ANTIBIOTIC RESISTANCE AND PLASMID PROFILING OF *ESHERICHIA COLI* ISOLATES OF DRINKING WATER

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By

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ABSTRACT

E. coli has a central role in water microbiology due to its indicator value of faecal pollution. Antibiotic resistance and its dissemination in water is a serious public health issue. The objective of the study was to explore the occurrence and distribution of *E. coli* in drinking water, their susceptibility to antibiotics and plasmid profiling.

This study was conducted at Laboratory of Central Department of Microbiology, from January to August 2011. A total of 66 water samples from tap, well and spring sources were bacteriological parameters. Disc diffusion was followed for antibiogram and plasmid DNA of thermotolerent *E. coli* was extracted by mini alkaline lysis following gel electrophoresis.

Type of water sources were not significantly associated with the presence of coliform (P=0.155) and thermotolerant coliform (P=0.235) and the significant association in thermotolerant coliform and thermotolerant *E. coli* was found for all sources tap (P=0.029), well (P=0.028), spring (P=0.05) but total coliform and *E. coli* association was found for well (P=0.01). Average count of thermotolerant *E. coli* was found higher than *E. coli* in all sources. All *E. coli* were found sensitive towards Ofloxacin, Chromphenicol and Cotrimixazole. Resistance to Cefexime, Amikacin and Nalidixic acid, Amoxicillin, Tetracycline were 54.8%, 29% 35.5%, 80.6%, 93.5% and 57.6%, 36.4%, 39.4%, 94%, 100% was observed in *E. coli* and thermotolerant *E. coli* respectively. High MDR 25 (75.8%) of thermotolerant *E. coli* was observed than *E. coli* 22 (70.9%). In gel electrophoresis, single band of plasmid were observed in three MDR isolates and one non-MDR isolate and size varied from 2kb to >10kb. All NAR thermotolerant *E. coli* were found to harbor plasmid.

Drinking water of Kathmandu is contaminated with drug resistance *E. coli* and plasmid mediated resistance to Nalidixic acid has emerged indicating possible outbreak of drug resistance enteric bacteria.

Key- words- *Thermotolerant, Association, Nalidixic acid, Plasmid*

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ABBREVATIONS

ADB	:	Asian Development Bank
APHA	:	American Public Health Association
AR	:	Antibiotics Resistance
ATCC	:	American Type Culture Collection
BPB	:	Bromophenol Blue
CLSI	:	Clinical and Laboratory Standards Institute
CFU	:	Colony Forming Unit
DNA	:	Deoxyribonucleic Acid
EMB	:	Eosin Methylene Blue Agar
EDTA	:	Ethylenediaminetetraacetic Acid
ENPHO	:	Environment and Publichealth organization
EPA	:	Environmental Protection Agency
EtOH	:	Ethanolhydroxide
HPC	:	Heterotrophic Plate Count
Kb	:	Kilobase Pair
LB	:	Luria-Bertani
MDR	:	Multi Drug Resistance
MF	:	Membrane Filter
MHA	:	Mullar Hinton Agar
MLD	:	Million Liters per Day
MoH	:	Ministry Of Health
MS	:	Microsoft
MUG	:	4-Methylumbelliferone Glucuronide
NZMoH	:	Ministry of Health, New Zealand
NAR	:	Nalidixic acid Resistance
NWSC	:	Nepal Water Supply Corporation
R	:	Resistance
R^+	:	Resistance Factor
RTF	:	Resistance Transfer Factor
SDS	:	Sodium Dodecyl Sulphate
SPSS	:	Statistical Package for Social Science
STP	:	Sewage Treatment Plant

UNEP	:	United Nations Environment Programmes
UV	:	Ultraviolet
WHO	:	World Health Organization
WTP	:	Water Treatment Plants
WWWT	:	Waste Water Treatment Plant

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CHAPTER I

INTRODUCTION

1.1 Introduction

Drinking water is the most important single source of gastroenteric diseases, mainly due to the faecally contaminated raw water, failures in the water treatment process or recontamination of treated drinking water (WHO, 2003). With the exception of a few key chemicals (such as arsenic, lead and fluoride) the risks of illness and death from chemicals are low, mostly speculative and unproven (Sobsey, 2006). The most common and widespread health risk associated with drinking-water is microbial contamination, the consequences of which mean that its control must always be of paramount importance (WHO, 2010).

Safe drinking water has defined as water with microbial, chemical and physical characteristics that meet guidelines (WHO and national standards) on drinking water quality (WHO, 2003). Therefore "safe" refers to a water supply that poses no any significant risk to health over a lifetime of consumption, including different sensitivities that may occur between life stages (WHO, 2010). In contrast, pathogenic microbes continue to be a major cause of waterborne disease globally, and they cause documented illness and death in the United States and worldwide (Sobsey, 2006).

The majority of large-scale waterborne disease outbreaks in the past have been attributed to human contamination or inadequacies at water treatment plants. Waterborne outbreaks, upon contact with contaminated recreational water bodies, are attributed to human fecal contamination or sewage (Arnone and Walling, 2007).In developing countries, problems such as a high population density with an increased number of persons in limited space, an increase in solid waste generation, unhygienic surroundings and an increased demand for water supply and sanitation facilities are one of the main causes of suffering from waterborne diseases such as diarrhoea, typhoid, dysentery and cholera (Pokhrel and Viraraghavan, 2004).

In Nepal, the incidence of diarrhoea in children under five years was 222 per thousand population and case fatality rate were 0.2 per thousand population in 2003/04 total diarrhoeal visits were 787,094 and total diarrhoeal deaths were 194 (MoH., 2003/04). Diarrhoeal diseases are still recognized as a major problem for Nepalese children, being recorded as the second most prevalent diagnosis in out-patient services. High prevalence of the nationwide disease burden (Nepal), 72% is related to poor quality of drinking water, and around 75 children die each day from diarrhoea alone (Sherpa, 2003) and a yearly minimum death of 30,000 and morbidity of 3.3 episodes per child was estimated due to diarrhoea (Pokhrel and Viraraghavan, 2004). In Nepal, the causes of outbreak of diarrhoeal diseases were found to be associated with the poor condition or absence of a sanitary system (Rai et al., 1997; Matsumura et al., 1998) and pathogenic *E. coli* was found surprisingly higher with the diarrheal patients (Sherchand et al., 2007; Ono et al., 2001).

In water presence of *E. coli* indicate recent faecal contamination (WHO, 2010)) and *E. coli* as a commensal pathogen in the human gut and in animal populations, resistance in *E. coli* may be a sensitive indicator of distinct therapeutic and nontherapeutic, appropriate and inappropriate uses of antimicrobial drugs (Stelling et al., 2005).

The emergence of bacteria resistant to antimicrobials is common in areas where antimicrobials are used (Ibezim, 2005) and enteric bacteria in humans and husbandry animals treated with antibiotics may develop and proliferate resistance to these substances (Russell, 2002) where human and animal faeces are the direct sources of this resistance (Mensink and Montforts, 2007). The prevalence of antimicrobial resistance has increased during the recent decades (Threlfall et al., 2000) and the presence and persistence of AR bacteria particularly MDR bacteria is a serious threat to mankind (Petersen, 2006) as antibiotic resistance determinants can be transferred to bacteria of human clinical significance (Blake et al., 2003). The emergence of antimicrobial resistance to members of the Enterobacteriaceae family (Ashok, 2008) and the occurrence of antimicrobial resistant bacteria is increasing in aquatic environments (Schwatz et al.,2003; Onyango et al.,2009) which may be due to numerous types of anthropogenic activity, other nonhuman applications of antibiotics, and waste disposal, create major environmental reserves of resistance and, quite probably, of virulence genes and the organisms that harbor them (Davies and Davies, 2010).

Understanding the molecular epidemiology of resistance plasmids has been a major issue since scientists became aware of its role in the spread of antimicrobial drug resistance (Jan et al., 2009). Plasmids are a major mechanism for the spread of antibiotic resistant genes in bacterial populations (Smalla et al., 2000) and release of antibiotics and other antibacterials into the environment is going to enrich R-plasmids, integrons and MDR (Hawkey and Jones, 2009). In faecally polluted water, *E. coli* is generally accepted as the predominant vehicle for the dissemination of resistance genes and vectors due to its abundance in such environments and has been reported to transfer the antibiotic resistant genes to enteric pathogenic and normal flora bacteria (Ozgumus et al., 2007; Platt et al., 1986) and acquisition and transfer of antibiotic resistance and virulence factor genes by the bacteria via horizontal transfer of the resistance (R) plasmids, transposons and integrons are increasing problems in infectious diseases (Leverstein-van et al., 2001; Tenover, 2006) and R^+ factor in *E. coli* found in environment when ingested transfer their resistance gene via colonizing the gastrointestinal tract or pass their plasmids to the resident faecal flora and consequently to *Salmonella* and *Shigella* (Corliss et al., 1981).

Antibiotics or their residues in water can create an environment that is hostile to bacteria, susceptible to these antibiotics and expression of ARG is vital for survival (Mensink and Montforts, 2007).

The widespread occurrence of drug resistant *E. coli* and other pathogens in our environment has necessitated the need for regular monitoring of antibiotics susceptibility trends to provide the basis for developing rational prescription programs, making policy decisions and assessing the effectiveness of both (Idia et al., 2006)

The aims of the present study were to investigate the antibiotic susceptibility pattern of both *E. coli* and thermotolerant *E. coli* and antibiotic resistance in relation to their plasmids profile of thermotolerant *E. coli*. Such information will be of use to expand the present knowledge on drug resistance in environment and the factors that aggravate the drug-resistance problem and provide information on the appropriate choice of antimicrobial agents.

CHAPTER II

OBJECTIVES

2.1 General objective

To assess the prevalence of multidrug resistance *E. coli* and thermotolerant *E. coli* and plasmid profiling of *E. coli* isolated from water samples.

2.2 Specific objectives

I) To detect total coliform and thermotolerant coliform.

II) To detect and enumerate *E. coli* and thermotolerant *E. coli.*

III) To find relation of *E. coli*, thermotolerant *E. coli*, coliform, thermotolerant coliform with sources and within sources.

IV) To describe antibiotic susceptibility pattern for *E. coli* and thermotolerant *E. coli.*

V) To analyze plasmid profiling of *E. coli* isolates from tap water.

CHAPTER III
LITERATURE REVIEW

3.1 Water distribution system in Kathmandu valley

Piped water system was established in 1895 in Kathmandu and Bhaktapur followed by Lalitpur in 1904. The water distribution system had been installed and expanded at various times and subsequently expanded in 1960s. More comprehensive development and expansion at Kathmandu and Lalitpur took place during 70's and 80's. The water supply serves about 1.5-2.5 million people in 5 Municipalities in the valley (Kathmandu, Lalitpur, Bhaktapur, Kritipur and Thimi-Madhyapur) and in some rural areas close to its transmission mains. The water supply serves during the monsoon season from a number of sources and streams, where necessary water is subsidized from tube wells. The total water potentially available from the surface and groundwater sources during the dry season is about 100 MLD (100,000 m3/day) and the wet season is 150 MLD (150,000 m3 /day). However, the volume of water available for consumption is estimated to be only 62 MLD during the dry season and 93 MLD in the wet season. The 38% difference is due to estimated process and system losses due to wastage of filter backwashing and distribution leakages. The current estimated average demand of 280 MLD for the Kathmandu Valley but only 1/3 of the demand is being met from the public water system. At present, there are 30 surface sources being tapped for water supply in the valley. There is considerable seasonal fluctuation in water discharge; the majority of them reduce flow up by 30 to 40% with some up to 70% in the dry season. Almost all the sources have some potential to yield more in the wet season. The total wet season supply is 137.7 MLD which reduces in the dry season to 70.5 MLD. (ADB I, 2009)

Deep tube wells are the main means of extracting groundwater for use in the water supply system. Out of 73 existing deep tube-wells only 54 are in operation at present. Most of the tube wells electro-mechanical parts are in a bad condition with most flow meters missing or broken. The tube wells used to be operated only in the dry season in order to supplement reducing surface water sources, but, due to demand exceeding supply, they are now also used in the wet season. Total dry season rated production is 40.6 MLD with a reduced wet season production of 2.2 MLD. (ADB II, 2009)

At present, there are 21 water treatment plants (WTPs) in the system with a total treatment capacity of about 85 MLD treating surface water and groundwater due to high iron content. The largest is at Mahankal Chaur with a treatment capacity of 9,900,000m3/annum and the smallest is at Kuleshwor with a treatment capacity of 40,000 m3/annum. Most of the WTPs are in poor condition and none has operational flow meters or properly operating chlorination equipment. (ADB I, 2009; ADB II, 2009)

The features and existing approximate capacities of the major water treatment plants in the Kathmandu Valley Water Supply System were listed. (Table-1)

Table-1: Major water treatment plants in Kathmandu valley water supply system

S.no	Name of water treatment plant	Type of treatment facilities	Capacity (MLD)	Area served
1	Sundarijal Treatment Plant	Aeration, Sedimentation, Filtration and Disinfection	>12	Kathmandu
2	Mahankal Chaur Treatment Plant	Biological treatment, Aeration, Sedimentation, Filtration and Disinfection	>27	Kathmandu
3	Bansbari Treatment Plant	Biological treatment, Sedimentation, Filtration and Disinfection	>12	Kathmandu
4	Balaju Treatment Plant	Flocculation/ Sedimentation Filtration	>7	Kathmandu
5	Bode Treatment Plant	, Sedimentation, Filtration and Disinfection	>8	Bhaktapur and Thimi
6	Sundarighat Treatment Plant	Flocculation/ Sedimentation Filtration	4	Kirtipur and Laitpur
7	Sainbu Treatment Plant	Filtration	6	Lalitpur

Source: ADB II, 2009

At present, the total length of pipelines including transmission mains, pumping mains and distribution lines is about 12,500 kms with pipe diameter varying from 50mm to 800mm. The pipe materials used include Galvanized Iron (GI), Cast Iron, Steel Iron (SI), Ductile Iron (DI), High Density Polythene Pipe (HDPE) and Polyvinyl Chloride

(PVC). The majority type of pipe used is 50mm diameter GI. The system has approximately 1260 valves of different diameters. There are many problems in the distribution system. The problems include: ad hoc laying of pipes and valves, involvement of users' group and their intervention in the operation of valves, spaghetti pipelines connections, direct pumping from distribution from transmission mains and few have operating consumer meters. (ADB II, 2009)

3.2 Sources and access of drinking water in Kathmandu

Nearly all of the surface sources and ground water sources have been exploited. The growing imbalance between supply and demand has led to chronic shortages and competition that have resulted in pollution and environmental degradation. Apart from quantitative shortages, the quality of drinking water in the Kathmandu Valley is becoming a serious public health issue for the past few years. The quality of water for drinking has deteriorated because of untreated sewage into rivers and inefficient management of the piped water distribution system. (UNEP, 2001)

Not all households and people in the valley receive safe drinking water. The dependency of households for drinking water on a variety of sources can be seen from table below.

Table-2: Sources and access of drinking water

	Kathmandu		**Lalitpur**		**Bhaktapur**		**Kathmandu Valley**	
	HH	%	HH	%	HH	%	HH	%
Tap	197851	84.1	57237	83	30755	73.5	285843	82.6
Well	14714	6.3	6745	9.8	4843	11.6	26302	7.6
Tube Well	13478	5.7	825	1.2	2977	7.1	17280	5.0
Spout	6082	2.6	3099	4.5	2632	6.3	11813	3.4
River/Stream	195	0.1	113	0.2	29	0.1	337	0.1
Others	1616	0.7	477	0.7	277	0.7	2370	0.7
Not Stated	1381	0.6	425	0.6	339	0.8	2145	0.6
Total	235317	100	68921	100	41852	100	346090	100
Key: HH= household								

Source: NWSC, 2005

3.3 Drinking water as a vehicle of diseases

For pathogens transmitted by the faecal–oral route, drinking-water is only one vehicle of transmission and the greatest risk from microbes in water is associated with consumption of drinking- water that is contaminated with human and animal excreta, although other sources and routes of exposure may also be significant (WHO, 2010).

Enteropathogenic microbes are usually adapted to multiplying in the intestines of humans and animals and surface water is only a niche in their circulation through the environment and human or animal populations (Medema et al., 2003).

Most bacterial pathogens potentially transmitted by water infect the gastrointestinal tract and are excreted in the faeces of infected humans and other animals. However, there are also some waterborne bacterial pathogens, such as *Legionella*, *Burkholderia pseudomallei* and atypical mycobacteria, that can grow in water and soil. The routes of transmission of these bacteria include inhalation and contact (bathing), with infections occurring in the respiratory tract, in skin lesions or in the brain (WHO, 2010).

The most important bacterial diseases transmitted through water are listed in Table 3.

