BACTERIOLOGICAL QUALITY OF DRINKING WATER IN KATHMANDU, NEPAL AND ANTIBIOTIC SUSCEPTIBILITY PATTERN OF *Escherichia coli* FROM WATER

A PROJECT WORK SUBMITTED TO THE

DEPARTMENT OF MICROBIOLOGY

AMRIT CAMPUS

INSTITUTE OF SCIENCE AND TECHNOLOGY

TRIBHUVAN UNIVERSITY

NEPAL

FOR THE AWARD OF

BACHELOR OF SCIENCE (B.Sc.) IN MICROBIOLOGY

BY

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RECOMMENDATION

This is recommend that **Manisha Gautam**, (Symbol No: 500330086, T.U. Registration No: 5-2-33-182-2017), has carried out project work entitled **"Bacteriological Quality of Drinking Water in Kathmandu, Nepal and Antibiotic Susceptibility Pattern of *Escherichia coli* From Water"** for the requirement to the project work in Bachelor of Science (B.Sc.) degree in Microbiology under our supervision in the Department of Microbiology, Amrit Campus, Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal.

To our knowledge this work has not been submitted for any other degree.

She has fulfilled all the requirements laid down by the Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal for the submission of the project work for the partial fulfillment of Bachelor of Science (B.Sc.) degree.

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Asst. Prof. **Ushana Shrestha Khwakhali**
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Date: 7/ 22/ 2022

DECLARATION

The project work entitled **"Bacteriological Quality of Drinking Water in Kathmandu, Nepal and Antibiotic Susceptibility Pattern of *Escherichia coli* From Water"** is being submitted to the Department of Microbiology, Amrit Campus, Institute of Science and Technology (IoST), Tribhuvan University (T. U.), Nepal for the partial fulfillment of the requirement to the project work in Bachelor of Science (B.Sc.) degree in Microbiology. This project work is carried out by me under the supervision of Asst. Prof. Ushana Shrestha Khwakhali and co-supervision of Mr. Prashanna Maharjan in the Department of Department of Microbiology, Amrit Campus, Institute of Science and Technology (IoST), Tribhuvan University (T.U), Nepal.

This work is original and has not been submitted earlier in part or full in this or any other form to any University or institute, here or elsewhere, for the award of any degree.

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Date: 7/ 22/ 2022

On the recommendation of **Asst. Prof. Ushana Shrestha Khwakhali** and **Mr. Prashanna Maharjan,** this project work is submitted by **Manisha Gautam**, symbol No. 500330086, T.U. Registration No. 5-2-33-182-2017, entitled **"Bacteriological Quality of Drinking Water in Kathmandu, Nepal and Antibiotic Susceptibility Pattern of *Escherichia coli* From Water"** is forwarded by the Department of Microbiology, Amrit Campus, for the approval to the Evaluation Committee, Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal.

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BOARD OF EXAMINATION AND CERTIFICATE OF APPROVAL

The project work entitled **"Bacteriological Quality of Drinking Water in Kathmandu, Nepal and Antibiotic Susceptibility Pattern of *Escherichia coli* From Water"** by Manisha Gautam (Symbol No: 500330086 and T.U. Registration No: 5-2-33-183-2017) under the supervision of Asst. Prof. Ushana Shrestha Khwakhali and co-supervision of Prashanna Maharjan in the Department of Department of Microbiology, Amrit Campus, Institute of Science and Technology (IoST), Tribhuvan University (T.U.), is hereby submitted for the partial fulfillment of the Bachelor of Science (B.Sc.) degree in Microbiology. This report has been accepted and forward to the controller of Examination, Institute of Science and Technology, Tribhuvan University, Nepal for the legal procedure.

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ABSTRACT

Water is the basic human need and one of the important factors for growth of human as well as living beings. Microbial pollution of drinking water causes waterborne diseases. In this study, bacteriological quality of drinking water in Kathmandu was assessed during March-May 2022 and antibiotic susceptibility pattern of *Escherichia coli* isolates from water was determined. A total of 39 water samples including samples from water shop (n=15) and drinking water from restaurants (n=24) were collected and assessed for total coliform and fecal coliform by using membrane filtration (MF) technique. The identification of bacteria was done by biotyping and antibiotic susceptibility testing of *E. coli* was carried out by using Kirby-Bauer disc diffusion method. According to bacteriological water quality, 93.3% of sample from water shop and 91.6% of sample from restaurants exceeded total coliform count whereas 53.3% of water sample from water shop and 41.6% of drinking water sample from restaurants exceeded fecal coliform count according to WHO guideline value of 0 CFU/100 mL. Maximum total coliform and maximum fecal coliform count of water from water shop were 310 CFU/100 mL and 190 CFU/100 mL respectively. Maximum total coliform and maximum fecal coliform count of drinking water from restaurants were 585 CFU/100 mL and 325 CFU/100 mL respectively. Out of 92 bacterial isolates obtained at 35°C±0.5°C from drinking water, higher percentage of isolates was found to be *E. coli* (31.5%) followed by *Klebsiella* spp (19.6%), *Enterobacter* spp (8.7%), *Citrobacter* spp. (5.4%), *Salmonella* spp. (13.0%), *Shigella* spp. (3.3%), *Pseudomonas* spp. (6.5%), *Proteus* spp. (9.8%) and unidentified Gram negative rods 2.2%. Similarly, out of 30 isolates of thermotolerant bacteria, 53.3% of isolates were *E. coli*. Antibiotic susceptibility pattern of *E. coli* (n= 29) isolates from drinking water showed 100% susceptibility towards Cotrimoxazole, Gentamycin and Amikacin where as 41.4%, 20.7%, 17.2%, 6.9%, 6.9% and 3.4% resistance were defected towards Ampicillin, Nitrofurantoin, Ceftriaxone, Tetracycline, Ciprofloxacin and Chloramphenicol respectively. Thermotolerant *E. coli* (n=16) showed resistance towards Ampicillin (37.5%), Nitrofurantoin (25%), Ceftriaxone (18.8%), Chloramphenicol (18.8%), Tetracycline (12.5%), Cotrimoxazole (6.3%) and Nalidixic Acid (6.3%). *E. coli* (10.3%) and thermotolerant *E. coli* (12.5%) where also detected as multiple antibiotic resistances (MAR) bacteria. From questionnaire survey, 72% of respondents in Kathmandu used jar water for drinking and 100% of respondents had toilet facilities and hand washing practices. This suggests that the bacteriological quality of drinking water in Kathmandu was unsatisfactory for drinking as well as household purposes. Awareness on drinking water quality, water disinfection and health impact of contaminated water has to be generated among the consumers.

Keywords: Antibiotic susceptibility pattern, *E. coli*, Fecal Coliform, Membrane filtration technique, Total coliform

शोधसार

पानी मानवको आधारभूत आवश्यकता हो । मानव र जीवित प्राणीहरूको विकासको लागि महत्त्वपूर्ण कारकहरू मध्य पानी पनि एक हो । पिउने पानीमा भएको माइक्रोबियल प्रदूषणले पानीजन्य रोगहरू निम्त्याउँछ । यस अध्ययनमा मार्च-मे २०२२ मा काठमाडौंमा पिउने पानीको ब्याक्टेरियोलोजिकल गुणस्तर मूल्याङ्कन गरिएको थियो र पानीबाट अलग गरिएका *Eschrichia coli* को एन्टिबायोटिक संवेदनशीलता ढाँचा परीक्षण गरिएको थियो। पानीको पसल (n=15) र रेस्टुरेन्टहरू (n=24) बाट पिउने पानीको नमूनाहरू संकलन गरी membrane filtration (MF) प्रविधि प्रयोग गरी total coliform र fecal coliform को मूल्याङ्कन गरिएको थियो। ब्याक्टेरियाको पहिचान बायोटाइपिङद्वारा गरिएको थियो र *E. coli* को एन्टिबायोटिक संवेदनशीलता परीक्षण Kirby-Bauer disk diffusion विधि प्रयोग गरी गरिएको थियो । ब्याक्टेरियोलोजिकल पानीको गुणस्तर अनुसार, पानी पसलबाट नमूनाको 93.3% र रेस्टुरेन्टबाट ९१.६% सङ्कलीत नमूनामा total coliform WHO गणना भन्दा बढी छ, जबकि 53.3% पानी पसलको र 41.6% रेस्टुरेन्टको पिउने पानीमा fecal coliform को गणना WHO निरदेशिका भन्दा बढी पाइएको थिया । पानी पसलबाट सङ्कलीत नमूना र रेस्टुरेन्टबाट सङ्कलीत नमूनामा अधिकतम total coliform र अधिकतम fecal coliform को गणना क्रमशः 310 CFU/100 mL ,190 CFU/100 mL र 585 CFU/100 mL , 325 CFU/100 mL थियो । सङ्कलीत सबै पिउने पानीको नमूनाबाट आइसोलेट ९२ ब्याक्टेरियल आइसोलेट्स मध्य सबै भन्दा उच्च प्रतिशत *E. coli* (३१.५%) थियो । त्यसलगायत *Klebsiella* spp. (१९.६%), *Enterobacter* spp. (८.७%)। *Citrobacter* spp. (५.४%), *Salmonella* spp. (१३.०%), *Shigella* spp. (३.३%), *Pseudomonas* spp. (६.५%), *Proteus* spp. (९.८%) र unidentified Gram negative rods २.२% पाइएको थियो। त्यसैगरी, थर्मोटोलरेन्ट ब्याक्टेरियाका ३० वटा आइसोलेट्समध्ये ५३.३% आइसोलेट्स *E. coli* थिए । *E. coli* (n=29) को antibiotic susceptibility pattern गर्दा Cotrimoxazole, Gentamycin र Amikacin तर्फ १००% संवेदनशीलता र Ampicillin, Nitrofurantoin, Ceftriaxone, Tetracycline, Ciprofloxacin र Chloramphenicol तर्फ ४१.४%, २०.७%, १७.२%, ६.९%, ६.९% र ३.४% resistance देखिएको थियो । थर्मोटोलरेन्ट *E. coli* (n=16) ले Ampicillin, Nitrofurantoin, Ceftriaxone, Chloramphenicol, Tetracycline, Cotrimoxazole, Nalidixic Acid विरुद्ध ३७.५%, २५%, १८.८%, १२.५%, ६.३%, ६.३% प्रतिरोध देखिएको थियो । १०.३% *E. coli* र (१२.५%) थर्मोटोलरेन्ट *E. coli* मा multiple antibiotic resistances (MAR) देखिएको थियो । प्रश्नावली सर्वेक्षणबाट, काठमाडौंको ७२% उत्तरदाताहरूले पिउनका लागि जारको पानी प्रयोग गरिएको पाइयो । १००% उत्तरदाताहरूले शौचालय प्रयोग गरिपछि हात धुने अभ्यासहरू गरेको भेटियो । यस अध्ययनमा काठमाडौंको पिउने पानीका स्रोतको ब्याक्टेरियोलोजिकल गुणस्तर तथा घरायसी प्रयोजनका लागि प्रयोग हुने पानी सन्तोषजनक नभएको देखाउँछ। पिउने पानीको गुणस्तर, पानीको कीटाणुशोधन र दूषित पानीले मानव स्वास्थ्यमा पार्ने असरबारे उपभोक्तामा चेतना जगाउनु पर्ने देखिन्छ ।

Keywords: Microbial quality, Water quality, Coliform, Membrane filtration technique, Antibiotic susceptibility.

LIST OF ACRONYMS AND ABBREVIATIONS

AMR	Antimicrobial Resistance
APHA	American Public Health Association
AST	Antibiotic Susceptibility Test
ATCC	American Type Culture Collection
BF	Bank Filtrations
CFU	Colony Forming Units
CLSI	Clinical and Laboratory Standards Institute
CWF	Ceramic Water Filter
DoHS	Department of Health Services
DWSS	Department of Water Supply and Sewerage
EMB	Eosin Methylene Blue agar
ETEC	Enterotoxigenic *E. coli*
FDA	Food and Drugs Administration
GoN	Government of Nepal
HIV	Human Immunodeficiency Virus
HPC	Heterotrophic Plate Count
JMP	Joint Monitoring Program
KUKL	Kathmandu Upatyaka Khanepani Limited
MBC	Minimum Bactericidal Concentration
MDG	Millennium Development Goals
MF	Membrane Filtration
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
MLD	Millions of Liter per Day
MPN	Most Probable Number

MR	Methyl Red
NDWQS	National Drinking Water Quality Standards
PCR	Polymerase Chain Reaction
POU	Point of Use
SDG	Sustainable Development Goals
SIM	Sulfide Indole Motility
SODIS	Solar Water Disinfection System
STEC	Shiga Toxin producing *E. coli*
TSIA	Triple Sugar Iron Agar
UN	United Nations
UNEP	United Nations Environment Programme
UNICEF	United Nations International Children's Emergency Fund
VP	Voges-Proskauer
WAN	Water Aid in Nepal
WASH	Water Sanitation and Hygiene
WHO	World Health Organization
WWF	World Wide Fund for Nature
ZOI	Zone of Inhibition

LIST OF SYMBOLS

%	Percentage
°C	Degree centigrade / Celsius
µm	Micrometer
gm	Gram
L	Liter
mg/L	Milligram per Liter
mL	Milliliter
mm	Millimeter
µg/ mL	Microgram per milliliter

LIST OF TABLES

LIST OF FIGURES

TABLE OF CONTENTS

CHAPTER 1

1. INTRODUCTION

1.1 General introduction

Pure drinking water is universally recognized as a basic human need and one of the basic or important factors for human growth as well as social and economic existence (WHO, 2007). Freshwater is essential for human health, agriculture, industry and natural ecosystems, but it is becoming scarce in many parts of the world (WWF, 1998). The availability of clean water determines quality of life, as water is one of the most important public health needs and water is a fundamental requirement of life. About 1.2 billion people worldwide lack access to safe drinking water (Wilkes, *et al*., 2009).

The major sources of water are rainfall, glaciers, rivers, lakes, ponds and ground water. Surface water is the major sources for drinking water in Nepal and ground water is an important source in terai region and Kathmandu valley. The major options for sources of drinking water in Nepal are tap or piped water, tube well or hand pumped water, covered and uncovered well water, stone, spout, river and other includes pond, rain water and stream water (ADB/ICIMOD, 2006 ; WAN,2011).

Preserving the quality of fresh water is important for the drinking water supply, food production and recreational water use. Water quality can be compromised by the presence of infectious agent, toxic chemicals and radiological hazards (WHO, 1993). In practice, a Point of use technology should be effective, easy to operate, with low maintenance requirement, economically viable, environmentally sustainable and socio-culturally acceptable. Chlorination with safe storage, combined coagulant-chlorine disinfection systems, solar disinfection, ceramic water filter (CWF) and bio sand filter are examples of current POU technologies (Sobsey, *et al*., 2008; Murphy et al., 2010; Kallman, *et al*., 2011; Geremew, *et al*., 2018). Microbial water quality is analyzed by membrane filtration technique and multiple tube fermentation method. Membrane filtration technique is particularly valuable in determining the number of bacteria in a large sample that has a small number of viable cells (WHO, 1997). Multiple tube ferment technique is the only procedure that can be used if water samples are very turbid or if semi-solids such as sediments or sludge are to be analyzed (WHO, 1996).

According to data from the Department of Health service in Nepal, about 3500 children die each year due to water- borne diseases (Aryal, *et a*l., 2012). The most common

intestinal helminthes among Nepalese children reported are *Ascaris lumbricoides*, hookworm, and *Trichuris trichiura*, with manifestations physical growth (Tandukar, *et al*., 2013).

Diarrheal diseases caused by poor hygiene and dirty water due to hygiene are responsible for a wide range of morbidity and mortality, especially among children in developed and developing countries. (Qadri *et al*., al., 2005; Beatty *et al*., 2006; Ram, *et al*., 2008). In many developing countries, including India, Bangladesh and Pakistan, large numbers of people drink, bathe and wash untreated water from rivers, lakes, wells and other surface water resources. For drinking, bathing, washing, recreation and other household purposes. Depends on (Ashbolt, *et al*., 2004; Obi *et al*., 2004; Qadri, *et al*., 2005). The potential sources of fecal contamination of surface water in these countries are domestic and wild animal defecation, malfunctioning of septic trenches, storm water drainage, and municipal wastes industrial effluents (Ahmed, *et al*., 2005).

Coliform bacteria are commonly used as an indicator for fecal pollution of water (Wolf, 1972; APHA, 1998). The coliform group consists of several genera of bacteria belonging to the family Enterobacteriaceae. These genera included *Escherichia* spp, *Citrobacter* spp, *Enterobacter* spp. and *Klebsiella* spp. initially. However, based on the modern taxonomical criteria, the group is heterogeneous and includes non-fecal lactose fermenting bacteria as well as other species which are rarely found in faeces but are capable of growing and multiplying in water (WHO, 1993).

Antimicrobial agent including naturally occurring antibiotics, synthetic derivatives of naturally occurring antibiotics (semi-synthetic antibiotics) and chemical antimicrobial compounds (Chemotherapeutic agents). Generally, however the term 'antibiotic' is used to describe antimicrobial agent (usually antibacterial) that can be used to treat infection. Compare with antibacterial agents, fewer antiviral and antifungal agents have beendeveloped. Many antiviral agents have serious side effects e.g. those used to treat HIV infection (Cheesbrough, 2006).

Kathmandu has become the most crowed and busiest city in Nepal because of its' haphazard urbanization and industrialization and rapid growth of population. This has brought adverse impacts on ground water system. The Kathmandu valley has its population growth rate of 4.7%. This growth rate exceeds the population growth rate of Nepal by more than double. The valley faces a daily demand of more than 360 million liters of water (Udmale, *et al*., 2016). Kathmandu Upatyaka Khanepani Limited

(KUKL), the government's authentic operator, is fulfilling the water demand of the valley people by only around 140 and 90 million liters per day in wet and dry seasons respectively. At present, more than 70% of households in the valley rely on municipal tap water as rest on ground water resources and others. Besides, more than 14% of households in the valley are receiving water supply from private drinking water tankers (Santosh, *et al.*, 2006). The geographical map of Kathmandu district is given in Appendix-A.

Today, the demand for pure water for drinking and hygienic purposes is more urgent than ever. The challenge is to create incentives in people share human values of common responsibility not to pollute or overuse the water sources (Johannsen, *et al.*, 2011). Clean water and sanitation are human rights, essential to life (Water Aid, *et al.*, 2005). The qualitative service indicators including coverage, quantity, continuity, cost and quality continuity, cost and quality are the vital elements to improve and maintain public health through provision of safe and adequate water supply (WHO, 1997).

