OCCURRENCE AND DISTRIBUTION OF GRAM NEGATIVE BACTERIA IN CHLORINATED DRINKING TAP WATER AND THEIR SUSCEPTIBILITY TO CHLORINE AND ANTIBIOTICS

A

DISSERTATION

SUBMITTED TO THE CENTRAL DEPARTMENT OF MICROBIOLOGY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DEGREE OF MASTER OF SCIENCE IN MICROBIOLOGY (ENVIRONMENT AND PUBLIC HEALTH)

BY

SURAJ BISHANKHA

Central Department of Microbiology

Tribhuvan University

Kirtipur, Kathmandu

Nepal

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RECOMMENDATION

This is to certify that **Mr. Suraj Bishankha** has completed his dissertation work entitled "**Occurrence and distribution of gram negative bacteria in chlorinated drinking tap water and their susceptibility to chlorine and antibiotics**" as a partial fulfillment of Master of Science Degree in Microbiology under our supervision. To our knowledge this work has not been submitted for any other degree.

Dr. Dwij Raj Bhatta, M. Sc., Ph. D.	**Mr. Dev Raj Joshi**	**Ms. Tista Prasai Joshi**
Associate Professor	Lecturer	Scientific Officer
Central Department of Microbiology	Central Department of Microbiology	Nepal Academy of
Tribhuvan University	Tribhuvan University	Science and Technology
Kirtipur, Kathmandu	Kirtipur, Kathmandu	Khumaltar, Lalitpur

Date: - 2010-11-30

CERTIFICATE OF APPROVAL

On the recommendation of **Dr. Dwij Raj Bhatta**, Associate Professor, **Mr. Dev Raj Joshi**, Lecturer and **Ms. Tista Prasai Joshi**, Scientific Officer, this dissertation work of **Mr. Suraj Bishankha** entitled "**Occurrence and distribution of gram negative bacteria in chlorinated drinking tap water and their susceptibility to chlorine and antibiotics**" has been approved for the examination and is submitted to the Tribhuvan University in partial fulfillment of the requirements for M. Sc. Degree in Microbiology.

Dr. Dwij Raj Bhatta, M. Sc., Ph. D.
Head of Department
Central Department of Microbiology
Tribhuvan University
Kirtipur, Kathmandu
Nepal

Date: - 2010-11-30

BOARD OF EXAMINERS

Recommended by:

Dr. Dwij Raj Bhatta, M. Sc., Ph. D.
Supervisor

Mr. Dev Raj Joshi
Supervisor

Ms. Tista Prasai Joshi
Supervisor

Approved by:

Dr. Dwij Raj Bhatta
Head of Department

Examined by:

External Examiner
Mr. Gyanendra Bahadur Karki
Chief of Water Quality Section
KUKL

Internal Examiner
Mr. Binod Lekhak
Lecturer

Date: - 2010-12-29

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Suraj Bishankha

Date: - 2010-11-30

ABSTRACT

Viable microorganisms are often recovered from potable chlorinated water distribution systems; suggesting the presence of chlorine-resistant microorganisms. Chlorine and antibiotic resistant microorganisms pose a global concern in sanitation and public health. The objective of the study was to explore the occurrence and distribution of gram negative bacteria in chlorinated drinking tap water and their susceptibility to chlorine and antibiotics.

This study was conducted at Environment and Climate Change Laboratory of Nepal Academy of Science and Technology from April 2009 to August 2010. A total of 107 water samples were collected randomly from Bansbari (52) and Mahankalchaur (55) treatment and distribution plant. Physico-chemical parameters of drinking water like pH, turbidity, conductivity, temperature, free residual chlorine, combined residual chlorine and total residual chlorine and microbiological parameters like heterotrophic plate count and total coliform count were conducted by standard methods. Chlorine resistance and antibiotic susceptibility test of selected isolates were performed by liquid assay and modified Kirby Bauer disc diffusion method respectively.

Sixty (56.1%) water samples crossed the permissible limit of WHO guideline value in heterotrophic plate count and total coliform count each. Ten different genera of gram negative bacteria were recovered in which *E. coli* was predominant followed by *Citrobacter* spp., *Shigella* spp., *Enterobacter* spp., *Providencia* spp., *Klebsiella* spp., *Salmonella* spp., *Pseudomonas* spp., *Proteus* spp. and *Edwardsiella* spp. Higher the temperature of water sample, higher the bacterial growth was obtained ($p= 0.002$) and similarly higher level of free residual chlorine in water reduced the bacterial growth ($p= 0.037$) whereas increase or decrease of pH ($p= 0.454$), turbidity ($p= 0.164$) and conductivity ($p= 0.969$) didn't affect the microbial growth. A negative correlation ($r= -0.162$) between heterotrophic plate count and free residual chlorine was observed however without statistical significance ($p= 0.096$). Similarly, a negative correlation $r= -0.383$) between total coliform count and free residual chlorine was observed with statistical significance ($p= 0.001$). In chlorine assay, all tested eight different genera of gram negative bacteria were found to be chlorine resistant at 0.2 mg/lt for a contact time of 30 minutes. All of the tested gram negative chlorine resistant bacteria were found to be antibiotic resistant to at least one antibiotic. Average time required for $T_{99.9}$ (3-log) and $T_{99.99}$ (4-log) reduction of viable isolates from initial population of 2×10^6 cells/ml were found to be less than 30 minutes and greater than 60 minutes respectively. Log inactivation of various bacterial isolates with chlorine concentration of Nepal Standard (0.2 mg/lt for a contact time of 30 minutes) were found to be ranged from 3 to 3.5-log. Among the isolates, Ampicillin was found to be 100% resistant followed by Amoxycillin, Cotrimoxazole, Tetracycline, Ceftazidime, Ceftriaxone, Chloramphenicol, Gentamicin and Ofloxacin. The isolates of water samples were found to 78.7% Antibiotic resistant (AR) and 27.7% Multiple antibiotic resistant (MAR).

Emergence of chlorine and antibiotic resistant organisms in drinking water probably demands alternate disinfection or mitigation strategy.

Keywords: Antibiogram, Chlorine Resistance, Drinking Water, Disinfection, Gram Negative Bacteria

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ABBREVIATIONS

ADB	:	Asian Development Bank
AOB	:	Ammonia Oxidizing Bacteria
APHA	:	American Public Health Association
AR	:	Antibiotics Resistance
ATCC	:	American Type Culture Collection
CBS	:	Central Bureau of Statistics
CDC	:	Centre for Disease Control
CFU	:	Colony Forming Unit
CRC	:	Combined Residual Chlorine
DBP	:	Disinfection By-Product
DoHS	:	Department Of Health Services
DPD	:	*N, N*-Diethyl-*P*-Phenylenediamine
DWSS	:	Department Of Water Supply and Sewerage
ENPHO	:	Environment and Public Health Organization
EPA	:	Environmental Protection Agency
EPS	:	Extra-Polymeric Substances
FRC	:	Free Residual Chlorine
HAA	:	Haloacetic Acid
HPC	:	Heterotrophic Plate Count
KUKL	:	Kathmandu Upatyataka Khanepani Limited
MAR	:	Multiple Antibiotic Resistance
MF	:	Membrane Filter
MHA	:	Mueller Hinton Agar
MLD	:	Million Liters per Day
MoH	:	Ministry Of Health
MoPE	:	Ministry Of Population and Environment

MPN	:	Most Probable Number
MPPW	:	Ministry of Physical Planning and Works
MR	:	Methyl Red
NA	:	Nutrient Agar
NAST	:	Nepal Academy of Science and Technology
NB	:	Nutrient Broth
NTU	:	Nephelometric Turbidity Unit
NWSC	:	Nepal Water Supply Corporation
O.D.	:	Optical Density
PPM	:	Parts Per Million
SIM	:	Sulphide Indole Motility
TCBS	:	Thiosulphate Citrate Bile Salt Sucrose Agar
TCC	:	Total Coliform Count
THM	:	Trihalomethane
TRC	:	Total Residual Chlorine
TSB	:	Triptic Soy Broth
TSI	:	Triple Sugar Iron Agar
TSS	:	Total Suspended Solids
UN	:	United Nation
UNESCO	:	United Nation Education and Science Cultural Organization
UNICEF	:	United Nation International Children's Emergency Fund
UV	:	Ultra Violet
VP	:	Voges Proskauer
WHO	:	World Health Organization
WSP	:	Water Safety Plan
XLD	:	Xylose Lysine Deoxycholate Agar
ZOI	:	Zone Of Inhibition

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CHAPTER I

1. INTRODUCTION

Life on earth depends on water. Our planet is only one where water is known to exist in liquid form; water is a unique solvent that carries the nutrients essential for life. How people use the land and change ecosystems affects the quality, movement and distribution of water (Beazley, 1993).

Safe water is essential for life itself. Around 1.2 billion people around the world lack access to safe drinking water, and twice that many lack adequate sanitation. As a result, the World Health Organization estimates that 3.4 million people, mostly children, die every year from water-related diseases (WHO, 2002). Many of water borne diseases can be prevented with appropriate water treatment and proper sanitation and hygiene practices. Increasing access to safe water can improve more than public health.

In Nepal, the total cases of Diarrhoea was 1,398,106 and among them 206 diarrhoeal death occurred in the fiscal year (FY) 2064/065(2007/2008). Incidence of the diarrhoea per 1000 below 5 years population was 378. Among top ten reasons for hospitalization, on FY 2064/065(2007/2008), diarrhoeal and gastroenteritis of presumed infectious origin becomes the 2nd leading causes and accounts 5.40% of total patients hospitalized. Increase in the incidence of diarrhoeal diseases starts from the month of march and continues till july and then starts to decline gradually, this trend was observed similar for FY 062/63,063/64 and 064/65 (DoHS, 2007/2008).

Kathmandu Valley, the Capital city of Nepal is the most populated and has most of the facilities a modern city demands, but water borne epidemics including cholera are almost regular here. A total of 306 persons, out of 4,724 admitted for acute gastroenteritis from mid-April to end of July 2007 in various hospitals of Kathmandu were confirmed of cholera cases. Five of them including three children lost their lives due to cholera (MPPW, UN HABITAT and GUTHI, 2007). Although almost 90%

Kathmanduties have access to municipalities' piped water supply, people are still suffering from diarrhoeal diseases (KUKL, 2010).

Disinfection is an important final step in drinking water treatment and is the principle barrier for preventing pathogen breakthrough into water supply systems. Additionally, a disinfectant residual has to be maintained in water distribution systems to inhibit pathogen growth or survival. Coliforms have served as indicator organisms for pathogen in drinking waters for decades (Goel and Bouwer, 2004).

Chlorination is the most widely used method of disinfection for community water distribution systems and reservoirs; however there may be an increased resistance of bacterial strains to chlorine inactivation. Furthermore, bacteria may be partially damaged by exposure to sublethal levels of chemical biocides. This may manifest as the inability to grow and form colonies and result in an underestimation of bacterial contamination (Ridgway and Oslon, 1982 and Howard and Inglis, 2003).

Detection of coliforms in high-quality treated waters is due to ineffective disinfection and/or bacterial regrowth in distribution system. Factors that can influence disinfection efficacy include disinfectant concentration and contact time (Ct), temperature, p^H, chemical composition of raw and treated waters, the presence of sediments and biofilms, the physiological state of microbes, the presence of excessive amounts of polysaccharide, growth rate and nutritional status. Research has shown that dilution of nutrient media and the presence of surfaces for bacterial growth can lead to greater bacterial resistance to disinfection (LeChevallier, 1990; Stewart and Oslon, 1992; Power *et al.,* 1997; Le Dantec *et al.,* 2002 and Srinivasan and Harrington, 2007).

Bacteria from chlorinated systems were more resistant to chlorine than those from the unchlorinated waters (Geldreich, 1996). Chlorination at the usual levels results in the low levels of bacteria in suspension, but this kind of treatment is not enough to prevent growth and development of microbial biofilms on the inner surfaces of the water distribution system (Codony *et al.*, 2005).

Free chlorine has been suggested to be the water quality parameter that reacts with the widest range of contaminants (USEPA, 2003). Chlorination when properly designed and operated, the process is well-developed, inexpensive and efficient. However several drawbacks of chlorine-based disinfection systems have been identified, including the formation of (potentially) hazardous disinfection by-products and the discovery of waterborne microbial pathogens that are relatively resistant to chlorine inactivation. These factors define the risks to be balanced in the design of disinfection systems, and dictate (new) optimization strategies for chlorination (Shang and Blatchley, 2001).

Antibiotic-resistant bacteria (mainly *Enterobacteriaceae*) and antibiotics are discharged in various amounts in the environment as a result of the increasing and indiscriminate use of antibiotics in medical, veterinary and agricultural practices. River waters are the main receptacle for these pollutants, since they receive the sewage of urban effluents. As rivers are one of the major sources of water, directly or indirectly, for human and animal consumption, this pollution may contribute to the maintenance and even the spread of bacterial antibiotics resistance (Goni-Urriza *et al*., 2000).

The percentage of multiple antibiotic resistant (MAR) bacteria was found to be significantly greater among isolates from distribution water samples than that of bacteria in corresponding untreated source waters. The selective factors operating in the aquatic environment of a water treatment facility can act to increase the proportion of antibiotic resistant members of the heterotrophic plate count (HPC) bacterial population in treated drinking water (Armstrong *et al*., 1982).

Therefore this study was carried out to assess the physicochemical and bacterial quality of drinking water and to explore susceptibility of gram negative bacterial isolates in municipal drinking water supplies of Kathmandu Metropolitan City to chlorine and antibiotics.

CHAPTER II

2. OBJECTIVES

2.1 General objective

To study the occurrence and distribution of gram negative bacteria in chlorinated drinking tap water and their susceptibility to chlorine and antibiotics.

2.2 Specific objective

1. To assess the physicochemical parameters (temperature, p^H, conductivity, turbidity, free residual chlorine, combined residual chlorine and total residual chlorine) and bacterial quality of drinking tap waters.
2. To describe frequency of gram negative bacteria in drinking tap waters.
3. To determine chlorine resistance and antibiotic susceptibility among bacterial isolates.
4. To correlate chlorine resistance at 0.2 mg/lt concentration for a contact time of 30 minutes and multiple antibiotic resistance pattern of bacteria.

CHAPTER III

3. LITERATURE REVIEW

3.1 Safe Drinking water

Safe drinking water has been one of the proud achievements of developed countries and an important milestone that less developed countries are trying to achieve. According to rough estimates, more than 15 million deaths worldwide result annually from waterborne infections. Diarrhoeal diseases of small children alone result in more than 2 million deaths annually in developing countries (Young, 1996). The outbreak of cholera in South America in 1991-1995 resulted in more than 1 million cases and over 10,000 fatalities.

Cholera, typhoid fever and amoebic dysentery can easily kill otherwise healthy adults. Milder forms of waterborne infections, spread likewise by fecal contamination of drinking water, normally cause only discomfort and less serious illness in healthy adults, but they can be serious and easily fatal in very young children, in elderly and in persons debilitated by other illnesses, especially AIDS.

