Biomedical Waste Analysis

BY

Kiran Bhatt

(20236023)

Shital Nagarkoti

(20236020)

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

Department of Mathematics and Natural Sciences (MNS)

Brac University

October, 2024

Declaration

It is hereby declared that

1. The thesis submitted is our own original work while completing the 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 main sources of help.

Student's Full Name and Signature:

_____

Kiran Bhatt
(20236023)

_____

Shital Nagarkoti
(20236020)

Approval

The thesis/project titled Biomedical Waste Analysis submitted by Kiran Bhatt (20236023) and Shital Nagarkoti (20236020) of Fall 2020 has been accepted as a satisfactory partial fulfillment of the Bachelor of Science in Biotechnology degree requirements on 15 October 2024.

Examining Committee:

Supervisor: _____

Dr. Munima Haque
Associate Professor,
Department of Mathematics and Natural Sciences Brac University

Program Director: _____

Dr. Munima Haque
Associate Professor,
Department of Mathematics and Natural Sciences Brac University

Departmental Head: _____

Dr. Md Firoze H. Haque
Associate Professor & Chairperson,
Department of Mathematics and Natural Sciences Brac University

Acknowledgment:

We would like to express our deepest gratitude to God for guiding us and giving us the strength to complete our thesis.

Our sincerest appreciation goes to our supervisor Dr. Munima Haque, Director of the Biotechnology Program, Department of Mathematics and Natural Sciences, for her guidance, expertise and unwavering support throughout the journey of our thesis.

Furthermore, we would like to express our gratitude to Dr. Md Firoze H. Haque, Associate Professor & Chairperson, Department of Mathematics and Natural Sciences Brac University for approving our project.

We would also like to thank the thesis lab officers, Shamima Akter Chowdhury and Md. Mahmudul Hasan for their guidance and insights on the various lab instruments and protocols. In addition, we would like to thank the lab assistants, for always being there and assisting us in operating the lab equipment.

Finally, a heartfelt thanks to our friends & family without whose unwavering support, this thesis would not have been completed.

.

Abstract:

The potential for biomedical waste to transmit infectious illnesses and include microorganisms resistant to antibiotics makes it a severe problem. The purpose of this study was to determine the microorganisms like *Pseudomonas, Salmonella*, *Shigella*, and *E.coli* were in biomedical waste The study was conducted from June 2023 to June 2024. A total of 39 samples were gathered from five different hospitals in Dhaka. From the total samples, 87 isolates of our targeted organism were identified. To identify presumed bacterial isolates, biochemical assays such as Triple Sugar Iron test, Oxidase test, Citrate Utilization test, Gram staining, and Catalase test were employed in combination with selective media. Furthermore, molecular identification of the 87 bacterial isolates was held using PCR. In addition to this, an antibiotic susceptibility test was carried out to find the resistance of these isolates to different antibiotics. The results from the tests showed a surprising number of confirmed targeted species. To begin with, approximately, 65% of the isolates were multi-drug resistant (MDR), with *Pseudomonas* species exhibiting the greatest level of resistance. In addition, it was found that *Escherichia coli* was present in 28% of the samples, and 65% of the isolates showed signs of multi-drug resistance (MDR). Also in about 22% of the samples, *Shigella* was found and every isolate had multiple drug resistance. Ultimately, 20% of the samples had Salmonella detected in them, and half of the isolates had multi-drug resistance.

Overall, the results show a concerning frequency of infections that are resistant to several drugs in biomedical waste, underscoring the pressing need for improved infection control and waste management procedures to protect the public's health and stop the spread of these harmful organisms.

Keywords: Biomedical waste, microbial analysis, antibiotic resistance

Table of Contents

Chapter 1: Introduction **1**

1.1 Overview of biomedical waste and its significance. 1

1.2 Importance of proper biomedical waste management: 1

1.3 Medical Waste Management in Bangladesh 4

1.4 Objectives of the Study 6

The primary objective of this project was: 6

1.5 Hypothesis: 6

Chapter 2: Literature Review **6**

2. 1 Introduction: 6

2.2 Types of Biomedical Waste 10

2.2.1 General waste: 11

2.2.2 Pathological waste: 11

2.2.3 Infectious waste 12

2.2.4 Sharps: 13

2.2.5 Pharmaceutical waste: 13

2.2.6 Chemical waste: 14

2.2.7 Radioactive waste: 15

2.2.8 Cytotoxic waste: 15

2.3 Potential health hazards from biomedical waste: 16

2.4 Environmental impact of improper waste disposal: 17

2.5. Isolation of Pathogens from Biomedical Waste: 18

2.6 Overview of Key Pathogens 19

2.6.1 Escherichia coli 20

2.6.2. Pseudomonas 20

2.6.3 Salmonella spp. 21

2.6.4 Shigella spp. 22

2.7. Molecular Techniques in Pathogen Detection 23

2.8. Microbial Tests for Confirmation 24

2.8.1 Gram staining 25

2.8.2 Biochemical tests (oxidase test, catalase test, sugar fermentation tests) 25

2.8.2.1 Methyl Red Test (MR-Test) 26

2.8.2.2 Voges-Proskauer (VP) Test 26

2.8.2.3 Triple Sugar Iron (TSI) Test 26

2.8.2.4 Catalase Test 26

2.8.2.5 Oxidase Test 27

2.8.2.6 Citrate Test 27

2.8.2.7 Motility Test 27

2.9 Antibiogram tests 28

2.10 Conclusion 31

Chapter 3: Methodology **31**
3.1. Research Design 31
3. 2. Microbial analysis 32
3.2.1 Preparation of Peptone buffer 33
3.2.2 Preparation of selective media and inoculation of microorganisms 33
3.2.2.1 Xylose Lysine Deoxycholate (XLD) media 34
3.2.2.2 Cetrimide media 36
3.2.2.3 UTI (Urinary tract infection) Media 37
3.2.2.4 Nutrient agar 39
Principle: 39
3.3 Extraction of the bacterial DNA 40
3.3.1 Preparation of Nutrient broth and inoculation of the bacterial samples 40
3.3.2 Preparation of TE buffer 41
3.3.3 Extraction of the bacterial DNA 43
3.4 Polymerase chain reaction (PCR) 45
3.5 Biochechemical Tests 48
3.5.1 Triple Sugar Iron (TSI) 48
3.5.2 Citrate test 50
3.5.3 Methyl Red/ Voges (MR VP Test) 51
3.5.3.1 Methyl red(MR) Test 51
3.5.3.2 Voges-Proskauer (VP) test 52
3.5.4 Mobility test 55
3.5.5 Catalase test 56
Principle: 56
3.5.6 Oxidase test: 57
3.5.7 Gram Staining 58
3.6 Antibiotic test 61
4. Chapter 4: Observation and Results **62**
4.1 Isolation of Bacterial Strains 62
4.2 Selective and Differential Media 64
4.3 Isolation of Pseudomonas 66
4.3.1. Polymerase Chain Reaction (PCR) Results: 66
4.3.2 Identification of Pseudomonas using Biochemical tests 67
4.3.2.1 Gram staining of isolated Pseudomonas 68
4.3.2.2 Identification by Triple Sugar Iron (TSI): 70
4.3.2.3 Identification using Citrate Utilization Test 71
4.3.2.4 Identification using Catalase Test 73
4.3.2.5 Identification using Oxidase Test 74
4.3.2.6 Identification using MR test: 76
4.3.2.7 Identification using VP test 78
4.3.2.8 Identification using Motility Test for Pseudomonas isolates 79

4.3.3 Antibiogram test result for Pseudomonas 81
4.4 Isolation of Salmonella 83
4.4.1 PCR and Gel electrophoresis: 83
4.4.2 Identification of Salmonella by Using Biochemical Tests 85
4.4.2.1 Gram staining: 86
4.4.2.2 Identification by Triple Sugar Iron (TSI) 87
4.4.2.3 Identification using Citrate Utilization Test 89
4.4.2.4 Identification using Catalase Test 91
4.4.2.5 Identification using Oxidase Test 93
4.4.2.6 Identification using MR test: 94
4.4.2.7 Identification using VP test 97
4.4.2.8 Identification using Motility Test 98
4.4.3 Antibiogram test for Salmonella 100
4.5 Isolations of Shigella 101
4.5.1 PCR and Gel electrophoresis 101
4.5.2 Identification of Shigella by Using Biochemical Tests 102
4.5.2.1 Gram staining 103
4.5.2.2 Identification by Triple Sugar Iron (TSI) 104
4.5.2.3 Identification using Citrate Utilization Test 106
4.5.2.4 Identification using Catalase Test 107
4.5.2.5 Identification using Oxidase Test 109
4.5.2.6 Identification using MR test 110
4.5.2.7 Identification using VP test 112
4.5.2.8 Identification using Motility Test: 114
4.5.3 Antibiogram Test for Shigella 115
4.6 Isolation of E. coli 117
4.6.1 PCR and Gel electrophoresis 117
4.6.2.Identification of E. coli using Biochemical Tests 118
Table: 4.6.1 119
4.6.2.1 Gram staining 119
4.6.2.2 Identification by Triple Sugar Iron (TSI) 120
4.6.2.3 Identification using Citrate Utilization Test 121
4.6.2.4 Identification using Catalase Test 124
4.6.2.5 Identification using Oxidase Test 125
4.6.2.6 Identification using MR test: 127
4.6.2.7 Identification using VP test: 129
4.6.2.8 Identification using Motility Test 131
4.6.3 Antibiogram test result for E.coli 132
Chapter 5: Discussions **134**
5.1 Prevalence of Pseudomonas 134
5.2 Prevalence of Salmonella 136

5.3 Prevalence of Shigella 138
5.4 Prevalence of E. coli 141
Limitation **143**
Conclusion **144**
References **146**

Biomedical Waste Analysis

Chapter 1: Introduction

1.1 Overview of biomedical waste and its significance.

According to the World Health Organization (WHO), medical waste is the waste generated from healthcare activities, that ranges from used syringes and needles to diagnostic blood samples, medical devices, bandages, dressings, radioactive materials, and body parts. The proper management of such kinds of waste is an important factor in healthcare. This is because improper management of such wastes can lead to various infections, injuries as well and toxic effects to the healthcare workers, the waste handler, and the community. Along with this, it can also contribute to environmental pollution affecting the whole ecosystem.

Approximately 15 percent of the total waste generated by healthcare facilities has been considered hazardous materials, which could be chemical, infectious, or radioactive (WHO, n.d). According to the data sources, the unsafe disposal of the injections led to 315,000 hepatitis C infections, 1.7 million hepatitis B infections, and 33,800 new HIV infections in the year 2010 (WHO, n.d). Thus to avoid adverse health outcomes associated with poor practices, including exposure to infectious agents and toxic substances, the proper management of health care waste requires increased attention and diligence. For long-term improvement, universal and sustained improvement, government commitment and support are needed.

In 2014, WHO developed the first global and comprehensive guidance document for the safe management of waste from healthcare activities. To illustrate, the guide addresses aspects such as regulatory framework, planning issues, minimization of waste and recycling, handling, storage and transportation, treatment and disposal options, and training.

1.2 Importance of proper biomedical waste management:

The term "biomedical waste management" describes the appropriate processing, storage, transportation, treatment, and disposal of waste produced by medical facilities, research facilities, and other establishments that perform biological or medical operations. To begin with, the waste produced during the diagnosis, treatment, or immunization of humans or animals is

categorized as biological waste. About 75-90% of the generated biomedical waste is found to be harmless and non-hazardous. While the remaining 10-25% is hazardous and can be injurious to the environment, humans, and as well as other organisms (Pasupathi et al., n.d.). These kinds of wastes can be home to various infectious agents, sharp objects, chemicals, radioactive materials, and other potentially dangerous elements. If improperly managed, these materials could endanger public health and the environment.

Therefore, organizing waste into distinct categories, such as chemical, pathological, infectious, and sharps, is essential to efficient biomedical waste management. Following that, the garbage is gathered, stored, and transported in compliance with particular laws and policies. The waste is rendered harmless by treatment techniques like autoclaving, burning, or chemical disinfection before being disposed of in a way that poses the fewest threats to the environment and public health. To improve healthcare waste management at the national, regional, and local levels, WHO (World Health Organization: WHO, 2018) states that setting goals and making plans to attain them is crucial. The recommendations made by Agenda 21 for appropriate waste management might be summed up as follows (World Health Organization: WHO, 2018): Waste should be treated by the final residues by landfilling in small, properly planned sites. (a) Prevent and minimize waste creation. (b) Reuse or recycle the trash to the degree practicable.

Thus, avoiding health risks and creating a welcoming hospital atmosphere are necessary for effective hospital waste management. The World Health Organization (World Health Organization: WHO, 2018) has guidelines that hospitals must follow to guarantee the safe management of medical waste. These are (a) waste minimization at the source through stock management, reuse, and recovery; (b) waste segregation according to categories and sharps; (c) waste identification through color coding for various wastes; (d) waste collection and storage through regular programs; and (e) waste transfer by using enclosed vehicles and the "chalked pathway from generation to disposal sites." (f) Options for both burn and non-burn treatment: To manage their medical waste, the northern countries have already embraced innovative technologies and management approaches. Currently, these countries are now taking a more environmentally friendly approach to handling their trash problems. They constantly monitor and assess their systems while creating new policies and implementing new technologies. The government of Bangladesh is working to create a fresh, contemporary strategy to deal with

medical waste, as it is still a relatively new occurrence in the country. Bangladesh lacks a national policy for the management of medical waste. The government should prioritize developing a framework for the appropriate and scientific handling of medical waste. In addition, the current laws are antiquated and can penalize offenders with little to no consequences. Thus, widespread education about this problem and the implementation of stricter legislation may be able to shield the public and the environment from hazardous medical waste.

A Hospital Waste Pocket Book was created by the Department of Environment in 2004 and updated in June 2010. Medical Waste Management Rules 2008 (Dana, 2011) provide support for this publication. All of the basic operating procedures for an appropriate management system for any type of healthcare facility are included in the pocketbook. The book provides color-coded guidelines for trash segregation along with a detailed illustration of the many sorts of hospital waste. Hospital waste handlers' responsibilities are spelled out. It also specifies how garbage is to be moved and kept on hospital property. This manual guarantees, at the very least, adherence to the most recent regulations and serves as an example of best practices.

However, it merely addresses choices for treatment, recycling, transportation, and disposal; it makes no mention of an acceptable, safe, or economical plan. In Bangladesh, healthcare facilities such as clinics, hospitals, nursing homes, and dentistry hospitals, among others, lack proper waste management systems, endangering both the environment and public health. The officials of the hospital and the government don't give this issue enough thought. Without any form of treatment, almost all hospitals and clinics dispose of their hazardous and non-hazardous garbage in the adjacent municipal dustbins or by the side of the road. Consequently, the hospitals and the surrounding areas have an unhealthy and dangerous atmosphere that impacts patients, hospital employees, and any individuals who come into contact with these conditions. Inadequate medical waste management practices are present across the nation, according to a 2005 study (Dana, 2011). It was also discovered that, although some hospitals separate their infected and non-infectious waste streams at the point of production, the wastes are disposed of in municipal dustbins where they are mixed (Dana, 2011).

1.3 Medical Waste Management in Bangladesh

The inadequate treatment and disposal techniques of medical waste pose significant threats to human health and the environment, making medical waste management a global concern. To illustrate, the handling of contaminated objects like bandages, syringes, and needles is a mismanagement that puts workers' health at risk for infections and other ailments. Furthermore, it also contaminates water supplies, produces air pollution, and adds to environmental contamination. As a result, the ecological equilibrium can be upset, public trust in healthcare institutions can be damaged, and governments and communities may incur long-term financial consequences as a result of poor management.

Poor medical waste management can result in long-term financial expenditures as well as infections, illnesses, health concerns, environmental degradation, distrust, and ecological imbalances (*Medical Waste Management in Bangladesh – A2i*, n.d.). Bangladesh, which is also facing a similar problem, has the opportunity to change its medical waste management system by utilizing technology breakthroughs, particularly in the areas of artificial intelligence (AI) and the Internet of Things (IoT) (*Medical Waste Management in Bangladesh – A2i*, n.d.). To solve Bangladesh's problems with medical waste, we can investigate and promote the integration of photo-pioneering technological projects from Asia and other developing regions.

Bangladesh is currently facing dire consequences as a result of insufficient regulation and a lack of a waste management plan. In Dhaka, medical waste generation is 1.63-1.99 kg per bed per day, and it grew dramatically after COVID-19, according to research released in 2022. The nation has had, to put it mildly, insufficient management for a very long time, lacking a secure system for the collection, transportation, handling, and disposal of garbage (Mim et al., 2024). This problem is also common in areas with a predominately vulnerable populace. Between 2015 and 2021, the ICRC built five medical waste management facilities in the Cox's Bazar area, including the Teknaf, Ukhiya, Chakaria, Ramu, and Pekua health complexes (*Medical Waste Management in Bangladesh – A2i*, n.d.). As part of this commitment, the ICRC also set up medical waste management operations to assist the Cox's Bazar district's healthcare complexes. And yet, the issue persists, impacting individuals all over Bangladesh.

Many organizations in Bangladesh are currently working to stop the risks associated with improperly managed medical waste, including Waste Concern, PRISM Bangladesh, the International Center for Diarrheal Disease Research (ICDDR, B), the Center for Sustainable Development (CSD), and the Environment and Social Development Organization (ESDO) (*Medical Waste Management in Bangladesh – A2i*, n.d.). An NGO called PRISM Bangladesh works to improve the environment and health in Bangladesh by making sure that medical waste is managed properly. They offer medical waste collection, transportation, treatment, and disposal services in addition to awareness-raising and training. Additionally, they support strong frameworks and a waste management culture.

But even with the greatest will on the part of organizations such as PRISM Bangladesh and other important players, it is difficult to guarantee appropriate medical waste management in Bangladesh (*Medical Waste Management in Bangladesh – A2i*, n.d.). With the appropriate resources and methods, we could discover the way to a long-term fix.

Using blockchain, IoT, AI, and big data can transform medical waste management in Bangladesh as we move toward a tech-driven future. Blockchain provides accountability and transparency, while AI helps optimize waste processes through picture recognition and predictive analytics. To avoid overflows, Internet of Things devices provide GPS tracking and real-time fill level monitoring. Big Data analytics offer perceptions into compliance and waste generation trends. By combining these technologies, risks to the environment and public health can be reduced while efficiency is increased.

The medical waste management industry has seen a radical change in recent years, as has the adoption of technology in many other areas of the global economy (Mohamed et al., 2023). One notable example is the AI- and face-recognition-powered medical waste monitoring system in China, which is built on the Internet of Things. To increase transparency, Turkey's solution combines blockchain technology with IoT. India uses artificial intelligence (AI) to classify residential waste, whereas Thailand uses an image recognition system to improve waste sorting. IoT is used in Singapore to track garbage levels in real time (*Medical Waste Management in Bangladesh – A2i*, n.d.-b). By evaluating local needs, customizing technology, integrating AI, educating stakeholders, guaranteeing regulatory compliance, launching pilot projects, forming public-private partnerships, and maintaining continuous development, Bangladesh may think

about embracing these advancements in tech-driven medical waste management. Through partnerships, awareness-raising, policy frameworks, capacity building, and pilot initiatives, the government may bring about change. It is necessary to do something disruptive. The severe risks that could arise from Bangladesh's improper handling of medical waste must be reduced, and this requires an innovative solution.

The management of medical waste in Bangladesh needs to be addressed right now. Bangladesh can transform trash management while preserving the environment and public health by utilizing AI, IoT, and other technology (*Medical Waste Management in Bangladesh – A2i*, n.d.-b). Initiatives from the government can accelerate this transformation, encourage cooperation, and provide stakeholders the authority to accept and maintain these creative solutions. Bangladesh's sustainable medical waste management is going to need to undergo a technology makeover (Nafiz, 2023).

1.4 Objectives of the Study

The primary objective of this project was:

●To identify the pathogenic microorganisms from medical waste

●To identify Biomedical waste limitations and management in Bangladesh

1.5 Hypothesis:

- Null hypothesis: Biomedical waste does not consist of any pathogenic microorganisms that may risk human health and the environment.
- Alternative hypothesis: Biomedical waste consists of pathogenic microorganisms that may risk human health and the environment.

Chapter 2: Literature Review

2. 1 Introduction:

Biomedical Wastes are the different types of waste produced by clinics, hospitals, or any medical waste. To begin with, any type of waste produced during research activities in health facilities is used in the diagnosis, treatment, or immunization of humans and animals. Such wastes include a

variety of materials used like syringes, needles, bandages, tissues, body fluids, as well as pharmaceutical products. Furthermore, Hospitals, research institutions, health care teaching institutes, clinics, laboratories, blood banks, animal houses, and veterinary institutions generate biomedical waste (Sharma M, 2002). These biomedical wastes carry the potential of transmission of diseases posing the risk of infection or harm to humans, animals, and the environment so, it must be disposed of with proper regulations. Moreover, Biomedical wastes can be classified based on various solid and liquid wastes into pressured, radioactive, chemical, sharps, infectious, as well as pharmaceuticals.

According to the WHO (1999) that, approximately 85% of health hazards to health workers, public and air hospital waste are non-hazardous, 10% are infective and 5% are zonal flora which are non-infective but hazardous. However, the scope of Biomedical waste is extended to various sources that include hazardous items from healthcare facilities, including infectious and pathological materials, sharps, chemical, pharmaceutical, and cytotoxic waste. It includes non-harmful waste from residences and diagnostic facilities. Proper classification and management are crucial to protect ecological systems, public health, and the environment from pollution and health risks. Such healthcare waste referred to as medical waste has a high chance of contamination by different types of blood, body fluids, and other infectious materials (*Medical Waste | US EPA*, 2024). According to the rough estimation, it was found that out of 4 kg, 1 kg of waste was infected also, based on the statistical data of the Environmental Protection Agency of America and Japan, it was found that 1 to 1.5 kg/day/ bed of volume for hospitals (Manasi et al., 2014). In addition, due to rapid population growth as well as global warming and pollutants such as air, water, soil, etc, more people are getting sick which increases the production of biomedical wastes.

S.N	**Name of the country**	**Quantity of waste generated (Kg/bed/day)**	**S.N**	**Name of the country**	**Quantity of waste generated (Kg/bed/day)**
1.	USA	2.5	5.	Spain	3.0
2.	UK	4.5	6.	Nepal	1.5

3.	France	2.5	7.	Bangladesh	1.18
4.	India	1.5			

Source: (Manasi et al., 2014)

According to the research conducted in different hospitals in Bangladesh, it was found that the average waste of non-hazardous waste was 0.957 kg/bed/day while hazardous waste was 0.229kg/bed/day (Basak et al., 2019).

Furthermore, the United States is the third largest country to discard more than two million of waste annually in the world. Also, developed countries' hospitals parcel tons of medical waste which are sent to developing countries to get rid of it, whereas in developing third-world countries customers buy those reprocessed items after sterilization at relatively low prices. Not to mention, in developing countries, the proper regulations of waste management problems are associated with a lack of awareness, proper funding as well as regulations for disposal of waste. In addition, African countries lack proper sanitary landfills so they are exempted from using incinerators to a large extent as well and many unscientific dump sites are found. Also, these incinerators more than 1000 are not properly operated. Similarly, some African countries such as Ghana, Lesotho, and Eritrea lack legislation for the management of healthcare waste while other countries like Kenya, Gambia, and Nigeria have passed some important laws in this regard (Manasi et al., 2014).

Medical wastes should be handled and disposed of properly according to the World Health Organization and Environmental Protection Agency. The collection, handling, removal, and disposal of such different hazardous as well as non-hazardous waste from public places to dumping grounds are the responsibility of municipal authorities in Bangladesh. Also, the management of hospital waste is under the guidance of municipal authorities which is being neglected and might cause different potential environmental hazards and public health risks (Dana, 2014). Various countries including Bangladesh face major health hazards around 5.2 million people with 4 million of them being children due to mismanagement and improper

disposal of medical waste according to the United Nations Conference on Environment and Development (Dhaka Tribune, 2023).

Individuals who handle trash that is infected or improperly discarded sharps run the risk of getting diseases like HIV or hepatitis from contaminated garbage, which poses a major danger to public health. Even though this practice has serious health implications, some indigent scavengers in developing countries City usually gather medical waste (like syringes-needles, saline bags, and blood bags) for resale purposes. Such practices have huge health risks leading to a serious disease burden. Moreover, collecting non-reusable healthcare equipment (such as needles), reselling them, and even using them again without disinfection could be one of the main reasons behind the disease burden. The concept of sharing or reusing syringes is known to spread some diseases like hepatitis and AIDS (Tamplin et al., 2005). Likewise, the medical professionals, waste disposal workers, and other citizens living around these hospitals are all in danger. Different unmetabolized or non-metabolized antibiotics released by patients through stool and urine might lead to the growth of antibiotic-resistant bacteria in the environment (Chowdhury & Uddin, 2022). Also, the presence of multidrug-resistant bacteria in biomedical waste further complicates this issue. Further, disposal methods for expired or old antibiotics particularly in public dustbins, animal husbandry farms, and pharmaceutical industries lead to the emergence of multidrug-resistant bacteria. Such emergence of multidrug resistance has caused treatment issues in developed as well as developing countries (Sitotaw et al., 2024). To prevent the spread of germs, provide safety to health workers, and minimize pollution, it is very important to manage biological waste correctly. Inappropriate disposal methods might cause the dissemination of drug-resistant infections and therefore stringent waste management protocols must be observed by healthcare institutions to protect the atmosphere as well as society at large.

Proper management of biomedical wastes is very important as well as crucial to prevent the risk or harm of transmission of diseases. The management of waste in an appropriate safety procedure is an integral part of the healthcare system (Ministry of Health and Family Welfare of Bangladesh, 2016). In addition, mismanagement of such wastes can cause widespread environmental damage by contamination of water, air, and soil as well as the health of humans and animals. Different waste management practices like collection, treatment, disposal, transportation, storage, and segregation, serve as means of maintaining a healthcare system that

is devoid of hazards and thus protecting public health at large. Also, the proper management of biomedical wastes prevents infection as well as minimizes the risks. Not only healthcare workers, and patients are protected by proper disposal of biomedical wastes but also, the patient visitors, and people collecting the waste, everyone as well as the environment are safeguarded. Hence, proper management of biomedical wastes has a significant long-term impact to prevent and minimize the transmission of infectious diseases (Hassan et al., 2008).

2.2 Types of Biomedical Waste

Different sources of waste are hospitals, research laboratories, veterinary clinics, Dental clinics, blood banks, pharmaceutical companies as well as healthcare facilities. Some other sources are slaughterhouses, cosmetic services, blood donation camps, and funeral services. In addition, Hospital waste or medical waste are of a variety from general waste like paper, and food to chemical, radioactive, and hazardous waste. Primary sources of waste are hospitals, nursing homes, clinics, and medical laboratories whereas research centers, industries, and households are other sources (Babu et al., 2009). Furthermore, biomedical wastes are characterized as non-hazardous and Hazardous waste based on the source and types of waste. Non-hazardous types of waste are approximately 75-90% which are harmless, whereas hazardous waste is 10-25% which are harmful but when such non-hazardous types of waste are mixed with hazardous waste it becomes harmful (Hirani et al., 2014).

Foods from hospital kitchens or cafeterias, peels of fruits, paper products, plastic like water bottles, containers, and glass as well as textiles that are not exposed to patients are included in non-hazardous types of waste. In addition, hazardous wastes that are produced by healthcare facilities pose serious risks to public health and the environment as such types of waste are infectious, toxic, and poisonous. Moreover, biomedical waste is categorized based on its origin and chemical, physical, and biological characteristics. Hence, the WHO has classified biomedical waste 8 categories into the following types:

2.2.1 General waste:

General waste is a type of non-hazardous waste material that includes materials like packaging, office paper, and uncontaminated food waste. Furthermore, these wastes do not contain any chemicals, biological, radioactive, or infectious hazards. These wastes contain recyclable waste, Non-recyclable waste, and Biodegradable waste. Recyclable wastes are any type of waste that can be recycled and be used again, non-recyclable waste can be recycled to be used again and Biodegradable wastes are wastes such as kitchen waste, trimmings from yard, food scraps (World Health Organization, n.d.).

Hospitals and other health facilities, laboratories, and research centers are the major sources of healthcare waste. This type of waste resembles our home trash but comes in larger amounts and needs to be disposed of appropriately to avoid any risk to public health. Among them are packaging materials, discarded office supplies, and food residues which are thrown away after meals. To ensure that they do not turn into public health threats these wastes must be disposed of properly.

2.2.2 Pathological waste:

Pathological waste includes human tissues, organs, and body fluids that follow medical interventions. Mostly, operation theaters produce human anatomical waste which is later sent to pathological laboratories for diagnosis. Furthermore, these materials are fragile and may harbor infectious germs. Pathological waste that can be decontaminated is disposed of as general waste (National Institutes of Health [NIH], n.d.). Moreover, Pathological waste is usually treated by incineration with the aim of destruction and minimization of possible contamination or infection. Mishandling can lead to serious health problems.

Some examples of pathological wastes are any human organs or fluids, amputated limbs, removed tumors or placentas, tissues, unused blood products, and fetuses (Padmanabhan & Barik, 2019). Among 15-20% of hazardous waste, pathological wastes are found to be 1%. In addition, this pathological waste consists of body fluid which is the major source of transmission of infections and pathogens. Due to the presence of infectious agents and bodily fluids such types of waste cannot be stored or disposed of. Such wastes have a higher risk of causing disease and infection to the individuals who come in direct contact with the waste. Hence, such wastes should be disposed of properly to minimize disease transmission (*What Is Pathological Waste?* 2024).

2.2.3 Infectious waste

Infectious healthcare waste can be defined as biomedical waste that contains infective pathogens, with enough virulence that can infect a vulnerable host and cause infectious diseases. Varieties of biomedical waste have been classified as infectious wastes that consist of materials contaminated with blood, bodily fluids, or other potentially hazardous substances. It puts individuals at serious risk of contracting diseases, particularly in health institutions where patients and medical practitioners interact. Examples include used surgical gloves, contaminated dressings, and laboratory cultures among other disposable sharp objects used on patients (Padmanabhan & Barik, 2019b).To prevent these risks, it is important to observe the right disposal procedures like autoclaving, incineration, or chemical disinfection.

Moreover, the reckless disposal of infectious waste can lead to contamination of water bodies, soil, and air which in turn results in increased numbers of organisms responsible for infectious diseases in our environment. This leads to the release of such harmful pollutants like dioxins and furans through the incineration of untreated contagious waste resulting in long-term ecological damage. Also, it has adverse negative effects on the health of living beings (World Health Organization: WHO, 2018). Furthermore, inadequate management of such infectious wastes may result in disease transmission affecting health care personnel including those responsible for waste disposal as well as members of the public who are exposed to a variety of bloodborne pathogens including hepatitis B or HIV/AIDS virus infections and hepatitis C infections. Poorly managed even when used antibiotics become their carrier bacteria resistant to antibiotics, they are likely to complicate treatment options and lead to increased outbreaks (Kawade & Kokare,

2023). Commonly, such biohazard infectious waste is known as red bag waste in healthcare as Red bags are used for the collection of blood waste, laboratory waste as well as human body fluids. However, they are not appropriate for chemotherapy waste, pathological waste, or radioactive waste which must be incarcerated. These biohazard infectious wastes are treated primarily by autoclaving and sent to special landfills (Ryberg, n.d.). Hence, it is important to handle, treat, and dispose of infectious waste in a very careful way so that the community and the environment do not suffer.

2.2.4 Sharps:

A sharp is a kind of biomedical garbage, which can cut or puncture the skin and consequently endanger both physical trauma and the transmission of infectious diseases. The most common examples include needles, syringes, scalpels, lancets, and broken glassware employed in healthcare processes (U.S. Food and Drug Administration [FDA], 2021). Furthermore, improper disposal of any type of sharps can injure and spread injections among people and the environment. Mostly waste handlers, healthcare workers as well and the general public could become infected with blood-borne pathogens like HIV, Hepatitis B, or Hepatitis C virus types due to improper disposal of sharps. Also, serious infections as well as long-term health implications that are expensive to treat may arise because of injury caused by sharps.

2.2.5 Pharmaceutical waste:

Pharmaceutical wastes are the wastes usually generated from expired medicines and drugs that are produced by hospitals, and laboratories that have been tainted and can be no longer utilized again. Furthermore, the source of such pharmaceutical wastes is from hospitals, pharmaceutical stores, nursing home facilities as well as households. Also, these wastes can be derived from medications of patients who partially used or unused forms of medicine and outdated pharmaceutical products accumulated in hospital and pharmacy dispensaries. Moreover, Pharmaceutical waste production issues are increased due to lack of awareness of proper management of waste, shortage of human as well as financial resources, and ignorance of its

adverse effects on our health (Kawade & Kokare, 2023b). It is crucial to understand that pharmaceutical waste excludes items like empty glass ampoules, medications, and other metabolic products that patients who are receiving treatment excrete, as well as empty pill bottles and strip packages from which the medication or capsules have already been removed. Improper disposal of this kind of waste can severely impact public health

2.2.6 Chemical waste:

Chemical wastes are any type of reagents, solvents, or chemicals used in medical institutions for conducting various medical procedures, cleaning processes, and laboratory work. Disinfectants, sterilant, batteries, lab reagents, and heavy metals present in different medical devices are some examples of chemical waste (World Health Organization: WHO, 2018b). These chemicals may be toxic, corrosive, flammable, or reactive hence posing serious dangers when not handled well. These are wastes that are any products used from solid, liquid, or gaseous chemicals in different treatments, experiments as well as diagnostic purposes. Moreover, chemical wastes have three characteristics which include flammability, corrosion, and poisoning by interaction with other substances. Because of such characteristics, such wastes are disposed of by keeping them underground lined with non-reactive materials inside concrete or metal drums (Aljamali et al., 2020).

Mishandling or improper disposal can give rise to respiratory problems, skin burns, or poisoning which are some of the public health issues affecting individuals. In addition, mainly health workers and waste employees become at risk if they do not have adequate protection equipment and training on how to handle them well. On the other hand, improper storage or chemical spills can result in hazardous situations like fires and toxic gas emissions. Also, different health problems as well as cross-infection can be a direct disturbance to patients and people living in the community. Further, chemical waste leads to pollution of soils, waters, and air which eventually impacts negatively on both aquatic species as well as human beings (Abidar et al., 2020). Hence, it is crucial to use proper disposal methods for chemical waste management such as neutralization, specialized incineration, or safe storage so that such risks may be minimized to protect human life.

2.2.7 Radioactive waste:

Radioactive wastes refers to the wastes generated by the use of radioactive sources in hospitals. To begin with, for diagnosis and therapy radioactive isotopes are used in hospitals and healthcare organizations. Such wastes consist of contaminated materials including syringes, gloves and patient excreta, vials, cotton swabs. One of the best techniques to cure deadly diseases such as cancer is Radionuclide therapy. Such therapy generates waste which consists of a substantial amount of radioactive substances (Banerjee, 2013). Furthermore, radioactive waste comprises solid, liquid, and gaseous waste which are contaminated with radionuclides that are generated from different diagnostic and treatment methods. The majority of radioactive waste is liquid as compared to solid and gaseous waste. Patients' clothing and utensils from when they were given large doses of radioisotopes like I 131 make up the solid radioactive waste material. So, safe disposal of discarded radioactive materials and any waste contaminated with them is crucial for biomedical waste management (Khan et al., 2010).

In most cases, radioactive waste is handled differently from infectious waste when pathological waste is contaminated with radioactive substances (*Types of Medical Waste*, n.d.). Ionizing radiation has serious effects on public health since it may induce tissue damage as well as increase the chances of having cancers. Moreover, improper disposal of radioactive waste leads to serious long-term or short-term diseases. Also, it can result in soil pollution, water pollution, and air pollution leading to irreversible damage over time with permanent consequences for the environment. Hence, for its proper disposal containment at shielded facilities or deep geological repositories should be used aimed at preventing contamination of soil water as well as air and thus ensuring the safety of those living around through avoidance of any interaction with radioactive wastes present in it.

2.2.8 Cytotoxic waste:

Cytotoxic waste is a type of waste materials that include substances with toxic effects on cells mostly associated with cancer treatment. According to the World Health Organization, they are highly hazardous having genotoxic properties such as carcinogenic, mutagenic as well as teratogenic. The main component of this waste is cytotoxic drugs. Cytotoxic drugs are mainly

used in the treatment purpose of cancer based on their ability to kill or inhibit the cancerous cells. Such wastes have adverse effects on both living beings and the environment. Moreover, certain cytostatic drugs and their residues in urine, stools, vomit, and radioactive and chemical materials are included as cytotoxic waste. Improper disposal or exposure to such wastes can create safety problems leading to cancers, mutations, infertility, and dermatitis (Ghasemi et al., 2018). Also, the hospitals need to take necessary steps to minimize exposure of cytotoxic agents to the hospital staff. Further, cytotoxic waste should be segregated from other wastes at the source to prevent the contamination of other waste sources and minimize risks to the health workers as well as waste employees (*Cytotoxic Waste Disposal & Management | Cleanaway*, 2024). Hence, safe disposal of cytotoxic waste is very important for the prevention of harmful toxic exposure to large-scale populations.

2.3 Potential health hazards from biomedical waste:

Several different potential health hazards can be possessed due to the diverse and dangerous harmful contents of biomedical wastes. Furthermore, improper disposal of such biomedical waste creates potential risks to the environment and living beings. Infectious waste composed of materials contaminated with blood and other body fluids can transmit diseases such as hepatitis or HIV. Also, Sharps, chemical waste, pharmaceutical waste, pathological waste, and radioactive waste have potential health risks that can be acute and chronic at the same time. Various infections like skin, eye, AIDS, meningitis, gastrointestinal, and tetanus are caused when biomedical wastes are not discarded properly (H. Singh et al., 2024). Moreover, the countries having transition economies are reported to suffer from infectious diseases like typhoid, hepatitis, cholera, and respiratory problems due to the open dumping of biomedical wastes. Management of biomedical waste is globally concerning because of its potential public health hazards to living beings and the environment (Singh et al., 2021). Apart from this, in third world countries healthcare-generated wastes are managed by untrained, unaware cleaners without any protection and proper segregation without any proper treatment of wastes. Subsequently, waste collectors are at greater risk if disposed materials consist of infectious waste (Rahman et al., 2020). Infectious waste including those with blood-contaminated materials cause pathogens

transmission while sharps lead to physical injury. Hence, proper management, treatment, and disposal of biomedical waste can reduce these health risks significantly.

2.4 Environmental impact of improper waste disposal:

The inappropriate disposal of biomedical waste such as infectious, chemical, and pharmaceutical wastes has far-reaching effects on the ecosystem. This has the potential to cause soil and water contamination which affects the quality of drinking water while at the same time endangering the survival of local flora and fauna. Furthermore, infectious pathogens transmission and contamination affect the food chain. Also, mosquitoes, flies and rodents which are disease-carrying vectors increase the risk of diseases like dengue fever, malaria, and leptospirosis as improper waste disposal provides breeding grounds (Adewoyin, 2017). Moreover, human food chains are disrupted as inappropriate waste disposal disturbs the ecosystem. Dioxins, furans, and heavy metals are released into the atmosphere by air pollution which are harmful emissions caused by improper disposal of waste. Wildlife are also at potential risk when they come into contact with such biomedical waste (Aryan, 2023). Such pollutants can affect large areas with adverse negative impacts on nearby communities as well as far away from their source. This might lead to the extinction of wildlife which can be a great problem soon due to the disturbance of the ecosystem and the environment.

According to Schenck et al. (2022), mismanagement of biomedical wastes has global impacts on social, environmental, and economic issues. Moreover, an increase in antibiotic-resistant bacteria may result from improper disposal of biomedical wastes. Drug-resistant bacteria are produced by careless dumping of antibiotic manufacturing wastes and their residues as well as the overuse of antibiotics in animal husbandry (Hheadmin, 2019). Antibiotics among other medicines get released into the environment because they were never disposed of correctly along with hazardous items which leads to creating conditions suitable for bacteria's resistance development. Later, the spread of such resistant bacterial strains throughout the habitat makes it difficult to treat infections besides being threatening to people's lives and animals (Kraemer et al., 2019). Hence, to minimize such adverse effects on the environment effective waste

management plans must be put in place which will ensure all possible safeguards are put in order against dangers threatening public health as well as ecology.

2.5. Isolation of Pathogens from Biomedical Waste:

Isolation of pathogens from Biomedical waste involves systematic procedures for identifying bacteria and confirming the presence of specific bacteria.

- **Sampling methods for biomedical waste, culturing, and Biochemical testing for pathogen identification**

In this research, we obtained different biomedical samples from 5 different hospitals following the proper protocol and safety considerations. Different samples like syringes, bandages with blood, samples with blood swabs, testing kits, cotton, pipes and taps, gloves, surgical needles as well as bottle caps were collected from 5 different hospitals. Furthermore, while collecting the samples gloves and aprons were used for safety considerations. Those samples were packed in different Ziplock bags to prevent cross-contamination. Such samples were handled carefully and transported to the laboratory under controlled conditions in an ice box.

Biomedical wastes were collected and categorized based on their type and potential hazard. Then, these collected samples were cut into small pieces to culture in the sterile peptone buffer for 24 hours to ensure the uniform growth of microorganisms. Further, selective media like XLD, Cetrimide, and UTI were used to detect the pathogens. XLD media was used to detect salmonella and Shigella whereas Cetrimide and UTI media were used to detect the E. coli and Pseudomonas pathogens. Samples that have been plated were incubated under standard temperatures (35-37 °C), for about a day to ensure that colonies get time to grow. Later, colonies were examined for morphological characteristics of the target pathogens. Salmonella colonies appeared red with black centers on XLD, red or pinkish color colonies were found for shigella in XLD, and yellow colonies were present for E. coli pathogens in XLD whereas Greenish yellow colonies were

present for Pseudomonas in cetrimide media. After incubation in selective media, such colonies were plated in nutrient agar plates and incubated for 24 hours at 35°C.

Furthermore, DNA was extracted from such pathogens to perform PCR and gel electrophoresis. As well as different Biochemical tests were performed to confirm the identity of the pathogens. Also, different Biochemical tests were performed to further confirm the identity of pathogens present in the biomedical samples. Methyl Red (MR) Test, Voges-Proskauer (VP) Test, Triple Sugar iron, catalase test, Oxidase test, Citrate test, and Motility test are some of the biochemical tests performed in this research. Gram staining method was used to classify whether the bacteria was gram-positive or gram-negative. This categorization relies on the composition of the bacterial cell wall, as well as its capacity to hold onto a particular coloring agent. All the results were recorded accordingly in detail based on the relevant observations.

In addition, an antibiogram test was performed to determine the susceptibility of isolated pathogens from wastes to different antibiotics. In the case of biowaste, where improper disposal may lead to significant public health issues and affected persons, the understanding of such patterns is important. Because resistant microorganisms can be passed on to humans and other organisms as well. This antibiogram test helps to identify potential risks to mitigate the spread of antibiotic-resistant pathogens to the environment. In this test, pathogens cultured on Nutrient Agar were taken and cultured in nutrient broth for 2-3 hours then by using the spread plate method plated on Milluer Hinton agar media by adding diffusion of antibiotics from the discs into the agar and incubated for 18-24 hours at 35-37°C for bacterial growth. After incubation, a zone of inhibition was measured which is the area where bacterial growths are inhibited forming the clear areas. Based on the size of the inhibition zone, the effectiveness of the antibiotics against targeted bacteria is determined as sensitive, intermediate, and resistant.