1.2 Rationale of the study

Since, water is life but still there are so many people who are not getting pure drinking water. Lack of access to safe drinking water increases the risk of developing water-borne diseases such as diarrhea, cholera, typhoid fever, hepatitis A, and amebiasis (WHO/UNICEF, 2000). People are not aware of microbial water quality and many diseases are associated with water. So, this study will help to know about bacteriological water quality, prevalence and control of waterborne diseases of study area. This study will help to know about awareness of bacterial water quality and importance of water treatment. This may also help in control of waterborne diseases. Furthermore, this results helps to know about antibiotic sensitivity of bacteria isolate from water sample. Point of use (POU) systems, which improve drinking water quality at the household level, represents a more affordable and effective way to produce water free from contamination (Yang, *et al.,* 2020). Rijal, et al., (2012) reported that the water from different sources is heavily polluted with multiple drug resistant coliform bacteria and detection of *Salmonella* spp indicated potential health risk to human.

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1.3 Objective

1.3.1 General objective

- To analyzed bacteriological quality of drinking water in Kathmandu, Nepal and antibiotic susceptibility testing of *E. coli* isolates from water.

1.3.2 Specific objectives

- To conduct pre-structure questionnaire survey on drinking water sources, sanitation, hygiene, water treatment and waterborne diseases in the study area.
- To isolate and enumerate total coliform and fecal coliform from drinking water sources (water shop and restaurants).
- To identify total coliform, fecal coliform and other bacteria from drinking water sources.
- To perform antibiotic susceptibility testing of *E. coli* isolates from drinking water sources.

CHAPTER 2

2. LITERATURE REVIEW

Water is one of the most important elements of all life forms. It is essential for the maintenance of life on earth also important for building and updating cells. Despite of this, human beings are continuing to pollute water sources resulting in provoking water related illnesses (MOH, 2004; WHO, 2008).

2.1 Sources of water

The sources of water to the Kathmandu valley drinking water systems are springs, streams and groundwater. Springs are used as sources in some higher elevation areas and both springs and perennial streams feed some treatment plants (GoN, 2011). Another water sources for individual and treatment plants are tube wells. Both shallow and deep tube wells are used in the Terai and Kathmandu valley. Some small communities who do not have gravity fed springs or pumping system harvest rainwater for drinking purposes (Andrea and Wolfe, 2000).

The Kathmandu valley constitutes the country's single largest economy and has a population of 1.5 million. The current piped water supply of underwater and surface water in the dry seasons varies between 65 and 85 MLD. Even in the wet season, the supply only reaches 140 MLD (Khatiwada, *et al.*, 2002). Due to this, approximately 50% of the urban water supply of Kathmandu valley is obtained from groundwater sources and is also widely exploited for private, domestic and industrial uses (Pant, *et al.*, 2010). The water quality of Kathmandu valley is in degraded state and not in agreement with the WHO (Prasai, *et al.*, 2007).

2.2 Use of water

Water is the basic and primary need for all vital life processes. With increasing industrialization and population growth, water sources, available for various purposes, such as drinking, hand washing, personal bathing, cooking, cleaning and laundering clothes, recreation, aquaculture, agriculture, have been adulterated with industrial as well as animal and human waste (Aneja, 2018; Cheesbrough, 2006).

2.3 Different sources of pollutant in drinking water

Water pollution has become a global problem today, requiring continuous assessment of water resources policies to address this issue. Mortality and disease occur worldwide from water pollution and about 14,000 people die every day from water pollution (Chaudhary, *et al*., 2017). Some factors of drinking water pollution are:

2.3.1 Point source pollution

Pollution is referred to as a point source when the cause of water pollution is known or when pollutants entering the water come from an identifiable source such as a gutter, industrial pipe, rainwater drain pipe, or sewage treatment plant. It can be distinguished from other pollutants (Chaudhary, *et al*., 2017).

2.3.2 Non- point source pollution

Pollution is referred to as a non-point source when the source of the water contamination is unknown or when the contamination does not originate from a single source (Brian, *et al*., 2008). It is very difficult to control and can come from different sources such as pesticides, industrial fertilizer waste and more. Nonpoint sources are the leading cause of water pollution in the United States (Chaudhary, *et al*., 2017).

2.3.3 Agricultural pollutants

Because rural areas are sparsely populated, they mainly contain fertilizers, pesticides and soil erosion, and these pollutants enter water bodies with runoff after rains and floods. Agricultural runoff causes eutrophication of freshwater bodies (Tyagi, *et al*., 2015). Half of America's lakes are eutrophic. Phosphate is a major factor in eutrophication and its high concentration promotes the growth of blue-green algae and algae, ultimately reducing the amount of dissolved oxygen in the water. Harmful toxins that accumulate in the food chain are formed as a result of flowering cyanobacteria. Nitrogen fertilizer compounds cause a lack of dissolved oxygen in rivers, lakes and coastal areas, which has a devastating effect on marine wildlife (Chaudhary, *et al*., 2017). Nitrogen (N) in the form of nitrates is a common contaminant in both surface and groundwater. Nitrate-N readily leaches beyond the root zone of agricultural soils and can enter soil and surface water. At latitudes, phytoplankton productivity is stimulated by nitrate-N, leading to eutrophication, resulting in widespread hypoxia and anoxia, loss of biodiversity and harmful algal blooms that can damage pristine marine environments and fisheries such as historic coral reefs (Singh, *et al*., 2006).

2.3.4 Atmospheric pollutants

Atmospheric pollutants are those small particles present in the air which reaches to water bodies through rain. It consists of carbon dioxide produced by burning fossil fuels as it combines with water molecules to form sulfuric acid. Industries and volcanoes produced sulfur dioxide which combines with water molecules to form sulfuric acid. Burning coal and petroleum products also produced Sulfur dioxide. Nitric acid was formed by the combination of nitrogen dioxide with water (Letchinger, *et al*., 2000; Brian, *et al*., 2008).

2.3.5 Pathogens

Pathogenic are microorganisms which can cause the diseases under right conditions. Most of the bacteria found in nature are not pathogenic or beneficial, only few bacteria are pathogenic in nature which is *Shigella* spp. (dysentery), *Salmonella* spp. (gastrointestinal illness) and *Pseudonomas auerognosa* (swimmer's itch) *Cryptosporidium parvum*, *Burkholderia pseudomallei, Giardia lamblia*, Norovirus, *Salmonella* and parasitic worms like Schistosoma (Chaudhary, *et al*., 2017). Cholera, typhoid fever and "staph" infections are caused by some subspecies. Single cell organisms which are motile in nature are known as protozoa. Giardia and cryptosporidium are the two common pathogens of protozoans (Thomas, *et al*., 2000).

2.3.6 Pesticides and herbicides

Herbicides and insecticides are used to control pests and weeds. Both the herbicides and insecticides contribute to water pollution. Ground water is also get polluted by leaching process. Soil texture, pesticide properties, irrigation and rain fall are the influenced factors of leaching (Chaudhary, *et al*., 2017).

2.3.6 Chemical pollutants

The toxic chemical waste came from factories, it is a material used as a by-product in the manufacturing process and it also plays a vital role in polluting water sources. Solid, liquid or gases are the hazardous chemicals waste. Corrosiveness, flammability, toxicity and reactivity of material are the properties that make material hazardous. Industrial chemical waste cannot be treated by wastewater treatment plants but can be treated using special waste treatment facilities (Chaudhary, *et al*., 2017).

2.4 Water and sanitation

Water is so important for all living beings that it should always remain pure but unfortunately it gets polluted through various sources. All over the world water pollution is posing threat to human life both in rural and urban areas. Water pollution is the cause of many diseases and consequently of atmospheric pollution (WHO, 1996).

Drinking water from underground source (e.g. wells) can be consumed safely, but surface water from most lakes and rivers must be treated (Ingraham and Ingraham, 2004). Currently there are no any water sources that do not need treatment before consumption. The general rule is that water must be treated to remove potentially harmful microbes and to improve its clarity, odour and taste (Alcamo, *et al*., 2003).

Water hygiene and hygiene (WASH) are the basis of human development and well-being. World Health Organization / United Nations International Children's Emergency Fund (WHO/ UNICEF) joint monitoring program (JMP) for water supply and sanitation estimates that, in 2015, 663 million people lacked improved drinking water sources and 2.4 billion lacked improved sanitation facilities (WHO/UNICEF, 2015). Lack of access to safe drinking water increases the risk of contracting waterborne diseases including diarrhea, cholera, typhoid, hepatitis A and amoebic dysentery. Each year more than 2.2 million people dies among 4 million diarrheal patient and most of them are children under the age of 5 in developing and under-developed countries (WHO/UNICEF, 2000). Nepal faces a plethora of problems related to WASH issues (Prüss, *et al*., 2014; Warner, *et al*., 2008). In 2015, WHO/ UNICEF reported that 92% of Nepalese population had access to improved water, and hence, met this specific MDG target (WHO/UNICEF, 2015).

2.5 Water and sanitation in Nepal

Nepal has made rapid progress in sanitation coverage, aiming to achieve basic sanitation for all and eliminate open defecation in the country by 2020. A substantial proportion of the total population still lack basic drinking water services, depend on unreliable and unimproved sources provided water such as ponds, unprotected wells and streams (Budhathoki, *et al*., 2019). Nepal faces a number of issues related to water and sanitation (Prüss, *et al*., 2014, Warner, *et al*., 2008). In 2015, WHO/ UNICEF reported that 92% of Nepalese population had access to improved water, and hence, met this specific MDG target (WHO/UNICEF, 2015). However, it is not yet clear whether water classified as improved can be safely consumed. Although the range of hygiene was

46%, 37% of the population still has open defecation, which poses a serious risk of environmental pollution represented by open water source (WHO/UNICEF, 2015 and GON, 2011). According to data from the Department of Health service in Nepal, about 3500 children die each year due to water- borne diseases (Aryal, *et al*., 2012). The most common intestinal helminths among Nepalese children reported are Ascaris lumbricoides, hookworm, and Trichuris trichiura, with manifestations physical growth (Tandukar, *et al*., 2013). Several studies investigated heavy metal, such as lead and arsenic. With regard to lead, a study reported high concentrations (15-35 µg/L) in drinking water samples collected from different parts of Nepal (Shrestha, *et al*., 2009).

2.6 Waterborne diseases and waterborne pathogens

Waterborne pathogens and related diseases are a major public health problem worldwide, not only because of the morbidity and mortality they cause, but also because of the high costs of prevention and treatment (Castillo, *et al*., 2015). There are frequent report of spread of typhoid, diarrhea and cholera through water supply in Nepal. In each and every summer, water borne epidemics hit different parts of the country including Kathmandu valley. Drinking water is only the vehicle for the transmission of water borne diseases (WHO, 2002). Annual report from DoHS (2004/2005) showed that there were 2332 cases of typhoid, 18611 cases of diarrhea disease, 9322 cases of intestinal worms, 543 cases of jaundice and infectious hepatitis in Kathmandu valley.

According to WHO (2002), unsafe water supply, is a major problems and fecal contamination of water sources and treated water is a persistent problem worldwide. Globally, 1.1 billion people rely on unsafe drinking water sources from lakes, rivers and open wells. The majority of these are in Asia (20%) and sub-Saharan Africa (42%) (WHO/ UNICEF, 2000; WHO/UNICEF, 2005).

According to (WHO), water-related diseases are estimated to cause 1.8 million deaths each year, mostly in developing countries and have been the major cause of mortality and morbidity (WHO, 2004). In Nepal, diarrheal disease ranks second in the list of top-ten diseases (Ministry of Health, 2005). The most common intestinal helminthes among Nepalese children reported are Ascaris lumbricoides, hookworm and Trichuris trichiura with manifestations physical growth (Trandukar, *et al*., 2013).

Pathogenic bacteria such as *Salmonella* spp., *Shigella* spp., *Escherichia coli* and *Campylobacter* spp. responsible for most of the waterborne disease burden (Griffith,

2017).Waterborne organism are bacteria (*Burkholderia pseudomallei.Campylobacter jejuni, C. coli, Escherichia coli*- pathogenic, *E. coli* Enterohaemorrhagic, *Legionella* spp. , Non- *tuberculous mycobacteria*, *Pseudomonas aerug 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., Cryptosporidiumparvum, Cyclospora cayetanesis, Entamoeba histolytica, Giardia intestinalis, Naegleria fowleri and Toxoplasma gondii) and Helminths (Drancunculus medinensis and Schistosoma spp.) (WHO, 2006).

2.7 Water related problems in Nepal

Water pollution is a worldwide problem and poses a serious threat to human life. For most Nepalese, obtaining sufficient water is a greater concern than obtaining safe water. Department of Water Supply and Sewerage (DWSS) reported that around 86% of the Nepalese population has access to basic water supply facility as of mid – 2015 (DWSS, 2015).

Maharjan, *et al*., (2018) evaluated the quality assurance of treated water in the Kathmandu Valley, in which 243 samples were taken from different sources such as filtered water, bottled water, tanks and bottled water. Analyzed microbiological parameters of the 243 samples processed, 160 (66%) samples were contaminated with coliform bacteria. More than 92% of water samples, 77% of water tank samples, and 69% of filtered water samples had total coliform counts higher than the NDWQS guidelines. Additionally, 20% of bottled water is contaminated with coliform bacteria.

Each year, especially during dry season Kathmandu valley suffers a severe drinking water supply crisis. The drinking water supply in the cities of the valley is intermittent whereas, in urban areas water supplies are often 24 hours a day. In major cities around the world like, Delhi, Karachi, Dhaka similar statistic on water supply can be seen as Kathmandu i.e. Less than 1.0% peoples with 24 hours water supply (Mcintosh, *et al*., 2003).

Gyawali, *et al*., (2007) conducted a study on Microbial and chemical quality of water available in Kathmandu with 6 samples of tap and river from Sundarighat upstream and

found that the physiochemical parameters were below WHO standards except the chloride along with bacteriological contamination of 900 CFU/100 mL in average. Like many developing countries, Nepal faces many challenges in terms of both water quality and availability of drinking water. Throughout Nepal, people face serious health threats from water pollution from sewage, agriculture and industry. Due to the effects of sewerage, typhoid fever, dysentery and cholera are prevalent every summer (Khadka, *et al*., 1993).These diseases account for 15% of all illness and 8% of total deaths, but those numbers increase to 41% of all illness and 32% of all deaths in children up to 4 years old (Sharma, *et al*., 1990).

In the Kathmandu Valley, main concern is contamination of surface or ground water is from sewage lines, septic tanks, open pit toilets or direct disposal of sewage waste (Jha, *et al*., 1997; Khadka, 1992; Karn and Harada 2001). Direct disposal of industrial waste to the surface water is also major problem in Kathmandu valley which might lead to contamination of exiting shallow aquifer inside Kathmandu (Khadka, *et al*., 1992, Karn and Harada, 2001).

Burlakoti, *et al*., (2020) analyzed the quality of drinking water from different public locations in Kathmandu, where a total of 50 water samples were analyzed. The number of microorganisms varied from 0 CFU to >300 CFU/100 mL. The results showed that more than 76% and 92% of samples were contaminated with E. coli bacteria and Total coliform bacteria, which exceeded WHO guidelines and national drinking quality.

Approximately 50% of the water supply in the Kathmandu Valley is derived from groundwater sources (Jha, *et al*., 1997; Khatiwada, *et al*., 2002). Time and again drinking water quality in Kathmandu valley was found to be very unsatisfactory, both considering microbiological water quality as well as physiochemical quality, furthermore, chlorinated water were also found to be heavily contaminated with fecal coliform (Jha and Lekhak, 1999). In Nepal, more than 33,000 people die every year by gastro-enteritis caused by drinking water contamination and poor sanitation (Anonymous, *et al*., 2004).

As of 3rd July 2022, a total of 17 cases of cholera has been reported which is sporadic in occurrence out of 62 water sample taken and tested showed contamination with fecal *E. coli* (MOHP, 2022).

2.8 Water treatment methods

Water is one of the basic human needs, but a large part of the people of Nepal does not have access to an adequate and safe source of drinking water. According to the Nepal Water Supply and Sewerage Department, although about 80% of the total population has access to potable water, it is not safe (DWSS, 2015).

A slow sand filtration system is one of the earliest processes used to removing contaminants from surface waters to produce drinking water (Rachwal, *et al.*, 1986; Montiel, *et al.*, 1988). Slow sand filters because of their simplicity, efficiency and economy are appropriate means of water treatment, particularly for community water supply in developing country (Visscher, *et al.*, 1988).

In practice, a Point of use technology should be effective, easy to operate, with low maintenance requirement, economically viable, environmentally sustainable and socio-culturally acceptable. Chlorination with safe storage, combined coagulant-chlorine disinfection systems, solar disinfection, ceramic water filter (CWF) and bio sand filter are examples of current POU technologies (Sobsey, *et al.*, 2008; Murphy *et al.*, 2010; Kallman, *et al.*, 2011; Geremew, *et al.*, 2018).

Bank filtration (BF) applied to a river or lake, is a reliable, natural and multi-objective treatment process which removes particles, biodegradable organic compounds, trace organics, microorganisms as well as ammonia and nitrate to some extent. Furthermore, it also dampens temperature peaks and concentration peaks associated with spills. BF could replace or support other treatment process, thus providing a robust barrier within a multi-barrier system and also decreases the costs of water treatment. BF is a traditional, efficient and well accepted method of surface water treatment in Europe. For more than 100 years, river bank filtration has been used in Europe for public and industrial water supply along Rhine, Elbe, and Danube rivers (Grischek, *et al.*, 2002).

There are many methods available for household-level disinfection of drinking water, including chlorination, iodine, filtering and solar disinfection. Each of these methods or combinations of methods has trade-offs in terms of effectiveness, convenience and cost (Sobsey, *et al.*, 2002).

Solar Water Disinfection system (SODIS) uses solar energy in the form of ultraviolet radiation and, to a lesser extent, infrared heat, to inactivate or destroy pathogenic microorganisms in the water (EAWAG/SANDEC, *et al.*, 2002). Other techniques, such

as filtration, chemical disinfection and exposure to ultraviolet radiation (including solar UV) have been demonstrated in an array of randomized control trials to significantly reduce levels of water-borne diseases among users in low-income countries (Classen, *et al*., 2007), but this suffer the same problem as boiling methods.

2.9 Water quality

To ensure safety and hygiene, it is necessary to ensure water quality before drinking. Safe drinking water is defined as water with microbial, chemical and physical properties that meet the WHO guidelines for national standards for drinking water quality (WHO, 2007).

2.10 Physicochemical parameters of water

According to APHA (1998), the physicochemical parameters of water are pH, temperature, turbidity, electrical conductivity, total hardness, iron, ammonia, nitrate, nitrite, arsenic, chloride, free residual chlorine etc. which determined the quality of water for drinking purpose.

2.11 Microbial parameters of water quality

Testing for bacteria in the water can confirm whether the water source is contaminated with feces. Fecal coliforms (sometimes called thermotolerant coliform organisms or *E. coli*) are the most appropriate indicator of fecal contamination. *E. coli* counts are the most useful test for detecting fecal contamination of water supplies during water quality testing. There are two main techniques for counting coliforms in stool (Cheesbrough, 2006).

