Identification of *Pseudomonas aeruginosa* from Post-Traumatic Wounds in Dhaka and their Antimicrobial Susceptibility Profiles: A single-center cross-sectional study

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of **Bachelor of Science in Biotechnology / Microbiology**

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Declaration

It is hereby declared that,

1. The submitted thesis is my/our own original work while completing a degree at BRAC University.

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

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

4. I/We have acknowledged all main sources of help.

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Ethics Statement

The permission to conduct this study was obtained from the departmental review board at National Institute of Traumatology and Orthopaedic Rehabilitation (NITOR) hospital Dhaka, Bangladesh with swab samples of post-traumatic wound infections from the patients and consent was taken in agreement to use the samples for thesis purposes. The patient's privacy and the data's confidentiality were strictly protected during data collection.

Abstract/Executive Summary:

The purpose of the experimental study was to isolate the target organisms *Pseudomonas aeruginosa* and *Staphylococcus aureus*, from patients' wounds, which were generally recognized as post-traumatic wounds. All 45 patients were selected for post-traumatic wound cases, with 41 female and 4 male patients, and samples were taken from wound-infected areas. Next, the samples were cultured in two selective media cetrimide agar and mannitol salt agar (MSA) for these two specific organisms. After that, subculture was done on nutrient agar. Following this, DNA extraction was conducted in order to do the polymerase chain reaction (PCR) and agarose gel electrophoresis. Lastly, Kirby- Bauer Disc Diffusion method was used and CLSI guideline was followed for Antibiotic Susceptibility Test (AST). We found 18 isolates of *Pseudomonas aeruginosa* but no growth rate of the other targeted organism, *Staphylococcus aureus*, in 45 samples. Even though all the isolates from the patient group showed some percentage of resistance toward the antibiotics. Moreover, gram-negative isolates of the patient group exhibited high resistance to imipenem, gentamicin, cefepime, ampicillin and susceptible were seen against amikacin, meropenem, and ciprofloxacin. Furthermore, gram-negative *Pseudomonas aeruginosa* is mostly resistant to cephalosporins, aminoglycoside, and carbapenem group of antibiotics. The findings of this study highlight the significance of regular surveillance for patterns of antibiotic resistance in wound infections, specifically in cases of post-traumatic wounds.

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1. Introduction

1.1. Wound Infection:

An infection may develop if bacteria or other organisms get into a wound. Redness, swelling, and rising pain are classic signs of a wound infection. Infection may occur in any sort of cut. However, wounds can be either due to trauma or surgical (a cut created during a treatment). Traumatic wounds may arise from accidents, fights, attacks, or weapons. An infection is more prone to occur in some kinds of wounds. However, infections from wounds can be prevented.

The presence of replicating organisms in a wound that causes host or tissue damage is referred to as wound infection (Robson, 1997). Wound infection may occur as a complication of surgery, trauma, or disease that may interrupt a mucosal or skin surface. Infection of the wound happened due to entry of the pathogens through breached skin. These pathogens stop healing and produce signs and symptoms. From the beginning of civilization, scientists have been fighting against infection. Wound infection is a challenging situation in recent times. For wound infection, mainly Methicillin-resistant staphylococcus aureus (MRSA) which is a form of contagious bacterial infection, and Extended-spectrum beta-lactamases (ESBL) producing gram-negative bacteria are of major concern (Maier et al., 2011). In general, there are two main types of wound infections. One is skin and other one is soft tissue infections.

Post-traumatic wounds are mainly defined as sudden and unplanned cut or puncture wounds that damage both skin and the underlying tissues caused by various reasons such as accidental cases or any kind of violence including surgical incisions involving minimal tissue damage, abrasions involving deep scrapes of any part of the body, lacerations involve in trauma overflow intrinsic tissue strength or a deep cut or flesh in skin, contusions involves in extensive damaged of tissue for blast or severe blunt, large open wounds involves in injury that causes external or internal excessive tissue damage or break into the tissue and more on. (Leaper & Harding, 2006)

Therefore, wound infection is very challenging to health care and a continuous burden nowadays. It's contagious and can spread from one person to another and from the hand to other body parts. There is a high chance of wound infection due to pathogenic attachment and growth at damaged skin where pathogens multiply in wound-damaged tissue and disrupt the healing process. (Harding, 2008)

Research showed that many bacteria present in the wounds that cause infection after traumas. Some of the most common pathogens are *Pseudomonas aeruginosa* and *Staphylococcus aureus*. However, *Pseudomonas* which has more than 140 species is mostly known as saprophytic and 25 species are directly related to humans and the reason for various diseases. Most pathogens that cause disease in humans are opportunistic infections including *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. cepacia*, *P. stutzeri*, *P. maltophilia*, and *P. putrefaciens*. *Pseudomonas aeruginosa* and *P. maltophilia* are the two species found in clinical specimens 80% of the time. So for the frequency of finding *Pseudomonas aeruginosa* which is a gram-negative opportunistic pathogen in most of the specimens, it gets a lot of attention and is one of the main target organisms. It causes disease mostly in unhealthy individuals and seldom infections in healthy cases. So it's a major threat to hospitalized patients rather than healthy people. It can be isolated from a healthy person's throat, stools, or other parts of the skin and is mostly motile with single polar flagellum some other strains contain double or triple flagella commonly colonize in the hospital's food, and water, tap, mops, and respiratory equipment and can spread from person to person by direct contiguity or consuming contaminated food or water in the hospital. The high mortality rate resulting from these infections is caused by a combination of weakened human defenses, antibiotic resistance, and the development of extracellular bacterial enzymes and toxins. (Iglewski, 1996)

Staphylococcus aureus is gram-positive spherically shaped bacteria belonging to the Bacillota family common member of the body's microbiota frequently found in the skin or the nose and upper respiratory tract and colonizes the nasal passage and axillae. It is one of a major pathogens directly related to human that causes wide range of diseases and clinical infections in hospitals and community. For instance bacteremia and infective endocarditis, superficial skin lesions (boils, styes) and localized abscesses in other sites, deep-seated infections such as osteoarticular, skin and soft tissue, pleuropulmonary, toxic shock syndrome by release of superantigens into the bloodstream and device-related infections. *Staphylococcus aureus* is another main cause of nonsocial or hospital-acquired infection of surgical wounds, as well as infections related to indwelling healthcare devices. It has grown into one of the most serious medical issues worldwide as a result of its expanding resistance to several antibiotics, rendering treatment more challenging. As multiple antibiotic resistance is common in the case of *Staphylococcus aureus*,

methicillin resistance is a sign of multiple resistance, which may result in hospital outbreaks and epidemics. (Foster, 1996; Wertheim et al., 2005)

1.2. Nosocomial Infection:

An infection acquired at a hospital or other health care center is referred to as a nosocomial infection or hospital-acquired infection. Patients receiving medical care could be exposed to nosocomial infections, which are also referred to as diseases related to hospitals. These infections can be found anywhere, in developed as well as developing countries. Infections from nosocomial bacteria are responsible for 7% of cases in developed countries and 10% in developing nations. These infections result in more prolonged hospital stays, impairments, and financial burdens.

Among the nosocomial pathogens include fungi, viruses, and bacteria. About 15% of all patients in hospitals experienced or suffered from these infections, according to the World Health Organization (WHO). Patients are exposed to infections while they are in the hospital through the environment, healthcare workers, and other hospitalized patients who are infected.

1.3. Global Situation of Wound Infection:

Wound infection can occur in several ways. For instance, cuts, lacerations and also during surgical intervention. Wound infection is caused by both bacteria and viruses. Mostly nosocomial bacteria are responsible for causing infection to the wound. Bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus aureus* are considered to be nosocomial bacteria. According to (Mengistu et al., 2023), the global rate of wound infection was 2.5%. The most affected region of wound infection was Africa with 7.2% and the least affected region was Western Pacific with 0.6%.