2.6 Overview of Key Pathogens

In this research, 4 bacteria i.e. E. coli, Salmonella, Shigella, and Pseudomonas were targeted from different hospital wastes like bandages, syringes, blades, tips, and gloves. Here, the characterization, significance, and potential health risks of these 4 targeted pathogens are described.

2.6.1 Escherichia coli

Escherichia coli (E. coli) is a Gram-negative, rod-shaped bacterium that is part of the normal flora in the human and warm-blooded animals' intestines. Furthermore, most strains are harmless and assist in digestion while producing necessary vitamins such as Vitamin K. However, some strains are pathogenic and lead to serious diseases. Severe diarrhea is usually caused by pathogenic E coli infection (Onyeaka & Nwabor, 2022). E coli has become a focal point in microbiology research, food safety, and public health surveillance initiatives due to its importance in both health and disease. Despite being the illness by E. coli is self-limiting, it may lead to serious threats to life especially in young children and the elderly by causing hemolytic uraemic syndrome (HUS) conditions that can cause kidney failure. Severe foodborne disease is caused by Shiga toxin-producing E coli which are transmitted to humans due to the consumption of raw, undercooked meats, milk, and vegetables as well as contaminated foods (World Health Organization: WHO, 2018a).

E. coli's evolution can be traced back to 1885 when German bacteriologist Theodor Escherich first discovered it from the intestines of neonates which were slightly motile as well as gram-negative. It is still one of the most important pathogens today concerning foodborne diseases and severe illness (Ullmann, 2011). E.coli can be transmitted via contaminated food or water, person-to-person contact as well as surface contamination with fecal matter containing bacteria from animals or humans who carry it without showing symptoms such as diarrhea or abdominal cramps. Also, gram-negative bacteria such as E. coli are detected in the emergence of multi-drug resistance like carbapenem resistance which poses serious threats and infections to health (Gashaw et al., 2024).

2.6.2. Pseudomonas

Pseudomonas aeruginosa is the most clinically important one because it causes infections mostly among immunocompromised people like those suffering from cystic fibrosis, burns, or patients admitted to intensive care units. *Pseudomonas aeruginosa* is the most commonly affecting species of Pseudomonas causing infections among humans. Furthermore, blood, lung, and urinary tract infections as well as multidrug resistance can be caused by *Pseudomonas aeruginosa (About Pseudomonas Aeruginosa, 2024).* Pseudomonas is a Gram-negative,

rod-shaped bacteria commonly found in soil, water, and other humid environments. *Pseudomonas aeruginosa* is an opportunistic pathogen that can adapt to different conditions and is highly resistant to many antibiotics. This bacterium poses a significant threat in hospitals because it is related to nosocomial infections. Moreover, it produces proteases, elastases, and toxin A which are virulence factors that inhibit the synthesis of protein. Such formation of virulence factors protects host immune response as well as antibiotic therapy. Also, these *Pseudomonas* pathogens are resistant to antimicrobial drugs which have spontaneous or induced resistance (Cullinane et al., 2006).

Improper disposal of biomedical wastes and the spread of these bacteria leads to serious threats to public health and the environment. The dangers posed by *Pseudomonas aeruginosa* regarding health are particularly serious because of its intrinsic resistance to numerous antibiotics together with the ability to obtain resistance through various means. Endocarditis, meningitis, pneumonia, malignant external otitis, and septicemia are the most serious infections caused by *Pseudomonas aeruginosa*. Between 10-20% of infections in hospitals are caused by this species (Bodey et al., 1983). As a result, treatment of infections becomes difficult leading to prolonged hospitalization and increased healthcare expenses. In addition, *Pseudomonas* are isolated more in number from the sinks of hospitals as compared to home. If such pathogens mix with other wastes, then many people including health workers, patients, patient visitors as well waste handlers are at great risk of getting infection (Ayliffe et al., 1974). Hence, this bacterium *Pseudomonas* poses great threats to public health as well as the environment.

2.6.3 *Salmonella spp.*

Salmonella are gram-negative, rod-shaped bacteria species that are mainly present in the intestines of both humans and animals. They can survive in all conditions as they are capable of surviving without oxygen or with a small amount of it. According to the European Food Safety Authority. (2024, March 21), yearly 91,000 people in the EU are infected with *Salmonella* causing salmonellosis which leads to an economic burden as high as €3 billion a year. Furthermore, foodborne disease outbreaks are commonly caused by *Salmonella* so in the EU, it is the second most common zoonotic disease after campylobacteriosis. This group of bacteria can be divided into two major types: *Salmonella enterica* and *Salmonella bongori* which further consists of 1800 known serovars. O, H, and Vi antigens are present which are characterized by

flagellated facultatively anaerobic bacilli. In addition, pathogenic Salmonellae are ingested in food that survives through the gastric acid barrier that later produces toxins after invading mucosa of the small and large intestines (Giannella, 1996).

American veterinary pathologist Daniel Elmer Salmon made the first discovery of *Salmonella* in 1885 by his assistant Dr. Theobald Smith which was initially isolated from pigs infected with cholera and named the genus after him (Sspf, 2019). *Salmonella Typhimurium* and *Salmonella Enteritidis* are the most frequent pathogenic strains which account for the better part of salmonellosis cases worldwide. In addition to this, there are quite severe strains like *Salmonella Typhi* that cause typhoid fever, an illness that poses danger to human lives as well as being among the main health concerns in many poor nations today. Due to consumption of infected food products or water, *Salmonella* continues to be a major risk concerning food hygiene. It causes life-threatening infections as in the bloodstream they multiply and spread after ingestion (World Health Organization: WHO, 2023). The presence of *Salmonella* within biomedical waste increases its ability to spread diseases if the waste is not properly handled and disposed of properly. However, with the emergence of strains that resist antibiotics, treating infections caused by *Salmonella* has become more difficult thus making infection prevention and control measures imperative. Therefore, to prevent outbreaks of diseases associated with this bacterium and safeguard people's health there should be monitoring systems put in place to control its spread particularly in hospitals and within biomedical waste management systems.

2.6.4 *Shigella spp.*

Shigella is a member of the Enterobacteriaceae family characterized as a Gram-negative, rod-shaped bacteria, which is responsible for shigellosis. Shigellosis is a severe form of dysentery known for its symptoms such as inflammation of the intestines and bloody diarrhea. There are four main species of *Shigella*: *Shigella dysenteriae* with 12 serotypes, *Shigella flexneri* with 6 serotypes, *Shigella boydii* with 18 serotypes, and *Shigella sonnei* with 1 serotype. Pathogenesis by *Shigella* is initiated by ingestion of the bacterium by fecal-oral usually through contaminated food, water, or direct person-to-person transmission. Colonic epithelium and inflammatory colitis are invaded by bacteria which is the hallmark of Shigellosis (Hale & Keusch, 1996). Furthermore, *Shigella* does not have any animal reservoirs as it infects humans only. Bacillary dysentery is caused by *Shigella* which produces exotoxin, Shiga toxin which is

capable of killing the host cells by inhibiting the synthesis of protein. These Shiga toxins act as enterotoxins, neurotoxins, and cytotoxins that bind to the surface of host cells (Mumy, 2024).

The Japanese microbiologist Kiyoshi Shiga first identified shigella in 1897. It has since then been recognized as one of the leading causes of bacterial dysentery globally. Throughout its history, *Shigella's* main associations have been with poverty, war, or natural calamities where unsatisfactory sanitation and overpopulation provide perfect conditions for its spread. Severe complications like hemolytic uremic syndrome are caused by Shiga toxin-producing the most virulent species of *Shigella* (Lampel et al., 2018). *Shigella* poses a serious public health risk, especially in poor countries. Treatment has become difficult due to the increasing resistance of *Shigella* species to antibiotics resulting in increased sickness and death rates. Further, the mixing of such pathogens with medical waste poses serious threats to public health. Hence, enhancing water quality, sanitation, and hygiene practices, developing vaccines and effective treatment methods, and proper disposal of infected waste should be managed to protect public health and control outbreaks of this disease-causing organism.

2.7. Molecular Techniques in Pathogen Detection

The polymerase chain reaction (PCR) is a molecular process that assists amplification of targeted DNA sequences making it effortless for identification and analysis of genetic material from different sources. Developed by Kary Mullis in 1983, this method has changed much in the area of molecular biology, particularly in pathogen detection. This technique is used to denature and renature Deoxyribonucleic acids or Ribonucleic acids by using DNA polymerase I enzyme (Khehra et al., 2023). Furthermore, primers which as short synthetic DNA fragments are used in PCR to amplify the targeted genome segment and then after multiple cycles of DNA synthesis amplify the targeted sequence (*Polymerase Chain Reaction (PCR)*, 2024). It utilizes cycles of repeated heating and cooling to separate strands of DNA, bind primers, and elongate new strands resulting in an increased amount of the desired piece of DNA. Moreover, PCR has a wide range of applications in Medical diagnostics, forensic sciences, and research extensively due to its sensitivity, and specificity thereby allowing detection even at trace levels. TaqDNA polymerase is the first and commonly used polymerase from *Thermis aquaticus* whereas PfuDNA

polymerase from *Pyrococcus furiosus*. These enzymes are widely used due to their heat resistance capacity and ability to generate new target DNA strands as well as increased fidelity in DNA replication (*Polymerase Chain Reaction (PCR)*, n.d.-b).

Polymerase chain reaction is an inexpensive, quick, and easy technique that generates unlimited copies of target DNA fragments. It identifies specific pathogens accurately without resulting in false positive results which happen when using traditional culture methods that involve prolonged incubation time while waiting for the growth of culture microorganisms present in samples collected from sick people's body organs such as blood or tissues until they grow into colonies large enough that one can see them with naked eyes. Also, it can detect minute amounts of viruses or bacteria present within any given sample which is important for timely diagnosis and avoiding the spread of contagious diseases. For instance, to detect *Pseudomonas* PAGS primer targeting virulence-associated genes is used. To identify this pathogen ECO primer targeting the uidA gene unique for *E. coli* is a reliable tool. INVA primer helps *Salmonella* detection through amplification of the invA gene which plays a significant role during host cell invasion by bacteria. IPAH primers targeting the ipaH gene responsible for invasion and disease pathogenesis are used in *Shigella* detection. The application of these specific primers makes it possible to accurately and efficiently identify these pathogens even from complex samples such as those found in biomedical waste. Consequently, the importance of PCR in both clinical diagnostics and public health initiatives particularly concerning biomedical waste management has to do with how effectively and rapidly it identifies these germs across the board.

2.8. Microbial Tests for Confirmation

Confirmation microbial tests are laboratory methods employed to detect and establish the existence of specific microorganisms in a sample. It plays an important role in diagnosing infections, enhancing the accuracy of microbiological studies, and verifying the identities of microorganisms from different sources (Kuriyama et al., 2014). Gram staining, biochemical methods, molecular methods such as PCR, and culturing techniques are common examples of microbial tests. Furthermore, these tests contribute to microbiology advancements by providing insights into the characteristics and behavior of microorganisms in research settings. The importance of microbial confirmation tests comes from their ability to give exact identification of pathogens which is important for proper treatment, control against infections as well and

public health management. This is because as well as these tests are highly sensitive, quick results and accurate identification aid the selection of appropriate antimicrobial therapy thus improving patient outcomes and reducing infectious disease spread (Marina, 2022).

2.8.1 Gram staining

Gram staining is one of the crucial techniques for classifying Gram-positive and Gram-negative bacteria. It was first introduced in 1882 by Danish bacteriologist Hans Christian Gram to identify bacteria causing pneumonia. This technique involves four main steps that include application of crystal violet (primary stain), application of iodine solution (mordant), decolorization with alcohol or acetone (decolorization), and counterstaining with safranin (Tripathi & Sapra, 2023).

Crystal violet is the primary stain that makes all bacterial cells look purple. The smear is heat-fixed and flooded with crystal violet for about 1 minute. In Gram-positive bacteria, the crystal violet-iodine complex is more tightly bound because of the thicker peptidoglycan layer, while in Gram-negative bacteria it is loosely bound. The decolorization process does not remove the crystal violet-iodine complex from Gram-positive but does so from Gram-negative bacteria. The decolorized slide is rinsed with a decolorizing agent for a few seconds causing Gram-positive to maintain their purple color while in the case of Gram-negative, they lose their stain and become colorless.

As a result, Gram-positive bacteria appear purple under the microscope because of the retention of crystal violet-iodine complex in their thick layer of peptidoglycan whereas on the other hand, Gram-negative ones appear pink or red after uptake of safranin after being decolorized (Erkmen, 2021). Hence, gram staining enables rapid differentiation of bacterial species and indicates treatment alternatives based on their cell wall structure.

2.8.2 Biochemical tests (oxidase test, catalase test, sugar fermentation tests)

By examining the metabolic characteristics of bacterial species, biochemical tests play an important role in their identification and confirmation.

2.8.2.1 Methyl Red Test (MR-Test)

One way bacterium that produces end products of the rapid acid that is stable is the Methyl Red (MR) Test. This method assists in distinguishing between those that undergo mixed acid fermentation from other forms. To perform this test, bacterial isolate is taken and inoculated in a tube of MR-VP broth then incubated at 37°C for 24 – 48 hours after which 5-6 selected drops of methyl red indicator are added. If it turns red, this means that its pH is 4.4 or less, however, if it turns yellow then it has a higher pH than this value (Aryal, 2022).

2.8.2.2 Voges-Proskauer (VP) Test

The mechanism of VP tests allows for indicting the bacterium that synthesizes acetoin as a metabolite from glucose through fermentation; this is the 2,3-butanediol precursor. To perform this test, a test tube is inoculated with a culture containing bacteria and incubated at 37°C for 24 to 48 hours. Then, Barritt's reagents A and B are added into the mixture which is shaken softly before observing any change in coloration of the solution. A positive result suggests that acetoin is present; however, a negative result implies a lack of acetoin. In addition, this test can be used to identify and differentiate Gram-negative bacteria as well as actinobacteria (Dahal, 2023).

2.8.2.3 Triple Sugar Iron (TSI) Test

Bacterial classifications under the TSI test are based on their sugar fermentation activities, H2S formation, and gas production. This involves the suspension of a bacterial isolate in TSI agar, streaking it, and incubating it for twenty-four hours at thirty-seven degrees Celsius. Findings cover glucose fermentation, lactose/sucrose fermentation, and no fermentation along with their ability to produce hydrogen sulfide gas. Furthermore, the change in color of the pH indicator from red to yellow along with the production of gas indicates carbohydrate fermentation. Also, based on sugar fermentation patterns, this test is used to differentiate among Enterobacteriaceae (Aryal, 2022b).

2.8.2.4 Catalase Test

The catalase test represents a means of distinguishing between bacterial organisms capable of producing catalase enzymes, which are responsible for decomposing hydrogen peroxide into water and oxygen. The test serves to distinguish between, for instance, Staphylococcus species

that are catalase positive and Streptococcus species that are catalase negative. To carry out this procedure place one drop of 3% hydrogen peroxide on the glass slide, then introduce a tiny portion from a bacterial isolate. Also, it is used in differentiating genera of the bacteria (*Catalase Test*, 2010).

2.8.2.5 Oxidase Test

The use of oxidase test is a technique for identifying bacterial species by detecting cytochrome c oxidase, which is an enzyme found in this bacterial electron transport chain. It differentiates between oxidase-positive and oxidase-negative bacteria such as Pseudomonas and Enterobacteriaceae respectively. A drop of oxidase reagent is applied onto the filter paper to carry out such a test, followed by smearing a small amount from the isolated bacteria on it and then watching for any color changes that may occur within 30 seconds (Aryal, 2022b).

2.8.2.6 Citrate Test

The citrate test is a technique used to establish the ability of bacteria to utilize citrate as their exclusive carbon source, hence, aiding in the distinction of Enterobacteriaceae family members. It involves infecting a Simmons citrate agar slope with the bacterial isolate and incubating at 37°C for 24-48 hours while assessing any color changes that may arise (*Citrate Test Protocol*, 2009).

2.8.2.7 Motility Test

The mobility test is crucial in identifying and differentiating motile organisms like E. coli from non-motile ones. This entails inoculating the bacterial isolate into a semi-solid agar medium, incubating it at 37°C for 24-48 hours then observing the pattern of bacteria growth. Positive results reveal an evident growth that spreads away from the stab line while negative results depict growth just along this line (Davis & Pezzlo, 2023).

2.9 Antibiogram tests

Different antibiotics used for Pseudomonas, Salmonella, Shigella, and E. coli are given below along with their interpretative categories and zone of inhibition (CLSI, 2020):

Pseudomonas

Name of Antibiotics	Disk Concentration (µg)	Symbol	Interpretive Categories and Zone Diameter		
			Sensitive (S)	Intermediate (I)	Resistant (R)
Aztreonam	30	ATM	≥22	16-21	≤15
Meropenem	10	MEM	≥19	16-18	≤15
Amikacin	30	AK	≥17	15-16	≤14
Gentamicin	10	CN	≥15	13-14	≤12
Piperacillin-tazobactam	110	TZP	≥21	15-20	≤14
Colistin	10	CT	-	-	-
Levofloxacin	5	LEV	≥22	15-21	≤14
Ciprofloxacin	5	CIP	≥25	19-24	≤18
Ceftazidime	30	CAZ	≥18	15-17	≤14
Clindamycin	10	CL	-	-	-
Azithromycin	30	AT	-	-	-

Salmonella and Shigella

Name of Antibiotics	Disk Concentration (µg)	Symbol	Interpretive Categories and Zone Diameter		
			Sensitive (S)	Intermediate (I)	Resistant (R)
Ciprofloxacin	5	CIP	≥31	21-30	≤20
Norfloxacin	10	NOR	≥17	13-16	≤12
Aztreonam	30	ATM	≥21	18-21	≤17
Tetracycline	30	TE	≥15	12-14	≤11
Gentamicin	10	CN	≥15	13-14	≤12
Ampicillin	25/10	AMP	≥15	12-14	≤11
Chloramphenicol	30	C	≥18	13-17	≤12
Imipenem	10	IPM	≥23	20-22	≤19
Azithromycin	15/30	AZM	≥13	-	≤12
Azithromycin	30	AT	≥13	-	≤12
Piperacillin-tazobactam	110	TZP	≥21	14-16	≤13
Erythromycin	15	E	-	-	-

E. coli

Name of Antibiotics	Disk Concentration (µg)	Symbol	Interpretive Categories and Zone Diameter		
			Sensitive (S)	Intermediate (I)	Resistant (R)
Piperacillin/Tazobactam	110	TZP	≥21	18-20	≤17
Gentamicin	10	CN	≥15	13-14	≤12
Amikacin	30	AK	≥17	15-16	≤14
Aztreonam	30	ATM	≥21	18-20	≤17
Tetracycline	30	TE	≥15	12-14	≤11
Meropenem	10	MEM	≥23	20-22	≤19
Chloramphenicol	30	C	≥18	13-17	≤12
Trimethoprim/Sulfamethoxazole	25	SXT	≥16	11-15	≤10
Ampicillin	10	AMP	≥15	12-14	≤11
Ciprofloxacin	5	CIP	≥31	21-30	≤20
Ceftazidime	30	CAZ	≥21	18-20	≤17

2.10 Conclusion

Thus, biomedical waste is a highly generic and dangerous category of waste generated in healthcare organizations, research institutions, and laboratories and may comprise syringes or needles, tissues, body fluids, and drugs. If not controlled, these wastes can become hazardous sources of infection transmission and risks to humans, animals, and the environment. In this respect, required action needs to be taken so that the regulatory measures are pushed here, and the proper method of disposal is exercised. There is therefore the need to categorize biomedical waste according to; Infectious waste, pathological waste, sharps, pharmaceutical waste, chemical waste, and radioactive wastes among which treatment and disposal methods to avoid disease transmission and pollution.

In this study, a comprehensive analysis was conducted using PCR to target *Pseudomonas, Salmonella, Shigella,* and *E. coli* with specific primers: *Salmonella:* INVA, *Pseudomonas:* PAGS, *Escherichia coli:* ECO, and *Shigella:* IPAH. Subsequently, the biochemical tests done were Gram staining, oxidase, catalase, motility, MR-VP, and antibiotic sensitivity tests. From the five participating hospitals, in total 87 isolates were isolated and studied toward the microorganism profile of hospital waste. Consequently, this study emphasizes the need to employ microbiological screening and good biomedical waste disposal systems to avoid infections and potential public health risks.

Chapter 3: Methodology

3.1. Research Design

- Type of research (qualitative, quantitative, or mixed-methods).

Our project was a quantitative-based research.

A) Quantitative phase

The quantitative phase was concerned with finding the target population of pathogenic microorganisms from the collected biomedical waste.

1. Sample collection:

   The samples were collected from 5 different hospitals. Different samples were collected from different departments of the hospital and were kept in different zip-log bags, to avoid cross-contamination. Ice bags were used while transporting the sample to the laboratory, to preserve the viability of the microorganisms.

2. Analysis of the microbial groups: Different selective microbial culture media were used to grow only our targeted group of pathogenic microorganisms. Furthermore, PCR was also performed to ensure that the selected microbes were present in the sample.

3. Biochemical tests: To further characterize the microbes present in our samples, various tests including MRVP, Catalase, Oxidase, gram staining and soon were also performed.

4. Antibiogram test: Antibiotic tests for the selected group of microbes were performed to determine their reaction towards a group of antibiotics.

5. Data analysis: The results were then interpreted.

3. 2. Microbial analysis

The microbial analysis was the longest phase of the project. To illustrate, different buffers, media, and many different reagents were prepared and used to support the growth of our targeted population of microbes. A step-by-step preparation method was followed as described below:

3.2.1 Preparation of Peptone buffer

Before growing the bacteria in the selective media, they are first grown in a peptone buffer. To begin with, various factors such as heating, freeze drying, or simply freezing physiologically injure or weaken the microorganisms which negatively impacts attempts at detection and enumeration of some microorganisms(Escobar et al., 2023). Therefore, to isolate microbes, the standard protocol includes the pre-enrichment of the microbial sample in a non-selective broth before inoculating them in a selective media (Escobar et al., 2023).

Peptone buffer is simply a pre-enrichment media that allows the growth of microbes. To illustrate, the pre-enrichment medium is well-buffered pH: 7.2±0.2, free from the inhibitors, and also provides appropriate conditions for the growth of the microbes (HiMedia Laboratories Technical Data, 2023).

Procedure:

For 1 L of peptone buffer, 1gm of peptone was taken, along with 3.56 gm of potassium dihydrogen phosphate, 7.23 gm of disodium hydrogen phosphate, 4.3 gm of sodium chloride mixed with 1 L of distilled water. The final pH was adjusted to 7.

3.2.2 Preparation of selective media and inoculation of microorganisms

Selective media were prepared for the growth of the targeted population of microbes, i.e. *Salmonella, Shigella, Pseudomonas, and Escherichia coli* (Libretexts, 2022). To begin with, culture media are simply media fused with many nutrients and water that help in the growth of microbial cultures. According to their uses, the culture media are differentiated into three different groups: nutritive, selective, and differential media. As the name suggests, the nutrient media allows the growth of all kinds of microorganisms without differentiating their species or genera. On the other hand, selective media are the type of media that supports the growth of only certain, selective microorganisms (Libretexts, 2022). To illustrate, the selective media can consist

of certain substances that inhibit the growth of all the micrograms except our interested bacteria. For example, salt can be present in media in high amounts, allowing only the growth of halophiles (salt-loving bacteria). Moreover, antibiotics can also be present in the media and only the specific bacterial group that is resistant to those antibiotics can survive there. Finally, the differential media allows for distinguishing between different species or genera of bacteria. To exemplify, it allows the growth of different bacterial groups, but the presence of certain compounds allows them to visualize different colonies. The microorganisms interact with the compound and give a different visualization than the other colonies (Why Differential & Selective Media Remain Invaluable Tools | ASM.org, n.d.).

In our experiment, Xylose Lysine Deoxycholate (XLD) agar, Cetrimide agar, and UTI agar were used. XLD is a selective media and it was used for the isolation of *Escherichia coli, Shigella,* and *Salmonella* species. The cetrimide media, also a selective media, was used to isolate the *Pseudomonas* species. On the other hand, as a differential media, UTI is a chromogenic differential media that is used to identify, differentiate, and confirm different enteric bacteria from the given sample (Sigma Aldrich media).

3.2.2.1 Xylose Lysine Deoxycholate (XLD) media

Xylose Lysine Deoxycholate (XLD) agar is a selective media that is primarily used to isolate the *Shigella* and *Salmonella* species from the given samples (Aryal, 2022c). The required vitamins, nitrogen, and nutrients are provided by the yeast extract present in the media. The presence of sodium chloride in the media balances the osmotic balance. The XLD is both selective and differential. To begin with, the presence of sodium Deoxycholate helps the selective growth of gram-negative bacterial groups only. On the other hand, the presence of Xylose, sucrose, and lactose ensures that the fermentable carbohydrate is provided. On the fermentation of the carbohydrate, acid is produced that changes the pH and the color of the media to yellow. This differentiates between the enteric pathogens from Shigella, as *Shigella* is an enteric pathogen that does not ferment the Xylose (Aryal, 2022c).

On the other hand, *Salmonella* species quickly ferments the Xylose and with the help of the enzyme lysine decarboxylase, the lysine is decarboxylated (Park et al., 2012). As a result, amines

are produced and increase in pH, with the return of media to an alkaline. Thus, large amounts of lysine and sucrose in the media are added to produce more acid. The colonies change from yellow to red from the lysine decarboxylation and production of the amines (Aryal, 2022c). Furthermore, black-centered colonies are formed from the organisms that can produce hydrogen sulfide from sodium thiosulfate, as in the presence of Hydrogen sulfide, the ammonium citrate precipitates.

The non-pathogenic Hydrogen sulfide (H2S) producing bacteria do not decarboxylate lysine, thus preventing the formation of black-centered colonies(Sigma Aldrich media)

Cultures:

Overnight-grown samples were inoculated in a peptone buffer.

Procedure:

1. The agar was weighed in electronic balance and poured into a conical flask.
2. Distilled water was used to dissolve the media completely.
3. The media was heated until it was completely dissolved.
4. Then it was kept for autoclaves.
5. After the autoclaving was done, the media was poured into sterilized medium Petri dishes inside the laminar flow and left to set.
6. The plates were then labeled with sample name, media name, and date.
7. The peptone buffer inoculated samples were then streaked into the agar plates with the help of inoculating loops.
8. After the inoculation in all of the media plates was done, they were kept in the incubator for growth for 24 hrs.
9. After the completion of 24 hrs, the plates were observed for different colonies.

10. The colonies were differentiated, and each selected single colony was then again streaked in different nutrient agar plates.

3.2.2.2 Cetrimide media

Principle:

To selectively isolate the *Pseudomonas aeruginosa* from the sample, a selective media called cetrimide agar was used (Aryal, 2023). To begin with, various yellow-brown, yellow-green, or fluorescent pyoverdines are produced by the *Pseudomonas aeruginosa*. Now this pyoverdine combines with the water-soluble blue pyocyanin, giving a bright green color that characterizes *Pseudomonas aeruginosa*. Moreover, the production of pyocyanin and pyoverdin is stimulated by the additional potassium sulphate and magnesium chloride (Ezeador et al., 2020).

Furthermore, the other constituents of the media such as the pancreatic digest of gelatin provide additional carbon, nitrogen, and other required nutrients to the *Pseudomonas aeruginosa* (Aryal, 2023). Thus the cetrimide, cetyltrimethylammonium bromide, is a selective media that works as a detergent to remove the other bacteria by releasing phosphorus and nitrogen and doesn't allow their growth except the *Pseudomonas aeruginosa.*

Cultures:

Overnight-grown samples were inoculated in a peptone buffer.

Procedure:

1. The agar was weighed in electronic balance and poured into a conical flask.
2. Distilled water was used to dissolve the media completely.
3. The media was heated until it was completely dissolved.
4. Then it was kept for autoclaves.
5. After the autoclaving was done, the media was poured into sterilized medium Petri dishes inside the laminar flow and left to set.

6. The plates were then labeled with sample name, media name, and date.
7. The peptone buffer inoculated samples were then streaked into the agar plates with the help of inoculating loops.
8. After the inoculation in all of the media plates was done, they were kept in the incubator for growth for 24 hrs.
9. After the completion of 24 hrs, the plates were observed for different colonies.
10. The colonies were differentiated, and each selected single colony was then again streaked in different nutrient agar plates.

3.2.2.3 UTI (Urinary tract infection) Media

To isolate and differentiate the bacteria from the urinary tract, UTI differential media is used (*Oxoid - Product Detail*, n.d.). To begin with, this media is used for the differentiation of both gram-positive as well as gram-negative bacteria. The media works through the production of different colors because of the presence of certain chromogenic substances in that media (*Oxoid - Product Detail*, n.d.). According to the color produced, we can easily characterize the pathogen present in the sample.

Several organisms are isolated from the UTI. One of the most prominently found organisms in such infection is Escherichia coli which is either found solely or in group with other organisms (*Oxoid - Product Detail*, n.d.). Different microorganisms produce different enzymes and act on the chromogenic substrates present in the media.

Some of the colors produced by some organisms are:

1. *Escherichia coli* produces magenta colonies, and no further testing is required.
2. *Pseudomonas* species produce light green or yellow, translucent colonies.
3. *Klebsiella, Enterobacter*, and *Serratia* spp, produce deep blue colonies.

Cultures:

Overnight-grown samples were inoculated in a peptone buffer.

Procedure:

1. The agar was weighed in electronic balance and poured into a conical flask.
2. Distilled water was used to dissolve the media completely.
3. The media was heated until it was completely dissolved.
4. Then it was kept for autoclaves.
5. After the autoclaving was done, the media was poured into sterilized medium Petri dishes inside the laminar flow and left to set.
6. The plates were then labeled with sample name, media name, and date.
7. The peptone buffer inoculated samples were then streaked into the agar plates with the help of inoculating loops.
8. After the inoculation in all of the media plates was done, they were kept in the incubator for growth for 24 hrs.
9. After the completion of 24 hrs, the plates were observed for different colonies.
10. The colonies were differentiated, and each selected single colony was then again streaked in different nutrient agar plates.

Once the colonies were obtained in these selective and differential media, they were isolated. Each colony for the targeted microbe was then streaked in individual nutrient agar plates.

3.2.2.4 Nutrient agar

Principle:

Nutrient agar is one of the most commonly used nutrient media that allows the growth of different groups of bacteria (Sapkota, 2022). To begin with, the media consists of beef extract, agar, and peptone, along with a trace amount of other required nutrients. The beef extract in the media works as a sole source of carbon and assists in the carbohydrate formation of the bacteria. Furthermore, the extract also contains trace amounts of minerals, essential nutrients, salts, and organic compounds, that help in the growth of the bacterial species(Sapkota, 2022).

Moreover, the agar acts as the solidifying agent and provides ground for the enumeration of colonies and observation of bacterial colony morphology (Sapkota, 2022). The peptone provides the nitrogen source that forms the amino acid formation and eventually protein production. In addition to this, salt is added to the media, which stabilizes the pH and maintains an osmotic balance of the media, which is essential for bacterial growth.

Cultures:

Overnight grown bacterial colonies inoculated in XLD, cetrimide, and UTI Medias.

Procedure:

1. The agar was weighed in electronic balance and poured into a conical flask.
2. Distilled water was used to dissolve the media completely.
3. The media was heated until it was completely dissolved.
4. Then it was kept for autoclaves.
5. After the autoclaving was done, the media was poured into sterilized medium Petri dishes inside the laminar flow and left to set.
6. The plates were then labeled with sample name, media name, and date.
7. The overnight-grown bacterial colonies isolated from each sample were then streaked into the individual agar plates with the help of inoculating loops.

8. After the inoculation in all of the media plates was done, they were kept in the incubator for growth for 24 hrs.
9. After the completion of 24 hrs, the plates were observed for different colonies.

3.3 Extraction of the bacterial DNA

After the growth of the targeted bacterial samples, our next step was the extraction of their DNA for our PCR.

To isolate the bacterial DNA, we needed:

- 24 hrs grown bacterial sample inoculated in the liquid broth.
- TE buffer

3.3.1 Preparation of Nutrient broth and inoculation of the bacterial samples

Principle:

To cultivate a variety of non-fastidious and fastidious organisms, the nutrient broth is used (*Nutrient Broth*, n.d.). To begin with, the presence of yeast extract and peptone provides the amino acids, nitrogen, and vitamin B complex in addition to other nutrients required for bacterial growth. Furthermore, the glucose present in the broth provides the source of fermentable carbohydrates while the osmotic balance is maintained by sodium chloride(*Nutrient Broth*, n.d.).

The formulation of both the nutrient agar and the nutrient broth is the same, except the agar is excluded in the nutrient broth. In testing some food samples or dairy products, nutrient broth is preferred as a medium for pre-enrichment (*Nutrient Broth*, n.d.). To exemplify, several microorganisms are present in the processed or dried food. The *Salmonella* species can be low in numbers and sub-lethal, as the other bacteria can hinder its growth. Thus, this non-elective pre-enrichment medium is used for the recovery and growth of the species *Salmonella*.

The nutrient broth dilutes the inhibitory substances or toxic substances, repairing the damage as well as providing nutrients for the recovery of the *Salmonella* species (Nutrient Broth, n.d.). Thus the pre-enrichment medium is used as a standard protocol in testing any dairy products, food, or samples.

Procedure:

1. The accurate amount of nutrient broth powder was measured using an electronic balance.
2. The measured amount was transferred to a conical flask and distilled water was added to it.
3. It was stirred until everything dissolved completely.
4. The broth was then given for autoclaving.
5. A single colony of the targeted bacterial population was inoculated in the nutrient broth, and it was left for 24 hrs in the incubator at 37°C.

3.3.2 Preparation of TE buffer

Tris EDTA or TE buffer is one of the most commonly used buffers in DNA RNA storage procedures (Sharebiology, 2023). To begin with, TE buffers consist of two major components: Tris and EDTA, in the ratio of their working concentration of 10mM and 1mM. In the buffer, the EDTA works as a chelating agent, binds and inactivates the metal ions thus inhibiting the DNase and RNase enzymes, which could degrade the DNA and RNA. On the other hand, the Tris maintains the buffering capacity (Sharebiology, 2023).

Thus when working with DNA or RNA, instead of distilled water, TE buffer is preferred because of its properties. The buffer pH is 8, which means alkaline, and stabilizes the RNA and DNA.

Furthermore, as it also inactivates the DNA degrading components like RNase and DNase with the help of EDTA, it is preferred more in DNA and RNA storage (Sharebiology, 2023).

Procedure:

For 500ml of 10x TE buffer:

1. 50 ml of stock Tris-Cl (pH 8) was added to the 500 ml conical flask.
2. 10 ml of 0.5M EDTA (pH 8) was then added to it.
3. Now the distilled water was added to it until it was 500ml.
4. pH was then measured and adjusted using concentrated HCl and NaOH.

For 500ml of working 1x TE buffer:

Calculations:

Initial concentration (C1) = 10x

Final volume (V2) = 500 ml

Initial volume(V1) = x

Final concentration (C2) = 1x

So, we know,

C1V1= C2V2

Or, V1= (C2V2)/C1

=> X= (1 X 500)/10

V1= 50ml

Therefore for preparing 500ml of 1x working buffer;

1. 50 ml of initial 10x stock was taken in a 500 ml of conical flask.
2. Distilled water was then added to it until the final volume was 500ml.
3. pH was measured and adjusted.

3.3.3 Extraction of the bacterial DNA

Principle:

To determine the shape and size of the DNA, isolation and purification are crucial steps (Dimitrakopoulou et al., 2020). To begin with, lysosomes are usually used to digest the cell wall, with the use of detergents to disrupt the nucleic acid-protein interactions and to remove the proteins, phenol-chloroform is employed to isolate the bacterial genomic DNA. Furthermore, different chaotropic agents and specific detergents are used depending on the physiological structure of the cells of the bacteria. To exemplify, bacteria with large amounts of glycocalyx or capsules that consist of large amounts of polysaccharides are generally more difficult to lysis.

Most of the methods of DNA extraction are laborious and time-consuming, with occasional breakage/chopped DNA, which cannot be used in the downstream process like polymerase chain reaction (PCR) (Dimitrakopoulou et al., 2020). Thus, one of the most feasible methods is the boiling method, where lysis of the cell is easier, and more purified DNA can be obtained. The process starts with a culture grown in liquid media, and requires frequent centrifugation steps, to separate the cell debris from the DNA. Repipeting with the TE buffer, where the Tris provides a balanced pH that stabilizes the DNA, while the EDTA acts as a chelating agent and destabilizes

the enzyme DNase that could cause DNA lysis. The DNA thus isolated can be kept in storage at -20°C or can be directly used for PCR.

Procedures:

1. 1 ml of the nutrient broth-grown bacterial samples were taken in microcentrifuge tubes.
2. The samples were then balanced and kept for centrifugation at a speed of 13000 rpm for 10 minutes.
3. The supernatant thus obtained was discarded, and the pellet was re-pipetted with 200 µL of TE buffer.
4. The tubes were then again kept for centrifugation at 13000 rpm for 10 minutes.
5. The obtained supernatant was then again discarded, and the pellets were again re-pipetted with 200 µL of TE buffer.
6. Now the tube samples were kept in a hot water bath, for the cell lysis to complete at 100°C for 10 minutes.
7. After 10 minutes the tubes were kept at room temperature for about 5 minutes.
8. After the cooling, the tubes were then again kept for centrifugation at 13000 rpm for 10 minutes.
9. Finally, the supernatants with our genomics DNA were carefully transferred to the fresh autoclave microcentrifuge tubes.
10. The DNA was then stored at -20°C for PCR.

3.4 Polymerase chain reaction (PCR)

Principle:

An in vitro technique that is based on the principle of DNA polymerization reaction by which a particular DNA sequence can be amplified and made into multiple copies is known as Polymerization Chain Reaction (The Editors of Encyclopaedia Britannica, 2024). Thermal cycling consists of repeated cycles of heating and cooling of the reaction for DNA melting and enzymatic replication of DNA using thermostatic DNA polymerase, Primer sequences(complementary to the target region) and dNTPs impact the polymerase chain reaction. Moreover, a specific DNA sequence can be amplified as many as one billion times. Although some techniques allow for the amplification of fragments up to 40kb in size, most PCR methods can amplify DNA fragments of up to ~ 10 kilo base pairs (kb (The Editors of Encyclopaedia Britannica, 2024)). Also, the availability of substrates in the reaction, which becomes limited as the reaction progresses determines the number of products amplified. PCR cycles are a series of 20-40 repeated temperature changes and each cycle consists of a series of 2-3 discrete temperature steps. Depending on the varieties of parameters, the temperature used, and the length of time applied in each cycle. The parameters consist of the enzymes used for DNA synthesis, the concentration of dialect ions and dNTPs in the reaction, and the melting temperature (™) of the primers (The Editors of Encyclopaedia Britannica, 2024).

Initialization step: It is only required for DNA polymerase that requires heat activation by hot-start PCR. The reaction is heated to a temperature of 94-96^{0} C (or 98^{0}C if the polymerase used is extremely thermostable) which is held for 1-9 minutes in this step (*Polymerase Chain Reaction Research*, n.d.).

Denaturation step: It is the first regular cycling event and, in this step, the reaction is heated to 94-98^{0}C for 20-30 seconds. Also, by disrupting the hydrogen bonds between the complementary bases, DNA melting of the DNA template is caused yielding single-stranded DNA molecules.

Annealing step: Annealing of the primers to the single-stranded DNA template is allowed by lowering the reaction temperature to 50-65^{0}C for 20-40 seconds. For hybridization of the primer to the strand, the temperature must be low enough; however, it also must be high enough for specific hybridization, i.e, the primer should only bind to a perfectly complementary part of the template (*Polymerase Chain Reaction Research*, n.d.). Moreover, the primer will bind imperfectly if the temperature is too low and the primer will not bind if the temperature is too high. Typically, the annealing temperature should be about 3-5^{0}C below the melting temperature the primers used. When the primer sequence is very closely matched to the template sequence, the DNA-DNA hydrogen bonds are only formed. Upon the binding of the polymerase to the primer-template hybrid, DNA formation begins.

Extension/elongation step: Depending on the DNA polymerase used, the temperature at this step varies. When Taq polymerase is used, commonly a temperature of 72^{0}C is used as its optimum activity temperature lies at 75-80^{0}C. In this step, by adding the dNTPs that are complementary to the DNA template strand in a 5' to 3' direction the DNA polymerase synthesizes a new DNA strand, condensing the 5'-phosphate group of the dNTPs with 3'- hydroxyl groups at the end of the nascent (extending) DNA strand (*Polymerase Chain Reaction Research*, n.d.). The DNA polymerase used and the length of the DNA fragment to amplify influence the extension time. The DNA polymerase polymerizes a thousand bases per minute at its optimum temperature, as a rule of thumb. At each extension step, the amount of DNA target is doubled, leading to exponential(geometric) amplification of the specific DNA fragment if there are no limitations due to the limiting substrates or reagents under optimum temperatures.

Final elongation: At a temperature of 70-74^{0}C(this is the temperature needed for optimal activity for most polymerase used in PCR) this single step is occasionally performed for 5-15 minutes after the last PCR cycle ensuring that any remaining single-stranded DNA is fully extended (*Polymerase Chain Reaction Research*, n.d.).

Final hold: at 4-15^0 C for an indefinite time, this step may be employed for short-term storage of the reaction.

For the amplification, the total volume of 13 µl was taken, where 6.5 µl was the master mix, 1 µl was the forward primer, 1 µl was the reverse primer, and 2.5µl of nuclease-free water (NFW) finally 2 µl of the isolated DNA was added.

PCR primers used for the experiment along with their band size product are given below:

Name of primer	**Primer Nucleotide Sequence**	**Targeted bacteria**	**Band Size**	**Reference**
PA-GS	F: GACGGGTGAGTAATGCCTA R: CACTGGTGTTCCTTCCTATA	*Pseudomonas aeruginosa*	618bp	(Azemin et al., 2022)
invA	Forward:GTG AAA TTA TCG CCA CGT TCGGGC AA Reverse:TCA TCG CAC CGT CAA AGG AAC C	*Salmonella*	284bp	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6431798/pdf/Vetworld-12-170.pdf
ipaH	Forward: GCTGGAAAAACTCAGTCCT Reverse: GCTGGAAAAACTCAGTCCT	*Shigella*	108bp	(Makabanyane et al., 2015)

ECO	Forward:GACCTCGGTTTAGTTCACAGA Reverse:CACACGCTGACGCTGACCA	*E. coli*	585bp	(J. Hassan et al., 2014)

3.5 Biochechemical Tests

3.5.1 Triple Sugar Iron (TSI)

Principle:

Bacteria usually possess the ability to ferment carbohydrates, like sugars. Depending on the type of bacteria, it could ferment only certain sugars (Aryal, 2022c). Thus, the ability of bacteria to be able to ferment some sugars while not being able to ferment others helps in the identification and characterization of the bacteria. The test, Triple Sugar Iron, is used to determine the ability of microorganisms to ferment sugars and also to produce H2 and O2 along with hydrogen sulfide (H2S) production (Aryal, 2022c).

To begin with, the slant of agar consists of three different sugars, glucose 0.1%, lactose 1%, and sucrose 1%. In addition to this, the media also consists of phenol red dye which is pH sensitive. Once the fermentation starts and acid is produced, the pH of the media dops, and the phenol changes its color from red to yellow.