2.11.1 Total coliform

Coliform bacteria are commonly used as an indicator for fecal pollution of water (Wolf 1972; APHA, 1998). The coliform group consists of several genera of bacteria belonging to the family Enterobacteriaceae. Traditionally these genera included *Escherichia*, *Citrobacter*, *Enterobacter* and *Klebsiella*. However, based on the modern taxonomical criteria, the group is heterogeneous and includes non-fecal lactose fermenting bacteria as well as other species which are rarely found in faeces but are capable of multiplication in water (WHO, 1993). These indicators, used to assess the potential public health risk of drinking water, are key elements of most drinking water quality guidelines (WHO, 1997). Certain strains of *Escherichia coli*, the widely used 'indicator' of the microbiological quality, have virulence properties that may account

for life- threatening urinary tract infection, haemolytic colitis, neonatal meningitis, nosocomial septicaemia, haemolytic uraemic syndrome and surgical site infections because of the presence of genes specific for a pathotype (Kuhnert, *et al*., 2000). Currently, six *E. coli* pathotypes are recognized (Turner, *et al*., 2006) that can causes diarrhea in humans: entero-pathogenic *E. coli*, enteroinvasive *E. coli*, enterohaemorrhagic *E. coli* or shiga toxin producing *E. coli* (STEC), enteroaggregative *E. coli*, enterotoxigenic *E. coli* (ETEC) and diffusely adhering *E. coli*. Very low studies provided information on the occurrence of pathogenic *E. coli* harbouring genes specific for different pathotypes of *E. coli* in surface water (Obi, *et al*., 2004; Begum *et al*., 2005; Higgins *et al*., 2005; Hamelin, *et al*., 2006; Shelton, *et al*., 2006; Ram, *et al*., 2008). The microbiological quality of drinking water has attracted great attention worldwide because of implied public health impacts (Amira, *et al*., 2011).

The term "total coliform" refers to a large group of Gram-negative, rod-shaped bacteria that share several characteristics. The group includes thermotolerant coliform and bacteria of fecal origin, as well as some bacteria that may be isolated from environmental sources (Singh, *et al.,* 2018). In the laboratory total coliform are grown in or on a medium containing lactose, at a temperature of 35 or 37°C with in a 24- 48 hours. They are provisionally identified by the production of acid and gas from the fermentation of lactose (UNEP/WHO 1996).

2.11.2 Thermotolerant (fecal) coliform

The term "fecal coliform" has been used in aquatic microbiology to refer to coliform organisms that grow at 44 or 44.5°C within 24- 48 hours and ferment lactose to produce acids and gases (UNEP/WHO, 1996). Coliform that come from fecal matter can tolerate higher temperature then most environmental coliform, so those that ferment lactose and produce gas at 45.5°C are called thermotolerant, or fecal coliform. These are more closely associated with fecal pollution then total coliform. The most specific indicator of fecal contamination is E. coli, which, unlike some fecal coliform, never breeds in aquatic environments. Either coliform or fecal coliform is an acceptable indicator species. The fecal coliform has many characteristics of excellent indicator species. They are universally abundant in feces, are not pathogenic in their own right, and are relatively easy to measure with a simple and inexpensive device (UNEP/WHO 1996). One drawback of coliform indicator is that they are significantly more susceptible to chlorine then other pathogens (e.g. Cryptosporidium, viruses). Also some treatment

processes may remove coliform but not viruses, which are much smaller. For these reasons, water without E. coli or fecal coliform should be seen as a low risk, rather than completely safe (UNICEF, 2008).

2.11.3 Other indicator organisms

Traditionally, microbiological indicators have been used to suggest the presence of pathogens. Today, however, we understand the multitude of possible reasons for the presence of indicators and the absence of pathogens, or vice versa. In summary, there is no direct correlation between the number of markers and enteric pathogens (Ashbolt, *et al.*, 2001). Other indicator organism sometimes used include fecal enterococci as an indicator of fecal pollution, and heterotrophic plate count (HPC) measurements, which are useful in assessing the effectiveness of treatment and distribution systems. Clostridia prefringens is a type of bacteria that can survive in the environment, and is resistant to contaminated with fecal material (UNICEF, 2008). Most Streptococci are parasites of human and animals, and several species are pathogenic. A few examples of them are *S. pyogenes, S. mutans, S. faecalis, S. lactics and S. cremoris* and *S. pneumoniae* (Pelczar, 2009).

Microbiologists often prefer to detect the presence of indicator organisms (coliforms) as an indication that water may be contaminated with human pathogens. Their presence indicates the possibility of fecal contamination in the water source. This water is considered unsanitary and must be treated well to remove indicators and pathogens (WHO, 2011).

2.12 Bacteriological water quality analysis techniques

2.12.1 Most probable number method (MPN)

Multiple tube ferment technique is the only procedure that can be used if water samples are very turbid or if semi-solids such as sediments or sludge are to be analyzed. It is customary to report the results of the multiple fermentation tube test for coliform as a Most Probable Number (MPN) index (WHO,1996).

The accuracy of each test depends on the number of tubes used. The most satisfying result is obtained when the largest sample inoculum tested contains gas in some or all tubes and the smallest sample inoculum contains no gas in all or few. Bacterial density can be estimated from a given formula or from a table using the number of positive tubes with multiple dilutions. The number of sample pieces selected depends on the

desired accuracy of the result. The MPN table is based on the assumption of Poisson distribution (random variance). However, if the sample is not shaken sufficiently before removing some, or if bacterial cells aggregate, the MPN value will not represent the actual bacterial density (APHA, 1998).

Testing is performed sequentially in three phases: assumption, validation, and termination. Lactose broth tubes were inoculated with different volumes of water in a hypothetical test. Gas-positive tubes were inoculated into brilliant green lactose bile juice in the validated test, and positive tubes were used to calculate the most likely number of coliforms in the water sample. The completed test, consisting of inoculation of EMB agar plates, skewing of nutrient agar and brilliant green lactose bile broth and preparation for Gram stain and spore slide from an angle, was used for determination (Aneja, 2018).

2.12.2 Membrane filtration method (MF)

Membrane Filtration technique is particularly valuable in determining the number of bacteria in a large sample that has a small number of viable cells (WHO, 1997).

A water sample is passed through a thin sterile Membrane Filter (pore size 0.45µm) which is kept in a special filter apparatus contained in a suction flask. The filter disc that contains the trapped microorganism is aseptically transferred to a sterile petriplate having an absorbent pad saturated with a selective, differential liquid medium, and the colonies which develop, following incubation are counted. This method enables a large volume of water to be tested more economically, results obtained are more accurate and are obtained more quickly than by the multiple-tube technique (Aneja, 2018).

Membrane Filter (MF) technology is reproducible and can be used to test relatively large samples (100 mL at a time), usually providing faster numerical results than multi-tube fermentation methods. MF technology is useful for monitoring drinking water and various natural waters. However, MF techniques have limitations, especially when testing turbid water and large numbers of bacterial (coliform or non-coliform bacteria). If interference occurs during enumeration and isolation due to high number of bacterial isolates (coliform or non-coliform bacteria), the sample results is considered as invalid and a new sample should be tested again (APHA, 1998).

This technique is not suitable for natural waters containing very high levels of suspended solids, sludge and sediment, all of which can clog the filter before sufficient

water has passed through. When small amounts of sample need to be tested, it is necessary to dilute a portion of the sample in a sterile diluent to ensure sufficient volume for filtration (UNEP/WHO, 1996).

2.12.3 Heterotrophic Plate Count (HPC) Enumeration

Heterotrophs are generally defined as microorganisms that require organic carbon to grow. These include bacteria, yeasts, and molds. A series of simple culture-based tests aimed at the recovery of a wide variety of microorganisms in water are collectively known as the "Heterotrophic Plate Count" or "HPC test" procedure. HPC test methods cover a variety of test conditions that lead to a wide range of quantitative and qualitative results. The test itself does not specify the organisms detected. Only a small percentage of the metabolically active microorganisms present in a water sample can grow and be detected under a given set of HPC test conditions, and recovered populations will vary considerably depending on method used. The actual organisms recovered in the HPC test can also vary widely from site to site, from season to season, and from successive samples at the same site. Microorganisms recovered through HPC testing typically include those that are part of the water's natural (usually non-hazardous) micro biome; in some cases, they may also include organisms originating from different pollution sources (Bartram, *et al*., 2013).

2.12.4 Molecular method

Molecular methods can also be applied to detect antibiotic resistance, which studies the presence of resistance genes or point mutations. The different molecular methods for drug resistance are Polymerase Chain Reaction (PCR), AMR Gene Detection, and Microarray (Anjum, *et* al., 2017).

2.13 Identification of the bacterial isolates

For the identification of bacteria isolated, identification of pure culture of bacteria is done by of colony morphology, biotyping, serotyping, molecular techniques (APHA 1998 ;Forbes *et al.,* 2007; Cheesbrough 2006).

2.14 Drinking water quality standard

According to the water quality of WHO bacteriological drinking water, coliform should not be detected in 100 mL samples of treated water in the distribution system. Total coliform should not be detected in 100 mL samples (WHO, 1997).

Table 1: Classification scheme for thermotolerant coliform or *E. coli* in water

Count per mL	Category	Remarks
0	A	In conformity with WHO guidelines
1-10	B	Low risk
10 – 100	C	Intermediate risk
100 – 1000	D	High risk
>1000	E	Very high risk

Source: WHO (1997)

According to the water quality of National drinking water quality standard NDWQS bacteriological drinking water, coliforms should not be detected in 100 mL samples of treated water in the distribution (NDWQS, 2005).

Table 2: Standard of drinking water given by National Drinking Water Quality Standard (NDWQS)

S.N.	Category	parameters	Units	Concentrations Limits
1.	Microbiological	*E. coli*	MPN/100mL	0
2.		Total coliform	MPN/100mL	0 in 95% samples

Source: National Drinking Quality Standards and Directives, 2005

2.15 Drugs resistance

Much of the intractable resistance to some infectious diseases is due to the fact that the widespread use and abuse of antibiotics has contributed to the emergence and persistence of resistant microbial strains. Commonly resistant strains are *Staphylococcus aureus, Gonorrhea, Meningococcus, Pneumococcus, Enterococci*, Gram-negative (eg *Salmonella* spp., *Shigella* spp., *Klebsiella* spp., *Pseudomonas* spp.) and *M. tuberculosis* (Cheesbrough, 2006).

2.16 Multiple drugs resistance

Antibiotic resistance occurs when bacteria adapt and multiply in the presence of antibiotics. The development of resistance depends on how often antibiotics are used. Because many antibiotics belong to the same class of drug, resistance to a particular antibiotic can lead to resistance to the entire related class. Resistance that develops in organisms and loci, for example, exchanges genetic material between different bacteria and affects antibiotic treatment of various infections and diseases, but can spread rapidly and unpredictably. Drug-resistant bacteria can circulate through human and animal populations through food, water and the environment, and infections are affected by

trade, travel, human and animal migration. Resistant bacteria are found in livestock and foods intended for human consumption (WHO, 2015).

Multiple Drug Resistance (MDR) is defined as the insensitivity or resistance of microorganisms to administered antimicrobials (is structurally unrelated and has different molecular targets), and these drugs are ineffective (Singh, *et al.*, 2013). The presence of MDR pathogens in animal-derived foods such as milk and meat has increased dramatically due to their ability to evolve (Rodriguez and Taban, 2019).

2.17 Antimicrobial susceptibility technique (AST)

The main goal of antibiotic susceptibility testing is to detect potential drug resistance of common pathogens and ensure susceptibility to selected drugs for the immediate disease or problem. The most widely used methods for testing antibiotic susceptibility include broth dilution test, antimicrobial gradient method, and disc diffusion method (Barth, et al., 2009). Antibiotic susceptibility testing is performed by the commonly used agar diffusion method to determine the minimum amount of antibiotic required to inhibit microbial growth. The value obtained is called the minimum inhibitory concentration (MIC) and is determined by measuring the diameter of the growth inhibitory zone (inner region) surrounding the antibiotic disc (Aneja, 2018). Susceptibility testing for common organisms or contaminants should never be performed, as this would mislead the clinician and could lead to the patient receiving antibiotic therapy. Ineffective and unnecessary use of antibiotics may causes possible side effects and may induce resistant properties to the same or potential pathogens (Cheesbrough, 2006). Standard bacterial cultures, the American Type Culture Collection (ATCC), have been used for decades as a reference, some of which are required or recommended in both FDA regulations (2012) and the standard is published by Clinical and Laboratory Standards Institute (CLSI) (Simione, *et al.*, 2011). Different methods that can be applied to detect antibiotic resistance are the Kirby-Bauer disc diffusion method and the molecular method.

2.18 Types of antimicrobial susceptibility technique

Laboratory tests for antibiotic susceptibility can be performed using:

- Dilution Technology
- Disc diffusion Technology

2.18.1 Dilution technology

Manual or semi-automated dilution susceptibility testing performed in a microbiological reference laboratory for epidemiological purposes or when a patient is not responding to the anticipated appropriate treatment. The dilution method measures the minimum inhibitory concentration (MIC). They can also be used to determine the minimum bactericidal concentration (MBC), which is the lowest concentration of an antimicrobial agent required to kill an antimicrobial agent. The dilution test is performed by adding a diluted solution of the antimicrobial agent to the culture solution or agar. A standardized inoculum of the test organism was then added. After overnight incubation, the MIC was reported to be the lowest concentration of antimicrobial agent needed to prevent visible growth. Clinical response can be assessed by comparing MIC values to known achievable drug concentrations in serum or other body fluids (Cheesbrough, 2006).

2.18.2 Disc Diffusion technology

The disc diffusion technique is used by most laboratories for routine testing of antibiotic susceptibility. An absorbent paper plate impregnated with known volume and the appropriate concentration of antimicrobial agent, and this substance is placed on a susceptibility test agar plate homogenized with the test organism. The antimicrobial agent diffused from the dish into the medium and growth of the test organism was inhibited at the distance from the dish (among other factors) to the susceptibility of the organism. Sensitive strains susceptible to antibiotic were inhibited from the disc while resistant strain had a zone of inhibition smaller than or grew to the edge of the plate. For clinical and surveillance purposes and to promote reproducibility and comparability of results between laboratories, WHO recommends the modified Kirby-Bauer disc diffusion technique (Cheesbrough, 2006).

2.18.2.1 Modified Kirby- Bauer susceptibility testing technique

Modified Kirby- Bauer disc diffusion test method is a reference method that could be used as a routine technique to test the stability of a bacterial isolates in clinical laboratory. It is standard method that can be used as a regular method to accesses the susceptibility test to a bacterial isolate in the clinical laboratory. The validity of this carefully standardized technique, for each identified species, is subject to the use of a plate with the correct antibacterial content, the inoculum producing confluent growth and Mueller agar Reliable Hinton. The test method must be followed precisely in every detail. After incubation at 35 °C for 16-18 hour, the zone size was measured and

interpreted using the CLSI standards. These are derived from a correlation that exists between region size and MIC (Cheesbrough, 2006; CLSI, 2018).

2.18.2.2 Stokes disc diffusion technique

In this plate technique, test and control organisms were inoculated with in the same plate. Area size of test organism was directly compared with that of control. This method is not as standardized as the Kirby-Bauer technique and is used inlaboratories, especially when the exact amount of antimicrobial agent in the plate cannot be guaranteed due to difficulty in obtaining the plates and storing them properly or when other conditions required for the Kirby-Bauer technique cannot be met. That laboratories in developing countries using Stokes technique can convert to technique which is comparable to the WHO Kirby-Bauer recommended technique using Rosco Diagnostic antibiotic tablets have high stability instead of which is less stable than paper discs (Cheesbrough, 2006).

2.18.2.3 Etest

Identification of an anomalous pattern of resistance can be done using the Etest method. Etest is a quantitative method to determine the minimum inhibitory concentration (MIC) of an antimicrobial agent against a microorganism and to identify mechanisms of resistance. It consists of a predefined antibiotic concentration gradient for a specific antibiotic on a plastic strip. When the Etest strip is applied to the surface of the inoculated agar, i.e., the Mueller-Hinton gradient of the antimicrobial agent is immediately transferred to the medium. After overnight or longer incubation, a symmetrical inhibition ellipse (elliptic region) was formed in the center along the band. The MIC was read directly in micrograms per milliliter (µg/mL) scale at the point where the edge of the inhibition ellipse intersects the band (Cheesbrough, 2006).

2.19 Antibiotic resistance pattern of *E. coli* isolated in water

Jayana, *et al*., (2009) reported that among total isolates of *E. coli* , resistance was directed to 79.5% Erythomycin, 62.67% Penicillin G, 61.9% Amoxycillin, 34.5% Ampicillin, 21.1% Tetracycline, 15.4% Ceftriaxone, 14.7%, Amikacin, 14.7%, Cephotaxine, 5.6% Chloramphenicol, 5.6% Ofloxacin respectively .

Rijal, *et al*., (2012) reported, the antibiotic susceptibility pattern among *E. coli* isolates showed that all the tested isolates were sensitive towards Chloramphenicol, Ofloxacin

and Co-trimoxazole, 93.5 % isolates were resistant to tetracycline followed by Amoxycillin 80.6 %, Cefexime 48.4%, Nalidixic Acid 25.8%, Amikacin 25.8%.

Chaudary, *et al*., (2011) found that the antibiotic susceptibility pattern among *E. coli* isolates were sensitive towards Chloramphenicol, Ofloxacin and Co-trimoxazole, 93.5 % isolates were resistant to tetracycline followed by Amoxycillin 80.6 %, Cefexime 48.4%, Nalidixic Acid 25.8%, Amikacin 25.8%.

Nazia, *et al*., (2021) reported that tap water samples collected from different towns of Lahore, the antibiotic pattern of *E. coli* showed the highest sensitivity towards towards Imipenem (IPM) which was 96.3% (n=26) followed by Meropenem (MEM) 92.6%, and Tazocin (TZP) 81.48% respectively and the highest resistance shown by the organism was towards Ampicillin (AMP) 81.48%, Augmentin (AMC), and Ceftazidime (CAZ) 51.85% respectively.

Subba, *et al*., (2013) found that the Thermotolerant *E. coli* isolates were more resistant than *E. coli* isolates towards Amoxicillin, Amikacin, Cefexime and Nalidxic acid though Ofloxacin, Chloramphenicol and Cotimoxazole were equally inhibitory to both *E. coli*. Tetracycline resistance was found in all thermotolerant *E. coli* isolates and two *E. coli* from well water did not show resistivity to Tetracycline. Higher proportion of thermotolerant *E. coli* 75.8% isolates expressed multiple drug resistance whereas, 70.9% *E. coli* isolates expressed Multiple Drug Resistance.

