# CHAPTER - I

## 1. INTRODUCTION

Water has a major impact on the quality of life. The ensuring of good quality drinking water is a basic factor in guaranteeing public health, the protection of the environment and sustainable development. Improved health is possible by means of adequate, safe drinking water and good sanitation provision (Park, 2005). Water quality has direct influence on public health. Water intended for human consumption should be safe and wholesome. Safe drinking water is defined as water with microbial, chemical and physical characteristics that meet WHO guidelines or national standards on drinking water quality (WHO, 2005).

The growing imbalances between supply and demand have resulted in pollution and environmental degradation. 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). Changes in water quality are reflected in its physical, biological, and chemical conditions; and these in turn are influenced by physical and anthropogenic activities (ICIMOD, 2006). When water gets contaminated with various pathogenic as well as opportunistic microflora and toxic chemical compounds, it serves as the vehicle of transmission of a number of infectious diseases. Unfortunately, over a billion people in the developing world do not have access to safe water supply. The WHO has estimated that up to 80% of all sickness and disease in the world is caused by inadequate sanitation, polluted or unavailability of water. The pollution of drinking water is responsible for a large number of mortalities and morbidities due to water-borne diseases like typhoid, cholera, dysentery, hepatitis as well a many protozoan and helminthic infestations (WHO, 1996).

Majority of water borne diseases in Nepal are due to consumption and usage of contaminated water. In the past pathogenic microorganisms like *Salmonell*a serotypes and *Vibrio* spp, were isolated from drinking water which suggests the pressing concern for the inhabitants of Kathmandu Valley. Indicator bacteria like coliforms provide a good assessment of fecal contamination of drinking water. Diarrhoeal diseases are still recognized as a major problem for Nepalese children, being recorded as the second most prevalent diagnosis in out-patience services. Today 72% of the nationwide disease burden is related to poor quality of drinking water, and around 75 children die each day from diarrhoea alone (Sherpa, 2003). On the whole, the incidence of diarrhoea is on the increase. 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). A report obtained from Teku Hospital in Kathmandu shows that 16.5% of all deaths were due to water-borne diseases (Metcalf and 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.). Water pollution is the most serious public health issue in Nepal. The rivers have become major places for urban solid waste disposal and dumping, and for industrial effluents, all of which are responsible for deteriorating the river-water quality and contributing to waterborne diseases e.g. diarrhoea, dysentery, cholera and typhoid resulting from consumption of contaminated water. These water-related diseases are generally caused by poor sanitation and poor water quality (DOHS, 2004/2005).

The emergence of antibiotic resistant (AR) bacteria has become a growing public health concern in recent years. The presence and persistence of AR bacteria, particularly multiple-antibiotic resistant (MAR) bacteria is a serious threat to mankind. Antibiotic susceptibility testing is an essential component of the practice. It allows physicians to make accurate

choices with all forms of antimicrobial therapy, prophylaxis, and pathogen directed therapy. Without surveillance for the development of antibiotic resistance, serious infections may lead to death due to inadequate antibiotic therapy.

Population growth and advances in technology have lead to increased discharge of industrial effluents replete with heavy metals such as mercury, zinc, cobalt, cadmium, lead and chromium into our environment. Heavy metals in low concentration play indespensible roles in cell growth and metallic functions but become inhibitory at relatively high concentrations. Nevertheless, some bacteria are able to resist some of the heavy metals at toxic levels (Gadd and Griffiths 1978). The resistance may be mediated by genetic factors, production of chelating agents, binding by cell surface slime and/or oxidative detoxification. Metal resistant microorganisms may be useful indicator of potential toxicity to other forms of life. Hg, Pb, and Cd are a serious concern because they are non biodegradable, highly toxic and are present in a variety of waste streams that contaminate the environment.

Over the past decade, the consumption of metals and chemicals in the process industries has increased dramatically. Industrial uses of metals such as metal plating, tanneries, industrial processes utilizing metal as catalysts, have generated large amount of aqueous effluents that contain high levels of heavy metals. These heavy metals include cadmium, chromium, cobalt, copper, iron, manganese, mercury, molybdenum, nickel, silver, and zinc. Metalpolluted industrial effluents discharged into sewage treatment plants could lead to high metal concentrations in the activated sludge. Microbial populations in metal polluted environments contain micro-organisms which have adapted to toxic concentrations of heavy metals and become “metal-resistant”.

At present, metal-polluted industrial effluents are mostly treated by the chemical methods, such as chemical precipitation, electrochemical treatment and ion exchange. These methods provide only partially effective treatment and are costly to implement, especially when the metal concentration is low. The alternative use of microbe-based biosorbents for the removal and recovery of toxic metals from industrial effluents can be economical and effective methods for metal removal.

# CHAPTER - II

## 2. OBJECTIVES

### 2.1 General Objective

To isolate and identify *E. coli* from drinking water of Kathmandu valley and to study the antibiotic susceptibility pattern and to determine the Minimum Inhibitory Concentration of various heavy metals against isolates.

### 2.2 Specific Objectives

- To enumerate indicator organisms (total coliform and thermotolerant coliform) in drinking water.
- To determine physico-chemical parameters (temperature and pH) of drinking water.
- To isolate and identify *E .coli* from drinking water.
- To study antibiotic susceptibility pattern of the isolates.
- To study heavy metals resistance pattern of bacterial isolates.

# CHAPTER – III

## 3. LITERATURE REVIEW

### 3.1 Water pollution

Water pollution is defined as the result of undesirable change caused by pollutants in the physical, chemical and biological characteristics of natural water that adversely affect man and his environment. Water being so important to all life, including man, the pollution of water is a treat to the survival and existence of life itself. The inadequate sewerage system and direct disposal of household waste into the water course has tremendously increased the water pollution. The pollutants thus being fed into the tributary streams have rendered the river useless and serve just as a wastewater drain. Rapid urbanization and absence of reliable wastewater treatment facilities are the major causes of river pollution. Drastic action is required to restore our waterways to their original glory. Thus protection of water supplies from contamination is the first line of defence. Source protection is almost invariably the best method of ensuring safe drinking water (WHO,1996).

Drinking water is the basic minimum requirement of all the human beings. Access to safe and adequate drinking water is the commitment of the government. Therefore, the government needs to raise the awareness of the people on sanitation and promote, operate and maintain domestic as well as industrial wastewater treatment plants to reduce the pollution of surface water and safeguard the public health.

Water pollution is the most serious public health issue in Nepal. Many human diseases in Nepal are due to polluted water, caused by lack of sanitation and to some extent by the inappropriate establishment of industries. Water-borne diseases such as diarrhea, dysentery, cholera and skin diseases are among the top ten dreadful leading diseases. The quality of both surface and ground water sources used for drinking water purpose is deteriorating mainly due to both natural and anthropogenic contaminations. Discharge of untreated domestic sewers and industrial effluents into rivers, and landslides, soil erosion and floods pollute the river water. The ground water in the Kathmandu valley is polluted with fecal contamination.

### 3.2 Sources of water pollution

The term point-source pollution refers to pollutants discharged from one discrete location or point, such as an industry or municipal wastewater treatment plant. Municipal sewage treatment plant point sources can contribute pollution in the form of oxygen-depleting nutrients and in the form of pathogens that cause serious health hazards in drinking water and swimming areas. Industrial point sources can contribute pollution in the form of toxic chemicals and heavy metals.

The term non-point-source pollution refers to pollutants that cannot be identified as coming from one discrete location or point. Examples are oil and grease that enter the water with runoff from urban streets, nitrogen from fertilizers and pesticides, and animal wastes that wash into surface waters from agricultural lands.

### 3.3 Effects of Water Pollution

Water pollution is the most serious public health issue in Nepal. There is a vital connection between water and health. Water pollution has been seriously affecting the life of humans, plants as well as animals. The eco-system of rivers, streams, lakes, seas and oceans is also

getting deteriorated due to the contamination of water, through various sources. This condition also leads to the outbreak of numerous diseases, majority of them being lethal and contagious. Pathogenic bacteria that have been transmitted by water or wastewater include *Salmonella* spp. causing typhoid fever, *Shigella* spp. causing bacillary dysentery, *Campylobacter*, enteropathogenic *Escherichia coli*, v*ibrio cholerae*, *Leptospira* and *Yersinia.*

Pollution affects the chemistry of water. The pollutants, including toxic chemicals, can alter the acidity, conductivity and temperature of water. Diseases affecting the heart, poor circulation of blood and the nervous system and ailments like skin lesion are often linked to the harmful effects of water pollution. Carcinogenic pollutants found in polluted water might cause cancer. Another effect is the acceleration of eutrophication processes of water. It is aging of a lake by biological enrichment. With time, streams draining into lake, introduce nutrient as nitrogen and phosphorous which encourage growth of aquatic organisms.

### 3.4 Water Supply System of Kathmandu valley

Kathmandu Valley is home to about 1.3 million people mostly living in urban environments, of which Kathmandu (population 800,000) is the largest. The water supply situation in Kathmandu Valley is critical, especially during the dry season, when most households receive water for only 1-2 hours per day, and many people have to rely on small quantities of water brought by tankers. In the Kathmandu Valley, the drinking water supply sources are varied and water quality often changes dramatically as it travels through the distribution system. As noted above, 58% of the water supply in urban areas is piped. Piped water is distributed in taps on the street or on individual dwellings.

There are also places where water is collected directly from a source, such as a tube well or spring, and either consumed on the spot or stored for future consumption. The KUKL is responsible for all treatment plants and supply systems in the Kathmandu Valley. Additionally, many other international nongovernmental organizations and Nepali NGOs are also interested in water quality. Some NGOs such as ENPHO play a role in water quality monitoring for the water supply and distribution system. Since water supplies are intermittent throughout the day, water is stored for future use. The National Water Supply Policy recently developed for Nepal spelled out urban and rural water supply. Kathmandu Upatyaka Khanepani Limited(KUKL)is a semi-government agency that supplies water mainly in the urban areas. This supply is only about 120 million litres per day (mld) during the rainy season, 80 mld during dry season of the estimated daily demand of 170 mld (NWSC 2001). However, since the supply of water by the public agency alone is not adequate, the additional requirement of domestic water is being supplied by the private sector. In addition, households also draw water by themselves from groundwater. The water drawn from the water source is not suitable for drinking purpose without treatment. The water treatment has been carried out in all the existing water supply systems. Treatment varies from bleaching powder chlorination in Chapagaon system to conventional treatments like sedimentation, filtration and chlorination in Bansbari and Sundarijal systems.

The status of the existing water treatment plants has been studied in detail. The study showed that most of the water treatment plants are in poor condition and very poorly maintained. Besides, the operational data are not properly recorded and kept. The treated water quality deteriorates in rainy season and does not appear to be suitable for drinking as per WHO guideline. The maintenance personnel are not well trained to operate and maintain the treatment plants. The maintenance budget is too low for normal maintenance of the treatment plants. Among the existing water treatment plants, the Mahankalchaur water treatment plant is working most satisfactorily.

### 3.5 Outbreak of water borne diseases in Nepal

Water related diseases remains one of the major health concern in the world. diarrheal diseases, which are largely derived from poor water and sanitation, accounted for 1.8 million death in 2002 and contributed around 62 million disability (WHO,2004).This health burden is primarily borne by the population in developing countries. The WHO has estimated that up to 80% of all sickness and disease in the developing countries is caused by inadequate sanitation, polluted water, or unavailability of water (Cheesebrough, 1993).

The number of outbreaks that has been throughout the world demonstrates that transmission of pathogens by drinking water remains a significant cause of illness. However, estimates of illness based solely on detected outbreaks are likely to be underestimating the problem. Out breaks of waterborne epidemic is rampant in Nepal as in most of the third world countries. Mortality and morbidity due to such disease still top the list. Every year the onset of the epidemics comes also with the monsoon (Sharma, 2002). Many outbreaks of waterborne diseases probably are not recognized; therefore, their incidences are not reported. But there are real incidents of waterborne disease, in which improvements in drinking water quality could have saved many lives.

As mentioned in the UNICEF situation analysis (UNICEF, 1987), in Nepal water and hygiene related diseases, are responsible for 15% of all cases and 8% of all deaths in the general population. In 1985, over 50% of hospital patients in Nepal were found to be suffering from gastro-intestinal disorder normally caused by waterborne pathogens. In 1990, cholera outbreak during summer hit different parts of the country including the capital city and caused an enormous loss of lives (DISVI, 1990). In Pokhara, more than 50% of the leading diseases causing morbidity were recorded to be waterborne. In

Emergency Department of Pokhara Hospital, 322 persons were registered only due to cholera in between June-July, 1990. In 1990, Public Health Division recorded 23,888 gastro-enteritis cases in 39 districts with maximum There was outbreak of gastro-enteritis in Bhaktapur district during summer in 1995 (ENPHO/DISVI, 1995).

Kathmandu Valley is affected by the severe water crises. The shortage of water is a problem in itself, but besides the quantitative shortage, the risk of waterborne diseases has become an even serious problem. People are not having access to hygienic drinking water. Outbreak of diseases like typhoid fever, cholera, dysentery, worm infestation, hepatitis and many other are spread by contaminated water, and are prevalent in urban and rural areas. A report from HMG Ministry of Health, 0.27% of patients had typhoid, 1.63% from Diarrhoea, and 0.07% from Jaundice and Hepatitis in Kathmandu in 2002/2003. In Lalitpur: typhoid cases were 0.58%, diarrhea 2.60%, and jaundice and infective hepatitis 0.09% and in Bhaktapur: typhoid 0.37%, diarrhea 1.53%, and jaundice and infective hepatitis 0.08%. Due to outbreak of diarrhoea in Jajarkok district; 1,368 cases were reported which gives an average attack rate of 4.4% and case fatality rate of 8.5 % with 122 deaths (between 15 May and 17 July 2009).

The outbreak of a diarrhoeal epidemic in some districts of the Mid-Western and Far Western Development Regions has yet to come under control. At least five to six persons are still dying of the disease in the districts everyday, and the total death toll has reached 142, with 122 deaths recorded in Jajarkot district alone, according to official media reports. And there is little hope of the epidemic coming under control within this week, which means the toll will only rise further.

### 3.6 Physicochemical parameters of water

The ordinary consumer judges the water quality by its physical characteristics. The provision of drinking water that is not only safe but also pleasing in appearance, taste and odor is a matter of high priority (Park, 2000). The chemical parameter is used to ascertain the presence of inorganic matter, soluble salts of organic matters in water. The problems associated with chemical constituents of drinking water arise primarily from their ability to cause health effects after prolonged periods of exposure of contaminants that have cumulative toxic properties, such as heavy metals, and substances that are carcinogenic (WHO, 1996).

### 3.6.1 Temperature

Temperature is one of the important parameter of water and is basically important for its effects on the chemistry and biological reactions in the organisms in the water. A rise in temperature of the water leads to the speeding of chemical reactions, enhanced growth of microorganisms, reduction in solubility of gases and amplify tastes and odour (Tridevi and Goel, 1986). Temperature of surface water is influence by latitude, altitude, season, time of day, air circulation, cloud cover and the flow and depth of water body (Chapman, 1999).

### 3.6.2 pH

pH is the negative log10 of the hydrogen ion concentration in a solution. Measurement of pH is the most important and frequently used tests in water chemistry (APHA, 1998). It is the major of the intensity of acidity or alkalinity and measures the concentration of hydrogen ions in water. pH less than 7 may cause corrosion and encrustation in the distribution system where as the disinfection with chlorine is less effective if pH of water exceeds 8.0 (WHO,1993). The pH value of drinking water from any sources should be within range, 6.5-8.5 (Trivedy and Goel, 1986). The pH of the water entering the distribution system must be controlled to minimize the corrosion of water mains and pipes

in house hold water systems. Alkalinity and calcium management also contribute to the stability of water and control its aggressiveness to pipe and appliance (WHO, 2004).

### 3.7 Water purification

Water purification is the process of removing undesirable chemicals, materials, and biological contaminants from raw water. The primary risk of consuming untreated water is the transmission of communicable diseases by pathogenic organisms. The goal is to produce water fit for a specific purpose. Most water is purified for human consumption (drinking water) but water purification may also be designed for a variety of other purposes, including to meet the requirements of medical, pharmacology, chemical and industrial applications. According to a 2007 World Health Organization report, 1.1 billion people lack access to an improved drinking water supply, 88% of the 4 billion annual cases of diarrheal disease are attributed to unsafe water and inadequate sanitation and hygiene, and 1.8 million people die from diarrheal diseases each year. The WHO estimates that 94% of these diarrheal cases are preventable through modifications to the environment, including access to safe water. Simple techniques for treating water at home, such as chlorination, filters, and solar disinfection, and storing it in safe containers could save a huge number of lives each year.

The important steps involved in the treatment of raw water are pretreatment, aeration, coagulation, flocculation, sedimentation, filtration and disinfection. Disinfection of water is the most important step in obtaining hygienically safe water. Chlorination is the most widely used method of disinfection in municipal drinking water treatment that destroys disease causing microorganisms and, the use of chlorine is often the only affordable mean of disinfecting drinking water in developing countries . Water is chlorinated to contain 0.1 to 0.2 ppm of residual chlorine after 20 minutes of addition. Dealing with the mechanism, often reacting with water chlorine is converted into hypochlorous acid, which in turn

release nascent oxygen, which oxidizes the cellular components of microorganisms as well as organic matter. Increasing pH reduces effectiveness of chlorine and lower temperature causes delay in disinfection.

### 3.8.Microbial Indicators of Water Quality

The Detection of all the disease causing organism is not possible for the high cost involving and time consuming techniques. Therefore some means are necessary to confirm that drinking water is safe. It is not usually practical to examine water for the various pathogenic organisms that may be present. So, some "indicator organisms" are chosen for the examination of bacteriological quality of the water. The major indicator organisms of faecal pollution *Escherichia coli*, the thermotolerant and other coliform bacteria, the faecal streptococci and spores of sulfite reducing clostridia (WHO,1993).

### 3.8.1.Coliform bacteria

Coliform bacteria have been recognized as a suitable microbial indicator of drinking water quality. They make up around 10 percent of the intestinal microflora of the human and animal intestine. The term coliform organism refers to gram negative, oxidase negative, non sporing rods capable of growing aerobically on agar medium containing bile salts and able to ferment lactose within 48 hours at 35-37$^0$C with the production of both acid and gas (Cheesebrough, 1985 and Anderson and Davidson, 2002). The coliform group includes genus, *Escherichia, Klebsella, Enterobacter* and *Citrobacter*. The levels of coliform organisms present in the drinking water should not exceed the maximum permissible value of less than one cell per 100 ml of water (WHO, 1993).

### 3.8.2 Thermotolerant coliform bacteria

These are the group of coliform organisms that are able to ferment lactose at $44.5^0$C in 24 hours and comprise genus *Escherichia* and to a lesser extent species of *Klebsiella*, and *Enterobacter.*

### 3.8.3 Faecal streptococci

Fecal streptococci regularly occur in feces, but in much smaller numbers than *E. coli.* In doubtful cases, the finding of fecal streptococci in water is regarded as important confirmatory evidence of recent fecal pollution of water. They are highly resistant to drying and may be valuable for routine control testing often laying new means in distribution system or for detecting pollution by surface run-off to ground or surface waters. These all give a positive reaction with lancefield Group D antisera.

