ASSESSMENT ON THE MICROBIAL STATUS OF POTABLE WATER IN DHAKA CITY AND THE COMPARATIVE ANALYSIS OF DIFFERENT REGIONS OF THE CITY ON ANTIBIOTIC RESISTANCE

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of Bachelors of Science in Biotechnology

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DEDICATION

The completion of this thesis would not have been possible without the support of our beloved and honorable supervisor Akash Ahmed. Thank you for all your support .

DECLARATION

It is hereby declared that

1. The thesis submitted is my original work while completing degree at BRAC University.

2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.

3. The thesis does not contain material that has been accepted or submitted, for any other degree or diploma at a university or other institution.

4. I have acknowledged all of the main sources of help.

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Abstract

Dhaka City is home to nearly 22,478,000 people, but accessible drinking water sources are limited. Moreover, the minimal available water sources are polluted, rendering the Dhaka City population victim of water-borne health hazards. The key water source examined in this study is the community water from street food vendors all over Dhaka City, which is consumed by general people on a regular basis. A total of 47 water samples were collected from tea stalls, street food vendors, and roadside restaurants and the samples were processed within 2 hours of collection. A combination of spread, pour and streak plating methods were used on a variety of selective media, such as MAC, XLD, MSA, TCBS, M-FC, etc. Gram staining and an array of biochemical tests were also performed to identify the target organisms. Antibiotic Susceptibility was further performed to examine the level of antibiotic resistance. In this study, *Bacillus spp* was the most prevalent (28.57%) bacteria isolated from drinking water. *E.coli* was found to be the highest one (12%) among a myriad of other enteric pathogens. However, a distressing number of fecal coliforms were found in more or less all the samples The isolates showed highest resistance to Penicillin 80.67% followed by Colistin 50.42%, Cefepime 23.52%, Gentamicin 42.01%, Cefuroxime 43.69%, Erythromycin 24.36%. Diseases related to the serious contamination of drinking water in Dhaka city constitute a major burden on human health and can cause a leading risk factor for infectious diseases including cholera, diarrhea, dysentery and typhoid.

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Chapter 1: Introduction

Water is both the source of life and a necessary resource for human survival. A vital requirement for good health, as well as a fundamental human right, is access to clean drinking water. All other human rights are acknowledged as being built on the right to access clean water. We are fortunate to be able to live on this planet thanks to water. Humans, from cave dwellers to city dwellers, have been using water for a variety of significant purposes since the birth of civilization, including drinking, washing, watering animals, and land irrigation. The fact that drinking water quality is still in danger, though, is a serious concern.

1.1 Consequences of contaminated drinking water

Drinking polluted water can have a number of negative health effects. The following list of waterborne ailments is not exhaustive and includes some of the more often reported issues caused by drinking polluted water: gastrointestinal issues, nausea, vomiting, aches and pains in the abdomen, dehydration, and even death. Additionally, it must be remembered that just because there are no immediate indications or symptoms, it does not necessarily mean that there won't be any long-term consequences

The main bacterial diseases transmitted through drinking water

Disease	Causal bacterial agent

Cholera	*Vibrio cholerae, serovarieties 01 and 0139*
Gastroenteritis caused by vibrios	*Mainly Vibrio parahaemolyticus*
Typhoid fever and other serious salmonellosis	*Salmonella enterica subsp. enterica serovar Paratyphi* *Salmonella enterica subsp. enterica serovar Typhi* *Salmonella enterica subsp. enterica serovar Typhimurium*
Bacillary dysentery or shigellosis	*Shigella dysenteriae* *Shigella flexneri* *Shigella boydii* *Shigella sonnei*
Acute diarrheas and gastroenteritis	*Escherichia coli, particularly serotypes such as 0148, 0157 and 0124*

1.1.1 Cholera

The cholera incubation period is between one and three days. Acute and extremely severe diarrhea that can surpass one liter per hour is the disease's symbol. Patients with cholera experience thirst, muscle aches, general weakness, and symptoms of oliguria, hypovolemia, 13-14, and anuria. Blood potassium levels fall to extremely low levels. Patients experience fatigue. Finally, cyanosis, circulatory collapse, and dehydration take place.

1.1.2 Salmonellosis

Typhoid, paratyphoid, and gastroenteritis are the two kinds of salmonellosis that Salmonellae, a pathogen, can produce. Less than 1,000 infective doses are required to produce clinical symptoms. Infants and newborns with salmonellosis can exhibit a range of clinical signs, from a moderate infection to a severe sickness with septicemia and typhoid-like symptoms. Typhoid fever cases decline as a country's level of development increases (for example, regulated water sewage systems and pasteurization of milk and dairy products). The likelihood of fecal contamination of food and water is high where basic hygienic standards are absent, and typhoid fever incidence is also high there. Salmonellae infections allow people to carry the bacteria in their guts without showing any symptoms of illness, unlike cholera. Humans with the infection can keep the bacteria in their bodies for long periods of time. For months or even years after being clinically healed of typhoid fever, about 5% of patients are still carriers. These individuals make up the primary reservoir of bacteria in the environment and may be long-term carriers of the bacteria in the gut.

1.1.3 Shigellosis or Bacillary Dysentery

This illness takes one to four days to incubate. Typically, symptoms of the illness include fever, anorexia, lethargy, and malaise. Patients frequently experience pains in their stomachs as well as

tiny, bloody feces that can occasionally be obscenely purulent. After 12 to 36 hours, diarrhea transforms into dysentery, and the volume of the stools reduces (to no more than 30 mL of fluid per kg per day). Blood, mucus, and pus then develop in the feces.

1.1.4 *Escherichia coli*

Infantile gastroenteritis can be brought on by certain ETEC serotypes (enterotoxigenic *E. coli*). Although there are relatively few reports of its incidence in industrialized nations, it is a major cause of diarrhea in underdeveloped regions with inadequate access to clean water and subpar sanitation. Despite impressive progress in improving access to water supply and sanitation, around 41% of all improved water sources in Bangladesh are polluted with E. coli (Shaibur et al. 2019b). Everyone, regardless of wealth or location, is equally affected by *E. coli* (Nabi 2018). ETEC is a disease that results from consuming contaminated food or water. It is characterized by severe diarrhea that lasts for many days and frequently causes dehydration and malnutrition in young children. According to the WHO, in Bangladesh, the mortality rate from diarrhea is 7.5 deaths per 100,000 people, and it is anticipated that 829 000 people will die from it annually in 2022 as a result of unsafe drinking water, poor sanitation, and poor hand hygiene.

Shigellae and enteroinvasive *E. coli* (EIEC) share several characteristics. In human beings, they have the capacity to invade and proliferate in the intestinal epithelial cells of the distal large bowel. Constipation, vomiting, diarrhea, fever, chills, a widespread malaise, and the presence of blood and mucus in the feces of infected people are symptoms of the sickness.

1.2 Methods of water contamination

Worldwide, at least 2 billion people consume water that has been contaminated with feces,

according to the WHO. Ingestion of water that has been contaminated with human or animal excrement, poses the biggest microbiological dangers. Microbial contamination caused by feces contamination is the greatest threat to the safety of drinking water. Microbial analyses of water are commonly performed to identify primarily fecal coliforms (Azizillah et al. 2011). Fecal coliforms are typically discovered in the excrement of several warm-blooded species, including people and domestic animals (Geldreich, 1996). The presence of microorganisms that cause human diseases and are spread through water is used by scientists as a sign of water pollution (Shiekh, 2006). Drinking water that has been contaminated by microorganisms can spread diseases like diarrhea, cholera, dysentery, typhoid, and polio, as well as cause 485 000 diarrhea-related deaths annually. The use of hygiene, a crucial step in preventing not only diarrheal illnesses but also acute respiratory infections and several neglected tropical diseases, is made easier by the availability of safe and sufficient water.

Fecal microorganisms, including pathogens, are primarily obtained from the discharge of wastewater into freshwaters and coastal seawaters. In our environment, including groundwater and surface water, bacteria are present everywhere. Many of these microorganisms have the potential to affect people's health. People can get sick from drinking water that contains pathogens, which are bacteria, viruses, or parasites that cause disease. Testing for every sort of pathogen in drinking water is not possible; however, coliform bacteria can be easily detected. Coliform bacteria can be a sign that there are potentially dangerous diseases in the water. The following contaminants may be present in water sources: *E. coli* bacteria, pharmaceuticals, feces, microbial pathogens, parasites, viruses, and coliform bacteria. Water supplies can become contaminated in a number of ways. The taste or smell of the water may occasionally change. However, a lot of contaminants are tasteless and odorless so there is no trace of contamination left behind.

When the water has just left the treatment facility, the quality of the pipeline water may be adequate. However, once the water has passed through the distribution system, a number of physical, chemical, and biological modifications may take place (Lahlou 2002). Diarrhea could be brought on by a cross-connection between municipal supply water and sewage due to leaky joints in water treatment facilities and distribution systems (Shaibur et al. 2019c), which can be a serious threat to public health (Semenza et al. 1998).

1.3 Susceptibility to Antibiotics

Antibiotics are drugs used to treat bacterial illnesses in both humans and animals. They do this by either killing the germs or making it difficult for them to develop and reproduce. They do not work well against viral infections, either. In contrast to bacteria, which have cell walls that may be attacked by antibiotics, viruses have a protective protein coating around them. In terms of structure, viruses differ from bacteria. Viruses are unable to survive outside of the human cell, where they can only exist and reproduce. In order to proliferate, viruses splice their genetic material into the DNA of human cells. Antibiotics are therefore ineffective against viruses. Antibiotics therefore cannot kill viruses since they differ from bacteria in terms of structure and mode of replication. Bacteria that are susceptible to antibiotics cannot proliferate in the presence of the medication. This indicates that the antibiotic is efficient in combating the bacteria, but resistance indicates that the bacteria are able to survive in the presence of the antibiotic and is a symptom of an ineffective antibiotic.

When bacteria adapt to the use of antibiotics, antibiotic resistance develops. In essence, it can happen when bacteria develop resistance to the antibiotic. Antimicrobial resistance occurs when bacteria, fungi, and other microorganisms learn to resist the medications meant to kill them. That implies that the germs survive and develop. Both humans and animals are susceptible to infection from these germs, and their infections are more difficult to treat than those brought on by non-resistant bacteria. Antibiotic usage is the primary contributor to antibiotic resistance. While some bacteria die when humans take antibiotics, resistant bacteria can live and even proliferate. Antibiotic usage increases the prevalence of microorganisms with resistance. Bacteria are more likely to develop antibiotic resistance as antibiotics are used more frequently. Antibiotics function by disrupting the bacterial cell wall and preventing the reproduction of bacteria but many of these medications have been overused, misused, or used often for a very long time.