Table 3: The main bacterial disease transmitted through water

Disease	Causal bacterial agent
Cholera	*Vibrio cholerae*, serovarieties O1 and O139
Gastroenteritis caused by vibrios	Mainly *Vibrio parahaemolyticus*
Typhoid fever and other serious salmonellosis	*Salmonella enterica* subsp. *enterica* serovar Paratyphi *Salmonella enterica* subsp. *enterica* serovar Typhi *Salmonella enterica* subsp. *enterica* serovar Typhimurium
Bacillary dysentery or shigellosis	*Shigella dysenteriae* *Shigella flexneri* *Shigella boydii* *Shigella sonnei*
Acute diarrheas and gastroenteritis	*Escherichia coli*, particularly serotypes such as O148, O157 and O124

Source: Cabral, 2010

3.4 Physical aspects

Application of an adequate concentration of disinfectant is an essential element for most treatment systems to achieve the necessary level of microbial risk reduction. Taking account of the level of microbial inactivation required for the more resistant microbial pathogens through the application of the Ct concept (product of disinfectant

concentration and contact time) for a particular pH and temperature ensures that other, more sensitive microbes are also effectively controlled (WHO, 2010).

3.4.1 pH

Although health-based guideline value has been proposed for pH and no direct impact on consumers it is one of the most important operational water quality parameters. For effective disinfection with chlorine, the pH should preferably be less than 8; however, lower-pH water (approximately pH 7 or less) is more likely to be corrosive. The pH of the water entering the distribution system must be controlled to minimize the corrosion of water mains and pipes in household water systems. The optimum pH required will vary in different supplies according to the composition of the water and the nature of the construction materials used in the distribution system, but it is usually in the range 6.5–8.5. (WHO, 2010) but survival of bacteria in soil (and concomitantly in groundwater) is enhanced by low temperatures with neutral or alkaline soil pH (Medema et al., 2003).

3.4.2 Temperature

Water in the temperature range of 7^0C to 11^0C has pleasant taste and is refreshing (WHO, 2006). Cool water is generally more palatable than warm water, and temperature will impact on the acceptability of a number of other inorganic constituents and chemical contaminants that may affect taste. High water temperature enhances the growth of microorganisms and may increase taste, odour, colour and corrosion problems (WHO, 2010). 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 (Trivedy and Goel, 1986) High temperatures of distributed water in warm climate areas and difficulty in maintaining disinfectant residuals during transport over long distances may lead to microbial aftergrowth, depending on nutrient availability (WHO, 2010) however low temperatures have been observed in the growth of fecal coliform and *E. coli* (Medema et al., 2003; Smith et al., 1994; Bogosian et al., 1996, Sampson et al.,2006).

3.5 Indicator microbes and water quality

Microbiological water analysis is mainly based on the concept of fecal indicator bacteria (Cabral, 2010). Since the analysis of various enteropathogens can be laborious and require special analytical techniques, fecal indicator organisms are routinely used for the assessment of presence absence of pathogen and water quality testing (Rompre et al., 2002; Cabral and Marques, 2006, Maier et al., 2000, Grabow, 1996; Pletschke et

al., 2006). In the late 1800s the concept of total heterotrophic plate count (HPC) had already been used to assess drinking water quality and > 100 bacteria in a 1-ml sample was noted as unacceptable (Medema et al., 2003a)

The HPC is no longer used as a faecal indicator of drinking water quality (WHO, 2004) and are considered to be harmless but some studies have proposed that they may constitute a health risk, especially for immunocompromised individuals (Pavlov et al., 2004).

An ideal indicator organism for faecal contamination must be present whenever enteric pathogens are present and should have a longer survival time than most enteric pathogens. It should not proliferate in natural water, and must be easily, reliably and cheaply detectable. The density of the indicator organism should have a direct relationship with the degree of faecal pollution and must be a member of the intestinal microflora of warm-blooded animals (Grabow, 1996; Maier et al., 2000; Pletschke et al., 2006). In addition, must be present in greater numbers than the pathogenic microorganisms, respond to natural environmental conditions and water treatment processes in a manner similar to that of the pathogens (Medema et al., 2003a) and should not be pathogenic microorganism (WHO, 2010; Medema et al., 2003).

3.5.1 Total coliform

Total coliform bacteria include a wide range of aerobic and facultatively anaerobic, Gram-negative, non-spore-forming bacilli capable of growing in the presence of relatively high concentrations of bile salts with the fermentation of lactose and production of acid or aldehyde within 24 hours at 35–37 °C. As part of lactose fermentation, total coliforms produce the enzyme β-galactosidase.Traditionally, coliform bacteria were regarded as belonging to the genera *Escherichia, Citrobacter, Klebsiella* and *Enterobacter*, but the group is more heterogeneous and includes a wider range of genera, such as *Serratia* and *Hafnia*. The total coliform group includes both faecal and environmental species (WHO, 2010).

The definition of total coliforms belong within the family Enterobacteriaceae have changes on the basis of fermentation of lactose and changes from fermentation lactose with gas and acid , to formation of acid only from lactose and recently extended to a genotypic definition based on the recognition that in order to ferment lactose, organisms must possess β-galactosidase activity. Using this approach total

coliforms are defined as members of a genus or species within the family Enterobacteriaceae possessing β –galactosidase enzyme (APHA et al., 1998; Rompre et al., 2002; Stevens et al., 2003).

Table 4: Coliform members by evolving Definition

Acid and Gas from Lactose	Acid from Lactose	Enzyme-based β-Galactosidase
Escherichia	*Escherichia*	*Escherichia*
Klebsiella	***Klebsiella***	***Klebsiella***
Enterobacter	***Enterobacter***	***Enterobacter***
Citrobacter	***Citrobacter***	***Citrobacter***
	Yersinia	***Yersinia***
	Serratia	***Serratia***
	Hafnia	***Hafnia***
	Pantoea	***Pantoea***
	Kluyvera	***Kluyvera***
		Cedecea
		Ewingella
		Moellerella
		Leclercia
		Rahnella
		Yokenella

bold type = coliforms which can be present in the environment as well as in human faeces.

bold and underline = coliforms which are considered to be primarily environmental

Source: Stevens et al., 2003.

Leclerc et al., (2001) describes *Enterobacteriaceae* as three groups of bacteria with very different roles in the environment. Group I harbored only *E. coli*. Since this species usually do not survive for long periods outside this environment, it was considered a good and reliable indicator of fecal pollution (both animal and human). Group II, the —ubiquitar group, encompassed several species of *Klebsiella* (*K. pneumoniae* and *K. oxytoca*), *Enterobacter* (*Enterobacter cloacae* subsp. *cloacae*, *E. aerogenes*) and *Citrobacter* (*C. amalonaticus*, *C. koseri* and *C. freundii*). These bacteria live in the animal and human gut, but also in the environment, and are easily isolated from the soil, polluted water and plants. Their presence in polluted waters does not necessarily indicate fecal pollution. Finally Group III was composed of *Raoultella planticola*, *R. terrigena, Enterobacter amnigenus* and *Kluyvera intermedia*

(*Enterobacter intermedius*), *Serratia fonticola*, and the genera *Budvicia*, *Buttiauxella*, *Leclercia*, *Rahnella*, *Yersinia*, and most species of *Erwinia* and *Pantoea*. These bacteria live in fresh waters, plants and small animals. They grow at 4 °C, but not at 41 °C. They are not indicators of fecal pollution, although can be detected in the total coliform test.

The detection of β-D-galactosidase activity (at 37°C) is usually a good marker for total coliforms in environmental waters, since most of these bacteria display this enzymatic activity (WHO, 2010; George et al, 2001; George et al., 2000; Rompré et al., 2002; VanPoucke and Nelis, 2000) but most strains of *Proteus*, *Salmonella* and *Edwardsiella* strains do not display β-galactosidase (Kämpfer et al., 1991; Tryland et al., 1998).

3.5.2 Limitations to using total coliforms as indicators

Coliforms have been found to grow in drinking water distribution systems; be normal inhabitants of soil, water and plants; and not always be present during waterborne disease outbreaks. (Stevens et al., 2003)

Because of the biofilms established in the piping and other surfaces where optimum physico - chemical permit growth of total coliform (Stevens et al., 2003; Gauthier et al., 2000) and give no indication on faecal coliform even with the presence of *E. coli* (Gauthier et al., 2000).

The existence heterogeneous group that fit the definitions of coliform bacteria limits the applicability of this group as an indicator of faecal pollution (Laclrec et al., 2001; Payment et al., 2003).

Total coliform counts are not necessarily a measure of fecal pollution and have no relation with fecal contamination (NZMoH., 2000; WHO, 2010; Grabow 1996; Medema et al., 2003; Payment et al., 2003).

3.5.3 Thermotolerant colioforms

Thermotolerant coliforms are now the preferred designation for the group of bacteria previously referred to as faecal coliforms (WHO, 1996). The term 'faecal coliforms', although frequently employed, is not correct: the correct terminology for these organisms is 'thermotolerant coliforms' (Payment et al., 2003). Thermotolerant coliforms are defined as the group of total coliforms that are able to ferment lactose at

44-45°C (Payment et al., 2003; WHO, 2010). This group includes members of the genera *Escherichia*, *Klebsiella*, *Enterobacter*, and *Citrobacter* (Health Canada, 2006). Thermotolerant coliform (faecal coliform) are considered more specific indicator to faecal pollution in drinking water, but do not distinguished from animal of human contamination (Maier et al., 2000) however, thermotolerant coliform species other than *E. coli* can include environmental organisms and originate from organically enriched water such as industrial effluents or from decaying plant materials and soils (Payment et al., 2003).

Thermotolerant coliform group in subtropical or tropical waters or those enriched with organic wastes does not necessarily suggest faecal contamination by humans. However, their presence in treated waters should not be ignored, as the basic assumptions that pathogens may be present and that treatment has been inadequate still hold good (Payment et al., 2003). And the presence of thermotolerant coliform bacteria is thought to correlate with the presence of enteric pathogens in the environment (Bulson et al., 1984).

3.5.4 *E. coli* as an indicator organism

Escherichia coli is a taxonomically well defined member of the family Enterobacteriaceae, and is characterised by possession of the enzymes β-galactosidase and β-glucuronidase. It grows at 44-45ºC on complex media, ferments lactose and mannitol with the production of acid and gas, and produces indole from tryptophan. However, some strains can grow at 37ºC but not at 44-45ºC, and some do not produce gas. *E. coli* does not produce oxidase or hydrolyse urea (Payment et al., 2003). *E. coli* is the only coliform that is an exclusive inhabitant of the gastrointestinal tract (Edberg et al., 2000), in fresh faeces it can be present at concentrations of 10^9 CFU/g (Payment et al., 2003) and in human faeces, 96.8% and in animal faeces 94% of *E. coli* were present among coliforms (Stevens et al., 2003).

Water temperatures and nutrient concentrations are not generally elevated enough within the distribution system to support the growth of *E. coli* (or enteric pathogenic bacteria) in biofilms. Thus, the presence of *E. coli* should be considered as evidence of recent faecal contamination (WHO, 2010) however it has been detected that low temperatures favours the growth of *E. coli* (Medema et al., 2003; Brettar and Hofle 1992; Smith et al. 1994; Bogosian et al. 1996, Sampson et al, 2006). And *E. coli* is not long lived organism like many pathogen, makes it ideal for identifying recent fecal

contamination (Anderson et al., 2005; Leclerc et al.2001*;* Medema et al, 2003) and the possible presence of enteric pathogens (Geissler et al., 2000; US EPA, 2002).

E. coli is still considered to be superior as an indicator of faecal contamination and hygienic quality of drinking water (Dufour, 1977, Leclerc et al., 2001; Edberg et al., 2000, WHO, 2010) and easy to distinguish from other member of faecal coliform group with absence of urease and presence of β- glucuroinidase (Maier et al., 2000).

In safety evaluation of drinking water, thermotolerant *E. coli* which grows at elevated temperature of 45°C is used as indicator of fecal contamination of water sources (APHA, 1992). This procedure utilizing incubation at 45°C was found more sensitive than incubation at 37°C (Bolton et al., 1996; Tewari et al., 2003).The detection of β-D-glucuronidase activity (at 44.5 °C) is, generally, a good marker for fecal coliforms in environmental polluted waters and very specific for *E. coli* (Ramamurthy et al., 2003; Cabral and Marcus , 2006; Manafi et al., 1991; Rompré et al, 2002; George et al., 2001; George et al, 2000; Farnleitner et al., 2001; Eccles et al., 2004) and exceptionally found in some *Salmonella* and *Shigella* strains (Edberg et al., 1986; Manafi et al., 1991; Tryland et al., 1998) *Aeromonas*, *Citrobacter*, *Enterobacter*, non-*coli Escherichia*, *Hafnia*, *Klebsiella*, *Proteus*, *Serratia*, *Vibrio*, *Yersinia*, and most *Salmonella* strains do not display β-glucuronidase activity (Tryland et al., 1998; Farnleitner., 2001; Edberg et al., 1986). β-D-glucuronidase activity in fecal bacteria other than *E. coli* (*Bacteroides*, bifidobacteria, clostridia, enterococci and *Lactobacillus*) is very limited (Saarela et al., 2002).

Leclerc et al., (2001) concluded that: (1) in the enterobacteria, *E. coli* is the only true and reliable indicator of fecal pollution in environmental waters; (2) the traditional total coliform test should be abandoned, since it can detect bacteria that have no connection with fecal pollution; (3) the detection of fecal coliforms must be carried out at 44.5 °C, and positive results confirmed by identification to species levels in order to exclude false positives such as *K. pneumoniae*.

NZMoH, (2000) revised it guide line of drinking water on detection of *E. coli* for fecal pollution and not rely on fecal coliform and total coliform. As both total coliforms and faecal coliforms can be found in natural waters and their presence in drinking water does not necessarily indicate a health risk (Stevens M et al., 2003; NZMoH, 2000).

3.6 Water-environment and persistence

In surface water aquatic environments, *E. coli* may retain growth potential and metabolic activity (Tanaka et al., 2000), however for what length of time is unknown. In one study, *E. coli* O157 in river water fell more than 10^6 organisms per gram to undetectable levels within 27 days (Maule, 2000).

Survival of bacteria in groundwater is influenced by several factors, namely the survival in soil, since in order to reach the groundwater bacteria have to percolate through the soil. Generally, survival in soil (and concomitantly in groundwater) is enhanced by low temperatures, high soil humidity, neutral or alkaline soil pH and the presence of organic carbon (Medema et al., 2003).

General aquifer and groundwater properties that influence microbial transport rates include flow velocity, grain/pore size of the aquifer material, amount of solid organic carbon content, pH, and temperature (Robertson and Edberg, 1997).

The disappearance rates in groundwater are lower than in surface water. *E. coli* disappearance rate (per day) in ground water *E. coli* is 0.063 - 0.36 and in surface water is 0.23 - 0.46 (Medema et al., 2003). , In non-sterile conditions (closer to true environmental conditions), the elimination rate of all bacteria was considerably faster. Total coliforms survived the longest and *E. coli* the shortest (Baudisova, 1997).

E. coli can survive in drinking water for four to twelve weeks depending on environmental conditions (Edberg et al., 2000).

E. coli O157:H7 survive better in municipal water versus surface water and may enter a viable but non-culturable state in both municipal and environmental water (Wang et al., 1998).

3.7 Significance of *E. coli* in water

As an indicator: *E. coli* is a member of the total coliform group of bacteria and is the only member that is found exclusively in the faeces of humans and other animals. Its presence in water indicates not only recent faecal contamination of the water but also the possible presence of pathogen. The detection of *E. coli* should lead to the immediate issue of boil water advisory and to corrective actions being taken. However, because *E. coli* is not as resistant to disinfections as intestinal viruses and protozoa, its absence does not necessarily indicate that intestinal viruses and protozoa are also absent (Health Canada, 2006).

The presence of *E. coli* (or, alternatively, thermotolerant coliforms) provides evidence of recent faecal contamination, and detection should lead to consideration of further action, which could include further sampling and investigation of potential sources such as inadequate treatment or breaches in distribution system integrity (WHO, 2010).

As a Pathogen:

Pathogenic *E. coli* has been spread through the fecal contamination and causing disease has been well documented (Maedema et al., 2003; WHO, 2010) and the higher proportion of pathogenic *E. coli* found manly in livestock (Medema et al., 2003; WHO, 2010). Waterborne transmission of pathogenic *E. coli* has been well documented for recreational waters and contaminated drinking-water and conventional testing for *E. coli* (or, alternatively, thermotolerant coliform bacteria) provides an appropriate indicator for the enteropathogenic serotypes in drinking-water (WHO, 2010).

3.8 Antibiotic Resistance

Antibiotics are a compound or substance that either kills or inhibits the growth of a microorganism, such as bacteria, fungi and protozoa. Antibiotics have three major sources of origin: (i) naturally isolated; (ii) purely chemically synthesized; or (iii) semi-synthetically derived. Antibiotics are also defined according to their mechanism for targeting and identifying microorganisms –broad-spectrum antibiotics are active against a wide range of microorganisms; narrow-spectrum antibiotics target a specific group of microorganisms by interfering with the metabolic process specific to those particular organisms (Mossialos et al., 2010).