### 3.8.4 *Clostridium perfringens*

They also occur regularly in faeces, however in much smaller number than *E. coli*. They are anaerobic, spore forming organisms; the spores are capable of surviving in water for a longer time than organisms of the colifrom group and usually resist chlorination at the dose normally used in water works practices. The presence of spores of *Clostridium perfringens* in natural water suggests that faecal pollution has occurred and their presence suggests that fecal contamination occurred at some remote time.

### 3.8.5 *Escherichia coli*

*Escherichia coli* is a member of the family Enterobactericeae and is characterized by possession of the enzymes β-glactosidase and β-glucononidase. The Enterbacteriaceae is are gram negative bacilli that are either motile with peritrichus flagella or non motile, grow both anaerobically and an aerobically on simple laboratory media and on MacConkey bile

salt Lactose agar, are oxidase negative, catalase positive and reduce nitrates to nitrites. They ferment glucose in peptone water with the production of either or acid and gas. The characters of *Escherichia coli* that distinguish it from other enterobacteria are that it motile forms, gas from glucose, ferments lactose, produce indole, gives a positive methyl red reaction and a negative Voges-Prokauser reaction and does not utilize citrate.

*Escherichia coli* is abundant in human or animal faeces, and it is found in sewage, effluents and all natural waters and soil that are subject to recent faecal contamination. It is the commonest cause of urinary tract infection (UTI) and is commonly present in appendix abscess, peritonitis, cholecystitis, Septic wounds and bedsores. They may infect the lower respiratory passages or cause bacteria and endotoxic shock of gastroenteritis (Diarrhea). It grows at 44-45$^0$C on complex media, lactose fermenter and mannitol fermenter with production of acid and gas. *E. coli* is abundant in human and animal faeces where it may attain concentrations in fresh faeces of $10^9$ per gram (WHO, 1993).

**Significance of *E. coli* in drinking water**

As a member of the Enterobacteriaceae family, *E. coli* is naturally found in the intestines of humans and warm-blooded animals. Unlike other bacteria in this family, *E. coli* does not usually occur naturally on plants or in soil and water. Within human and animal faeces, *E. coli* is present at a concentration of approximately $10^9$ per gram (Edberg et al., 2000) . Although *E. coli* are part of the natural faecal flora, some strains of this bacterium can cause gastrointestinal illness along with other, more serious health problems. Of the coliforms, *E. coli* is generally the most sensitive to environmental stresses. Its survival time in the environment is dependent on many factors, including temperature, exposure to sunlight, presence and types of other microflora, and the type of water involved (e.g., groundwater, surface water, or treated distribution water). In general terms, *E. coli* survives for about 4-12 weeks in water containing a moderate microflora at a temperature of 15-

$18^0$C ( Edberg et al., 2000). The inability of *E. coli* to grow in water, combined with its short survival time in water environments, means that the detection of *E. coli* in a water system is a good indicator of recent faecal contamination.

**Role of *E. coli* as an indicator of microbiological safety**

Although modern microbiological techniques have made the detection of pathogenic bacteria, viruses, and protozoa possible, it is currently not practical to attempt to routinely isolate them from drinking water. Reasons for this include the large number of possible pathogens, the lack of inclusion of previously unknown pathogens, and the time and expense associated with routine monitoring of all pathogens. Instead, microbial indicators are used, since it is less difficult, less expensive, and less time consuming to monitor indicators than to monitor individual pathogens. Simple, inexpensive techniques encourage a higher number of samples to be tested, giving a better overall picture of the water quality and therefore better protection of public health. Newer molecular methods may provide an easy, inexpensive, and quick method for the detection of pathogens or indicators; to date, however, this is not the case.

An appropriate health-based indicator of microbial pathogens should possess qualities of an ideal indicator. The life span of *E. coli* in water environment is short, so the detection of *E. coli* in water sources indicates the recent fecal contamination of the water bodies. The indicator should always be present when the pathogen is present and should not be detected when the pathogen is absent; it should have a life span similar to that of the pathogen of concern; it should be present in large numbers and should be readily detected by simple and inexpensive methods; and it should not multiply in the environment once it has been shed by the host. It should not grow in the natural water. Based on these qualities, if the

indicator is isolated from the water supply, this infers that pathogenic organisms could be present; if the indicator is absent, pathogenic organisms are probably also absent.

Of the contaminants that may be found in drinking water, those present in human and animal faeces pose the greatest danger to public health. For this reason, the ability to detect faecal contamination in drinking water is a necessity for ensuring public safety. As early as the 19th century, *E. coli* was recognized as a good indicator of faecal contamination. It was identified as the only species in the coliform group found exclusively in the intestinal tract of humans and other warm-blooded animals and subsequently excreted in large numbers in faeces (approximately $10^9$ per gram) (Department of National Health and Welfare, 1977). In addition to being faecal specific, *E. coli* do not usually multiply in the environment and have a life span on the same order of magnitude as those of other enteric bacterial pathogens, both of which are qualities of an ideal indicator. As mentioned previously, they are also excreted in the faeces in high numbers, making detection possible even when greatly diluted.

### 3.8.6 Other members of coliform group

*Klebsiella* spp belongs to the family Enterobacteriaceae. They are Gram-negative, non-motile, capsulated rods, aerobes, facultative anaerobes and can grow an optimum temperature of $37^0$C. *Klebsiella* are lactose fermenting, producing mucoid colonies on MacConkey agar (Cheesbrough, 2000). D-glucose and other carbohydrates are catabolized with the production of acid and gas. Acetoin production and citrate utilization reaction vary among species. Several species hydrolyze urea and $H_2S$ is not produced (Holt et al., 1994). *Klebsiella* spp causes chest infections and occasionally severe bronchopneumonia with lung abscess. Infections are often opportunistic, occurring in those with existing chest disease or diabetes mellitus, or in malnourished person.

*Enterobacter* spp. can be found in the intestinal tract of humans and animals and in soil, sewage, water and dairy products. They are one of the commonest opportunistic pathogens isolated from urine, respiratory tract and blood (Cheesbrough, 1993).

*Citrobacter* spp. is isolated from human or animal faces, food, water, sewage, soil, etc. they are opportunistic pathogens and occasionally isolated from urine, blood, pus and other specimens (Cheesbrough, 1993). *Citrobacter freundii* and *Citrobacter diversus* have been associated with cases of diarrhoea (WHO, 2004).

### 3.8.7 Other common intestinal pathogens contaminating drinking water

***Salmonella spp.***

*Salmonella* are ubiquitous in the environment and can be detected at low concentration in most surface water. It belongs to the family Enterobacteriaceae. It is Gram-negative, straight rod shaped, non capsulated, non-sporing with peritrichous flagella, aerobic and facultative anaerobic bacilli. They grow between 15-45$^0$C with an optimum temperature of 37$^0$C. XLD is best selective media and selenite F broth is probably the best enrichment media for it. Most strain produces $H_2S$ in TSI with production of acid and gas (Collee et al., 1996). These organisms are usually present in small numbers compared to coliform (APHA, 1998). *S.* Typhi is mainly water borne (Cheesebrough, 2000). It causes enteric fever (typhoid and paratyphoid) and other *Salmonella* spp causes diarrhoeal disease. At present, 107 types can be distinguished by phage typing which is the value in epidemiologic studies. For paratyphoid fever, three bioserotypes of *S. enteridis* are recognized, paratyphoid A, B and C (Benenson, 1995).

***Shigella spp.***

It belongs to the family Enterobacteriaceace. The isolation of *Shigella* from drinking water indicates recent faecal contamination (WHO, 1996). Transmission is mainly by the faecal oral route with poor sanitation, unhygienic conditions and overcrowding, facilitating the rapid spread of infection. Only small numbers of organisms are required to cause disease (Cheesbrough, 2002). It is Gram-negative non-spore forming, non-motile rods, capable of growth under both aerobic and anaerobic conditions. They are non lactose fermenting and non citrate utilizing. All *Shigella* form acid from glucose, but except for a few serotypes, not gas. Colonies are pale and yellowish in MacConkey agar. XLD is probably the best selective medium and producing red colonies (Collee et al., 1996).

***Vibrio cholerae***

*Vibrio* species are primarily aquatic occurring in fresh water, estuarine and marine habitats in association with aquatic organisms include example copepods (Singleton et al., 2001). *V. cholerae* is transmitted by the faecal oral route with most epidemics occurring when water supplies become faecally contaminated (Cheesbrough, 2000). It belongs to the family Vibrionacea. *Vibro* spp are Gram negative, motile by one or more polar flagella, non-sporing, slightly curved rods with a single polar flagellum (Holt et al, 1994). TCBS agar is an excellent selective medium for the primary isolation of vibrionaceae, in which sucrose-fermenting yellow colonies of diameter 2-3 mm after overnight incubation at 35-37$^0$C (Cheesbrough, 2000). Some of the major emerging and re-emerging water-borne agents are: *V. cholerae* biotype ElT or Serotype 0139. This microorganism is responsible for cholera, which is a painless form of diarrhea, characterized by rice-watery stool (Anderson and Davidson, 2002).

***Proteus vulgaris***

It belongs to the family Enterobacteriaceae. They are Gram- negative, actively motile, non capsulated, pleomorphic rod. Motility is not as easily observed at 35-37$^0$C (Cheesebrough, 2000). *Proteus* are Methyl red positive, they hydrolyse urea rapidly, $H_2S$ positive (Collee et al., 1996). *P. vulgaris* are found widely distributed in soil, polluted water, intestine of healthy man and animals. *P. vulgaris* may cause urinary infection.

**3.9 Study of physico-chemical quality of water in Nepal**

Several studies on the physico-chemical parameters were made in water of Kathmandu valley and several parts of Nepal. Sharma (1986) studied chemical parameters of tap water samples from 51 different localities in Kathmandu and observed very little variation in the chemical content of drinking water supplied to different localities in Kathmandu. The pH content of all samples was reported to be within the permissible level (6.5-8.5) ranging from 6.5-7.5.

ENPHO/DISVI (1990) tested water of tube wells of rural areas of the Eastern Development Region of Nepal and reported most of the physicochemical parameters within the WHO permissible level except iron and hardness. DISVI (1990 b) also studied the chemical parameters of water in Eastern Nepal (Illam) and found that samples from six sources were acidic with pH values below the acceptable limit set by WHO.

A study conducted by Bottino et al., (1991) in Kathmandu revealed that most water samples were within the permissible value regarding the chemical parameters except ammonia, nitrate and iron. Pradhananga et al., (1993) recorded the lower pH value of most water samples from the Pashupati area.

Ghimire (1996) assessed 11 groundwater samples form Patan area in two seasons. In rainy season, the pH and temperature ranged from 5.6-6.3 and $19.7^0$C-$22.5^0$C respectively. In summer season, pH and temperature ranged from 5.9-6.7 and $21^0$C-$22.7^0$C respectively.

Thapa (1997) also examined water quality in Kathmandu valley during three seasons, from different sources and reported most of the parameters including temperature and pH to be within the safety limits set by WHO except for BOD value of some drinking water samples.

Maharjan (1998) reported that the temperature of water samples ranged from$19.01^0$C to $22.0^0$C, $19.2^0$C to $20.5^0$C, $19.0^0$C to $25.0^0$C, $18.1^0$C to $22^0$C and $17.5^0$C to $21.4^0$C for shallow pumps, shallow wells, stone spouts, protected pumps and unprotected pumps respectively. Most of the sources (88.6 %) were recorded within permissible limit of pH value while some (11.4%) were found with pH values slightly less than the WHO guideline value.

Shrestha (2002) analyzed water samples from various sources and reported the physicochemical parameters of most of the samples lying within the WHO guideline value except for conductivity, turbidity and iron. Similarly, Prasai (2002) also assessed water samples of Kathmandu city from different sources and reported 8.3 % of water samples to have crossed the guideline value for pH.

JICA/ENPHO (2005) assessed the physicochemical parameters in pre-monsoon and reported one- third of the water samples (34.3%) to be below permissible value.

Bajracharya (2007) showed that the temperature showed marked variation ranging from $23.2^0$C to $30.2^0$C, $21.1^0$C to $30.1^0$C and $18.0^0$C to $30.1^0$C for stone spouts, tap water and tube well respectively. Similarly, a notable variation in the pH value was observed ranging

from 6.1 in tube well to 8.3 in tap water. A comparison of pH value with WHO standard revealed that 85.09% of the samples were within standard, 14.91% below the standard and 0% above the standard. Comparing to the sources, all the samples from the tap were within standard.

Gyewali (2007) assessed the physicochemical parameters of water samples taken from seven different sources. Temperature of the water samples were found approximately near value depending upon the season of sample collection ranging from $14^0$C to $17^0$C. The pH value of all the water samples was found within the WHO guideline value (7.0-8.5) ranging from 7.08 to 7.80.

Jayana (2007) collected a total of 105 samples of drinking water from different sources of Madhyapur Thimi. A distinct variation in the temperature of tested water samples was recorded ranging from $12^0$C to $30.3^0$C in well water, $12.2^0$C to $29.8^0$C in tap water and $18.8^0$C to $29.2^0$C in stone spout. The pH value of water range from 5.3 to 8.8 in well water, 6.6 to 8.5 in tap water and 6.2 to 7.2 in stone spout. 89 (84.76 %) samples were within the permissible limit while 14 (13.33 %) samples showed below the limit and 2 (1.9%) samples crossed the upper limit of WHO guideline value.

Gopali (2008) showed that the temperature of water recorded from Balaju and Sundarighat reservoirs did not show variability with values lying between $23.2^0$C and $30.2^0$C. Also from the 73 water samples of water distributed by Balaju reservoir, the pH value was found to be ranging from 7.05 to 8.08. Similarly, in Sundarighat reservoir, 38 samples showed the pH value ranging from 7.40 to 7.83. Thus the samples from both the reservoirs showed pH values within the WHO guideline value (6.5-8.5).

Shrestha (2008) collected a total of one hundred and seventy water samples from different places in Kathmandu city. The physicochemical analysis revealed the variation in the temperature as the lowest and the highest temperature recorded being $23.2^0$C and $29.6^0$C respectively. The pH value of the water samples ranged from 6.70 to 8.50.

**3.10 Study on microbial quality of drinking water of Nepal**

In poor and developing countries like Nepal poor water quality, water pollution and their consequences have always been the topic of sensational story. The vital connection between the water and health though been established already, is given little emphasis in government policy (UNICEF, 1987). It has been reported that 66% of the rural people and more than 80% of the urban people has access to the piped water with the per capita water consumption in the rural area being far less than that in urban area of 45 liter and 60 liter respectively (Pradhan, 2004).

The study in water pollution and water quality in Nepal seems to have been started since last three decades and most of the studies show that the water quality in several parts of the country is unacceptable and worsening with time. DISVI, an Italian INGO and ENPHO, a national NGO have been conducting an extensive research on water quality since 1985.

The early study conducted on water quality of Kathmandu valley by Sharma (1978) reported the coliform densities in drinking water of Kathmandu valley ranging from 4 to 450/100 ml of water. In a follow up study done by Sharma (1986), reported the coliform bacteria count range from 0 to 4800/100 ml during the rainy season, 0 to 75/100 ml in winter and 0 to 460/100 ml in the summer months. In this time interval from 1978-1986, almost 10 fold increase in the coliform density was reported in the drinking water of Kathmandu. The study also showed the season wise variation in the coliform density in the water sample.

Adhikari et al., (1986) carried out coliform tests in 100 samples of drinking water collected from different sources and areas of valley found that most of the water samples had unsatisfactory coliform density exceeding 1800 coliform/100 ml of water and CEDA (1989) tested the drinking water samples from different localities in Kathmandu and found that all samples were contaminated with faecal material and crossed the WHO guideline value for coliform indicating the unsafeness of the water.

ENPHO/DISVI (1990) conducted a survey on water quality of 21 stone spouts of Kathmandu and reported that samples from all the spouts had been contaminated and the faecal coliform densities were found ranging from 1 to 37,602 coliform/100ml of water.

DISVI (1990) carried out bacteriological tests of drinking water in seven rural areas of Ilam in Eastern Nepal. Samples from 36 households were collected from different water sources which reported the unacceptable levels of faecal coliform bacteria ranging from 2 to 2,400 coliform/100 ml.

ENPHO/DISVI (1992) conducted a one-year monitoring on microbiological quality of water supply in Kathmandu City. 39 samples from 5 treatment plants and 172 samples from 37 public taps were examined from different localities. 7 samples i.e. 18 % from treatment plants were found contaminated with an average faecal coliform of 4 coliform/ 100 ml. Similarly 50% samples from public taps were found contaminated. The bacterial densities in contaminated watersamples ranged from 1 to TMTC coliform/100 ml.

Pradhananga et al., (1993) examined water samples from 6 different stone spouts around Pashupati and reported average value of coliform at different stone spouts ranging from 8 to 88 coliform/100ml. The studies suggest that the water of the stone spouts of Kathmandu valley is unsafe to drink without proper treatment.

Thapa (1997) examined water quality of different sites near Kathmandu during three seasons from different sources and found that the samples were contaminated during all seasons. The bacterial densities ranged from 43 to 210 cfu/100 ml during winter, 75 to 240 coliform/100 ml during summer and 150 to 460 coliform/100 ml during rainy season.

Maharjan (1998) examined 70 water samples randomly collected from different sources in urban area of Patan city. Out of these, 85.6% of samples showed the presence of total coliform and 68.6 % contained faecal coliform. In this study, 120 enteric bacteria were isolated from 49 samples. Recovery of *Enterobacter* spp. was found maximum followed by *E. coli, Citrobacter* spp and *Salmonella* spp.

Karki (2001) studied the solar disinfection of 35 drinking water samples which were artificially contaminated with *E. coli* ATCC 25922. Out of these, 28 water samples showed more than 99.99% disinfection, 2 water samples showed 60-70% and 5 water samples showed only unto 25% disinfection. Similarly for *S.* Typhi, among 20 water samples tested, 15 water samples showed more than 99.99% disinfection, 2 water samples showed 70-80 % disinfection and 3 water samples showed only up to 30 % disinfection.

Rai et al., (2001) analyzed a total of 57 drinking water samples, 41 from natural water sources and 16 from piped tap water collected from different areas in Kathmandu Valley. 43(75%) samples had one or more species of organisms, out of which 22(51%) were *E. coli*. Among the 17 *E. coli* strains isolated, over two-thirds (15) were EPEC.

Prasai (2002) examined a total of 132 samples of drinking water of Kathmandu valley from different sources where 92.4% of samples showed the presence of coliform bacteria with the highest recovery of *E. coli* (26.4%) followed by *Citrobacter* spp.(22.6%), *P. aeruginosa*

(6.3%), *Klebsiella* spp.(5.4%), *Shigella* spp.(3.78%), *Salmonella* Typhi (3.3%), *Proteus vulgaris* (2.9%) , *Serratia* spp. (2.52%), *Vibrio cholerae* (0.0845%).