1.4. The Situation of Wound Infection in Bangladesh:

A study of wound infection was also conducted in hospitals in Bangladesh. From the study conducted by (Wound Infection in BSMMU, 2014) at Bangabandhu Sheikh Mujibur Rahman Medical University, 20.16% of patients were wound infected out of 496 patients. Following this study, the most isolated bacteria were *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Wound infections represent a substantial healthcare concern in Bangladesh, as evidenced by multiple research studies conducted in various medical settings across the country. (Wound Infection in BSMMU, 2014) conducted a study at Bangabandhu Sheikh Mujibur Rahman Medical University, where they found that 20.16% of 496 patients surveyed were afflicted with wound infections, underscoring the significant prevalence of this condition. Furthermore, (M Nur-e-elahi, 2014) identified *Staphylococcus aureus* and *Pseudomonas aeruginosa* as the most commonly isolated bacteria in wound infections, indicating the diverse range of pathogens contributing to the problem.

In a subsequent study, (Giacometti et al., 2000) conducted research at a tertiary care hospital in Bangladesh, analyzing 1709 samples to understand the landscape of wound infections in greater detail. Their findings revealed that a substantial proportion (72%) of the samples yielded growth of organisms, emphasizing the pervasive nature of wound infections within the healthcare system. Of particular concern is the predominance of gram-negative bacteria, which constituted 86.4% of the isolates, compared to 13.6% of gram-positive bacteria. *Pseudomonas spp* emerged as the most commonly isolated organism, comprising 43.8% of the samples obtained from both pus and wound swabs, followed closely by *Staphylococcus aureus*, constituting 11.8% of the isolates.

Moreover, additional research conducted by (Mama, 2014) underscored the importance of antibiotic susceptibility patterns in managing wound infections in Bangladesh. Their study highlighted the need for tailored treatment strategies based on local bacterial epidemiology and resistance patterns. Addressing the prevalence of wound infections in Bangladesh necessitates a comprehensive approach, including enhanced infection control measures, judicious antibiotic prescribing practices, and continuous surveillance to monitor bacterial resistance. The situation of wound infection in Bangladesh presents a significant public health challenge, requiring

concerted efforts from healthcare professionals, policymakers, and researchers to effectively manage and mitigate its impact on patient outcomes.

1.5. The Risk of Infection in Post-Traumatic Wound Infection Patients:

In the community of individuals with post-traumatic wounds, the potential for bacterial infection is a major concern that needs to be addressed because it can have serious consequences for the patient's overall health and the effectiveness of their treatment. Patients who have an infection from a post-traumatic wound frequently have slight redness or swelling around the area, bleeding, and possibly even a fever (Int Wound J., 2008). As a result, these individuals become more susceptible to bacterial colonization and subsequent health issues.

It is important to note that opportunistic microorganisms such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, have been identified as prevalent causative agents in causing infections. The complex interaction among bacterial infections, the primary disease, and its treatments emphasizes the significance of immediate treatment, specialized antimicrobial approaches, and comprehensive infection monitoring to reduce the negative impact of bacterial infections on the health of patients.

Infections caused by bacteria can deteriorate a patient's overall health, which could lead to an overall decrease in their functional level and a reduced capacity to tolerate intensive treatments. Furthermore, the use of antibiotics for bacterial infections may reduce the effectiveness of therapeutic medical treatments. In addition, bacterial infections can damage tissue and produce inflammation, which may prevent the healing of wounds and could even influence the outcome of surgical procedures (David J Leaper, 2006)

1.6. Opportunistic Microbes in Post-traumatic Wound Infection Patients:

One of the most important aspects of post-traumatic wound infections has been identified as the complex relationship that develops between individuals with wound infections and opportunistic microorganisms (Manal M Khan, 2020). People who have been diagnosed with wound infections are more susceptible to opportunistic bacterial colonization and subsequent infections. These

microorganisms utilize the weaknesses of the weakened immune system and modify the infected surroundings that are essential to the illness and its treatment approaches.

According to the Indian Journal of Medical Microbiology, various species of opportunistic microbes can be found in post-traumatic wound infection patients, including *Staphylococcus spp,* pseudomonas spp, etc. However, *Pseudomonas aeruginosa* among gram-negative bacteria was commonly found in post-traumatic wound infection sites in this study.

1.6.1. *Pseudomonas spp:*

Pseudomonas spp are a varied and medically significant category of gram-negative bacteria. It is commonly found in a variety of ecological habitats. Differentiating itself from other species, *Pseudomonas aeruginosa* is a very important and adaptable opportunistic pathogen. The pathogen is well known for its remarkable ability to induce a diverse range of illnesses, varying in severity, with a particular preference for individuals with impaired immune systems. *Pseudomonas aeruginosa* is a prominent etiological agent that is responsible for nosocomial infections, encompassing surgical site infections, and bloodstream infections (Vu & Carvalho, 2011)

1.6.2. *Staphylococcus spp:*

The gram-positive *Staphylococcus* species constitute a diverse category of bacteria. Among these kinds of organisms, *Staphylococcus aureus* stands out for being a multipurpose pathogen with a broad spectrum of pathogenicity and having undergone extensive investigation (Vu & Carvalho, 2011) This pathogen is a major cause of both community- and hospital-acquired infections. It can present in a wide range of ways, from infections of the bare skin and soft tissues to more invasive, serious illnesses like osteomyelitis, bacteremia, and endocarditis. Despite being widely accepted as a commensal organism, *Staphylococcus* epidermidis has emerged as a notable opportunistic infectious agent associated with medical device infections, particularly among patients with weak immune systems or those who recently underwent surgical treatment (Cuong Vuong, 2002)

1.7. Objective:

1.7.1. General objective:

- This study aimed to identify and isolate the microorganisms from post-surgical accidental patients from NITOR hospital to analyze their antibiotic resistance profile or to identify their susceptibility against widely used antibiotics.

1.7.2. Specific objectives:

- To identify the common microorganism e.g. *Pseudomonas aeruginosa,* and *Staphylococcus aureus* from post-traumatic wounds of patients.

- To identify the pathogens that cause wound infection in hospital patients.

- To determine the resistance of these two specific organisms against different antibiotics.

Other than that, this study investigated the antibiotic resistance profile and identified multi-drug-resistant bacteria in light of the increasing prevalence of multi-drug-resistant organisms. Lastly, this investigation aimed to conduct a comprehensive survey to estimate the epidemiological, etiological, and socio-economic characteristics of wound infection patients in Bangladesh.

2. Materials & Methods

2.1. Sample Collection Site:

The study was conducted at the National Institute of Traumatology & Orthopaedic Rehabilitation (NITOR), which is situated in Sher-e-Bangla Nagar, Dhaka. NITOR, also known as RIHD (Rehabilitation Institute and Hospital for the Disabled), is a prominent orthopedic hospital and undergraduate & postgraduate institute hospital in Bangladesh. It has been dedicated to providing specialized orthopedic care and rehabilitation services to patients with musculoskeletal injuries and disabilities. The establishment and evolution of orthopedic surgery in Bangladesh have strong historical ties to significant events such as the liberation war and the contributions of freedom fighters.

NITOR operates as a comprehensive orthopedic center equipped with hospital facilities, including an Intensive Care Unit (ICU), to cater to the diverse needs of patients requiring orthopedic treatment and rehabilitation services. Situated in the heart of Dhaka, NITOR serves as a beacon of hope for individuals suffering from traumatic injuries, disabilities, and orthopedic conditions, offering state-of-the-art medical care and advanced treatment modalities. The rest of the experiments were conducted at Brac University Thesis Laboratory.

