ASSESSMENT OF BACTERIOLOGICAL QUALITY OF UNDERGROUND WATER FROM KATHMANDU, NEPAL AND ANTIBIOTIC SUSCEPTIBILITY PATTERN OF *Escherichia coli*

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

NILU KUMARI SHAH

SYMBOL No: 500330102

T.U. REGISTRATION No: 5-2-33-159-2017

JULY, 2022

Tribhuvan University
Institute of Science & Technology
AMRIT CAMPUS
P O Box No. 1021, Thamel, Kathmandu, Nepal
E-mail: amritcampus@ntc.net.np
☎ 4411627
4412438

Ref No

Date

Amrit Campus

RECOMMENDATION

This is to recommend that **Ms. Nilu Kumari Shah** has carried out her project work entitled **"Assessment of Bacteriological Quality of Underground Water from Kathmandu, Nepal and Antibiotic Susceptibility Pattern of *Escherichia coli*"** 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 the Bachelor of Science (B.Sc.) degree.

.....

Asst. Prof. Ushana Shrestha Khwakhali
Supervisor
Department of Microbiology,
Amrit Campus
Tribhuvan University
Thamel, Kathmandu, Nepal

.....

Mr. Atmaz Kumar Shrestha
Co-Supervisor
Department of Microbiology,
Amrit Campus
Tribhuvan University
Thamel, Kathmandu, Nepal

22 JULY, 2022

DECLARATION

This project work entitled **"Assessment of Bacteriological Quality of Underground Water from Kathmandu, Nepal and Antibiotic Susceptibility Pattern of *Escherichia coli*"** 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. Atmaz Kumar Shrestha in the 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 my degree.

.....

Signature

Nilu Kumari Shah

Symbol No: 500330102

T.U. Registration No: 5-2-33-159-2017

22 JULY, 2022

Tribhuvan University
Institute of Science & Technology
AMRIT CAMPUS
P. O. Box No. [illegible], Thamel, Kathmandu, Nepal
E-mail: [illegible]@ntc.net.np
☎ 4411637
4413408

Ref No

Date

Institute of Science & Technology
Amrit Campus

LETTER OF FORWARD

Date: 22/07/2022

On the recommendation of **Asst. Prof. Ushana Shrestha Khwakhali** and **Mr. Atmaz Kumar Shrestha** this project work is submitted by **Nilu Kumari Shah**, Symbol No: 500330102, T.U. Registration No: 5-2-33-159-2017, entitled "**Assessment of Bacteriological Quality of Underground Water from Kathmandu, Nepal and Antibiotic Susceptibility Pattern of *Escherichia coli***" 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.

She has fulfilled all the requirements laid down by the Institute of Science and Technology (IoST), Tribhuvan University (T.U.), Nepal for the project work.

.....

Asst. Prof. Suchitra Thapa
Head of Department
Department of Microbiology
Amrit Campus
Tribhuvan University

Tribhuvan University
Institute of Science & Technology
AMRIT CAMPUS
P O Box No 10[illegible] Thamel, Kathmandu, Nepal
E-mail amritcampus@ntc.net.np
☎ 441[illegible]
441[illegible]

Institute of Science & Technology
Amrit Campus

Ref No

Date

BOARD OF EXAMINATION AND CERTIFICATE OF APPROVAL

This project work entitled "**Assessment of Bacteriological Quality of Underground Water from Kathmandu, Nepal and Antibiotic Susceptibility Pattern of *Escherichia coli***" by Nilu Kumari Shah (Symbol No: 500330102 and T.U. Registration No: 5-2-33-159-2017) under the supervision of Asst. Prof. Ushana Shrestha Khwakhali and co-supervision of Mr. Atmaz Kumar Shrestha in the 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 Sciencee (B.Sc.) degree in Microbiology. This report has been accepted and forwarded to the Controller of Examination, Institute of Science and Technology, Tribhuvan University, Nepal for the legal procedure.

Asst. Prof. Ushana Shrestha Khwakhali
Supervisor
Department of Microbiology
Amrit Campus
Tribhuvan Univer sity

Mr. Atmaz Kumar Shrestha
Co-Supervisor
Department of Microbiology
Amrit Campus
Tribhuvan University

Asst. Prof. Puspa Raj Dahal
External Examiner
Department of Microbiology
Tri-Chandra Multiple Campus
Tribhuvan University

Asst. Prof. Suman Rai
Internal Examiner
Department of Microbiology
Amrit Campus
Tribhuvan University

Asst. Prof. Suchitra Thapa
Head of Department
Department of Microbiology
Amrit Campus
Tribhuvan University
28 JULY, 2022

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Nilu Kumari Shah

Symbol No: 500330102

T.U. Registration No: 5-2-33-159-2017

July, 2022

ABSTRACT

Water is a major resource in ecosystem as it supports life of all living organisms. Microbial contamination of underground water has been an important cause of waterborne epidemics. This study was carried out to assess the bacteriological quality of underground water in Kathmandu Metropolitan city during March to May 2022 and determine the antibiotic susceptibility pattern of *Escherichia coli* isolated from water. A total of 36 water samples were collected of which 32 were untreated and 4 were treated underground water. Total coliform and fecal coliform count were done using Membrane Filtration (MF) technique. The identification of bacteria from underground water was done by biotyping and Antibiotic Susceptibility Testing (AST) was done by Kirby-Bauer disc diffusion method. In this study, 87.5% of underground water were found to be used without any filtration and disinfection. Total coliform as well as fecal coliform count exceeded WHO guideline value of 0 CFU/100 mL in 84.4% and 25% of untreated underground water respectively. In treated underground water, 100% of samples were found to be contaminated with total coliform whereas fecal coliform was not detected. Of 75 isolates of bacteria obtained at 35°C±0.5°C from underground water, *E. coli* (37.3%), *Klebsiella* spp. (32%), *Enterobacter* spp. (9.3%), *Citrobacter* spp. (2.7%), *Salmonella* spp. (6.7%), *Pseudomonas* spp. (2.7%), *Proteus* spp. (4%) and unidentified gram-negative rods (5.3%) were identified. Among 16 isolates of thermotolerant bacteria, 50% of isolates were *E. coli*. According to antibiotic susceptibility pattern, *E. coli* (n=28) isolates from water were found to be 100% sensitive to Cotrimoxazole, Gentamycin and Amikacin while resistance towards Ampicillin (60.7%), Nitrofurantoin (53.6%), Ciprofloxacin (25%), Ceftriaxone (17.9%), Nalidixic acid (10.7%), Chloramphenicol (3.6%) and Tetracycline (3.6%) were detected. Thermotolerant *E. coli* (n=8) showed resistance towards Nitrofurantoin (25%), Ampicillin (12.5%), Chloramphenicol (12.5%), Ceftriaxone (12.5%) and Tetracycline (12.5%). *E. coli* (21.4%) and thermotolerant *E. coli* (12.5%) were also found to be Multiple Antibiotic Resistant (MAR) bacteria. Questionnaire survey revealed that underground water was used for drinking by 4% of respondents and household activities by 81% of respondents. All the respondents had toilet facility as well as habit of handwashing. Most of the underground water sources were contaminated with total coliform and fecal coliform. Awareness on quality of underground water should be generated to the public. An appropriate treatment and disinfection methods has to be implemented to maintain quality of underground water and prevent waterborne diseases.

Keywords: Antibiotic Susceptibility Pattern, Fecal Coliform, Membrane Filtration Technique, Total Coliform, Underground water.

शोधसार

पानी वातावरणको महत्वपूर्ण अङ्ग हो । यो सबै जीवित जीवहरूको जीवनमा अतिआवश्यक तत्व हो । जमिनमुनिको पानीको माइक्रोबियल प्रदुषण जलजन्य महामारीको प्रमुख कारण भएको छ । मार्च देखि मे २०२२ सम्म काठमाण्डौ सहरमा भूमिगत पानीका स्रोतहरूको ब्याकटेरियोलोजिकल गुणस्तरको मूल्यांकन गर्न र पानीमा पाइने *Escherichia coli* को एन्टिबायोटिक संवेदनशीलता परीक्षण गर्न यो अध्ययन गरिएको थियो । कुल ३६ पानीको नमूना संकलन गरिएको थियो जसमा ३२ वटा प्रशोधन नगरिएको र ४ वटालाई प्रशोधन गरिएको थियो । टोटल कोलिफर्म र फेकल कोलिफर्म गणना मेम्ब्रेण फिल्ट्रेशन प्रविधि प्रयोग गरी गरिएको थियो । जमिनमुनिको पानीबाट ब्याक्टेरियाको पहिचान बायोटाइपिङ गरेर गरिएको थियो र एन्टिबायोटिक संवेदनशीलता परीक्षण किर्बी-बाउर डिस्क डिफ्युजन विधि द्वारा गरिएको थियो । यस अध्ययनमा ८७.५% जमिनमुनिको पानी बिना फिल्टर र कीटाणुशोधन प्रयोग भएको पाइयो । अप्रसोधित भूमिगत पानीमा टोटल कोलिफर्म र फेकल कोलिफर्म गणनाले 0 CFU/100 mL को WHO निर्देशिका मानलाई क्रमश: ८४.४% र २५%ले पार गर्यो । प्रशोधित भूमिगत पानीमा १००% नमुनामा टोटल कोलिफर्म दूषित भएको पाइएको थियो भने फेकल कोलिफर्म फेला परेको थिएन । भूमिगत पानीबाट 35°C±0.5°C मा प्राप्त ब्याक्टेरियाका ७५ पृथकहरु मध्य *E. coli* (३७.३%), *Klebsiella* spp. (३२%), *Enterobacter* spp. (९.३%), *Citrobacter* spp. (२.७%), *Salmonella* spp. (६.७%), *Pseudomonas* spp. (२.७%), *Proteus* spp. (४%) र अज्ञात ग्राम नेगेटिभ रडहरु (५.३%) पहिचान गरिएको थियो । थर्मोटोलरेन्ट ब्याक्टेरियाका १६ पृथक नमुनाहरु मध्ये ५०% आइसोलेट्स *E. coli* थिए । एन्टिबायोटिक संवेदनशीलता परीक्षण अनुसार, पानीबाट *E. coli* (n=२८) पृथक नमुनाहरु, Cotrimoxazole, Gentamycin र Amikacin को लागि १००% संवेदनशील पाइयो भने Ampicillin (६०.७%), Nitrofurantoin (५३.६%), Ciprofloxacin (२५%), Ceftriaxone (१७.९%), Nalidixic acid (१०.७%), Chloramphenicol (३.६%) र Tetracycline (३.६%) को रेसिस्टेन्ट । थमीटोलरेन्ट *E. coli* (n=८) ले Nitrofurantoin (२५%), Ampicillin (१२.५%), Chloramphenicol (१२.५%), Ceftriaxone (१२.५%) र Tetracycline (१२.५%) विरुद्ध रेसिस्टेन्स देखायो । *E. coli* (२१.४%) र थर्मोटोलरेन्ट *E. coli* (१२.५%) दुवै मल्टिपल एन्टिबायोटिक प्रतिरोधी (MAR) पाइयो । प्रश्नावली सर्वेक्षणले ४% उत्तरदाताहरुले भूमिगत पानी पिउन र ८१% उत्तरदाताहरुले घरायसी प्रयोजनका लागि प्रयोग गरेको खुलेको छ । सबै उत्तरदाताहरु (१००%) सँग शौचालयको सुविधा र हात धुने बानी थियो । अधिकांश भूमिगत पानीका स्रोतहरूको टोटल कोलिफर्मले दूषित थिए । भूमिगत जलस्रोतको गुणस्तरबारे जनतामा चेतना जगाउनु पर्दछ । भूमिगत पानीको गुणस्तर कायम राख्न र जलजन्य रोगहरू रोक्न उपयुक्त उपचार र कीटाणुनासक विधिहरू लागू गर्नु पर्दछ ।

कीवर्डहरु : एन्टिबायोटिक संवेदनशीलता परीक्षण, फेकल कोलिफर्म, मेम्ब्रेण फिल्ट्रेशन, टोटल कोलिफर्म र भूमिगत पानी ।

LIST OF ACRONYMS AND ABBREVIATIONS

APHA	American Public Health Association
ARG	Antibiotic Resistant Genes
AST	Antibiotic Susceptibility Testing
CBS	Central Bureau of Statistics
CFU	Colony Forming Units
CLSI	Clinical and Laboratory Standards Institute
DoHS	Department of Health Services
EMB	Eosin Methylene Blue
HWT	Household Water Treatment
KUKL	Kathmandu Upatyaka Khanepani Limited
MCM	Million Cubic Meters
MF	Membrane Filtration
MIC	Minimum Inhibitory Concentration
MLD	Million Liters per Day
MPN	Most Probable Number
MR	Methyl Red
NDWQS	National Drinking Water Quality Standards
Nos	Numbers
OF	Oxidative Fermentative
SDGs	Sustainable Development Goals
SIM	Sulphide Indole Motility
TSIA	Triple Sugar Iron Agar
UNICEF	United Nations International Children's Emergency Fund
VP	Voges-Proskauer
WAN	Water Aid in Nepal
WHO	World Health Organization
µg/mL	Micrograms per Milliliter
gm/L	Grams per Liter
mg/L	Milligrams per Liter

LIST OF SYMBOLS

°C	Degree Centigrade
%	Percentage
+	Positive
–	Negative
±	Equal or less

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CHAPTER 1

1.INTRODUCTION

1.1 General introduction

Water is one of the most important elements for living creatures. The majority of the water is either in the ocean or in glaciers in the Himalayas (Thapa, *et al*., 2019). Water has a significant impact on human health and the quality of the water supplied is critical in determining individual and community health (Jayana, *et al*., 2009).

Approximately 663 million people in the world lack access to improved drinking water sources, while 1.8 billion consume fecally contaminated water. Goal 6 of the 17 United Nations Sustainable Development Goals (SDGs) aims to provide everyone with universal and equal access to safe and inexpensive drinking water, as well as suitable sanitation facilities by 2030. In resource-poor developing countries, achieving this goal will be more difficult (Udmale, *et al*., 2016).

Several big rivers flow south from the Northern Himalayas, including around 6,000 rivers and rivulets that flow through Nepal, making it one of the world's most water-rich countries. Despite this abundance, water security has been a growing concern for Nepal and other South Asian countries (Pandey, *et al*., 2019).

According to the Department of Water Supply and Sanitation (DWSS), 86.45% of Nepal's population has access to drinking water, however only 15% of that population is served with good quality drinking water. In metropolitan places, the impoverished and socially alienated have little or no access to both basic quantity and quality drinking water. A sizable population in both urban and rural areas must rely on untreated water from nearby springs, streams, tube wells, and small brooks, and may spend hours collecting water (Pandey, 2021).

The Kathmandu Valley is one of the historical cities with many UNESCO-designated world heritage sites. It is centrally placed in Nepal's middle hills. Kathmandu district is located in Bagmati Province of Nepal, in the Kathmandu Valley covering an area of 413.69 Km^2. It is the most densely inhabited district in Nepal, with the population of 2,017,532 in 2021 (CBS, 2022). Kathmandu Metropolitan city, which is also Nepal's capital, serves as the district's headquarters and is the country's oldest metropolitan city. Due to rapid population growth and increased urbanization, the valley suffers from

acute water scarcity and deteriorated water quality. Low availability of potable water in the valley has resulted from rapid and mainly unplanned urban and population growth, a lack of sustainable water sources, severe land use changes, socioeconomic transition, and a poor management system (Vaidya, 2017 and Udmale, *et al*., 2016).

Groundwater resources are often regarded as a dependable source of water for a variety of purposes. The Kathmandu valley, Nepal's major metropolitan hub, has seen substantial population increase in recent years, and about half of the valley's water supply comes from groundwater. Because of the large imbalance between water demand and supply in the valley, the majority of families have a bore hole or a well to draw ground water. This puts a greater strain on the valley's groundwater quality and quantity (Khatiwada, *et al*., 2002).

For the 1.7 million people who live in the Kathmandu Valley, getting safe water is incredibly challenging. Even for individuals who have access to tap water in their houses, the municipal system is only available periodically, especially since the 7.8-magnitude earthquake in April 2015. As a result, people collect and drink water from a variety of alternative sources, including dug wells, tube wells, and groundwater-accessing stone spouts (dhunge dharas) (Sarkar, *et al*., 2022). The erosion of surface and shallow groundwater quality is said to have encouraged people to draw deep groundwater in pursuit of a more secure and consistent source (Ghartimagar, *et al*., 2020). In Kathmandu valley, the groundwater is widely used for drinking and is rapidly increasing in industries, hotels, agriculture and other activities (Maharjan, *et al*., 2020).

