BIOMEDICAL WASTE IN HEALTHCARE: A COMPREHENSIVE STUDY ON MICROBIAL ANALYSIS, RADIOACTIVE ANALYSIS AND PREVALENCE OF OPPORTUNISTIC BACTERIA

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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 B.Sc. in Biotechnology

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Declaration

It is hereby declared that

1. The thesis submitted is our own original work while completing a 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. We have acknowledged all of the main sources of help.

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ABSTRACT

Proper biomedical waste (BMW) management is one of the crucial steps in ensuring public health. Covid 19 has given an emergence to maintain proper waste management. Microbial analysis and radioactivity tests are two objectives of this paper. Microbial analysis in BRAC University wet lab and radioactivity tests in Bangladesh Atomic Energy Center (BAEC) has been performed with three tertiary public hospitals' waste disposals including solid and liquid wastes. The waste has been collected from central waste collection bins of different departments including - pathology, urology, surgery, cardiology and hospital ward. The collected waste has been in contact with human beings and affects the human immune system labeled as infectious and hazardous. In this regard, opportunistic bacteria search is also included in this research. The organisms that have been found in waste are - Escherichia coli, Enterococcus faecalis, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus. Some other biochemical tests are in process for the recognition of further characteristics. Hence this research paper has been conducted following normal safe ranges of alpha, beta, gamma ray and microbial population in human contact for radioactive analysis. The research process is carried out with analysis and preparing proper management techniques through bio-statistical analysis using SPSS. It also intends to create awareness among the people involved in the health care unit further.

Chapter 2

INTRODUCTION

2.1. Introduction :

The biomedical waste management system requires the procedure of disposing, dealing and handling of the waste materials that are produced throughout the healthcare spectrum. This process is a comprehensive work of protocols for ensuring the safety and proper maintenance of the disposals. Biomedical waste management systems are involved in minimizing the potential risks that are associated with the waste. These wastes have severe effects on human health as well as on the environment. Furthermore, people can get affected by these wastes both directly and indirectly through the environment since the wastes can be hazardous, infectious as well as harmful. All over the world the biomedical waste management system is operated with utmost caution because there's a great possibility that the inappropriate management of the biomedical waste management system can cause infections to the healthcare workers, the patients visiting the facilities and the surrounding environment and community. (Bansod, et. al.,2023) For this project, we have collected biomedical waste from three of the renowned public hospitals in Bangladesh which will be referred to as A, B & C hospitals. These samples were found outside of these hospitals, opened in the environment without proper disposal. Therefore to know the infections and radioactivity that might remain in these waste samples, biochemical tests and radioactivity tests, bio-statistical analysis have been conducted. Significance of biomedical waste management and what might occur if the wastes are not disposed properly are the main objectives we've tried to show in this project.

2.2.What are the biomedical wastes and key components of the Biomedical Waste Management :

2.2.a. Biomedical wastes and their classifications :

According to WHO(World Health Organization), biomedical wastes contain a diverse range of materials. The classifications are described below -

Infectious wastes : These wastes contain blood and other bodily fluids such as - infectious agents of the laboratories, discarded diagnostic samples, cultures and stocks like wastes produced from autopsies and infected animals. These wastes also might come from the patients with infections like swabs, bandages and disposable medical devices.

Pathological wastes : These wastes include human organs, tissues, fluids, body parts, contaminated animal carcasses etc.

Sharps wastes : These wastes include syringes, needles, disposable scalpels, blades etc.

Chemical wastes : These wastes include the solvents and reagents that are used in laboratory sterilants, disinfectants. Also these wastes contain the heavy metals which contain medical devices like mercury in broken thermometers and batteries.

Pharmaceutical wastes : These wastes include expired and contaminated drugs and vaccines.

Cytotoxic wastes : These wastes include the highly hazardous substances that are mutagenic, teratogenic such as cytotoxic drugs used in cancer treatment and their metabolites.

Radioactive wastes : These wastes include the products which are contaminated by the radionuclides including radioactive diagnostic material or radiotherapeutic materials.

Non-hazardous or general waste: These are such types of wastes that do not pose any specific biological, chemical, radioactive or physical hazard. (World Health Organisation, WHO, 2018)

2.2.b. Key components of the Biomedical Waste Management :

Biomedical waste management system is a well organized set of procedures which is required for assuring the safe and reliable disposal of waste produced in the healthcare spectrum. The goal of waste management is to minimize or reduce the risk either it be hazardous or infectious. Biomedical waste management system involves -

- Awareness training
- Generation
- Segregation
- Disposal
- Storage
- Transportation(Ramcharan, et. al.,2015)

Awareness training : Through various seminars, workshops and conferences personnel associated with healthcare sectors get trained on how to dispose of the wastes and segregate those including how to perform the management protocols with proper safety and ensuring minimal accidents.(Ramcharan, et. al.,2015)

Generation or production of wastes : Wastes produced from healthcare are various types of wastes including sharp wastes, plastic wastes, radioactive wastes, pathological wastes, pharmaceutical wastes and so on.(Ramcharan, et. al.,2015)

Segregation: Biomedical wastes are minutely sorted at the time of production of the wastes. Wastes are kept into different categories which means different bins are for different sorts of wastes. Such as there are different types of bins in the hospitals for pathological wastes, radioactive wastes, sharp containers, plastic wastes etc.(Ramcharan, et. al.,2015)

Disposal of the wastes : There are numerous disposal methods which are implemented depending on the type of the waste materials. Autoclaving, incineration are two most popular methods which are followed for disposing of the medical wastes. Other than these chemical disinfection, specialized recycling methods are also followed.(Ramcharan, et. al.,2015)

Transportation : For transporting the wages, designated containers provided for the hospitals are used. Expert people related with the transportation collect the waste and move them following safety protocols so that the risk gets reduced.(Ramcharan, et. al.,2015)

Effective implementation of Medical waste management in the public and private hospitals is a key determinant of improvement of hospital environment and providing quality service. (*4th Health, Population and Nutrition Sector Programme (4th HPNSP), Republic of Bangladesh Government.)*

2.3. The effects of biomedical wastes on human health

Health-care wastes are composed of the harmful microorganisms which can infect the hospital patients, health workers and public.(*World Healthcare Organisation, WHO, 2018).* Other hazards may be the drug-resistant microorganisms which are spreaded from the health sectors into the environment. Furthermore, poor handling of biomedical wastes pose health hazards to the hospital staff, rag-pickers, municipal workers and the community.(Meshram, et. al.,2022). All biomedical waste has the potential to endanger human health. The most common types of potentially infectious biomedical waste includes sharps waste, pharmaceutical/chemical waste, cytotoxic waste, and body part waste.

As such, improper disposal of biomedical waste can cause -

- Spread of HIV, hepatitis B, hepatitis C and other viral illnesses
- Parasitic infections
- Tuberculosis
- ALung infections such as asthma, lung cancer or chronic obstructive pulmonary disease (COPD)
- Skin infections
- Cholera (Wakelam,et. al.,2021)

According to WHO(World Healthcare Organisation*)*, the health consequences by the biomedical wastes as well as their by-products include -

- Sharps inflicted injuries
- Toxic effects of pharmaceutical products such as antibiotics, cytotoxic drugs. These get released into the surrounding environment. Also substances like mercury, dioxins can lead to severe diseases.
- Chemical burns arising for the disinfection, sterilization or waste treatment activities may cause several diseases.

- Arising of air pollution as a consequence of release of particulate matter during medical waste incineration.
- Thermal injuries may occur due to open burning and the operation of medical waste incinerators.
- Radiation burns.

Injections with contaminated needles and syringes in low- and middle-income countries have reduced substantially in recent years, partly due to efforts to reduce reuse of injection devices. Despite this progress, in 2010, unsafe injections were still responsible for as many as 33 800 new HIV infections, 1.7 million hepatitis B infections and 315 000 hepatitis C infections. Moreover a person who experiences one needle stick injury from a needle used on an infected source patient has risks of 30%, 1.8%, and 0.3% respectively of becoming infected with HBV, HCV and HIV.(*World Healthcare Organisation, WHO, 2018).*

2.4. The effects of biomedical wastes on the environment :

Biomedical wastes have detrimental effects on the environment if they are not disposed of properly. These are the effects that occur in the environment due to the biomedical wastes -

- Pollution through wastewater is a danger, as is the release of dangerous emissions during incineration. (Wakelam,et. al.,2021)
- Groundwater contamination and polluted streams and river ways cause unique dangers and damage to aquatic life, wildlife, and agricultural irrigation systems. (Wakelam,et. al.,2021)

- Used syringes, soiled dressings, and chemical substances, can infiltrate soil and water bodies, leading to the degradation of ecosystems. (Webber,et. al.,2024)
- Improper disposal of biomedical waste that ends up in landfills has the potential to transfer to stray animals, rodents, and birds that can then spread parasites and bacterial infections through animal populations. (Wakelam,et. al.,2021)
- Air pollutants generated by on-site or off-site incineration where emission standards are not maintained has the potential to contaminate and spread potentially dangerous airborne particles that affect not only those diagnosed with lung or breathing issues, but all life. (Wakelam,et. al.,2021)
- If not properly contained and monitored, diagnostic technologies releasing radioactive particles into the air have the potential to travel long distances to other areas, disseminating air pollutants over large expanses of inhabited and uninhabited land that can cause illness and disease.(Wakelam,et. al.,2021)
- Incineration and other disposal processes release greenhouse gasses and toxic pollutants, exacerbating air quality issues. (Webber,et. al.,2024)
- Inadequate incineration often leads to the release of pollutants in the air as well as in the ash residue production. Also incinerated materials which are treated with chlorine produce dioxins and furans. These are human carcinogens and have a range of adverse health effects. Incineration of heavy metals or materials with high metal content like lead, mercury, cadmium can lead to spread of the toxic metals into the environment.(World Health care Organisation, WHO, 2018)
- Waste materials, such as plastics and sharps that enter oceans and rivers become lethal hazards for aquatic organisms (Webber,et. al.,2024)

Chapter 3

Literature Review

3.1. Biomedical wastes management in Bangladesh

Inadequate data availability and lack of enough research in this field in Bangladesh, made this critical topic gain less attention from GoB, the media and the mass people. Most of the studies that are available, are scattered and lack proper research procedures.

Till 2020, Bangladesh had around 14,770 HCFs of which 654 public hospitals, 5055 private hospitals and clinics along with 9061 diagnostic centers and pathological labs. A total of 141,903 beds were available for the patients who were producing a huge amount of medical wastes throughout the country (Rahman et al., 2020).

As one of the fastest urbanizing economies in South Asian countries, Bangladesh has been facing a rapid growth of waste generation for the last few decades. Waste volume has doubled every 15 years in the last three decades. An average of 55% of solid waste remains uncollected in urban areas, with a variation of collection efficiency from 37% to 77%. (Sirajul, et. al.,2021) Studies that were conducted across Bangladesh about the medical waste management scenario found that most of the HCFs didn't follow the color-coded segregation method which were suggested by DGHS and WHO. Open dumping and mixing up with the waste bin for MSW are found to be common phenomena. All of the HCFs didn't have any personal treatment facilities and they depend on a few NGOs who take care of medical wastes such as - PRISM, Swapno, Protidin, BASA etc. Moreover, in Bangladesh, medical wastes are mainly managed through NGOs that have a partnership with the government and they get support from some international organizations for the safe disposal of generated medical wastes.(Musfekur, et. al.,2023)

PRISM Bangladesh is an NGO which started its operation in 1989 with the help of the UN organization and now it collects medical wastes from 1121 HCFs in Dhaka city. PRISM has only fully developed medical waste management procedures, treatment facilities and disposal systems in Bangladesh. This NGO engaged in the medical waste management system in 2004 in Dhaka and has currently expanded its operation to other major districts of Bangladesh. From the source of waste production, 56 % of clinical medical wastes are disposed of in the municipal wastes with the remaining 44 % managed by PRISM Bangladesh. PRISM mainly collects three types of medical wastes which are - infectious, plastic and sharp wastes. After final management, these medical wastes are treated according to their type.(PRISM 2020)

- Infectious wastes are treated through autoclaving, incineration and burial methods. Infectious wastes are sterilized in an autoclave at 135–140°C and 3 atm pressure for 45 min. After that, these treated medical wastes are disposed of into landfill sites. The highly infectious wastes are treated in two chamber incinerators. The minimum operating temperature for the first and second chambers are 850°C and 1050°C respectively. The ash formed in incineration and body parts from operation theaters is finally disposed of by concrete deep burial method.
- Sharp wastes are treated through autoclaving and deep burial. For sharp wastes, concrete tanks, specially constructed to prevent soil and water pollution after adding chemicals.
- Lastly, the plastic wastes are treated through chemical disinfection, shredding and recycling. Plastic wastes are disinfected with NaOCl and Cl2 and then recycled. (PRISM, 2022)

Three types of tanks are used in this treatment process. In the first chlorination tank, plastic wastes are submerged for 45 min then these partially treated wastes are again submerged into the second chlorination tank for 20 min for the final chemical disinfection process. After that, in the third tank, these wastes are washed with clean water and dried in sunlight and at last, by a mechanical shredder machine, they are recycled by cutting them down into small pieces (PRISM, 2022)

However, the existing healthcare waste management in Bangladesh is far behind the sustainable waste management concept. To achieve an effective waste management structure, Bangladesh

has to implement life cycle assessment (LCA) and circular economy (CE) concepts in this area. However, inadequate data and insufficient research in this field are the primary barriers to the establishment of an efficient medical waste management system in Bangladesh. (Musfekur, et. al.,2023). Despite the introduction of the Medical Waste Management and processing rules in 2008, no safe system has yet been developed to manage the health-care waste generated daily in hospitals and clinics. Waste generated inside Bangladeshi hospitals is often collected without any separation by untrained, unprotected, and unaware cleaners, and disposed of in unauthorized places without any separation or proper treatment. (Md Mostafizur, et.al.,2020)

Study region	**The number or type of HCFs considered**	**Year**	**Key findings**
Dhaka	69 HCFs: 4 hospitals, 21 clinics, and 44 diagnostic/pathological centers	2006	- Waste generation: 0.28 kg/bed/day (H), 37 ± 5 ton/day (T) - 21% of the sample contained hazardous substances (48.4% in diagnostic centers, 22.5% in clinics and hospitals) - Only 10% of HCFs followed proper guidelines for waste disposal and 25% followed partial treatment processes
Dhaka (DSCC)	1012 HCFs: 17% hospitals, 16% clinics, 21% diagnostic centers, and 46% dental clinics	2012	Waste generation: 1.99 kg/bed/day (T), 27 ton/day (T), 7.13 ton/day (H) - 26.4% of hazardous waste found - Hospitals generated more waste compared to non-residential labs and diagnostic centers - Only 7% of total HCFs managed waste properly, 30% followed partial processes, while the rest was disposed without proper management
DNCC	Not specified	Not specified	- Waste generation: 1.63 kg/bed/day (T), 22.7 ton/day (T), 5.8 ton/day (H) - 25.6% of total medical waste was hazardous - Only 12% of HCFs managed waste properly, 36% managed partially

Table 3.1 : The key findings of biomedical wastes of three different hospitals of Dhaka, Bangladesh.