In conclusion, the following are the six primary causes of antibiotic resistance:

Antibiotic overuse in livestock and fish aquaculture, excessive antibiotic prescribing, inadequate

infection control in healthcare facilities, poor hygiene and sanitation, and the lack of new antibiotic discoveries are all factors.

One of the major risks to modern development, food security, and global health is antibiotic resistance. Antibiotic resistance can affect anyone, at any age, in any country. The abuse of antibiotics in both humans and animals is hastening the occurrence of antibiotic resistance, which is a natural phenomenon. Antibiotic resistance causes increased mortality, longer hospital stays, and higher medical expenses. Antibiotic prescribing and use must change immediately around the world. In every region of the world, antibiotic resistance is increasing to dangerously high levels. New resistance mechanisms are emerging and spreading globally, threatening our ability to treat widespread infectious illnesses. As antibiotics lose their effectiveness, a rising number of infections, including gonorrhea, blood poisoning, pneumonia, and tuberculosis, become difficult to cure and occasionally incurable. The establishment and spread of resistance are accelerated in situations where antibiotics are available for purchase without a prescription for human or animal use. Similar to this, in nations lacking standardized treatment recommendations, veterinarians and medical professionals frequently overprescribe antibiotics, and the general public frequently overuses them. Without immediate action, we risk entering a post-antibiotic world where ordinary diseases and minor wounds can again be fatal. Aquatic habitats are known to be a reservoir for antibiotic-resistant bacteria, and the presence and spread of these germs are serious issues for global public health. Antibiotic-resistant bacteria and tiny amounts of antibiotics in drinking water are developing concerns for the general public and may have a significant impact on public health.

1.4 Bangladesh's drinking water quality

In many regions of the world, there are already limited supplies of safe drinking water. Due to rising population, urbanization, and climate change, it will become much more restrictive in the following century (Jackson et al., 2001). The availability of clean water to drink is a major

concern. Bangladesh, one of the most densely populated nations, is struggling with severe water pollution and scarcity, just like the rest of the Third World. Despite the fact that 97% of the population has access to water, the purity of the water is never guaranteed (WHO, 2018). Although 98% of Bangladesh's population has access to water from modern sources, the quality of that water is still subpar (Islam et al., 2015; Shaibur et al., 2019e, f). Many individuals have trouble getting access to clean water. In developed nations like Bangladesh, where access to clean water and sanitation is not the norm, waterborne diseases are highly prevalent. Clean, treated water supplies may be the standard in Europe and North America, but they are not in developing nations like Bangladesh. More than 1.2 million people die each year as a result of contaminated water (Ritchie & Roser, 2021). One of the main risk factors is unsafe drinking water. Malnutrition makes infectious diseases like cholera, diarrhea, dysentery, hepatitis A, typhoid, and polio worse. In developing nations, acute microbial diarrheal diseases are a significant public health issue. The majority of microbial diseases that are spread through water affect children under five, primarily in Asian and African nations.

Despite being the capital of Bangladesh, Dhaka still struggles with a lack of access to clean drinking water for the majority of its residents. As a result, they continue to rely on unprotected water sources, which are more prone to contamination. More precisely, Bangladesh's restaurants' water supply is subpar. As public awareness of waterborne diseases (da-Silva et al. 2008) progressively grows, the microbiological safety of drinking water has recently become a concern (Shaibur et al. 2012). The conventional drinking water that is provided to customers in hotels is microbially contaminated as a result of improper handling and serving procedures (Tambekar et al. 2006). Despite the fact that street food is so popular and inexpensive (Islam et al. 2017b), the majority of Dhaka city residents suffer from dysentery, diarrhea, and gastrointestinal disorders (Faruque et al. 2010).

Currently, people are more drawn to foods found on the streets or in restaurants. In Dhaka, there are many different types of hotels, restaurants, and food stands where people of various social classes eat. They prefer street food when dining, which is problematic given that the food vendors sometimes serve subpar water in Dhaka for drinking purposes. Due to the taste, low cost, accessibility, wide variety, and, most importantly, the requirements of the homeless, a significant portion of Dhaka's population prefers to eat at roadside stalls or restaurants (Islam et

al. 2017b). When having breakfast, lunch, or supper in a restaurant, most patrons opt to drink water. The majority of the time, water is kept in an enormous plastic drum, an earthen pot (a kola), or a lidless plastic tank. Customers are occasionally given access to tube well water or locally sourced water (Shaibur et al. 2012). Additionally, customers may be given access to stored water or tap water in some eateries. Most people don't want to pay for drinking water, so when they suddenly get thirsty, they drink the contaminated water that is readily available. As a result, after drinking contaminated water, individuals frequently experience numerous waterborne illnesses such as diarrhea, dysentery, and gastritis.

The objectives of this study were to evaluate the microbiological drinking water quality in Dhaka's street food vendors and restaurants and to calculate the prevalence of antibiotic resistance. This study also attempted to determine whether or not the supplied water is suitable for a consumer's health. Another goal of this study was to raise awareness among city residents so that the authorities could take the necessary action to ensure that the quality of our drinking water is improved

Chapter 2: Materials and Methods

2.1 Equipment

- Autoclave machine
- Laminar Air Flow
- Incubator
- Vortex machine
- Culture freeze
- Fresh freeze
- Water filtration

2.2 Media

Media of different types and categories were used for the selective identification, growth, and enrichment of different organisms from the samples

- Mueller Hinton agar (MHA)
- M-faekal Coliform Agar (MFC)
- Different Biochemical mediums
- MacConkey agar (MAC),

- Mannitol Salt Agar (MSA),
- Nutrient Agar (NA),
- Xylose Lysine Deoxycholate (XLD),
- Thiosulfate-citrate-bile salts-sucrose (TCBS),
- Eosin methylene blue (EMB)

2.3 Sample collection

2.3.1 Description of the sampling sites

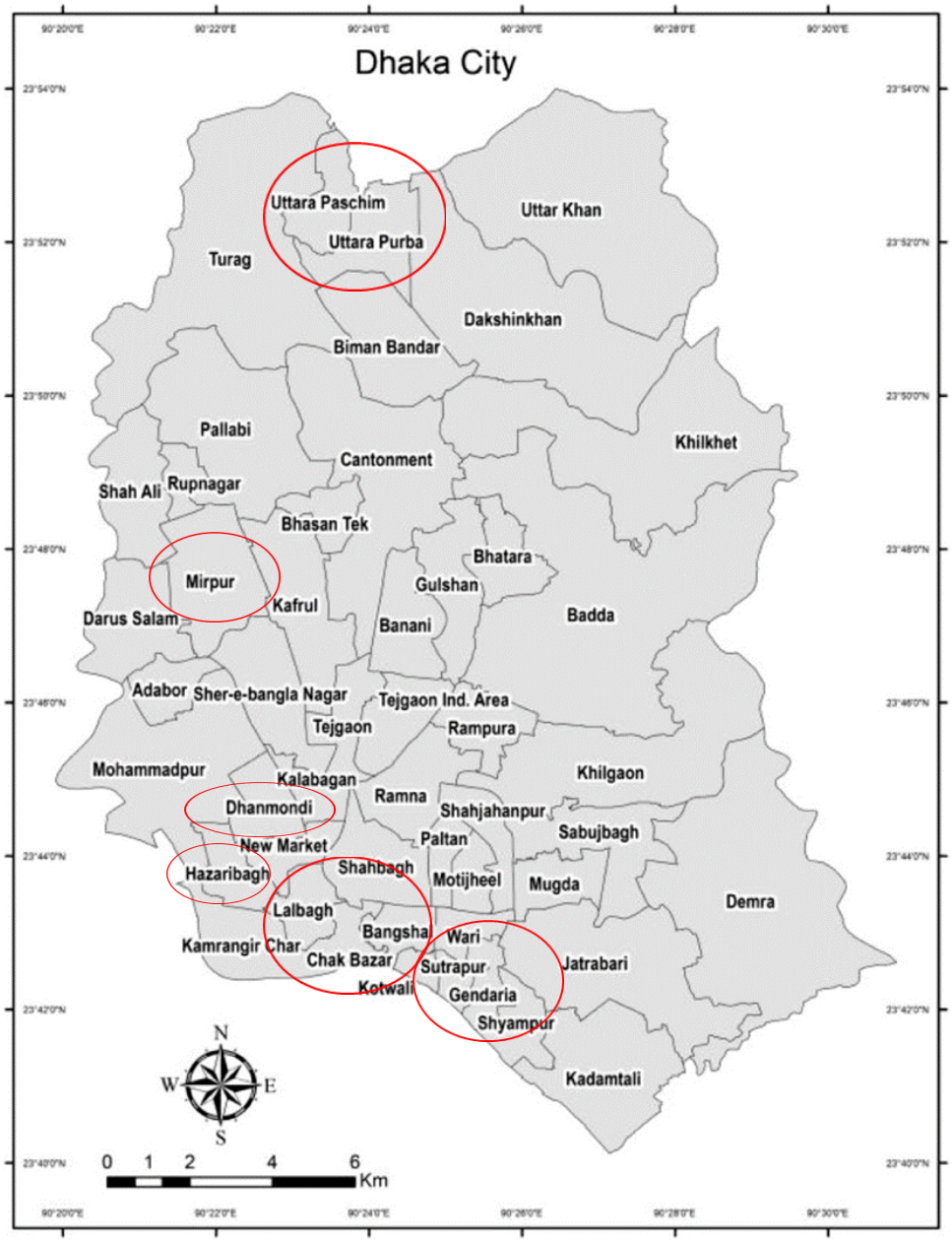


Figure- I

In total, 47 water samples were collected in autoclaved plastic bottles from different sorts of food stalls and restaurants from different regions of Dhaka city. The sample collection procedure

started from the end of December 2021 and lasted until the beginning of August 2022.

2.3.2 Description of the food stalls

All the samples were collected from tea stalls, street side fast food stalls or different types of restaurants. These food stalls provide locally SW, TW, DTW or stored water to their consumers.

