ANTIBIOTIC SUSCEPTIBILITY PATTERN OF *Pseudomonas aeruginosa* ISOLATED FROM CLINICAL SPECIMENS AND DETECTION OF *toxA*, *exoY* AND *oprL* VIRULENCE GENES

A Dissertation submitted to the Department of Microbiology

St. Xavier's College

(Affiliated to Tribhuvan University)

In Partial Fulfillment of the Requirements for the Award of the Degree of Master of Science in Microbiology

(Medical)

By

TEK RAJ OJHA

T.U Regd. No: 5-2-37-981-2014

TU Roll No: MB 1406/075

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RECOMMENDATION

This is to certify that **Mr. Tek Raj Ojha** has completed this dissertation work entitled "**Antibiotic susceptibility pattern of *Pseudomonas aeruginosa* isolated from clinical specimens and detection of *toxA, exoY* and *oprL* virulence genes"** as a partial fulfillment of M.Sc. Degree in Microbiology **(Medical)** under our supervision. To our knowledge, this is an original research work of him and has not been submitted to any other degree.

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CERTIFICATE OF APPROVAL

On the recommendation of **Ms. Pramila Parajuli** and **Mr. Anil Kumar Sah,** this dissertation work of **Mr. Tek Raj Ojha** entitled **"Antibiotic susceptibility pattern of *Pseudomonas aeruginosa* isolated from clinical specimens and detection of *toxA*, *exoY* and *oprL* virulence genes"** has been approved for the examination and is submitted to the Tribhuvan University in partial fulfillment of the requirements for **M.Sc. degree in Microbiology (Medical)**.

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Tek Raj Ojha

Date: ……

ABSTRACT

Pseudomonas aeruginosa has become one of the most common cause of hospital and community acquired infections. It is hazardous to worldwide public health because of its great potential to develop antibiotic resistance and also it contain several virulence genes. The objective of this research was to determine the prevalence of *P. aeruginosa* in clinical settings, examine its susceptibility to antibiotics and detect the presence of virulence genes (*toxA*, *exoY* and *oprL*) in all *P. aeruginosa* isolates in clinical specimens (urine, sputum, blood, CSF, body fluid, tissue, pus, swab, catheter tip and stool) collected from patients visiting Annapurna Neurological Institute and Allied Sciences. It was a cross-sectional study carried out from June, 2022 to December, 2022. Total 1356 clinical specimens were collected and tested bacteriologically using Gram staining, culture techniques and biochemical tests. The modified disk diffusion method was applied to determine the antibiotic susceptibility pattern and conventional PCR was used to evaluate the virulence genes in all the confirmed *P. aeruginosa* isolates. 40.5% specimens showed bacterial growth in which only 3.02% were identified as *P. aeruginosa.* Among them, 61% were multidrug resistant and 29.2% were β-lactamase producers. Aztreonam (70.7%) was the most effective antibiotic. The result highlighted that 56.1% isolates were *toxA* positive, 68.3% isolates were *exoY* positive and 61% were *oprL* positive. The strategic interventions are required to stop the emergence and spread of antimicrobial resistance as a result of the isolation of multidrug resistant (MDR) isolates carrying these virulence genes. This study provides data about phenotypic and genotypic properties of *Pseudomonas aeruginosa* and can be beneficial for the medical staff and the entire community to build a surveillance system and enhance infection control procedures.

Keywords: *Pseudomonas aeruginosa*; antimicrobial resistance; virulence genes

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ABBREVIATIONS

ANIAS	Annapurna Neurological Institute and Allied Sciences
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection
BA	Blood Agar
BP	Base Pair
CLSI	Clinical and Laboratory Standard Institute
DDST	Double Disk Synergy Test
DNA	Deoxyribonucleic acid
EDTA	Etheylenediamine tetraacetic acid
ESBL	Extended Spectrum Beta-Lactamase
ICU	Intensive Care Unit
LPS	Lipopolysaccharide
MA	MacConkey Agar
MBL	Metallo beta-lactamase
MDR	Multidrug resistant
MHA	Mueller Hinton Agar
NA	Nutrient Agar
OPD	Out-patient Department
PBPs	Penicillin-binding protein
PCR	Polymerase Chain Reaction
SPSS	Statistical Package for Social Science
WHO	World Health Organization

CHAPTER I

INTRODUCTION AND OBJECTIVES

1.1 BACKGROUND

Pseudomonas aeruginosa are Gram negative, rod-shaped, aerobic and non-spore forming bacteria belonging to Pseudomonadaceae family (Gyawali et al 2020). It is one of the most prevalent opportunistic pathogen which is responsible for 18 to 63% of infections globally (Chand et al 2021). It can be differentiated from other *Pseudomonas* species by its ability to grow at 42℃ (El Zowalaty et al 2015). The majority of the *P. aeruginosa* strains produce water soluble pigments, such as pyocyanin (blue-green), pyorubin (red-brown), pyoverdine (yellow-green and fluorescent) and pyomelanin (Chand et al 2021; Wu et al 2014). It has been assigned to persist in both hospital and community settings due to its capability to grow in nutrient deficient conditions and to resist different physical conditions like disinfectant solutions (Arora et al 2011; Wisplinghoff and Seifert 2018). They have potential to cause a variety of infections including critical illness and hospital-acquired infections that are common among the patients (Zeb et al 2017). Since the bacteria are resistant to different class of antibiotics, the treatment of infection caused by the bacterium has become challenging and leads to higher rate of mortality, morbidity as well as higher medical cost (Fair & Tor 2014).

P. aeruginosa is extremely diverse and has potential to cause variety of illnesses, such as bacterial infection, wound infection, urinary tract infection, endocarditis and even death (Japoni et al 2009; Moradali et al 2017). It is an opportunistic pathogens that spreads quickly among the immunocompromised patients, especially those with cystic fibrosis, cancer or AIDS (Jurado-Martín et al 2021). The anatomical sites such as, such as the epidermis, sub-cutaneous tissue, bones, ears, urinary tract, respiratory tract and cardiac valves are susceptible to *Pseudomonas* infections. The site of infections may depends on portal of entry and patient's susceptibility (Gyawali et al 2020). The ability of *P. aeruginosa* to cause disease is due to the emergence of an antibiotic

resistance, the creation of biofilms and the production of virulence factors (Ullah et al 2016). Due to the lipopolysaccharide in its outer membrane which serves as a permeability barrier, it is generally resistant to many antibiotics (Mahaseth et al 2020). The development of antibiotics resistance in *P. aeruginosa* involves other various mechanisms, including MDR, efflux pumps, biofilm formation, aminoglycoside modifying enzymes and β-lactamase production (Yadav et al 2017). *P. aeruginosa* can form biofilm that protect it from environmental stress and prevent phagocytosis, enabling it to colonize and persists for long period (Thi et al 2020). The presence of different intercellular and extracellular virulence factors may contribute to the pathogenicity of the organism (Choy et al 2008). The colonization of these virulence factor cause invasive bloodstream infections, extensive tissue damage and spread throughout the body (Chand et al 2021).

P. aeruginosa has a numbers of virulence genes such as *toxA, exoY, exoS, oprL, oprI, oprD, lasA, lasB, alg, plcH, plcN* and *nan1* (Alonso et al 2020; Chand et al 2021). These genes encodes different virulence factors which helps in its pathogenicity (Ciamak 2022). The outer membrane lipoprotein of *P. aeruginosa* are encoded by *oprL, oprI* and *oprD* genes and are also used as marker for the identification of *Pseudomonas* infections (Chand et al 2021; Mapipa et al 2021). *oprL* gene maintains the cellular integrity of bacteria and protects against oxidative stress (Ciamak 2022). The *toxA* gene encodes exotoxin A which is a cytotoxic agent that inhibits the protein synthesis by stopping the elongation of polypeptide chains, leading to host tissue damage (Dong et al 2015). Similarly, *exoY* gene encodes exotoxin Y that is responsible for producing enzyme called adenylate cyclase which increases the level of cyclic nucleotides, specially cyclic AMP (cAMP) inside the cell (Hritonenko et al 2011; Kloth et al 2018).

For the treatment of *Pseudomonas* infection, eight different categories of antibiotics are used including aminoglycosides, carbapenems, cephalosporins, fluoroquinolones, penicillin with β-lactamase inhibitors, monobactams fosfomycin and polymyxins (Bassetti et al 2018). B-lactam antibiotics are considered safe and most effective antibiotics for many types of bacterial infections and are commonly prescribes by the healthcare provider around the world (Bradford 2001). However, some bacteria have developed a defense

mechanism against beta-lactam antibiotics called beta-lactamase enzyme (Thenmozhi et al 2014). This enzyme break down the beta-lactam ring in the structure of beta-lactam antibiotics, as a result antimicrobial properties of antibiotics is completely lost (Tekiner & Özpınar 2016). Among the identified beta lactamase, Extended-Spectrum-β-lactamases (ESBL) and Metallo-β-lactamases (MBL) are most clinically significant (Zhao & Hu 2010).

Antibiotic resistance is a significant global issue, particularly with the rise of multi-drug resistant *P. aeruginosa* infection that can cause frequent nosocomial and chronic infections (Moradali et al 2017). The increasing trend of antimicrobial resistant bacteria in both clinical and environmental setting is a growing health concern (Chand et al 2021). Thus, the knowledge of bacterial antibiotic susceptibility profile is crucial for selecting the most effective empirical therapy, as it helps to determine the proper choice of antibiotics against bacterial infections (El Zowalaty et al 2015). The production of beta lactamases is causing many commonly used antipseudomonal antibiotics to become ineffective for treatment (Bush & Bradford 2016). The combination of virulence gene and antibiotic resistance genes makes the bacterial isolates more difficult to treat and control (Schroeder et al 2017). In Nepal, there is lack of standardized system for monitoring and reporting antibiotic resistance patterns which make it difficult to identify the trends in resistance and to develop appropriate treatment strategies. Thus, this study is performed to know the current resistance pattern of *P. aeruginosa* against different class of antibiotics and to determine the involvement of some virulence genes in various clinical specimens which ultimately aids in selection of appropriate antibiotics for the treatment of *Pseudomonas* infection.

1.2 OBJECTIVES

1.2.1 General objective

To detect the antibiotic susceptibility pattern of *P. aeruginosa* and presence of *toxA, exoY* and *oprL* virulence genes among them.

1.2.2 Specific objectives

1. To isolate and identify *Pseudomonas aeruginosa* from different clinical specimens.
2. To assess antibiotic susceptibility pattern of *P. aeruginosa* isolated from clinical specimens by disc diffusion method.
3. To detect MDR, ESBL and MBL phenotypically.
4. To detect the presence of *toxA, exoY* and *oprL* virulence genes in all *P. aeruginosa* isolated from clinical specimens.
5. To determine a significant association of virulence genes (*toxA, exoY* and *oprL*) with MDR, ESBL and MBL.

CHAPTER II

LITERATURE REVIEW

2.1 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a motile, rod-shaped, gram negative bacteria measuring 0.5 to 0.8 µm by 1.5 to 3.0 µm belonging to the bacterial family Pseudomonadaceae and class Gamma Proteobacteria (Todar 2020). It is frequently isolated from soil, water, plants and animals including human (Chong 2009). It is an opportunistic pathogen that causes the infections which are most severe, particularly in people whose immune system is compromised and associated with high death and morbidity rate (Moradali et al 2017; Odumosu et al 2013).

P. aeruginosa was first isolated by Carle Gessard in 1882 from the wounds of patients (Lister et al 2009). It is associated with healthcare-related infection in hospitalized patients such as pneumonia, urinary tract infection, skin and soft tissue infection, and other disorders. Additionally, this microbe has potential to colonize on implanted medical equipment such as catheters, heart valves, ventilators and dental implants which could lead to a nosocomial infection connected with the device (El-kholy et al 2012; Zorgani et al 2015). This bacterium frequently displays multi-resistant strains, which is associated with faster onset of infection and mortality (Bassetti et al 2018).

Due to the production of water soluble pigments, such as pyocyanin (blue-green), pyoverdine (yellow-green and fluorescent), pyorubin (red-brown) and pyomelanin (brown), *P. aeruginosa* can be easily detected on agar (Kaszab & Dura 2016; Lamont & Martin 2003; Wu et al 2014). It can grow at high temperature up to 42℃ and is classified as strictly aerobic but has the ability to evolve under anaerobic conditions with suitable terminal electron acceptors such as nitrite or arginine (Suer 2021; Dworkin 2006). Typically, *Pseudomonas aeruginosa* can be identified by its shiny appearance, production of grape like odor in culture media, ability to grow at high temperature and production of fluorescein and pyocyanin (Ghane & Azimi 2014). The ability of this bacterium

to cause disease is enhanced by several virulence factors such as the release of enzymes like alkaline protease, elastase and gelatinase, production of toxins such as hemolysin, exotoxin A, S and T, as well as having flagella, pili, lipopolysaccharide, outer membrane protein and pyocyanin present on their cell wall (Jiang et al 2014; Parija 2012).

2.2 Clinical significance of *Pseudomonas aeruginosa*

P. aeruginosa has a large genome with many regulatory genes that enable it to adapt easily to new environments (Silby et al 2011). It possesses multiple mechanisms that help to increase antibiotic resistance and virulence (Fichant et al 2013; Suer 2021). In a sessile community arrangement, *P. aeruginosa* has ability to persist in environments with host defense systems, dryness, disinfectants, antibiotics and UV light (Hogardt & Heesemann 2011). It is considered as an opportunistic bacterium that can cause acute and chronic infections in both humans and animals (Kidd et al 2012). The most common way of infection is through contact with external sources. The bacterium can cause community-acquired infections like keratitis, skin infections, and otitis media and externa, as well as nosocomial infections (Driscoll et al 2007; Suer 2021). For the therapy of these infections, the selection of appropriate antipseudomonal drug is crucial to achieve the best possible result (Tümmler 2019).

Respiratory tract infections

Respiratory tract infection caused by *P. aeruginosa* which occurs almost in those individuals with immunodeficiency. Exposure to a hospital setting, specifically in an ICU where respiratory inhalation equipment is used and prior use of antibiotics, raises the chances of contracting these infections (Parija 2012).

Skin infections

P. aeruginosa is capable of causing several types of skin infections, including those affecting burn wounds, chronic paronychia, infected toe web,

pseudomonal folliculitis, and pseudomonal cellulitis. Among these, infection of burn wounds is the most frequently identified condition associated with *P. aeruginosa* (Parija 2012).

Urinary tract infections

P. aeruginosa is responsible for urinary tract infections in individuals who have indwelling urinary catheters or who undergo instrumentation and surgery of the urinary tract. These infections typically occur in hospital settings and are a result of medical procedures (Parija 2012).

Ear infections

P. aeruginosa is a frequent cause of external otitis, particularly in individuals with a history of swimming. Additionally, this bacterium is responsible for malignant external otitis, a severe and aggressive form of the condition that primarily affects patients with diabetes, acquired immunodeficiency syndrome (AIDS), and the elderly (Parija 2012).

Eye infections

P. aeruginosa can lead to a condition known as pseudomonal endophthalmitis. This condition can arise after trauma, intraocular surgery, or when there is a posterior perforation of a corneal ulcer. Individuals who wear contact lenses are at a higher risk of developing a pseudomonal infection (Parija 2012).

Endocarditis

Pseudomonal infectious endocarditis is a type of cardiovascular infection that affects both normal and abnormal heart valves (tricuspid, aortic, and mitral valves) on both sides of the heart. This infection causes damage to the heart valves, leading to heart failure. It is most commonly observed in individuals who misuse intravenous drugs such as pentazocine and triphenylamin (Hassan & Al-Riyami 2012).

Nosocomial infections

Most of the infection caused by *P. aeruginosa* are opportunistic infections seen in immunocompromised patients (Parija 2012). In recent times, hospital-acquired infections caused by *P. aeruginosa* have become a significant concern. This is primarily due to the bacterium's natural resistance to numerous types of antibiotics and its ability to develop resistance to virtually all effective antibiotics through practical means (Fazeli & Momtaz 2014).

2.3 Antibiotics used to treat *Pseudomonas* infections

For the treatment of the infection caused by *Pseudomonas aeruginosa,* mainly eight different classes of antibiotics are used (Bassetti et al 2018). These includes penicillins (piperacillin), penicillins with β-lactamase inhibitor (ticarcillin and piperacillin plus clavulanic acid or tazobactam), cephalosporins (ceftazidime, cefepime), monobactams (aztreonam), carbapenems (imipenem, meropenem, doripenem), polymyxins (colistin or polymyxin B), aminoglycosides (gentamicin, amikacin) and fluoroquinolones (ciprofloxacin, levofloxacin) (Bassetti et al 2018; El Zowalaty et al 2015; Mapipa et al 2021).