The production of hydrogen sulfide was detected by the ferrous ammonium sulfate and sodium thiosulfate present in the media and indicated by the black butt color of the tube.

Furthermore, glucose is in very low concentration compared to that of sucrose and lactose. Thus, the glucose is fermented very quickly by the bacteria, and the color of the medium changes back to red to alkaline pH. On fermentation of the other sugars, the medium remains in color.

The agar is set at an angle, giving it a slanted shape that provides surfaces for either exposure to an aerobic environment (oxygen), or an anaerobic environment (no exposure to air)(Aryal, 2022c). This helps to understand and observe the fermentation patterns of the organisms.

Bacterial cultures used:

Overnight freshly grown bacteria: *Salmonella, Escherichia coli, Pseudomonas and Shigella.*

Procedure:

1. The agar was weighed in electronic balance and poured into a conical flask.
2. Distilled water was used to dissolve the media completely.
3. The media was heated until it was completely dissolved.
4. Then it was kept for autoclaves.
5. After the autoclaving was done, the media was poured into sterilized tubes, inside the laminar flow and left to set in a slanted angle.
6. A straight inoculation needle was taken to pick the isolated colony.
7. The colonies were then first stabbed through the center of the medium to tube bottom and then streaked on the agar slant surface.
8. The cap was left on loosely and the tubes were incubated at 37 degree Celsius for 24 hrs.
9. The results were then interpreted.

3.5.2 Citrate test

Principle: To determine the ability of microorganisms to utilize citrate as a source of energy, a citrate test is used (Aryal, 2022b). To begin with, as a source of nitrogen, ammonium salts ($NH_4H_2PO_4$) are used while for the carbon source, citrate is used.

To begin with, citrate-permease enzymes are produced by the bacteria that can grow in this media (Aryal, 2022b). As a result, this bacterium converts the citrate to pyruvate. Thus produced pyruvate enters the metabolic cycle of the bacteria to generate energy. Furthermore, the alkalinity of the media increases the metabolization of the citrate and on production of ammonia from the ammonium salts. An indicator, bromothymol blue, which is added to the media indicates the change in color from green to blue, with the shift in pH(Aryal, 2022b).

Bacterial cultures used:

Overnight freshly grown bacteria: *Salmonella, Escherichia coli, Pseudomonas and Shigella.*

Procedure:

1. The agar was weighed in electronic balance and poured into a conical flask.
2. Distilled water was used to dissolve the media completely.
3. Bromothymol blue was added as an indicator.
4. The media was heated until it was completely dissolved.
5. Then it was kept for autoclaves.
6. After the autoclaving was done, the media was poured into sterilized tubes, inside the laminar flow and left to set in a slanted angle.
7. A straight inoculating needle was taken to pick the isolated colony.

8. The colonies were then first stabbed through the center of the medium to tube bottom and then streaked on the agar slant surface.
9. The cap was left on loosely and the tubes were incubated at 37 degrees Celsius for 24 hrs.
10. The results were then interpreted.

3.5.3 Methyl Red/ Voges (MR VP Test)

To determine which fermentation pathway is used by the organisms to utilize the glucose, the MR VP test is performed. MR and VP are two different tests where the Methyl red test or MR test looks for the mixed acid fermentation pathway of glucose while the Voges-Proskauer test of VP test is used for glucose fermentation through the butanediol fermentation pathway.

3.5.3.1 Methyl red(MR) Test

To begin with, some species of bacteria produce a mixture of fermentation products through mixed-acid fermentation pathways (Libretexts, 2023). The products can vary within strains as well as among species, depending on the enzymes that do the reactions. Some of the products of mixed fermentation are lactate, formate, and acetate as well as byproducts like ethanol. The methyl red test consists of glucose liquid media, where the bacteria are inoculated and allowed to grow. Methyl red is added as an indicator to sense the change in pH on the production of the mixed acid (Libretexts, 2023). The result is shown by the red color of the medium, which means a positive result. If the color does not change to red, it means that the mixed acids are not produced.

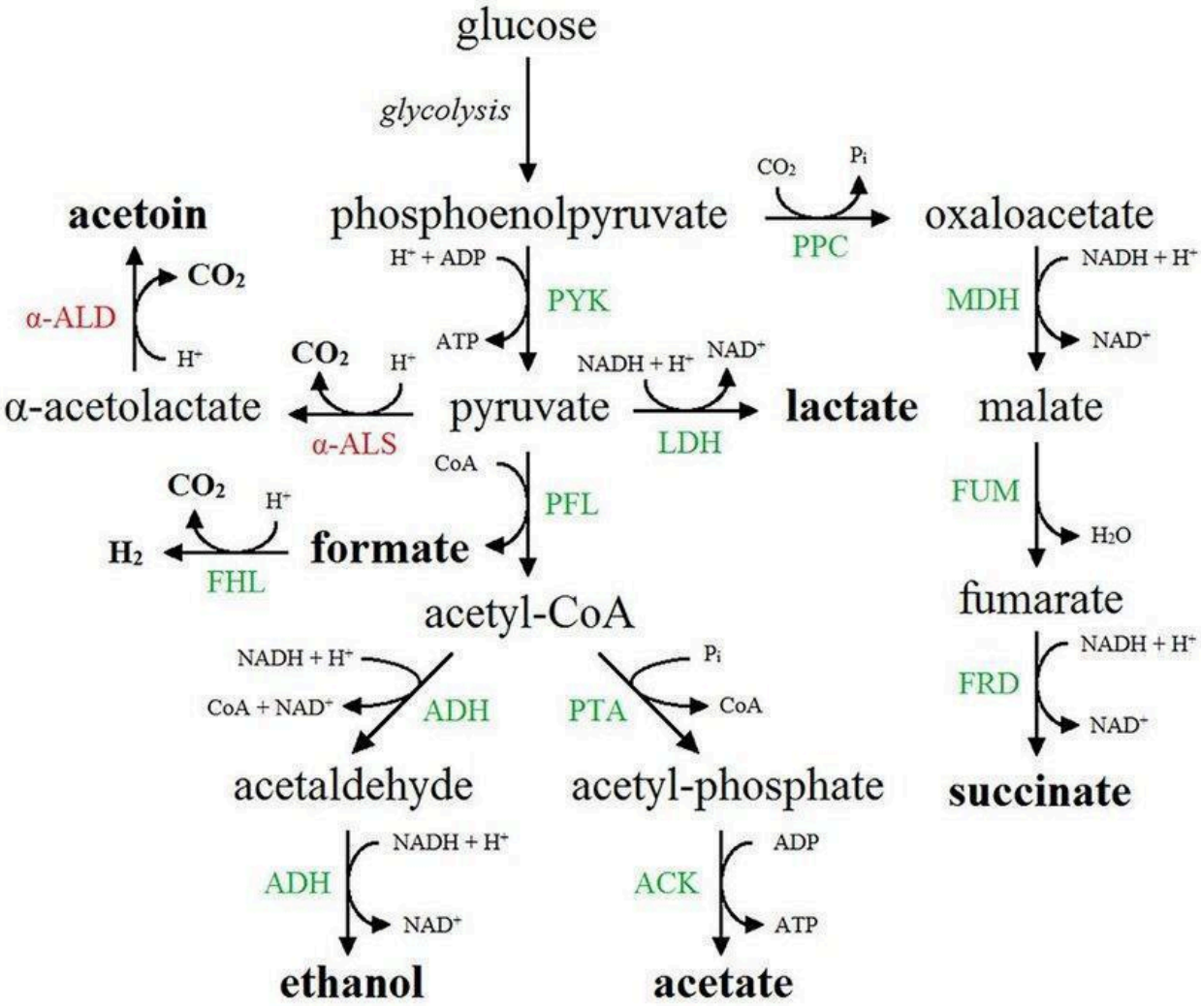


Fig: Glucose fermentation into mixed acids fermentation (Libretexts, 2023).

3.5.3.2 Voges-Proskauer (VP) test

To determine whether or not the end product of the glucose fermentation is 2,3-butanediol, a VP test is used (Libretexts, 2023). The VP test also uses the same broth i.e. MRVP broth, which is

separated before adding the MR reagent. After the inoculation of the bacteria in the broth for about 48 hrs, Barritt's A reagent is added followed by Baritt's reagent B. On mixing the reagents and letting them set for a certain amount of time, a layer is formed on the upper part of the tube that gives the results of our test (Libretexts, 2023). To illustrate, a positive Voges-Proskauer test is indicated by a red-brown or red color in the top layer, which indicates the fermentation to 2,3-butanediol. Whereas, an absence of the red color i.e. presence of a yellow color indicates that no fermentation of 2,3-butanediol is there in the medium.

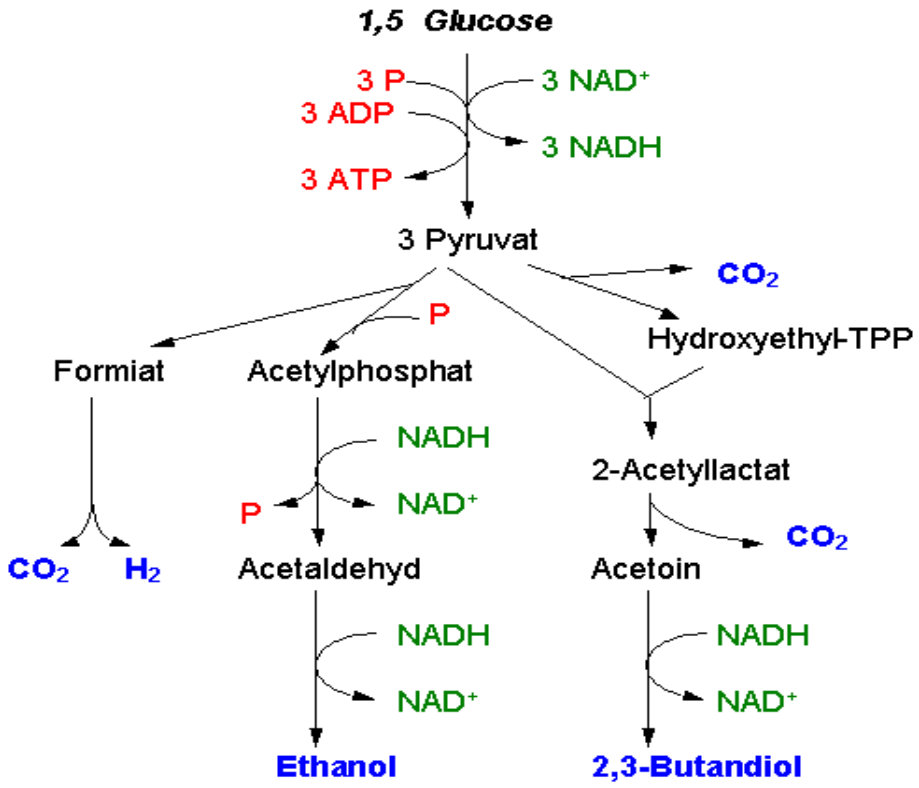


Fig: Glucose fermentation into 2,3-butanediol (Libretexts, 2023).

Bacterial cultures used:

Overnight freshly grown bacteria: *Salmonella, Escherichia coli, Pseudomonas and Shigella.*

Procedure:

1. Appropriate amounts of the reagents were measured using an electronic balance.
2. The reagents were then added to a conical flask and were dissolved in distilled water.
3. A magnetic stirrer was used for the complete dissolution of the reagents.
4. The media was kept for autoclaving.
5. After the completion of autoclaving, the media was poured into test tubes which were also autoclaved.
6. The well-isolated colonies were then incubated in the test tubes in an incubator for about 48 hrs at 37 degrees Celsius.

The procedure of the MR test:

1. After 48 hrs of incubation, the samples were ready for the MR test.
2. 4-5 drops of MR reagent (Methyl red) were added to each tube.
3. The color change was observed.

The procedure of the VP test:

1. After 48 hrs of incubation, the samples were ready for the VP test.

2. At first 400 µL Barritt's A (Alpha-naphthol) was added to the tubes, followed by 200 µL of Barritt's B (40% KOH).
3. The tubes were shaken so that the reagents could mix properly and were let to rest for 30 minutes.
4. After the resting phase, the tubes were observed for pink-red color, which gave a positive result. The absence of the pink-red color indicated a negative result.

3.5.4 Mobility test

To differentiate between non-motile and motile bacteria, a mobility test is performed (Aryal, 2022c). To begin with, the ability of an organism to use some special features like fibrils or propellers like flagella to move itself is known as a mobility test. The motile bacteria usually use flagella which can be single or multiple flagella. These flagella are helical in shape with a rigid structure, and their rotation mimics the movement of a boat propeller (Aryal, 2022c). The media used for the mobility test is a semi-solid SIM (Sulphide Indole Motility), which is a differential media that performs a combination of tests like; the indole test, motility, and sulfur reduction test. To allow the migration of the bacteria, this media is in soft consistency and the cloudiness of the media suggests the motility. The bacteria is stabbed in the center of the agar using a sterile inoculating needle (Aryal, 2022c). After the inoculation the motile bacteria migrate from the stabbed line, showing a zone of growth. Some of the bacteria migrate to small areas while the other can grow throughout the medium. On the other hand, the non-motile bacteria will grow only in the stabbed line.

Bacterial cultures used:

Overnight freshly grown bacteria: *Salmonella, Escherichia coli, Pseudomonas and Shigella.*

Procedure:

1. The agar was weighed in electronic balance and poured into a conical flask.
2. Distilled water was used to dissolve the media completely.
3. The media was heated until it was completely dissolved.
4. Then it was kept for autoclaves.
5. After the autoclaving was done, the media was poured into sterilized tubes, inside the laminar flow and left to set.
6. A straight inoculation needle was taken to pick a well-isolated colony.
7. The colonies were then single-stabbed through the center of the semi-solid medium to the tube bottom.
8. The tubes were incubated at 37 degrees Celsius for 24 hrs.
9. The results were then interpreted.

3.5.5 Catalase test

Principle:

The catalase test is performed to determine whether or not bacteria have an enzyme catalase that can convert Hydrogen peroxide into oxygen (Libretexts, 2023a). To begin with, this test is used to differentiate between catalase enzyme-producing bacteria and the ones that cannot produce this enzyme. When the bacterial colonies are inoculated in Hydrogen peroxide (H2O2), small bubbles are produced (Libretexts, 2023a). In a catalase-positive test, even a small amount of colony or inoculum gives a good amount of bubbles, whereas, in the case of catalase-negative results, weak bubbles or no bubbles could be seen. Furthermore, when doing this experiment, the sample needs to be fresh (within 24 hours of grown culture). The bacteria thus protect themselves from the harmful effects of hydrogen peroxide (Aryal, 2022).

Bacterial cultures used:

Overnight freshly grown bacteria: *Salmonella, Escherichia coli, Pseudomonas and Shigella.*

Procedure:

1. A sterilized glass slide was taken.
2. A drop of hydrogen peroxide was added to the glass slide using a dropper.
3. With a sterilized inoculating loop, the bacteria were placed on the hydrogen peroxide and smeared.
4. The results were observed: bubbles showed a positive result while the no bubbles showed a negative result.

3.5.6 Oxidase test:

To determine the capability of bacteria to synthesize the oxidase enzyme cytochrome C, an oxidase test is performed (Aryal, 2022b). To begin with, this large transmembrane protein, Cytochrome C, works to transfer the last electron from cytochrome c to an oxygen molecule in the electron transport chain of aerobic bacterial and mitochondrial respiration systems. To illustrate, this enzyme is present in some bacteria, and when it is present, the bacteria can successfully transfer a terminal electron to molecular oxygen; in other cases, the bacteria may use another cytochrome to accomplish the same task. It is crucial to distinguish between bacteria that contain and do not possess the cytochrome c oxidase enzyme to properly define and biochemically identify microorganisms (Aryal, 2022b). Moreover, indophenol oxidase which is an iron-containing hemoprotein or cytochrome oxidase, works in the electron transport from NADH donors to Oxygen which is the electron acceptor. The reagent used in this experiment is N, N, N, N-tetramethyl-p-phenylenediamine dihydrochloride, which works as an electron

acceptor (artificial) for the oxidase enzyme. Later the colored substance, indophenol blue, is formed by the oxidizing reagent (Aryal, 2022b).

Bacterial cultures used:

Overnight freshly grown bacteria: *Salmonella, Escherichia coli, Pseudomonas and Shigella.*

Procedure:

1. 1.0 gm of Kovac's reagent was added to 100ml distilled water and mixed thoroughly.
2. A strip of Whattman's filter paper was taken and soaked in freshly prepared reagent (1% solution).
3. A well-isolated colony from the fresh culture was taken with a sterilized inoculating loop and smeared on the reagent-soaked filter paper.
4. The color change was then observed within 60 seconds of inoculation.

3.5.7 Gram Staining

Principle:

The uses of at least three chemical reagents are required in differential staining and are applied sequentially to a heat-fixed smear. To begin with, the first stain is called the primary stain and its function is to impart color to all cells. Furthermore, a second reagent, a decolorizing agent is used to establish a color contrast The decolonizing agent may or may not remove the primary stain from the entire cell or only certain cell structures, based on the chemical components of the cellular constituents. Finally, a counter stain which has a contrasting color to that of the primary stain is the final reagent. After decolorization, the counter stam cannot be absorbed if the primary stam is not washed out and the color of the primary stain will be retained by the cell or its components If the primary stain is removed, the contrasting color of the counterstain will be

accepted by the decolonized cellular components. Hence based on the stain that is retained, the cell types and structure can be distinguished (Kalet, 2013).

The Gram stain, named after Dr. Christian Gram, is the most important differential stain used in bacteriology. It divided the bacteria into two principal groups of bacteria: Gram-positive and Gram-negative. Hence this method is essential for the classification and differentiation of microorganisms (Kalet, 2013). Furthermore, four different reagents are used in the gram staining and their descriptions and mechanism of their action are as follows.

A) Primary stain: The violet stain, Crystal violet is used at first and all cells stain violet.

B) Mordant: Gram's iodine is the reagent used as a mordant. To begin with, by binding to the primary stain it forms an insoluble complex. Hence obtained crystal violet-iodine complex (CV-1) acts to intensify the stain's color and at this point, the cells appear purple-black. Furthermore, in the case of the gram-positive cells, the magnesium ribonucleic acid component of the cell wall is bounded only by the CV-1 complex. As compared with the smaller CV-1 complex, the resultant magnesium ribonucleic acid-crystal violet-iodine complex (Mg-RNA-CV-I) complex is more difficult to remove (Tripathi & Sapra, 2023).

C) Decolorizing agent: Ethyl Alcohol (95%) serves a dual function i.e., as a protein dehydrating agent and as a lipid solvent. The lipid concentration of the microbial cell walls determines its action. In the case of Gram-positive cells, for the retention of the Mg-RNA-CV-I complex, a small amount of lipid concentration is important. Hence, by the action of alcohol, a small amount of lipid content is readily dissolved by the formation of minute cell wall pores, and then these are closed by the effects of alcohol dehydrating. Whereas it is difficult to remove the tightly bound stain, and the cells remain purple. On the other hand, in Gram-negative cells, the high lipid concentration found in the outer layers of cells does not close appreciably on the dehydration of cell wall proteins and it results in the release of the unbound CV-1 complex. This leads to the cells being colorless or unstained (Tripathi & Sapra, 2023).

D) Counterstain: The previously decolorized cells are stained by the final reagent Safranin. This counterstain is absorbed only by the gram-negative cells because they undergo decolorization. The purple color of the primary stain is retained by the gram-positive cells (Tripathi & Sapra, 2023).

Bacterial cultures used:

Overnight freshly grown bacteria: *Salmonella, Escherichia coli, Pseudomonas and Shigella.*

Procedure:

1. A clean glass slide was taken.
2. A smear of the given organism was made using a sterile technique. This was done by placing a drop of water on the slide and then transferring the given organism to the drop of water with a sterilized and cooled loop. Then the organism was mixed and spread by the inoculation loop in a circular motion.
3. The smear was allowed to air dry and then was heat fixed.
4. Now the smear was flooded with crystal violet and was left to stand for about 1 minute.
5. The reagent was then washed with tap water.
6. Then gram iodine mordant was flooded on the smear and was left to stand for 1 minute.
7. Again the reagent was washed with tap water.
8. Now decolorization was done with the help of Ethyl Alcohol (95%). Over decolorization was avoided. The reagent was added drop by drop until the crystal violet failed to wash from the smear.
9. Then it was washed with tap water.
10. After that counter stain with safranın was applied for about 45 seconds. And then it was washed with tap water.
11. Finally with the help of bibulous paper blot drying was done and examination was performed under oil immersion.

3.6 Antibiotic test

Principle:

In general definition, an antimicrobial chemical substance that is produced by one microorganism as a defense from a different microorganism is known as an antibiotic. Thus humans have been successfully utilizing these antimicrobial substances to protect themselves from pathogens and diseases caused by them.

The Kirby Bauer disc diffusion method for antibiotic susceptibility is one of the standard protocols that was developed during the 1950s. Later was standardized and used worldwide from 1961. This test is used to determine the ability of the chemicals to inhibit certain species of microorganisms and whether or not that antimicrobial chemical could be prescribed to patients in their treatments (Libretexts, 2024). To illustrate, the pathogens are first grown in Mueller Hinton agar- which is a non-differential and non-selective media, with loose agar, that allows proper diffusion of the antibiotic throughout the plate and gives clear zones of inhibition. In addition to this, the agar also consists of starch, which acts as a source of energy on hydrolysis and also absorbs any inhibitors produced by the bacteria such as toxins, so that they could not tamper with the working of the antibiotics. In this agar, the bacteria are swapped, and the antibiotic discs are then placed with gentle pressure so that they remain attached to the agar. Then the antibiotic starts its work, and diffuses itself from the disc in the agar, with decreasing diffusion with increasing distance from the disc. A zone of inhibition is formed in immediate regions around the disc, with no growth of bacteria, showing that the bacteria are killed or inhibited by the antibiotic concentration(Libretexts, 2024). For each antibiotic, there is a standard chart that shows the zone sizes and gives results whether it falls under intermediate, resistant, or susceptible. Along with the antibiotic name, there is also a column for the dosage of that antibiotic and the result if interpreted(Libretexts, 2024). Based on the chart, it is concluded whether or not certain antibiotics should be prescribed to the patient for treatment or not.

Culture used:

4-6 hrs NB grown freshly grown bacteria: *Salmonella, Escherichia coli, Pseudomonas and Shigella.*

Procedures:

1. The well-isolated colonies were first inoculated in nutrient broth for 4-6 hrs and kept in for incubation at 37 degrees Celsius.
2. After incubation, with the help of sterile swabs, the inoculum was taken and the excess dip was removed by pressing the swab to the tube wall.
3. By using a technique called lawn culture, the inoculum was swabbed throughout the plate surface by rotating the plate, ensuring that there was no gap.
4. The surface of the plate was let to dry for at least 5 minutes for the inoculum to be absorbed by the media.
5. Now with the help of sterilized forceps, the discs were placed in the medium. The sterility of the forceps was maintained by dipping it in alcohol and lightly flaming it.
6. The discs were then slightly touched with the help of an inoculating loop so that they would be in good contact with the medium.
7. The plates were then kept in for incubation at 37 degrees Celsius in an upside-down manner.

Chapter 4: Observation and Results

This section presents the findings from the isolation and identification of E. coli, Pseudomonas, Salmonella, and Shigella from biomedical waste samples. The results include PCR analysis and microbial tests, supported by images to illustrate key points.

4.1 Isolation of Bacterial Strains

Samples were collected from various biomedical waste sources

Different samples were collected from 5 different hospitals by using safety protocol to prevent contamination and risks from such waste. All the wastes collected were packed in different ziplock bags with proper labeling. Such different categorization of wastes helps to prevent cross-contamination.

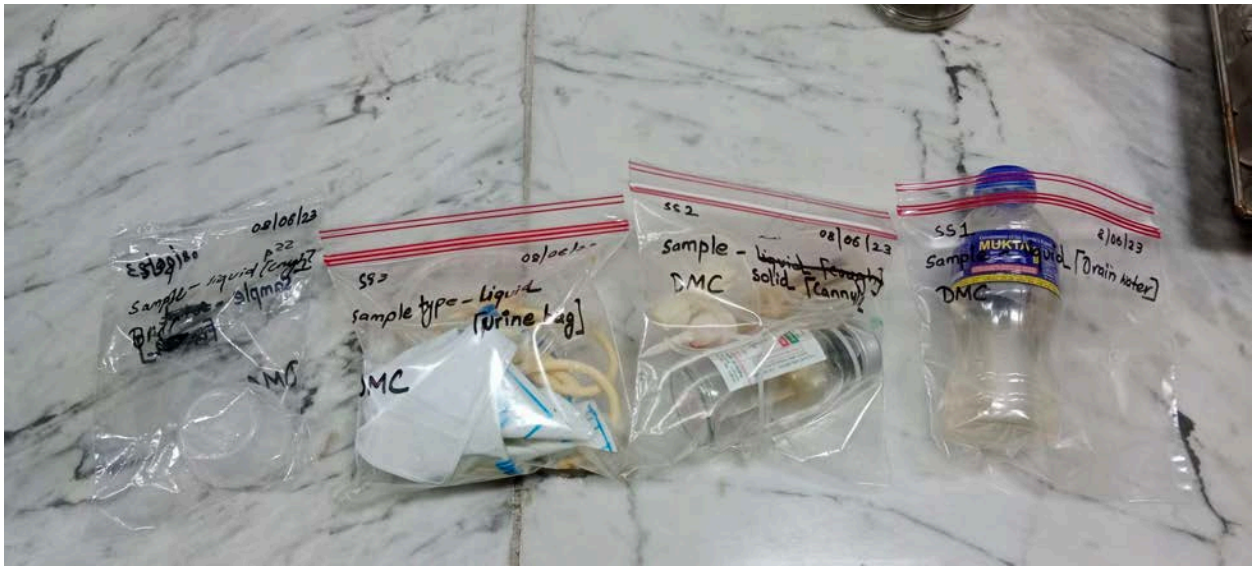


Fig 4.1.1: Wastes from the hospital collected in different bags with proper labeling

Further, the samples were inoculated in a peptone buffer for 24 hours.

Fig 4.1.2: Samples inoculated in peptone buffer with proper labeling

4.2 Selective and Differential Media

Bacteria were further isolated and identified using selective and differential media. In this research, XLD, cetrimide, and UTI were used as selective media. Further, cultures from the peptone buffer after 24 hours were grown on selective and differential media to isolate target bacteria. In this experiment, *Salmonella, Shigella, Pseudomonas, and E. coli* were isolated as targeted bacteria for Biomedical Analysis.

XLD and cetrimide media were used as selective media in Hospital 1, Hospital 3, and Hospital 4 whereas XLD and UTI media were used as selective media in Hospital 2 and 5. Further, In XLD media, 2 *Salmonella* and 2 *Shigella* isolates were present whereas from Cetrimide media 2 *E. coli* and 3 *Pseudomonas* isolates were isolated from Hospital 1. In XLD media, 3 *Salmonella* and 4 *Shigella* isolates were present whereas from UTI media 7 *E. coli* strains and 5 *Pseudomonas* isolates were isolated in Hospital 2. Also, in XLD media, 3 *Salmonella,* and 2 *Shigella* isolates were present whereas from Cetrimide media 7 *E. coli* and 6 Pseudomonas isolates were isolated from Hospital 3 whereas, in XLD media, 4 Salmonella and 6 Shigella isolates were present whereas from Cetrimide media 8 *E. coli* isolates and 5 Pseudomonas isolates were isolated from Hospital 4. Later, In XLD media, 1 *Salmonella,* 2 *Shigella* isolates, and 7 *E. coli* isolates were present whereas from UTI media 10 Pseudomonas strains were isolated from Hospital 5.

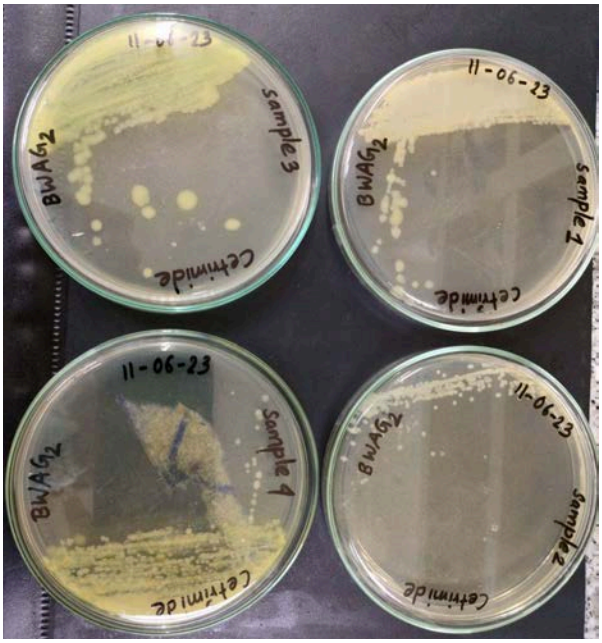


Fig 4.2.1: Hospital samples in cetrimide media

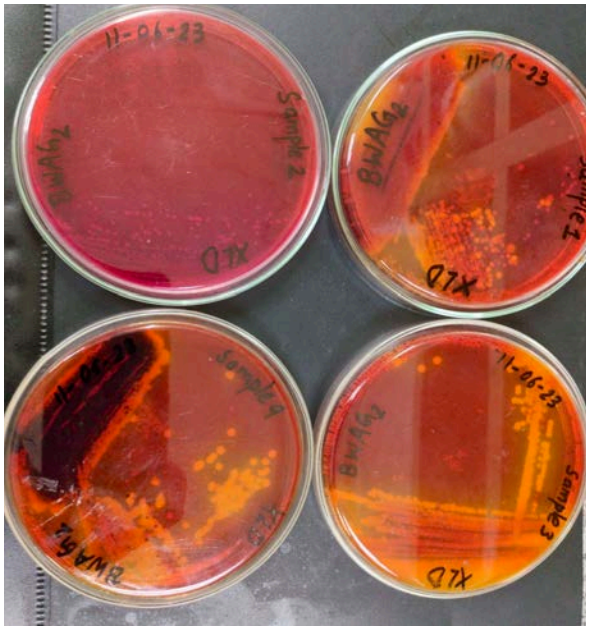
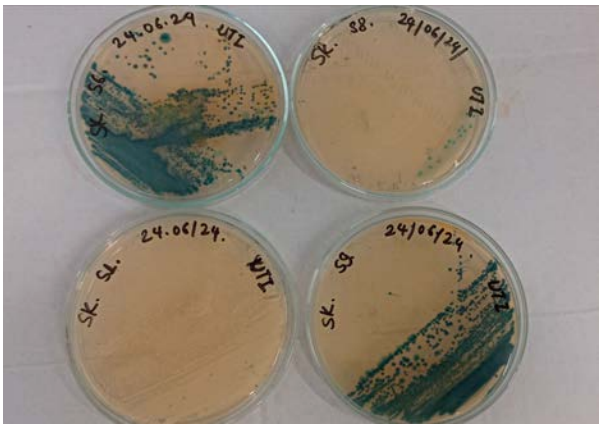


Fig 4.2.2: Hospital samples in XLD media



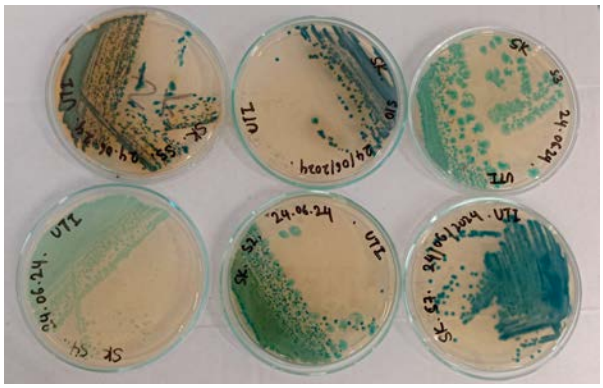


Fig 4.2.3: Hospital samples in UTI media

4.3 Isolation of Pseudomonas

4.3.1. Polymerase Chain Reaction (PCR) Results:

Identification of Pseudomonas isolates by PCR Assay and Gel electrophoresis

From the total number of 87 isolates, 29 of the isolates were found to be *Pseudomonas*. To confirm this, biochemical presumptive tests were done, along with Polymerase Chain reaction (PCR). For the PCR, the PAGS was used, which is specific for the 16s rDNA of *Pseudomonas* species, with a product size of 618bp (Azemin et al., 2022). The results indicated a 33.33 % prevalence of *Pseudomonas* in the biomedical wastes collected from the dustbins of the hospitals.

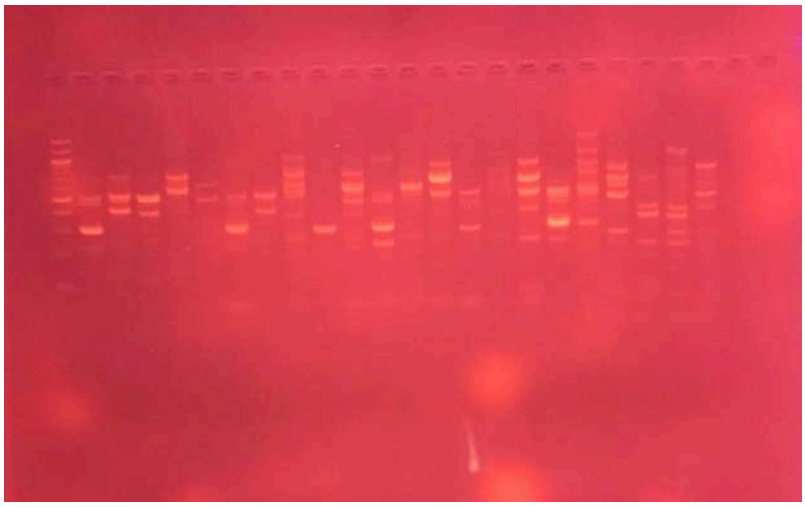


Fig 4.3.1.1: PCR amplified products of Pseudomonas

The identified 29 isolates of *Pseudomonas* underwent the PCR process later followed by gel electrophoresis. 1% agarose gel was used to run the PCR products, as described earlier. The isolates showed positive results for the PA-GS gene, resulting in a band product of size 618bp as shown under UV transilluminator. From the total isolates, 20 of the isolates showed clear bands, indicating the presence of the *Pseudomonas*-specific genes in the collected samples.

Further, the distributions of 33.33% isolates among 5 different hospitals are shown in Fig.

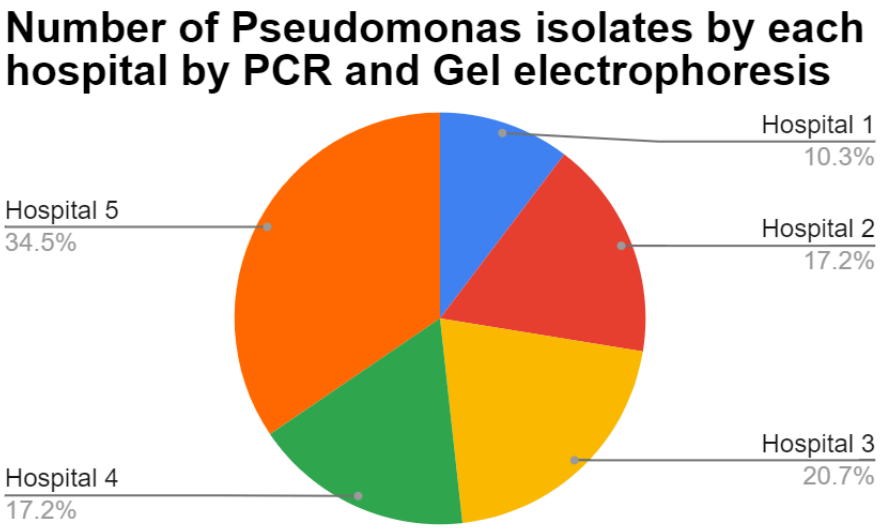


Fig 4.3.1.2: Pie chart showing the distribution of *Pseudomonas* across the different hospitals.

4.3.2 Identification of Pseudomonas using Biochemical tests

Here, the basic characteristics of different biochemical tests for *Pseudomonas* are mentioned in Table 4.3.2.1.

S.N	Different Test	Results for *Pseudomonas*
1.	Gram staining	Negative (Pink)
2.	MR Test	Negative (No red color)
3.	VP Test	Positive (Red color)
4.	Catalase Test	Positive (Bubbles present)
5.	Oxidase Test	Positive (Color change)

6.	**Citrate Test**	**Positive (Growth on medium)**
7.	**Motility Test**	**Positive (Diffusion from stab line)**

Table: 4.3.2.1

4.3.2.1 Gram staining of isolated *Pseudomonas*

The presumptive 29 isolates of *Pseudomonas* underwent the process of Gram staining. The results of the Gram staining characteristics were found to be consistent with the gram-negative *Pseudomonas*. To illustrate, all of the 29 presumed isolates indicated lipopolysaccharide cell walls by giving pink coloration and retaining the safranin dye, as visualized under the microscope. Thus, these results set a path in the identification and characterizing of the *Pseudomonas* species. The result of one of the gram-negative samples of *Pseudomonas* under an electron microscope is shown below.

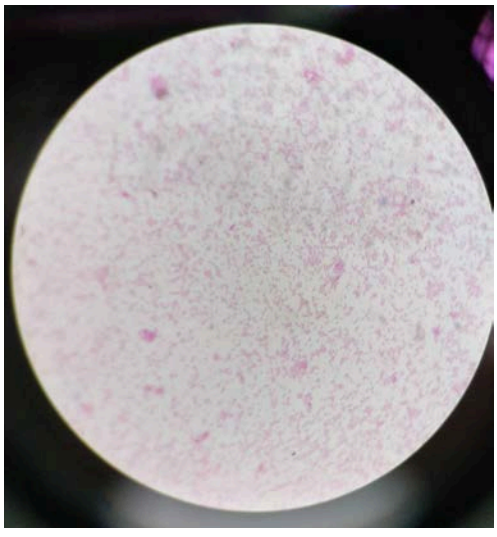


Fig 4.3.2.1.1: Gram staining of *Pseudomonas* showing Gram-negative result

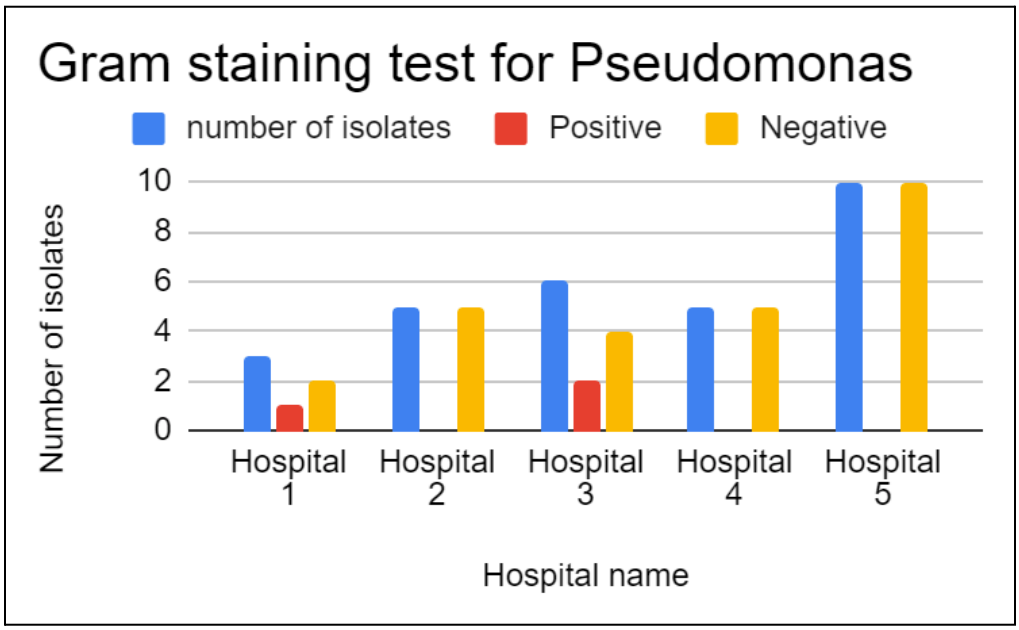


Fig 4.3.2.1.2: Bar graph showing results of Gram staining test for *Pseudomonas*

4.3.2.2 Identification by Triple Sugar Iron (TSI):

After 24 hours of incubation of the 28 isolates, the results were interpreted. The result showed that neither the butt nor the slant turned yellow indicating that there was no acid production. Furthermore, there was an absence of production of gasses H2 & CO2, and also H2S gas was absent. This aligned with the characters of *Pseudomonas*.

Fig 4.3.2.2.1: Triple Sugar Iron test in identifying *Pseudomonas*

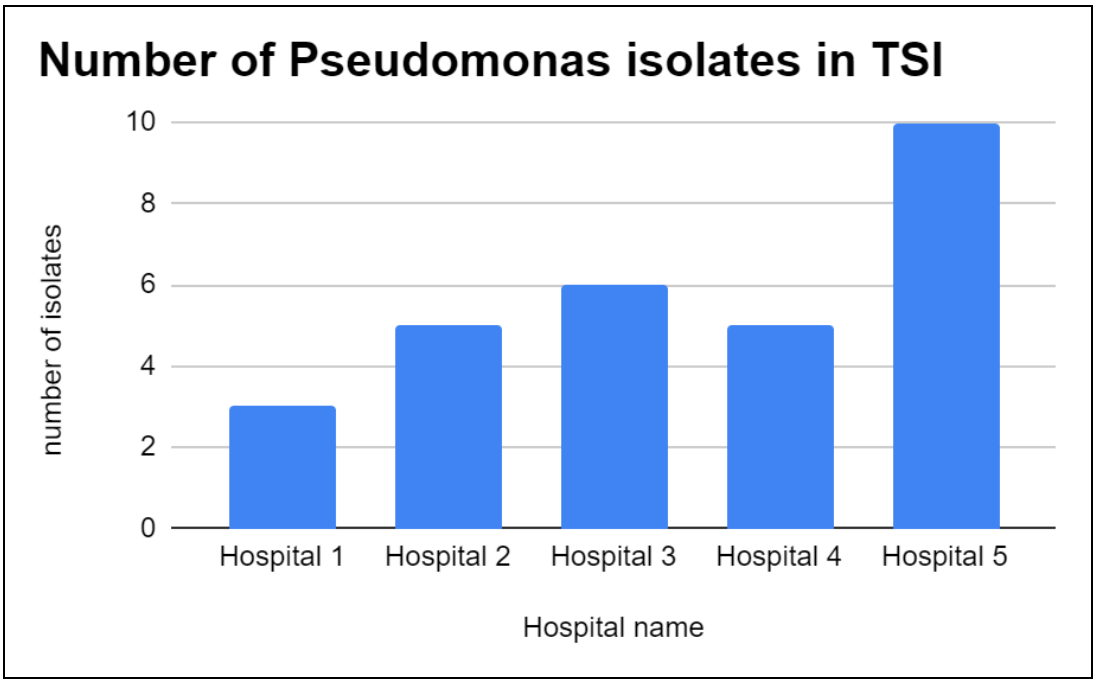


Fig 4.3.2.2.2: Bar graphs showing the number of *Pseudomonas* isolates in TSI

4.3.2.3 Identification using Citrate Utilization Test

For the citrate Utilization test, out of 29 presumptive isolates of *Pseudomonas,* 24 of the isolates showed a positive result. As described earlier in this paper, the breakdown of citrate to oxaloacetate and acetate and its further break down to pyruvate and CO2 was found in the samples. Furthermore, in case of the positive results, these metabolic products further break down due to the presence of alkaline pH, and finally, the indicator Bromothymol blue gives a Prussian Blue color. On the other hand, 5 of the isolates showed no change in color. This indicated the absence of metabolic breakdown; the color of the media remained unchanged (green) as shown below:

Fig 4.3.2.3.1: Citrate Utilization Test results for *Pseudomonas* isolates

That means 82.75% were found to be positive, which aligns with the standard characteristics of the *Pseudomonas*. Furthermore, the hospital-wise comparison showed that the hospital 5 with a value of 10 isolates had the greatest number of positive *Pseudomonas*. Followed by Hospital 4 with 5 isolates, hospital 3 with 4 isolates, Hospital Two with 3 isolates, and Hospital 1 with just 2 positive isolates of *Pseudomonas.* The bar graph shows the distribution of *Pseudomonas* across different hospitals.