The water samples that were collected from the tea stalls are usually found on the road side. Usually, they store water in the earthen pot or plastic tank with or without a lid. Often, the environmental conditions are very filthy or greasy in or around the tea stalls. On the other hand, street side fast food stalls are moving stalls. Traditionally, they serve food on a mobile van. Few cheap delicious foods, e.g., fuchka, chotpoti, burgers, French fries, jhalmuri, sandwiches, juices, milkshakes etc. are sold here, and the vendors keep the drinking water in plastic or earthen container. However, samples were also collected from restaurants where typically, bread (ruti), fried bread (parata), singara, puri, rice, curries, fishes, drinks, sweet or savory items are sold. Moreover, a few samples were also collected from restaurants which have sitting arrangement, e.g., chair, table or bench.

Figure II: Sample collection plastic bottle

2.4 Processing of Water Sample

The water samples were brought back to the lab within 2 hours for further processing. At first, MAC, MSA, NA, XLD, TCBS, EMB agar media were made and autoclaved. Then, all the media was poured into petri dishes using the pour plate method and labeled accordingly.

2.4.1 Serial Dilutions

Each of the water samples was used to serially dilute to concentrations of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} in order to spread on the MAC, NA and EMB media plates. Only MAC, NA and EMB media plates were used for serially diluted water samples along with the raw water samples. The rest of the media plates which are XLD, TCBS and MSA, were only used for the raw water samples, no serial dilution was done to spread in these media. For doing the serial dilution method, at first 9 ml of autoclaved saline water (0.9% NaCl) was taken in each of the test tubes and then 1 ml of

water was added to it, making a total volume of 10 ml. This test tube was labeled 10- 1 dilution, then, 1ml of this solution was transferred to another test tube containing 9 ml of saline water, and labeled 10-2 . This is how the water samples were diluted ten folds up to 10-4. Then, each of the serially diluted water samples was mixed using the vortex machine.

2.5 Spread plate method

Petri dishes containing NA, XLD, MSA, MAC, TCBS and EMB media were taken for both of the raw and diluted water samples. After labeling each plate properly, from each of the water samples, 60 µl of the solution were added onto their corresponding, labeled plates using a micropipette. Then, using a spreader, the samples were spread onto the plates and incubated for 24 hours- 48 hours at 37 ^{o} C. After the incubation, the plates were observed for any growth of organisms and were noted and used for further study. The selection of the isolates was done based on their morphology. Among the numerous colonies growing in the media, only the colonies that had different colony sizes, color, and textures were taken forward for the study. The colonies which looked similar to the naked eyes were discarded. The individual selected colonies were subcultured in the NA media.

2.6 Identification

2.6.1 Microscopic view of the bacteria

Gram stain Gram staining was done to differentiate between two principal groups of bacteria: gram positive and gram negative. Gram positive bacteria give a purple stain while Gram negative bacteria give a pink stain. The morphology of the bacteria can also be checked using this method. There are four steps involved in Gram staining. Firstly, a smear of bacteria is prepared onto a glass slide and heat treated. Gram's iodine is then added to the smear. If the bacteria are gram positive their thick peptidoglycan layers retain the purple color. Secondly, mordant is applied which stabilizes the purple stain. Thirdly, 95% ethanol is used to wash the stain; if the bacterium is negative its thin peptidoglycan layer is washed off. Finally, a counter dye Safranin is added

which is retained by gram negative bacteria. Lastly, the slide is viewed under a compound microscope to analyze the color and shape of the bacteria (Cappuccino and Sherman, 2005).

2.6.2 Biochemical characterization of the bacteria

Biochemical analysis is based on the different biochemical activities of the various bacterial species and is used for the identification of these specific species. Several biochemical tests were carried out in order to have a presumptive identification of the potential bacteria chosen before. Most of the methods were done according to the microbiology laboratory manual (Cappuccino & Sherman, 2005). The biochemical tests performed were Triple sugar iron agar test, IMViC test (Indole production test, Methyl red test, Voges- Proskauer test, and Citrate utilization test), MIU test (Motility test, Indole test, and Urease test), and Catalase test.

Triple Sugar Iron Agar test

Triple sugar iron test was done to differentiate among the different groups or genera of the Enterobacteriaceae based on the ability to reduce sulfur and ferment carbohydrates. Triple sugar iron slants were prepared in the test tubes by autoclaving at 15 psi 121^0 C. Using sterile technique; a small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the tubes by means of a stab and streak inoculation method with an inoculating needle. So, to conduct the test, a straight inoculating needle was used to pick an isolated colony and inoculate the TSI slant by first stabbing the butt down to the bottom, and then streaking the surface of the slant. Then, the tubes were incubated for 24 hours at 37^0 C (Cappuccino & Sherman, 2005).

1. If lactose (or sucrose) is fermented, a large amount of acid is produced, which turns the phenol red indicator yellow both in the butt and in the slant. Some organisms generate gases,

which produce bubbles/cracks in the medium.

2. If lactose is not fermented but the small amount of glucose is, the oxygen-deficient butt will be yellow (remember that butt has comparatively more glucose than slant i.e. more media and more glucose), but on the slant, the acid produced (less acid produces in slant as media in slant is less) will be oxidized to carbon dioxide and water by the organism and the slant will be red (alkaline or neutral pH).

3. If neither lactose/sucrose nor glucose is fermented, both the butt and the slant will be red. The slant can become a deeper red-purple (more alkaline) as a result of the production of ammonia from the oxidative deamination of amino acids (remember peptone is a major constituent of TSI agar).

4. If H_2S is produced, the black color of ferrous sulfide is seen.

Methyl red test

Methyl red test was done to determine the ability of the bacteria to oxidize glucose with the production and stabilization of high concentration of acid end products. MR-VP broth of 9 ml in each test tube was prepared by autoclaving at 15 psi 121^0 C. Using sterile technique, small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the tubes by means of a loop inoculation method with an inoculating loop and the tubes were incubated for 24 hours at 37◦C. After 48-hour incubation, for the MR test, about 5 drops of methyl red indicator solution are added to the MR test tubes. A positive reaction is indicated if the color of the broth changes to red within a few minutes. Appearance of a cherry red color indicates positive results, orange color indicates inconclusive results and yellow indicates negative results (Cappuccino & Sherman, 2005).

Voges Proskauer test

Voges Proskauer test was done to determine the capability of the organism to produce non acidic or neutral end products such as acetylmethylcarbinol. The reagent used in this test is Barritt's reagent, consisting of a mixture of alcoholic α-naphthol and 40% potassium hydroxide solution. Detection of acetyl methyl carbinol requires this end product to be oxidized to a diacetyl compound. This reaction will occur in the presence of the α-naphthol catalyst and a guanidine group that is present in the peptone of the MR-VP medium. Test tubes containing 5 ml of MRVP broths were inoculated with the samples and incubated at 37oC overnight. To the aliquot of MR-VP broth after 24-hour incubation, 0.6 ml (12 drops) of 5% alpha-naphthol (reagent A) was added followed by 0.2 ml (4 drops) of 40% KOH (reagent B). The tube was gently moved to expose the medium to atmospheric oxygen (30 seconds-1 minute) and the medium was allowed to remain undisturbed for 10-15 minutes. As a result, a pink complex is formed, imparting a rose color to the medium. The test was read, but not beyond, one hour following the addition of the reagent. Development of a deep rose color in the culture 15 minutes following the addition of Barritt's reagent is indicative of the presence of acetyl methyl carbinol and represents a positive result. The absence of rose color is a negative result (Cappuccino & Sherman,2005).

Citrate utilization test

Citrate utilization test was done to differentiate among enteric organisms on the basis of their ability to ferment citrate as a sole source of carbon by the enzyme citrate permease. This ability depends on the presence of citrate permease enzyme that facilitates the transport of citrate in the cell. The citrate agar was prepared and 2 ml media was added to clean vials. The vials were autoclaved at 15 psi 121^0 C and then left to cool at a slanted position in order to create a butt and slant. Using sterile technique, a small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the vials by means of a streak inoculation method with an inoculating needle and the vials were incubated for 48 hours at 37^0 C (Cappuccino & Sherman, 2005). The color of the media was observed after incubation. Color change to blue is considered to be positive and no color change was considered to be negative.

MIU (Motility- Indole- Urease) test

MIU test was done to simultaneously determine the ability of the bacteria to produce indole, check motility and degrade urea by means of the enzyme urease. This medium contains peptones that consist of carbon and nitrogen needed for bacterial growth. At the same time, the microorganisms which produce urease, is provided with a source of nitrogen. The demonstration of motility is allowed due to the low agar concentration in the media by showing diffused growth away from the stab inoculated line (Westmann, 2018). For this test, the MIU was made, boiled and autoclaved. Some empty test tubes were autoclaved as well. On a separate flask, 40% urea solution was made and filtered. MIU media was prepared by autoclaving at 15 psi 121^0 C and after autoclave, the media was cooled to about 50-55^0 C and 100 ml of urea solution was added aseptically to 900 ml base medium. After that, 6 ml solution was transferred to each sterile test tube and the media was left to cool down completely until it had a semi solid consistency. Using sterile technique, a small amount of the experimental bacteria from 24-hours old pure culture was inoculated into the tubes by means of a stab inoculation method with an inoculating needle and the needle was then withdrawn and taken out in a vertical manner. The tubes were then incubated for 24 hours at 37^0 C (Cappuccino and Sherman, 2005). After that, the tubes were checked for any change in color and bacterial growth.

Motility test

A positive reaction is shown by clouding of the medium or by growth extension from the inoculating line. A negative reaction is seen when the growth is restricted to the inoculating line. So, motility was indicated by a spiraling growth away from the site of inoculation. Non-motile bacteria generally give growths that are confined to the stab-line, have sharply defined margins and leave the surrounding medium clearly transparent. On the other hand, motile Bacteria typically give diffuse, hazy growths that spread throughout the medium rendering it slightly opaque (Acharya, 2015)

Urease test

If a microorganism produces urease enzyme, the color of the slant changes from light orange to magenta or pink. If microorganism does not produce urease the agar slant and butt remain light orange (medium retains original color) (Acharya, 2015). Therefore, pink color indicated positive for utilization of urea whereas no color change indicates a negative reaction.

Indole Production test

The indole production test was done to determine the ability of the bacteria to degrade the amino acid tryptophan by the enzyme tryptophanase. In order to test for indole production, 5 drops of Kovac's reagent was added directly into the tubes. Finally, it was observed for color changes to determine whether the result is positive (cherry red ring) or negative (yellow) (MacWilliams, 2009).