3.2. Biomedical waste management in Bangladesh amid COVID-19:

According to the Environment and Social Development Organization, 14,165 tons of wastes from single-use plastic was generated in 26 March- 25 April 2020, roughly the first month of COVID-19 infection in Bangladesh(Rahman, et. al.,2020) The maximum amount of waste (5,877 tons) was generated from used hand gloves including 3,039 tons from plastic gloves and 2,838 tons from surgical gloves. In addition, 5,796, 1,592, and 900-tons medical wastes were generated from polythene shopping bags, surgical masks, and used hand sanitizer containers (The New Nation, 2020)

But unfortunately the disposal system was very poor. During the lockdown period, the number of operational waste collectors reduced by almost 50.0% in Dhaka. (Asian Development Bank, ADB, 2020) This voluminous amount of medical wastes generated in the country remained poorly managed, and thus, posing a potential environmental threat, creating a prolonged and unwanted public health hazard and being a potential source of re-emerging infection.

Figure 1 : Poor management of medical wastes amid COVID 19 situation in Bangladesh(Faisal, er. al., 2021) Challenges in medical waste management amid COVID-19 pandemic in a megacity Dhaka, J Adv Biotechnol Exp Ther. 2021; 4(1): 106-113. doi:10.5455/jabet.2021.d111

Furthermore, most of the hospitals dispose of their COVID-19 related medical wastes by mixing them with general waste without sterilization. Moreover, the untrained, unprotected, and unaware

cleaners collect the medical wastes and dispose of it in unauthorized places without any separation or proper treatment(Rahman, et. al., 2020)

Also, owing to lack of the established protected areas for the disposal of medical wastes, the wastes were disposed of in canals or open dumping zones, ultimately polluting the environment and contaminating the food chain. In addition to MW, SARS-CoV-2 had been detected in excreta (feces and urine) of infected people, and therefore, wastewater and sewage sludge from infected areas might contain SARS-CoV-2 RNA.(Collivignarelli, et. al., 2020) In Dhaka city, all sewage and domestic wastewater were discharged to the river Buriganga through various open canals. It posed an additional risk of spreading COVID-19 to Dhaka city.

The estimated generation of medical waste in Bangladesh without considering the surge due to Covid-19 and other unusual medical emergencies would be approximately 50,000 tons (1.25 kg/bed/day) in 2025, out of which 12,435 tons were predicted to be hazardous waste. However, calculation estimated that a total of 82,553, 168.4, and 2300 tons of medical waste was generated only from handling of covid patients, test kits and vaccination from March 2021 to May 2022. (Musfekur, et. al.,2023). Approximately 40 000 informal waste collectors working across the country were at high risk of getting infected by SARS-CoV-2 because they worked without adequate protection. There might be a serious risk of spreading SARS-CoV-2 if used masks, gloves and other personal protective equipment were not managed and disposed of properly. Additionally, household waste such as tissues, masks, gloves put waste management workers at increased health risk.

In case of the collection of medical wastes by waste collectors or NGOs from the HCFs, it was done in open vans, open drums or garbage trucks and also the vehicles which were used for transporting the waste are not managed properly(Tayeb et. al.,2020). Medical wastes were frequently found in municipal bins(Razzak et. al.,2020). The openly piled wastes are then picked up by waste scavengers.(Tayeb, et. al.,2020) This is increasing the risk on the environment and the health of waste handlers as they do not use proper protective gears(Ahmad, et. al.,2020). Considering the risk, workers involved with the biomedical waste management system

reportedly left their jobs or are unwilling to collect, resulting in a significant reduction in the number of waste handlers in Bangladesh (Amin, et. al.,2020)

Legal notice had been served in early June 2020 to the Government to take necessary measures for the medical waste management amidst the Covid-19 pandemic(Rahman et. al.,2020). On 22 June 2020, Dhaka North and South City Corporations mentioned that they will start the process in collaboration with the concerned NGO. However, till mid-July, there was no visible progress in this. Other municipalities were unable to develop Covid-19 WM mechanisms.(Ahmad, et. al.,2020).

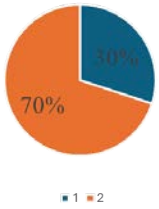
Thus, reflection and application of the policy guidelines were not observed at any step of the medical waste management system in the Covid-19 situation in Bangladesh.

CHAPTER 4

Methodology

4.1 Sample Guidelines

4.1.a Sample Type



70% solid waste and 30% liquid waste have been collected from hospital premises and open places. Solid wastes are- needles, gauze, syringe, urine test cap, bandage, tissue,saline bag, cotton, and cannula. Liquid wastes are- drain water, urine, lung waste(indoor care unit bins)

4.1.b Sample Site

Samples were collected from indoor and outdoor areas. Indoor areas included- cardiology, gynecology, medicine, pathology, hystopathology, radiology. Outdoor areas included- patients' waiting rooms, Outdoor Cafeterias, Walking Paths etc.

Figure 2: Sample collection from outdoor areas

Figure 3: Sample collection from indoor areas

4.1.c. Sample Collection

Samples were collected in zipper bags and were carried in an ice box to keep the specimen alive. The zipper bags were labeled as type of sample, name of the sample, hospital code name, sample no, and date.

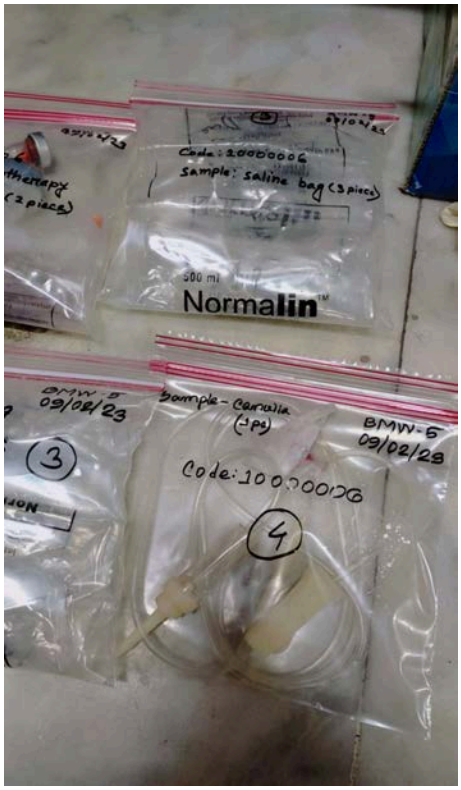



Figure 4 & 5: Samples are being collected in zipper bags from hospital premises

4.1.d. Sample Processing

Samples were taken for two different tests- one for radioactive analysis and another for microbial analysis. For radioactive analysis, the samples were kept for one month, dried and then analyzed in the machine. And for microbial analysis, samples were treated with peptone buffer and then kept in shaker incubator for 24h.

4.2 Microbial Analysis

Microbial analysis has been performed at Brac University Nanotechnology and Radiation Biotechnology Lab. Microbial analysis has been intended to identify and study microorganisms found in biomedical waste collected from hospitals. Microbial analysis needs to go through several steps to find the microbes.

4.2.a Sample Preparation

Step 1: Suspension of microbial samples in peptone buffer:

Peptone buffer is usually used in microbial analysis for the preparation, dilution, and resuspension of microbial samples. Here's how it is used:

Ingredients:

- Peptone: Provides nutrients like peptides and amino acids for microbial growth.
- Sodium chloride (NaCl): Maintains osmotic balance.
- Distilled water: Used to dissolve the ingredients.

Procedure:

- The required amount of peptone and NaCl has been dissolved in distilled water.
- Then, the pH has been adjusted to the desired level (usually around pH 7.0).
- After that, the solution has been sterilized by autoclaving at 121°C for 15-20 minutes.
- It needs to be cooled before use

Suspension:

The samples were cut into small pieces and then suspended in the peptone buffer, dissolved and put in the shaker incubator for 48h to collect microorganisms for analysis. Here the peptone buffer has been used as the pre-enrichment medium to support the initial growth of stressed or injured microorganisms before transferring them to selective or differential media.

Figure 6 & 7: Samples suspension in peptone buffer

Shaker Incubator

Shaker incubator combines the functions of shaking and incubating, providing a controlled environment for growing and maintaining microbial cultures, cell cultures, and other biological samples.

Key Features

1. **Controlled Temperature :** Precisely controlled temperature for optimal growth conditions for various types of microorganisms and cells.
2. **Shaking Mechanism:** As the incubator was set at a fixed speed, it would give a uniform mixture.
3. **Adjustable Shaking Speed:** According to the requirements of the culture, the user can set the shaking speed.
4. **Humidity Control:** Some shaker incubators have another optional feature which is humidity control. This helps to prevent the evaporation of culture media.
5. **CO_2 Control:** Another optional feature is CO_2 control which helps in the growth of cells by providing required levels.
6. **Capacity:** Shaker incubator comes in different sizes where one can accommodate different numbers of flasks, tubes or plates.

Operational Aspects

1. **Preparation:**
    - Ensure that the shaker incubator is clean and properly calibrated.
    - The culture media has been prepared and inoculated with the microorganism or cells of interest.
2. **Loading Samples:**
    - The flasks, tubes, or plates are placed securely on the shaking platform.
    - The load needs to be balanced to ensure even shaking and prevent mechanical issues.
3. **Setting Parameters:**
    - The desired parameters like- temperature, shaking speed, and, if applicable, CO_2

and humidity levels needs to be set

4. **Monitoring:**
    - The incubator needs to be checked periodically to ensure it maintains the set conditions and the samples are properly mixed.
5. **Harvesting Samples:**
    - After the incubation period, the samples are removed for further analysis or downstream applications.

4.2.b. Culture Techniques

After getting the samples from the shaker incubator, these are now cultured in differential media and then transferred to the selective media/general-purpose solid media.

The culture techniques require some media preparation, plate sterilization and streaking methods. These are performed as below-

1. **Media preparation:** First the samples are needed to be transferred to a differential media to find the organisms based on the indicators.

    Differential media: Differential media are specialized types of growth media used in microbiology to distinguish between different types of microorganisms based on their biological characteristics. These media contain specific indicators that reveal differences in microbial metabolic activities, such as fermentation, enzyme production, or gas formation. Here are key aspects and examples of differential media:

    #### Key Features of Differential Media

    1. **Indicators:**
        - Contain substances like dyes, pH indicators, or substrates that undergo visible changes (color changes, precipitate formation) when acted upon by microbial enzymes.
    2. **Selective Agents (Optional):**
        - May include selective agents to inhibit the growth of certain microorganisms, allowing for better differentiation among those that do grow.

3. **Nutrient Components:**
    - Provide essential nutrients to support the growth of a wide range of microorganisms.

Mannitol Salt Agar (MSA):

- **Indicator:** Phenol red.
- **Differentiation:** Detects mannitol fermentation.
    - **Mannitol Fermenters:** Yellow colonies with a yellow halo (e.g., Staphylococcus aureus).
    - **Non-Fermenters:** Red or pink colonies (e.g., Staphylococcus epidermidis).

Chromogenic UTI Media:

- **Purpose:** Recommended for presumptive identification and confirmation of microorganisms mainly causing urinary tract infections, can also be used for testing water, food, environmental and other clinical samples. (Collee J.G et al, 1996)
- **Indicators:**
    - **Enzyme Activity:** Chromogenic substrates produce colored compounds when metabolized by specific enzymes.
- **Differentiation:**
    - **E. coli:** Typically blue or green colonies.
    - **Enterococcus:** Blue or turquoise colonies.
    - **Proteus, Morganella, Providencia:** Brown colonies.
    - **Klebsiella, Enterobacter, Serratia:** Pink or purple colonies.
    - **Pseudomonas:** Colorless or yellow colonies.
- **Advantages:** Provides rapid and easy identification based on colony color, reducing the need for further biochemical testing.

  For our sample analysis, UTI media has been used to find the target organisms E.coli , Pseudomonas, Staphylococcus, Enterococcus.

UTI Media preparation

Media Composition:

Ingredients	**Gms/Litre**
Peptone, special	15.000
Chromogenic mixture	2.450
Agar	15.000
Final pH (at 25°C)	6.8±0.2

Directions:

1. 32.45 grams of UTI needs to be suspended in 1000 ml purified /distilled water. Then the media is heated to dissolve properly
2. Then the media is sterilized by autoclaving it at 15 lbs pressure (121°C) for 15 minutes. Cool it to 45-50°C. After that, the media is poured into sterile petri plates.

Selective media: Selective media is used in the detection of one or more organisms while inhibiting other microorganisms. That is why it is called selective as it selectively identifies microorganisms.

Key Features

1. **Selective Agents**: These are compounds added to the media that inhibit the growth of certain microorganisms while allowing others to grow. Examples include antibiotics, dyes, or specific chemicals.
2. **Nutrient Composition**: The nutrients in the media are tailored to support the growth of

the desired microorganisms.

3. **pH Level**: The pH of the media can be adjusted to favor the growth of certain microorganisms over others.
4. **Indicator Compounds**: Some selective media contain indicators that change color when certain biochemical reactions occur, aiding in the identification of specific microorganisms.

