DETECTION OF METALLO BETA-LACTAMASES PRODUCING *Pseudomonas aeruginosa* FROM DIFFERENT CLINICAL SAMPLES

A dissertation submitted to the **Department of Microbiology, National College,** Tribhuwan University, Kathmandu Nepal in the Partial Fulfillment of the requirement for the award of Degree of Masters of Science in Microbiology

Medical

By

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DECLARATION

This dissertation entitled **"DETECTION OF METALLO BETA-LACTAMASES PRODUCING *Pseudomonas aeruginosa* FROM DIFFERENT CLINICAL SAMPLES"** has been submitted to the Department of Microbiology, National College, Tribhuvan University (T.U.), for the partial fulfillment of the requirements to the degree of Master of Science in Microbiology. This dissertation is conducted under the supervision of **Dr. Era Tuladhar and Dr Ranajana Parajuli** This is an original report of my research, has been conducted entirely by myself, and has not been submitted for any other degree or professional qualification. I have followed Tribhuvan University's current research ethics guidelines and accept responsibility for the conduct of the procedure in accordance with the University's rules and regulations.

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RECOMMENDATION

This is to certify that Mr. Bonny Shrestha has completed the dissertation entitled **"DETECTION OF METALLO BETA-LACTAMASES PRODUCING *Pseudomonas aeruginosa* FROM DIFFERENT CLINICAL SAMPLES"** as a partial fulfillment of the requirements of Tribhuvan University for the completion of Master's Degree in Microbiology (Medical) under our supervision. To our knowledge, this work has not been submitted for any other degree.

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ABSTRACT

*Pseudomonas aeruginosa*is an opportunistic bacteria that have an ability to cause nosocomial infection in patient with diverse range of infection such as urine infection, respiratory infection, bacteremia, wound and other central nervous system. Increase in antibiotic resistant has been a nuisance in the hospital settings. More ever, increase in prevalence of metallo beta lactamase producing *P. aeruginosa* leads to increase in morbidity and mortality due to the limited number of choice of drugs for treatment. This study aims in isolating the *P. aeruginosa* from different clinical samples and detection of multidrug resistant as well as screening of MBL producing *P. aeruginosa*. The hospital based cross sectional study was done in Grande International Hospital, Kathmandu from January – June 2024. A total of 1500 different clinical samples such as urine, blood, sputum, pus, tissue, wound etc. were processed during the study time, where 126(8.2%) of *Pseudomonas aeruginosa* were isolated from different samples of different ages and gender, of either inpatient or outpatient. 34.6% isolates were isolated form urine,31.7% from sputum, 19% from blood, 1.6% from pus, 4.8% from wound swab and 0.8% form tissue were obtained from 66.1% of male and 38.9% of female infected patients. MBL prevalence was found to be 16.6% which were more from ICU (47.6%/) followed by 33.3% from general ward. Levofloxacin had higher sensitive pattern(82.4%) and Nitrofurantoin was more resistant (90.2%).In MBL isolates, Aztrenam was found to be least resistant (33.3%). Infection by *P. aeruginosa* can cause a medical threat. A multidrug resistant isolates can even be more difficult to treat. Increased MBL isolates has been a nuisance in hospital settings. Therefore, routine evaluation should be done to ensure the control of these isolates.

Key words: *Pseudomonas aeruginosa*, Multidrug resistant,Metallo beta-lactamases (MBLs),

शोधसार

स्यूडोमोनास एरुगिनोसा एक अवसरवादी ब्याक्टेरिया हो जसमा विभिन्न प्रकारका संक्रमणहरू जस्तै पिसाबको संक्रमण, श्वासप्रश्वासको संक्रमण, ब्याक्टेरेमिया, घाउ र अन्य केन्द्रीय स्नायु प्रणाली भएका बिरामीहरूमा नोसोकोमियल संक्रमण गराउने क्षमता हुन्छ। एन्टिबायोटिक प्रतिरोधी वृद्धि अस्पताल सेटिङहरूमा एक उपद्रव भएको छ। पहिले भन्दा बढि, *P. aeruginosa* उत्पादन गर्ने Metallo beta lactamase को प्रचलनमा भएको वृद्धिले उपचारको लागि सीमित संख्यामा औषधि छनोटको कारणले गर्दा बिरामी र मृत्युदरमा वृद्धि हुन्छ। यस अध्ययनको उद्देश्य विभिन्न क्लिनिकल नमूनाहरूबाट *P. aeruginosa* लाई अलग गर्नु र बहुऔषधि प्रतिरोधी पत्ता लगाउनु साथै *P. aeruginosa* उत्पादन गर्ने MBL को स्क्रीनिंग गर्नु हो। अस्पतालमा आधारित क्रस सेक्शनल अध्ययन ग्रान्डी इन्टरनेशनल अस्पताल, काठमाडौंमा जनवरी-जुन २०२४ सम्म गरिएको थियो। अध्ययन अवधिमा पिसाब, रगत, थुक, पिप, तन्तु, घाउ आदि जस्ता कुल १५०० विभिन्न क्लिनिकल नमूनाहरू प्रशोधन गरिएको थियो। स्यूडोमोनास एरुगिनोसा को (%ङाद्द) ज्ञद्दट विभिन्न उमेर र लिङ्गका विभिन्न नमूनाहरूबाट अलग गरिएको थियो, कि त इनपेशेन्ट वा बाहिरी बिरामी। ३४.६ %आइसोलेट्स पिसाबबाट, ३१.७ %खकारबाट, १९ %रगतबाट, १.६ %पिपबाट, ४.८ %घाउ स्वाबबाट र ०.८ %टिस्यु , ६६.१ %पुरुष र ३८.९ %महिला संक्रमित बिरामीबाट प्राप्त भएको थियो। MBL व्याप्तता %ज्ञटाट फेला पर्यो जुन ICU ((%द्धठाटबाट बढी थियो र त्यसपछि %घघाघ सामान्य वार्डमा थियो। Levofloxacin उच्च संवेदनशील ढाँचा (%डद्दाद्ध) थियो र Nitrofurantoin अधिक प्रतिरोधी (%ढणद्द) थियो। MBL पृथकहरूमा, Aztreonam कम प्रतिरोधी (%घघाघ) फेला पर्यो। *P. aeruginosa* को संक्रमणले मेडिकल खतरा निम्त्याउन सक्छ। एक बहुऔषधि प्रतिरोधी आइसोलेट्स उपचार गर्न अझ गाह्रो हुन सक्छ। बढेको MBL आइसोलेट्स अस्पताल सेटिङहरूमा एक उपद्रव भएको छ। तसर्थ, यी अलगावहरूको नियन्त्रण सुनिश्चित गर्न नियमित मूल्याङ्कन गरिनुपर्छ।

मुख्य शब्दहरू: *Pseudomonas aeruginosa*, बहुऔषधि प्रतिरोधी, मेटालो बीटा-लैक्टमेसेस (MBLs)

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Abbreviation

P. aeruginosa:	*Pseudomonas aeruginosa*
MDR:	Multi Drug Resistant
MBL:	Metallo Beta Lactamase
AST:	Antibiotic Sensitive Test
ESBL:	Extended Spectrum Beta Lactamase
ICU:	Intensive Care Unit
MA:	Mac Conkey Agar
MHA:	Muller Hilton Agar
BA:	Blood Agar
CLED:	Cysteine Lactose Electrolyte Deficient Agar
CLSI:	Clinical and Laboratory

CHAPTER I
INTRODUCTION

1.1 Background

Pseudomonas aeruginosa is one of the most common Gram-negative, non-fermentative bacteria which can cause nosocomial infections. This organism is able to form biofilm, that causes resistant to many antimicrobial agents.As *Pseudomonas aeruginosa* is ubiquitous in nature, it colonizes multiple environmental niches and utilizes those compounds present in environment as energy sources(Shrestha. 2018).

Even *P. aeruginosa* is found ubiquitous in nature, it can also cause infection to humans. Infection may be transmitted from environmental settings, soils, during accidents due to opening of wounds and trauma, in hospitals through medical appliances, prosthetic devices and (Shrestha , 2018)poor sanitization It is one of the prominent nosocomial infection causing agent in hospital settings(Wang & Wang, 2020). The infection may range from local infection to septicemia. Infections such as Urinary tract infection, wound infection, tissue infection, respiratory tract infection, blood infection etc are some of the infection frequently occurring to the patients due to this bacterium. The bacteria present different mechanisms to adhere in different environmental situations. Presence of different antigens, production of toxins, formation of biofilms and quorum sensing, efflux properties and secretion system helps them to escape host defense mechanism and antibiotics(Mahaseth et al., 2020a). These properties also help the organism to naturally acquire immune to some of the antibiotics group. Thus, the choices of antibiotics have become limited in treating the infection. Multidrug resistant *P aeruginosa* is a problem in hospital settings as it increase in morbidity, mortality and healthcare costs. Treatment remains challenge and will most probably require some high dose antibiotics that has more side effects(Ansari and Aryal, et al., 2021).