Catalase test

This test was done to demonstrate the production of catalase enzyme by detecting the ability of microorganisms to breakdown hydrogen peroxide and produce oxygen and water. A very small amount of fresh bacterial colony was transferred into a clean and sterile glass slide using a sterile wooden stick. To that, a drop of 3% H2O2 was placed on top of the bacterial colony by using a dropper and mixed with the toothpick. For positive results, bubbles were observed in a few seconds (Aditi et al., 2017).

Oxidase Test

The oxidase test detects bacteria that produce cytochrome c oxidase, which is an enzyme of the bacterial transport system. All aerobic bacteria are oxidase positive. In this procedure, Kovacs

Oxidase Reagent was used. Its composition is 1% tetra-methyl-p-phenylenediamine dihydrochloride, in water. A filter paper was saturated with oxidase reagent using dropper. Next, a portion of the paper was heavily smeared with the sample using a sterile toothpick. Finally, the smear was checked for any change in colour. Organisms possessing the cytochrome c oxidase enzyme gave positive tests. No color change or presence of light pink color of the colonies is indicative of the absence of oxidase activity and is a negative result. In positive cases, a deep blue or purple stain appears within 5-10 seconds (Cappuccino and Sherman, 2005).

2.7 Preservation of the isolates

In order to store the samples, colonies from subcultures were taken and inoculated in T1N1 media. After 24 hours incubation at 37^0 C, paraffin oil was added to the media and stored at - 20^0 C.

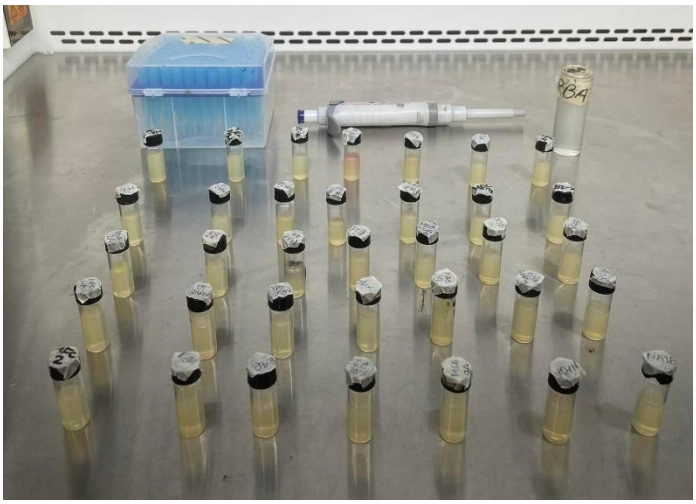


Figure III: Adding paraffin oil in T_1N_1 media

2.8 Antibiotic resistance and susceptibility analysis

This analysis aims to check the performance of antimicrobial susceptibility of certain isolated bacteria for particular infections and as well as the possible drug resistance in pathogenic organisms. It is one of the most basic and important tasks in a microbiology laboratory. This test is performed manually and the possible methods include the disk diffusion and the gradient diffusion methods.

2.8.1 Disk Diffusion Test

The disc diffusion test is one of the classic microbiology techniques and because of its simplicity, effectiveness, and efficiency; it is still most widely used for determining the antibiotic resistance pattern of microorganisms. At first, Mueller-Hinton agar plates are prepared and a suspension of isolates of approximately $3X10^8$ CFU/ml, which was prepared to a McFarland standard of 1.0, was spread evenly onto the agar plates. Five different antibiotic discs were placed on the surface of a single plate and were incubated for 16-24 hours at 35°C. The results were observed and their zone of inhibition around each antibiotic disc was measured in millimeter. The size or the diameter of the no-growth zone is dependent on the inoculum density as well as the diffusion rate in the agar. The size of the zone can be compared with the reference table and can be interpreted as whether the organism is susceptible, intermediate, or resistant to a particular antibiotic (Christenson and Relich, 2018).

Chapter 3: Results

Biochemical identification

Several colonies with different morphologies and colors were found in each plate after spread plating during the study period. Colonies were chosen based on their unique morphology. 119 isolates were selected to conduct several biochemical tests to determine the probable organisms. It was necessary to depict the graveness of the threat of the isolates by identifying the pathogens. For identification of the isolates, seven biochemical tests (Oxidase test, Catalase test, TSI (Triple Sugar Iron), MIU (Motility Indole Urea), MR (Methyl Red), Vp (Voges–Proskauer), Citrate) were performed. The results obtained from the biochemical tests were analyzed using the **Microrao** software.

Biochemical tests and observation revealed the identities of 25 different types of organisms among the 119 isolates. The majority of the isolates from drinking water were belonging to the Gram-negative group including *E. coli* (13), *Serratia marcescens* (8), *Providencia spp.* (6), *Pseudomonas spp.* (4), *Vibrio spp.* (4), *Shigella dysenteriae* (4), *Salmonella spp.* (3), *Klebsiella pneumonia* (3), *Proteus mirabilis* (3), *Yersinia spp.* (2), *Citrobacter spp.* (1), *Shigella sonnei* (1), *Vibrio salmonicida* (1), *Hafnia alvei* (1), *Acinetobacter spp.* (1), *Hafnia spp.* (1), and *Proteus spp.* (1). The remaining 8 isolates belonged to Gram-positive group including *Bacillus spp.* (34), *Staphylococcus spp.* (10), *Micrococcus spp.* (6), *Streptococcus spp.* (4), *Staphylococcus aureus* (4), *Listeria spp.* (2), *Arthrobacter spp.* (1), and *Lactobacillus spp.* (1).

The most prevalent organism in the water samples was *Bacillus spp.* (34), representing 28.57% of the total number of isolates obtained. The highest number of *Bacillus spp.* (10) was found in the water of the Uttara and Dhanmondi areas. The next most prevalent organism, accounting for 10.92% of all isolated organisms, was *E. coli*. This was followed by *Staphylococcus spp.* (8.40%), *Serratia marcescens* (6.72%), *Micrococcus spp.* (5.04%), and *Providencia spp.*

(5.04%). 3.36% of the organisms were *Streptococcus spp., Pseudomonas spp., Vibrio spp., Shigella dysenteriae* and *Staphylococcus aureus*. In addition, *Salmonella spp.*, *Klebsiella pneumoniae*, and *Proteus mirabilis* were also found, representing only 2.52% of the total number of organisms. Furthermore, the presence of *Yersinia spp*. and *Listeria spp*. was detected in 1.68% of samples. The least found organisms were *Citrobacter spp*., *Shigella sonnei, Vibrio salmonicida, Arthrobacter spp., Hafnia alvei, Lactobacillus spp., Acinetobacter spp., Hafnia spp., and Proteus spp.* (0.84%).

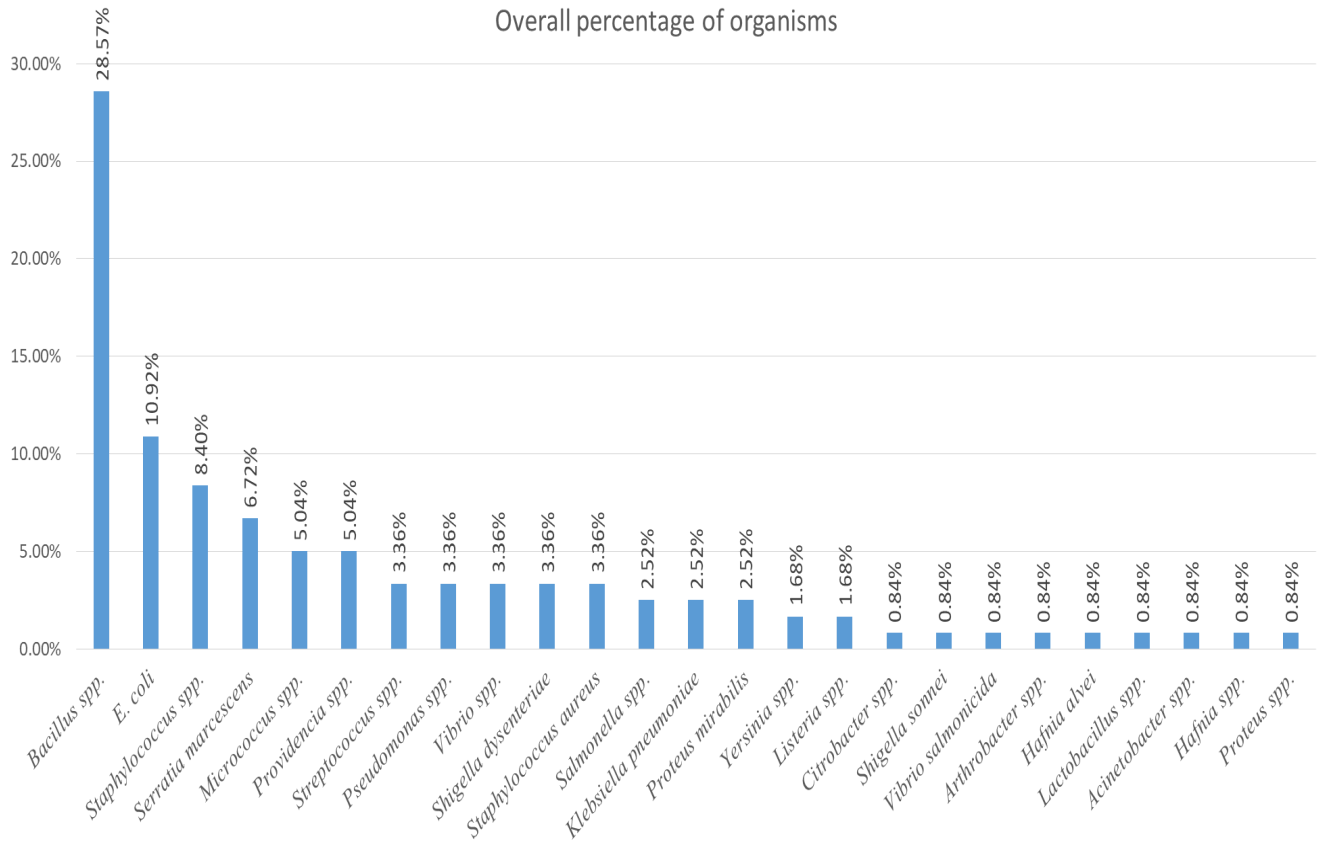


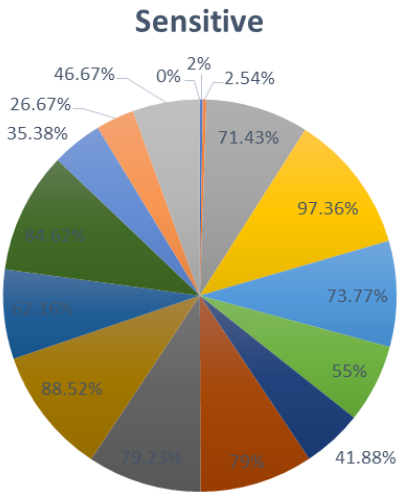
Table (I)- Overall percentage of all the organisms isolated from the water sample