Providing safe drinking water is a complex problem that goes far beyond a treatment step. In many countries, the quality of the distribution system is so poor that the initially chlorinated water becomes unsafe before it reaches the consumer. The upgrading of the distribution system is a much more complex and expensive problem than the initial treatment of the water. In addition to the physical upgrading of treatment and distribution systems, education, sanitary engineering and monitoring of water safety are essential elements in upgrading water quality (Atlas and Bartha, 2000).

3.2 Water Distribution System in Kathmandu Valley

Piped water system was established in 1895 in Kathmandu and Bhaktapur followed by Lalitpur in 1904 (UN HABITAT, 2008). 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 m^3/day) and the wet season is 150 MLD (150,000 m^3 /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.

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.

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 9,900,000m^3/annum and the smallest is at Kuleshwor with a treatment capacity of 40,000 m^3/annum. Most of the WTPs are in poor condition and none has operational flow meters or properly operating chlorination equipment. A study called "Improvement Measures for Water and Wastewater Treatment Process" carried out under the TA-7007 by Eng. Chistopher Hagenbruch (September 2008) states that there are 26 water treatment plants that supply water to the Kathmandu Valley using both surface and groundwater sources. The total estimated treatment capacity of the plants under KUKL is reported to be 85 MLD. Table 3.1 summarizes the features and existing approximate capacities (design) of the major water treatment plants in the Kathmandu Valley Water Supply System.

Table 3.1 Major water treatment plants in Kathmandu Valley water supply system

S.N.	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	MAHANKALCHAUR 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 LALITPUR
7	SAINBU FILTRATION PLANT	FILTRATION	6	LALITPUR

Source: "Improvement Measures for Water and Wastewater Treatment Process" Eng. Chistopher Hagenbruch (September 2008)

There are a total of 43 service reservoirs in the water supply system with capacities ranging from 4,500m^3 down to 50 m^3. Most of the reservoirs are in reasonable condition but two are leaking. The storage capacity is 40,910m^3.

There are 31 water supply pumping stations in the system that are used to draw water from sump wells to treatment plants or service reservoirs, and to fill up reservoirs located on higher ground or overhead tanks. Of these only 11 are in satisfactory condition. Few have operational flow meters or pressure gauges.

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 I, June 2009 and ADB II, November 2009).

3.3 Study of Physicochemical Parameters of Drinking Water

Ridgway and Olson (1982) studied a chlorine resistance pattern of bacteria from two drinking water distribution systems in which the mean free residual chlorine, temperature, pH, conductivity and turbidity were found to be 0.73±0.14ppm, 17.5±4.96 ^oC, 7.77±0.44, 836±246.0 µS/cm and 0.42±0.19 NTU respectively of Irvine system.

Bajracharya *et al.* (2007) assessed the drinking water quality of Kathmandu Metropolitan Area. Out of 114 water samples, 38 samples were of tap water in which the mean temperature, pH, conductivity and turbidity were found to be 26.86 ^oC, 7.07, 132.37 µS/cm and 5.98 NTU respectively. Conductivity of 5.26% samples and turbidity

of 36.84% samples were found to be above WHO guideline value but pH of 100% samples was found to within WHO guideline value.

Jayana (2007) assessed the drinking water quality of Madhyapur Thimi. Out of 105 water samples, 45 (42.85%) were of tap water in which the mean temperature, pH, conductivity and turbidity were found to be 21^{o}C, 7.3, 368 µS/cm and 16.4 NTU respectively. pH of 3 (6.66%) were found to be below the WHO guideline value. Conductivity of 5 (11.11%) and turbidity of 7 (15.55%) were found to be above the WHO guideline value.

Prasai *et al.* (2007) conducted a microbiological analysis of drinking water of Kathmandu valley. Of 132 water samples, 17 samples were of tap water in which the mean temperature, pH, conductivity and turbidity were found to be 24.1^{o}C, 6.8, 198.29 µS/cm and 4.9 NTU respectively.

Diwakar *et al.* (2008) assessed the drinking water quality of Kathmandu Metropolitan Areas. Of 114 water samples, 31 samples were of public tap water in which the mean temperature, pH, conductivity and turbidity were found to be 24.32^{o}C, 7.36, 197.073 µS/cm and 12.7 NTU respectively. pH of 100% samples was found to be within WHO guideline value.

Gopali (2008) studied the microbial quality of chlorinated water of Kathmandu. Out of 114 tap water samples, pH and conductivity of 114 (100%) samples were found to be above WHO guideline value. Turbidity of 15.17% samples was found to be above WHO guideline value. Free residual chlorine of 18.42% samples fall within, 2.63% samples above and 78.94% below WHO guideline value among 38 samples from Sundarighat reservoir.

Aryal (2009) studied drinking water of Kathmandu Valley. Out of 102 tap water samples, the mean temperature and pH were found to be 18.69 ^{o}C and 7.37 respectively.

Manandhar (2009) assessed the drinking water quality of sub-urban regions of Kathmandu. Of 102 samples, 40 (40.8%) samples were of tap water in which the mean temperature, pH, conductivity and turbidity were found to be 18.88^{o}C, 7.49, 190.80 μS/cm and 2.8 NTU respectively.

Shrestha (2009) studied drinking water samples of Urban Water Supply System of Kathmandu district. Of 86 tap water samples, the mean temperature and pH were found to be 19.25^{o}C and 7.5 respectively. pH of 5(5.81%) samples was below WHO guideline value and 6 (6.98%) samples was above the WHO guideline value.

3.4 Water borne organisms

Water borne organisms are **Bacteria** (*Burkholderia pseudomallei, Campylobacter jejuni, C. coli, Escherischia coli-Pathogenic, E. coli Enterohaemorrhagic, Legionella* spp*., Non-tuberculous mycobacteria, Pseudomonas aeruginosa, Salmonella* Typhi*, other Salmonellae, Shigella* spp*., Vibrio cholerae and Yersinia enterocolitica*), **Viruses** (Adenoviruses, Enteroviruses, Hepatitis A viruses, Hepatitis E viruses, Noroviruses and sapoviruses and Rotaviruses), **Protozoa** (*Acanthamoeba* spp*., Cryptosporidium parvum, Cyclospora cayetanesis, Entamoeba histolytica, Giardia intestinalis, Naegleria fowleri and Toxoplasma gondii*) and **Helminths** (*Drancunculus medinensis* and *Schistosoma* spp.) (WHO, 2006).

Different study showed that the intestinal pathogens found in contaminated drinking water are *Escherichia coli*, *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp., *Salmonella* spp., *Shigella* spp., *Proteus* spp*., Pseudomonas aeruginosa* and *Vibrio* spp. which revealed that Gram negative were found predominant over the Gram positive bacteria (Bajracharya *et al*., 2007; Jayana, 2007; Kafle, 2007; Prasai *et al*., 2007; Gopali, 2008; Aryal, 2009 and Shrestha, 2009). Norton and Lechevallier (2000) also found that Gram reactions from all four distribution systems showed similar ratios of gram-positive organisms (average, 30%) to gram-negative organisms (average, 70%).

3.5 Water Disinfection

Water disinfection destroys harmful microorganism pathogens including bacteria, viruses, and protozoa. While certain pathogens can be eliminated through sedimentation and natural die-off by storage in open tanks, this is often unacceptable because of the resulting growth of algae and other sources of contamination. Disinfection is an important final step in drinking water treatment and is the principal barrier for preventing pathogen breakthrough into water supply systems.

There is the use of different disinfectant such as: i) Chlorination which is applied to water in one of three forms: Elemental chlorine (chlorine gas), Hypochlorite solution (bleach) or dry Calcium hypochlorite, ii) Chlorine-based alternative disinfectants such as Chloramines and Chlorine dioxide and iii) Non-chlorine alternative disinfectants such as Ozone and UV.

In the context of Nepal, majority of the population can't afford to use expensive technologies like UV radiation and Ozonation for water disinfection. Buying a filtration unit would mean an additional cost burden in their daily expenses and moreover a regular maintenance is necessary for its effective functioning. Similarly boiling water consumes more energy and time. Under such condition chlorination could be one of the best option of water disinfection overcoming the barriers of high cost, time and energy.

3.5.1 Disinfection using chlorine

Strategies for disinfection vary depending on the source – groundwater, surface water, or rainwater. Groundwater usually contains low levels of organics, consistent quality, and higher amounts of inorganic compounds. Rainwater is usually relatively clean, with the main contaminants coming from the atmosphere and to a lesser extent the roof or other collection surface (WHO, 2007). However, quality may still be deteriorated during harvesting and storage. Surface waters often have high levels of suspended materials and organics, variable quality, and higher levels of pathogens (M20 Manual of Water Supply Practices, 2006). Surface water treatment is also the most complicated of the three. The elevated levels of organics require greater chlorine concentrations and

longer contact times, and care must be taken to ensure harmful chlorination byproducts are either not formed or properly removed afterwards. Chlorine resistant organisms such as *Giardia* and *Cryptosporidium* further complicate treatment and ideally necessitate a combination strategy of coagulation, filtration, and disinfection. In order to measure disinfection efficacy, the concept of "Ct" is used. "C" is the free chlorine residual (in mg/L) and "t" is time (in minutes); the two terms are multiplied together to obtain a value. For instance, if water contains a 2 mg/L solution of free chlorine for 30 minutes, then the Ct value is 60 mg/L-min. Figure 3.1 shows an example of a Ct chart of disinfection of various microorganisms with a two-log disinfection efficiency (99% removal).

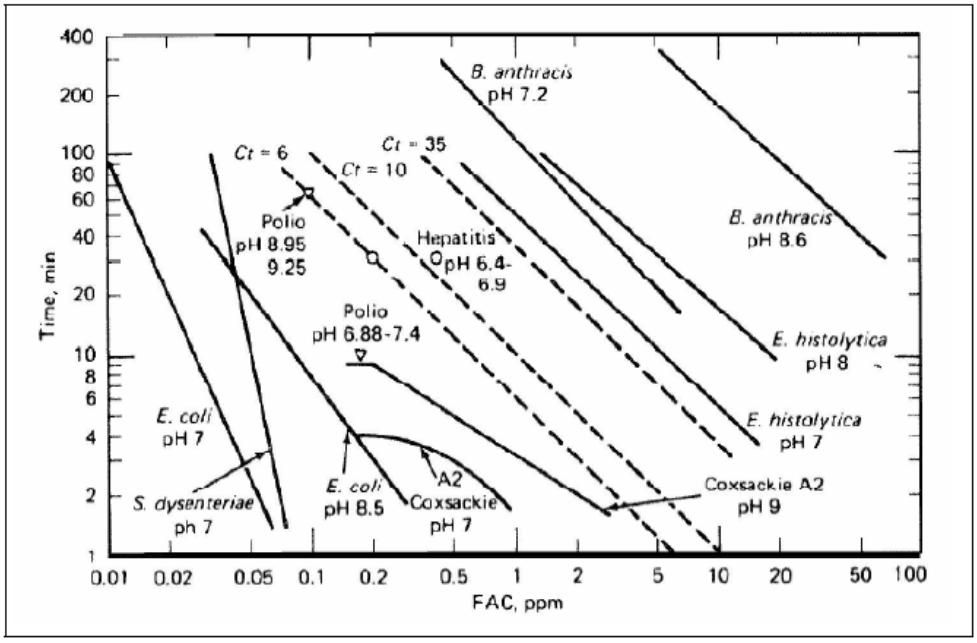

Figure 3.1 Disinfection (2 Log) of Selected Microorganisms Using Free Residual Chlorine (FRC)

3.5.2 Chlorine Chemistry

When chlorine is added in water, the two main disinfectant species formed are hypochlorous acid (HOCl) and hypochlorite ion (OCL^-). Together, they are called "free available" chlorine. Of the two, hypochlorous acid is the more effective disinfectant but

can be dissociated at high pH values. The following two equations show the relationships between solutions of calcium hypochlorite.

$$Ca(OCl)_2 + 2H_2O \longrightarrow Ca^{2+} + 2HOCl + 2OH^-$$
$$HOCl \longrightarrow H^+ + OCl^{--}$$

Any ammonia present in natural waters will react with the hypochlorous acid and hypochlorite ions to form chloramines. The chlorine demand of such reactions with ammonia is called the "combined chlorine." These reactions are important because chloramines are a comparatively poor disinfectant and thus alter the desired dosage requirements. The free and combined chlorine values are summed together to form "total chlorine". This assumption is applicable if no organic matter, phenols, or organic nitrogen are present in the water. If this is not the case, stable chlorine compounds can be formed and this relationship is invalid (USEPA, 1997).

3.5.3 Breakpoint

The presence of ammonia and the formation of chloramines also lead to break-point chlorination curves. As stated, chloramines are ineffective disinfectants. Thus the desired free chlorine concentration cannot be obtained until all ammonia has been converted.

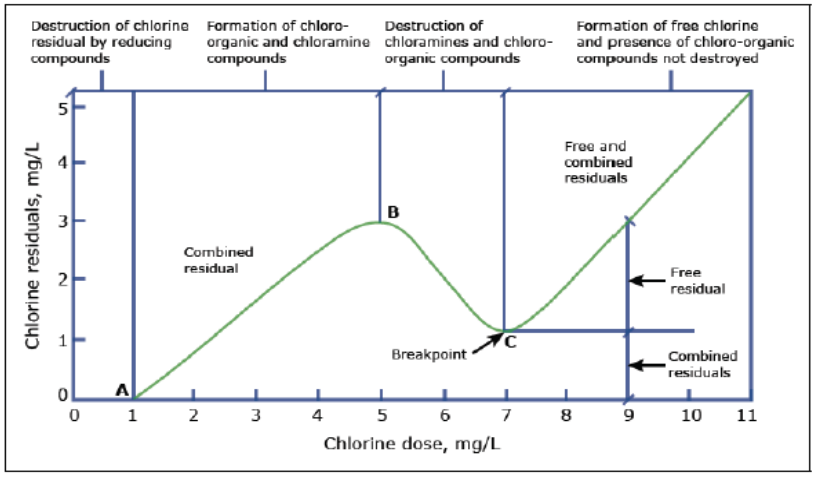

Figure 3.2 Typical Break-point Chlorination Curve (Metcalf & Eddy, 2003)

As shown, chlorine residual concentrations initially increase with chlorine dosing as chloramines are formed (A to B) but then begin to drop as these compounds are slowly destroyed (B to C). The point where all the ammonia has been converted is called the "break-point" (C). After this, additional chlorine leads to higher values of free chlorine (hypochlorous acid and hypochlorite). Each water source will have its own specific break-point based on pH, water temperature, ammonia concentrations, and contact time. Understanding this relationship is critical to proper disinfection, as the chlorine on the right side of the break-point is 25 times more potent than that of the left side (Harp, 1995). Many waters do not have a pronounced dip in the residual curve, but regardless of the shape of the breakpoint curve the presence of free residual chlorine is an indicator of adequate disinfection.