Microorganisms, chemicals, and other solid waste products can contaminate water. Coliform bacteria are often employed as indicators of food and water hygienic status. They are classified as rod-shaped Gram-negative non-spore forming motile or non-motile bacteria that ferment lactose with the production of acid and gas when incubated at 35–37°C. Coliform bacteria can be found in the aquatic environment, in soil, and on vegetation, and they are found in great numbers in the feces of warm-blooded animals universally (Mian, *et al*., 2020)

The ground surface is covered in concrete, which prevents regular ground water recharging. Pollution of ground water is also caused by pollutants on the soil and sewage leaking from sewers (Vaidya, 2017). In the Kathmandu Valley, the microbiological condition of groundwater is a major public health concern. Several earlier investigations have demonstrated severe fecal contamination and microbiological

pollution of groundwater in the Kathmandu Valley (Shrestha, *et al*., 2014). The pH, ammonia, turbidity, electrical conductivity, and arsenic levels for drinking water also differ from WHO norms and Nepal standards. Infants and small children, persons who are disabled, and the elderly are all at danger of contracting a waterborne disease, especially if they live in unsanitary surroundings (Maharjan, *et al*., 2018).

Drinking water polluted with human feces is the leading source of a variety of water-borne diseases (diarrhoea, dysentery, typhoid fever, hepatitis, and others) caused by bacteria, viruses, and parasites. Every year, three and a half million people die as a result of contaminated water and inadequate sanitation. Diarrhoea is responsible for 4% of all deaths and 5% of all health-related disability in children, especially in underdeveloped nations. Until recently, deadly diarrhoeal infections dominated the list of "top ten diseases" and were one of Nepal's most serious public health issues. According to a 2011 demographic health survey, two-thirds of hospital outpatient department visiting patients have diseases related to water and sanitation. Diarrhoea affects 12 percent of children (Rai, *et al*., 2012).

Antibiotics are frequently used in humans, animals, food, and plants to treat infections. Antibiotic resistance microorganisms have emerged as a result of the unregulated and widespread use of antibiotics against pathogens (Padmini, *et al*., 2017). According to the WHO (2020), antimicrobial resistance will cause more than 350 million deaths globally by 2050 if current trends continue (Toranzos, *et al*., 2020). Antibiotic resistance is one of the most serious threats to global health today, and it is mostly caused by antibiotic misuse. Antibiotic usage increased by 65 percent between 2000 and 2015, and is anticipated to rise by 200 percent by 2030 unless significant policy adjustments are adopted. Antibiotic consumption has risen as a result of greater use in low and middle-income nations. Antibiotic resistance is increasing in poorer nations like Nepal due to inadequate infection control, irrational antibiotic usage, and a lack of adequate surveillance mechanisms for tracking antibiotic use and resistant bacterium patterns (Thakali, *et al*., 2021).

Antibiotic susceptibility testing is an important part of the process. It enables doctors to make informed decisions about all types of antimicrobial therapy without having to worry about antibiotic resistance developing. Inadequate antibiotic therapy can result in death from serious illnesses (Mian, *et al*., 2020).

Household water treatment (HWT), which treats water at the point of use with methods such as boiling and ceramic filtering, solar disinfection systems, and chlorination, are both inexpensive and effective ways to address the problem of non-potable water in the household. However, studies have revealed that, despite being necessary and potentially beneficial to one's health, households do not use HWT on a regular basis. As a result, its potential health benefits are diminished. These avoidance practices are both effective and cost-effective in preventing water-borne illnesses. Unfortunately, not all households implement these treatment methods, exposing them to health concerns as a result. Household water treatment can considerably reduce the risk of developing a waterborne disease, a greater knowledge of the factors that impact household treatment behavior is crucial. Poverty and a lack of understanding of waterborne diseases may be evident reasons why some households do not treat their drinking water (Daniel, *et al*., 2019 and Valley, *et al*., 2015)

1.2 Rationale

Coliform bacteria in underground water are the major contaminant by organic means through the discharge of sewage and domestic effluents into water sources (Maharjan, *et al*., 2020). The fecal contamination and pollution of water sources, unsanitary habits or a lack of sanitation causes waterborne epidemics (of diarrhoea, cholera, typhoid, etc.) spread throughout Nepal, including the Kathmandu Valley every summer and take a large number of lives (Magar, *et al*., 2019). Microbiological risk assessment must be carried out at the water treatment system to ensure effective pathogen removal (George, *et al*., 2015). Globally, antibiotic resistance has emerged as a severe public health issue (Chaudhary, *et al*., 2011). Drinking water is a potential route of Antibiotic Resistant Genes (ARG) exposure to humans (Thakali, *et al*., 2022). The therapeutic choices are now severely constrained due to the substantial rise in antimicrobial resistance among Enterobacteriaceae in recent years (Kayastha, *et al*., 2020). Therefore, the study of antibiotic resistant bacteria should be done. Thus, it is anticipated that the current investigation would clarify the bacteriological water quality by enumeration and identification of total coliform and fecal coliform which is essential for maintaining water quality and antibiotic susceptibility testing to prevent waterborne epidemic outbreak in Kathmandu.

1.3 Objectives

1.3.1 General objective:

- To assess the bacteriological quality of underground water from Kathmandu, Nepal and antibiotic susceptibility testing of *Escherichia coli* isolated from water.

1.3.2 Specific objectives:

- To conduct pre-structured questionnaire survey on water sources, sanitation, hygiene, water treatment and waterborne diseases in the study area.
- To enumerate total coliform and fecal coliform from underground water (treated water and untreated water) from Kathmandu Metropolitan city by Membrane Filtration technique.
- To identify total coliform, fecal coliform and other bacteria present in the underground water (treated water and untreated water).
- To carry out antibiotic susceptibility testing of *E. coli* isolated from underground water.

CHAPTER 2

2. LITERATURE REVIEW

Water is a vital component of all living things and is required for a variety of functions. Water is found in 70–90% of living organisms' cells, making it a crucial component of cell physiology (Mian, *et al*., 2020). At least 2 billion people throughout the world consume water that has been tainted with feces. Microbial contamination of drinking water as a result of fecal contamination provides the greatest threat to water safety. Drinking water that is microbiologically contaminated can spread diseases like diarrhoea, cholera, dysentery, typhoid, and polio and is estimated to cause 485,000 diarrhoeal fatalities per year (WHO, 2020).

2.1 Sources of water

Cities in the Kathmandu Valley were once built around cascading water distribution network of canals, ponds (phuku or pokhari), wells (inar), and stone spouts or stepwells (hiti or dhunge dhara). The dynamics of these water systems differ depending on the geography, but in general, they catch, store, and move water from rainfed springs in the Valley's surrounding woods to aquifers in the Valley floor. Stone spouts and wells are still used as critical water sources for the urban population today, in addition to its cultural heritage importance (Molden, *et al*., 2020). Private wells, groundwater pumping, stone spouts (traditional public tap-stands made of stone), water tankers, and jar water (water sold in 20 liter plastic containers from water vendors) are now filling the gap left by Kathmandu Upatyaka Khanepani Limited (KUKL), the authority responsible for water supply inside Kathmandu Valley (Ojha, *et al*., 2018).

2.2 Uses of water

Water has been utilized by humans for a variety of livelihoods throughout history, including drinking, washing, cooking, irrigating, manufacturing, and worship. They occasionally designate certain sources for specific purposes, but more frequently, they use the same sources for a variety of purposes. Water is also important for personal hygiene, food production and pharmaceutical use (Moriarty, *et al*., 2004 and WHO, 2004).

2.3 Water accessibility and availability

Because there is a scarcity of surface water for both drinking and non-drinking purposes, Kathmandu gets around half of its water from groundwater systems, which include shallow and deep aquifers. The main mechanisms of groundwater utilization include tube wells, excavated wells, and stone spouts. As a result of excessive use for domestic and industrial uses, the groundwater in Kathmandu Valley is under severe stress. The KUKL provides water to Kathmandu from 35 surface water and 57 groundwater sources. According to KUKL, the Kathmandu Valley's water demand in 2013 was 360 million liters per day (MLD), yet there was only 95–154 MLD available. According to a research, the valley's aquifers can sustain 26.3 MLD of groundwater removal. In 2012, the Ground Water Resource Development Board conducted a well inventory study and found 759 deep tube wells in Kathmandu. Private and community tube wells extract a total of 31.15 million cubic meters (MCM) of groundwater (GWRDB, 2012). This reveals that the yearly groundwater extraction from deep tube wells in Kathmandu is 69.44 MCM. Both analyses show a higher rate of groundwater withdrawal than is sustainable. As a result of excessive use for domestic and industrial uses, the groundwater in Kathmandu Valley is under severe stress. Groundwater reserves will be depleted significantly sooner than the 100-year timetable under such conditions and with restricted water supplies (Chinnasamy & Shrestha, 2019).

2.4 Water and sanitation in Nepal

Water and sanitation are the primary drivers of public health. Since access to clean water and sufficient sanitation facilities for all people, regardless of their living conditions is essential for both good health and social and economic development, we will have won a major war against all types of diseases (UNDP, 2006 and Pradhan, 2004).

Nepal faces a number of problems related to water and sanitation (Warner, *et al*., 2008). In 2015, WHO/UNICEF reported that 92% of Nepalese population had access to improved water though it is not yet clear if the water classified as improved can be safely consumed (WHO/UNICEF, 2015). About 3500 children die each year due to waterborne diseases according to the Department of health Service in Nepal (Aryal, *et al*., 2012).

The top 10 diseases in Nepal include waterborne infections, which can be reduced by implementing effective preventive measures such raising public awareness of health and sanitation issues, safeguarding water sources, and preventing contamination. By 2020, Nepal aimed to provide basic sanitation for everyone and end open defecation nationwide (Budhathoki, 2019).

2.5 Waterborne diseases

World Health Organization (WHO) has estimated that up to 80% of all illness and disease in underdeveloped nations is brought on by poor sanitation, contaminated water, or a lack of access to clean water (Cheesebrough, 2006). The amount of outbreaks that have occurred globally shows that contaminating drinking water with microorganisms is still a major source of disease. Estimates of illness based solely on identified outbreaks, however, are probably an underestimate of the issue. Waterborne epidemic outbreaks are common in Nepal as well as most third-world nations. Such disease-related mortality and morbidity continue to top the list. The most significant risk from waterborne microbes is related to drinking water consumption that is contaminated with human and animal excreta, although other sources and routes of exposure may also be significant for pathogens transmitted by the faecal-oral route. Drinking water is only one vehicle of transmission for these pathogens (WHO, 2010). Polluted water can cause diarrhoea, dysentery, typhoid and paratyphoid which may cause mortality (Upreti, 2017).

According to WHO (2017), unsafe water supply is a major problem and fecal contamination of water sources and treated water is a global problem. At least 2 billion people use drinking water source contaminated with feces worldwide. 785 million people lack even a basic drinking water service. Households drinking groundwater from shallow wells followed by those drinking tanker water with different enteropathogenic *E. coli* have highest potential risk of diarrhoea. In 2073/74, 11,620 cases of diarrhoea were reported in Nepal in which 106 were death caused due to diarrhoea (DOHS, 2018). In 2076/77, 27,176 waterborne diseases morbidity cases were reported while 491 were morbidity death cases (DOHS, 2020). As of 20th July 2022, a total of 34 cases of Cholera has been reported in Kathmandu (MOHP, 2022).

2.6 Microbial parameter of water quality

Drinking water should ideally be free of pathogenic germs and bacteria associated with fecal contamination. The presence of fecal indicator bacteria in drinking water has been discovered. Water gives an extremely sensitive technique of determining quality. It is impossible to test water for every possible contaminant or pathogen that might be present. Recent investigations have revealed that the groundwater in the Kathmandu Valley contains significant microbiological pollution that surpasses the World Health Organization (WHO) drinking water quality standard (Prasai, *et al*., 2007).

2.6.1 Total coliform

Coliform bacteria refer to gram-negative, oxidase-negative, non-sporing rods that can ferment lactose in 48 hours at 35–37°C while producing both gas and acid. They may grow aerobically on agar medium containing bile salts (Cheesbrough, 2006). Traditionally, coliform bacteria were classified to the genera *Escherichia*, *Citrobacter*, *Klebsiella* and *Enterobacter*, but the group is more heterogeneous and includes a wider range of genera, such as *Serratia* and *Hafnia*. Total coliform should not be present following disinfection, and their presence suggests insufficient treatment. Total coliform can indicate regrowth, potential biofilm formation, or pollution from the intrusion of foreign materials like soil or plants in distribution systems and stored water sources (WHO, 2008).

2.6.2 Fecal (Thermotolerant) coliform

Thermotolerant coliform are any total coliform bacteria that can ferment lactose at 44°C-45°C (Cheesbrough, 2006). Although *Escherichia* is typically the dominating genus in aquatic environments, several varieties of *Citrobacter, Klebsiella*, and *Enterobacter* are also thermotolerant. The capacity to synthesize indole from tryptophan or the ability to manufacture the enzyme β-glucuronidase allow *Escherichia coli* to be distinguished from other thermotolerant coliforms. Even though there is some evidence of development in tropical soils, *Escherichia coli* is typically only detected in very high concentrations in human and animal feces. Thermotolerant coliform species other than *E. coli* can include environmental organisms (WHO, 2008).

2.6.3 Indicator organisms

Coliform bacteria, in particular *E. coli*, are almost always found in large numbers in the human intestine. An average human excretes billions of these organisms every day,

according to estimates. In general, these organisms survive in water longer than intestinal pathogens. Pathogenic organisms are typically not excreted by healthy individuals, but if an intestinal tract infection occurs, the pathogen is likely to show up in the feces. As a result, the presence of coliform in water is seen as a sign that it has been exposed to potentially dangerous pollution. *Clostridium perfringens* and *Enterococcus faecalis* are the other indicator microbes (Pelczar, *et al*., 2012).

Other waterborne pathogens are bacteria (*Campylobacter jejuni*, *C. coli*, *Escherichia coli*-pathogenic, *E. coli* Enterohaemorrhagic, *Legionella* spp., Non-tuberculous mycobacteria, *Pseudomonas aeruginosa*, *Salmonella* Typhi and other *Salmonella* and *Shigella* spp.), viruses (*Adenovirus*, *Enterovirus*, Hepatitis A viruses, Hepatitis E viruses, *Norovirus* and *Rotavirus*), protozoa (*Acanthamoeba* spp., *Cryptosporidium parvum*, *Cyclospora cayetanesus*, *Entamoeba histolytica*, *Giardia intestinalis* and *Toxoplasma gondii*) and helminths (*Drancunculus* and *Schistosoma* spp.) (WHO, 2006).

Microbiological pollution of drinking water supplies is the biggest concern to the public health. Drinking water microbiological characteristics are determined by the types and quantity of bacteria present. In drinking water, numerous bacteria may be present. The majority of these bacteria are harmless, although pathogens could be present if the water is polluted. The pathogens could include bacteria, viruses, protozoa, etc., and when consumed with drinking water, they can result in a variety of health issues. However, the main concern is infectious diarrheal disorders spread by the feco-oral route. Due to issues with complexity, expense, and the timeliness of obtaining results, it is challenging to determine the presence of specific pathogens and is not typically done. As a result, only the testing of indicator organisms which are used to give an indication of the posssible presence of pathogens is done. There are different types of indicator organisms. The most commonly used indicator organisms for drinking water quality assessment are total coliform and fecal coliform (UNICEF, 2008 and WAN, 2011).

2.7 Drinking water quality standard

According to WHO bacteriological quality of drinking water, *E. coli* or any thermotolerant coliform must not be detectable in 100 mL treated water sample in the distribution system and total coliform bacteria must not be detectable in any 100 mL water samples. In case of large supplies where sufficient samples are examined total

coliform must not be present in 95% of samples taken throughout any 12 months period (WHO 1997).

The guideline value of physiochemical and bacteriological water quality is given in **Appendix-C**.