A globally arising problem is the increasing strains of metallo beta lactamase producing *P. aeruginosa*. Metallo beta-lactamases are a various set of enzymes that hydrolyses broad range of beta- lactam drugs including

carbapenems(Reynolds & Kollef, 2021).To control the spread of resistance and provide proper treatment of patients, particularly chronically ill and nosocomial infections, due to its broad-spectrum activities carbapenem are used. Metallo – beta lactamase is the enzyme that inactivates carbapenems, and has prevented the use of these antibiotics. These enzymes are inactivated by chelating agents such as ethylene diamine tetra acetic acid (EDTA)(Rajput et al., 2012).

Due to biofilm formation, *P aeruginosa* can cause severe chronic infections and development of antimicrobial resistant that makes difficult to treat the patients (Shrestha et al., 2019)Most prevalent families of MBL resistant genes are IMP (inactivate imipenem), which was first reported in *Pseudomonas aeruginosa* strain from Japan in 1988) and another gene includes VIM (Verona Integron-encoded Metallo beta-lactamase), which was found in Europe then emerged to other countries.MBL enzymes seen among Pseudomonas *aeruginosa* strains are matters of important concern in future for antimicrobial chemotherapy(Bahmani, 2019)

Prevalence rate of Metallo beta-lactamases (MBLs) forming *P. aeruginosa* are increasing globally. It has been an alarming threat in treatment of clinically ill patients with MBLs. Treatment in patient with MBL producer *Pseudomonas aeruginosa* is being challenging for many physicians as they are resistance to broad range of beta- lactam antibiotics including carbapenems. Main aim of this study is to observe antimicrobial susceptibility pattern, phenotypical detection of MBL using combine disk method.

1.2OBJECTIVES

1.2.1 General objectives

- To detect metallo beta-lactamase producing *Pseudomonas aeruginosa* from various clinical isolates.

1.2.2 Specific objectives

- To isolate *Pseudomonas aeruginosa* from different clinical samples
- To perform Antibiotic susceptibility Test (AST) of obtained isolates.
- To screen Multidrug Resistance (MDR) and extensively drug resistant (XDR) *Pseudomonas aeruginosa.*
- To confirm MBL producer *Pseudomonas aeruginosa* phenotypically by combined disc method.

CHAPTER II

LITERATURE REVIEW

2.1Background:

Pseudomonas aeruginosa:

P. aeruginosa is a gram-negative bacterium that is found ubiquitous in nature. It is found in water, soils, plants and even in human gut as a normal flora. It is a motile bacterium. In binomial nomenclature, it lies under domain Bacteria and family Pseudomonadaceae. The word *Pseudomonas* is derived from Greek work which terms as 'false unit'. Naturally, these bacteria are pigment forming bacteria. Normally four types of pigmentations are found: pyocyanin(blueish), pyoverdine(yellowish-green), pyorubin(red) and pyomelanin (brownish). *P.aeruginosa* normally produces pyocyanin pigments which are water soluble bluish green colour (Chand et al., 2021).

2.2 Epidemiology

P. aeruginosa is ubiquitously found in nature like in soils, water sources etc. It is almost found in every part of the world. Infection in outdoors are due the contact with these sources from accidents, abrasions, work related infections. Normally, it causes mild infection which can be effectively treated. However, the organism has been reported to develop a resistant pattern to many antibiotics. Every year, an increasing rate of antibiotic resistant has been reported in many research article(Shrestha et al., 2019)(Neupane et al., 2017.). The problems are serious when the infection source is from the hospitals, there are number of cases where the hospital acquired *P. aeruginosa* infection are increasing to the admitted patients. Cases are more to them who has to use prosthetic devices for a longer period of time. Cystic fibrosis patients, immune compromised patients, patients from ICUs and those have to use catheters are more prone to the infections. Another alarming problem is the development of multidrug resistant mechanism in the bacteria, As the hospital settings have to use the antibiotics frequently almost all the time, the bacteria may have evolved to resist the antibiotics. As a result, treatment has been a challenging part in hospitals.

A property to produce biofilm is one the challenging phase to tackle in the hospitals. The production of biofilm helps the organism to adhere in the surfaces and survive in harsh environmental condition. The biofilm producer organisms are found to be resisting to much more antibiotics. A study done by (Gupta et al., 2016) shows the production of biofilm by 60% of the isolates. A research done by (Shrestha et al., 2019) and (Kunwar et al., 2021) reveals the biofilm producing *P. aeruginosa* by 26%. Study done in Iran by (Kamali et al., 2020) shows a higher isolates of biofilm producers by 83%. Another study done in Nepal by (Neupane et al., 2017.) shows 76% percent of *P. aeruginosa* produces bio films. These data show the variable prevalence of biofilm producing *P. aeruginosa*. This result may be due to different geographical reasons or the size and difference in study periods. Nonetheless, the biofilm producing *P. aeruginosa* might contribute to persistent infection in immune compromised patients and selecting the effective antibiotic therapy(Elfadadny et al., 2024).

Antibiotics has been a key role in treating the infection caused by bacteria. Inappropriate use of antibiotics by humans are causing development of antibiotic resistant. Nonetheless multidrug resistant strains are developing in bacterial population. These strains can transfer the resistant gene to other bacteria as well evolving the resistant bacteria over the period of time (Pathak et al., 2017.). Increase in resistance in *P aeruginosa* is a leading cause of threat to patient care due to its limited therapeutic options. Multi drug resistance in *P aeruginosa* is being a major clinical problem and many physicians are facing serious problems in treatment of patients with hospital acquired infections, especially those admitted to ICUs to whom medical devices are indwelled (Varaiya et al., 2008.). These organisms can acquire resistant through mutation and acquisition of new foreign resistant gene. These are intrinsic to many classes of drugs. β- lactam drugs are choices of drugs for therapeutics in hospitals as they have broad spectrum range, well tolerable and usually safe to administer compared to other drugs. β- lactam drugs like cephalosporin, penicillin, monobactams, carbapenems are widely used. (Acharya et al., 2017; Chand et al., 2020). Carbapenems such as Imipenem, Meropenem etc. are the chosen antibiotics while treating *P. aeruginosa* infection. However, there is a global rise in resistant to this breakthrough antibiotic groups(Kali et al., 2013).

The most frequent cause of carbapenem resistance in *Pseudomonas aeruginosa* is metallo-beta-lactamases (MBLs). Conventional beta-lactamase inhibitors such as clavulanic acid or sulbactam do not inhibit MBLs, which are broad-spectrum enzymes that hydrolyze the majority of beta lactam antibiotics, with the exception of monobactams(Manoharan et al., 2010). MBLs are a various set of enzymes which are emerging resistance determinants in *P aeruginosa.* Many MBL genes are detected now. The genes responsible for MBL such as IMP, VIM, NDM genes. The first MBL producing gene was discovered in Japan in 1988(Adam & Elhag, 2018). The isolates were *IMP (*Inactivated Imipenem) genes which is now ubiquitously found in worldwide now. Later *VIM* (Verona Integron encoded Metallo-β lactamase) gene was detected in Europe for the first time in 1999. Other MBL genes such as *SPM*(Sao Paulo Metallo-β lactamase) in Sao Paulo of Brazil in1997, *GIM* (Germany Imipenemase) in Germany in 2002 and *NDM (*New Delhi Metallo-β lactamase) in New Delhi of India in 2009 were identified. A novel gene *FIM* (Florence Imipenamase) was recently discovered in Florence of Italy in 2012 which has similarities upto 40% from *NDM* genes.(Hong et al., 2015). These MBL genes has been found around the world. A study done by (Takahashi et al., 2021) in Nepal isolated carbapenemase inactivating genes such as (*IMP-1, IMP-26, VIM-2, VIM-5, NMD-1)* in 39.5% of *P.aeruginosa* isolates. This study suggest that these genes may have been transmitted in Nepal by the tourists visiting Nepal who harbors the resistant gene encoded organism. Similarly, (Sharma et al., 2023a) isolated 16.7% of MBL producing *P. aeruginosa* harbouring bla_{NDM-1}. (Aryal, et al., 2021) also isolated 59.7% bla_{VIM} and 56.7% bla_{IPM} from total isolates of *P. aeruginosa*. These studies suggest that different metallo-β lactamase producing *P. aeruginosa* is also prevalent in Nepal and is a subject of concern and detail studies of these isolates is much needed

2.3 Virulence factor:

The infection mostly involves three steps: i) Adherence and Colonization ii) local invasion iii) septicemia. However, it does not need to be complete in

these steps. Due to host defense mechanism or administration of antibiotics might halt the process(Strateva & Mitov, 2011). For a successful antimicrobial therapy, the virulence factor and mechanism of infection should be thoroughly studied. In *P. aeruginosa* different virulence factor are present which are presented below.