3.5.4 Chlorine Dose

The chlorine dose needs to be sufficient to provide a desired free residual beyond the water's demand. The demand is related to the impurities in the water and can vary considerably. The relationship between the two is linear and can be estimated by the following equation (Cairncross and Feachem, 1983):

Chlorine Demand (mg/L) = Chlorine Dose (mg/L) – Chlorine Residual (mg/L)

One thing to note is that even clean water is likely to have a chlorine demand of 2 mg/L (Cairncross and Feachem, 1983). In addition, chlorine residual should generally be in the range of 0.3 – 0.5 mg/L with a contact time of 30 minutes (WHO, 1997). Taste problems may occur at higher concentrations, and a lower concentration does not provide adequate protection.

3.5.5 Disinfection By-Products

During the 1970s, it was discovered that reacting chlorine with organic material resulted in disinfection by-products (DBPs), and would most likely occur with turbid water. The organics are usually humic and fulvic acids that originate in agricultural runoff and natural vegetation. The two classes of byproducts are trihalomethanes (THMs) and haloacetic acids (HAAs), both of which are suspected to be carcinogenic (M20 Manual

of Water Supply Practices, 2006). The risk of such compounds has become a concern since 1970s and have resulted in the regulation of THMs and the consideration of alternatives to the use of chlorine for disinfection. However, the WHO has said that **"**the risks to health from these by-products are extremely small in comparison with the risks associated with inadequate disinfection, and it is important that disinfection not be compromised in attempting to control such by-products**"** (WHO, 1993).

3.5.6 Chlorine Appropriate Situations

Even without considering the health risks of DBPs, chlorine is only appropriate in certain situations. A suitable and steady supply of chlorine must be available. The equipment must be strictly controlled and well-maintained to prevent erratic dosages. There should also be enough time (at least 30 minutes) between the addition of chlorine and the consumption of water. Though residual chlorine will naturally degrade over time, care must be taken to ensure consumption occurs within acceptable ranges. This is likely to be successful when a community has a steady supply of water and adequate storage capacity. On the other hand, there are circumstances where chlorine disinfection is unsuitable. These include when a regular supply of chlorine compounds cannot be expected, when specific populations are sensitive to DBPs and are likely to encounter them due to the characteristics of the water supply, when elimination of cysts or viruses is desired, or when careful monitoring is not anticipated (Parr *et al*., 2007).

There are several major important influent parameters necessary for appropriate chlorination. The first is turbidity. As turbidity measurements begin to rise above 5NTU, the turbidity can begin to interfere with disinfection or give rise to a significant chlorine demand (WHO, 1997). As a result, chlorination is often used as the last step in a series of treatments which might include a filtration or biological process in order to ensure appropriate turbidity values. If chlorination is attempted with turbid water, not only will DBPs form, but it will require more chlorine to be as effective. To date there is no easy way to predict an appropriate chlorine dose based on only a known influent

turbidity value, although the disinfection process becomes increasingly retarded as the turbidity values rise.

A second important parameter is pH. As shown in Figure 3.4, by the time the pH has reached a value of 8, nearly 80% of the effective disinfection mechanism of HOCl has been lost. This results from the dissociation sensitivity of the calcium hypochlorite into the less-effective form, hypochlorite ion.

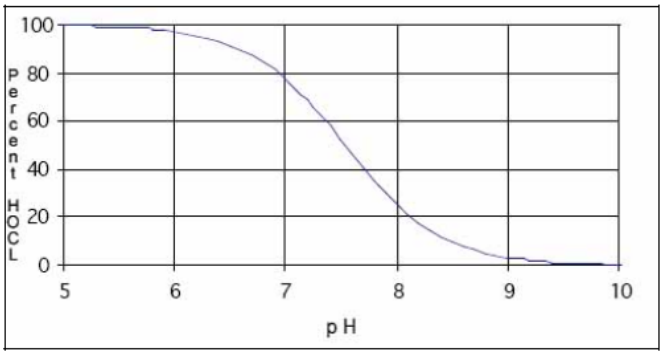

Figure 3.4 Effect of pH on Percent HOCL Formation (Meyer, 2004)

For safety reasons, additional constituents which are a concern if present include organic chemicals such as oils, greases, solvents, and other hydrocarbons. Chlorine reacts with many of these compounds in a violent or explosive way. In addition, chlorine compounds mixed with ammonia can cause potential explosions or toxic chlorine releases and should also be avoided (M20 Manual of Water Supply Practices, 2006).

3.6 Effect of Chlorine Disinfection on Distribution system

Lechevallier *et al*. (1981) found turbidity is a useful indicator of potential problems in drinking water. Components of turbidity associated with total organic carbon (TOC) exert a continuous interference with disinfection efficiency, maintenance of chlorine residual and microbiological determinations.

Ridgway and Olson (1982) compared the relative chlorine sensitivities of bacteria isolated from chlorinated and unchlorinated drinking water distribution systems and found that bacteria from the chlorinated systems were more resistant to both the combined and free forms of chlorine than those from the unchlorinated system, suggesting that there may be selection for more chlorine-tolerant microorganism in chlorinated waters. The most resistant microorganism were able to survive a 2 minutes exposure to 10 mg of free chlorine/lt include the gram positive spore forming bacilli, actinomycetes and some micrococci.

Calomiris *et al*. (1984) assessed the association of metal tolerance with multiple antibiotic resistance (MAR), bacterial isolates from the drinking water systems of an Oregon coastal community. Results suggested that simultaneous selection phenomena occurred in distribution water for bacteria which exhibited unique patterns of tolerance to Cu^{2+}, Pb^{2+} and Zn^{2+} and antibiotic resistance.

Kuchta *et al*. (1985) revealed that more natural populations of legionellae are much more resistant to cl_2 than their agar-passaged counterparts. The difference in resistance appears to be caused by physiological rather than genetic changes since even strains which have acquired greater cl_2 resistance by in-house chlorination lose resistance to levels comparable to organisms not previously exposed to cl_2. Changes in growth rate, cell density, differences in amounts or kinds of carbon and nitrogen sources and degree of nutrient limitation, in combination or with other factors probably contribute to the differences. It is likely that in actual practice higher dosages of cl_2 for longer contact times may be required to achieve disinfection of natural populations compared with agar medium- adapted strains.

Maki *et al*. (1986) enumerated heterotrophic bacteria from the Seattle drinking water and the highest bacterial recoveries were obtained by using a very dilute medium containing 0.01% peptone as the primary carbon source. Other factors favours increases recovery were the use of incubation temperatures close to that of the habitat and on extended incubation (28 days or longer provided the highest counts). The known genera

identified in the study were *Caulobacter, Hyphomonas, Aquaspirillum, Vibrio* and *Gluconobacter.*

Lechevallier *et al*. (1988) showed that the attachment of bacteria to surfaces provided the greatest increase in disinfection resistance. Other mechanisms which increased disinfection resistance included the age of the biofilm, bacterial encapsulation and previous growth condition which increased resistance to chlorine from 2 to 10-fold. These resistance mechanisms were multiplicative (i.e., the resistance provided by one mechanism could be multiplied by the resistance provided by a second mechanism).

K.C. (1992) analyzed drinking water quality of Shivapuri main source to its Panipokhari reservoir and treatment plant and its distribution systems of Kathmandu Valley. Altogether 27 samples from 7 different sectors were examined. In which sector 1, i.e. Shivapuri intake, Shivapuri Hill forest, Shivapuri Lipikot and Bishnumati Chisaney, FRC was zero because water was not chlorinated. Sector 2, i.e. water coming from Shivapuri source, boring water from Gangabu, both mixed water and treated water (both mixed) was found zero except treated water which was 0.1 ppm exceeding the WHO guideline but within Nepal standard. Sector 3, i.e. 4 different sub sites of Lazimpat showed 0.1, 0.1, 0, 0.1 ppm respectively. The entire sample crossed the WHO guidelines but three sites were within the Nepal standard. Sector 4, i.e. 2 sub sites of Bansbari showed nil being raw water and in 2 sub sites of Maharajgunj were also found to be nil exceeding the WHO guidelines. Sector 5, i.e. Tangal, Chandol, Chundevi and Lamgtanginii areas, FRC was 0.2, 0, 0 and 0 ppm respectively which showed that only Tangal was within Nepal standard but entire sample crossed WHO guideline. Sector 6, i.e. Mandikatar, Ringroad and Dhumbarahi areas, FRC were found nil. Sector 7, i.e. Bishalnagar, Nepal Rastra Bank, Russian Embassy and Balwatar areas, FRC was 0.2, 0.1, 0.1, 0.25 ppm respectively showed entire samples exceeded the WHO guidelines but within Nepal standard.

Hiraishi *et al.* (1995) conducted a research where strains of pink-pigmented methylotrophs which were isolated previously from various environments and assigned

to the genus *Methylobacterium.* Most of the isolates derived from Chlorinated water supplies exhibited resistance to chlorine, where 29 to 40 % of the isolates from air, natural aquatic environments and clinical materials were chlorine resistant. None of the tested authentic strains of *Methylobacterium* species obtained from culture collections exhibited chlorine resistance. Result demonstrates that there is phenotypic and genetic diversity among chlorine-resistant *Methylobacterium* strains within the genus.

Power *et al.* (1997) carried out a research in which a strain of *Klebsiella oxytoca* isolated from within Sydney's water distribution system was grown under a range of physiological conditions including batch cultures and continuous culture under both carbon and nitrogen limitation. Cells were then exposed to varying concentration of total chlorine. The initial growth condition affected the ability of cells to survive. Cells grown under batch condition in a medium containing minerals salts and yeast extract were the most resistant. The resistance to chlorine was not related to the presence of polysaccharide or differences in protein levels but to the formation of aggregates. The initial growth conditions also influenced the recovery of cells after the removal of chlorine stress.

Momba *et al*. (1999) revealed the effectiveness of chloramines in preventing the attachment of *E. coli* in developing biofilm.

According to Yildiz and Schooolnik (1999) the rugose colony variant of *Vibrio cholera O1 El Tor*, is shown to produce an exopolysaccharide, EPS ^{ETR} that confers chlorine resistant and biofilm forming capacity. EPS ^{ETR} production requires a chromosomal locus, Vps that contains sequences homologus to carbohydrate biosynthesis genes of other bacterial sps. Mutation within this locus yield chlorine-sensitive, smooth colony variants that are biofilm deficient.

Norton and Lechevallier (2000) conducted a pilot study in which a comparison of bacterial biofilms in pipe networks supplied with water containing either high level of biodegradable organic matter (BOM) or low levels of BOM (conventionally or

biologically treated, respectively). Prechlorination with free chlorine resulted not only in reduced plate count values but also in a dramatic shift in the composition of the bacterial population to predominately gram-positive bacteria. Chlorination of biologically treated water produced the same shifts towards gram-positive bacteria. Iron pipes stimulated the rate of biofilm development and bacterial levels on disinfected iron pipes exceeded those for chlorinated polyvinyl chloride pipes. The study showed that the iron pipes surface dramatically influenced the composition, activity and disinfection resistance of biofilm bacteria.

Shang and Blatchley III (2001) found that the pattern of residual chlorine decay following chlorination of the bacterial suspensions indicated rapid initial free chlorine consumption, followed by slow free chlorine consumption, with trace quantities of inorganic chloramine being formed.

Le Dantec *et al*. (2002) have reported that *Mycobacterium gordonae*, and atypical mycobacterium isolated from a water distribution system, is more resistant to chlorine in low-nutrient media, such as those encountered in water and that an increase in temperature (from 4^{o}C to 25^{o}C) and a decrease in pH result in better inactivation.

Howard and Inglis (2003) after exposure to chlorine, viable bacteria were undetectable by conventional plate count techniques; however, persistence of *Burkholderia pseudomallei* was verified by flow cytometry and bacteria were recoverable following a simple one-step broth procedure. Chlorine had a bacteriostatic effect only on *B. pseudomallei*, viable bacteria were recovered from water containing upto 1000 ppm free chlorine.

Lee and Kim (2003) conducted a research on bacterial species in biofilm and found that- a) the bacterial population in biofilm changed dramatically, b) the bacterial community was more diverse before the HPC level of the biofilm stabilized, c) *Micrococcus* sps and other gram-positive bacteria constituted a increase proportion of

the population and d) bacteria of potentially fecal origin may be accommodated and might grow in biofilm, especially in the end regions of water distribution systems.

Dussart *et al.* (2003) described the presence of *Pseudomonas oryzihabitans* adhering on suspended particulate matters recovered from Karst groundwaters. Adherent *P. oryzihabitans* cells displayed a high resistance to chlorine as compared with the organisms cultured in the planktonic mode. Results demonstrate that aquifer biofilms are potential environmental sources for water-born *P. oryzihabitans* infections and that bacterial attachment might affect drinking water purification.

According to Goel and Bouwer (2004) *Klebsiella pneumoniae* grown in dilutions of nutrient broth media were more resistant to chlorine and chloramines as compared to those grown in undiluted nutrient media, $C^*T_{99.9}$ values for chlorine increased > 20-fold and for chloramines increased 2.6 fold when nutrient broth solutions were diluted 100-fold (final TOC of 35-40 mg/lt). The impact of change in growth temperature was significant for chlorine but not for chloramines. Increase in growth temperature for unamended 2 NB cultures from 22.5^{o}C to 35^{o}C resulted in a 42 fold decrease in $C^*T_{99.9}$ values for chloramines.

Shrivastava *et al.* (2004) carried out a study in which chlorine resistant bacteria had mucoid colonies and grow better at 24^{o}C. Laboratory experiments using different strains of the *Pseudomonas aeruginosa* in distil water showed that only the resistant strain survived chlorine resistant at a dose of ≤ 500 mg/lt. Similar results were obtained when water collected from seven different sites on the River Gomti was treated with graded doses of chlorine.

Vitro *et al*. (2005) found out that extensive membrane damage is not a key event in the inactivation of bacteria by chlorine and confirm the observation of other authors that suggest that more subtle events, such as uncoupling of the electron chain or enzyme inactivation either in the membrane or in the cell interior, are involved in the bactericidal mechanism of chlorine.

Condony *et al*. (2005) carried out the study in which cell viability in the water phase in the presence of chlorine was low at the beginning of the experiment but increased 4 orders of magnitude after five neutralization periods. Therefore subsequent episodes of chlorine depletion may accelerate the development of microbial communities with reduced susceptibility to disinfection in real drinking water systems.

A study conducted by Moreno *et al*. (2007) revealed that *Helicobacter pylori* could survive to disinfection practices normally used in drinking water treatment in the viable but non culturable form (VBNC), which would allow them to reach final consumption points and at the same time, enable them to be undetectable by culture methods.

Helbling and VanBriesen (2007) determined that microorganism do exert a chlorine demand in proportion to their concentration. Chlorine resistant organisms also demand chlorine, and their resistance mechanisms are hypothesized to be attributed to extracellular material sacrificially reacting with free chlorine to reduce the concentration at the cell membrane or wall. In the organisms studied, chlorine resistant organisms demanded more chlorine than more sensitive organisms. Chlorine demand also increased with increasing free chlorine concentration. Maintenance of higher chlorine residuals in the distribution system will produce greater chlorine demand signals during contamination events and a greater chance of detecting biological contamination.

Bajracharya *et al*. (2007) showed that out of 38 tap sample of Kathmandu Metropolitan Areas, 73.68% of them have total coliforms.