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

Count per 100 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)

2.8 Global scenario of microbial quality of water

Suthar, *et al*. (2009) studied eighty six different water samples in rural areas of northern Rajasthan and found the total microbial load in drinking water was in the ranges of 8.3×10^4–28.3×10^4, in different localities. A total of ten bacterial species, i.e. *E. coli, Pseudomonas aeruginosa, E. aerogenes, Klebsiella* spp., *Proteus vulgaris, Alcaligenes faecalis, Bacillus cereus, Staphylococcus aureus, Streptococcus lactis* and *Micrococcus luteus* were identified in drinking water samples. The occurrences and distribution patterns of microbial species varied greatly among different villages of this region. Three bacterial species, e.g. *E. coli, P. vulgaris,* and *S. lactis* showed the maximum occurrences (recorded from 73.1% villages/towns) followed by *M. luteus, Klebsiella spp., S. aureus, P. aeruginosa, E. aerogenes, B. cereus* and *A. faecalis.*

Hannan, *et al*. (2010) reported *E. coli* was grown from 42% (n = 42) of water samples out of hundred samples studied in Lahore. Coliforms were grown from 8% (n = 8), 7% (n = 7) and 54% (n = 54) of water samples with a count of 1–3 CFU / 100 mL, 4–10 CFU / 100 mL and > 10 CFU / 100 mL respectively. Analysis of boiled water samples (n = 12) showed the growth of *E. coli* in 16.6% (n = 2) samples whereas coliforms were grown from 41.6% (n = 5) samples.

Ahmed, *et al*. (2013) collected seventy five drinking water samples in Dhaka and reported *E. coli* isolates which were further confirmed by their Gram reaction as Gram

negative, short rod cells visualized under bright field microscope and by observing the formation of gas in lactose fermentation broth. The presence of the indicator bacteria indicated the possible occurrence of fecal contamination. All samples contained total aerobic bacterial load ranging from 1×10^4 to 3×10^6 CFU/mL.

Bekuretsion, *et al*. (2018) sampled out 75 hand pump fitted boreholes in Northern Ethiopia and reported 15% (n=11) positive for total coliform with colony count of 20-140 CFU/100 mL. A total of 13 enteric bacteria were identified among which Klebsiella spp. was predominant followed by E. coli, Enterobacter spp. and Citrobacter spp..

Samie, *et al*. (2011) conducted a study on 6 different borehole water samples from schools in South Africa and reported total coliform count ranged between 11 and 6.92 $\times10^4$ CFU/100 mL and fecal coliform count ranged between 8 and 9 $\times10^3$ CFU/100 mL. The disease causing organisms such as *Klebsiella pneumonia*, *E. cloacae*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Serratia fonticola* and *Shigella* spp. were identified.

2.9 Microbial quality of water in Nepal

Jayana, *et al*. (2009) studied one hundred and five drinking water samples from Madhyapur thimi, Kathmandu and found total coliform in 64.76% of samples crossed the WHO guideline values. Well water and stone spout contained high numbers of coliforms (96% and 80% respectively) followed by tap water samples in which only 28.88% water samples contained coliform. A total of 142 enteric bacteria of 11 different types were isolated. Sourcewise distribution revealed that 106 (74.64%), 11 (7.74%), and 25 (17.6%) of isolates from well, tap and stonespout water samples respectively. The organisms identified include *Escherichia coli, Enterobacter* spp., *Citrobacter* spp., *P. vulgaries, P mirabilis., Pseudomonas aeruginosa, S.* Typhi, *S.* Paratyphi, *Shigella dysentery*, and *Vibrio cholerae*. *Enterobacter* spp. (29.5%) was 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%).

Prasai, *et al*. (2007) studied a total of 132 drinking water samples randomly collected from 49 tube wells, 57 wells, 17 taps and 9 stone spouts in different places of Kathmandu valley. The samples were analyzed for microbiological parameters. Total plate and coliform count revealed that 82.6% and 92.4% of drinking water samples

found to cross the WHO guideline value for drinking water. During the study, 238 isolates of enteric bacteria were identified, of which 26.4% were *Escherichia coli*, 25.6% were *Enterobacter* spp, 23% were *Citrobacter* spp, 6.3% were *Pseudomonas aeruginosa,* 5.4% were *Klebsiella* spp., 4.0% were *Shigella* spp., 3.0% were *Salmonella* Typhi, 3.0% were *Proteus vulgaris,* 3.0% were *Serratia* spp. and 1.0% were *Vibrio cholerae.*

Chaudhary, *et al*. (2011) reported higher number of stone spouts water samples 85% to be contaminated with coliforms followed by well water 79.2% than tap water 60.7% in different locations in Kathmandu valley. Similarly, the thermotolerant coliform growth was found in higher number of well water samples 87.5%, followed by stone spouts water 71.4% and tap water 67.9%. 97 different isolates of organisms were identified as *Escherichia coli* 32%, *Citrobacter* spp. 24.7%, *Enterobacter* spp. 21.6% and *Klebsiella* spp. 18.6% and *Salmonella* Typhi 3.1%.

Pant (2011) undertook a study to assess the quality of groundwaters in the Kathmandu Valley, Nepal. The groundwater samples were randomly collected from shallow well, tube well, and deep- tube wells located at different places of Kathmandu, Lalitpur, and Bhaktapur districts in the Kathmandu valley. Physical, chemical, and microbiological parameters of the samples were evaluated to estimate the groundwater quality for drinking water. It was found that the groundwater in the valley was vulnerable to drink due to presence of coliform bacteria. Total coliform bacteria enumerated in groundwater significantly exceeded the drinking water quality standard and observed maximum coliform (267 CFU/100 mL) in shallow wells.

Rai, *et al*. (2012) conducted a large scale study on contamination of drinking water covering all three ecological belts in all five development regions of Nepal during 2009-2011. Of the total 506 water samples studied, one-forth (25.1%; 127/506) were visually turbid. Bacteriologically, 88.5% samples were positive for total coliform whereas 56.5% were positive for fecal coliform (*E. coli*). Of the total 506, 335 were piped tap water, 129 were boring water, 16 natural tap (spout), 16 were well (shallow/deep well) and 10 were mineral/uroguard treated water. Total coliform positive rate was very high (81.2% to 100%) in different type water samples (piped tap: 90.1%; boring water: 85.2%; natural spout/tap: 81.2%; well water 100% and mineral water/uroguard treated water: 80%). Fecal coliform positive rate ranged from 0% in mineral water/uroguard treated water to 93.7% in well water samples.

Shrestha, *et al*. (2014) conducted a study of groundwater samples from dug wells that were collected during the dry and wet seasons from 2009 to 2012, and *Escherichia coli* and total coliform were analysed. Three wells were monitored each month for a year. Microbial concentrations in shallow groundwater were significantly higher during the wet season than during the dry season. *E. coli* concentrations in different seasons indicated that a high level of fecal material infiltration during the rainy season may have caused the seasonal variations in microbial quality. A moderate to strong relationship between *E. coli* concentrations and groundwater level suggested that the rise in groundwater levels during the wet season may be another reason for this variation.

Maharjan, *et al*. (2018) collected the treated drinking water from all over the Kathmandu valley and analyzed in terms of physicochemical and microbiological parameters over the period of one year from July 2017 to July 2018. The study found 66% of the water samples crossed the guideline value for total coliform count. Above 92% of jar water samples, 77% of tanker water samples and 69% of filtered water samples had the total coliform count exceeding the NDWQS. Moreover, 20% of bottled water was contaminated by coliform bacteria.

2.10 Methods for microbial analysis of water quality

2.10.1 Most Probable Number (MPN) method

The multiple tube fermentation method is a three stage procedure in which the results are statistically expressed in terms of Most Probable Number (MPN). A three tube set or a five tube set or a ten tube set is used for the three stages: Presumptive test, Confirmed test and Completed test (APHA, 1998).

2.9.1.1 Presumptive test

A series of lauryl tryptose broth primary fermentation tubes are inoculated with required quantities of sample to be tested and are then incubated at 35 ± 0.5°C for 24 hours. The tubes after incubation are examined for gas formation. If no gas is formed further incubation is done. At the end of 48 hours when examined any amount of gas formation is taken as a positive presumptive test.

2.10.1.2 Confirmed test

Brilliant green lactose bile broth (BGLB) for total coliform and EC broth for fecal coliform are inoculated with medium from all the primary fermentation tubes showing

gas formation. The inoculated BGLB tubes are then incubated for 48 hours at 35 ± 0.5°C and EC tubes are incubated at 44.5°C for 48 hours (Eijkman test). The formation of gas at any time indicates a positive confirmed test.

2.10.1.3 Completed test

The completed test is performed in all samples showing positive confirmed test. The plates of Eosin Methylene Blue (EMB) agar are streaked with the samples and are incubated for 24 hours at 35°C. One or more typical colonies are transferred to lauryl tryptose broth fermentation tube and nutrient agar slant. If gas formation occurs within 24-48 hours of incubation at 35°C the colonies are gram stained, spore stained and microscopically examined for the presence of coliform bacteria.

2.10.2 Membrane Filtration (MF) method

To assess the microbiological quality of water, other forms of fluid, and materials dissolved in the appropriate diluents, this procedure is commonly used in laboratories. Additionally, it can be used to trap organisms in the air stream. This method is very useful for counting the number of bacteria in a big sample with few viable cells (WHO,1997).

This approach involves passing a measured volume of the water sample through a membrane filter with pores just big enough to keep the indicator bacteria that need to be counted in place. Membrane filters can have reasonably precise pore sizes and are made of cellulose nitrate or cellulose acetate. The typical pore size is 0.45 micrometers, which can hold both yeast and bacterial cells. The result can be attained in a period of 24-48 hours. Suction is used to finish the filtering process in a particular filter apparatus (APHA, 1998). The membrane filter is then placed in a petridish with an appropriate selective culture medium (Eosin Methylene Blue agar) and incubated to 35°C±0.5°C and 44°C±0.2°C. After 24 to 48 hours, colonies of the bacteria that the culture media favors will grow and can be counted. Colony forming units (CFU) per 100 mL are used to express the results (APHA, 1998; Aneja, 2018 and Cheesbrough, 2006).

The method is not appropriate for natural water that has extremely high concentrations of suspended matter, sludges, and sediments because these materials could clog the filter before enough water has flowed through it. When small quantities of samples need to be tested, it is necessary to dilute the sample in sterile diluent (UNEP/WHO, 1996).

2.10.3 Presence-absence test

A modification of the multiple procedures with a low probability of positive outcomes is the Presence-absence test for the coliform group. It is a qualitative test that is supported by the idea that 100 mL of water samples shouldn't contain any coliform. These tests should not be used to analyze surface water, untreated small community supplies, or larger water supplies in countries where there may occasionally be problems with operation and maintenance (WHO, 1997 and APHA, 1998).

This technique involves vigorously shaking the sample water before inoculating 100 mL to a Presence-Absence (P-A) culture bottle containing triple-strength sterile P-A broth. To ensure that the medium is evenly distributed throughout the sample, the mixture is mixed thoroughly by inverting the bottle, and it is then incubated at 35°C±0.5°C. A prominent yellow color in the medium indicates a positive presumptive test (acid generation) after an incubation period of 24 to 48 hours requiring confirmation. The positive cultures are transferred to Brilliant Green Lactose Bile (BGLB) broth and incubated at 35°C±0.5°C for the confirmed test. The presence of coliform is confirmed by gas production in the BGLB broth culture after 48 hours. The results are reported as Presence-Absence test positive or negative for total coliforms in 100 mL of water samples (APHA, 1998).

2.11 Water treatment

Protecting customers from pathogens and pollutants in the water that could be undesirable or harmful to human health is the primary goal of water treatment. Filtration, ultrafiltration, reverse osmosis, sedimentation, flocculation, adsorption, ion-exchange, addition of conditioning agents or additives, degasification, oxidation, and disinfection are the main water treatment techniques used in practices (WHO, 2017).

Chlorination is mostly used to disinfect water from microbes. Chlorine can, however, also operate as an oxidant and can help eliminate or chemically convert some compounds. Ozone is typically used in conjunction with a further treatment, such as biological filtering to remove biodegradable organics, followed by a chlorine residual, as ozone does not give a disinfection residual, to prevent undesired bacterial development in distribution. Many different insecticides and other organic compounds can be degraded using ozone (WHO, 2022).

Rapid gravity, horizontal, preset, or slow sand filters can all be used to filter out particulate matter from raw waters. The other procedures of treatment are primarily physical, whereas slow sand filtering is largely a biological process. When maintaining head is required, pressure filters may be utilized to replace the need for pumping into the supply. A cylindrical shell encloses the filter bed. Glass-reinforced plastics are capable of being used to create small pressure filters with a treatment capacity of up to 15 m^3/h. Up to 4 m in diameter, larger pressure filters are made of steel that has been specifically coated. The solids are concentrated in the top layers of the bed as a result of the water's downward movement. Water that has been treated is collected via filter floor nozzles. Periodically, the accumulated solids are eliminated by backwashing with treated water, which is occasionally followed by air scouring of the sand. The result is a diluted sludge that needs to be disposed off. Dual-media or multimedia filters are used in addition to single-medium sand filters. When water travels through one of these filters, the structure changes from coarse to fine due to the incorporation of various materials. To keep the different layers separated after backwashing, materials with the right density are utilized (WHO, 2022).

Households in the Kathmandu Valley typically utilize the following treatment techniques: boiling, filtration, using chemical tablets, and combinations thereof. In the Kathmandu Valley, at least one water treatment technology is used by three out of every four houses (Valley, *et al*., 2015).

2.12 Antibiotic resistance

The treatment of infectious disorders has greatly improved since the 1940s, when penicillin and numerous other antimicrobials were discovered. Despite this significant accomplishment, the uncontrolled use of antibiotics has inevitably led to the development of resistance, and as a result, diseases and disease agents are now emerging in new forms resistant to antibiotic treatments, making the present medications used for treatment worthless. A bacterium is termed multiple antibiotic resistant (MAR) if it is found to be resistant to three or more antimicrobials (Titilawo, *et al*., 2015).

First-line antibiotics like Ampicillin, Tetracyclines, Chloramphenicol, and Sulphonamides have shown an increase in resistance in recent years. In addition, bacillary dysentery caused by *Shigella dysenteriae* 1, meningitis brought on by *Haemophilus influenzae* type b strains, and enteric fever caused by *Salmonella*

Typhi are all experiencing an increase in antibiotic resistance. Developing countries are often unable to afford costly second-line antibiotics to treat infections due to resistant organisms. This results in prolonged illness with longer periods of infectivity and the further spread of resistant strains (Cheesbrough, 2006).

2.12.1 Antibiotic resistance bacteria in water sources

Eight antibiotics were tested by Shrestha (2008) against 33 isolates from drinking water of Kathmandu. Only two strains showed partial susceptibility to Ampicillin, while the majority of isolates (93.9 %) were resistant to it. The following antibiotics were reported to have the highest levels of resistance among isolates: Ampicillin (93.93%), Tetracycline (27.27%), Nitrofurantoin (24.24%), Co-trimoxazole (18.18%), and Nalidixic acid (15.15%). While all *E. coli* isolates demonstrated susceptibility to Ciprofloxacin and Norfloxacin, they all demonstrated resistance to Ampicillin. *E. coli* had a multidrug resistance pattern in 28.5% of the cases.

Jayana (2009) tested ten antibiotics against the 142 total isolates of Kathmandu and reported the maximum resistannce towards Erythromycin (79.5%) and Penicillin G (62.67%). All the isolates were resistant to at least one antibiotic. *P aeruginosa* was found resistant to almost all antibiotics used. Of the total isolates, resistance towards Ampicillin (34.5%), Ofloxacin (5.6%), Chloramphenicol (5.6%), Amoxycillin (61.9%), Cephotaxime (41.7%), Amikacin (14.7%), Ceftriaxone (15.4%) and Tetracycline (21.1%) was shown.

Chaudhary, *et al*. (2011) reported higher Multiple Drug Resistance (MDR) isolates were *E. coli* 92.3% (n=28), *Citrobacter* spp. 62.5% (n=15), *Klebsiella* spp. 72.2% (n=13), *Enterobacter* spp. 47.6% (n=10) and *Salmonella* spp. 100% (n=3). The most of MDR were attributed to resistance towards Tetracycline and Amoxicillin.

2.13 Antibiotic susceptibility testing

In the treatment and control of infectious diseases, especially when caused by pathogens that are often drug resistant, susceptibility testing is used to select effective antimicrobial drugs. Susceptibility testing is not usually indicated when the susceptibility reactions of a pathogen can be predicted (Cheesbrough, 2006).