2.2. Study Duration:

This study's identification of pathogens from post-traumatic wounds in Dhaka and their antimicrobial susceptibility profiles or the duration of this research work was mainly conducted between 1st March 2023 to 30th November 2023.

2.3. Study Population:

Swab samples and data were collected from the post-traumatic injury patients treated at the National Institute of Traumatology & Orthopaedic Rehabilitation (NITOR). In this study, a total of 45 swab samples were thus taken from patients from patients infection sites. Patients who were selected for sample collection were mainly infected by bacteria due to the period of post-traumatic injury prior to surgery. In a total of 45 samples, there were 41 female patients and 4 male patients. The age group was from 20 to 60 years old. This distribution reflects the prevailing demographic pattern commonly observed in orthopedic trauma cases, where male patients tend to comprise the majority due to various factors such as occupational hazards, sports

injuries, and road traffic accidents. By focusing on samples collected from both male and female patients, the study aimed to provide a comprehensive understanding of the bacterial flora associated with post-traumatic wounds in the orthopedic hospital setting, thus contributing to improved clinical management and patient outcomes.

2.4. Sample Collection Technique:

All post-traumatic wound samples were collected in sterile saline where sterile cotton swabs were used to collect the samples from the infected area of the wounds. Therefore, patients were both male and female but there were significantly more male patients than female as well. In addition, the samples were collected and handled carefully in the lab under the supervision of medical professionals and after proper sample collection and handover to the lab, further procedures were conducted including specimen culture at specific media.

2.5. Bacterial Culture:

The bacterial culture process begins with the collection and transportation of samples while maintaining a cold chain to preserve sample integrity. Following sample dilution using saline, 10-4 fold samples are subcultured onto two specific media: mannitol salt agar (MSA) for *Staphylococcus aureus* and cetrimide agar for *Pseudomonas aeruginosa*. These plates are then incubated at 37°C for 24 hours (AL-Fridawy et al., 2020; Sharp & Searcy, 2006). The agar was placed within the laboratory setting and then incubated at 37 degrees Celsius to facilitate bacterial growth. Upon incubation, colonies are examined for their morphological characteristics. *Staphylococcus aureus* colonies on MSA appear as motile, small-circulated yellow colonies with a yellow zone, indicative of mannitol fermentation. In contrast, *Pseudomonas aeruginosa* colonies on cetrimide agar display motile, circular growth with a green to bluish-green coloration due to pyocyanin production. This preliminary identification based on colony morphology provides valuable insights into the presence of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in the sample, guiding further confirmatory tests and subsequent therapeutic interventions as necessary. The first identification of these two target organisms is based on their morphological structure, and the primary structure is given below.

Table 2: Colony Morphology of Specific Organism

Targeted Organism	Gram (+/-)	Media	Colony Shape	Expected Colony Morphology
Pseudomonas aeruginosa	Gram-negative (-)	Cetrimide agar	Motile, and circulated	Green to bluish-green, glows under UV ray
Staphylococcus aureus	Gram-positive (+)	Mannitol salt agar (MSA)	Motile and small-circulated	Yellow/white colonies surrounded with yellow zone

Table 2: Colony Morphology of Specific Bacteria on Selective Media

2.6. Culture Media Used for Bacterial Isolation:

Based on the complex requirements of microbiological investigation, several kinds of culture media are strategically used in the identification and isolation of microorganisms. The selection of culture media is influenced by several factors, including the individual bacteria being studied, their nutritional requirements, and the desired outcomes of the research goals or diagnosis. Different media compositions are created to meet the metabolic needs of distinct species of bacteria, increasing the growth and facilitating the isolation of these species.

2.6.1. Cetrimide Agar:

The technique of isolating gram-negative Pseudomonas bacteria involves the use of cetrimide agar. Production of pyocyanin can be observed, exhibiting a distinctive greenish color.

To create the solution 1 liter of distilled water and 46.7 grams of powder were mixed properly. After that, the powder was boiled to dissolve it in water, and the resulting mixture was covered

with aluminum foil and autoclaved afterward. The liquid medium was then put into sterile petri dishes that had been cleaned, dried, and disinfected beforehand.

2.6.2. Mannitol Salt Agar (MSA):

Mannitol Salt Agar (MSA) is a selective and differential medium that is commonly used in microbiology laboratories. It is mostly used to isolate and identify pathogenic *Staphylococcus* species— mainly *Staphylococcus aureus*. A type of selective medium called Mannitol Salt Agar (MSA) is used to detect and separate gram-positive bacteria, especially *Staphylococcus* species, additionally inhibiting the growth of other bacterial species. Mannitol, a sugar, and phenol red, a pH indicator are the components of the material. The microorganism exhibits the capacity to ferment mannitol, producing an acidic byproduct in the process. The medium of agar containing phenol red changes color due to this byproduct, becoming yellow instead of its original color. It is possible for *Staphylococcus aureus* to ferment mannitol.

Throughout of the study, 111.02 grams of MSA powder were dissolved in one liter of distilled water in a conical flask. The mixture subsequently underwent the boiling procedure. After covering the conical flask in aluminum foil, it was placed in an autoclave for sterilizing it. Following sterilization, the boiled liquid medium was gently placed into a petri dish.

2.6.3. Nutrient Agar:

Nutrient agar is a culture medium in which a variety of non-fastidious microorganisms can be cultivated. This medium is well-known as it facilitates the growth of a variety of bacteria and fungi. 28 grams of nutritional agar powder were measured for the preparation and then dissolved in one liter of distilled water in a conical flask. The ingredients then went through the boiling procedure. After wrapping the conical flask in aluminum foil, it was sterilized in an autoclave. Following sterilization, the heated liquid medium was carefully poured into a petri dish.

2.7. Molecular Detection:

The main goal of molecular detection is to use advanced molecular techniques to precisely and purposely amplify, observe, and confirm the presence of the target genes of interest between complex biological materials, when it comes to gene identification. The technique described in

this study makes use of the core ideas behind polymerase chain reaction (PCR) and nucleic acid hybridization to retrieve genetic information with high sensitivity and specificity.

Cultural and colony counting techniques are dominant traditional techniques for identifying pathogenic bacteria and are broadly used for regulatory purposes but the results can be falsely positive or negative, lack of differentiation between target and non-target microbes of the same samples whereas analysis of reliable results molecular the technique can be a better option as it is faster, more sensitive and more specific to detecting organisms missed by cultural technique. So after the cultural process, we performed PCR which is one of the most common molecular-based techniques to identify pathogens by targeting specific DNA sequences (Zhang et al., 2021).

2.7.1. DNA isolation:

The boiling method was performed to extract the genomic DNA from the identified isolates. For the boiling process, the isolates were streaked on nutrient agar media and incubated at 37°C for 24 hours. After that, 150 microliter of TE buffer was added in Eppendorf tubes, and using a vortex machine, one loopful of the selected isolate was thoroughly mixed into the buffer.

The sample was then further heat shock to 95°C for 20 minutes using a thermocycler as a heat block. The cell debris precipitated as a result of centrifuging the cell suspension for 10 minutes at 4°C and 10,000 rpm. Subsequently, the final supernatant was collected which contained the DNA.

We used stock culture for preserving the targeted organism in T1N1 media where a needle full of microbes was taken from an isolated colony from nutrient agar. After that, it was stabbed in T1N1 media 3-4 times for preservation which can be stored at room temperature or freeze at (2 to 8)°C for further procedure and experiment stock revives can proceed, streaking for bacterial growth at NA.

2.7.2. PCR Amplification:

After performing DNA extraction, we proceed to Polymerase Chain Reaction (PCR) for confirmation.

A standard PCR procedure is given below-

- ➢ Adding PCR master mix (which includes forward primer, reverse primer, master mix, and nuclease-free water) and lastly template DNA to a PCR tube.
- ➢ All the PCR tubes were centrifuged for 30 seconds, 3000 rpm (Short spin), and added mineral oil to prevent evaporation in a thermal cycler.
- ➢ After that amplifying every thermal cycle and primer parameter.
- ➢ Lastly evaluates amplified DNA by agarose gel electrophoresis.