Prasai *et al*. (2007) conducted a microbiological analysis of drinking water of Kathmandu valley. Of 132 samples, 17 were of tap water in which 14 (82.4%) and 17 (100%) samples were found to be above WHO guideline value for total count and total coliform count respectively.

Sarbatly and Krishnaiah (2007) found that despite the higher free residual chlorine concentration at the treatment plant and the intake point the results showed that the total coliform count was higher than the level suggested by Malaysian Water Association.

Diwakar *et al*. (2008) showed that out of 31 public tap of Bhaktapur Municipality, 11 were contaminated with total coliforms.

Gopali (2008) studied the microbial quality of chlorinated drinking water of Kathmandu and found that 65.78% of samples were above WHO guideline value for coliform in Sundarighat reservoir and 90.41% samples exceeded the standard permissible limit in Balaju reservoir. From the 38 water samples tested of sundarighat reservoir, the minimum FRC was 0 mg/lt in Teku whereas maximum FRC was 1.2 mg/lt in Kuleshwor. Among which 18.42% fall within, 2.63% was above and 78.94% below the WHO guideline value (0.2-0.5mg/lt). Similarly in water samples of Balaju reservoir the minimum FRC value was 0 mg/lt in 2 different areas i.e. MR campus, Tahachal and the maximum FRC was 0.09 mg/lt in Balaju which showed that all the samples had FRC value below the standard value set by WHO.

3.7 Antibiotic resistance among water borne bacterial isolates

Armstrong *et al*. (1981) analyzed drinking water from seven communities for multiply antibiotic resistant (MAR) bacteria. Overall, 33.9% of 2,653 standard plate count (SPC) bacteria for treated drinking waters were MAR. Isolates identification revealed that MAR gram-positive cocci (staphylococcus) and MAR gram negative, nonfermentative rods (*Pseudomonas, Alcaligenes, Moraxella*-like group M and *Acinetobacter*) were more common in drinking waters than in untreated source waters. Study concluded that the treatment of raw water and its subsequent distribution select for standard plate count bacteria exhibiting the MAR phenotype.

Armstrong *et al*. (1982) noted that the results showed rivers water contained microbial populations that were 15.8 and 18.2% MAR on dated when treated clear well water populations were 43.5 and 57.1% MAR respectively. Selection for bacteria exhibiting

resistance to streptomycin was achieved by chlorinating river water in the laboratory. We concluded that the selective factors operating in the aquatic environment of a water treatment facility can act to increase the proportion of antibiotic-resistant members of the SPC bacterial population in treated drinking water.

Ash *et al.* (2002) isolated antibiotic-resistant bacteria in freshwater samples from 16 U.S. Rivers at 22 sites. Over 40% of the bacteria resistant to more than one antibiotic had at least one plasmid. The most common resistant organisms belonged to the following genera: *Acinetobacter, Alcaligenes, Citrobacter, Enterobacter, Pseudomonas and Serratia.*

Shrivastava *et al*. (2004) undertook a study to investigate the spectrum of bacteria present in the River Gomti water before and after chlorination for drinking purposes. The strains of *Pseudomonas aeruginosa* that survived chlorination on three out of seven occasions were resistant to almost all the antibiotic tested.

Bhatta *et al*. (2006) examined 54 isolates of *Salmonella* spp. for resistance to different antibiotic. Many isolates of *Salmonella* Typhi, *Salmonella* Paratyphi A, *Salmonella* Typhimurium and *Salmonella* Enteritidis were multiple drug resistance (MDR) and also they were sensitive to ciprofloxacin and Ofloxacin. All the isolates of the *Salmonella* Enteritidis and four isolates of *Salmonella* Typhimurium were resistant to Ceftriaxone.

Kafle (2007) showed that out of 100 water samples 72% of sample had total coliforms and 625 contained fecal coliforms. Among the isolated bacteria, the Antibiotic Susceptibility Test results showed Ampicillin 100% resistant and Cephalexin 90% resistant. Multiple Antibiotic Resistant (MAR) as *E. coli* (20%), *Enterobacter* spp. (12%), *Citrobacter* spp. (5%), *Klebsiella* spp. (20%) and *Salmonella* spp. (25%).

Jayana (2007) assessed the drinking water quality of Madhyapur Thimi where out of 105 samples, 64.76% of samples crossed the WHO guideline value for total coliform count. Resistance of the isolates were found as 79.5% Erythomycin, 62.67 % Penicillin G, 61.9% Amoxycillin, 34.5% Ampicillin, 21.1% Tetracycline, 15.4% Ceftriaxone,

14.7% Amikacin, 14.7% Cephotaxime, 5.6% Chloramphenical, and 5.6% Ofloxacin respectively.

Shrestha (2008) assayed eight antibiotics against 33 isolates out of 296 from the drinking water of Kathmandu. The level of resistance exhibited by isolates to specific antibiotics was found as follows: Ampicillin (93.93%), Tetracycline (27.27%), Nitrofurantoin (24.24%), Cotrimoxazole (18.18%), Nalidixic Acid (15.15%).

Shrestha (2009) carriedout a studies on drinking water of Kathmandu district in which 86 (100%) samples crossed the WHO guideline value for coliform count and out of 86 water samples, 4 samples were positive for *Salmonella*. Antibiotic Susceptibility Test showed that all the isolates were 100% susceptibility to Tetracycline, Chloramphenicol, Cotrimoxazole, Nalidixic acid and Ciprofloxacin. 70% were resistant to amoxicillin, 20% to Cephalexin and 10% to Ceftizoxime.

Aryal (2009) revealed 86.1% and 19.6% of the samples contain total coliform and thermotolerant coliform respectively exceeding the WHO guideline. Antibiotic Sensitivity Test showed resistance of *E. coli* isolated towards Cephalexin (65%) followed by Amoxycillin (45%) and Tetracycline (15%).

CHAPTER IV

4. 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

The study was conducted from April 2009 to August 2010 in the Environment and Climate Change Laboratory of Nepal Academy of Science and Technology. The tap water samples were collected from 2 different drinking water distribution systems of Kathmandu which were Bansbari and Mahankalchaur treatment and distribution system.

4.2.1 Study 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^{o} 35' to 27^{o} 48' and longitude of 85^{o} 12' to 85^{o} 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. The length of Bagmati River within Kathmandu valley is 28 km. Bagmati, Manohara, Dhobikhola, Nagmati and Balkhu River are the main tributaries of Bagmati River. However, this study was conducted only in Kathmandu Metropolitan City.

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, June 2009; ADB II, November 2009).

4.2.2 Sample collection

Grab water samples were collected. Before the sample collection, any external fittings of tap if present were removed, the water was allowed to run to waste at a uniform rate for about 2-3 mins. Contamination was prevented during sampling. The grab water sample for microbial analysis was collected in sterilized plastic bottles containing sodium thiosulfate to a final concentration of 3% (w/v) to neutralize any free or residual chlorine where as for physicochemical analysis the water was collected in clean and dry plastic bottles. A total of one hundred and seven samples of drinking water were randomly collected from different place of Kathmandu Valley distributed from 2 different distribution points.

4.2.3 Sample transportation and preservation

The collected water samples were analyzed in Environment and Climate Change Laboratory of Nepal Academy of Science and Technology (NAST), Khumaltar, Lalitpur 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 ^{o}C.

4.2.4 Physico-chemical analysis of water sample

Analysis of most of the physico-chemical parameters of water were done by following "Standard Methods for the Examination of Water and Wastewater" (APHA, 1998). All the physicochemical parameters were analyzed in the Environment and climate change laboratory of NAST.

4.2.5 Temperature

Temperature was determined with the help of a standard mercury thermometer graduated up to 50^{0}C. Soon after collection of the sample, thermometer bulb was immersed into the water and noted the reading.

4.2.6 pH

Hydrogen ion concentration in the sample was measured with the help of a pH meter (TOA Electronics, Japan) by inserting the electrode into the water sample.

4.2.7 Turbidity

The turbidity of sample was measured with the help of a Nephelometer (Elico, India). The sample was put in the clean, free of any scratch sample tube taking a reference with standard turbidity suspension and noted the reading on the scale.

4.2.8 Conductivity

Conductivity was measured with the help of a conductivity meter (Toa Electronics Limited). The electrode was first rinsed with distilled water and then immersed into water sample, gently stirred and noted the reading.

4.2.9 Residual Chlorine

For the measurement of residual chlorine (Free, Total and combined residual chlorine) DPD Palintest$_{1000}$ CHLOROMETER (0-5.00 mg/l) was used.

The cell (glass) tube was thoroughly cleaned with distilled water and dried by blotting paper before use. Cell of 10ml was filled with the distilled water and it was placed in the cell holder. ON button was pressed until the display showed 0.00. Cell of 10ml was filled with the sample and DPD No.1 tablet for free residual chlorine (FRC) was added and crushed. Then it was placed in the cell holder and reading was noted after pressing ON button. Similarly for combined residual chlorine (CRC) and total residual chlorine (TRC), DPD No. 3 and 4 tablet was used respectively.

4.2.10 Microbiological analysis of water sample

4.2.10.1 Total coliform count

In this study, total coliforms were enumerated by the membrane filtration (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 M-Endo agar with the help of sterile forceps. Care was taken not to entrap air bubbles between the membrane and the medium. Then it was incubated for 24 hrs at 37^0C in inverted position. After proper incubation total colony forming unit (CFU) were counted. For this, all green metallic sheen-producing colonies were counted.

4.2.10.2 Heterotrophic plate count (HPC)

The water sample was thoroughly mixed and 0.1ml sample was aseptically pipetted onto surface of pre-dried agar plate. Sterile bent glass rod was used to distribute inoculums over surface of the medium by rotating the dish by hand. Inoculum was allowed to absorb completely into the medium and then incubated at 37ºC for 24-48 hrs. After the period of incubation colonies were counted. The selected colonies were cultured on Nutrient Agar for purification. From NA, Gram stain and biochemical tests were performed.

4.2.10.3 Pathogenic and opportunistic pathogenic bacteria detection and identification water in samples

4.2.10.3.1 *Salmonella and Shigella*

Five milliliters of water sample was enriched by inoculated into 45ml Selenite F broth. It was then incubated at 37°C for overnight, and a loopful of the upper part of the broth was subcultured on a selective enteric medium, XLD agar. The plate was incubated at 37°C for 24 hours (Collee *et al.*, 1996).

4.2.10.3.2 *Vibrio cholerae*

One milliliter of water sample was enriched by inoculated into 9ml of 1 % alkaline peptone water. It was then incubated at 37^0C for 6 to 8 hours. Then a loopful of the enrichment broth was streaked on TCBS agar medium plate. The pate was incubated at 37^0C for 24 hours (Collee *et al.*, 1996).

Green metallic sheen producing colonies and all colonies with different characteristics from M-Endo agar, XLD agar and TCBS agar were streaked onto NA to get pure culture. Bacteria isolated on respective selective or differential media were identified on the basis of their colonial characteristic, morphological characteristics and biochemical properties. Identification was carried out following Bergey's Manual of Systematic Bacteriology, 1994. Biochemical tests for identification of isolates were given in Appendix III.

4.2.11 Chlorine solutions

Chlorine solutions were prepared daily by mixing 3.5gm of calcium hypochlorite (a.v. 35%) with 100ml of chlorine demand free water. The stock solution thus formed was of 10,000ppm and which was diluted to produce solutions of final free chlorine concentrations of 0.1±0.02, 0.2±0.02, 0.3±0.02, 0.4±0.02, 0.5±0.02 and 1.25±0.02mg/lt. Final concentrations were verified by the palintest $_{1000}$ CHLOROMETER.

4.2.12 Determination of bacterial chlorine resistance

Bacteria were grown overnight in tryptic soy broth in an orbital shaker at 23^oC. The cells were harvested by centrifugation at 3,500×g for 15 minutes and were washed twice in sterile ice-cold 50mM monobasic potassium phosphate buffer (pH 7.0). After the final centrifugation, the bacteria were suspended in buffer to an optical density of 1.05 (measured at 580 nm), and 10µl of this suspension were transferred to each of 7 reaction tubes containing 10ml of the 50mM phosphate buffer. The final cell concentration in each tube was approximately 4.0×10^6 per ml. Equi-volume of sample and double

strength calcium hypochlorite solution were mixed to obtain the desired half concentration of both sample and calcium hypochlorite solution in next six reaction tubes. The last one tube served as an untreated control and received no chlorine but instead equi-volume of sample and sterile chlorine demand free distilled water was added. The applied calcium hypochlorite concentrations in the tubes were varied from 0 to 1.25 mg/lt. After time (t) = 0, 15, 30, 45 and 60 minutes at 23^{o}C, 100µl of sterile 1.0 M sodium thiosulfate was added to each tube. The tubes were chilled to 2^{o}C, and portions were plated on Plate Count Agar to rescue surviving bacteria. The plates were incubated at 35^{o}C for 48 hrs, at which time colonies were enumerated (Ridgway and Olson, 1982).

All glassware and reagents for chlorine exposure work were prepared as described in Standard Methods (APHA, 1998). The chlorine demand of glassware and magnetic fleas used in the assays was removed by soaking them in a strong chlorine solution for 48 hrs, and then the glassware was heated overnight at 160^{o}C. Chlorine demand free water (CDFW) was used in all assay solutions and for diluting the chlorine stock. Chlorine demand was removed from the water in a final concentration of 5 mg/lt of free chlorine. This solution was left for 2 days after which time the remaining chlorine was dissipated by exposure to ultraviolet light.

4.2.13 Antimicrobial sensitivity test

Antibiotic susceptibility of isolated enteric bacteria was assayed using a modified Kirby Bauer disc diffusion method (Bauer *et al.*, 1966).

Bacterial culture was grown at 37ºC in 5 ml of nutrient broth for about 4 hrs using pure cultures as inoculum having McFarland 0.5. A sterile cotton swab was taken and dipped into culture suspension of bacteria. The entire agar surface of each plate was inoculated, first in a horizontal direction and then in vertical direction to ascertain the even distribution of the organism over the agar surface using a swab. The agar surface was allowed to dry for 5 minutes. A sterile antibiotic disc was picked up by the outer edge using a flamed, sterile forceps and placed the disc near the edge of agar surface 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 30 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.

4.2.14 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. Control strains of ATCC were given in Appendix I used for the identification test and for the standardization of Kirby-Bauer test and also for correct interpretation of inhibition zones of diameter. Quality of sensitivity test was maintained by maintaining the thickness of MHA at 4mm 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.

4.2.15 Purity plate

Purity plate for each biochemical test was maintained to ensure the pure culture inoculums used as well as to assess that the biochemical tests were undertaken in an aseptic condition.

4.2.16 Statistical analysis

Statistical analysis of data was conducted using SPSS 16 (Statistical Packages for Social Science) and Microsoft Excel 2007. Student t-test, Chi-square test and Pearson Correlation were used where appropriate.