2.4 Mode of action of antibiotics

2.4.1 β-Lactam antibiotics

Beta lactam antibiotics are among the most widely used class of antibiotics in the infectious disease which acts as bactericidal agents by interrupting the process of cell wall formation (Bush & Bradford 2016; Ritter et al 2020). It includes four major class of antibiotics that are used in clinical practice worldwide. They are penicillin, cephalosporin, monobactam and carbapenems (Ritter et al 2020). These antibiotics contain beta lactam ring in their molecular structure and have similar mode of action (Meletis 2016). Beta lactam antibiotics kill susceptible bacteria by inactivating numerous bacterial protein called penicillin-binding proteins (PBPs), which are essential for the synthesis of the cell wall and maintenance of morphology of bacteria. This leads to the

inhibition of cell wall synthesis and can cause changes in the shape or destruction of susceptible bacteria (Ritter et al 2020).

2.4.2 Polymyxin

Colistin and polymyxin B (PMB) are the antibiotics of polymyxin group, now used in therapeutic settings. They are highly basic in nature and most powerful antimicrobial against Gram negative bacteria due to the presence of five free amino group (Vaara 2019). Polymyxin interact with lipopolysaccharide (LPS) of the outer membrane of Gram negative bacteria which causes the LPS to become destabilized. This destabilization leads to an increase in the permeability of the bacterial membrane, which can result in the release of the cytoplasmic contents and ultimately lead to the death of the cell (Zavascki et al 2007).

2.4.3 Fluoroquinolones

Antipseudomonal fluoroquinolones includes ciprofloxacin, ofloxacin, and levofloxacin (El Zowalaty et al 2015). They inhibit the replication of bacterial DNA by inhabiting the activity of DNA gyrase (topoisomerase II) and topoisomerase IV (Ritter et al 2020).

2.4.4 Aminoglycosides

Antipseudomonal aminoglucosides includes amikacin, tobramycin and gentamicin (El Zowalaty et al 2015). Aminoglycosides are antibiotics that have a positive charge and can attach to the outer membrane of bacteria, which has a negative charge. This attachment creates large pores in the membrane, allowing the antibiotic to penetrate into the bacterium. These antibiotics inhibit the synthesis of proteins in the bacteria by binding irreversibly to the 16S rRNA of the 30S subunit of the ribosome. This binding can cause errors in the reading of mRNA, which then prevents the protein synthesis (Kapoor et al 2017; Ritter et al 2020).

2.5 Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*

The emergence of antibiotic resistance is a significant medical issue that needs to be addressed by a great understanding of the antimicrobial susceptibility patterns, antibiotic resistant genes and antimicrobial resistance spread (Adel & Sabiha 2010). Antibiotic resistance in *P. aeruginosa* can be acquired through intrinsic or acquired mechanisms to various class of antibiotics through numerous method (C Reygaert 2018; Suer 2021). Bacteria shows intrinsic resistance through antibiotic inactivation, decreased antibiotic penetration and efflux, and changes in target sites (Dzidic et al 2008; Munita & Arias 2016). The mechanisms involved in acquired resistance include mutational resistance and horizontal gene transfer (Dzidic et al 2008).

2.5.1 Drug inactivation

Bacteria have two main methods through which bacteria inactivate drugs: either by degradation of the drug or by modifying it through the transfer of a chemical group to the drug (Dzidic et al 2008; Munita & Arias 2016).

Bacteria have the ability to generate enzymes that can attach specific chemical groups to antibiotic molecules. This modification prevents the drugs from binding to their intended targets. To modify antibiotics, chemical techniques such as acetylation (for aminoglycosides, chloramphenicol, and streptogramins), phosphorylation (for aminoglycosides and chloramphenicol), and adenylation (for aminoglycosides and lincosamides) are used (Llano-Sotelo et al 2002).

A lot of antibiotic molecules have chemical bonds like esters and amides that can be easily broken down by bacterial enzymes. This breakdown can occur before the antibiotic molecules reach their intended target site, which causes them to lose their ability to fight against bacteria (Džidić et al 2008). An example of this is the β-lactamases enzyme, which works on antibiotics that have a β-lactam group by breaking the amide bond of β-lactam. This process leads to the deactivation of this category of antibiotics (Thenmozhi et al 2014).

The broad usage of beta lactam antibiotics in medical environments has resulted in the emergence of several types of beta lactamases. In different parts of the world, there are increasing reports of extended-spectrum β-lactamases, AmpC-type β-lactamases, and metallo-β-lactamases (Oberoi et al 2013).

2.5.2 Restricted uptake and efflux pump

For all the primary antipseudomonal drug classes to be effective, they need to pass through the cell wall to reach their intended target. Antibiotics may fail to reach their intended site because of restricted permeability of the outer membrane and the effective removal of antibiotic molecules by efflux pumps (Lambert 2002). The outer membrane of *P. aeruginosa* exhibits a particular degree of permeability which is significantly lower, about 12 to 100 times less, than that of E. coli (Hancock 1998). This poor permeability acts as an obstacle for the entry of many molecules, partly contributing to the high intrinsic and induced resistance against several antibiotic classes, such as aminoglycosides, β-lactams, and quinolones (Chevalier et al 2017; Hancock 1998; Lambert 2002). *P. aeruginosa* also possesses multiple efflux pumps that eject antibiotics from the bacterial cell, which hinders or restricts the antimicrobial agents from reaching their intended target site (Lister et al 2009). Approximately twelve efflux pumps belonging to the resistance-nodulation-division (RND) family have been discovered in *P. aeruginosa*. Among these, four efflux pumps, namely mexAB-oprM, mexCD-oprJ, mexEF-oprN, and mexXY-oprM, contribute to antimicrobial resistance (Dreier & Ruggerone 2015). The mexAB-oprM and mexXY-oprM efflux pump systems are responsible for both acquired and intrinsic resistance, while the other two systems only assist in acquired resistance. Efflux pumps only provide moderate levels of resistance and usually work in tandem with other resistance mechanisms (Bassetti et al 2018).

2.5.3 Changes in target sites

Antimicrobial resistance can also occur through the modification of the target site of antibiotics. This can be achieved through various methods, such as protection of the target, modification of the target site, and mutation of the target that reduce its affinity for the antibiotic molecule (Munita & Arias 2016).

2.5.4 Mutational resistance

Mutation is a prevalent mechanism of resistance to various antibiotics. Typically, mutations causing antimicrobial resistance alter the antibiotic's efficacy through one of these mechanisms: (i) modification of the antimicrobial target, (ii) decrease in the drug uptake, or (iii) activating efflux mechanisms to expel the harmful molecule (Munita & Arias 2016).

2.5.5 Horizontal gene transfer (HGT)

Horizontal gene transfer (HGT) is a significant factor in the spread of antimicrobial resistance across diverse species, as it allows for the exchange of genetic material. The natural soil environment is a source of both antibiotics and antibiotic resistance genes. Bacteria that inhabit the same environment as these molecules possess resistance genes as a defense mechanism against antibiotics. This "environmental resistome" is believed to be the primary source of antibiotic resistance genes in clinically relevant pathogens. HGT can occur through three distinct mechanisms: transformation, transduction, and conjugation (Munita & Arias 2016). The spread of antibiotic resistance genes to other bacteria can occur through the involvement of mobile genetic elements such as plasmids, transposons, and integrons, which are considered to be key elements in the evolution and dissemination of such genes among bacteria of the same or different species (Allen et al 2010).

2.6 Multi-drug resistant *P. aeruginosa* (MDRPA)

MDR in *P. aeruginosa* is defined as resistance to three or more antipseudomonal antibiotics including penicillins (piperacillin), cephalosporin (ceftazidime), fluoroquinolones (ciprofloxacin), carbapenems (imipenem, meropenem and doripenem) and aminoglycosides (gentamicin, tobramycin or amikacin) (Defez et al 2004; Mahto et al 2021). Bacterial antibiotic resistance can be attended through intrinsic or acquired mechanism. Intrinsic mechanisms involves naturally occurring genes found on host chromosome while acquired mechanism involves mutation in genes targeted by the antibiotic or genetic materials (Levy & Bonnie 2004).

2.7 Extended-spectrum beta lactamases

ESBLs are the β-lactamases that are capable of conferring bacterial resistance to the penicillins, cephalosporins, aztreonam (but not cephamycins) and carbapenems by hydrolysis of these drugs and are inhibited by β-lactamase inhibitors (such as clavulanic acid). ESBLs are able to hydrolyze penicillins, narrow spectrum and third generation cephalosporins and monobactams with hydrolysis rate for ceftazidime, cefotaxime or aztreonam at least 10% that for benzyl penicillin (Bush, 2008; Paterson & Bonomo, 2005)

2.7.1 Types of ESBLs

1. SHV type: It is most frequently found ESBL type in clinical isolates than any other type.

2. TEM type: They are derivative of TEM-1 and TEM-2. Over 100 TEM type β-lactamases have been reported of which majority are ESBL.

3. CTX-M: CTX-M type ESBLs reflects the potent hydrolytic activity of β-lactamases toward cefotaximes than ceftazidime. The number of CTX type ESBLs are rapidely increasing.

4. OXA type: They are so named because of their oxavillin hydrolyzing activity. They are found predominantly in *P. aeruginosa.* (Horcajada et al 2019*;* Bonnet et al 2000).

2.8 Metallo-β-lactamases (MBL)

Amongst the various β-lactamases identified till date, the genetically mobile metallo-β-lactamases (MBLs) are most versatile one as they are able to hydrolyze all β-lactams except monobactam. MBL is a group of beta lactamase which requires divalent cations of zinc as cofactors for enzyme activity (Patzer et al 2009). MBL are classified into three classes at molecular level: class A, B and C. Class B metallo β-lactamases have a broad substrate spectrum and can catalyze the hydrolysis of virtually all β-lactam antibiotics except monobactams (Drawz & Bonomo 2010; Perez-Llarena & Bou 2009).

2.9 Virulence factors of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a pathogenic bacterium that synthesizes several virulence factors responsible for both acute and chronic infections, depending on the strain. These virulence factors include exoenzyme S, exotoxin A, phospholipase C, and pili, which are associated with acute infections in the body. Additionally, *P. aeruginosa* produces siderophores, alginate pseudo-capsules, and other components that protect the bacteria from antibiotics, phagocytosis, and dehydration. Two important virulence factors produced by *P. aeruginosa* that are specifically linked to pneumonia are elastase and the type III secretion system (Khalifa et al 2011).

The bacterium *P. aeruginosa* produces a toxin called exotoxin A which hinders the production of proteins in eukaryotic cells. This process involves the transportation of the molecule nicotinamide adenine dinucleotide (NAD) to a component called eukaryotic elongation factor 2 (EF-2). Exotoxin A inhibits the activity of EF-2, leading to the cessation of protein synthesis. Additionally, *P. aeruginosa* produces exoenzyme S, which is an ADP-ribosyl transferase encoded by the *exoS* gene. Another protein called elastase B, encoded by the *lasB* genes, is a zinc metalloprotease that can destroy immunological proteins such as cytokines. Furthermore, the alginate genes encode the alginate gene *algD*, which is necessary for defending against biofilm and the host immune response (Michalska & Wolf 2015).

P. aeruginosa is a bacterium that is known for its ability to cause infections through various virulence factors such as adhesions, biofilm formation, hemagglutinin synthesis, movement, phenazines, pyocyanin, rhamnolipids, siderophores, colonization, and the type III secretion system. The regulation of many of these factors is controlled by quorum sensing, indicating that a certain bacterial density is necessary for effective infections to occur. Pyocyanin, for instance, can inhibit cellular respiration, while elastase can disrupt blood vessels and epithelial cells and rhamnolipids destroy the cell which allow the bacteria to invade them easily (Johnson 2019; Suer 2021). *P. aeruginosa* creates toxins that impact the cells of the human immune system. It uses different virulence factors such as biofilm synthesis, elastase and hemolysis production in the host,

motility, pyocyanin formation, and rhamnolipids production to harm the host cells at different infection stages. Additionally, it produces toxins to eliminate the host cells (Karpagam et al 2013)

2.9.1 Cell surface virulence factors

Alginate is an exopolysaccharide produced by all types of *P. aeruginosa*, including mucoid variants, which is believed to aid in the adhesion and invasion of host cells. However, there is no significant difference in the ability of non-mucoid and mucoid bacteria to adhere or to invade host cells. The primary function of Alginate is to protect against opsonization and phagocytosis while also inhibiting the penetration of antimicrobial agents into the cell. Furthermore, when compared to other bacterial strains, *Pseudomonas aeruginosa* alginates have a higher tendency to persist in aerosol droplets under laboratory conditions (Thi et al 2020). The binding and subsequent internalization of *P. aeruginosa* to host cells are facilitated by the lipopolysaccharide cell surface (LPS). *P. aeruginosa* LPS comprises of polysaccharide O and core antigens, as well as lipids A composed of fatty acids, phosphates, and disaccharide-binding glucosamine. Eliminating the O polysaccharide side chains increases the risk of cells being destroyed by complement-mediated lysis, but it also has the potential to offer a selective advantage by providing protection to certain antibodies identified by host O specific antigen (Huszczynski et al 2020). Type IV pili (TFP) play a crucial role in the attachment of *P. aeruginosa*, consisting of a single protein, to host cell membranes and non-living surfaces, such as the ability to adhere to stainless steel. Researchers believe that TFP selectively adhere to CF respiratory epithelial cells (F508 CF) more than normal respiratory epithelium. Some studies indicate that glycoproteins on the outer membrane of CF respiratory epithelial cells (F508 CF) have reduced sialylation. Following adhesion mediated by LPS and TFP, *P. aeruginosa* can produce a large number of virulence factors that can cause cell damage and death (Laventie et al 2019).

2.9.2 Virulence genes in *Pseudomonas aeruginosa*

The inherent expression of genes that are responsible for virulence are different and contributes to varying levels of disease-causing potential in infected

individuals (Khattab et al 2015). The primary causes of these infections include the emergence of drug-resistant strains, the formation of biofilms, and the production of virulence factors. Biofilms are particularly difficult to eradicate due to their high resistance to the immune system and commonly used antibacterial treatments, leading to persistent infections. *Pseudomonas aeruginosa* is capable of producing pigments in various environmental conditions, and its outer membrane proteins (*OprI* and *OprL*) stimulated the immune responses of patients and became a strong candidate for developing *P. aeruginosa* vaccines (Ingle et al 2017). Environmental changes may enhance the production of elastase in these pathogens. *Pseudomonas aeruginosa* is capable of producing two types of phospholipase *plch*, with hemolytic activity being associated with the hydrolysis of Sphingomyelin and phosphatidyllin. Patients with lung infections tend to exhibit the virulence factor *toxA*, while those with cystic fibrosis (CF) are more likely to have *ExoS*. The expression of cellular and extracellular virulence factors in *P. aeruginosa* is regulated by signaling mechanisms within the cell. Understanding the close relationship between virulence genes and the source of infection can facilitate better community control of these infections (Khattab et al 2015). In *P. aeruginosa*, most research focuses on four major effector enzymes of type 3 secretion system (T3SS), *exoS*, *exoY*, *exoU* and *exoT* (Pena et al 2019).

The two main cytotoxins produced by this bacterium are *exoS* and *exoT*, which are involved in colonization, invasion, and spread throughout the host during infection. Both enzymes have ADP-ribosyltransferase activity, but *exoS* is more potent than *exoT*. The *exoS* targets small proteins similar to *Ras* that inhibit internalization, DNA synthesis, and apoptosis, while *exoT* targets kinases involved in host phagocytosis and focal adhesion and is linked to the spread of disease to the liver in mice (Suer 2021). Exoenzyme S is a ribosyltransferase ADP that is directly secreted into the cytoplasm of epithelial cells by the *exoS* gene. It is produced by the type III secretion system. The exotoxin injection of *exoU* cause demise of host cells within 1-2 hours that is marked through the damage of necrotic plasma membrane (Lee et al 2005)

CHAPTER III

MATERIALS AND METHODS

3.1. Materials

Materials, equipment and reagents used in this study have been included in the Appendix D-E.

3.2 Methodology

3.2.1 Study type

This research was a hospital based descriptive cross-sectional study.

3.2.2 Study area

The study was conducted in Annapurna Neurological Institute and Allied Sciences (ANIAS), Maitighar, Kathmandu, Nepal.

3.2.3 Study period

The study was carried out from June 2022 to December 2022.

3.2.4 Study population

The study was conducted among both inpatients and outpatients of all age groups and both sexes visiting ANIAS. The demographic parameters, clinical history, prior antibiotic used were recorded (Appendix C).

3.2.5 Sample types

All samples received during the study period were taken for the study. Specimens included urine, sputum, blood, CSF, body fluid, tissue, pus, swab, catheter tip and stool.