According to Abdullah, *et al*., (2011), *E. coli* resistance was most frequently observed to Ampicillin 76.9%, Chloramphenicol 53.8%, Streptomycin 65.4%, Tetracycline 50%, Co-trimoxazole 38.5%, Nalidixic Acid 42.3%, and Ciprofloxacin 34.6%.

CHAPTER 3

3. MATERIALS AND METHOD

3.1 Materials

All materials including glass ware, microbiological media and chemicals are as given in appendix- D.

3.2 Method and study design

3.2.1 Site survey

A site survey was conducted to analyze bacteriological quality of drinking water and available of treatment plants to carry out the project work. The study observed drinking water sources and consulted with residents of the Kathmandu metropolitan area to determine sampling points.

3.2.2 Types of study

The type of this study was cross sectional study.

3.2.3 Duration

All the site survey, water sampling, questionnaire survey, data collection and bacteriological analysis of the drinking water sample were performed during March 2022 to May 2022.

3.2.4 Sampling and sampling site

The locations of samples collection were Makhan, Bangemuda, Ason, Jamal, Jytha, Thamel, Nardevi and Chhetrapati.

3.2.5 Sampling size

During the study the total numbers of water samples collected were (N=39) where (n= 15) water samples from water shops and (n=24) drinking water samples from the restaurants.

3.2.6 Data sources and data collection technique

The study was carried out on the basis of primary source of data. The data was collected from questionnaire survey and laboratory analysis for the study. The secondary sources of data was also collected from population census and publication related to drinking water, antibiotic susceptibility and sanitation.

3.3 Questionnaire survey

Questionnaire survey was conducted by communicating with the population using shop water and restaurants water for drinking of Makhan, Ason, Jytha, Jamal, Bangemuda, Thamel, Nardevi and Chhetrapati. The survey was performed to collect data and gather the information about drinking water supply system, water disinfectants methods and hygiene, waterborne diseases, sanitation and hand washing practice of residents.

3.4 Analysis of bacteriological quality of drinking water

3.4.1 Methods of sample collection

The water samples were collected from different places inside Kathmandu. For the analysis of bacteriological quality of drinking water, the water sample was collected in sterile plastic bottles. During filling inner surface of the cap was not contaminated and the sample were taken in the bottle without rinsing up to neck of the bottle leaving air space and the sample was not overflowed from the sampling bottle. Sodium thiosulphate was used in sterile bottle to neutralize the chlorine or chloramines (APHA, 1998 and Cheesebrough, 2006).

3.4.2 Sample transportation

The ice box was used to transport the water sample to the Department of Microbiology, Amrit Campus and process with in 6 hour for bacteriological analysis of water. All the laboratory work was done in Microbiology laboratory of Amrit Campus (APHA, 1998).

3.4.3 Isolation and enumeration of coliform

Isolation and enumeration of coliform was done by membrane filtration technique (APHA, 1998). For Membrane Filtration technique, 100mL of water sample undiluted or dilluted was vacuum filtered through 0.45µm filter paper, which was then placed in Eosin Methylene Blue Agar (EMB) to suppress the growth of unwanted organism and promote the growth of coliform as it is a selective as well as differential media which suppress the growth of gram positive bacteria and it was incubated at 35°C±0.5 and 44°C± 0.2. Process shown in appendix-H (APHA,1998; Hi-Media 2021, Aneja, 2018).

3.4.4 Identification of bacteria isolates from water sample

For the identification of isolates on EMB agar plate, isolated colony was sub cultured on MacConkey agar for obtaining pure culture. After this the biochemical test was performed to identify the bacteria. Different biochemical test including Gram Staining, Oxidative-Fermentative test (OF), Sulfide Indole Motility test (SIM), Methyl Red (MR),

Voges –Proskauer test (VP), Triple Sugar Iron Agar test (TSIA), citrate utilization and urea hydrolysis test was performed (APHA, 1998; Cheesbrough, 2006 ; Forbes, *et al.,* 2007).

3.4.5 Antibiotic susceptibility testing

Antibiotic susceptibility of *E. coli* was carried out as described by Kirby-Bauer disc diffusion method and interpreted as susceptible, intermediates and resistance according to the CLSI guidelines. The inoculum will be prepared by suspending the *E. coli* into 2 mL of nutrient broth and the turbidity of these inoculums was adjusted to 0.5 McFarland standards. The lawn was prepared on Mueller Hinton Agar (MHA) media with sterile cotton swab. Ten antibiotics were taken which were: Amikacin, Ampicillin, Ceftriaxone, Chloramphenicol, Ciprofloxacin, Cotrimoxazole, Gentamycin, Nalidixic Acid, Nitrofruantoin, and Tetracycline. The antibiotic discs were placed on the plate and incubated at 35°C ± 2°C for 16 to 18 hour. The zone of inhibition (mm) was measured after 16 to 18 hour of incubation. The antibiotic susceptibility was interpreted based on CLSI guidelines (CLSI, 2021).

3.5 Quality control

Strict quality control was carried out to obtain accurate microbiological data. The equipment's used in the laboratory for analysis of water were calibrated and the bacteriological analysis of water quality was performed by following the standards method. *E. coli* ATCC 25922 cultures were taken as reference culture and a thickness of (4mm) kept for susceptibility test had maintained the quality of the sensitivity test. All procedures were performed under strict aseptic conditions (Appendix-J).

3.6 Data analysis

Quantitative data analysis was carried out by using MS-Excel 2010 as a means of table and graphs.

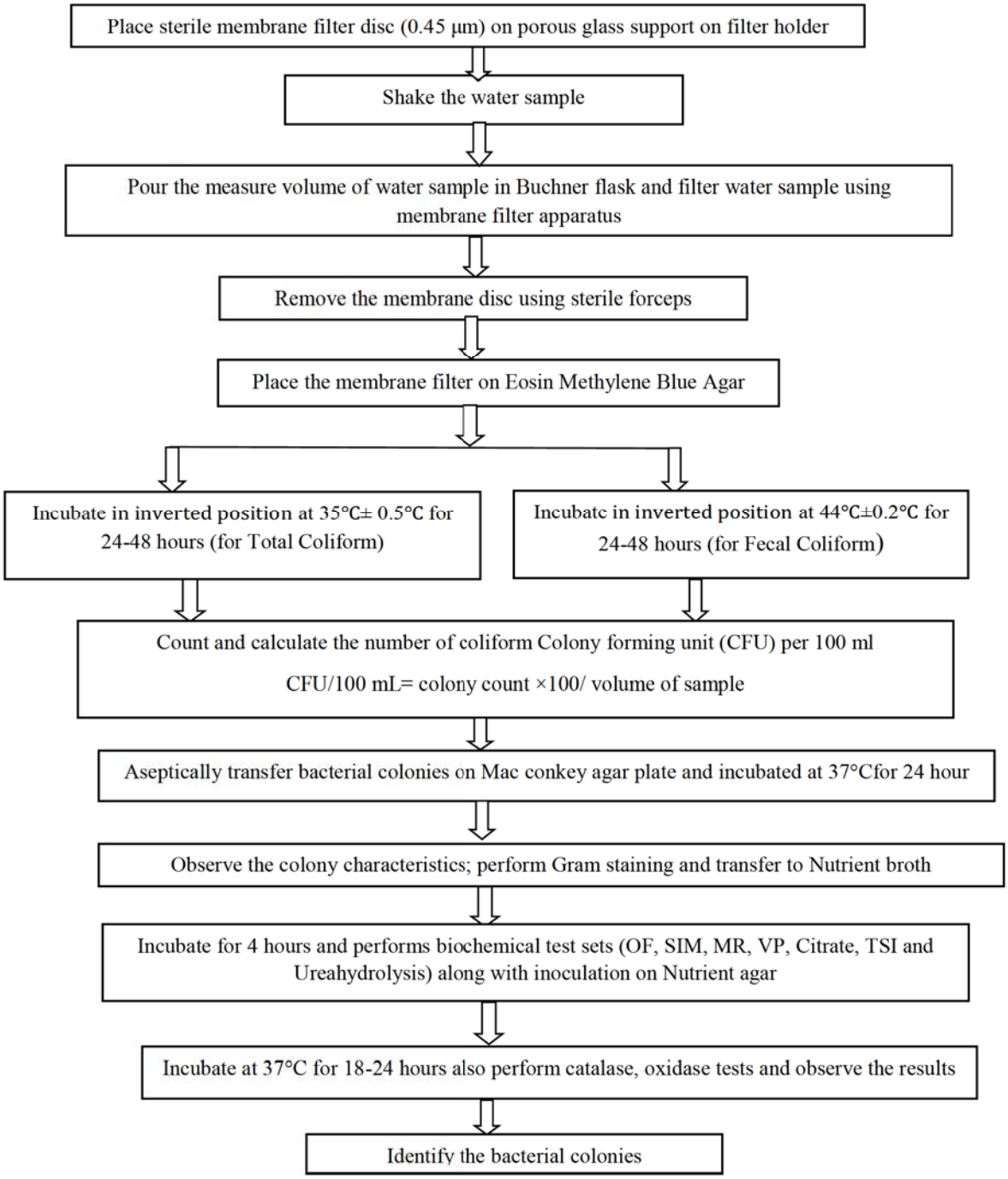


Figure 1: Flow diagram of membrane filter technique for detection of coliform and identification of bacterial isolates.

(APHA 1998; Aneja 2003; Cheesbrough 2006; Forbes, *et al.,* 2007).

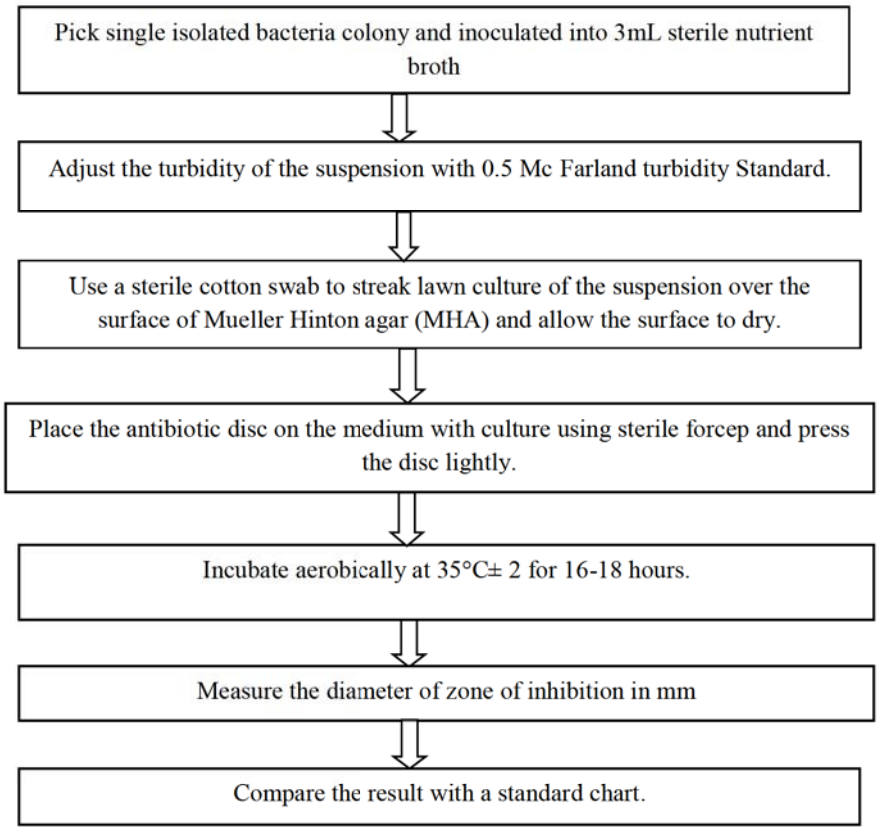


Figure 2: Flow diagram of antibiotic susceptibility testing of *E. coli*. (Cheesbrough, 2006 ;CLSI, 2021)

CHAPTER 4

4. RESULT AND DISCUSSION

4.1 Results

4.1.1 General information of the different water sources

The water sample was collected from different places inside the Kathmandu city which includes Makhan,Bangemuda, Ason, Jamal, Jytha, Thamel, Nardevi and Chhetrapati.

During field survey, it was found that Kathmandu city has the different water shops. Generally, shops were open in the morning 6 A.M. to 9 A.M. and in the evening 5 P.M. to 8 P.M. The cost of the water ranges from Rs.12 to Rs.20 per jar (20 liters). Different place have different price rate for one jar water. Similarly, drinking water samples were also collected from restaurants at point of use which mostly used jar water or jar water with dispenser.

4.1.2 Result of questionnaire survey on water quality and sanitation

Questionnaire survey conducted among 75 respondents showed that maximum number of people was aware of basic hygiene sanitation practice.

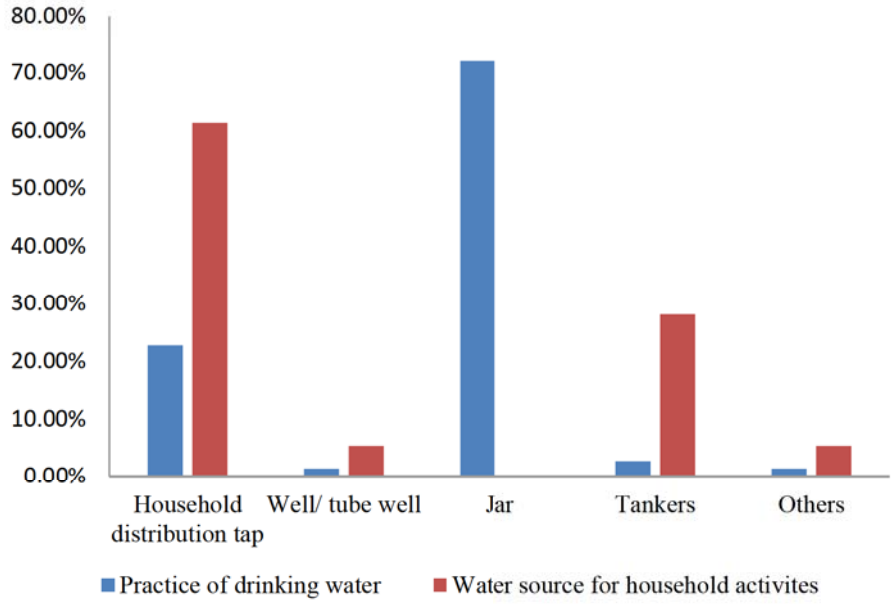


Figure 3: Different sources of water for drinking and other purpose

Majority of the respondent used jar water (72%) for drinking, 22.7 % used household distribution tap water, 2.7% used tanker water, 1.3% used well/ tube well water and 1.3% used other sources of water for drinking purposes. Whereas 61.3% of respondents

used the household distributed tap water for household activates, 5.3% used well/ tube well water, 28% used tanker water and 5.3% used other sources as shown in **Figure 3.**

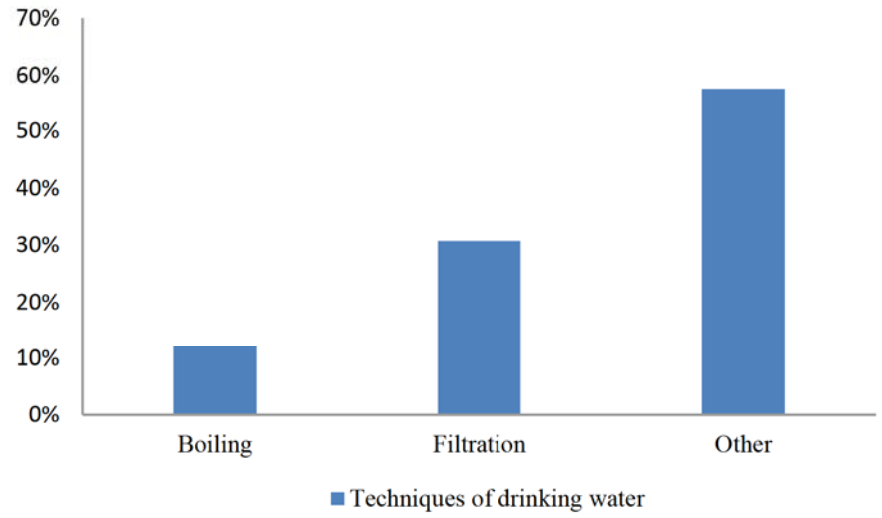


Figure 4 : Techniques of drinking water treatment

In this study it was found that 12% respondents used boiling technique, 30.7% used after filtration and 57.3% of respondents used other techniques before drinking as shown in **Figure 4**.

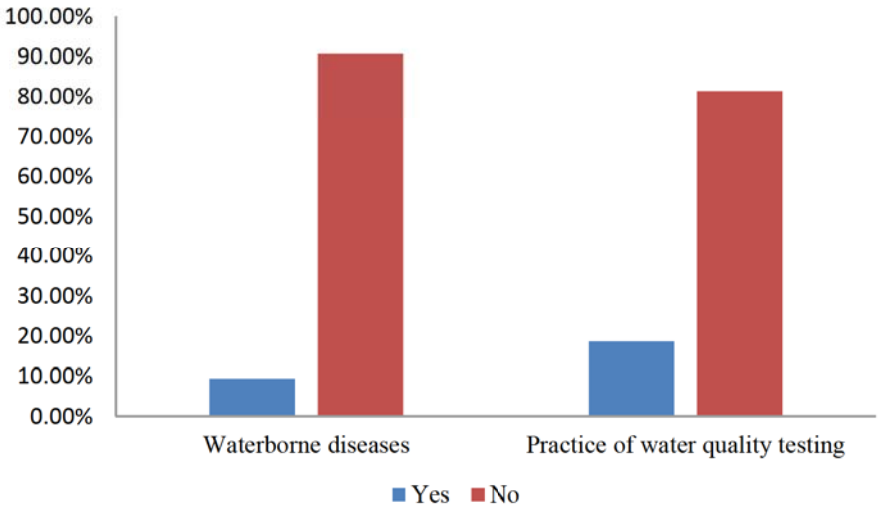


Figure 5 : Respondents having family member suffering from Water borne diseases and their practice of testing water quality.