The pathogen, the specimen, the variety of locally accessible antimicrobials and local prescribing guidelines will all influence the antimicrobials that will be used in susceptibility tests. It is necessary for laboratory, medical, and pharmacy staff to consult

with one another. The variety of first-choice medications should be restricted and periodically reviewed. Only on special request may additional medications be added. Only one representative from each group of connected antimicrobials needs to be chosen in cases where there is cross-resistance (Cheesbrough, 2006).

Laboratory antimicrobial susceptibility testing can be performed using a dilution technique or disc diffusion technique.

2.13.1 Modified Kirby-Bauer susceptibility testing technique

The identified isolate is taken for the antibiotic susceptibility test by Kirby-Bauer disc diffusion. The inoculum is prepared by suspending the isolated organism into 2 mL of nutrient broth and the turbidity of this inoculum is adjusted to 0.5 McFarland standards. The inoculum is prepared on Mueller Hinton agar (MHA) media with sterile cotton swab. The antibiotic disc is placed on the plate and incubated at 35° C for 16-18 hours. The zones of inhibition (mm) is then measured after incubation. The antibiotic susceptibility is interpreted based on CLSI guidelines (CLSI, 2018 and Cheesbrough, 2006).

2.13.2 Stokes disc diffusion technique

The test and control organisms are injected on the same plate using this disc approach. Zone sizes of the test and control organisms are clearly compared. When the precise amount of antimicrobial in a disc cannot be guaranteed due to difficulties obtaining discs and storing them correctly or when the other conditions required for the Kirby-Bauer technique cannot be met, laboratories use this method, which is less highly standardized than the Kirby-Bauer technique. Applying highly stable Rosco Diagnostica antibiotic tablets (Neo-Sensitabs) rather than less stable paper discs in developing nations the Stokes technique is performed (Cheesbrough, 2006).

2.13.3 Etest

Using the Etest approach, unusual resistance profiles can be confirmed. Etest is a quantitative method for determining out the Minimum Inhibitory Concentration (MIC) of antibiotics against bacteria and detecting resistance mechanisms. It consists of a plastic strip with a predefined gradient of antibiotic concentration for a particular antibiotic.

The gradient of antimicrobial agent is immediately transferred to the medium when the Etest strip is placed on an inoculated agar surface, such as Mueller Hinton. A symmetrical inhibitory elipse, or zone with an elliptical shape, is created after an overnight or extended incubation period. At the point where the inhibitory elipse edge contacts the strip, the MIC is directly read from the scale in micro grams per milliliter (µg/mL) (Cheesbrough, 2006).

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Materials

All the materials including glass wares, chemicals and bacteriological media are given in **Appendix D**.

3.2 Study area

Collection of underground water samples was done from the core area of Kathmandu Metropolitan city- Makhan, Bangemuda, Ason, Jamal, Jytha, Thamel, Nardevi and Chetrapati.

3.3 Methodology

3.3.1 Preliminary survey

Site survey was done for the implementation of the project work to analyze the groundwater quality and treatment facilities available. During the survey, underground water sources were observed and sampling points were determined in coordination with residents of Kathmandu Metropolitan city.

3.3.2 Type of study

The type of this study was cross-sectional.

3.3.3 Duration

All the preliminary survey, questionnaire survey, water sampling, data collection and laboratory analysis of underground water samples were performed during March 2022 to May 2022.

3.3.4 Sampling site

The random sampling technique was done for sampling of underground water. The water samples were collected from different underground water sources-Deep tubewells (2), Hand pumps (2) and Tubewells (32).

3.3.5 Sampling size

A total of 36 underground water samples were collected among which 32 were untreated and 4 were treated underground water samples. Both treated and untreated water samples were collected from the places where treatment facility was available.

3.3.6 Data source and data collection technique

The study was carried out based on primary source of data. The questionnaire survey, and laboratory analysis of water samples were used to collect the primary data for the study. However, the secondary sources of data such as population census and publications related to water consumption and sanitation were also collected.

3.3.6.1 Questionnaire survey

Questionnaire survey was conducted directly on-site by communicating with the local residents using underground water in the study area of Kathmandu Metropolitan city. This survey was designed to collect the data and gather information regarding drinking water supply, consumption of underground water sources for household activities, water treatment methods, waterborne diseases, water disinfection method, hygiene and sanitation and hand washing practices of locals via pre-structured questionnaire (**Appendix B**).

3.3.6.2 Water quality analysis

The study of water quality analyzed in laboratory was performed according to the APHA (1998). The Membrane Filtration (MF) technique was used to isolate,enumerate and identify the total coliform, fecal coliform and other bacteria in the laboratory of Amrit Campus, Thamel. In addition to this, bacterial isolates were identified according to Forbes, *et al.* (2007) and Cheesbrough (2006).

- **Method of sample collection**

For the bacteriological analysis of water samples were collected in 500 mL sterilized poly-reagent plastic bottles containing 0.5 mL of 3% sodium thiosulphate. During filling the sample was 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. Then the cap was replaced tightly and labelled with sample code, sampling time and date. Sampling was done following methods by APHA (1998).

- **Sample transportation**

The water samples were transported in an ice-cold box for the laboratory analysis to the Department of Microbiology, Amrit Campus, Thamel. The samples were processed in laboratory within 6 hours (APHA, 1998).

- **Isolation and enumeration of coliform**

Water samples processing for enumeration of total coliform and fecal coliform was performed using Membrane Filtration (MF) technique (**Appendix-H**) in laboratory of Microbiology Department, Amrit Campus. For isolation and enumeration of coliform, 100 mL of water samples (undiluted and diluted) were vacuum filtered through a 0.45 µm membrane filter paper, which was then placed on Eosin Methylene Blue (EMB) agar plate to suppress the growth of unwanted organisms and promote the growth of coliforms. The agar plate was then incubated at 35°C±0.5°C and 44°C±0.2°C for 24-48 hours for total coliform and fecal coliform respectively (APHA, 1998 and Hi-Media, 2021).

3.3.6.3 Identification of bacterial isolates

For the identification of bacterial isolates from water samples, Gram staining and various biochemical tests Catalase test, Oxidase test, Oxidation Fermentation (OF) test, Sulphide Indole Motility (SIM) test, Methyl Red (MR) test, Voges- Proskauer (VP) test, Citrate utilization test, Triple Sugar Iron Agar (TSIA) test and Urea hydrolysis test were performed (APHA, 1998, Cheesbrough, 2006 and Forbes, *et al*., 2007).

3.3.6.4 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 was prepared on Mueller Hinton Agar (MHA) and different antibiotic discs- Amikacin (30 mcg), Ampicillin (10 mcg), Ceftriaxone (30 mcg), Chloramphenicol (30 mcg), Ciprofloxacin (5 mcg), Cotrimoxazole (25 mcg), Gentamycin (10 mcg), Nalidixic acid (30 mcg), Nitrofurantoin (300 mcg) and Tetracycline (30 mcg) were placed on MHA and the plate was incubated at 35±2°C for 16-18 hours. The zone of inhibition was measured after incubation and results were interpreted based upon CLSI 2021 guidelines (**Appendix-H**).

3.3.7 Quality control

For water quality analysis all the equipments were caliberated. Laboratory analysis of water quality, identification of bacterial isolates from water and antibiotic susceptibility testing of bacterial isolates were carried out according to standard methods of APHA (1998), methods described in Forbes, *et al*. (2007) and Cheesbrough (2006). An *E. coli* ATCC 25922 was used as reference culture.

3.3.8 Data analysis

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

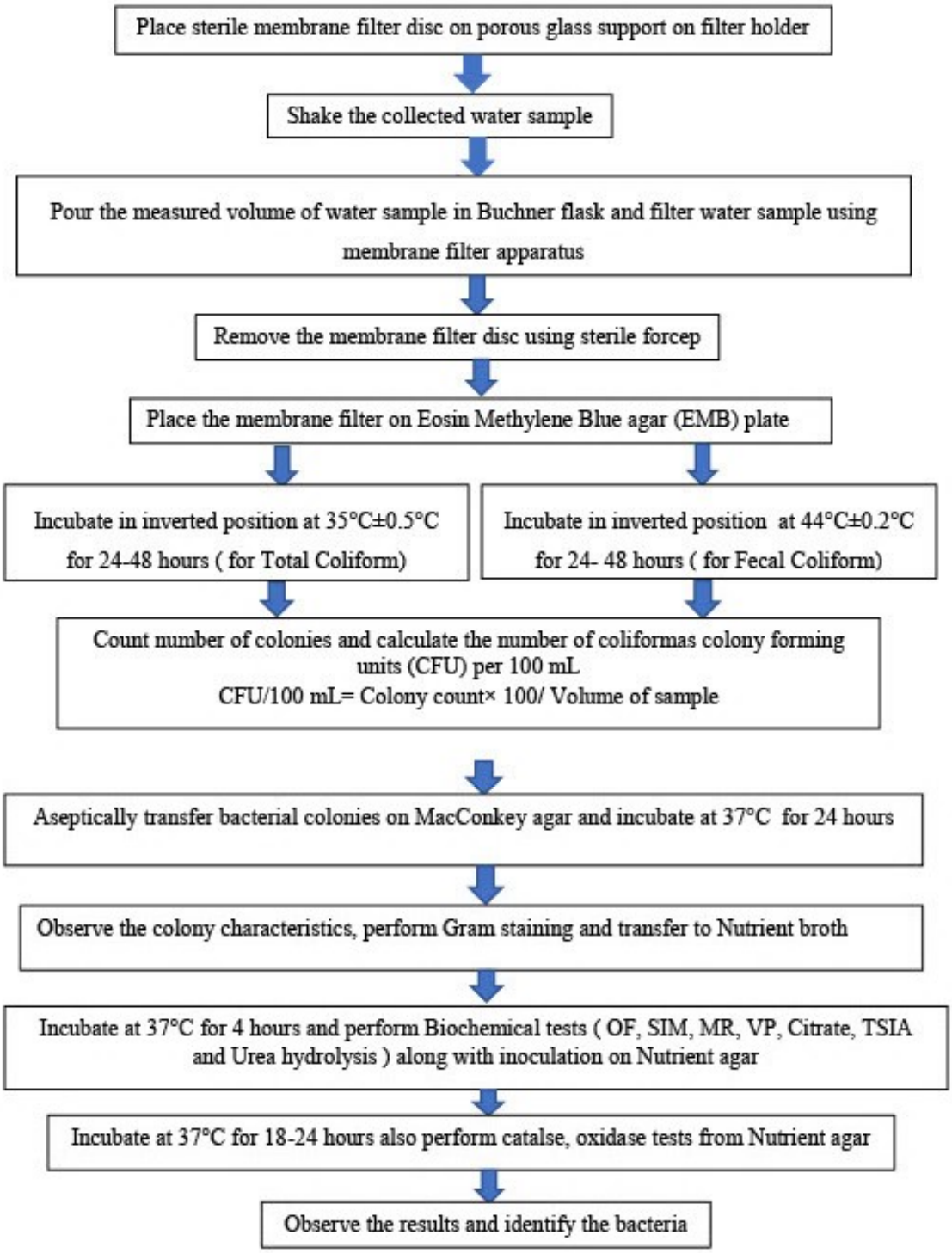


Figure 1: Flow Diagram of Membrane Filtration Technique for Water Quality Analysis and Identification of Bacteria Isolated from Water (APHA, 1998; Cheesbrough, 2006 and Forbes, *et al.*, 2007).

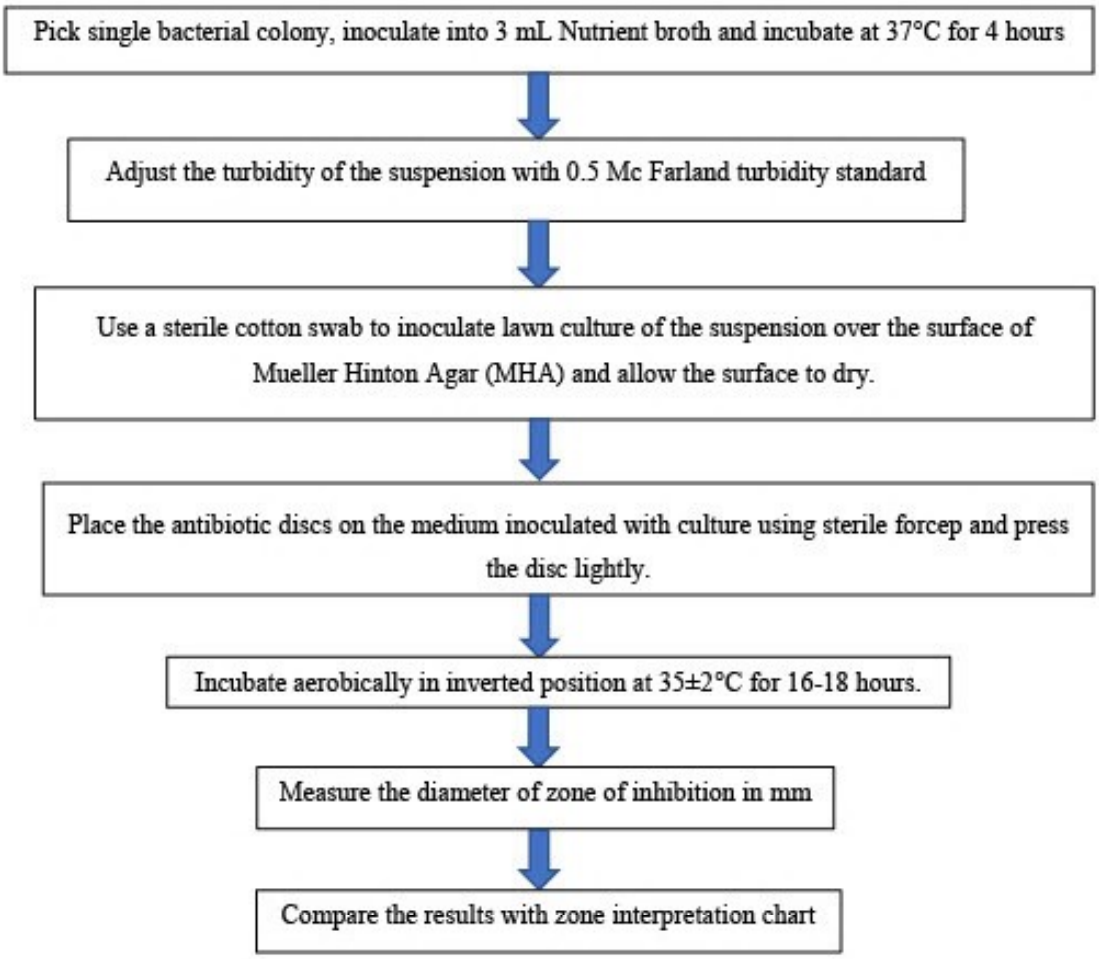


Figure 2: Flow Diagram of Antibiotic Susceptibility Testing of *Escherichia coli* (CLSI, 2021).

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1 Result

4.1.1 Result of questionnaire survey on water quality and sanitation

According to questionnaire survey done among 75 respondents during collection of underground water samples for laboratory analysis, maximum numbers of people were aware of basic sanitation practices.