3.2.6 Sample size

The sample size of the study was calculated as below:

$$n = \frac{(z_\alpha)^2 \times p\,(1 - p)}{e^2}$$ (Kothari, 2004)

Where,

n = total number of samples

p = expected prevalence (expressed as decimals)

z_α = z-value (1.96% for 95% confidence level) =1.96

e = acceptable error = 5% = 0.05

Since (Mahaseth et al., 2020) have reported the prevalence of *P. aeruginosa* as 11.29%,

$$n = \frac{1.96^2 \times 0.1129\,(1 - 0.1129)}{0.05^2} = 153.9 \approx 154 \text{ clinical specimens}$$

The statistical sample size significant to this study was 154, however a total of 1,356 clinical samples were included in this study.

The patient's demographic data such as age, sex, and gender of the patient were noted during sample collection. The specimens (urine, sputum, blood, CSF, body fluid, tissue, pus, swab, catheter tip and stool) collected during study were processed by standard microbiological techniques (Cheesbrough 2006). Isolation, identification, antibiotic susceptibility testing for MDR, ESBL and MBL were carried out at Annapurna Neurological Institute and Allied Sciences, Maitighar, Nepal. Furthermore, genetic experiments were carried out at the laboratory of Annapurna Research Centre, Maitighar, Nepal. Ethical approval for carrying out the study was obtained from Institutional Review Committee of Central Department of Microbiology prior to specimen collection.

3.2.7 Identification of *P. aeruginosa*

P. aeruginosa isolates were identified and confirmed using a combination of colony morphology, Gram staining and different biochemical tests. Non-lactose fermenting Gram negative bacteria were subjected to catalase test, oxidase test, indole test, methyl red test, Voges Proskauer test, triple sugar iron agar test, urease test and citrate test for the identification of *P. aeruginosa*. Furthermore,

growth on cetrimide agar, growth at 42°C and pigment production *P. aeruginosa* (Cheesbrough 2006; Tille 2014).

3.2.8 Antibiotic susceptibility test (AST)

Antimicrobial susceptibility testing of all collected isolates was carried out using modified Kirby-Bauer disc diffusion method according to the standard procedure of CLSI (CLSI 2021). Gentamicin (GEN, 10 µg), ciprofloxacin (CIP, 5 µg), piperacillin (PI, 100 µg), piperacillin/tazobactam (PIT, 100/10 µg), ceftazidime (CAZ, 30 µg), cefepime (CPM, 30 µg), imipenem (IPM, 10 µg) and aztreonam (AT, 30 µg) were tested for susceptibility.

Carpet culture of the test organism was done on the surface of Mueller-Hinton Agar (MHA) using a broth culture, which was compared to a 0.5 McFarland standard. Then, the selected antibiotic discs were placed on the surface of agar plate which was already inoculated with test strain. The plate were then incubated at 37°C for 24 hours (or overnight). After incubation, the diameter of zone of inhibition was measured and results were interpreted according to CLSI guidelines (CLSI 2021). *P. aeruginosa* ATCC 27853 was used for the quality control of the assay. The isolates showing resistance to at least one agent of three different class of antimicrobial agents were considered as multidrug resistant (Gyawali et al., 2020).

3.2.9 Phenotypic detection of extended spectrum β-lactamase (ESBLs)

Screening for ESBL production was done using cefotaxime and ceftazidime discs. *Pseudomonas aeruginosa* isolates exhibiting ≤22 mm zone of inhibition for ceftazidime and ≤27 mm zone of inhibition for cefotaxime were considered potential ESBL producers. The phenotypic confirmation of ESBL production on suspected isolates was done by combination disk test (CDT). In this method, a lawn culture of test strain was made on MHA and ceftazidime and ceftazidime plus clavulanic acid as well as cefotaxime and cefotaxime plus clavulanic acid were placed over the culture. The plates were incubated at 37°C for 18-24 hours. An increase in zone diameter of ≥5 mm in disc containing clavulanic acid in

comparison with the inhibition zone of antibiotic tested alone confirmed ESBL production (Sherchan et al., 2013).

3.2.10 Phenotypic detection of Metallo-β-lactamase (MBL)

Isolates exhibiting resistance to imipenem in Kirby Bauer disc diffusion method were considered potential MBL producer. Imipenem – EDTA disc method was employed for the phenotypic confirmation of MBLs in imipenem resistant *P. aeruginosa*. A test inoculum equivalent to 0.5 McFarland was inoculated onto plates of MHA. Two 10 µg imipenem discs were placed on the plates, and 10 µl of 0.5 M EDTA of p^H 8 was added to one of the discs to obtain 750 µg concentration. The distance between the center of imipenem and imipenem-EDTA discs was maintained to be 20 mm. after 16-18 hours of incubation in aerobic condition at 37℃, the inhibition zones of imipenem and imipenem-EDTA discs were measured and compared with each other. If the difference in inhibition zone between imipenem disk and imipenem-EDTA disk was ≥7 mm, the isolate was considered as MBL-producer (Al-Khudhairy & Al-Shammari 2020).

3.2.11 DNA extraction

The chromosomal DNA was extracted from *P. aeruginosa* by phenol chloroform method (Ghatak et al 2013). The extracted DNA was preserved in TE buffer at 4℃. Later, it was used for Polymerase Chain Reaction (PCR).

3.2.12 Detection of *toxA*, *exoY* and *oprL* virulence genes by Polymerase Chain Reaction (PCR)

The primers used for amplification of genes and PCR conditions are listed in Table 1- 4 below.

Table 1: Primers used for the amplification of genes

Virulence genes	Target gene	Primer name	Sequence (5' to 3'	Size of product
Exotoxin A	*toxA*	Forward	GACAACGCCCTCAGCATCACCAGC	352 bp
		Reverse	GACAACGCCCTCAGCATCACCAGC	(Chand et al 2021)
Exotoxin Y	*exoY*	Forward	CGGATTCTATGGCAG GGAGG	289 bp
		Reverse	GCCCTTGATGCACTCGACCA	(Gawish 2013)
Sialidase enzyme	*oprL*	Forward	ATGGGAATGCTGAAATTCGGC	500 bp
		Reverse	CTTCTTCAGCTCGACGCGACG	(Chand et al 2021)

Table 2: Polymerase Chain reaction steps and conditions for *toxA*

Steps	**Duration**	**Temperature**	**Number of cycles**
Initial denaturation	5 min	94˚C	1
Denaturation	1 min	94˚C	
Annealing	1 min	58˚C	35
Extension	1 min	72˚C	
Final extension	10 min	72˚C	1

Table 3: Polymerase Chain reaction steps and conditions for *exoY*

Steps	**Duration**	**Temperature**	**Number of cycles**
Initial denaturation	10 min	94°C	1
Denaturation	40 sec	94°C	
Annealing	30 sec	64°C	35
Extension	55 sec	72°C	
Final extension	10 min	72°C	1

Table 4: Polymerase Chain reaction steps and conditions for *oprL*

Steps	Duration	Temperature	Number of cycles
Initial denaturation	5 min	94℃	1
Denaturation	1 min	94℃	
Annealing	1 min	60℃	35
Extension	1 min	72˚C	
Final extension	10 min	72℃	1

PCR mix was prepared in a final volume of 25µl containing 12.5µl Master mix, 6.5µl D/W, 1µl primer and 5µl template DNA. PCR was performed as per the reactions described in above table. The final hold temperature is 4℃ during PCR. A reagent blank was included in every PCR reaction which contained all the components of reaction mixture except the bacterial DNA. *P. aeruginosa* ATCC 27853 was used as positive control (Chand et al 2021).

The amplified PCR products were resolved by electrophoresis on 1.5% agarose gel containing 0.5µg/ml ethidium bromide. The DNA bands were visualized under UV light using a gel documentation system and then photographed.

3.3 Quality control

Prior to use, the sterility of all culture media and biochemical media used in this study were confirmed. For the standardization of Kirby-Bauer test and for performance testing of antibiotics and MHA, control strain of *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were examined primarily to assess the quality. Quality of sensitivity tests was maintained by maintaining the thickness of MHA at 4 mm. Also, p^H of the MHA medium was maintained at 7.2 to 7.4.

3.4 Statistical analysis

All collected data were entered in the worksheet of Statistical Package for Social Science (SPSS) software (version 21). Chi-square test was used for data comparison and results were presented in the form of tables and figures. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant in all statistical data.

LABORATORY ANALYSIS

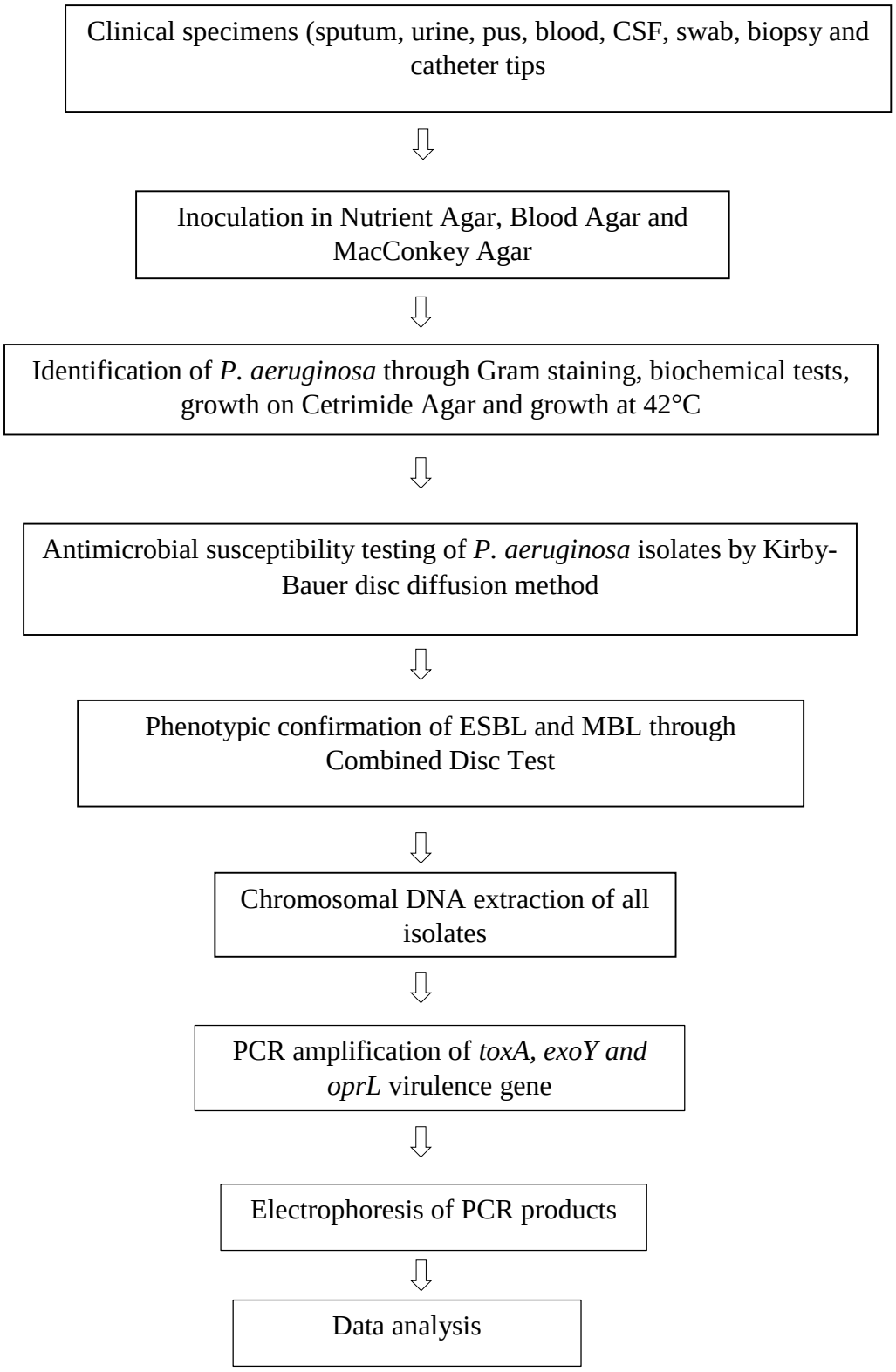


Figure 1: Flowchart of methodology

CHAPTER IV

RESULTS

4.1 Growth pattern of organisms

A total of 1356 clinical specimens were collected during the study period. Of the total specimens processed, 549 (40.5%) samples showed bacterial growth while 807 (59.5%) showed no growth (Figure 2).

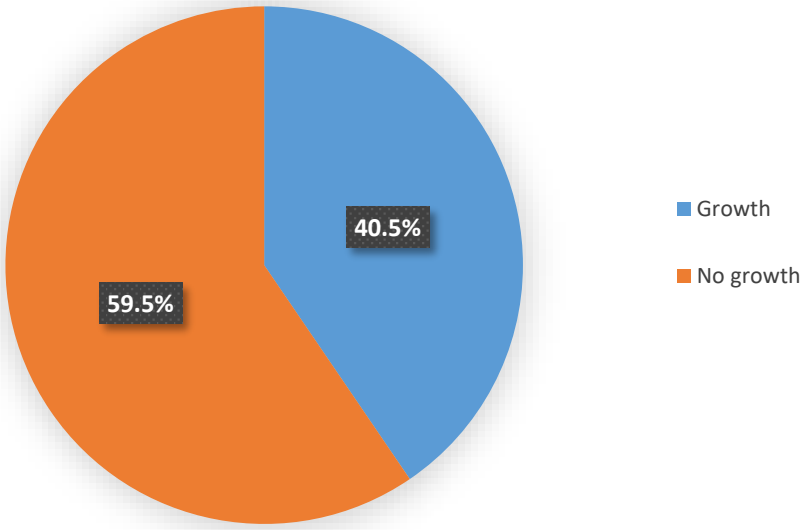


Figure 2: Distribution of bacterial growth among clinical samples

4.2 Culture positivity of specimens

Out of 549 total bacterial growth, 513 Gram negative bacteria were isolated from various clinical specimens in which 41 (3.02%) isolates were identified as *Pseudomonas aeruginosa* (Figure 3).

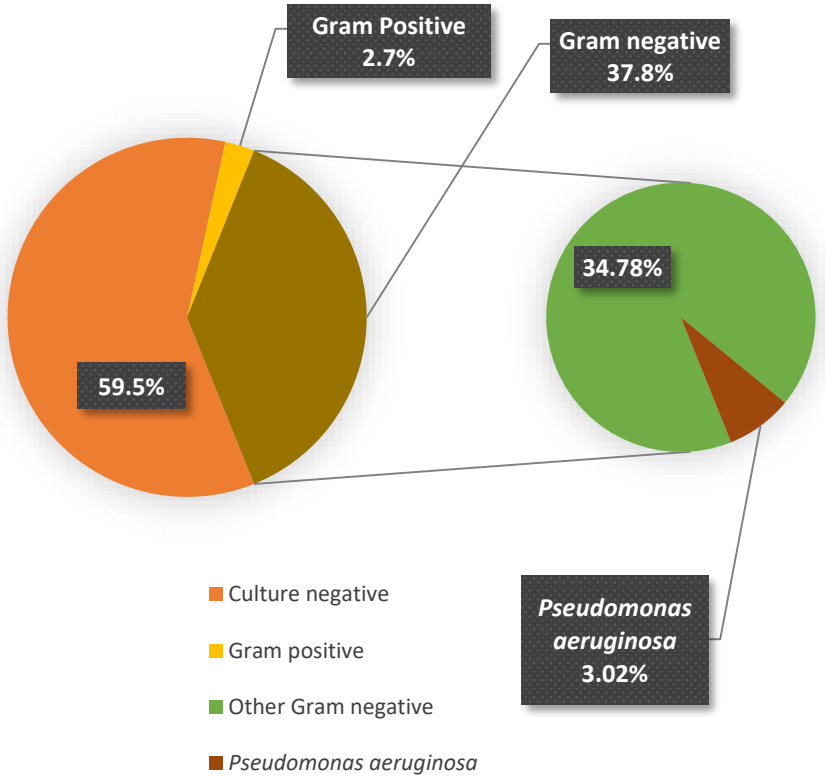


Figure 3: Growth of *P. aeruginosa* in the clinical specimens

4.3 Prevalence of *P. aeruginosa* according to age, sex, specimen type and patient types

Out of 41 *P. aeruginosa* isolates, more cases of Pseudomonal infection were seen in the age group 60 years and above (16, 39%) which was highest among all age group. It was followed by age group 40-59 years (11, 26.8%). Lowest number of isolates was observed in age group below 20 years (5, 12.2%). Of the total *P. aeruginosa* isolates, 63.4% (26) were from male patients while 36.6% (15) were from female patients. Sample wise distribution study revealed that *P. aeruginosa* was predominant in sputum samples (13, 31.7%), followed by urine (9, 22%) and the least from swab and biopsy. Department-wise distribution study shows that most *P. aeruginosa* strains were isolated from the inpatient department. Among 41 *P. aeruginosa* isolates, 26 (63.4%) were from inpatient department while rest 15 (36.6%) were from outpatient department (Table 5).