The process was started by taking an 11 microliter master mix, 2 microliter template DNA total volume of 13 microliter, product size 956 base pair, 3.5 microliters nuclease-free water, 0.5-microliter forward primer and reverse primer, and 6.5 microliter PCR master mix. The amplification was preceded by a rapid cycler (Idaho Technology Inc., Salt Lake City, Utah) thermocontroller. Therefore ,the PCR master mix contains equal amounts of thermostable DNA polymerase, optimized reaction buffers, dNTPs and MgCl2 (Spilker et al., 2004).

PCR Condition-

- Initial Denaturation- at 95°C for 2 Minute
- Denaturation- at 94°C for 20 seconds
- Annealing or Primer binding- at 58°C for 20 seconds
- Elongation- at 72°C for 40 seconds
- Final extension- at 72°C for 1 minute

To verify *Pseudomonas aeruginosa* the PCR amplification was done by pair PA-SS-F and PA-SS-R as it was designed to amplify only *Pseudomonas. Aeruginosa.* The gyrB gene is the target gene of this primer. This gene is a useful phylogenetic marker. In this study, our target organism is *Pseudomonas aeruginosa.*

Primer Sequence (5′-3′)

PA-SS-F- GGGGGATCTTCGGACCTCA

PA-SS-R- TCCTTAGAGTGCCCACCCG

2.7.3. Agarose Gel Electrophoresis:

After PCR performance we proceed to gel electrophoresis which is a common method to evaluate PCR success. By preparing the samples by various processes needed to prepare the agarose for the gel run which contains 1.5% agar at 100 ml TBE buffer and 35 ml ETBR was used to make the agarose. Need to be careful and wear gloves at the time of touching ETBR as it's a hazard and prone to cancer.

The procedure can be continued over a microwave or Bunsen burner. After agarose cool it down on the benchtop or by incubation in a 65°C water bath. Then place the gel tray in the casting device. Alternatively, one might build a mold by taping the open sides of a gel tray. To make the wells, place an appropriate comb into the gel mold. Pour the agarose into the gel mold and place it at room temperature. After removing the comb, the gel can be placed in the gel box and can be stored wrapped in plastic wrap and stored at 4°C until use.

Therefore, the samples were individually placed by using a pipette and adding a ladder. The PCR master mix contained an equal amount of dNTP, MgCl2, and Taq polymerase. The PCR products were analyzed by electrophoresis with 1% agarose gel concentration. The gel was stained with ethidium-bromide and visualized under a UV transilluminator. After proper procedure run the gel with electrophoresis. After completing the electrophoresis further observation proceeded (Gupta, 2019).

2.8. Antibiotic Resistance and Susceptibility Analysis:

An antibiotic susceptibility test (AST) is done to identify the sensitivity of bacteria to antibiotics. By doing so, physicians can easily prescribe the medicine to the patients. It is essential to evaluate the effectiveness of antibiotic susceptibility testing for significant isolates of bacteria. The whole purpose of the experiment was performed to identify the antibiotic profile of the bacteria from the collected samples. It was done by following the Kirby-Bauer disk diffusion formula and CLSI (Clinical and Laboratory Standards Institute) guidelines. Fourteen carefully selected antibiotics from commercial antimicrobial disks were used to evaluate the antimicrobial susceptibility pattern. These antibiotics have a variety of mechanisms of action, including targeting proteins, nucleic acids, and cell walls.

From the sub-cultured bacteria, an isolated colony was taken and mixed with 10 ml of saline. Then, the solution was matched with the “0.5 McFarland" standard to obtain the best result. The cotton swab was dipped into the inoculum and excess fluid was required to be removed by pressing the side of the tube.

2.8.1. Preparation of Muller Hinton Agar (MHA):

It is commonly known that Muller Hinton agar (MHA) is a helpful medium to evaluate antibiotic susceptibility testing (AST) Since this medium lacks differentiation and selectivity, all microbes that are introduced into it will proliferate. 38g of Mueller Hinton agar powder was dissolved in 1 liter of distilled stirring to create the media. To maintain sterility, aluminum foil was placed over the conical flask aperture and autoclaved. Following the process of sterilization, the liquid was poured into sterile petri dishes.

2.8.2. Bacterial Suspension Preparation:

The bacterial colony from the 24-hour-old culture was taken with a sterile loop and it was mixed with sterile 0.9% saline. The turbidity concentration was kept at 0.5 McFarland Standard solution.

Table 3: List of Antibiotics

Group Name	Antibiotics Name	Disc Code	Disc Potency (µg)	Susceptible (mm)	Intermediate (mm)	Resistance (mm)
	Amikacin	AK	30mcg	**≥17**	**15-16^**	**≤14**
Aminoglycoside	Gentamicin	GEN	10mcg	**≥15**	**13-14^**	**≤12**
	Netilmicin	NA	30mcg	**≥22**	**16-21^**	**≤15**
Peptide	Colistin	CL	10mcg	**≥25**	**19-24^**	**≤18**
Cephalosporins	Cefepime	FEP	30mcg	**≥18**	**15-17^**	**≤14**

	Cefixime	CFM	5mcg	**≥15**	**13-14^**	**≤12**
Fluoroquinolones	Ciprofloxacin	CIP	5mcg	**≥17**	**15-16^**	**≤14**
Tigecycline	Tigecycline	TGC	15mcg	**≥19**	**16-18^**	**≤15**
Tazobactam/ Piperacillin	Tazobactam/ piperacillin	TPZ	110mcg	**≥19**	**16-18^**	**≤15**
Monobactam	Aztreonam	ATM	30mcg	**≥18**	**15-17^**	**≤14**
Macrolides	Azithromycin	AZM	15mcg	**≥15**	**12-14^**	**≤11**
Carbapenem	Meropenem	MEM	10mcg	**≥22**	**18-21^**	**≤17**
Carbapenem	Imipenem	IMP	10mcg	**≥23**	**20-22^**	**≤19→**
Penicillin	Ampicillin	AMP	10mcg	**≥19**	**15-18^**	**≤14**