9.3% of respondents suffered from water borne diseases and 90.7% don't suffer from any kind of waterborne diseases. According to 81.3% respondents they don't have practice of water quality testing while 18.7% said they have tested the quality of water as shown in **Figure 5.**

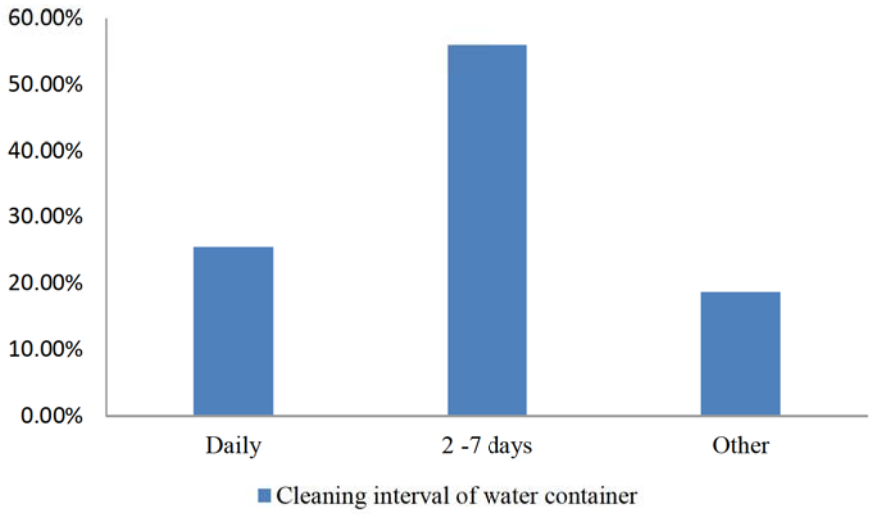


Figure 6 : Cleaning interval of water container

25.3% of respondents cleaned the water container daily, 56% cleaned in 2-7 days and 18.7% of peoples cleaned the container in other days as shown in **Figure 6.**

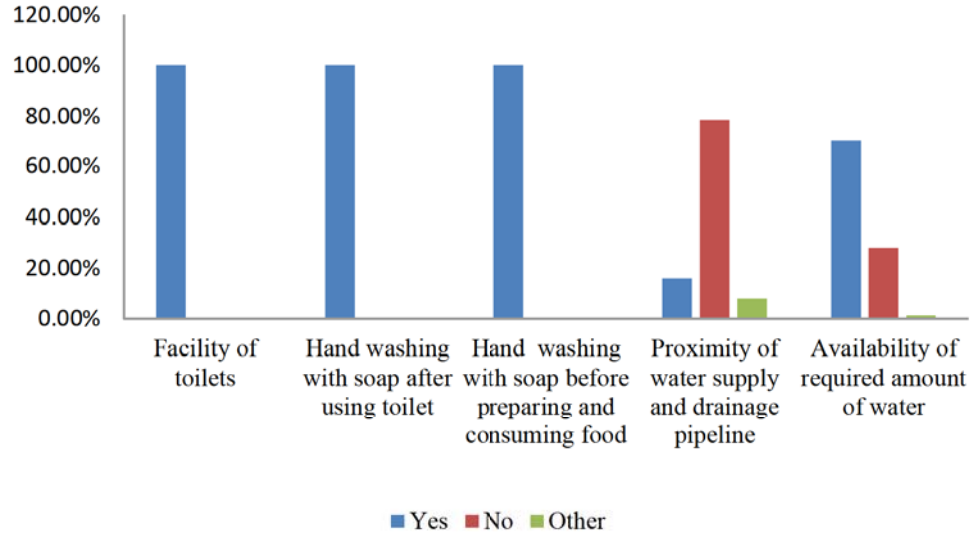


Figure 7: Respondents who have facility of toilets, hand washing habits before and after toilet, or preparation and consumption of food, proximity of water supply and availability of water.

100% people respondents that they use toilet, wash hands with soap after using toilet and they washed hand with soap before preparing and consuming food**.** According 16% respondents, pipeline of water and drainage was together, 78.7% respondents said that pipeline was not together and 8% respondents they don't know about this. According to 81.3% respondents they don't have practice of water quality testing while 18.7% said they have tested the quality of water. 70.7% of the respondent that the availability of

water was enough, 28 % respondent it was not enough while other 1.3% was given mixed review as shown in **Figure 7.**

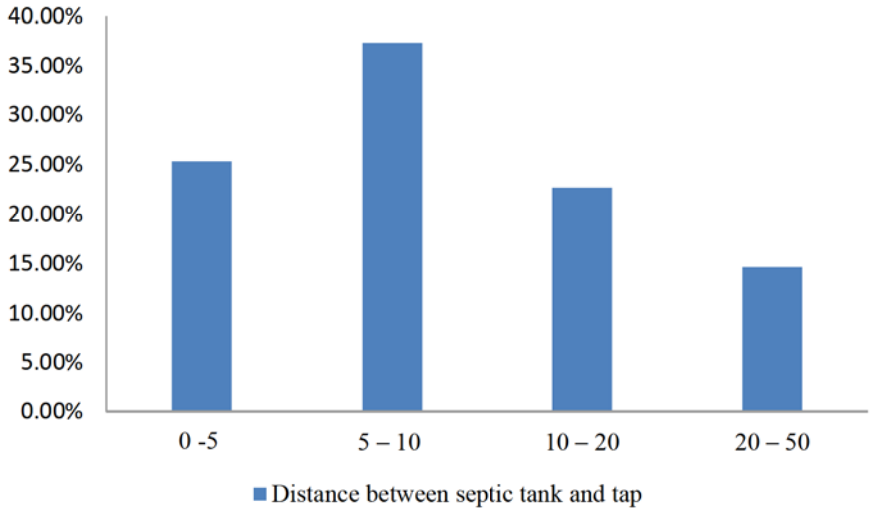


Figure 8: Distance between septic tank and tap

According to 25.3% respondent's safety tank is near to tap i.e., 0-5m distance, 37.3% respondents said that the distance between septic tank was 5-10m approximately,22.7% respondents said that the distance between septic tank was 10-20m whereas 14.7% said the distance was 20-50 m as shown in **Figure 8**.

4.1.3 Bacteriological quality of water

The results of bacteriological parameters and antimicrobial susceptibility pattern of the *E. coli* isolates from sample (N=39) in Kathmandu is described below.

4.1.3.1 Total coliform and fecal coliform count of water samples from water shop

Out of 15 water samples collected from water shop, 93.3% were found to be contaminated with total coliform and 53.3% with fecal coliform as shown in **Table 3**, which exceeded the WHO guideline value (0 CFU/ 100 mL).

Table 3: Total coliform and fecal coliform count of water sample from water shop

Coliform count	Total no of water samples (n)	Samples within WHO guideline value 0 CFU/ 100 mL		Samples exceeding WHO guideline value 0 CFU/100 mL	
		Nos	%	Nos	%
Total coliform count	15	1	6.7	14	93.3
Fecal coliform count	15	7	46.7	8	53.3

4.1.3.2 Coliform count of drinking water samples from different restaurants

Out of 24 water samples collected from restaurants 91.6% were found to be contaminated with total coliform and 41.6% with fecal coliform as shown in **Table 3**, which exceeded the WHO guideline value (0 CFU/ 100 mL).

Table 4: Total coliform and fecal coliform count of water sample from drinking water

Coliform count	No of samples (n)	Samples Within WHO guideline value 0 CFU/100 mL		Samples exceeding WHO guideline value CFU/100 mL	
		Nos	%	Nos	%
Total coliform count	24	2	8.4	22	91.6
Fecal coliform count	24	14	58.4	10	41.6

4.1.3.3 Maximum and minimum coliform count in water from water shop

The bacteriological quality of water sample from water shops revealed that the maximum count of total coliform and fecal coliform were 310 CFU/100 mL and 190 CFU/100 mL respectively. (**Table 4**)

Table 5: Total coliform and fecal coliform count from shop water sample

Coliform count	Total coliform (CFU/100 mL) (n=15)	Fecal coliform (CFU/100 mL) (n=15)
Maximum	310	190
Minimum	0	0

4.1.3.4 Maximum and minimum coliform count in drinking water samples from restaurants.

The bacteriological quality of drinking water from water sample revealed that the maximum counts of total coliform and fecal coliform were 585 CFU/100 mL and 324 CFU/100 mL respectively (**Table 5**).

Table 6: Total coliform and fecal coliform count from drinking water sample from restaurant

Coliform count	Total coliform (CFU/100 mL) (n=24)	Fecal coliform (CFU/ 100 mL) (n=24)
Maximum	585	324
Minimum	0	0

4.1.3.5 Distribution of water samples from water shop contaminated with coliform

There was 100% contamination of water samples with total coliform in different localities of Kathmandu which includes Makhan, Ason, Jytha, Nerdevi and Chhetrapati of the study area. The fecal contamination was not detected in water sample in Ason and Chhetrapati area as shown in the **Table 7**.

Table 7: Distribution of water samples from water shop contaminated with coliform in different localities of Kathmandu

Locations	No of samples (n)	Total coliform Positive sample		Fecal coliform Positive sample	
		Nos	%	Nos	%
Makhan	2	2	100	2	100
Bangemuda	3	2	66.7	1	33.3
Ason	2	2	100	0	-
Jytha	3	3	100	3	100
Nerdevi	3	3	100	2	66.7
Chhetrapati	2	2	100	0	-
Total (N)	15	14		8	

4.1.3.6 Distribution of water samples from restaurant contaminated with coliform

There was 100% contamination of water samples with total coliform in different localities of Kathmandu which includes Makhan, Bangemuda, Jamal, Jytha, Nerdevi and Chhetrapati of the study area. The fecal contamination was not detected in water sample from Thamel area as shown in the **Table 8**.

Table 8: Distribution of water samples from restaurant contaminated with coliform in different localities of Kathmandu

Locations	No of samples (n)	Total coliform Positive sample		Fecal coliform Positive sample	
		Nos	%	Nos	%
Makhan	3	3	100	2	66.7
Bangemuda	3	3	100	1	33.3
Ason	3	2	66.7	1	33.3
Jamal	3	3	100	2	66.7
Jytha	3	3	100	2	66.7
Thamel	3	2	66.7	0	0
Nerdevi	3	3	100	1	33.3
Chhetrapati	3	3	100	1	33.3
Total (N)	24	22		10	

4.1.3.7 Shop water sources contaminated with *E. coli*

Out of 15 sample collected from water shop, 73.3% (11) samples were contaminated with *E. coli* and 26.7% (4) sample were negative for *E. coli* while 53.3% (8) samples contain thermotolerant *E. coli* and 46.7% (7) sample were negative for thermotolerant *E. coli* as shown in **Table 9**.

Table 9: *E. coli* and thermotolerant *E. coli* count from shop water sample

Bacteria	No of samples (n)	Water samples contaminated with *E. coli*		water samples not contaminated with *E. coli*	
		Nos	%	Nos	%
E. coli	15	11	73.3	4	26.7
Thermotolerant *E. coli*	15	8	53.3	7	46.7

4.1.3.8 Drinking water sources contaminated with *E. coli*

Out of 24 sample collected from water shop 75% (18) samples were contaminated with *E. coli* and 25% (6) sample were negative for *E. coli* while 33.3% (8) samples contain

thermotolerant *E. coli* and 66.7% (16) sample were negative for thermotolerant *E. coli* as shown in **Table 10**.

Table 10: *E. coli* and thermotolerant *E. coli* count from drinking water sample

Bacteria	No of samples (n)	Water sample contaminated with *E. coli*		Water sample not contaminated with *E. coli*	
		Nos	%	Nos	%
E. coli	24	18	75	6	25
Thermotolerant *E.coli*	24	8	33.3	16	66.7

4.1.3.9 Identification of bacteria obtain from drinking water sources incubated at 37°C

Out of 92 bacterial isolates obtained from drinking water sources, incubated at 35°C±0.5°C, higher percentage of isolates was found to be *E. coli* (31.5%) followed by *Klebsiella* spp. (19.6%), *Enterobacter* spp. (8.7%), *Citrobacter* spp. (5.4%), *Salmonella* spp. (13.0%), *Shigella* spp. (3.3%), *Pseudomonas* spp. (6.5%), *Proteus* spp. (9.8%) and unidentified Gram negative rods 2.2%. Similarly, out of 30 isolates of thermotolerant bacteria, 53.3% of isolates were *E. coli* as shown in **Table 11.**

Table 11: Microbial profile of bacteria isolated from drinking water sources

Bacteria	Bacteria isolated			
	at 35±0.5°C		at 44°C±0.2°C	
	Nos	%	Nos	%
E. coli	29	31.5	16	53.3
Klebsiella spp.	18	19.6	7	23.3
Enterobacter spp.	8	8.7	1	3.3
Citrobacter spp.	5	5.4	1	3.3
Salmonella spp.	12	13.0	4	13.3
Shigella spp.	3	3.3	1	3.3
Pseudomanas spp.	6	6.5	-	-
Proteus spp.	9	9.8	-	-
Gram negative rods (unidentified)	2	2.2	-	-
Total (N)	92		30	-

4.1.2.10 Antibiotic susceptibility test of *E. coli* and thermotolerant *E. coli*

Antibiotic susceptibility pattern of *E. coli* (n= 29) isolates from drinking water sources showed 100% susceptibility towards Cotrimoxazole, Gentamycin and Amikacin and 41.4%, 20.7%, 17.2%, 6.9%, 6.9% and 3.4% resistance towards Ampicillin, Nitrofurantoin, Ceftriaxone, Tetracycline, Ciprofloxacin and Chloramphenicol respectively. Thermotolerant *E. coli* (n=16) showed resistance towards Ampicillin 37.5%, Nitrofurantoin 25%, Ceftriaxone 18.8%, Chloramphenicol 18.8%, Tetracycline 12.5%, Cotrimoxazole 6.3% and Nalidixic Acid 6.3%. *E. coli* 10.3% and thermotolerant *E. coli* 12.5% was also detected as multiple antibiotic resistances (MAR) bacteria as shown in **Table 12.**

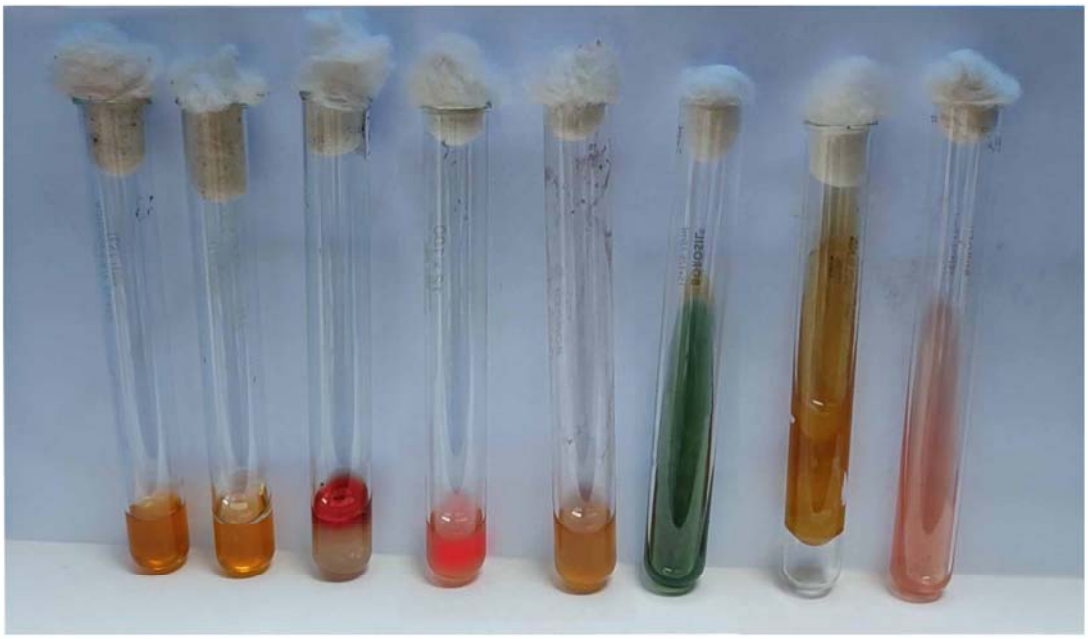
Table 12: Antibiotic susceptibility pattern of *E. coli* and thermotolerant *E. coli*

Antibiotics	*E. coli* (n=29)						Thermotolerant *E. coli* (n=16)					
	S		I		R		S		I		R	
	N	%	N	%	N	%	N	%	N	%	N	%
Amikacin (30mcg)	29	100	0	-	0	-	16	100	0	-	0	-
Ampicillin (10mcg)	11	37.9	6	20.7	12	41.4	9	56.2	1	6.3	6	37.5
Ceftriaxone (30 mcg)	20	69	4	13.8	5	17.2	11	68.7	2	12.5	3	18.8
Chloramphenicol (30mcg)	25	86.2	3	10.4	1	3.4	11	68.7	2	12.5	3	18.8
Ciprofloxacin (5 mcg)	22	75.9	5	17.2	2	6.9	14	87.5	2	12.5	0	-
Cotrimoxazole (25 mcg)	29	100	0	-	0	-	15	93.7	0	-	1	6.3
Gentamycin (10 mcg)	29	100	0	-	0	-	16	100	0	-	0	-
Nalidixic Acid (30 mcg)	26	89.7	3	10.3	0	-	12	74.9	3	18.8	1	6.3
Nitrofurantoin (3oo mcg)	20	69	3	10.3	6	20.7	9	56.2	3	18.8	4	25
Tetracycline (30 mcg)	26	89.7	1	3.4	2	6.9	13	81.2	1	6.3	2	12.5
Multiple antibiotic resistance	-	-	-	-	3	10.3	-	-	-	-	2	12.5

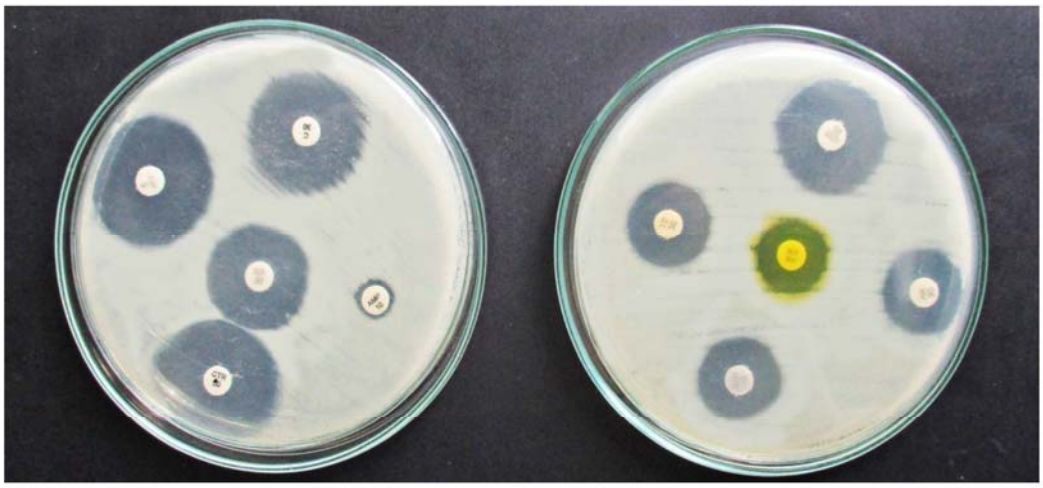
PHOTOGRAPHS

Photograph 1: Total coliform on EMB agar plate (Membrane Filtration Technique)

Photograph 2: *E. coli* on Mac Conkey agar plate



Photographs 3: Biochemical tests of *Escherichia coli* (Left to right: OF test (fermentative), SIM test (motile, indole (positive)), MR test (positive), VP test (negative), TSIA test (acid/acid, gas positive, h_2s negative), Citrate utilization test (positive), Urease test (negative).