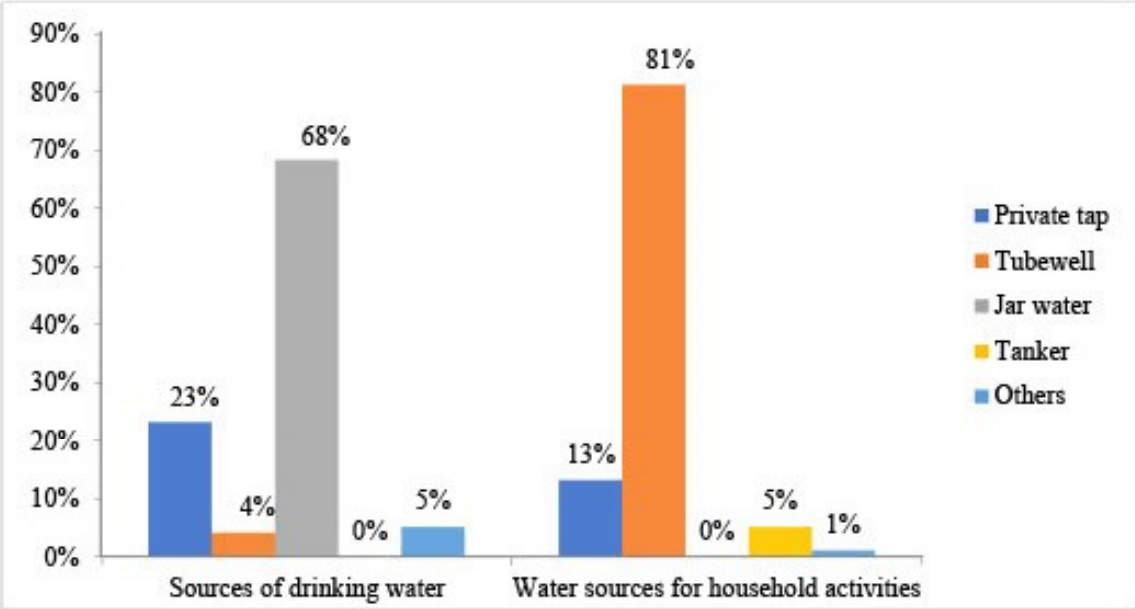


Figure 3: Sources of water for drinking and household activities

For drinking purpose majority of the respondents (68%) used jar water, 23% used private tap water, 4% used tubewell water and 5% used water from other sources while 81% of the respondents used tubewell water, 13% used private tap water, 5% used tanker water and 1% used water from other sources for household activities.

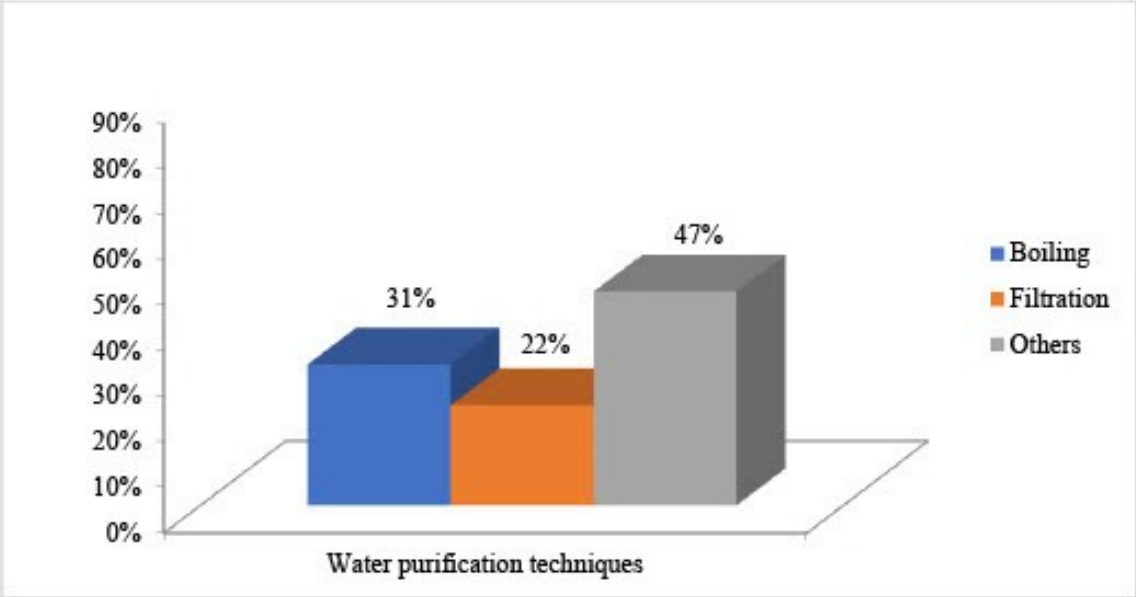


Figure 4: Basic techniques used for purification of water before drinking

In the survey, 31% of the respondents used boiling method, 22% used filtration and 47% used other techniques for purification of water.

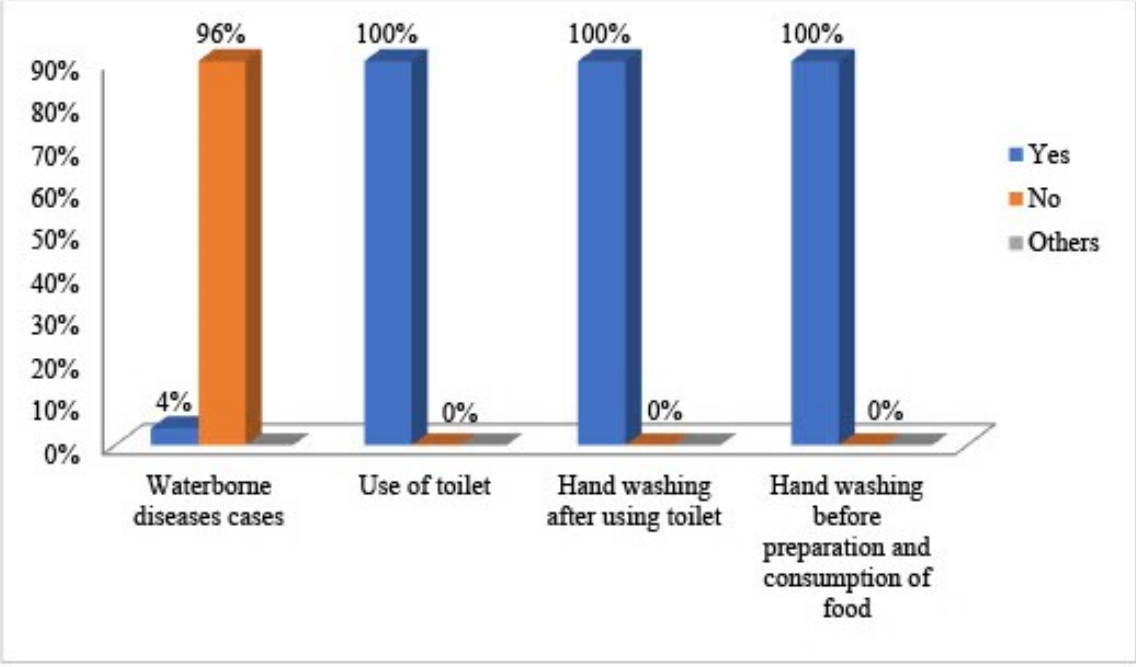


Figure 5: Waterborne diseases, sanitation and hygiene practice

Approximately 96% of the respondents did not suffer from any waterborne diseases within a year whereas 4% suffered from some of the waterborne diseases. In the survey 100% of the respondents were found to use toilets regularly. All (100%) of the respondents were found to wash hands with soap and water after using toilet. When

asked about the hand washing habit before preparing and after consuming food, 100% of the respondents were found to follow it.

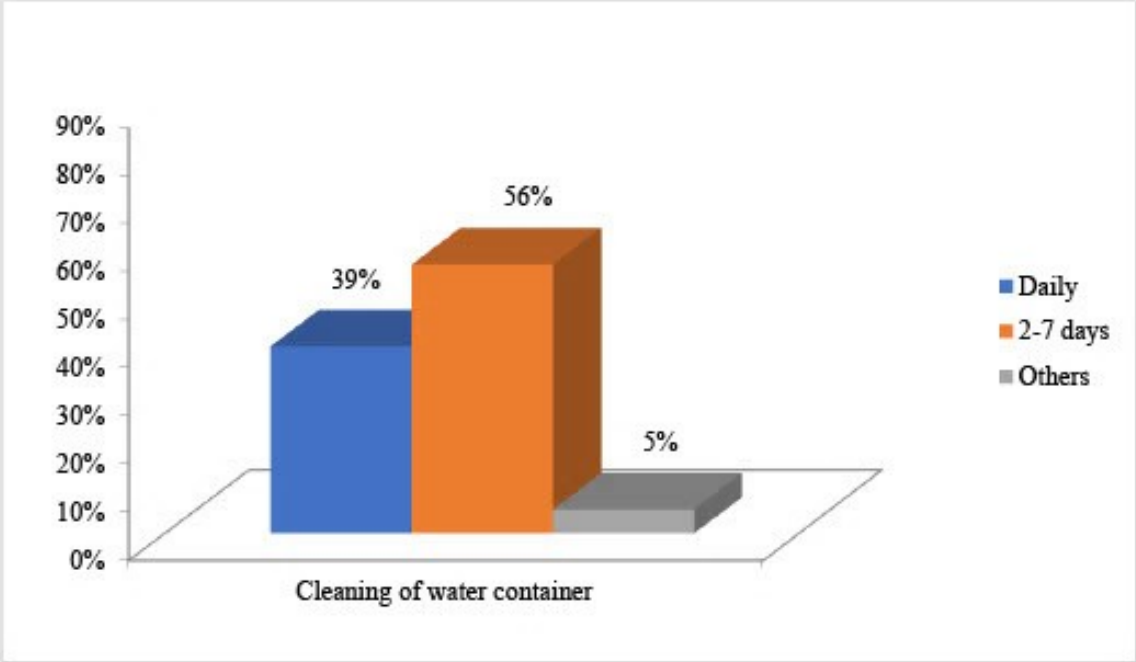


Figure 6: Frequency of cleaning water collecting container

When asked about the cleaning interval of water collecting containers 39% of the respondents cleaned daily, 56% cleaned in 2 to 7 days and 5% cleaned in other frequency.

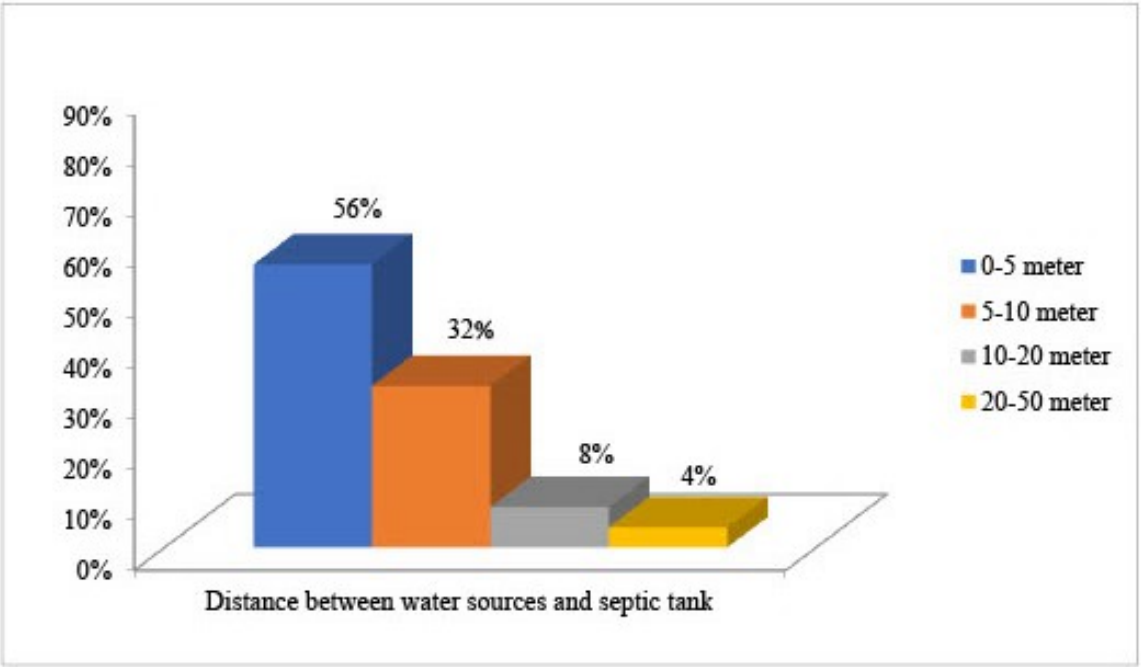


Figure 7: Distance between underground water sources and septic tank

According to the the 56% of the respondents the distance between underground water sources and septic tank was 0-5 meter whereas it was at 5-10 meter distance as said by 32% respondents. 8% of them said the distance was 10-20 meter and it was found at 20-50 meter by 4% respondents.

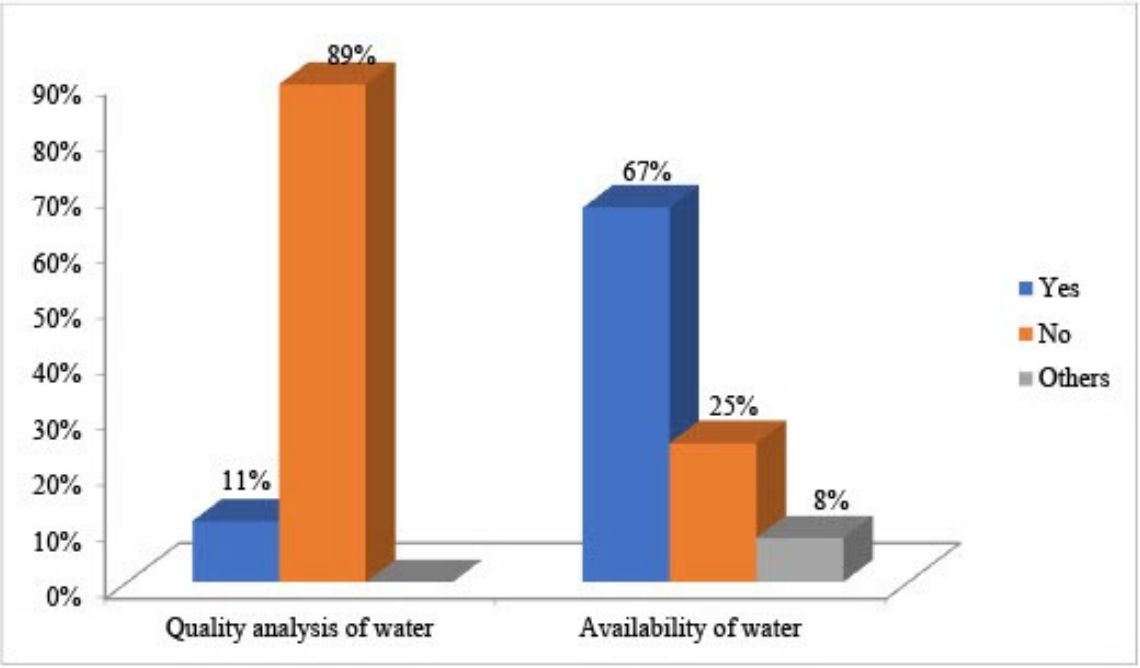


Figure 8: Water quality analysis and availability of water for domestic purposes

Majority (89%) of the respondents were not concerned about the practice of water quality testing. Only 11% of them had tested the quality of water. Sufficient amount of water was available to 67% of the respondents. 25% of them did not get required amount of water whereas in some season it is sufficient but in the other it is not as per 8% respondents.

4.1.2 Distribution of the underground water sources in Kathmandu

Out of 36 underground water samples collected from 8 different locations of Kathmandu- Makhan, Bangemuda, Ason, Jamal, Jytha, Thamel, Nardevi and Chetrapati, 32 were from untreated underground water and 4 were treated underground water. Among the 32 underground water sources, 12.5% (n=4) of underground water sources were found to have treatment facility whereas 87.5% (n=28) of underground water sources were used without any filtration and disinfection.

Table 2: Distribution of treated and untreated underground water sources in Kathmandu

Locations	Number of Samples (n=36)			
	Untreated Underground Water source		Treated Underground Water source	
	Nos	%	Nos	%
Makhan	4	100	0	0
Bangemuda	4	100	0	0
Ason	4	100	0	0
Jamal	4	80	1	20
Jytha	4	66.7	2	33.3
Thamel	4	80	1	20
Nardevi	4	100	0	0
Chetrapati	4	100	0	0
Total (N)	32	87.5	4	12.5

4.1.3 Enumeration of coliform from underground water in Kathmandu

Out of 32 underground water samples (untreated) collected from 8 different locations, 15.6% (n=5) of water samples were found to be within WHO guideline value of 0 CFU/100 mL for total coliform and 75% (n=24) of water samples were found to be within WHOguideline of 0 CFU/100 mL for fecal coliform whereas 84.4% (n=27) of water samples were found to be contaminated with total coliform and 25% (n=8) of water samples were found to be contaminated with fecal coliform exceeding WHO guideline value of 0 CFU/100 mL as shown in **Table 3**.