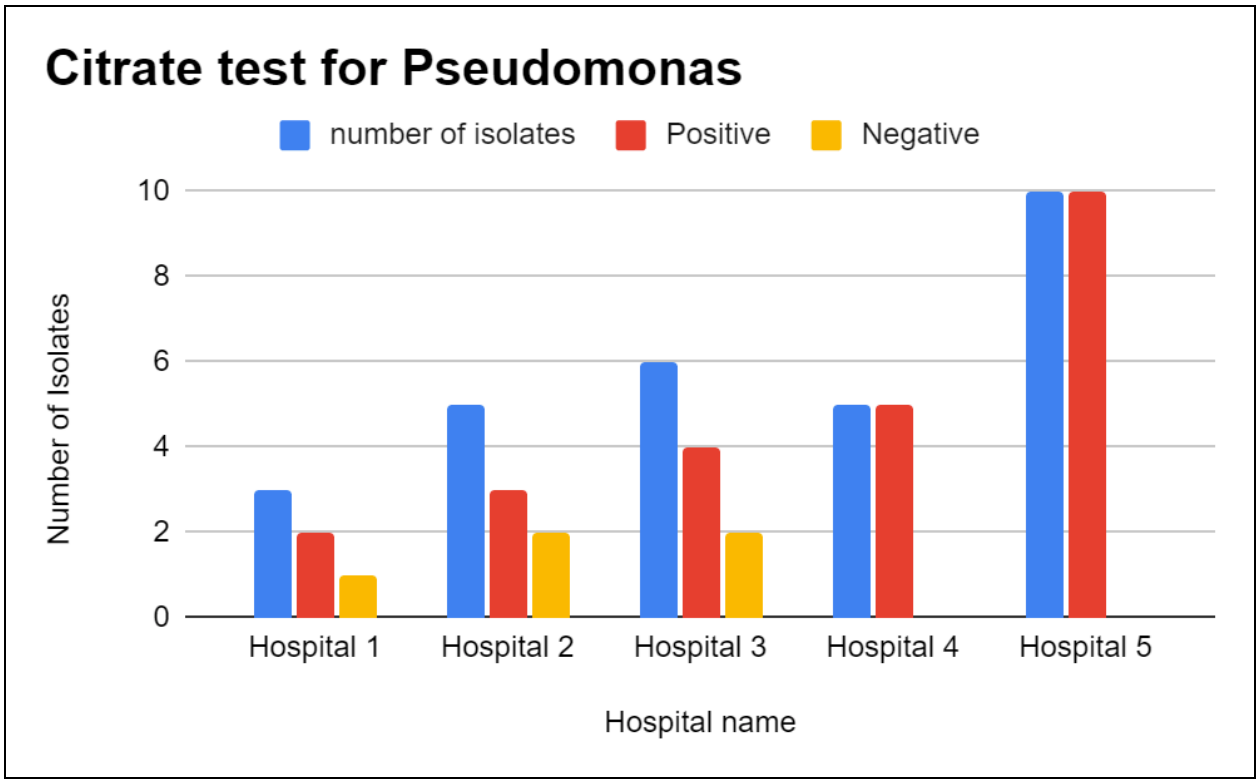


Fig 4.3.2.3.2: Bar graphs showing citrate tests for *Pseudomonas*

4.3.2.4 Identification using Catalase Test

Hydrogen peroxide, a toxic product is produced as a result of aerobic metabolism by bacteria. To stop the damage that could be caused by this toxicity, cells use an enzyme called catalase to break down the H_2O2. The species of bacteria that have this catalase are catalase positive, while those that lack the enzyme are negative to the test. To exemplify, the positive result is indicated by bubble production (Oxygen gas), which is simply an outcome of the breakdown of the H_2O2 by the enzyme catalase, into water and O2 gas. Our species *Pseudomonas,* has the enzyme catalase. As a result, all 29 isolates showed a positive test with the bubble formation. **Fig.4.3.2.4.1** shows the formation of bubbles on the addition of Hydrogen peroxide to the presumptive isolates.

Fig 4.3.2.4.1: Results of Catalase test for Pseudomonas

Furthermore, the bar graph shows the distribution of *Pseudomonas,* in the five hospitals the samples were collected from. The chart shows that positive isolates number varied among the hospitals. Hospital five with 10 numbers of positive isolates had the highest number, followed by Hospital Three, Hospital Four, Hospital Two with 5 positive isolates, and Hospital One with 3 positive isolates.

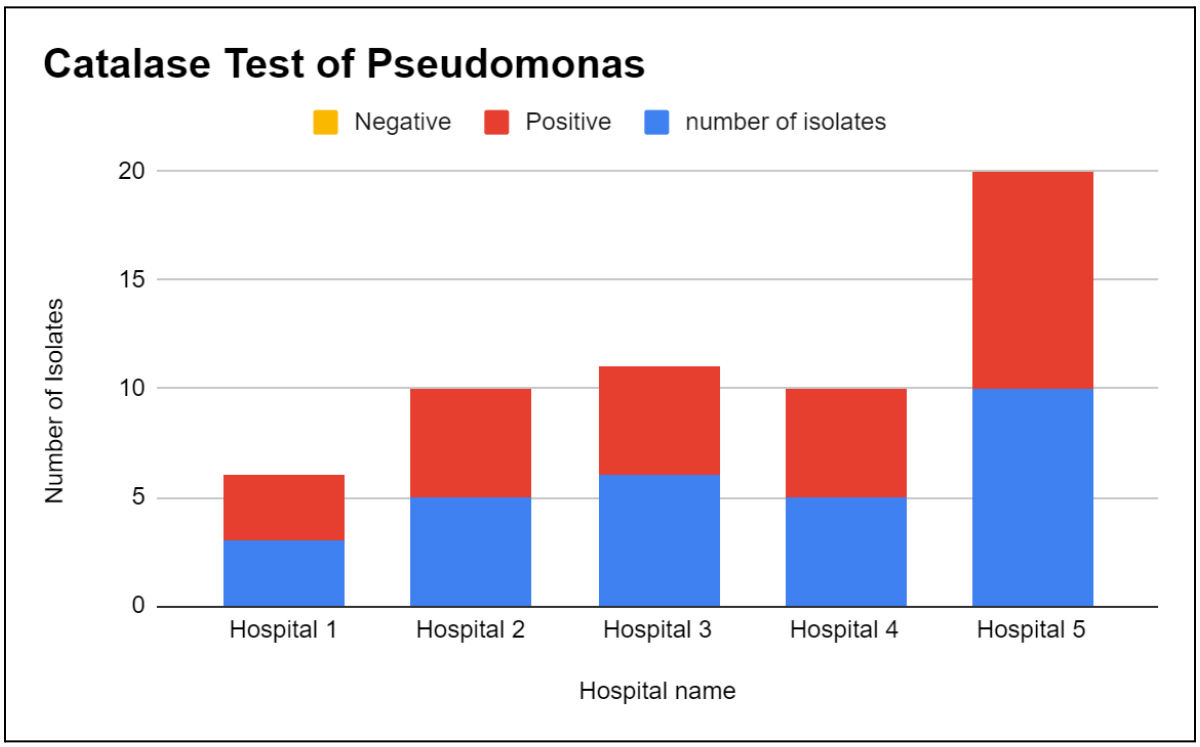


Fig4.3.2.4.2: Bar graphs showing Catalase tests for *Pseudomonas*

4.3.2.5 Identification using Oxidase Test

The enzyme cytochrome oxidase reduces oxygen at the end of the electron transport chain. In our experiment, a colorless redox reagent i,e. 1 % of tetramethyl-p-phenylenediamine was used to detect the presence of oxidase enzyme. The positive result was obtained because of the reagent used which acts as an electron acceptor and leads to the oxidation of our reagent into end product indophenols. This indophenol was responsible for the end purple color. From the 29 presumptive *Pseudomonas* isolates, for 25 of the isolates, there was a change in color to dark purple, concluding that these isolates do have cytochrome oxidase enzymes. On the other hand, four of the isolates showed a negative oxidase result.

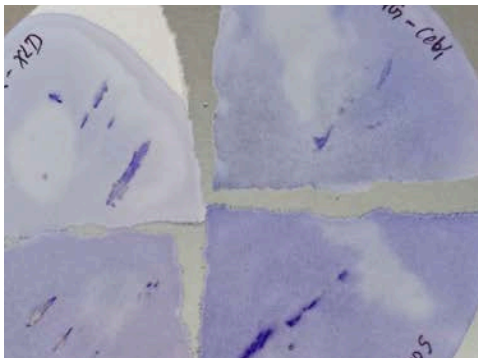


Fig 4.3.2.5.1: Oxidase test for *Pseudomonas*

Furthermore, the bar graph shows the distribution of *Pseudomonas* in the five hospitals the samples were collected from. The chart shows the percentage of positive and negative isolates number varied among the hospitals. Hospital five had the highest number 9 positive isolates followed by hospitals two and four with equal number 5 isolates and hospital three with four positive isolates followed by hospital one 3 positive isolates.

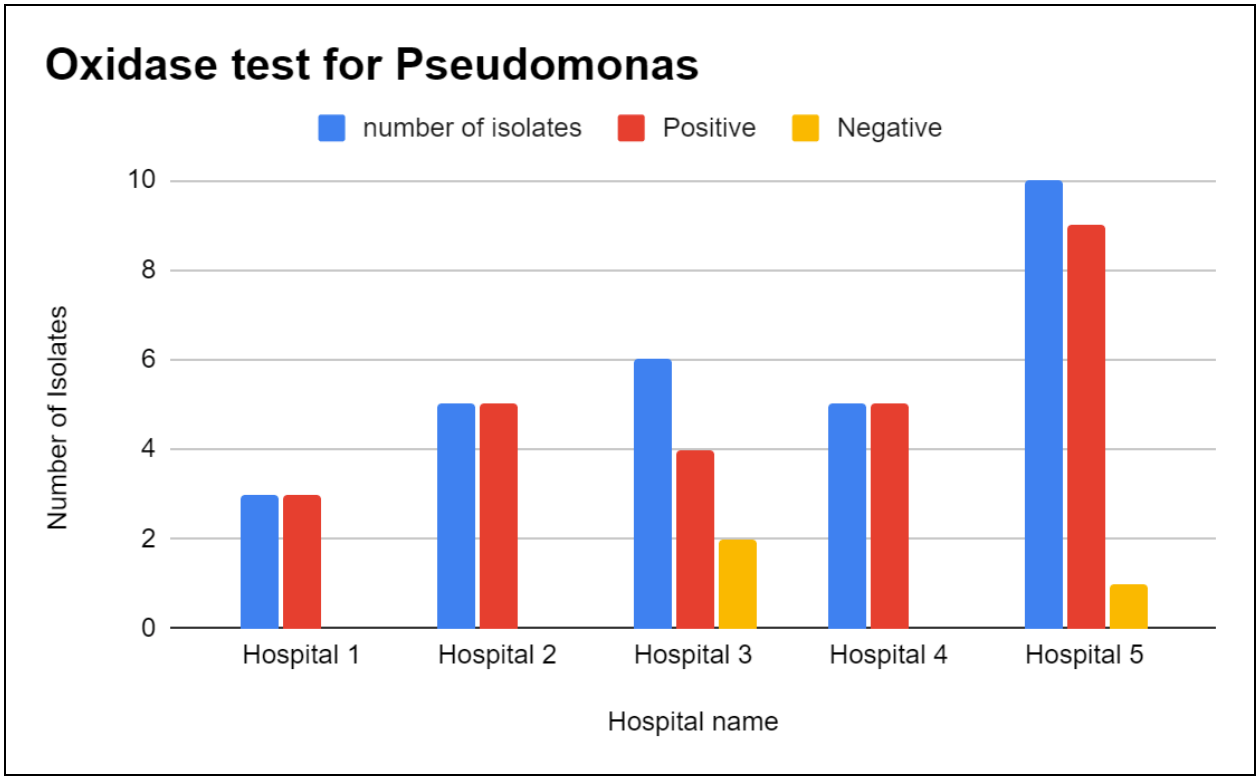


Fig 4.3.2.5.2: Bar graphs showing results of Oxidase test for *Pseudomonas* in 5 different hospitals

4.3.2.6 Identification using MR test:

For the MR test, the 29 isolates were inoculated in liquid broth for 48 hrs. On the addition of the reagent methyl red, a change in color to red was observed in 4 isolates. The resulting red color from the positive MR test indicated that the end product was acidic and had a pH of 4.4 or much less, showing that mixed acids were produced. Furthermore, 25 isolates on the addition of methyl red drops, indicated a yellow color showing the end product is not much acidic and the PH is 6.2. This yellow color indicates a negative MR Test.

Furthermore, the bar graph shows the MR test results of *Pseudomonas,* in the five hospitals the samples were collected from. The chart shows that the negative isolates number varied among the hospitals. Hospital five with 7 numbers of negative isolates had the highest number, followed by Hospital three with 6 isolates, Hospital four with 5 positive isolates, Hospital 2 with 4 negative isolates, and Hospital One with 3 positive isolates respectively.

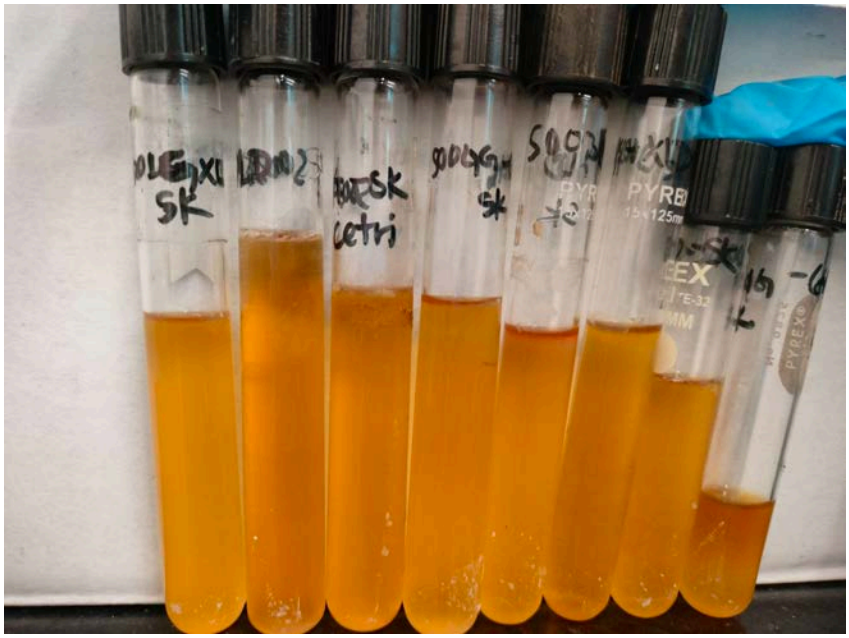


Fig 4.3.2.6.1: Methyl red tests for *Pseudomonas*

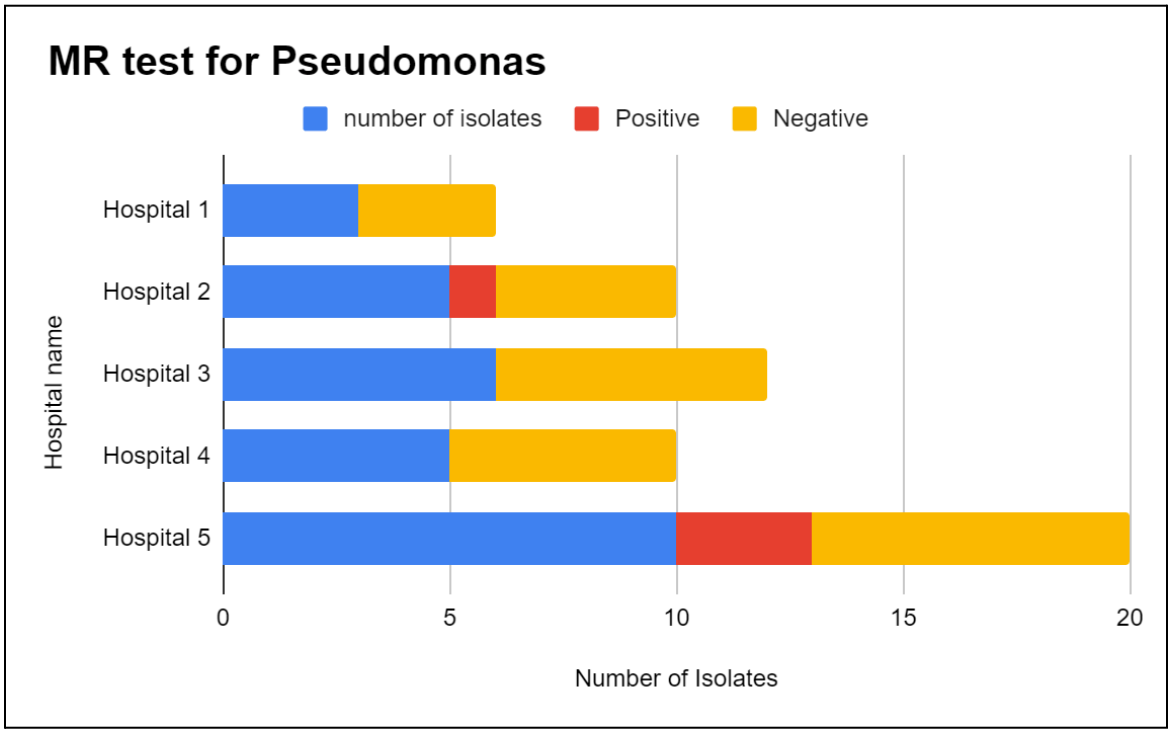


Fig 4.3.2.6.2: Bar graphs showing results of MR tests for *Pseudomonas* in 5 different hospitals

4.3.2.7 Identification using VP test

For the VP test also the samples were inoculated in the broth for 48 hrs. Therefore, for the VP test, 6 drops of Baritt's A and 2 drops of Barritt's B were added. After adding the reagents, the tubes were held for an hour, to show a maximum development in color. As a result, the 29 presumptive isolates of *Pseudomonas* showed negative results. The mild copper color produced indicated that it does not contain acetyl methyl carbinol. As a result of which the color change was not found. This result aligned with the characteristics of the *Pseudomonas* species.

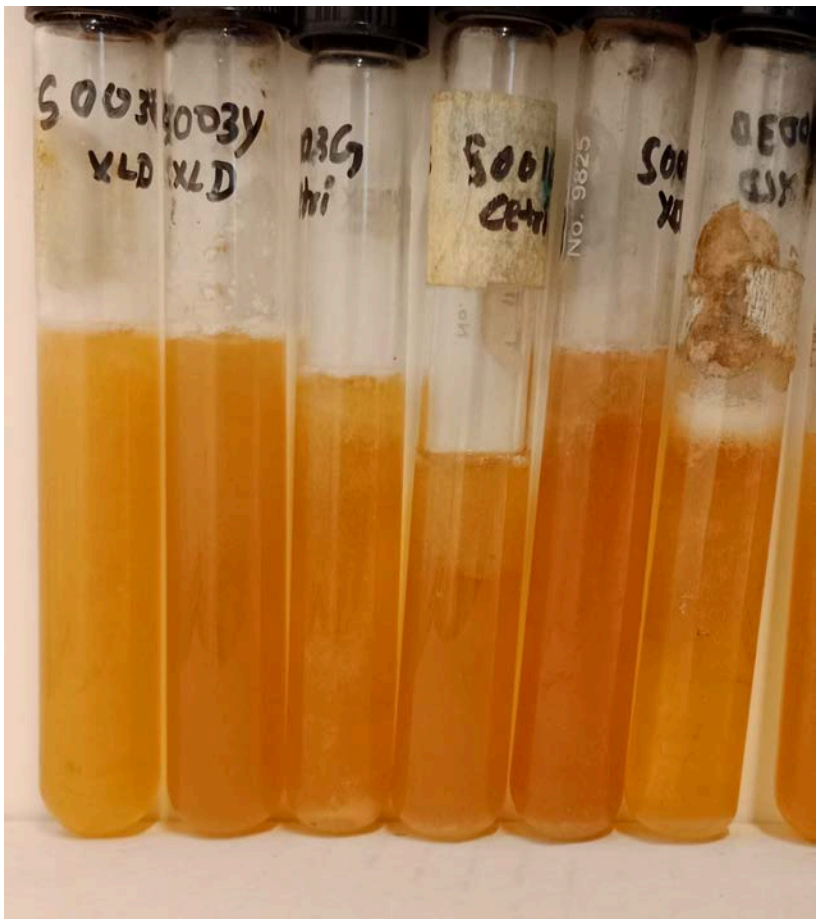


Fig 4.3.2.7.1: Showing the color of the isolates of *Pseudomonas* in VP tests

Furthermore, the bar graph shows the VP test results of *Pseudomonas,* in the five hospitals the samples were collected from. The chart shows that the negative isolates number varied among the hospitals. Hospital five with 10 numbers of negative isolates had the highest number,

followed by Hospital three with 6 negative isolates, hospital two and hospital four with 5 isolates, and hospital one with 3 isolates respectively.

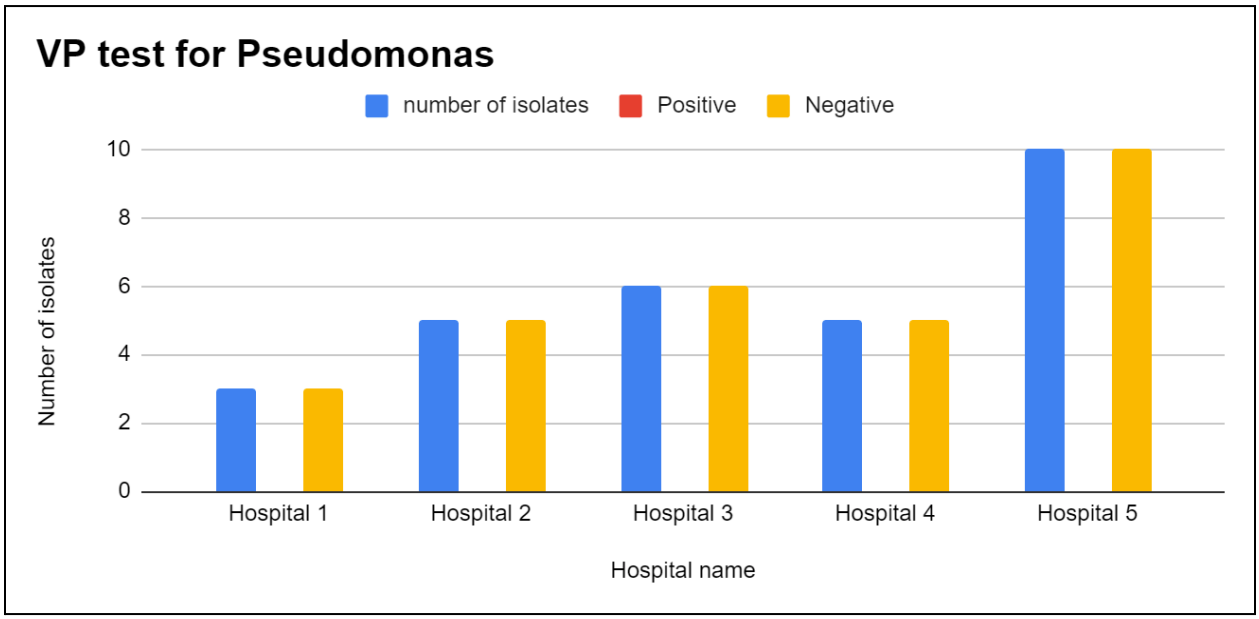


Fig 4.3.2.7.2: Bar graphs showing results of VP test for *Pseudomonas* in 5 different hospitals

4.3.2.8 Identification using Motility Test for Pseudomonas isolates

To differentiate between non-motile and motile bacteria, a mobility test is performed. Motile organisms have some special features like fibrils or propellers like flagella to move themselves towards regions with more concentration of oxygen or nutrients that favor their survival. From the 29 presumptive *Pseudomonas* isolates, all were found to be positive for motility tests.

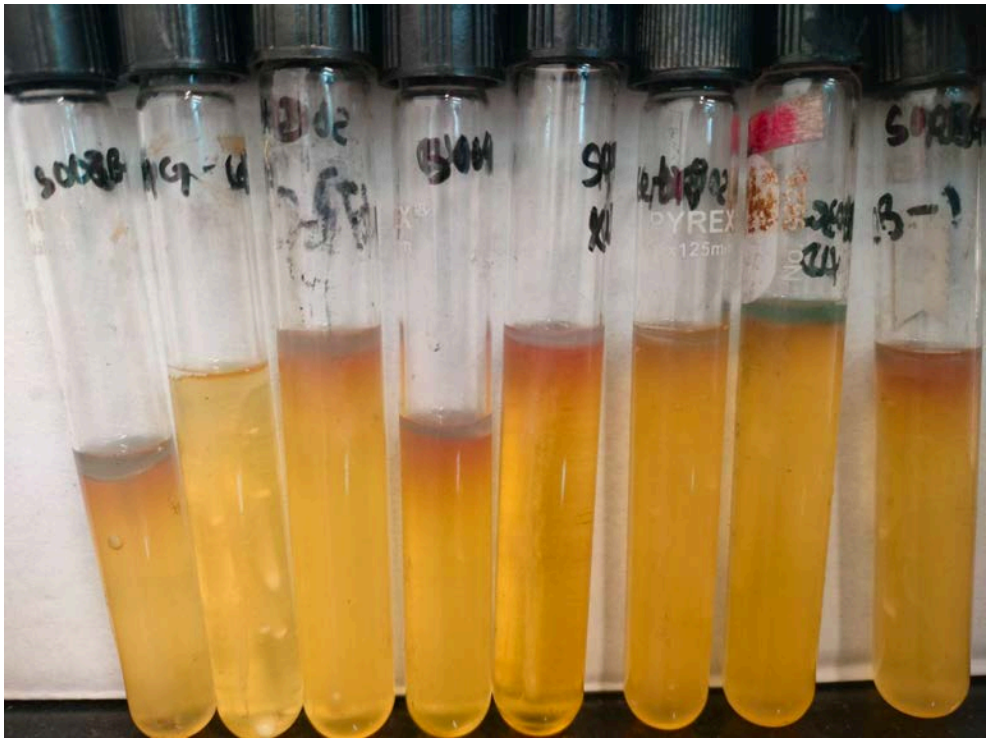


Fig 4.3.2.8.1: Motility tests for Pseudomonas isolates from 5 different hospitals

Furthermore, the bar graph shows the motility test results of *Pseudomonas,* in the five hospitals the samples were collected from. The chart shows that positive isolates number varied among the hospitals. Hospital Five with 10 numbers of positive isolates had the highest number, followed by Hospital Three with 6 positive isolates, Hospital Two and Hospital Four with 5 isolates, and Hospital One with 3 isolates respectively.

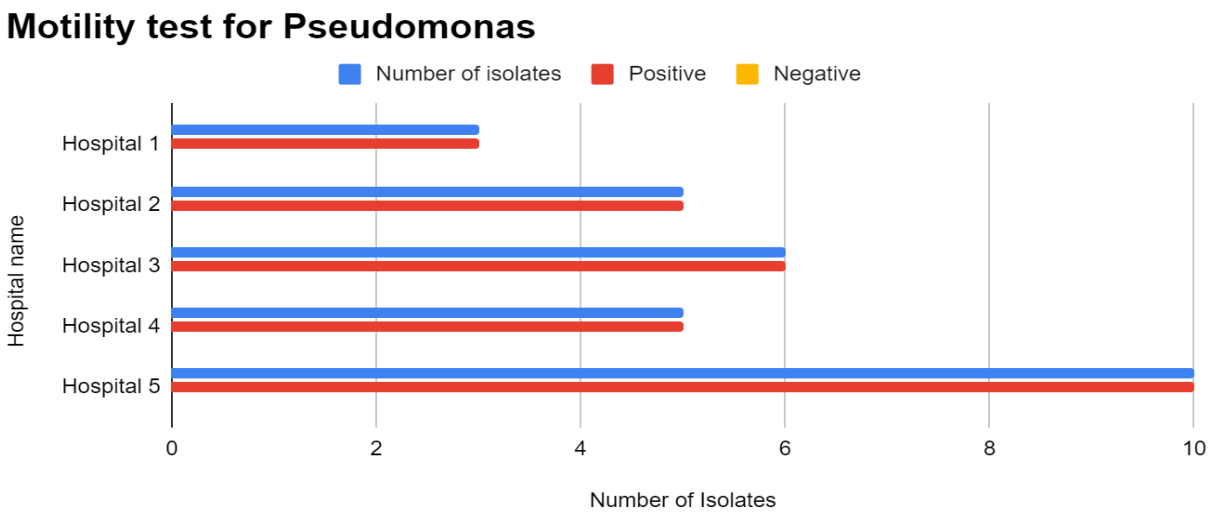


Fig 4.3.2.8.2: Bar graphs showing results of Motility test for *Pseudomonas* in 5 different hospitals

4.3.3 Antibiogram test result for *Pseudomonas*

The 29 isolates of *Pseudomonas* underwent the antibiogram test, with 11 antibiotics, under the disk diffusion protocol. The highest resistance was shown by meropenem (MEM10) with 31%, as well as Gentamicin (CN10) 31%, followed by ceftazidime (CAZ30) and levofloxacin (LEV5) with 27.6%, Aztreonam (ATM30), Amikacin (AK30) and Piperacillin-tazobactam (TZP110) having 17.2% then Ciprofloxacin (CIP5) with the lowest resistance rate of 13.8%.

Amikacin (AK30) showed the highest susceptibility value with 79.3% followed by Ciprofloxacin (CIP5) showing 69.5 % as the second highest susceptibility value. Then, Meropenem (MEM10) had a susceptibility of 65.5% followed by Aztreonam (ATM30) with 62.1%, Levofloxacin (LE5) with 58.6%, then Gentamicin (CN10) and Piperacillin (TZP110) with 55.2% and Ceftazidime with lowest susceptibility value 48.3%.

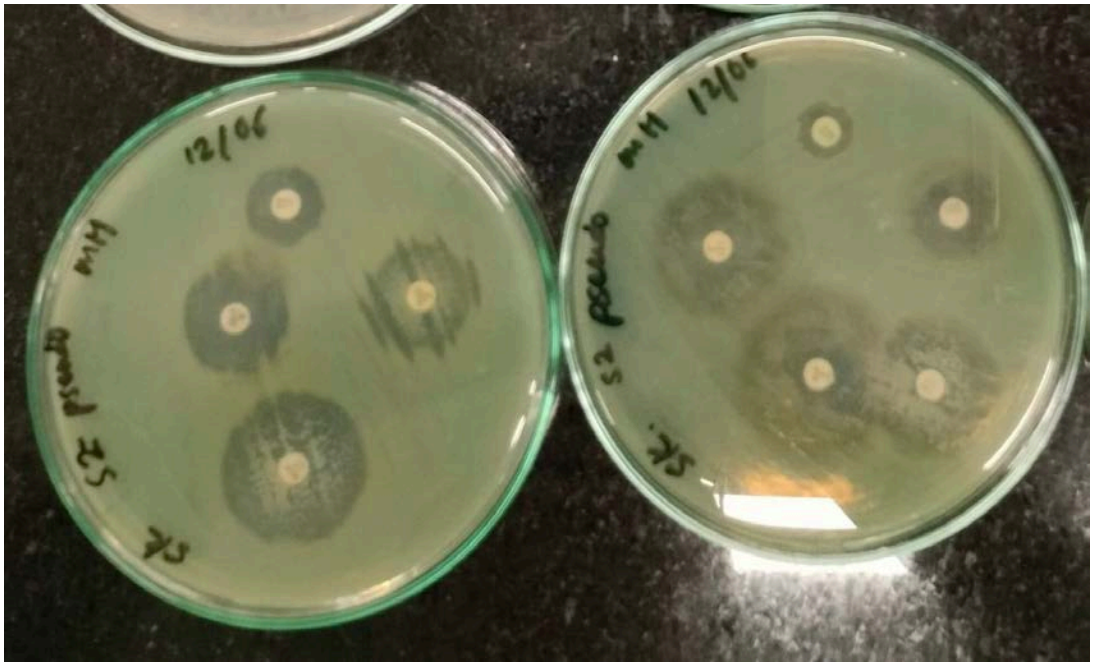


Fig 4.3.3.1: Results for Antibiotics test for *Pseudomonas*

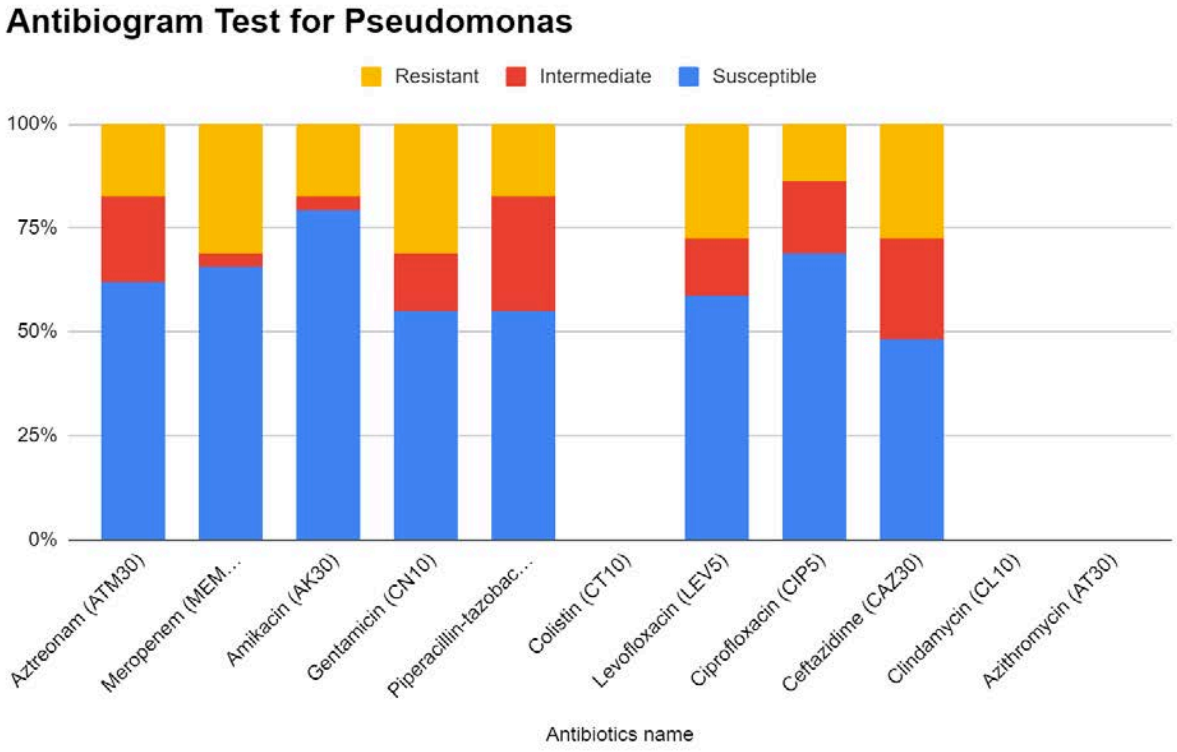


Fig 4.3.3.2: Bar graphs showing results of Antibiogram tests for *Pseudomonas* with comparisons between 11 antibiotics

4.4 Isolation of Salmonella

4.4.1 PCR and Gel electrophoresis:

From the total number of 87 isolates, 13 of the isolates were found to be *Salmonella*. To confirm this, biochemical presumptive tests were done, along with Polymerase Chain reaction (PCR). For the PCR, the target pair was the INVA gene found in *Salmonella*, which gives a product size of 284bp. After the completion of the PCR, the product went under the gel electrophoresis process. After the completion of the gel run from the initial 13 presumed isolates, only 11 of the isolates showed clear bands. This indicated the presence of *Salmonella* in the collected waste samples. The results indicated a 14.9 % prevalence of *Salmonella* in the biomedical wastes collected from the dustbins of the hospitals.

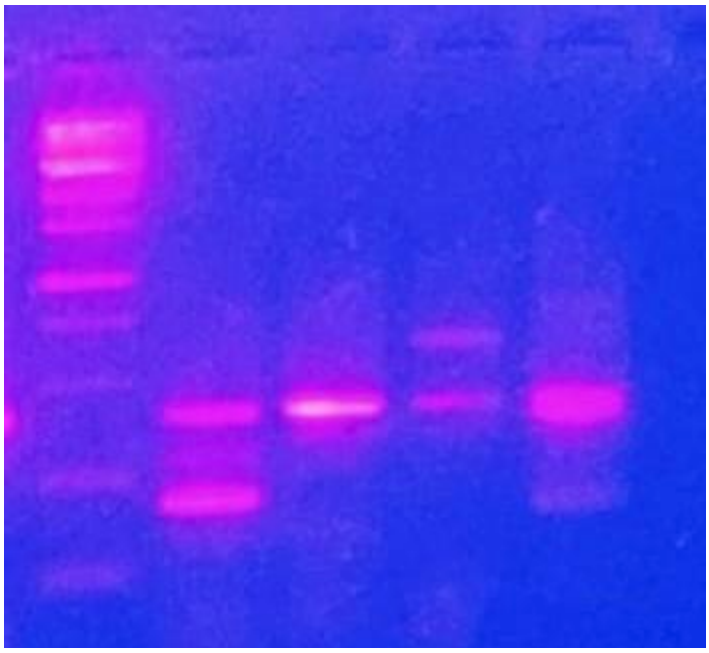


Fig 4.4.1.1- *Salmonella* PCR product bands under UV transilluminator.

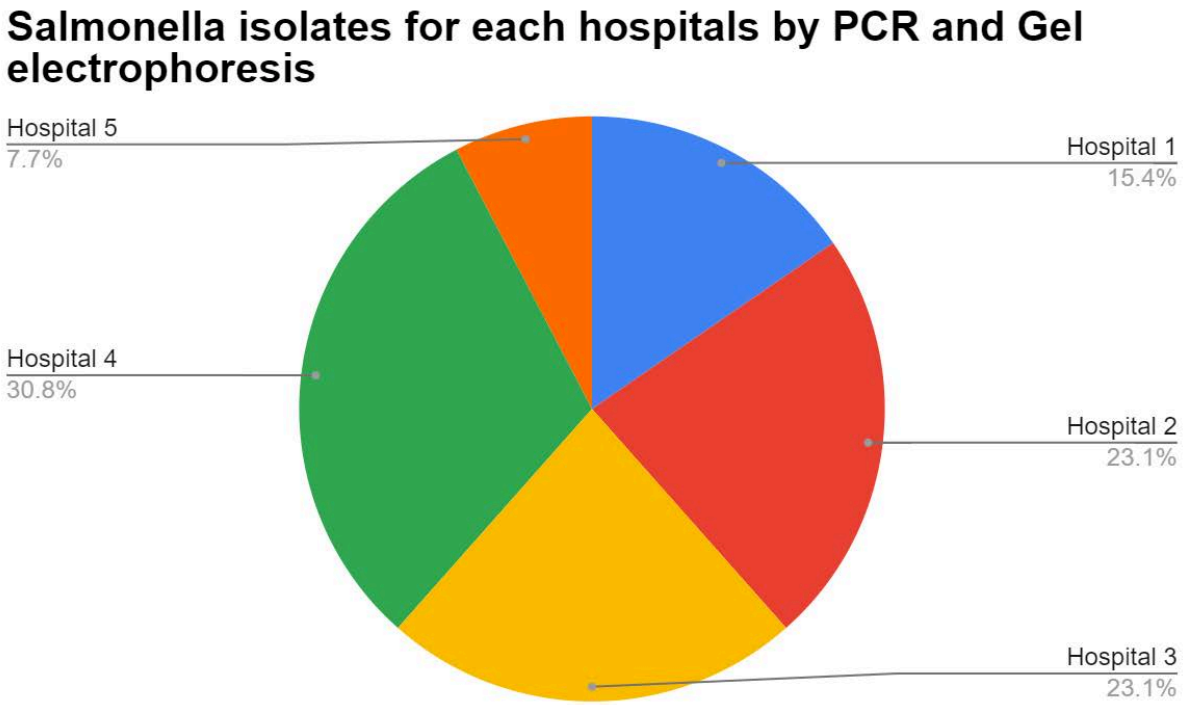


Fig 4.4.1.2: Pie chart showing the distribution of *Salmonella* across the different hospitals.