Photograph 4: Antibiotic susceptibility test of *Escherichia coli*

C= Chloramphenicol (30mcg), A=Ampicillin (10mcg), CTR=Ceftriaxone (30mcg), CIP= Ciprofloxacin (5 mcg), NA=Nalidixic Acid (30mcg) COT=Cotrimoxazole (25mcg), AK=Amikacin (30mcg), GEN=Gentamycin (10mcg), TE = Tetracycline (30mcg), NIT = Nitrofruantoin (300mcg).

4.2 Discussion

The most vital resource for all living creatures on this planet is unconditionally water. Safe drinking water is univocally a major concern for all since health and well-being of the human race is closely connected with the quality of water used (Sharma, *et al.*, 2005). World Health Organization estimated that up to 80% of all sicknesses and diseases in the world are caused by inadequate sanitation, polluted water or unavailability of water (WHO, 2004). Water which is used for human consumption should be safe and of good quality as it's directly influence public health concern. Safe drinking water is defined as water with microbial, chemical and physical characteristics that meet WHO guidelines or national standards on drinking water quality (WHO, 2005). As a member of the Enterobacteriaceae family, *E. coli* is naturally found in the intestines of humans and warm-blooded animals (Edberg, *et al.*, 2000).

According to questionnaire survey, out of 75 respondents, 72% people used jar water for drinking purposes, 1.33% used well/ tube well, 22.7% used household distribution tap, 2.7% used tanker water and 1.33% people used other sources of water. In this study, 30.7% people filter water before drinking while only 12% of people boiled water before drinking and 57.3% people used other methods to purify water. 61.3% of respondents used the household distributed tap water for household activates, 5.3% used well/ tube well water, 28% used tanker water and 5.3% used other sources for household activates. 9.3% of respondents suffered from water borne diseases like diarrhea as well as typhoid and 90.7% don't suffered from any kind of water borne diseases. 25.3% of respondents cleaned the water container daily, 56% cleaned in 2-7 days while 18.7% of peoples cleaned the container in other days. In question about facility of toilet, hand washing with soap after using toilet, before preparing and consuming food, 100% people respondents that they wash hand and used toilet. According to 81.3% respondents they don't have practice of water quality testing while 18.66% said they have tested the quality of water. 70.7% of the respondent that the availability of water was enough, 28 % respondent it was not enough while other 1.3% was given mixed review. Similar study was conducted by Subedi, et al., (2010) from Kathmandu in which 525 peoples were using jar water for drinking purposes

In this study, the 93.3% shop water was contaminated with total coliform and 53.3% fecal coliform whereas 91.6% total coliform and 41.6% fecal coliform in drinking water which was lower than that reported by Subba, *et al.*, (2013) in which all water

sources were contaminated with coliform and thermotolerant coliform. Result for total coliform contamination was more than Shakya, *et al.*, (2008) as he have reported 61.4% of the water samples were found to have coliform count above the recommended level of WHO guideline. This may be due to the fact that the drinking water sample was treated before consuming.

In this study 53.3% of water sample from water shop and 41.6% drinking water sample from restaurant were contaminated with thermotolerant coliform. Out of 105 water samples analyzed by Jayana, *et al.*, (2009), total coliform count showed 64.8% of samples crossed the WHO guideline. 10% brands of marketed bottled water within the acceptable range of bacterial load according to the national as well as WHO guideline. Although , slightly higher result was seen in Rai, *et al.*, (2015) in which 75% samples were found above WHO guidelines (0 CFU/ mL) in coliform, 25% samples were found within the WHO guideline (0 CFU/mL) in coliform. Nevertheless, Prasai *et al.*, (2002) and Shrestha, *et al.*, (2008) found all tested tap water samples contaminated with total Coliform. However, Bajracharya, *et al.*, (2007) and Aryal, *et al.*, (2009) reported 73.7% and 86.2% samples contaminated with total coliform respectively. Among the 70 samples that contain total coliform bacteria, 15.7% were found to be contaminated with thermotolerant type coliform bacteria (Shakya, *et al.*, 2008). Presence of *E. coli* in drinking water may be due to insufficient or in adequt treatment capacity of the water samples. As it indicates there is a fecal contamination so, there is chance of pathogen present. Drinking water contamination is a major public health problem in developing countries like Nepal.

In this study, highest bacterial load was 585 CFU/100 mL of total coliform and 324 CFU/100 mL of fecal coliform in drinking water and 310 CFU/100 mL total coliform and 190 CFU/100 mL fecal coliform in water shop was found but Magar, *et al.*, (2019) reported result was higher than this result. He reported that highest bacterial load observed on drinking water was (137×10^3) CFU/100 mL and least bacterial load observed was (28×10^3) CFU/100 mL. This may be due to the disinfection of raw water at treatment plants of raw water.

In this study during identification, 31.5% *E. coli* was identified in an isolated. Similarly, 19.6% *Klebsiella spp*, 5.4% *Citrobacter* spp, 13.0% *Salmonella* spp., 3.3% *Shigella* spp, 6.5% *Pseudomonas* spp., 9.8% *Proteus* spp. were identified along with 17% unidentified gram negative rods. Similarly, the fecal coliform identified were *E.*

coli 53.3%, *Klebsiella* spp. 23.3%, *Enterobacter spp.* 3.3% *Citrobacter* spp. 3.3%, *Salmonella spp*13.3% and *Shigella spp.* 3.3%. *E. coli* was found to be most common microorganism in studies done by Rijal, *et al.*, (2012). Similarly, Shidiki, *et al.*, (2016), also found most frequently isolated total coliform and fecal coliform revealed most frequently were *E. coli* and *Citrobacter*. But Nazia, *et al.*, (2021) reported higher highest frequency of *E. coli* than the study. Frequency of other coliform isolated from drinking water other than *E. coli* was *Klebsiella* spp 26%, *Pseudomonas* spp 27%, *Enterobacter* spp 7%, *Citrobacter* spp 8% and *Acinetobacter* spp 5%.Jayana, *et al.*, (2009), reported more percentage of organism where, Percentage of *Enterobacter* spp. 29.5%, found to be maximum followed by *E. coli* 24.6%, Citrobacter spp 20.4%, *P. vulgaris* 7.0%, *Klebsiella spp.* 5.6%, *P. mirabilis* 3.5%, *S. dysentery* 2.8%, *S.* Typhi 2.1%, *Pseudomonas aeruginosa* 2.1%, *Salmonella* Paratyphi 1.4%, and *V. cholerae* 0.7%.

E. coli isolated from water at 37°C was found to be 100% susceptible to Amikacin, Cotrimoxazole, Gentamycin and Nitrofurantoin, while susceptibility to other antibiotics were:- Tetracycline 89.7%, Chloramphenicolcol 86.2%, Ciprofloxacin 75.9%, Nitrofurantoin 69% , Ceftriaxone 69% and Ampicillin 37.9%. The thermotolerant *E. coli* was 100% susceptible to Amikacin and Gentamycin, while susceptibility to other antibiotics were in the following order, Cotrimoxazole 93.6%, Nalidixic acid 87.5%, Ciprofloxacin 87.5%, Chloramphenicol 87.5%, Tetracycline 81.3, Ceftriaxone 68.8% , Nitrofurantoin 56.3% and Ampicillin 56.3%. *E. coli* 10.3% and thermotolerant *E. coli* 12.5% was also detected as multiple antibiotic resistances (MAR) bacteria. However, Jayana, *et al.*, (2009) reported that among total isolates, resistance was directed to 79.5% Erythomycin, 62.67% Penicillin G, 61.9% Amoxycillin, 34.5% Ampicillin, 21.1% Tetracycline, 15.4% Ceftriaxone, 14.7%, Amikacin, 14.7%, Cephotaxine, 5.6% Chloramphenicol, 5.6% Ofloxacin respectively .Similar, study was conducted by Rijal, (*et al.*, 2012) where, the antibiotic susceptibility pattern among *E. coli* isolates showed that all the tested isolates were sensitive towards Chloramphenicol, Ofloxacin and Co-trimoxazole, 93.5 % isolates were resistant to Tetracycline followed by Amoxycillin 80.6 %, Cefexime 48.4%, Nalidixic acid 25.8%, Amikacin 25.8%. The E. coli are resistance to ampicillian because ampicillian work by inhibiting cell wall biosynthesis in the bacterial organism and these are the broadly used anibiotics. The cause of

ampicillian resistane might be due to production of beta lactamase (Denyer, et al., 2004).

But Chaudary, *et al*., (2011) found the antibiotic susceptibility pattern among *E. coli* isolates were sensitive towards Chloramphenicol, Ofloxacin and Co-trimoxazole, 93.5 % isolates were resistant to Tetracycline followed by Amoxycillin 80.6 %, Cefexime 48.4%, Nalidixic acid 25.8%, Amikacin 25.8%. The misuse of particular antibiotics, environmental conditions, over use of antibiotics and unsafe disposal of animal excreta and sewages may be the important factors in developing the antibiotic resistance which is concerns of great public health issues.

CHAPTER 5

5. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Microbiologically contaminated drinking water is the main problem in Kathmandu which threats the human health and cause waterborne diseases e.g. diarrhea, typhoid, cholera etc. In the present study, water samples from water shops showed 93.3% total coliform and 53.3% with fecal coliform contamination which exceeded the WHO guideline value (0 CFU/100 mL) while 91.6% of drinking water samples from restaurants were found to be contaminated with total coliform and 41.6% with fecal coliform. The drinking water in Kathmandu was contaminated with *E. coli, Klebsiella* spp., *Enterobactr* spp., *Citrobacter* spp., *Salmonella* spp., *Shigella* spp., *Pseudomona* spp. and *Proteus* spp. The isolates of *E. coli* from drinking water were 100% sensitive to Cotrimoxazole, Gentamycin and Amikacin and most of the *E. coli* isolates were resistant to Ampicillin, Nitrofurantoin, Ceftriaxone, Chloramphenicol and Nalidixic Acid. Multiple antibiotic resistance (MAR) isolates of *E. coli* and thermotolerant *E. coli* were also detected in drinking water sources which increased the risk of emergence of antibiotic resistant bacteria that may cause difficulty in treatment of waterborne diseases. More than 90% of the drinking water in Kathmandu was contaminated with coliform bacteria which are unsatisfactory for drinking as well as household purposes. Awareness on drinking water quality, water disinfection and health impact of contaminated water has to be generated among the consumers.

5.2 Novelty and national prosperity aspect of project work

Bacteriological quality of drinking water is an important public health concern and the quality of drinking water at point of use (POU) directly impacts the health of consumers. Therefore, this study will be helpful to know the quality of drinking water in Kathmandu and presence of MAR *E. coli* in drinking water sources. These findings will help the policy makers to maintain drinking water quality in Kathmandu which can be further implemented for prevention of waterborne diseases.

5.3 Limitation of the work

The physicochemical quality of drinking water in Kathmandu and the presence of pathogenic bacteria in drinking water were not analyzed due to lack of resources and the availability of time for the project work. The limited numbers of drinking water

samples were analyzed and water samples were collected from few locations of central Kathmandu as this is a cross-sectional study.

5.4 Recommendation for further work

On the basis of findings of this study on bacteriological quality of drinking water in Kathmandu and for antibiotic susceptibility testing following recommendations are suggested:

- Bacteriological quality of drinking water sources has to be assessed to reveal the water quality and maintain the quality of drinking water according to WHO guidelines and NDWQS.
- The physicochemical parameters of water quality including residual chlorine should be tested in drinking water in addition to bacteria and analysis.
- Awareness on drinking water quality and waterborne diseases should also be generated among the consumers to minimize the morbidity and outbreak of waterborne diseases.
- Basic educational programs on disinfection of drinking water, hygiene practices, sanitation and waterborne disease should be organized in the community.
- The quality analysis of drinking water at household level / Point of Use (POU) should be done to know the exact quality of water used by consumers.
- Antibiotic resistant bacteria in drinking water should also be detected to understand the emergence of antibiotic resistance bacteria in water.

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APPENDIX

APPENDIX-A

MAP OF KATHMANDU MUNCIPALITY

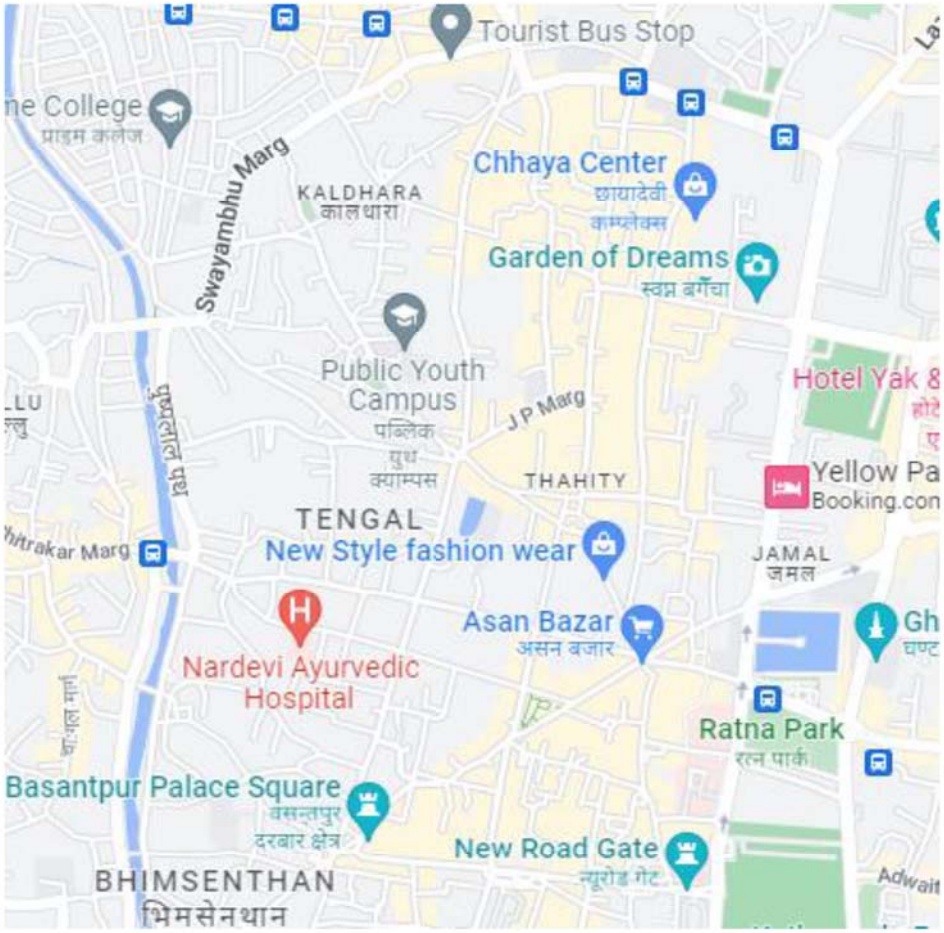


Figure 9: Map showing different sampling localities (Makhan, Bangemuda, Ason, Jamal, Thamel, Nerdevi and Chhetrapati)

APPENDIX-B

COMMUNITY SURVEY FORM

पिउने पानी र सरसफाई सम्बन्धी प्रश्नहरू

Tribhuvan University
Amrit Campus
Department of Microbiology

BACTERIOLOGICAL QUALITY OF DRINKING WATER IN KATHMANDU, NEPAL AND ANTIBIOTIC SUSCEPTIBILITY PATTERN *OF Escherichia coli* FROM WATER

नाम कोड:

लिङ्ग: पुरुष महिला

टोल: वडा नं: नगरपालिका: जन्ममिति/उमेर:

शिक्षा: पेशा:

परिवार संख्या: पुरुष महिला बालबालिका (पाँच बर्ष भन्दा मुनि)

१.तपाईले पिउनको निम्ति कुन पानीको स्रोत प्रयोग गर्नु हुन्छ?

क.बितरित खाने पानी ख. ट्युबवेल/ इनार ग. जारको पानी

घ. ट्यांकर ङ.खुल्ला खरिद गरेको पानी

२. तपाईले पिउने पानी घरमा प्रशोधन गर्न कुन माध्यम प्रयोग गनुहुन्छ?

क.उमालेर ख. फिल्टर गरेर ग. अन्य

३. तपाईले घरायसी प्रयोजनको लागि कुन पानीको स्रोत प्रयोग गनुहुन्छ?

क.वितरित खाने पानी ख. ट्युबवेल/ इनार ग.ट्याकर

घ.खुल्ला खरिद गरेको पानी

४. तपाई वा तपाईको परिवारमा बिगत एक बषभित्र कुनै पानीजन्य रोग लागेको छ?

क. छ ख. छैन

यदि छ भने कुन रोग लागेको छ?

क.झडापखला ख.टाइफाइड ग. जन्डिस

घ. आँउ ङ. हैजा च. अन्य कुनै

५. तपाईले पानी थाप्ने भाडा/ बोतल/ गाग्री/बाल्टिन कहिले- कहिले सफा गनुहुन्छ?

क.दिनहुँ ख. २-७ दिनमा ग.अन्य

६.तपाईको घरमा शौचालय छ ?

क. छ　　ख. छैन　　ग. अन्य

७. तपाईले शौचालयको प्रयोग गरिसकेपछि साबुन पानीले धनु हन्छ?

क.धुन्छु　　ख. धुदिन　　ग. अन्य

८. तपाईले खाना पकाउन/ खाना खान अघि साबुन पानीले हात धुनु हुन्छ?

क .धुन्छु　　ख.धुदिन　　ग. अन्य

९. तपाईको घरमा खानेपानी र ढलको निकासको पाइपलाइन सगै छ?

क. छ　　ख. छैन　　ग.अन्य

१०. तपाईको घरमा खानेपानीको धारा/ ट्युबवेल / इनार सेफ्टी ट्यांकी/ शौचालय बाट कति दुरिमा छ?

क. ०-५ मिटर　　ख. ५-१० मिटर　　ग. १०-२० मिटर

घ.२०-५० मिटर

११. तपाईले आफुले पिउने वा प्रयोग गर्ने पानीको गुणस्तर परीक्षण गराउनु भएको छ?

क. छ　　ख. छैन

छ भने कस्तो छ?

१२. तपाईलाई पिउन र घरायसी काममा चाहिने पानी सहजै रुपमा प्रयाप्त पाइराख्नु भएको छ?