It has been observed that antibiotic susceptibility of bacterial isolates is not constant but dynamic and varies with time and environment (Hassan, 1995) and changes in the incidence and levels of antibiotic resistance in natural population are not confined to particular segments of the bacterial population and reflect responses to the increased exposure of bacteria to antimicrobial compounds over the past several decades (Hound and Ochman, 2000).

Multiple drug resistance is defined as resistance to ≥3 antibacterial drug classes (Tenover, 2006). Multidrug resistance (MDR) can occur through the acquisition of extrachromosomal DNA, such as plasmids or transposons, via chromosomal mutations in genes coding for proteins targeted by the drug, or via altered expression

of intrinsic mechanisms, such as efflux pumps that expel multiple classes of antibiotics out of the cell (Alekshun et al., 2007). Although increased intrinsic resistance usually produces moderate resistance levels, it can generate resistance to a wide array of antibiotics and other toxic chemicals (Alekshun et al., 1997; Dzink-Fox and Oethinger, 2005). Xenobiotics, vietenery antibiotics, clinical antibiotics serves as the major sources for creating selective pressure and water is a profound vehicle for spreading for MDR genes in an interlocked environmental system for spread and horizontal gene transfer of MDR strain of *E. coli* (Hawkey and Jones, 2009).

3.9 Antibiotic resistance dissimilation in aquatic environment

As antibiotic resistance is commonly found in aquatic environments (Goni-Urriza et al., 2000, Biyela et al., 2004, Schwartz et al., 2006, Mensink and Montforts, 2007) and anthropogenic and autochthonous source leads to the antibiotic resistance in the environment and its dissemination (Mensink and Montforts, 2007). Hospital waste (Obst et al., 2006, Schwartz et al., 2006, Mensink and Montforts, 2007, Aminov et al., 2001) , Sewage from households, waste from buildings, manure of industrial farms with husbandry animals, water of facilities in the feed and food industries (eg., Slaughter houses), industrial effluents and application of antibiotics for the crop production are the major anthropogenic sources (Mensink and Montforts, 2007) have been found source for the antibiotic resistance dissemination in water. Sewage Treatment Plants and rivers play a major role in the spread of antibiotic resistance into the environment (Goni-Urriza et al., 2000, Biyela et al., 2004, Schwartz et al., 2006, Mensink and Montforts, 2007).

Water as hostile environment with anthropogenic antibiotic contamination, the expression of an ARG is then vital for the survival of bacteria (Mensink and Montforts, 2007).Tetracycline-encoding resistance genes have disseminated between different Aeromonas species and *E. coli* and between human and aquaculture environments in distinct geographical regions (Mensink and Montforts, 2007 ; Huys et al., 2000). Quaternary ammonium compounds, organic solvents and detergents are possible stressors to select for mutant bacteria with higher expressions of multiple antibiotic resistances (Alonso et al., 2001). In nature, *E. coli* as being a mutator (LeClerc et al., 1996) is advantageous to the organism when adapting to environmental changes or stressful situations, exposure to antibiotics etc. show 100–1000 fold increase mutation, for any given marker such as Rifampicin resistance (Jayaraman, 2009). The multiple antibiotic resistance (mar) locus at 34 min on the

E. coli chromosome controls the intrinsic susceptibility of *E. coli* strains to many structurally unrelated antibiotics, including Tetracycline, Chloramphenicol, β-lactams, and Quinolones (Cohen et al., 1993; Goldman et al., 1996) and Floroquinolones (Goldman et al., 1996).

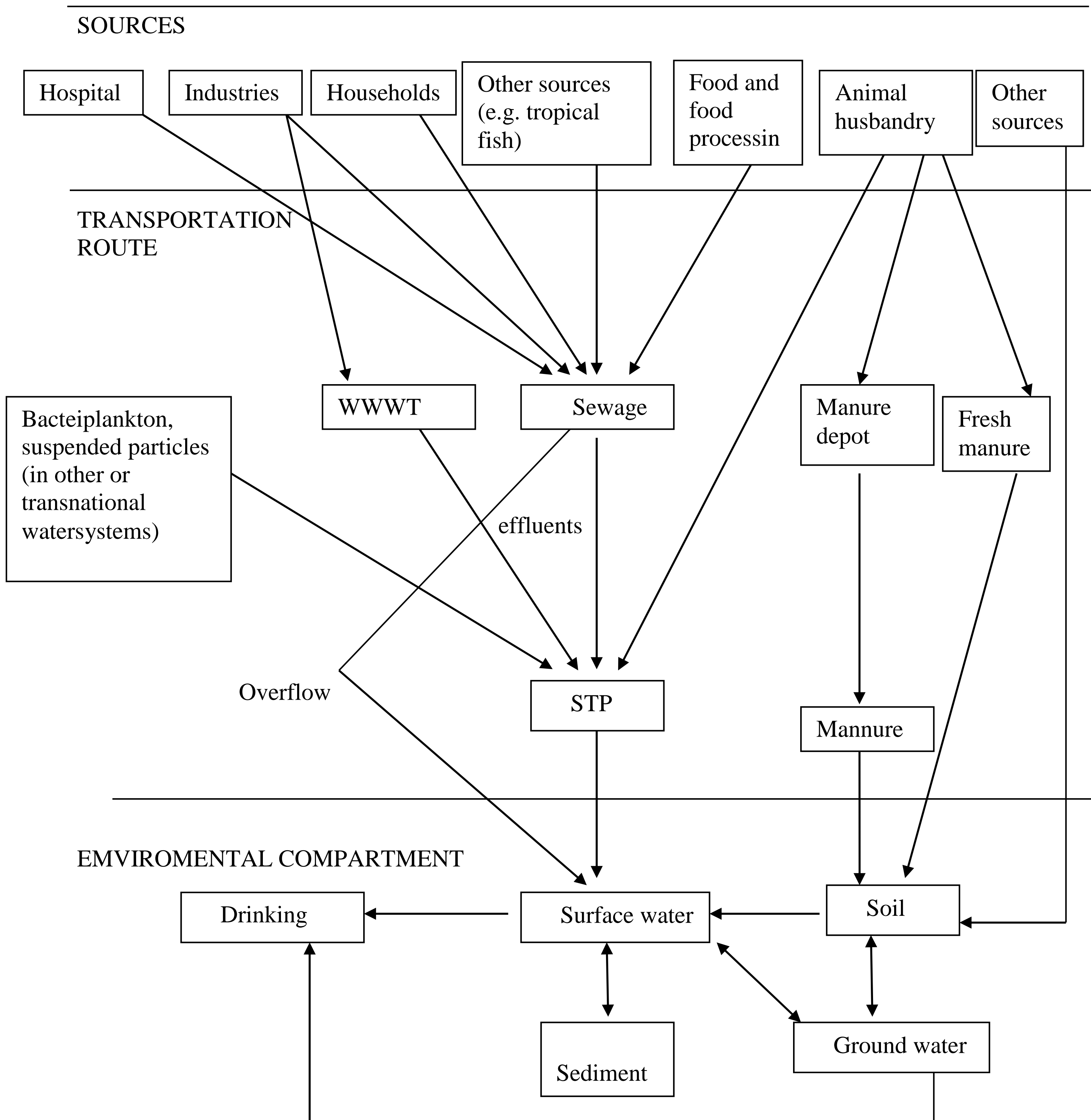


Figure-1: Transport routes for antibiotic resistance in drinking water via water pollution

Source: Mensink and Montforts, 2007

3.10 Transferable antibiotic resistance

Antibiotic resistance gene can be transmitted by horizontal gene flow achieved via conjugation, transformation or transduction of mobile DNA fragments (plasmids, transposons, and integrons) (Tenover, 2006; McManus, 1997; Davison, 1999; Mensink and Montforts,2007) and resistance determinants carried on the chromosome are transmitted vertically by clonal dissemination (Kyle et al., 2004; Tenover, 2006) in aquatic environment (Mensink and Montforts, 2007).

3.10.1 Transposon

Transposones are small, mobile DNA elements capable of mediating transfer of DNA by removing and inserting themselves into host chromosomal and plasmid DNA(Kyle et al.,2004), migrating between unrelated plasmids and/or the bacterial chromosomes independently of the normal bacterial recombination process (Greenhood, 2007). Many resistance genes are organized on transposons, which may have a broader host range than their parent plasmids (Normark, 2002).

3.10.2 Intergron

Intergrons are genetic class of element, form an esstential building block of transposons and allow the rapid formation and expression of new combination of antibiotic resistance genes in response to selection pressure (Greenhood, 2007). In aquatic environments class 1 and class 2 integrons have been found for the dissemination of antibiotic resistance gene in *E. coli* (Roe et al., 2003; Ozgumus et al., 2007).

3.10.3 Plasmid

Bacteria carry extrachromosomal, self-replicating genetic elements called plasmids. A plasmid is defined as a double-stranded, circular DNA molecule capable of autonomous replication. By definition, plasmids do not carry genes essential for the growth of host cells under nonstressed conditions (Thomas et al., 2005). Plasmids have systems which guarantee their autonomous replication but also have mechanisms controlling their copy number and ensuring stable inheritance during cell division and efficiently promote plasmid maintenance in the bacterial population, regardless of other selective pressure, and do not provide any apparent benefit to the bacterium hosting the plasmid (Carattoli, 2009). Many plasmids encode addiction systems generally based on toxin-antitoxin factors, which are able to kill daughter cells that do not inherit the plasmid during cell division (Hayes, 2003).

However, most of the plasmids confer positively selectable phenotypes with the presence of antimicrobial resistance genes (Carattoli, 2009). Plasmids are autonomously replicated DNA which are extra- chromosomally located in the micro-organisims. The plasmid mediated drug resistance is caused due to the presence of drug resistance gene(s) harboring on the plasmid DNA. These gene(s) confer the drug resistance phenomenon in the host organism (Meyer et al., 1976).

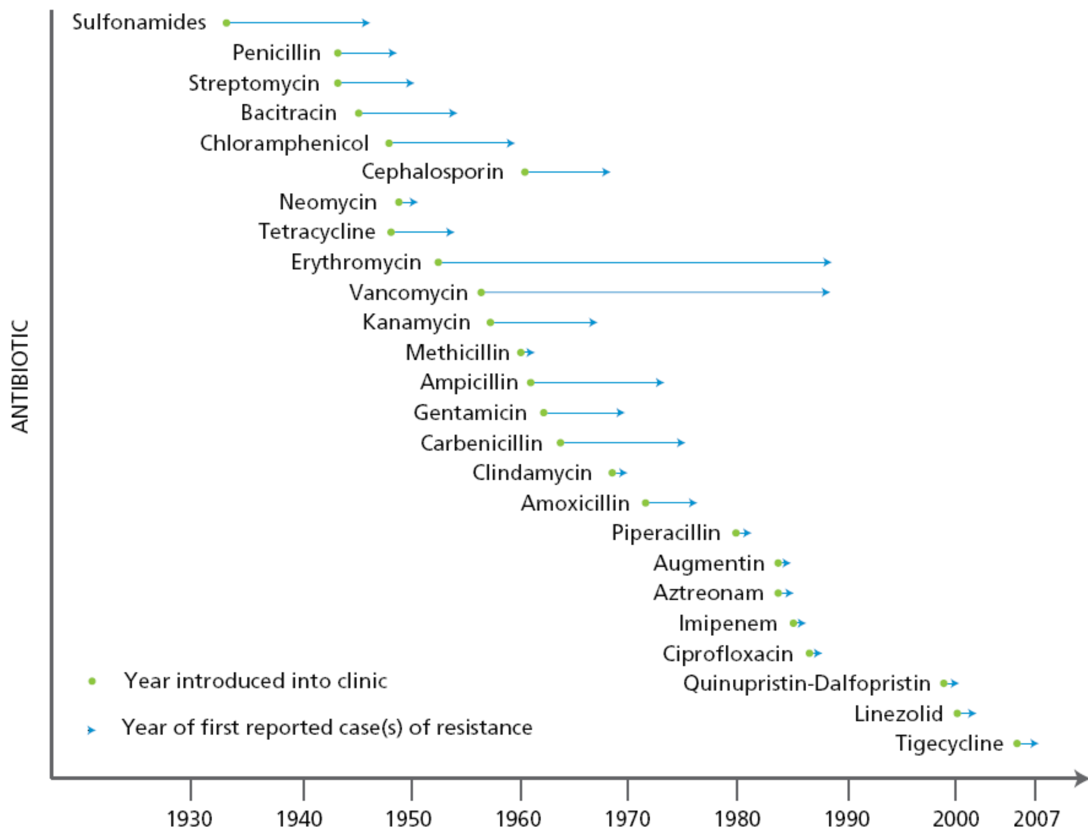
Plasmids carrying drug resistance phenotype are known as R-factor which is responsible for the spread of multiple drug resistance among bacteria. R-factor consists of two components i.e. resistance transfer factor (RTF) and resistance determinant 'r'. The complete plasmid (RTF+ r) is called R-factor (Patwary, 1994).

Resistance determinants on plasmids can also be transferred vertically, although plasmids may be lost from the bacterial population if they no longer confer a particular selective advantage. Plasmids also are capable of horizontal transfer by conjugation, although the efficiency of plasmid transfer both within and between species can vary tremendously (Kyle, 2004). Plasmid transfer between gram-positive and gram negative bacteria, once thought to be an unlikely event, can occur both in the laboratory and in the gastrointestinal tract of gnotobiotic mice, suggesting that such transfer events between even distantly related organisms may be important in nature (Courvalin, 1994).

R plasmids and other genetic elements conferring antibiotic resistance can be efficiently maintained and disseminated within this species by conjugation, transformation, and transduction (Boyd et al., 1997; Ochman, 2000; Hound and Ochman, 2000)

Multiple drug resistance traits may be transferred by conjugation from resistant to sensitive bacterium by means of plasmid, resulting in the development of new resistant species or strains (Buxton and Fraser, 1977). Not only are these plasmids rapidly dispersed within a bacterial species, but are very much responsible for the transposition of genes controlling resistance to antibiotics (and other drugs) from one molecule to another (Gardner et al., 1991). Considering that *E. coli* exists in large numbers in the intestinal flora, it strongly indicates that there is tremendous potential for plasmid dissemination in nature (Freeman, 1985). The importance of plasmids carrying multiple drug resistance (MDR) markers in *Shigella* spp. and *Escherichia coli* was first described in the seminal work of Watanabe in Japan over 40 years ago. Plasmids are capable of self transfer (conjugation) between strains and species and

have a mosaic structure that has arisen by recombination and transposition, which is responsible for the capture of different resistance genes, giving rise to the MDR phenotype (Leplae et al., 2006). R^+ *E. coli* environmental isolates to donate their plasmids to the resident *E. coli* strains via consumption of drinking water and of the resultant R^+ transconjugants to pass the R-plasmid to Salmonellae or Shigellae has been well documented (Corliss et al.,1981).



Note: some of the dates are estimates only **Source**: Mossialos, 2010

Figure-2: Time line of rapid rate of resistance

3.11 Antibiotic resistance and plasmid

Kalanter et al., (2011) studied the presence of plasmids of molecular sizes ranging from 1.4kb to 4.5kb among the acute diarrhea causing *E. coli* isolates showing resistance to Ampicillin, Chloramphenicol and Tetracycline and stated that these resistances are plasmid mediated.

From 54 *E. coli* isolates 24 different plasmid bands occurring in various combinations from the different antibiotic resistance phenotype Ampicillin and Streptomycin Sulphamethoxazole Trimethoprim resistant and sensitive to Ciprofloxacin while water

Tetracyclin Chloromphenicol and in broiler, layer, calves and cattle isolates *E. coli* to The size of these bands ranged from 0.5 to 40 kbp and no relation with antibiotic reseistance was detected (Alam et al., 2010).

The results from Jan et al., (2009) revealed that out of 76 *E. coli* isolates, 40 (52.6%) were found to possess plasmids. Some isolates possess single sized plasmid while other had multiple plasmids with different size ranged from different molecular size ranging from 2-3 kb to 6.5 kb and maximum 26 kb.

Idia et al., (2006) reported that over ninety percent of the strains were sensitive to Nitrofuratoin, 57% to Nalidxic acid, 51.2% percent to Gentamicin, 77.9% to Ofloxacin, 48.8% percent to Cotrimoxazole whereas 88.4% were resistant to Amoxicillins ,>90 % to Tetracycline and out of the 86 *E. coli* isolates, 54 (62.7%) were found to possess plasmids, which ranged in sizes from 2.322 kb to 23. 130 kb and some isolates possessed single sized plasmids while others had multiple plasmids and large sizes in the range of 6.557 – 23.130 kb are found in MDR cases.

Antibiotic susceptibility patterns from the healthy animals showed the isolates to be highly susceptible to the various antibiotics screened with a few showing multiple antibiotic resistance and the plasmid profiles revealed that 8/17 (47%) of the animal isolates harboured detectable plasmids ranging in size from 0.564 kb to >23 kb (Smith et al., 2003) .