Shrestha (2002) analyzed a total of 95 water samples for bacteriological parameters from various sources. The raw water samples and settled water samples showed 100% presence of coliform, whereas reservoir water and Kuleshwor water showed 53.33 % and 76% respectively. The overall study found 85.26% of the samples to have exceeded WHO guideline value for total coliform.

Joshi et al., (2004) analyzed 160 samples randomly collected from 86 tube wells and 77 open wells in urban areas and reported that more than 87% of analyzed ground water samples of tube well and open well were contaminated.

Bajracharya (2007) assessed the quality of drinking water of Kathmandu and reported 90.35% samples showing the presence of the coliform with ten different kinds of enteric bacteria with the highest recovery of *Citrobacter* spp.(26.22 %), followed by *E. coli* (25%), *Enterobacter* spp. (20.73%), *Shigella* spp.(8.54%), *Proteus vulgaris* (7.93%), *P. aeruginosa* (3.66%), *Salmonella* Paratyphi (3.05%), *Klebsiella* spp. (2.44%), *Proteus mirabilis* (1.83%) and *Salmonella* Typhi (0.61%) was reported.

Bhatta et al., (2007) studied the occurrence and diversity of *Salmonella* serovars in urban water supply systems of Nepal and detected the occurrence of *Salmonella* in 42 out of 300 water samples. A total of 54 isolates were identified to genus level by standard tests, subsequently confirmed by serotyping, phage typing and PCR detection of virulence genes. The predominant was *Salmonella* Typhimurium followed by *Salmonella* Typhi, *Salmonella* Paratyphi A and *Salmonella* enteritidis.

Jayana (2007) assessed a total of 105 drinking water samples from the different sources of Madhyapur Thimi and reported 64.76 % of the samples crossed the WHO guideline value for total coliform count. *Enterobacter* spp. was the most predominant organism (29.5%) followed by *E. coli* (24.6%), *Citrobacter spp.* (20.4%), *Proteus vulgaris* (7%), *Klebsiella* spp. (5.6%), *Proteus mirabilis* (3.5%), *Shigella dysentery* (2.8%), *Salmonella* Typhi (2.1%), *Pseudomonas* spp. (2.1%), *Salmonella* Paratyphi (1.4%) and *Vibrio cholerae* (0.7%).

Gyewali (2007) assessed the water quality of Kathmandu valley, taken from seven different sources. All the water samples showed the presence of coliform bacteria. The highest bacterial count was 6.4 X 106 CFU/ml in river water and lowest bacterial count was found to be 3.0 X 103 CFU/ml in Kuleshwor tap water. Similarly, the highest coliform count was 1100 CFU/100 ml and lowest coliform count was found to be 500 CFU/100 ml in sample taken from Sundharighat tank. Both the study showed that the most water get contaminated during storage or in the distribution system.

Warner et al., (2007) sampled water from over 100 sources in Kathmandu and examined for contamination from sewage, agriculture or industry. Total coliform and *E. coli* bacteria were present in 94% and 72% of all the water samples respectively.

Gopali (2008) assessed the microbial quality of chlorinated drinking water of Kathmandu valley. 111 water samples were collected from the 7 distribution points of Sundarightat reservoir (38) and 13 distribution points of Balaju reservoir (73). The microbiological analysis of water samples from Sundarighat reservoir revealed the presence of total coliform within WHO guideline value in 34.21% of total samples and 68.78% were found to contain total coliforms above the WHO guideline value in Sundarighat reservoir. In

Balaju only 9.58% were found to meet the WHO guideline value whereas the rest 90.41% exceeded the standard permissible limit.

Shrestha (2008) assessed the municipal drinking water quality of the Kathmandu from June to November 2007. A total of one hundred and seventy water samples were collected. 85.9% of the sample revealed the presence of heterotrophic bacteria (HPC) while 88.2% of the total sample showed the presence of total coliform. Fifteen different genera of enteric bacteria with the total of two hundred and ninety six isolates were identified, *E. coli* (22.6%) being the predominant followed by *Klebsiella oxytoca* (16.55%), *Pseudomonas aeruginosa* (11.14%), *Citrobacter freundii* (10.13%), *Enterobacter aerogenes* (9.8%), *Shigella* spp. (7.09%), *Salmonella* Paratyphi (6.75%), *Proteus mirabilis* (6.41%), *Proteus vulgaris* (3.04%), *Acenetobacter spp*. (2.70%), *Citrobacter diversus* (2.02%), Salmonella Typhi and *Vibrio* (0.67%), *Serratia* spp. and *Vibrio parahaemolyticus* (0.33%).

### 3.11 Antibiotics and bacterial resistance to antibiotics

Antibiotics refer to a metabolic product of one microorganism that in very small amounts exert antimicrobial activity. Some antibiotics are inhibitory to many different species ie. broad spectrum, while some are inhibitory to only a few species of microorganism. To determine the effectiveness of various antibiotics, susceptibility tests are performed. These tests are commonly done by following Kirby Bauer method in which small paper disc impregnated with antibiotics are placed on the surface of an agar plate heavily seeded with test organism. The plate is incubated for 24 hours at $37^0$C. A zone of inhibition surrounding the filter paper disc is observed if the test organism is sensitive to the antibiotic.

Some of the antibiotics are bactericidal i.e, they can kill the bacteria and others are bacteriostatic i.e, they inhibit the growth of bacteria. A way of classifying antibiotics is on

the basis of their mode of action. The major point of attack of antibiotics on microorganisms include:

i. Inhibition of cell wall synthesis: Penicillin, Cephalosporin, Cycloserine, Vancomyin, Bacitracin.
ii. Damage to cytoplasmic membrane: Polymyxins, Gramicidines, Tyrocidines.
iii. Inhibition of nucleic acid: Actinomycin D, Rifamycim, Mitomycin.
iv. Inhibition of protein synthesis: Tetracycline, Aminoglycosides, Chloramphenicol, Erythromycin.
v. Inhibition of specific enzymes: Sulfonamides.
vi. Antifungal antibiotics: Nystatin, Griseofulvin.

Antibiotic resistance is a specific type of drug resistance when a microorganism has the ability of withstanding the effects of antibiotics. Antibiotic resistance evolves via natural selection acting upon random mutation, but it can also be engineered by applying an evolutionary stress on a population. Once such a gene is generated, bacteria can then transfer the genetic information in a horizontal fashion (between individuals) by plasmid exchange. If a bacterium carries several resistance genes, it is called multi resistant or, informally, a superbug.

The widespread use of antibiotics both inside and outside of medicine is playing a significant role in the emergence of resistant bacteria. They are often used in animals but also in other industries which at least in the case of agricultural use lead to the spread of resistant strains to human populations. In human medicine the major problem of the emergence of resistant bacteria is due to misuse and overuse of antibiotics by doctors as well as patients. Other practices contributing towards resistance include the addition of antibiotics to the feed of livestock. Household use of anti-bacterials in soaps and other products, although not clearly contributing to resistance, is also discouraged (as not being

effective at infection control). Also unsound practices in the pharmaceutical manufacturing industry can contribute towards the likelihood of creating antibiotic resistant strains.

### 3.12 Studies on bacterial resistance to antibiotics

Most of the works about surveillance on antimicrobial resistance have been carried out in bacteria isolated from clinical samples; however, studies should also be expanded to those bacteria recovered from environmental samples in order to evaluate their role as possible reservoir of resistance genes and their capacity to transfer them to human pathogenic organism (Harakeh et al., 2006).

Hosny et al., (1988) examined 101 isolates from underground water in Cairo, Egypt for their resistance towards four antibiotics. He found 32 and 18 isolates resistant to Tetracycline and Chloramphenicol respectively.

Ramtete et al., (1991) studied the antibiotic resistance pattern among the coliform bacteria isolated from the drinking water and found that most of the isolates were resistant to Ampicillin. The agreement with the above result for the predominance of Ampicillin resistance was also added by the study conducted by Pandey and Musarrat (1993) who reported the antibiotic resistance among the coliform isolated from drinking water in the urban area of Aligarh city, India and found that the *E. coli* were resistant to Ampicillin and Tetracycline significantly.

Pathak et al., (1994) studied antibiotic resistance among the coliform isolates from 89 water samples in India. He found that 36.4% of the coliform isolates exhibited resistance towards the 12 antibiotics used. Resistance to Ampicillin (56.8%) was found maximum while almost 50% of the total isolates exhibited the multiple antibiotics resistance.

Bissonette et al., (1995) examined the total of 264 ground water isolates from the rural water supplies in West Virginia, USA against 16 antibiotics. The study showed that all of the non-coliforms and 87% of the total coliform isolates were resistant to at least one antibiotic. Similarly, 69.4%, 47.7%, 32.2%, 16.9% and 12% of the isolates were found resistant towards Ampicillin, Nitrofurantoin, Tetracycline, Chloramphenicol and Nalidixic acid respectively.

Parveen et al., (1997) studied association of multiple antibiotic resistance profiles with point and non-point sources of *E. coli* in Apalachicola bay. *E. coli* isolates from point sources showed significantly greater resistance ($P<0.05$) to antibiotics. MAR profiles included percentages of isolates with resistance to Chlortetracycline-sulfathiazole of 33.7% and to Chlortetracycline-penicillin G-sulfathiazole of 14.5% for point sources versus 15.4 % and 1.7% respectively for non point source isolates.

Maharjan (1998) studied the antibiotic resistance pattern from ground water samples randomly collected from Patan area and reported that 82.5% isolates were resistant to at least one antibiotic and 2.5% of the total isolates were resistant to five or more antibiotics. Resistance was more commonly directed towards Nitrofurantoin (68.3%) followed by Ampicillin (44.2%) and Tetracycline (28.3%). All the isolates were sensitive to Amikacin and Gentamicin. 71.4% of the *E. coli* isolates were resistant to at least one antibiotic and 23.8% were resistant to at least two antibiotics.

Lazar et al., (2002) performed antimicrobial susceptibility testing of 12 *E. coli* strains isolated from chronically polluted waters using Ampicillin, Tetracycline, Gentamicin, Kanamycin, Chloramphenicol, Ceftazidime and Cefotaxime. All strains were multiple antibiotic resistant, 16% of them being resistant to 3, 4 and 6 antibiotics, 32% to 5 and 8% to all 7 antibiotics, respectively.

Shrestha (2002) studied the antibiotic resistance pattern of *E. coli* isolated from five different sources. 10 different antibiotics were used against the *E. coli* isolates and found that all the *E. coli* isolates were resistant to Ampicillin followed by 51.8%, 11.11%, 14.8% and 7.40 % of the total isolates being resistant to Nitrofurantoin, Nalidixic acid, Co-trimoxazole and Kanamycin respectively. The study also reported 74.07% of the total isolates being resistant to at least one antibiotic and 3.07% of the total isolates were multiple antibiotic resistant.

Walia et al., (2004) found that 70%, 55% and 15% of *E. coli* (n=20) tested from drinking water in Michigan were resistant to Carbenicillin, Tetracycline and Streptomycin, respectively.

Tambekar et al., (2006) isolated 85 strains of thermotolerant *E. coli* from 1000 water samples from different sources. These 85 isolates showed maximum resistance to Ofloxacin (92%) followed by Novobiocin (86%) and Cefdinir (82%) and Ciprofloxacin (79%). The antibiotics such as Cefazolin (64%), Ceftriaxone (58%), Nitrofurantoin (51%) were moderately effective against the isolates. It was also observed that Azithromycin, Gentamycin, Amikacin, Chloramphenicol, Co-trimoxazole and Tetracycline were the most effective while the Ofloxacin, Novobiocin, Cefdinir and Ciprofloxacin were the least effective against the *E. coli* strains.

Roy et al., (2006) cultured 32 *E. coli* isolates from different sources in Tamilnadu state of India. All the *E. coli* isolates showed high resistance to multiple drugs with 100% resistance observed against Ampicillin/cloxacillin, Chloramphenicol, Tetracycline, and Co-trimoxazole followed by Ciprofloxacin (67.7%), Gentamicin (61.3%), and Nitrofurantoin (51.3%).

Alhaj et al., (2007) studied the prevalence of antimicrobial resistance in *E. coli* isolates from different sources. Seventy *E. coli* isolates from humans and environments were tested for susceptibility to 10 antimicrobial agents by diffusion method. Resistance was found in 61.2% of the isolates. The most prevalent resistances were to kanamycin and tetracycline (81.4%), followed by Chloramphenicol (75.7%) and Gentamicin, (74.3%). The low prevalent were to Cefetoxin (44.3%), Norfloxacin (27.1%) and Ciprofloxacin (24.3%).

Watkinson et al., (2007) determined the antibiotic resistance patterns of 462 *E. coli* isolates from different sources. The antibiotics chosen were Ampicillin, Cephalothin, Nalidixic acid, Sulfafurazole, Gentamicin and Tetracycline. The highest incidence of bacterial resistance recorded was that for Tetracycline (51%), followed by those for Cephalothin (41%) and Sulfafurazole (32%).

Lima-Bittencourt et al., (2007) performed antibiotic susceptibility test upon 102 enterobacterial isolates which included *E. coli* (n=8). Ten antibiotics were used: Ampicillin, Amoxicillin-clavulanic acid, Tetracycline, Chloramphenicol, Nalidixic acid, Rifampicin, Amikacin, Gentamicin, Kanamycin and Streptomycin. 93% showed resistance to at least one antimicrobial. *E. coli* was the most sensitive genus to antimicrobials because only 12.5% of the isolates demonstrated multiple resistance. During rainy season 100% *E. coli* isolates were resistant to Ampicillin, Amoxicillin-clavulanic acid, Tetracycline, Chloramphenicol, Nalidixic acid and Streptomycin and during dry season 71%, 14%, 14%, 57% and 100% were resistant to Ampicillin, Kanamycin and Rifampicin.

Yadav et al., (2007) isolated 49 *E. coli* strains from 100 samples of mutton collected from dressed sheep carcasses in India. Among the 15 isolates of *E. coli* tested for resistance against various antibiotics all the isolates (100%) were found to be resistant to

Erythromycin and Streptomycin, followed by Sulphadiazine (95.84%) and Cephaloridine (87.50%). Moderately high resistance was detected towards Cephalexin (41.69%), Penicillin G (37.60%), Ceftiofur and Norfloxacin (33.36% each), Enrofloxacin (27.40%) and Carbenicillin (25.30%). The isolates were least (16.70%) resistant to the antibiotics Amoxycillin and Oxytetracycline.

Lourenco et al., (2007) isolated enterobacterial strains from estuarine water of Sao Vicente,Brazil. Out of 142 isolates, *E. coli* (40.1%) was the predominant one. The isolates were subjected to antimicrobial susceptibility testing using seven different antibiotics. Analysis of the antimicrobial activity of the tested drugs against the isolates showed Gentamycin, Netilmicin and Ciprofloxacin with the highest activity (100%), followed by Cefepime (97.3%), Cefoxitin (84.2%), Amoxicillin/clavulanic acid (57.8%), and Ampicillin (47.3%).

Jayana (2007) assayed ten antibiotics against the total of 142 isolates of Kathmandu and reported the maximum resistance commonly directed toward Erythromycin (79.5%) and Penicillin G (62.67%). All the isolates were resistant to at least one antibiotic. *P aeruginosa* was found resistant to almost all antibiotics used. Of the total isolates, resistance towards Ampicillin (34.5%), Ofloxacin (5.6%), Chloramphenicol (5.6%), Amoxycillin (61.9%), Cephotaxime (41.7%), Amikacin (14.7%), Ceftriaxone (15.4%) and Tetracycline (21.1%) was shown.

Shrestha (2008) assayed eight antibiotics against 33 isolates out of 296 from the drinking water of Kathmandu. Most of the isolates (93.9%) were resistant to Ampicillin while only two strains showed the partial sensitivity towards Ampicillin. The level of resistance exhibited by isolates to specific antibiotics was found as follows: Ampicillin (93.93 %), Tetracycline (27.27%), Nitrofurantoin (24.24%), Co-trimoxazole (18.18 %), Nalidixic acid

(15.15 %).%). All *E. coli* isolates showed resistance towards Ampicillin while all *E. coli* showed sensitivity towards Ciprofloxacin and Norfloxacin. 28.5% of *E. coli* showed the multi drug resistance pattern.

### 3.13 Heavy metals and their effects on microorganisms

Heavy metals are often defined as a group of metals whose atomic density is greater than 5 g/cm3 .Metals play a vital role in the metabolic processes of the biota. Some of the heavy metals are essential and are required by the organisms as micro nutrients (cobalt, chromium, nickel, iron manganese and zinc etc.) and are known as 'trace elements'. They are involved in redox processes, in order to stabilize molecules through electrostatic interactions, as catalysts in enzymatic reactions, and regulating the osmotic balance. On the other hand some other heavy metals have no biological role and are detrimental to the organisms even at very low concentration (cadmium, mercury, lead etc.).However, at high levels both of the essential and non essential metals become toxic to the organisms.

These heavy metals influence the microbial population by affecting their growth, morphology, biochemical activities and ultimately resulting in decreased biomass and diversity. Heavy metals can damage the cell membranes, alter enzymes specificity, disrupt cellular functions and damage the structure of the DNA. Toxicity of these heavy metals occurs through the displacement of essential metals from their native binding sites or through ligand interactions. Also, toxicity can occur as a result of alterations in the conformational structure of the nucleic acids and proteins and interference with oxidative phosphorylation and osmotic balance.

Due to the selective pressure from the metal in the growth environment, microorganisms have evolved various mechanisms to resist the heavy metal stress. Several metal resistance

mechanisms have been identified: exclusion by permeability barrier, intra and extra cellular sequestration, active transport, efflux pumps, enzymatic detoxification, and reduction in the sensitivity of the cellular targets to metal ions. Heavy metal contamination in the environment has become a serious problem due to the increase in the addition of these metals to the environment. Natural sources as well as the anthropogenic sources account for this contamination, which has become a threat to public health. Cadmium, copper and zinc are among those heavy metals that are being released to the environment. In this perspective many approaches have been used to assess the risk posed by the contaminating metals in soil, water bodies etc. At present the tolerance of soil bacteria to heavy metals has been proposed as an indicator of the potential toxicity of heavy metals to other forms of biota. Therefore, there is a dramatic increase in the interest on studying the interactions of heavy metals with microorganisms. The favoured approach now is selecting the organisms that can be used to develop tools to assess the metal levels in the environment.

### 3.14 Human Health Effects of Heavy Metals

Heavy metals are individual metals and metal compounds that can impact human health. Generally, humans are exposed to these metals by ingestion (drinking or eating) or inhalation (breathing). Working in or living near an industrial site which utilizes these metals and their compounds increases ones risk of exposure, as does living near a site where these metals have been improperly disposed. Subsistence lifestyles can also impose higher risks of exposure and health impacts because of hunting and gathering activities. At higher concentration, essential heavy metals form unspecific complex compounds in the cell, which lead to toxic effects. Non-essential heavy metal cations like $Hg^{++}$, $Cd^{++}$, $Ag^{+}$ form extremely toxic complexes that are dangerous for any biological functions

**Mercury**

The anthropogenic source of mercury pollution in the surface water is the effluent from thermometer and barometers production plant, dental clinics, and battery manufacturing industries. Mercury is a nerve toxin and the main health concern is its effect on the brain, particularly in the growing foetus.