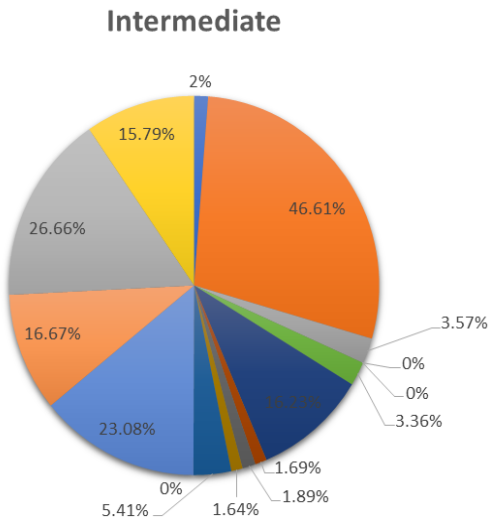
Antibiotic resistance/susceptibility pattern of bacterial isolates

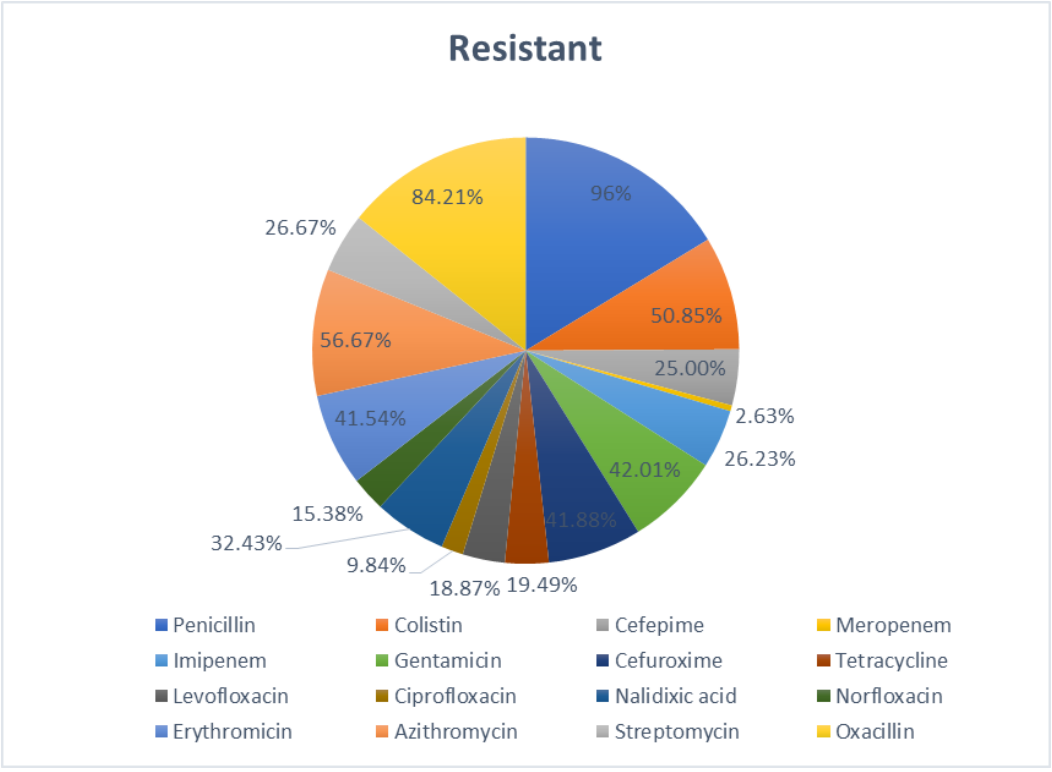
Antibiotic susceptibility test was done to assess the effectiveness of regularly prescribed antibiotics against the bacterial isolates.

All 119 isolates were tested against 16 different antibiotics from 8 groups, using Kirby–Bauer disc diffusion, standardized and evaluated by the methods of the National Committee for Clinical Laboratory Standards (T. Odonkor & K. Addo, 2018). Meropenem, Imipenem from the Carbapenem group; Penicillin G/V and Oxacillin from the Penicillins group; Erythromycin, Azithromycin from the Macrolides group; Streptomycin, Gentamicin from the Aminoglycosides group, and Cefuroxime, Cefepime from the Cephalosporin group were the disks used in our study. In addition, all the disks from the Quinolones/Fluoroquinolones group (Ciprofloxacin, Levofloxacin, Nalidixic Acid, and Norfloxacin) were used. The study also included one disk from the Polypeptides group (Colistin Sulfate) and one from the Tetracyclines group (Tetracycline).

The charts showing the percentage of isolates that were resistant, sensitive and intermediate to the disks are given below,







Results of the antibiotic sensitivity testing revealed that the isolates showed the highest resistance to Penicillin representing 96%, and Oxacillin representing 84.21%. This is followed by Azithromycin (56.67%), Colistin (50.85%), Gentamicin (42.01%), Cefuroxime (41.88%), and Erythromycin (41.54%). In addition, 32.43% of the isolates showed resistance to Nalidixic Acid, 26% to Imipenem and Streptomycin and 25% of the isolates to Cefepime. The organisms showed the lowest resistance to Tetracycline (19.49%), Levofloxacin (18.87%), Norfloxacin (15.38%), and Ciprofloxacin (9.84%). It was interesting to note that only 2.63% of the isolates were resistant to Meropenem.

In contrast, the isolates were most sensitive to Meropenem (97.36%), Ciprofloxacin (88.52%) and Norfloxacin (84.62%). 79% of the isolates showed sensitivity to Tetracycline and Levofloxacin. The isolates were also sensitive to Imipenem (73.77%), Cefepime (71.43%), Nalidixic Acid (62.16%). No isolate was sensitive to Oxacillin, and only 2% of the organisms were sensitive to Penicillin and Colistin.

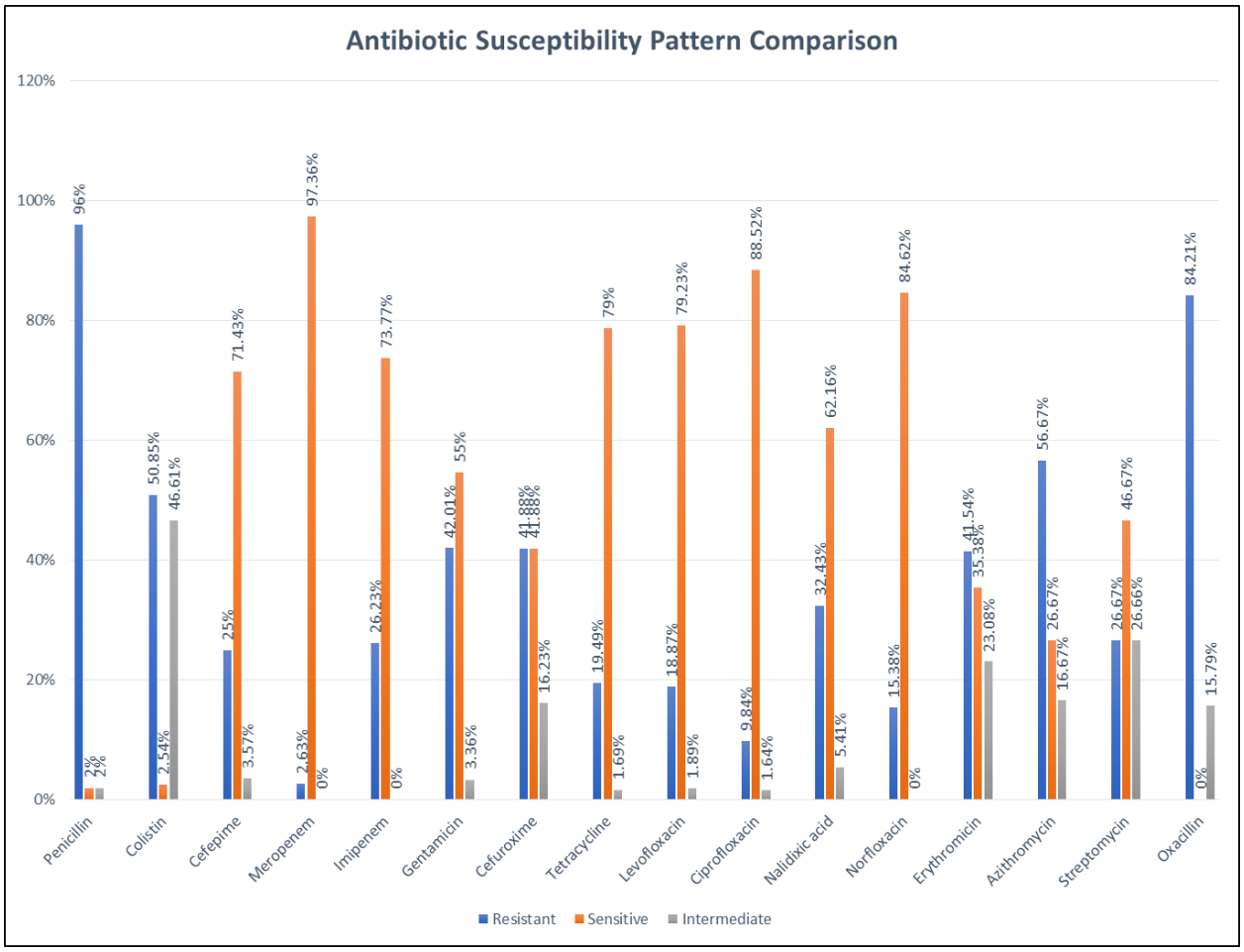


Table (II)- Antibiotic susceptibility patterns of all the organisms isolated from the water sample

Biochemical Test Results

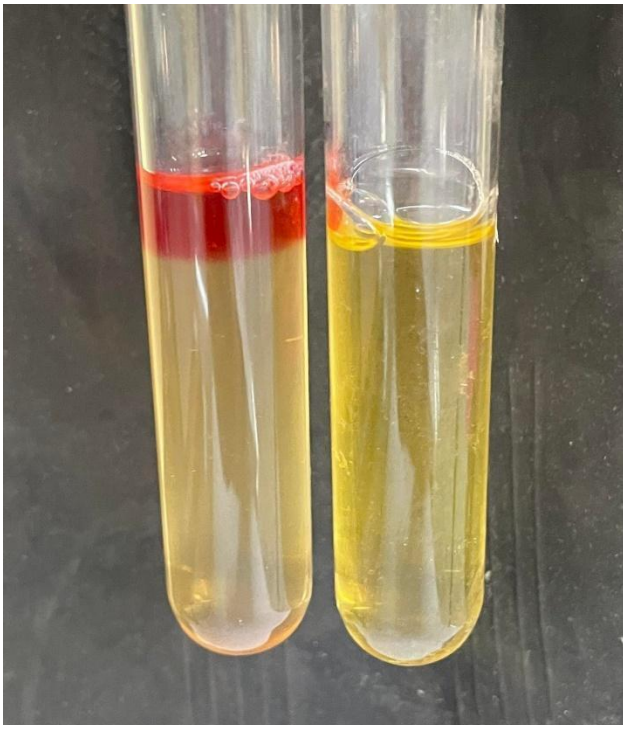


Figure (IV): Positive and negative test results for MR test.