Table 5: Distribution of *P. aeruginosa* according to demographic information of patients

Character	No. of cases	*P. aeruginosa* isolated (n=41)		
		No.	% of total *P. aeruginosa*	% of total specimens
Age group (in years)				
<20	122	5	12.2	0.37
20-39	427	9	22	0.66
40-59	371	11	26.8	0.81
60 and above	436	16	39	1.18
Gender				
Male	737	26	63.4	1.91
Female	619	15	36.6	1.11
Specimen types				
Sputum	214	13	31.7	0.96
Urine	498	9	21.9	0.66
Pus	68	5	12.2	0.37
Blood	172	4	9.8	0.29
CSF	243	3	7.3	0.22
Swab	84	2	4.9	0.15
Biopsy	22	2	4.9	0.15
Catheter tip	22	3	7.3	0.22
Others	33	0	0	0
Patient type				
Inpatient	1063	26	63.4	1.9
Outpatient	293	15	36.6	1.12
Total	**1356**	**41**	**100**	**3.02**

*Others: Body fluid and stool

4.4 Antibiotic susceptibility pattern of *P. aeruginosa*

Antibiotic susceptibility test (AST) was performed by modified Kirby Bauer disc diffusion method. A total of 10 antibiotic discs from eight different class of antibiotics were used in AST along with piperacillin, piperacillin-tazobactam, ceftazidime, aztreonam, imipenem, gentamicin, ciprofloxacin, amikacin and levofloxacin. Aztreonam was most effective antibiotics in this study and its antimicrobial activity was reported to be 70.7% of the *P. aeruginosa* isolates. Similarly, ceftazidime was found to be least effective drug which was found to be effective against 31.7% of the *P. aeruginosa* isolates.

Table 6: Antibiotic susceptibility pattern of *P. aeruginosa*

Antibiotic discs	Susceptibility Patterns		
	Resistant N (%)	Intermediate N (%)	Sensitive N (%)
PI (100 µg)	13 (31.7)	10 (24.4)	18 (43.9)
PIT (100/10 µg)	8 (19.5)	9 (22)	24 (58.5)
CAZ (30 µg)	24 (58.5)	4 (9.8)	13 (31.7)
CPM (30 µg)	15 (36.6)	1 (2.4)	25 (61)
AT (30 µg)	12 (29.3)	0	29 (70.7)
IPM (10 µg)	17 (41.5)	1 (2.4)	23 (56.1)
GEN (10 µg)	15 (36.6)	4 (9.8)	22 (53.7)
CIP (5 µg)	20 (48.8)	7 (17.1)	14 (34.1)
AK (30 µg)	9 (22)	7 (17)	25 (61)
LE (5 µg)	20 (48.8)	4 (9.8)	17 (41.5)

*Abbreviations: PI: Piperacillin; PIT: Piperacillin-tazobactam; CAZ: Ceftazidime; CPM: Cefepime; AT: Aztreonam; IPM: Imipenem; GEN: Gentamicin; CIP: Ciprofloxacin; AK: Amikacin; LE: Levofloxacin

4.5 Distribution of multidrug resistance pattern in *P. aeruginosa* isolates

Among 41 *P. aeruginosa* strains subjected to antimicrobial susceptibility testing, 25 (61%) isolates were resistant to three or more classes of antibiotics and thus were considered as multidrug resistant (MDR). Non-MDR constituted 39% of the total isolates. The maximum number of MDR *P. aeruginosa* (MDRPA) was isolated from sputum (26.8%). There is statistically significant association found between prevalence of MDR *P. aeruginosa* in various specimens with MDR *P. aeruginosa* ($p<0.05$).

Maximum MDR isolates were from inpatients (48.8%). There is significant association between MDR *P. aeruginosa* and type of patients. Table 3 represents the distribution of MDR *P. aeruginosa*.

Table 7: Distribution of MDR *P. aeruginosa* among different clinical specimens and type of patient

Character	No. of cases	*P. aeruginosa* isolated			p-value
		MDR	Non-MDR	Total	
		No. (%)	No. (%)	No. (%)	
Specimen types					
Sputum	214	11 (26.8)	2 (4.9)	13 (31.7)	
Urine	498	2 (4.9)	7 (17.1)	9 (22)	
Pus	68	2 (4.9)	3 (7.3)	5 (12.2)	
Blood	172	4 (9.8)	0	4 (9.7)	0.015
CSF	243	2 (4.9)	1 (2.4)	3 (7.3)	
Swab	84	1 (2.4)	1 (2.4)	2 (4.9)	
Biopsy	22	0	2 (4.9)	2 (4.9)	
Catheter tips	22	3 (7.3)	0	3 (7.3)	
Others	33	0	0	0	
Patient type					
Inpatient	1063	20 (48.8)	6 (14.6)	26 (63.4)	0.006
Outpatient	293	5 (12.2)	10 (24.4)	15 (36.6)	
Total	**1356**	**25**	**16**	**41 (100)**	

The significant association is represented by $P < 0.05$ (significant), while $P > 0.05$ (insignificant)

4.6 β-lactamase production in *Pseudomonas aeruginosa* isolates

For the detection of β-lactamase production, ESBL and MBL were tested among the *P. aeruginosa* isolates. Out of 41 *P. aeruginosa*, 12 (29.2%) were found to produce at least one type of β-lactamase. Among them, 3 (7.3%) were ESBL producer only and 3 (7.3%) were MBL producer only. Co-production of ESBL and MBL was observed in 6 (14.6%) isolates (Figure 4).

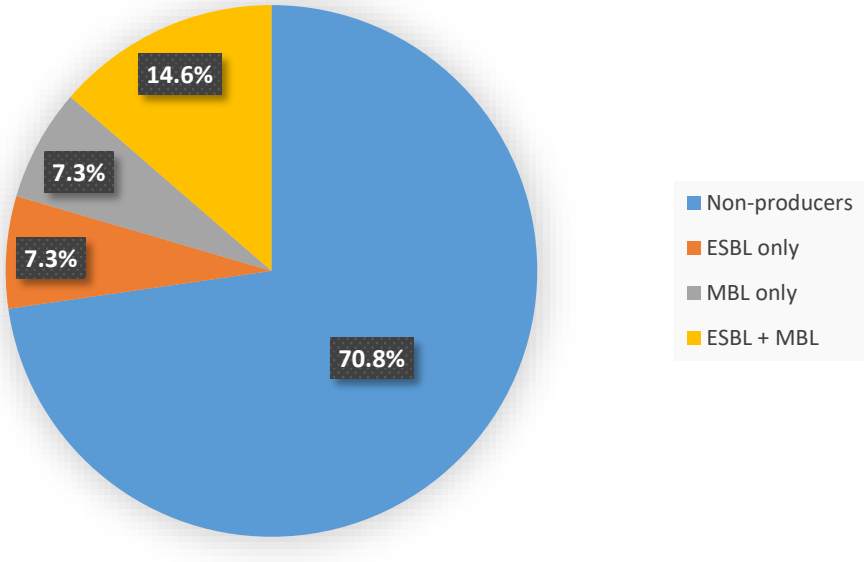


Figure 4: Beta lactamase production in *P. aeruginosa* isolates

4.7 β-lactamase production among MDR *P. aeruginosa*

Among 41 *P. aeruginosa* isolates, 17% MDR isolates were ESBL producer while 19.5% MDR isolates were MBL producer. Statically, it is found that ESBL production in MDR isolates were insignificant. However, MBL producing *P. aeruginosa* shows significant association with MDR *P. aeruginosa.*

Table 8: β-lactamase production among MDR *P. aeruginosa*

Tests	ESBL producing *P. aeruginosa*		MBL producing *P. aeruginosa*	
	Positive	**Negative**	**Positive**	**Negative**
	N (%)	**N (%)**	**N (%)**	**N (%)**
MDR	7 (17)	18 (43.9)	9 (21.9)	16 (39)
Non-MDR	2 (4.9)	14 (34)	0 (2.4)	16 (39)
Total	**9 (21.9)**	**22 (77.9)**	**9 (21.9)**	**32 (78)**
p-value	**0.242**		**0.007**	

The significant association is represented by $P < 0.05$ (significant), while $P > 0.05$ (insignificant)

4.8 Prevalence of virulence genes (*toxA*, *exoY* and *oprL*) in *Pseudomonas aeruginosa* isolates

Out of 41 total isolates, the polymerase chain reaction (PCR) result showed the prevalence of virulence genes in *P. aeruginosa* in which 23 (56%) isolates were positive for *toxA* gene, 28 (68.3%) isolates were positive for *exoY* gene and 25 (61%) isolates were positive for *oprL* gene. The maximum number of virulence genes were found in sputum followed by urine sample. No significant association was found between occurrences of virulence genes with prevalence of *P. aeruginosa* in various clinical specimens ($P > 0.05$).

Table 9: Prevalence of virulence genes (*toxA*, *exoY* and *oprL*) in *P. aeruginosa* isolated from various clinical specimens

Character	Presence of virulence genes among *P. aeruginosa* isolates								
	toxA gene			*exoY* gene			*oprL* gene		
	No.	**%**	**p-value**	**No.**	**%**	**p-value**	**No.**	**%**	**p-value**
Sputum	9	22		8	19.5		10	24.4	
Urine	5	12.2		7	12.2		4	9.8	
Pus	2	4.9		4	9.8		3	7.3	
Blood	3	7.3	0.852	2	4.9	0.551	1	2.4	0.14
CSF	1	2.4		3	7.3		3	7.3	
Swab	1	2.4		2	4.9		0	0	
Biopsy	1	2.4		2	4.9		2	4.9	
Catheter tip	1	2.4		1	2.4		2	4.9	
Others	0			0			0	0	
Total	**23**	**56.1**		**28**	**68.3**		**25**	**61**	

The significant association is represented by $P < 0.05$ (significant), while $P > 0.05$ (insignificant)

4.9 Occurrence of virulence genes (*toxA*, *exoy* and *oprL*) among multidrug resistant and β-lactamase producing *P. aeruginosa* isolates

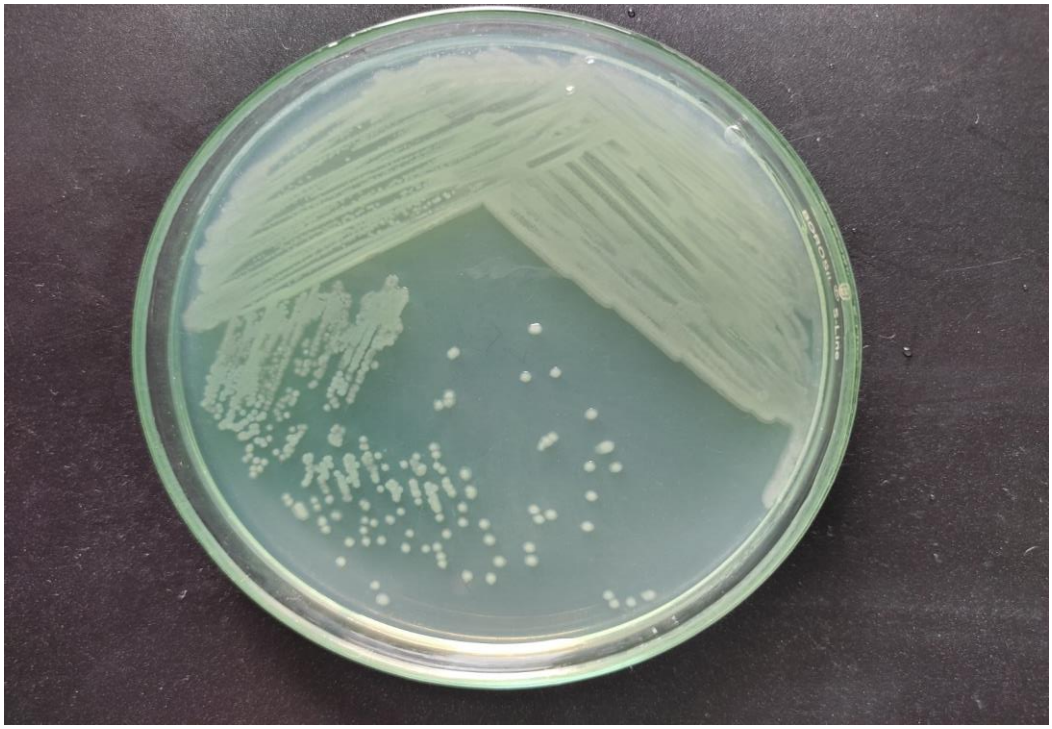
Among 25 MDR *P. aeruginosa*, 36.6% shows *toxA* gene and *exoY* gene while 41.5% shows *oprL* gene. No significant association was found among occurrence of virulence genes in MDR *P. aeruginosa* (p>0.05).

Among 9 ESBL producing *P. aeruginosa*, 17.1% shows the presence of *toxA* gene, 14.6% shows *exoY* gene and 7.3% shows *oprL* gene. Among 9 MBL producing *P. aeruginosa*, 17.1 shows *toxA* gene and *exoY* gene while 12.2% *oprL* shows gene. No significant association was found among the occurrence of virulence gene with β-lactamase producing *P. aeruginosa* (p>0.05).

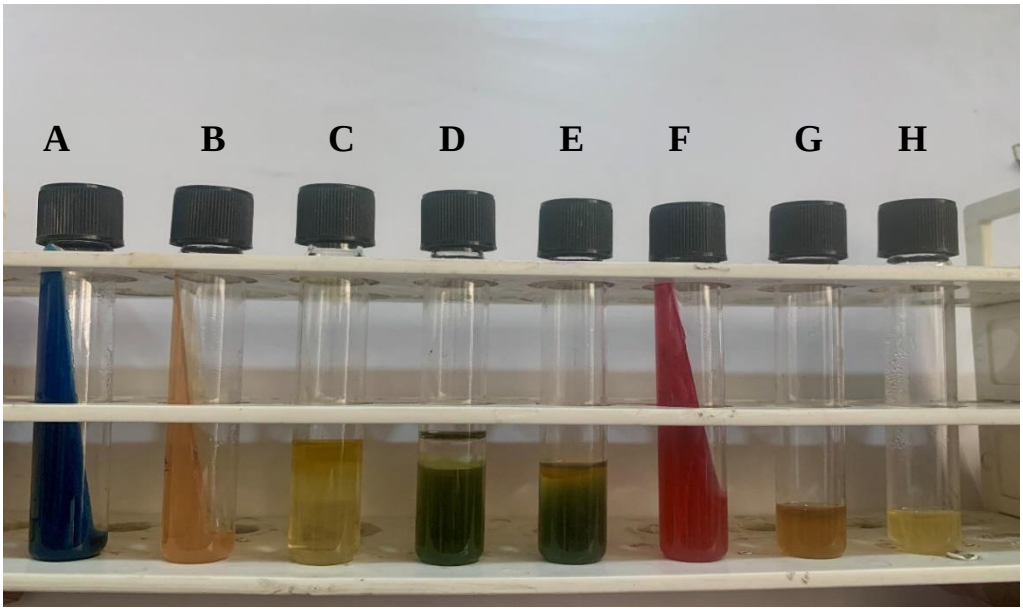
Table 9: Occurrence of *toxA*, *exoY* and *oprL* virulence genes among multidrug resistant and β-lactamase producing *P. aeruginosa* isolates

Drug spectrum	Presence of virulence genes among *P. aeruginosa* isolates								
	***toxA* gene**			***exoY* gene**			***oprL* gene**		
	No.	**%**	**p-value**	**No.**	**%**	**p-value**	**No.**	**%**	**p-value**
MDR	15	36.6	**0.529**	15	36.6	**0.154**	17	41.5	**0.249**
Non-MDR	8	19.5		13	31.7		8	19.5	
ESBL	7	17.1	**0.138**	6	14.6	**0.906**	3	7.3	**0.054**
Non-ESBL	16	39		22	53.7		22	53.7	
MBL	7	17.1	**0.138**	7	17.1	**0.489**	5	12.2	**0.706**
Non-MBL	16	39		21	51.2		20	48.8	
Total	**23**	**56.1**		**28**	**68.3**		**25**	**61**	

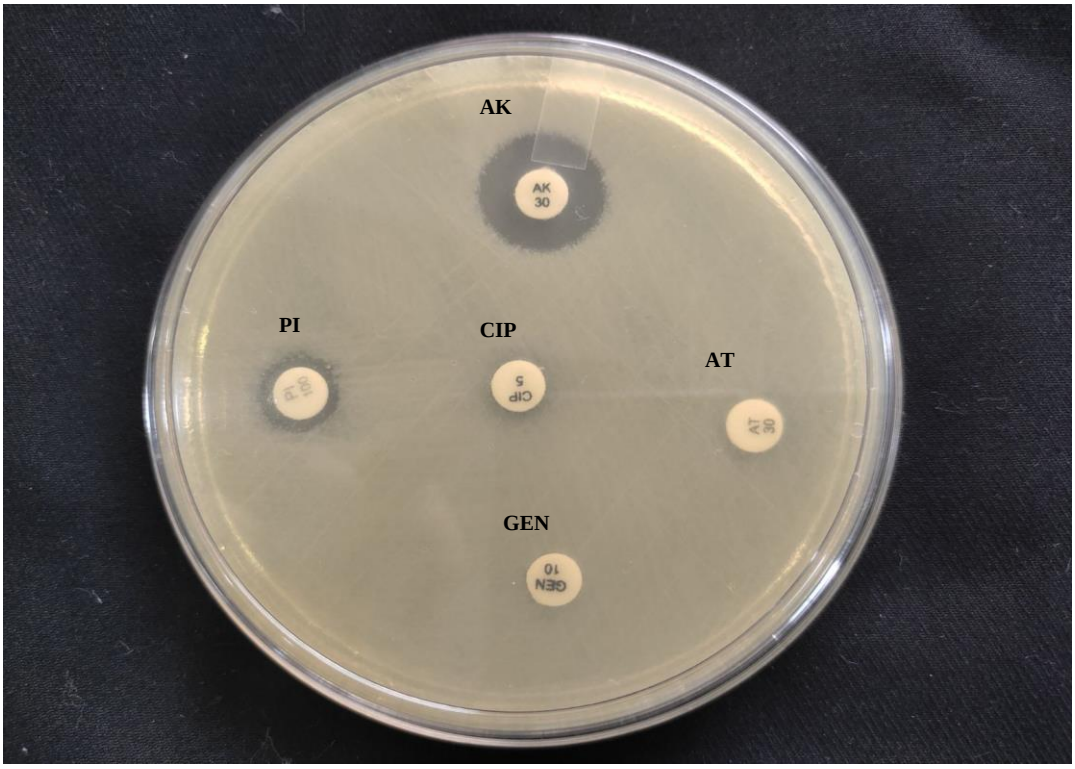
The significant association is represented by $P < 0.05$ (significant), while $P > 0.05$ (insignificant).