Uma et al., 2009 reported about 90% of *E. coli* strains isolates were resistant to Ampicillin, Imipenem and Cotrimoxazole and were sensitive to Amikacin from from the pediatric diarrhea. The resistance to antibiotics shows 29 different antibiotic resistance patterns. About 67 (64%) strains of *E. coli* isolates harbored plasmids. ranged from 1.0 to 25 kb and A-Imipenem (IP)-Cotrimoxazole (Co) resistance among *E. coli* isolates, obtained from patients below five years of age with diarrhea, was encoded by 4.8 kb plasmid, based upon the fact that same plasmid was found in the transconjugants, conferring similar antibiotic resistance pattern..

Enabulele, 2006 observed plasmids ranged from ≤2.9kbp to ≤5.5 kbp in quinolone resistance bacterial isolates and concluded that movement of genetic materials including *qnr* resistant genes between bacteria *Pseudomonas aeruginosa* ,*E. coli* , *Klebsiella pneumoniae* , *Salmonella* Typhi , *Shigella dysenteriae* ,*Proteus mirabilis* and *Serratia marcescens* species occur via plasmids.

Antimicrobial resistance to Tetracycline, Kanamycin and Nalidxic acid was noted among avian *E. coli* isolates and showed to presence one or more plasmid bands between 2 kb and ≥ 12 kb (Miles et al., 2006).

Ahmadi et al., (2008) reported to harbour single plasmid with size ranges from of 9.162 to 13.000 Kb were present in *E. coli* strains which were resistance to Ampicillin, Amoxicillin, Neomycin, Kanamycin, Streptomycin, Tetracycline, Nalidxic acid, Flumequine, Erythromycin and Enrofloxacin.

In the study of Al-Bahry et al., 2006 the highest frequency in *E. coli* isolates was with Tetracycline (97.9%) followed by Nalidxic acid (78.7%), Streptomycin (68.1%) and Kanamycin (59.6%). None of the strains were resistant to Amikacin and Cephotaxin and plasmid occurrence rate of 100% was observed in all resistant strains and harbored 1-5 small size plasmids with molecular weight in the range 2.9-66 kb where strains 31.9% had one plasmid, 27.7% had two plasmids, 19.1% had three plasmids, 17% strains had four plasmids and 4.3% harbored five plasmids. In general, strains resistant to one antibiotic contained one plasmid.

From the study of pond system of Kathmandu, Adhikari et al, (2000) reported that 55.5% of the 9 *E. coli* isolates having resistance pattern to Ampicillin Tetracycline Trimethoprim, possessed conjugative types of plasmids of size 34 to 98 MDa, and single plasmid has been transferred and found possess at least two resistance markers, viz. Ampicillin, Tetracycline, Trimethoprim and Ampicillin, Trimethoprim in conjugents.

Ozakabir et al., (2010) revealed that single plasmid was most frequently present in Ampicillin–resistant *E. coli* along with Ciprofloxacin resistant and in significant relation with MDR.

Nandy et al., (2010) showed that a number of smaller plasmids (<20 kb) with distinct patterns have been observed for several years in predominant subtypes where the majority of *Shigella* isolates (81.0%) were multidrug resistant to commonly used drugs like Ampicillin, Tetracycline, Cotrimoxazole and Nalidxic acid. As emergence of fluoroquinolone (FQ)-resistant *S. dysenteriae* type 1 (100.0%) in 2002–2003 was followed by frequent isolation (>25.0%) of FQ-resistant *S. flexneri* 2a, and *S. flexneri* 3a in 2004.

CHAPTER IV
MATERIALS AND METHODS

4.1 Materials

The materials, equipments, media and reagents used in this study are systematically listed in Appendix I.

4.2 Methods

4.2.1 Study duration

The study was conducted from January 2011 to August 2011.

4.2.2 Laboratory setting

Laboratory setting was done in the Central Department of Microbiology Laboratory.

4.2.3 Sample collection area

Kathmandu the capital of Nepal lies in Bagmati zone of the Central Development Region of Nepal. The Kathmandu valley is composed of three districts namely Kathmandu, Lalitpur and Bhaktapur. It consists of five municipalities which are: Kathmandu Metropolitan City, Lalitpur Submetropolitan city, Bhaktapur municipality, Madhyapur Thimi Municipality and Kirtipur Municipality. There are more than 57 VDCs in Kathmandu valley. Geographically, the district lies between 27° 35' to 27° 48' and longitude of 85° 12' to 85° 33' E. The altitude of the district ranges between 1372-2732 m above mean sea level. The major rivers flowing in the district are Bagmati River, Bishnumati River and Manohara River. In Kathmandu valley, drinking water demand is fulfilled by Kathmandu Upatakya Khanepani Limited through 21 treatment plants and it has 165,000 private house connections which are linked to system legally (ADB I, 2009).

4.2.4 Sampling method and sample size

Simple random sampling method was applied for the collection of samples and total of 66 samples of drinking water, 28 from tap water; 24 from well water and 14 from spring water were collected from different place of Kathmandu.

4.2.5 Sample collection and transport

Sample collection was carried out according ‘Standard Methods for the Examination of Water and Wastewater’ (APHA, 1998).

For tap water: Grab water samples were collected. Before the sample collection, any external fittings of tap if present were removed, tip was wiped with 70% alcohol then water was allowed to run to waste at a uniform rate for about 2-3 minutes.

Contamination was prevented during sampling. The grab water sample for microbial analysis was collected in sterilized bottles containing sodium thiosulfate to a final concentration of 3% (w/v) to neutralize any free or residual chlorine.

For well water: Water samples were collected using sterilized bottle fitted with a weight at the base and care was taken to avoid contaminating samples by any surface scum. Sodium thiosulfate treatment was done for chlorinated well water samples.

For spring water: The grab water sample for microbial analysis was collected in sterilized bottles.

The collected water samples were analyzed on the same day immediately after its delivery and always within 6 hours of collection. In some cases when immediate analysis was not possible, the samples were preserved at 4°C.

4.2.6 Laboratory analysis

4.2.6.1 Microbiological analysis of water sample

I. Total coliform and thermotolerant coliform

In this study, total coliforms and thermotolerant coliform were enumerated by the membrane filter (MF) technique as described by APHA, 1998. Initially, 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. The sample of water was well mixed by inverting the bottle several times, 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, onto a plate of EMB agar with the help of sterile forceps. Care was taken not to entrap air bubbles between the membrane and the medium. The procedure was performed for duplicate samples. One of the plates was incubated at 37°C and another was incubated at 44.5°C for 24 hrs in inverted position. After incubation total colony forming unit (CFU) were counted. For this, all green metallic sheen-producing colonies were counted.

II. Enumeration and conformation of *E. coli* and thermotolerant *E. coli*

A green metallic sheen-producing colony from EMB Agar from the total coliform and thermotolerant coliform were streaked on McConkey Agar and incubated for 24 - 48 hours at 37°C and 44.5°C respectively. Typical colony was further sub cultured on Nutrient Agar and again incubated at 37°C and 44.5°C respectively for 24-48 hours.

E. coli was identified by colony morphology, gram staining and biochemical test according to Bergey's Manual of Determinative Bacteriology, 1994 including MUG hydrolysis test. MUG (4-Methylumbelliferone glucuronide) hydrolysis test was performed for Glucouronidase production. MUG being a fluorogenic substrate, which provides fluorescent end–product methylumbelliferone after the interaction with enzyme β-glucuronidase found in *E. coli*. The end product was detected with long wave ultraviolet (UV) light. For the thermotolerant *E. coli* MUG test was performed in 44.5°C while for non-thermotolerant *E. coli* it was performed in 37°C.

4.2.6.2 Antimicrobial susceptibility test

Antibiotic susceptibility of isolated enteric bacteria was assayed using a modified Kirby Bauer disc diffusion method (Vandepitte et al., 2003) with eight different antibiotics Ofloxaxin (OF, 5µg), Chloramphenicol (C, 30µg), Cotrimoxazole (Co, 25µg), Amoxycillin (Am, 10µg), Cefixime (Cfx, 5µg), Tetracycline (T, 30µg), Amikacin (Ak, 30µg) and Nalidxic acid (NA, 30µg).

Single well isolated colonies from the culture of bacteria grown on non selective was inoculated on 5 ml of Mueller hinton broth at 37°C for about 4 hrs. After incubation turbidity of inoculums was adjusted with the turbidity of 0.5 McFerland standard (1.5X 10^8 CFU/ml). A sterile cotton swab was taken and dipped into adjusted inoculum of bacteria. The entire agar surface of each plate was swabbed, three times at 60° angle to ascertain the even distribution of the organism over the agar surface. The agar surface was allowed to dry for 5 to 10 minutes but not more than 15 minutes. A sterile antibiotic disc was picked up by the outer edge using a flamed, sterile forceps and placed into agar surface with maximum distance between two antibiotic discs of inoculated plate. It was pressed gently with the sterile forceps to ascertain firm contact with the agar surface. A second disc was placed at the opposite side of the former one. The plate was allowed to stand at room temperature for 15 minutes for pre-diffusion and then incubated at 37ºC for 16 to18 hours. The diameter of the zone of inhibition was measured at the completion of the incubation period. Organisms were classified as sensitive, intermediate and resistant to an antibiotic according to the diameter of the inhibition zone surrounding each antibiotic disc following CLSI guideline (CLSI, 2007).

Based on the sensitivity pattern of the isolates, *E. coli* resistant three or more than three classes of antibiotics were considered as multiple drug resistant bacteria.

4.2.6.2 Plasmid profiling

Only, thermotolerant *E. coli* from the Tap water were subjected for the plasmid profiling.

I. Plasmid DNA isolation

The selected bacterial strain (single colony) was grown overnight in Luria-Bertani (LB) broth at 37°C with aeration using an orbital shaker and plasmid DNA from *E. coli* isolates was extracted through Mini alkaline lysis by SDS (Sambrook and Russell, 2001).

In brief, from overnight cultures of a single *E. coli* colony in about 10 ml Lauria-Bertani (LB) broth containing appropriate antibiotic, about 3 mL culture was pelleted down by centrifugation at maximum speed (20,000 rpm) for 1 minutes and the supernatant was removed. To resuspend the pellet, 100 µL of solution-I (refrigerated) containing 50 mM glucose, 25 mM Tris-Cl (pH 8.0) and 10mM EDTA (pH 8.0) was added and vortex.. Then 200 µL of freshly prepared solution-II containing 0.2 N NaOH, 1% (w/v) SDS was added to and mixed well by inverting gently four or five times followed by addition of 150 µL of solution-III containing Potassium acetate 3M, and mixed comprehensively. The tube was stored on ice for 3-5 minutes. The total mixture was centrifuged at 20,000 rpm for 10 minutes and 400 µL of the clean supernatant was taken in fresh eppendorf tube. DNA was precipitated by adding 900 ul of 95% EtOH. After standing the tube at -20°C for 30 minutes, the mixture was centrifuged at 20,000 rpm for 30 minutes, and pellet was suspended with 1000 µL with 70% (v/v) ethanol and allowed to sit for 3 minutes and centrifuged at 20,000 rpm for 3 minutes. Supernatant was discarded and the pellet was air dried fir 10 minutes. Finally the plasmid DNA was dissolved in 50 µL of TE buffer and stored at -20°C.

II. Agarose gel electrophoresis of plasmid DNA

The plasmid concentration and size was estimated by agarose gel electrophoresis (Sambrook and Russell, 2001). Electrophoresis was carried out in a horizontal gel apparatus. Electrophoresis was conducted in agarose (0.8%) gel containg Ethilium bromide (EtBr). Supermix 1kb DNA marker (GeNei Pvt. Ltd., Bangalore, India) was used as a reference marker. The plasmid size was estimated carefully comparing with the DNA marker.

In brief, 0.8% agarose gel was dissolved in 1 X TAE buffer and dissolved completely in oven and cool to about 60°C. Then ETBr (33 µl of 1 mg/ml stock) was added and mixed well. The molten gel was poured into gel mould in horizontal surface. Immediately, the comb was positioned and allowed to stand for about half an hour. TAE buffer (1X) was poured into the buffer tank into the electrophoresis set. Then, gel mould was positioned into the set. The comb was removed carefully. In first lane 2µl of 1 kb ladder DNA marker was loaded. Plasmid DNA sample, 10 µl per well (in a ratio of 4 µl gel loading dye + 20µl DNA solution) were loaded. The apparatus was closed and the electrodes were attached to power pack and at 110 volt, the electrophoresis was run for 1 hour. And then, gel was taken out in gel tray and visualized into UV- illuminator and the photograph was taken.

4.2.7 Quality control

Strict quality control was maintained to obtain reliable microbiological results. The quality of each agar plate prepared was maintained by incubating one plate of each batch in the incubator. A control strain of ATCC was given in Appendix I used for the identification test, standardization of Kirby-Bauer test, correct interpretation of inhibition zones of diameter and for plasmid profiling. Quality of sensitivity test was maintained by maintaining the thickness of MHA at 4 mm and the pH of 7.2-7.4. Similarly antibiotics discs having correct amount as indicated was used. Strict aseptic condition was maintained while carrying out all the procedures.

4.2.8 Statistical analysis

Chi-square test for Source verses coliform, Source verses thermotolerant coliform, Source verses *E. coli* and Source verses thermotolerant *E. coli;* Fisher exact test for coliform verses thermotolerant coliform, coliform verses presence of *E. coli,* Thermotolerant coliform verses presence of Thermotolerant *E. coli,* organism verses MDR, organism verses NAR and MDR verses NAR were calculated using SPSS 16 (Statistical package for social science). Average value for temperature, pH and enumeration of *E. coli*, thermotolerant *E. coli* were calculated using MS EXCEL 2007. Semi log graph was used for plasmid size determination (CIBT, 2008).

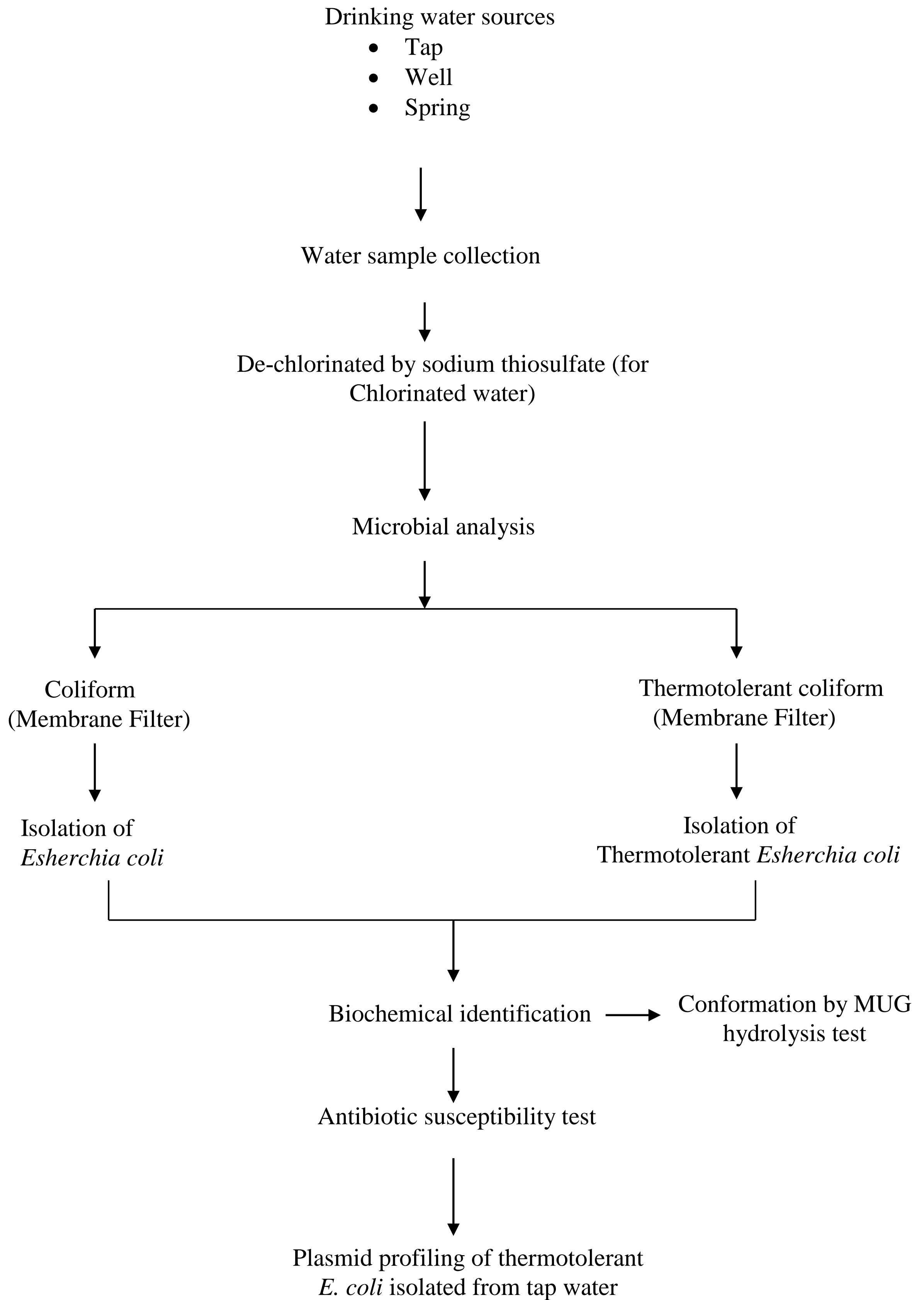


Figure-3: FLOW CHART OF THE METHODS

CHAPTER V

RESULT

A total of 66 water samples from tap, well and spring sources from different places of Kathmandu were collected and analysed for bacteriological parameters (enlisted in Appendix- IV). *E. coli* and thermotolerent *E. coli* isolates were subjected for antibiogram and plasmid profiling of thermotolerent *E. coli* was done.