4.4.2 Identification of Salmonella by Using Biochemical Tests

Here, the basic characteristics of different biochemical tests for Salmonella are mentioned in **Table: 4.4.2.1**

S.N.	Different test	Results for Salmonella
1.	Gram Staining	Negative (pink)
2.	MR Test	Positive (red color)
3.	VP Test	Negative (no red color)
4.	Catalase Test	Positive (bubbles present)
5.	Oxidase Test	Negative (no color change)
6.	Citrate test	Positive (growth on medium)
7.	Motility Test	Positive (diffusion from stab line)

Table: 4.4.2.1

4.4.2.1 Gram staining:

The presumptive 13 isolates of *Salmonella* underwent the process of Gram staining. The results of the Gram staining characteristics were found to be consistent with the gram-negative *Salmonella*. To illustrate, all of the 13 presumed isolates indicated lipopolysaccharide cell walls by giving pink coloration and retaining the safranin dye, as visualized under the microscope. Thus, these results set a path in the identification and characterizing of the *Salmonella* species. The result of one of the gram-negative samples of *Salmonella* under an electron microscope is shown in

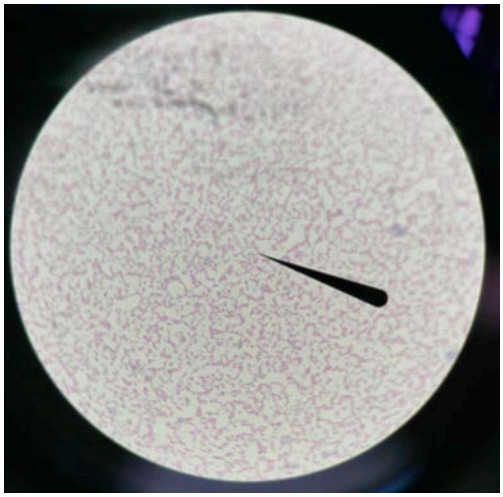


Fig 4.4.2.1.1: Gram staining result for *Salmonella.*

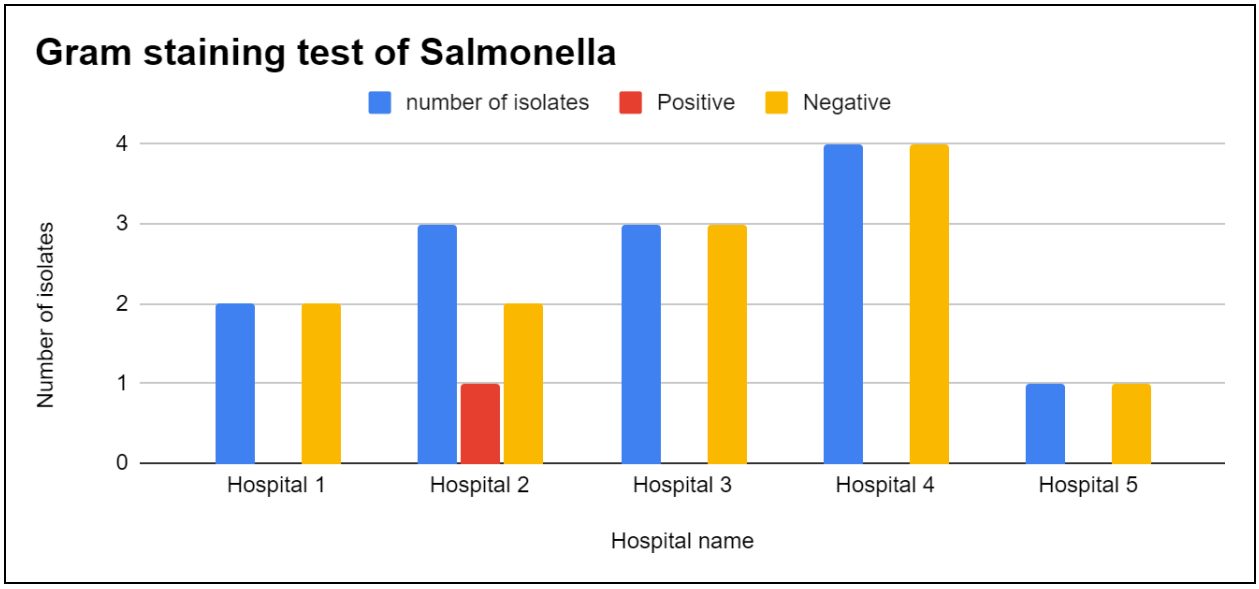


Fig 4.4.2.1.2: Hospital-wise comparison of *Salmonella* isolates based on gram staining.

4.4.2.2 Identification by Triple Sugar Iron (TSI)

After 24 hours of incubation of the 13 isolates, the results were interpreted. The result showed that only the butt turned yellow indicating that acid produced is in small quantities, and only glucose is fermented. The yellow color indicates that fermentation did occur as acids have been produced hence phenol red turned yellow. There was no fermentation of sucrose or lactose. Furthermore, there was the production of gasses H2 & CO2 in samples, with the presence of the bubble formation and cracking of the media. In addition to this black color precipitate also indicated the presence of FeS (Ferrous Sulfide), a black compound formation, and H2S gas formation. In addition to this, the number of positive isolates of *Salmonella* varied from hospital to hospital. To exemplify, hospital 4 showed the maximum number of positive isolates of *Salmonella* i.e. 4, followed by hospital 2 and hospital 3 with 3 positive isolates, hospital 1 had 2 isolates while hospital 5 only had one positive isolate of *Salmonella*.

Fig 4.4.2.2.1: Results of TSI test for *Salmonella*

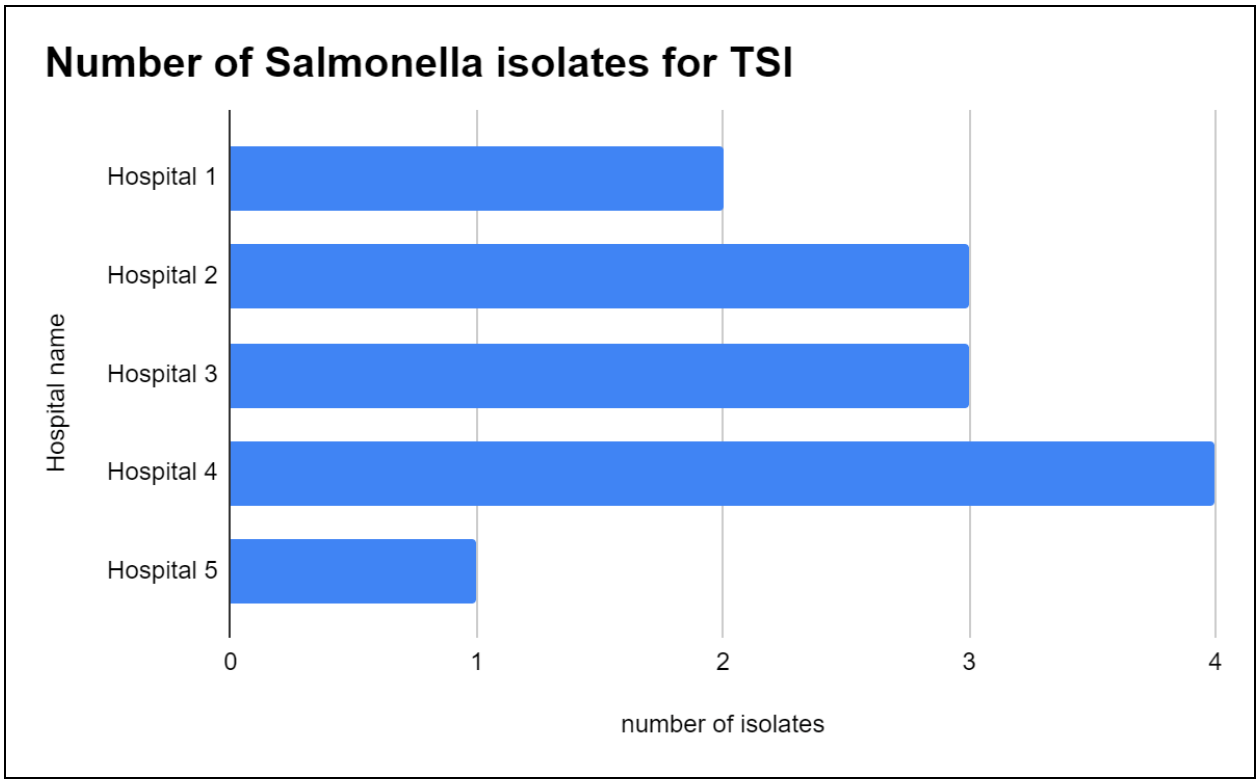


Fig 4.4.2.2.2: Bar graphs showing the distribution of *Salmonella* across the different hospitals for TSI test.

4.4.2.3 Identification using Citrate Utilization Test

For the citrate Utilization test, out of 13 presumptive isolates of *Salmonella,* four of the isolates showed a positive result. As described earlier in this paper, the breakdown of citrate to oxaloacetate and acetate and its further breakdown to pyruvate and CO_2 was found in the samples. Furthermore, in case of the positive results, these metabolic products further break down due to the presence of alkaline pH, and finally, the indicator Bromothymol blue gives a Prussian Blue color. On the other hand, 9 of the isolates showed no change in color. This indicated the absence of the metabolic breakdown; the color of the media remained unchanged (green) as shown in the figure below.

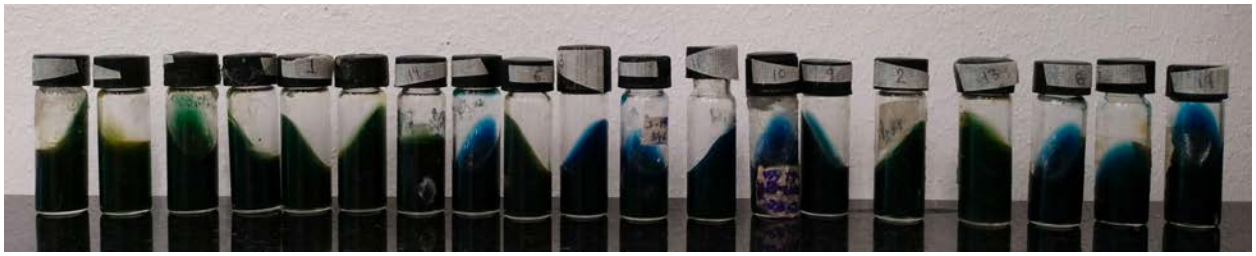


Fig 4.4.2.3.1: Results of Citrate utilization test for *Salmonella*

That means 69.23% were found to be negative, which aligns with the standard characteristics of *Salmonella*. Furthermore, hospital-wise comparison showed that the hospital 4 with a value of 4 isolates had the most negative *Salmonella*. Followed by hospitals one and two with 2 isolates in each of them and finally hospital three with 1 of *Salmonella.* Surprisingly no negative tested isolates of *Salmonella* were seen in hospital 5.

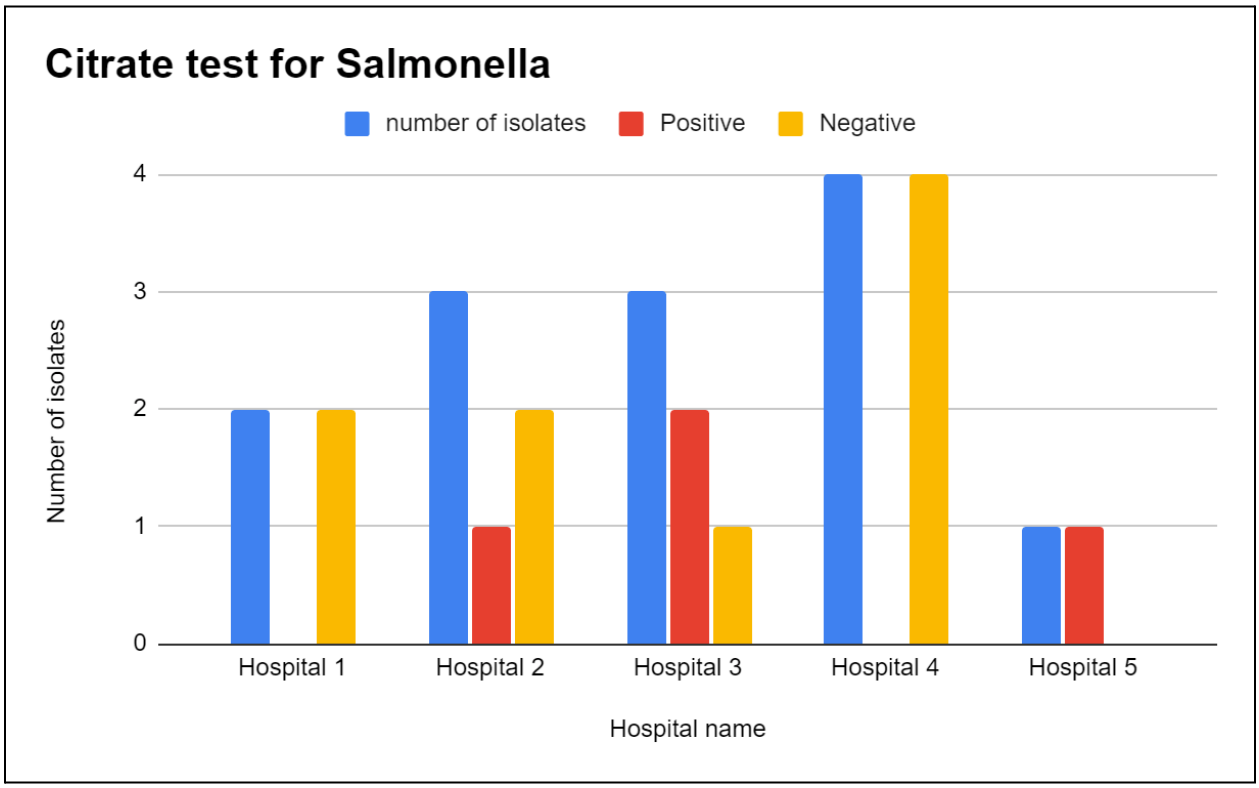


Fig 4.4.2.3.2: The bar graph shows the distribution of *Salmonella* across different hospitals for the Citrate Utilization test.

4.4.2.4 Identification using Catalase Test

Hydrogen peroxide, a toxic product is produced as a result of aerobic metabolism by bacteria. To stop the damage that could be caused by this toxicity, cells use an enzyme called catalase to break down the H_2O_2. The species of bacteria that have this catalase are catalase positive, while those that lack the enzyme are negative to the test. To exemplify, the positive result is indicated by bubble production (Oxygen gas), which is simply an outcome of the breakdown of the H_2O_2 by the enzyme catalase, into water and O_2 gas. Our species *Salmonella* has the enzyme catalase. As a result, all 11 isolates showed a positive test with the bubble formation. **Fig.4.4.2.4.1** shows the formation of bubbles on the addition of Hydrogen peroxide to the presumptive isolates.

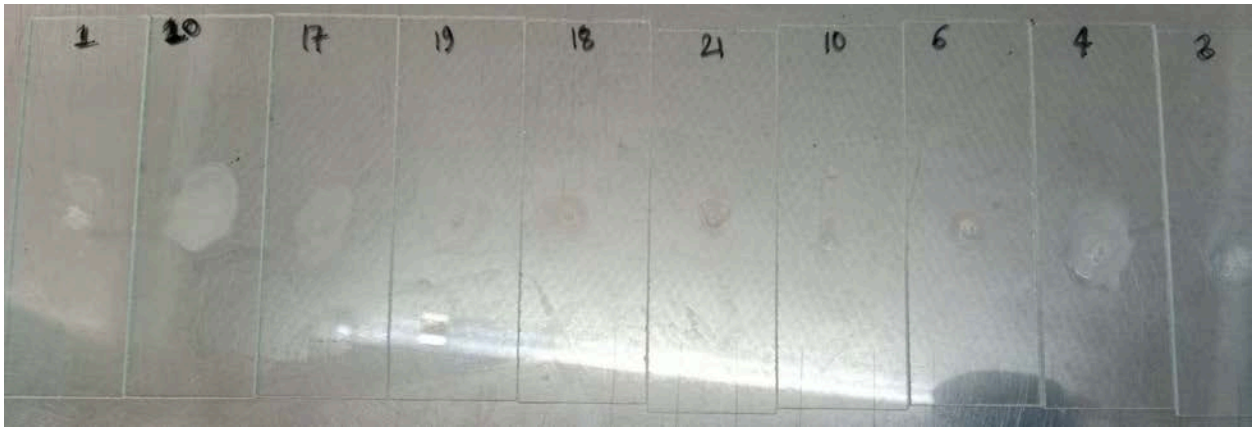


Fig.4.4.2.4.1 Results of Catalase test for *Salmonella*

Furthermore, the bar graph shows the distribution of *Salmonella* in the five hospitals where the samples were collected from. The chart shows that positive isolates number varied among the hospitals. Hospital Four with 4 number with positive isolates had the highest number, followed by hospitals two and three with 3 positive isolates, hospital one had two positive isolates and finally, hospital five had 1 catalase-positive isolate.

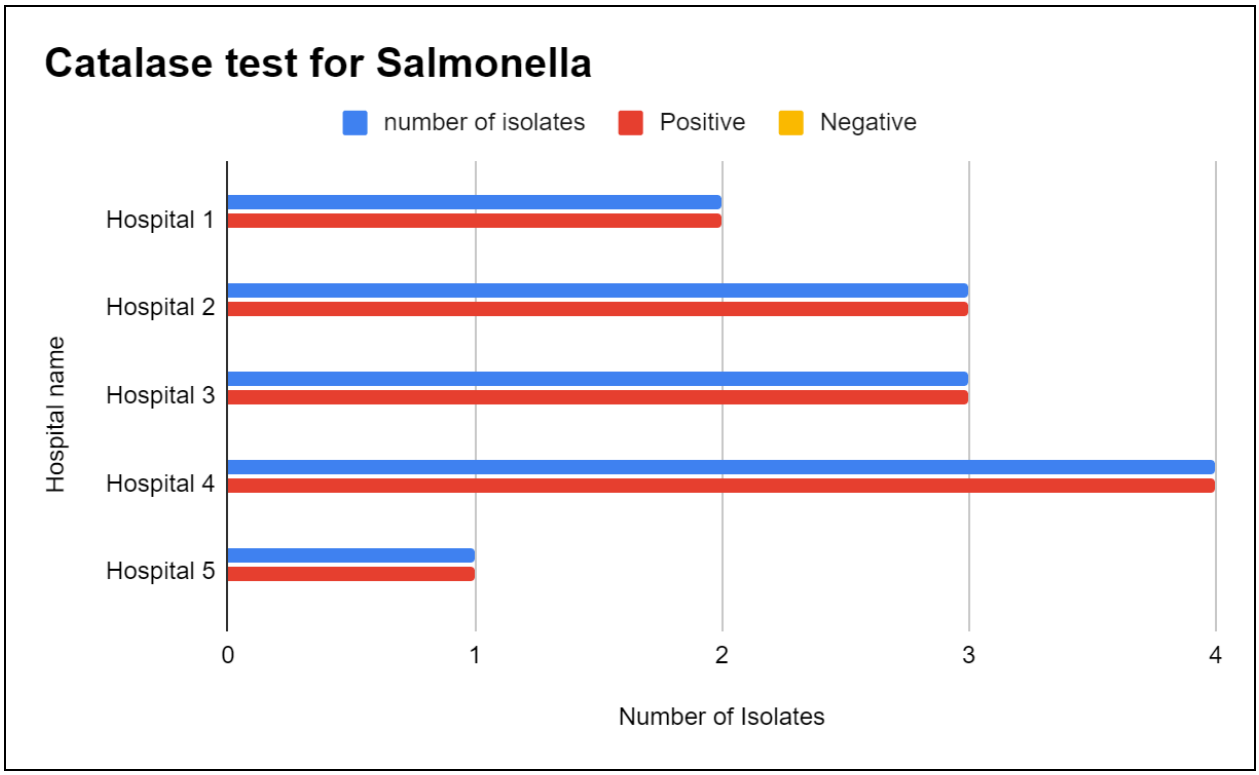


Fig 4.4.2.4.2: Bar graphs showing the distribution of *Salmonella* across the different hospitals in the catalase test.

4.4.2.5 Identification using Oxidase Test

The enzyme cytochrome oxidase reduces oxygen at the end of the electron transport chain. In our experiment, a colorless redox reagent i.e. 1 % of tetramethyl-p-phenylenediamine was used to detect the presence of oxidase enzyme. The positive result was obtained because of the reagent used which acts as an electron acceptor and leads to the oxidation of our reagent into end product indophenols. This indophenol was responsible for the end purple color. For our 11 presumptive *Salmonella* isolates, only 2 isolates were found to be positive for the oxidase test with a change in color to dark purple. While the remaining 11 isolates showed a negative result with no color change. It concluded that these isolates do not have cytochrome oxidase enzymes that align with the standard characteristics of *Salmonella.*

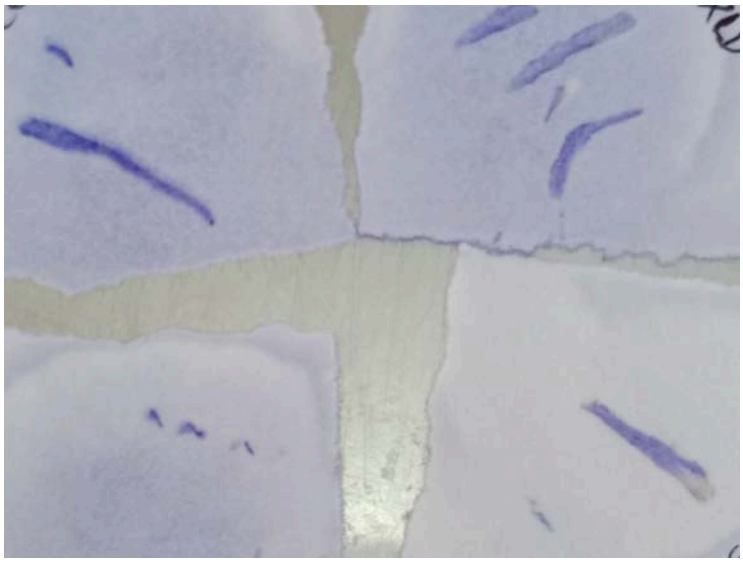


Fig 4.4.2.5.1: Oxidase test for *Salmonella*

Furthermore, the bar graph shows the distribution of *Salmonella in* the five hospitals where the samples were collected from. The chart shows that the negative isolates number varied among the hospitals. Hospital four with 4 numbers of negative isolates had the highest number, followed by hospital two with 2 isolates, hospitals one and three had 2 negative isolates each, and finally, hospital 5 had 0 negative isolates.

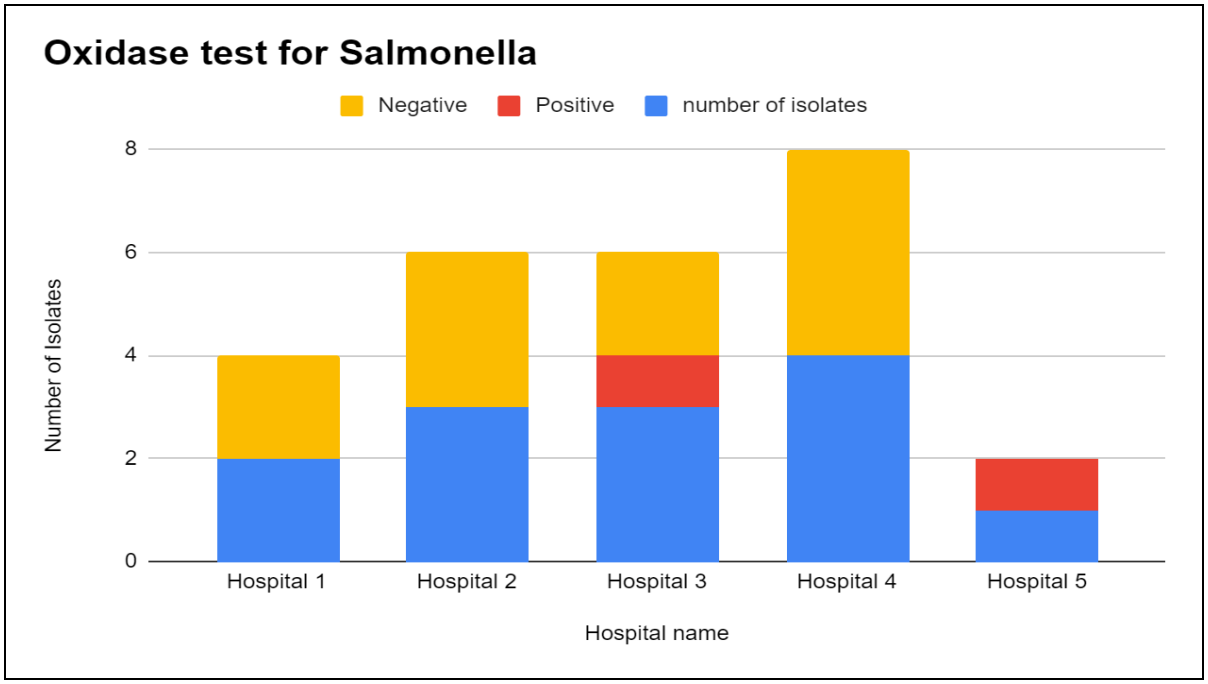


Fig 4.4.2.5.2: **Bar graphs showing the results of the Oxidase test for Salmonella in 5 different Hospitals**

4.4.2.6 Identification using MR test:

For the MR test, the 13 isolates were inoculated in liquid broth for 48 hrs. On the addition of the reagent methyl red, a change in color to red was observed in 10 isolates. The resulting red color from the positive MR test indicated that the end product was acidic and had a pH of 4.4 or much less, showing that mixed acids were produced. Furthermore, 3 isolates on the addition of methyl red drops, indicated a yellow color showing the end product is not much acidic and the PH is 6.2. This yellow color indicates a negative Test MR Test.

Fig 4.4.2.6.1: Results of *Salmonella* in MR test

Furthermore, the bar graph shows the MR test results of *Salmonella,* in the five hospitals the samples were collected from. The chart shows that positive isolates number varied among the hospitals. Hospitals 4 and Three with 3 numbers of positive isolates had the highest number, followed by Hospital 2 and Hospital 5 with 1 positive isolate, and Hospital 1 with 2 positive isolates. The 3 negative isolates were found to be present in Hospital 2 and Hospital 4 respectively.

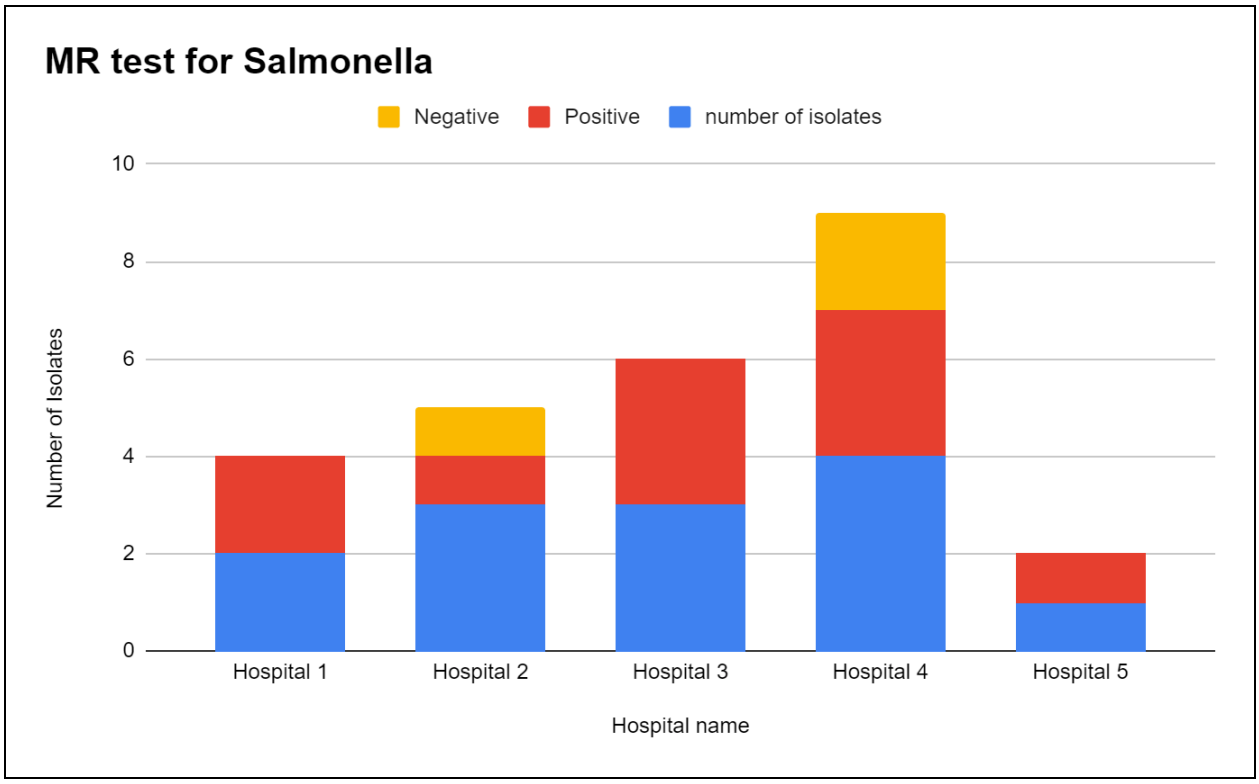


Fig 4.4.2.6.2:Bar graphs representing the results of MR tests for *Salmonella* in 5 different hospitals

4.4.2.7 Identification using VP test

For the VP test also the samples were inoculated in the broth for 48 hrs. Therefore, for the VP test, 6 drops of Baritt's A and 2 drops of Barritt's B were added. After adding the reagents, the tubes were held for an hour, to show a maximum development in color. As a result, the positive test was considered to be pinkish-red, indicating the presence of acetyl methyl carbinol which is responsible for the fermentation of glucose. Of the 13 isolates of *Salmonella,* all showed positive results. Showing pinkish-red color of the isolates of *Salmonella.* This result aligned with the characteristics of the *Salmonella* species.

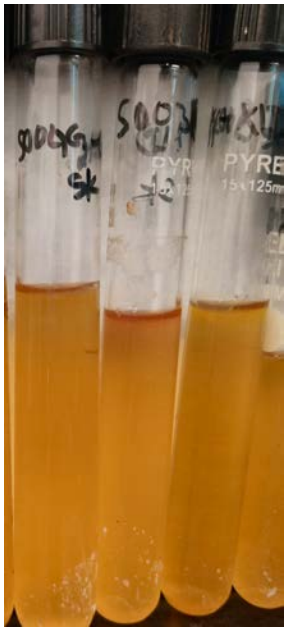


Fig 4.4.2.7.1: VP test for *Salmonella*

Furthermore, the bar graph shows the VP test results of *Salmonella,* in the five hospitals the samples were collected from. The chart shows that positive isolates number varied among the hospitals. Hospital four with 4 numbers of negative isolates had the highest number, followed by hospital two and three with 3 negative isolates, hospital one had two negative isolates and finally hospital five had 1 VP negative isolate.

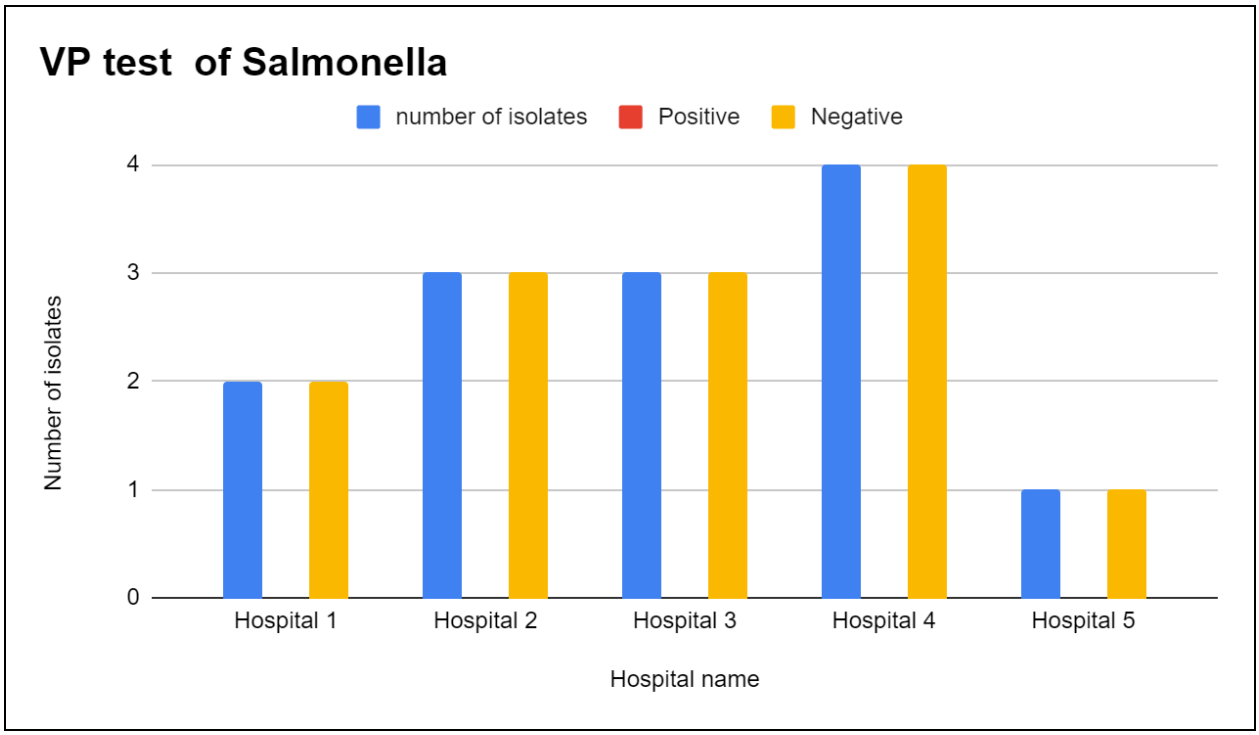


Fig 4.4.2.7.2: Bar graphs representing VP tests for *Salmonella* in 5 different hospitals

4.4.2.8 Identification using Motility Test

To differentiate between non-motile and motile bacteria, a mobility test is performed. Motile organisms have some special features like fibrils or propellers like flagella to move themselves towards regions with more concentration of oxygen or nutrients that favor their survival. From the 13 presumptive *Salmonella* isolates, all were found to be positive for the test.

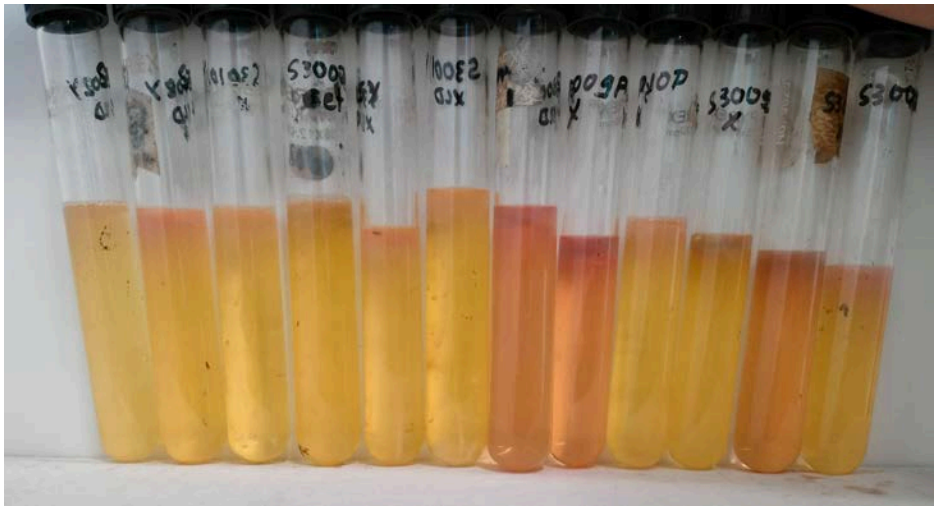


Fig 4.4.2.8.1: Results of Motility test for *Salmonella*

Furthermore, the bar graph shows the motility test results of *Salmonella,* in the five hospitals the samples were collected from. The chart shows that positive isolates number varied among the hospitals. Hospital Four with 4 numbers of positive isolates had the highest number, followed by Hospitals 2 and Three with 3 positive isolates, hospital one had two positive isolates and finally Hospital Five had 1 catalase-positive isolate.

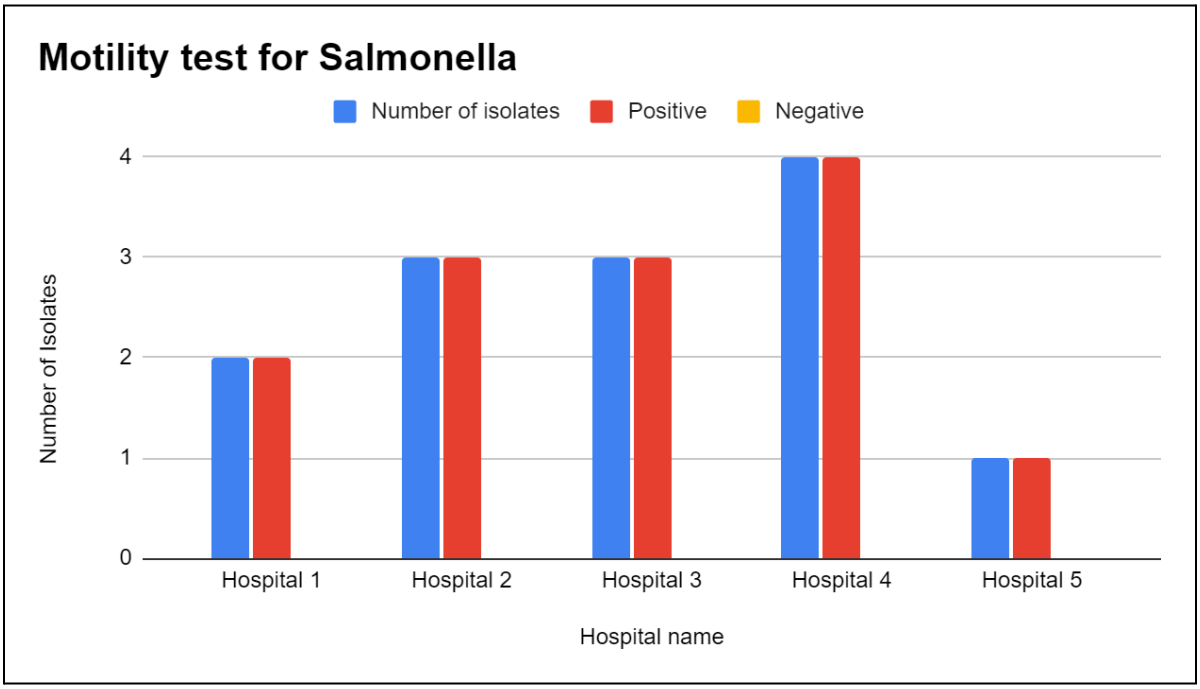


Fig 4.4.2.8.2: Bar graphs representing motility tests for *Salmonella* in 5 different hospitals

4.4.3 Antibiogram test for *Salmonella*

The 13 isolates of *Salmonella* underwent the antibiogram test, with 12 antibiotics, under the disk diffusion protocol.

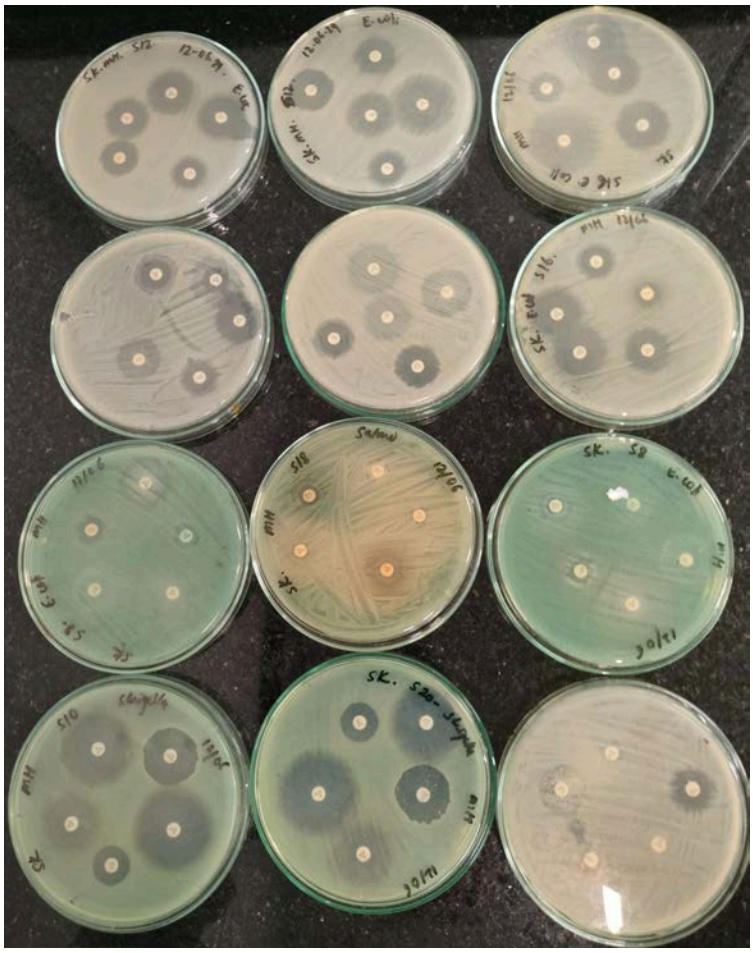


Fig 4.4.3.1:Antibiotic test results for *Salmonella.*

The results showed that ampicillin-25 and erythromycin (E15) had the greatest resistance rates (100%), followed by tetracycline with 76.9%, chloramphenicol and aztreonam with 53.8%, Azithromycin-15 with 61.5%, Norfloxacin 10 with 38.5%, Ciprofloxacin-5 and imipenem with 30.8%, lowest resistant rates gentamicin-10 with 15.4%, piperacillin (TZP110) with 23.1% respectively.

On the other hand, azithromycin with 92.3% showed the highest susceptibility followed by piperacillin (TZP110) showed 69.2% susceptibility, followed by gentamicin with 61.5%,

aztreonam, azithromycin, and ciprofloxacin with 38.5% susceptibility, erythromycin (E15) with 46.2%, azithromycin (AZM 15) with 38.5%, chloramphenicol with 30.8%, imipenem with 15.4% and the lowest susceptibility was given by tetracycline (TE30) with 7.7%.

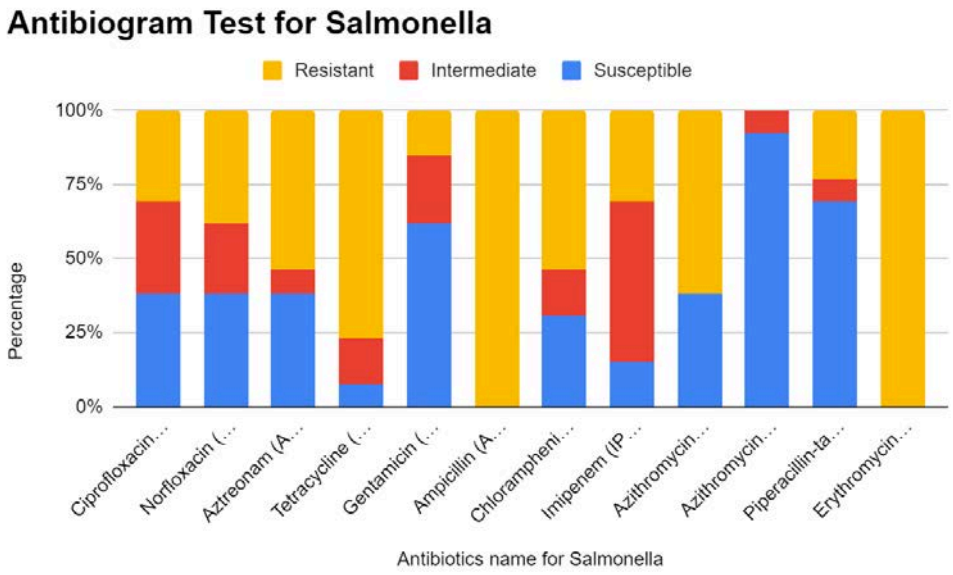


Fig 4.4.3.1: Bar graphs showing results of Antibiogram tests for *Salmonella* with comparisons between 12 antibiotics.

4.5 Isolations of Shigella

4.5.1 PCR and Gel electrophoresis

From the total number of 87 isolates, 17 of the isolates were found to be *Shigella*. To confirm this, biochemical presumptive tests were done, along with Polymerase Chain reaction (PCR). For the PCR, the target pair was the IPAH gene, which is an invasion plasmid antigen H gene sequence (*ipaH)* and is found in all 4 species of *Shigella* (Makabanyane et al., 2015). The product size of this gene is 108bp. From the 17 presumed isolates of *Shigella*, 15 showed positive results by displaying clear bands. The results indicated a 19.5 % prevalence of *Shigella* in the biomedical wastes collected from the dustbins of the hospitals. Fig-

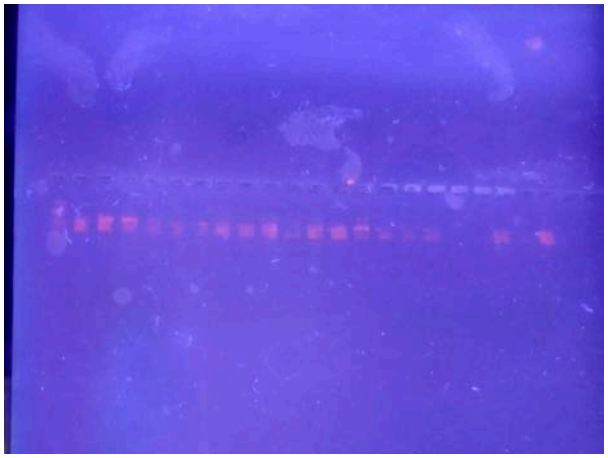


Fig 4.5.1.1: PCR products band under UV transilluminator.