2.3.1 Bacterial Surface:

The surface of the bacteria different adhesins that helps in the attachment to the host cells. Pili are the projected spikes like structure that helps in attachment. Flagella helps in twitching motility of the bacterium. These structures also trigger the host cells immune response releasing different immunogenic compounds(Pollack, 1984.).

Presence of lipopolysaccharide is one the important virulence that can cause inflammatory reactions, tissue degenerations and damages and other immune responses in host. The lipopolysaccharide contains three domains: lipid A, core oligosaccharide and O antigen or O polysaccharide .It is also responsible for producing endotoxins as well (King et al., 2009).

2.3.2 Secretion System:

The bacteria involve different secretion systems that has role in the inflammatory response of the host cells by releasing various secretion. The bacteria produce different toxins that can cause serious damage to host cells cell necrosis, bleeding, circulatory damage etc. The secretion system plays part in facilitating the escape route from host immune system and enable the bacterial colonization. (de Sousa et al., 2021). Five different types of Secretion system has been studies till now in *P. aeruginosa*.These secretion systems are responsible for delivering the virulence factors such as toxins, proteases, lipases, elastases enzymes that alters the host immune defenses(Streeter & Katouli, 2016). Type I secretion system (TISS) is responsible for acute virulent role in inflammation period. They produce alkaline proteases and involve in heme acquisition protein secretion for utilizing iron. T2SS posses secretion of extracellular toxins and exo proteins such as Proteanase IV, Elastase A and B, Lipase A and C and Phopholipase C as well a alkaline phosphatase. Type 3

Secretion System contains a needle like projections which helps in attachment and helps to inject the effector proteins of *P. aeruginosa* into the host cells. This system helps to invade and infect the host that helps the pathogenicity, clinical manifestation and severity in the host cells. Type 5 Secretion system helps in adhesion and formation of biofilm. They produce large virulence protein that helps to escape immune response and helps in bacterial attachment. Type 6 Section system helps in endocytosis into host cells (Liao et al., 2022)(Alhazmi, 2015).

2.3.3 Secreted factors:

P. aeruginosa secrets different toxins and enzymes that helps in the virulence factor. These secretions alter the host immunity and helps in the pathogenicity of the infection.

2.3.3.1 Exopolysaccharides:

These are the substance produced by *P. aeruginosa* that enhance the bacterial tolerance to the harsh environmental condition such as desiccation, oxidizing agent and also tolerate the host defense. Basically, they are sugar based extracellular macromolecules. They are one of the important compositions of the biofilm production that also helps in adhesins and are virulent in nature.

2.3.3.2 Siderophores:

They are the iron chelating compounds. It is secreted by bacteria to help in the accumulation of iron. *P. aeruginosa* secretes two types of Siderophore; peptidic pyoverdine (pvd) and pyochelin (pch). They both chelates the iron and also responsible for the bacterial growth and transfer iron through secretion system.

2.3.3.3 Proteases:

Alkaline Proteases (Apr A) is a virulent factor which is secreted by the Type 1 secretion system It is controlled by quorum sensing pathway. It can degrade the complement components as well as INF-γ and TNF-α. Therefore, alters the host

defense mechanism and exacerbate infection in the host body. Similarly, Elastase A and B plays a role in destroying the elastin; a component present in the pulmonary tissue and blood vessels. The results end with the impairing the lungs function and pulmonary hemorrhage. The enzyme also damages the host tissue and enhances the bacterial infection by degrading the fibrinogen, lactoferrin, transferrin and elastin.

2.3.3.4 Toxins

P. aeruginosa secretes different types of protein via secretion system. The toxins such as ExoS, ExoT, ExoU and Exo Y are produced by Type 3 Secretion system. ExoS and Exo T are bifunctional in nature and are cytotoxins. They disrupts the hosts cytoskeleton that interfere the cell to cell adhesion. The results end in the apoptosis of hosts cell. Exo U is a potent phospholipase and causes rapid necrotic cell death. Exl A(Exolysin) is a pore forming tissue that also have cytolysin activity. It helps in inducing the increase in permeability in the plasma membrane resulting in necrotic cell death. Exotoxin A inhibits the host protein synthesis by catalyzing ADP ribosylation of cell elongation. Induced programmed cell death is a result of exotoxin A. Another major extracellular toxin section is Lipase A. It degrades the major lungs tissue as well a host cell membrane. It interacts with alginate in extracellular biofilm matrix production that contributes in the bacterial antimicrobial resistant.

Similarly, toxins such as Phospholipase, Lipoxygenase, Leukocidin and Pyocyanin are other toxins produced by *P. aeruginosa* that plays a role in tissue degradation and altering the hosts defense making it easy for the organism to invade the host cell.

2.4 Quorum Sensing:

Quorum sensing is a mechanism in which the bacteria detects the change in surrounding environment and allows to respond the information by regulating various genes around its bacterial population. It also helps in exchanging the

mutating genes among the surrounding bacterial population, from which the bacteria can gain the resistant gene to certain antibiotics resulting in distribution of Antibiotics resistant organism.(Rumbaugh et al., 2000). Quorum sensing has a role in releasing major virulence factor as well as helping in maturing the formation of biofilms(Liao et al., 2022).

2.5 Biofilm:

P. aeruginosa produces a extracellular polymeric substances around the bacterial cell such as exopolysaccharide, matrix proteins and extracellular DNA. These substance wrap around the bacterial cells to form a biofilm. The formation of biofilm helps the organism to adhere in the hospital settings and prosthetic devices helping in colonization(Brindhadevi et al., 2020). Later when used, the bacteria may transfer to the patient resulting in the infection. This is one the reason of nosocomial infection in hospital settings. The formation of biofilm leads to the resistant to multiple antibiotics, narrowing in the gap in treatment of infection(Kunwar et al., 2021).

Table No. 1: Figure shows the detail virulence factor of *P. aeruginosa*(Liao et al.,2022).

Categories	Virulence factors	Functions
Bacterial surface structures		
surface appendages	Type IV pili	Attachment to host cells, bacterial twitching and swarming motility, biofilm formation
	Flagella	Swarming motility, biofilm formation, bacterial adhesion and other pathogenic adaptations
Outer membrane component	Lipopolysaccharide	Stimulation of host inflammatory response, resistance to serum killing and phagocytosis
Secretion systems	Type 1 secretion system (T1SS)	Secretion of alkaline proteases, utilization of iron, heme uptake
	Type 2 secretion system (T2SS)	Secretion of various lytic enzymes
	Type 3 secretion system (T3SS)	Injection of virulent effectors into host cells
	Type 5 secretion system (T5SS)	Secretion of proteins related to biofilm formation and adhesion
	Type 6 secretion system (T6SS)	Delivery of toxins to neighboring bacteria, translocation of effectors to host cells, biofilm formation
Secreted factors		
Exopolysaccharide	Alginate	Biofilm formation, Immune evasion, bacterial adhesion, mostly existed in strains isolated from infected patients
	Pel and Psl	Biofilm formation, Immune evasion, bacterial adhesion, mostly existed in strains isolated from environment
Siderophores	Pyoverdine	Chelating irons, promoting bacteria growth, contributing to bacterial virulence
	Pyochelin (Pch)	Chelating irons, promoting bacteria growth, contributing to bacterial virulence
Protease	Alkaline protease (AprA)	Regulation of quorum sensing, protection of bacteria from host defense
	Elastase A and B (LasA and LasB)	Degrading proteins in host tissues, causing tissue damage
	Protease IV	Degrades host proteins that involve in immunity against infection
Toxin	T3SS effectors (ExoS, ExoT, ExoU and ExoY)	Disruption of host actin cytoskeleton, interference of cell-to-cell junctions, induction of host cell apoptosis
	Exolysin (ExlA)	Pore-forming on the host cell membrane
	Exotoxin A (PEA)	Inhibition of protein synthesis resulting in cell death
	Lipase A (LipA)	Immunomodulator, damaging host tissue
	Phospholipase C (PLC)	Degrading the phospholipid surfactant, damaging host cells
	Lipoxygenase (LoxA)	Interference of the host lipid signaling, regulation of bacterial invasion process
	Leukocidin	Inhibition of host immune functions
	Pyocyanin (PCN)	Suppresses immune response, cytotoxic to host cells
Bacterial cell-to-cell interaction		
Quorum-sensing (QS)	–	Regulation of the production virulence factors, integration of the environmental stress, modulating production of biofilm and swarming and twitching motilities
Biofilm	–	Escape from host immune responses, resistance against antibiotics, persistency of bacteria under harsh conditions

2.6 Antibiotic Resistant:

Antibiotic resistant in *P. aeruginosa* has been a topic of concern in present world. Resistant to antibiotic mechanism may have been due over use of antibiotics, naturally adapting feature to certain antibiotics or transfer of resistant gene from other bacterial population to the bacteria(Alhazmi, 2015). Some mechanisms are discussed below:

2.6.1 Intrinsic Resistant:

P. aeruginosa has develop resistant to some of the drugs naturally. It may have exhibit intrinsic resistant by the means of mechanical efflux pumping of antibiotic molecules, decrease in permeability of cell wall or due to the production of special enzymes that can degrade the antimicrobial effect of antibiotic(Moore & Flaws, 2011).