5.1 Total coliform and thermotolerant coliform presence in water sample

All water sources were contaminated with coliform and thermotolerant coliform.

Total coliform growth was found higher in spring water (85.7%), followed by well water (79.2%) then tap water (60.7%). Similarly, the thermotolerant coliform growth was found higher in well water (87.5%) followed by spring water (71.4%) the tap water (67.9%). (Table-5)

Table-5: Total coliform and thermotolerant coliform in drinking water sources

Source	Total Coliform	Thermotolerant Coliform
	Growth (Percentage)	Growth (Percentage)
Tap(n=28)	17 (60.7)	19 (67.9)
Well(n=24)	19 (79.2)	21 (87.5)
Spring(n=14)	12 (85.7)	10 (71.4)

5.2 Recovery of *E. coli* and thermotolerant *E. coli*

In well and spring water sources 66.7% and 64.3% of samples was contaminated with *E. coli* and thermotolerant *E. coli* both where as 21.4% and 50% tap water was detected with *E. coli* and thermotolerant *E. coli* respectively. Consequently, 47% *E. coli* and 50 % thermotolerant *E. coli* were detected from the total coliform and thermotolerant coliform respectively. (Table- 6)

Table -6: *E. coli* and thermotolerant *E. coli* in water sources

Source	*E. coli* (Percentage)	Thermotolerant *E. coli* (Percentage)
Tap water (n=28)	6 (21.4)	8 (50)
Well water (n=24)	16 (66.7)	16 (66.7)
Spring water (n=14)	9 (64.3)	9 (64.3)
Total (n= 66)	31 (47)	33 (50)

5.3 Enumeration of *E. coli* and thermotolerant *E. coli*

Minimum *E. coli* and thermotolerant *E. coli* concentration was detected in tap water and maximum in well water. Thermotolerant *E. coli* load was minimum $\log_{10}0.90309$ in tap water and maximum $\log_{10}2.65896$ in well water. Average count of *E. coli* was $\log_{10}1.549518$, $\log_{10}1.959942$ and $\log_{10}2.13545$ and thermotolerant *E. coli* was $\log_{10}1.992076$, $\log_{10}2.054940$ and $\log_{10}2.179505$ in tap, well and spring water sources respectively. (Table-7)

Table-7: Enumeration of *E. coli* and thermotolerant *E. coli*

S.no	Source	Organism	Min($\log_{10}$)	Max($\log_{10}$)	Average($\log_{10}$)
1	Tap(n=28)	*E. coli*	0.954243	2.313867	1.549518
		Thermotolerant *E. coli*	0.90309	2.614970	1.992076
2	Well(n=24)	*E. coli*	1.397940	2.667453	1.959942
		Thermotolerant *E. coli*	1.55068	2.65896	2.054940
3	Spring(n=14)	*E. coli*	1.57978	2.59770	2.135345
		Thermotolerant *E. coli*	1.54407	2.62839	2.179505

5.4 Relation of organism with source

There was no significant relation between water sources with presence of coliform (P=0.155) and thermotolerant coliform (P=0.235) but significant difference on presence of *E. coli* (P=0.002) and thermotolerant *E. coli* (P=0.011) occurs among sources. (Table-8)

Table-8: Relation of organism with source

Source / Organism	Tap (n=28)	Well (n=24)	Spring (n=14)	Chi square test Source verses Organism
Total coliform	17	19	12	P=0.155
Thermotolerant coliform	19	21	10	P=0.235
E. coli	6	16	9	P=0.002
Thermotolerant *E. coli*	8	16	9	P=0.011

5.5 Relation of organisms within source

There was no significant relation on presence of coliforms and thermotolerant coliforms within sources, for well (P=0.521) and spring (P=0.505) but significant relation occurs in tap water (P=0.010) and no significance difference on presence of coliforms and presence of *E. coli* for tap (P=0.055), for spring water (P=0.110) but significance difference occurs in presence of coliforms and presence of *E. coli* for well water (P=0.01). The significant relation in presence of thermotolerant coliform and presence of thermotolerant *E. coli* was found for all sources (P=0.029) in tap, (P=0.028) in well, (P=0.05) in spring water. (Table-9)

Table-9: Relation of coliform and thermotolerant coliform with *E. coli*, thermotolerant *E. coli* and within source.

Source	Coliform	Thermotolerant coliform	Fisher exact test Coliform verses thermotolerant coliform	Presence of *E. coli*	Fisher exact test Coliform verses presence of *E. coli*	Presence of thermo-tolerent *E. coli*	Fisher exact test Thermotolerant Coliform verses presence of Thermotolernt *E. coli*
Tap (n=28)	17	19	P=0.010	6	P=0.055	8	P=0.029
Well (n=24)	19	21	P=0.521	16	P=0.01	16	P=0.028
Spring (n=14)	12	10	P=0.505	9	P=0.110	9	P=0.05

5.6 Antibiotic susceptibility pattern of *E. coli* and thermotolerant *E. coli*

All *E. coli* and thermotolerant *E. coli* isolates were subjected to the antibiotic susceptibility test to eight different antibiotics. The individual antibiotic susceptibility pattern of both *E. coli* and thermotolerant *E. coli* were presented accordingly with source. Higher resistance pattern were found in well water followed by spring water and tap water and all *E. coli* isolates and thermotolerant *E. coli* isolates were resistance to Tetracycline with exception of two *E. coli* isolates from well water and most of MDR isolates had resistance to Tetracycline, Amoxicillin, Cefixeme respectively. (Table-10; Table-11; Table-12; Table-13; Table-14; Table-15)

Table-10: Antibiotic susceptibility of *E. coli* isolated from tap water

S.no	Source	Sample code	Of	C	Am	Ak	Cfx	Na	Co	T
1	Tap	KT1	S	S	R	I	R	S	S	R
2	Tap	KT3	S	S	R	R	R	S	S	R
3	Tap	KupT3	S	S	R	S	I	I	S	R
4	Tap	BalT1	S	S	I	I	I	S	S	R
5	Tap	BalT2	S	S	R	I	R	R	S	R
6	Tap	BhtT3	S	S	R	S	I	I	S	R

Table-11: Antibiotic susceptibility of themotolerant *E. coli* isolated from tap water

S.no	Source	Sample code	Of	C	Am	Ak	Cfx	Na	Co	T
1	Tap	TKT1	S	S	R	I	R	I	S	R
2	Tap	TKT2	S	S	R	R	R	S	S	R
3	Tap	TKT3	S	S	R	S	I	R	S	R
4	Tap	TKT5	S	S	S	S	I	R	S	R
5	Tap	T KupT3	S	S	R	R	I	I	S	R
6	Tap	TKupT4	S	S	R	I	I	I	S	R
7	Tap	TBalT2	S	S	R	I	R	S	S	R
8	Tap	TBhtT2	S	S	R	S	I	R	S	R

Table-12: Antibiotic susceptibility of *E. coli* isolated from well water

S.no	Source	Sample code	Of	C	Am	Ak	Cfx	Na	Co	T
1	Well	KW1	S	S	R	S	R	I	S	R
2	Well	KW2	S	S	R	I	R	R	S	R
3	Well	KW3	S	S	I	S	I	S	S	I
4	Well	KW4	S	S	R	R	R	I	S	R
5	Well	KupW1	S	S	R	I	R	S	S	R
6	Well	KupW2	S	S	R	R	R	R	S	R
7	Well	JhmW1	S	S	R	S	I	R	S	R
8	Well	JhmW3	S	S	I	I	I	S	S	R
9	Well	SW2	S	S	R	S	R	I	S	R
10	Well	SW3	S	S	I	R	I	I	S	R
11	Well	BalW1	S	S	R	I	R	R	S	I
12	Well	BalW2	S	S	R	S	I	I	S	R
13	Well	BalW3	S	S	R	R	R	S	S	R
14	Well	BhtW1	S	S	R	S	I	R	S	R
15	Well	BhtW2	S	S	R	R	R	I	S	R
16	Well	BhtW3	S	S	R	I	S	R	S	R

Table-13: Antibiotic susceptibility of themotolerant *E. coli* isolated from well water

S.no	Source	Sample code	Of	C	Am	Ak	Cfx	Na	Co	T
1	Well	TKW1	S	S	R	R	R	S	S	R
2	Well	TKW3	S	S	R	S	I	R	S	R
3	Well	TKW4	S	S	R	R	R	I	S	R
4	Well	TKupW1	S	S	R	S	I	S	S	R
5	Well	TKupW2	S	S	R	I	R	I	S	R
6	Well	TKupW3	S	S	R	R	R	I	S	R
7	Well	TJhmW2	S	S	R	I	R	R	S	R
8	Well	TJhmW3	S	S	R	S	R	R	S	R
9	Well	TJhmW1	S	S	R	I	R	I	S	R
10	Well	TSW3	S	S	R	S	I	I	S	R
11	Well	TSW5	S	S	R	I	R	I	S	R
12	Well	TBalW2	S	S	R	R	I	R	S	R
13	Well	TBalW3	S	S	R	S	I	I	S	R
14	Well	TBhtW1	S	S	R	S	R	R	S	R
15	Well	TBhtW2	S	S	R	R	R	I	S	R
16	Well	TBhtW3	S	S	I	R	R	R	S	R

Table-14: Antibiotic susceptibility of *E. coli* isolated from spring water

S.no	Source	Sample code	Of	C	Am	Ak	Cfx	Na	Co	T
1	Stone Spout	KD2	S	S	I	S	I	S	S	R
2	Stone Spout	KupD1	S	S	R	R	R	S	S	R
3	Stone Spout	KupD2	S	S	R	R	R	R	S	R
4	Stone Spout	SD2	S	S	R	S	I	I	S	R
5	Stone Spout	BalD1	S	S	R	S	I	R	S	R
6	Stone Spout	BalD2	S	S	R	I	R	I	S	R
7	Stone Spout	BhtD1	S	S	R	I	I	R	S	R
8	Stone Spout	BhtD2	S	S	R	R	R	I	S	R
9	Stone Spout	BhtD3	S	S	R	I	R	R	S	R

Table-15: Antibiotic susceptibility of tormotolerent *E. coli* isolated from spring water

S.no	Source	Sample code	Of	C	Am	Ak	Cfx	Na	Co	T
1	Stone Spout	TKD2	S	S	R	S	R	I	S	R
2	Stone Spout	TKD3	S	S	R	R	I	R	S	R
3	Stone Spout	TKupD1	S	S	R	R	R	R	S	R
4	Stone Spout	TKupD2	S	S	R	R	R	I	S	R
5	Stone Spout	TSD2	S	S	R	S	I	S	S	R
6	Stone Spout	TBalD1	S	S	R	I	I	R	S	R
7	Stone Spout	TBalD2	S	S	R	S	I	R	S	R
8	Stone Spout	TBhtD1	S	S	R	R	R	I	S	R
9	Stone Spout	TBhtD2	S	S	R	I	R	S	S	R

5.7 Antibiotic susceptibility of *E. coli* and thermotolerant *E. coli*

All *E. coli* and thermotolerant *E. coli* isolates were susceptible to Ofloxacin, Chloramphenicol and Cotrimixazole. Resistance to Cefexime, Amikacin and Nalidixic acid, Amoxicillin, Tetracycline were 54.8%, 29% 35.5%, 80.6%, 93.5% and 57.6%, 36.4%, 39.4%, 94%, 100% was observed in *E. coli* and thermotolerant *E. coli* respectively. (Table-16)

Table-16: Antibiotic susceptibilities of *E. coli* and thermotolerant *E. coli*

Antibiotics	*E. coli* (*N* = 31)			Thermotolerant *E. coli* (*N* = 33)		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Ofloxacin	31 (100)	0 (0)	0 (0)	33 (100)	0 (0)	0 (0)
Chloramphenicol	31(100)	0 (0)	0 (0)	33 (100)	0 (0)	0 (0)
Amoxicilin	0 (0)	6 (19.4)	25 (80.6)	1 (3)	1 (3)	31 (94)
Amikacin	11 (35.5)	11 (35.5)	9 (29)	12 (36.4)	9 (27.2)	12 (36.4)
Cefexime	1 (3.2)	13 (42)	17 (54.8)	0 (0)	14 (42.4)	19 (57.6)
Nalidxic acid	8 (25.8)	12 (38.7)	11 (35.4)	6 (18.2)	14 (42.4)	13 (39.4)
Cotrimoxazole	31 (100)	0 (0)	0 (0)	31 (100)	0 (0)	0 (0)
Tetracycline	0 (0)	2 (6.5)	29 (93.5)	0 (0)	0 (0)	16 (100)

Comparatively, thermotolerant *E. coli* isolates were observed more resistant than *E. coli* isolates in Amoxicillin, Amikacin, Cefexime and Nalidxic acid but Ofloxacin, Chloramphenicol and Cotimoxazole were active against both *E. coli*. Tetracycline resistance was found in all thermotolerant *E. coli* isolates and two *E. coli* from well water did not show resistivity to Tetracycline. MDR *E. coli* and thermotolerant *E. coli* were found maximum due to the resistance to Tetracycline and Amoxicillin followed by Cefexime. (Table-17)

Table-17: Comparative AST pattern for *E. coli* and thermotolerant *E. coli*

Source	Organisms	Of	C	Am			Ak			Cfx			Na			Co	T	
		S	S	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Tap	*E. coli* (n=6)	6	6		1	5	2	3	1		3	3	2	3	1	6		6
	Thermotolerant *E. coli* (n=8)	8	8	1		7	3	3	2		5	3	2	3	3	8		8
Well	*E. coli* (n=16)	16	16		3	13	6	5	5	1	6	9	4	6	6	16	2	14
	Thermotolerant *E. coli* (n=16)	16	16		1	15	6	4	6		5	11	2	8	6	16		16
Spring	*E. coli* (n=9)	9	9		1	8	3	3	3		4	5	2	3	4	9		9
	Thermotolerant *E. coli* (n=9)	9	9			9	3	2	4		4	5	2	3	4	9		9

5.8 Multiple drug resistance and Nalidxic acid resistance

Among isolates, 22 (70.9%) and 11 (35.5%) *E. coli* isolates expressed MDR and NAR, whereas, 25 (75.8%) and 13 (39.4%) of thermotolerant *E. coli* isolates expressed MDR and NAR. There was no significance relation between thermo tolerance property of *E. coli* with MDR (P=0.779), and with NAR (P=0.800). (Table-18)

Table-18: Correlation between MDR, NAR and NAR-MDR with thermo tolerance property of *E. coli*

Organism	MDR (%)	Fisher exact test Organism*MDR	NAR (%)	Fisher exact test Organism verses NAR
E. coli(N=31)	22 (70.9)	P=0.779	11 (35.5)	P=0.800
Thermotolerant *E. coli* (N=33)	25 (75.8)		13 (39.4)	

MDR isolates were found higher in well and spring water than tap water but thermotolerant *E. coli* isolates had high MDR 62.5% and 81.2% than *E. coli* isolates in tap and well water respectively. Among the sources, 44.4% of MDR-NAR *E. coli* and thermotolerant *E. coli*, were found in spring water followed by 37.5% (*E. coli*) well water and 25% (thermotolerant *E. coli*) and 16.7 % (*E. coli* and thermotolerant *E. coli*) in tap water respectively. (Table-19)

Table-19: MDR, NAR and NAR-MDR *E. coli* and thermotolerant *E. coli*

Source	Organisms	MDR (%)	NAR (%)	NAR-MDR (%)
Tap	*E. coli* (N=6)	3 (50)	1 (16.7)	1 (16.7)
	T. *E. coli* (N=8)	5 (62.5)	3 (37.5)	2 (25)
Well	*E. coli* (N=16)	12 (75)	6 (37.5)	6 (37.5)
	T. *E. coli* (N=16)	13 (81.2)	6 (37.5)	6 (37.5)
Spring	*E. coli* (N=9)	7 (77.8)	4 (44.4)	4 (44.4)
	T. *E. coli* (N=9)	7 (77.8)	4 (44.4)	4 (44.4)

The significant relation between the MDR and NAR expression of *E. coli* (P=0.012) and thermotolerant *E. coli* (P=0.012) was detected. (Table-20)

Table-20: Correlation between MDR and NAR

MDR *E. coli*	NAR-*E. coli*	Fisher Exact test MDR verses NAR
22	11	P=0.012
MDR thermotolerant *E. coli*	NAR thermotolerant *E. coli*	
25	13	P=0.012

5.9 Plasmid analysis of thermotolerant *E. coli*

All tap water thermotolerant *E. coli* isolates where subjected for plasmid analysis. Single band of plasmid were observed in three MDR isolates and one non-MDR isolate and size varies from 2 kb to >10 kb were obtained. All NAR thermotolerant *E. coli* were found to harbor plasmid. (Table-21)

Table-21: Plasmid profiling of thermotolerant *E. coli*

Organism code	Antibiotic Resistance pattern	No. of Plasmid band	Plasmid size
Tkt1	Am, Cfx, T	-	-
Tkt2	Am, Ak ,Cfx, T	-	-
Tkt3	Am, Na, T	1	2 kb
Tkt5	Na, T	1	>10kb
T kupt3	Am, Ak, T	1	2.1 kb
Tkupt4	Am, T	-	-
TBalT2	Am, Cfx, T	-	-
TBhtt2	Am, Na, T	1	2 kb

CHAPTER VI

DISCUSSION AND CONCLUSION

6.1 Discussion

Water is essential to life, due to lack of safe drinking water and many die of waterborne bacterial infections**.** There is constant risk of spread of antibiotic resistance in aquatic environments. Therefore, detection of *E. coli* as indicators of faecal contamination and the spread of antibiotic resistance determinants has become a great concern is very important to protect public health.