Figure (V): Positive and negative test results for VP test.

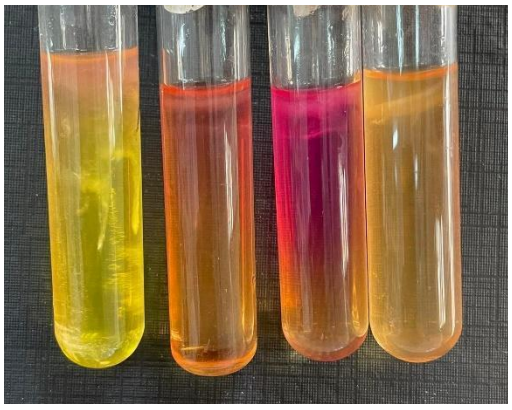


Figure (VI): MIU test result

Figure(VII): Positive (blue) and negative (green) result for Citrate test

Figure VIII: TSI test results

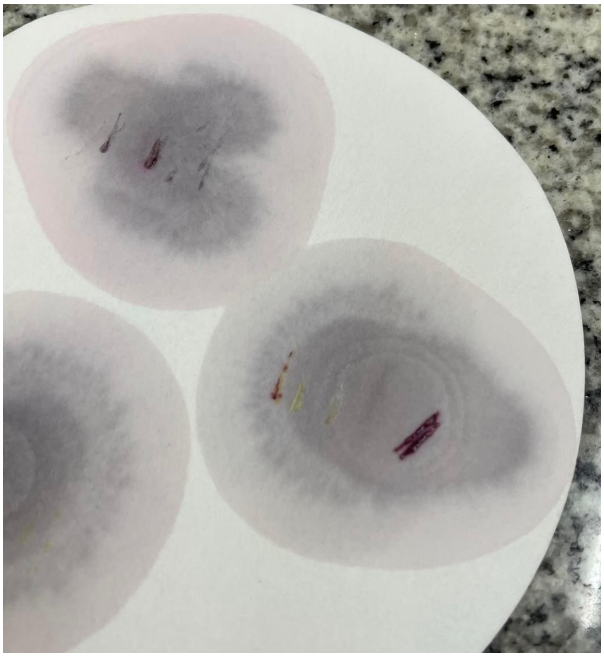


Figure (IX): Catalase test (Positive).

Figure (X): Positive and negative Oxidase test result.

Antibiotic sensitivity test

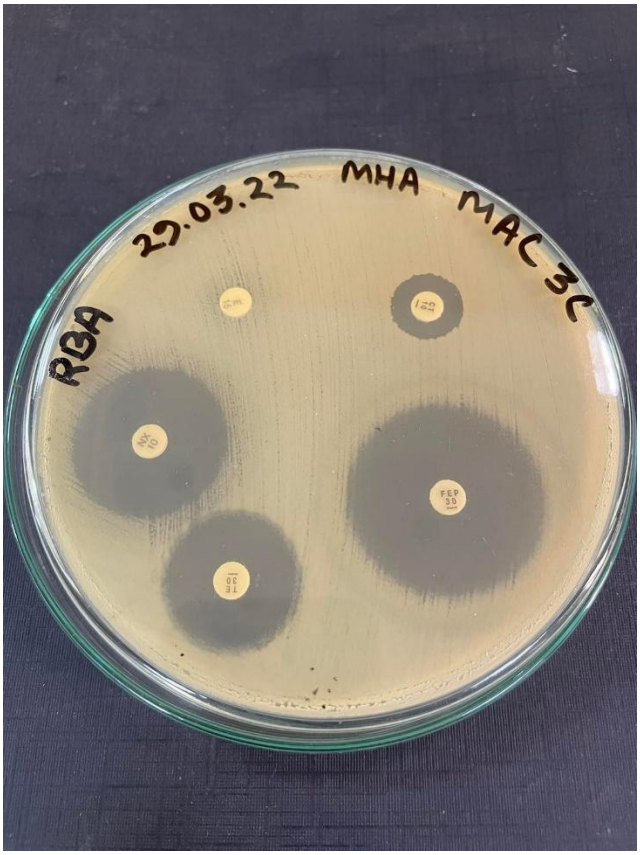


Figure XI: Organisms showing susceptibility and resistance to different antibiotics on Mueller Hinton Agar plates.

Chapter 4: Discussion

Safe drinking water is a necessity for all living things. In Bangladesh, using contaminated surface water is a major cause of waterborne illnesses. This study's objective was to assess the quality of the city of Dhaka's drinking water in various areas. Samples were collected from various parts of the city of Dhaka. These mostly consist of the old town, Mohakhali, Uttara, Mirpur, and Dhanmondi. These areas were put together in five separate batches. 26 water samples were collected and from these 119 isolates were separated. These indicated that the drinking water from these parts of Dhaka city had poor bacteriological quality. 25 different organisms have so far been identified from the 119 different isolates taken from the five separate batches, and they are: *Salmonella spp, Serratia marcescens, Micrococcus spp, E.coli, Staphylococcus spp, Proteus mirabilis, Sterptococcus spp., Citrobacter spp, Basicllus spp, Shigella sonnei, Pseudomonas spp., Klebsiella pneumoniae, V. salmonicida, Arthrobacter sp, Yersinia spp, Hafnia alvei, Vibrio spp, Lactobacillus spp, Shigella dysenteriae, Acinetobacter spp, Hafnia spp, Providencia spp, Staphylococcus aureus, Proteus spp, Listeria spp.*

Surface water, groundwater, and rainfall are Bangladesh's three main water sources. Across Bangladesh, the Buriganga, Brahmaputra, Meghna, and Shitolakha river system discharges a significant volume of surface water, some of which seeps into the ground to create groundwater. The source of 93% of the stream flow that flows through the nation is not Bangladesh. Bangladesh has a total of three sources of water: rainfall, groundwater recharged by a portion of the infiltrated rainwater, and surface runoff from the remaining rainwater. (Acharjee et al., 2011)

Bangladesh's capital, Dhaka, has struggled to provide its residents with clean drinking water, and as the only provider, DWASA (Dhaka Water Supply & Sewerage Authority) is in charge of supplying water to Dhaka city. Since 78% of DWASA water is below ground, groundwater is gradually being depleted. Due to leaks, contamination from old and damaged sewerage pipes, and an infrequent water delivery, the majority of the distribution system has been weak. Water-borne illnesses such as cholera, paratyphoid fever, dysentery, jaundice, and others may be caused by microbial contamination of drinking water along the Dhaka WASA supply chain's distribution lines. WASA provided drinking water in and around Dhaka City, where it found poisonous PCP contamination and pathogenic bacterial contamination that is concerning for the general public's health. (Hossain et al., 2021). In a study conducted by Hossain et al.(2021) they found that 50% of the water in the distribution line and 5% of the tap water were both total coliform-contaminated. According to the questionnaire study, 41% of the respondents had waterborne illnesses within the previous year (2020). Due to odor, 48% of the respondents did not drink the water provided by DWASA. About 56% of those surveyed concurred that the main cause of water contamination is a defective water distribution line (Hossain et al., 2021). From various WASA water supply chain outlets, a total of 45 samples were taken. 29 of these samples were taken from household taps, 5 from street pipe line taps, and 11 from WASA source pumps. 57.78% of the water samples tested positive for coliform, while 51.11% of the samples tested positive for E. coli bacterium (Mahbub et al., 2012). In another study, bacteriological Quality of Drinking Water Samples were tested across Bangladesh. A total of 106 water samples (tube well, deep well, surface, and municipal supplies) from 37 Bangladeshi districts were examined. Of these, 34% of the samples contained fecal coliform and 80% of the samples contained coliform contamination. Twenty of the 86 bacterial isolates were identified as *Enterobacter aerogenes* and twenty as *Escherichia coli* (parvez et al., 2016). In another study conducted by Razzak et al. they

focussed on the concerns of the existence of coliform, fecal coliform, and other pathogens in both tap water and commercially available bottled water. They found that each sample of tap water was highly contaminated with various microorganisms. The samples were fecal coliform-contaminated, indicating fecal contamination and a possible danger of additional microbial infections. *E. coli* and *Klebsiella spp.* were found along with *Salmonella spp*. Which was present in more than half of the samples. *Shigella spp*. were present in almost every sample of tap water. Other samples had *Pseudomonas spp.*, which may become opportunistic in favorable conditions. Other samples also contained *Vibrio spp. Staphylococcus spp.* It was clear that the tap water samples' microbiological quality was subpar for consumption and could seriously endanger customers' health.

