	—	—	—	—
	When testing *Streptococci*			—	—	17	—	—	—	—	—	—	20-27	—

Note :
For control limits of Gentamicin 120 mcg (SD190) use *E. faecalis* ATCC 29212, standard 16-23 mm.
For control limits of Streptomycin 300 mcg (SD236) use *E. faecalis* ATCC 29212, standard 14-20 mm.

In accordance to Performance Standards for Antimicrobial Disk Susceptibility Tests, CLSI (formerly NCCLS).
Not included in CLSI chart: FDA approved performance standards for Antimicrobial Discs obtained from drug manufacturers.
For *E. coli*, *S. aureus*, *P. aeruginosa* : Mueller Hinton Agar (MHA). For *Haemophilus* spp. : Haemophilus Test Medium. For *S. pneumoniae* : Mueller Hinton Agar with 5% sheep blood.
For *N. gonorrhoeae* : GC Agar Base with 1% defined growth supplement.
Determination in Oxacillin disc content is best assessed with QC Organism *S. aureus* ATCC 25923, with an acceptable zone diameter of 18-24 mm.

References: 1. Bauer, Kirby, Sherris and Turck, 1966, Am. J. Clin. Path., 45 : 493.
2. Performance Standards for Antimicrobial Disk Susceptibility Tests, CLSI Vol. 28 No. 1, Jan. 2008. For more details refer to this volume.