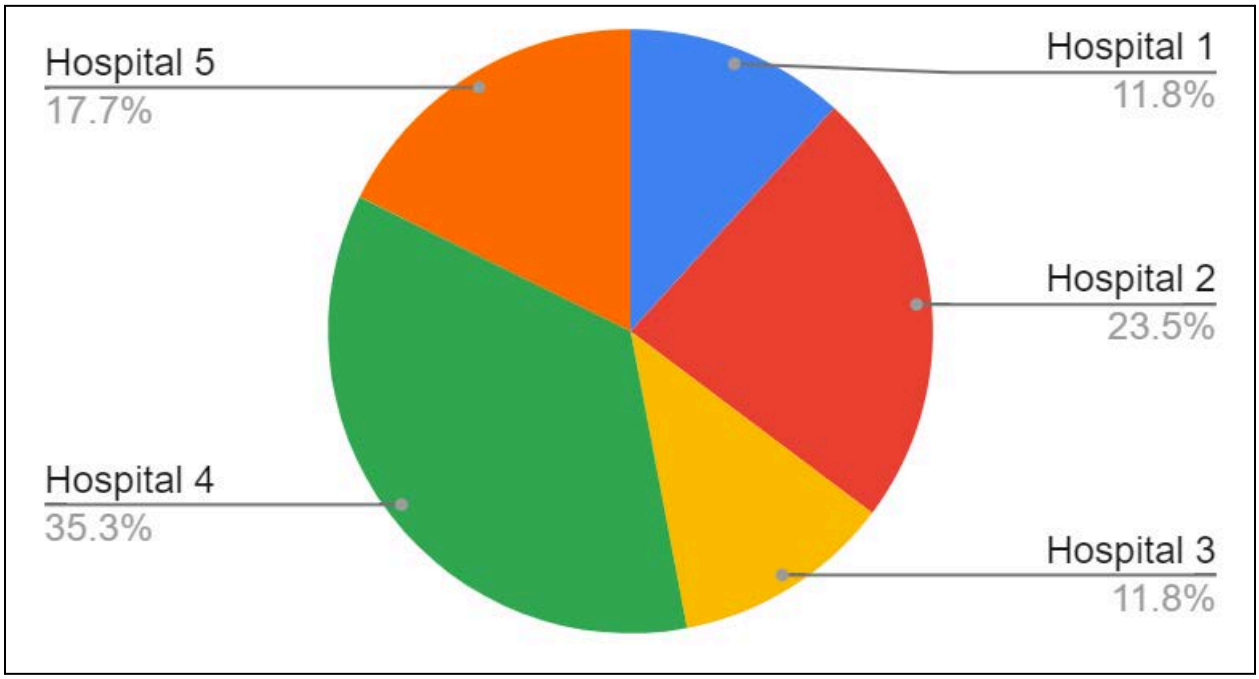


Fig 4.5.1.2: Pie chart showing the distribution of presumptive *Shigella* isolates in different hospitals.

4.5.2 Identification of Shigella by Using Biochemical Tests

The fundamental characteristics of Shigella for different biochemical tests are mentioned below:

S.N	Different test	Results for Shigella
1.	Gram Staining	Gram-negative (pink)
2.	MR Test	Positive (red color)
3.	VP Test	Negative (no red color)
4.	Catalase Test	Positive (bubbles present)
5.	Oxidase Test	Negative (no color change)
6.	Citrate test	Negative (no growth)
7.	Motility Test	Negative (no diffusion)

Table:4.5.1

4.5.2.1 Gram staining

The presumptive 17 isolates of *Shigella* underwent the process of Gram staining. The results of the Gram staining characteristics were found to be consistent with the gram-negative Shigella. To illustrate, all of the 17 presumed isolates indicated lipopolysaccharide cell walls by giving pink coloration and retaining the safranin dye, as visualized under the microscope. Thus, these results set a path in the identification and characterizing of the *Shigella* species. The result of one of the gram-negative samples of *Shigella* under an electronic microscope is shown in Fig **4.5.2.1.1**.

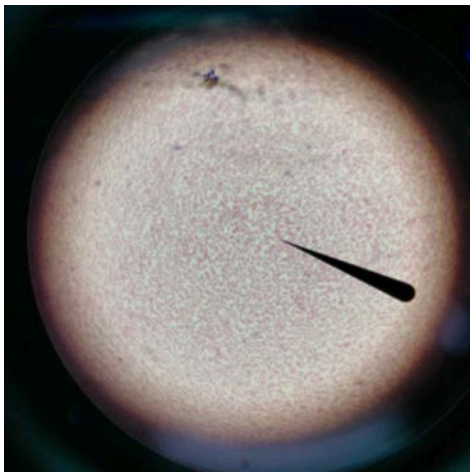


Fig 4.5.2.1.1: Gram staining test result for *Shigella*.

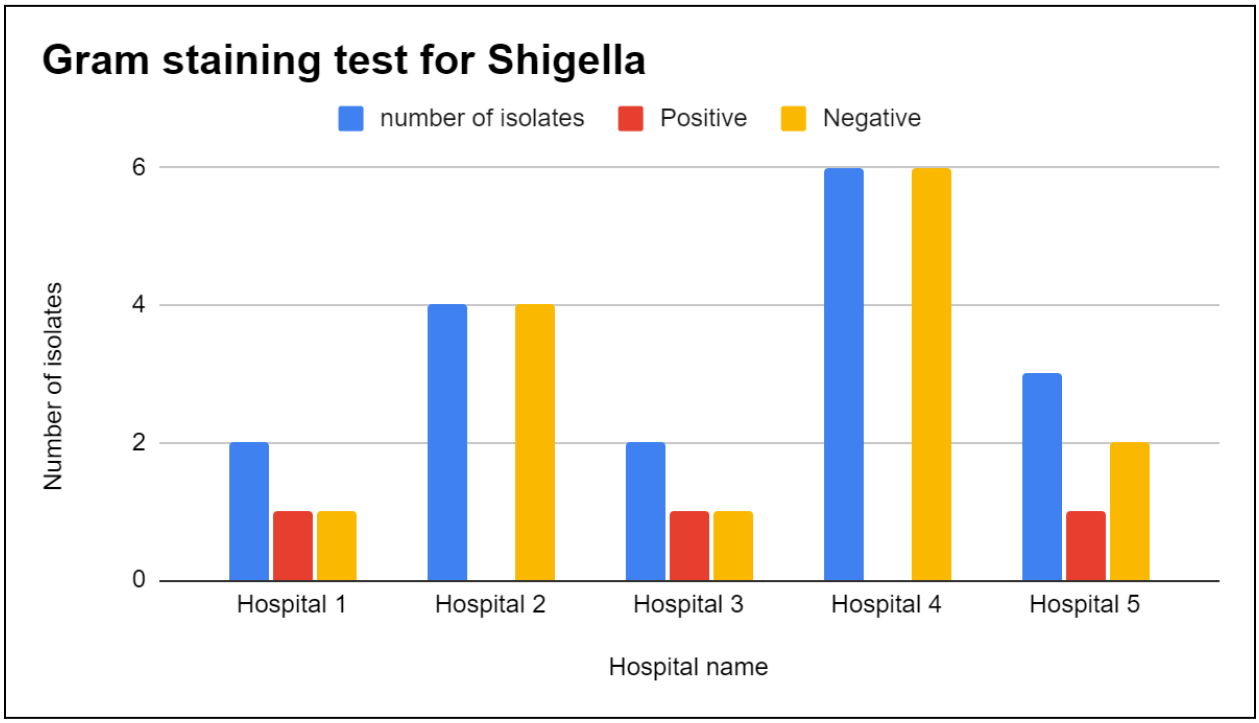


Fig 4.5.2.1.2: Hospital-wise distribution of presumptive *Shigella* based on gram staining.

4.5.2.2 Identification by Triple Sugar Iron (TSI)

After 24 hours of incubation of the 17 isolates, the results were interpreted. The result showed that only the butt turned yellow indicating that acid produced is in small quantities, and only glucose is fermented. The yellow color indicates that fermentation did occur as acids have been produced hence phenol red turned yellow. There was no fermentation of sucrose or lactose. Furthermore, no signs of the production of gasses H2 & CO2 in samples, with the absence of bubble formation and cracking of the media. No black color precipitate indicating the absence of FeS (Ferrous Sulfide), a black compound formation. In addition to this, the number of positive isolates of shigella varied from hospital to hospital. To exemplify, Hospital 4 showed the maximum amount of positive isolates of *Shigella* i.e. 6, followed by Hospital 2 with 4 positive isolates, Hospital 5 had 3 isolates while both Hospitals 1 and 3 were found to have the same number of isolates i.e 2.

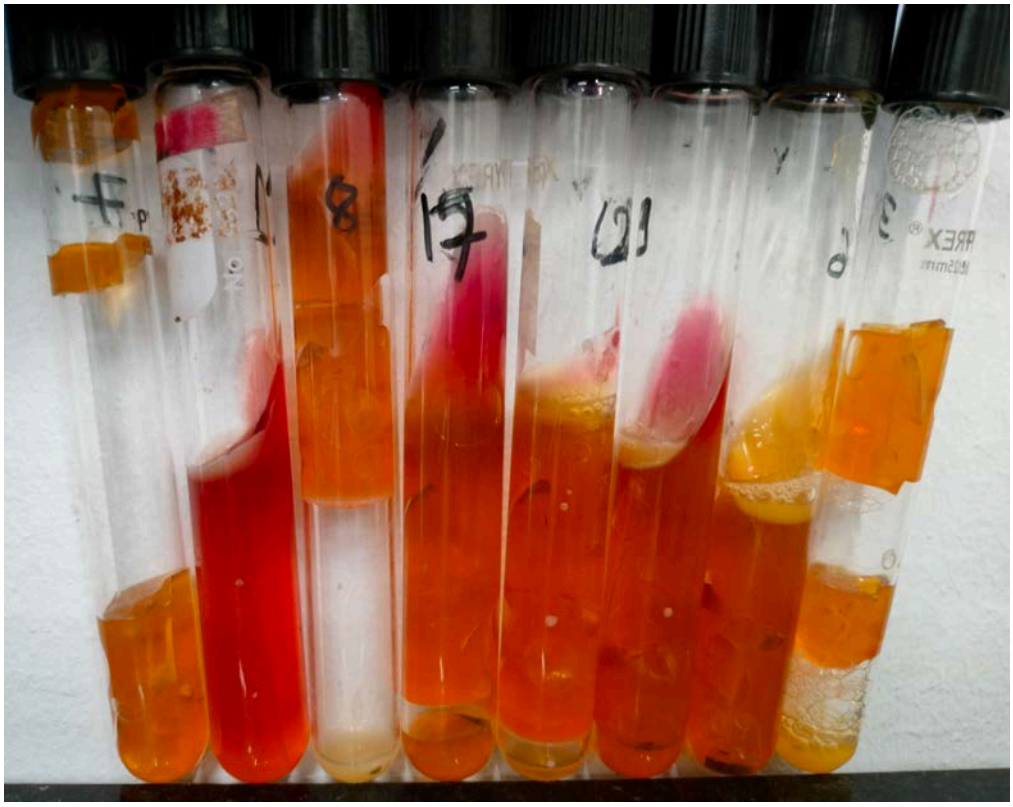


Fig 4.5.2.2.1: TSI result for *Shigella.*

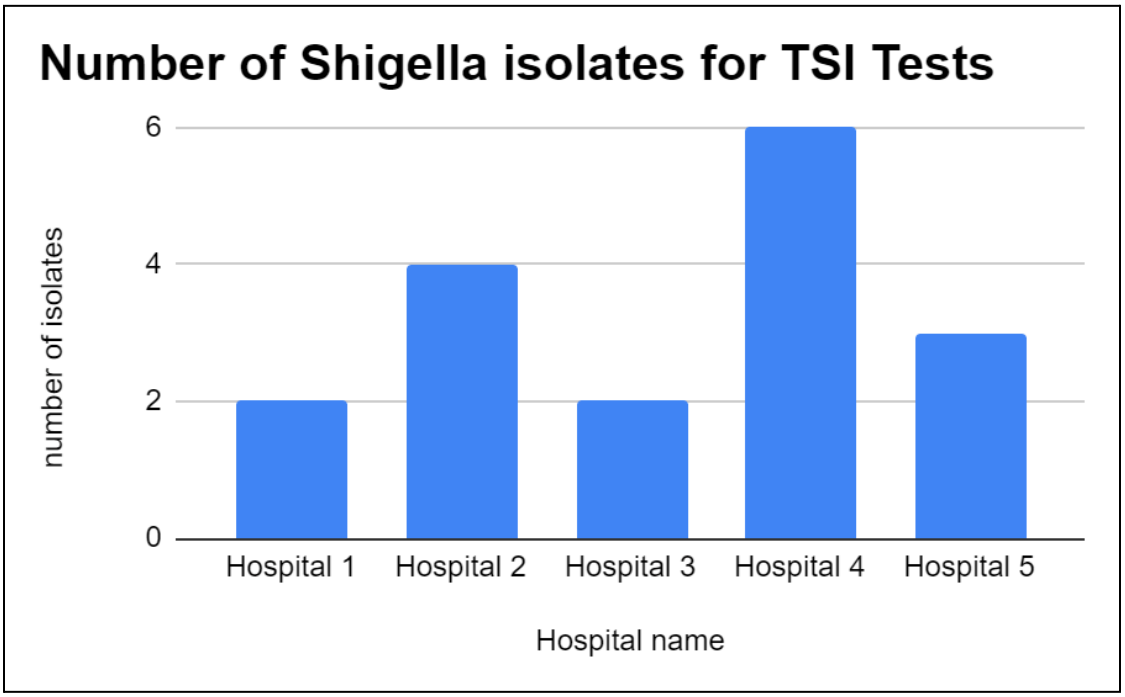


Fig 4.5.2.2.2: Hospital-wise distribution of isolates of *Shigella.*

4.5.2.3 Identification using Citrate Utilization Test

For the citrate Utilization test, out of 17 presumptive isolates of *Shigella,* five of the isolates showed a positive result. As described earlier in this paper, the breakdown of citrate to oxaloacetate and acetate and its further breakdown to pyruvate and CO2 was found in the samples. Furthermore, in case of the positive results, these metabolic products further break down due to the presence of alkaline pH, and finally, the indicator Bromothymol blue gives a Prussian Blue color. On the other hand, 12 of the isolates showed no change in color. This indicated the absence of the metabolic breakdown; the color of the media remained unchanged (green) as shown in the figure below.

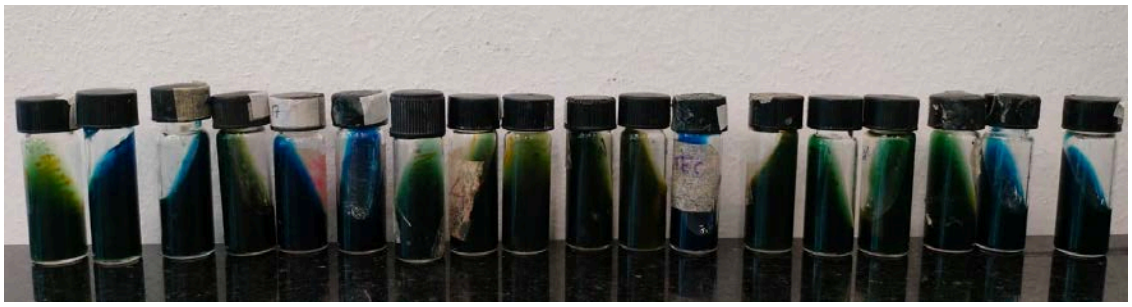


Fig 4.5.2.3.1: Citrate utilization test for *Shigella*.

That means 70.59% were found to be negative, which aligns with the standard characteristics of the *Shigella*. Furthermore, the hospital-wise comparison showed that the Hospital 4 with a value of 6 isolates had the most negative *Shigella*. Followed by Hospital 5, with 3 isolates, and the remaining Hospitals with just 1 negative isolate of *Shigella.* Fig: The bar graph shows the distribution of *Shigella* across different hospitals.

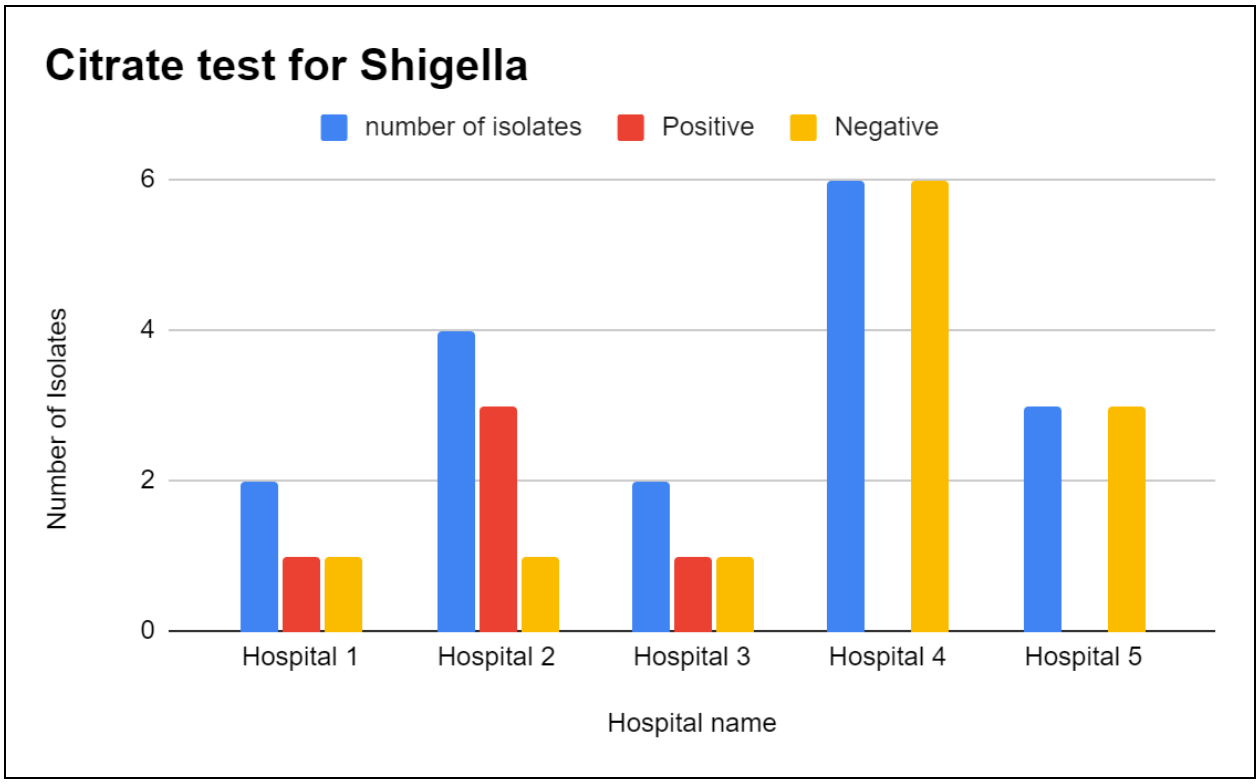


Fig 4.5.2.3.2: Bar graph showing the distribution of positive and negative isolates of *Shigella* in different hospitals.

4.5.2.4 Identification using Catalase Test

Hydrogen peroxide, a toxic product is produced as a result of aerobic metabolism by bacteria. To stop the damage that could be caused by this toxicity, cells use an enzyme called catalase to break down the H2O2. The species of bacteria that have this catalase are catalase positive, while those that lack the enzyme are negative to the test. To exemplify, the positive result is indicated by bubble production (Oxygen gas), which is simply an outcome of the breakdown of the H2O2 by the enzyme catalase, into water and O2 gas. Our species *Shigella,* has the enzyme catalase. As a result, all 17 isolates showed a positive test with the bubble formation. Fig **4.5.2.4.1** shows the formation of bubbles with the addition of Hydrogen peroxide to the presumptive isolates.

Fig 4.5.2.4.1: Isolates of *Shigella* showing positive results for catalase test.

Furthermore, the bar graph shows the distribution of *Shigella,* in the five hospitals the samples were collected from. The chart shows that positive isolates number varied among the hospitals. Hospital four with 6 numbers of positive isolates had the highest number, followed by Hospital two, Hospital five, Hospital one, and three.

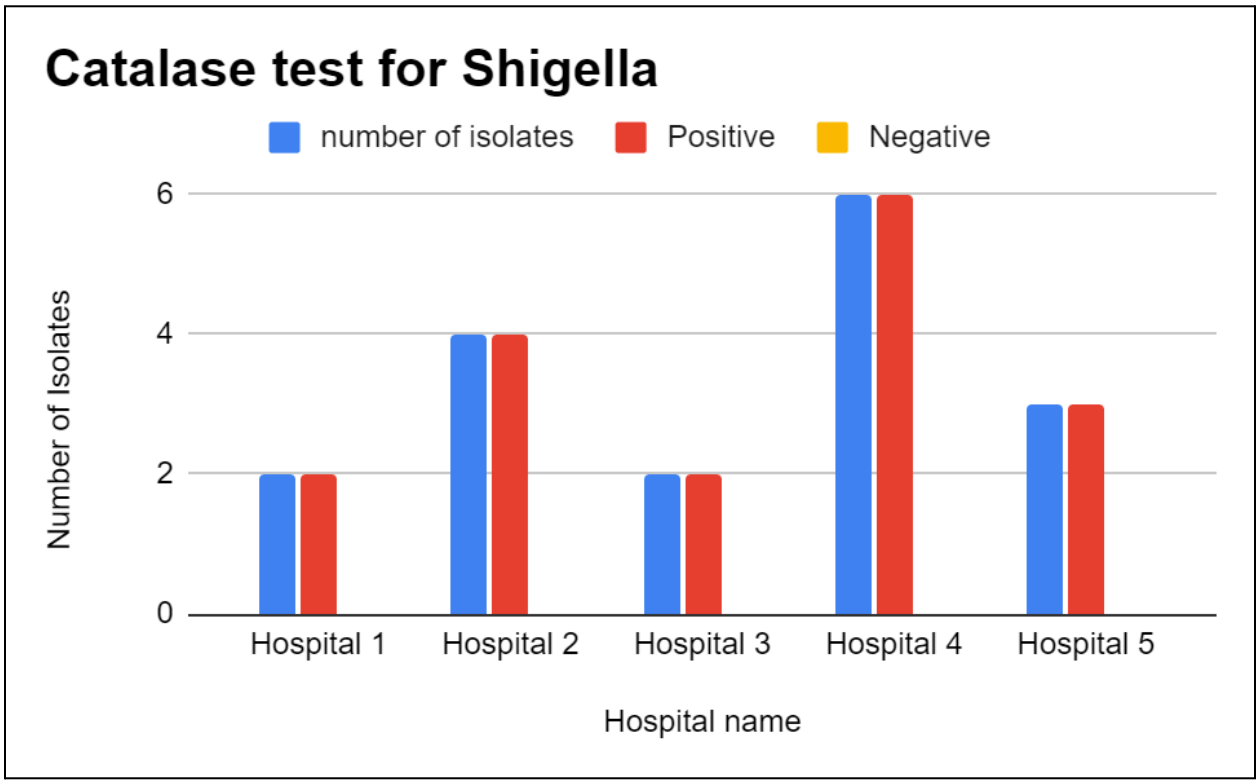


Fig 4.5.2.4.2: Bar diagram for catalase hospital-wise test result of *Shigella*.

4.5.2.5 Identification using Oxidase Test

The enzyme cytochrome oxidase reduces oxygen at the end of the electron transport chain. In our experiment, a colorless redox reagent i,e. 1 % of tetramethyl-p-phenylenediamine was used to detect the presence of oxidase enzyme. The positive result was obtained because of the reagent used which acts as an electron acceptor and leads to the oxidation of our reagent into end product indophenols. This indophenol was responsible for the end purple color. For our 17 presumptive *Shigella* isolates, there was no change in color. It concluded that the isolates do not have cytochrome oxidase enzymes, giving a 100 percent negative oxidase result.

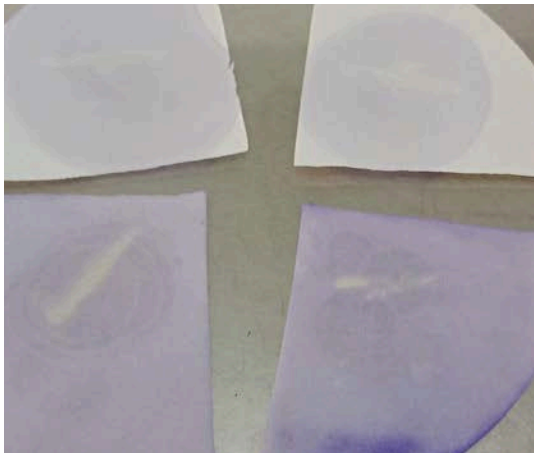


Fig 4.5.2.5.1: Oxidase test results for *Shigella*.

In addition, the bar graph shows the distribution of *Shigella,* in the five hospitals the samples were collected from. The chart shows that the 100 percent negative isolates number varied among the hospitals. Hospital four with 6 numbers of negative isolates had the highest number, followed by Hospital Two, Hospital Five, Hospital One, and Three.

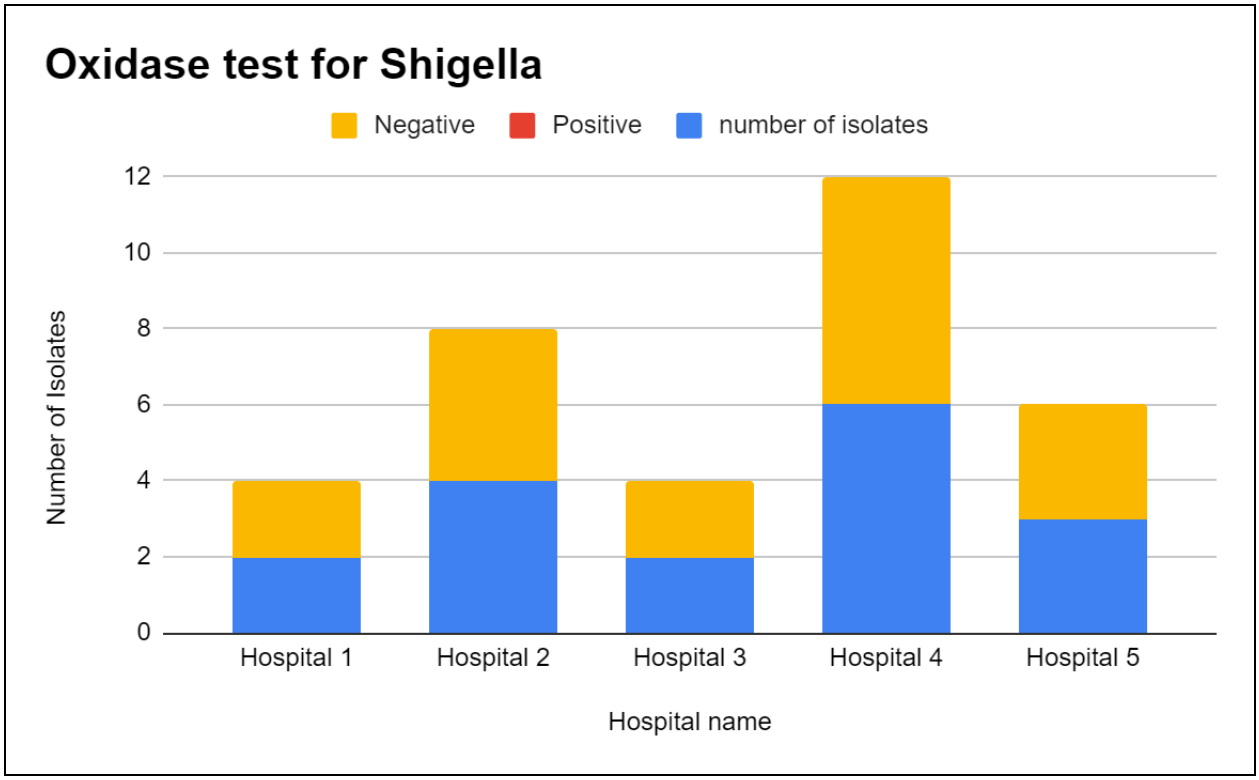


Fig 4.5.2.5.2: Bar diagram showing the hospital-wise positive and negative results for *Shigella*.

4.5.2.6 Identification using MR test

For the MR test, the 17 isolates were inoculated in liquid broth for 48 hrs. On the addition of the reagent methyl red, a change in color to red was observed in 15 isolates. The resulting red color from the positive MR test indicated that the end product was acidic and had a pH of 4.4 or much less, showing that the mixed acids were produced. Furthermore, 2 isolates on the addition of methyl red drops, indicated a yellow color showing the end product is not much acidic and the PH is 6.2. This yellow color indicates a negative MR Test.

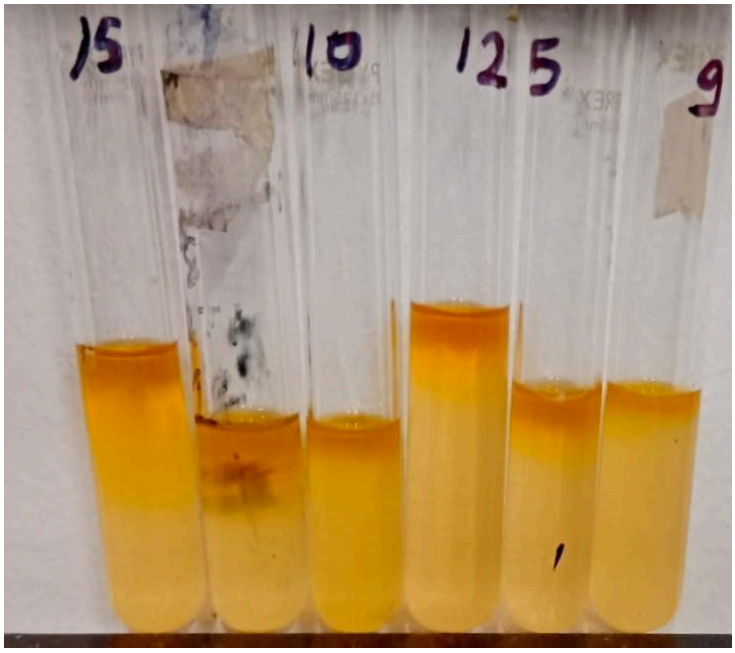


Fig 4.5.2.6.1: Results of MR test for identification of *Shigella*

Furthermore, the bar graph shows the MR test results of *Shigella,* in the five hospitals the samples were collected from. The chart shows that positive isolates number varied among the hospitals. Hospital four with 5 numbers of positive isolates had the highest number, followed by Hospital Two and Hospital 5 with 3 positive isolates, and Hospital One and Three with 2 positive isolates. The two negative isolates were found to be present in Hospital 2 and Hospital 4 respectively.

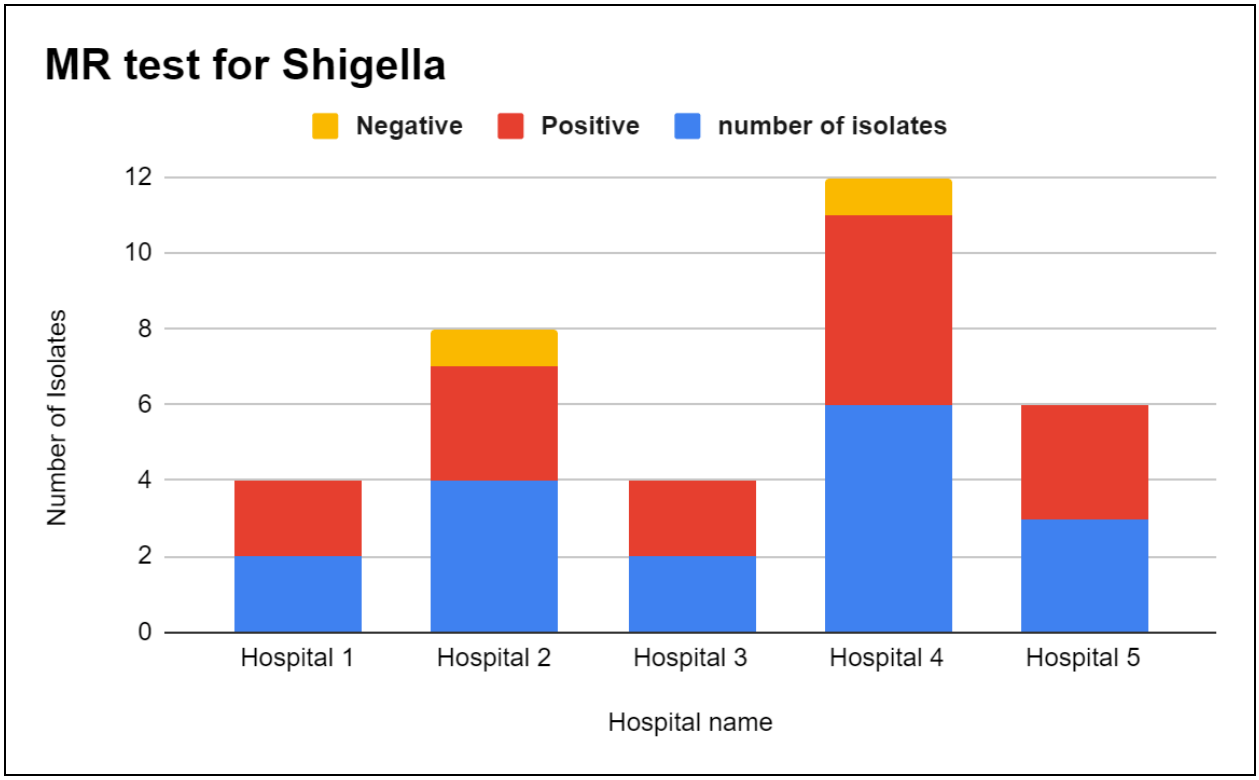


Fig 4.5.2.6.2: Bar graph showing hospital-wise MR test result for *Shigella*.

4.5.2.7 Identification using VP test

For the VP test also the samples were inoculated in the broth for 48 hrs. Therefore, for the VP test, 6 drops of Baritt's A and 2 drops of Barritt's B were added. After adding the reagents, the tubes were held for an hour, to show a maximum development in color. As a result, the positive test was considered to be pinkish-red, indicating the presence of acetyl methyl carbinol which is responsible for the fermentation of glucose. From the 17 isolates of *Shigella,* 3 isolates showed positive results. Showing pinkish-red color of the isolates of *Shigella.* On the other hand, 14 of the isolates showed negative results, with a mild copper color produced indicating that it does not contain acetyl methyl carbinol. As a result of which the color change was not found. This result aligned with the characteristics of the *Shigella* species.

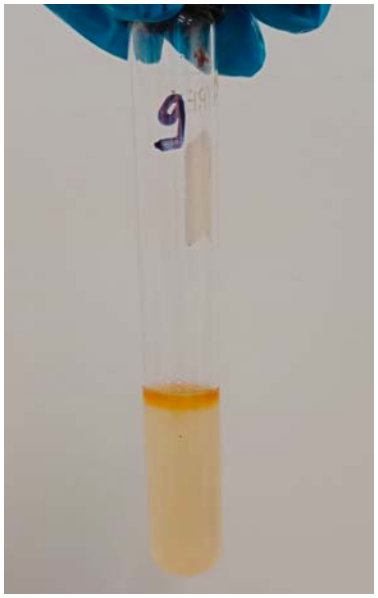


Fig 4.5.2.7.1: VP negative test result for *Shigella*.

Furthermore, the bar graph shows the VP test results of *Shigella,* in the five hospitals the samples were collected from. The chart shows that the negative isolates number varied among the hospitals. Hospital Four with 4 numbers with positive isolates had the highest number, followed by Hospital Two and Hospital Five with 3 negative isolates, and Hospital One and Three with 2 negative isolates. The two positive isolates were found to be present in Hospital 4 and one positive isolate was found in Hospital two respectively.

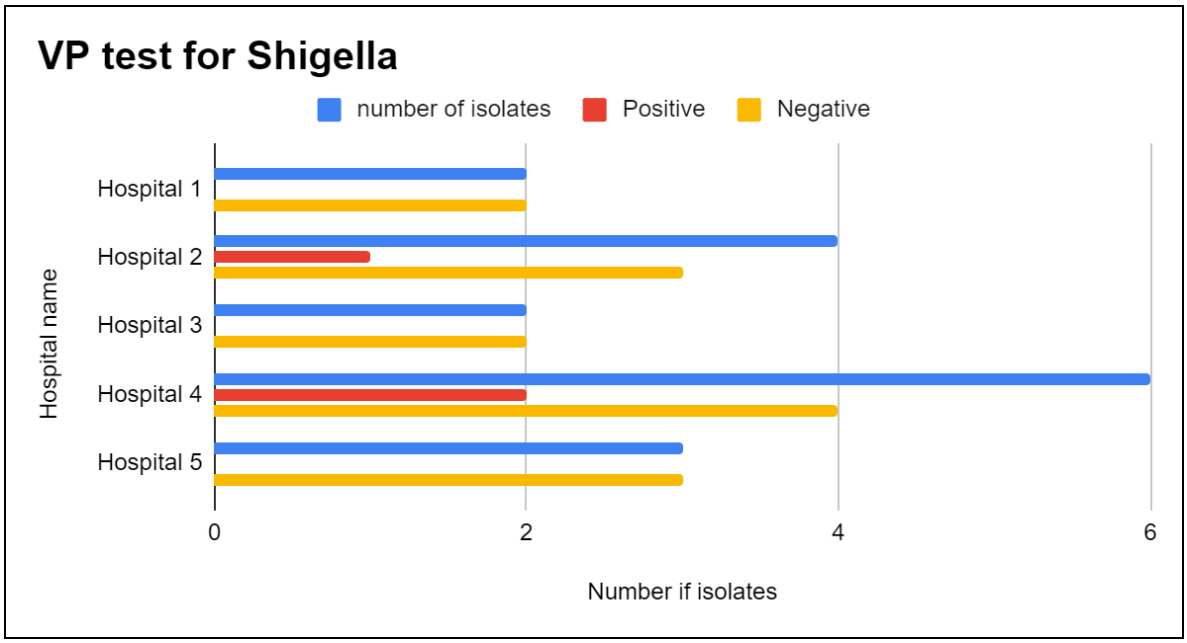


Fig 4.5.2.7.2: Bar graph showing hospital-wise test result of VP test for *Shigella*.

4.5.2.8 Identification using Motility Test:

To differentiate between non-motile and motile bacteria, a mobility test is performed. Motile organisms have some special features like fibrils or propellers like flagella to move themselves towards regions with more concentration of oxygen or nutrients that favor their survival. From the 17 presumptive S*higella* isolates, 12 isolates were found to be negative for the motility test, while 5 of the isolates were positive for the test.

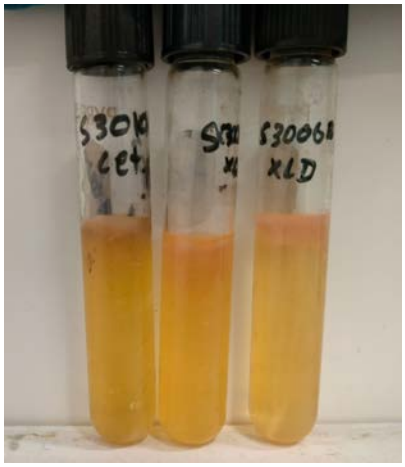


Fig 4.5.2.8.1: Motility test results for *Shigella*.

Furthermore, the bar graph shows the motility test results of *Shigella,* in the five hospitals the samples were collected from. The chart shows that the negative isolates number varied among the hospitals. Hospital Four 4 numbers positive isolates had the highest number, followed by Hospital Two and Hospital Five with 3 negative isolates, and Hospital Three with 2 negative isolates. Surprisingly, no negative isolates were found in Hospital One. The two positive isolates were found to be present in Hospital Four and Hospital One, and then one positive isolate was found in Hospital Two respectively.

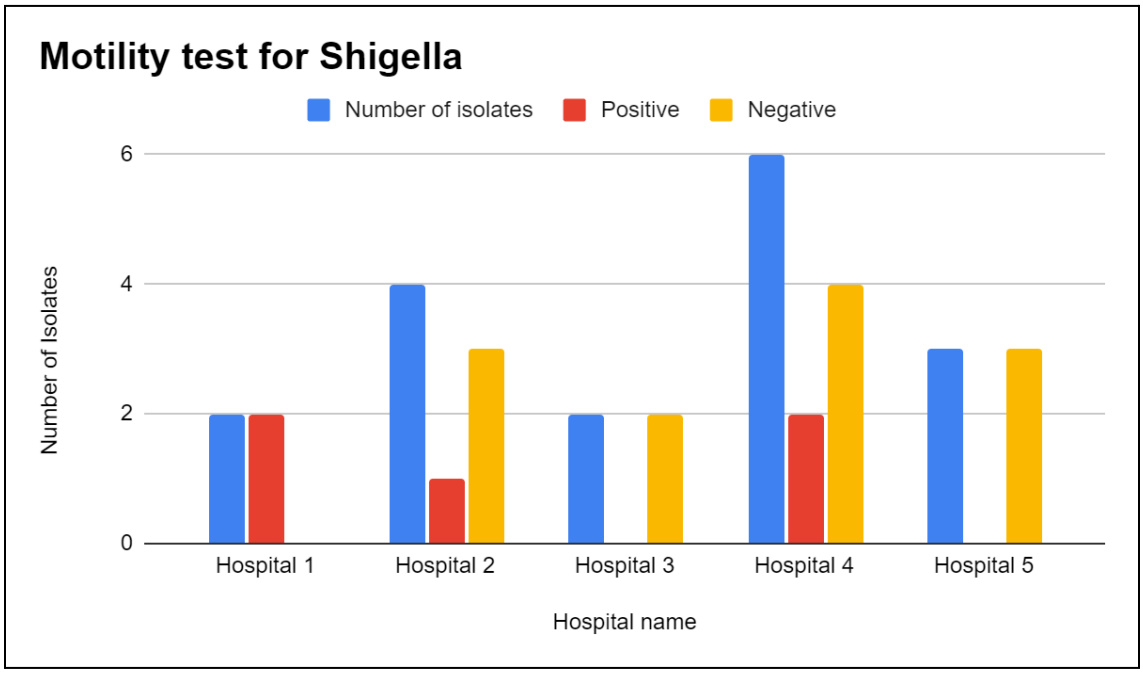


Fig 4.5.2.8.2: Hospital-wise comparison of presumptive *Shigella* isolates using motility test.

4.5.3 Antibiogram Test for *Shigella*

The CDC reports that the percentage of Shigella infections in the US resistant to all known antibiotic treatments increased from 0% in 2015 to 0.4% in 2019, and surged to 5% last year. (*CDC Issues Health Alert for Extensively Drug-Resistant Shigella*, n.d.). The 17 isolates of *Shigella* underwent the antibiogram test, with 12 antibiotics, under the disk.