Many antibiotics have to cross the bacterial cell wall and trigger the destined targets to demonstrate its efficacy of antimicrobial features. As they require porins to travel all the way from the cell wall component, the *Pseudomonas* cell wall has evolved to reduce the size of the pores, through which the larger component of the antibiotics cannot pass through it cell wall. This results in the ineffectiveness of the antibiotics.(Lambert, 2002.)

Efflux pumping is also responsible in resisting the antibiotics. In this system, antibiotics are effluxed through porin channels from cell membrane. The multidrug efflux pumping mechanism consists of three protein component(Moore & Flaws, 2011). A resistant nodulation cell division (RND) exporter protein that is embedded in the cytoplasmic membrane of *P. aeruginosa* is an energy dependent pumping system that push antimicrobial molecules outside the bacterial cell. The inner membrane protein connects the RND protein to the third protein. These three efflux protein combine to form a mechanism to remove the antibacterial properties from the cytoplasmic membrane.(Lambert, 2002.)

2.6.2 Acquired Resistant:

Resistant in *P. aeruginosa* can also be acquired through mutation in the chromosomal genes. The mutation in the genes that regulates in the expression to the targeted resistant gene causes overexpression and develop resistant to such antibiotics. Similarly, acquiring the resistant gene present in the plasmid also produce resistant to several antibiotics.

2.7β – lactam resistant:

Beta lactam drugs are the group of antibiotics that contain beta lactam rings in their chemical composition. These group of antibiotics works by inhibiting the cell wall biosynthesis. It inhibits the synthesis of peptidoglycan layer being unable to form the cell wall of bacteria causing leaking of cytoplasmic material resulting in the cell death. The group includes Penicillin derivatives, Cephalosporins, monobactams and carbapenems (Glen & Lamont, 2021). They are considered as wonder drug and widely used in treating different infection. The overuse of these antibiotics has results in developing resistant to these

antibiotics. The organism produces beta lactamase enzymes which hydrolyses the beta lactam drugs being unable to function(Tooke et al., 2019)(Davies, 1996). Bacteria has evolve to develop a mechanism to resist the beta lactam antibiotics by producing the beta lactamase enzyme known as extended spectrum beta lactamase (ESBL). These enzymes hydrolyses the beta lactam drugs like Penicillin and cephalosporins(Neupane et al., 2018). The bacterial cell wall contains Penicillin binding protein (PBP). These protein have high affinity to Penicillin antibiotics. Due to high binding affinity, the beta lactam binds to PBPs instead of hydrolyzing the cell of bacteria. As a result, the beta lactam drug is unable to function as antimicrobial therapy. Other reasons for resistant are due the low permeability of cell walls, over expression of efflux pumping, production of extracellular secretion, quorum sensing and biofilm formation(Aghamiri et al., 2014).

Different mechanism of beta lactamase has been found. These different beta lactamase targets the different class of antibiotics. The result of other beta lactam drugs being resistant, carbapenems are most widely used antibiotics for the multidrug resistant infection. Carbapenem such as Imipenem and Meropenem are choice for those cases. However, resistant pattern has been developed against these antibiotics as well. The resistant to these antibiotics are called metallo beta lactamase. The resistant mechanism is dependent on zinc ions hence called metallo beta lactamase. The MBL producer can hydrolyze all other beta lactams except Monobactams. Hence, carbapenemase producing *P aeruginosa* are mostly resistant to other beta lactam group of antibiotics.

Table No. 2: Figure showing different β-lactamase enzymes and their action against antibiotics

Functional mechanism	Ambler class	Bush (Groups)	Examples	Substrates
Serine-β-lactamases	Class A-penicillinases	(2a,2b,2c)	Broad-spectrum β-lactamases: TEM-1, TEM-2, SHV-1	Benzylpenicillin (penicillin), aminopenicillins (amoxicillin, ampicillin), carboxypenicillins (carboxypenicillin, ticarcillin), narrow-spectrum cephalosporins (cefzolin, cefuroxim and others)
		(2be)	Expanded-spectrum-β-lactamases (ESBL):TEM family and SHV-family	Substrates of the broad- spectrum group β-lactamases plus cloxacillin, methicillin and oxacillin
			Others: BES-1,GES/IBC family, PER-1,PER-2, SFO-1, TLA-1, VEB-1/2	Same as for TEM and SHV family
		(2br)	TEM family (TEM-30, TEM-31) IRTs*	Same as for TEM and SHV family and * inhibitors resistant
		(2e)	CTX-family	Substrates of the expanded-spectrum-β-lactamases group, for some enzymes, cefepime
		(2f)	Carbapenemases: (KPC-1, KPC-2 and KPC-3; GES-1, GES-2)	Substrates of the expanded-spectrum-β-lactamases group plus cephamycins and carbapenems (ertapenem, meropenem, imipenem)
Metallo-β-lactamases	Class B-metallo-β-lactamases (zinc)	(3a,3b,3c)	Carbapenemases: IMP family, VIM-family, SPM-1, SPM-2, GIM-1, and L1, CcrA	Same as for carbapenemases class A
Serine-β-lactamases	Class C-cephalosporinases	(1)	AmpC-type: AAC-1, ACT-1, CFE-1, CMY-family, DHA-1, DHA-2, FOX-family, LAT-family, MIR-1, MOX-1, and MOX-2	Substrates of the expanded-spectrum-β-lactamases group plus cephamycins
Serine-β-lactamases	Class D-cloxacillin-hydrolyzing enzymes (OXA)	(2d)	Most of OXA family	Substrates of the broad- spectrum group plus cloxacillin, methicillin and oxacillin
			Other OXA: OXA-23 → OXA-27,and OXA-40, OXA-48	Same as for IMP family, VIM-family, SPM-1, SPM-2 and GIM-1
Unknown		(4)	AVS-1	Miscellaneous or unsequenced/ uncharacterized enzymes that do not fit into any function or molecular group

2.8 Role of *P. aeruginosa* in Urine infection:

Urinary Tract Infection is an infection of any parts of the urinary system by micro-organism. The system includes the urinary bladder, ureter, urinary bladder and kidneys. The infection is predominant in females than in males. The infection may be localized or may disseminate upto the kidney in chronic condition. Different micro-organisms are responsible for causing UTI such as *E.coli, Klebsiella pneumonia, Proteus vulgaris, Pseudomonas aeruginosa*. *P. aeruginosa* is most common in nosocomial urinary tract infection. During a prolonged stay in hospital, patient acquire the infection through the contaminated devices or surroundings. The case is even common in patients who are catheterized. Catheterization causes the bacteria form a biofilm and adhere in the surface of catheter. That lead to disseminate the organism into the urinary tract system. In catheterized patients, antibiotics resistant *P. aeruginosa* is most detected. The dissemination of these strain causes the patient difficult to treat. The case worsen if the organism is found to be metallo betalactamase producer as they are notorious and hard to treat because they are resistant to most of other antibiotics. Adverse side effects and high dosage drugs like Polymixin and Colistin are the alternative choice of drugs for treatment. Mortality and morbidity are high among those isolates

2.9 Role of *Pseudomonas aeruginosa* infection in respiratory tract:

Infection of *Pseudonomas aeruginosa* in lungs is observed in patients with immunosuppression and chronic lungs disease such as asthma, COPD, cystic fibrosis etc. In hospital settings mainly, in ICU and patient with positive pressure ventilation and endotracheal tubes, the infection by *P. aeruginosa* can be acquired through nosocomial settings. The pneumonia may be a primary infection following the aspiration of organism from upper respiratory tract infection(Li et al., 2015). Alternatively, the infection might occur through the result of bacterial septicemia into lungs. *P. aeruginosa's* flagella and pili are essential for both motility and respiratory infection because they allow attachment to respiratory epithelium via respiratory mucins and the glycolipid asialoGM1, P.(Pollack, 1989b)An important stage in the infection process is bacterial adherence to the respiratory epithelium, which is achieved by

interactions between bacterial adhesins and host receptors. The single flagellum, which is required for motility, adhesion to cells, and biofilm formation, and type IV pili, which are appendages made of pilin polymers and which enable the bacteria to move across surfaces in addition to playing important roles in biofilm formation and respiratory epithelial cell attachment, are the main adhesins for *P. aeruginosa* infections. Subsequently, *P. aeruginosa* starts to release an extracellular matrix, resulting in the formation of a biofilm. A biofilm is a bacterial cell-encased structural matrix that sticks to the respiratory epithelium.By forming biofilms, bacteria not only offer resistance against antibiotics and neutrophil phagocytosis, but they can also function in concert with one another. A few bacteria may escape from the biofilm and spread to infect other lung regions thereafter(Rossi et al., 2022).