**Chromium**

The sources of chromium are industrial effluents coming from carpet industries, metal finishing industries , tanning industries. Continuous exposure to hexavalent chromium by ingestion of chromium-contaminated water can cause severe gastrointestinal system disturbance, circulatory system damage or kidney damage (U.S EPA 1998). Another health concern regarding the ingestion of Cr-VI is its absorption by the cells where it binds to genetic materials and cause mutation (U.S EPA 1998).

**Cadmium**

Sources of cadmium are metal and refining industries , electroplating plants etc. the toxic effect of cadmium consumption is its accumulation in kidneys, liver and lungs and metabolic anomalies caused by enzyme inhibition (IOCC 2002). The outbreak of itai-itai disease in Japan was due to ingestion of cadmium-contaminated shellfish and water.

**Cobalt**

Cobalt is trace element essential for biological activity. However cobalt can be equally toxic if it is found in high concentration (IOCC 1996). Effluents from fertilizer industries and paint industries add cobalt to natural water system. Exposure to high levels of cobalt can result in lung and heart effects and dermatitis (ATSDR 2003).

**Lead**

Industrial sources of lead are batteries manufacturers, metal-processing industries. health effects of lead consumption are more serious in children than adult. If not detected early, children with high levels of lead in their bodies can suffer from damage to brain and nervous system, retarded growth, hearing problems, headaches (U.S EPA 2003).

**Copper**

Copper is another essential heavy metal that is required for good health. However, very large single or daily intakes of copper can cause diarrhea, vomiting, abdominal cramps and nausea. Chronic high intakes of copper can cause liver and kidney damage and even death (Ohio EPA 2002).

**Nickel**

The sources of nickel pollution in water are mainly metal finishing industries, electroplating industries, battery industries. The adverse health effect of nickel is an allergic reaction. People can become sensitive to nickel when it comes in direct contact with the skin. Such sensitized persons are more susceptible to adverse health effect by drinking nickel-containing water. The effect of consumption of nickel includes stomach aches and severe effect on blood and kidney (ATSDR 1997).

### 3.15 Microbial resistance to heavy metals

Micro-organisms require the presence of a number of metals that play essential biochemical roles such as catalysts, enzyme co-factors, activity in redox processes and stabilizing protein structures (reviewed by Bruins *et al*., 2000). Metals may accumulate above normal physiological concentrations by the action of unspecific, constitutively expressed transport systems, whereby they become toxic. Intracellular metals can exert a toxic effect by forming co-ordinate bonds with anions blocking functional groups of enzymes, inhibiting transport systems, displacing essential metals from their native binding sites and disrupting

cellular membrane integrity (Nies, 1999). To survive under metal-stressed conditions, bacteria have evolved several types of mechanisms to tolerate the uptake of heavy metal ions. These mechanisms include the efflux of metal ions outside the cell, accumulation and complexation of the metal ions inside the cell, and reduction of the heavy metal ions to a less toxic state (Nies, 1999). There are five basic mechanisms that convey an increased level of cellular resistance to metals: (1) efflux of the toxic metal out of the cell; (2) enzymic conversion; (3) intra- or extracellular sequestration; (4) exclusion by a permeability barrier; and (5) reduction in sensitivity of cellular targets.

**a. Metal exclusion by permeability barrier**

Alterations in the cell wall, membrane, or envelope of a microorganism are examples of metal exclusion by a permeability barrier. This mechanism is an attempt by the organism to protect metal-sensitive, essential cellular components. A prominent example is the exclusion of cupric ion resulting from altered production of the membrane channel protein porin by *E. coli* (Rouch et al., 1995). Another example is the nonspecific binding of metals by the outer membrane or envelope. This offers limited metal protection due to the possibility of saturation of binding sites (Hoyle et al., 1984). Although not fully proven, it is believed that some forms of copper resistance are based on periplasmic binding (Silver et al., 1994; Saxena et al., 2004).

**b. Active transport of the metal away from the microorganism**

Active transport or efflux systems represent the largest category of metal resistance systems. Microorganisms use active transport mechanisms to export toxic metals from their cytoplasm. These mechanisms can be chromosomal or plasmid-encoded. Non essential metals normally enter the cell through normal nutrient transport systems but are rapidly exported. These efflux systems can be non-ATPase or ATPase-linked and highly specific for the cation or anion they export.

**c. Sequestration of metals by protein binding**

Intracellular sequestration is the accumulation of metals within the cytoplasm to prevent exposure to essential cellular components. Metals commonly sequestered are copper, cadmium and zinc. Two examples exist for this form of metal resistance: metallothionein production and cysteine-rich proteins (Silver et al., 1996). Metal resistance based on extracellular sequestration has been found in several species of yeast and fungi and has been hypothesized for bacteria (Joho et al., 1995).

**d. Enzymatic detoxification of a metal to a less toxic form**

Resistance to mercury may be achieved by enzymatic detoxification in both gram-positive and gram-negative bacteria. A set of genes encodes for the production of a periplasmic binding protein and membrane-associated transport proteins. The periplasmic binding protein collects $Hg^{2+}$ from the surrounding environment and transport proteins take it to the cytoplasm where it is neutralized by redox chemistry (Bruins et al., 2000). Metal ion-specific reducing enzyme systems function in the cell surface layer of microorganisms. Metal ion reductases are also found in the cytoplasm (Wakatsuki, 1995).

**e. Decrease in metal sensitivity of cellular targets**

Some microorganisms adapt to the presence of toxic metals by altering the sensitivity of essential cellular components. Protection is achieved by mutations that decrease sensitivity but do not alter basic function or by increasing production of a particular cellular component to keep ahead of metal inactivation. DNA repair mechanisms also provide limited protection to plasmid and genomic DNA. The microorganism may also protect itself by producing metal-resistant components or alternate pathways in an effort to bypass sensitive components. Adaptation has been found in *E. coli*. Upon exposure to cadmium,

unadapted *E. coli* demonstrate considerable DNA damage; however, after subculture, the same organisms show resistance.

### 3.16 Studies on microbial heavy metals resistance

Calomiris et al. (1984) studied the association of metal tolerance with multiple antibiotic resistance of bacteria isolated from drinking water. Bacterial isolates from drinking water system of an Oregan coastal community were examined to assess the association of metal tolerance with antibiotic resistance. Positive correlation between tolerance to high levels of $Cu^{2+}$, $Pb^{2+}$, and $Zn^{2+}$ and multiple antibiotic resistance were noted bacteria from distribution waters but not among bacteria from raw bacteria from raw waters. Tolerance to higher levels of $Al^{3+}$ and $Sn^{2+}$ were demonstrated more often by raw water isolates which were not typically multiple antibiotic resistant.

Teitzel etal. (2003) Studied the effects of heavy metal copper, lead, and zinc on biofilm and planktonic *Pseudomonas aeroginosa.* It was determined that biofilm were 2 to 600 times resistant to heavy metal stress than free- swimming cells. When planktonic cells at different stages of growth were examined, it was found that logarithmically growing cells were more resistant to copper and lead stress than stationary phase.

Rajbansi (2003) analyzed 60 samples for bacteriological quality of effluent in different seasons. A total 10 heavy metal resistant bacteria were isolated and identified as *Staphylococcus* spp, *E. coli*, *Citrobacter* spp, *Acinetobacter* spp, *Flavobacterium* spp, *Psedomonas* spp, *Bacillus* spp, *Methylobacterium* spp. All the 10 heavy metal resistant bacteria were found to be resistant to antibiotics and among these 90% were found to be multi antibiotic resistant.

Farazmand and Ghajourian (1996) isolated 50 bacterial strains resistant to heavy metals from metal melting and electroplating effluent.

Shakoori et al., (1998) isolated cadmium resistant *Enterobacter* spp and *Klebsiella* spp from industrial effluent and studied their possible role on cadmium detoxification.

Clausen (1998) isolated metal-tolerant bacteria capable of removing copper, chromium and arsenic from treated woods. A total of 12 non resistant species and 7 metal resistant species including both gram positive (*Bacillus*) and gram negative (*Pseudomonas)* bacteria and one yeast strain were isolated. The strains were capable of removing significant amounts of copper and lead.

Sharma et al., (2002) isolated highly cadmium resistant *Klebsiella planticola* strain from reducing salt marsh sediments. The strains was found to grow in upto 15mM $CdCl_{2,}$ under wide range of NaCl concentration at acidic or neutral pH.

Matyar et al., (2008) studied the susceptibility patterns to 15 different antibiotics and six heavy metals in *Aeromonas* spp. and *Pseudomonas* spp. isolated from Iskenderun Bay, Turkey (northeast Mediterranean Sea). A high percentage of *Aeromonas* isolates showed resistance to cefazolin (66.6%) and trimethoprim–sulphamethoxazole (66.6%). Amongst the *Pseudomonas* isolates, there was a high incidence of resistance to nitrofurantoin (86.2%), cefazolin (84.8%) and cefuroxime (71.7%). Most isolates showed tolerance to different concentrations of heavy metals, and minimal inhibition concentrations ranged from 25 to >3,200 μg/ml. The *Aeromonas* spp. and *Pseudomonas* spp. showed high resistance to copper of 98.3% and 75.4%, respectively, and low resistance to lead of 1.7% and 7.2%, respectively.

# CHAPTER – IV

# 4. MATERIALS AND METHODS

## 4.1 MATERIALS

All the materials used to accomplish this study are given in the Appendix-I and Appendix II.

## 4.2 METHODS

### 4.2.1 Study area

A total of 102 tap water samples were collected randomly from different localities of Kathmandu. The study period was from August 2008 to March 2009. Water sources sites selected for the study were Kuleshwor, Balkhu, Bagbazaar, Maitidevi, Handigaon, Kirtipur, Gaushala, Kamalpokhari, Kalikaistan, Teku and Kalopul.

### 4.2.2 Collection of water samples

Water samples for bacteriological analysis were collected in pre-sterilized bottles (15 lbs at $121^0$C for 15 minutes) of 500 ml capacity containing 0.3 ml of 0.025 N sodium thiosulphate solution added prior to the sterilization. Bottles were tightly capped to prevent contamination. For physico-chemical examination water samples were collected in clean plastic bottles, washed and rinsed 3-4 times with water to be tested. The water was sampled for bacteriological analysis as given by Cheesbrough (2000).Firstly external fittings from the tap were removed and tap was sterilized using alcohol and ignited. Then tap was allowed to cool by running the water to waste for 2 minutes. Sample bottle was filled from a gentle flow of water and cap of the bottle was replaced. A code number, sampling date and sampling time was written to each bottle using a marker.

### 4.2.3 Transportation and preservation of sample

Immediately after collection, samples were transported to the laboratory holding in an ice-box at $4^o$C and examined as soon as possible. In some cases when immediate analysis was not possible, the samples were preserved at $4^o$C. . If microbial examination is not possible within one hour the

samples should be keep in refrigerator. Sometimes if required samples should be preserved at 4°c upto 6 hours. In this study samples were subjected to microbial examination within four hour of collection .

#### 4.2.4. Analysis of physico- chemical parameters of water samples

Standard Methods for the Examination of Water and Wastewater (APHA, 1998) was followed to analyze physico-chemical parameters of water. The reading of temperature and pH of water samples were taken at the site during sampling period. Temperature was determined with the help of a standard mercury thermometer graduated up to $50^0$C.

Hydrogen ion concentration in the sample was measured with the help of the $p^H$ meter by inserting the electrode into the water sample.

### 4.2.5. Total coliform and thermotolerant coliform count

In this study, total coliform and thermotolerant coliform were enumerated by the membrane filtration (MF) technique as described by APHA, 1998. First of all, sterile filter holder with stopper was assembled on the filter flask. Using sterile blunt- edged forceps, a sterile membrane filter of pore size 0.45 µm (grid side up) was placed over the porous disc in such a way that it overlapped the entire circumference of sintered filterable area. The sterile funnel was securely placed on the filter base and then 100 ml of the water sample was poured into the funnel. The sample was slowly filtered under partial vacuum by using electric vacuum pump. The funnel was removed and the membrane was directly transferred, keeping its upper side upwards, on to a plate of M- Endo agar with the help of sterile forcep. Care was taken not to entrap air bubbles between the membrane and the medium. Then it was incubated at $37^0$C for 24 hours for total coliform and $44.5^0$C for thermotolerant coliform in an inverted position. After proper incubation total colony forming unit (CFU) were counted.

#### 4.2.6. Isolation and identification of *E. coli*

Greenish metallic sheen producing colonies from M-Endo agar were sub-cultured onto MacConkey agar and incubated at $44.5^0$C for 24 hours. All lactose fermenting colonies were subcultured on Nutrient agar for pure culture. Identification of *E. coli* was done based on colony characteristics, morphological characteristics and biochemical properties on respective media according to Bergey's Manual of Determinative Bacteriology, 1986. Cell morphology was studied using gram's reaction under oil immersion. Biochemical tests are based on the ability of microorganisms to produce enzymes thus utilizing different substrates as described by Cheesbrough,1993. The isolated pure colonies were inoculated into different biochemical media for different tests which are as follows:

**Table4.1 Biochemical tests performed for identification of *E. coli* isolates**

| S.N. | Tests | Biochemical Media |
|---|---|---|
| 1. | Catalase | 3% $H_2O_2$ |
| 2. | Oxidase | 10% Tetramethyl - p- phenyle diamine dihydrochloride. |
| 3. | Indole Production | Sulfide- Indole- Motility medium (SIM) |
| 4. | Methyl Red test | Glucose phosphate peptone water or MR-VP medium. |
| 5. | Voges- Proskauer test | Glucose phosphate peptone water or MR-VP medium. |
| 6. | Citrate utilization test | Simmon's citrate agar. |
| 7. | Fermentation of glucose, lactose and sucrose, $H_2S$ and gas production | Triple sugar Iron Agar (TSIA) |

| | | |
|---|---|---|
| 8. | Aerobic or anaerobic utilization of carbohydrate | Biochemical Media Hugh and Leifson Medium. |
| 9. | Urease Production | Urea base agar. |

**4.2.7 Study of antibiotic susceptibility of *E. coli* isolates**

Antibiotic susceptibility of isolated *E. coli* strains was assayed using a modified Kirby-Bauer disk diffusion method (Bauer et al., 1966). Cells were grown at 37$^{o}$C in 5ml of nutrient broth for about 4 hours using pure cultures as inoculum. The turbidity developed was compared with that of standard barium sulphate. A sterile cotton swab was dipped into the properly prepared inoculum and firmly rotated against the upper inside wall of the tube to expel excess fluid, and then swabbed onto Muller-Hinton agar. During swabbing the plate was streaked with the swab three times turning the plate 60$^{o}$C between each streaking to achieve a lawn of confluent bacterial growth. The plate was kept at room temperature for 5 to 10 minutes, but no longer than 15 minutes to dry the inoculum. Sensitivity discs from their respective vials were carefully placed in the plate with the help of a flamed forceps, at equal distance and sufficiently separated from each other to avoid the overlapping of the inhibition. The discs were lightly pressed with the forceps to make complete contact with the surface of the medium. The plate was allowed to stand at room temperature for 30 minutes for pre diffusion and then incubation periods at 37$^{0}$C for 24 hrs. The diameter of the zone of inhibition was measured at the end of the incubation period. Organisms were classified as sensitive or resistant to an antibiotic according to the diameter of the inhibition zone surrounding each antibiotic disk as listed by manufacturer. Organisms considered to be intermediate resistance were scored as sensitive.

**4.2.7.1 Preparation of swab**

Cotton wool swabs on wooden applicator sticks should be prepared. They were sterilized in tubes either in the autoclave or by dry heat.

### 4.2.7.2 Preparation of barium sulphate standard:

- Preparation of 1% v/v $H_2SO_4$

1 ml of conc. $H_2SO_4$ was added to 99 ml of distilled water and mixed well.

- Prepaarion of 1% w/v $Bacl_2 .2H_2O$

0.5 gram of $Bacl_2 .2H_2O$ was dissolved in 50 ml of distilled water.

- 0.6 ml of $Bacl_2 .2H_2O$ was mixed with 99.4 ml of $H_2SO_4$ and dispensed in capped tube
- It was stored in well sealed container in dark at room temperature.

### 4.2.8 Study of heavy metals resistance of *E .coli* isolates

The resistance of *E. coli* isolates to heavy metals was investigated by agar dilution method (Cervante, et.al 1986). The preparation of stock solution of different heavy metal salts is given in appendix I. In agar dilution method, plates were prepared freshly by supplementing agar medium with different concentrations of metal salts. The metal salts used in the study were $CuSO_4.5H_2O$, $ZnCl_2$, $Pb(NO_3)$, $CdCl_2$ and $HgCl_2$. The media was prepared by incorporating different concentrations of each metals separately. For metal resistance profile, isolated bacterial strains were inoculated on metal incorporated nutrient agar plates by streaking. Then the culture were incubated at $37^0$C for 24 hours and growth in each concentration was recorded. The concentration of each metal was increased till the isolates failed to grow on media plates. Then the Minimum Inhibitory Concentration was noted when the isolates failed to grow on the plates.

# CHAPTER-V

### 5. RESULTS

This study was carried out from August 2008 to March 2009. During the study period physico-chemical parameters and bacteriological assessment along with isolation and identification of the isolates, in addition, antibiotic susceptibility and heavy metals resistance pattern of the isolates was determined.

### 5.1. Physico-chemical parameters of water

#### 5.1.1 Temperature

The seasonal variation in temperature was observed with the highest and the lowest temperature being $25.7^0$C and $11.8^0$C respectively and the average temperature being $18.7^0$C. The highest temperature was recorded in the month of August from Kuleshwor while the lowest temperature was recorded in the month of December from Teku.

#### 5.1.2 pH

No variation was seen in the pH values of the water samples with all the values lying close to the neutral pH and within the WHO recommended limit (6.5-8.5). The pH value of the water ranged from 6.9 in Gaushala to 7.9 in Balkhu with an average value of 7.4.

**Table5. 1: Location wise result for physico-chemical parameters**

| S.N. | Date | Location | Mean temperature ($^0$C) | Mean pH |
|---|---|---|---|---|
| 1. | 29-08-2008 | Kuleshwor | 25.5 | 7.4 |
| 2. | 09-09-2008 | Balkhu | 24.3 | 7.7 |
| 3. | 18-09-2008 | Bagbazaar | 23.8 | 7.6 |
| 4. | 05-11-2008 | Maitidevi | 20.5 | 7.4 |
| 5. | 24-11-2008 | Handigaon | 16.4 | 7.2 |
| 6. | 17-12-2008 | Kirtipur | 13.6 | 7.6 |
| 7. | 23-12-2008 | Teku | 12.1 | 7.5 |
| 8. | 03-01-2009 | Gaushala | 13.6 | 6.9 |

| | | | | |
|---|---|---|---|---|
| 9. | 10-02-2009 | Kalopul | 14.9 | 7.2 |
| 10. | 24-02-2009 | Kamalpokhari | 18.7 | 7.1 |
| 11. | 25-03-2009 | Kalikastan | 22.2 | 7.5 |

**5.2 Bacteriological quality of water**

A total of 102 water samples were analyzed for the presence of total coliform and thermotolerant coliform by millipore membrane filtration method.