Table 3: Coliform count of untreated underground water samples

Coliform Count	Number of 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	32	5	15.6	27	84.4
Fecal Coliform Count	32	24	75	8	25

Among 4 treated underground water samples collected from 3 different locations of Kathmandu, 100% (n=4) of water samples were found to be contaminated with total

coliform which exceeded the WHO guideline value (0 CFU/100 mL) and none were found to be contaminated with fecal coliform as shown in **Table 4**.

Table 4: Coliform count of treated underground water samples

Coliform Count	Number of 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	4	0	0	4	100
Fecal Coliform Count	4	4	100	0	0

The bacteriological quality of the untreated underground water samples (n=32) on enumeration revealed that maximun count of total coliform was 915 CFU/100 mL, minimum total coliform count was 0 CFU/100 mL. The maximum fecal coliform count was 665 CFU/100 mL and the minimum fecal coliform count was 0 CFU/100 mL.

Table 5: Maximum and minimum coliform count from untreated underground water samples (n=32)

Coliform Count	Total Coliform Count (CFU/100 mL) (n=32)	Fecal Coliform Count (CFU/100 mL) (n=32)
Maximum	915	665
Minimum	0	0

Among 4 treated underground water samples all were contaminated with total coliform as shown in **Table 6** below. The maximum total coliform count was 54 CFU/100 mL and minimum total coliform count was 3 CFU/100 mL.

Table 6: Maximum and minimum coliform count from treated underground water samples (n=4)

Coliform Count	Total Coliform Count (CFU/100 mL) (n=4)	Fecal Coliform Count (CFU/100 mL) (n=4)
Maximum	54	0
Minimum	3	0

4.1.4 Distribution of water samples contaminated with coliform in different locations of Kathmandu

The distribution of untreated underground water samples contaminated with coliform in different locations of Kathmandu is shown in the **Table** 7. Among 32 untreated underground water samples from different locations of Kathmandu 100% water samples

in each Makhan (n=4), Jytha (n=4), Thamel (n=4), Nardevi (n=4) and Chetrapati (n=4) were found to be contaminated with total coliform whereas 75%, 50% and 50% of water samples from Jamal (n=4), Bangemuda (n=4) and Ason (n=4) were found to be contaminated with total coliform. The water samples from Jytha and Chetrapati were not contaminated with fecal coliform whereas 50% of water samples from Makhan, 50% from Nardevi and 25% of water samples from Bangemuda, 25% from Ason, 25% from Jamal and 25% from Thamel were found to be contaminated with fecal coliform.

Table 7: Distribution of untreated underground water samples contaminated with coliform in different locations of Kathmandu

Locations	Number of Samples (n)	Total Coliform Positive Sample		Fecal Coliform Positive Sample	
		Nos	%	Nos	%
Makhan	4	4	100	2	50
Bangemuda	4	2	50	1	25
Ason	4	2	50	1	25
Jamal	4	3	75	1	25
Jytha	4	4	100	0	0
Thamel	4	4	100	1	25
Nardevi	4	4	100	2	50
Chetrapati	4	4	100	0	0
Total (N)	32	27	84.3	8	25

All 100% underground treated water samples from Jamal (n=1), Jytha (n=2) and Thamel (n=1) were found to be contaminated with total coliform whereas the fecal coliform was zero.

Table 8: Distribution of treated underground water samples contaminated with coliform in different locations of Kathmandu

Locations	Number of Samples (n)	Total Coliform Positive Sample		Fecal Coliform Positive Sample	
		Nos	%	Nos	%
Jamal	1	1	100	0	0
Jytha	2	2	100	0	0
Thamel	1	1	100	0	0
Total (N)	4	4	100	0	0

4.1.5 Identification of bacterial isolated from underground water

In this study, total coliform and fecal coliform from underground water samples were isolated and identified. Of 75 isolates of bacteria obtained at 35°C±0.5°C from underground water, *E. coli* (37.3%), *Klebsiella* spp. (32%), *Enterobacter* spp. (9.3%), *Citrobacter* spp. (2.7%), *Salmonella* spp. (6.7%), *Pseudomonas* spp. (2.7%), *Proteus* spp. (25%) Among 16 isolates of thermotolerant bacteria, 50% of isolates were *E. coli*, *Klebsiella* spp. (25%), *Enterobacter* spp. (6.3%), and *Salmonella* spp. (18.7%) were identified.

Table 9: Identification of bacteria isolated from underground water sources

Bacteria	Bacteria isolated at 35°C±0.5°C		Bacteria isolated at 44°C±0.2°C	
	Nos	%	Nos	%
Escherichia coli	28	37.3	8	50
Klebsiella spp.	24	32	4	25
Enterobacter spp.	7	9.3	1	6.3
Citrobacter spp.	2	2.7	-	-
Salmonella spp.	5	6.7	3	18.7
Pseudomonas spp.	2	2.7	-	-
Proteus spp.	3	4	-	-
Gram negative rods (Unidentified)	4	5.3	-	-
Total (N)	75	100	16	100

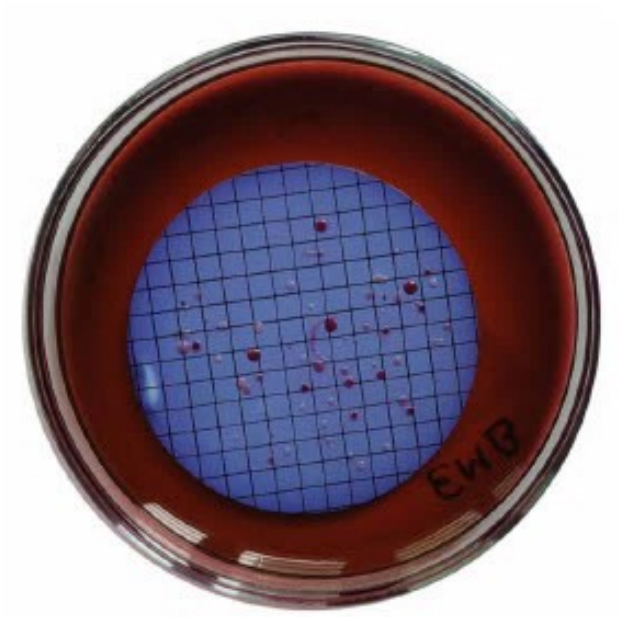
4.1.6 Antibiotic susceptibility pattern of *E. coli* isolates from underground water

As given in **Table 10**, among the 28 isolated *E. coli*, 100 % sensitivity was found to the antibiotics Cotrimoxazole, Gentamycin and Amikacin. 96.4 % isolates were susceptible towards Tetracycline followed by 89.3% towards Chloramphenicol, 78.6 % towards Nalidixic acid, 75% towards Ceftriaxone, 39.3% towards Ciprofloxacin, 35.7% towards Ampicillin and 25% towards Nitrofurantoin. Altogether 53.6% of *E.coli* were resistant to Nitrofurantoin, 60.7% to Ampicillin followed by 25% to Ciprofloxacin, 17.9% to Ceftriaxone, 10.7% to Nalidixic acid and 3.6% to both Chloramphenicol and Tetracycline.Among 8 thermotolerant *E. coli*, resistant was seen towards Nitrofurantoin (25%) followed by 12.5% towards Ampicillin, Chloramphenicol (12.5%), Ceftriaxone (12.5%) and Tetracycline (12.5%) whereas all isolates were sensitive towards Nalidixic acid, Ciprofloxacin, Cotrimoxazole, Gentamycin and Amikacin. Multiple antibiotic resistance (MAR) was detected in 21.4% of *E. coli* at 37°C and also in 12.5% of thermotolerant *E. coli*.

Table 10: Antibiotic susceptibility pattern of *Escherichia coli* and thermotolerant *E. coli*

Antibiotics	*Escherichia coli* (n=28)						Thermotolerant *E. coli* (n=8)					
	S		I		R		S		I		R	
	N	%	N	%	N	%	N	%	N	%	N	%
Amikacin (30 mcg)	28	100	0	0	0	0	8	100	0	0	0	0
Ampicillin (10 mcg)	10	35.7	1	3.6	17	60.7	5	62.5	2	25	1	12.5
Ceftriaxone (30 mcg)	21	75	2	7.1	5	17.9	4	50	3	37.5	1	12.5
Chloramphenicol (30 mcg)	25	89.3	2	7.1	1	3.6	6	75	1	12.5	1	12.5
Ciprofloxacin (5 mcg)	11	39.3	10	35.7	7	25	8	100	0	0	0	0
Cotrimoxazole (25 mcg)	28	100	0	0	0	0	8	100	0	0	0	0
Gentamycin (10 mcg)	28	100	0	0	0	0	8	100	0	0	0	0
Nalidixic acid (30 mcg)	22	78.6	3	10.7	3	10.7	8	100	0	0	0	0
Nitrofurantoin (300 mcg)	7	25	6	21.4	15	53.6	6	75	0	0	2	25
Tetracycline (30 mcg)	27	96.4	0	0	1	3.6	7	87.5	0	0	1	12.5
Multiple Antibiotic Resistance (MAR)	-	-	-	-	6	21.4	-	-	-	-	1	12.5

LIST OF PHOTOGRAPHS



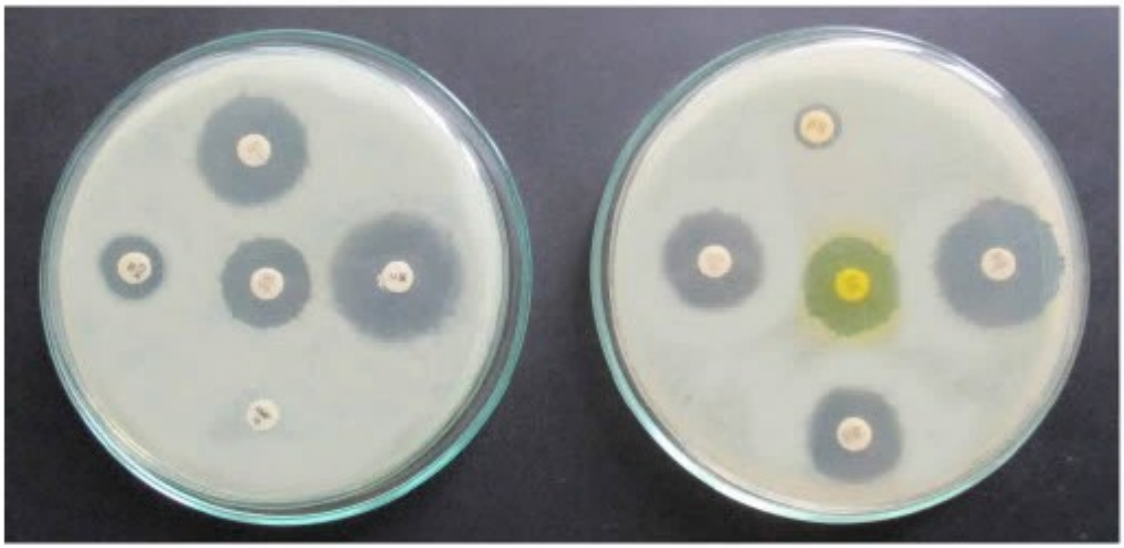
Photograph 1: Total Coliform on Eosin Methylene Blue agar plate

(Membrane Filtration Technique)

Photograph 2: *E. coli* on MacConkey agar plate

Photograph 3: Biochemical tests of *E. coli*

(OF test=Fermentative; Sulphide Indole Motility test=Sulphide Negative, Indole Positive and Motility Positive; Methyl Red test= Positive; Voges-Proskauer test=Negative; Citrate Utilization test=Negative; Triple Sugar Iron Agar test=Acid/Acid, H_2S Negative, Gas Positive; Urea Hydrolysis test=Negative; from left to right)



Photograph 4: Antibiotic susceptibility testing of *E. 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

Water is necessary for life, and everyone needs access to a sufficient (appropriate, safe) supply of it. Enhancing access to clean water for drinking can have real positive effects on health. Every effort should be made to get drinking water that is as safe as practicable. Although other sources and modes of exposure may also be important, drinking water that has been contaminated with human and animal excreta poses the greatest risk to the public's health from microbes (WHO, 2022).

Coliform in drinking water is seen as a potential concern or as an indication that the microbiological water quality is deteriorating. The total number of coliforms should not be detected in 100 mL of drinking water, according to WHO standards (2017) and NDWQS (2005). Most of the untreated and all the treated underground water samples used in this investigation had coliform contamination, making it unfit for consumption.

In this study, 84.4% untreated underground water samples showed contamination of total coliform that exceeded the WHO guideline value which was less compared to 100% reported by Diwakar, *et al.* (2008), 97.37% in tubewell water reported by Bajracharya, et al. (2007) and 94.7% in well water reported by Prasai, *et al.* (2007). 100% underground treated water were found to be contaminated with total coliform where Maharjan, *et al.* (2018) report on treated jar water in Kathmandu valley revealed that 92% of jar water, 77% of tanker water crossed the guideline value for total coliform count which might be due to the inadequate treatment or post treatment contamination. However, 15.6% and 75% of the untreated water samples were not contaminated with total and fecal coliform respectively which was higher compared to 4% of well water within the WHO guideline as reported by Jayana, *et al.* (2009).

In the present study, 25% untreated underground water was found to be contaminated with fecal coliform which was less than the contamination on 30% of samples from ground water of Kathmandu valley as reported by Ghimire, *et al.* (2013). However, this report showed less contamination of fecal coliform than Pandey and Shakya, (2012) who reported 97%. The treated underground water samples were within the WHO guideline value where previous study by Koju *et al.* (2014) reported 36% of treated water samples contained coliform bacteria indicating possible contamination of fecal origin.

The maximum total coliform count in untreated underground water samples was 915 CFU/100 mL while that in treated underground water was 54 CFU/100 mL. The maximum fecal coliform count in untreated underground water was 665 CFU/100 mL. The bacterial contamination of underground water is mostly due to watershed erosion and drainage from sewage and swamps. Due to space limitations, crowding, lack of proper drainage network and also unsanitary septic tank constructed near underground water sources in the study area, seepage from the unfiltered sewage might have polluted the underground water sources.

Among total coliform 37.3% of *E. coli* was identified followed by 32% *Klebsiella* spp., 9.3% *Enterobacter* spp., 2.7% *Citrobacter* spp., 6.7% *Salmonella* spp., 2.7% *Pseudomonas* spp. And 4% *Proteus* spp.. Likewise, from fecal coliform, 50% of the bacteria identified was *E. coli,* 25% was *Klebsiella* spp. and 6.3% was *Enterobacter* spp.. *E. coli* was found to be most predominant microorganism and apart from total and fecal coliform other microorganisms (*Salmonella* spp.) that are pathogenic to human were also detected in this study which is supported by various other studies done by Jayana, *et al*. (2009), Pant, *et al*. (2016), Chaudhary, *et al*. (2011) and Shakya, (2013).

Similar study evaluating different water resources conducted by many researchers reported the presence of coliform bacteria from water. Chaudhary, *et al*. (2011) isolated 32% of *E. coli*, 24.7% *Citrobacter* spp, 21.6% *Enterobacter* spp., 18.6% *Klebsiella* spp. and 3.1% *Salmonella* spp..Similarly, Jayana (2009) isolated 142 enteric bacteria of different types of which 11 different enteric bacteria were isolated. *Enterobacter* spp. was found most predominant which was 29.5% followed by 24.6% *E. coli*, 20.4% *Citrobacter* spp., 7% *P. vulgaris*, 5.6% *Klebsiella* spp., 3.5% *P. mirabilis*, 2.1% *Salmonella* Typhi, 2.1% *Pseudomonas aeruginosa* and 1.4% *Salmonella* Paratyphi.

The treatment of infectious diseases is seriously threatened by antibiotic resistance, which is also a major public health issue in the twenty-first century. Ground water has been found to be contaminated with antibiotic resistant *E. coli* in various studies. Antimicrobial resistance may be more prevalent in humans as a result of drinking water polluted with antibiotic-resistant microorganisms.

In this study, *E. coli* isolates were taken for antibiotic susceptibility testing against different antibiotics. Among the tested antibiotics, 60.7% of the isolates tested showed resistance towards Ampicillin while 53.6% of the isolates were resistant towards Nitrofurantoin and 25% of the isolates were resistant towards Ciprofloxacin. The

thermotolerant *E. coli* isolates were 100% sensitive towards Nalidixic acid, Ciprofloxacin, Cotrimoxazole, Gentamycin and Amikacin and 87.5% of the thermotolerant isolates were sensitive towards Tetracycline.