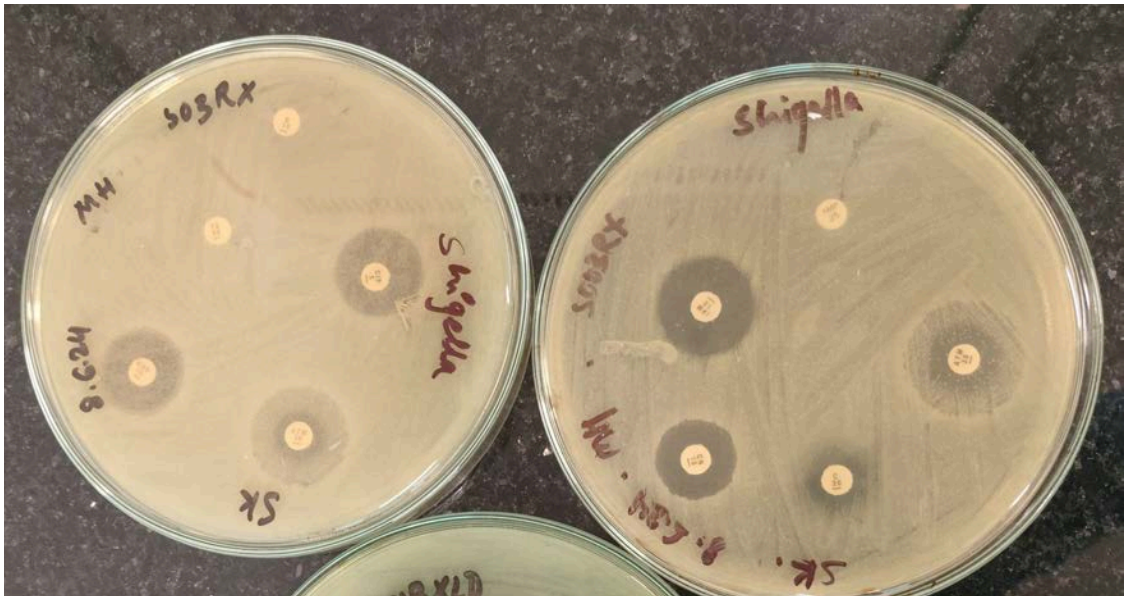


Fig 4.5.3.1: Kirby-disc test results for *Shigella.*

The results showed that ampicillin-25 and erythromycin-15 had the greatest resistance rates (100%), followed by tetracycline with 47.1%, chloramphenicol with 35.3%, Azithromycin-30 with 23.5%, Azithromycin-15 with 25.0%, gentamicin-10 and Norfloxacin 10 with 17.6%, Ciprofloxacin-5 with 11.8% and last aztreonam-30 and imipenem-10 with 5.9% lowest resistant rates.

On the other hand, piperacillin-TZP110 showed 100% susceptibility, followed by aztreonam with 88.2% susceptibility, gentamicin with 82.4%, azithromycin with 75%, ciprofloxacin with 70.6%, imipenem with 64.7%, Norfloxacin and azithromycin with 58.8% and chloramphenicol with 47.1% susceptible rates and tetracycline with 29.4% patterns of susceptibility.

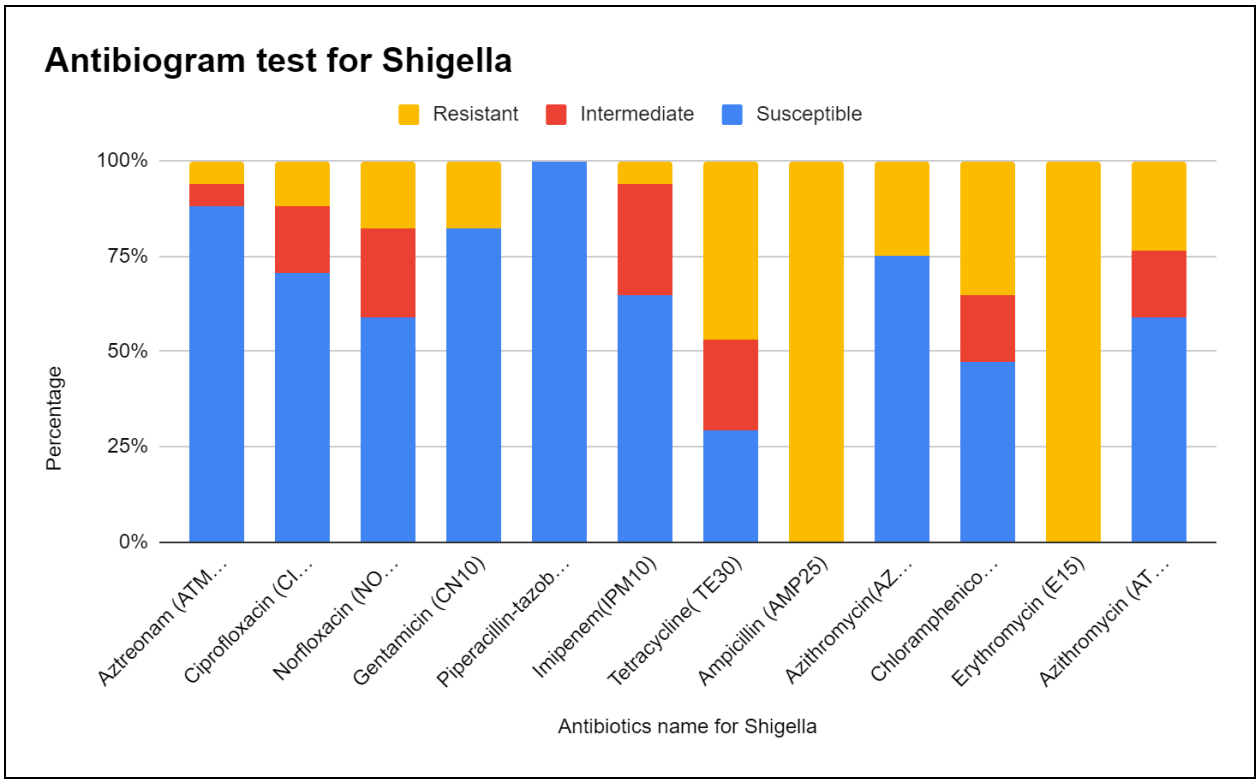


Fig 4.5.3.1: Bar graphs showing results of Antibiogram tests for *Shigella* with comparisons between 12 antibiotics.

4.6 Isolation of E. coli

4.6.1 PCR and Gel electrophoresis

From the total number of 87 isolates, 28 of the isolates were found to be *E.coli*. To confirm this, biochemical presumptive tests were done, along with Polymerase Chain reaction (PCR). For the PCR, the eco-f and eco-r were used, which are specific for the *E.coli* species. Only 23 isolates showed positive bands for *E.coli* from the total strains. On the other hand, the absence of bands in some of the wells suggests that that sample lacks the gene specific to *Escherichia coli*, thus serving as a negative control for the experiment. The results indicated a 32.2 % prevalence of *E.coli* in the biomedical wastes collected from the dustbins of the hospitals.

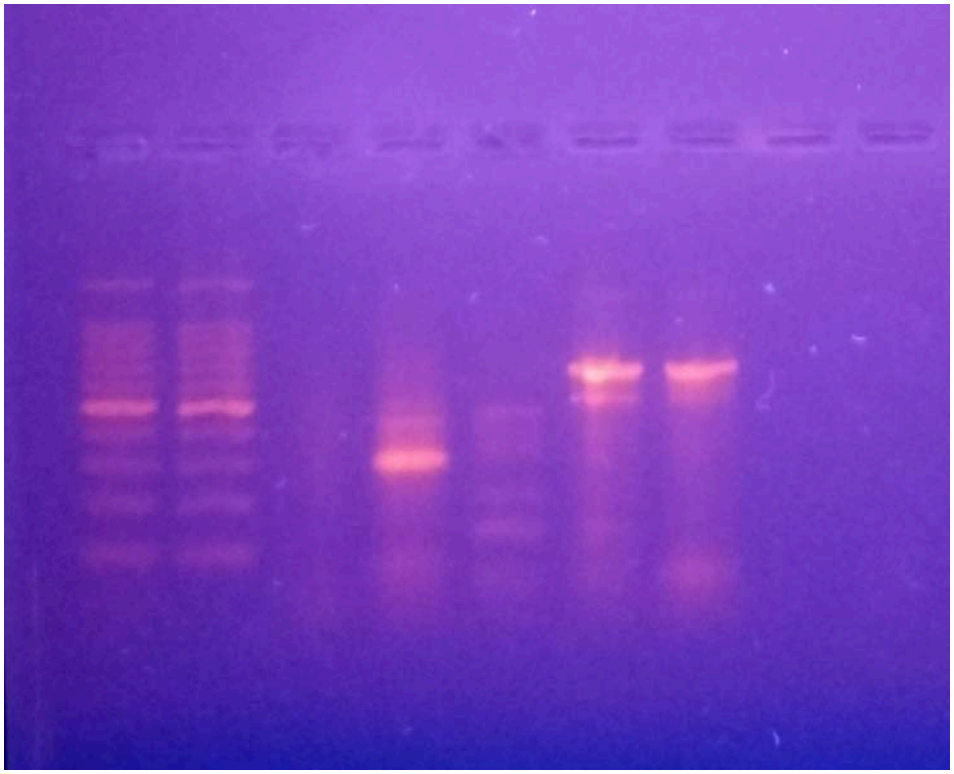


Fig **4.6.1.1**: Gel electrophoresis of *E.coli* isolates.

4.6.2.Identification of E. coli using Biochemical Tests

The fundamental characteristics of E. coli for different biochemical tests are mentioned here.

S.N	**Different test**	**Results for E.coli**
1.	Gram Staining	Negative
2.	MR Test	Positive (red color)
3.	VP Test	Negative
4.	Catalase Test	Positive (bubbles present) |
5.	Oxidase Test	Negative (no color change)

S.N	Different test	Results for E.coli
6.	Citrate test	Negative (no growth)
7.	Motility Test	Positive (diffusion from stab line

Table: 4.6.1

4.6.2.1 Gram staining

The presumptive 28 isolates of *E.coli* underwent the process of Gram staining. The results of the Gram staining characteristics were found to be consistent with the gram-negative *E.coli*. To illustrate, all of the 28 presumed isolates indicated lipopolysaccharide cell walls by giving pink coloration and retaining the safranin dye, as visualized under the microscope. Thus, these results set a path in the identification and characterizing the *E.coli* species. The result of one of the gram-negative samples of *E.coli* under an electron microscope is shown in the figure below.

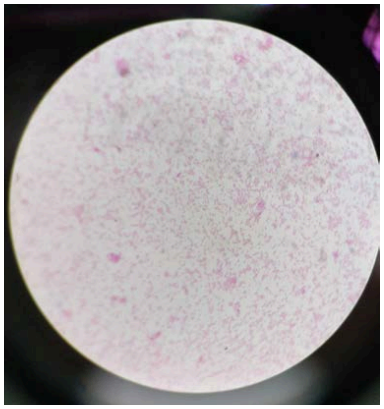


Fig 4.6.2.1.1: Gram staining result for *E.coli.*

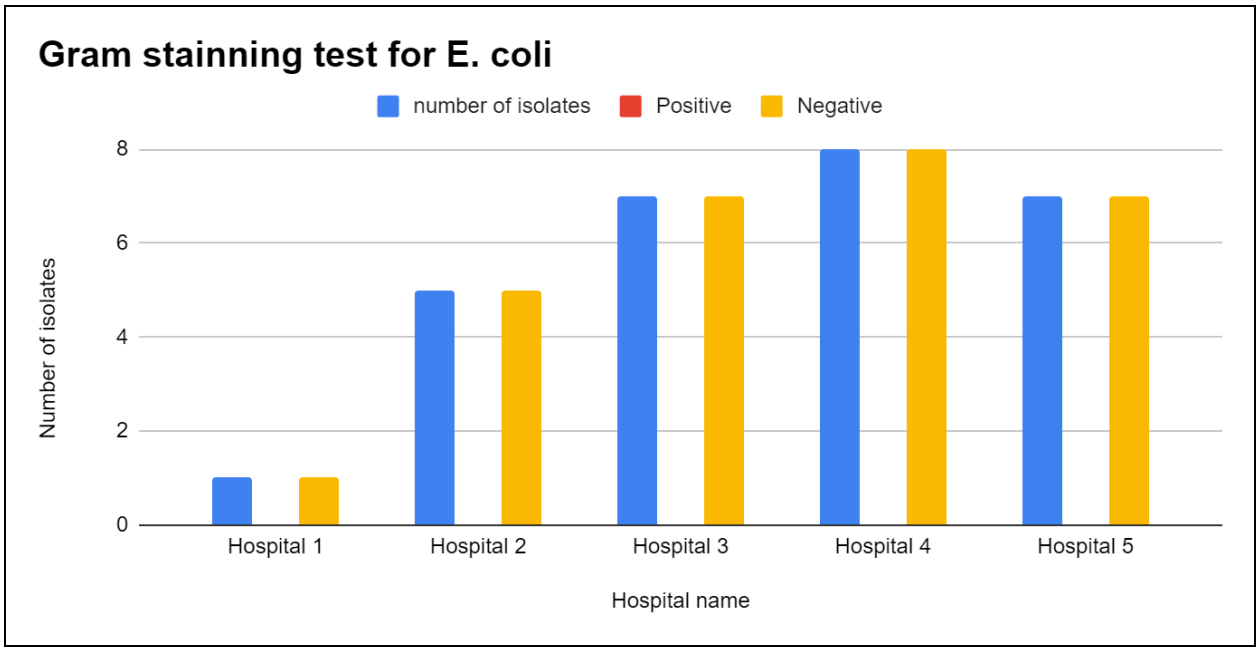


Fig 4.6.2.1.2: Bar graph showing the gram staining results of *E.coli.*

4.6.2.2 Identification by Triple Sugar Iron (TSI)

After 24 hours of incubation of the 28 isolates, the results were interpreted. The result showed that the butt and slant both turned yellow indicating that the acid produced is in large quantities. There was fermentation of sucrose and lactose along with glucose. The yellow color indicates that fermentation did occur as acids have been produced hence phenol red turned yellow. Furthermore, the production of gasses H2 & CO2 was indicated by the bubble formation and cracking of the media. No black color precipitate indicating an absence of FeS (Ferrous Sulfide), a black compound formation. In addition to this, the number of positive isolates of *E.coli* varied from hospital to hospital. To exemplify, hospital 4 showed the maximum amount of positive isolates of *E.coli* i.e 8, both hospitals 5 and 3 were found to have the same number of the isolates 7 positive isolates followed by hospital 2 showed five positive isolates, and hospital 1 with only one positive isolate.

Fig 4.6.2.2.1: TSI test result for *E.coli.*

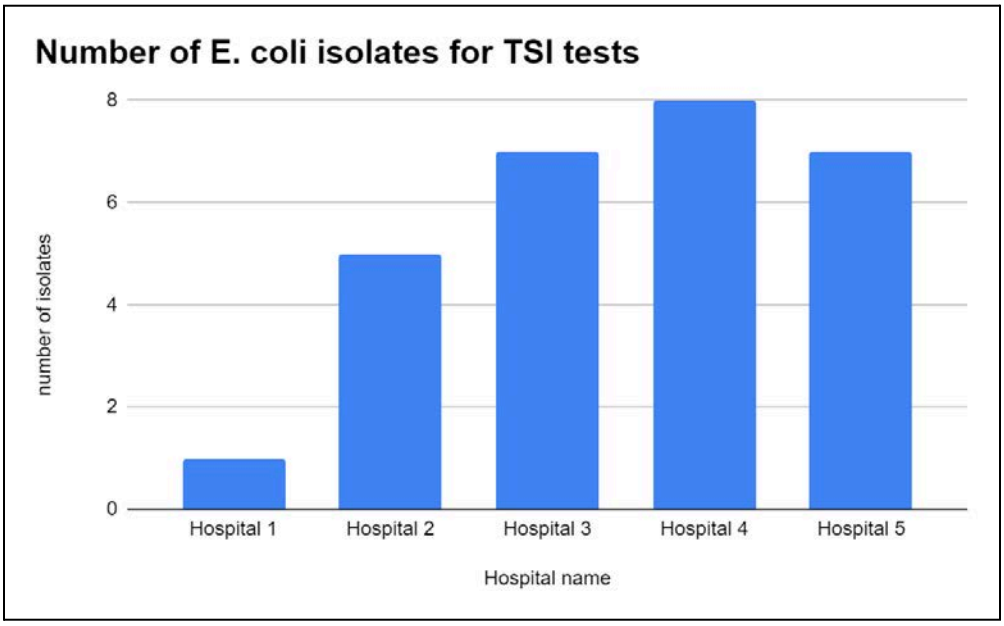


Fig 4.6.2.2.2: Distribution of E.coli among different hospitals based upon the TSI results.

4.6.2.3 Identification using Citrate Utilization Test

For the citrate Utilization test, out of 28 presumptive isolates of *E.coli,* nine of the isolates showed a positive result. As described earlier in this paper, the breakdown of citrate to oxaloacetate and acetate and its further breakdown to pyruvate and CO2 was found in the

samples. Furthermore, in case of the positive results, these metabolic products further break down due to the presence of alkaline pH, and finally, the indicator Bromothymol blue gives a Prussian Blue color.

Fig 4.6.2.3.1: Citrate utilization test result for *E.coli*.

On the other hand, 19 of the isolates showed no change in color. This indicated the absence of metabolic breakdown; the color of the media remained unchanged (green) as shown in the figure above. That means 67.86% were found to be negative, which aligns with the standard characteristics of the *E.coli*. Furthermore, the hospital-wise comparison showed that the hospital 4 with a value of 7 isolates had the most number of positive *E.coli*. Followed by Hospital 3, with 5 isolates, Hospitals 2 and 5 with 3 isolates, and Hospital 1 with just 1 negative isolate of *E.coli.*

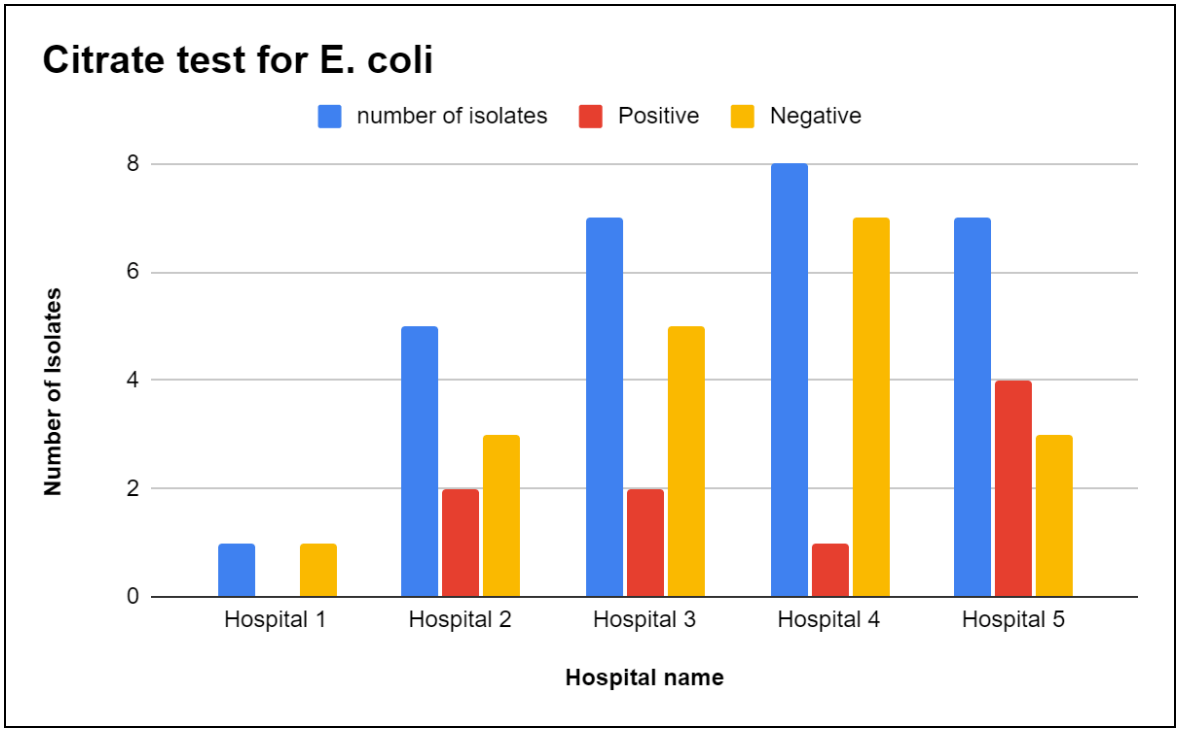


Fig 4.6.2.3.2: The bar graph shows the distribution of *E.coli* across different hospitals.

4.6.2.4 Identification using Catalase Test

Hydrogen peroxide, a toxic product is produced as a result of aerobic metabolism by bacteria. To stop the damage that could be caused by this toxicity, cells use an enzyme called catalase to break down the H_2O2. The species of bacteria that have this catalase are catalase positive, while those that lack the enzyme are negative to the test. To exemplify, the positive result is indicated by bubble production (Oxygen gas), which is simply an outcome of the breakdown of the H_2O2 by the enzyme catalase, into water and O2 gas. Our species *E.coli,* has the enzyme catalase. As a result, all 17 isolates showed a positive test with the bubble formation. **Fig 4.6.2.4.1:** shows the formation of bubbles on the addition of Hydrogen peroxide to the presumptive isolates.

Fig 4.6.2.4.1: Isolates showing catalase positive result on addition of hydrogen peroxide.

Furthermore, the bar graph shows the distribution of *E.coli,* in the five hospitals the samples were collected from. The chart shows that positive isolates number varied among the hospitals. Hospital four with 8 numbers of positive isolates had the highest number, followed by Hospital three, Hospital five, Hospital two, and One.

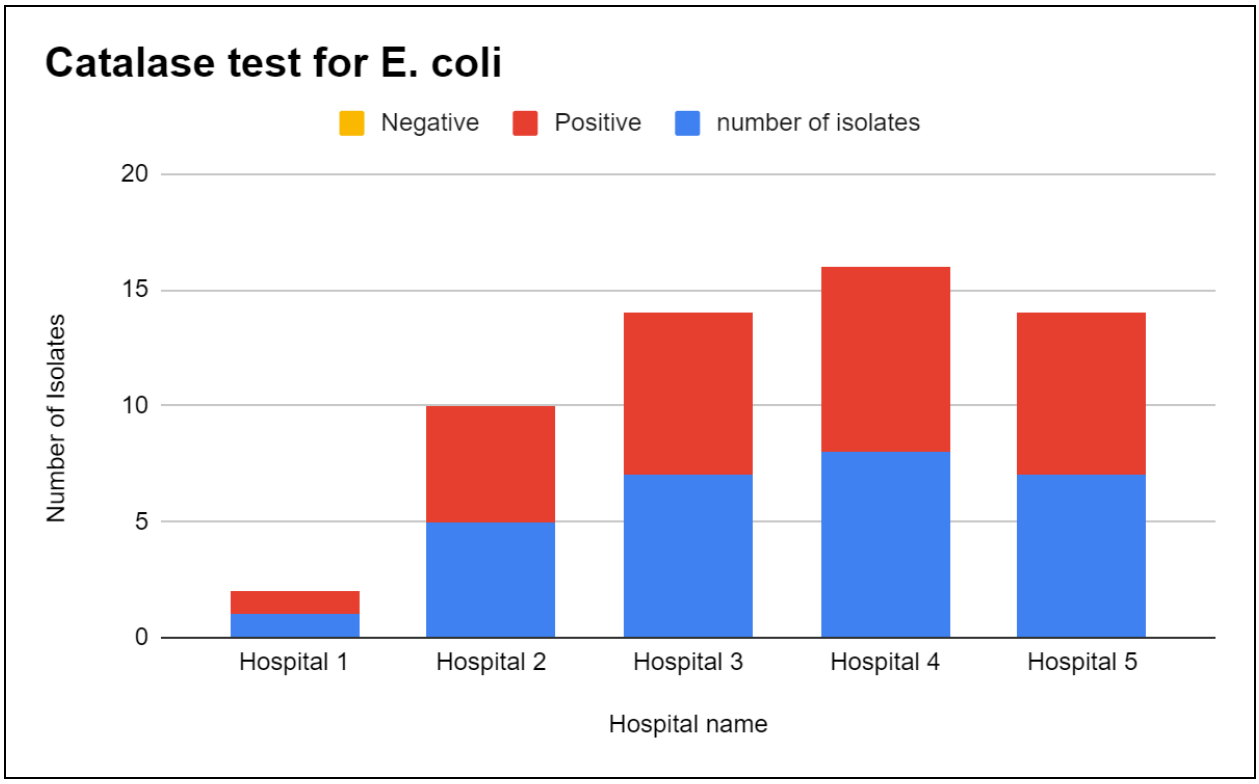


Fig 4.6.2.4.2: Comparison of hospital-wise distribution of positive and negative isolates based on catalase test.

4.6.2.5 Identification using Oxidase Test

The enzyme cytochrome oxidase reduces oxygen at the end of the electron transport chain. In our experiment, a colorless redox reagent i,e. 1 % of tetramethyl-p-phenylenediamine was used to detect the presence of oxidase enzyme. The positive result was obtained because of the reagent used which acts as an electron acceptor and leads to the oxidation of our reagent into end-product indophenols. This indophenol was responsible for the end purple color. From the 28 presumptive *E.coli* isolates, for 25 of the isolates, there was no change in color, concluding that these isolates do not have cytochrome oxidase enzymes. On the other hand, three of the isolates showed a positive oxidase result.

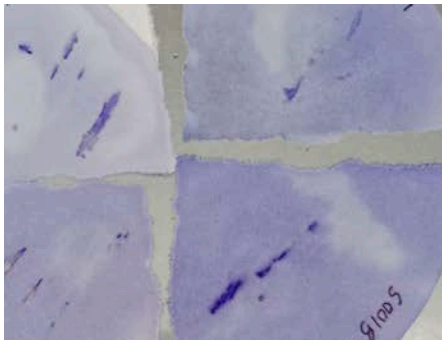


Fig 4.6.2.5.1: Oxidase test result for *E. coli* isolates.

Furthermore, the bar graph shows the distribution of *E.coli* in the five hospitals where the samples were collected from. The chart shows the percentage of positive and negative isolates number varied among the hospitals. Hospital five had the highest number,7 negative isolates followed by hospitals three and four with an equal number (6) numbers of negative isolates, followed by Hospital 2 and lastly Hospital One.

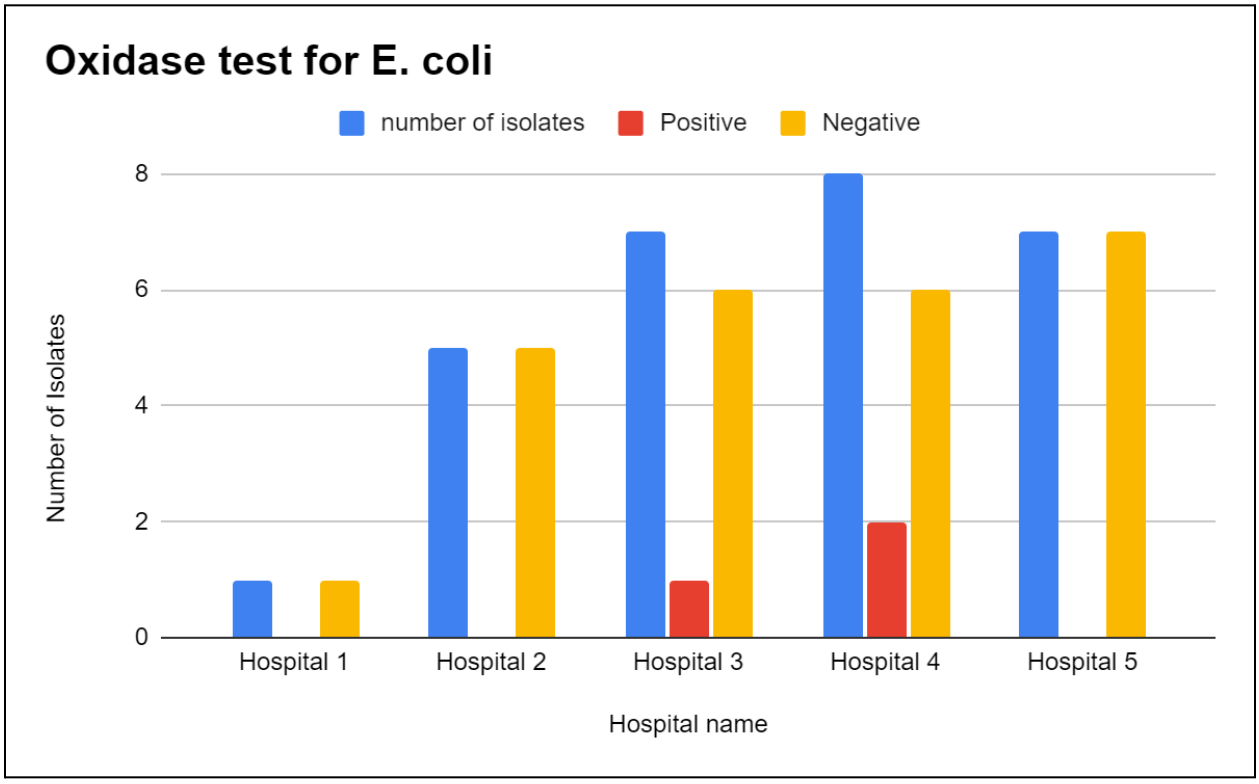


Fig 4.6.2.5.2: Distribution of presumptive E.coli based on Oxidase test.

4.6.2.6 Identification using MR test:

For the MR test, the 28 isolates were inoculated in liquid broth for 48 hrs. On the addition of the reagent methyl red, a change in color to red was observed in 22 isolates. The resulting red color from the positive MR test indicated that the end product was acidic and had a pH of 4.4 or much less, showing that mixed acids were produced. Furthermore, 5 isolates on the addition of methyl red drops, indicated a yellow color showing the end product is not much acidic and the PH is 6.2. This yellow color indicates a negative MR Test.

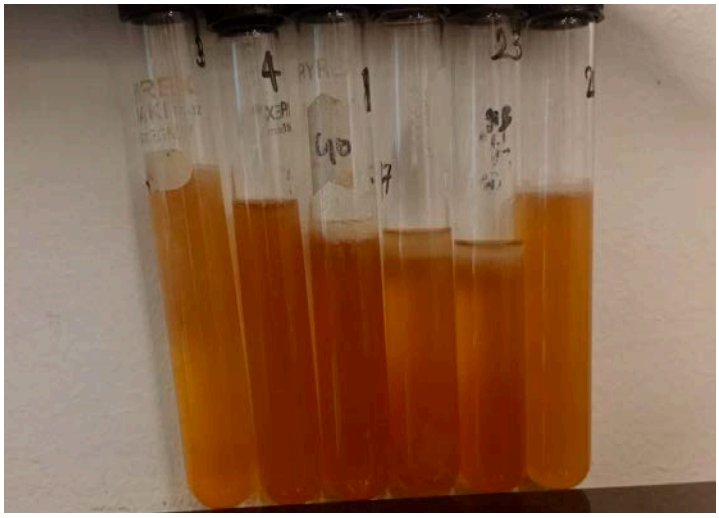


Fig 4.6.2.6.2: Results of MR tests for *E. coli*

Furthermore, the bar graph shows the MR test results of *E.coli,* in the five hospitals the samples were collected from. The chart shows that positive isolates number varied among the hospitals. Hospitals 4 and Three with 6 numbers of positive isolates had the highest number, followed by Hospital 2 with 5 isolates, and hospital 5 with 4 positive isolates, hospital 1 with 1 positive isolate. The negative isolates were found to be present in Hospital 3, Hospital 4, and Hospital 5 respectively.

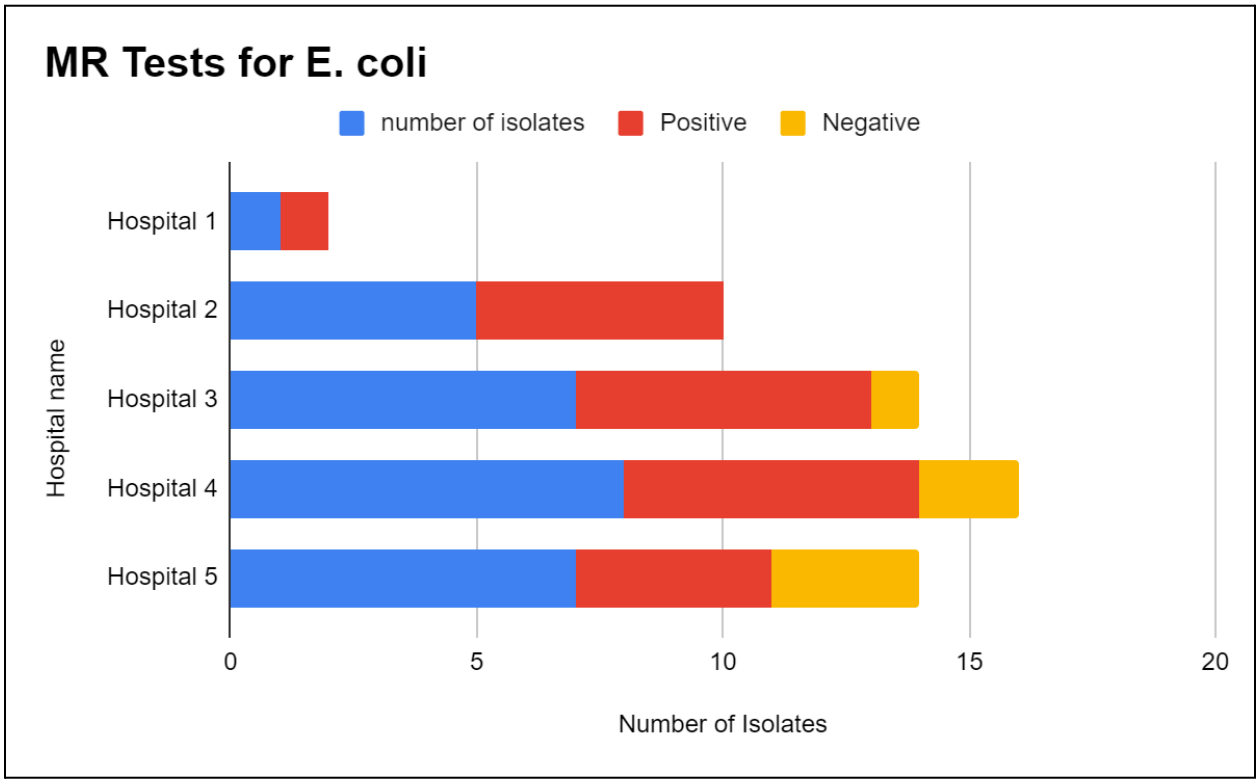


Fig 4.6.2.6.2: Presumptive *E.coli* distribution based on MR test.

4.6.2.7 Identification using VP test:

For the VP test also, the samples were inoculated in the broth for 48 hrs. Therefore, for the VP test, 6 drops of Baritt's A and 2 drops of Barritt's B were added. After adding the reagents, the tubes were held for an hour, to show a maximum development in color. As a result, the 28 presumptive isolates of *E.coli,* showed negative results. The mild copper color produced indicated that it does not contain acetyl methyl carbinol. As a result of which the color change was not found. This result aligned with the characteristics of the *E.coli* species.

Fig 4.6.2.7.1: VP-Test of *E. coli* showing a negative result

Showing the color of the isolates of *E.coli.* Furthermore, the bar graph shows the VP test results of *E.coli,* in the five hospitals the samples were collected from. The chart shows that the negative isolates number varied among the hospitals. Hospital Four with 8 numbers of negative isolates had the highest number, followed by Hospital Three and Hospital Five with 7 negative isolates, Hospital Two with 5 isolates, and Hospital One with 1 isolate respectively.

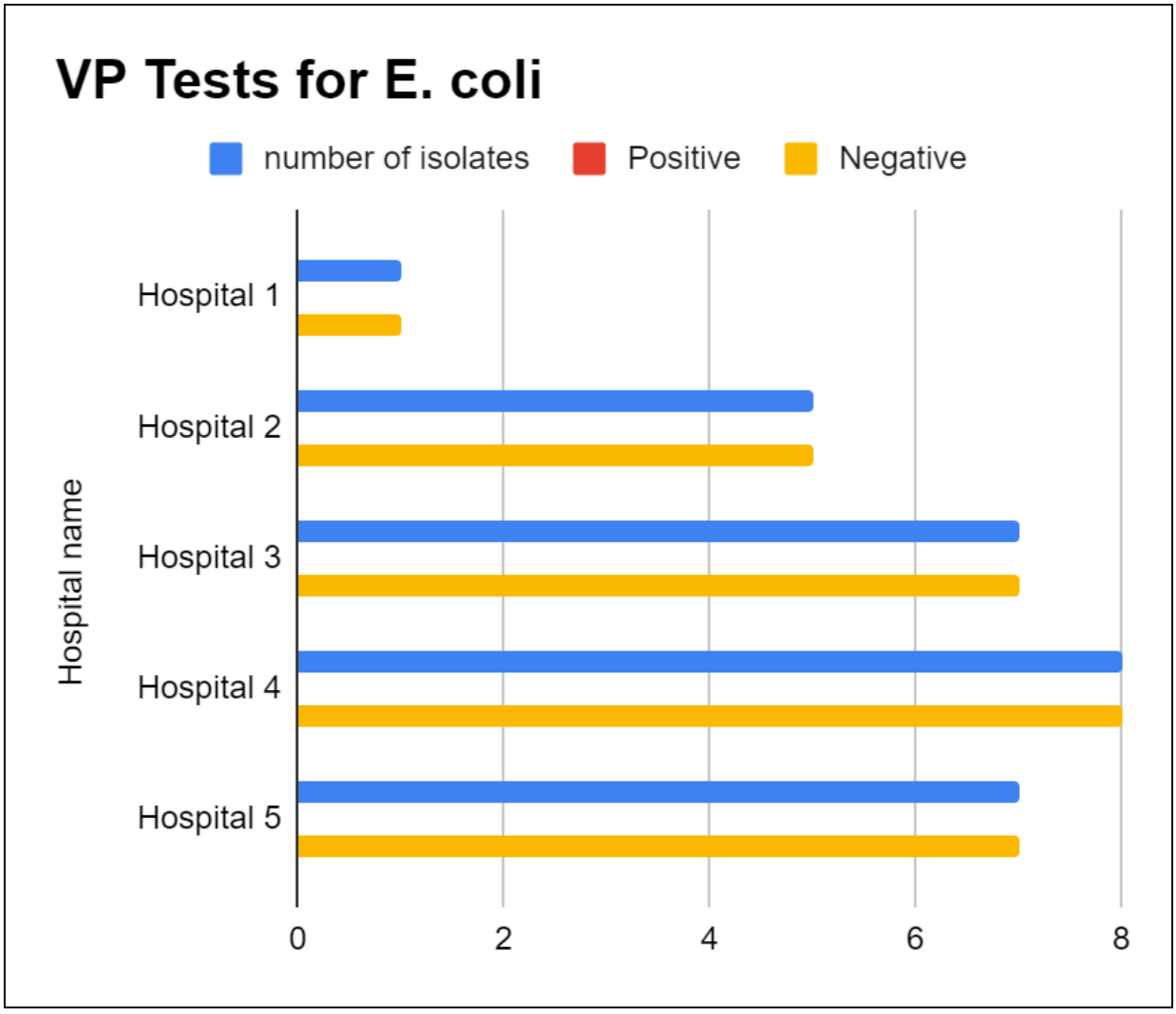


Fig 4.6.2.7.2: Distribution of E.coli based on VP test.

4.6.2.8 Identification using Motility Test

To differentiate between non-motile and motile bacteria, a mobility test is performed. Motile organisms have some special features like fibrils or propellers like flagella to move themselves towards regions with more concentration of oxygen or nutrients that favor their survival. From the 28 presumptive *E.coli* isolates, 3 isolates were found to be negative for the motility test, while 25 of the isolates were positive for the test. Fig:

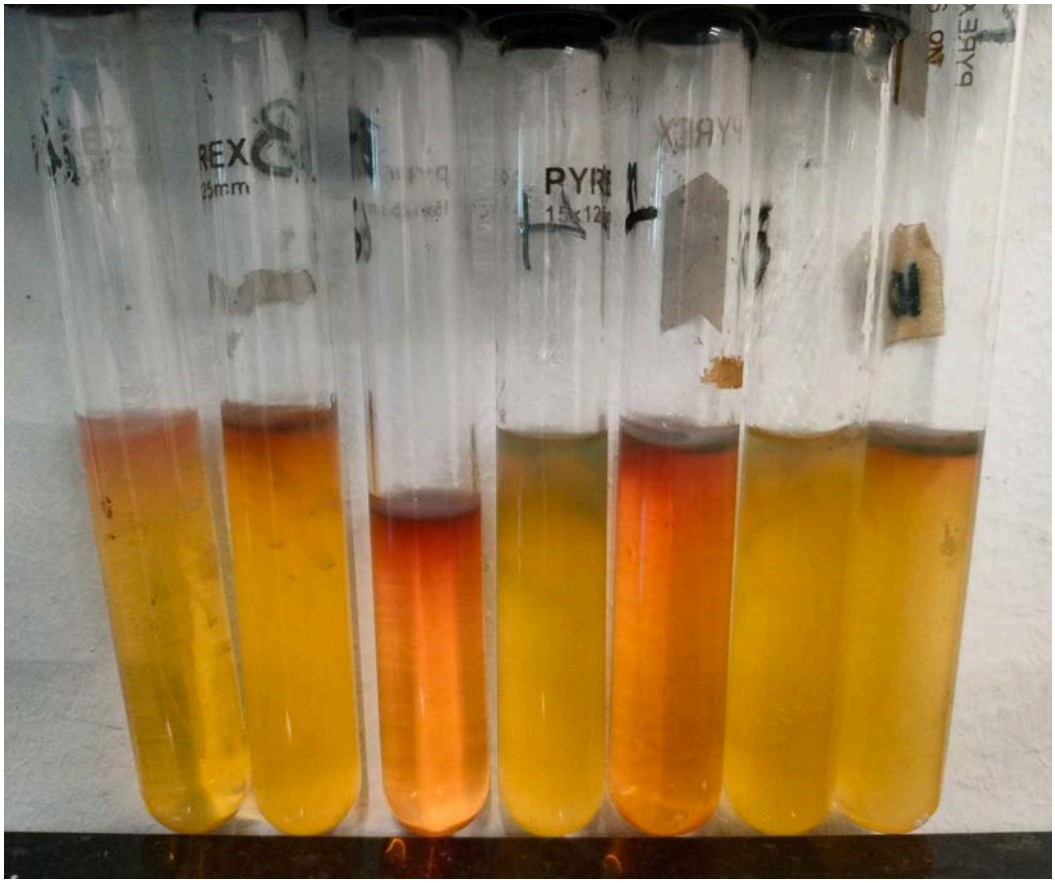


Fig 4.6.2.8.1: Motility test results for presumptive isolates of *E.coli*.