**5.2.1 Total Coliform count and Thermotolerant Coliform count:**

Out of 102 water samples, 88 samples (86.2%) were found to contain the total coliform beyond the WHO permissible limit (0 cfu/100 ml), while the rest of the water samples i.e., 14 (13.7%) meet the recommended limit. The coliform count ranged from 0 to >300 cfu/100 ml.

Similarly, 20 samples (19.6%) were found to contain thermotolerant coliform beyond the guideline value as recommended by the WHO (0 cfu/100 ml) while rest of the samples i.e., 82 (80.3%) were found to meet the WHO guideline. The thermotolerant count ranged from 0 to 52 cfu/100 ml.

**Table5. 2: Total coliform count exceeding WHO guideline value**

| Total no. of samples | No. of samples exceeding WHO guideline value | % |
|---|---|---|
| 102 | 88 | 86.2 |

**Table 5.3: Location wise total coliform count exceeding WHO guideline value**

| Location | No. of sample | No. of sample exceeding WHO guideline | % |
|---|---|---|---|
| Kuleshwor | 10 | 10 | 100 |
| Balkhu | 10 | 10 | 100 |
| Bagbazaar | 8 | 8 | 100 |
| Maitidevi | 10 | 10 | 100 |
| Handigaon | 8 | 4 | 50 |
| Kirtipur | 10 | 4 | 40 |
| Teku | 10 | 10 | 100 |
| Gaushala | 10 | 10 | 100 |
| Kalopul | 8 | 4 | 50 |
| Kamalpokhari | 10 | 10 | 100 |
| Kalikastan | 8 | 8 | 100 |

**Table 5.4: Thermotolerant coliform count exceeding WHO guideline value**

| Total no. of sample | No of sample exceeding WHO guideline value | % |
|---|---|---|
| 102 | 20 | 19.6% |

**Table5. 5: Location wise thermotolerant coliform count exceeding WHO guideline value**

| Location | No. of sample | No. of sample exceeding WHO guideline | % |
|---|---|---|---|
| Kuleshwor | 10 | 2 | 20 |
| Balkhu | 10 | 3 | 30 |
| Bagbazaar | 8 | 5 | 62.5 |
| Maitidevi | 10 | 3 | 30 |
| Handigaon | 8 | 0 | 0 |
| Kirtipur | 10 | 0 | 0 |

| | | | |
|---|---|---|---|
| Teku | 10 | 0 | 0 |
| Gaushala | 10 | 0 | 0 |
| Kalopul | 8 | 0 | 0 |
| Kamalpokhari | 10 | 3 | 30 |
| Kalikastan | 8 | 4 | 50 |

### 5.2.2 Isolation and identification of *E. coli*

*E. coli* were isolated and identified using the conventional biochemical method from the water samples that were tested positive for thermotolerant coliform. Thus, a total of 20 *E. coli* isolates were obtained.

**Table5. 6: Location wise isolation of *E. coli***

| S.N. | Location | Total no. of samples | No. of isolates | % recovery |
|---|---|---|---|---|
| 1. | Kuleshwor | 10 | 2 | 20 |
| 2. | Balkhu | 10 | 3 | 40 |
| 3. | Bagbazaar | 8 | 5 | 87.5 |
| 4. | Maitidevi | 10 | 3 | 40 |

| | | | | |
|---|---|---|---|---|
| 5. | Handigaon | 8 | 0 | 0 |
| 6. | Kirtipur | 10 | 0 | 0 |
| 7. | Teku | 10 | 0 | 0 |
| 8. | Gaushala | 10 | 0 | 0 |
| 9. | Kalopul | 8 | 0 | 0 |
| 10. | Kamalpokhari | 10 | 3 | 20 |
| 11. | Kalikastan | 8 | 4 | 50 |

**5.3 Antibiotic susceptibility pattern of *E. coli***

20 *E. coli* isolates were subjected to the antibiotic susceptibility test to ten different antibiotics using Kirby-Bauer disk diffusion method. The antibiotics used were Tetracycline(T), Co-trimoxazole(Co), Amikacin(AK), Cephalexin(Cp), Chloramphenicol(C), Amoxycillin(Am), Ceftriaxone(Ci), Ciprofloxacin(CF), Nalidixic Acid(NA) and Gentamicin(G). Resistance of the isolates was directed towards Cephalexin(65%) followed by Amoxycillin (45%) and Tetracycline(15%). All the isolates were sensitive to Co-trimoxazole**,** Amikacin, Chloramphenicol**,** Ceftriaxone, Ciprofloxacin, Nalidixic Acid and Gentamicin. Isolates that were resistant to at least one antibiotic were (n=14 i.e, 70%) followed by those resistant to at least two or more (n=10 i.e, 50%) while only one (n=1 i.e, 5%) was resistant to three antibiotics.

**Table 5.7: Antibiotic susceptibility pattern of *E. coli***

| Isolate no. | Antibiotics | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | T | Co | Ak | Cp | C | Am | Ci | CF | NA | G |
| E1. | S | S | S | R | S | I | S | S | S | S |
| E2. | S | S | S | R | S | R | S | S | S | S |
| E3. | S | S | S | R | S | R | S | S | S | S |
| E4. | S | S | S | S | S | I | S | S | S | S |
| E5. | S | S | S | R | S | R | S | S | S | S |
| E6. | S | S | S | R | S | R | S | S | S | S |
| E7. | S | S | S | I | S | I | S | S | S | S |
| E8. | R | S | S | R | S | I | S | S | S | S |
| E9. | S | S | S | R | S | R | S | S | S | S |
| E10. | S | S | S | I | S | I | S | S | S | S |
| E11. | S | S | S | I | S | I | S | S | S | S |
| E12. | R | S | S | R | S | R | S | S | S | S |
| E13. | I | S | S | R | S | R | S | S | S | S |
| E14. | S | S | S | I | S | I | S | S | S | S |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| E15. | R | S | S | R | S | I | S | S | S | S |
| E16. | S | S | S | R | S | R | S | S | S | S |
| E17. | I | S | S | I | S | R | S | S | S | S |
| E18. | S | S | S | I | S | I | S | S | S | S |
| E19. | S | S | S | R | S | S | S | S | S | S |
| E20. | S | S | S | R | S | S | S | S | S | S |

N.B: T-Tetracycline (30 µg), Co-Co-trimoxazole (25 µg), Ak-Amikacin (30 µg), Cp-Cephalexin (30 µg), C-Chloramphenicol(30 µg ), Am-Amoxicillin(10 µg ),

Ci-Ceftriaxone (30 µg ), CF- Ciprofloxacin(5 µg), NA- Nalidixic Acid(30 µg ), G-Gentamicin(30 µg)

**Table5. 8: Percentage of antibiotic susceptibility of *E. coli* isolates**

| Antibiotic used | Sensitive(S) | | Intermediate(I) | | Resistant(R) | |
|---|---|---|---|---|---|---|
| | No. of isolates | % | No. of isolates | % | No. of isolates | % |
| Tetracycline (30 µg) | 15 | 75 | 2 | 10 | 3 | 15 |
| Co-trimoxazole (25 µg) | 20 | 100 | 0 | 0 | 0 | 0 |
| Amikacin (30 µg) | 20 | 100 | 0 | 0 | 0 | 0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Cephalexin (30 µg) | 1 | 5 | 6 | 30 | 13 | 65 |
| Chloramphenicol(30 µg) | 20 | 100 | 0 | 0 | 0 | 0 |
| Amoxicillin(10 µg) | 2 | 10 | 9 | 45 | 9 | 45 |
| Ceftriaxone(30 µg) | 20 | 100 | 0 | 0 | 0 | 0 |
| Ciprofloxacin(5 µg) | 20 | 100 | 0 | 0 | 0 | 0 |
| Nalidixic Acid(30 µg) | 20 | 100 | 0 | 0 | 0 | 0 |
| Gentamicin(30 µg) | 20 | 100 | 0 | 0 | 0 | 0 |

**Table5.9: Frequency of antibiotic and multiple-antibiotic resistance among *E. coli***

| Total no. of isolates | AR | | MAR | |
|---|---|---|---|---|
| 20 | No. of isolates | % | No. of isolates | % |
| | 14 | 70 | 10 | 50 |

AR= resistant to at least one antibiotic

MAR= resistant to at least two or more antibiotics

**5.4 Minimum Inhibitory Concentration of heavy metals to isolates**

In the present study 12 *E. coli* isolates were assayed for determination of MIC of various heavy metals on solid medium. The metal salts used in the study were $CuSO_4.5H_2O$, $ZnCl_2$, $Pb(NO_3)_2$, $CdCl_2$ and $HgCl_2$. The media was prepared by incorporating different concentrations of each metals separately. Among them 8 (66.6%) isolates that exhibited resistance to more than one antibiotics has high MIC values for a set of heavy metals. All isolates exhibited high resistance to Zinc with average MIC 679.2 µg/ml and low resistance to Mercury with average MIC 58.3 µg/ml.

**Table5.10: Minimum Inhibitory Concentration of heavy metals to isolates**

| Bacterial isolates no. | Minimum Inhibitory Concentration (MIC) of : (µg/ml) | | | | |
|---|---|---|---|---|---|
| | $Hg^{2+}$ | $Cu^{2+}$ | $Pb^{2+}$ | $Zn^{2+}$ | $Cd^{2+}$ |
| E1 | 25 | 250 | 125 | 500 | 50 |
| E2 | 25 | 100 | 50 | 550 | 75 |
| E3 | 50 | 200 | 100 | 700 | 100 |
| E4 | 50 | 100 | 50 | 800 | 50 |
| E5 | 50 | 100 | 150 | 1000 | 125 |
| E6 | 75 | 125 | 125 | 700 | 150 |
| E7 | 50 | 200 | 175 | 650 | 125 |
| E8 | 25 | 100 | 200 | 700 | 125 |
| E9 | 100 | 300 | 150 | 800 | 150 |

| | | | | | |
|---|---|---|---|---|---|
| E10 | 50 | 200 | 125 | 500 | 125 |
| E11 | 100 | 250 | 125 | 550 | 125 |
| E12 | 100 | 250 | 100 | 700 | 150 |
| Average MIC | 58.3 | 181.2 | 122.9 | 679.2 | 104.2 |

# CHAPTER- VI

# 6. DISCUSSION AND CONCLUSION

## 6.1 DISCUSSION

This study was undertaken with an aim to investigate drinking water quality of Kathmandu. Drinking water samples were analyzed for bacteriogical quality of water of different places of Kathmandu valley along with the measurement of some physicochemical parameters (pH and temperature). Antibiotic susceptibility pattern and heavy metals resistance pattern against *E. coli* isolates were also studied**.**

Temperature has its effect on the chemistry and biological reactions in the organisms in water. A rise in the temperature of the water leads to the speeding up of the chemical reactions in water, reduces the stability of gases and amplifies taste and odour and corrosion problem however there is no standard guideline value regarding the temperature. The maximum temperature recorded in the study was $25.7^0$C and minimum temperature was $11.8^0$C. This variation in temperature has also been reported by others in the previous studies. In the studies conducted by Joshi et al., (2004) and Ghimire (1996), the temperature ranged from $14.7^0$C to $27.4^0$C and $19.7^0$C to $22.5^0$C respectively. Bajracharya (2007) showed that the temperature ranged from $23.2^0$C to $30.2^0$C, $21.1^0$C to $30.1^0$C and $18.0^0$C to $30.1^0$C for stone spouts, tap water and tube well respectively. Similarly, Jayana (2007) reported a distinct variation in the temperature of tested water samples ranging from $12^0$C to $30.3^0$C in well water, $12.2^0$C to $29.8^0$C in tap water and $18.8^0$C to $29.2^0$C in stone spout. Variation in the temperature of water from two reservoirs was reported by Gopali (2008) lying between $23.2^0$C and $30.2^0$C. Similarly, Shrestha (2008) revealed the variation in the temperature as the lowest and the highest temperature recorded being $23.2^0$C and $29.6^0$C respectively.

pH is the measure of the intensity of acidity or alkalinity and measures the concentration of hydrogen ions in water. pH of the water gets changed with time due to exposure to air, biological activity and change in temperature. In drinking water, acidic pH may cause corrosion of metal pipes in the distribution system and alkaline pH adversely affect the disinfection process. Chlorination may markedly less effective in increasing pH values.

The pH value of the water ranged from 6.9 to 7.9 with an average value of 7.5. Thus, all the samples (100%) were within the permissible limit as recommended by the WHO (6.5-8.5). Similar result was obtained in the studies conducted by Sharma (1996), Bottino et al., (1991), Thapa (1997), Maharjan (1998), Gyewali (2007), Gopali (2008) and Shrestha (2008) who reported that the pH of the water samples lie within the permissible limit. However, other studies conducted previously have shown a deviation in the pH values of water from the normal value. Pradhananga et al., (1993) recorded the lower pH value of the most water samples from the Pashupati area. Ghimire (1996) reported that the pH ranged from 5.6-6.3 in rainy season and 5.9-6.7 in summer season. Prasai (2002) reported 8.3 % of water samples to have crossed the guideline value for pH. Bajracharya (2007) reported that 85.09% of the samples were within standard, 14.91% below the standard and 0 % above the standard with regard to pH. Jayana (2008) reported that 84.76 % samples were within the permissible limit while 13.33 % samples showed below the limit and 1.9% samples crossed the upper limit of WHO guideline value.

The bacteriological quality of the water samples revealed the presence of total coliform in 86.2% of the samples to be beyond the WHO permissible limit (0 cfu/100 ml), while the rest of the water samples i.e, 13.7% meet the recommended limit. The coliform count ranged from 0 to >300 cfu/100 ml. Similarly, 19.6% of the samples were found to contain thermotolerant coliform beyond the guideline value as recommended by the WHO (0

cfu/100 ml) while rests of the samples i.e., 80.3% were found to meet the WHO guideline. The thermotolerant count ranged from 0 to 52 cfu/100 ml.

This study along with the previous studies carried till date have shown that the drinking water quality of Kathmandu is deteriorating with time in all types of sources. The result of this study in terms of microbial quality of drinking water is in agreement with the previous studies.

Sharma (1978) found only 50% samples contaminated with faecal material in Kathmandu but in a follow up study, Sharma (1986) found that the level of coliform contamination had significantly increased by 10 fold in between nine years. Manandhar *et al*., (1986) examined the quality of drinking water in the Kathmandu valley and reported 85.6% of the total sample crossed the WHO permissible level. Likewise, CEDA (1989) recorded that all samples were contaminated with faecal matter and none of the tap and ground water sources were safe for drinking. ENPHO/DISVI (1992) revealed that 50% samples from public taps were contaminated after one-year monitoring on microbiological quality of water supply in Kathmandu City. Prasai (2002) showed that majority of water samples (82.6%) crossed the guideline value as prescribed by WHO and only 17.39 % samples were safe. Shrestha (2002) found 85.26% of the samples to have exceeded WHO guideline value for total coliform.

Bajracharya (2007) assessed the quality of drinking water of Kathmandu and reported 90.35% samples showing the presence of the coliform. Gyewali (2007) assessed the water quality of Kathmadu valley, taken from seven different sources. All the water samples showed the presence of coliform bacteria. Warner et al., (2007) sampled water from over 100 sources in Kathmandu and found that total coliform and *E. coli* bacteria were present in

94% and 72% of all the water samples respectively. Gopali (2008) assessed the microbial quality of chlorinated drinking water from two reservoirs. The microbiological analysis of water samples from Sundarighat reservoir revealed the presence of total colifrom in 68.78% of the samples above the WHO guideline value in Sundarighat reservoir while 90.41% exceeded the standard permissible limit in the Balaju reservoir. Shrestha (2008) assessed the municipal drinking water quality of the Kathmandu in which 88.2% of the total sample showed the presence of total coliform.

Altogether 20 *E. coli* isolates were obtained from 102 water samples. The data obtained from the studies on water quality in Nepal and other countries suggest that *E. coli* was the predominant enteric bacteria isolated from faecally contaminated water. Sharma (1993) and Thapa (1997) both isolated *E. coli* as predominant bacteria along with *Salmonella* spp and *Klebsiella* spp from water samples. Shrestha (2002) obtained a total of 27 *E. coli* isolates from 95 drinking water samples. Similarly, Prasai (2002) reported the highest recovery of *E. coli* (26.4%) from drinking water samples of Kathmandu followed by other enteric bacteria. Bajracharya (2007) isolated ten different kinds of enteric bacteria with the high recovery of *E. coli* (25%). Jayana (2007) isolated 24.6% *E.coli* isolates among the total enteric bacterial population. Warner et al., (2007) found 72% of all the water samples contain *E. coli.* Shrestha (2008) isolated fifteen different genera of enteric bacteria with the total of two hundred and ninety six isolates, *E. coli* (22.6%) being the predominant.

Out of 102 samples, 20 samples were found to contain thermotolerant coliforms. The term “fecal coliforms” although frequently employed, is not correct: the correct terminology for these organisms is “thermotolerant coliforms”. Thermotolerant coliforms are defined as the group of total coliforms that are able to ferment lactose at 44-45°C. They comprise the genus *Escherichia* and, to a lesser extent, species of *Klebsiella, Enterobacter,* and *Citrobacter.* Of these organisms, only *E. coli* is considered to be specifically of faecal

origin, being always present in the faeces of humans, other mammals, and birds in large numbers and rarely, if ever, found in water or soil in temperate climates that has not been subject to faecal pollution (although there is the possibility of regrowth in hot environments, (Fujioka et al., 1999).

The principal reason of the bacteriological pollution of drinking water are due to the use of unrepaired old pipeline systems for distribution, parallel arrangement of the drinking water pipeline with that of the drainage system and irregular supply of the drinking water in the pipeline. Beside that the contamination may be due to infiltration of contaminated water (sewage) through cross connection and leakage points. Thus deteriorating water quality is the major problem and it has created serious treat to human health and environment. The quality of water has deteriorated due to poor management and no monitoring of water quality.