2.8.3. Inoculation and Disc Diffusion:

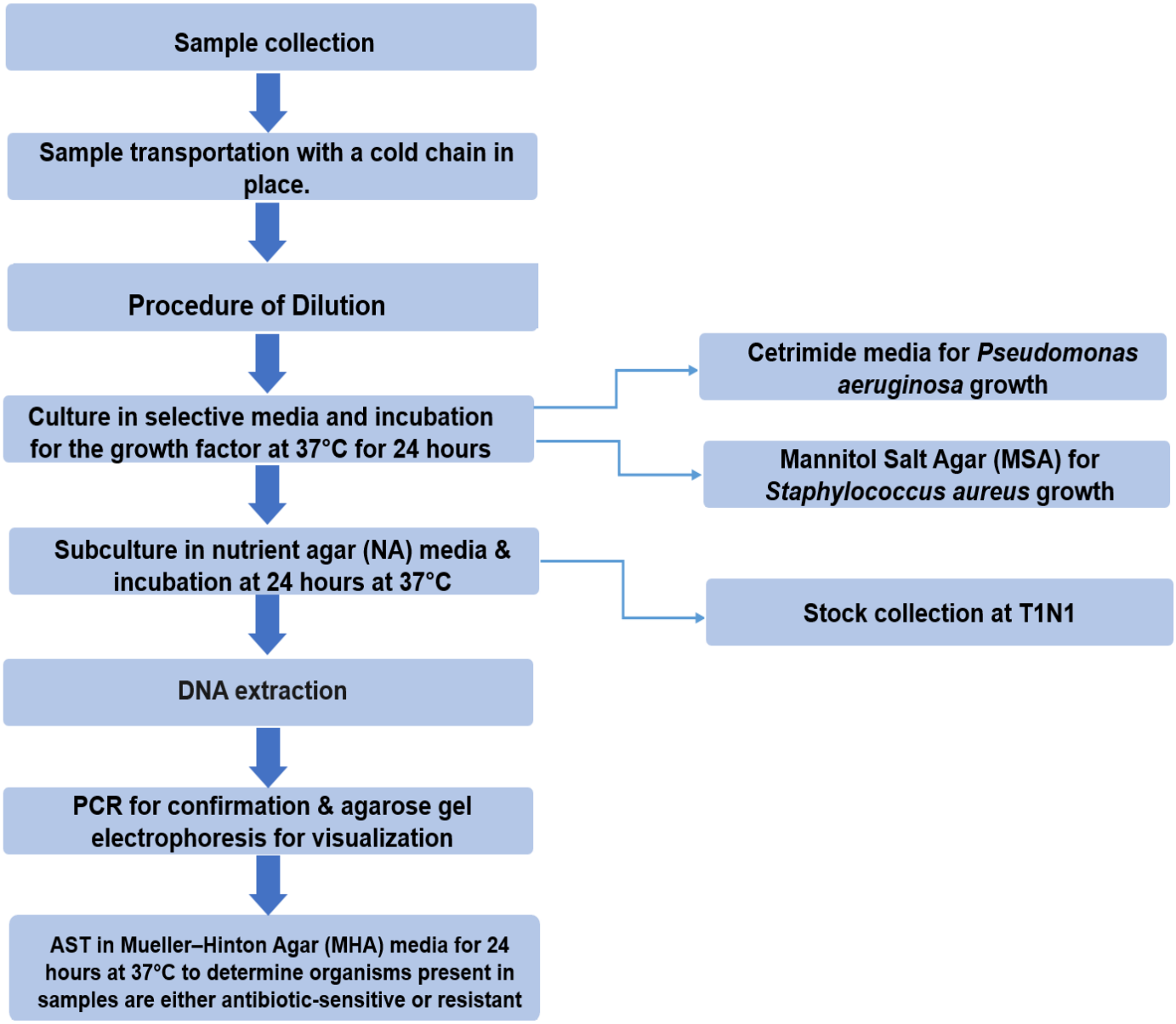
An autoclaved cotton swab was used to inoculate a Muller Hinton Agar (MHA) plate. The suspension of the bacterial mixture was submerged in the autoclaved cotton swab. Then, to create an evenly distributed grass culture, the swab was equally spread throughout the Mueller-Hinton Agar (MHA) plate surface. After the streaking process, the plate was allowed to dry for five minutes. Afterward, sterile forceps were used to properly place the antibiotic discs on the plate. The discs were placed to guarantee that there would be no overlap and that the area would be evenly distributed. After the discs were inserted, the plates were turned over and placed in the incubator for 24 hours at 37°C. Following the incubation period, the zone of diameter was measured and from the measurement of diameter, the result was determined.

2.9. Multi-drug Resistance (MDR) :

Multidrug resistance (MDR) is the ability of bacteria to withstand the effects of numerous antibiotic categories, limiting the efficacy of existing treatment options and posing a substantial problem in clinical settings. Multidrug-resistant *Pseudomonas aeruginosa,* a Gram-negative bacteria, has become a serious problem in healthcare institutions around the world due to its capacity to cause severe infections, especially in immunocompromised individuals and those with underlying health disorders (Tacconelli et al., 2018). Several risk factors influence the establishment and spread of multidrug-resistant *Pseudomonas aeruginosa* infections. Primary risk factors include prolonged hospitalization, broad-spectrum antibiotic exposure, invasive medical procedures including mechanical ventilation and indwelling catheters, immunocompromised conditions, and chronic disorders such as cystic fibrosis (Rodríguez-Martínez et al., 2021). These conditions promote the selection and development of multidrug-resistant pathogens, resulting in higher misery and fatality rates. Multidrug-resistant *Pseudomonas aeruginosa* infections have a significant clinical impact, as they are associated with longer hospital stays, higher treatment costs, increased rates of treatment failure, and higher mortality rates when compared to infections caused by susceptible strains (Tacconelli et al., 2018). Furthermore, multidrug-resistant Pseudomonas aeruginosa infections present considerable hurdles to infection control and prevention efforts in healthcare institutions, as they are easily transmitted between patients and healthcare personnel. To effectively manage multidrug-resistant *Pseudomonas aeruginosa* infections, a multifaceted approach is needed, including antimicrobial stewardship programs, enhanced infection control measures, resistance surveillance, and the development of new therapeutic strategies like combination therapies and alternative antimicrobial agents (Tacconelli et al., 2018); (Rodríguez-Martínez et al., 2021). Healthcare providers can better understand and mitigate the challenges posed by these formidable pathogens by addressing both the risk factors that contribute to multidrug resistance and the clinical impact of multidrug-resistant *Pseudomonas aeruginosa* infections, ultimately improving patient outcomes and lowering the burden of antimicrobial resistance in healthcare settings.

2.10. Experimental Workflow:

The following chart summarizes all of the processes that took place throughout the experimental study.


Sample collection
Sample transportation with a cold chain in place.
Procedure of Dilution
Culture in selective media and incubation for the growth factor at 37°C for 24 hours
Cetrimide media for *Pseudomonas aeruginosa* growth
Mannitol Salt Agar (MSA) for *Staphylococcus aureus* growth
Subculture in nutrient agar (NA) media & incubation at 24 hours at 37°C
Stock collection at T1N1
DNA extraction
PCR for confirmation & agarose gel electrophoresis for visualization
AST in Mueller–Hinton Agar (MHA) media for 24 hours at 37°C to determine organisms present in samples are either antibiotic-sensitive or resistant

3. Results

Result:

In our study conducted at the National Institute of Traumatology and Orthopaedic Rehabilitation (NITOR), we collected a total of 45 wound samples from orthopedic trauma patients. Among these samples, 91.1% were from female patients, while only 8.9% were from male patients.. Despite our extensive microbiological investigations, including bacterial culture and PCR analysis, none of the samples yielded growth of *Staphylococcus aureus,* indicating its absence in the wound samples.

Table 1: Patient age distribution

Women - 41 & Male - 4

Age Range	No of Patients
20 - 25	5
26 -30	7
31 - 35	10
36 - 40	6
41 - 45	7
46 - 50	3
51 - 55	4
56 - 60	3

Table 1: Patients Age Distribution

Gender and Age of Patients:

This experimental study was conducted at NITOR hospital to find out *Pseudomonas aeruginosa* of trauma patients. This microorganism is contagious and cause disease all over the world. This study included a total of 45 patients and our main targeted patients were those who were infected with bacterial flora reason stated as post-traumatic wounds. Mainly we collected

samples from legs infected with sterile cotton swabs. Overall patients were 45 but there were 41 female patients and 4 male patients with different age groups from 20 to 60 years old. (Figure 1).