Furthermore, the bar graph shows the motility test results of *E.coli,* in the five hospitals the samples were collected from. The chart shows that positive isolates number varied among the hospitals. Hospital 4 with 8 numbers of positive isolates had the highest number, followed by Hospital 3 with 7 isolates, Hospital 5 with 6 positive isolates, and Hospital 3 with 3 positive isolates. Surprisingly, no negative isolates were found in hospital one. The three negative isolates were found to be present in Hospital 2 and Hospital 5 respectively.

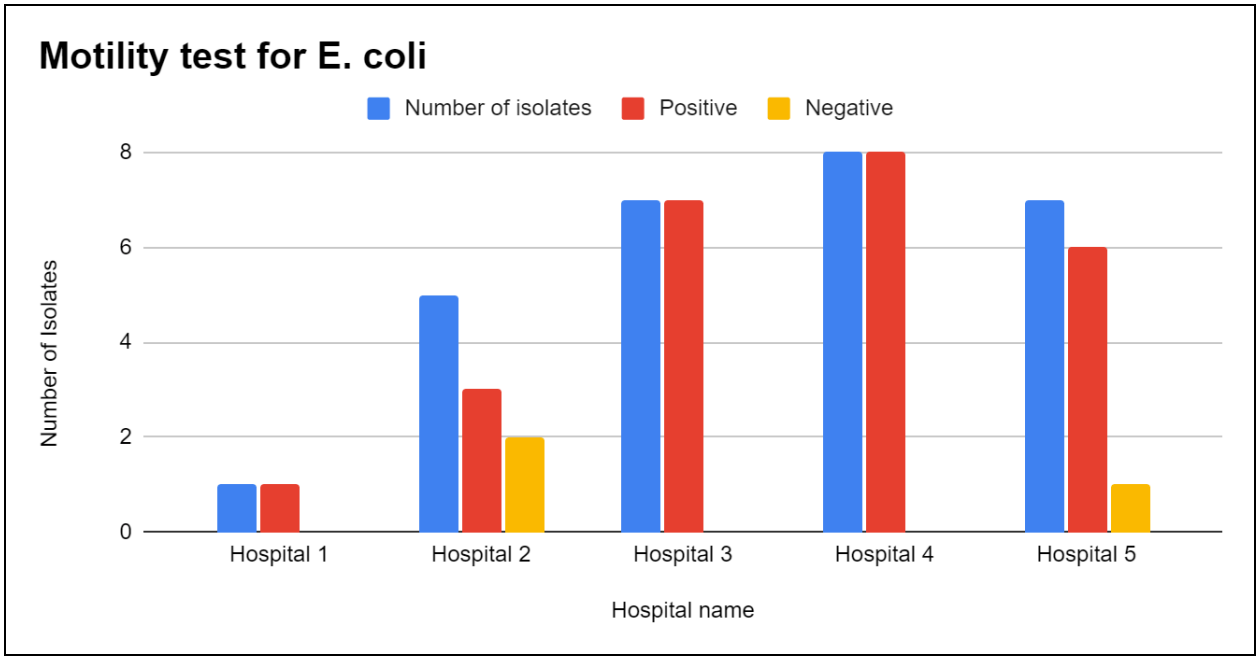


Fig 4.6.2.8.2: Bar graph comparing the distribution of E.coli in different hospitals.

4.6.3 Antibiogram test result for *E.coli*

The 28 isolates of *Pseudomonas* underwent the antibiogram test, with 11 antibiotics, under the disk diffusion protocol. The highest resistance was shown by ampicillin (AMP10) at 67.9%, followed by ciprofloxacin (CIP5) at 35.7%, Trimethoprim (SXT25), and tetracycline at 32.1%. Meropenem and ceftazidime at 28.6%, Chloramphenicol (C30) at 20.7%, and Piperacillin at 21.4%, the lowest resistance activity.

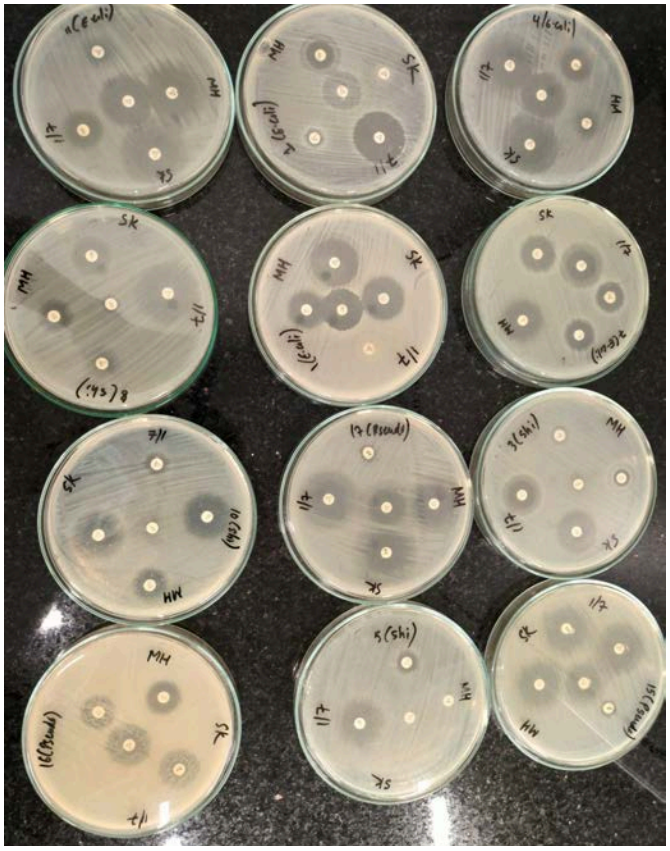


Fig 4.6.3.1: Antibiotics test result for *E.coli*.

On the other hand, gentamicin (CN10) showed highest susceptibility (100%), followed by amikacin (AK30) showed 82.1% susceptibility, piperacillin (TZP110) with 75.0% aztreonam (ATM 30) with 69.7%, trimethoprim with 67.9%, and, ceftazidime (CAZ) with 50.0%, and, tetracycline with 57.1%, meropenem(MEM 10) with 46.4%, ampicillin with 25.0% and ciprofloxacin (CIP5) with 17.9% low rates of susceptibility.

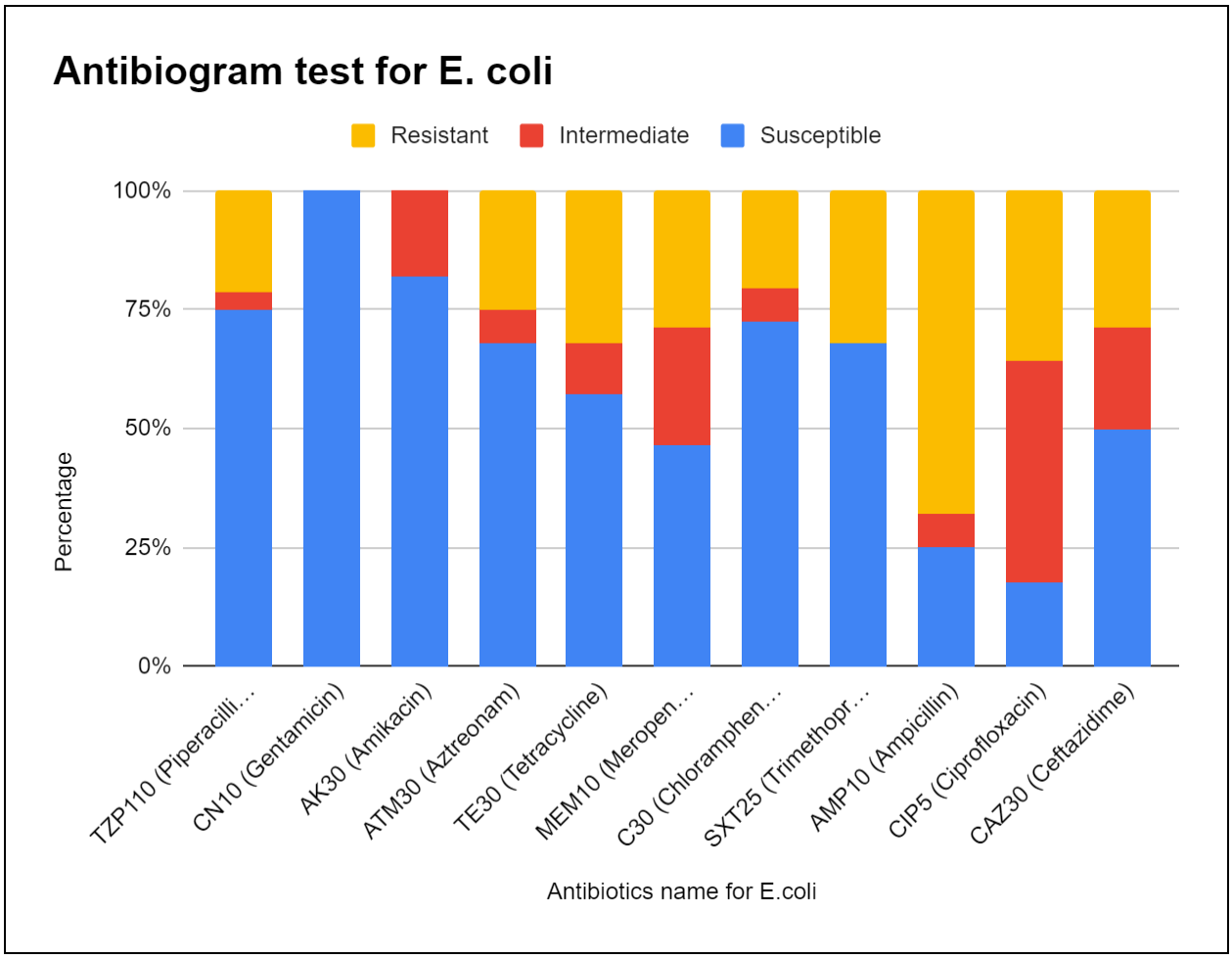


Fig 4.6.3.2: Bar graphs showing results of Antibiogram tests for *Shigella* with comparisons between 11 antibiotics.

Chapter 5: Discussions

5.1 Prevalence of Pseudomonas

This study confirmed that *Pseudomona*s is predominant in biomedical waste samples collected from five hospitals with a percentage frail of 33.33% with 29 isolates out of 87 samples. This was further confirmed by PCR analysis using PAGS primers, which are specific to the *Pseudomonas* genus; thus, hospital waste from the study area is suitable for the growth of this opportunistic pathogen. However, the higher levels of *Pseudomonas* present several problems, because these bacteria are well known to cause infections, especially in immune-compromised

individuals. This is in agreement with similar previous studies whereby hospital-related context is acknowledged as suitable for *Pseudomonas* owing to their versatility and resistance to unfavorable conditions.

After performing the gram staining procedure, all 29 presumptive *Pseudomonas* isolates proved to be gram-negative since they retained the safranin dye to give pink coloration. Before going through this observation, it is crucial to note that *Pseudomonas* had lipopolysaccharides in the cell wall. Even though the results of the Gram staining of the suspected *Pseudomonas* species were obtained from a different source, they were in harmony with the previously known qualities of *Pseudomonas*. In addition to gram staining, The Triple Sugar Iron (TSI) test which was done gave negative results for both acid and gas that supported the non-fermentative characteristic of these isolates. Also, the citrate utilization test came out positive meaning that 82.75% of the isolates were able to metabolize citrate. On this test, the number of times citrate-positive isolates was highest in hospital 5 and this may mean that the hospitals were having differences in their waste management systems.

The metabolic patterns of the *Pseudomonas* isolates were also elucidated by the catalase and oxidase tests. All these isolates were positive to catalase since they are aerobic and 86.2 % were positive to oxidase since they use oxidative metabolic pathways. The motility test served an extra role in confirming the identity of *Pseudomonas* because the motility of all the isolates was an added advantage in their ability to infect tissues and compete. The motility given by flagella enables *Pseudomonas* to adhere to a variety of substrates successfully infecting surfaces in hospitals among them.

Finally, the antibiogram of the 29 *Pseudomonas* isolates showed that they have different levels of resistance and sensitivity toward antibiotics. A comparatively high resistance frequency was detected for meropenem (31%) and gentamicin (31%). This was succeeded by Ceftazidime (27.6%) and levofloxacin (27.6%). Among those ANTdB, amikacin received the highest susceptibilities of 79.3%, and ciprofloxacin was the second best with 69.5%. The observed patterns of resistance trends for antibiotics underscore the importance of appropriate choice of

antibiotics when treating *Pseudomonas* infections, especially in areas where Multi-Drug Resistant (MDR) *P. aeruginosa* is prevalent.

Therefore, this research shows that 60% of the hospitals had *Pseudomonas*-contaminated biomedical wastes with significant differences in distribution and resistance patterns between the studied hospitals. The molecular and biochemical data together with antibiogram results imply the pathogenicity and antibiotic resistance of these isolates. Therefore, raising infection control standards enrollment, and systemic antimicrobial monitoring in hospitals appears of crucial importance to control the spread of multidrug-resistant *Pseudomonas* strains.

5.2 Prevalence of Salmonella

The topic of identification of *Salmonella* isolates from biomedical waste throws light on frequency, identification, and resistance to antibiotics. Seven out of the 87 total isolates were presumptively identified as *Salmonella* through biochemical tests, and all seven samples returned positive results after being confirmed using PCR with the INVA gene. This led to a total average percentage of 14.9% of *Salmonella* in the hospital biomedical waste and this is very alarming since *Salmonella* is pathogenic, especially in a healthcare setting. The PCR results are consistent with the biochemical results and highlight the value of improving waste disposal to control such pathogens.

Characterization of the presumptive *Salmonella* isolates based on Gram staining was also done to check whether they belonged to the gram-negative group or not, all 13 isolates were pink in color due to the retention of safranin dye. This view is in line with the lipopolysaccharide cell wall features that characterize all the *Salmonella* species that lead to their pathogenicity. These results were then followed by biochemical tests which offered stronger support to the success of the identification procedure.

The biochemical characterization using the Triple Sugar Iron (TSI) test confirmed that all the isolates fermented glucose with the formation of dark/black precipitate indicative of hydrogen sulfide (H_2S), however, none of the isolates was able to ferment lactose or sucrose. Moreover, the production of gas in the form of bubbles and rather a cracking of the media was also recorded. That indicates the degree of hospitality contracted differed between different hospitals, hospital four had four positive *Salmonella* isolates, hospitals Two and Three both had three positive

Salmonella isolates, while hospitals One and Five had two and one positive *Salmonella* isolates, respectively.

A positive result in the citrate utilization test was recorded in only 4 of the 13 isolates: the presence of Prussian blue coloration suggests the further metabolic breakdown of the citrate in the medium. The large majority of the isolates fell within this category, 69.23% to be exact and all these conform to the basic salmonella profiles. The distribution of positive and negative isolates in the hospitals where Hospital 4 had the highest number of negative isolates while Hospital 5 had no negative isolates.

The Catalase test was positive in all *Salmonella* isolates where bubbles were produced due to the cracking of toxic hydrogen peroxide by the catalase enzyme. The findings reflected other general attributes of *Salmonella* thus supporting the identification of the results found during analysis. In the distribution of different hospitals, the percentage of catalase-positive isolates again seems high in hospital 4.

Likewise, the oxidase test to confirm the presence of cytochrome oxidase was also positive for two of the isolates while the other 11 were negative. This finding corresponds well with the general underlying fact regarding the Salmonella species which are generally oxidase-negative. The presence of isolates that were positive for oxidase can be blamed on slight genetic or perhaps environmentally related differences in the strains.

By using the Methyl Red (MR) test, it confirmed that 10 out of the total *Salmonella* isolates showed positive results with the red coloration result elaborating that the strain possesses its ability to ferment mixed acid and have a low pH value. The other three isolates taken through the MR test, produced a yellow color as a result of the high pH levels as indicated by a negative MR test. The distribution of MR-positive isolates was not the same, hospitals 4 and 3 had more frequencies of MR-positive isolates.

All the 13 isolates were Voges-Proskauer positive where the formation of a pinkish red color indicated the formation of acetoin from glucose. This is in agreement with the expected metabolic profile of *Salmonella* which validates the identification process.

Molecular Assay: There was a motility test which displayed that all 13 Isolates of *Salmonella* are Motile, they contain flagella a species character of *Salmonella*. The overall motile isolates count was also followed with the rest of the tests and showed that a hospital 4 had the highest frequency of motile isolates.

This was deduced from the antibiogram test result since the levels of antibiotic resistance exhibited by *Salmonella* isolates were extremely high. The highest resistance profile was compared to ampicillin with 100% followed by tetracycline at 76.9% and chloramphenicol at 53.8%. Such high resistance rates therefore imply that the management of infections caused by *Salmonella* is posing great concerns within the health care facilities. For the other antibiotics, the susceptibility rate was azithromycin (92.3 percent), piperacillin, and gentamicin. These features should alert a continuous monitoring of antibiotic resistance and the urgent requirement for effectively coordinated appeal for the appropriate usage of antimicrobials to stop the emergence of such resistant forms of *Salmonella.*

Last but not least, it is imperative to contend that the presence of *Salmonella* in biomedical wastes from the involved hospitals, and its detection through a series of tests, is dangerous for human beings, and in this regard, there is a dire need to improve biomedical waste disposal and infection control measures. This is also because different and many kinds of waste are managed in many different ways in hospitals because of which cross-contamination is possible. In total, the high antibiotic resistance detected calls for an immediate reaction regarding the problems associated with MDR Salmonella strains.

5.3 Prevalence of Shigella

What the study also showed was that *Shigella* was present in a sample of biomedical wastes taken from dustbins of hospitals and out of 87 samples examined for the study, 17 were *Shigella* thus the prevalence rate of Shigella was 19.5%. It also confirms a high detection of *Shigella* in the hospital wastes suggesting that there is a health risk to the general public through contact with such wastes save for proper disposal methods. These isolates underwent identification through PCR in which the IPAH gene was again amplified; this gene is universal to all four species of *Shigella*. The identity of the bacterial species detected in both PCR methods was further confirmed using gel electrophoresis.

The following identification of the isolates was done by Gram staining in which all the seventeen isolates were observed to be Gram-negative: a feature that is inherent in *Shigella.* The pink color under the microscope suggested a lipopolysaccharide cell wall which is characteristic of Gram-negative organisms. The first test was crucial in the exclusion of the general groups of bacteria because after that biochemical tests were conducted to produce narrower results.

The biochemical tests also played an added advantage in the confirmation of identification of the isolates as *Shigella*. TSI lower control showed that all isolates fermented glucose, and others did not go through fermentation of lactose and sucrose which is in accord with *Shigella'*s species' Biochemical activities. No formation of the gas or hydrogen sulfide was produced in this case which tally with the normal biochemical characteristic of *Shigella*. The number of positive isolates from the selected hospitals and identified *Shigella* isolates presented discrepancies in the number of isolates thereby suggesting differences in waste contamination most notably in Hospital 4 with the highest number of isolates.

The citrate utilization test revealed that only five of the seventeen isolates are capable of using citrate as its carbon source, while the rest of the isolates have no discerned citrate metabolic ability. This is in accord with the typical non-citrate utilizing nature of *Shigella*. The test that we performed to assess the presence of the catalase enzyme amongst the isolates agreed with the above observation as all the samples tested positive for catalase and the formation of bubbles was noticed after the addition of hydrogen peroxide. That means *Shigella* can metabolize with hydrogen peroxide, which in actuality is a toxic substance that is produced during the process of aerobic respiration.

The oxidase test, though, revealed no color change in any of the isolates, hence supporting the finding that *Shigella* does not possess the cytochrome oxidase enzyme, which differentiates this genus. This negative result on the oxidase test was in harmony with the identification of the isolates as *Shigella*.

Further characterization of the metabolic profiles of the isolates was made by performing the Methyl Red (MR) and Voges-Proskauer (VP) tests. The result of the MR test also affirmed this as 15 out of 17 isolates assumed a red color in the presence of the indicator, confirming the

production of stable acidic byproducts from glucose fermentation typical of *Shigella*. The VP test however revealed that only 3 isolates were VP positive, a common negativity for *Shigella* which is in agreement with previous experiments. These tests were very critical in establishing the fermentation behavior of the bacterial strains.

As expected, the motility test showed that only 5 out of 17 isolates were motile, which is consistent with the characteristics of *Shigella*, a genus of bacteria that do not possess flagella. The remaining 5 isolates in which the motility was observed at least to some degree can represent phenotypic variants or species closely related to the genus.

Making a comparison with the antibiogram, the resistance pattern of the *Shigella* isolates awoke a concern. The highest percentage resistance recorded was from both Ampicillin and erythromycin both of which had zero sensitivity; implying that they cannot be used in the treatment of *Shigella* infections from biomedical wastes. Tetracycline and chloramphenicol also revealed moderate resistance rates among the seventy-nine isolated bacteria. However, the level of resistance to some of the antibiotics including the piperacillin-tazobactam was either low or negligible at some times and might be useful in treatment. The differentiation of the hospitals shows how the antibiotic resistance is different, and therefore the antibiotics' use should be observed and reconsidered from time to time while handling *Shigella*.

Therefore, these studies bring the discovery of the high incidence of *Shigella* in biomedical waste and promote the measures that can be adopted to improve the sanitary and safety measures of waste disposal to prevent probable diseases caused by the pathogen. This is evidenced by the constant appearance of resistance to such a broad-spectrum antibiotic in outpatient clinic populations; therefore, strict antibiotic stewardship is necessary to curtail the spread of such resistant *Shigella* species.

5.4 Prevalence of *E. coli*

Of the 87 bacterial isolates studied, 28 contained *E. coli*, which reflects a prevalence rate of 32.2% in bacterial samples from hospital dustbins. This recognition was validated with biochemical tests and Polymerase Chain Reaction (PCR) that used primers specific to *E. coli*, targeting eco-f and eco-r. The findings of PCR were confirmed by gel electrophoresis, which confirmed the presence of *E. coli* in the waste samples.

The identification as Gram-negative bacteria was supported by the Gram staining of the 28 *E. coli* isolates. All isolates showed the anticipated pink color, reflecting the presence of a lipopolysaccharide cell wall, characteristic of gram-negative microorganisms such as *E. coli.* Microscopic observations of the safranin dye retained in these isolates provided an important step for identifying the bacterial species, setting the stage for additional tests.

The Triple Sugar Iron test (TSI) indicated that all 28 *E. coli* isolates fermented glucose, lactose, and sucrose, causing the butt and slant to turn yellow. The yellow color of the medium signaled that fermentation processes within it had been completed. In the same vein, the formation of bubbles and cracks indicates that gasses (H_2 and CO_2) are present, while the absence of a black precipitate points to an absence of hydrogen sulfide (FeS). The allocation of positive E. coli isolates in hospitals was inconsistent, with Hospital 4 featuring the greatest number of these isolates.

From the results of the Citrate Utilization Test, it became clear that 9 of the 28 isolates appeared blue, which suggested that citrate had split into oxaloacetate, acetate, and CO_2. The leftover 19 isolates reported no color transformation, which meant that there was no citrate metabolism present. These observations are compatible with the typical characteristics of E. coli, as they frequently do not acquire their carbon from citrate. The highest quantity of positive isolates was found at Hospital 4, then at Hospitals 3, 2, and 5.

Every one of the 28 *E. coli* isolates in the catalase Test showed bubble formation, revealing a positive catalase reaction. The presence of hydrogen peroxide breakdown bubbles (into water and oxygen) served to confirm that there were catalase enzymes in these isolates. The outcome from catalase testing corresponds with the recognized metabolic qualities of E. coli.

According to the Oxidase Test results, 25 of the 28 isolates showed a negative result, showing no change in color because they lacked the cytochrome oxidase enzyme. The three remaining isolates indicated a positive readout, producing a purple color because of the oxidation of tetramethyl-p-phenylenediamine. These results more specifically characterized E. coli in contrast to other bacteria that have cytochrome oxidase.

Of the 28 isolates tested in the Methyl Red (MR) test, 22 produced a positive outcome, transforming red once methyl red reagent was added. This demonstrated the creation of stable acidic byproducts typical of mixed-acid fermentation by *E. coli.* The last six isolates changed to yellow, highlighting a less acidic context and an MR test result negative.

The Voges-Proskauer (VP) test found that every one of the 28 *E. coli* isolates tested gave negative results, exhibiting no red color when adding Barritt's A and B reagents. The copper coloration identified confirmed that there was no acetyl methyl carbinol, following the recognized metabolic profile of *E. coli.*

A total of 25 from the 28 tested isolates were shown to be motile, possessing flagella that helped them to move toward advantageous environments. The final three isolates were immotile, and deficient in movement, potentially providing evidence of phenotypic variations within the species. Hospital 4 had the greatest total of motile E. coli isolates.

Ultimately, tests for the antibiogram indicated that the 28 E. coli isolates showed different qualities of resistance and susceptibility to antibiotics. The maximum resistance was noted with ampicillin (67.9%), then by ciprofloxacin (35.7%) and finally trimethoprim (32.1%). By comparison, gentamicin showed the highest susceptibility, with figures of 100%, respectively. The observed pattern of antibiotic resistance stresses the critical necessity for cautious antibiotic selection in the management of E. coli infections derived from hospital waste.

These findings underscore the prominent nature of *E. coli* in hospital waste management and again emphasize the critical demand for effective waste management and infection control systems. The varying antibiotic resistance profiles stress the essential nature of surveillance and management of E. coli infections in healthcare sites.

Limitation

When conducting this thesis, several limitations were observed that might have affected the generality of the analysis. The first and major drawback was the limited sample size due to 87 isolates obtained from five hospitals. These samples were beneficial in providing more insight, but may not generalize to the wide variability in microbial contamination in different healthcare or geographic regions. Variability in waste management practices within the selected hospitals can also act as a challenge since such variability can complicate the information being collected. Of course, the limited sample size would not allow making some more generic conclusions regarding the kinds of microbial contamination, which biomedical waste may contain.

Another significant limitation of this study was the scope of bacterial identification, which focused on only four bacterial genera: *Pseudomonas, Salmonella, Shigella, and Escherichia coli.* However, as powerful indicators of infection and contamination risks in biomedically generated waste, these are not the only potentially dangerous microbes that could have been detected but were not. Furthermore, the Antibiotic susceptibility tests performed here were more comprehensive only within the chosen panel of agents, but the susceptibility testing was not done for all potential antibiotics; therefore, the results might underestimate the total level of resistance, including multidrug resistance. It also lacks molecular approaches like detection of certain gene resistance or the incapability of identifying the certain driving mechanism of antibiotic resistance.

Finally, they discuss some of the limitations that pertain to the methodology of the study. As a conventional diagnostic technique employed in the identification and characterization of bacteria, the PCR and biochemical tests have limitations to their use. This suggests that, for advanced stages of infection, the use of qPCR alone may not give a view of the entire picture without the use of other molecular techniques to analyze resistance gene expression and function.

Moreover, a cross-sectional study means that the data collected in the study only gives a picture of bacterial contamination and resistance at one given period without realizing changes on either a short or long-term basis. That is why using this cross-sectional approach, it is possible to identify current levels of contamination at best but cannot monitor how bacterial populations or antibiotic resistance might change. A more extended analysis of data collection conducted in

several months or years would reveal new patterns or trends in the development of resistance, which is necessary to consider the threat of biomedical waste. Such limitations should be suitably addressed in future studies to gain enhanced insight into the risks associated with biomedical wastes as well as the bacterial species concerned.

Conclusion

The results of the investigated biomedical waste samples from different hospitals as done above have provided information concerning more often occurring and typical pathogenic bacteria such as *E. coli, Pseudomonas, Salmonella, and Shigella.* The prevalence rate of *E. coli* was recorded at 32.2%, taking the first four positions in the medium category among all the isolates. This was further affirmed through the PCR method coupled with biochemical tests to assess the ability of these methods to reveal the kinds of bacteria in waste.

The biochemical tests that are conducted here support the identification of *E.coli* as a microbial belonging to the Gram-negative category and also able to ferment the sugars and produce acid. The findings and analysis revealed variations in the count of *E. coli* isolates across the different hospitals, although one of the hospitals had the highest number of the bacteria. Such differences justify calls for more intensified infection control measures in hospitals that are associated with relatively high bacterial loads, because they may cause higher infection risks in the concerned hospitals.

Another organism that was considered in the study was *Pseudomonas*, and this showed fairly high antibiotic resistance, especially to ampicillin and tetracycline. From the antibiogram tests, it emerged that the majority of the isolates exhibited resistance, a factor that makes the treatment of infection emanating from this bacterium a very sensitive operation. The presence of high resistance levels shows the need to prevent and control the development of resistant strains in healthcare facilities more aggressively through the use of sound antimicrobial stewardship programs.

Also, *Salmonella* and *Shigella* health threats are evident from their detection within biomedical waste hence underlining the need for pathogen checks. Both bacteria are also familiar with contracting gastrointestinal diseases and can pose a threat to life. The presence of these pathogens in hospital waste sheds light on the risks they pose to healthcare workers, patients, and

the community at large; hence the need for time-tested waste disposal strategies to avoid polluting the environment.

In conclusion, this study has brought out important findings on microbial risk factors in biomedical waste which should be useful in formulating surveillance and management measures. The research recommends enhanced awareness and practice by facilities in the health sector regarding the disposal of biomedical waste and disposal to avoid a negative impact on public health. Further investigations to combat the threats from antibiotic-resistant pathogens and implementation of corresponding policies essential to minimize the dangerous effects of biomedical waste in healthcare settings are the further directions of research.

References

Abidar, N., Tiskat, S., & Zohra, M. (2020). Chemical waste management in hospital; impact on environment and health. *Journal Wetenskap Health*, *1*(2), 36–41. https://doi.org/10.48173/jwh.v1i2.36

About Pseudomonas aeruginosa. (2024, April 11). *Pseudomonas Aeruginosa*. https://www.cdc.gov/pseudomonas-aeruginosa/about/index.html

Adewoyin, Y. (2017). Gender mainstreaming, solid waste management and urban health in Nigeria. *ResearchGate*. https://www.researchgate.net/publication/321670712_Gender_Mainstreaming_Solid_Waste_Management_and_Urban_Health_in_Nigeria

Aljamali, N. M., Alfatlawi, I. O., Mohammed, N. S., & Almosawy, M. G. A. A. (2020). Review on Effect of Chemical Waste on Environment. *ResearchGate*. https://www.researchgate.net/publication/342614035_Review_on_Effect_of_Chemical_Waste_on_Environment

Aryal, S. (2022, August 10). *Methyl Red (MR) test- principle, procedure, and result interpretation*. Microbiology Info.com. https://microbiologyinfo.com/methyl-red-mr-test-principle-procedure-and-result-interpretation/

Aryal, S. (2022b, August 10). *Oxidase Test- Principle, uses, procedure, types, result interpretation, examples, and limitations*. Microbiology Info.com. https://microbiologyinfo.com/oxidase-test-principle-uses-procedure-types-result-interpretation-examples-and-limitations/

Aryal, S. (2022c, August 10). *The Triple Sugar Iron (TSI) Test – Principle, Procedure, Uses, and Interpretation*. Microbiology Info.com. https://microbiologyinfo.com/triple-sugar-iron-tsi-test/

Aryan, A. (2023). *Efficient Household Biomedical Waste Management - SRIRAMs IAS*. https://www.sriramsias.com/upsc-daily-current-affairs/household-biomedical-waste-management/

Babu, B. R., Parande, A., Rajalakshmi, R., Suriyakala, P., & Volga, M. (2009). *Management of biomedical waste in India and other countries: a review*. https://www.semanticscholar.org/paper/Management-of-Biomedical-Waste-in-India-and-Other-A-Babu-Parande/a17dec1eebad2145b729b0258b4e8ff0d160b16b

Banerjee, S. (2013). *Radioactive and Hospital Waste Management : A review*. https://www.semanticscholar.org/paper/Radioactive-and-Hospital-Waste-Management-%3A-A-Banerjee/7b8a6b6fe3cddd0cae6fe733f1f37f89ccae1506

Basak, S. R., Mita, A. F., Ekra, N. J., & Alam, M. J. B. (2019). A STUDY ON HOSPITAL WASTE MANAGEMENT OF SYLHET CITY IN BANGLADESH. *International Journal of Engineering Applied Sciences and Technology*, *04*(05), 36–40. https://doi.org/10.33564/ijeast.2019.v04i05.006

Catalase test. (2010). ASM.org. https://asm.org/protocols/catalase-test-protocol#:~:text=The%20catalase%20test%20facilitates%20the,speciation%20of%20certain%20gram%20positives.

Chowdhury, A. M. M. A., & Uddin, K. N. (2022). Analysis of the Occurrence of Antibiotic-Resistant Bacteria in the Hospital's Effluent and its Receiving Environment. *Microbiology Insights*, *15*, 117863612210782. https://doi.org/10.1177/11786361221078211

Citrate test protocol. (2009). Citrate Test Protocol. https://asm.org/protocols/citrate-test-protocol

Clinical and Laboratory Standards Institute. (2020). *CLSI 2020 guidelines*. National Institute of Health. https://www.nih.org.pk/wp-content/uploads/2021/02/CLSI-2020.pdf

Cullinane, A. A., Barr, B., Bernard, W., Duncan, J., Mulcahy, G., Smith, I., & Timoney, J. (2006). Infectious diseases. In *Elsevier eBooks* (pp. 1–111). https://doi.org/10.1016/b978-0-7020-2769-7.50006-x

Cytotoxic Waste Disposal & Management | Cleanaway. (2024, September 5). Cleanaway. https://www.cleanaway.com.au/waste/cytotoxic-waste/

Dahal, P. (2023, October 6). *VP Test: Principle, reagents, procedure, results, uses*. Microbe Notes. https://microbenotes.com/voges-proskauer-vp-test/

Dana, T. (2014). Hospital Waste Management: Bangladesh. *www.academia.edu*. https://www.academia.edu/3308726/Hospital_Waste_Management_Bangladesh

Davis, R., & Pezzlo, M. (2023). 3.17.30 Motility Tests. In *Clinical Microbiology Procedures Handbook*. ASM Press. https://www.clinmicronow.org/doi/10.1128/9781683670438.CMPH.ch3.17-30

Dhaka Tribune. (2023, September 12). The legal side of medical waste disposal. *Dhaka Tribune*. https://www.dhakatribune.com/opinion/op-ed/333088/the-legal-side-of-medical-waste-disposal

Erkmen, O. (2021). Gram staining technique. In *Elsevier eBooks* (pp. 99–105). https://doi.org/10.1016/b978-0-323-91017-0.00028-7

European Food Safety Authority. (2024, March 21). *Salmonella*. https://www.efsa.europa.eu/en/topics/topic/salmonella

Gashaw, M., Gudina, E. K., Ali, S., Gabriele, L., Seeholzer, T., Alemu, B., Froeschl, G., Kroidl, A., & Wieser, A. (2024). Molecular characterization of carbapenem resistance in Gram-negative isolates obtained from clinical samples at Jimma Medical Center, Ethiopia. *Frontiers in Microbiology*, *15*. https://doi.org/10.3389/fmicb.2024.1336387

Ghasemi, L., Yousefzadeh, S., Rastkari, N., Naddafi, K., Far, N. S., & Nabizadeh, R. (2018). Evaluate the types and amount of genotoxic waste in Tehran University of Medical Science's hospitals. *Journal of Environmental Health Science and Engineering*, *16*(2), 171–179. https://doi.org/10.1007/s40201-018-0305-7

Giannella, R. A. (1996b). *Salmonella*. Medical Microbiology - NCBI Bookshelf. https://www.ncbi.nlm.nih.gov/books/NBK8435/

Hale, T. L., & Keusch, G. T. (1996). *Shigella*. Medical Microbiology - NCBI Bookshelf. https://www.ncbi.nlm.nih.gov/books/NBK8038/

Hassan, J., Parvej, M. S., Rahman, M. B., Khan, M. S. R., Rahman, M. T., Kamal, T., & Nazir, K. N. H. (2014). Prevalence and Characterization of Escherichia coli from Rectal Swab of Apparently Healthy Cattle in Mymensingh, Bangladesh. *Microbes and Health*, *3*(1), 12–14. https://doi.org/10.3329/mh.v3i1.19775

Hassan, M. M., Ahmed, S. A., Rahman, K. A., & Biswas, T. K. (2008). Pattern of medical waste management: existing scenario in Dhaka City, Bangladesh. *BMC Public Health*, *8*(1). https://doi.org/10.1186/1471-2458-8-36

Hheadmin. (2019, December 9). Waste disposal in healthcare and effects on AMR. *Hospital Healthcare Europe*. https://hospitalhealthcare.com/latest-issue-2016/waste-disposal-in-healthcare-and-effects-on-amr/

Hirani, D. P., Villaitramani, K. R., & Kumbhar, S. J. (2014). Biomedical Waste: An Introduction to its Management. *International Journal of Innovative Research in Advanced Engineering (IJIRAE)*, *1*(8), 82–83. https://www.nswai.org/docs/Biomedical%20Waste%20An%20Introduction%20to%20its%20Management.pdf

Kawade, H. M., & Kokare, D. M. (2023). Development strategies for pharmaceutical waste management: given healthcare perspectives. In *Elsevier eBooks* (pp. 97–121). https://doi.org/10.1016/b978-0-323-90909-9.00006-x

Kawade, H. M., & Kokare, D. M. (2023b). Development strategies for pharmaceutical waste management: given healthcare perspectives. In *Elsevier eBooks* (pp. 97–121). https://doi.org/10.1016/b978-0-323-90909-9.00006-x

Khan, S., Syed, A., Ahmad, R., Rather, T. A., Ajaz, M., & Jan, F. (2010, January 1). *Radioactive waste management in a hospital*. PubMed Central (PMC). https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3068798/

Kuriyama, T., Karasawa, T., & Williams, D. W. (2014). Antimicrobial chemotherapy. In *Elsevier eBooks* (pp. 209–244). https://doi.org/10.1016/b978-0-12-397043-5.00013-x

Lampel, K. A., Formal, S. B., & Maurelli, A. T. (2018). A brief history of Shigella. *EcoSal Plus*, *8*(1). https://doi.org/10.1128/ecosalplus.esp-0006-2017

Manasi, S., Umamani, K. S., & Latha, N. (2014). *Biomedical Waste Management: Issues and concerns - A ward level study of Bangalore City*. http://arks.princeton.edu/ark:/88435/dsp01rj430680k

Marina. (2022, June 2). *What is the purpose of microbiological testing? What types of tests are included?* Trung Tâm Y Khoa Diag. https://diag.vn/en/thongtinyte/what-is-the-purpose-of-microbiological-testing/#:~:text=This%20method%20has%20many%20advantages,yet%20found%20a%20specific%20antigen.

Medical Waste | US EPA. (2024, May 17). US EPA. https://www.epa.gov/rcra/medical-waste

Ministry of Health and Family Welfare of the People's Republic of Bangladesh: Guideline for Medical Waste Management. Ministry of Health and Family Welfare of Bangladesh, Dhaka; 2016.

Mumy, K. L. (2024). Shigella. In *Elsevier eBooks* (pp. 501–503). https://doi.org/10.1016/b978-0-12-824315-2.01000-9

National Institutes of Health. (n.d.). *Medical pathological waste*. https://nems.nih.gov/environmental-programs/Pages/Medical-Pathological-Waste.aspx

Onyeaka, H. N., & Nwabor, O. F. (2022). Microbial food contamination and foodborne diseases. In *Elsevier eBooks* (pp. 19–37). https://doi.org/10.1016/b978-0-323-85700-0.00002-2

Padmanabhan, K., & Barik, D. (2019). Health Hazards of Medical Waste and its Disposal. In *Elsevier eBooks* (pp. 99–118). https://doi.org/10.1016/b978-0-08-102528-4.00008-0

Padmanabhan, K., & Barik, D. (2019b). Health Hazards of Medical Waste and its Disposal. In *Elsevier eBooks* (pp. 99–118). https://doi.org/10.1016/b978-0-08-102528-4.00008-0

Polymerase chain Reaction (PCR). (2024). Genome.gov. https://www.genome.gov/genetics-glossary/Polymerase-Chain-Reaction

Rahman, M. M., Bodrud-Doza, M., Griffiths, M. D., & Mamun, M. A. (2020). Biomedical waste amid COVID-19: perspectives from Bangladesh. *The Lancet Global Health*, *8*(10), e1262. https://doi.org/10.1016/s2214-109x(20)30349-1

Ryberg, A. (n.d.). *MNTAP – infectious waste*. http://www.mntap.umn.edu/industries/facility/healthcare/waste/infectious/

Sharma M (2002): Hospital waste management and its monitoring, (1st ed.), Jaypee Brothers Medical Publication

Singh, H., Yt, K., Mishra, A. K., Singh, M., Mohanto, S., Ghumra, S., Seelan, A., Mishra, A., Kumar, A., Pallavi, J., Ahmed, M. G., Sangeetha, J., & Thangadurai, D. (2024). Harnessing the foundation of biomedical waste management for fostering public health: strategies and policies for a clean and safer environment. *Deleted Journal*, *6*(3). https://doi.org/10.1007/s42452-024-05735-2

Singh, N., Ogunseitan, O. A., & Tang, Y. (2021). Medical waste: Current challenges and future opportunities for sustainable management. *Critical Reviews in Environmental Science and Technology*, *52*(11), 2000–2022. https://doi.org/10.1080/10643389.2021.1885325

Sitotaw, B., Ayalew, F., Girma, A., Geta, K., Tadesse, B., & Birhanu, A. G. (2024). High prevalence of antibiotic resistance bacteria isolated from Bahir Dar City Municipal solid Waste Dumpsite, North West Ethiopia. *Environmental Health Insights*, *18*. https://doi.org/10.1177/11786302241260508

Sspf. (2019, February 12). Daniel Elmer Salmon | Inducted 1918. *SaddleandSirloin*. https://www.saddleandsirloinportraitfoundation.org/post/daniel-elmer-salmon-inducted-1918

Tripathi, N., & Sapra, A. (2023, August 14). *Gram staining*. StatPearls - NCBI Bookshelf. https://www.ncbi.nlm.nih.gov/books/NBK562156/

Types of medical waste. (n.d.). https://www.malsparo.com/types.htm

U.S. Food and Drug Administration. (2021). *Safely using sharps (needles and syringes) at home, at work, and on travel*. https://www.fda.gov/medical-devices/consumer-products/safely-using-sharps-needles-and-syringes-home-work-and-travel

Ullmann, A. (2011). Escherichia coli and the Emergence of Molecular Biology. *EcoSal Plus*, *4*(2). https://doi.org/10.1128/ecosalplus.1.1.2

What is Pathological Waste? (2024, January 26). Daniels Health. https://www.danielshealth.com/knowledge-center/what-pathological-waste

WHO (1999, December 20). *Guidelines for safe disposal of unwanted pharmaceuticals in and after emergencies*. https://www.who.int/publications/i/item/guidelines-for-safe-disposal-of-unwanted-pharmaceuticals-in-and-after-emergencies

World Health Organization. (n.d.). *Training modules in healthcare waste management: Module 9 – Classification of healthcare waste*. https://cdn.who.int/media/docs/default-source/wash-documents/wash-in-hcf/training-modules-in-health-care-waste-management/module-9--classification-of-health-care-waste.pdf

World Health Organization: WHO. (2018, February 8). *Health-care waste*. https://www.who.int/news-room/fact-sheets/detail/health-care-waste

World Health Organization: WHO. (2018a, February 7). *E. coli*. https://www.who.int/news-room/fact-sheets/detail/e-coli