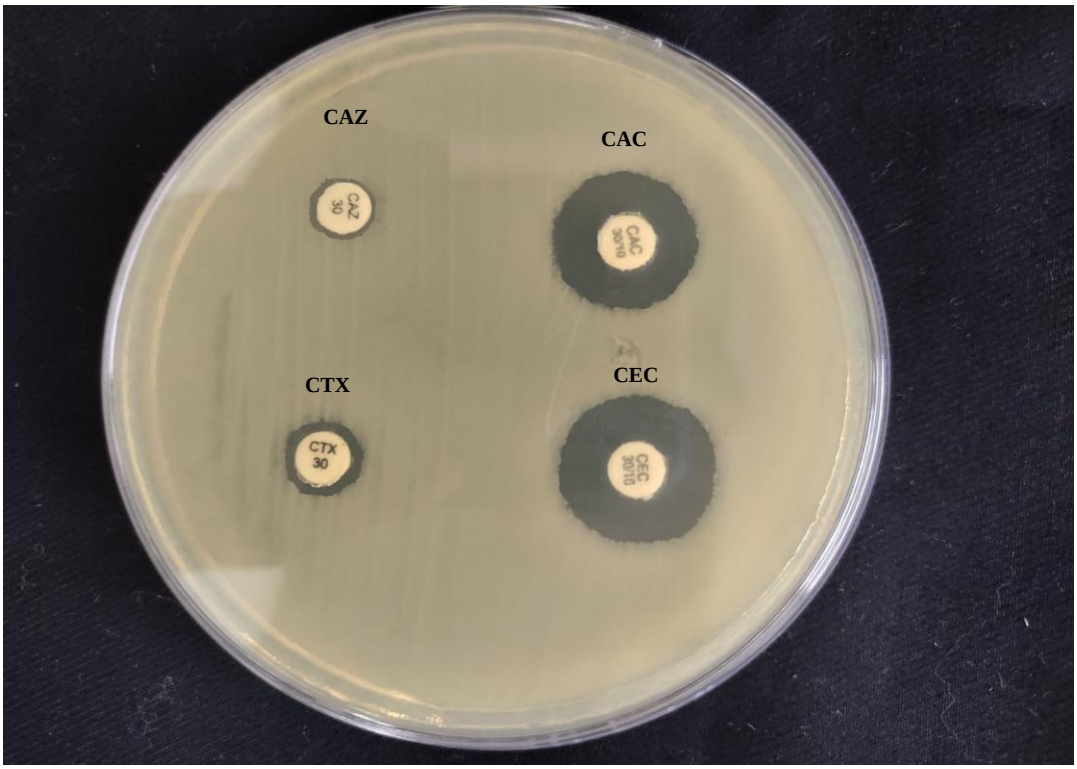
Photograph 1: Pigmented colonies of *Pseudomonas aeruginosa* on Nutrient Agar after 24 hours of incubation at 37°C



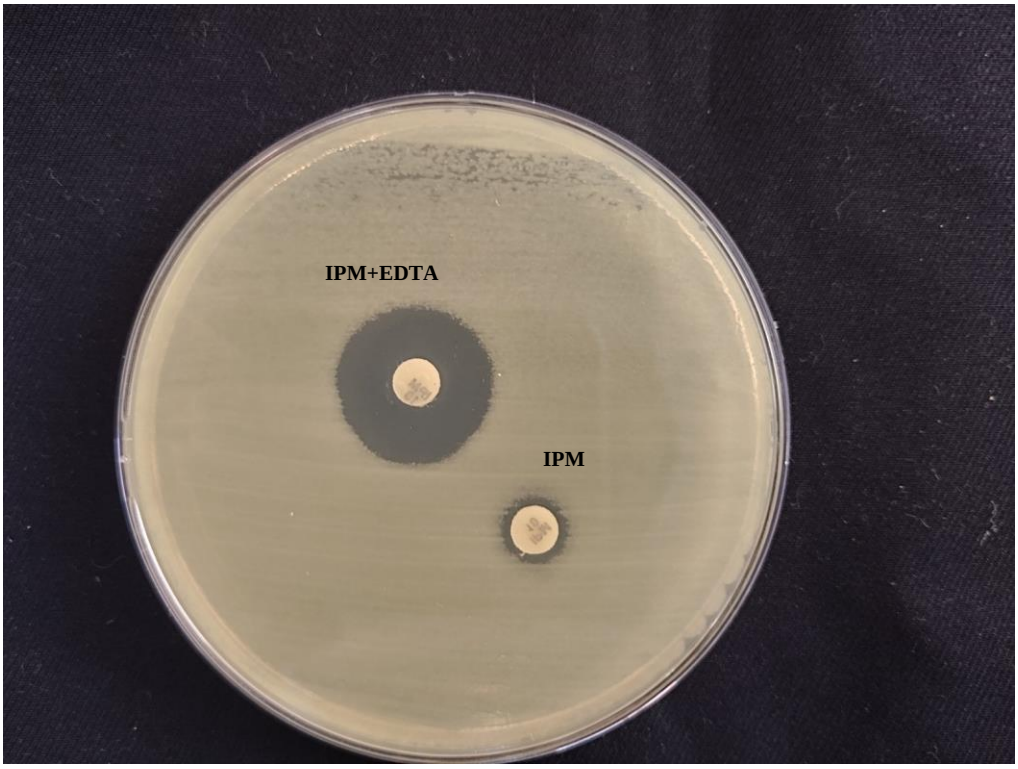
Photograph 2: Biochemical tests of *P. aeruginosa* (A: Citrate utilization positive; B: Urease production negative; C: Indole production negative and motility positive; D: Fermentative test negative; E: Oxidative test positive; F: TSI: alkaline/no change, lactose non-fermenter; H_2S production negative, gas production negative; G: Voges-Proskauer test negative; H: Methyl red test negative).



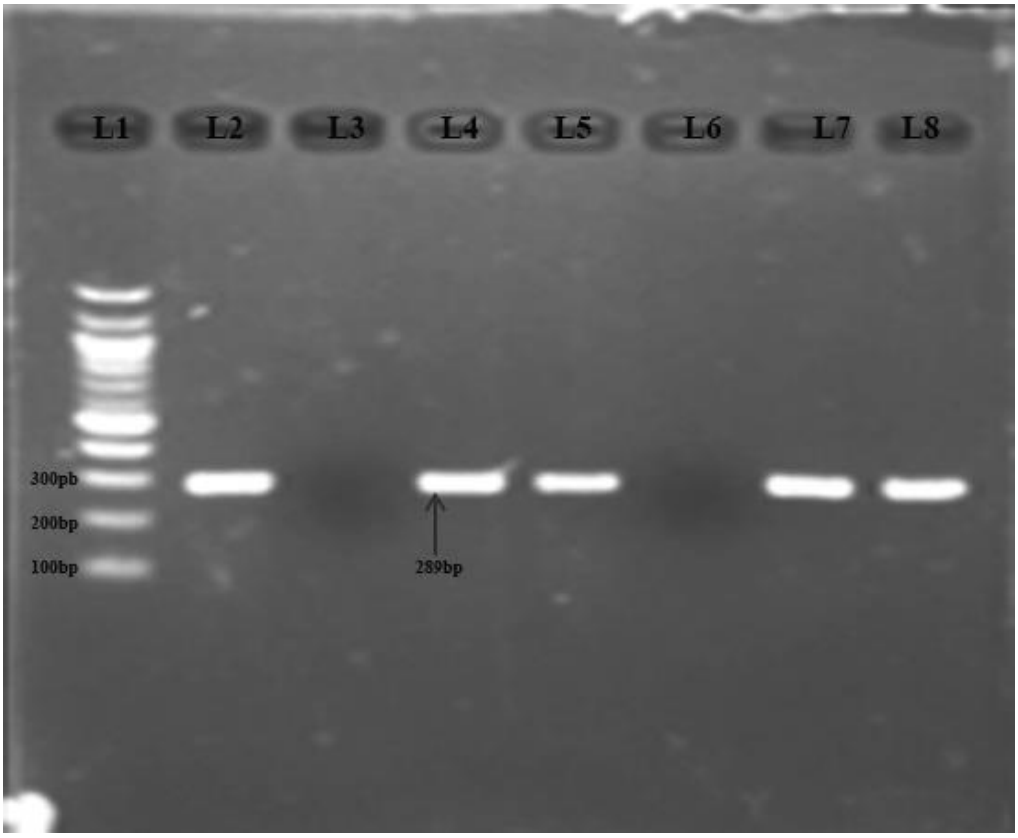
Photograph 3: A: Multidrug resistant *P. aeruginosa* on MHA (AK: Amikacin; PI: Piperacillin; CIP: Ciprofloxacin; AT: Aztreonam; GEN: Gentamicin; CAZ: Ceftazidime)



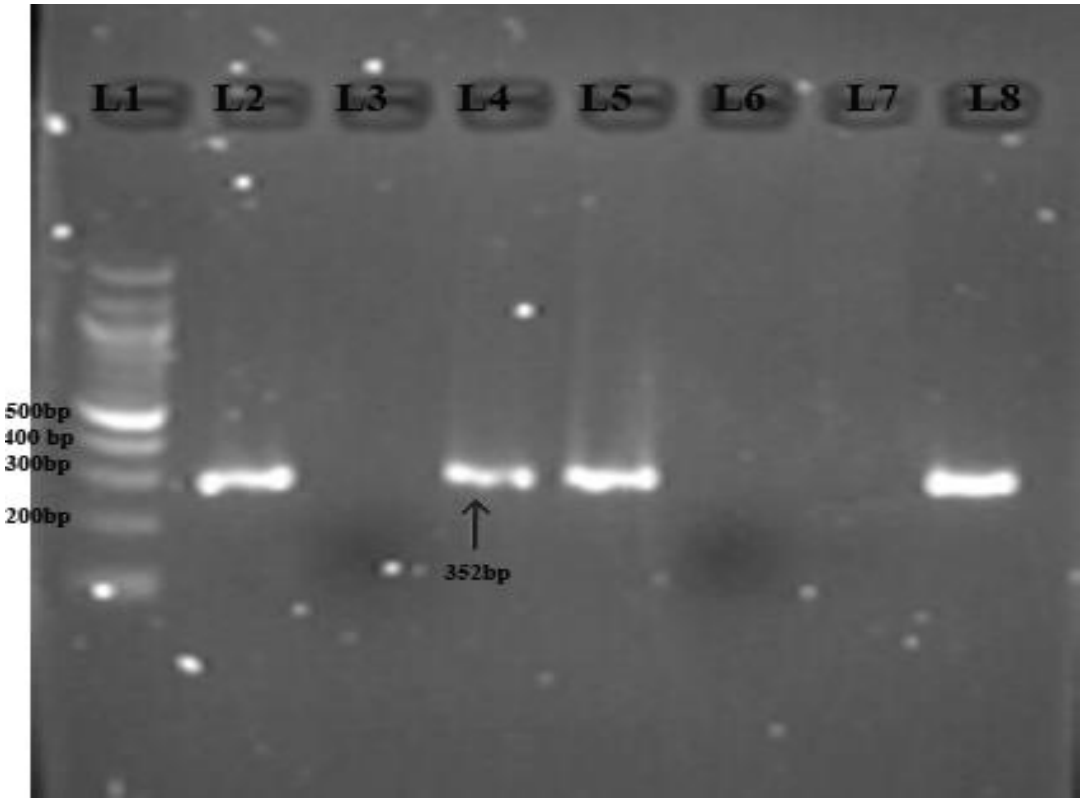
Photograph 4: Phenotypic detection of ESBL by Combined Disc Test (ESBL producer) (CAZ: Ceftazidime; CAC: Ceftazidime/Clavulanic acid; CTX: Cefotaxime; CEC: Cefotaxime/Clavulanic Acid)



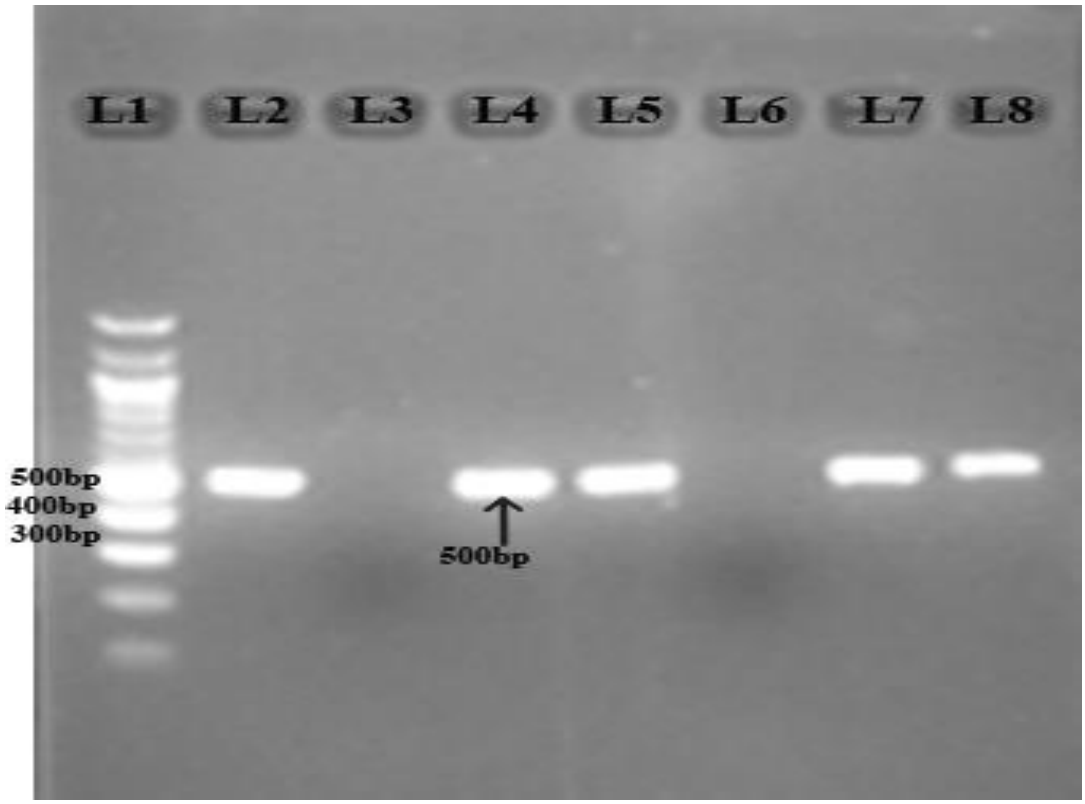
Photograph 5: Phenotypic detection of MBL by Imipenem-EDTA method (MBL producer) (IPM: Imipenem; EDTA: Ethylene Diamine Tetra-acidic Acid)



Photograph 6: PCR amplification of *exoY* gene in *P. aeruginosa* isolates. Lane L1: Molecular marker (ladder 100 bp), Lane L2: Positive control for *exoY* gene (289 bp), Lane L3: Negative control for *exoY* gene, Lane L4-L8 represents samples 1-5 (sample number 1, 2, 4 and 5 were positive).