2.10 Role of *Pseudomonas aeruginosa* infection in wound

Pseudomonas aeruginosa are normally opportunistic in nature. Human skin is non-penetrable and is one of the first line of defense mechanism. Breaching of the skin through cut, abrasion, accidents, tissue injury or wounds due to surgical procedure might lead in opportunity for the organism to invade and colonize into the tissue through those openings. (Maharjan et al., 2020)*P. aeruginosa* then adhere the tissue through adhesins and secretion system and degrade the surrounding tissues via production of toxins and extracellular enzymes. Production of biofilms and quorum sensing helps the organism to survive through host immune defense and some antibiotics. Infection by the bacteria may cause formation of pus, tissue damage and even septicemia if left untreated. The infection may lead to inflammation of the local area, pain, pus formation. In hospitals, nosocomial infection of *P. aeruginosa* is common in surgical sites through the surgical materials, blades and other devices. Infection may also increase if the wounds are not treated aseptically. (Saffari et al., 2016)

2.11Role of *Pseudomonas aeruginosa* infection in blood

There are several known risk factors that put people at risk factor for *Pseudomonas aeruginosa* bacteremia. In most of the cases, prior exposure to antimicrobial treatment, the integrity of the typical physical barriers is disrupted by vascular catheters, urinary catheters, drainage tubes and endotracheal intubation devices, which are foreign entities that *P. aeruginosa* easily colonizes. Another reason for causing bacteremia in nosocomial infection is due to the dissemination of organism from local infection that are left untreated into the blood stream.(Rossi et al., 2022) Dissemination of organism from local infection may also be due to the multidrug resistant isolates. When the antibiotics could not inhibit or suppress the infection of *P. aeruginosa*, it migrates from local infection into whole body degrading the host tissue. Thus later, resulting in the mortality and morbidity of the patients. (Qin et al., 2022)

CHAPTER III

METHODOLOGY:

3.1 Materials

Appendix A includes a list of the supplies, tools, and different reagents that were employed during the course of this investigation. Flow chart 1 summarizes the process for identifying *Pseudomonas aeruginosa* that produces Metallo Beta Lactamase (MBL).

3.2 Study site and settings

The cross-sectional investigation was done from January 2024 to June 2024 in the Microbiology lab of the Grande international hospital in Dhapasi, Kathmandu. Inpatients and outpatients of all ages from Grande international hospital were involve in this study. Different clinical samples from hospital patients were taken for routine culture, microscopic examination, and antibiotic susceptibility testing in the hospital laboratory.

3.3 Sample size and sample population

The study employed 1500 clinical samples, including urine, wound, swabs, pus, blood, feces, and diverse body fluid samples from male and female patients of all ages. Samples that were collected in a pristine, leak-proof container with no obvious contamination and those were appropriately labeled with patients required information were accepted; otherwise, a second sample was required for further investigation.

3.3.1Inclusion criteria:

All clinical samples of patients requested by clinician of any ages are included in this study. The isolates of *P. aeruginosa* were included.

3.3.2 Exclusion Criteria

The isolates of bacteria other than *P. aeruginosa* were excluded from the study.

3.3.3 Sample size and sampling technique

Sample size calculation was estimated by using Hajian-tilaki method(Hajian-Tilaki, 2014)

$$N=\frac{z^2PQ}{d^2}$$

where,

P=4.6% estimated from previous finding ((Sharma et al., 2023b)

Q= (1-P)

Margin of error(d) =4%

Now, the final sample size was calculated as follow,

Applying the formula,

$$N=\frac{z^2PQ}{d^2}$$

$$N=\frac{(1.96)^2\times0.046\times(1-0.046)}{(0.04)^2}$$

$$=\frac{3.84\times0.046\times0.954}{0.0016}$$

=105

Now, Adding 20% isolates due to mislabeled, leaked or contaminated sample,

n=20% of 105

$$=\frac{20}{100}\times105$$

=0.2×105

=21

Therefore, the total estimated sample size was found to be

g(N+n) =105+21

=126

Hence, 126 number of *Pseudomonas* isolates was taken as sample size.

3.4 Collection of Specimen

Medical staff members working in the wards assisted in the aseptic collection of the clinical samples in a sterile, leak-proof, and tightly-capable container. The pus was removed from the affected area using a sterile cotton swab. All necessary samples, such as sputum, blood, pus, wound swabs (throat, mucosal), bodily fluids (pleural fluid, epigastric fluid, ascetic fluid), cerebrospinal fluid, and endotracheal tubes, were taken aseptically and forwarded to the microbiology lab for routine culture and antibiotic susceptibility testing.

3.4.1 Urine sample

Patients were told to collect 10-20 ml of midstream urine in a dry, sterile, wide-necked and leak-proof container. After that, the container was accurately labeled and sent as soon as possible to the laboratory with a request for more processing. When rapid delivery was not possible, the specimen was refrigerated around 4C. The urine was treated with 1.8% wt/vol of boric acid as a preservative when a delivery delay of more than two hours was anticipated. (Vandepitte J., 2003)(Forbes, 2016)

3.4.2Pus specimen

Pus samples were collected from wounds that were either clinically infected or deteriorating and taking a long time to heal. While an open wound was debrided and then carefully cleaned with sterile saline before to collecting a pus sample, where as a closed wound and aspirator were disinfected using 2% chlorhexidine followed by an iodine solution. Instead of superficial debris, pus samples should contain the most advanced stage of the lesion or exudates (Forbes et al 2007)

3.4.3 Sputum specimen

The sputum sample was taken in a disposable, sterile, leak-proof container. Prior to sample collection, the patient was taught not to stand up, gargle with non-sterile water, use mouthwash, or rinse their mouth with non-sterile water. They were also told to obtain a sample from a deep cough rather than from saliva or postnasal drip. The container was then properly labeled and brought as quickly as possible to the laboratory (Forbes et al 2007).

3.4.4 Sample of body fluids (bile, ascetic fluid, peritoneal fluids)

These samples were collected by percutaneous aspiration under the guidance of skilled medical professionals, taking care to avoid contamination with commensal bacteria. In order to prevent patient infection or sample contamination, the needle puncture site was cleansed with 70% ethanol and disinfected with iodine solution. After properly labeled, 3-5 ml of the sample were collected and taken to the lab.

3.5 Culture of specimen

3.5.1 Urine specimen

Cysteine Lactose Electrolyte Deficient Agar (CLED) plates were used to culture each sample. On CLED agar, an inoculating loop was used to streak a loop full of urine sample. The plates conducted a 24-hour aerobic incubation at 37 °C following inoculation. The bacterial count was reported as

- Less than 10^4 cfu/ml urine-Not significant
- 10^4-10^5cfu/ml urine- Doubtful Significant
- More than 10^5cfu/ml urine-Significant bacteriuria (Cheesbrough, 2006). Low count bacteriuria was also taken into consideration if there was any indication which could lower the concentration in urine, e.g. patient under treatment with diabetes, chronic kidney disease were concentrating power of kidney is low, obstruction in ureter due to tumor or stone, etc,

If the culture showed the existence of two uropahogens that were growing significantly, both of their identifications and microbial susceptibility tests were carried out; however, if there were three or more pathogens, the situation was described as numerous bacterial morphotypes, and the proper remembrance of the sample was requested (Forbes et al 2007).

3.5.2 Pus specimen

Pus samples were inoculated into Blood Agar and MacConkey agar plates using a sterile loop, streaked, and incubated at 37 °C overnight. Pus and wound swabs were smeared with the use of a sterile inoculating loop on the side of Blood Agar and MacConkey Agar plates (WHO 2013) and

incubated at 37 °C for 18 to 24 hours.

3.5.3 Sputum specimens

Sputum samples were inoculated onto Blood Agar and MacConkey Agar plates. Inoculation was done using a sterile inoculating loop and by quadrant streaking. They were inoculated at 37 °C in an aerobic environment all night long.

3.5.4 Other specimens

With the use of a sterile inoculating loop and quadrant streaking, additional specimens like endotracheal tips, drain tips, stool samples, tissue and blood samples were obtained and inoculated in Blood Agar and MacConkey Agar plates. They were cultured in an aerobic environment at 37 °C for the entire night.

3.6 Identification of Isolates

According to Bergey's Manual of Systematic Bacteriology, pure colonies of isolated bacteria were identified using conventional microbiological and biochemical procedures. The methods include examining colony morphology, staining reactions, and other biochemical characteristics (Cheesbrough, 2006).

3.6.1 Identification using pure culture

Prior to running biochemical tests and other analyses, each of the organisms was isolated in its purest form. A primary culture was used to perform gram staining on an isolated colony. A single isolated colony from MacConkey agar was added to nutrient broth and cultured for two hours at 37 °C to detect Gram-negative organisms. After that, it was sub cultured on a dried Nutrient agar plate and incubated for 24 hours at 37 °C. Catalase, oxidase, and other biochemical assays were conducted using the organism culture that had been collected and overnight incubated. In Appendix D, the Gram staining process is described.