Examples

1. **MacConkey Agar**: It is only selective for Gram-negative bacteria as it only grows gram-negative bacteria inhibiting gram-positive bacteria. The media contains bile salts and crystal violet which inhibits gram-positive bacteria. (Andrea Prinzi, 2020)
2. **Mannitol Salt Agar (MSA)**: Selective for Staphylococci, with high salt concentration inhibiting most other bacteria. (Sagar Aryal, 2022)

Advantages

1. **Isolation of Specific Microorganisms**: Helps in isolating and identifying specific types of microorganisms from mixed cultures. (Sagar Aryal, 2022)
2. **Enhanced Growth Conditions**: Provides optimal growth conditions for the desired microorganisms while suppressing unwanted ones. (Sagar Aryal, 2022)
3. **Diagnostic Value**: Useful in clinical settings for identifying pathogens and in environmental microbiology for isolating organisms from samples. (Sagar Aryal, 2022)
4. **Efficiency**: Reduces time and effort needed to isolate specific microorganisms compared to using general media. (Sagar Aryal, 2022)

Preparation

1. **Base Media Selection**: A nutrient base that supports the growth of the desired microorganisms was selected (e.g., tryptic soy agar, nutrient agar). (Sagar Aryal, 2022)
2. **Selective Agents Addition**: Selective agents such as antibiotics, dyes, or specific chemicals were incorporated into the media, ensuring they were evenly distributed.

(Sagar Aryal, 2022)

3. **pH Adjustment**: The pH was modified as necessary to create an environment favorable for the target microorganism. (Sagar Aryal, 2022)
4. **Sterilization**: The media were sterilized using autoclaving to eliminate any unwanted microorganisms. (Sagar Aryal, 2022)
5. **Plate Pouring**: The sterilized media were poured into sterile petri dishes under aseptic conditions and allowed to solidify. (Sagar Aryal, 2022)
6. **Storage**: The prepared media were stored under appropriate conditions, usually in a refrigerator, until use. (Sagar Aryal, 2022)

MSA media has been used as selective media to selectively find the staphylococci. Alongside that, one general-purpose solid media has been used which is Nutrient agar media or NA in short. After microorganisms grow in differential media, these are then transferred to nutrient agar media plates to selectively grow the individual organisms. Later it was transferred to NB and T1N1 broth for storage.

Nutrient Agar media:

Nutrient agar acts as a general-purpose medium which supports the growth of a wide variety of microorganisms. It is one of the most commonly used media in microbiology laboratories due to its simplicity and effectiveness. Here's a comprehensive overview of nutrient agar, including its key features, composition, preparation, and uses. (Anupama Sapkota, 2022)

Key Features

1. **Non-selective Nature**: Suitable for the growth of a broad range of bacteria without selective inhibition. (Anupama Sapkota, 2022)
2. **Simple Composition**: Contains basic nutrients that promote the growth of many different microorganisms. (Anupama Sapkota, 2022)
3. **Versatility**: Used in various microbiological applications, including culture maintenance, isolation, and enumeration of bacteria. (Anupama Sapkota, 2022)

4. **Clear Medium**: Provides a clear background that helps in observing colony morphology. (Anupama Sapkota, 2022)

Composition

The standard composition of nutrient agar per liter of distilled water is:

- **Peptone**: 5.0 g
- **Beef Extract**: 3.0 g
- **Sodium Chloride**: 5.0 g
- **Agar**: 15.0 g

Preparation

1. **Ingredients Mixing**: The powdered ingredients are weighed and mixed together.
2. **Dissolving**: The mixture is dissolved in distilled water.
3. **pH Adjustment**: The pH is adjusted to 7.0 ± 0.2 if necessary.
4. **Sterilization**: The medium is sterilized by autoclaving at 121°C for 15 minutes.
5. **Pouring Plates**: The sterilized medium is poured into sterile petri dishes and allowed to solidify.
6. **Storage**: The prepared plates are stored in a refrigerator at 2-8°C until use.

Advantages

1. **Ease of Preparation**: Simple to prepare with basic laboratory equipment.
2. **Broad Spectrum**: Supports the growth of many different types of bacteria and fungi.
3. **Clear Background**: Provides a clear medium for observing colony characteristics.
4. **Cost-effective**: Relatively inexpensive compared to specialized media.

Limitations

1. **Non-selective**: Does not inhibit the growth of unwanted microorganisms, which can be a limitation when isolating specific bacteria. (Anupama Sapkota, 2022)

2. **Nutrient Limitations**: This may not support the growth of fastidious microorganisms that require specific nutrients or growth factors. (Anupama Sapkota, 2022)

2. Media Plate Sterilization:

Before pouring the media into media plates, it needs to be sterilized first. The clean media plates after the dishwasher need to be sterilized by putting them the autoclave machine. Mostly the plates get sterilized within 3h in the autoclave machine.

Pouring Plates:

- Allow the sterilized media to cool to about 45-50°C to prevent condensation inside the petri dishes.
- Pour the media into sterile petri dishes (approximately 20-25 ml per dish) under aseptic conditions in a laminar flow hood.
- Allow the agar to solidify at room temperature.

Storage:

- Once solidified, the agar plates can be stored upside down (to prevent condensation from dripping onto the agar surface) in plastic bags or containers to prevent drying.
- Store at 2-8°C until ready for use. For storage, one needs to be careful to keep the media plates separate from culture plates. Otherwise it will get contaminated.

3. Streaking method:

Streaking is a method which is used to obtain a pure culture from a single microorganism species. To perform the method, the quadrant streaking method has been used. (Prashant Dahal, 2024)

Procedure:

- The inoculating loop was flame sterilized until it was red-hot and allowed to cool.
- The loop was dipped into the bacterial culture or a small number of bacteria was picked from a colony. (Prashant Dahal, 2024)

- The loop was streaked across one quadrant of the agar plate in a back-and-forth motion. (Prashant Dahal, 2024)
- The loop was flame sterilized again and allowed to cool.
- The plate was turned approximately 90 degrees, and the loop was streaked from the edge of the first quadrant into the second quadrant. (Prashant Dahal, 2024)
- The process was repeated for the third and fourth quadrants, flaming the loop and cooling it between each streaking.
- The plate was incubated upside down at the appropriate temperature for the microorganism being cultured. (Prashant Dahal, 2024)

Figure 8: UTI media plate after being cultured

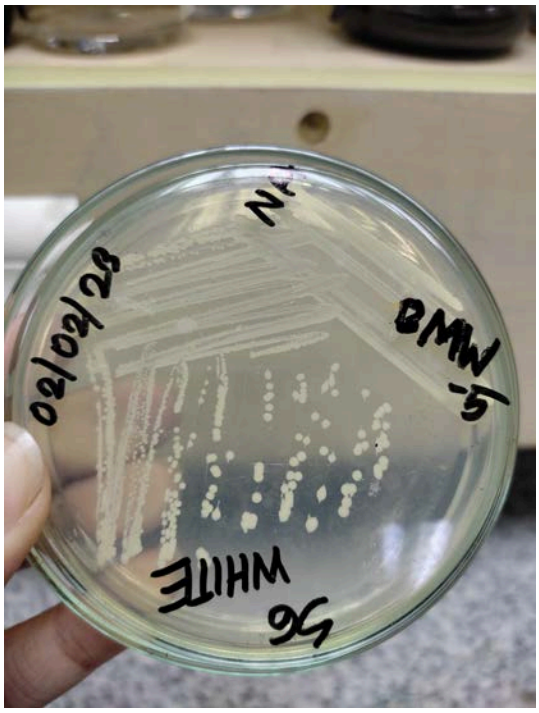


Figure 9: NA media plate after being cultured

Notes on Streaking:

- **Sterilization**: The inoculating loop was flame sterilized and cooled between streaks to avoid cross-contamination.
- **Pressure**: Gentle pressure was applied to avoid gouging the agar surface.
- **Aseptic Technique**: Aseptic conditions were maintained to prevent contamination of the media and cultures.
- **Incubation**: Plates were incubated in an inverted position to prevent condensation from dripping onto the agar surface.

These procedures ensured proper isolation and growth of microorganisms, facilitating accurate identification and study.

Streaking method is used to get the single colonies. As-

Accurate Identification: Single colonies derived from a single cell or a group of identical cells ensure that the resulting culture is pure, which is crucial for accurate identification and characterization. (Prashant Dahal, 2024)

Avoid Contamination: Ensures that the observed characteristics are due to the intended microorganism and not influenced by contaminants. (Prashant Dahal, 2024)

Biochemical Tests: Accurate biochemical testing requires pure cultures to ensure the validity of the results, such as antibiotic susceptibility testing.

4.2.c. Biochemical Tests

The purpose of biochemical tests in microbiology is to identify and differentiate microorganisms based on their biochemical activities. Several biochemical tests have been performed to identify and detect the presence of specific microorganisms.

1. **Gram Staining:** Gram staining is performed to find the two main groups of bacteria- gram positive and gram negative. It is based on the structural properties of their cell walls. Danish Bacteriologist Hans Christian Gram was the person who introduced this technique in 1884. The differentiation is based on the differences in cell wall structure and composition of bacteria. Gram staining and differentiation are based on variations in the cell wall structure and composition of bacteria. Bacteria with a thick peptidoglycan layer retain the primary stain, remaining violet or purple after decolorization. In contrast, bacteria with a thin peptidoglycan layer and fewer cross-links lose the primary stain during decolorization and take up the counterstain, appearing pink or red. (Tripathi N et al, 2023)

Gram Staining Reagents

- Crystal Violet-the primary stain
- Iodine-the mordant
- Decolorizer- made of acetone and alcohol (95%)
- Safranin-the counterstain

Procedure of Gram Staining

1. A clean, and grease-free slide was prepared.
2. A loopful of the sample was used to create a smear of the suspension on the slide.
3. The smear was allowed to air-dry, then heat-fixed. (Tripathi N et al, 2023)
4. Crystal violet was applied to the smear and left for 30 seconds to 1 minute before being rinsed with water.
5. Gram's iodine was added for 1 minute and then washed off with water.
6. The slide was decolorized with 95% alcohol or acetone for 10-20 seconds, followed by a water rinse. (Tripathi N et al, 2023)
7. Safranin was applied for 1 minute, then rinsed with water.
8. The slide was air-dried, and examined under a microscope. (Tripathi N et al, 2023)

Interpretation

Gram Positive: Blue/Purple Color

Cell Wall Structure: A thick layer of peptidoglycan that holds onto the crystal violet-iodine complex.

Gram Negative: Red Color

Cell Wall Structure: Thin peptidoglycan layer surrounded by an outer membrane that loses the crystal violet during decolorization but takes up the safranin counterstain. (Tripathi N et al, 2023)

Examples

Gram Positive Bacteria: Bacillus, Actinomyces, Enterococcus, Clostridium, Lactobacillus, Corynebacterium, Gardnerella, Listeria, Staphylococcus, Mycoplasma, Nocardia,, Streptococcus, Streptomyces etc. (Tripathi N et al, 2023)

Gram Negative Bacteria: Escherichia coli (E. coli), Salmonella, Shigella, and other Enterobacteriaceae, Pseudomonas, Moraxella, Helicobacter, Stenotrophomonas, Bdellovibrio, acetic acid bacteria, Legionella etc. (Tripathi N et al, 2023)

2. **MR Test:** The Methyl Red (MR) test determines an organism's ability to produce and maintain stable acidic byproducts from glucose fermentation, signifying mixed acid fermentation. It is performed alongside the Voges-Proskauer (VP) test on MRVP broth. Organisms that metabolize pyruvic acid via the mixed acid pathway produce significant acids like lactic and acetic acids, lowering the pH and causing the medium to remain red when methyl red indicator is added. In contrast, MR-negative organisms convert these acids into neutral products like acetoin, raising the pH and turning the medium yellow, indicating a negative test. The test is particularly useful for differentiating members of the Enterobacteriaceae family. (Sagar Aryal, 2022)

Methyl Red (MR) Test Media and Reagents

MRVP broth

Ingredients needed per liter of deionized water:

- buffered peptone= 7.0 gm
- glucose= 5.0 gm

- dipotassium phosphate= 5.0 gm

Methyl red solution, 0.02% was prepared by dissolving 0.1 g of methyl red in 300 ml of 95% ethyl alcohol. Sufficient distilled water was added to make a final volume of 500 ml, and the solution was stored at 4 to 8°C in a brown bottle, ensuring stability for up to 1 year. (Sagar Aryal, 2022)

Procedure of Methyl Red (MR) Test:

1. The medium was allowed to equilibrate to room temperature prior to inoculation.
2. The medium was lightly inoculated with organisms from a pure culture that had been grown for 18 to 24 hours. (Sagar Aryal, 2022)
3. It was then incubated aerobically at 37°C for 24 hours.
4. After this period, 1 ml of the broth was transferred into a clean test tube, while the remaining broth was incubated for an additional 24 hours. (Sagar Aryal, 2022)
5. To the aliquot, 2 or 3 drops of methyl red indicator were added.
6. The broth was then observed immediately for the development of a red color.

Result Interpretation

Positive Reaction: A clear red color (A). Examples include *E. coli* and *Yersinia* species. (Sagar Aryal, 2022)

Negative Reaction: A yellow color (B). Examples include *Enterobacter aerogenes* and *Klebsiella pneumoniae*. (Sagar Aryal, 2022)

A weak positive result appears red-orange. If an orange color is observed, incubate the remaining broth for up to 4 days and repeat the test after additional incubation. It may also be beneficial to prepare a duplicate broth at 25°C. (Sagar Aryal, 2022)

3. VP Test: Although most bacteria can ferment glucose, the end products of glucose metabolism can vary between different bacterial species. After glycolysis, some bacteria use the mixed acid fermentation pathway, converting pyruvate into a stable mixture of organic acids. Others follow the butylene glycol pathway, producing acetylmethylcarbinol (acetoin) and butanediol. The Voges-Proskauer (VP) test is a biochemical assay that identifies a bacterium's ability to metabolize pyruvate into a neutral intermediate compound known as acetylmethylcarbinol, or acetoin. (Prashant Dahal, 2023)

Voges–Proskauer (VP) Test Media and Reagents

MRVP Broth

Ingredients per liter of deionized water:

1. buffered peptone= 7.0 gm
2. glucose= 5.0 gm
3. dipotassium phosphate= 5.0 gm

Voges-Proskauer Reagent A: Barritt's reagent A

- Alpha-Naphthol, 5% 50 gm
- Absolute Ethanol 1000 ml

Voges-Proskauer Reagent B: Barritt's reagent B

- Potassium Hydroxide 400 gm
- Deionized Water 1000 ml

Procedure for the Voges–Proskauer (VP) Test:

1. The medium was allowed to equilibrate to room temperature before inoculation.
2. The medium was lightly inoculated with organisms from a pure culture that had been grown for 18 to 24 hours. (Prashant Dahal, 2023)
3. It was then incubated aerobically at 37°C for 24 hours.
4. After incubation, 2 ml of the broth was transferred into a clean test tube, while the remaining broth was re-incubated for another 24 hours. (Prashant Dahal, 2023)
5. Six drops of 5% alpha-naphthol were added to the aliquoted broth and mixed thoroughly to introduce air. (Prashant Dahal, 2023)
6. Following this, two drops of 40% potassium hydroxide were added, and the mixture was aerated through vigorous mixing.
7. The surface of the mixture was observed for the development of a pink-red color within 30 minutes. The tube was shaken vigorously during the 30-minute period. (Prashant Dahal, 2023)

Result Interpretation

Positive Reaction: A pink-red color appears at the surface.