Photograph 7: PCR amplification of *toxA* gene in *P. aeruginosa* isolates. Lane L1: Molecular marker (ladder 100 bp), Lane L2: Positive control for *toxA* gene (352 bp), Lane L3: Negative control for *toxA* gene, L4-L8 represents samples (sample number 1, 2 and 5 were positive).



Photograph 8: PCR amplification of *oprL* gene in *P. aeruginosa* isolates. Lane L1: Molecular marker (ladder 100 bp), Lane L2: Positive control for *oprL* gene (500 bp), Lane L3: Negative control for *oprL* gene, Lane L4-L8 represents samples (sample number 1, 2, 4 and 5 were positive).

CHAPTER V

DISCUSSION

Pseudomonas aeruginosa is a well-known harmful microbe that can cause different illnesses, especially in individuals with weak immune systems (Ansari et al 2016). The bacteria developing resistance to multiple antibiotics is a growing trend that poses a significant global challenge and is the primary cause of treatment failures, which can lead to severe illness and even death (Ansari et al 2016; Bassetti et al 2018; El Zowalaty et al 2015). In developing countries like Nepal self-medication is a common practice which could potentially contribute significantly to the emergence of antibiotic resistance in clinical isolates (Acharya & Wilson 2019). The improper use of antibiotics and the involvement of unqualified healthcare providers can contribute to the development of antibiotic resistance in bacteria. Other factors such as expired medications, counterfeit drugs, and insufficient hospital infection control measures can also promote the emergence of resistance in clinical strains (Ayukekbong et al 2017).

P. aeruginosa is an opportunistic pathogen that can cause infection in humans which involves adhering to a surface, colonization, local tissue invasion, and spreading throughout the body to cause a systemic disease (Chand et al 2021). Its infections can cause serious illness and death because this bacterium can adapt quickly to changing environments, develop antibiotic resistance rapidly, and produce many different virulence factors (Strateva & Mitov 2011). The objective of this research was to investigate the impact of *Pseudomonas aeruginosa* in clinical settings, examine its susceptibility to antibiotics and detect the presence of virulence genes (*toxA, exoY* and *oprL*) in all isolated samples.

This study was conducted at Annapurna Neurological Institute and Allied Sciences (ANIAS), Maitighar, Kathmandu, Nepal. During the study period, different types of clinical specimens (sputum, urine, pus, blood, CSF, swab, biopsy and Catheter's tip) were collected and processed for the primary isolation and identification of *P. aeruginosa*.

Among 1356 clinical specimens received, 549 samples (40.5%) showed bacterial growth whereas 807 samples (59.5%) showed no growth. Similar results were shown in study conducted by Mahaseth et al (2020) in Chitwan, Nepal which showed 43.9% culture positivity among various clinical samples. Different other studies conducted in Nepal by Bhandari et al (2022), Pokharel et al (2019), Thakur et al (2012) and Thapa et al (2016) showed the culture positive rate of 13 to 58% in clinical isolates. Such variation in the bacterial isolation rate might be due to the difference in sample size involved in study, geographical condition, climatic condition, host factor and lack of good hygiene practice (Adhikari et al 2019; Maharjan et al 2020).

In this study, Gram negative bacteria (37.8%) isolated from total clinical specimens were more prevalent than Gram positive bacteria (2.7%). Similar findings have been reported by Bhandari et al (2022) and Adhikari et al (2019) in Nepal and Moyo et al (2010) in Tanzania. Several studies conducted in Nepal have shown an increase in the number of Gram-negative bacterial isolates in clinical specimens, indicating that these bacteria are becoming a significant concern in healthcare-associated infections (Koirala et al 2021; Sah et al 2021).

A total of 41 strains of *P. aeruginosa* was isolated from various clinical specimens. The prevalence of *P. aeruginosa* in this investigation was 3.02%. The lower prevalence found in this study is supported by Sathyavathy & Madhusudhan (2020), Chand et al (2021) and Bhandari et al (2022) which reported the frequency of *P. aeruginosa* as 2.2%, 4.29% and 1.09% respectively. On contrary, in study conducted by Adhikari et al (2020), Maharjan (2022), Mahaseth et al (2020) and Shrestha et al (2018) , the prevalence was reported as 8.6%, 6.48%, 11.29% and 7.9% respectively. The variation in prevalence rate of *P. aeruginosa* in these studies might be due to difference in the type of clinical specimens received, study population, study duration and type of hospital (Chand et al 2021).

It was observed that majority of the isolates were obtained from the age group 60 years and above with a prevalence rate of 39% which reflects that the infection caused by *P. aeruginosa* is more common in patient of old age group. Chand et al (2021) and Shrestha et al (2016) in Nepal and Gautam & Gopi

(2019) in India also found most of the cases of Pseudomonal infection belonging to this age group. This age group is highly vulnerable for infection and also longer duration of stay at the hospital, decrease immunity and other associated co-morbidities could be the reason for infection this age group (Chand et al 2021; Gautam & Gopi 2019).

Gender-wise, majority of the isolates were from male patients (63.4%) while 36.6% isolates were from female patients. This finding correlates with various previous reports from Nepal. Baral et al (2019), Shrestha et al (2018), Shrestha et al (2019), Sathyavathy & Madhusudhan (2020), Maharjan (2022), Mahaseth et al (2020) reported highest percentage of male patients which are 61%, 55.6%, 60%, 57.1%, 51.47% and 62.25% respectively. But in contrast to the finding by Bhattarai et al (2016) and Anil & Mohammad Shahid (2013) in Nepal and Mohammadzadeh et al (2017) in Iran showed maximum infection in female patients by 80.08%, 55.17% and 68.4% respectively. This may be due to the differences in various factors like variances in the immune system, lifestyle choices, and occupational performance among individuals who contract with this opportunistic pathogen (Mohammadzadeh et al 2017).

Sample-wise distribution study revealed sputum as the most common source of the isolates accounting for 31.7% of the total *P. aeruginosa* growth. Previous studies from Nepal have also reported higher proportion of *P. aeruginosa* from sputum samples in which Ansari et al (2016), Baral et al (2019), Mahaseth et al (2020) and Shrestha et al (2019) showed the prevalence of *P. aeruginosa* by 38.2%, 65.8%, 45.47% and 33.3% respectively. On contrary, some studies have reported maximum isolation from other specimens such as Anil & Mohammad Shahid (2013) and Shrestha et al (2019) in Nepal reported maximum isolates from pus samples. The occurrence of *P. aeruginosa* in various specimens may differ across with each hospital, since each hospital has its own unique environment that can influence bacterial distribution (Manandhar et al 2018).

In this study, the higher prevalence rate of *P. aeruginosa* was observed from clinical specimens of inpatients (75.6%) as compared to outpatients (24.4%). Similarly, higher growth rate of *P. aeruginosa* in hospitalized patients rather than outpatients were observed in study carried out by Ansari et al (2016),

Mahaseth et al (2020) and Sujakhu et al (2018). The majority of the isolates were identified from inpatients since the duration of hospital stay is directly proportional to the infection (Mahaseth et al 2020). In contrast, another study carried out by Chand et al (2021) shows majority of *P. aeruginosa* growth in outdoor patients. This may be due to the frequent exposure of those patients with infected surrounding (Chand et al 2021).

Knowledge on the antibiogram of pathogen is vital for clinicians in their routine works (Truong et al 2021). *P. aeruginosa* isolates in this study were tested for susceptibility to ten different antibiotics by modified Kirby-Bauer disc diffusion method. Aztreonam (AT/30 µg) with sensitivity 70.7% were found to be most potent antibiotics. Mahaseth et al (2020) and Chand et al (2021) also reported high sensitivity of aztreonam as 62.25% and 66.66% respectively. The limited use of aztreonam in this hospital could be the reason behind its high sensitivity.

The spread of MDR *P. aeruginosa* pose a huge threat in the treatment of Pseudomonal infection. This study revealed that more than half (61%) of our isolates were MDR. This is quite worrisome as MDR strains are difficult and expensive to treat. The percentage of MDR in the study is lower than previously reported rate of 82.97% by Maharjan et al (2020). Yet lower number of MDR have also been noted by various researchers 20.69% by Anil & Mohammad Shahid (2013) and 32.2% by Shrestha et al (2019). The high percentage of resistance may be due to indiscriminate use of antibiotics resulting in the failure of commonly used drugs for the management of Pseudomonal infection. Furthermore, mutation or acquisition of resistant genes through horizontal gene transfer can occur during antibiotic therapy resulting in the over expression of endogenous beta lactamases, efflux pump genes and expression of specific porins. This could contribute to high level of resistivity (Honoré et al 1989). Self-medication by patients and incomplete course of treatment are also probable contributing factors (Acharya & Wilson 2019). Highest frequency of MDR *P. aeruginosa* were identified in sputum sample (26.8%) and from inpatient department (48.8%). Statistically, there was found significance association between occurrence of *P. aeruginosa* with type of patient and nature of specimen.

The issue of β-lactamases production in bacteria is increasing throughout the world and has become a huge concern to therapeutic treatment. All the isolates in this study were tested for ESBL and MBL production. It was found that 29.2% isolates showed β-lactamases production. This finding is supported by Ansari et al (2016) and Shrestha et al (2018) which report 33.1% and 36% ESBL producer *P. aeruginosa* respectively. However, in some studies such as Poudyal et al (2011) and Bhandari et al (2015) did not find any ESBL producing *Pseudomonas aeruginosa*. Similarly, this study showed 29.2% MBL producer isolates which is supported by the study done by Acharya et al (2017) who had reported 30.9% MBL producing *P. aeruginosa*. Shrestha et al (2018) have reported that 8% *P. aeruginosa* as MBL producer which is much lower compared to our study. The high prevalence of β-lactamase production is may be due to the long-term hospital stay, maximum use of β-lactam antibiotics and horizontal transfer of β-lactamases encoding genes.

Detection of β-lactamase producing multidrug-resistant (MDR) bacteria is crucial as it poses challenges in therapeutic management and restricts available treatment choices. Among 25 MDR *P. aeruginosa*, 17% were ESBL producer and 21.9% were MBL producers. Statistical analysis showed that there was significant association in prevalence of MDR and MBL producer isolates ($p<0.05$) while there was found no significant association of prevalence of MDR with ESBL producer isolates ($p>0.05$). This result is supported by (Shrestha et al 2017) which showed 34.9% ESBL producer among MDR *P. aeruginosa* with no significant association between them.

In this present study, PCR showed that 56% *Pseudomonas aeruginosa* isolates showed *toxA* gene positive of amplicon size 352bp, 68.3% isolates were *exoY* gene positive of amplicon size 289 bp and 61% isolates showed *oprL* gene positive of 500 bp amplicon size. Mapipa et al (2021), Chand et al (2021) and Mohammadzadeh et al (2017) showed the prevalence of *toxA* gene as 100%, 95.4% and 100% respectively. Bogiel et al (2021) and Rodrigues et al (2020) showed the prevalence of *exoY* as 99.1% and 75.9% respectively. Chand et al (2021) and Aslani et al (2012) showed prevalence rate of *oprL* gene as 100% and 96% respectively. The possible reason for low prevalence rate of these three virulence genes (*toxA, exoY* and *oprL*) in this study might be because during

infection the bacterium may undergo genomic reduction. This process may lead to the loss of virulence factors that are no longer necessary for survival in the host. As a result, the bacterium may become less virulent but better adapted to persist in the host environment (Elmouaden et al 2019; Lee et al 2006). This study also revealed that there is no significant association between presences of virulence gene with prevalence of *P. aeruginosa* in various clinical specimens. This result are compatible to the research conducted by Elmouaden et al (2019) in Morocco and Pirnay et al (2009) in Belgium. The prevalence of *P. aeruginosa* and its virulence genes depends upon nature of place, degree of contamination, immunity of patients and virulence of strains (Chand et al 2021).

Most of the previous studies like Garey et al (2008) and Breidenstein et al (2011) have separately discussed on antibiotic resistance or virulence. This study assessed the potential relationship between virulence genes and antibiotic resistance parameters. Among 25 MDR, the prevalence of *toxA*, *exoY* and oprL gene are 36.6%, 36.6% and 41.5% respectively. Among 9 ESBL, prevalence of these genes are 17.1%, 14.6% and 7.3% respectively while among 9 MBL, the prevalence of these genes are 17.1%, 17.1% and 12.2% respectively. In this research, it was found that there is no significance association between virulence genes with multidrug resistant and β-lactamase producing *P. aeruginosa* ($p>0.05$). Research conducted by Elmouaden et al (2019) and Gajdács et al (2021) also showed no association between virulence gene and antibiotic resistance.

This study provides the data about phenotypic and genotypic characteristics of *P. aeruginosa* which could be helpful for health worker to improve infection control measures and to establish a surveillance system. This effort may contribute to control the emergence and transmission of MDR bacteria in clinical setting. Additionally, this study could be a significant reference for further study on prevalence of virulence genes in *P. aeruginosa.*

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study reported 40.5% bacterial growth in various clinical specimens and the prevalence rate of *P. aeruginosa* was 3.02%. Aztreonam was found to be effective antibiotics for treatment of infection caused by *P. aeruginosa* followed by imipenem, gentamicin and amikacin. The study reveal that more than half of *P. aeruginosa* isolates harbor at least one of virulence genes, namely *toxA, exoY* and *oprL*. Out of total *P. aeruginosa* isolates, majority (61%) of them were MDR while only 29.2% showed β-lactamase production. Additionally, the study also suggests that there is no association of virulence genes of *Pseudomonas aeruginosa* with MDR and β-lactamase production. Multidrug resistant *P. aeruginosa* have been considered as one of the challenging nosocomial as well as community acquired pathogen. The higher rate of MDR is worrisome and special attention is required in regular surveillance of antibiotic susceptibility patterns. Similarly, the presence of intrinsic virulence and pathogenicity of bacteria is indicated by existence of virulence genes such as *toxA*, *oprL* and *exoY*. Therefore, the detection of these genes by PCR is highly recommended.

6.2 Recommendations

1. Aztreonam could be the drug of choice for the management of *P. aeruginosa* infections.
2. Identification of *P. aeruginosa* from clinical isolates is needed along with detection of virulence genes so that pathogenicity of bacteria can be known.
3. Since MDR isolates of *P. aeruginosa* are significantly associated with type of patients, it is crucial to prioritize strict infection control measures, raise awareness regarding appropriate use of antibiotics and implement surveillance program for early diagnosis and treatment.
4. Screening of MDR, ESBL and MBL encoding genes is recommended.