3.7 Antimicrobial Susceptibility testing (AST)

On isolates from various species of Gram-negative bacteria, the antimicrobial susceptibility test was carried out using the disc diffusion method, also known as the Kirby- Bauer method, in accordance with the clinical and Laboratory Standard Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS) guidelines as follows:

- A standard volume of 25 ml of medium was used to produce MHA at a uniform depth of 4 mm.
- A single isolated colony whose susceptibility pattern has to be determined was handled and inoculated into a Mueller Hinton broth tube using a sterile wire loop. The colony was then incubated at 37 °C for 24 hours.
- Following incubation, the turbidity of the suspension was compared to the McFarland turbidity standard.
- Using the carpet culture approach, a plate was infected with the bacterial suspension using a sterile swab. The agar surface was allowed to dry on the plate for about five minutes.
- Appropriate antimicrobial discs of HI media, measuring 6 mm in diameter, were evenly distributed on the infected plates, with a maximum of 6 discs per petri dish with a 90 mm diameter.

The plates incubated overnight at 37 °C were examined next day to ensure confluent growth. Diameter of each zone of inhibition (mm) was measured and compared with standardized interpretative chart provided by the company.

The pattern of antibiotic susceptibility of the bacterial isolates was used to identify multidrug resistance (MDR), and only Gram-negative isolates that showed resistance to two or more classes of antibiotics, as defined by the Clinical Laboratory Standards Institute (CLSI 2020) standards, were included in this study.

3.8 Detection of MBL producers

Many phenotypic techniques, including the MBL E-test, combined disc

assay, Hodge test, Double-disc synergy test (DDST), and micro-dilution, have been developed for the identification of MBL. The basis of each of these tests is the MBL activity's capacity to be inhibited by chelating agents, EDTA, and thiol based substances (Andrade et.al 2007; Walsh et.al 2005). Here, EDTA-Imp was employed to identify MBL producers in MDR *Pseudomonas aeruginosa* in this investigation.

Preparation of 0.5 McFarland standards

A 0.5 McFarland dilution of the isolated in 5 ml of broth or saline was prepared.

The screening test

- · Using a sterile cotton swab, the isolated compared with 0.5 McFarland dilutions were inoculated onto a Mueller Hinton Agar plate.
- · The organism covered the entire Mueller Hinton Agar plate in streaks. Two or three times, the procedure was repeated while rotating the plate each time.
- · The agar plates' rim was finally swabbed.
- · The plate was left to dry for three to seven minutes.
- · The antibiotic disc containing imipenem (10µg) was positioned on the plate.
- · The plate was then incubated for 24 hours at 37 °C.

3.8.1 Phenotypic detection

To detect the possible MBL producing organism, the suspected carbapenemases producing isolates from screening test were tested by phenotypic detection technique as described below:

Metallo-beta-lactamase (MBL) detection by Combined Disk (CD) assay

The tested organisms was inoculated on an MHA plate using the normal techniques, as advised by the CLSI recommendations. The surface of the

agar plate was covered with two imipenem (10 g) disks, one of which had 5 µl of 0.5 M EDTA solution added to it. After 18 hours of aerobic incubation at 37°C, the outcome was analyzed by contrasting the inhibition zones of imipenem and imipenem-EDTA disks. The zone of inhibition increased by ≥ 7mm with imipenem-EDTA disks compared to imipenem alone, which was seemed to be MBL Positive (Maharjan et al. 2020).

All the process of the sample was presented in flow chart 1

Flow diagram

All clinical samples (Urine, Blood, Swabs, Pus, Tissues, Sputum,etc)

⇩

Inoculate and incubate on CLED, MA and BA plates

⇩

Gram stain of the isolates

⇩

Subculture the colonies on NA incubate at 37°C for 24 hours

⇩ ⇩ ⇩

Gram's stain Colony characteristics Biochemical test

↘ ⇩ ↙

Identification of possible pathogens (Pseudomonas *aeruginosa)*

⇩

Antibiotic susceptibility testing and screening of MBLs producing Pseudomonas *aeruginosa*

⇩

Phenotypic confirmation of MBL producing (*Pseudomonas aeruginosa)* by combined disc method

Flow chart No. 1: Flow chart showing the process for sample processing and insolation of organism

CHAPTER IV

RESULTS

4.1 Bacterial Isolates

During the study period, a total of 1500 various clinical samples were processed. A total of 126 isolates of *P. aeruginosa* was obtained. The prevalence of *P. aeruginosa* was found to be 8.4%. A total of 126 isolates of *P. aeruginosa* were isolated from the clinical samples, 44 Were from Urine samples, 40 from sputum, 6 from swab, 2from pus, 24 from blood samples.9 from fluid and 1 from tissue . Out of these, 27 isolates were meropenem resistant which were screened for metallo beta lactamase producer. A total of 21 isolates were found to be MBL producer by Phenotypic detection using combined disc diffusion method.

4.2 Distribution of isolates on the basis of clinical samples:

A total of 8.4% of *Pseudomonas aeruginosa* isolates were obtained from various clinical sample. Out of which 34.9% were isolated from Urine, 31.7% from Sputum, 19% from Blood, 1.6% from pus, 4.8% from wound swabs, 7.1% from body fluids and 0.8% from tissue. The highest number of isolates were obtained from urine sample followed by Sputum. The least number of isolates was obtained from tissue sample which was 1 in number.

Table No 3 : Distribution of Isolates of *P. aeruginosa* on the basis of different clinical samples.

Samples	**Number of *P. aeruginosa* isolates**	**Isolates in percentage(%)**
Urine	44	34.9
Sputum	40	31.7
Blood	24	19
Pus	2	1.6
Swabs	6	4.8
Body Fluids	9	7.1

Tissue	1	0.8

4.3 Gender wise distribution of isolates:

Out of total *P. aeruginosa* isolates, more organisms were isolated from male patient than in female. In male patients, *Pseudomonas aeruginosa* isolates were found more in sputum sample following urine sample. Least were found in Tissue sample about 1 isolate. In female, more isolates were found in urine and lest were found in swab. No isolates of *P. aeruginosa* were found in pus and tissue sample. Following Figure number 1is the graphical representation of each isolates obtained from different clinical isolates on the basis of gender.

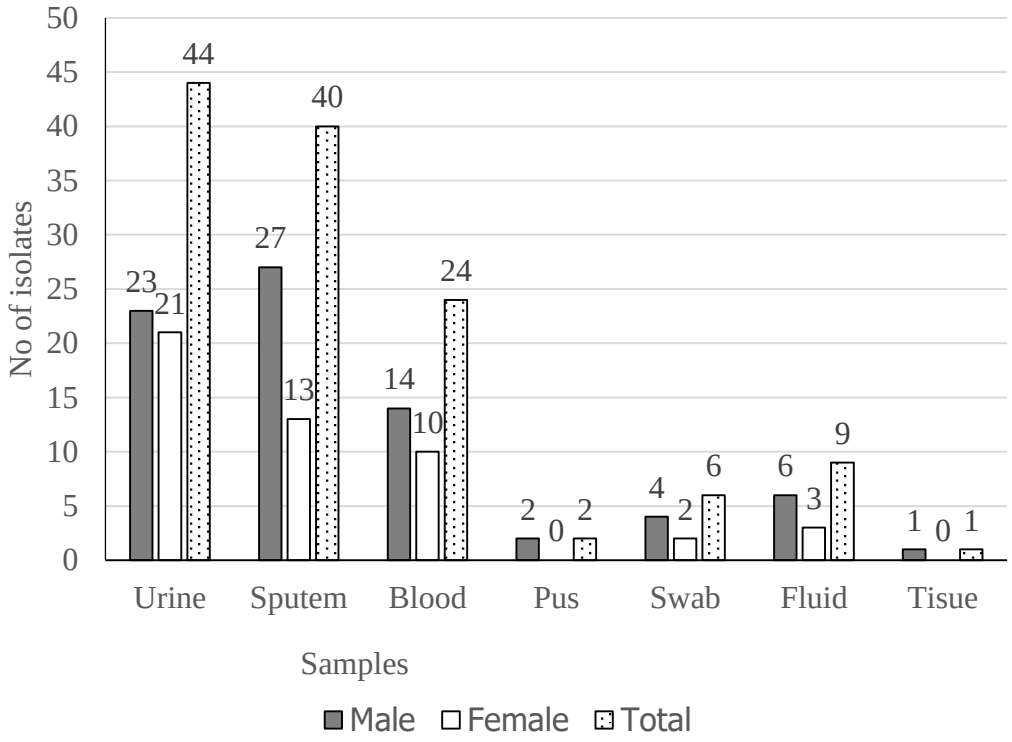


Figure No. 1: Distribution of *P. aeruginosa* of clinical isolates based on Gender

4.4 Age wise distribution of isolates:

Among the total *P. aeruginosa* isolates, most number of the isolates were found in age group more than 70 years while least isolates were obtained from age group between 20-30 years. About 42 of *P. aeruginosa* were isolated from patients above age group 70. Similarly, 24 number of isolates were from age group 60-70, 20 and 17 isolates were obtained from age group 50-60and age group 40-50. Lower number of *P. aeruginosa* were isolated from sample of age group from 20-30 and 30-40, which were 5 and 7 in number of isolates. Following figure number 2. shows the distribution of number of *Pseudomonas aeruginosa* isolates according to different age group.