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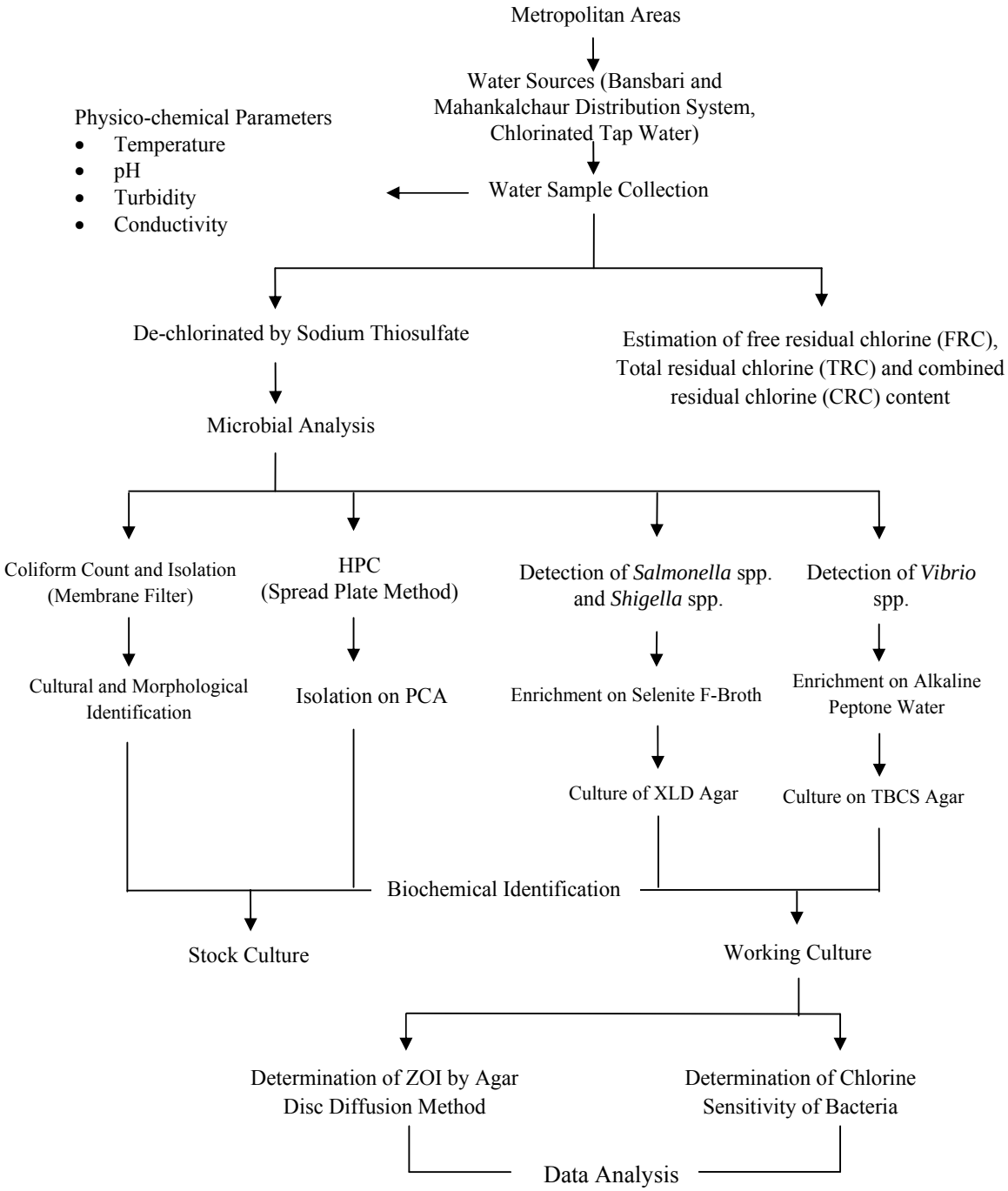


CHAPTER V

5. RESULTS

5.1 Sample distribution and Association between growth of organism and location of sample

Of 107 water samples, 52 (48.6%) were collected from Bansbari treatment and distribution plant and 55 (51.4%) from Mahankalchaur treatment and distribution plant. In which, Mahankal showed the maximum growth of 69.1%. But there was no significant difference between the growth of organism and location of sample (p=0.548) (Table 5.1).

Table 5.1 Sample distribution and Association between growth of organism and location of sample

Variables	Growth of Organism		Total (N=107)		p-value[b]
	n	%	n	%[a]	
Location of sample					
Bansbari	33	63.5	52	48.6	0.548
Mahankal	38	69.1	55	51.4	

a: Column percentage to indicate the distribution of overall characteristics

b: Compare the significance difference in growth patterns of organism and location of sample

5.2 Physico-chemical parameter of water

The mean conductivity, pH, temperature and turbidity of 107 samples were found to be 58.34 µS/cm, 7.221, 15.833 ^{o}C and 1.413 NTU respectively. Conductivity was found to be 107 (100%) within the WHO guideline value. In case of pH and turbidity, 103 (96.3%) and 100 (93.5%) were found to be within the WHO guideline value (Table 5.2).

Table 5.2 Physico-chemical according to WHO guideline value

S.No.	Parameters	Min.	Max.	Mean	WHO guideline		
					Below	Within	Above
1	Conductivity	12 µS/cm	130 µS/cm	58.34±24.468		107 (100%)	
2	pH	6.3	8.3	7.221±0.423	4 (3.7%)	103 (96.3%)	
3	Temperature	10.2 ^{o}C	26.3 ^{o}C	15.833±3.563			
4	Turbidity	0 NTU	39 NTU	1.413±3.999		100 (93.5%)	7 (6.5%)

5.3 Residual chlorine

The mean free residual chlorine, combined residual chlorine and total residual chlorine were found to be 0.2764, 0.0015 and 0.2779 ppm respectively. Where 91 (85.1%) were below and 3 (2.8%) were above and 13 (12.1%) were found to be within WHO guideline value. The maximum free residual chlorine was found to be 1.75 ppm (Table 5.3).

Table 5.3 Residual chlorine according to WHO guideline value

S.No.	Parameter	Min. (ppm)	Max. (ppm)	Mean	WHO guideline		
					Below	Within	Above
1	Free Residual Chlorine	0	1.75	0.2764	91 (85.1%)	13 (12.1%)	3 (2.8%)
2	Combined Residual Chlorine	0	0.08	0.0015			
3	Total Residual Chlorine	0	1.75	0.2779			

5.4 Bacteriological analysis of samples

5.4.1 Heterotrophic plate count of water sample

Out of 107 water samples, 47 (43.9%) were within and 60 (56.1%) were found to be above the WHO guideline value. The mean HPC was found to be 9×10^{2} cfu/ml (Table 5.4).

Table 5.4 Heterotrophic plate count according to WHO guideline value

Variables	No. of Samples	Percentage of Samples (%)
HPC		
Within WHO guideline (<10cfu/ml)	47	43.9
Above WHO guideline (>10cfu/ml)	60	56.1
Total	**107**	**100**

5.4.2 Total coliform count of water sample

Out of 107 water samples, in total coliform count, 47 (43.9%) were within and 60 (56.1%) were above the WHO guideline value. The mean total coliform was found to be 86 cfu/100ml (Table 5.5).

Table 5.5 Total coliform count according to WHO guideline value

Variables	No. of Samples	Percentage of Samples (%)
TCC		
Within WHO guideline (0 cfu/100ml)	47	43.9
Above WHO guideline (>0 cfu/100ml)	60	56.1
Total	**107**	**100**

5.4.3 Isolation and identification of Bacteria

In this study a total of 134 bacteria of 10 different genera were isolated and identified. Among 107 water samples, *E. coli* (21.6%) was found to be maximum followed by *Citrobacter* spp. (20.9%), *Shigella* spp. (13.4%), *Enterobacter* spp. (11.2%), *Providencia* spp. (9.7%), *Klebsiella* spp. (8.2%), *Salmonella* spp. (6%), *Pseudomonas* spp. (4.5%), *Proteus* spp. (3%) and *Edwardsiella* spp. (1.5%) (Table 5.6).

Table 5.6 Types of organism in growth positive samples

S.No.	Organisms	No. of organisms	Percentage (%)
1	*Citrobacter* spp.	28	20.9
2	*Escherichia coil*	29	21.6
3	*Edwardsiella* spp.	2	1.5
4	*Enterobacter* spp.	15	11.2
5	*Klebsiella* spp.	11	8.2
6	*Proteus* spp.	4	3
7	*Providencia* spp.	13	9.7
8	*Pseudomonas* spp.	6	4.5
9	*Salmonella* spp.	8	6
10	*Shigella* spp.	18	13.4
Total		**134**	

5.5 Difference of Physico-chemical parameters and the growth of organism

There was no significant difference between conductivity, pH and turbidity with that of growth of organism **($p>0.05$)** but there was significance difference between temperature and growth of organism **($p<0.05$)** (Table 5.7).

Table 5.7 Difference of Conductivity, pH, Temperature and Turbidity between the growth of organism

Variables	**Growth** (N=71)	**No growth** (N=36)	**p-value**
Conductivity	58.28±24.499	58.47±24.754	0.969
pH	7.199±0.413	7.264±0.444	0.454
Temperature	16.596±3.962	14.328±1.885	0.002
Turbidity	1.797±4.870	0.656±0.350	0.164

5.6 Free residual chlorine level and the growth of organism

Out of 107 water samples, 36 (33.6%) samples were found to be below Nepal guideline (BNG) for free residual chlorine, 27 (25.2%) were within Nepal guideline (WNG), 28 (26.2%) were below WHO guideline (BWG), 13 (12.1%) were within WHO guideline (WWG) and remaining 3 (2.8%) were above the WHO guideline (AWG). Growth of the organism was found to be maximum in samples with free residual chlorine level below Nepal guideline value (80.6%). There was significance difference between growth of organism and free residual chlorine level **(p = 0.037)** (Table 5.8).

Table 5.8 Association between the growth of organism and free residual chlorine

Variables	Growth of Organism		Total (N=107)		p-value[b]
	n	%	n	%[a]	
Free residual chlorine					
BNG[*] (<0.1ppm)	29	80.6	36	33.6	
WNG (0.1-0.2 ppm)	19	70.4	27	25.2	0.037
BWG (0.21-0.59 ppm)	17	60.7	28	26.2	
WWG (0.6-1 ppm)	4	30.8	13	12.1	
AWG (>1 ppm)	2	66.7	3	2.8	

a: Column percentage to indicate the distribution of overall characteristics

b: Compare the significance difference in growth patterns of organism and free residual chlorine

Note:[*]BNG = Below Nepal Guideline Value, WNG = Within Nepal Guideline Value, BWG = Below WHO Guideline Value, WWG = Within WHO Guideline Value and AWG = Above WHO Guideline Value

5.7 Relation between Free residual chlorine and microbial parameters

5.7.1 Relation between Free residual chlorine and Heterotrophic plate count

The correlation between Heterotrophic plate count (HPC) and free residual chlorine (FRC) was that as FRC decreases, bacterial count increases. The correlation was found insignificant **(p=0.096)** with negative correlation **(r= -0.162)** (Figure 5.1).

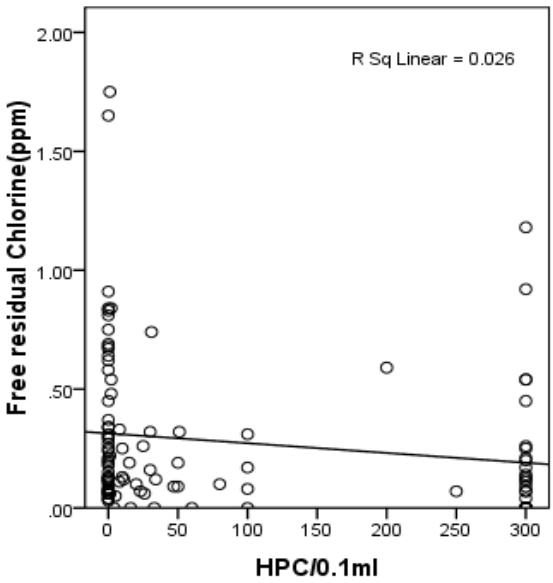


Figure 5.1 Relation between Free residual chlorine and Heterotrophic plate count

5.7.2 Relation between Free residual chlorine and Total coliform count

The correlation between Total coliform count (TCC) and free residual chlorine (FRC) was that as FRC decreases, bacterial count increases. The correlation was found significant **(p= 0.001)** with negative correlation **(r = -0.383)** (Figure 5.2)

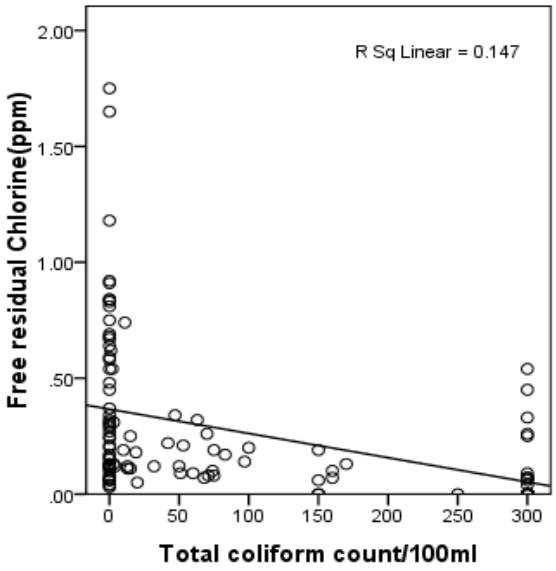


Figure 5.2 Relation between Free residual chlorine and Total coliform count

5.8 Chlorine assay

In chlorine assay, eight different isolates namely *Escherichia coli, Enterobacter* spp., *Citrobacter* spp., *Klebsiella* spp., *Shigella* spp., *Salmonella* spp., *Providencia* spp. and *Pseudomonas* spp. were tested and found to be chlorine resistant at concentration of 0.2 mg/lt for a contact time of 30 minutes.

5.9 Time required for a $T_{99.9}$ (3-log) and $T_{99.99}$ (4-log) reduction in the number of viable isolate from the initial population after exposure in drinking water to increasing concentration of free chlorine

Time required to reduce the organisms by 3-log ($T_{99.9}$) at Nepal standard of 0.2 mg/lt of residual chlorine concentration were found to be less than 30 minutes for all eight different genera of organisms tested. Similarly, time required reducing the organisms by 4-log ($T_{99.99}$) at 0.2 mg/lt of residual chlorine concentration were found to be greater than 60 minutes for all eight different genera of organisms tested except *Citrobacter* spp. which was found to require only 45 minutes (Table 5.9).