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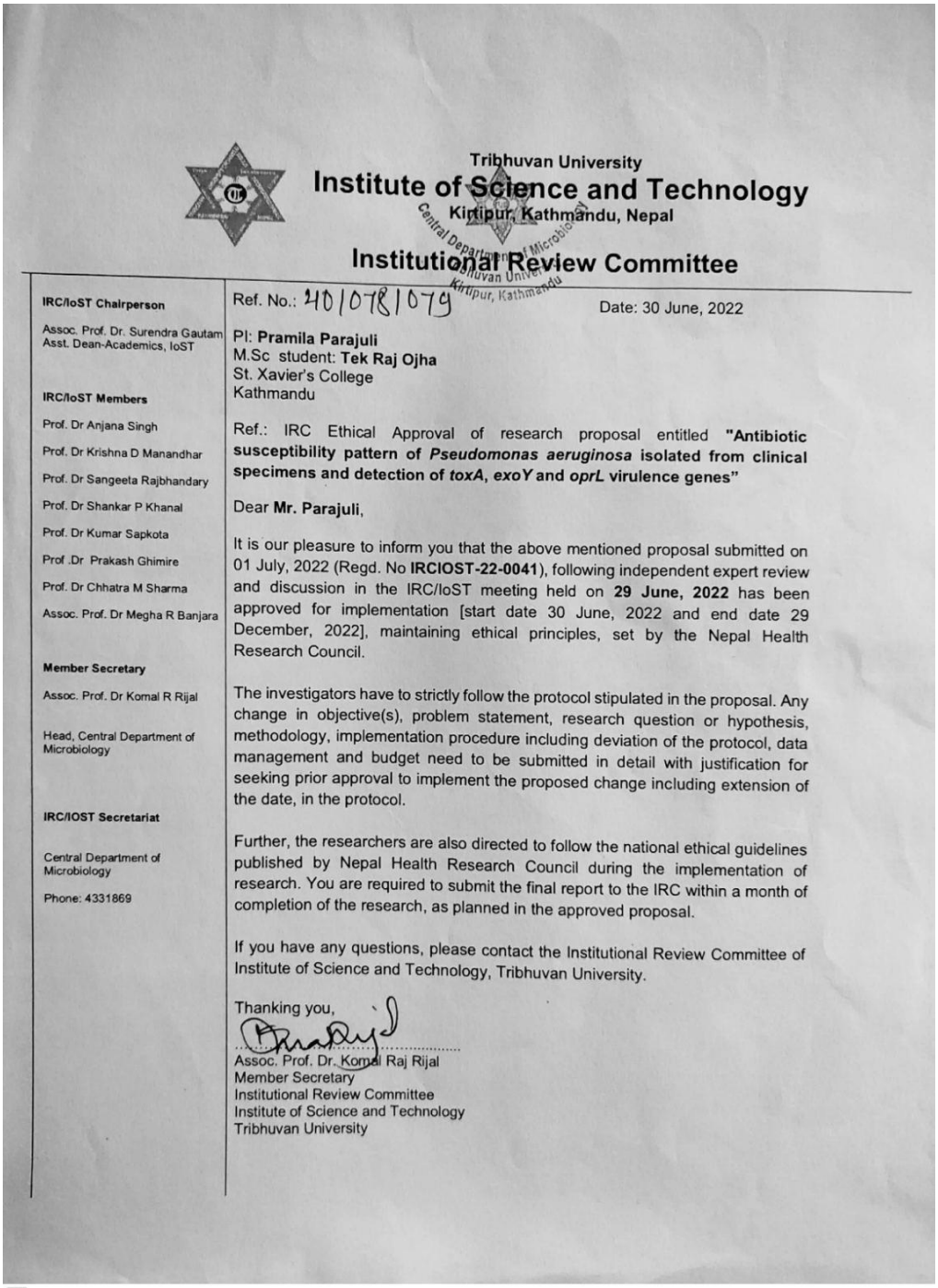
APPENDIX A

ETHICAL APPROVAL



Tribhuvan University
Institute of Science and Technology
Kirtipur, Kathmandu, Nepal

Institutional Review Committee

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Ref. No.: 210/078/079 Date: 30 June, 2022

PI: **Pramila Parajuli**
M.Sc student: **Tek Raj Ojha**
St. Xavier's College
Kathmandu

Ref.: IRC Ethical Approval of research proposal entitled **"Antibiotic susceptibility pattern of *Pseudomonas aeruginosa* isolated from clinical specimens and detection of *toxA*, *exoY* and *oprL* virulence genes"**

Dear **Mr. Parajuli**,

It is our pleasure to inform you that the above mentioned proposal submitted on 01 July, 2022 (Regd. No **IRCIOST-22-0041**), following independent expert review and discussion in the IRC/IoST meeting held on **29 June, 2022** has been approved for implementation [start date 30 June, 2022 and end date 29 December, 2022], maintaining ethical principles, set by the Nepal Health Research Council.

The investigators have to strictly follow the protocol stipulated in the proposal. Any change in objective(s), problem statement, research question or hypothesis, methodology, implementation procedure including deviation of the protocol, data management and budget need to be submitted in detail with justification for seeking prior approval to implement the proposed change including extension of the date, in the protocol.

Further, the researchers are also directed to follow the national ethical guidelines published by Nepal Health Research Council during the implementation of research. You are required to submit the final report to the IRC within a month of completion of the research, as planned in the approved proposal.

If you have any questions, please contact the Institutional Review Committee of Institute of Science and Technology, Tribhuvan University.

Thanking you,

Assoc. Prof. Dr. Komal Raj Rijal
Member Secretary
Institutional Review Committee
Institute of Science and Technology
Tribhuvan University

APPENDIX B

INFORMED CONSENT FORM

Consent form for sample testing in patients

This is to inform that I have been counseled about the samples test to be conducted on me and have been explained about the implications of the test results. I have given the return date of my test results. I also understand that I am free to refuse the test and still get the help I need from this center without being discriminated against.

I hereby give my consent for the test to be conducted in order for me to know my status.

Hospital no. of patient: …….
Signature of patient: ……..
Date: …/ ……/……

APPENDIX C

QUESTIONNAIRES

CLINICAL AND MICROBIOLOGICAL PROFILES OF PATIENTS

Clinical profile

Name of the patient: …….. Address: ……

Age/Sex: ……

In patients/Out patients: ……..

Lab No: ……..

Date: ……

Brief clinical history:

Patients on antibiotics: a. Yes b. No

If Yes, antibiotic(s) taken: 1) ……. 2) ……

Duration of treatment ……..

Immunosuppressive disease present: a. Yes b. No

If yes, what: a) Kidney transplant patient b) Diabetes c) Others

Microbiological profile

Day 1 (……. /……. /……)

Specimen: ……

Type of collection: ……

Method of sample collection: ……

Microscopic Observation:

Color: ….. Appearance: … pH: ….. Others: …….

Direct microscopic examination (if necessary):

Culture on: 1)…… 2)……3)…….

Incubation: 1) Aerobic 2) Anaerobic 3) Microaerophilic

Incubation temperature and period: …..

Day 2 (……../……/……..)

Reading of culture plates

Colony characteristics

Media used	Shape	Size	Color	Texture	Opacity	Consistency

Gram staining results: …..

Catalase: …….

Oxidase: …….

Coagulase: …….

Others: …….

Provisional identification of organism: ……..

Day 3 (……../……/……..)

Identification of organisms

Biochemical Tests:

MR: …

VP: ….

SIM: …

Citrate: …

TSIA: ….

Urease:

Organism identified as: …….

Day 4 (……../……/……..)

Antibiotic Sensitivity Test: Kirby-Bauer Method

Antibiotics used	Zone of inhibition (mm)	Interpretation

Day 5 (……../……/……..)

Confirmatory test for ESBL production

Disk used	**Increase in size of zone of inhibition (mm)**	**Interpretation**
Ceftazidime-clavulanate (CAC)		

Confirmatory test for MBL production

Disk used	**Increase in size of zone of inhibition (mm)**	**Interpretation**

Comments on drug resistance pattern: MDR/ No MDR

Resistance to …… number of antibiotics

Performed by:

……

Checked by:

……

Appendix D

List of materials and equipment

Equipment

Autoclave
Bunsen burners
Centrifuge
Electrophoresis tank
Gel documentation system
Heater
Hot air oven
Incubator
Microscope
p^H meter
Refrigerator
Thermo cycler
Vortex
Water bath
Weighing machine

Glassware

Beakers
Blood culture bottles
Conical flasks
Glass slides
Measuring cylinder
Microscopic slide
Petri plates
Test tubes
Volumetric flasks
Cover slip
Glass rod

Microbiological media

Blood Agar Base
Cetrimide Agar

Chromogenic UTI Agar
MacConkey Agar
Muller Hinton Agar
Nutrient Agar
Nutrient Broth
Tryptic Soya Broth

Biochemical media

MacConkey Agar
Blood Agar
Muller Hinton Agar
Nutrient Agar
Nutrient Broth
MR-VP Broth
Simmon's Citrate Agar
Sulphur Indole Motility Agar
Urea Broth
Triple Sugar Iron Agar

Antibiotic Discs

Aztreonam (30 mcg)
Cefepime (30 mcg)
Ceftazidime (30 mcg)
Cefotaxime (30 mcg)
Cefotaxime/Clavulanic acid (30/10 mcg)
Ciprofloxacin (5 mcg)
Gentamicin (10 mcg)
Ceftazidime/Clavulanic acid (30/10 mcg)
Imipenem (10mcg)
Piperacillin (100 mcg)
Piperacillin/Tazobactam (100/10 mcg)

Chemicals and reagents

100bp DNA marker
70% ethanol
95% ethanol
3% H_2O_2
Absolute alcohol
Crystal violet (1%)
Distilled water
Gram's Iodine
Kovac's reagent
Loading dye
Methyl red reagent
Nuclease free water
Oxidase reagent
Agarose gel powder
Barritt's reagent
Blood
Bromophenol blue
Gram's decolorizer
Ethidium Bromide
Glycerol
Paraffin oil
Reagents for master mix (PCR)
Safranin
Tris borate buffer
Tris-EDTA
Urea

Quality control strains

1. *Pseudomonas aeruginosa* ATCC 27853
2. *Escherichia coli* ATCC 25922
3. *Staphylococcus aureus* ATCC 25923

Miscellaneous

Aluminum foil
Cotton swabs
Dropper
Eppendorf tubes
Inoculating loop
Inoculating needle
Microscope oil
Spatula
Staining rack
Sterile forceps
Sterile wooden sticks
Test tube rack

Appendix E

Microbiological media and reagents

A. Composition and preparation of different culture media

1. Blood Agar Base (Infusion Agar)

Blood agar base (infusion agar) + 5-10% sheep blood

Ingredients	**gm/liter**
Beef heart infusion	500.0
Tryptose	10.0
Sodium Chloride	5.0
Agar	15.0
Final pH (at 25ºC)	7.3±0.2

Preparation: 42.5 grams of the blood agar base medium was suspended in 1000 ml distilled water and sterilized by autoclaving at 121ºC (15 lbs pressure) for 15 minutes. After cooling to 40-50ºC, 50 ml sterile defibrinated blood was added aseptically and mixed well before pouring.

2. MacConkey Agar

Ingredients	**gm/liter**
Peptone (meat and casein)	3.0
Pancreatic digest of gelatin	17.0
Lactose monohydrate	10.0
Bile salts	1.5
Sodium chloride	5.0
Crystal violet	0.001
Neutral Red	0.04
Agar	20.0
Final pH (at 25ºC)	7.4±0.2

Preparation: As directed by the manufacturer, 55 grams of the medium was suspended in 1000 ml of distilled water. Then the medium was sterilized by autoclaving at 121ºC (15 lbs pressure) for 15 minutes. The sterilized media was then poured in sterile petri plates and allowed to cool.

3. Chromogenic UTI Agar

Ingredients	**gm/liter**
Peptone, Special	15.0
Chromogenic Mixture	2.45
Agar	15.0
Final pH (at 25°C)	6.8 ± 0.2

Preparation: 32.45g of powder was suspended in 1000ml D/W and then boiled with frequent agitation to dissolve the powder completely. The medium was then sterilized by autoclaving at 121ºC (15 lbs pressure) for 15 minutes. It was then cooled to 50°C and poured into sterile petri plates.

4. Cetrimide Agar

Ingredients	**gm/liter**
Gelatin peptone	20.00
Magnesium chloride	1.400
Potassium sulphate	10.000
Cetrimide	0.300
Agar	15.000
Final pH (at 25°C)	7.2±0.2

Preparation: 46.7 grams of powder was dissolved in 1000 ml distilled water, heated to boiling to dissolve the medium completely and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. It was then cooled to 50°C and poured into sterile Petri plates.

5. Muller Hinton Agar

Ingredients	**gm/liter**
Beef, Infusion form	300.0
Casein Acid Hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH (at 25ºC)	7.4±0.2

Preparation: 38 grams of the medium was suspended in 1000 ml distilled water and the medium was warmed to dissolve the medium completely. The media was then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The sterilized media was then poured in sterile petri plates and allowed to cool.

6. Nutrient Agar

Ingredients	**gm/liter**
Peptone	10.0
Sodium Chloride	5
Beef Extract	10.0
Yeast Extract	1.5
Agar	12.0
Final pH (at 25ºC)	7.4±0.2

Preparation: 37 grams of the medium was suspended in 1000 ml of distilled water. The media was then sterilized by autoclaving at 121ºC (15 lbs pressure) for 15 minutes. The sterilized media was then poured in sterile petri plates and allowed to cool.

7. Nutrient Broth

Ingredients	**gm/liter**
Peptone	5.0
Sodium Chloride	5.0
Beef Extract	1.5
Yeast Extract	1.5
Final pH (at 25ºC)	7.4±0.2

Preparation: 13 grams of the medium was dissolved in 1000 ml distilled water, suspended into tubes, and autoclaved at 121ºC for 15 minutes. The sterilized media was then poured in sterile petri plates and allowed to cool.

8. **Tryptone Soya Broth (Soyabean Casein Digest Medium)**

Ingredients	**gm/liter**
Pancreatic digest of casein	5.0
Pancreatic digest of soyabean meal	3.0
Sodium Chloride	5.0
Dextrose (Glucose)	2.5
Dipotassium Hydrogen Phosphate	2.5
Final pH (at 25ºC)	7.3±0.2

Preparation: 30 grams of the medium was suspended in 1000 ml of distilled water and suspended into tubes and autoclaved at 121ºC for 15 minutes. The sterilized media was then poured in sterile petri plates and allowed to cool.

B. Biochemical Test Media

1. Sulphide Indole Motility (SIM) medium

Ingredients	**gm/liter**
Beef Extract	3.0
Peptone	30.0
Peptonized Iron	0.2
Sodium Thiosulphate	0.025
Agar	3.0
Final pH (at 25ºC)	7.3±0.2

Preparation: 36 grams of the medium was suspended in 1000 ml distilled water and dissolved completely. Then it was distributed in tubes to a depth of about 3 inches and sterilized by autoclaving at 121°C for 15 minutes.

2. Simmons Citrate Agar

Ingredients	**gm/liter**
Magnesium Sulfate	0.2
Mono-ammonium Phosphate	1.0
Dipotassium Phosphate	1.0
Sodium Citrate	2.0
Sodium Chloride	5.0
Agar	15.0
Bromothymol Blue	0.08
Final pH (at 25ºC)	6.8±0.2

Preparation: 24.2 grams of the medium was dissolved in 1000ml distilled water. 3ml medium was distributed in test tubes and sterilized by autoclaving at 121ºC for 15 minutes. After autoclaving tubes containing medium were tilted to form slant.

3. Triple Sugar Iron (TSI) Agar

Ingredients	gm/liter
Peptone	10.0
Tryptone	10.0
Yeast Extract	3.0
Beef Extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous Sulphate	0.2
Sodium Chloride	5.0
Sodium Thiosulphate	0.3
Phenol Red	0.024
Agar	12.0
Final pH (at 25ºC)	7.4±0.2

Preparation: 65 grams of the medium was dissolved in 1000ml of distilled water and sterilized by autoclaving at 15 lbs (121ºC) pressure for 15 minutes. The medium was allowed to set in sloped form with a butt about 1 inch of thickness.

4. Urea Agar Base

Ingredients	gm/liter
Peptone	1.0
Dextrose	1.0
Sodium Chloride	5.0
Dipotassium Phosphate	1.2
Mono-potassium Phosphate	0.8
Phenol Red	0.012
Agar	15.0
Final pH (at 25ºC)	7.4±0.2

Preparation: 24 grams of the medium was suspended in 950 ml distilled water and sterilized by autoclaving at 121ºC for 15 minutes. After cooling to about 45ºC, 50 ml of 40% urea was added and mixed well. Then 5 ml was dispensed into sterile test tubes and set at slant position.

5. MR-VP Medium

Ingredients	**gm/liter**
Peptone	5.0
Dextrose	5.0
Dipotassium phosphate	5.0
Final pH (at 25°C)	7.3±0.2

Preparation: 15gm powder was dissolved in 1000 ml of distilled water & mixed well. 1 ml of medium was distributed in each test tube and autoclaved at 121°C for 15 minutes.

C. Composition and preparation of different reagents

1. Gram's stain

i. Crystal violet solution

Crystal violet	20g
Ammonium Oxalate	9 g
Ethanol or Methanol	95 ml
D/W	1 L

Preparation:

20 g crystal violet was weighed and transferred into a clean brown bottle. Then, 95 ml ethanol was added and mixed until the dye was completely dissolved. To the mixture, 9g ammonium oxalate dissolved in 200 ml D/W was added. Final volume was made 1 L by adding distilled water.

ii. Lugol's iodine

Potassium iodide	20 g
Iodine	10 g
Distilled water	1000 ml

Preparation:

To 225 ml of D/W, 20 g Potassium iodide was added and mixed well. Then, 10 g of iodine was added to it until it was dissolved completely. Final volume was made 1L by adding D/W.

iii. Acetone-Alcohol Decolorizer

Acetone	500 ml
Ethanol	475 ml
Distilled Water	25 ml

Preparation:

To 25 ml D/W, 475 ml of absolute alcohol was added, mixed, and transferred into clean bottle. Then immediately, 500 ml acetone was added to the bottle and mixed well.

iv. Safranin

Safranin	10 g
Distilled water	1000 ml

Preparation:

In a clean piece of paper, 10 g safranin was weighed and transferred to a clean bottle. Then 1 L D/W was added to the bottle and mixed well until safranin dissolved completely.