In our project we found *E.coli* in 10.92%, *Bacillus spp.* In 28.57%, *Staphylococcus spp* in 8.40%, *Serratia marcescens* 6.72%. *Micrococcus spp* 5.04%, *Providencia spp* 5.04%, *Streptococcus spp.* 3.36%. *Pseudomonas spp.* 3.36%, *Vibrio spp.* 3.36%, *Shigella dysenteriae* 3.36%, *Staphylococcus aureus* 3.36%, *Salmonella spp.* 2.52%, *Klebsiella pneumoniae* 2.52%, *Proteus mirabilis* 2.52%, *Yersinia spp.* 1.68%, *Listeria spp* 1.68%, *Citrobacter spp* 0.84%, *Shigella sonnei* 0.84%, *V. salmonicida* 0.84%, *Arthrobacter spp*. 0.84%, *Hafnia alvei* 0.84%, *Lactobacillus spp.* 0.84%, *Acinetobacter spp*. 0.84%, *Hafnia spp*. 0.84%, *Proteus spp.* 0.84%. Among these 25 organisms *Bacillus spp* was found in the highest range. The remaining organisms were not found in considerable amounts. Only a small fraction of the urban population in Bangladesh relies on open water sources such ring wells, ponds, and rivers for their daily water needs, whereas 8 to 9% of the population is predominantly fed by hand-operated tube wells (Acharjee et al., 2011). The purpose of a recent research of Acharjee et al. (2011) was to classify the bacterial ecology in river and canal water and identify harmful microorganisms. 18 water samples were collected from various rivers and canals. Total coliform, faecal coliform, *Escherichia coli, Klebsiella spp., Salmonella spp., Shigella spp., Vibrio cholerae, Aeromonas spp.,* and Fungi were discovered to be present in all water sources. *Escherichia coli, Salmonella species,* and *Vibrio cholera* were discovered in tap water from the southern Isfahan province during a research effort in Iran. *Salmonella species, Vibrio cholera,* and *E. coli* species were each detected in 3.47%, 2.08%, and 26.38 percent of the population. By doing tests on river water, the positive samples for *V. cholerae* were acquired. In the southern part of Isfahan, tap water samples

tested positive for *E. coli, Salmonella spp., and V. cholera* in proportions of 44.44%, 5.55%, and 8.33%, respectively. In addition, there were much more polluted tap water samples in the months of July and August than in the months of November and December. *E. coli* was detected in 10.92% of our research, *Salmonella spp*. in 2.52%, and *Vibrio* in 3.36%. This range matches our rate, most likely as a result of the countries' ecological similarities (Momtaz et al., 2013).

As part of our study, we gathered drinkable water off the side of the road. These water sources can come from tube wells, rivers, canals, or WASA. It is pretty evident that the water we had collected will have microbial contamination because other research has identified microbial contamination in these sources.

In our study, 16 different types of antibiotic disk had been used to run antimicrobial susceptibility test and they are- Penicillin, Oxacillin, Colistin, Cefuroxime, Cepfepime, Meropenem, Imipenem, Ciprofloxacin, Norfloxacin, Levofloxacin, Nalidixic acid, Streptomycin, Gentamicin, Erythromycin, Azithromycin and Tetracycline. Percentage of antibiotic resistance among bacteria from our study is- Penicillin 96%, Colistin 50.85%, Cefepime 25%, Gentamicin 42.01%, Cefuroxime 41.88%, Erythromycin 41.54% are notable, rest of them were not that considerable. The isolates showed highest resistance to Penicillin.

Razzak (S.B.A et al.,2021) examined the tap and bottled water used for the study and discovered that all strains of *E. coli, Klebsiella spp., Salmonella spp., Shigella spp., Vibrio spp., Pseudomonas spp., and Staphylococcus spp.* were 100% Penicillin G resistant. Additionally, his research showed that erythromycin was 50% resistant to *Salmonella spp.* and 100% resistant to *E. coli, Vibrio spp., Shigella spp., Pseudomonas spp., Staphylococcus spp.,* and *Klebsiella spp.* Additionally, Tetracycline was 50% resistant to *Klebsiella spp.* and 100% resistant to *E. coli, Salmonella spp., Shigella spp., Vibrio spp., Pseudomonas spp.,* and *Staphylococcus spp.*. And Streptomycin, Gentamicin, Nalidixic acid and Azithromycin was not resistant to any of these mentioned isolates. However, 96% of the *E. coli, Salmonella spp., Vibrio spp., Pseudomonas spp.,* and *Staphylococcus spp.* bacteria we examined in our study lacked penicillin resistance.

Resistance was also found in *Salmonella spp., Vibrio spp., Pseudomonas spp., Staphylococcus spp.,* and *E. coli*, as well as to 41.54% Erythromycin, 42.01% Gentamicin, 32.43% Nalidixic acid, 26.67% Streptomycin, 56.67% Azithromycin, and 19.49% Tetracycline.

Furthermore, according to Anowar (AK et al.,2016), 13.33% Tetracycline, 6.67% Ciprofloxacin, 6.66% Levofloxacin was resistant to 45 different isolates. However, in our project, 19.49% Tetracycline, 9.84% Ciprofloxacin, 18.87% Levofloxacin was resistant to 119 different isolates. Between 2016 and 2022, the amount of antimicrobial resistance rose for Tetracycline and Levofloxacin but declined for Ciprofloxacin.

In addition, according to Samer (Swedan & Alrub.,2019), a study was carried out in Jordan with 109 confirmed *E.coli* isolates recovered from drinking water sources, and among them, 18% of the isolates were resistant to Ciprofloxacin and 16% to Levofloxacin, but all of the isolates were sensitive to imipenem and meropenem as well as other antibiotic disks. However, we found *E. coli* in the water of Dhaka City, where it was resistant to 9.84% Ciprofloxacin, 18.87% Levofloxacin, and 26.23% Imipenem and didn't cross even 5% for Meropenem.

Another study from Ghana, Stephen (Odonkor & Addo., 2018) reported, multiple resistance is defined for *E.coli* from different source of drinking water and identified 28% Cefuroxime, 8% Ciprofloxacin, 23% Erythromycin, 5% Gentamicin, 4% Nalidixic acid, 21% Tetracycline and 32% Penicillin were found to be resistant in the water of Ghana. And according to our study, the resistance rate in Dhaka city's water in those mentioned antibiotic disks are- 41.88% Cefuroxime, 9.84% Ciprofloxacin, 41.54% Erythromycin, 42.01% Gentamicin, 32.43% Nalidixic acid, 19.49% Tetracycline and 96% Penicillin was found resistant to *E.coli* .

Finally, due to the presence of bacteria like *E. coli, Klebsiella pneumoniae, Salmonella spp.,* and others, drinking water from here and there after the current work is not advised at all. Another bacteria called *Pseudomonas spp.* has been discovered in this area; it may be an opportunistic

pathogen and endanger the health of children, the elderly, and people with compromised immune systems. Contamination of the environment, improper management, lack of information and training can all lead to contaminated drinking water.

Chapter 5: Conclusion

Diseases related to contamination of drinking-water constitute a major burden on human health. Interventions to improve the quality of drinking-water provide significant benefits to health and save millions of lives. Water is essential to sustain life hence an adequate, safe and accessible supply must be available to all (Ayenew 2004). After analyzing, the results of this study states that the drinking water used in street side food stalls and restaurants of Dhaka did not meet the

requirements for potable drinking water quality standards as it was contaminated with pathogens and bacteria and so the water is not good for public health at all. In order to evaluate the risk to the public's health, it is crucial to comprehend the severity of the matter and its causes. Every effort should be made in order to provide the safest drinking-water quality. The great majority of evident water-related health problems are the result of microbial (bacteriological, viral, protozoan or other biological) contamination (Ayenew 2004). Antibiotic resistance is accelerated by incorrect and excessive antibiotic use and inadequate infection control and precautions. At all societal levels steps can be taken to mitigate the consequences and halt the spread of resistance. While there are some new antibiotics in development, it is not anticipated that any of them will be effective against the most dangerous forms of bacteria that are resistant to antibiotics. Given the ease and frequency with which people now travel, antibiotic resistance is a worldwide issue that needs to be tackled by all nations and numerous sectors.When infections can no longer be treated by first-line antibiotics, more expensive medicines must be used. The progress of contemporary medicine is now under threat due to antibiotic resistance. Procedures including organ transplants, chemotherapy, and cesarean deliveries become noticeably riskier without effective antibiotics for the prevention and treatment of infections.

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APPENDIX-I

Media Composition

For our project, NA, MAC, EMB, MSA, XLD, TCBS, MHA, T1N1 media were used. All the media were measured based on the amount needed to use for our experiment and then the ingredients were boiled to dissolve agar. After the media properly dissolved, the media were autoclaved at 121°C for 15 minutes except XLD and TCBS.

The composition of all media used in the project have been given below,

● Nutrient Agar

Ingredients	Amount (g/L)
Beef extract	3.0 g
Peptone	5.0 g
Sodium chloride	8.0 g
Agar	15.0 g
Distilled water	1 L
Final pH	6.8 ± 0.2

- **MacConkey agar (MAC)**

Ingredients	Amount (g/L)
Peptone	20 g
Lactose monohydrate	10 g
Bile salts	1.5 g
Sodium chloride	5 g
Neutral red	0.03 g
Crystal Violet	0.001 g
Agar	13.5 g
Distilled Water	1 L
Final pH	7.1 ± 0.2

● Eosin Methylene Blue (EMB) agar

Ingredients	Amount (g/L)
Peptone	10 g
Lactose	5 g
Sucrose	5g
Dipottasium,PO4	2g
Eosin Y	0.4g
Methylene blue	0.065g
Agar	13.5g
Distilled Water	1 L
Final pH	7.1 ± 0.2

● Mannitol Salt Agar (MSA)

Ingredients	Amount (g/L)
Proteose peptone	10 g
Sodium chloride	75 g
D- mannitol	10 g
Beef extract	1 g
Phenol red	0.025 g
Agar	15 g
Distilled Water	1 L

pH final	7.4 ± 0.2

● Xylose Lysine Deoxycholate (XLD) agar

Ingredients	Amount (g/L)
Lactose	7.5 g
Sucrose	7.5 g
Sodium Thiosulfate	6.8 g
L-Lysine	5.0 g
Sodium Chloride	5.0 g
Xylose	3.75 g
Yeast Extract	3.0 g
Sodium Deoxycholate	2.5 g
Ferric Ammonium Citrate	0.8 g

Phenol Red	0.08 g
Agar	15.0 g
Distilled Water	1 L
Final pH	7.4±0.2

- **Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar**

Ingredients	**Amount (g/L)**
Proteose peptone	10 g
Yeast extract	5 g
Sodium thiosulphate	10 g
Sodium citrate	10 g
Bile	8 g
Sucrose	20 g

Sodium chloride	10 g
Ferric citrate	1 g
Bromothymol blue	0.04 g
Thymol blue	0.04 g
Agar	15 g
Distilled Water	1 L
Final pH	7.4±0.2

● Mueller Hinton Agar (MHA)

Ingredients	**Amount (g/L)**
Beef Extract	2 g
Acid Hydrolysate of Casein	17.5 g
Starch	1.5 g

Ingredients	Amount (g/L)
Agar	17 g
Distilled Water	1000 ml
Final pH	7.3 ± 0.1

● Tryptone Salt Agar (T1N1 Agar)