In present study, total coliform growth was found higher in spring water (85.7%), followed by well water (79.2%) then tap water (60.7%) and comparative with other report which shows 80% to 100% contamination of water sources (Panta, 2011; Diwakar et al , 2008; Prasai et al., 2007; ENPHO, 2007).

Similarly, the thermotolerant coliform growth was found higher in well water (87.5%) followed by spring water (71.4%) the tap water (67.9%) and accordance with Shakya, 2008. Both *E. coli* and thermotolerant *E. coli* was found 66.7% and 64.3% of in well and spring water sources where as 21.4% of *E. coli* and 50% of *E. coli* was found in tap water and comparative to previous results ranging from 20.6% to 70.6% contamination (Prasai et al., 2007; ENPHO, 2007).

Higher thermotolerant coliform growth than total coliform growth in drinking water is in agreement with the study of India (Gaur et al., 1992) and high coliform and thermotolerant coliform growth in winter season was found in accordance with Al-khatib et al., (2005) who reported that the bacteriological contamination was higher in winter months than in summer months in drinking water.

From different water samples, minimum *E. coli* load was $\log_{10}$ 0.95423 in tap water and maximum load was $\log_{10}$2.667453 in well water. Thermotolerant *E. coli* load was minimum $\log_{10}$0.90309 in tap water and maximum $\log_{10}$2.65896 in well water. Average count of *E. coli* was $\log_{10}$1.549518, $\log_{10}$1.959942 and $\log_{10}$2.13545 and thermotolerant *E. coli* was $\log_{10}$1.992076, $\log_{10}$2.054940 and 2 $\log_{10}$. 179505 in tap, well and spring water sources respectively. As the sampling was done in winter season (January to February), several studies reported that cooler water temperatures can increase the ability of fecal bacteria and *E. coli* to survive in a variety of aquatic conditions (Brettar and Hofle 1992; Smith et al . 1994; Bogosian et al . 1996, Medema et al., 2003; Sampson et al ,2006), presence of sand ,other particles, or green algae, in

the water environment may enhance survival of *E. coli* in lower temperatures (Brettar and Hofle 1992; Bogosian et al., 1996; Whitman et al., 2003; Sampson et al., 2006). This may be the reason for the high number of occurrence of *E. coli* and coliform in water environment.

Thus, this study indicates that most of the water sources are highly faecally contaminated. Water quality thus indicates that pollution of water is increasing alarmingly and it has created serious threat to human health and environment.

High contamination on stone spout and groundwater may be due to direct discharge of untreated sewage or municipal wastes into surface waters or in open places near to sources, which was observed in most of the places. Contamination with in such milieu due to such unusual practices, contaminants can easily leach down to groundwater table leading high microbial contamination to shallow water.

Maximum thermotolerant *E. coli* along with thermotolerant coliform detected in shallow water is probably due to poor drainage facility and improper construction pattern of septic reservoirs, infiltration of domestic or wild animal fecal matter Construction of septic tank close to the groundwater sources may be a reason of high microbial contamination to groundwater, which was common almost in all the places as observed. As a consequence, the effluent from septic tank can easily percolate down to groundwater and leads high microbial contamination to groundwater table.

The current water distribution system in the city of Kathmandu dates back to 1895 (Shakya and Sharma, 1996).Water is piped from the treatment plants to distribution points in underground pipelines. These pipelines are often quite old and lie in the same vicinity as the sewage network (Shrestha and Sharma, 1995). Discontinuous supply of the drinking water in the pipeline cause the risk of back siphonage into the distribution network is increased when pipes are at lower pressure than the surroundings soil, which often contains leaked out effluent from leaking sewers. Unrepaired old pipeline, parallel arrangement with that of the drainage system and irregular supply and failure of the disinfections of the raw water at the treatment plant or because of the infiltration of contaminated water (sewage) through cross-connection and leakage points with the connected premises were major reasons for contamination in piped distribution.

In this study, the higher thermotolerant coliforms were found in the well water and spring water may be the result from the human or animal faeces and anthropogenic activities. Eventually higher human activities near the premises of the natural water

sources leads to contamination they could be washed by rain water as run-off into the spring and thus contaminate it as the natural waters are neither treated nor protected and 50% of tap water had detected with the presence of thermotolerant *E. coli*, it is clear that the piped drinking water in the Kathmandu valley was contaminated fecal source. The problems of clean drinking water and lack of proper sanitation are closely related. Pathogen-laden human and animal wastes, food and garbage pile up near homes and drain into waterways, contaminating the water sources. According to the WHO, the lack of safe water supply and of adequate means of sanitation is blamed for as much as 80 % of all diseases in developing countries.

In this study, there was no significant relation between water sources with presence of coliform (P=0.155) and thermotolerant coliform (P=0.235) was agreement with result from Turkey (Ozgumus et al., 2007) but significant difference occurs on presence of *E. coli* (P=0.002) and thermotolerant *E. coli* (P=0.011) with source. There was no significant relation on presence of coliforms and thermotolerant coliforms within sources, for well (P=0.521) and spring (P=0.505) but significant relation in tap water (P=0.010) which indicates that all sources were contaminated with fecal pollution and also the piped water system were exposed to contamination.

In present study, no significance association on presence of coliforms and presence of *E. coli* for tap (P=0.055) and for spring water (P=0.110) and significance relation occurred for well water (P=0.01). The significant relation in presence of thermotolerant coliform and presence of thermotolerant *E. coli* was found for all sources (P=0.029) in tap, (P=0.028) in well, (P=0.05) in spring water. The presence of coliforms at these stations could also be a result of direct contamination caused by human activities (anthropogenic) and indirect effect caused by ecological disturbances.

The result indicates that thermotolerant *E. coli* (alternatively thermotolerant coliform) provides the best indication for fecal pollution and must be detect for all water sources.

In *Drinking Water Standards for New Zealand 2000, E. coli* was suggested for the faecal pollution indicator and no rely on total coliforms and faecal coliforms as they can be found in natural waters and their presence in drinking water does not necessarily indicate a health risk (Stevens et al , 2003).

E. coli isolates appeared to show higher levels of resistance or reduced susceptibility to some specific antibacterial agents. This may have been the result of resistance

factors that are readily retained by *E. coli*, the easy acquisition of resistance factors horizontally. The current practice of the use of antibiotics needs to be changed, otherwise emergence of resistant *E. coli* strains will occur.

In present study, all *E. coli* and thermotolerant *E. coli* isolates were susceptible to Ofloxacin, Chloramphenicol and Cotrimoxazole. Resistance to Cefexime, Amikacin and Nalidixic acid, Amoxicillin, Tetracycline were 54.8%, 29% 35.5%, 80.6%, 93.5% and 57.6%, 36.4%, 39.4%, 94%, 100% was observed in *E. coli* and thermotolerant *E. coli* respectively. High MDR isolates 70.9% and 75.8% *E. coli* and thermotolerant *E. coli* were prevalent and the result is in comparative with the study of Chigor et al., (2010); Patoli et al., (2010); Olaniran et al., (2009); Alhaj et al., (2007) and Idia et al., (2006) which shows MDR cases ranging from 61.2% to 97.1% in aquatic isolates.

High level of resistance to Tetracycline is in accordance to results from the studies (Jackson et al , 2011, Sifuna et al ,2008, Chigor et al., 2010 ; Onyuka et al, 2011) from water isolate and (Tabatabaei et al., 2003, Roy et al., 2006, Tabatabaei et al.,2010) from the chicken isolates. And high level of resistance to Amoxicilin was detected in chicken isolates (Tabatabaei et al., 2010; Poudel et al., 2009; Bogaard et al*.,* 2001). High level of Tetracycline, Amoxcilin, and Nalidxic acid had found form buffalo faeces (Ahmadi et al., 2008). Bayat et al.,(2011) reported 100% resistance to Amoxicillin , Tetracycline, Cefexime was found in the hospital patients and Sayah et al., (2005) observed that the *E. coli* were much more resistance to Tetracycline from different samples including farm animal environment, wild life faeces and surface water and MDR association with Tetracycline which is similar to this result. The result found contradicts to Alam et al., (2010) in resistivity of Tetracycline from water isolate.

Simialr results of sensitivity of *E. coli* against Chloramphenicol (Alam et al., 2010; Olaniran et al., 2009; Tambekar et al., 2005) and Ofloxacin (Tambekar et al., 2005; Egri-Okwaji, 1996 Kesah et al., 1999) were documented but result contradicts to Umala et al., (2009) reported 22.1% resistance to Ofloxacin from UTI patients.

In this study it may be because of restricted use Chloramphenicol (Goni-Urriza et al., 2000) and higher resistance to the Cefexime than Amikacin has been observed and may be the cause of reduced use of Amikacin and in which prolonged use affects kidney and auditory nerves leading to deafness (Goni-Urriza et al., 2000). In this study, Amikacin resistance found is higher than India (Tambekar et al., 2010) who observed only 8% resistance in drinking water.

Tetracycline is a naturally derived compound; bacteria can be exposed to these agents in nature and outside any human use for disease treatment, for prophylaxis, or for livestock growth promotion and often disseminated in environment by domestic faeces (Miles, 2006). Kariuki et al., (1999) reported Tetracycline is one of the broad-spectrum antibiotics that are available in feed supplements, and its improper use led to the development of multiple antibiotic resistances. Antibiotics may be administered to whole flocks rather than individual animals, and antimicrobial agents may be continuously fed to food animals such as broilers and turkeys as antimicrobial growth promoters. Therefore the antibiotic selection pressure for resistance in bacteria in poultry is high and consequently their faecal flora contains a relatively high proportion of resistant bacteria (Caudry et al., 1979). There is strong evidence that the use of antimicrobial agents can lead to the emergence and dissemination of resistant *E. coli* (Linton et al., 1977; Bogaard et al., 2001), which can then be passed onto people via food or through direct contact with animals and there are increasing numbers of reports detailing circulation and amplification of antimicrobial resistance genes (Adhikari et al., 2000), which could facilitate the emergence and spread of antibiotic resistance in bacteria.

Much of the antibiotic used in humans and animals remains unmetabolized and thus a significant amount is added to the environment via excretion. This ultimately contributes to the residues of antibiotics in recipient waters. Antibiotics might also be added to the environment from pharmaceutical plants and as a result of the dumping of unused antibiotics (Kummerer, 2009 and Rooklidge, 2004)

Idia et al., (2006) reported that 43% of *E. coli* were resistance to Nalidxic acid and Johnson et al., (2003) reported 37% in United states and which is accordance with this result and found lower than Hydrabad ,India (92.6%) in drinking water (Patoli et al., 2010). *E. coli* isolates were resistant to Nalidxic acid is important considering that the fluoroquinolones are used to treat a range of *E. coli* infections in humans (Thielman and Gurrent, 1999) and mainly used in the treatment of urinary tract infections (Olaniran et al., 2009).

There was no significance relation between thermo tolerance property of *E. coli* with MDR (P=0.779), and with NAR (P=0.800). MDR isolates were found higher in well and spring water than tap water where thermotolerant *E. coli* isolates had high MDR 62.5% and 81.2% than *E. coli* isolates in tap and well water respectively. Among the sources high MDR-NAR were found in spring water followed by well water and tap

water respectively. MDR *E. coli* and thermotolerant *E. coli* were found maximum due to the resistance to Tetracycline and Amoxicillin followed by Cefexime where the significant relation between the MDR and NAR expression of *E. coli* (P=0.012) and thermotolerant *E. coli* (P=0.012) was detected.

The increased antimicrobial used in animal husbandry may results the high level of resistance towards them and faeces from animal, chicken serves as the source of antibiotic resistance organisms as use of antibacterial agents creates selective pressure for the emergence of resistant strains. Similarly, the contamination by hospital waste to water sources, indiscriminate use of antibiotic in human and agriculture and unmanaged disposal of antibiotics in environment emerges the resistance bacteria in nature and aquatic environment and water serves as genetic pool for antibiotic resistance microorganism and transformation. Most multidrug-resistant isolates exhibited resistance to a combination of antimicrobial agents that included Tetracycline and Amoxicillin which may suggest that *E. coli* strains that are Tetracycline resistant are also at increased risk for becoming resistant to additional antimicrobial agents. Resistance to Tetracycline may be conserved in bacterial populations over time, regardless of selection pressure, which might result in an overall increase in resistance over time.

Antibiotic residues in hospital effluent and in other environmental niches have been conducted mostly in high-income countries, while studies in low- and middle-income settings are few and sparsely distributed (Kummerer, 2009; Duong et al, 2008), so must be recommended.

Increased incidence level of resistance of *E. coli* to Nalidxic acid and Amoxicillin, Cefexime and Tetracycline can create the public health problem and heralds in the therapeutic treatment of infections and outbreak of drug resistance.

In plasmid profiling of thermotolerant *E. coli* isolated from tap water, single band of plasmid were observed in three MDR isolates and one non-MDR isolate and size varied from 2kb to >10kb were obtained. All NAR thermotolerant *E. coli* were found to harbor plasmid. All NAR *E. coli* had found to contain single plasmid. Single >10kb plasmid was found in the thermotolerant *E. coli* having resistance to Nalidxic acid and Tetracycline. Thermotolerant *E. coli* from the two different places, Kirtipur and Bhakatpur having common antibiotic resistance to Amoxicilin, Nalidxic acid and Tetracycline were found to contain single 2 kb plasmid. And the thermotolerant *E. coli* having resistance to Amikacin, Amoxcillin and Tetracycline has found to contain

2.1 kb plasmid. Some isolates were resistant to antibiotics but they did not possess any plasmid band. In Amoxillin and Tetracycline resistance thermotolerant *E. coli* and 2 thermotolerant *E. coli* having resistance to Amoxicillin, Cefexime and Tetracycline, plasmids were not detected. Thermotolerant *E. coli* resistance to four antibiotics, Amoxcilin, Amikacin, Cefexime and Tetracycline plasmid was not detected.

Similar result was documented as some carried single plasmid and some carrying no plasmids which correlates with the results of Rahman et al., (2008); Lee et al., (2000) and Ozakabir et al., (2010). Ahmadi et al., (2008), reported that each of the twenty drug resistant *E. coli* harboured a single plasmid from the MDR cases with Nalidxic acid, Tetracycline with different sizes (Ahmadi et al., 2008) from Amikacin resistant (Ozakabir et al., 2010).

In the study of Alam et al., (2010) plasmid of 0.5 to 40 kb in size from *E. coli* different sources isolates having one or more were detected and among the 8 isolates of water 4 isolates showed plasmid bands. Similarly, One or more plasmid bands between 2 kb and $\geq$ 12 kb had been detected (Miles et al , 2006) of plasmids of molecular sizes ranging from 1.4kb to 4.5kb among the acute diarrhea causing *E. coli* (Kalanter et al . 2011).Smaller size of Nalidxic acid resistance plasmid had found correlate with results of (Enabulele, 2006) who reported molecular weight of the plasmids ranged from $\leq$2.9 kbp to $\leq$5.5 kbp from the Qunolone resitance organism along with single plasmid of size 2.9 and 3 kb in Nalidxic acid resistance *E. coli* and Nandy et al., (2010) reported that a smaller plasmids (<20 kb) with distinct patterns are found in Fluoroquinolone (FQ)-resistant *S. dysenteriae* which are frequently isolated yearly (2000-2007) in Kolkata.

In present study, thermotolerant *E. coli* resistance to four antibiotics and two and three antibiotics but did not harbor any plasmid. This supposition has been supported by the finding that the plasmidless strains may also be resistant to one or more antibiotics. Therefore, there was no noticeable correlation between antibiotic resistance patterns and plasmid patterns and accordance with (Miles et al., 2006; Alam et al., 2010; Rahman et al , 2008).