In the previous study performed by Jayana (2009) 34.5% of the isolates were resistant towards Ampicillin whereas 49.2% of the isolates were sensitive towards Tetracycline and 85.2% of the isolates were sensitive towards Amikacin which was less compared to the present study. Another study by Chaudhary, et al. (2011) showed that *E. coli* isolates were sensitive towards Chloramphenicol and Cotrimoxazole whereas 93.5% of the isolates were found resistant towards Tetracycline, followed by 25.8% of the isolates resistant towards both Nalidixic acid and Amikacin.

From this study 21.4% *E. coli* and 12.5% thermotolerant *E. coli* were found to be Multiple antibiotic resistance (MAR). In a study performed by Subba, *et al.*(2013) revealed 70.9% of *E. coli* and 75.8% of thermotolerant *E. coli* were Multiple drug resistant and prevalent in drinking waters which when compared to this result was found very high. Chaudhary, *et al.* (2011) reported 92.3% of *E. coli* isolates to be MAR which was also greater than the result of this study.

Bacterial strains that are resistant to antibiotics may evolve when bacteria are exposed to them repeatedly. Acquired resistance to β-lactam antibiotics can occur by three different mechanisms; inactivation of antibiotic, alteration of target site and reduced permeability. Resistant strains are also a result of the production of enzymes that metabolize or alter drugs as well as modifications to bacterial cell membranes that block the uptake of an antibiotic. Most β-lactam antibiotics like Ampicillin work by inhibiting cell wall biosynthesis in the bacterial organism and are the most widely used group of antibiotics. The possible cause of resistance of towards Ampicillin may be due to production of Beta lactamase. Beta Lactamase hydrolysis of penicilins form the corresponding penicilloic acid, which is antibacterially inactive (Denyer, et al., 2004).

Due to insufficient antibiotic therapy, serious infections may result in mortality. Major infectious diseases can spread and cause significant epidemics due to the decreasing efficacy of medications and the lack of substitute antimicrobials. Therefore, a correct selection of antimicrobial therapy depends on the results of an antibiotic susceptibility test.

According to questionnaire survey done in the study area, it was found that 23% of the people used private water taps, 4% of the people used tube well and 68 % used jar water for drinking purpose. According to respondents of this study 81% used tubewell water for household activities. MoHP (2014) report showed about 50 % received water distributed through pipes and 30 % received water from hand pumps. A study carried out by the CBS showed that 59 % of the surveyed households did not have adequate piped water supply (Uprety, 2017) whereas this study revealed 25% people not getting required amount of water. In this study, 31% people followed boiling, 22% followed filtration and 47% followed other techniques for purification of water. Similar study conducted by Gaire, *et al*. (2017) reported 74.5% of the people followed various water disinfecting practices such as boiling, filtration and chlorination in Kavrepalanchowk. Majority (56%) people cleaned the water collecting container in 2-7 days interval. The data from questionnaire survey revealed that 100% of respondents were aware of basic hygienic, sanitation practices whereas a report on water, sanitation and hygiene by Budhathoki (2019) revealed that Kathmandu had the 99 coverage of basic sanitation facility. 56% people had the distance between the water source and septic tank less than 5 meter. The findings also showed that 4% of people suffered from waterborne diseases within last year.

The detection of coliform in water sources is directly related to public health concerns. In this study, 84.4% of the underground untreated water was found to be contaminated with total coliform and 25% of the untreated water was contaminated with fecal coliform. This shows a higher risk of waterborne epidemics but due to the different purification techniques of water followed by the respondents they are less likely to suffer from waterborne diseases. The respondents used tubewell water and jar water for drinking purposes and for household activities tubewell water and tanker water other than private tap water was used due to the unavailability of water as said by 25% of them. Majority of the respondents cleaned the water collecting container in 2-7 days while 5% of them cleaned the container in more than 7 days which might lead to contamination of water causing water pollution and waterborne diseases. Almost all respondents are aware of sanitation and also follow hygiene practice due to which there is less risk of waterborne epidemics.

CHAPTER 5

5. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusions

The routine bacteriological examination of water quality is of great concern as waterborne diseases are caused due to the fecal contamination of water and unhygienic practices. However, the treatment of water helps to maintain water quality and reduce waterborne diseases cases. According to this study, 84.4% of underground water was contaminated with total coliform which indicated the poor bacteriological quality of underground water in central Kathmandu and these water sources were unsatisfactory for drinking as well as household activities. The identified bacteria were *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Salmonella* spp., *Pseudomonas* spp. and *Proteus* spp.. Majority of the *E. coli* isolated from underground water sources were resistant to Ampicillin, Nitrofurantoin, Ciprofloxacin, Ceftriaxone and Chloramphenicol but all the isolates were sensitive to Cotrimoxazole, Gentamycin and Amikacin. Multiple antibiotic resistant (MAR) isolates of *E. coli* and thermotolerant *E. coli* were also detected in the study that increased the risk of emergence of antibiotic resistant bacteria which may threat the treatment of waterborne diseases by antibiotic resistant bacteria. Awareness on quality of underground water should be generated to the public. An appropriate treatment and disinfection methods has to be implemented to maintain quality of underground water and prevent waterborne diseases.

5.2 Novelty and national priority aspect of project work

As this study aim to detect coliform bacteria and Multiple Antibiotic Resistant (MAR) *E.coli* in underground water, the findings of this study have revealed the present status of underground water quality in Kathmndu Metropolitan city at point of use (POU). Thus, this study will be useful to understand the quality of underground water in Kathmandu Metropolitan city, availability of treatment facilities, efficacy of water treatment systems as well as existence of MAR bacteria in water. This study will also help the policy makers to design policy for maintaining water quality and implement action for water quality control and prevention of waterborne diseases in Kathmandu Metropolitan city.

5.3 Limitations of the study

This study is confined to analyse bacteriological quality of underground water and determine Multiple Antibiotic Resistant (MAR) isolates of *E. coli* from water. The physicochemical quality of underground water and the presence of pathogenic bacteria in untreated and treated water samples were not assessed due to lack of resources and the availability of time for the research work. The number of water samples and the study area in the Kathmandu Metropolitan City was also limited as this is a cross-sectional study.

5.4 Recommendations

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

- Assessment of bacteriological quality of underground water has to be done for utilization of water for drinking as well as domestic purposes.
- The purification and disinfection by chlorination of underground water sources should be done to maintain the water quality at point of use (POU).
- Source protection of underground water sources has to be done and there should be construction of sealed platform and management of wastewater drainage system.
- Susceptibilty of bacteria from water sources towards the antibiotic should be tested to understand the antibiotic resistance bacteria in water.
- Monitoring of underground water quality and water quality control has to be done to prevent waterborne diseases.
- Public awareness on quality of water at point of use, water treatment, personal hygiene and sanitation and waterborne diseases should be generated along with the knowledge on water quality and health risk.

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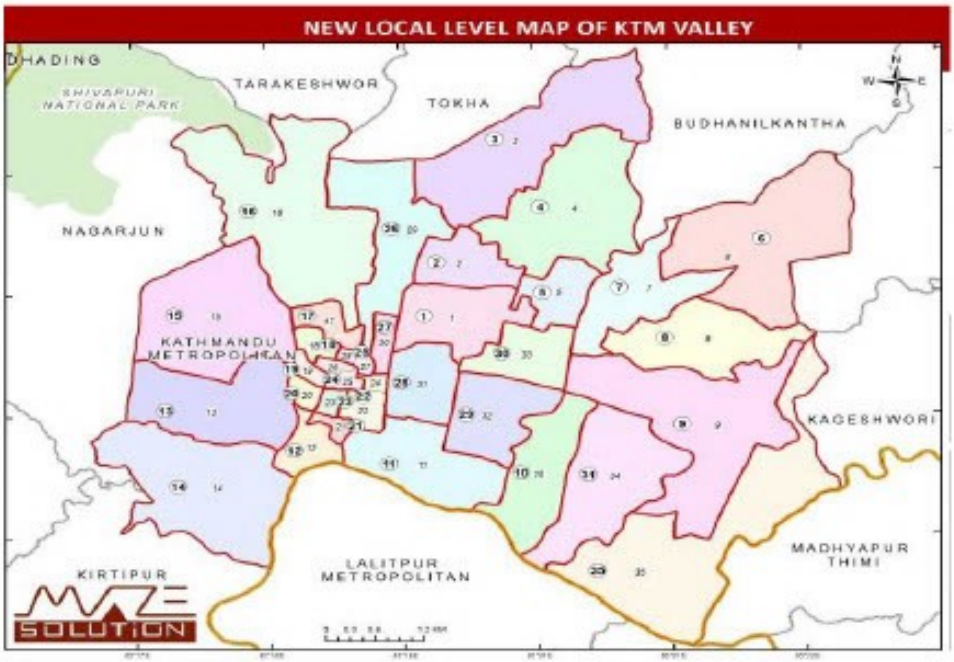
APPENDIX

APPENDIX-A

GEOGRAPHICAL MAP OF KATHMANDU METROPOLITAN CITY



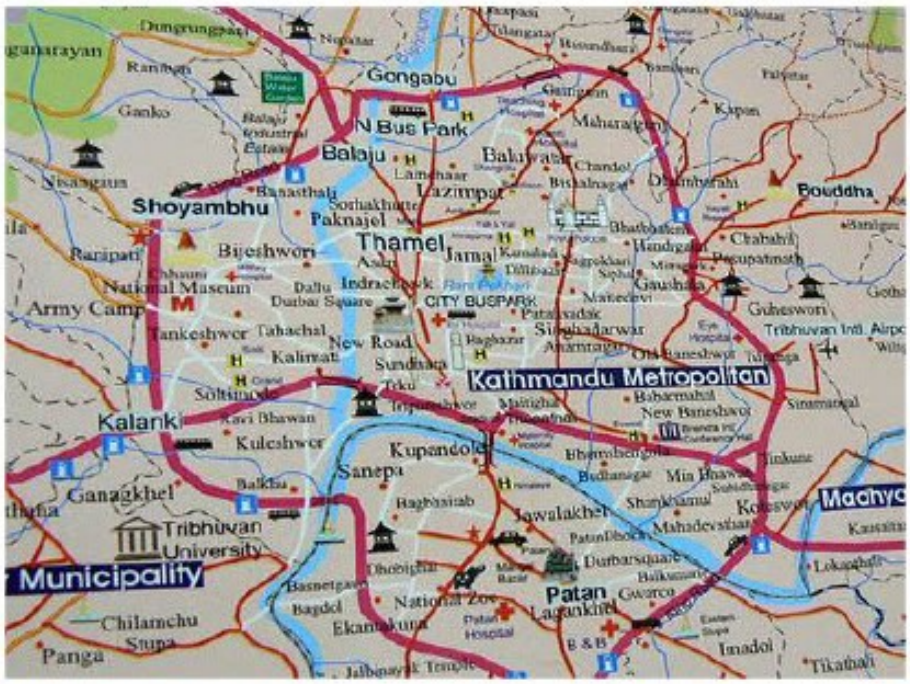


Figure: Map of Kathmandu Metropolitan city showing different sampling locations

APPENDIX-B

COMMUNITY SURVEY FORM

खानेपानी तथा सरसफाई सम्बन्धी प्रश्नावली

TRIBHUVAN UNIVERSITY
AMRIT CAMPUS
Department of Microbiology
B.Sc. 4th year

ASSESSMENT OF BACTERIOLOGICAL QUALITY OF UNDERGROUND WATER FROM KATHMANDU, NEPAL AND ANTIBIOTIC SUSCEPTIBILITY PATTERN OF *Escherichia coli*

नाम कोड:.....

लिङ्ग:..... नगरपालिका:..... टोल: .....

जन्ममिति / उमेर: ..... शिक्षा: .....

परिवार संख्या: पुरुष..... महिला..... बालबालिका (५ वर्षभन्दा मुनि).....

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

क.बितरित खानेपानी 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.ट्यांकर

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.झाडापखला 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 guideline 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 $CaCo_3$	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		*E. coli*	MPN/100 mL	0	0	
26		Total coliform	MPN/100 mL	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

1. Equipments

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

2. 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

3. Chemicals

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

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

1. Eosin Methylene Blue agar (EMB)
2. MacConkey agar
3. Mueller Hinton agar
4. Nutrient broth
5. Nutrient agar
6. Hugh and Leifson's media
7. Sulphide indole motility media
8. MR/VP medium
9. Simmon's citrate agar
10. Triple sugar iron agar
11. Urea agar

5. 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)

6. Antibiotic discs (Hi-Media Laboratories Pvt. Ltd.)

1. Amikacin (30 mcg)
2. Ampicillin (10 mcg)
3. Ceftriaxone (30 mcg)
4. Chloramphenicol (30 mcg)
5. Ciprofloxacin (5 mcg)
6. Cotrimoxazole (25 mcg)
7. Gentamycin (10 mcg)
8. Nalidixic acid (30 mcg)

9. Nitrofurantoin (300 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
16. Applicator sticks

APPENDIX-E

COMPOSITION AND PREPARATION OF DIFFERENT STAINING REAGENT AND CHEMICALS FOR MICROBIAL TESTS

1. Gram staining reagent

a) Crystal violet

Solution A

Composition

Ingredients

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

b) Gram's Iodine

Composition

Ingredients

Iodine	1.0 gm
Potassium iodide	2.0 gm
Distilled water	300.0 mL

Preparation

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

c) Acetone-alcohol decolorizer

Composition

Ingredients

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

Preparation

25 mL distilled water was mixed with 475 mL absolute ethanol. Then, 500 mL acetone was added immediately to the alcohol solution and mixed 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

10 mL safranin was added to 100 mL distilled water and mixed well. Then it was transferred to clean reagent bottle.

2. Catalase reagent

Composition

Ingredients

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

Preparation

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

3. Oxidase reagent

Composition

Ingredients

Tetra methyl paraphenylenediamine dihydrochloride	5.0 gm
Distilled water	50 mL

Preparation

This reagent was prepared by dissolved by 5.0 gm of reagent in 50mL of distilled water. To that solution stripes of Whatman No. 1 filter paper was soaked and drained for about 30 sec. Then these stripes were completely dried and stored in dark bottle tightly sealed with a screw cap.

4. 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 gm of reagent was dissolved in clean brown bottle. Then 25 mL of concentrated HCl was added to it and mixed well.

5. Methyl Red reagent

Composition

Ingredients

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

Preparation:

0.04 gm of methyl red was dissolved in 40 mL of ethanol. Then, 60 mL distilled water was added and mixed well.

6. Voges-Proskauer reagent (Barritt's reagent)

a) VP reagent A

Composition

Ingredients

Alpha-naphthol	5 gm
Ethanol (absolute)	100 mL

Preparation

To 28 mL distilled water, 5 gm of alpha naphthol was dissolved and transferred to a clean brown bottle. Then final volume was made 100 mL by adding distilled water.

b) VP reagent B

Composition

Ingredients

Potassium hydroxide	40 gm
Distilled water	100 mL

Preparation:

40 gm Potassium hydroxide was dissolved and transferred to a clean brown bottle. Then, final volume was made 100 mL by adding distilled water. (Source: Aneja, 2018 and Cheesbrough, 2006)

7. Sodium thiosulphate

Preparation

24.82 gm of $Na_2S_2O_3.5H_2O$ was mixed with boiled distilled water to make 1 Litre 0.4 gm of NaOH. Borax or pellet was added as a stabilizer. This was 0.1 N stock solution. For 4 times dilution, bolied distilled water was used to prepare 0.025N solution. It was kept in brown glass stoppered bottle.

8. Composition of diluent stock phosphate buffer solution

Composition

Ingredients

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

Preparation

The pH of the solution was adjusted to 7.2 with 1N NaOH and dilute to a volume of 1000 mL with distilled water. The solution was autoclaved at 121°C in 15 lbs pressure.

APPENDIX-F

A. COMPOSITION AND PREPARATION OF DIFFERENT CULTURE MEDIA

1. Eosin Methylene Blue (EMB) agar

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

37.4 grams of EMB agar was suspended in 1000 mL distilled water and heated to dissolve the medium completely. It was then sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes. The media was cooled to 45-50ºC and was shaken in order to oxidize the methylene blue and to suspend the precipitate. The media was mixed well and was poured into sterile petriplates.