क. छ　　ख. छैन　　ग. अन्य

APPENDIX- C

NATIONAL DRINKING WATER QUALITY STANDARDS - 2005 AND WHO GUIDELINE VALUES

S.N.	Category	Parameters	Units	Maximum concentration limit		Remarks
				NDWQS-2005	WHO guidelines values	
1	Physical	Turbidity	NTU	5(10)	5	
2		pH		6.5- 8.5*	6.5-8.5	
3		Color	TCU	5(15)	15	
4		Taste and odor		Non-objectionable	Non-objectionable	
5		TDS	Mg/L	1000	1000	
6		EC	µS/cm	1500	-	
7	chemical	Iron	Mg/L	0.3(3)	0.3	
8		Manganese	Mg/ L	0.2	0.1	
9		Arsenic	Mg/ L	0.05	0.01	
10		Cadmium	Mg/ L	0.003	0.003	
11		Chromium	Mg/ L	0.05	0.05	
12		Cyanide	Mg/ L	0.07	0.07	
13		Fluoride	Mg/ L	0.5- 1.5*	1.5	
14		Lead	Mg/ L	0.01	0.01	
15		Ammonia	Mg/ L	1.5	1.5	
16		Chloride	Mg/ L	250	250	
17		Sulphate	Mg/ L	250	50	
18		Nitrate	Mg/ L	50	50	
19		Copper	Mg/ L	1	2	
20		Total hardness	Mg/ L as caco3	500	500	
21		Zinc	Mg/ L	3	3	
22		Mercury	Mg/ L	0.001	0.006	
23		Aluminium	Mg/ L	0.2	0.1- 0.2	
24		Residual chlorine	Mg/ L	0.1- 0.2*	0.2-0.5	In system using chlorination
25	Microbial	*E. **coli***	MPN/100mL	0	0	
26		Total coliform	MPN/100mL	0 in 95% samples	0 in 95% samples	

* These values show lower and upper limits

( ) Values in parenthesis refers the acceptable values only when alternative is not available.

APPENDIX-D

MATERIALS USED

Equipment's

1. Autoclave (Life, India)
2. Incubator (Leader,UK)
3. Incubator (Memmert, Germany)
4. Hot air oven (Ambassador)
5. Binocular microscope (COSLAB, India)
6. Refrigerator (LG, India)
7. Electronic weighing balance (Phoenix instrument, Germany)
8. Bunsen burner
9. Ice box (Marina 24S)
10. Laminar air flow (ACCO, India)

Glass-wares / Plastic-wares

1. Beakers
2. Sampling bottles
3. Conical flasks
4. Petriplates
5. Pipettes
6. Measuring cylinders
7. Membrane filtration apparatus
8. Test tubes

Chemicals

1. Sodium thiosulphate
2. Lysol
3. Ethanol
4. Distilled water
5. Phosphate buffer

Microbiological media (Hi-Media Laboratories Pvt. Ltd.)

1. Eosin Methylene Blue agar (EMB)
2. MacConkey agar
3. Nutrient broth
4. Nutrient agar

5. Hugh and Leifson's media
6. Sulphide Indole Motility (SIM) media
7. MR/VP medium
8. Simmon's citrate agar
9. Triple Sugar Iron (TSI) agar
10. Urea agar base
11. Mueller Hinton Agar (MHA)

Staining reagent / Biochemical reagents

1. Crystal violet
2. Gram's iodine
3. Acetone alcohol decolorizer
4. Safranin
5. Catalase reagent
6. Oxidase reagent
7. Kovac's reagent
8. Methyl red reagent
9. Voges-Proskauer reagent (Barrits reagent)

Antibiotics discs (Hi-Media Laboratories Pvt. Ltd.)

1. Amikacin (30mcg)
2. Ampicillin (10mcg)
3. Ceftriaxone (30 mcg)
4. Chloramphenicol (30mcg)
5. Ciprofloxacin (5 mcg)
6. Cotrimoxazole (25 mcg)
7. Gentamycin (10 mcg)
8. Nalidixic Acid (30 mcg)
9. Nitrofurantoin (3oo mcg)
10. Tetracycline (30 mcg)

Miscellaneous

1. Membrane filter (Sartorius, Germany)
2. Inoculating loop and inoculating needle
3. Forceps
4. Pipette filler

5. Paraffin oil
6. Labelling tags
7. Cotton
8. Aluminium foil
9. Paraffin tape
10. Tissue paper
11. Tray
12. Test tube rack
13. Record book and pencils
14. Pens / Pencils
15. Distilled water

APPENDIX- E

PREPARATION OF CHEMICALS FOR MICROBIAL TESTS

Sodium thiosulphate

Preparation:

The distilled water was boiled and mixed with 24.82 grams of $Na_2S_2O_3.5H_2O$ to make 1 Liter. 0.4 grams of NaOH borax or a pellet was added as a stabilizer. This was 0.1N stock solution. Boiled distilled water was used to diluted for 4 times to prepare 0.025 N solution and brown glass stoppered bottle was used to keep it.

Phosphate Buffer solution

Composition

Ingredients

Potassium Dihydrogen Phosphate (KH_2PO_4)	34.0 gm
Distilled water	500 mL.

Preparation

Adjust the pH of the solution to 7.2 with 1N NaOH and diluted to a volume of 1000 mL with distilled water. Autoclave for 15 minutes at 121°C (15 lbs pressure).

(Source: APHA, 1998)

Preparation of MacFarland

McFarland turbidity standards

McFarland standard No.	1.0% Barium chloride (mL)	1.0% Sulfuric acid (mL)	Approx. cell density (1×10¬8 CFU/mL
0.5	0.05	9.95	1.5
1	0.1	9.9	3.0
2	0.2	9.8	6.0

Preparation of 0.5 McFarland standards

1. A 1% solution of sulfuric acid is prepared by adding 1 mL of concentrated sulfuric acid to 99 ml of water.

2. A 1% w/v barium chloride solution was prepared by dissolving 0.5grams of dehydrated barium chloride ($BaCl_2.2H_2O$) in 50 mL of distilled water.
3. Add 0.6 mL of barium chloride solution to 99.4 mL of sulfuric acid solution and mix.
4. A small volume of the cloudy solution is then transferred to a screw capped flask.
5. To standardize the inoculum density for susceptibility testing, the BaCl turbidity standard, equivalent to 0.5 McFarland standards was used.

APPENDIX-F

COMPOSITION AND PREPARATION OF DIFFERENT STAINING AND BIOCHEMICAL REAGENTS

I. Gram staining reagents

A. Crystal violet (Hucker's modification)

Composition

Ingredients

Solution A	
Crystal violet (90% dye content)	2.0 gm
Ethanol (90%)	20.0 mL
Solution B	
Ammonium oxalate	0.8 gm
Distilled water	80.0 mL

Preparation

In 2 grams of crystal violet, 20 mL of ethyl alcohol was dissolved and in 80 mL of distilled water, 0.8 grams of ammonium oxalate was added. Both the solutions A and B were mixed and were kept in clean reagent bottle.

B. Gram's Iodine

Composition

Ingredients

Iodine	1.0 gm
Potassium iodide	2.0 gm
Distilled water	300.0 mL

Preparation

1 grams of iodine and 2 grams of potassium iodide was added to 300 mL of distilled water and mixed well to dissolve then, it was transferred to clean reagent bottle.

C. Acetone-alcohol decolorizer

Composition

Ingredients

Acetone	500 mL
Ethanol (absolute)	475 mL
Distilled water	25 mL

Preparation

475 mL absolute ethanol was mixed with 25 mL of distilled water. Then, immediately 500 mL acetone was added to the alcohol solution and mixed it well and was transferred to the clean reagent bottle.

D. Safranin

Composition

Ingredients

Safranin (2.5% solution in 95% ethyl alcohol)	10.0 mL
Distilled water	100.0 mL

Preparation

In 100 mL distilled water 10 mL of safranin was added and mixed well. Then it was kept in clean reagent bottle.

II. Biochemical reagents

Catalase reagent

Composition

Ingredients

Hydrogen peroxide (6%)	50 mL
Distilled water	50 mL

Preparation

50 mL hydrogen peroxide (6%) and 50 mL of distilled water was added and mixed well.

Oxidase reagent

Composition

Ingredients

Tetra methyl para-phenylenediamine dihydrochloride	5.0 gm
Distilled water	50 mL

Preparation

To prepare this reagent in 50 mL of distilled water, 5.0 grams of reagent was added. To that solution stripes of Whatman No. 1 filter paper was soaked and drained for about 30sec. Then these stripes were left for completely dried and stored in dark bottle tightly sealed with a screw cap.

Kovac's reagent

Composition

Ingredients

P-dimethyl amino benzyldehyde	5.0 gm
Amyl alcohol	75.0 mL
Concentrated HCl	25.0 mL

Preparation

In 75 mL of amyl alcohol, 5 grams of reagent was dissolved with 75 mL of amyl alcohol in clean brown bottle. Then, 25 mL of concentrated HCl was added and mixed well.

Methyl Red reagent

Composition

Ingredients

Methyl red	0.04 gm
Ethanol (absolute)	40 mL
Distilled water	60 mL

Preparation:

In 40 mL of ethanol, 0.04 grams of methyl red was dissolved. Then, it was mixed with 60 mL distilled water.

Voges- Proskauer reagent (Barritt's reagent)

VP reagent A

Composition

Ingredients

Alpha-naphthol	l5 gm
Ethanol (absolute)	100 mL

Preparation

5 grams of alpha naphthol was dissolved in 28 mL of distilled water and transferred to a clean brown bottle. Then distilled water was added to made final volume 100 mL.

VP reagent B

Composition

Ingredients

Potassium hydroxide	40 gm
Distilled water	100 mL

Preparation:

40 grams Potassium hydroxide was dissolved and transferred to a clean brown bottle. Then, distilled water was added to made final volume 100 mL.

(Source: Aneja 2003; Cheesbrough 2006).

APPENDIX - G

A. COMPOSITION AND PREPARATION OF DIFFERENT CULTURE MEDIA

Eosin Methylene Blue agar (EMB)

Composition

Ingredients	**gm/liter**
Peptone	10.0
Di-potassium hydrogen phosphate	2.0
Lactose	10.0
Eosin-Y	0.4
Methylene blue	0.065
Agar	15.0

Final pH (at 25ºC) 7.1±0.2

Preparation

In 1000 mL distilled water 37.4 grams of EMB agar was suspended and heated it until medium dissolve completely. Then it was sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes. The media was allows to cooled at 45-50ºC and was shaken properly in order to oxidize the methylene blue and to suspend the precipitate. After that the media was mixed properly and was poured into sterile petri plates.

MacConkey agar W/0.15% bile salts, CV and NaCl

Composition

Ingredients	**gm/liter**
Peptic digest of animal tissue	1.50
Casein enzyme hydrolysate	1.50
Pancreatic digest of gelatin	17.0
Lactose	10.0
Bile salts	1.50
Sodium chloride	5.0
Crystal violet	0.001
Neutral red	0.03
Agar	15.0

Final pH (at 25ºC) 7.1±0.2

Preparation

In 1000 mL of distilled water 55.0 grams was suspended and heated to boiling until the medium dissolve completely. Then it was sterilized by autoclaving at 15lbs pressure (121ºC) for 15 minutes. After that the medium was cooled to 45-50ºC and was poured into sterile petri plates.

Mueller Hinton Agar (MHA)

Composition

Ingredients	**gm/liter**
HM infusion B from	300.00
Acicase	17.50
Starch	1.50
Agar	17.00

Final pH (at 25°C) 7.3±0.1

Preparation

38.0 grams was suspended in 1000 mL distilled water and heated to dissolve the medium completely. The medium was then sterilized by autoclaving at 15 lbs. pressure (121°C) for 15 minutes.

Nutrient agar

Composition

Ingredients	**gm/liter**
Peptic digest of animal tissue	5.00
Sodium chloride	5.00
Beef extract	1.50
Yeast extract	1.50
Agar	15

Final pH (at 25ºC) 7.4± 0.2

Preparation:

In 1000 mL distilled water 28 grams nutrient agar was suspended and was boiled to dissolve the medium completely. Then, sterilized by autoclaving at 15 lbs. pressure (121ºC) for 15 minutes and cooled to 45-50ºC then it was mixed well and poured into sterile petri plates.

Nutrient broth

Composition

Ingredients	**gm/liter**
Peptic digest of animal tissue	5.00
Yeast extract	1.50
Beef	1.50
Sodium chloride	5.00

Final pH (at 25ºC) 7.4±0.2

Preparation

In 1000 mL distilled water 13.0 grams of nutrient broth was suspended and heated until the medium dissolve completely. Then the medium was sterilized by autoclaving at 15lbs pressure (121ºC) for 15 minutes.

B. COMPOSITION AND PREPARATION OF DIFFERENT BIOCHEMICAL MEDIA

Hugh and Leifson's medium

Composition

Ingredients	**gm/liter**
Casein enzymic hydrolysate	2.00
Sodium chloride	5.00
Dipotassium phosphate	0.30
Bromo thymol blue	0.08
Agar	2.00

Final pH (at 25ºC) 6.8±0.2

Preparation

Suspend 9.38 grams of Hugh and Leifson medium in 1000 ml of distilled water and boil until the medium is completely dissolved. A 100 ml volume was dispensed, 1 grams of dextrose solution was aseptically added to the first 100 ml sterile basal medium, and then the medium was sterilized by autoclaving at 110 °C for 15 minutes. The entire solution was mixed and dispensed twice in 5 mL volumes into sterile tubes for aerobic and anaerobic fermentation.

Sulphide Indole Motility medium

Composition

Ingredients	**gm/liter**
Peptic digest of animal tissue	30.00
Beef extract	3.00
Peptonized iron	0.20
Sodium thiosulphate	0.025
Agar	3.00

Final pH (at 25ºC) 7.3±0.2

Preparation

In 1000 mL distilled water 36.23 grams of medium was suspended and heated it until medium dissolve completely with boiling. Then the medium is dispensed in tubes and sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes. The tubes were left to cool in upright position.

MR-VP medium

Composition

Ingredients	**gm/liter**
Buffered peptone	7.0
Dextrose	5.00
Dipotassium phosphate	5.00

Final pH (at 25ºC) 6.9±0.2

Preparation

In 1000 mL of distilled water 17.0 grams was suspended and heated (if necessary) to dissolve the medium completely. The medium was then distributed in 100 amounts in test tubes and sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes.

Simmon`s Citrate agar

Composition

Ingredients	**gm/lite**
Magnesium sulphate	0.20
Ammonium dihydrogen phosphate	1.00
Dipotassium phosphate	1.00
Sodium citrate	2.00
Sodium chloride	5.00
Bromo-thymol blue	0.08
Agar	15.00

Final pH (at 25ºC) 6.8±0.2

Preparation

24.28 grams were suspended in 1000 ml of distilled water and heated to completely dissolve the medium. The medium was then dispensed into tubes or bottles as needed and autoclaved for 15 minutes at 15 lbs of pressure (121 ° C).

Triple Sugar Iron agar (TSI)

Composition

Ingredients	**gm/liter**
Peptic digest of animal tissue	10.00
Casein enzymic hydrolysate	10.00
Yeast extract	3.00
Beef extract	3.00

Lactose	10.00
Sucrose	10.00
Dextrose	1.00
Sodium chloride	5.00
Ferrous sulphate	0.20
Sodium thiosulphate	0.30
Phenol	0.024
Agar	12.00

Final pH (at 25ºC) 7.4±0.2

Preparation

64.52 grams was suspended in 1000 ml of distilled water and boiled to completely dissolve the medium. The medium was thoroughly mixed, dispensed into tubes and autoclaved for 15 minutes at a pressure of 15 lbs (121 °C). The media could be set in a slanted shape with one end approximately 1 inch long.

Urea Agar base (Autoclavable)

Composition

Ingredient	**gm/liter**
Peptic digest of animal tissue	1.00
Dextrose	1.00
Sodium chloride	5.00
Disodium phosphate	1.20
Mono potassium phosphate	0.80
Phenol red	0.012
Agar	15.00

Final pH (at 25ºC) 6.8±0.2

Preparation

24.0 grams were suspended in 950 mL of distilled water and boiled to completely dissolve the medium. The medium was then sterilized by autoclaving for 20 minutes at a pressure of 10 pounds (115°C). The medium was cooled to 50°C, aseptically added to 47.5 mL of sterile 40% urea solution and mixed well. The medium was dispensed into a sterile tube and set in an inclined position.

APPENDIX- H

ISOLATION, ENUMERATION AND IDENTIFICATION OF BACTERIA FROM WATER SAMPLES

1. Membrane Filtration (MF) technique

Principle

In this method, the water sample is passed through a thin sterile membrane filter (pore size 0.45 µm) and stored in a special filter device inside a suction flask. Aseptically transfer the filter disc containing the captured microorganisms to a sterile Petriplate containing selective eosin methylene blue (EMB) agar medium and count the colonies that develop after incubation. Using this method, large volumes of water can be tested more economically, with more accurate and faster results than multi-tube technology (APHA, 1989; Aneja; 2003; Cheesbrough, 2006).

Calculation of Coliform bacteria in colony forming unit

$$(\text{CFU}/100\ \text{mL}) = \frac{\text{Number of colonies} \times 100}{\text{Volume of sample}}$$

Procedure

1. Assembled filtration unit and suction device.
2. A sterile membrane filter disc (0.45 µm) was placed on the porous glass support of the filter holder of the membrane filter device.
3. The bottle was flipped several times to thoroughly mix the water sample.
4. A 100 mL water sample was poured into the funnel and filtered under vacuum.
5. The membrane filter from the filtration unit was aseptically removed using sterile blunt forceps and placed on EMB medium in a Petri plate.
6. Sealed Petriplate and labeled the code number of the water sample.
7. Similarly, 100 mL of the same water sample was filtered through a membrane filter and transferred to EMB medium.
8. Next, one Petriplate was incubated at 35 ± 0.5 ° C and the other Petri plate at 44 ± 0.2°C for 24 hours.
9. After incubation, the number of colonies on each plate was counted and CFU / 100 mL were calculated.

2. Gram staining

Principle

Gram stain is a very useful stain for identifying bacteria and classifying them into two major groups: Gram-positive and Gram-negative. The immobilized bacterial smear is sequentially exposed to four different reagents: crystal violet (primary stain), iodine solution (stain), alcohol (bleaching agent), and safranin (counterstain). Bacteria that retain a primary stain (dark blue or purple) are called Gram-positive, and bacteria that lose crystal violet and are counterstained by safranin (red/pink) are called Gram-negative. Differences in staining response to Gram stain may be related to chemical and physical differences in the cell wall. The cell walls of Gram-negative bacteria are thin, complex, multi-layered and contain relatively high lipid content in addition to proteins and mucopeptides. In contrast, the cell walls of Gram-negative bacteria are thick, chemically simple, and composed primarily of proteins and cross-linked mucopeptides (Aneja, 2003; Cheesbrough, 2006 and Forbes *et al.,* 2007).