Human infectious diseases are among the most serious effects of water pollution, especially in developing countries, where sanitation may be inadequate or non-existent. The emerging and re-emerging infectious diseases have recently proven to be the biggest health threat worldwide and they contribute between 70-80% of health problems in developing countries. Waterborne diseases occur when parasites or other disease-causing microorganisms are transmitted via contaminated water, particularly water contaminated by pathogens originating from excreta. These include typhoid, intestinal parasites, and most of the enteric and diarrheal diseases caused by bacteria, parasites, and viruses. Among the most serious parasitic diseases are amoebiasis, giardiasis, ascariasis, and hookworm. The conservation of water sources is very important to provide safe water. The ultimate objective for monitoring the microbiological quality of water is to identify and then minimize the public health risk from consuming water intended for drinking and from

exposure to recreational water. So, the conservation, management and establishment of sanitary practices for the disposal of sewage and the increasing use of filtration and chlorination of drinking water resulted in a dramatic decrease in waterborne diseases, designed to protect public water supplies from contamination and providing safe drinking water.

A total of 20 *E. coli* isolates were assayed against ten antibiotics. Resistance was found most commonly directed toward Cephalexin (65%) and Amoxicillin (45%) while only 15% exhibited resistance towards Tetracycline. All the isolates were sensitive to Co-trimoxazole, Amikacin, Ceftriaxone, Nalidixic acid and Gentamycin. 70% isolates exhibited resistance to at least one antibiotics followed by 50% were resistant to more than one antibiotics. Similar pattern of resistance of *E. coli* and other coliforms towards these widely used antibiotics has also been reported in the studies conducted within and outside the country. Similarly, Shrestha (2002) reported that all the *E. coli* isolates were resistant to Ampicillin followed by 51.8%, 11.11%, 14.8% and 7.40 % of the total isolates being resistant to Nitrofurantoin, Nalidixic acid, Co-trimoxazole and Kanamycin respectively.74.07% of total *E. coli* isolates were resistant to at least one antibiotics and MAR was found in 3.07%. Jayana (2007) assayed ten antibiotics against the total of 142 isolates of Kathmandu and reported the maximum resistance commonly directed toward Erythromycin (79.5%) and Penicillin G (62.67%). Of the total isolates, resistance towards Ampicillin (34.5%), Ofloxacin (5.6%), Chloramphenicol (5.6%), Amoxycillin (61.9%), Cephotaxime (41.7%), Amikacin (14.7%), Ceftriaxone (15.4%) and Tetracycline (21.1%) was shown. Shrestha (2008) reported that all the *E. coli* isolates showed resistance towards Ampicillin while all the *E. coli* showed sensitivity towards Ciprofloxaxin and Norfloxacin. 28.5% of *E. coli* showed the multi drug resistant pattern.

Antibiotics are used substantially to reduces the threat posed by infectious diseases but the extensive use and misuse of these drugs have lead to the emergence of the resistant microbes. Besides the use and misuse, there are various other factor that cause the microbes to be more resistant include repeated exposure of bacteria of microbes to antibiotics, production of enzymes that inactivate or modify antibiotics and changes in bacterial membrane that prevent the uptake of antibacterial agents also lead to the bacterial cells to be more resistant against the antibiotics used (Lin et al.,2004).. Serious infections may lead to death due to inadequate antibiotic therapy. The increasing ineffectiveness of drugs combined with the unavailability of alternative antimicrobials can contribute to the spread of major infectious diseases causing serious epidemics. So, antibiotics susceptibility test is a crucial for accurate choices of antimicrobial therapy.

Nowadays, metals and antibiotic resistance among bacterial population is becoming a major global concern. In the present study 12 *E. coli* isolates were assayed for determination of MIC of various heavy metals on solid medium. Among them 8 (66.6%) isolates that exhibited resistance to more than one antibiotics has high MIC values for a set of heavy metals. All isolates exhibited high resistance to Zinc and low resistance to Mercury. Multiple metal resistant bacterial isolates have exhibited high resistance to a group of antibiotics (Vajiheh et al, 2003). The use of heavy metals in industries creates the selective pressure for the survival of bacteria in a contaminated environment. Thus in a multiple stressed environment, bacterial cells acquire these resistance by a change in the genetic makeup either by mutation or by transfer of resistant genes between bacteria.

In all selected isolates, the highest resistance was found against Zinc and less resistance against Mercury. We hypothesized that bacteria resistant to high concentrations of heavy metal salts would have potential capacities to tolerate or possibly degrade a variety of toxic materials and thus, would be important in environmental pollution bioremediation. The fact that 66.6% of the bacteria resistant to antibiotics are also resistant to heavy metals suggests

that transfer of resistance takes place via plasmids. These heavy metals influence the microbial population by affecting their growth, morphology, biochemical activities and ultimately resulting in decreased biomass and diversity. Heavy metals can damage the cell membranes, alter enzymes specificity, disrupt cellular functions and damage the structure of the DNA. Toxicity of these heavy metals occurs through the displacement of essential metals from their native binding sites or through ligand interactions . Also, toxicity can occur as a result of alterations in the conformational structure of the nucleic acids and proteins and interference with oxidative phosphorylation and osmotic balance.

Due to the selective pressure from the metal in the growth environment, microorganisms have evolved various mechanisms to resist the heavy metal stress. Several metal resistance mechanisms have been identified: exclusion by permeability barrier, intra and extra cellular sequestration, active transport, efflux pumps, enzymatic detoxification, and reduction in the sensitivity of the cellular targets to metal ions.

Heavy metal contamination in the environment has become a serious problem due to the increase in the addition of these metals to the environment. Natural sources as well as the anthropogenic sources account for this contamination, which has become a threat to public health. Cadmium, copper and zinc are among those heavy metals that are being released to the environment. In this perspective many approaches have been used to assess the risk posed by the contaminating metals in soil, water bodies etc. At present the tolerance of soil bacteria to heavy metals has been proposed as an indicator of the potential toxicity of heavy metals to other forms of biota. Therefore, there is a dramatic increase in the interest on studying the interactions of heavy metals with microorganisms.

## 6.2 CONCLUSION

From this study ,86% were total coliforms and 19.6% were found to contain thermotolerant coliforms out of 102 water samples . 20 *E. coli* isolates were subjected to antibiotic susceptibility test to ten antibiotics . Resistance of the isolates was directed towards Cephalexin (65%) followed by Amoxycillin(45%) and Tetracycline (15%). 12 *E. coli* isolates were assayed for determination of MIC of various heavy metals on solid medium. Among them 66.6% isolates that exhibited resistance to more than one antibiotics had high MIC values for a set of heavy metals. All isolates exhibited high to resistance Zinc and low resistance to Mercury. The deteriorating water quality may contribute different types of water-borne diseases at any time. Quality of water consumed is critical in controlling infectious diseases and the level of diseases can be decreased significantly by implementing simple strategies, such as proper waste and water management and education on maintainance of hygienic condition. So, this study will help in proper water management and also checks water resources from going further pollution. Antibiotic sensitivity test was also performed which help to make accurate choice of antimicrobials. The heavy metal tolerance property of the isolates could be further exploited for means of bioremediation as well as processes like bioleaching for the economical recovery of important metals like Fe and Cu. Heavy metal pollution of the environment is a problem for which bioremediation by microorganisms is a natural, viable and economic solution.

# CHAPTER-VII

## 7. SUMMARY AND RECOMMENDATIONS

### 7.1 SUMMARY

The examination of water quality is of great concern as it is directly linked with the healthy life. All the water borne diseases are caused by fecal contamination of water resources and unhygienic practices. Nevertheless, the incidence of disease can be minimized by safe disposal of waste, hygienic and good sanitary practices, additionally, by protecting water resources. The bacteriological and physicochemical quality of urban drinking water of Kathmandu valley was studied. Moreover, antibiotic susceptibility and determination of Minimum Inhibitory Concentration of various heavy metals on *E. coli* isolates was also performed.

1. A total of 102 water samples were randomly collected from different areas of Kathmandu city. Physico-chemical and bacteriological parameters were analyzed to assess the drinking water quality.
2. The temperature of water samples was noted which ranges from $25.7^0$C to $11.8^0$C. The pH values of the water samples was not varied lying close to the neutral and the WHO recommended limit (6.5-8.5).
3. Out of 102 water samples, 88 samples (86.2%) were found to contain the total coliform while 20 samples (19.6%) were found to contain thermotolerant coliform beyond the guideline value as recommended by the WHO (0 cfu/100 ml).
4. A total of 20 *E. coli* isolates were obtained from 20 water samples tested positive for thermotolerant coliform.
5. Antibiotic resistance of the isolates was predominantly directed towards Cephalexin (65%) followed by Amoxycillin (45%) and Tetracycline (15%). All the isolates were 100% sensitive to Co-trimoxazole, Amikacin, Chloramphenicol, Ceftriaxone, Ciprofloxacin, Nalidixic Acid and Gentamicin.
6. Study on heavy metals resistance of the isolates was performed. Most of the antibiotics resistant isolates was found to be grown at the higher concentration of metals. All the isolates exhibited high resistance to Zinc and low resistance to Mercury.

## 7.2 RECOMMENDATIONS

On the basis of the findings of this study, following recommendation can be enunciated.

1. Basic education programmes regarding consciousness arousing on hygienic practice and sanitary practice; simple and cheap water treatment methods such as boiling,

filtration, solar disinfection; safe disposal of waste and prevention of waterborne disease should be carried out.

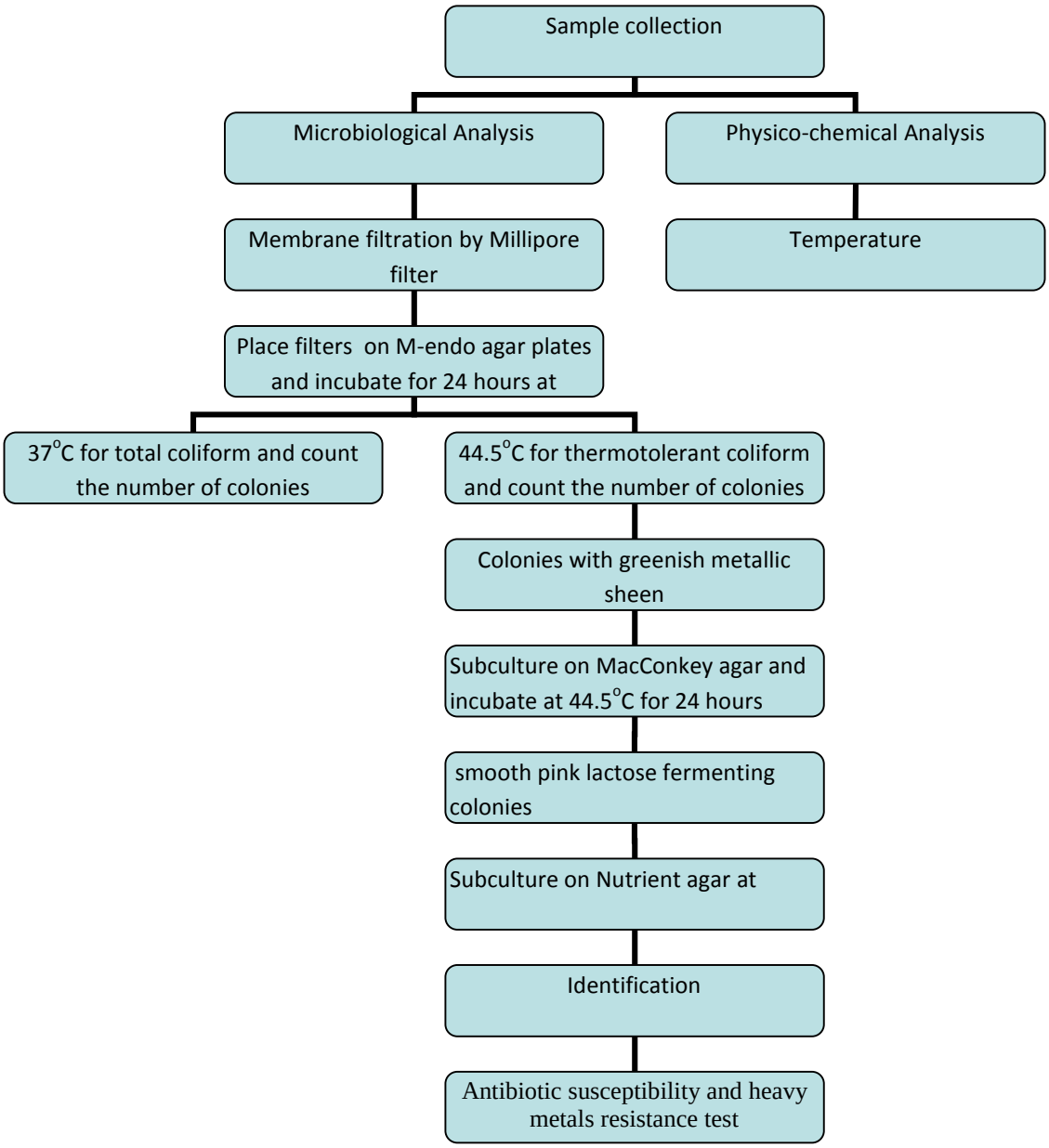
2. Antibiotic susceptibility testing of isolates should be carried out to make accurate choice of the antibiotic to be used.

3. Since the MDR *E. coli* isolate is seen, the possible contamination from the hospital waste should be monitored.

4. Serotyping of *E .coli* should be performed to eliminate the possibility of the presence of enteropathogenic strains.

5. The growth of isolates on higher concentration of metals indicates the heavy metals pollution on water sources. Further research should be conducted on these organisms for the study of their ability to bioaccumulate heavy metals and their possible use in bioremediation.

## Flowchart for general sample processing


Sample collection
Microbiological Analysis
Physico-chemical Analysis
Membrane filtration by Millipore filter
Temperature
Place filters on M-endo agar plates and incubate for 24 hours at
37°C for total coliform and count the number of colonies
44.5°C for thermotolerant coliform and count the number of colonies
Colonies with greenish metallic sheen
Subculture on MacConkey agar and incubate at 44.5°C for 24 hours
smooth pink lactose fermenting colonies
Subculture on Nutrient agar at
Identification
Antibiotic susceptibility and heavy metals resistance test

# APPENDIX – I

## List of materials

### Equipments

1. Autoclave
2. Electric balance
3. Hot air oven
4. Incubators
5. Membrane filter apparatus-Millipore
6. Microscope
7. pH meter
8. Refrigerator

### 1.2 Glassware

Beakers
Conical flasks
Petri dishes
Glass rods
Graduated cylinders
Microscopic-slides
Pipettes
Reagent bottles
Screw capped test tubes

### 1.3 Miscellaneous

Aluminum foil
Blotting paper
Cotton role
Cotton-swab
Dropper
Forceps
Immersion oil
Inoculating loop
Membrane Filter Paper
Measuring scale
Sampling bottles
Detergent
Labelling tape
Transport tray etc

**1.4 Chemicals/Reagents**

Lysol

ℑ-Naphthol

Methyl red

Kovac's reagent

Paraffin
Barium Chloride

Potassium hydroxide

Crystal violet

Safranin

Ethanol

Gram's iodine

Conc. Sulfuric acid

Hydrogen peroxide

- Tetramethyl-p-phenylenediamine dihydroxide
- Sodium thiosulphate

**1.5 Microbiological media**

- M-endo Agar (Hi-media)
- Nutrient Agar (Hi-media)
- Muller Hinton Agar (Hi-media)

Nutrient Broth (Hi-media)
McConkey agar (Hi-media)

**1.6 Biochemical media**

Sulphide Indole Motility Medium (Hi-media)

MR-VP Broth(Hi-media)
Simmon Citrate Agar (Hi-media)

Triple Sugar Iron Agar(Hi-media)
Urease Agar (Hi-media)
Hugh-Leifson’s Agar(Hi-media)

**Composition and preparation of bacteriological media**

**I. Culture media**

**1.Nutrient Agar (NA)**

| Composition | (Gram/Litre) |
|---|---|
| Peptone | 5.0 |
| Sodium Chloride | 5.0 |
| Beef extract | 1.5 |
| Yeast extract | 1.5 |
| Agar | 15.0 |
| Final pH (at $25^0$C) | 7.4±0.2 |

Procedure

28 gms of media was dissolved in 1000 ml of distilled water and heated to dissolve the media. The media was autoclaved at 15 lbs pressure at $121^0$C for 15 minutes.

**2. Nutrient Broth (NB)**

| Composition | (Gram/litre) |
|---|---|
| Peptone | 5.0 |
| Sodium Chloride | 5.0 |
| Beef extract | 1.5 |
| Yeast extract | 1.5 |
| Final pH (at $25^0$C) | 7.4±0.2 |

Sterilized by autoclaving at 15 lbs pressure ($121^0$C) for 15 minutes.

**3. M-Endo Agar**

| Composition | (Gram/Litre) |
|---|---|
| Yeast extract | 6.0 |
| Peptone | 20.6 |
| Lactose | 25.0 |
| Dipotassium phosphate | 7.0 |
| Basic fuchsin | 1.0 |
| Sodium sulphite | 2.5 |
| Agar | 15.0 |
| Final pH (at $25^0$C) | 7.5±0.2 |

Preparation

5.1 grams of media was dissolved water containing 2ml of 95% ethanol. It was boied to dissolve the medium completely. It was cooled to $45^0$C and poured in petriplates.

Note: The medium was not autoclaved.

**4. McConkey agar (MA)**

| Composition | gm/lit |
|---|---|
| Peptone | 2.0 |
| Lactose | 20.0 |
| Bile salt | 5.0 |
| Sodium chloride | 5.0 |
| Neutral Red | 0.075 |
| Agar | 12.0 |
| Final pH (at 250 C) | 7.4 ± 0.2 |

51.5 grams of media was dissolved in 1ooo ml of distilled water and heated to dissolve the media. The media was autoclaved at 15 lbs pressure for 15 min.

**5. Mueller Hinton Agar (MHA)**

| Composition | (Gram/Litre) |
|---|---|
| Beef Infusion Broth | 300.0 |
| Casein Acid Hydrolysate | 17.0 |
| Starch | 1.0 |
| Agar | 17.0 |
| Final pH | 7.0±0.2 |

Preparation:

3.8 gm of media was suspended in 100 ml distilled water, boiled to dissolve and sterilized by autoclaving at $121^0$C for 15 minutes. It was poured while at 45-$55^0$C in sterile 9 cm diameter plates in 25 ml quantities. To ensure the uniformity in depth of medium, the plates were placed over level surface and the medium was poured into it.

## II. Composition of stains and reagents

### 1. Gram's staining

i. Crystal violet

| | |
|---|---|
| Solution A | |
| Crystal Violet | 2.0gm |
| 95% ethyl alcohol | 20.0ml |
| Solution B | |
| Ammonium oxalate | 0.8gm |
| Distilled water | 30.0ml |

Crystal violet was dissolved in ethyl alcohol and ammonium oxalate in distilled water. Then solution A and B are mixed.

ii. Gram's Iodine

| | |
|---|---|
| Iodine | 1.0gm |
| Potassium iodide | 2.0ml |
| Distilled water | 300.0ml |

Iodine and potassium iodide were dissolved in distilled water.

iii. Ethyl Alcohol (95%)

| | |
|---|---|
| Absolute alcohol | 95.0ml |
| Distilled water | 5.0ml |

iv. Safranin

| | |
|---|---|
| Safranin | 10.0ml |
| (2.5 % solution in 95% ethyl alcohol) | |
| Distilled water | 100.0ml |

Procedure

Heat fixed smear of bacterial culture was flooded with crystal violet for one minute and excess stain was washed out. The slide was treated with Gram's Iodine for 1 minute and washed. It was flooded with decolorize alcohol and immediately washed with water. Then smear was treated with safranin for 1 minute and washed with water. It was dried and observed under microscope.