2. 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

55.0 grams was suspended in 1000 mL of distilled water and heated to boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 15lbs pressure (121ºC) for 15 minutes. Then the medium was cooled to 45-50ºC and was poured into sterile petriplates.

3. Nutrient broth

Composition

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

Final pH (at 25ºC) 7.4±0.2

Preparation

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

4.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

**Formula adjusted, standardized to suit performance parameters

- Equivalent to Beef infusion from

- Equivalent to Casein acid hydrolysate

Preparation

38.0 grams was suspended in 1000 mL of ditilled water and heated to boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The medium was then cooled to 45-50°C and was poured into sterile petriplates.

B. COMPOSITION AND PREPARATION OF DIFFERENT BIOCHEMICAL MEDIA

1. 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 25°C) 6.8±0.2

Preparation

9.38 grams was suspended in 1000 mL distilled water and heated to boiling to dissolve the medium completely. 100 mL amounts were dispensed and to the first 100 mL of sterile basal medium, 1gm of dextrose solution was added aseptically then medium was sterilized by autoclaving at 110°C for 15 minutes. All the solution was mixed and was dispensed in 5 mL amounts in sterile tubes in duplicate for aerobic and anaerobic fermentation.

2. 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 25°C) 7.3±0.2

Preparation

36.23 grams of medium was suspended in 1000 mL distilled water and heated to boiling to dissolve the medium completely. The medium was then dispensed in tubes and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The tubes were allowed to cool in the upright position.

3. MR-VP medium

Composition

Ingredients	**gm/liter**
Buffered peptone	7.00
Dextrose	5.00
Dipotassium phosphate	5.00

Final pH (at 25ºC) 6.9±0.2

Preparation

17.0 grams was suspended in 1000 mL of distilled water 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.

4. Simmons Citrate agar

Composition

Ingredients	**gm/liter**
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 was suspended in 1000 mL distilled water and was heated to dissolve the medium completely. The medium was then dispensed as desired in tubes or flasks and sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes.

5. Triple Sugar Iron agar

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 red	0.024
Agar	12.00

Final pH (at 25ºC) 7.4±0.2

Preparation

64.52 grams was suspended in 1000 mL distilled water and heated to boiling to dissolve the medium completely. The medium was mixed well and was distributed into test tubes and sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes. The medium was allowed to set in sloped form with a butt about 1 inch long.

6. 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 was suspended in 950 mL distilled water and heated to boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 10 lbs pressure (115ºC) for 20 minutes. The medium was cooled to 50 ºC and 47.5 mL of

sterile 40% urea solution aseptically and mixed well. The medium was dispensed in sterile tubes and allowed to set in the slanting position.

APPENDIX-G

PREPARATION OF MACFARLAND TURBIDITY STANDARDS

McFarland turbidity standards

McFarland standard No.	1.0% Barium Chloride (mL)	1.0% Sulphuric 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 turbidity standard equivalent to 0.5 McFarland

1. 1% V/V solution of Sulphuric acid was prepared by adding 1 mL of concentrated Sulphuric acid to 99 mL water and mixing it well.
2. 1% W/V solution of Barium Chloride was prepared by dissolving 0.5 gm of dihydrate Barium Chloride ($BaCl_2.2H_2O$) in 50 mL of distilled water.
3. 0.6 mL of the Barium Chloride solution was then added to 99.4 mL of the Sulphuric acid and mixed.
4. A small volume of the turbid solution was then transferred to a screw capped bottle.
5. To standardize the inoculum density for a susceptibility test, a $BaCl_2$ turbidity standard, equivalent to 0.5 McFarland standard was used.

APPENDIX-H

PROCEDURE FOR ENUMERATION AND IDENTIFICATION OF BACTERIA IN WATER SAMPLE

1. Membrane Filtration (MF) Technique

Principle

In this method, 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 filer disc that contains the trapped microorganisms is aseptically transferred to a sterile Petriplate having a selective Eosin Methylene Blue agar medium and the colonies which developed, 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 (APHA, 1998; Aneja, 2018 and Cheesbrough, 2006).

Calculation of Coliform bacteria in colony forming units

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

Procedure

1. The filtration unit and suction device was assembled.
2. A sterile membrane filter, grid-side uppermost was placed using sterile blunt ended forcep on the filter base.
3. The sample of water was mixed thoroughly by inverting the bottle several times.
4. To draw the water sample, suction was applied through the filter membrane. Dilutionof the water sample was done as per the necessity.
5. The membrane from the filtration unit was removed aseptically using sterile blunt-ended forcep and was placed on the culture medium pad in the petriplates keeping the grid side uppermost.
6. The petriplates were closed and labelled with the code number of water sample.
7. After one hour, the petriplates were incubated at 35±0.5°C and 44±0.2°C for 24 hours.
8. After incubation, the number of colonies were counted and identified.

2. Gram Staining

Principle

Gram staining is a very useful stain for identifying and classifying bacteria into two major groups: the gram positive and gram negative. In this process, the fixed bacterial smear is subject to four different reagents in the order: crystal violet (primary stain), iodine solution (mordant), alcohol (decolorizing agent) and safranin (counter stain). The bacteria which retain the primary stain (appear dark blue or violet) are called gram positive, whereas those that lose the crystal violet and counter stained by safranin (appear red/pink) are referred as gram negative (Aneja, 2018; Cheesbrough, 2006 and Forbes, *et al*., 2007).

Procedure

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

- The material on the slide was heated fixed and allowed to cool before staining.
- The slide was flooded with crystal violet stain and allowed to remain without drying for 1 minute.
- The slide was rinsed with tap water, shaking of excess.
- The slide was flooded with iodine solution and allowed to remain on the surface without drying for 1 minute.
- The slide was rinsed with tap water, shaking off excess.
- The slide was flooded with alcohol acetone decolorizer for 15 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolorizer.
- The slide was flooded with counter stain (safranin) for 1 minute and washed off with tap water.
- The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 100X.

3. Antibiotic Susceptibility Testing

Principle

The Kirby-Bauer test is an assay whereby discs of filter paper impregnated with a single concentration of different antibiotics or any chemicals that will diffuse from the disc into the agar. The selected antibiotic discs are placed on the surface of an agar plate which has already been inoculated with test bacteria. During the incubation period the

antibiotics/ chemicals diffuse outward from the discs into the agar. This will create a concentration gradient in the agar which depends on the solubility of the chemical and its molecular size. The absence of growth of the organism around the antibiotic discs indicates that the respected organism is susceptible to that antibiotic and the presence of growth around the antibiotic disc indicates the organism is resistant to that particular antibiotic. This area of no growth around the disc is known as a zone of inhibition, which is uniformly circular with a confluent lawn of growth in the media (CLSI, 2021 and Cheesbrough, 2006).

Procedure

A sterile non- toxic cotton swab on a wooden applicator stick was dipped into standardized inoculum (turbidity so adjusted, as to obtain confluent growth on the petriplate).

- The soaked swab was rotated firmly against the upper inside wall of the tube to express excess fluid.
- The entire agar surface of the plate was streaked three times, turning the plate at 60° angle between each streaking.
- The inoculum was allowed to dry for 5-15 minutes with lid in place.
- The disc was then applied using aseptic technique. The discs with centres at least 24 mm apart should be deposited.
- Incubation was done immediately at 35 ± 2°C and examination was done after 16-18 hours.
- The zone of inhibition was measured and compared with the standard chart.

APPENDIX-I

PRINCIPLE AND PROCEDURE OF BIOCHEMICAL TEST

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

1. Catalase test

Principle

The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. The presence of the enzyme in a bacterial isolate is evident when a small inoculum is introduced into hydrogen peroxide, and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. The culture should not be more than 24 hours old. This test is used to differentiate those bacteria that produce the enzyme catalase, such as Staphylococci, from non-catalase producing bacteria such as streptococci.

Procedure

1. Using wooden stick, a colony was transferred to the surface of clean, dry glass slide.
2. A drop of 3% H_2O_2 was placed on the glass slide and mixed.
3. Then effervescence of the gas was marked by bubbles in case of positive result within 10 seconds.

2. Oxidase test

Principle

The oxidase test is used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. The ability of an organism to produce the cytochrome C oxidase can be determined by using the reagent tetramethyl-p-phenylenediamine dihydrochloride impregnated in filter paper. The reagent serves as an artificial substrate donating electrons and thereby becoming oxidized to a deep purple compound in the presence of the enzyme oxidase and free O_2. Development of pink, then maroon and finally dark purple coloration after rubbing the organism in the oxidase disc containing the reagent indicates positive reaction. The positive reaction involves the conversion of colorless, reduced tetramethyl-p-phenylenediamine to oxidized form into deep purple color in presence of Cytochrome C oxidase. No color change is indicative of the negative test result.

Procedure

1. A piece of filter paper was soaked with the substrate tetramethyl-p-phenylenediamine dihydrochloride was taken.
2. The colony to be tested was picked with wooden stick and made smear in the filter paper.
3. The area of paper was inoculated with the colony for a color change to deep blue or purple and observed within 10-30 second.

3. Oxidative-Fermentative (OF) test

Principle

The oxidative-fermentative test determines if certain gram-negative rods metabolize glucose by fermentation or aerobic respiration (oxidatively). During the anaerobic process of fermentation, pyruvate is converted to a variety of mixed acids depending on the type of fermentation. The high concentration of acid produced during fermentation will turn the bromothymol blue indicator in OF media from green to yellow in the presence or absence of oxygen.

Certain non-fermenting gram-negative bacteria metabolize glucose using aerobic respiration and therefore only produce a small amount of weak acids during glycolysis and Krebs cycle. The decrease amount of peptone and increase amount of glucose facilitates the detection of weak acids thus produced. Dipotassium phosphate buffer is added to further promote acid detection. OF Test is used to determine if gram-negative bacteria metabolize carbohydrates oxidatively, by fermentation, or are non-sacchrolytic (have no ability to use the carbohydrate in the media).

Procedure:

1. Two tubes containing Hugh and Leifson's medium were taken and the organism was stabbed into both media using sterile inoculating wire.
2. One of the tubes was sealed with paraffin oil to create anaerobic condition.
3. Both the tubes were incubated at 37°C for 24-48 hours and observed for color change in both the tubes.

4. Sulphide Indole Motility (SIM) test

Principle

SIM (Sulphide Indole Motility) medium is useful for the differentiation of gram-negative enteric bacilli. SIM test helps to isolate the organisms on the basis of sulphide production and motility. The medium having the constituent's ferrous ammonium sulphate and sodium thiosulphate, that together serve as indicators for the production of hydrogen sulphide (H_2S). Hydrogen sulphide production detects when ferrous sulphide, a black precipitate, is produced as a result of ferrous ammonium sulphate reacting with hydrogen sulphide gas. Casein peptone of this medium is rich in tryptophan. Organisms having the enzyme tryptophanase degrade tryptophan to indole. Indole detection is achieved after the addition of Kovac's reagent following incubation of the inoculated medium. Indole combines with p-dimethyl amino benzaldehyde and produces a red band at the top of the medium. A negative indole test produces no color change after the addition of Kovac's reagent i.e., Yellow color of Kovac's reagent. Lower concentration of agar added to the medium provides a semi-solid structure allowing for the detection of bacterial motility. The bacterial growth diffuse from the stab line and produce turbidity or cloudiness throughout the medium. Growth of non-motile bacteria is restricted along the stab line and leave the surrounding medium clear. Another constituent, animal tissue of this medium which provides amino acids and nutrients necessary for bacterial growth.

Procedure

1. The test organism was inoculated in test tube containing SIM medium.
2. Then, it was incubated at 37°C for 24 hours.
3. After incubation, test for indole was done by adding 0.5 mL of Kovac's reagent and shaken gently.
4. Red color in the surface layer was examined within 10 minutes.

5. Methyl Red (MR) test

Principle

In methyl red test, the test bacteria are grown in a broth medium (MR media) containing glucose. The bacteria with the ability of utilizing glucose producing stable acid, change the color of methyl red media to red in addition of methyl red reagent.

Procedure

1. MR-VP broth was aseptically inoculated with the organism with the help of sterile inoculating loop and incubated at 37°C for 24 hours.
2. Then 5-6 drops of Methyl red reagent were added to the incubated test tube.
3. The positive test is indicated by the development of red color of the indicator.

6. Voges-Proskauer (VP) test

Principle

The Voges-Proskauer (VP) test is used to determine if 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. The α-naphthol was not part of the original procedure but was found to act as a color intensifier by Barritt and must be added first. The diacetyl and quinidine-containing compounds found in the peptones of the broth then condense to form a pinkish red polymer.

Procedure

1. The bacterial suspension was inoculated aseptically into the MR-VP broth tube and incubated at 37°C for 24 hours.
2. Then the Barrit's reagent I and II were added in the ratio of 3:1 drops and the tube was shaken.
3. The positive test is indicated by the development of red color.

7. Citrate Utilization test

Principle

Citrate agar is used to test an organism's ability to utilize citrate as a source of energy. The medium contains citrate as the sole carbon source and inorganic ammonium salts ($NH_4H_2PO_4$) as the sole source of nitrogen. Bacteria that can grow on this medium produce an enzyme, citrate-permease, capable of converting citrate to pyruvate. Pyruvate can then enter the organism's metabolic cycle for the production of energy. Growth is indicative of utilization of citrate, an intermediate metabolite in the Krebs cycle.

When the bacteria metabolize citrate, the ammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH turns the bromothymol blue indicator in the medium from green to blue above pH 7.6.

Procedure

1. The organism was streaked aseptically on the surface of the Simmon's citrate agar and incubated at 37°C for 24 hours.
2. Change in the color of the medium was observed.

8. Triple Sugar Iron Agar (TSIA) test

Principle

Triple Sugar Iron agar (TSIA) medium is composed of three sugars; lactose, sucrose and glucose, iron (ferrous sulfites) and phenol red as an indicator. The indicator is employed for the detection of fermentation of sugar indicated by the change in color of the medium due to the production of organic acid and hydrogen sulphide (H_2S). If an organism ferments any of three sugar or any combination of them, the medium will become yellow due to the production of acid as end product of fermentation. Glucose utilization occurs both aerobically on slant where O_2 is available and in the butt where the condition is anaerobic. Change of color slant and butt indicates. Production of gas from the fermentation of sugar by an organism is indicated by the appearance of bubbles in the butt or pushing up of the entire slant from the two third of the tube. Hydrogen sulphide (H_2S) production by an organism is indicated by the reduction of ferrous sulfites of the medium to ferric sulphides, which is manifested as a black precipitate.

Procedure

1. With a sterilized straight inoculation needle a well-isolated colony was touched.
2. Inoculation on TSIA by first stabbing through the center of the medium to the two third of the tube and streaking on the surface of the agar slant was done.
3. The tube was incubated at 37°C for 18 hours.
4. The change in color of the medium was observed.

9. Urea Hydrolysis test

Principle

Urea hydrolysis test is used to determine the ability of certain microorganism to produce the enzyme urease. Organism capable of producing enzyme urease catalyzes the

breakdown of urea into ammonia and carbon dioxide which cause the rise in the pH of the medium. As the pH increases, the color of the phenol red changes from an 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.

APPENDIX-J

BIOCHEMICAL CHART FOR THE IDENTIFICATION OF BACTERIA

Tests	Bacteria						
	E. coli	*Klebsiella* spp	*Enterobacter* spp	*Citrobacter* spp	*Salmonella* spp	*Pseudomonas* spp	*Proteus* spp
Catalase	+	+	+	+	+	+	+
Oxidase	_	_	_	_	_	+	_
Oxidative Fermentative	F	F	F	F	F	O	F
Motility	+	_	+	+	+	+	+
Indole	+	_	_	_	_	_	+/_
Methyl Red	+	_	_	+	+	_	+
Voges Proskauer	_	+	+	_	_	_	+/_
Citrate Utilization	_	+	+	+	_	+	+
TSI	Y/Y	Y/Y	Y/Y	Y/Yor R/Y	R/Y	R/R	R/Y
	H_2S_	H_2S_	H_2S_	H_2S+	H_2S+/_	H_2S_	H_2S+
	Gas+	Gas+	Gas+	Gas+	Gas+/_	Gas_	Gas+
Urea Hydrolysis	_	+ Slow	_	+/_	_	+/_	+

+ = Positive ; _ = Negative ; F = Fermentative ; O = Oxidative ; Y = Yellow (Acid Reaction) and R = Red-pink (Alkaline Reaction)

Source: Cheesbrough (2006)