2. Biochemical Reagents

i. Catalase reagent

Hydrogen peroxide	3 ml
Distilled Water	97 ml

Preparation:

To 97 ml of distilled water, 3 ml of hydrogen peroxide (H_2O_2) was added and mixed well. The bottle was labelled and stored at room temperature.

ii. Oxidase reagent

Tetramethyl-p-phenylene diamine dihydrochloride (TDP)	1 g
Distilled water	100 ml

Preparation:

1g of TDP was dissolved in 100 ml D/W. To that solution, strips of Whatmann's No. 1 filter paper were soaked and drained for about 30 seconds. Then the strips were freeze dried and stored in dark bottles sealed with screw cap.

iii. Kovac's reagent

Isoamyl alcohol	30 ml
p-dimethyl amino benzaldehyde	2 g
Conc. Hydrochloric acid	10 ml

Preparation

In 30 ml of isoamyl alcohol, 2 g of p-dimethyl amino-benzaldehyde was dissolved and transferred to a clean brown bottle. Then to it, 10 ml conc. HCl was added and mixed well.

iv. Methyl red solution

Methyl red	0.05 g
Ethyl alcohol	28 ml
Distilled water	22 ml

Preparation:

0.05 g methyl red was dissolved in 28 ml ethanol and 22 ml distilled water. The solution was then transferred to a clean brown bottle and stored in dark at room temperature.

v. Barritt's reagent

Solution A

α-naphthol	5 g
Ethyl alcohol	95 ml

Preparation:

5 g of alpha naphthol was dissolved in 95 ml ethanol with constant stirring.

Solution B

Potassium Hydroxide	40 g
Distilled Water	1000 ml

Preparation:

To 25 ml D/W, 40 g of KOH was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding D/W.

3. Phenyl boronic acid

Phenyl Boronic acid	60 mg
Dimethyl sulfoxide (DMSO)	1.5 ml
Distilled water	1.5 ml

Preparation:

60 mg phenyl boronic acid was dissolved in 1.5 ml DMSO. 1.5ml distilled water was added to the above mixture.

4. 0.5 McFarland standards

Barium Chloride	0.5 ml
Sulfuric Acid	99.5 ml

Preparation:

0.5 ml of 1.175% (w/v) solution of Barium Chloride solution was added to 99.5 ml of 0.36 N Sulfuric acid solution. The mixture was shaken well. This stock solution was stored in a well-sealed container in the dark. A small amount of solution was taken a time in a clean test tube to compare the turbidity of the inoculums.

5. Preparation of reagents for molecular tests

i. 1 M Tris-HCl (50 ml)

Tris-HCl	6.057 g
D/W	50 ml

Preparation:

6.057 g Tris-HCl was dissolved in 50 ml distilled water, mixed well in a volumetric flask, and labelled properly.

ii. 0.5 M EDTA (pH 8)

Disodium EDTA	18.61 g
D/W	100 ml
NaOH	(as per requirement)

Preparation:

About 18.61 g EDTA was weighed and dissolved in 80 ml deionized water using magnetic stirrer. Then, NaOH pellets were added to it to adjust the pH to 8. The volume was then adjusted to 100 ml using distilled water.

iii. Preparation of TE buffer

1 M Tris-HCL	10 ml
0.5 M EDTA (pH 8)	2 ml
D/W	1000 ml

Preparation:

1. Tris-HCl (1 ml) and 2 ml of 0.5 M EDTA were transferred to a beaker and distilled water was added to it to make volume 1000 ml.
2. The solution was mixed well and sterilized by autoclaving.

iv. Preparation of TBE buffer

a) 10X TBE buffer (stock solution)

0.5 M EDTA (pH 8)	40 ml
Tris-base	108 g
Boric acid	55 g
D/W	1000 ml

Preparation:

1. About 108 g of Tris base and 55 g boric acid were dissolved in 800 ml water.

2. Then, 40 ml of 0.5 M EDTA was added.
3. The volume was adjusted to 1000 ml by adding distilled water.

b. 1X TBE (Working solution)

TBE (10X)	50 ml
D/W	450 ml

Preparation:

3. In the volumetric flask, 50 ml TBE (10X) was taken.
4. Then, distilled water was added to it to make the volume 500 ml.
5. It was mixed well and used for electrophoresis.

v. Preparation of 1.5% agarose gel

Agarose	1.5 g
1X TBE	100 ml

Preparation:

1. About 0.9 gm dry agarose was dissolved in 60 ml of 1X TBE buffer solution and boiled until the formation of transparent solution.
2. About 0.5 µl of ethidium bromide (0.5 µg/ml) solution was mixed with the boiled agarose and the solution was poured into plastic tray fitted with the comb. The agarose gel was then allowed to solidify for 30 minutes.

vi. Ethidium bromide solution

Ethidium Bromide	0.2 g
Distilled water	20 ml

Preparation:

0.2 g ethidium bromide was dissolved in 20 ml distilled water, mixed well, and stored at 4ºC in the dark (stock solution was 10 mg/ml concentration). 10 µl of stock solution was dissolved in 200 ml of distilled water to prepare working solution (0.5µg/ml).

vii. Loading dye for gel electrophoresis

Bromophenol blue	0.025 g
Sucrose	4 g
D/W	10 ml

Preparation:

Calculated amount of sucrose and bromophenol blue were dissolved in 8 ml distilled water and the final volume was made 10 ml. It was stored at 4°C.

6. Glycerol stock preparation

Tryptic Soy Broth	2 ml
Glycerol	2 ml

Preparation:

a) Each isolate was cultured in Tryptic Soy Broth for overnight at 37℃.
b) With the help of pipette 500µl of TSB culture was transferred to three of sterile vials aseptically.
c) With the help of pipette 500 µl of sterile glycerol was added to each vial and mixed thoroughly.
d) The triplicate stocks were preserved at -20℃.

Appendix F

Protocol for Gram staining and biochemical tests

1. Gram-staining

Gram staining is a differential staining technique which differentiates all bacterial species into two groups: Gram-positive and Gram-negative bacteria. Following are the steps involved in Gram staining.

Procedure:

1. A thin bacterial smear was prepared on a clean grease-free slide, and it was allowed to dry in air.
2. The smear was heat fixed by passing the slide quickly over the Bunsen burner flame three times.
3. The slide was flooded with crystal violet stain and left for 1 minute.
4. The slide was rinsed with tap water to remove excess stain.
5. The slide was flooded with iodine solution and left for 1 minute. It was then rinsed with tap water.
6. The slide was flooded with Gram's decolourizer for 20 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolourizer.
7. The slide was flooded with safranin for 1 minute and washed off with tap water.
8. The slide was air dried and examined microscopically under oil immersion at 100X.

2. Catalase test

During aerobic respiration, in the presence of oxygen, microorganisms produce hydrogen peroxide, which is lethal to the cell itself. Catalase enzyme breaks down hydrogen peroxide into water and oxygen. The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria, the main exception being S*treptococcus* spp.

Procedure:

A small amount of a culture from Nutrient Agar plate was taken in a clean glass slide and about 2-3 drops of 3% H_2O_2 was put on the surface of the slide. The positive test is indicated by the formation of oxygen bubbles within 10 seconds. A false positive reaction may be obtained if the culture medium contains catalase (e.g., Blood Agar) or if an iron wire loop is used.

3. Oxidase test

This test is performed for the detection of cytochrome c oxidase enzyme in bacteria which is the terminal electron acceptor in aerobic respiration. The ability of an organism to produce cytochrome c oxidase can be determined by using the reagent tetramethyl-p-phenylenediamine. This reagent serves as an artificial electron donor thus being oxidized into a deep purple color in the presence of cytochrome c oxidase. Development of purple color indicates positive result.

Procedure:

The colony of the test organism was smeared on oxidase disc and observed for the development of purple coloration. The positive test is indicated by the appearance of purple color within 10 seconds.

4. Indole production test

This test detects the ability of the organism to produce an enzyme: 'tryptophanase'. This enzyme hydrolyzes the amino acid tryptophan into indole, pyruvic acid, and ammonia. Indole production is detected using Kovac's reagent which contains HCl and dimethylaminobenzaldehyde dissolved in amyl alcohol. Indole, if present, combines with the aldehyde present in the reagent to give a red color in the alcohol layer.

Procedure:

A smooth bacterial colony was stabbed on SIM (Sulphide Indole Motility) medium by a sterile inoculating wire and the inoculated media was incubated at 37°C for 24 hours. After 24 hours incubation, 4-8 drops of Kovac's reagent was added. Appearance of cherry red color on the top of media indicates indole positive.

5. Citrate utilization test

This test is performed to detect whether an organism utilizes citrate as a sole source of carbon for metabolism with resulting alkalinity.

Procedure:

A loopful of test organism was streaked on the slant area of Simmon's Citrate Agar medium and incubated at 37°C for 24 hours. A positive test was indicated by the growth of organism and change of media from green to blue due to alkaline reaction. The pH indicator bromothymol blue has a pH range of 6.0-7.6, i.e., above pH 7.6; a blue color develops due to alkalinity of the medium.

6. Motility test

Sulphide Indole Motility media is used for the detection of motility of bacteria. Motile organisms migrate from the stab line and diffuse into the medium causing turbidity whereas non-motile bacteria show the growth along the stab line, and the surrounding media remains colorless and clear.

Procedure:

The organisms were inoculated into SIM media by stabbing with a sterile inoculating wire and the inoculated media was incubated at 37°C for 24 hours. Appearance of turbidity or the movement of organisms from stab line indicates motility.

7. Triple Sugar Iron (TSI) Agar

The TSI agar is used to determine the ability of an organism to utilize specific carbohydrate incorporated in the medium (glucose, sucrose, and lactose in concentrations of 0.1%, 1.0% and 1.0% respectively), with or without the production of gas (indicated by cracks in the media as well as an air gap at the bottom of the tube) along with determination of possible hydrogen sulfide production (detected by production of black color in the medium). Acid production limited only to the butt region of the tube is indicative of glucose utilization, while acid production in slant and butt indicates sucrose or lactose fermentation. Phenol red is the pH indicator which gives yellow reaction at acidic pH, and red reaction to indicate an alkaline surrounding.

Procedure:

The test organism was streaked and stabbed on the surface of TSI and incubated at 37°C for 24 hours. The media was then examined for color change in the slant and butt region, gas production and H_2S production.

8. Urea hydrolysis test

This test demonstrates the urease activity present in certain bacteria which decomposes urea, releasing ammonia and carbon dioxide. With the release of ammonia, the medium becomes alkaline as shown by a change in color of the indicator to pink.

Procedure:

The test organism was inoculated in a medium containing urea and the indicator phenol red. The inoculated medium is incubated at 37°C overnight. Positive organism shows pink-red color due to the breakdown of urea to ammonia.

Table 12: Tests for the identification of *P. aeruginosa*

Tests	**Results**
Gram stain	Gram negative rod
MacConkey Agar	Non-lactose fermenter (pale colonies)
Cetrimide Agar	Pigmentation
Growth at 42°C	Positive
Catalase test	Positive
Oxidase test	Positive
Citrate test	Positive
Indole test	Negative
Motility test	Motile
H_2S production	Negative
TSIA	Red/Red
O/F test	Oxidative
Urease	Negative
Methyl red test	Negative
Voges-Proskauer test	Negative
Odor	Fruity smell

APPENDIX G

Disc diffusion method for antimicrobial susceptibility testing

A. Preparation of 0.5 Mc Farland Standard

Add 0.5 ml of 0.048 M $BaCl_2$ (1.17% w/v $BaCl_2.2H_2O$) to 99.5 ml of 0.18M H_2SO_4 (1% v/v) with constant stirring.

B. Preparation of inoculum

By touching 2-3 morphologically similar colonies with sterile loop, inoculate into MHB or NB and incubate at 37°C until turbidity matches with that of 0.5 Mc Farland Standard. Direct colony suspension method can also be used.

C. Inoculation of agar plates

1. The agar plates, canister of discs are brought to room temperature before use. It should be made sure that the agar surface doesn't have any moisture, if so should be dried by keeping it in incubator.
2. Using a sterile swab, a plate of Mueller-Hinton agar is inoculated with the bacterial suspension using carpet culture technique. The plate is left for about 5 minutes to let the agar surface dry.
3. Using sterile forceps, appropriate antimicrobial discs (6 mm diameter) is placed, evenly distributed on the inoculated plates, not more than 6 discs are placed on a 90 mm diameter Petri plate.

4. Within 30 minutes of applying the discs, the plates are incubated at 35℃ for 16-18 hours.
5. After overnight incubation, the plates are examined to ensure confluent growth. Using a measuring scale, the diameter of each zone of inhibition in mm is measured and results are interpreted.

Table 11: Zone size interpretative chart

Antibiotics	Symbol	Disc content	Susceptibility pattern		
			Sensitive	Intermediate	Resistant
Amikacin	AK	30 mcg	17	15-16	14
Gentamicin	GEN	10 mcg	15	13-14	12
Ciprofloxacin	CIP	5 mcg	25	19-24	18
Levofloxacin	LE	5 mcg	22	15-21	14
Piperacillin	PI	100 mcg	21	15-20	17
Piperacillin-Tazobactam	PIT	100/10 mcg	21	15-20	17
Ceftazidime	CAZ	30 mcg	18	15-17	14
Cefepime	CPM	30 mcg	18	15-17	14
Aztreonam	AT	Mcg	22	16-21	15
Meropenem	MRP	10 mcg	19	16-18	15
Imipenem	IPM	10 mcg	19	16-18	15

APPENDIX H

A. Screening for ESBL Producers

Clinical and Laboratory Standards Institute (CLSI) has develop disk diffusion screening test for possible ESBL production. The possible ESBL production can be detected by noting specific zone diameters which indicates a high level of suspicion for ESBL production. Cefpodoxime, ceftazidime, cefotaxime, ceftriaxone, or aztreonam can be used for screening, however, use of more than one of these agents for screening improves the sensitivity of detection

B. Phenotypic confirmatory test for ESBL production

The CLSI has recommended phenotypic confirmatory test for the suspected ESBL producers. The phenotypic confirmation of ESBL production on suspected isolates was done by combination disk test (CDT). In this method, a lawn culture of test strain was made on MHA and ceftazidime and ceftazidime plus clavulanic acid as well as cefotaxime and cefotaxime plus clavulanic acid were placed over the culture. The plates were incubated at 37°C for 18-24 hrs. An increase in zone diameter of ⩾5mm in disc containing clavulanic acid in comparison with the inhibition zone of antibiotic tested alone confirmed ESBL production.

C. Screening of MBL producers

Isolates exhibiting resistance to imipenem in Kirby Bauer disc diffusion method were considered potential MBL producer

D. Phenotypic confirmatory test for MBL producers

Imipenem – EDTA disc method was employed for the phenotypic confirmation of MBLs in imipenem resistant *P. aeruginosa*. A test inoculum equivalent to 0.5 McFarland was inoculated onto plates of MHA. Two 10 µg imipenem discs were placed on the plates, and 10 µl of 0.5 M EDTA of pH 8 was added to one of the discs to obtain 750 µg concentration. The distance between the center of the imipenem and imipenem-EDTA discs was maintained to be 20 mm. After 16-18 hours of incubation in aerobic condition at 37°C, the inhibition zones of imipenem

and imipenem-EDTA discs were measured and compared with each other. If the difference in inhibition zone between imipenem disk and imipenem - EDTA disk was ≥ 7mm, the isolate was considered as MBL-producer.

Appendix I

Chromosomal DNA extraction protocols

1. 1.5 ml of overnight grown bacterial culture was taken into microfuge tube.
2. The bacterial suspension was centrifuged at 10,000 rpm for 5 minutes at room temperature and the supernatant was discarded.
3. 567 µl 1X TE buffer was added and mixed well by vortexing.
4. 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K was mixed and incubated at 37℃ for 1 hour.
5. After incubation, 100 µl 5M NaCl was added and mixed.
6. 80 µl CTAB/NaCl was added and incubated for 10 minutes at 65℃.
7. Equal volume of chloroform: isoamyl alcohol was added and mixed thoroughly.
8. Then it was centrifuged for 5 mins at 10,000 rpm.
9. Upper aqueous phase was transferred to new microfuge tube (not to transfer the interface).
10. Equal volume of P:C:I was added and centrifuged at 14,000 rpm for 5 mins.
11. The supernatant was transferred to new tubes.
12. The solution was treated with 600 µl isopropanol.
13. It was centrifuged at 12000 rpm for 5 mins and the isopropanol was removed carefully.
14. 100 µl of 70% ethanol was added and centrifuged at 12000 rpm. Then, the ethanol was discarded.
15. It was air dried and resuspended in 100 µl TE buffer.
16. The extracted DNA was stored at 4℃.