In this study, thermotolerant *E. coli* from the two different places, Kritipur and Bhakatpur which lies very apart in Kathmandu having common antibiotic resistance to Amoxcilin, Nalidxic acid and Tetracycline were found to contain single 2 kb plasmid which may suggest of common polluting source in piped water system, as Nalidxic acid is resistance which may be the hospital waste contamination.

The transmission of resistance plasmids of *Escherichia coli* from poultry to human intestines commonly occurs (Tabatabaei et al., 2003) vial direct handling or through food and water to resident gut flora and to *Salmonella* and *Shigella* has been found (Corliss et al.,1981) which creates emerging public health problem .

In this study, plasmid were found in all Nalidxic acid resistance organism and it is against quinolone resistance involves chromosomal mutations that reduce membrane permeability and decrease drug accumulation or alter DNA topoisomerases, resistance to Fluoroquinolones is most associated with mutations in DNA gyrase (Webber et al., 2001) and suggest that plasmid encoded resistance to Nalidxic acid is emerging issue to the Nepal and World. This finding highlights the need to monitor Quinolone resistant bacteria as emergence is important public health concern and resistance dissemination.

6.2 Conclusion

In conclusion, tap water of some places and most of the well and spring sources in Kathmandu is contaminated with drug resistance *E. coli* and plasmid mediated resistance to Nalidixic acid has emerged indicating possible outbreak of drug resistance enteric bacteria.

CHAPTER VII

SUMMARY AND RECOMMENDATIONS

7.1 SUMMARY

1. This study was conducted at Laboratory of Central Department of Microbiology from January 2011-August 2011. A total of 66 water samples were collected randomly from different drinking water sources, 28 from tap water, 24 from well water and 14 from spring water.
2. Total coliform growth was found higher in spring water (85.7%), followed by well water (79.2%) then tap water (60.7%). Similarly, the thermotolerant coliform growth was found higher in well water (87.5%, followed by spring water (71.4%) the tap water (67.9%).
3. In well and spring water sources 66.7% and 64.3% of samples was contaminated with *E. coli* and thermotolerant *E. coli* both where as 21.4% and 50% tap water was detected with *E. coli* and thermotolerant *E. coli* respectively.
4. From the total coliform and thermotolerant coliform 64.6 % *E. coli* and 66 % thermotolerant *E. coli* were detected respectively.
5. Minimum *E. coli* load was $\log_{10}0.95423$ in tap water and maximum load was $\log_{10}2.667453$ in well water. Thermotolerant *E. coli* load was minimum $\log_{10}0.90309$ in tap water and maximum $\log_{10}2.65896$ in well water. Average count of *E. coli* was $\log_{10}1.549518$, $\log_{10}1.959942$ and $\log_{10}2.13545$ and thermotolerant *E. coli* was $\log_{10}1.992076$, $\log_{10}2.054940$ and $\log_{10}2.179505$ in tap, well and spring water sources respectively.
6. There was no significant relation between water sources with presence of coliform (P=0.155) and thermotolerant coliform (P=0.235) but significant difference on presence of *E. coli* (P=0.002) and thermotolerant *E. coli* (P=0.011).
7. There was no significant relation on presence of coliforms and thermotolerant coliforms within sources, for well (P=0.521) and spring (P=0.505) but significant relation in tap water (P=0.010). There was no significant association on presence of coliforms and presence of *E. coli* for tap (P=0.055) and for spring water (P=0.110) but significant association in presence of coliforms and presence of *E. coli* for well water (P=0.01). The significant relation in presence of thermotolerant

coliform and presence of thermotolerant *E. coli* was found for all sources (P=0.029) in tap, (P=0.028) in well, (P=0.05) in spring water.

8. All *E. coli* and thermotolerant *E. coli* isolates were susceptible to Ofloxacin, Chloramphenicol and Cotrimixazole. Resistance to Cefexime, Amikacin and Nalidixic acid, Amoxicillin, Tetracycline were 54.8%, 29% 35.5%, 80.6%, 93.5% and 57.6%, 36.4%, 39.4%, 94%, 100% was observed in *E. coli* and thermotolerant *E. coli* respectively. Twenty two (70.9%) and 11 (35.5%) *E. coli* isolates expressed MDR and NAR, whereas, 25 (75.8%) and 13 (39.4%) of thermotolerant *E. coli* isolates expressed MDR and NAR. There was no significance relation between thermo tolerance property of *E. coli* with MDR (P=0.779), and with NAR (P=0.800)

9. MDR isolates were found higher in well and spring water than tap water but thermotolerant *E. coli* isolates had high MDR 62.5% and 81.2% than *E. coli* isolates in tap and well water respectively. Among the sources high MDR-NAR were found in spring water followed by well water and tap water respectively.

10. The significant relation between the MDR and NAR expression of *E. coli* (P=0.012) and thermotolerant *E. coli* (P=0.012) was detected.

11. All tap water thermotolerant *E. coli* isolates where subjected for plasmid analysis. Single band of plasmid were observed in three MDR isolates and one non-MDR isolate and size varied from 2 kb to >10 kb were obtained. All NAR thermotolerant *E. coli* were found to harbor plasmid.

7.2 RECOMMENDATION

1. For all sources thermotolerant *E. coli* (or alternatively thermotolerant) coliform is reliable indicator for faecal pollution.
2. Since MDR enteric bacterial load is high in potable water therefore antibiotic residue must be monitor in drinking water, sewage and treatment plants.
3. Nalidxic acid along with Amoxicillin and Tetracycline must be reduced in use for husbandry.
4. NAR *E. coli* must be taken under surveillance as it emerges with plasmid resistance.
5. In future, the gene for the NAR resistance in *E. coli* should be sequenced.

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APPENDIX- I

LIST OF MATERIALS

1. Equipments Used

1. Autoclave - Life steriware, India
2. Electric balance - Explorer
3. Hot air oven - Indoexim
4. Incubator- Indoexim
5. Laminar airflow cabinet – Gindal instruments
6. Membrane filter apparatus -
7. Microscope - Olompus
8. pH meter – Hanna (H198107)
9. Refrigerator - Godrej
10. Water double distillation plant - JSGW
11. Water bath shaker – Grant, OLS 200
12. Cold Centrifuge - Ependroff
13. Micropipette – Status, Thermoelectron
14. UV light apparatus(Multiimage™ Light Cabinet)– Alpha, Innotech Corporation
15. Horizontal gel documentation apparatus - IBI
16. Voltage generator- Fisher Scientific (FB 300)
17. Membrane filtration apparatus - Millipore

2. Microbiological / Biochemical Media

1. Nutrient Agar (Hi-Media)
2. EMB Agar (Hi-Media)
3. McConkey Agar (Hi-Media)
4. Sulphide Indole Motility Medium (Hi-media)
5. MR-VP Broth(Hi-media)
6. Simmon Citrate Agar (Hi-media)
7. Triple Sugar Iron Agar (Hi-media)
8. Urease Agar (Hi-media)
9. Mueller Hinton Agar (Hi-Media)
10. Mueller Hinton Broth (Hi- Media)
11. Peptone (Hi-Media)
12. Yest extract (Hi-Media)

13. Tryptone (Hi-Media)

14. Agarose (Hi-Media)

3. Chemicals and Reagents

1. Lysol
2. Gram's iodine
3. Oxidase reagent
4. Kovac's reagent
5. Alpha –naphthol
6. Potassium hydroxide
7. Conc. Sulfuric acid
8. Crystal violet
9. Saffranin
10. Hydrogen peroxide
11. Methyl red
12. Ethanol
13. Barium Chloride
14. Sodium Chloride

4. Extraction / Lysis buffer and Solution

Alkaline lysis Solution I: 100 ml

	(Mol. Wt)	(for 100 ml)
Tris (25 mM)	121.1	0.303 gm
EDTA (10 mM)	372.0	0.372 gm
Glucose (50 mM)	180.16	0.901 gm

Alkaline lysis Solution II:

NaOH 0.2 N (Freshly prepared from 10 N stock solution)

SDS 1.0%

Alkaline lysis Solution III :

5 M potassium acetate	60 ml
Glacial acetic acid	11.5 ml
H2O(dd)	28.5 ml

TE buffer (1 x) from 10 X stock solution/ liter

100 mM Tris-Cl (pH 8.0)

1 mM EDTA (pH 8.0)

Electrophoresis buffer

TAE buffer (1X) from 50x stock solution/ liter

Tris- base – 242 gm

Glacial acetic acid- 57.1 ml

EDTA – 100 ml of 0.5 M (pH 8)

EtBr (1 mg/ml) Stock solution

EtBr- 1 mg

dd H2O- 10 ml

Gel loading dye (6X)

0.25% (W/V) Bromophenol blue

0.25% (W/V) Xylan cyanol FF

30% (V/V) Glycerol in dd water

5. Glassware (Borosil)

1. Pipette
2. Funnels
3. Beakers
4. Test Tubes
5. Petri dishes
6. Conical Flasks
7. Glass Rod
8. Reagent Bottle
9. Microscopic Slides
10. Graduated Cylinders
11. Screw Capped Test Tubes

6. Miscellaneous

1. Transport Tray
2. Forceps
3. Immersion Oil
4. Dropper
5. Measuring Scale
6. Cotton Role
7. Aluminium Foil
8. Tissue Paper
9. Inoculating Loop
10. Cotton-Swab
11. Membrane Filter Paper
12. Blotting Paper
13. Sampler (Sample Collecting Bottles)
14. Centrifuge tubes
15. Colony counter
16. Detergent
17. Labelling tape

7. Standard organisms

Escherichia coli ATCC 25922

APPENDIX- II

METHODOLOGY OF BIOCHEMICAL TESTS USED FOR IDENTIFICATION OF BACTERIA

A. Catalase test

This test is performed to demonstrate the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide. During aerobic respiration, in the presence of oxygen, microorganisms produce hydrogen peroxide, which is lethal to the cell itself. The enzyme catalase splits hydrogen peroxide to water and oxygen.The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria, the main exception being *Streptococcus* spp.

Procedure: A small amount of a culture from Nutrient Agar plate was taken in a clean glass slide and about 2-3 drops of 3% H2O2 was put on the surface of the slide. The positive test is indicated by the formation of active bubbling of the oxygen gas. A false positive reaction may be obtained if the culture medium contains catalase (e.g. Blood Agar) or if an iron wire loop is used.

B. Oxidase test

This test is performed for the detection of cytochrome oxidase in bacteria which catalyzes the transport of electrons between electron donors. In the presence of redox dye Tetramethyl-*p*-phenylene diamine dihydrochloride, the cytochrome oxidase oxidizes it into a deep purple colored end product Indophenol which is detected in the test. The test is used for screening species of *Neisseria, Alkaligenes, Aeromonas, Vibrio, Campylobacter* and *Pseudomonas* which give positive reactions and for excluding the Enterobacteriaceae, all species of which give negative reactions.

Procedure: A piece of filter paper was soaked with few drops of oxidase reagent (Whatman's No. 1 filter paper impregnated with 1% tetramethyl-p-phenylene diamine dihydrochloride). Then the colony of the test organism was smeared on the filter paper. The positive test is indicated by the appearance of blue-purple color within 10 seconds.

C. Indole Production test

This test detects the ability of the organism to produce an enzyme: 'tryptophanase' which oxidizes tryptophan to form indolic metabolites: indole, skatole (methyl indole) and indole acetic acid. The enzyme tryptophanase catalyses the deamination reaction

attacking the tryptophan molecule in its side chain and leaving the aromatic ring intact in the form of indole.

Procedure: A smooth bacterial colony was stabbed on SIM (Sulphide Indole Motility) medium by a sterile stab wire and the inoculated media was incubated at 37°C for non thermotolerent and 44.5°C for thermotolerent organism for 24 hours. After 24 hours incubation, 2-3 drops of Kovac's reagent was added. Appearance of red color on the top of media indicates indole positive. Indole if present combines with the aldehyde present in the reagent to give a red color in the alcohol layer. The color reaction is based on the presence of the pyrrole structure present in indole.

D. Methyl Red test

This test is performed to test the ability of an organism to produce and maintain stable acid end product from the fermentation of glucose to give a red color with the indicator methyl red and to overcome the buffering capacity of the system. Medium used in the study was Clark and Lubs medium (MR/VP broth, pH 6.9). Methyl red is an indicator which is already acid and will denote changes in degree of acidity by color reactions over a pH range of 4.4- 6.0.

Procedure: A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37°C for non thermotolerent and 44.5°C for thermotolerent organism for 24 hours. After incubation, about 5 drops of methyl red reagent was added and mixed well. The positive test was indicated by the development of bright red color, indicating acidity and negative with yellow color.

E. Voges-Proskauer (VP) test

The principle of this test is to determine the ability of some organisms to produce an acetyl methyl carbinol, a neutral end product (acetoin) or its reduction product 2, 3-butanidiol during fermentation of carbohydrates. An organism of the Enterobacteriaceae group is usually either methyl red positive and Voges- proskauer-negative or methyl red negative and Voges-Proskauer positive. The Voges proskauer test for acetoin is used primarily to separate *E. coli* from *Klebsiella* and *Enterobacter* species.

Procedure: A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37°C for non thermotolerent and 44.2 °C for thermotolerent organism for 24 hours. After incubation, about 5 drops of Barritt's reagent was added and shaken well for maximum aeration and kept for 15 minutes, positive test is indicated by the development of pink red color.

F. Citrate Utilization test

This test is performed to detect whether an organism utilizes citrate as a sole source of carbon for metabolism with resulting alkalinity. The medium used for citrate fermentation (Simmon's Citrate medium) also contains inorganic ammonium salts. Organisms capable of utilizing citrate as its sole carbon source also utilizes the ammonium salts present in the medium as its sole nitrogen source, the ammonium salts are broken down to ammonia with resulting alkalinity.

Procedure: A loopful of test organism was streaked on the slant area of Simmon's Citrate Agar medium and incubated at 37°C for non thermotolerent and 44.5°C for thermotolerent organism for 24 hours. A positive test was indicated by the growth of organism and change of media by green to blue, due to alkaline reaction. The pH indicator bromothymol blue has a pH range of 6.0-7.6, i.e. above pH 7.6; a blue color develops due to alkalinity of the medium.

G. Motility test

This test is done to determine if an organism was motile or non-motile. Bacteria are motile by means of flagella. Flagella occur primarily among the bacilli; however a few cocci forms are motile. Motile bacteria may contain single flagella. The motility media used for motility test are semisolid, making motility interpretations macroscopic.

Procedure: Motility of organism was tested by hanging drop and cultural method. In cultural method, the test organism was stabbed in the SIM medium and incubated at 37ºC for 48 hours. Motile organisms migrate from the stabline and diffuse into the medium causing turbidity. Whereas non-motile bacteria show the growth along the stabline, and the surrounding media remains colorless and clear.

H. Triple Sugar Iron (TSI) Agar Test

The TSI agar is used to determine the ability of an organism to utilize specific carbohydrate incorporated in the medium (glucose, sucrose and lactose in concentrations of 0.1%, 1.0% and 1.0% respectively), with or without the production of gas (indicated by cracks in the media as well as an air gap at the bottom of the tube) along with determination of possible hydrogen sulfide production (detected by production of black color in the medium). A pH indicator (phenol red) included in the medium can detect acid production from fermentation of these carbohydrates and it gives yellow reaction at acidic pH, and red reaction to indicate an alkaline surrounding.

Procedure: The test organism was streaked and stabbed on the surface of TSI and incubated at 37°C for non thermotolerent and 44.5°C for thermotolerent organism for 24 hours. Acid production limited only to the butt region of the tube is indicative of glucose utilization, while acid production in slant and butt indicates sucrose or lactose fermentation. The results are interpreted as follows:

Yellow (Acid)/ Yellow (Acid), Gas, H_2S → Lactose/ Sucrose fermenter, H_2S producer.

Red (Alkaline) / Yellow (Acid), No Gas, No H_2S → Only Glucose, not lactose/ Sucrose fermenter, not aerogenic, No H_2S production.

Red (Alkaline) / No Change → Glucose, Lactose and Sucrose non-fermenter.

Yellow (Acid)/ No Change → Glucose- Oxidiser.

No Change / No Change →Non-fermenter.

I. Urea Hydrolysis test:

This test demonstrates the urease activity present in certain bacteria which decomposes urea, releasing ammonia and carbon dioxide. Ammonia thus produced changes the color of indicator (phenol red) incorporated in the medium.

Procedure: The test organism was inoculated in a medium containing urea and the indicator phenol red. The inoculated medium was incubated at 37°C for non thermotolerent and 44.5°C for thermotolerent organism overnight. Positive organism shows pink red color due to the breakdown of urea to ammonia. With the release of ammonia the medium becomes alkaline as shown by a change in color of the indicator to pink.

J. MUG Hydrolysis Test

This test demonstrates the β- Glucuronidase activity present in certain bacteria which decomposes MUG (4-Methylumbelliferone glucuronide) releasing fluorescent end–product methylumbelliferone. The end product was detected with long wave ultraviolet (UV) light.

Procedure: The test organism was inoculated in a medium containing flurogenic substrate MUG (4-Methylumbelliferone glucuronide). The inoculated medium was incubated at 37°C for non thermotolerent and 44.5°C for thermotolerent organism overnight.