Examples include: Viridans group streptococci (excluding *Streptococcus vestibularis*), *Listeria*, *Enterobacter*, *Klebsiella*, *Serratia marcescens*, *Hafnia alvei*, *Vibrio eltor*, *Vibrio alginolyticus*, and others. (Prashant Dahal, 2023)

Negative Reaction: A lack of a pink-red color

Examples: Streptococcus mitis, Citrobacter sp., Shigella, Yersinia, Edwardsiella, Salmonella, Vibrio furnissii, Vibrio fluvialis, Vibrio vulnificus, and Vibrio parahaemolyticus etc. A copper color should be considered negative. A rust color is a weak positive reaction. (Prashant Dahal, 2023)

4. The Triple Sugar Iron Test (TSI): Most bacteria can ferment carbohydrates, especially sugars, but each bacterium can only ferment certain sugars while being unable to ferment others. This selective ability to ferment specific sugars is a distinctive trait of bacteria and serves as a key factor in their identification. Triple Sugar Iron (TSI) agar is a culture medium that tests a microorganism's ability to ferment different sugars and produce hydrogen sulfide. (Sagar Aryal, 2022)

Purpose of the TSI Test:

1. **Differentiation of Enteric Bacteria:** The primary purpose of the TSI test is to distinguish between organisms which fall uder Enterobacteriaceae family and Gram-negative rods based on their carbohydrate fermentation patterns and gas production. (Sagar Aryal, 2022)
2. **Fermentation of Sugars:** The TSI medium contains three sugars: glucose (0.1%), and sucrose (1%), lactose (1%). The test helps determine if an organism can ferment these sugars and if it can do so aerobically (on the slant) or anaerobically (in the butt). (Sagar Aryal, 2022)
3. **Hydrogen Sulfide Production:** The test also detects the production of hydrogen sulfide (H_2S) gas. The medium contains ferrous sulfate, which reacts with H_2S to form a black precipitate, indicating the presence of hydrogen sulfide production. (Sagar Aryal, 2022)

4. **Gas Production:** Gas production during sugar fermentation is indicated by the presence of bubbles or cracks in the agar. (Sagar Aryal, 2022)

Media Preparation:

TSI Agar

For 1000ml-

- Enzymatic digest of casein - 5 g
- Rnzymatic digest of animal tissue - 5 g
- Yeast enriched peptone - 10 g
- Dextrose - 1 g
- Lactose - 10 g
- Sucrose - 10 g
- Ferric ammonium citrate - 0.2 g
- NaCl - 5 g,
- Sodium thiosulfate - 0.3 g
- Phenol red - 0.025 g
- Agar - 13.5 g

Method

1. A straight inoculation needle was gently used to touch the surface of a well-isolated colony.
2. The Triple Sugar Iron (TSI) medium was inoculated by stabbing the needle through the center to the bottom of the tube, followed by streaking the surface of the agar slant.
3. The tube was loosely capped and incubated at 35°-37°C in ambient air for 18 to 24 hours.
4. The reaction of the medium was then examined.

Expected Results

- **Alkaline/acid reaction (red slant/yellow butt):** Indicates fermentation of dextrose only.
- **Acid/acid reaction (yellow slant/yellow butt):** Suggests fermentation of dextrose, lactose, and/or sucrose.
- **Alkaline/alkaline reaction (red slant/red butt):** No carbohydrate fermentation has occurred.
- **Blackening of the medium:** Indicates the presence of hydrogen sulfide (H_2S).
- **Gas production:** Bubbles or cracks in the agar signify the production of carbon dioxide (CO_2) and hydrogen (H_2). (Sagar Aryal, 2022)

5. Catalase Test: This test detects the presence of catalase, an enzyme that facilitates the breakdown of hydrogen peroxide (H_2O_2), releasing oxygen. It helps distinguish bacteria that produce catalase, like staphylococci, from those that do not, such as streptococci. Typically, 3% H_2O_2 is used for routine cultures, while 15% H_2O_2 is employed for detecting catalase activity in anaerobes. The catalase test is useful in differentiating morphologically similar bacteria, such as catalase-negative Enterococcus or Streptococcus, and catalase-positive Staphylococcus. (Sagar Aryal, 2022)

Procedure of the Test

Slide Method

1. A small sample of the colony was placed onto the surface of a clean, dry glass slide using a loop or a sterile wooden stick.
2. A drop of 3% hydrogen peroxide (H_2O_2) was applied to the slide.
3. The slide was observed for the evolution of oxygen bubbles. (Sagar Aryal, 2022)

Interpretation

Positive result: Vigorous bubbling with the production of many bubbles.

- Examples: Staphylococci, Klebsiella, E. coli, Enterobacter, Pseudomonas.

Negative result: Little to no bubble formation

- Examples: Enterococcus, and Streptococcus species. (Sagar Aryal, 2022)

6. Indole Test

The indole test is conducted on bacterial organisms to detect their ability to produce indole from tryptophan through the action of enzymes known as tryptophanase. The indole test is important in identifying various bacteria, including *Escherichia coli*, *Proteus*, *Morganella*, and others. This test remains a traditional method for distinguishing indole-positive *E. coli* from indole-negative *Klebsiella and Enterobacter* species. The capability of an organism to produce indole from the amino acid tryptophan is due to the presence of the enzyme tryptophanase. This enzyme breaks down tryptophan by removing its amino group, a process that also requires pyridoxal phosphate as a coenzyme. The breakdown results in the formation of indole, pyruvic acid, ammonium (NH_4^+), and energy. Indole, if produced, can react with specific reagents to produce visible color changes. For example, when indole interacts with benzaldehyde reagent, it forms a pink to red-violet compound, while with cinnamaldehyde reagent, it produces a blue to green color. In the commonly used Kovac's reagent test, indole reacts to create a yellow or cherry red color, forming a distinct oily layer on top of the broth due to the water-insoluble nature of the amyl alcohol in the reagent. (Maria. P, 2009)

Reagents used in Indole Test

Ingredients per liter:

Indole Kovacs Reagent:

p-Dimethylaminobenzaldehyde	50.0 gm
Hydrochloric Acid, 37%	250.0 ml
Amyl Alcohol	750.0 ml

Procedure of Indole Test

1. Sterilized test tubes with 4 ml of tryptophan broth were taken.
2. Aseptically, growth from an 18 to 24-hour culture was introduced into the tube.

3. The tube was incubated at 37°C for 24 to 28 hours.
4. After incubation, 0.5 ml of Kovac's reagent was added to the broth culture.
5. The presence or absence of a colored ring at the top of the broth was then observed.

Result Interpretation

Positive: The appearance of a pink to red color ("cherry-red ring") in the reagent layer at the top of the medium occurs within seconds after the reagent is added.

Examples: Escherichia coli, Haemophilus influenzae, Klebsiella oxytoca, Proteus sp. (not P. mirabilis and P. penneri), Plesiomonas shigelloides,Pasteurella multocida, Pasteurella pneumotropica, Enterococcus faecalis, and Vibrio sp.

Negative: If there is no color change after the reagent is added, it indicates a negative result.

Examples: Actinobacillus spp., Aeromonas salmonicida, Alcaligenes sp., most Bacillus sp., Bordetella sp., Enterobacter sp., Lactobacillus spp., most Haemophilus sp., most Klebsiella sp., Neisseria sp., Pasteurella haemolytica, Pasteurella ureae, Proteus mirabilis, P. penneri, Pseudomonas sp., Salmonella sp., Serratia sp., Yersinia sp.

7. Oxidase Test

The oxidase test is designed to detect the presence of a cytochrome oxidase system, which catalyzes the transfer of electrons between bacterial electron donors and the redox dye tetramethyl-p-phenylenediamine. This dye is reduced to a deep purple color. The test aids in identifying bacteria such as Pseudomonas, Neisseria, Alcaligenes, Aeromonas, Campylobacter, Vibrio, Brucella, and Pasteurella, all of which produce the enzyme cytochrome oxidase. Various reagents can be used for this test. (Sagar Aryal, 2022)

The reagent employed in this case is:

Kovacs Oxidase Reagent: In water- 1% tetra-methyl-p-phenylenediamine dihydrochloride

The wet filter method has been utilized for this test.

Procedure:

1. First a strip of filter paper was soaked with a freshly prepared 1% solution of the reagent.
2. A small amount of culture was rubbed onto the paper using a platinum loop.
3. A positive reaction was indicated by an intense deep-purple hue that appeared within 5-10 seconds, a "delayed positive" reaction was noted by color appearing within 10-60 seconds, and a negative reaction was observed by the absence of color or color appearing after 60 seconds. (Sagar Aryal, 2022

Interpretation

Oxidase Positive Bacteria: Neisseria gonorrhoeae, Neisseria spp., Pseudomonas aeruginosa, Aeromonas spp., Vibrio spp., Brucella spp., Moraxella spp., Micrococcus spp., Bordetella pertussis, Campylobacter spp., etc.

Oxidase Negative Bacteria: E. coli and all Enterobacteriaceae except Plesiomonas shigelloides, Staphylococcus spp., Streptococcus spp., Pseudomonas maltophilia, Mycoplasma spp., Bordetella parapertussis, Listeria spp., etc.

8. Citrate Test

The **citrate test** is a biochemical assay used in microbiology to determine if an organism can use citrate as its sole carbon source and ammonium salts as its sole nitrogen source. It is particularly useful for differentiating members of the *Enterobacteriaceae* family, such as *Escherichia coli* and *Enterobacter* species.

Principle:

The test is based on the ability of bacteria to utilize citrate when it is the only available carbon source. When bacteria can metabolize citrate, they convert it to oxaloacetate and acetate through the enzyme **citrate lyase**. The oxaloacetate is further broken down, and ammonia is released, increasing the pH of the medium.

The test is typically performed using **Simmons Citrate Agar**, a medium containing citrate, ammonium phosphate, and the pH indicator bromothymol blue.

Procedure:

1. **Inoculation**: A sterile loop is used to lightly streak the bacterial sample onto the surface of the Simmons Citrate Agar slant.
2. **Incubation**: The inoculated tube is incubated at 35-37°C for 24 to 48 hours.
3. **Observation**: After incubation, results are interpreted based on color change.

Interpretation of Results:

- **Positive Citrate Utilization**: If the bacterium can utilize citrate, the medium's pH rises, and bromothymol blue turns from green (neutral pH) to **blue** (alkaline pH).
- **Negative Citrate Utilization**: If the bacterium cannot use citrate, the medium remains green, indicating no growth or pH change.

Applications:

- Used to differentiate *Klebsiella* (citrate positive) from *Escherichia coli* (citrate negative).
- Helps in the identification of other genera within the Enterobacteriaceae family and non-fermentative Gram-negative rods.

4.3. Radioactive Analysis

When measuring the concentration of radioactive elements like Uranium-238 (U-238), Thorium-232 (Th-232), and Potassium-40 (K-40) in environmental samples or geological materials, gamma spectroscopy has been employed. Gamma spectroscopy is a common method for detecting and quantifying radioactive isotopes by measuring the energy and intensity of gamma photons emitted during radioactive decay. Gamma spectroscopy is a powerful analytical technique used to measure and identify gamma-emitting radionuclides in a sample. It's widely used in fields like nuclear physics, environmental monitoring, geology, and medicine due to its ability to provide both qualitative and quantitative information about radioactive materials. (E.S Joel et al, 2021)

1. Principle of Gamma Spectroscopy

Gamma rays are high-energy photons emitted from the nucleus of an atom during radioactive decay. When these gamma rays interact with a detector, they produce signals that can be

analyzed to determine the energy of the photons and the intensity (or number) of photons at each energy level. This information is used to identify the specific radionuclides present and to quantify their concentrations. (E.S Joel et al, 2021)

2. Components of a Gamma Spectroscopy System

- **Detector**: The heart of the gamma spectroscopy system. The most common types are:
    - **High-Purity Germanium (HPGe) Detector**: Known for its excellent energy resolution, allowing for precise identification of gamma-ray energies.
    - **NaI(Tl) Scintillation Detector**: Sodium iodide detectors doped with thallium are more cost-effective and have higher efficiency, but lower resolution compared to HPGe detectors. (E.S Joel et al, 2021)
- **Preamplifier**: Connects directly to the detector and converts the small charge pulses from the detector into a voltage signal.
- **Amplifier**: Further amplifies the signal and shapes it for easier processing by the multichannel analyzer (MCA).
- **Multichannel Analyzer (MCA)**: A device that sorts the pulses according to their amplitude (which corresponds to gamma-ray energy) and stores them in channels to create an energy spectrum.
- **Data Acquisition System and Software**: Used to collect, display, and analyze the gamma spectra. It includes software for peak identification, energy calibration, and quantification. (E.S Joel et al, 2021)

3. How Gamma Spectroscopy Works

1. **Interaction with the Detector**:
    - When gamma rays enter the detector, they interact with the material inside (e.g., germanium or sodium iodide) and produce ionization events or scintillation (light flashes).
    - The amount of energy deposited by the gamma-ray in the detector material is proportional to the energy of the gamma-ray. (Young Jun Jung et al, 2020)
2. **Signal Processing**:
    - The energy deposited by the gamma rays is converted into electrical pulses by the detector.
    - These pulses are then amplified and sent to the MCA, which sorts them into channels based on their amplitude. (Young Jun Jung et al, 2020)
3. **Spectrum Formation**:
    - The MCA generates a gamma-ray energy spectrum, which is a plot of the number of detected pulses (counts) versus the energy of the pulses.