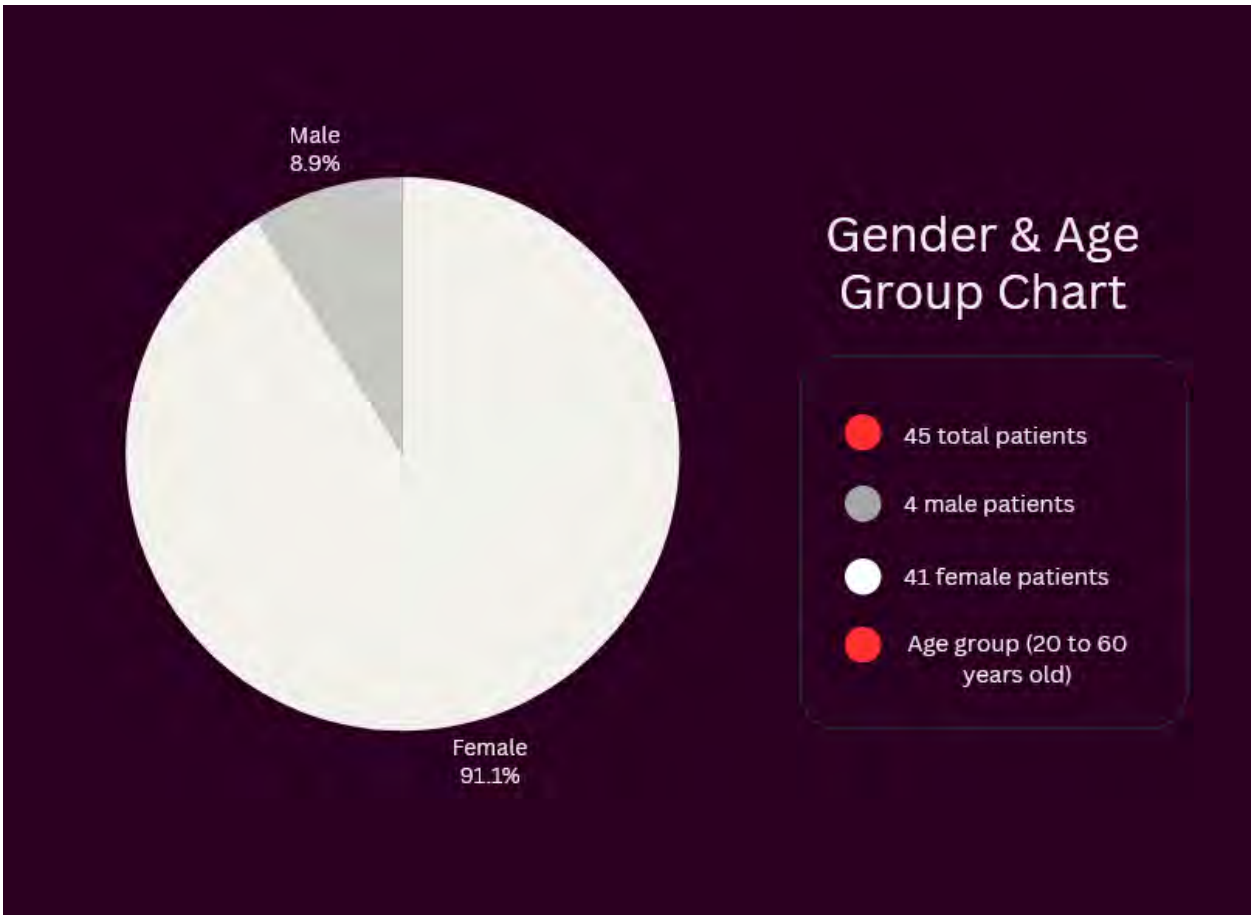


Figure 1: Gender and Age Group of the Patients

Saline dilution was employed, followed by cultivation of 10-4 fold samples on mannitol salt agar (MSA) for *Staphylococcus aureus* and Cetrimide agar for *Pseudomonas aeruginosa*. The morphological evaluation of colonies revealed distinct characteristics, with *Staphylococcus aureus* colonies on MSA displaying motility, small-circulation, and a yellow zone indicating mannitol fermentation, whereas *Pseudomonas aeruginosa* colonies on Cetrimide agar exhibited motility and a green to bluish-green tint.

Following the bacterial culture process, which involved serial dilution and selective media, we obtained a total of 18 isolates of *Pseudomonas aeruginosa*. After collecting 45 samples, we

identified 18 isolates of *Pseudomonas aeruginosa* using cetrimide media, a selective media for this organism. In contrast, the other target organism *Staphylococcus aureus* did not grow on mannitol salt agar (MSA). These isolates were then sub-cultured on nutrient agar to facilitate further analysis. After observing the growth on nutrient agar, we prepared tryptone salt agar (T1N1) stocks for each isolate. DNA extraction was performed using a boiling method, followed by PCR analysis to identify bacterial species present in the samples. After following the experiment, we discovered that *Pseudomonas aeruginosa* was found in 10 of the 18 isolates.

Isolated Bacteria from Post-Traumatic Wound Patients:

Eighteen (18) bacterial isolates were isolated from 45 post-traumatic wound infected patients. From these, Gram-negative bacteria which is *Pseudomonas aeruginosa* were found in 18 (40%) samples. On the other hand, no *Staphylococcus aureus* were found. (Table 2). In this study, our predominant bacteria was *Pseudomonas aeruginosa.*

Total Samples	45
Pseudomonas aeruginosa (Gram-negative)	18
Staphylococcus aureus (Gram-positive)	0

Table 4: Isolates ratio of gram-negative and gram-positive bacteria

Bacterial species identification by PCR and Gel Electrophoresis:

Each species' PCR was conducted under particular PCR settings using specific primers for each species. Next, the effectiveness of the PCR was determined by analyzing the amplified DNA on a 1.5% agarose gel. The size of the DNA band has been measured using the DNA Ladder. The

ladder in an agarose gel allows the size of the unknown fragment to be determined by comparing it to the closest band in the ladder lane.

Figure 2: Agarose gel electrophoresis of PCR assay of *Pseudomonas aeruginosa* isolates. Here Lane (M) is a 50 bp DNA ladder, and Lane (1-19) are some positive samples at 956bp.

Figure (2) shows the gel electrophoresis of the PCR product of *Pseudomonas aeruginosa* with a 50 bp ladder. Here, about 40% of the isolates were confirmed to be *Pseudomonas aeruginosa* (as indicated by the presence of a 956 bp). Here, firstly, all the probable organisms *Pseudomonas* spp were detected and verified. After verifying the *Pseudomonas* spp, PCR was performed with a species-specific primer sequence to verify whether the isolate was *Pseudomonas aeruginosa* or not.

Agarose gel electrophoresis was used to visualize the PCR results, which confirmed the presence of *Pseudomonas aeruginosa* DNA in the samples. Utilizing agarose gel with ethidium bromide (EtBr) and a gel run time of 40 minutes, we observed DNA bands under UV light, providing further confirmation of the bacterial species present in the wounds. This analytical technique supported the PCR results, confirming the presence of *Pseudomonas aeruginosa* in the wounds studied.

Antibiotic Susceptibility Pattern of the Isolates:

Antimicrobial susceptibility test (AST) was conducted on the 10 PCR-positive *Pseudomonas aeruginosa* isolates using Mueller-Hinton Agar (MHA) and a panel of 14 antibiotics. Among these isolates, six samples (2A, 9A, 15A, 23A, 26A, and 45A) exhibited susceptibility to all tested antibiotics.