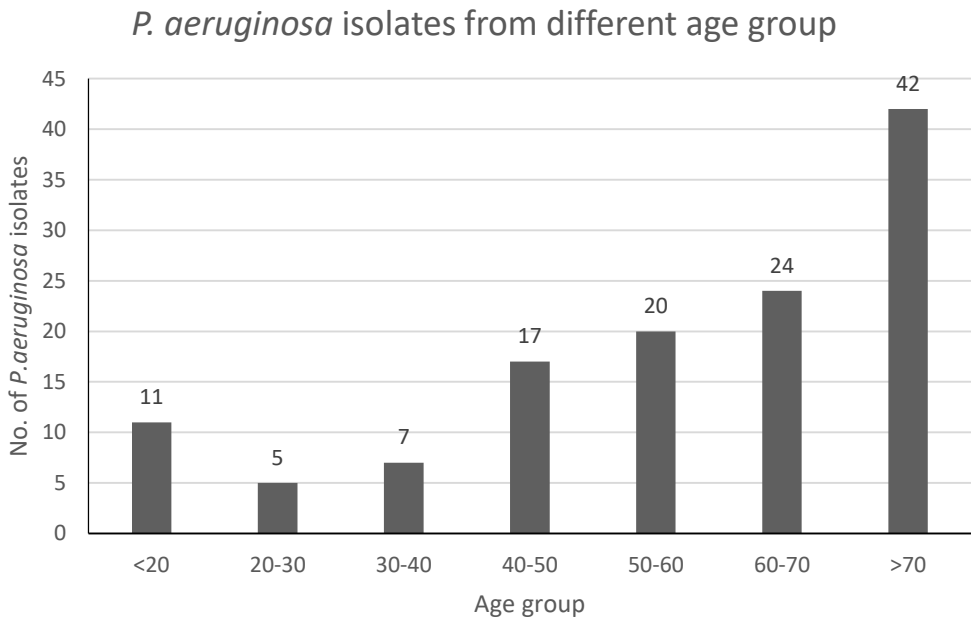


Figure No. 2: Distribution of *P. aeruginosa* of clinical isolates based on different Age group

4.5 Antibiotic Susceptibility Pattern:

On testing susceptibility test of the isolates, most of the isolates were found to be susceptible to Meropenem (78.6%), Levofloxacin (82.4%), Cefoperazone sulbactam (76.4%) and Piperacillin Tazobactam (73%). Most of the resistant cases where found in Nitrofurantoin(90.2%) and Azithromycin (48.8%). Ceftazidime and Cefepime shows the resistant percentage of 33.6% and 30.8% respectively, which are the choice of drugs for *P. aeruginosa* infection. After performing disc diffusion method in MHA plate, the reports of sensitive patterns of different antibiotics are shown in following Table No 4.

Table no. 4: Table showing distribution of sensitive patterns of different antibiotics of isolated *P. aeruginosa.*

Antibiotics	**Sensitive**	**%**	**Resistant**	**%**	**Total**
Amikacin(30mcg)	86	69.4	38	30.6	124
Aztreonam(30mcg)	21	50.0	21	50.0	42
Cefepime(30mcg)	83	69.2	37	30.8	120
Cefoperazone sulbactam(75/100 mcg)	94	76.4	29	23.6	123
Ceftazidime(30mcg)	81	66.4	41	33.6	122
Meropenem(10mcg)	99	78.6	27	21.3	126
Levofloxacin(5mcg)	70	82.4	15	17.6	85
Piperacilin Tazobactam(100/10mcg)	92	73.0	34	27.0	126
Nitrofurantion(300mcg) (For Urine Isolates)	4	9.8	37	90.2	41
Norfloxacin(10mcg) (For urine isolates)	26	63.4	15	36.6	41
Azithromycin(15mcg) (for sputum isolates)	22	51.2	21	48.8	43

4.6 Detection of multidrug resistant and extremely drug resistant:

Out of total isolates of *P. aeruginosa,* 43 samples were resistant to more than three antibiotics which were termed as multidrug resistant and 10 were detected as extreme drug resistant. The distribution is shown is following Figure no. 3 pie chart below.

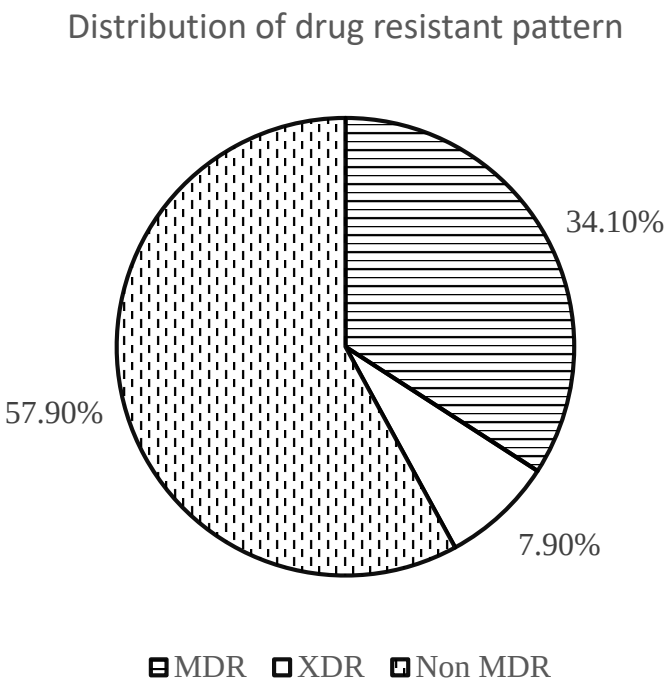


Figure No. 3: Pie Chart showing the percentage of multidrug resistant and extremely drug resistant isolates.

4.7 Detection of Metallo β lactamase Producer *P. aeruginosa*

The 27(21.30%) Meropenem resistant isolates obtained after performing antibiotic susceptibility test were tested for metallo beta lactamase producer. Out of which only 21(16.6%) isolates were found to be positive for metallo beta lactamase producer by Combined disc method using Imipenem and Imipenem impregnated with EDTA separated by distance of 20mm.

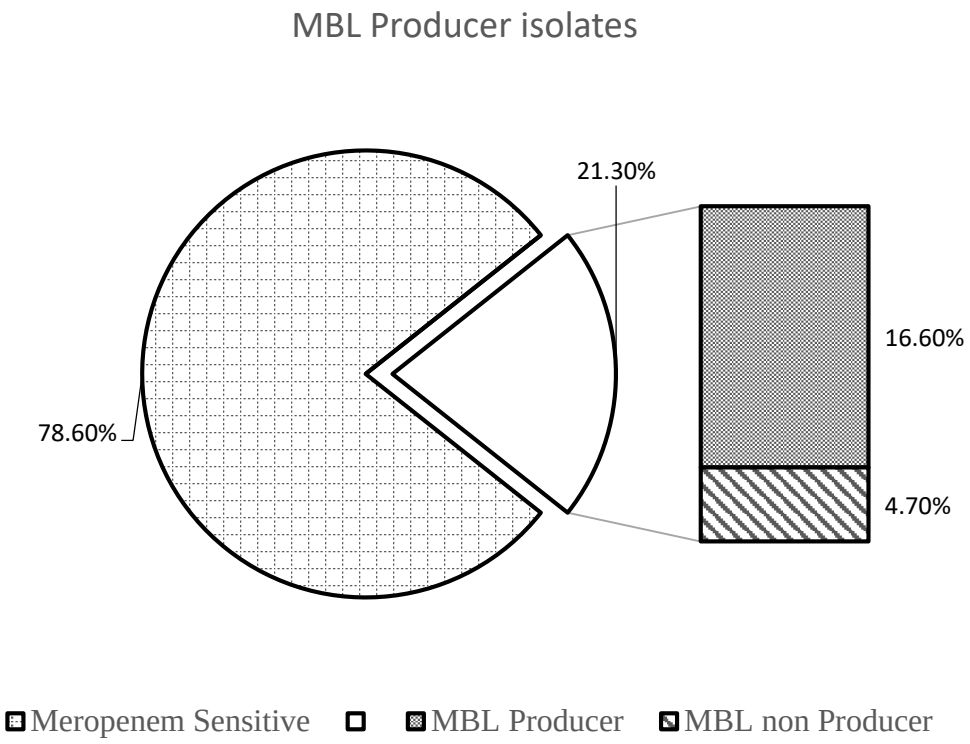


Figure No 4: Pie chart showing distribution of Meropenem Susceptibility pattern

4.8 Distribution of metallo betalactamse producing *P. aeruginosa* on the basis of type of patients.

A total of 21 isolates of *P. aeruginosa* were confirmed as MBL producer by phenotypic detection method. Among them 10(47.6%) isolates were obtained from ICU patients, 7(33.3%) isolates from general wards and 4(19.1%) isolates were obtained from out patients. The above information is represented in pie chart below in Figure No. 5.