    - Peaks in the spectrum correspond to specific gamma-ray energies, which are characteristic of particular radionuclides.
4. **Analysis**:
    - The energy of the peaks is used to identify the radionuclides present (qualitative analysis).
    - The area under each peak is proportional to the number of gamma rays detected and is used to determine the concentration of the radionuclide (quantitative analysis). (Young Jun Jung et al, 2020)

4. Energy Calibration

Before analyzing samples, the gamma spectrometer must be calibrated. This involves:

- **Using Calibration Sources**: Known radioactive sources with well-defined gamma-ray energies (e.g., Cs-137, Co-60) are used to calibrate the energy scale of the MCA.
- **Creating a Calibration Curve**: The relationship between the channel number and the gamma-ray energy is established, typically resulting in a linear calibration curve. (Sagar Aryal, 2022)

5. Quantitative Analysis

- **Efficiency Calibration**: The detector's efficiency varies with gamma-ray energy and sample geometry. Efficiency calibration involves determining how well the detector can measure gamma rays of different energies.
- **Activity Calculation**: The activity of the radionuclide (usually expressed in becquerels or curies) is calculated using the counts under the peak, the detector efficiency, and the sample's geometry. (Sagar Aryal, 2022)

6. Applications of Gamma Spectroscopy

- **Environmental Monitoring**: Measuring radionuclide concentrations in soil, water, and air samples.
- **Nuclear Industry**: Analyzing radioactive waste and monitoring radiation in nuclear facilities.
- **Geology**: Dating rocks and minerals through the measurement of naturally occurring radionuclides.
- **Medicine**: In the production and quality control of radiopharmaceuticals. (Sagar Aryal, 2022)

Figure 10: Gamma Spectroscopy

7. Advantages and Limitations

- **Advantages**:
  - Non-destructive technique.
  - Capable of identifying multiple radionuclides in a single sample.
  - Provides both qualitative and quantitative information.
- **Limitations**:
  - Requires expensive and sensitive equipment (e.g., HPGe detectors).
  - Lower sensitivity for low-energy gamma rays and in complex matrices.
  - Requires careful calibration and data analysis to ensure accuracy.

Gamma spectroscopy is a versatile and powerful tool, essential for many scientific and industrial applications involving radioactive materials. The detector measures the gamma rays, and software is used to analyze the spectrum and quantify the presence of U-238, Th-232, and K-40. And it has been conducted at Bangladesh atomic energy center (BAEC) under the supervision of experts.

3.4. Opportunistic Bacteria Analysis

Opportunistic bacteria are defined as non-pathogenic until they attack the weak immune system and become pathogenic. Example: E.coli, Pseudomonas aeruginosa, Enterobacteria, Klebsiella spp. E.coli cause urinary tract infections (UTIs). Pseudomonas aeruginosa can cause pneumonia or bloodstream infections in patients with cystic fibrosis. To detect the opportunistic bacteria, samples were collected using cotton swabs from indoor areas - patients bed, blood, urine cap tissue used by patient, catheter. Culture tests are performed to identify the bacteria.(Zheng Pang et al, 2019)

CHAPTER 5

RESULT ANALYSIS

5.1 MICROBIAL TEST RESULT:

5.1.1 GRAM STAINING RESULT

We conducted the Gram staining method as the very first step of our microbial analysis. Due to privacy issues we will be naming those hospitals as - **Hospital A, Hospital B, Hospital C**. The method of gram staining was used very first to determine the gram-negative and gram-positive microorganisms of collected samples from hospital A, hospital B and hospital C. this simple differentiation process helped broadly to differentiate the found organisms into these two categories.

Below, hospital-wise results are provided:

HOSPITAL A

GRAM STAINING

SAMPLE	COLOR	OBSERVED SHAPE	INTERPRETATION
2	PURPLE	ROD SHAPE	+
6	PURPLE	ROD SHAPE	+
4B	PURPLE	ROD SHAPE	+
5P	PURPLE	DOT SHAPE	+
10P	PURPLE	ROUND SHAPE	+
10B	PURPLE	DOT SHAPE	+
9B	PURPLE	SPIRAL SHAPE	+
9P	PURPLE	ROD SHAPE	+
1	PURPLE	SPIRAL SHAPE	+

Table 1: Gram staining results of samples of Hospital A.

HOSPITAL B

GRAM STAINING

SAMPLE	COLOR	OBSERVED SHAPE	INTERPRETATION
2B	PURPLE	SPIRAL	+
2W	PURPLE	DOT	+
3G	PINK	DOT	-
3W	PINK	SPIRAL	-
1W	PURPLE	ROUND	+

2G	PINK	DOT	-
1B	PINK	SPIRAL	-

Table 2: Gram staining result of samples of Hospital B.

HOSPITAL C

GRAM STAINING

SAMPLE	COLOR	OBSERVED SHAPE	INTERPRETATION
6B	PINK	NO SHAPE	-
6W	PURPLE	ROUND	+
8B	PURPLE	SPIRAL	+
8W	PINK	SPIRAL	-

Table 3: Gram staining results of samples of Hospital C.

5.2 BIOCHEMICAL TEST RESULT

We conducted in total seven biochemical tests on the collected samples from three renowned hospitals of Dhaka. From those tests we came to hypothesize some microbial organisms present based on the test results. Due to privacy issues we will be naming those hospitals as - **Hospital A, Hospital B, Hospital C**. The seven conducted for identification of the microorganisms were-

Methyl Red Test, Voges Proskauer Test, Indole Test, Citrate Utilization Test, Triple Sugar Iron Test, Oxidase test, Catalase Test.

Below hospital-wise test results are provided:

HOSPITAL A

METHYL RED TEST

Sample	Media Color	Interpretation	Hypothesized Organisms
9P	RED	+	E.COLI, SALMONEILA SPP. SHIGELLA SPP. PROTEUS VULGARIS
5P	RED	+	
2P	RED	+	
10P	RED	+	
4B	RED	+	
6P	RED	+	
10B	RED	+	
1P	RED	+	
9B	RED	+	

Table 4: Methyl Red Test results of samples of Hospital A.

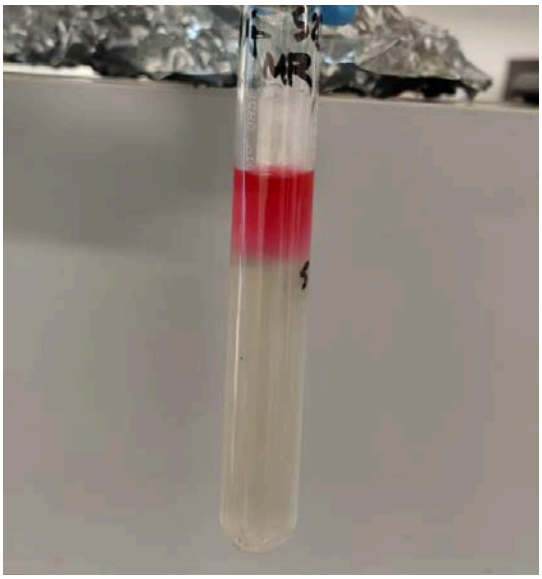


Fig 11: Methyl Red Positive Result.

VOGES PROSKAUER TEST

Sample	Media Color	Interpretation	Hypothesized Organisms
1P	YELLOW	NEGATIVE(-)	E.coli
2P			
4B			
5P			
6P			
9B			
9P			

10B			
10P			

Table 5: Voges Proskauer test result of the samples of Hospital A.

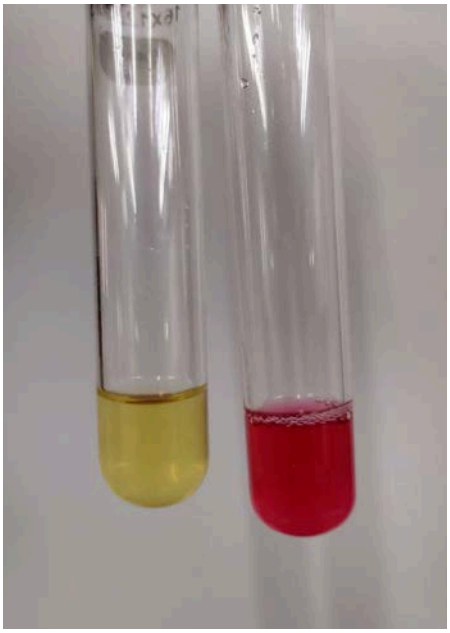


Fig 12: Voges Proskauer test positive and negative results.

INDOLE TEST

Sample	Media Color	Interpretation	Hypothesized Organisms
9B	YELLOW	-	E.coli
4B	PINK/ORANGE	+	KLEBSIELLA PSEUDOMONAS
2P	LIGHT PINK	+	
6P	V.LIGHT PINK	+	KLEBSIELLA PSEUDOMONAS
9P	YELLOW	-	E.coli
10P	YELLOW	-	
1P	PINK	+	KLEBSIELLA PSEUDOMONAS
10B	YELLOW	-	E.coli
5P	YELLOW	-	

Table 6: Indole test results of samples of Hospital A.

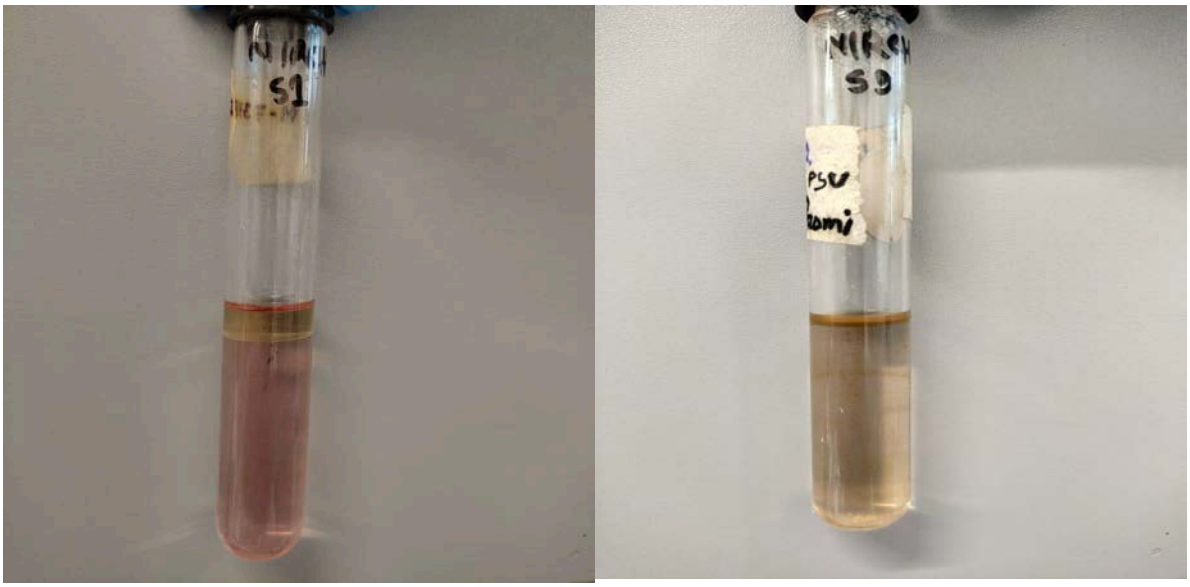


Fig 13 & 14: Indole positive & negative.

CITRATE UTILIZATION TEST

SAMPLE	MEDIA COLOR	INTERPRETATION	HYPOTHESIZED ORGANISM
6P	GREEN	-	E.COLI

1P	GREEN	-	
9B	GREEN	-	
5P	BLUE	+	KLEBSIELLA, PSEUDOMONAS
10P	BLUE	+	
9P	GREEN	-	E.COLI
2P	BLUE	+	KLEBSIELLA, PSEUDOMONAS
10B	GREEN	-	E.COLI
4B	GREEN	-	

Table 7: Citrate utilization test results of samples of Hospital A.

Fig 15: Citrate test.

OXIDASE TEST

SAMPLE	COLOR	INTERPRETATION	HYPOTHESIZED ORGANISMS
9B	PURPLE	+	PSEUDOMONAS AERUGINOSA

1P	NO COLOR	-	E.COLI, STREPTOCOCCUS
2P	NO COLOR	-	E.COLI, STREPTOCOCCUS
9P	PURPLE	+	PSEUDOMONAS AERUGINOSA
10B	NO COLOR	-	E.COLI, STREPTOCOCCUS
4B	PURPLE	+	PSEUDOMONAS AERUGINOSA
5P	PURPLE	+	PSEUDOMONAS AERUGINOSA
6P	NO COLOR	-	E.COLI, STREPTOCOCCUS
10P	NO COLOR	-	E.COLI, STREPTOCOCCUS

Table 8: Oxidase test result of samples of Hospital A.

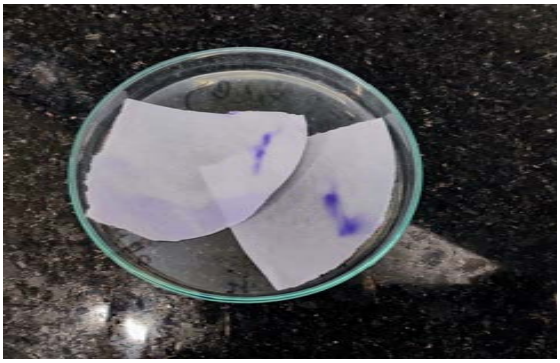


Fig 16: Oxidase Positive

CATALASE TEST

SAMPLES	RESULT	INTERPRETATION	HYPOTHESIZED ORGANISMS
10P	BUBBLES	+	E. coli, Enterobacter, Klebsiella, Staphylococcus
6P	BUBBLES	+	
4B	BUBBLES	+	
5P	BUBBLES	+	
10B	BUBBLES	+	
9P	BUBBLES	+	
2P	BUBBLES	+	
1P	BUBBLES	+	
9B	LOW BUBBLES	-	Streptococcus and Enterococcus spp

Table 9: Catalase test result of samples of Hospital A

Fig 17: catalase positive

TRIPLE-SUGAR-IRON TEST

SAMPLE	SUGAR FERMENTATION		H2S (Blackening)	GAS PRODUCTION	HYPOTHESIZED ORGANISMS
	Slant/Butt Condition	FERMENTED SUGAR			
1P	A/A	GLUCOSE, SUCROSE, LACTOSE	+	-	1. ENTEROCOCCUS FAECALIS 2.SALMONELLA (APPEARED)
2P	A/A	GLUCOSE, SUCROSE, LACTOSE	-	+	E.COLI

4B	A/A	GLUCOSE, SUCROSE, LACTOSE	-	+	1.ENTEROCOCCUS 2.KLEBSHIELLA PNEUMONIAE
5P	A/A	GLUCOSE, SUCROSE, LACTOSE	-	+	E.COLI
6P	A/A	GLUCOSE, SUCROSE, LACTOSE	-	-	PSEUDOMONAS
9B	A/A	GLUCOSE, SUCROSE, LACTOSE	-	+	ENTEROBACTER
9P	A/A	GLUCOSE, SUCROSE, LACTOSE	-	+	E.COLI
10B	K/A	GLUCOSE	-	+	SALMONELLA PARATYPHI
10P	A/A	GLUCOSE, SUCROSE, LACTOSE	-	+	E.COLI

Table 10: TSI test result of samples of Hospital A.