Procedure

1. A thin film of the material to be tested was prepared and dried.

2. The material on the slide was heated, fixed, cooled and then dyed.

3. The slides were stained with crystal violet and left undried for 1 minute.

4. The slide was rinsed with tap water and shaken excessively.

5. The slide was dipped in iodine solution and placed on the surface for 1 minute without drying.

6. Rinse the slide with tap water and shake off excess water.

7. The slide was soaked in alcohol-acetone decolorizer for 15 seconds and immediately rinsed with tap water until the color stopped flowing from the slide with the decolorizer. Thicker smears require more aggressive decolorization.

8. The slides were soaked in counterstain (safranin) for 1 minute and washed with tap water.

9. The slide was sucked between two clean blotting papers and examined under a microscope under 10x, 40x and 100x oil immersion.

APPENDIX-I

PRINCIPLE AND PROCEDURE OF BIOCHEMICAL TESTS

Principle and procedure for biochemical tests were followed according to Cheesbrough, 2006; Forbes, *et al.,* 2007.

Catalase test

Principle

The enzyme catalase is involved in the breakdown of hydrogen peroxide into oxygen and water. The presence of the enzyme in the bacterial strain was clearly isolated when a small inoculum was introduced into hydrogen peroxide, and rapid oxygen bubble formation occurred. The lack of catalase is evident by the lack or production of few bubbles. The culture medium should not be longer than 24 hours. This test is used to distinguish bacteria that produce the enzyme catalase, such as staphylococcus, from bacteria that do not produce catalase, such as Streptococcus.

Procedure

1. Using an applicator, a colony is transferred to the surface of a clean, dry slide.

2. Place one drop of 3% H_2O_2 on the slide and mix.

3. After that, the effervescence of the gas is marked with bubbles in the case of a positive result within 10 seconds.

Oxidase test

Principle

Oxidative fermentation test determines whether certain Gram-negative bars metabolize glucose by fermentation or aerobic (oxidative) respiration. During anaerobic fermentation, pyruvate is converted to various mixed acids depending on the type of fermentation. The high concentration of acid produced during fermentation will cause the bromothymol blue indicator in OF medium to change from green to yellow in the presence or absence of oxygen. Some non-fermentative Gram-negative bacteria metabolize glucose by aerobic respiration and thus produce only small amounts of weak acids during glycolysis and the Krebs cycle. The decrease in the number of peptones and the increase in the amount of glucose facilitate the detection of the weak acids produced. Potassium phosphate buffer is added to further aid in acid detection. The OF test is used to determine if Gram-negative bacteria metabolize carbohydrates either

oxidatively, by fermentation, or without sacchrolytic (lack of the ability to utilize carbohydrates in the medium).

Procedure

1. Remove a piece of filter paper soaked in tetra-methyl-p-phenylenediamine dihydrochloride substrate.
2. Colonies to be examined are removed with an applicator and spread in filter paper.
3. The inoculated paper area changes color to dark green or purple which is observed within 10-30 seconds.

Oxidative-Fermentative test (OF)

Principle

Oxidative fermentation test determines whether certain Gram-negative bars metabolize glucose by fermentation or aerobic (oxidative) respiration. During anaerobic fermentation, pyruvate is converted to various mixed acids depending on the type of fermentation. The high concentration of acid produced during fermentation will cause the bromothymol blue indicator in OF medium to change from green to yellow in the presence or absence of oxygen. Some non-fermentative Gram-negative bacteria metabolize glucose by aerobic respiration and thus produce only small amounts of weak acids during glycolysis and the Krebs cycle. The decrease in the number of peptones and the increase in the amount of glucose facilitate the detection of the weak acids produced. Potassium phosphate buffer is added to further aid in acid detection. The OF test is used to determine if Gram-negative bacteria metabolize carbohydrates either oxidatively, by fermentation, or without sacchrolytic (lack of the ability to utilize carbohydrates in the medium).

Procedure:

1. Two tubes containing OF medium were removed and the organism was pierced with both media with sterile inoculation.
2. One of the tubes is sealed with paraffin oil to create anaerobic conditions.
3. Both tubes were incubated at 37°C for 24-48 hour and color change was observed in both tubes.

Sulfide Indole Motility (SIM) test

Principle

SIM medium (Sulphide, Indole, Motility) is useful to differentiate Gram-negative bacilli in the intestine. SIM testing helps isolate organisms based on sulfide production, indole formation, and motility. The medium contains ammonium ferric sulfate and sodium thiosulfate, which together serve as indicators for the production of hydrogen sulfide (H_2S). Hydrogen sulfide production was discovered when black sulfide, a black precipitate, was formed by the reaction of colored ammonium sulfate with hydrogen sulfide gas. The casein peptone of this medium is rich in tryptophan. Organisms possessing the enzyme tryptophanase break down tryptophan into indole. Detection of indole was obtained after the addition of Kovac reagent after incubation of the culture medium. Indole combines with p-dimethyl amino benzaldehyde and produces a red band at the middle end. Negative indole test does not produce color change after addition of Kovac reagent i.e. yellow color of Kovac reagent. The lower concentration of agar added to the medium produces a semi-solid structure that allows detection of bacterial motility. Motile organisms diffuse away from the puncture line and create turbidity or cloudiness throughout the medium. Non-motile bacterial growth is restricted along the puncture line and the surrounding environment is ventilated. Another component, the animal tissue of this medium, provides the amino acids and nutrients needed for bacterial growth.

Procedure

1. Inoculate the test organism into a test tube containing the SIM medium.
2. It was then incubated at 37°C for 24 h.
3. After incubation, the indole test was performed by adding 0.5 mL of Kovac reagent and gently shaking it.
4. The red color of the surface layer is checked for 10 minutes.

Methyl Red (MR) test

Principle

In the methyl red test, the bacteria under test are cultured in broth (MRVP medium) containing glucose. Bacteria are capable of using glucose to produce a stable acid; formic, acetic, lactic and succinic (end products) of glucose, the medium will remain red

(test positive) after adding methyl red a pH indicator (i.e. pH remains below 4.4). In other organisms, methyl red will turn yellow (test negative) due to an increase in pH above 6.0 due to enzymes that convert organic acids (produced during glucose fermentation) into end products the same non-acids as ethanol and acetoin (acetylmethylcarbinol).

Procedures

1. MR-VP medium was inoculated aseptically with the organism using a sterile loop and incubated at 37°C for 24 h.

2. Next add 5-6 drops of methyl red reagent to the incubated test tube.

3. A positive test is indicated by the development of the red color of the indicator.

Voges-Proskauer (VP) test

Principle

The Voges-Proskauer (VP) test is used to determine whether an organism produces acetyl methyl carbinol from glucose fermentation. If present, acetyl methyl carbinol is converted to diacetyl in the presence of α-naphthol, strong alkali (40% KOH) and atmospheric oxygen. α-naphthol was not part of the initial process but was discovered by Barritt to act as a color enhancer and should be added first. Compounds containing diacetyl and quinidine are found in the broth peptone which then condenses to form a pink-red polymer.

Procedure

1. The bacterial suspension was inoculated aseptically into MR-VP broth tubes and incubated at 37 °C for 24 h.

2. Next, Barrit reagents I and II were added in a 3:1 droplet ratio and the tube was shaken.

3. A positive test is indicated by the development of a red-pink color.

Citrate utilization test

Principle

Citrate agar is used to test an organism's ability to use citrate as an energy source. The medium contained citrate as the source and inorganic ammonium salts ($NH_4H_2PO_4$) as the sole nitrogen source. Bacteria that can grow on this medium produce an enzyme, citrate-permease that converts citrate to pyruvate. The pyruvate can then enter the body's metabolism to produce energy. Growth indicates utilization of citrate, an intermediate metabolite in the Krebs cycle. When bacteria metabolize citrate, ammonium salts are

broken down into ammonia, increasing alkalinity. The pH change changes the bromothymol blue indicator in the medium from green to blue above pH 7.6.

Procedure

1. Organisms were inoculated aseptically on Simmon's citrate agar and incubated at 37°C for 24 h.

2. A positive test is indicated by the growth of Prussian blue and the growth of the organism.

Triple Sugar Iron Agar (TSIA) test

Principle

TSIA (Triple Sugar Iron Agar) medium consists of three sugars; Lactose, sucrose and glucose, iron (color sulfide) and phenol red as indicators. The indicator used to detect sugar fermentation is indicated by the change in color of the medium due to the production of organic acids and hydrogen sulfide (H2S). If an organism ferments any of the three sugars or their combination, the medium will turn yellow due to the production of acids which are the end products of fermentation. Glucose utilization occurs aerobically in the presence of O2 and in anaerobic conditions. Oblique and end-to-end color change indicates. The generation of gas from the fermentation of sugar by an organism is indicated by the appearance of bubbles in the buttock or the upward thrust of the entire 2/3 inclination of the tube. The production of hydrogen sulfide (H2S) by an ionized organism by the reduction of ferric sulfides in the medium to ferric sulfide manifests as a black precipitate

Procedure

1. With a sterilized straight needle, touch the tip of a well isolated colony.
2. Inoculate TSIA agar by first inserting the medium up to two-thirds of the tube and by marking the surface along the slope of the agar.
3. This tube was incubated at 37°C for 18 hour.
4. A mid-color change has been observed.

Urea hydrolysis test

Principle

The urea hydrolysis test is used to determine the ability of certain microorganisms to produce the enzyme urease. Organisms capable of producing the enzyme urease

catalyze the breakdown of urea in TSIA (Triple Sugar Iron Agar) medium consists of three sugars; Lactose, sucrose and glucose, iron (color sulfide) and phenol red as indicators. The indicator used to detect sugar fermentation is indicated by the change in color of the medium due to the production of organic acids and hydrogen sulfide (H_2S). If an organism ferments any of the three sugars or their combination, the medium will turn yellow due to the production of acids which are the end products of fermentation. Glucose utilization occurs aerobically in the presence of O_2 and in anaerobic conditions. Oblique and end-to-end color change indicates. The generation of gas from the fermentation of sugar by an organism is indicated by the appearance of bubbles in the buttock or the upward thrust of the entire 2/3 inclination of the tube. The production of hydrogen sulfide (H_2S) by an ionized organism by the reduction of ferric sulfides in the medium to ferric sulfide manifests as a black precipitate.to ammonia and carbon dioxide, causing the pH of the environment to rise. As the pH increases, the red color of phenol changes from orange to deep pink.

Procedure

1. The test organism was streaked on the surface of the urea agar slant.
2. The tube was incubated at 37°C for 24-48 hours.
3. The change in color of the medium was observed (deep pink).

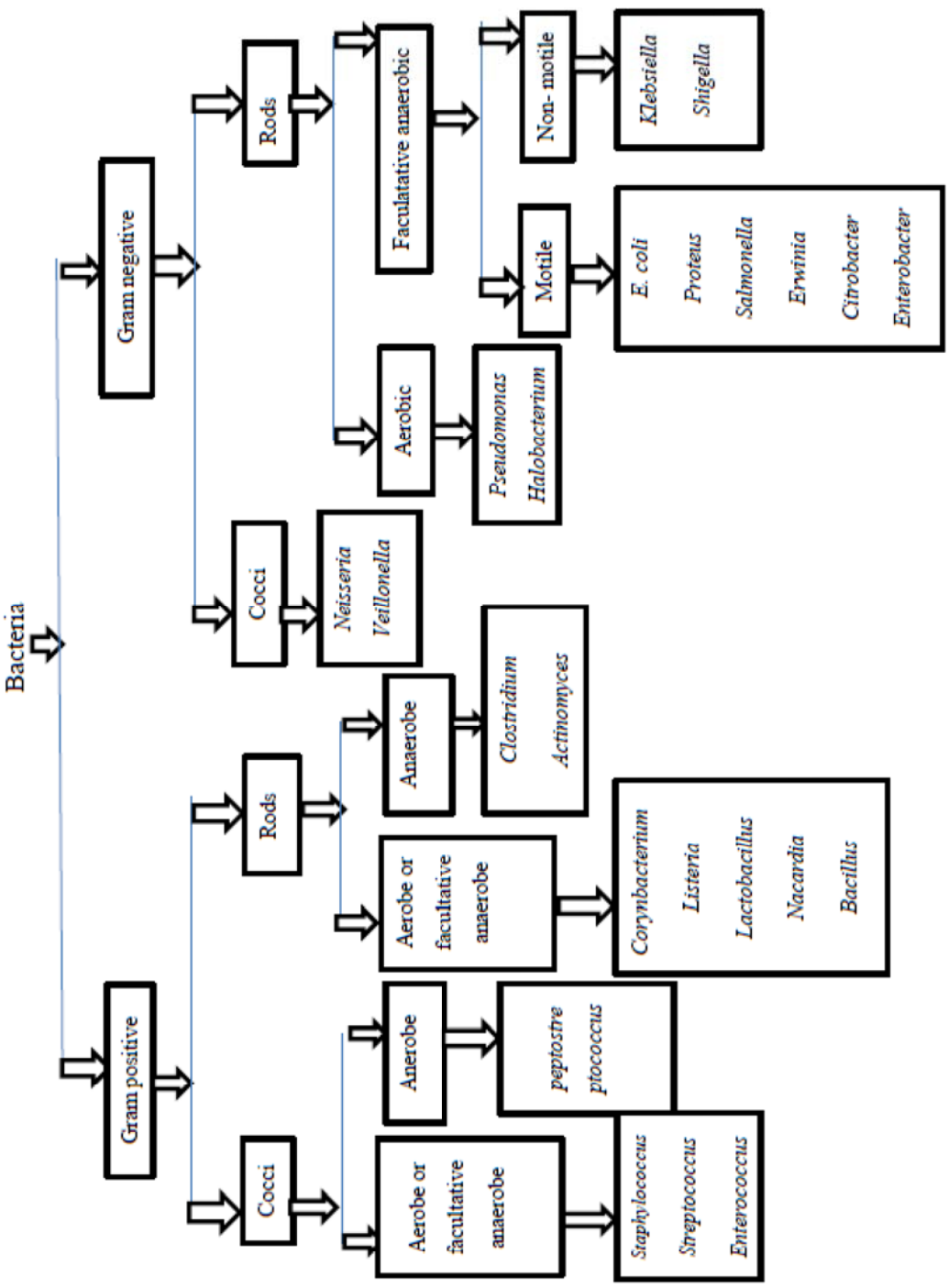
Biochemical tests for identification of bacteria

Tests	**bacteria**					
	E. coli	***Klebsiella*** **spp.**	***Enterobacter*** **spp.**	***Citrobacter*** **spp.**	***salmonella*** **spp.**	***Shigella*** **spp.**
Catalasc tcst	+	+	+	+	+	+
Oxidase test	_	-	-	-	-	-
Oxidative fermentative test	F	F	F	F	F	F
Motility test	+	-	+	+	+	-
Indole test	+	+	-	-	-	+
Methyl red test	+	-	-	+	+	+
Voges- Proskauer test	_	+	+	-	-	-
Citrate utilization test	_	+	+	+	+	-
TSI test	Y/Y H_2S- Gas+	Y/Y H_2S- Gas+	Y/Y H_2S- Gas+	R or Y/ Y D Gas +	R/Y H_2S – Gas +	R/Y H_2S- Gas-
Urease hydrolysis test	_	+	-	+	-	-

+ = Positive, - = Negative, F= Fermentative, H_2S= Hydrogen sulphide (blackening), Y= Yellow (acid reaction), R= red-pink (alkaline reaction).

Source**:** Chakraborty (2019), Cheesebrough (2006)

Bacterial classification chart



Source: Cheesebrough (2006), Aneja(2018

APPENDIX- J

1.Antibiotic susceptibility testing of bacteria isolated from water

Antimicrobial agents include natural antibiotics, synthetic derivatives of natural antibiotics (semi-synthetic antibiotics) and chemical antibacterial compounds (chemotherapy). In general, however, the term "antibiotics" is used to describe antibacterial (usually antibacterial) substances that can be used to treat infections. Compared with antibacterial agents, fewer antiviral and antifungal agents have been developed. However, most exhibit sufficient selective toxicity to be useful in the treatment of diseases caused by microorganisms. Antimicrobial agents can be grouped according to their mode of action, i.e. their ability to inhibit the synthesis of bacterial cell walls, cell membranes, proteins and nucleic acids. The Kirby-Bauer test is a test in which a disc of filter paper is impregnated with a different concentration of antibiotic or any chemical that diffuses from the plate into the agar. The selected antibiotic plates are placed on the surface of the agar plate that has been inoculated with the bacteria to be examined. During incubation, the antibiotics/chemicals will diffuse out of the plates in the agar. This will create a concentration gradient in the agar that depends on the solubility of the chemical and its molecular size. The absence of organism growth around the antibiotic disc indicates that the organism is susceptible to that particular antibiotic, and the presence of growth around the antibiotic disc indicates that the organism has resistance to that particular antibiotic. The zone of non-growth around this disc, called the zone of inhibition, is uniformly circular with a confluent lawn in the center (CLSI 2021).

Procedure

1. Using a sterile loop, a single colony of test organism was inoculated into 2-3 mL of nutrient broth and incubated at 37 °C for 4 hour.
2. Then, the turbidity of the prepared inoculum was compared and corrected for
3. Using the 0.5 Mac Farland turbidity standards.
4. Next, a sterile cotton swab was dipped in nutrient medium containing inoculum. Excess inoculum was removed by pressing and rotating the gauze pad against the side of the tube wall above the level of the suspension.

5. The inoculum was then evenly dipped onto the surface of MHA medium in three directions, rotating the plate approximately 60° at to ensure even distribution to obtain the forage medium.
6. With the Petriplate covered, the agar surface is allowed to dry for 3-5 minutes.
7. The antibiotics are then placed on the agar surface with sterile forceps and gently pressed.
8. Within 30 min of applying the plates, the plates were inverted and then incubated at 35 ± 2°C for 16-18 h.
9. Characterization of susceptibility recorded after overnight incubation by measuring the inhibitory region as susceptible, intermediate, and resistant.

2. Zone size interpretative chart

For Enterobacteriaceae.

Antibiotic disc	**Code**	**Disc content(mcg)**	**Zone of inhibition (mm)**			***E. coli* ATCC 25922**
			Sensitive	**Intermediate**	**Resistance**	
Amikacin	AK	30	17	15-16	14	19-26
Ampicillin	A	10	17	14-16	13	15-22
Ceftriaxone	CTR	30	23	20-22	19	29-35
Chloramphenicol	C	30	18	13-17	12	21- 27
Ciprofloxacin	CIP	5	21	16-20	15	29-37
Cotrimoxazole	COT	25	16	11-15	10	23-29
Gentamycin	GEN	10	15	13-14	12	19- 26
Nalidixic Acid	NA	30	19	14-18	13	22-28
Nitrofruantoin	NIT	300	17	15-16	14	20-25
Tetracycline	TE	30	15	12-14	11	18-25

(Source: CLSI, 2021)