Table 5.9 Time required for a $T_{99.9}$ (3-log) and $T_{99.99}$ (4-log) reduction in the number of viable isolate from the initial population after exposure in drinking water to increasing concentration of free chlorine*

Free Chlorine (mg/lt)	*Escherichia coli* ($T_{99.9}$)	*Enterobacter* spp .($T_{99.9}$)	*Citrobacter* spp. ($T_{99.9}$)	*Klebsiella* spp. ($T_{99.9}$)	*Shigella* spp. ($T_{99.9}$)	*Salmonella* spp .($T_{99.9}$)	*Providencia* spp. ($T_{99.9}$)	*Pseudomonas* spp. ($T_{99.9}$)
0.1	< 30 min.	< 30 min.	< 30 min.	< 30 min.	< 30 min.	< 30 min.	< 15 min.	< 30 min.
0.2	< 30 min.	< 15 min.	< 15 min.	< 15 min.	< 15 min.	< 30 min.	< 15 min.	< 30 min.
0.3	< 15 min.	20 sec.	< 15 min.	20 sec.	< 15 min.	< 15 min.	< 15 min.	< 15 min.
0.4	< 15min.	< 20 sec.	< 15 min.	< 20 sec.	< 15 min.	< 15 min.	< 15 min.	< 15 min.
0.5	< 15 min.	< 20 sec.	< 15 min.	< 20 sec.	< 20 sec.	< 15 min.	< 15 min.	< 20 sec.
1.25	< 15 min.	< 20 sec.	< 15 min.	< 20 sec.	< 20 sec.	< 20 sec.	< 20 sec.	< 20 sec.
Free Chlorine (mg/lt)	***Escherichia coli*** **($T_{99.99}$)**	***Enterobacter* spp.** **($T_{99.99}$)**	***Citrobacter* spp.** **($T_{99.99}$)**	***Klebsiella* spp.** **($T_{99.99}$)**	***Shigella* spp.** **($T_{99.99}$)**	***Salmonella* spp.** **($T_{99.99}$)**	***Providencia* spp.** **($T_{99.99}$)**	***Pseudomonas* spp.** **($T_{99.99}$)**
0.1	> 60 min.	> 60 min.	> 60 min.	60 min.	< 60 min.	> 60 min.	> 60 min.	> 60 min.
0.2	> 60 min.	< 60 min.	45 min.	< 60 min.	< 60 min.	> 60 min.	> 60 min.	> 60 min.
0.3	< 60 min.	< 60 min.	< 45 min.	< 60 min.	< 60 min.	< 60 min.	> 60 min.	< 60 min.
0.4	< 45 min.	< 60 min.	< 45 min.	< 45 min.	< 45 min.	< 60 min.	< 60 min.	< 60 min.
0.5	30 min.	< 30 min.	< 30 min.	< 30 min.	< 30 min	45 min.	< 60 min.	< 45 min.
1.25	< 15 min.	< 20 sec.	< 15 min.	< 20 sec.	< 20 sec.	< 15 min.	< 15 min.	< 15 min.

*Treatments were performed at density 2×10^6cells/ml

5.10 Log inactivation of various organisms with the residual concentration of Nepal Standard

The minimum free residual chlorine and contact time as prescribed by the potable water provider in Nepal was found insufficient to reduce the organism populations by more than 3 log. The highest reduction was found to be of *Citrobacter* spp. (3.54-log) and the lowest was found to be of *E. coli* (3-log) (Table 5.10).

Table 5.10 Log inactivation of various organisms with chlorine concentration of 0.2 mg/lt for a contact time of 30 minutes

S.No.	Organisms	Log inactivation
1.	*Escherichia coli*	3.097
2.	*Enterobacter* spp.	3.190
3.	*Citrobacter* spp.	3.545
4.	*Klebsiella* spp.	3.426
5.	*Shigella* spp.	3.260
6.	*Salmonella* spp.	3.125
7.	*Providencia* spp.	3.240
8.	*Pseudomonas* spp.	3.240

5.11 Antibiotic sensitivity test of water sample

Ten different antibiotics were used to assess the antimicrobial susceptible pattern of the isolated 9 different bacteria from water sample. Among them, only nine were found to be resistant in which Ampicillin was found to be 100% resistant followed by Amoxycillin, Cotrimoxazole, Tetracycline, Ceftazidime, Ceftriaxone, Chloramphenicol, Gentamicin and Ofloxacin. (Table 5.11)

Table 5.11 Antibiotics sensitivity test of bacterial isolates from water samples

Antibiotics	Sensitivity pattern	*Citrobacter* spp.	*Escherichia coli*	*Enterobacter* spp.	*Klebsiella* spp.	*Proteus* spp.	*Providencia* spp.	*Pseudomonas* spp.	*Salmonella* spp.	*Shigella* spp.	Total Percent
Amoxycillin	S	20	0	0	0	0	25	20	33.3	16.7	13
	I	40	44.4	33.3	16.7	0	25	0	0	50	26
	R	40	55.6	66.7	83.3	100	50	80	66.7	33.3	61
Ampicillin	S	0	0	0	0	0	0	0	0	0	0
	I	0	0	0	0	0	0	0	0	0	0
	R	100	100	100	100	100	100	100	100	100	100
Cefriaxone	S	80	88.9	100	100	100	100	83.3	50	83.3	84.8
	I	0	11.1	0	0	0	0	16.7	33.3	16.7	10.9
	R	20	0	0	0	0	0	0	16.7	0	4.3
Ceftazidime	S	100	71.4	100	100	100	100	100	50	50	80.6
	I	0	14.3	0	0	0	0	0	0	50	9.7
	R	0	14.3	0	0	0	0	0	50	0	9.7
Chloramphenicol	S	100	100	100	100	100	75	100	66.6	100	93.5
	I	0	0	0	0	0	25	0	16.7	0	4.3
	R	0	0	0	0	0	0	0	16.7	0	2.2
Ciprofloxacin	S	80	100	100	100	50	100	83.3	100	100	93.6
	I	20	0	0	0	50	0	16.7	0	0	6.4
	R	0	0	0	0	0	0	0	0	0	0
Cotrimoxazole	S	50	88.9	100	100	100	100	50	50	100	81
	I	0	0	0	0	0	0	0	0	0	0
	R	50	11.1	0	0	0	0	50	50	0	19
Gentamicin	S	100	100	100	83.3	100	100	100	100	100	97.9
	I	0	0	0	0	0	0	0	0	0	0
	R	0	0	0	16.7	0	0	0	0	0	2.1
Ofloxacin	S	75	100	100	100	100	100	100	100	100	97.9
	I	0	0	0	0	0	0	0	0	0	0
	R	25	0	0	0	0	0	0	0	0	2.1
Tetracycline	S	50	60	100	100	100	0	0	50	66.7	61.9
	I	0	20	0	0	0	50	100	50	33.3	23.8
	R	50	20	0	0	0	50	0	0	0	14.3

Note: All values are in percent and S= Sensitive, I= Intermediate, R= Resistant

5.12 Antibiotic resistant and Multiple antibiotic resistant from water

Among the isolates, 78.70% were found to be Antibiotic resistant (AR) and 27.70% were found to be Multiple antibiotic resistant (MAR) (Figure 5.3).

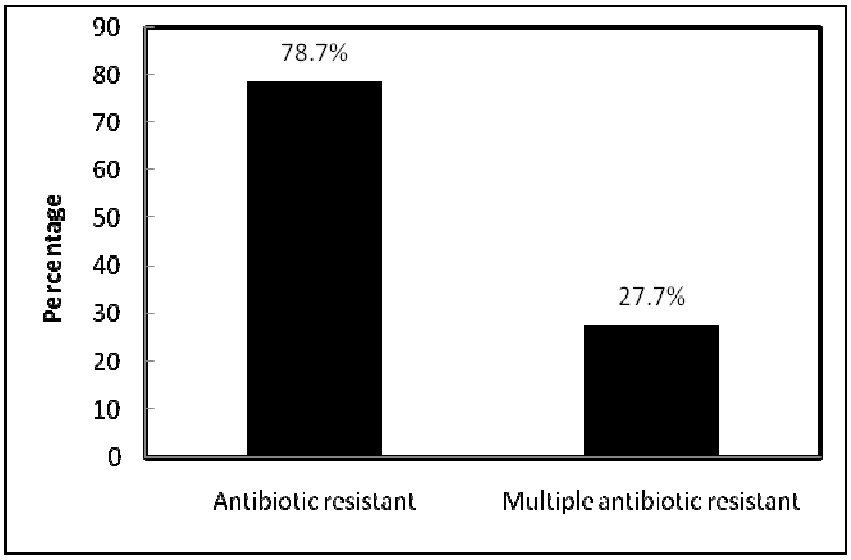


Figure 5.3 Frequency of Antibiotics Resistance and Multiple Antibiotics Resistance from water

Note: Antibiotic Resistance (AR): At least resistance to one antibiotic
Multiple Antibiotic Resistance (MAR): At least resistance to two different classes of antibiotics

5.13 Antibiotics Resistance and Multiple Antibiotics Resistance among isolates from water

Among the isolates, Antibiotic resistance (AR) was found to be maximum in *Klebsiella* spp. (83.3%) and least was found to be in *Pseudomonas* spp. and *Salmonella* spp. (16.7%) each. Multiple antibiotic resistant (MAR) was found to be maximum in *Salmonella* spp. (83.3%) and was found to be absent in *Enterobacter* spp., *Proteus* spp. and *Shigella* spp (Table 5.12).

Table 5.12 Frequency of Antibiotic Resistance and Multiple Antibiotic Resistance among isolates from water

S.No.	Organisms	AR (%)	MAR (%)
1	*Citrobacter* spp.	60	20
2	*Escherichia coli*	44.4	22.2
3	*Enterobacter* spp.	66.7	0
4	*Klebsiella* spp.	83.3	16.7
5	*Proteus* spp.	100	0
6	*Providencia* spp.	50	25
7	*Pseudomonas* spp.	16.7	50
8	*Salmonella* spp.	16.7	83.3
9	*Shigella* spp.	50	0

5.14 Relation between chlorine resistance and antibiotic susceptibility test

All the eight different bacterial isolates namely *Escherichia coli, Enterobacter* spp.*, Citrobacter* spp.*, Klebsiella* spp., *Shigella* spp.*, Salmonella* spp.*, Providencia* spp. and *Pseudomonas* spp. were found to be chlorine resistant at concentration of 0.2 mg/lt for contact time of 30 minutes and simultaneously, these chlorine resistant organisms were found to be antibiotic resistance on antibiotic susceptibility test (Table 5.13).

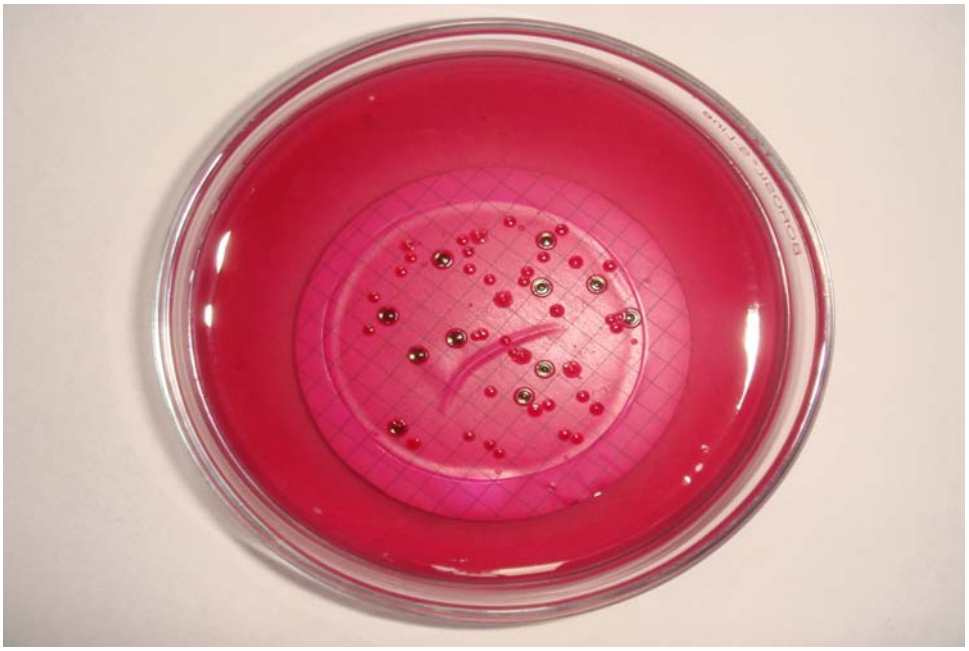
Table 5.13 Relation between chlorine resistance and antibiotic susceptibility test among eight different isolates from drinking water

S.No.	Organism	Chlorine assay[a]	Antibiotic assay[b]
1.	*Escherichia coli*	Resistance	Resistance
2	*Enterobacter* spp.	Resistance	Resistance
3	*Citrobacter* spp.	Resistance	Resistance
4	*Klebsiella* spp.	Resistance	Resistance
5	*Shigella* spp.	Resistance	Resistance
6	*Salmonella* spp.	Resistance	Resistance
7	*Providencia* spp.	Resistance	Resistance
8	*Pseudomonas* spp.	Resistance	Resistance

a: If the growth of organism takes place at free residual chlorine of at least 0.2 mg/lt after minimum contact time of 30 minutes then it is called chlorine resistant organism.

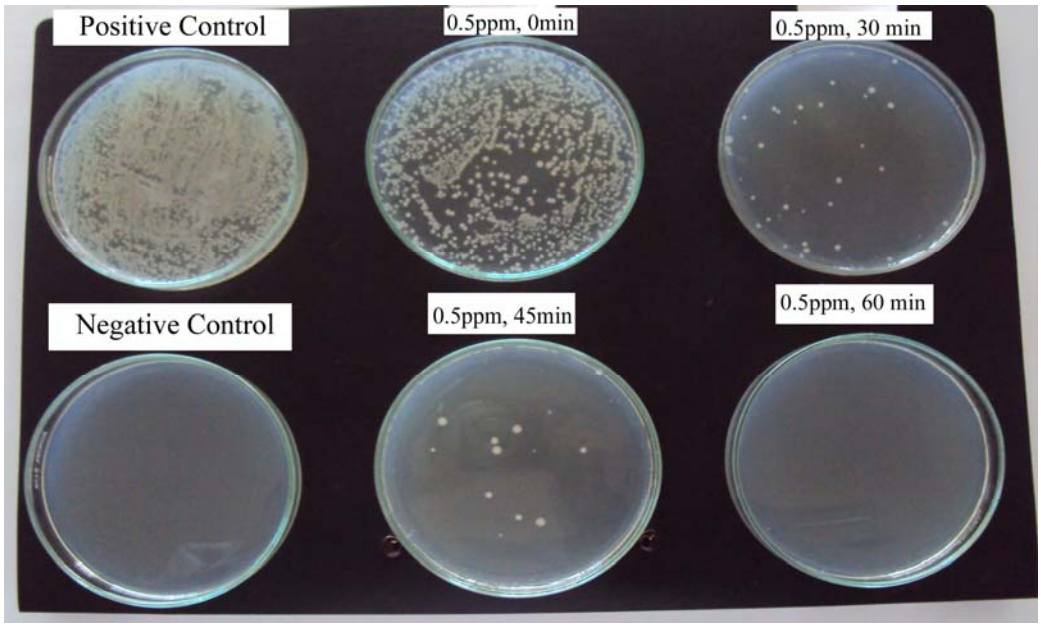
b: If the organism is resistant to at least one antibiotic then it is called antibiotic resistant.

Glimpses of Photographs



PHOTOGRAPH 1 : M-Endo Agar with Green Metallic sheen colonies

PHOTOGRAPH 2 : Chlorine determination by CHLOROMETER



PHOTOGRAPH 3 : Chlorine sensitivity test of *Escherichia coli* (0.5ppm at different time intervals)

PHOTOGRAPH 4 : Antibiotic susceptibility test of *Pseudomonas* spp.