Fig 18 & 19: TSI test Result of E.coli & Salmonella.

HOSPITAL B

METHYL RED TEST

SAMPLE	MEDIA COLOR	INTERPRETATION	HYPOTHESIZED ORGANISMS
1W	YELLOW	-	1. ENTEROBACTER 2. KLEBSIELLA SPP.
1B	YELLOW	-	
3G	YELLOW	-	
2B	RED	+	1. E.COLI 2. SALMONELLA SPP. 3. SHIGELLA SPP. 4. PROTEUS VULGARI
2G	YELLOW	-	3. ENTEROBACTER 4. KLEBSIELLA SPP.
2W	YELLOW	-	
3W	YELLOW	-	

Table 11: MR test results of samples of Hospital B.

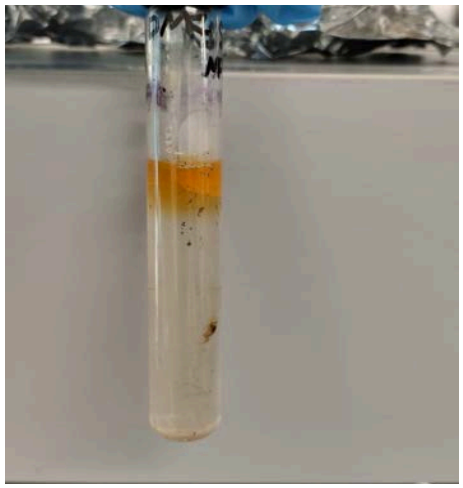


Fig 20: MR negative result.

VOGES PROSKAUER TEST

SAMPLE	MEDIA COLOR	INTERPRETATION	HYPOTHESIZED ORGANISMS
1W	Red	+	Klebsiella, Enterobacter
1B			
3G			
3W			
2B	Yellow	-	E.coli
2G	Red	+	Klebsiella, Enterobacter
2W			

Table 12: VP test results of samples of Hospital B.

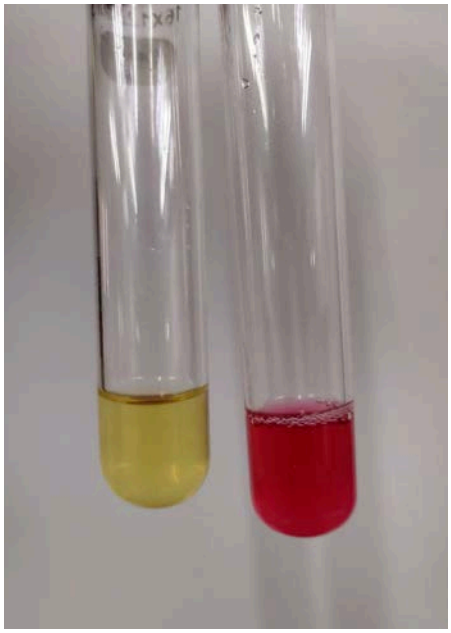


Fig 21: VP Positive and Negative test results.

INDOLE TEST

SAMPLE	MEDIA COLOR	INTERPRETATION	HYPOTHESIZED ORGANISMS
2W	YELLOW	-	1. KLEBSIELLA 2. PSEUDOMONAS
3W	YELLOW	-	
3G	YELLOW	-	
1W	YELLOW	-	
2G	YELLOW	-	
1B	YELLOW	-	
2B	PINK	+	E.COLI

Table 13: Indole test results of samples of Hospitals B.

Fig 22: Indole test negative.

CITRATE UTILIZATION TEST

SAMPLE	MEDIA COLOR	INTERPRETATION	HYPOTHESIZED ORGANISMS
1W	GREEN	-	E.coli
1B	GREEN	-	
3G	GREEN	-	
3W	BLUE	+	Klebsiella, Pseudomonas
2B	GREEN	-	E.coli
2W	GREEN	-	
2G	GREEN	-	

Table 14: Citrate utilization test results of samples of samples of Hospital B.

Fig 23: Citrate utilization test results (positive & negative)

OXIDASE TEST

SAMPLE	COLOR	INTERPRETATION	HYPOTHESIZED ORGANISMS
2B	PURPLE	+	PSEUDOMONAS AERUGINOSA
2W	PURPLE	+	PSEUDOMONAS AERUGINOSA
3G	NO COLOR	-	E.COLI, STREPTOCOCCUS
3W	PURPLE	+	PSEUDOMONAS AERUGINOSA
1W	PURPLE	+	PSEUDOMONAS AERUGINOSA
2G	PURPLE	+	PSEUDOMONAS AERUGINOSA
1B	NO COLOR	-	E.COLI, STREPTOCOCCUS

Table 15: Oxidase test results of samples of Hospital B.

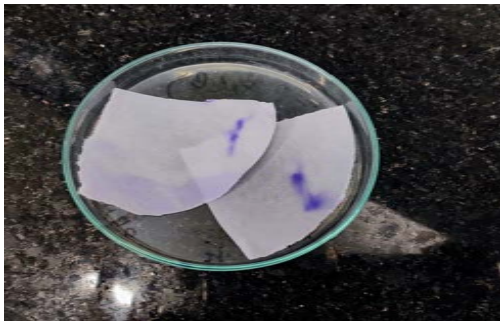


Fig 24: Oxidase test

CATALASE TEST

SAMPLE	RESULT	INTERPRETATION	HYPOTHESIZED ORGANISM
1B	BUBBLES	+	E. coli, Enterobacter, Klebsiella, Staphylococcus
2G	BUBBLES	+	
1W	BUBBLES	+	
3W	BUBBLES	+	
3G	BUBBLES	+	
2W	BUBBLES	+	
2B	BUBBLES	+	

Table 16: Catalase test results of samples of Hospital B.

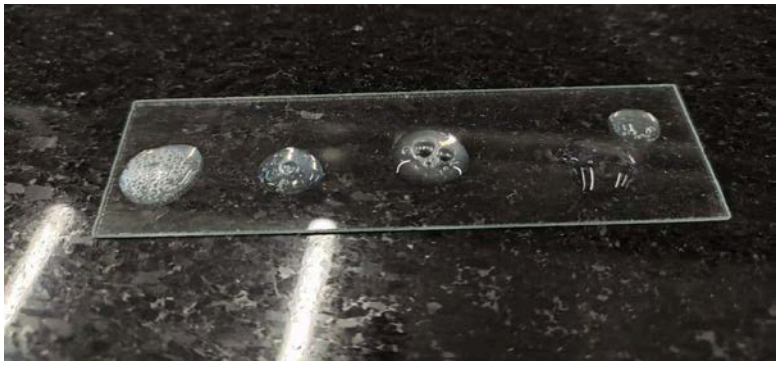


Fig 25: Catalase Positive

TRIPLE-SUGAR-IRON TEST

SAMPLES	SUGAR FERMENTATION		H2S (BLACKENING)	GAS PRODUCTION	HYPOTHESIZED ORGANISMS
	SLANT/BUTT CONDITION	FERMENTED SUGAR			
1B	K/A	GLUCOSE	-	-	PSEUDOMONAS AERUGINOSA ENTEROCOCCUS PSEUDOMONAS AERUGINOSA
2B	K/A	GLUCOSE	-	-	
2G	K/K	-	-	-	

3G	A/A	GLUCOSE, SUCROSE, LACTOSE	-	-	
2W	K/K	-	-	-	
1W	A/A	GLUCOSE, SUCROSE, LACTOSE	-	+	E.COLI
3W	K/K	-	-	-	PSEUDOMONAS AERUGINOSA

Table 17: TSI test results of samples of Hospital B.

Fig 26: TSI Test Results.

HOSPITAL C

METHYL RED TEST

SAMPLE	MEDIA COLOR	INTERPRETATION	HYPOTHESIZED ORGANISMS
6W	YELLOW	-	1. ENTEROBACTER 2. KLEBSIELLA SPP. 3. PSEUDOMONAS SPP. 4. ENTEROCOCCUS
8B	RED	+	1. E.COLI 2. SALMONELLA SPP. 3. SHIGELLA SPP. 4. PROTEUS VULGARI
8W	RED	+	
6B	RED	+	

Table 18: MR test results of samples of Hospital C.

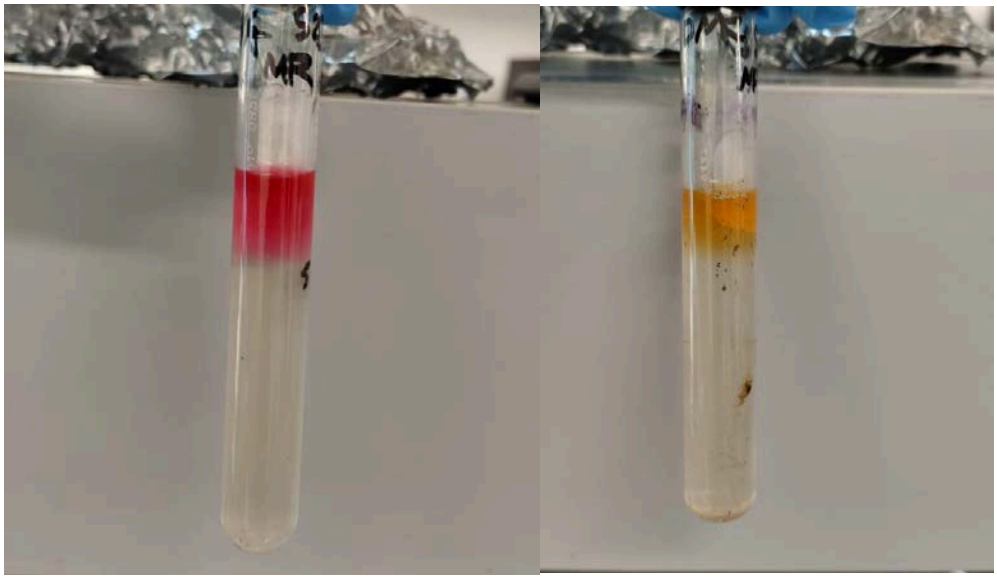


Fig 27 & 28: Methyl Red positive & negative

VOGES PROSKAUER TEST

SAMPLE	MEDIA COLOR	INTERPRETATION	HYPOTHESIZED ORGANISMS
6W	Red	+	Klebsiella, Enterobacter
6P	Yellow	-	E.coli
8W			
8P			

Table 19: VP test results of samples of Hospital C.

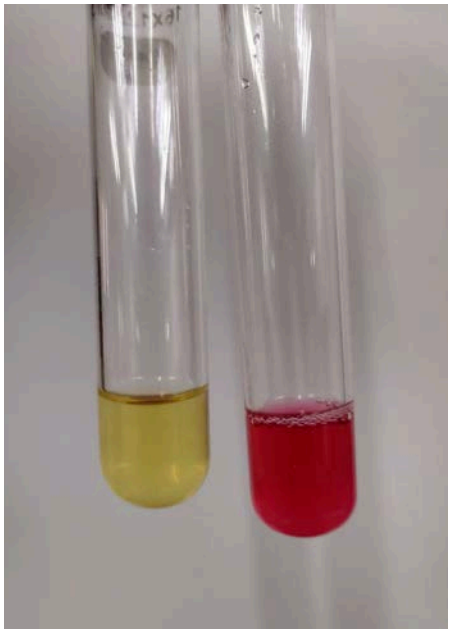


Fig 29: VP Test positive and negative result.

INDOLE TEST

SAMPLE	MEDIA COLOR	INTERPRETATION	HYPOTHESIZED ORGANISMS
8B	YELLOW	-	KLEBSIELLA PSEUDOMONAS
6B	YELLOW	-	
8W	YELLOW	-	
6W	PINK	+	E.COLI

Table 20: Indole test results of samples of Hospital C.

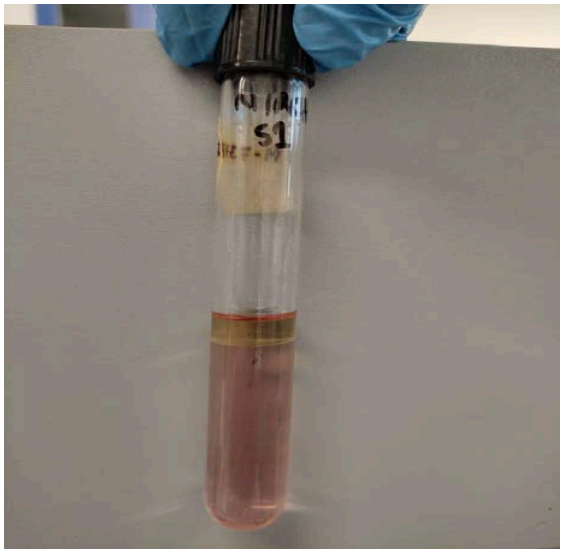


Fig 30: Indole test positive result.

CITRATE UTILIZATION TEST

SAMPLE	COLOR	INTERPRETATION	HYPOTHESIZED ORGANISMS
8W	GREEN	-	E.COLI
6B	BLUE	+	KLEBSIELLA, PSEUDOMONAS
8B	BLUE	+	
6W	BLUE	+	

Table 21: Citrate Utilization test results of samples of Hospital C.