**2.Catalase Test**

Catalase test is done to test the presence of enzyme catalase. The enzyme catalase splits hydrogen peroxide to water and oxygen.

Reagents: (3 % Hydrogen peroxides).

Composition

| | |
|---|---|
| Concentration hydrogen peroxide | 3ml |
| Distilled water | 97ml |

Procedure

3ml of 3 % hydrogen peroxide was taken in a test tube and colony of bacteria to be tested was picked up from nutrient agar with the help of glass rod and inserted into the tube-containing reagent. The production of gas bubbles immediately indicates positive catalase test.

**3.Oxidase Test**

Oxidase test is done to determine the presence of the oxidase enzyme. Oxidase reaction is due to the presence of a cytochrome oxidase system.

Oxidase reagent

Whatman No. 1 filter paper was cut into strips of 6-8 cm in diameter. It was soaked in the reagent till saturation. The paper strips were drained and freeze dried and stored in a dark tightly sealed bottle.

Procedure
The Oxidase test paper was moistened with distilled water. A colony was picked using glass rod and rubbed to the paper. Development of violet colour within 10 seconds is an indicative of positive test.

**Composition and preparation of biochemical media**

**1. Sulfide-Indole-Motility Medium (SIM)**

Sulfide-Indole-Motility is a semi solid medium used for the determination of sulfide production, Indole formation and motility of enteric bacteria.

| Composition | (gm/litre) |
|---|---|
| Beef extract | 3.0 |
| Peptone | 30.0 |
| Peptonized iron | 0.2 |
| Sodium thiosulfate | 0.025 |
| Agar | 3.0 |
| Final pH (at $25^0$C) | 7.3±0.2 |

Preparation
Thirty-six grams was suspended in 100ml-distilled water. It was heated to boil to dissolve the medium completely. It was dispense in tubes and sterilized by autoclaving for 15 minutes at 15 lbs pressure ($121^0$C). The medium was allowed to solidify in vertical position.

Reagent: Kovac's reagent

| Composition | gm/lit |
|---|---|
| P-Dim ethyl aminobenzaldehyde | 5.0 |
| ISO amyl alcohol | 75.0 ml |
| Conc. Hydrochloric acid | 25.0 ml |

Procedure
The test organisms was stabbed into the medium and incubated at $37^0$C for 24 hours. Motile organism show diffuse growth or turbidity away from the line of inoculation and non-motile grows only along the line of inoculation. Blackening along the line of inoculation indicates H2S positive test.
0.2ml of Kovac's reagent was added to the tube and allowed to stand for 10 minutes. A dark red colour in the reagent indicates a positive indole test.

**2.Methyl Red test**

The methyl red test is done to test the ability of an organism to produce and maintain stable acid products from glucose fermentation and to overcome the buffering capacity of the system.

MR-VP medium (glucose-phosphate broth).

| Composition | (gm/litre) |
|---|---|
| Buffered peptone | 7.0 |
| Dextrose | 5.0 |
| Tripotassim phosphate | 5.0 |
| Final pH (at $25^0$C) | 6.9±0.2 |

Preparation

Seventeen grams was dissolved in 1000ml-distilled water. It was distributed in test tubes in 10 ml amount and sterilized by autoclaving at 15 lbs pressure for 15 minutes.

Reagent – Methyl Red

| Composition | (gm/litre) |
|---|---|
| Methyl red | 0.04gm |
| Ethyl alcohol | 40.0ml |
| Distilled water | 60.0ml |

Preparation

Methyl red was dissolved in ethyl alcohol and water was added.

Procedure

The glucose phosphate broth was inoculated with culture to be tested and incubated at $37^0$C for 48 hours. Methyl red indicator was added to the culture and development of red colour indicates positive test while yellow colour indicates negative test.

**3.Voges – Proskauer Test**

Voges – Proskauer test determine the ability of organism to produce a neutral end product, acetylmethylcarbinol from glucose formation.

Medium -MR-VP medium (Glucose – phosphate broth)

| | |
|---|---|
| Solution A | |
| ά-naphthol | 5.0gm |
| Ethyl alcohol (95%) | 100.0ml |
| Solution B | |
| Potassium hydroxide | 40.0gm |
| Distilled water | 100.0ml |

Procedure

Sterile broth was inoculated with fresh culture medium and inoculated with fresh culture medium and incubated at $37^0$C for 48 hours. Development of pink-red

colour within 30 minutes after adding of ά-naphthol and 40% potassium hydroxide in 1:3 proportions was recorded as positive test.

**4.Citrate Utilization Test**

Citrate utilization test is performed to determine if an organism is capable of utilizing citrate as the sole source of Carbon for metabolism with resulting alkalinity.

Medium – Simmon's Citrate Agar

| Composition | (Gram/Litre) |
|---|---|
| Monoammonium phosphate | 1.0 |
| Dipotassium phosphate | 1.0 |
| Sodium Chloride | 5.0 |
| Sodium Citrate | 2.0 |
| Magnesium Sulphate | 0.2 |
| Bromothymol blue | 0.08 |
| Agar | 15.0 |
| Final pH (at $25^0$C) | 6.8±0.2 |

Preparation

24.2 grams was suspended in 1000ml-distilled water. It was heated to boil to dissolve the medium completely. It was distributed in tubes and sterilized by autoclaving at 15 lbs pressure ($121^0$C) for 15 minutes. The mediums in tubes were solidified in slanted position.

Procedure

The slant was streaked with test organism and incubated at $37^0$C for 48 hours. Growth of organism with an intense blue colour on slant is the indicative of positive test. No growth no change in colour (green) is the negative test.

**5.Triple Sugar Iron Agar Test**

The test is done to determine the ability of an organism to utilize specific Carbohydrate incorporated in the medium, with or without the production of gas, along with determination of possible hydrogen sulfide production

| Composition | (gm/litre) |
|---|---|
| Peptone | 10.0 |
| Tryptone | 10.0 |
| Yeast extract | 3.0 |
| Beef extract | 3.0 |
| Lactose | 10.0 |
| Sucrose | 10.0 |
| Dextrose | 1.0 |
| Ferrous sulfate | 0.2 |

| | |
|---|---|
| Sodium Chloride | 5.0 |
| Sodium Thiosulfate | 0.3 |
| Phenol- red | 0.024 |
| Agar | 12 |
| Final pH (at $25^0$C) | 7.4±0.2 |

Preparation

6.5 grams was suspended in 1000ml-distilled water. It was boiled to dissolve completely. It was distributed in tubes and sterilized by autoclaving at 15 lbs pressure ($121^0$C) for 15 minutes. The medium was allowed to set in slopped from with a butt about 1 inch long.

Procedure

The test organism was stabbed in the butt and streaked on the slant. The tubes were incubated at $37^0$C for 24 hours. Black colouration of butt was indicative of $H_2S$ formation. The change in colour of butt, slant and gas formation was also noted and recorded as alkali/alkali, alkali/acid and acid/acid for the growth of fermenters and all sugar fermenters.

**6. Urease Test**

Urease test demonstrates the ability of an organism to split forming two molecules of ammonia by the action of the enzyme urease.

Medium – Urea agar base.

| Composition | (gm/Litre) |
|---|---|
| Peptone | 1.0 |
| Dextrose | 1.0 |
| Sodium Chloride | 5.0 |
| Disodium phosphate | 1.2 |
| Monopotassium phosphate | 0.8 |
| Phenol red | 0.012 |
| Agar | 15.0 |
| Final pH (at $25^0$C) | 6.8±0.2. |

Preparation

Twenty-four grams urea agar base was suspended in 950ml of distilled water. It was boiled to dissolve completely and sterilized by autoclaving at 10 Lbs pressure ($115^0$C) for 20 minutes. It was cooled down to $55^0$C and aseptically introduced 50ml of sterile 40% area solution and mixed well. It was distributed in sterile test tubes and allowed to solidify in slanted position.

Procedure

Fresh culture of test organism was streaked heavily on the slant and incubated at $37^0$C for overnight. Change in colour of medium to pink indicates positive test and no change in colour indicate negative test.

**7.Hugh and Leifson Medium**

| Composition | (Gram/Litre) |
|---|---|
| Peptone | 20.0 |
| Sodium Chloride | 50.0 |
| Dipotassium Phosphate | 3.0 |
| Agar | 20.0 |
| Bromo Thymol Blue | 0.5 |
| Glucose | 100.0 |
| Final pH | 7.1±0.2 |

Preparation

193 grams of media was dispensed in 1000ml of distilled water. It was boiled to dissolve the medium completely and distributed into tubes in duplicate. The medium was then sterilized by autoclaving at 15 lbs at $121^0$C for 15 minutes.

**Preparation of stock solution of heavy metal salts**

The stock solution was made 1000ppm concentration of all the heavy metal salts.

Calculation,

We know, 1mg/ml = 1ppm

i.e., 0.1g/100ml =1000ppm

Since all the salts except copper salt were in anhydrous form, so, these salts were weighed 0.1 gram and dissolved in 100ml water to make the concentration of 1000ppm.

Note : If the concentration of salt is required less than 1000ppm it is diluted according to the required concentration.

In case of $CuSO_4.5H_2O$, then,

Weight of copper salt required = $\frac{\text{Mol.wt.of } CuSO_4.5H_2O}{\text{Mol.wt.of } CuSO_4.5H_2O - \text{Mol.wt.of water of crystallization}} * 0.1$ gram

$= \frac{249.68}{249.68 - 5*18} * 0.1$ gram

$= \frac{249.68}{159.68} * 0.1$ gram

$= 0.15$ gram

So, 0.15 gram of copper salt is dissolved in 100ml water to make the concentration of 1000ppm.

N.B: Mol.wt = Molecular weight, 18 = Molecular weight of water

# APPENDIX-II

## Sampling sites and their codes

| S.N | Date | Location | Address | Code |
|---|---|---|---|---|
| 1 | 27-08-2008 | Kuleshwor | Shridishi galli, House no. 66/6 | KW1 |
| 2 | 27-08-2008 | Kuleshwor | Gaurishankar Marga, House no.371 | KW2 |
| 3 | 27-08-2008 | Kuleshwor | Gaurishankar Marga, House no.451 | KW3 |
| 4 | 27-08-2008 | Kuleshwor | Gaurishankar Marga, House no.666 | KW4 |
| 5 | 27-08-2008 | Kuleshwor | Lik Marga, House no. 417 | KW5 |
| 6 | 27-08-2008 | Kuleshwor | Lik Marga, House no. 500 | KW6 |
| 7 | 27-08-2008 | Kuleshwor | Lik Marga, House no. 605 | KW7 |
| 8 | 27-08-2008 | Kuleshwor | Lik Marga, House no. 725 | KW8 |
| 9 | 27-08-2008 | Kuleshwor | 25 Maharjan House no. 4 | KW9 |
| 10 | 27-08-2008 | Kuleshwor | Parijat margha House no.129 | KW10 |
| 11 | 09-09-2008 | Balkhu | Viswabidyalaya path 277/19 | B1 |
| 12 | 09-09-2008 | Balkhu | Viswabidyalaya path 219 | B2 |
| 13 | 09-09-2008 | Balkhu | Viswabidyalaya path 345 | B3 |
| 14 | 09-09-2008 | Balkhu | Viswabidyalaya path 290/9 | B4 |
| 15 | 09-09-2008 | Balkhu | Khadya kirana bazaar marg 127 | B5 |
| 16 | 09-09-2008 | Balkhu | Khadya kirana bazaar marg 108 | B6 |

| | | | | |
|---|---|---|---|---|
| 17 | 09-09-2008 | Balkhu | Khadya kirana bazaar marg 75/30 | B7 |
| 18 | 09-09-2008 | Balkhu | Viswabidyalaya path 315 | B8 |
| 19 | 09-09-2008 | Balkhu | Viswabidyalaya path 347/20 | B9 |
| 20 | 09-09-2008 | Balkhu | Hanagul marg 69 369 | B10 |
| 21 | 18-09-2008 | Bagbazaar | Bagbazaar Sadak 55 | BB1 |
| 22 | 18-09-2008 | Bagbazaar | Bagbazaar Sadak 549 | BB2 |
| 23 | 18-09-2008 | Bagbazaar | Siddhartha Marg 34 | BB3 |
| 24 | 18-09-2008 | Bagbazaar | Bajrabir Marg 48 | BB4 |
| 25 | 18-09-2008 | Bagbazaar | Bajrabir Marg 262 | BB5 |
| 26 | 18-09-2008 | Bagbazaar | Dharmachakra Galli 144 | BB6 |
| 27 | 18-09-2008 | Bagbazaar | Dharmachakra Galli 318 | BB7 |
| 28 | 18-09-2008 | Bagbazaar | Adyait Marg 478 | BB8 |
| 29 | 05-11-2008 | Maitidevi | Maitidevi marg 360 | MD1 |
| 30 | 05-11-2008 | Maitidevi | Panchakumari Marg 22 | MD2 |
| 31 | 05-11-2008 | Maitidevi | Panchakumari Marg 204 | MD3 |
| 32 | 05-11-2008 | Maitidevi | Devi Marg 79 | MD4 |
| 33 | 05-11-2008 | Maitidevi | Devi Marg 282 | MD5 |
| 34 | 05-11-2008 | Maitidevi | Janagoreto Galli 178 | MD6 |
| 35 | 05-11-2008 | Maitidevi | Shanti Galli 4 | MD7 |
| 36 | 05-11-2008 | Maitidevi | Mahakabi Marg 463 | MD8 |

| | | | | |
|---|---|---|---|---|
| 37 | 05-11-2008 | Maitidevi | Janata Marg 251 | MD9 |
| 38 | 05-11-2008 | Maitidevi | Iswari Marg 69 | MD10 |
| 39 | 24-11-2008 | Handigaon | Gulaaf Marg 26 | HG1 |
| 40 | 24-11-2008 | Handigaon | Gulaaf Marg 141 | HG2 |
| 41 | 24-11-2008 | Handigaon | Handigaon Marg 104 | HG3 |
| 42 | 24-11-2008 | Handigaon | Handigaon Marg 227 | HG4 |
| 43 | 24-11-2008 | Handigaon | Charnarayan Marg 76 | HG5 |
| 44 | 24-11-2008 | Handigaon | Charnarayan Marg 222 | HG6 |
| 45 | 24-11-2008 | Handigaon | Munal Marg 16 | HG7 |
| 46 | 24-11-2008 | Handigaon | Kotal Marg 98 | HG8 |
| 47 | 17-12-2008 | Kirtipur | Bhajangal ward no. 18 | KP1 |
| 48 | 17-12-2008 | Kirtipur | Bhajangal ward no. 17(242-08-053/1) | KP2 |
| 49 | 17-12-2008 | Kirtipur | Bhajangal ward no. 17(241-18-401) | KP3 |
| 50 | 17-12-2008 | Kirtipur | Bhajangal ward no. 17(242-08-065) | KP4 |
| 51 | 17-12-2008 | Kirtipur | Bhajangal ward no. 17(242-08-045) | KP5 |
| 52 | 17-12-2008 | Kirtipur | Bhajangal ward no.17(242-08-36) | KP6 |
| 53 | 17-12-2008 | Kirtipur | Bhajangal ward no. 17(242-08-47) | KP7 |
| 54 | 17-12-2008 | Kirtipur | Bhajangal ward no.17(244-20-027) | KP8 |
| 55 | 17-12-2008 | Kirtipur | Bhajangal ward no.17(244-19-1) | KP9 |
| 56 | 17-12-2008 | Kirtipur | TU quarter house no.1 | KP10 |

| | | | | |
|---|---|---|---|---|
| 57 | 23-12-2008 | Teku | 40- Ujamo galli, Jyabahal | T1 |
| 58 | 23-12-2008 | Teku | 132/37 Jamana, Gubhajub Marg | T2 |
| 59 | 23-12-2008 | Teku | 132/73 Jamana, Gubhajub Marg | T3 |
| 60 | 23-12-2008 | Teku | 21/17 - Ujamo galli, Jyabahal | T4 |
| 61 | 23-12-2008 | Teku | Public tap, Jyabahal | T5 |
| 62 | 23-12-2008 | Teku | 107 Jamana, Gubhajub Marg | T6 |
| 63 | 23-12-2008 | Teku | 132/42 Jamana, Gubhajub Marg | T7 |
| 64 | 23-12-2008 | Teku | 134 Jamana, Gubhajub Marg | T8 |
| 65 | 23-12-2008 | Teku | 131 Jamana, Gubhajub Marg | T9 |
| 66 | 23-12-2008 | Teku | 44 Jyabaha Marg | T10 |
| 67 | 03-01-2009 | Gaushala | Abhibyakti Marg 10 | G 1 |
| 68 | 03-01-2009 | Gaushala | Abhibyakti Marg 90 | G 2 |
| 69 | 03-01-2009 | Gaushala | Abhibyakti Marg 131 | G 3 |
| 70 | 03-01-2009 | Gaushala | Abhibyakti Marg 186 | G 4 |
| 71 | 03-01-2009 | Gaushala | Dharma Marg 123 | G 5 |
| 72 | 03-01-2009 | Gaushala | Dharma Marg 348 | G 6 |
| 73 | 03-01-2009 | Gaushala | Dharma Marg 585 | G 7 |
| 74 | 03-01-2009 | Gaushala | Dharma Marg 750 | G 8 |
| 75 | 03-01-2009 | Gaushala | Punya Marg 24 | G 9 |
| 76 | 03-01-2009 | Gaushala | Punya Marg 152 | G 10 |

| | | | | |
|---|---|---|---|---|
| 77 | 10-02-2009 | Kalopul | Rudramati Marg 120 | K1 |
| 78 | 10-02-2009 | Kalopul | Rudramati Marg 312 | K2 |
| 79 | 10-02-2009 | Kalopul | Rudramati Marg 555 | K3 |
| 80 | 10-02-2009 | Kalopul | Jamuna Marg 27 | K4 |
| 81 | 10-02-2009 | Kalopul | Jamuna Marg 150 | K5 |
| 82 | 10-02-2009 | Kalopul | Jamuna Marg 297 | K6 |
| 83 | 10-02-2009 | Kalopul | Lamo Galli 118 | K7 |
| 84 | 10-02-2009 | Kalopul | Lamo Galli 222 | K8 |
| 85 | 24-02-2009 | Kamalpokhari | Kamal Marg 42 | KAM1 |
| 86 | 24-02-2009 | Kamalpokhari | Kamal Marg 98 | KAM2 |
| 87 | 24-02-2009 | Kamalpokhari | Kamal Marg 145 | KAM3 |
| 88 | 24-02-2009 | Kamalpokhari | Kamal Marg 182 | KAM4 |
| 89 | 24-02-2009 | Kamalpokhari | Kamal Marg 118/26 | KAM5 |
| 90 | 24-02-2009 | Kamalpokhari | Dhobidhara Marg 221 | KAM6 |
| 91 | 24-02-2009 | Kamalpokhari | Dhobidhara Marg 93 | KAM7 |
| 92 | 24-02-2009 | Kamalpokhari | Dhobidhara Marg 351 | KAM8 |
| 93 | 24-02-2009 | Kamalpokhari | Dhobidhara Marg 468 | KAM9 |
| 94 | 24-02-2009 | Kamalpokhari | Dhobidhara Marg 265 | KAM10 |
| 95 | 25-03-2009 | Kalikastan | Kalika Marg 256 | KLK1 |
| 96 | 25-03-2009 | Kalikastan | Kalika Marg 332 | KLK2 |

| | | | | |
|---|---|---|---|---|
| 97 | 25-03-2009 | Kalikastan | Kalika Marg 451 | KLK3 |
| 98 | 25-03-2009 | Kalikastan | Kalika Marg 550 | KLK4 |
| 99 | 25-03-2009 | Kalikastan | Kalika Marg 618 | KLK5 |
| 100 | 25-03-2009 | Kalikastan | Tirendra Marg 428 | KLK6 |
| 101 | 25-03-2009 | Kalikastan | Tirendra Marg 276 | KLK7 |
| 102 | 25-03-2009 | Kalikastan | Goswara Marg 14 | KLK8 |