CHAPTER VI

6. DISCUSSION AND CONCLUSION

6.1 Discussion

The impact of water quality on the efficacy of disinfection has been recognized for several decades. Disinfectants such as chlorine are used at very high concentration in order to attain a rapid rate of killing. However, the use of such high concentration increases the risk of formation of potentially hazardous by-products or the production of off-tastes and odors. Effective microbial control by chlorine requires appropriate disinfection design criteria to ensure protection of public health and to minimize unwanted effects of the chlorination process (Vitro *et al.,* 2005). Despite these advances in knowledge concerning the physiological mechanisms of chlorine disinfection, all of the physico-chemical and biological parameters which influence the bactericidal properties of chlorine in the environment are not yet fully understood. Large numbers of viable microorganisms, many of which have been shown to be human secondary opportunistic pathogens, can often be recovered from potable water distribution systems maintaining free chlorine residuals of 0.5 to 1.0 mg/lt. Thus, specific mechanisms may exist for the survival of certain bacteria and viruses in waters containing relatively increased concentration of chlorine (Ridgway and Olson, 1982).

The chlorination process attributes as the disinfection agent and prevents the re-growth of microbial was found to be unsuccessful and a high free residual chlorine concentration at the treatment plant and the intake point was not guaranteed to eliminate the microbial (Sarbatly and Krishnaiah, 2007). Antibiotic-resistant bacteria and antibiotics are discharged in various amounts in the environment as a result of the increasing and often indiscriminate use of antibiotics in medical, veterinary, and agricultural practices. The urban effluent resulted in an increase of the rates of resistance to antibiotics in the riverine bacterial populations (Goni-urriza *et al.,* 2000).

The purpose of this study was to investigate occurrence of microorganisms in drinking tap water of Kathmandu and the relationship between chlorine resistance and antibiotic susceptibility pattern of the bacterial isolates. In this study, 107 water samples from 2 distribution systems namely Bansbari (52) and Mahankalchaur (55) treatment and distribution plant.

Microbial quality of drinking water was assessed by physico-chemical and microbial parameters. The temperature of the analyzed water varied between 10.2 to 26.3^{o}C with the mean temperature 15.83^{o}C. High temperature has negatively impact on water quality by enhancing microbial growth and may increase taste, odor, colors and corrosion problem, though taste and odor can arise from inorganic and organic compounds in water sources occurring naturally or as a result of human activity (UNICEF, 2008). However, previously average temperature of drinking water of Kathmandu was slightly higher ranging 18.7-19.2^{o}C (Shrestha, 2009 and Aryal, 2009). Bajracharya *et al.* (2007) revealed that water bodies undergo temperature variation along with normal climatic fluctuations and seasonal variations. Analysing conductivity, all tested water samples (N=107) were found to be within WHO guideline value (≤ 1500 µS/cm). In agreement to this study, Gopali (2008) also reported all 111 water samples having conductivity within WHO guideline value. As for drinking water, it should remain constant; a sudden increase of conductivity indicates pollution from industrial and domestic waste water. The pH ranged from 6.3 to 8.3 with 96.3% samples was within and 3.7% below the WHO guideline value (6.5-8.5). Jayana (2007) reported in agreement with this study that 6.66% samples were below WHO guideline value. Previously, Ridgway and Olson (1982), K.C. (1992), Prasai *et al.* (2007), Bajracharya *et al.* (2007) and Gopali (2008) found that 100% water samples were within WHO guideline value. pH of water gets changed with time due to exposure to air, biological activity and temperature changes (UNICEF, 2008). Turbidity of 93.5% samples was within WHO guideline value (≤ 5NTU) and varied upto 39 NTU. Similarly, Bajracharya *et al.* (2007) revealed that the turbidity varied up to 48 NTU. Gopali (2008) reported that 84.21% were within WHO guideline value which ranged upto 20 NTU. Turbidity is caused by suspended particles

in water which may result from insufficient filtration or mobilization of sediments, mineral precipitates or biomass within the distribution system. Changes in turbidity following rainfall may indicate contamination with untreated water and may contain pathogens. Increase level of turbidity can shield the pathogens from disinfectants so effective disinfection requires the turbidity less than 1 NTU (UNICEF, 2008).

In this study, the free residual chlorine ranged up to 1.75 mg/lt with 85.1% samples below, 12.1% within and 2.8% above the WHO guideline value (0.6-1 mg/lt). However, two decades ago, K.C. (1992) found the FRC level varied up to 0.25 mg/lt in water samples of Kathmandu valley. Likewise, Gopali (2008) reported that free residual chlorine ranged up to 1.2 mg/lt in which 78.94% samples were below, 18.42% samples within and 2.63% samples above the WHO guideline value. The result indicates insufficient chlorination in drinking tap water.

This study showed that the HPC ranged up to 3×10^3 cfu/ml with the mean HPC 9×10^2 cfu/ml where 56.1% samples have exceeded the WHO guideline value ($\leq$ 10 cfu/ml). In 2007, Prasai *et al.* revealed that 82.4% samples crossed the WHO guideline value for HPC and Gopali (2008) reported that 65.79% samples in Sundarighat reservoir and 87.68% samples in Balaju reservoir crossed the WHO guideline value for HPC. This indicates the decreasing microbial contamination. HPC has become an indicator of general water quality within distribution system. Heterotrophic microorganisms are indigenous to water (and biofilms) and are always present in greater concentration than coliform bacteria in distribution and storage system. An increase in HPC indicates treatment breakthrough, posttreatment contamination, growth within the water conveyed the distribution system or the presence of deposits and biofilms in the system. A sudden increase in HPC above the historic baseline values should trigger actions to investigate and necessary remediate the situation. There is no evidence that heterotrophic microorganism in distribution systems are responsible for public health effects in the general population through ingestion of drinking water (WHO, 2003).

In this study, 56.1% water samples were above the WHO guideline value (0 coliform/100ml) for total coliform count. Likewise, Prasai *et al*. (2007) and Shrestha (2009) reported that all water samples crossed the WHO guideline value. Gopali (2008) found that 65.78% samples were above the WHO guideline value in Sundarighat reservoir whereas 90.41% samples exceeded the permissible limit in Balaju reservoir. Aryal (2009) showed that 86.2% samples crossed the permissible limit for coliform. This indicates the decreasing trend of microbial contamination. Coliform have been used extensively as the basis for regulating the microbial quality of drinking water. Good quality drinking water can suffer serious contamination in distribution systems because of breaches in the integrity of pipelines and storage reservoirs. There are many sources for the contamination of water sources as the treatment plant non-operational, operates intermittently (e.g. broken equipment, no treatment chemicals) or inadequately maintained and supervised (e.g. process control tests not carried out regularly, record keeping inadequate, poorly trained operators, incorrect storage of chemicals), cracked storage and reservoirs, tank access covers or vents improperly sealed, improper cleaning of storage tanks and reservoirs, broken or leaking pipes, exposed pipes due to erosion or poor construction, service interruptions causing pressure less and thus potentially allowing the entry of contaminated surface and groundwater into system via pipes and fittings, standing water around tap stands (stand pipes) due to poor drainage, open defecation near tap stands (UNICEF, 2008).

Physico-chemical parameters influence the growth of microorganism in water (Lechevallier *et al.,* 1981). In the present study, temperature (p= 0.002) was significantly associated with bacterial growth, where as pH (p= 0.454), Turbidity (p=0.164), Conductivity (p= 0.969) were not found to be associated with microbial growth. The association between the growth of organisms and the FRC was found to be significant (p= 0.037). The correlation between the FRC and HPC was found to be negatively correlated (r= -0.162) and insignificant (p= 0.092). But the correlation between the FRC and total coliform count (TCC) was found to be negatively correlated (r= -0.383) and significant (p= 0.001). Similarly, Gopali (2008) reported that the

correlation between FRC and HPC was negatively correlated and was found to be insignificant at ($p<0.05$) in Balaju reservoir but was found significant in Sundarighat reservoir. Also the correlation between FRC and TCC was found to be negatively correlated and was significant in Sundarighat reservoir but was found insignificant in Balaju reservoir.

In this study, a total of 134 bacterial isolates were recovered of 10 different genera from growth positive water samples. *E. coli* was found to be highest (21.6%), followed by *Citrobacter* spp. (20.9%), *Shigella* spp. (13.4%), *Enterobacter* spp. (11.2%), *Providencia* spp. (9.7%), *Klebsiella* spp. (8.2%), *Salmonella* spp. (6%), *Pseudomonas* spp. (4.5%), *Proteus* spp. (3%) and *Edwardsiella* spp. (1.5%). In drinking tap water of Kathmandu, *E. coli* is the most predominant indicator organism (Bajracharya *et al.,* 2007; Jayana, 2007; Kafle, 2007; Prasai *et al.,* 2007; Gopali, 2008; Aryal, 2009). However, in this study *Providencia* spp. and *Edwardsiella* spp. was isolated which was not previously reported. Besides, *Serratia* spp. and *Vibrio cholerae* were not recovered in the present study.

There are four types of cross contamination as: i) infiltration in which contaminated sub-surface water is drawn into the distribution system. In order for this to happen three conditions must be in place, firstly contaminated water must be present in the sub-surface material surrounding the distribution system, possibly from a leaking sanitary, storm or combined sewers, secondly there must be an adjacent low-pressure zone within the system. These zones can arise through high water usage resulting from fire fighting or other peak demands, decreased flow arising from restrictions in the system, pump failures or intermittent operation of the treatment plant. More recent evidence points to the role of pressure surges, in otherwise properly operated systems, in creating transient low pressure that may lead to the ingress of contaminants (Lechevallier, 1999). Thirdly, there must be a route for contaminated water to enter the systems. This can occur through pinholes caused by corrosion, cracks or outright breaks or leaking joints in the wall of the mains, ii) Back siphonage in which faecally contaminated surface water is

drawn into the distribution system or storage reservoir through a back flow mechanism. Firstly, there must be a reduction in line pressure as described above; secondly there must be a physical link between contaminated water and the storage and distribution system. Open taps connected to hoses that are submerged in pools of water may provide the link, iii) Open drinking water storage reservoir where microbial contamination can also be introduced into the distribution system through open treated-water storage reservoirs (Geldreich, 1996) and iv) Line construction and repair in which during the existing mains are repaired or replaced or when new water mains are installed strict protocols involving disinfection and flushing must be followed to prevent the introduction of contaminated soil or debris into the system.

Chlorine assay among water isolates was assayed as described by Ridgway and Olson (1982). In the chlorine assay, eight different genera of bacterial water isolates tested were found to be chlorine resistant at concentration of 0.2 mg/lt of residual chlorine for a contact time of 30 minutes (Nepal Standard). Present Nepal guideline (0.2 mg/lt) didn't seem to be sufficient for disinfection at a contact time of 30 minutes so WHO guideline value (0.6-1 mg/lt) can be adopted for effective disinfection (Table 5.9). The inactivation of 3 to 3.5- logs was demonstrated at Nepal guideline value (Table 5.10). Variables influencing the efficacy of disinfection are variables influencing the activity of the disinfectant chemical, interfering with the availability of the disinfectant in water and interfering with the accessibility of the disinfectant to the target organism (Barbeau *et al*., 2005). There are several major important influent variables necessary for appropriate chlorination. They are turbidity, pH, organic chemicals such as oils, greases, solvents, and other hydrocarbons. Chlorine reacts with many of these compounds in a violent or explosive way so they should be avoided (WHO, 1997 and M20 Manual of Water Supply Practices, 2006).

Sarbatly and Krishnaiah (2007) found that despite the high FRC at the treatment plant and the intake point the total coliform count was higher than the level suggested by Malaysian Water Association. Gopali (2008) found that total coliform were present in

those samples which were within guideline value for free residual chlorine. Likewise, Bhatta *et al.* (2006); Kafle (2007); Bajrachary *et al.* (2007); Prasai *et al.* (2007); Jayana (2007); Diwakar *et al.* (2008); Shrestha (2008); Shrestha (2009) and Aryal (2009) also found the high load of coliform count in chlorinated drinking tap water samples. In agreement with this study, Shrivastava *et al.* (2004) also observed that the strains of *Pseudomonas aeruginosa* survived under chlorination on three out of seven occasions tested. Similarly, Hiraishi *et al.* (1995); Power *et al.* (1997); Le Dantec *et al*. (2002); Howard and Inglis (2003); Condony *et al*. (2005) and Moreno *et al*. (2007) also found the chlorine resistant organisms from chlorinated water distribution systems.

Ridgway and Olson (1982) found that bacteria from the chlorinated systems were more resistant to both the combined and free forms of chlorine than those from the unchlorinated system. Murray *et al.* (1984) revealed that of the nine antibiotics tested, chlorination resulted in an increased proportion of bacteria resistant to some. Lechevallier *et al*. (1988) showed that the attachment of bacteria to surfaces provided the greatest increase in disinfection resistance. Other mechanisms which increased disinfection resistance included the age of the biofilm, bacterial encapsulation and previous growth condition which increased resistance to chlorine from 2 to 10-fold. These resistance mechanisms were multiplicative (i.e., the resistance provided by one mechanism could be multiplied by the resistance provided by a second mechanism). According to Yildiz and Schoolnik (1999) the rugose colony variant of *Vibrio cholera O1 El Tor*, was shown to produce an exopolysaccharide, EPS ^{ETR} that confers chlorine resistant and biofilm forming capacity. Condony *et al*. (2005) carried out the study in which cell viability in the water phase in the presence of chlorine was low at the beginning of the experiment but increased 4 orders of magnitude after five neutralization periods. Therefore subsequent episodes of chlorine depletion may accelerate the development of microbial communities with reduced susceptibility to disinfection in real drinking water systems. Helbling and VanBriesen (2007) found that chlorine resistant organisms demanded more chlorine than more sensitive organisms. Chlorine demand also increased with increasing free chlorine concentration.