Fig 30: Citrate Test

OXIDASE TEST

SAMPLE	COLOR	INTERPRETATION	HYPOTHESIZED ORGANISMS
6B	PURPLE	+	PSEUDOMONAS AERUGINOSA

	(AFTER 10S)	(DELAYED)	
6W	NO COLOR	-	E.COLI, STREPTOCOCCUS
8B	PURPLE (AFTER 46S)	+ (DELAYED)	PSEUDOMONAS AERUGINOSA
8W	PURPLE (AFTER 24S)	+	PSEUDOMONAS AERUGINOSA

Table 22: Oxidase test results of samples of Hospital C.

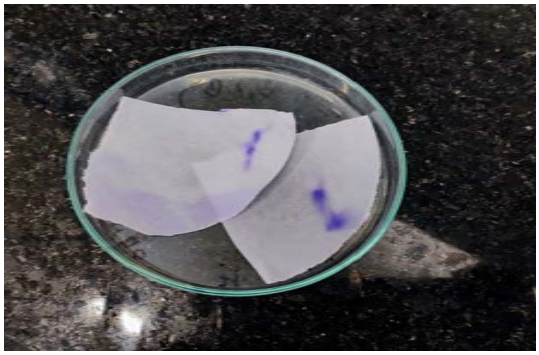


Fig 31: oxidase test

CATALASE TEST

SAMPLE	RESULT	INTERPRETATION	HYPOTHESIZED ORGANISMS
8w	BUBBLES	+	E. coli, Enterobacter, Klebsiella, Staphylococcus
8b	LOW BUBBLES	+	
6w	BUBBLES (A LOT)	+	
6b	LOW BUBBLES	+	

Table 23: Catalase test results of samples of Hospital C.

Fig 32: Catalase positive

TRIPLE-SUGAR-IRON TEST

SAMPLE	SUGAR FERMENTATION		H2S (BLACKENING)	GAS PRODUCTION	HYPOTHESIZED ORGANISMS
	SLANT/BUTT CONDITION	FERMENTED SUGAR			
6B	A/A	GLUCOSE, SUCROSE, LACTOSE	-	+	E.COLI
6W	K/K	-	-	-	PSEUDOMONAS AERUGINOSA
8B	A/A	GLUCOSE, SUCROSE, LACTOSE	-	+	E.COLI
8W	A/A	GLUCOSE, SUCROSE, LACTOSE	-	-	ENTEROCOCCUS

Table 24: TSI test results of samples of Hospital C.

Fig 33:TSI test result of Pseudomonas Aeruginosa

5.3. RADIOACTIVITY TEST RESULT

After collecting samples from the three hospitals- Hospital A, Hospital B, hospital C, we prepared the samples for radioactive analysis which took place in the Bangladesh atomic Center. In total we sent 18 samples from the three hospitals for their radioactivity analysis. The samples were mainly needles, gauge, urine etc.

	HOSPITAL A					
			Concentration of Radioactivity in Bq/kg			
	Sample No.	**Sample Name**	**U-238**	**Th-232**	**K-40**	
	1	Urine test cap	2.53±0.43	2.21±0.41	143±4.47	
	2	Drain water	1.89±0.43	1.32±0.35	87±2.64	
	3	Needles (X-ray)	2.32±0.42	1.49±0.43	44±6.14	
	4	Gauge (Radioactive)	2.46±0.42	1.95±0.36	102±6.23	
	5	Lung Waste	1.98±0.43	2.08±0.42	41±6.36	
	6	Urine	2.44±0.45	2.70±0.43	243±6.16	
	HOSPITAL C					
			Concentration of Radioactivity in Bq/kg			
	Sample No.	**Sample Name**	**U-238**	**Th-232**	**K-40**	
	1	Urine test cap	2.25±0.41	2.51±0.44	131±2.46	
	2	Drain water	2.44±0.56	2.09±0.49	134±5.36	

	3	Needles (X-ray)	2.84±0.62	2.91±0.42	93±3.56	
	4	Gauge (Radioactive)	3.09±0.45	1.99±0.43	207±4.52	
	5	Lung Waste	2.45±0.42	1.23±0.39	47±2.55	
	6	Urine	2.94±0.61	1.65±0.43	55±3.71	
	HOSPITAL B					
			Concentration of Radioactivity in Bq/kg			
	Sample No.	**Sample Name**	**U-238**	**Th-232**	**K-40**	
	1	Urine test cap	2.54±0.42	2.78±0.58	109± 3.06	
	2	Drain water	2.71±0.66	2.89±0.61	145±7.34	
	3	Needles (X-ray)	2.73±0.42	2.10±0.72	115±2.02	
	4	Gauge (Radioactive)	2.52±0.39	1.94±0.33	186±2.00	
	5	Lung Waste	1.95±0.37	2.00±0.34	179±1.87	
	6	Urine	2.97±0.32	2.46±0.42	107±2.00	

Table 25: Radioactivity test results of the samples of respectively Hospital A, C & B.

5.4. OPPORTUNISTIC BACTERIA TEST RESULT

The analysis of the identification of opportunistic bacteria has been carried out by performing TSI and Citrate Utilization tests. The following samples have been taken using cotton swabs. Out of 4 Samples, some samples did not give any results in the UTI plates. Hence, some samples did not grow any organism on UTI plates. Later, the TSI and Citrate tests were carried out with samples that had grown organisms on UTI plates.

Hospital serial	Sample No	Sample Name	TSI Result	Citrate Result	Hypothesized Organism
Hospital A	1P	Tissue(blood stained)	A/A, H2S +, Gas-	Negative, Green	E.coli, Enterobacter
	2P	Urine Cap	A/A, H2S-, Gas+	Positive, Blue	Klebsiella, E.coli
	9P	Catheter	A/A, H2S-, Gas+	Negative, Green	E.coli
	9B		A/A, H2S-, Gas+	Negative, Green	E.coli, Enterobacter
Hospital C	6W	Urine Cap	K/K, H2S-, Gas-	Positive, Blue	Klebsiella, Pseudomonas
	6B		A/A, H2S-, Gas+	Positive, Blue	E.coli
Hospital B	1B	Tissue (Blood stained)	K/A, H2S-, Gas-	Negative, green	Klebsiella, Pseudomonas
	1W		A/A, H2S-, Gas+	Negative, green	Klebsiella, Pseudomonas, E.coli
	3W	Catheter	K/K, H2S-, Gas-	Positive, Blue	E.coli, Pseudomonas
	3G		A/A, H2S-, Gas-	Negative, Green	Pseudomonas

Table 26: Opportunistic bacteria analysis and outcome from Hospital A, B & C

CHAPTER 6

DISCUSSION

6.1 MICROBIAL RESULT-DISCUSSION

6.1.1 Gram staining Result-Discussion

The Gram staining results from Hospitals A, B, and C highlight notable differences in the bacterial species present across the samples and the Gram staining method's utility for classification.

- **Hospital A Result-Discussion:**

In **Hospital A** (Table 1), all nine samples stained purple, indicating that they are Gram-positive bacteria. These results confirm that the bacterial species in these samples retain the crystal violet stain due to the thick peptidoglycan layer in their cell walls. The samples exhibit a variety of morphologies, including rod shapes (7 samples), dot shapes (2 samples), spiral shapes (2 samples), and round shapes (1 sample).

The predominance of rod-shaped bacteria suggests the presence of *bacilli*, which are commonly associated with both aerobic and anaerobic bacteria in human infections. Notable genera that exhibit this shape include *Bacillus* and *Clostridium*, which can be involved in skin, gastrointestinal, or respiratory infections.(Tripathi & Sapra, 2023)

- **Hospital B Result-Discussion**

In **Hospital B** (Table 2), there is greater variation in Gram stain results compared to Hospital A. Out of seven samples, four are Gram-positive (purple) and three are Gram-negative (pink). The Gram-negative samples are characterized by either dot or spiral shapes, which may represent pathogenic Gram-negative bacteria such as *Neisseria* (round shape) or *Helicobacter* (spiral shape). The Gram-positive bacteria in this hospital were observed as spiral, dot, and round shapes, with a distribution similar to Hospital A.

The presence of Gram-negative bacteria in this set, including dot-shaped and spiral-shaped bacteria, raises concerns as these bacteria typically possess an outer membrane that makes them more resistant to antibiotics.(Tripathi & Sapra, 2023). These organisms might include species such as *Escherichia coli* (dot shape) or *Helicobacter pylori* (spiral shape), both of which are relevant in hospital settings due to their role in gastrointestinal and urinary tract infections.

- **Hospital C Result- Discussion**

From Hospital C (Table 3)we have collected a small amount of samples, but it still reveals important information. The data show two Gram-positive and two Gram-negative samples, with a similar pattern of spiral and round bacterial shapes for the Gram-positive bacteria, and no specified shapes for the Gram-negative samples. The absence of shape information for the Gram-negative samples makes it challenging to determine their exact identity, but the negative result in two cases could be indicative of environmental Gram-negative bacteria or opportunistic pathogens, such as members of the Enterobacteriaceae family.

Notably, the spiral-shaped Gram-positive bacteria in sample 8B suggest a similar bacterial type as those found in Hospital A and B, while the round-shaped Gram-positive bacteria (sample 6W) could again suggest organisms like *Staphylococcus* or *Streptococcus* species, (Tripathi & Sapra, 2023) both of which are frequently encountered in clinical infections.

6.2. BIOCHEMICAL TEST RESULT-DISCUSSION

The results from the biochemical tests conducted in Hospitals A, B, and C provide significant insights into the microbial diversity and metabolic activities of the samples. The tests include Methyl Red, Voges-Proskauer, Indole, Citrate Utilization, Oxidase, Catalase, and Triple-Sugar-Iron (TSI) tests. These tests serve to identify bacterial species based on their biochemical properties, which can be crucial for clinical diagnostics, especially in detecting pathogenic organisms.

1. **Methyl Red Test**

The **Methyl Red Test** detects the ability of organisms to perform mixed acid fermentation, producing stable acids that lower the pH of the medium.

- ➔ In **Hospital A**, all samples tested positive (red media color), suggesting that organisms such as *Escherichia coli* (E. coli), *Salmonella spp.*, *Shigella spp.*, and *Proteus vulgaris* are present (Table 4).(Osagie et al., 2016) These organisms are significant pathogens, often involved in gastrointestinal infections, with E. coli being particularly associated with urinary tract infections (UTIs) and *Salmonella* with foodborne illness.
- ➔ In **Hospital B**, the results were more varied. One sample (2B) tested positive, indicating the possible presence of *E. coli*, while the majority of samples were negative (yellow media), indicating that these organisms do not perform mixed acid fermentation. This could suggest the presence of organisms like *Enterobacter* or *Klebsiella spp.*, which typically do not lower the pH as much (Table 11).
- ➔ In **Hospital C**, three samples tested positive, suggesting similar organisms to those in Hospital A, while one sample (6W) was negative, potentially indicating *Klebsiella* or *Enterobacter* (Table 18)(Osagie et al., 2016).

The predominance of MR-positive organisms in Hospitals A and C suggests a higher prevalence of mixed acid fermenters, which are often associated with more aggressive pathogenic behavior.

2. Voges-Proskauer Test

The **Voges-Proskauer (VP) Test** identifies organisms capable of producing acetoin from glucose fermentation, which is typical of butanediol fermentation.

- ➔ In **Hospital A**, all samples were negative, indicating the absence of organisms that utilize the butanediol pathway, such as *Klebsiella* or *Enterobacter* (Table 5).
- ➔ In **Hospital B**, two samples (1W and 2G) tested positive, indicating the possible presence of *Klebsiella* or *Enterobacter*, while other samples were negative (Table 12).
- ➔ In **Hospital C**, one sample (6W) was positive, suggesting the presence of *Klebsiella* or *Enterobacter* (Table 19).

The presence of VP-positive organisms in Hospitals B and C suggests a microbial environment

with organisms that may thrive in less acidic conditions compared to those detected in Hospital A.

3. Indole Test

The **Indole Test** determines an organism's ability to convert tryptophan into indole, an important metabolic activity for identifying *E. coli*.

- ➔ In **Hospital A**, four samples tested positive for indole production, strongly indicating the presence of *E. coli*. In contrast, the other samples tested negative, suggesting organisms like *Klebsiella* and *Pseudomonas* (Table 6).
- ➔ - In **Hospital B**, only one sample (2B) tested positive, indicating *E. coli,* while the majority of samples were negative, suggesting *Klebsiella* or *Pseudomonas* (Table 13).
- ➔ - In **Hospital C**, only one sample (6W) tested positive, indicating *E. coli*, while others were negative (Table 20).

The consistent presence of indole-positive *E. coli* in all hospitals suggests it is a common pathogen in the clinical environments sampled.

4. Citrate Utilization Test

The Citrate Utilization Test assesses the ability of organisms to use citrate as their sole carbon source

- ➔ In **Hospital A**, three samples tested positive for citrate utilization, indicating the presence of organisms like *Klebsiella* and *Pseudomonas.* The remaining samples were negative, suggesting organisms such as *E. coli* (Table 7).
- ➔ In **Hospital B,** only one sample (3W) tested positive, indicating the presence of *Klebsiella* and *Pseudomonas* while most were negative, suggesting the absence of

citrate-utilizing organisms- *E.coli.* (Table 14).

- ➔ In **Hospital C**, three samples tested positive, indicating *Klebsiella* or *Pseudomonas,* one negative(Table 21).

The citrate utilization results indicate that the hospitals are managing microbial populations that include both citrate-utilizing and non-utilizing organisms, with *Klebsiella* and *Pseudomonas* being potential opportunistic pathogens in some environments.

5. **Oxidase Test**

The **Oxidase Test** helps identify organisms that produce the enzyme cytochrome c oxidase, indicative of aerobic metabolism.

- ➔ In **Hospital A**, four samples tested positive for oxidase activity, indicating the presence of *Pseudomonas aeruginosa,* a known pathogen that causes respiratory infections, particularly in immunocompromised individuals. The remaining samples were negative, likely indicating *E. coli* or *Streptococcus* (Table 8).
- ➔ In **Hospital B**, the majority of samples tested positive for oxidase activity, again suggesting *Pseudomonas aeruginosa* (Table 15).
- ➔ In **Hospital C**, three samples tested positive for oxidase, again pointing to the presence of *Pseudomonas aeruginosa* (Table 22).

The oxidase test results suggest that *Pseudomonas aeruginosa*, a highly resistant pathogen, is a recurring microorganism in the clinical settings of all hospitals.