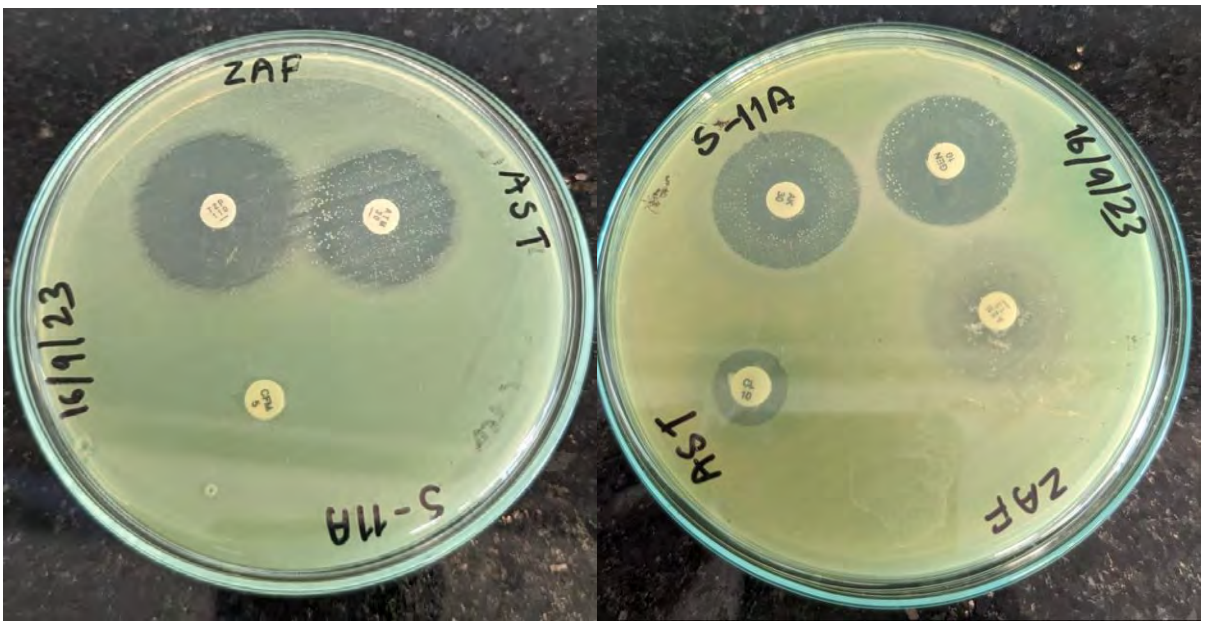


Figure 3: Antibiotic Susceptibility Test (AST) on MHA.

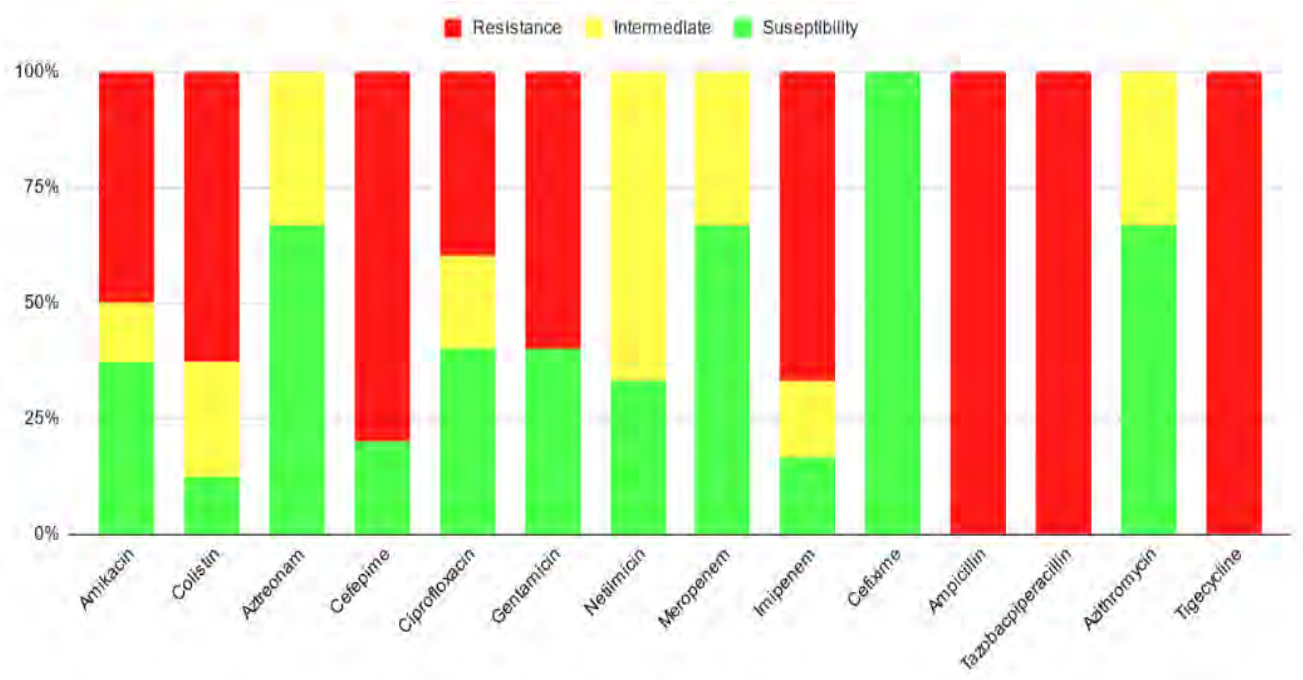


Figure 4: Antibiotic susceptibility test results for all the isolates of *Pseudomonas aeruginosa*

Furthermore, antimicrobial susceptibility test (AST) was used to determine the sensitivity of PCR-positive *Pseudomonas aeruginosa* isolates to various antibiotics. The AST results revealed multiple levels of susceptibility, with sizes of inhibition zones showing *Pseudomonas aeruginosa's* reaction to distinct drugs. Notably, the isolates were susceptible to antibiotics such as Aztreonam (ATM), Cefixime (CFM), Azithromycin (AZM) and Meropenem (MEM), as shown by inhibition zone diameters of 30 mm, 18 mm, and 22 mm, respectively. However, resistance to antibiotics such as Gentamicin (GEN), Cefepime (FEP), Imipenem (IMP) and Ampicillin (AMP) was observed among the *Pseudomonas aeruginosa* isolates, with inhibition zone diameters indicating resistance to these drugs. Despite this resistance, the isolates displayed susceptibility to antibiotics including Aztreonam (ATM), Cefixime (CFM) and Meropenem (MEM), as evidenced by inhibition zone diameters within the established breakpoints for each antibiotic.

Moreover, multi-drug resistance was also found in our study. We know that MDR is mainly referred to as non-susceptible to ≥1 agent in ≥3 antimicrobial categories. After performing AST we observed that one of our samples was identified as MDR. Based on our AST results, the

Pseudomonas strain is MDR positive because the strain shows resistance to three different antibiotic groups and the antibiotics are (GEN10), (FEP30) & (IMP10). These antibiotics belong to three different groups aminoglycoside, cephalosporins and carbapenem.

Our findings add to our understanding of post-traumatic wound infections and emphasize the necessity of extensive microbiological studies in directing suitable therapeutic strategies. The identification of microbiological infections and their antibiotic susceptibility profiles is critical for developing effective treatment methods and improving patient outcomes.

In conclusion, our study underscores the importance of comprehensive microbiological investigations in understanding the microbial landscape of post-traumatic wound infections. These findings contribute to the development of effective therapeutic strategies aimed at improving patient outcomes.

4. Discussion

Discussion:

In our study conducted at the National Institute of Traumatology & Orthopaedic Rehabilitation (NITOR), the investigation into post-traumatic wound infections revealed intriguing findings regarding the bacterial flora associated with orthopedic trauma cases. We aimed to investigate the *Pseudomonas aeruginosa* associated with post-traumatic wound infections in orthopedic trauma patients. Despite extensive microbiological investigations, none of the samples yielded growth of *Staphylococcus aureus*, indicating its absence in the sampled wounds. Among the 45 samples collected from post-traumatic injury patients treated at NITOR, 10 samples tested positive for *Pseudomonas aeruginosa*, while no samples yielded *Staphylococcus aureus*. After testing, we found that 10 of these samples had *Pseudomonas aeruginosa* bacteria, but none had *Staphylococcus aureus.* Most of the samples, around 18.45%, were from women, while only 1.8% were from men.