In the antibiotic susceptibility test of isolates, Gentamicin showed 97.9% sensitivity by all types of isolates except *Klebsiella* spp. which was found to be 83.3% sensitive. Similarly, Ofloxacin was found to be 97.9% sensitive by all types of isolates except *Citrobacter* spp. which was 75% sensitive. Followed by Ciprofloxacin (93.6%), Chloramphenicol (93.5%), Ceftriaxone (84.8%), Cotriomoxazole (81%), Ceftazidime (80.6%), Tetracycline (61.9%), and Amoxycillin (13%). Ampicillin was found to be 100% resistant. 27.7% of the isolates were found to be multiple antibiotics resistant (MAR) whereas 78.7% were found to be Antibiotic resistant (AR). The highest AR was shown by *Proteus* spp. (100%) followed by *Klebsiella* spp. (83.3%), *Enterobacter* spp. (66.7%), *Citrobacter* spp. (60%), *Shigella* spp. and *Providencia* spp. (50%) each, *E. coli* (44.4%) and finally the lowest *was* found to be *Pseudomonas* spp. and *Salmonell*a spp. (16.7%) each. Similarly, MAR was found highest in *Salmonella* spp. (83.3%), followed by *Pseudomonas* spp. (50%), *Providencia* spp. (25%), *E. coli* (22.2%), *Citrobacter* spp. (20%) and *Klebsiella* spp. (16.7%). In agreement with this study, Armstrong *et al.* (1981) also found that 33.3% of 2,653 standard plate count (SPC) bacteria for treated drinking water were MAR. Armstrong *et al.* (1982) reported that the selective factors operating in the aquatic environment of a water treatment facility can act to increase the proportion of antibiotic-resistant members of the SPC bacterial population in treated drinking water. Calomiris *et al*. (1984) revealed that simultaneous selection phenomena occurred in distribution drinking water systems for bacteria which exhibited unique patterns of tolerance to Cu^{2+}, Pb^{2+} and Zn^{2+} and antibiotic resistance. Shrivastava *et al.* (2004) observed that the strains of *Pseudomonas aeruginosa* that survived chlorination on three out of seven occasions were resistant to almost all the antibiotic tested. Bhatta *et al.* (2006) study showed that many isolates of *Salmonella* were Multi Drug Resistant (MDR) and were found to be sensitive to Ciprofloxacin and Ofloxacin. Likewise, Kafle (2007) also showed that Ampicillin was 100% resistant. AR was found to be *Salmonella* spp. (100%), *Klebsiella* spp. (80%), *E. coli* (70%), *Enterobacter* spp. (20%), and *Citrobacter* spp. (5%). MAR was found to be *Salmonella* spp. (25%), *E. coli* (20%), *Klebsiella* spp. (20%), *Enterobacter* spp. (12%), and *Citrobacter* spp. (5%). Jayana (2007) also found that Ofloxacin was 92.9% sensitive in agreement with this study.

Antimicrobial resistance is a global problem. It is now generally accepted as major public health issue and has significant implication on health and patient care. Resistance to antimicrobial drugs is associated with high morbidity and mortality, high health-care cost and prolonged hospitalization. The problem of antimicrobial resistance is more troublesome to developing countries. Among drinking-water isolates, the emergence of resistance and decreasing levels of susceptibility to a wide spectrum of antibiotics is a matter of concern because it may limit the availability of antibiotics for clinical management of waterborne outbreaks in the future (Siya Ram *et al.,* 2008). Acquired antibiotic resistance in developing countries is serious because antibiotics can be obtained outside of recognized treatment centers and taken without medical authorization. Guidelines regarding the selection of drugs and information about drug resistance are not communicated to those prescribing antimicrobial especially in rural areas (Kafle, 2007).

All the eight different isolates were found to be both chlorine resistant at concentration of 0.2 mg/lt for contact time of 30 minutes and antibiotic resistant to at least one antibiotic (Table 5.13). In agreement with this study, Murray *et al.* (1984) revealed that of the nine antibiotics tested, chlorination resulted in an increased proportion of bacteria resistant to some, but a decrease in the proportion resistant to the remainder. Similarly, Shrivastava *et al.* (2004) also observed that the strains of *Pseudomonas aeruginosa* survived under chlorination on three out of seven occasions were resistant to almost all challenged antibiotic tested. Likewise, Armstrong *et al*. (1981), Bhatta *et al.* (2006), Kafle (2007), Jayana (2007), Shrestha (2008), Shrestha (2009) and Aryal (2009) found gram negative bacteria in chlorinated drinking tap water samples and simultaneously antibiotic resistant.

Acquisition of resistance towards disinfectant and antibiotic may follow similar mechanism. Which resistance occurred first, either chlorine or antibiotic and which occurred later?

6.2 Conclusion

Gram negative organisms were isolated from chlorinated drinking tap water of Kathmandu and these were found to be both chlorine resistance at 0.2 mg/lt for a contact of 30 minutes and antibiotic resistance to at least one antibiotic tested.

CHAPTER VII

7. SUMMARY AND RECOMMENDATIONS

7.1 SUMMARY

1. This study was conducted at Environment and Climate Change Laboratory of Nepal Academy of Science and Technology from April 2009 to August 2010. A total of 107 water samples were collected randomly from two distribution system namely Bansbari (52) and Mahankalchaur (55) treatment and distribution plant.

2. In physico-chemical parameter, conductivity was found to be 107 (100%) within the WHO guideline value. But pH of 4 (3.7%) samples below and turbidity of 7 (6.5%) samples above the WHO guideline value. Likewise, free residual chlorine of 91 (85.1%) and 3 (2.8%) samples were found to be below and above the WHO guideline value respectively.

3. Out of 107 water samples, 47 (43.9%) were found to be within and 60 (56.1%) were found to be above the WHO guideline value for total coliform and heterotrophic plate count each. The mean total coliform and HPC was found to be 86 cfu/100ml and 9×10^2 cfu/ml respectively.

4. Ten different genera of organism were found in growth positive samples in which *E. coli* (21.6%) was found to be highest followed by *Citrobacter* spp. (20.9%), *Shigella* spp. (13.4%), *Enterobacter* spp. (11.2%), *Providencia* spp. (9.7%), *Klebsiella* spp. (8.2%), *Salmonella* spp. (6%), *Pseudomonas* spp. (4.5%), *Proteus* spp. (3%) and *Edwardsiella* spp. (1.5%).

5. In chlorine assay, eight different genera of organism were found to be both chlorine resistant at 0.2 mg/lt for a contact time of 30 minutes.

6. Time required for $T_{99.9}$ (3-log) and $T_{99.99}$ (4-log) reduction of viable isolates from initial population of 2×10^6 cells/ml was found to be less than 30 minutes and greater than 60 minutes respectively.

7. Log inactivation of various organism with chlorine concentration of Nepal Standard (0.2 mg/lt for a contact time of 30 minutes) were found to be highest for *Citrobacter* spp. (3.545-log), followed by *Klebsiella* spp. (3.426-log), *Shigella* spp. (3.260-log), *Providencia* spp. and *Pseudomonas* spp. (3.240-log

each), *Enterobacter* spp. (3.190-log), *Salmonella* spp. (3.125-log) and the lowest was found to be *E. coli* (3.097-log).

8. Ten different types of antibiotics were used for antibiotic susceptibility test of the isolates. Antibiotic resistant (AR) and multiple antibiotics resistant (MAR) were found to be 78.7% and 27.7 % respectively.

9. Ten different antibiotics were tested and only nine were found to be resistant among water isolates in which Ampicillin was found to be 100% resistant followed by Amoxycillin, Cotrimoxazole, Tetracycline, Ceftazidime, Ceftriaxone, Chloramphenicol, Gentamicin and Ofloxacin.

10. The above mentioned, chlorine resistant bacteria were found to be antibiotic resistant to at least one antibiotic.

7.2 RECOMMENDATIONS

Based on the study the following recommendations have been made.

1. Either concentration or contact time of chlorination can be increased for effective disinfection.
2. Necessity for the alternate disinfection.
3. Control of the urban effluent discharge into river, mainly from medical, veterinary, agricultural practices and different industries where indiscriminate antibiotics are often used.
4. Study should be done, which resistance developed first either chlorine or antibiotic?

CHAPTER VIII

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APPENDIX I

1. List of Materials

1.1 Equipments Used

1. Autoclave- Life steriware, India
2. Conductivity meter- Toa Electronics Ltd
3. Electric balance- OHAUS GA 2000
4. Hot air oven- Universal, India
5. Incubator- Universal, India
6. Laminar airflow cabinet- Tosiba, India
7. Membrane filter apparatus- MILLIPORE
8. Microscope- Olympus, Japan
9. Nephelometer- Elico, India
10. pH meter- TOA pH meter HM-10P
11. Refrigerator- Samsung
12. Cyclo mixer-CM 101, REMI
13. Spectrophotometer- Jenway
14. Water distillation plant- Ogawa seiki
15. Water bath shaker- Narang, India
16. Centrifuge- FD 80-2, China
17. Micropipette

1.2 Microbiological Media

1. Nutrient Agar (Hi-Media)
2. M- Endo Agar (Hi-Media)
3. MR-VP Broth (Hi-Media)
4. Plate Count Agar (Hi-Media)
5. Mueller Hinton Agar (Hi-Media)
6. Hugh- Leifson's Agar (Hi-Media)
7. Salmonella-Shigella Agar (Hi-Media)
8. Sulphide Indole Motility Medium (Hi-Media)
9. Brillant Green Lactose Bile Broth (Hi-Media)
10. Xylose Lysine Deoxycholate Agar (Hi-Media)
11. Thiosulphate Citrate Bile Salt Sucrose Agar (Hi-Media)
12. Peptone (Hi-Media)
13. Nutrient Agar (Hi-Media)
14. Urea Agar Base (Hi-Media)
15. Selenite F Broth (Hi-Media)
16. Tryptic Soy Broth (Hi-Media)
17. Triple Sugar Iron Agar (Hi-Media)

1.3 Glassware

1. Sticker
2. Glass Rod
3. Reagent Bottle
4. Durham's Tube
5. Microscopic Slides
6. Graduated Cylinders
7. Screw Capped Test Tubes
8. Pipette
9. Funnels
10. Burette
11. Beakers
12. Test Tubes
13. Petri dishes
14. Conical Flasks

1.4 Miscellaneous

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

1.5 Chemicals And Reagents

2. Acetic acid
3. Alpha -naphthol
4. Barium chloride
5. Calcium hypochlorite (av. 35%)
6. Chlorine demand free water
7. Conc. Hydrochloric acid
8. Crystal violet
9. Ethanol
10. Gram's iodine
11. Monobasic Phosphate buffer
12. Oxidase reagent
13. Potassium iodide
13. Methyl red
14. Kovac's reagent
15. Iso-amyl alcohol
16. Iodine
17. Sodium thiosulfate
18. Hydrogen peroxide
19. Potassium hydroxide
20. Phenol red
21. Paraffin
22. Sodium hydroxide
23. Saffranin
24. Sulfuric acid

1.6 Antibiotic Discs

All the antibiotics discs used for the susceptibility tests were from Hi-Media Laboratories Pvt. Limited, Bombay, India. The antibiotics used were as follows:

1. Amoxycillin (10 mcg)
2. Ampicillin (10 mcg)
3. Ceftazidime (30 mcg)
4. Ceftriaxone (30 mcg)
5. Chloramphenicol (30 mcg)
6. Ciprofloxacin (5 mcg)
7. Cotrimoxazole (25 mcg)
8. Gentamicin (10 mcg)
9. Ofloxacin (5 mcg)
10. Tetracycline (30 mcg)

Test organisms

Escherichia coli ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumonia* ATCC 700603 and *Pseudomonas aeruginosa* (ATCC 27853).

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 catalyzes 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 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% H_2O_2 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 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 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 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 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 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 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.

APPENDIX III

DISTINGUISHING REACTIONS OF THE COMMONER AND PATHOGENIC ENTEROBACTERIACEAE

Table: Distinguishing reactions of the commoner and pathogenic Enterobacteriaceae

Species	Test/ substrate1											
	lac	**mot**	**gas**	**ind**	**VP**	**cit**	**PDA**	**ure**	**lys**	**H_2S**	**inos**	**ONPG**
Escherichia coli	+	+	+	+	-	-	-	-	+	-	-	+
Shigella groups A, B, C	-	-	-	±	-	-	-	-	-	-	-	-
Sh. sonnei	-	-	-	-	-	-	-	-	-	-	-	+
Salmonella (most serotypes)	-	+	+	-	-	+	-	-	+	+	±	-
S. Typhi	-	+	-	-	-	-	-	-	+	+	-	-
S. Paratyphi A	-	+	+	-	-	-	-	-	-	-	-	-
Citrobacter freundii	±	+	+	-	-	+	-	±	-	±	-	+
C. koseri	±	+	+	+	-	+	-	±	-	-	-	+
Klebsiella pneumoniae	+	-	++	-	+	+	-	+	+	-	+	+
K. oxytoca	+	-	++	+	+	+	-	+	+	-	+	+
Enterobacter aerogenes	+	+	++	-	+	+	-	-	+	-	+	+
E. cloacae	+	+	+	-	+	+	-	±	-	-	-	+
Hafnia alvei	-	+	+	-	+	-	-	-	+	-	-	+
Serratia marcescens2	-	+	±	-	+	+	-	-	+	-	±	+
Proteus mirabilis	-	+	+	-	±	±	+	++	-	+	-	-
P. vulgaris	-	+	+	+	-	-	+	++	-	+	-	-
Morganella morganii	-	+	+	+	-	-	+	++	-	±	-	-
Providencia rettgeri	-	+	-	+	-	+	+	++	-	-	+	-
P. stuartii	-	+	-	+	-	+	+	±	-	-	+	-
P. alcalifaciens	-	+	+	+	-	+	+	-	-	-	-	-
Yersinia enterocolitica3	-	-	-	±	-	-	-	±	-	-	±	+
Y. pestis	-	-	-	-	-	-	-	-	-	-	-	±
Y. pseudotuberculosis	-	-	-	-	-	-	-	+	-	-	-	±

[1] lac, inos, fermentation of lactose, inositol; mot, motility; gas, gas from glucose; ind, indole production; VP, Voges-Proskauer; cit, Citrate utilization (Simmons'); PDA, phenylalanine deaminase; ure, urease; lys, lysine decarboxylase; H_2S, H_2S produced in TSI agar; ONPG, metabolism of *o*-nitrophenyl-β-D-galactopyranoside.

[2] Some strains of *Serratia marcescens* may produce a red pigment

[3] *Yersinia* are motile at 22°C.{Key: +, ≥85% of strains positive; -, ≥ 85% of strains negative; ±, 16-84% of strains are positive after 24-48 hour at 36°C}

(Source: Collee *et al.* 1996)

APPENDIX IV

Zone Size Interpretative Chart of Antibiotic Susceptibility Testing

Antibiotics used	Symbol	Disc content (mcg)	Diameter of zone of inhibition		
			Resistant (mm or less)	Intermediate (mm)	Sensitive (mm or more)
Amoxycillin	Am	10	13	14-17	18
Ampicillin	A	10	13	14-16	17
Ceftazidime	CA	30	14	15-17	18
Ceftriaxone	Ci	30	13	14-20	21
Chloramphenicol	C	30	12	13-17	18
Ciprofloxacin	Cf	5	15	16-20	21
Cotrimoxazole	Co	25	10	11-15	16
Gentamicin	G	30	12	13-14	15
Ofloxacin	OF	5	12	13-15	16
Tetracycline	T	30	14	15-18	19

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

APPENDIX V

Nepal Drinking Water Quality Standards (2005) and WHO Guidelines

List of Parameters and concentration limits

S.No.	Category	Parameters	Units	WHO Guidelines For Potable Drinking Water	Nepal Drinking Water Quality Standard (June 2005)
				Concentration Limits	Concentration Limits
1.	Physical	Turbidity	NTU	5	5(10)
2.		pH		6.5-8.5	6.5-8.5
3.		Taste and Odour		Non-objectionable	Non-objectionable
4.		Electrical Conductivity	µS/cm	500	1500
5.	Chemical	Residual Chlorine	mg/lt	0.6-1	0.1-0.2
6.	Microbiological	Total Coliform	MPN/ 100ml	0	0 in 95% samples
7.		Thermotolerant Coliform	MPN/ 100ml	0	0
8.		Total Count	cfu/ml	≤10	