Ingredients	Amount (g/mL)
Trypticase or Tryptone	10 g
NaCl	10 g
Agar	20 g
Distilled water	1000 mL
Final pH	7.6-7.8

- **MR (Methyl Red)-VP broth (Voges-Proskauer)**

Ingredients	Amount (g/mL)
Peptone	7 g
Dextrose (D-glucose)	5 g
di-potassium hydrogen phosphate	5 g
Distilled water	1000 mL
Final pH	6.9 ± 0.2

- **Triple Sugar Iron Agar (TSI)**

Ingredients	Amount (g/mL)
Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	20.0 g

Glucose	1.0 g
Lactose	10.0 g
Sucrose	10.0 g
Ferrous sulfate or ferrous ammonium sulfate	0.2 g
NaCl	5.0 g
Sodium thiosulfate	0.3 g
Phenol red	0.024 g
Agar	13.0 g
Distilled water	1,000 mL
Final pH	7.3 ± 0.1

- **Simmons Citrate Agar**

Ingredients	Amount (g/mL)
Ammonium dihydrogen phosphate	1 g
Dipotassium phosphate	1 g
Sodium chloride	5 g
Sodium citrate	2 g
Magnesium sulfate	0.20 g
Agar	15 g
Bromothymol blue	0.08 g

Distilled water	1 L
Final pH	6.8±0.2

APPENDIX-II

Reagent Composition

1. Gram-Staining reagent:

- **Gram's iodine:**

Ingredients	Amount (g/mL)
Iodine	1g

Potassium iodine	2g
Distilled water	300 mL

The ingredients are measured by measuring machine and then grinded in a morter pastel with little amount of water. The reagent need to be stored in a light resistant amber bottle in room temperature.

- **Safranin:**

Ingredients	Amount (g/mL)
Safranin	2.5 g
95% ethanol	10 mL
Distilled water	90 mL

- **Crystal Violet:**

i) 2 g Crystal Violet is dissolved into 20 mL 95% Ethyl Alcohol.
ii) 0.8 g Ammonium Oxalate Mohohydrate into 80 mL distilled water.

Solution (i) and (ii) are mixed together and allowed them to stand overnight at room temperature.

2. Oxidase Test reagent:

- **Kovac's reagent:**

Ingredients	Amount (g/mL)
Tetramethyl-p-phenylenediamine dihydrochloride	0.1 g
Distilled water	10 mL

4. Vp reagent:

- **Barritt's A:**

Ingredients	Amount (g/mL)
Alpha naphthanol (5%)	50 g
Distilled water	1000 mL

- **Barritt's B:**

Ingredients	Amount (g/mL)
Potassium hydroxide (40%)	400 g
Distilled water	1000 mL

5. MR Test reagent:

- **Methyl red:** (50 ml)

Ingredients	Amount (g/mL)
Methyl red	1 g
95% ethanol	30 mL
Distilled water	20 mL

Supplementary

Growth of bacteria in different media plate

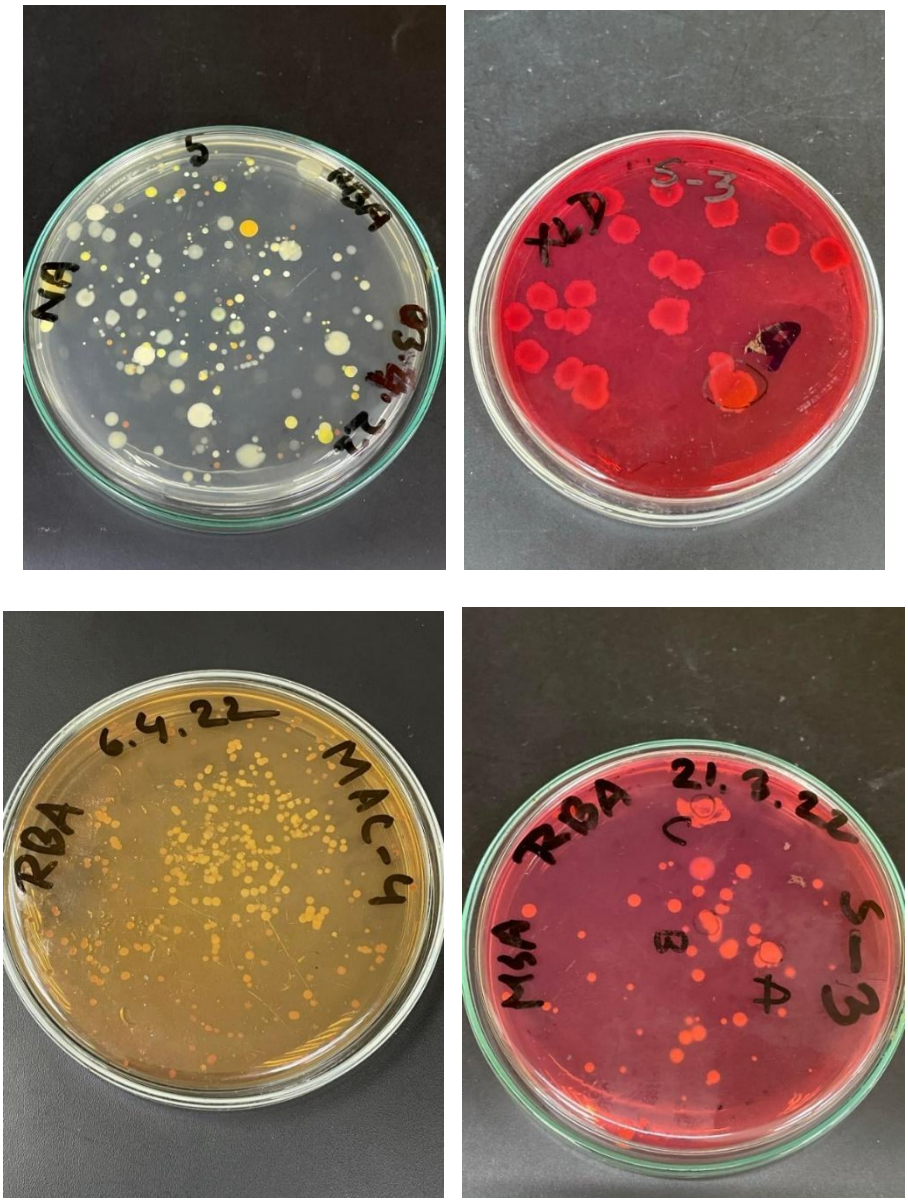

Figure XII: Bacterial growth on media plate

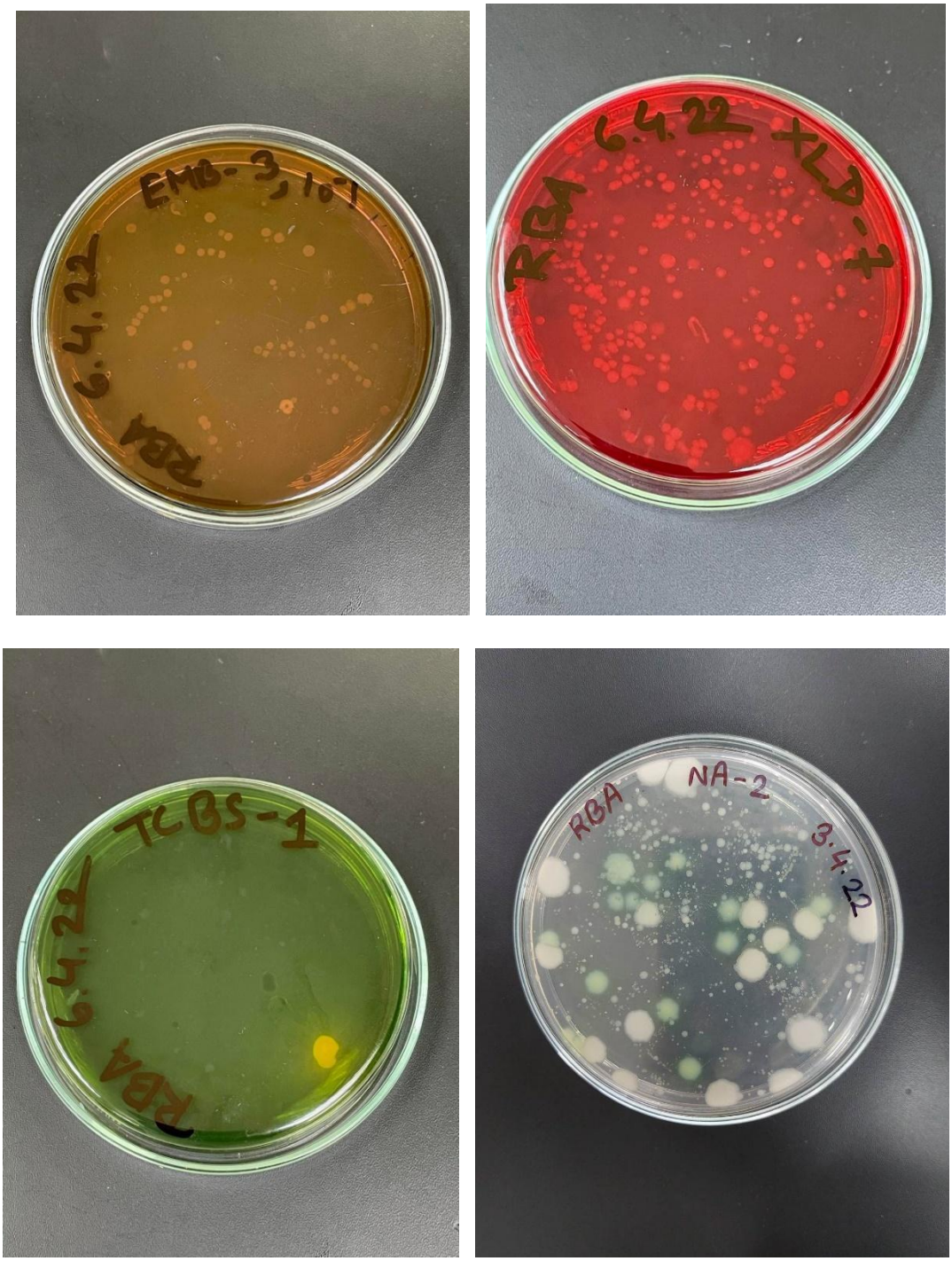

Figure XIII : Bacterial growth on media plate

Figure XIV: Filter paper with bacterial growth