6. **Catalase Test**

The **Catalase Test** detects the presence of the enzyme catalase, which breaks down hydrogen peroxide into water and oxygen, helping differentiate between *Staphylococci* and *Streptococci*.

- ➔ - In **Hospital A**, all samples except one (9B) were catalase-positive, indicating the

presence of organisms such as *E. coli, Staphylococcus*, and *Klebsiella* (Table 9).

- ➔ - In **Hospital B**, all samples were catalase-positive, suggesting a dominance of catalase-positive organisms such as *E. coli*, *Enterobacter*, and *Staphylococcus* (Table 16).
- ➔ - In **Hospital C**, all samples were catalase-positive, again indicating the presence of organisms like *E. coli*, *Enterobacte*r, and *Klebsiella* (Table 23).

The widespread presence of catalase-positive organisms across the hospitals reflects the prevalence of aerobic or facultatively anaerobic bacteria, common in nosocomial infections.

7. Triple-Sugar-Iron (TSI) Test

The **TSI Test** is a differential medium used to assess an organism's ability to ferment sugars and produce hydrogen sulfide (H2S) or gas.

- ➔ In **Hospital A,** most samples fermented all three sugars (glucose, sucrose, and lactose), with one sample producing H2S, indicating organisms like *E. coli, Enterococcus*, and *Salmonella* (Table 10).
- ➔ In **Hospital B**, one sample (1W) fermented all sugars and produced gas, indicating *E. coli,* while the other samples showed limited sugar fermentation (Table 17).
- ➔ In **Hospital C**, most samples showed full sugar fermentation without H2S production, indicating *E. coli* or *Enterococcus* (Table 24).

The TSI results indicate a high prevalence of enteric organisms like *E. coli* and *Enterococcus*, which are common in hospital-acquired infections.

6.3. RADIOACTIVITY TEST RESULT-DISCUSSION

The radioactivity results from Hospitals A, B, and C provide insights into the presence of radioactive isotopes in various medical and environmental samples. Specifically, the concentrations of uranium-238 (U-238), thorium-232 (Th-232), and potassium-40 (K-40) were measured in samples like urine test caps, drain water, X-ray needles, radioactive gauges, and lung waste. The activity concentrations of natural radionuclides U-238, Th-232 and non chained K-40 were measured using gamma-ray spectrometry. (UNSCEAR 2000)

From Hospital A, a total of 6 samples were collected. These are- urine test cap, drain water, Gauge (Radioactive), Lung waste, urine. The test result for U-238 identifies that the average value of these sample's are respectively 2.53±0.43 , 1.89±0.43 , 2.32±0.42, 2.46±0.42 , 1.98±0.43 , 2.44±0.45. And according to UNSCEAR 2000, the world average value for U-238 is 35 Bq/kg. This means that all of the samples average concentrations is lower than the average worldwide value. It means that the samples do not contain any high concentration of radioactivity. So, it is not harmful for the environment and public health. The average activity concentration of Th-232 of these samples is - 2.21±0.41 ,1.32±0.35 ,1.49±0.43 ,1.95±0.36 ,2.08±0.42 ,2.70±0.43. The world average value of Th-232 is 30Bq/kg. This means that the activity concentration of Th-232 is much lower than the world average value.(Ramli et al., 2004) The activity concentration of K-40 is 143±4.47 , 87±2.64 , 44±6.14 ,102±6.23 ,41±6.36 , 243±6.16 respectively whereas the world average value of K-40 is 400Bq/kg. Here, sample 6-urine has the highest average value though it is much lower than the world average value. So, samples collected from hospital A pose no threat to the environment and public health as the values are much lower than the world average value(Haque.M et al, 2017).

From Hospital B, The result interpretation for the same six samples is the same as Hospital A. The activity concentration of U-238, Th-232 and K-40 is much lower than the world average value.(Ramli et al., 2004). Here, the urine test cap and urine pose a high concentration of K-40 which are - 109± 3.06, 107±2.00 respectively. However, it is much lower than the world average value of K-40. From the Hospital A samples it is also seen that the urine samples have much higher activity concentration than other samples. The other two radionuclides U-238 and Th-232 have not been in high concentration in Hospital B samples. All of them were below 3Bq/kg whereas the world average value is 30 and 35 respectively (Haque.M et al, 2017).

Lastly, **from Hospital C**, we have seen that the activity concentration of urine and urine test cap is much lower than from Hospital A and B which are -55±3.71, 131±2.46. Hence, the activity concentration of the sample 4- Gauge which had been collected from the radioactive department is 207±4.52. Though this is much lower than the K-40 world average activity level which is 400Bq/kg, it is quite high compared to other samples.(Ramli et al., 2004). The other two radionuclides U-238 and Th-232 have not been in high concentration in Hospital C samples. All of them were below 4Bq/kg whereas the world average value is 30 and 35 respectively.(Haque.M et al, 2017)

According to UNSCEAR 2000, the levels of radioactivity observed in these samples are relatively low but not insignificant. Chronic exposure to even low levels of radiation can pose health risks, particularly in sensitive environments like hospitals, where patients may have compromised immune systems. The presence of U-238 and Th-232 in biological samples (e.g., urine and lung waste) suggests potential bioaccumulation, which could have long-term effects on hospital staff or patients if proper waste management protocols are not strictly followed.

6.4. Opportunistic Bacteria Analysis:

As mentioned before, opportunistic organisms are those that normally do not cause infection in a healthy host rather they choose a weakened immune system to cause infection or illness. These organisms take advantage of certain organisms and create illness in the patient's body. Many opportunistic organisms are part of the normal flora of the body such as bacteria in the gut, skin, or respiratory tract. Others can be found in the environment. Here, a weakened immune system means- underlying diseases such as diabetes, chronic lung disease, malnutrition, etc. The immune system normally keeps the opportunistic bacteria in check. However, when the immune system is weakened, these organisms can multiply and cause infection. Opportunistic bacteria can also occur when the physical barriers are compromised such as skin, and mucosal surfaces. Imbalance of the normal flora can also reduce the normal bacterial population causing opportunistic bacteria to thrive. So, these bacteria are usually not harmful but they can cause illness or disease when the defense mechanism is weak. (Niccolo Riccardi et al, 2019)

Some examples of these bacteria are- E.coli, Klebsiella, Enterobacter and Pseudomonas aeruginosa. E.coli causes urinary tract infections. Pseudomonas aeruginosa cause lung infection in people with cystic fibrosis. Enterobacter is present in the normal flora or gut of the host which causes disease when the system is weakened. Along with UTIs, these also cause respiratory tract infections, wound infections, and sepsis. These are associated with medical devices like- catheters. Klebsiella causes pneumonia, UTI, and wound infection. Enterococcus are also opportunistic organisms that cause UTI, abdominal infection, endocarditis, and wound infection. Pseudomonas also causes UTI, wound infection, and respiratory tract infections as it is associated with burn infection, and ear infections. These are also called hospital-acquired infections(HCIs). (Niccolo Riccardi et al, 2019)

Three samples were collected from Hospital A: blood-stained tissue, urine cap, and catheter. These three samples were associated with patients. After culturing them on UTI media, we collected a total of 4 samples. These are 1P, 2P, 9P, 9B. TSI Test and Citrate Utilization Test have been performed to find out the hypothesized organisms. After performing both of these tests, 1P gives positive results in the Citrate test and TSI test, it shows A/A in butt/slant, H2S production no gas production. From this, we can conclude that the hypothesized organism can be E.coli, Enterobacter. After analyzing the results for 2P, we can also conclude that the hypothesized organisms are- Klebsiella and E.coli. The opportunistic organisms found in 9P are E.coli and 9B organisms can be interpreted as E.coli, Enterobacter.

The concern fact of opportunistic bacteria is that they can be the reason of antibiotic resistance which means the patients would face hardships in treating the diseases as they show resistance towards most of the antibiotics. So, the infections caused by opportunistic bacterias get more difficult to treat. (Niccolo Riccardi et al, 2019)

From Hospital B, 4 samples have been found out from UTI tests. These are - 1B, 1W, 3W, 3G. Sample 1 is blood-stained tissue and sample 3 is catheter. These two samples concluded the opportunistic organisms named as- Klebsiella, Pseudomonas, Enterobacter, and E.coli.

Lastly, from Hospital C, a total of 2 samples have been collected- 6W, 6B. These two samples are the outcome of sample 6 which is urine cap. These two samples gave opportunistic bacterias - Klebsiella, Pseudomonas, E.coli.

By following the protocol, all of these organisms have been found by using TSI Test and Citrate Utilization test. All of the organisms found from these three hospitals are opportunistic bacterias.

These have become alarming these days because of their antibiotic resistance. Though these bacterias are harmless in normal individuals, these can be life-threatening in weak immune system of the patient's body.

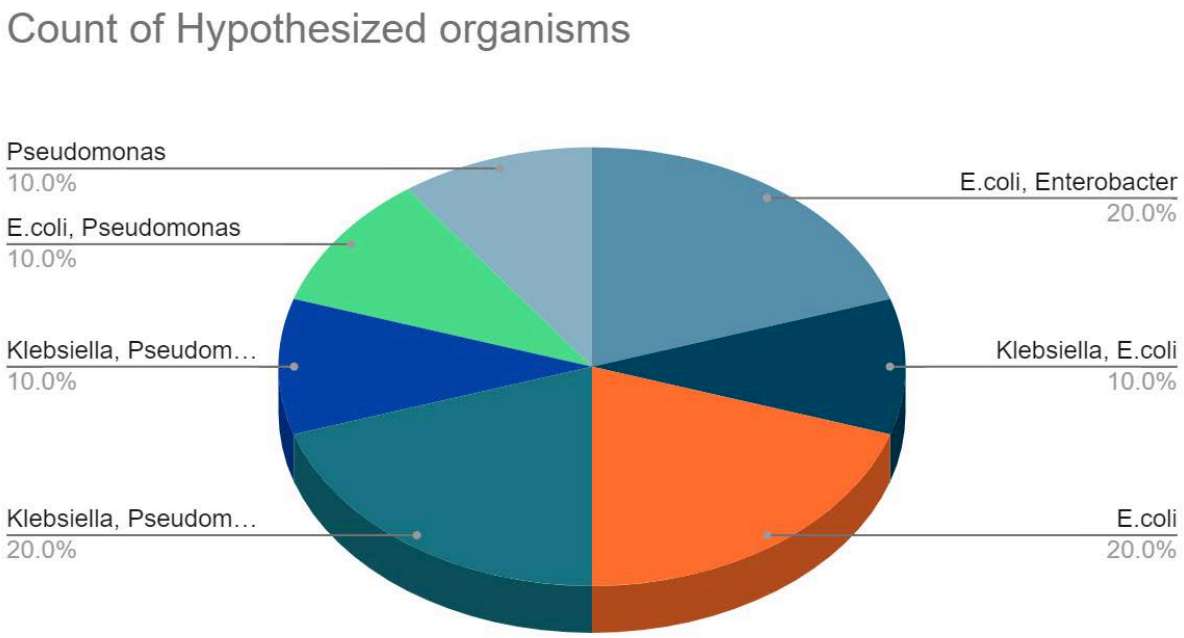


Figure 34: The pie chart of hypothesized opportunistic bacterias collected from Hospital A, B & C

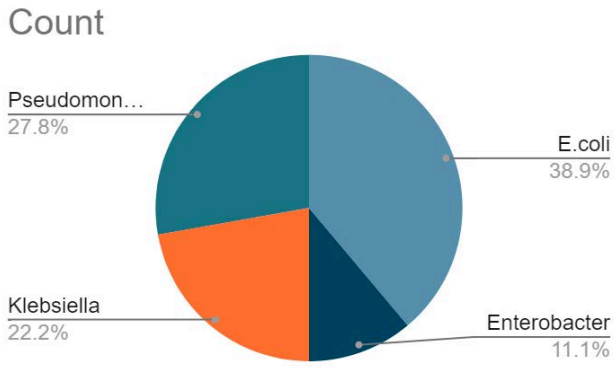


Figure 35: The most possible opportunistic bacteria found from 3 hospitals is E.coli. Then comes pseudomonas, Klebsiella, Enterobacter respectively.

Chapter 7

Future Prospects

Although this comprehensive study focuses on mainly the microbial, radioactive and opportunistic bacteria analysis, this study can further be done by putting focus on Biomedical Waste Management in the hospitals. This study can connect various fields together like the health impact of inadequate waste management, the impact of the exposure of opportunistic bacteria found there. Nonetheless, the impact of waste management during COVID-19 can help to draw a more effective pattern to deal with the waste management so that future crises can be handled more carefully.

- As the research continues to work on statistical analysis, there will be a data collection process from not only these three hospitals. This research targets at least 10 hospitals from where we will analyze how biomedical waste affects our environment and public health. This analysis will be carried out using SPSS.
- More sample collection using cotton swabs will be taken to carry out our opportunistic bacteria finding, the hypothesized analysis done on opportunistic bacteria will be useful later to prove the prevalence of harmful opportunistic bacteria.
- Health Impact of Inadequate Waste Management: Further research could examine the long-term health effects on healthcare workers and the general public caused by improper medical waste disposal, focusing on the role of infectious and antibiotic-resistant bacteria found in biomedical waste.
- Impact of Waste Management on Public Health During Pandemics: Research on the specific challenges posed by pandemics, like COVID-19, for biomedical waste management systems in Bangladesh would provide valuable insights into how to prepare for future health crises.

Chapter 8

Conclusion

Biomedical waste management concern was not talked about until COVID-19 affected our lives. The improper waste management system not only affects our health as well as our environment. The microbial analysis made us concerned about our well-being, these microbes found in the open places from the wastes can get contaminated with our daily life usage materials. From the radioactive analysis, the results of U-238, Th-232 and K-40 activity concentration are below the world average level according to UNSCEAR 2000. Hence, the radioactivity test ensures safety for public health and the environment. Later, the opportunistic bacteria analysis has made us concerned about how these organisms are very much involved with weak immune systems and how they are working for the new fear which is antibiotic resistance. This will surely lead us to a very alarming public health concern. So, the proper waste management system needs to be followed strictly to provide a safe environment for the people. A safety protocol needs to be followed strictly by the authorities as this proves to be weakening the health system and also affecting the environment. In the long run, a weakened safety protocol may cost us a lot.

Chapter 9

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