APPENDIX- III

ZONE SIZE INTERPRITATION CHART OF ANTIBIOTIC SUSCEPTIBILITY TESTING

Antibiotics used	Symbol	Disc content (mcg)	Diameter of zone of inhibition in mm		
			Resistant	Intermediate	Sensitive
Amoxycillin	Am	10	13	14-17	18
Amikacin	Ak	30	14	15-16	17
Cefexime	Cfx	5	15	16-18	19
Chloramphenicol	C	30	12	13-17	18
Cotrimoxazole	Co	25	10	11-15	16
Nalidixic Acid	NA	30	13	14-18	19
Ofloxacin	Of	5	15	16-20	21
Tetracycline	T	30	14	15-18	19

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

APPENDIX-IV

SAMPLE DISTRIBUTION

S.no	Place	Source	Date	Sample code	Coliform	Thermotolerent coliform	*E.coli* (log_{10}cfu /100ml)	Thermotolrent *E.coli* (log_{10}cfu /100ml)
1	Kritipur	Tap	02/01/11	KT1	+	+	2.31387	2.46982
2	Kritipur	Tap	02/01/11	KT2		+		2.51322
3	Kritipur	Tap	05/01/11	KT3	+	+	1.93952	2.02938
4	Kritipur	Tap	05/01/11	KT4	+	+		
5	Kritipur	Tap	05/01/11	KT5	+	+		2.6149
6	Kupandole	Tap	09/01/11	KupT1	+	+		
7	Kupandole	Tap	13/01/11	KupT2	+			
8	Kupandole	Tap	13/01/11	KupT3	+	+	0.95424	2.38917
9	Kupandole	Tap	13/01/11	KupT4		+		1.20412
10	Jhamshikel	Tap	16/01/11	JT1		+		
11	Jhamshikel	Tap	16/01/11	JT2				
12	Jhamshikel	Tap	16/01/11	JT3				
13	Jhamshikel	Tap	19/01/11	JT4	+	+		
14	Jhamshikel	Tap	19/01/11	JT5	+			
15	Swambhu	Tap	24/01/11	ST1				
16	Swambhu	Tap	24/01/11	ST2				
17	Swambhu	Tap	24/01/11	ST3	+	+		
18	Swambhu	Tap	27/01/11	ST4				
19	Swambhu	Tap	27/01/11	ST5				
20	Balkhu	Tap	06/02/11	BalT1	+	+	1.20412	
21	Balkhu	Tap	06/02/11	BalT2	+	+	1.80618	0.90309
22	Balkhu	Tap	10/02/11	BalT3	+	+		
23	Balkhu	Tap	10/02/11	BalT4				
24	Balkhu	Tap	10/02/11	BalT5		+		
25	Bhaktpur	Tap	17/02/11	BhtT1	+	+		
26	Bhaktpur	Tap	17/02/11	BhtT2	+	+		1.81291
27	Bhaktpur	Tap	22/02/11	BhtT3	+	+	1.07918	
28	Bhaktpur	Tap	22/02/11	BhtT4	+	+		
29	Kritipur	Well	02/01/11	KW1	+	+	2.10037	1.54407
30	Kritipur	Well	02/01/11	KW2	+	+	2.19312	
31	Kritipur	Well	06/01/11	KW3	+	+	1.79239	1.62325
32	Kritipur	Well	06/01/11	KW4	+	+	1.83885	1.4624
33	Kupandole	Well	09/01/11	KupW1	+	+	2.66745	2.65896
34	Kupandole	Well	11/01/11	KupW2	+	+	2.42975	2.45484

35	Kupandole	Well	11/01/11	KupW3		+		2.65514
36	Jhamshikel	Well	16/01/11	JW1	+	+	1.86332	2.46687
37	Jhamshikel	Well	16/01/11	JW2		+		1.54407
38	Jhamshikel	Well	18/01/11	JW3	+	+	1.5563	2.42325
39	Jhamshikel	Well	18/01/11	JW4		+		
40	Jhamshikel	Well	18/01/11	JW5	+	+		
41	Swambhu	Well	24/01/11	SW1				
42	Swambhu	Well	27/01/11	SW2	+		1.66276	
43	Swambhu	Well	27/01/11	SW3	+	+	1.39794	1.5682
44	Swambhu	Well	27/01/11	SW5	+	+		1.88081
45	Balkhu	Well	08/02/11	BalW1	+		1.57978	
46	Balkhu	Well	08/02/11	BalW2	+	+	2.09691	1.98227
47	Balkhu	Well	13/02/11	BalW3	+	+	2.0607	2.08279
48	Balkhu	Well	13/02/11	BW4	+	+		
49	Bhaktpur	Well	22/02/11	BhtW1	+	+	1.94939	2.07555
50	Bhaktpur	Well	22/02/11	BhtW2	+	+	2.02119	2.0569
51	Bhaktpur	Well	27/02/11	BhtW3	+	+	2.38202	2.39967
52	Bhaktpur	Well	27/02/11	BhtW4		+		
53	Kritipur	Spout	06/01/11	KD1				
54	Kritipur	Spout	06/01/11	KD2	+	+	2.33244	
55	Kritipur	Spout	06/01/11	KD3		+		2.29226
56	Kupandole	Spout	09/01/11	KupD1	+	+	2.5977	2.62839
57	Kupandole	Spout	11/01/11	KupD2	+	+	2.45637	2.21748
58	Kupandole	Spout	11/01/11	KupD3	+			
59	Swambhu	spout	24/01/11	SD1	+			
60	Swambhu	spout	24/01/11	SD2	+	+	2.02119	2.07188
61	Balkhu	Spout	14/02/11	BalD1	+	+	1.89763	1.716
62	Balkhu	Spout	15/02/11	BalD2	+	+	1.98227	1.54407
63	Bhaktpur	Spout	22/02/11	BhtD1	+	+	1.57978	2.42813
64	Bhaktpur	Spout	22/02/11	BhtD2	+	+	2.51188	2.53782
65	Bhaktpur	Spout	27/02/11	BhtD3	+		1.83885	
66	Bhaktpur	Spout	27/02/11	BhtD4	+	+		

APPENDIX- V

STATISTICAL ANALYSIS

Non parametetic test (Chi-Square test and Fisher exat test) was used in this study for the analysis and interpretation of data.

1. Source versus Total Coliform

Crosstab				
Count				
		Total Coliform		Total
		absent	present	
Source	Tap	11	17	28
	Well	5	19	24
	Spring	2	12	14
Total		18	48	66

Chi-Square Tests			
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	3.729[a]	2	.155
Likelihood Ratio	3.778	2	.151
Linear-by-Linear Association	3.416	1	.065
N of Valid Cases	66		
a. 1 cells (16.7%) have expected count less than 5. The minimum expected count is 3.82.			

2. Source verses Thermotolerent Coliform

Crosstab				
Count				
		Thermotolerent Coliform		Total
		absent	present	
Source	Tap	9	19	28
	Well	3	21	24
	Spring	4	10	14
Total		16	50	66

Chi-Square Tests			
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.896[a]	2	.235
Likelihood Ratio	3.108	2	.211
Linear-by-Linear Association	.354	1	.552
N of Valid Cases	66		
a. 1 cells (16.7%) have expected count less than 5. The minimum expected count is 3.39.			

3. Source verses *E. coli*

Crosstab				
Count				
		E. coli		Total
		absent	present	
Source	Tap	22	6	28
	Well	8	16	24
	Spring	5	9	14
Total		35	31	66

Chi-Square Tests			
	Value	Df	Asymp. Sig. (2-sided)
Pearson Chi-Square	12.757[a]	2	.002
Likelihood Ratio	13.355	2	.001
Linear-by-Linear Association	9.289	1	.002
N of Valid Cases	66		
a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 6.58.			

4. Source verses Thermotolerent *E. coli*

Crosstab				
Count				
		Thermotolerent *E. coli*		Total
		absent	present	
Source	Tap	20	8	28
	Well	8	16	24
	Spring	5	9	14
Total		33	33	66

Chi-Square Tests			
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	8.952[a]	2	.011
Likelihood Ratio	9.190	2	.010
Linear-by-Linear Association	6.460	1	.011
N of Valid Cases	66		
a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 7.00.			

5. Tap coliform verses Tap thermotolerent coliform

Crosstab				
Count				
		Tap thermotolerent Coliform		Total
		absent	present	
Tap coliform	absent	7	4	11
	present	2	15	17
Total		9	19	28

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	8.239[a]	1	.004		
Continuity Correction[b]	6.032	1	.014		
Likelihood Ratio	8.429	1	.004		
Fisher's Exact Test				.010	.007
Linear-by-Linear Association	7.945	1	.005		
N of Valid Cases[b]	28				
a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 3.54.					
b. Computed only for a 2x2 table					

6. Tap coliform verses Tap *E. coli*

Crosstab				
Count				
		Tap *E. coli*		Total
		Absent	present	
Tap coliform	absent	11	0	11
	present	11	6	17
Total		22	6	28

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	4.941[a]	1	.026		
Continuity Correction[b]	3.067	1	.080		
Likelihood Ratio	7.022	1	.008		
Fisher's Exact Test				.055	.033
Linear-by-Linear Association	4.765	1	.029		
N of Valid Cases[b]	28				
a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 2.36.					
b. Computed only for a 2x2 table					

7. Tap thermotolerent coliform verses Tap thermotolerent *E. coli*

Crosstab				
Count				
		Tap Thermotolerent *E. coli*		Total
		Absent	present	
Tap thermotolerent Coliform	absent	9	0	9
	present	11	8	19
Total		20	8	28

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	5.305[a]	1	.021		
Continuity Correction[b]	3.443	1	.064		
Likelihood Ratio	7.639	1	.006		
Fisher's Exact Test				.029	.024
Linear-by-Linear Association	5.116	1	.024		
N of Valid Cases[b]	28				
a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 2.57.					
b. Computed only for a 2x2 table					

8. Well coliform verses Well thermotolerent coliform

Crosstab				
Count				
		Well Thermotolrent Coliform		Total
		Absent	present	
Well coliform	absent	1	4	5
	present	2	17	19
Total		3	21	24

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.325[a]	1	.569		
Continuity Correction[b]	.000	1	1.000		
Likelihood Ratio	.294	1	.588		
Fisher's Exact Test				.521	.521
Linear-by-Linear Association	.311	1	.577		
N of Valid Cases[b]	24				
a. 3 cells (75.0%) have expected count less than 5. The minimum expected count is .63.					
b. Computed only for a 2x2 table					

9. Well coliform verses Well *E. coli*

Crosstab				
Count				
		Well *E. coli*		Total
		Absent	present	
Well coliform	absent	5	0	5
	present	3	16	19
Total		8	16	24

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	12.632[a]	1	.000		
Continuity Correction[b]	9.126	1	.003		
Likelihood Ratio	13.979	1	.000		
Fisher's Exact Test				.001	.001
Linear-by-Linear Association	12.105	1	.001		
N of Valid Cases[b]	24				
a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 1.67.					
b. Computed only for a 2x2 table					

10. Well thermotolerent coliform verses Well thermotolerent *E. coli*

Crosstab				
Count				
		Well Thermotolerent *E. coli*		Total
		absent	present	
Well Thermotolrent Coliform	absent	3	0	3
	present	5	16	21
Total		8	16	24

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	6.857[a]	1	.009		
Continuity Correction[b]	3.857	1	.050		
Likelihood Ratio	7.500	1	.006		
Fisher's Exact Test				.028	.028
Linear-by-Linear Association	6.571	1	.010		
N of Valid Cases[b]	24				
a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 1.00.					
b. Computed only for a 2x2 table					

11. Spring coliform verses Spring thermotolerent coliform

Crosstab				
Count				
		Spring Thermotolerent Coliform		Total
		Absent	present	
Spring Coliform	absent	1	1	2
	present	3	9	12
Total		4	10	14

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.525[a]	1	.469		
Continuity Correction[b]	.000	1	1.000		
Likelihood Ratio	.483	1	.487		
Fisher's Exact Test				.505	.505
Linear-by-Linear Association	.488	1	.485		
N of Valid Cases[b]	14				
a. 3 cells (75.0%) have expected count less than 5. The minimum expected count is .57.					
b. Computed only for a 2x2 table					

12. Spring coliform verses Spring *E. coli*

Crosstab				
Count				
		Spring *E. coli*		Total
		Absent	present	
Spring Coliform	absent	2	0	2
	present	3	9	12
Total		5	9	14

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	4.200[a]	1	.040		
Continuity Correction[b]	1.569	1	.210		
Likelihood Ratio	4.753	1	.029		
Fisher's Exact Test				.110	.110
Linear-by-Linear Association	3.900	1	.048		
N of Valid Cases[b]	14				
a. 3 cells (75.0%) have expected count less than 5. The minimum expected count is .71.					
b. Computed only for a 2x2 table					

13. Spring thermotolerent coliform verses Spring thermotolerent *E. coli*

Crosstab				
Count				
		Spring Thermolerent *E. coli*		Total
		absent	present	
Spring Thermotolerent Coliform	absent	4	0	4
	present	1	9	10
Total		5	9	14

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	10.080[a]	1	.001		
Continuity Correction[b]	6.541	1	.011		
Likelihood Ratio	11.748	1	.001		
Fisher's Exact Test				.005	.005
Linear-by-Linear Association	9.360	1	.002		
N of Valid Cases[b]	14				
a. 3 cells (75.0%) have expected count less than 5. The minimum expected count is 1.43.					
b. Computed only for a 2x2 table					

14. Organism verses MDR

Crosstab				
Count				
		MDR *E. coli*		Total
		Non MDR	MDR	
Organism	*E. coli*	9	22	31
	Thermotolerent *E. coli*	8	25	33
Total		17	47	64

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.188[a]	1	.665		
Continuity Correction[b]	.023	1	.880		
Likelihood Ratio	.188	1	.665		
Fisher's Exact Test				.779	.440
Linear-by-Linear Association	.185	1	.667		
N of Valid Cases[b]	64				
a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 8.23.					
b. Computed only for a 2x2 table					

15. Organism verses NAR

Crosstab				
Count				
		NAlidixic acid resistance		Total
		No NA resistance	NA resistance	
Organism	*E. coli*	20	11	31
	Thermotolerent *E. coli*	20	13	33
Total		40	24	64

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.104[a]	1	.747		
Continuity Correction[b]	.004	1	.949		
Likelihood Ratio	.104	1	.747		
Fisher's Exact Test				.800	.475
Linear-by-Linear Association	.103	1	.749		
N of Valid Cases[b]	64				
a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 11.63.					
b. Computed only for a 2x2 table					

16. MDR *E. coli* verses NAR *E. coli*

Crosstab				
Count				
		NAR-*E. coli*		Total
		NO-NAR	NAR	
MDR *E. coli*	NO MDR	9	0	9
	MDR	11	11	22
Total		20	11	31

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	6.975[a]	1	.008		
Continuity Correction[b]	4.962	1	.026		
Likelihood Ratio	9.826	1	.002		
Fisher's Exact Test				.012	.008
Linear-by-Linear Association	6.750	1	.009		
N of Valid Cases[b]	31				
a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 3.19.					
b. Computed only for a 2x2 table					

17. **MDR Thermotolerent *E. coli* verses NAR- Thermotolerent *E. coli***

Crosstab				
Count				
		NAR- Thermotolerent *E. coli*		Total
		NO-NAR	NAR	
MDR Thermotolerent *E. coli*	NO MDR	8	0	8
	MDR	12	13	25
Total		20	13	33

Chi-Square Tests					
	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	6.864[a]	1	.009		
Continuity Correction[b]	4.859	1	.028		
Likelihood Ratio	9.634	1	.002		
Fisher's Exact Test				.012	.009
Linear-by-Linear Association	6.656	1	.010		
N of Valid Cases[b]	33				
a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.15.					
b. Computed only for a 2x2 table					

APPENDIX- VI

SEMI LOG GRAPH FOR PLASMID SIZE DETERMINATION

Plasmid size was determined by semi log graph with according to (CIBT, 2008).

1. From the gel photo (or stained gel) the distance in millimeters measure from the well to each of the bands in the molecular weight markers.
2. The bottom axis labeled as the distance migrated. The units on this axis will be millimeters.
3. The Y-axis labeled molecular weight. Here the units will be kilo basepairs (kb) in log scale. Therefore, the paper is called semi-log because it is linear on one axis (the X-axis) and logarithmic on the other.
4. The standard curve "best fit curve" was drawn from the points.

APPENDIX- VII

NEPAL DRINKING WATER QUALITY STANDARDS (2005) AND WHO (2010) GUIDELINES

List of Parameters and concentration limits for potable dinking water

S.no.	Category	Parameters	Units	WHO Guideline Value for potable drinking water	National Drinking Water Quality Standard
				Conc. limits	Conc. limits
1	Physical	Temperature	^{o}C	-----	-----
2		pH	------	6.5-8.5	6.5-8.5
3	Microbiological	Total Coliform	CFU/100	0	0 in 95% samples
		Thermotolerant Coliform	CFU/100	0	0