# APPENDIX-III

**Physico-chemical analysis of tap water samples**

| S.N. | Date | Code | Temperature($^{o}$C) | pH |
|---|---|---|---|---|
| 1 | 27-08-2008 | KW1 | 25.6 | 7.2 |
| 2 | 27-08-2008 | KW2 | 25.4 | 7.5 |
| 3 | 27-08-2008 | KW3 | 25.5 | 7.3 |
| 4 | 27-08-2008 | KW4 | 25.4 | 7.5 |
| 5 | 27-08-2008 | KW5 | 25.4 | 7.4 |
| 6 | 27-08-2008 | KW6 | 25.4 | 7.4 |
| 7 | 27-08-2008 | KW7 | 25.5 | 7.5 |
| 8 | 27-08-2008 | KW8 | 25.6 | 7.5 |
| 9 | 27-08-2008 | KW9 | 25.5 | 7.4 |
| 10 | 27-08-2008 | KW10 | 25.7 | 7.4 |
| 11 | 09-09-2008 | B1 | 24.0 | 7.7 |
| 12 | 09-09-2008 | B2 | 24.5 | 7.6 |
| 13 | 09-09-2008 | B3 | 24.2 | 7.6 |
| 14 | 09-09-2008 | B4 | 24.3 | 7.7 |
| 15 | 09-09-2008 | B5 | 24.3 | 7.8 |
| 16 | 09-09-2008 | B6 | 24.6 | 7.5 |
| 17 | 09-09-2008 | B7 | 24.3 | 7.7 |
| 18 | 09-09-2008 | B8 | 24.3 | 7.8 |
| 19 | 09-09-2008 | B9 | 24.4 | 7.9 |
| 20 | 09-09-2008 | B10 | 24.5 | 7.7 |

| | | | | |
|---|---|---|---|---|
| 21 | 18-09-2008 | BB1 | 23.7 | 7.6 |
| 22 | 18-09-2008 | BB2 | 23.7 | 7.7 |
| 23 | 18-09-2008 | BB3 | 24.1 | 7.7 |
| 24 | 18-09-2008 | BB4 | 23.8 | 7.8 |
| 25 | 18-09-2008 | BB5 | 23.8 | 7.6 |
| 26 | 18-09-2008 | BB6 | 23.7 | 7.6 |
| 27 | 18-09-2008 | BB7 | 23.8 | 7.7 |
| 28 | 18-09-2008 | BB8 | 24.0 | 7.7 |
| 29 | 05-11-2008 | MD1 | 20.6 | 7.5 |
| 30 | 05-11-2008 | MD2 | 20.6 | 7.3 |
| 31 | 05-11-2008 | MD3 | 20.8 | 7.4 |
| 32 | 05-11-2008 | MD4 | 20.4 | 7.4 |
| 33 | 05-11-2008 | MD5 | 20.5 | 7.5 |
| 34 | 05-11-2008 | MD6 | 20.0 | 7.4 |
| 35 | 05-11-2008 | MD7 | 20.7 | 7.4 |
| 36 | 05-11-2008 | MD8 | 20.8 | 7.4 |
| 37 | 05-11-2008 | MD9 | 20.6 | 7.4 |
| 38 | 05-11-2008 | MD10 | 20.8 | 7.5 |
| 39 | 24-11-2008 | HG1 | 16.5 | 7.3 |
| 40 | 24-11-2008 | HG2 | 16.2 | 7.3 |

| | | | | |
|---|---|---|---|---|
| 41 | 24-11-2008 | HG3 | 16.1 | 7.2 |
| 42 | 24-11-2008 | HG4 | 16.1 | 7.3 |
| 43 | 24-11-2008 | HG5 | 17.0 | 7.2 |
| 44 | 24-11-2008 | HG6 | 16.2 | 7.3 |
| 45 | 24-11-2008 | HG7 | 17.0 | 7.3 |
| 46 | 24-11-2008 | HG8 | 16.4 | 7.3 |
| 47 | 17-12-2008 | KP1 | 13.6 | 7.6 |
| 48 | 17-12-2008 | KP2 | 13.6 | 7.6 |
| 49 | 17-12-2008 | KP3 | 13.5 | 7.8 |
| 50 | 17-12-2008 | KP4 | 13.6 | 7.6 |
| 51 | 17-12-2008 | KP5 | 13.8 | 7.6 |
| 52 | 17-12-2008 | KP6 | 13.8 | 7.7 |
| 53 | 17-12-2008 | KP7 | 13.9 | 7.6 |
| 54 | 17-12-2008 | KP8 | 13.5 | 7.8 |
| 55 | 17-12-2008 | KP9 | 13.6 | 7.6 |
| 56 | 17-12-2008 | KP10 | 13.6 | 7.7 |
| 57 | 23-12-2008 | T1 | 12.2 | 7.5 |
| 58 | 23-12-2008 | T2 | 12.0 | 7.5 |
| 59 | 23-12-2008 | T3 | 12.2 | 7.4 |
| 60 | 23-12-2008 | T4 | 12.4 | 7.6 |

| | | | | |
|---|---|---|---|---|
| 61 | 23-12-2008 | T5 | 12.2 | 7.5 |
| 62 | 23-12-2008 | T6 | 12.0 | 7.5 |
| 63 | 23-12-2008 | T7 | 12.0 | 7.7 |
| 64 | 23-12-2008 | T8 | 11.8 | 7.4 |
| 65 | 23-12-2008 | T9 | 12.2 | 7.5 |
| 66 | 23-12-2008 | T10 | 12.0 | 7.5 |
| 67 | 03-01-2009 | G 1 | 13.4 | 6.9 |
| 68 | 03-01-2009 | G 2 | 13.8 | 7.0 |
| 69 | 03-01-2009 | G 3 | 13.6 | 7.0 |
| 70 | 03-01-2009 | G 4 | 13.8 | 7.0 |
| 71 | 03-01-2009 | G 5 | 13.8 | 6.9 |
| 72 | 03-01-2009 | G 6 | 13.6 | 7.0 |
| 73 | 03-01-2009 | G 7 | 13.8 | 7.0 |
| 74 | 03-01-2009 | G 8 | 13.5 | 7.0 |
| 75 | 03-01-2009 | G 9 | 13.4 | 7.1 |
| 76 | 03-01-2009 | G 10 | 13.6 | 7.0 |
| 77 | 10-02-2009 | K1 | 15.0 | 7.2 |
| 78 | 10-02-2009 | K2 | 15.0 | 7.2 |
| 79 | 10-02-2009 | K3 | 14.9 | 7.1 |
| 80 | 10-02-2009 | K4 | 14.9 | 7.2 |

| | | | | |
|---|---|---|---|---|
| 81 | 10-02-2009 | K5 | 15.1 | 7.3 |
| 82 | 10-02-2009 | K6 | 14.8 | 7.2 |
| 83 | 10-02-2009 | K7 | 14.8 | 7.2 |
| 84 | 10-02-2009 | K8 | 14.8 | 7.2 |
| 85 | 24-02-2009 | KAM1 | 18.8 | 7.2 |
| 86 | 24-02-2009 | KAM2 | 19.0 | 7.2 |
| 87 | 24-02-2009 | KAM3 | 19.2 | 7.2 |
| 88 | 24-02-2009 | KAM4 | 18.2 | 7.1 |
| 89 | 24-02-2009 | KAM5 | 18.9 | 7.2 |
| 90 | 24-02-2009 | KAM6 | 18.0 | 7.0 |
| 91 | 24-02-2009 | KAM7 | 18.6 | 7.2 |
| 92 | 24-02-2009 | KAM8 | 18.6 | 7.2 |
| 93 | 24-02-2009 | KAM9 | 19.1 | 7.2 |
| 94 | 24-02-2009 | KAM10 | 19.0 | 7.1 |
| 95 | 25-03-2009 | KLK1 | 22.4 | 7.6 |
| 96 | 25-03-2009 | KLK2 | 22.3 | 7.6 |
| 97 | 25-03-2009 | KLK3 | 22.3 | 7.5 |
| 98 | 25-03-2009 | KLK4 | 22.3 | 7.6 |
| 99 | 25-03-2009 | KLK5 | 22.2 | 7.6 |
| 100 | 25-03-2009 | KLK6 | 22.3 | 7.5 |

| | | | | |
|---|---|---|---|---|
| 101 | 25-03-2009 | KLK7 | 22.2 | 7.6 |
| 102 | 25-03-2009 | KLK8 | 22.2 | 7.6 |

# Appendix-IV

## Bacteriological analysis of tap water samples

| S.N. | Date | Code | Total coliform (cfu/100ml) | Thermotolerant coliform (cfu/100ml) |
|---|---|---|---|---|
| 1 | 27-08-2008 | KW1 | >300 | - |
| 2 | 27-08-2008 | KW2 | 80 | - |
| 3 | 27-08-2008 | KW3 | >300 | - |
| 4 | 27-08-2008 | KW4 | >300 | - |
| 5 | 27-08-2008 | KW5 | >300 | 2 |
| 6 | 27-08-2008 | KW6 | >300 | - |
| 7 | 27-08-2008 | KW7 | >300 | - |
| 8 | 27-08-2008 | KW8 | 46 | - |
| 9 | 27-08-2008 | KW9 | 200 | - |
| 10 | 27-08-2008 | KW10 | >300 | 2 |
| 11 | 09-09-2008 | B1 | >300 | - |
| 12 | 09-09-2008 | B2 | 80 | - |
| 13 | 09-09-2008 | B3 | >300 | 2 |
| 14 | 09-09-2008 | B4 | >300 | 26 |
| 15 | 09-09-2008 | B5 | >300 | - |
| 16 | 09-09-2008 | B6 | 97 | - |
| 17 | 09-09-2008 | B7 | >300 | - |

| | | | | |
|---|---|---|---|---|
| 18 | 09-09-2008 | B8 | >300 | 2 |
| 19 | 09-09-2008 | B9 | 208 | - |
| 20 | 09-09-2008 | B10 | >300 | - |
| 21 | 18-09-2008 | BB1 | >300 | 18 |
| 22 | 18-09-2008 | BB2 | >300 | 28 |
| 23 | 18-09-2008 | BB3 | >300 | - |
| 24 | 18-09-2008 | BB4 | >300 | 25 |
| 25 | 18-09-2008 | BB5 | >300 | 4 |
| 26 | 18-09-2008 | BB6 | >300 | - |
| 27 | 18-09-2008 | BB7 | >300 | - |
| 28 | 18-09-2008 | BB8 | >300 | 52 |
| 29 | 05-11-2008 | MD1 | >300 | - |
| 30 | 05-11-2008 | MD2 | >300 | - |
| 31 | 05-11-2008 | MD3 | >300 | - |
| 32 | 05-11-2008 | MD4 | >300 | 2 |
| 33 | 05-11-2008 | MD5 | >300 | - |
| 34 | 05-11-2008 | MD6 | >300 | - |
| 35 | 05-11-2008 | MD7 | >300 | - |
| 36 | 05-11-2008 | MD8 | >300 | - |
| 37 | 05-11-2008 | MD9 | >300 | 2 |

| | | | | |
|---|---|---|---|---|
| 38 | 05-11-2008 | MD10 | >300 | 18 |
| 39 | 24-11-2008 | HG1 | 186 | - |
| 40 | 24-11-2008 | HG2 | 155 | - |
| 41 | 24-11-2008 | HG3 | - | - |
| 42 | 24-11-2008 | HG4 | - | - |
| 43 | 24-11-2008 | HG5 | - | - |
| 44 | 24-11-2008 | HG6 | 123 | - |
| 45 | 24-11-2008 | HG7 | - | - |
| 46 | 24-11-2008 | HG8 | 118 | - |
| 47 | 17-12-2008 | KP1 | 30 | - |
| 48 | 17-12-2008 | KP2 | - | - |
| 49 | 17-12-2008 | KP3 | - | - |
| 50 | 17-12-2008 | KP4 | - | - |
| 51 | 17-12-2008 | KP5 | 64 | - |
| 52 | 17-12-2008 | KP6 | - | - |
| 53 | 17-12-2008 | KP7 | - | - |
| 54 | 17-12-2008 | KP8 | 80 | - |
| 55 | 17-12-2008 | KP9 | 59 | - |
| 56 | 17-12-2008 | KP10 | - | - |
| 57 | 23-12-2008 | T1 | 155 | - |
| 58 | 23-12-2008 | T2 | 138 | - |

| | | | | |
|---|---|---|---|---|
| 59 | 23-12-2008 | T3 | >300 | - |
| 60 | 23-12-2008 | T4 | >300 | - |
| 61 | 23-12-2008 | T5 | 269 | - |
| 62 | 23-12-2008 | T6 | 288 | - |
| 63 | 23-12-2008 | T7 | >300 | - |
| 64 | 23-12-2008 | T8 | >300 | - |
| 65 | 23-12-2008 | T9 | 97 | - |
| 66 | 23-12-2008 | T10 | >300 | - |
| 67 | 03-01-2009 | G 1 | >300 | - |
| 68 | 03-01-2009 | G 2 | >300 | - |
| 69 | 03-01-2009 | G 3 | >300 | - |
| 70 | 03-01-2009 | G 4 | 217 | - |
| 71 | 03-01-2009 | G 5 | >300 | - |
| 72 | 03-01-2009 | G 6 | >300 | - |
| 73 | 03-01-2009 | G 7 | >300 | - |
| 74 | 03-01-2009 | G 8 | >300 | - |
| 75 | 03-01-2009 | G 9 | >300 | - |
| 76 | 03-01-2009 | G 10 | >300 | - |
| 77 | 10-02-2009 | K1 | 153 | - |
| 78 | 10-02-2009 | K2 | 118 | - |

| | | | | |
|---|---|---|---|---|
| 79 | 10-02-2009 | K3 | 149 | - |
| 80 | 10-02-2009 | K4 | - | - |
| 81 | 10-02-2009 | K5 | - | - |
| 82 | 10-02-2009 | K6 | - | - |
| 83 | 10-02-2009 | K7 | 97 | - |
| 84 | 10-02-2009 | K8 | - | - |
| 85 | 24-02-2009 | KAM1 | >300 | - |
| 86 | 24-02-2009 | KAM2 | >300 | - |
| 87 | 24-02-2009 | KAM3 | >300 | 16 |
| 88 | 24-02-2009 | KAM4 | >300 | 4 |
| 89 | 24-02-2009 | KAM5 | >300 | - |
| 90 | 24-02-2009 | KAM6 | >300 | - |
| 91 | 24-02-2009 | KAM7 | >300 | - |
| 92 | 24-02-2009 | KAM8 | >300 | - |
| 93 | 24-02-2009 | KAM9 | >300 | 8 |
| 94 | 24-02-2009 | KAM10 | >300 | - |
| 95 | 25-03-2009 | KLK1 | >300 | 28 |
| 96 | 25-03-2009 | KLK1 | >300 | 12 |
| 97 | 25-03-2009 | KLK3 | 206 | - |
| 98 | 25-03-2009 | KLK4 | >300 | - |

| | | | | |
|---|---|---|---|---|
| 99 | 25-03-2009 | KLK5 | >300 | - |
| 100 | 25-03-2009 | KLK6 | >300 | - |
| 101 | 25-03-2009 | KLK7 | >300 | 4 |
| 102 | 25-03-2009 | KLK8 | >300 | 25 |

# APPENDIX - V

## Chart for identification of *E. coli* isolates

| Organism | Biochemical Tests | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | SIM | | | TSI | | |
| | MR | VP | Urease | Citrate | OF | $H_2S$ | Indole | Motility | Slant | Butt | Gas |
| *E. coli* | +ve | -ve | -ve | -ve | F | -ve | +ve | +ve | Acid | Acid | +ve |

+ve : Positive

-ve : Negative

## APPENDIX – VI

WHO guideline value for bacteriological quality of drinking water

| | Organism value | Unit | Guideline |
|---|---|---|---|
| A. | **Piped water supplied** | | |
| | **Treated water entering distribution system** | | |
| | Thermotolerant coliform | Number/100ml | 0 |
| | Total coliform | Number/100ml | 0 |
| | **Untreated water entering distribution system** | | |
| | Thermotolerant coliform | Number/100ml | 0 |
| | Total Coliform | Number/100ml | 3 |
| | **Water in the distribution system** | | |
| | Thermotolerant coliform | Number/100ml | 0 |
| | Total coliform | Number/100ml | 3 |
| **B.** | **Unpiped water supplies** | | |
| | Thermotolerant coliform | Number/100ml | 0 |
| | Total coliform | Number/100ml | 10 |

# APPENDIX-VII

**Zone Size Interpretative Chart of Antibiotic Susceptibility Testing**

| Antibiotics used | Symbol | Disc content (mcg) | Diameter of zone of inhibition in mm | | |
|---|---|---|---|---|---|
| | | | Resistant | Intermediate | Sensitive |
| Tetracycline | T | 30 | 14 | 15-18 | 19 |
| Co-trimoxazole | Co | 25 | 10 | 11-15 | 16 |
| Amikacin | Ak | 30 | 14 | 15-16 | 17 |
| Chloramphenicol | C | 30 | 12 | 13-17 | 18 |
| Amoxycillin | Am | 10 | 13 | 14-17 | 18 |
| Ceftriaxone | Ci | 30 | 13 | 14-20 | 21 |
| Ciprofloxacin | Cf | 5 | 15 | 16-20 | 21 |
| Nalidixic Acid | NA | 30 | 13 | 14-18 | 19 |
| Gentamicin | G | 30 | 12 | 13-14 | 15 |

(Source: Product Information Guide, Hi-Media Laboratories Pvt. Limited, Mumbai, India).

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