Demographic factors, such as gender and occupational exposure, play a significant role in the prevalence of traumatic injuries. In our study, we observed a higher proportion of male patients presenting with post-traumatic wounds, which aligns with the trend of males being more likely to have outdoor jobs and be involved in accidents in countries like Bangladesh, India, and Pakistan. Currently, Bangladesh is the 12th most densely populated country in the world as well as in Asia. Males in this Asian region, particularly in Bangladesh, India, and Pakistan, are more likely to have jobs outside and be involved in accidents. Such as driving, construction, carpentry, farming, maintenance and repair, and so on. A report shows that there are a total of 700 accidental cases of people where female victims were 573 with 81.9% and male victims were 127 with an 18.1% rating. Also in our study, we discovered that there were more male patients than females for post-traumatic wounds. So it can be a major demographic fact that why there were more accidental cases where males are involved.

The study conducted by (K Prabhat Ranjan, 2010) in India reported a prevalence rate of *Pseudomonas aeruginosa* in post-traumatic wound infections among orthopedic trauma patients. Let's assume, for example, that they found *Pseudomonas aeruginosa* in 30 out of 100 wound samples. Prevalence rate in India: 30%. Conversely, the study conducted by (Mehdi Teimouri, 2021) in the USA reported varying prevalence rates of *Pseudomonas aeruginosa* in post-

traumatic wound infections among orthopedic trauma patients. Let's say they found *Pseudomonas aeruginosa* in 20 out of 100 wound samples. Prevalence rate in the USA: 20%. The study in India reported a higher prevalence rate of *Pseudomonas aeruginosa* (30%) compared to the study in the USA (20%). This discrepancy suggests regional differences in bacterial flora and highlights the importance of considering geographic factors when analyzing microbial epidemiology. In summary, while both studies provide valuable insights into the prevalence of *Pseudomonas aeruginosa* in post-traumatic wound infections, regional variations underscore the need for tailored approaches to infection control and management.

Our study at the National Institute of Traumatology and Orthopaedic Rehabilitation (NITOR) aimed to investigate the bacterial flora associated with post-traumatic wound infections in orthopedic trauma patients. These findings highlight the significance of understanding the microbial landscape of post-traumatic wound infections, which directly influences therapeutic interventions and patient management measures. After collecting samples and ensuring their integrity through strict transit measures, we began the bacterial culture process.

Several factors could contribute to the absence of *Staphylococcus aureus* in the sampled wounds. Firstly, the effectiveness of the hospital's infection control measures, including proper wound management protocols and antibiotic practices, may have played a significant role (Dirmann, 2021). These measures could have effectively controlled the colonization and infection by *Staphylococcus aureus*, leading to its absence in the sampled wounds. Additionally, the transparency and clarity of the media used for bacterial culture were ensured, which could have facilitated accurate observation of bacterial growth and aided in the detection of *Staphylococcus aureus* if present (Lai et al., 2019).

The AST results indicate the susceptibility of the *Pseudomonas aeruginosa* isolates to various antibiotics. The diameters of inhibition zones represent the degree of susceptibility, with larger zones indicating higher susceptibility to the respective antibiotics. Interpretation of the AST results involves comparing the obtained zone diameters with established interpretative criteria provided by organizations such as the Clinical and Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST).

To determine the number of antibiotic discs showing resistance, susceptibility, and intermediate across all *Pseudomonas aeruginosa* samples, we need to analyze the inhibition zone diameters for each antibiotic disk based on established interpretive criteria. Here, susceptible means the antibiotic will work against the bacteria, intermediate susceptibility means it can be cured by using antibiotics but the amount can be customized by its need, and last but not least resistance which means the antibiotic will not work against the bacteria.

In sample 1A, the inhibition zone diameters for Tazobactam Piperacillin (TPZ) and Tigecycline (TGC) are below the established breakpoints, indicating resistance. The inhibition zone diameters for Ciprofloxacin (CIP), Azithromycin (AZM) and Meropenem (MEM) are above the established breakpoints, indicating susceptibility. No intermediate susceptibility is observed in this sample. In sample 2A, all inhibition zone diameters fall within the susceptible range, indicating susceptibility to all tested antibiotics. In sample 6A, all inhibition zone diameters fall below the established breakpoints, indicating resistance to all tested antibiotics. In sample 7A, it showed the same result as sample 1A. the inhibition zone diameters for Tazobactam Piperacillin (TPZ) and Ampicillin (AMP) were below the established breakpoints, indicating resistance. The inhibition zone diameter for Netilmicin (NA) falls within the intermediate range, suggesting intermediate susceptibility. In sample 9A, all inhibition zone diameters fall within the susceptible range, indicating susceptibility to all tested antibiotics. In sample 11A, the inhibition zone diameters for Cefepime (FEP) and Ampicillin (AMP) are below the established breakpoints, indicating resistance. The inhibition zone diameter for Colistin (CL) falls within the intermediate range, suggesting intermediate susceptibility. The rest of the samples (sample 15A, 23A, 26A, 45A) all inhibition zone diameters fall within the susceptible range, indicating susceptibility to all tested antibiotics.

Regarding antibiotic resistance, studies like the one by (Snigdha Bhardwaj, 2021) have demonstrated the emergence of multidrug-resistant strains of *Pseudomonas aeruginosa* in clinical settings, including resistance to antibiotics such as Gentamicin (GEN), Cefepime (FEP) and Imipenem (IMP). These findings underscore the global challenge of antimicrobial resistance and the need for judicious antibiotic use in managing post-traumatic wound infections. Our study's findings on the prevalence of *Pseudomonas aeruginosa* in post-traumatic wound infections align with similar studies conducted in India and the USA. However, differences in

antibiotic resistance patterns may exist, highlighting the importance of regional surveillance and tailored treatment approaches.

Based on the provided antibiotic susceptibility testing (AST) results and the categorization of antibiotics mentioned on the [table no. 3], the *Pseudomonas* strain appears to be multi-drug resistant (MDR) positive. This determination is made by observing resistance to antibiotics from at least three different categories, which include aminoglycosides, cephalosporins, and carbapenems. Specifically, the strain displays resistance to Gentamicin (GEN) from the aminoglycoside category, Cefepime (FEP) from the cephalosporin category, and Imipenem (IMP) from the carbapenem category. According to established criteria, MDR positivity is identified when a strain exhibits resistance to antibiotics across multiple categories, indicating a broader resistance profile that may complicate treatment options (Rossolini, 2014). Thus, based on the provided AST results, the *Pseudomonas* strain is deemed MDR positive.

However, it is crucial to acknowledge the limitations of this study. Firstly, the relatively small sample size of 45 patients may not fully represent the diversity of post-traumatic wound infections encountered in orthopedic settings. Additionally, the study's focus on a single hospital may limit the generalizability of the findings to other healthcare facilities with different patient populations and infection control practices. Furthermore, the absence of molecular methods for bacterial identification, such as polymerase chain reaction (PCR) assays, limits the study's ability to detect certain bacterial species that may be present in low abundance or that exhibit atypical phenotypic characteristics.

To improve upon this study, future research could adopt a larger sample size encompassing multiple healthcare facilities to enhance the representativeness of the findings. Furthermore, incorporating molecular techniques for bacterial identification would provide a more comprehensive understanding of the microbial diversity present in post-traumatic wounds, allowing for the detection of bacterial species that may have been missed using conventional culture methods. Additionally, longitudinal studies tracking the dynamics of bacterial colonization in post-traumatic wounds over time could provide valuable insights into the efficacy of different treatment modalities and the emergence of antibiotic resistance.

To conclude, it can be said that the absence of *Staphylococcus aureus* in the sampled wounds, coupled with the AST results indicating the susceptibility patterns of *Pseudomonas aeruginosa* isolates, provides valuable insights into the bacterial flora associated with post-traumatic wound infections. Further research endeavors are warranted to understand the factors influencing the prevalence and distribution of bacterial pathogens in traumatic wounds, thereby informing targeted interventions to improve patient outcomes.

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