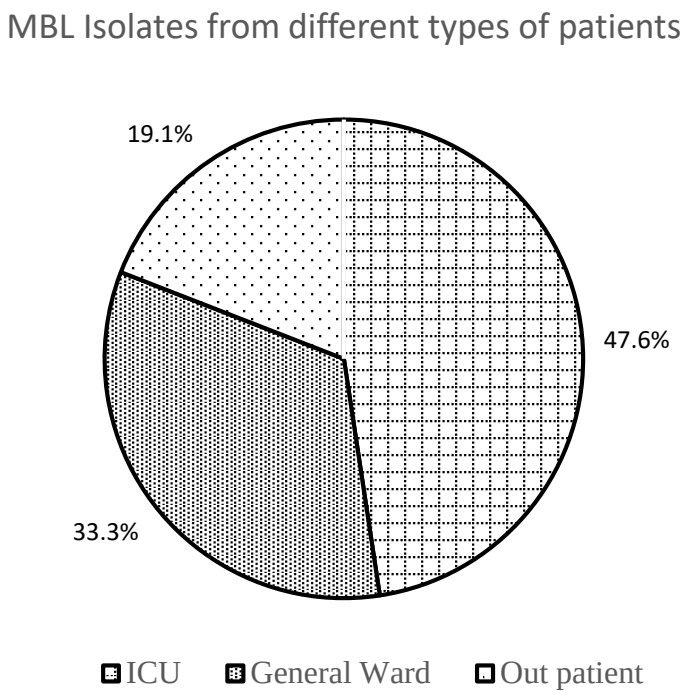


Figure No. 5 : Pie chart showing distribution of MBL producing *Pseudomonas aeruginosa* on the basis of patient type.

4.9 Distribution MBL producer according to the clinical samples

Out of 21 MBL isolates, 10(47.6%) were from urine, 6(28.6%) were from sputum, 3(14.3%) were from blood, 1(4.8%)each from pus and tissue sample. No mbl producers were detected from wound swab and body fluids. The information are shown in the following Figure No. 6 represented in a pie chart.

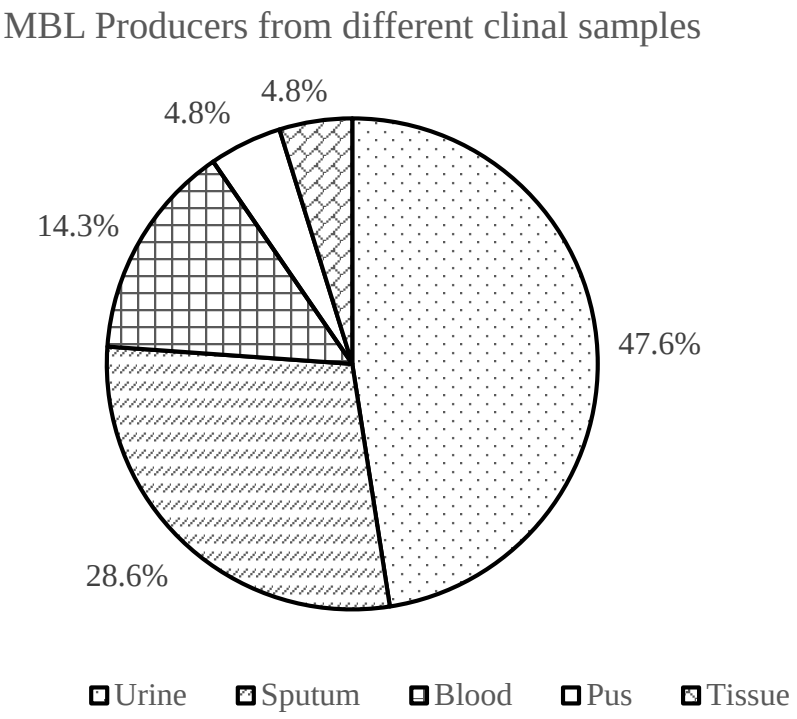


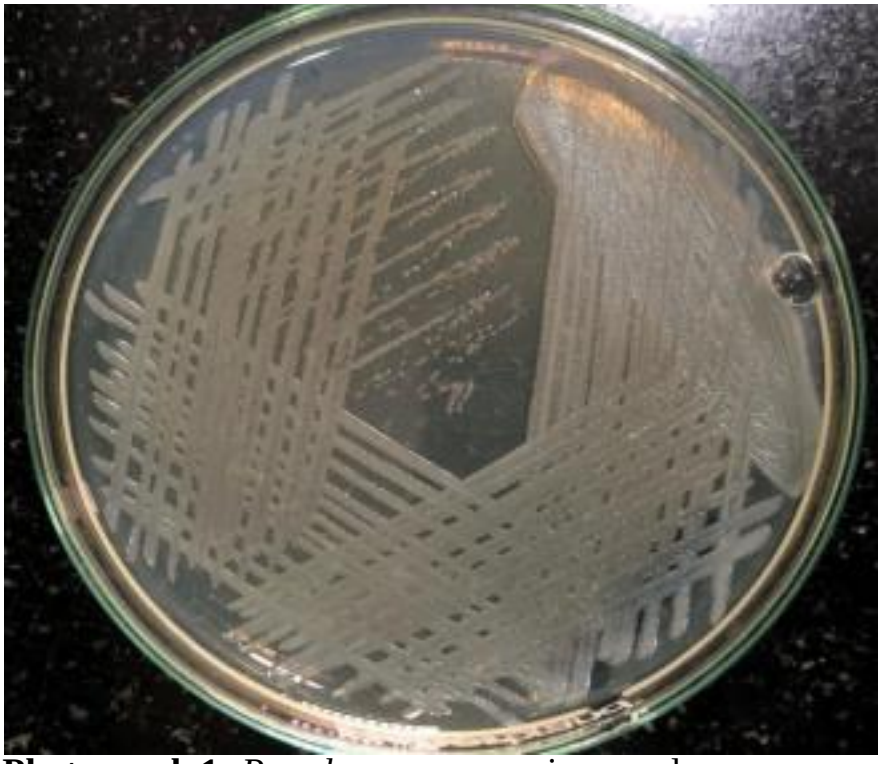
Figure No 6: Pie Chart showing distribution of MBL producer among different clinical samples.

4.10 Antibiotic Resistant pattern of MBL and Non MBL Producer:

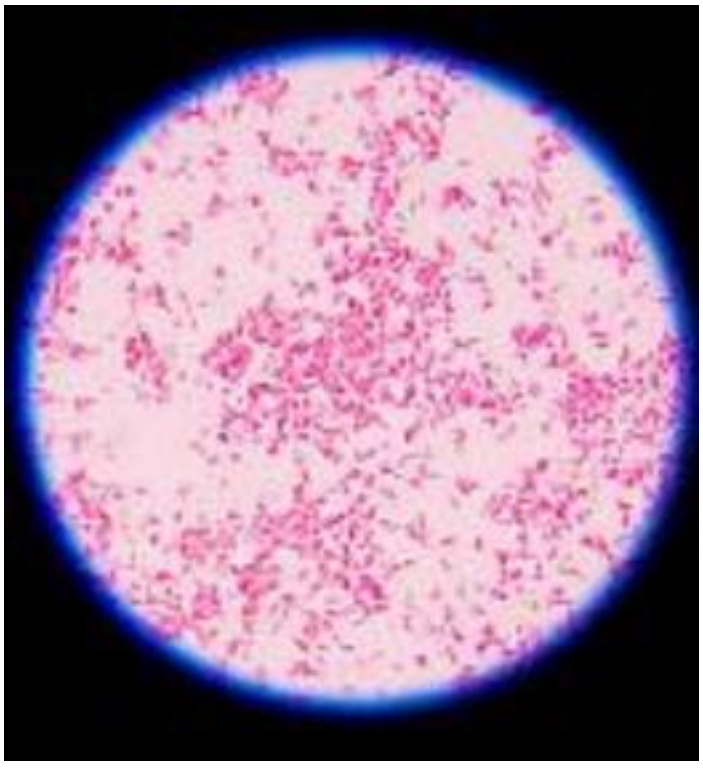
The resistant pattern of different antibiotics were compared. Cefoperazone sulbactam and Levofloxacin showed higher resistant in MBL producer which were 65.52% and 66.67% respectively. Aztreonam was least resistant which was found to be 33.3% resistant in MBL producer bacterial isolates. Following tables shows the distribution of resistant pattern of different antibiotics with MBL producer and non MBL producer.

Table No. 5: Table representing comparison of different resistant patterns of antibiotics of MBL and Non-MBL *P. aeruginosa*

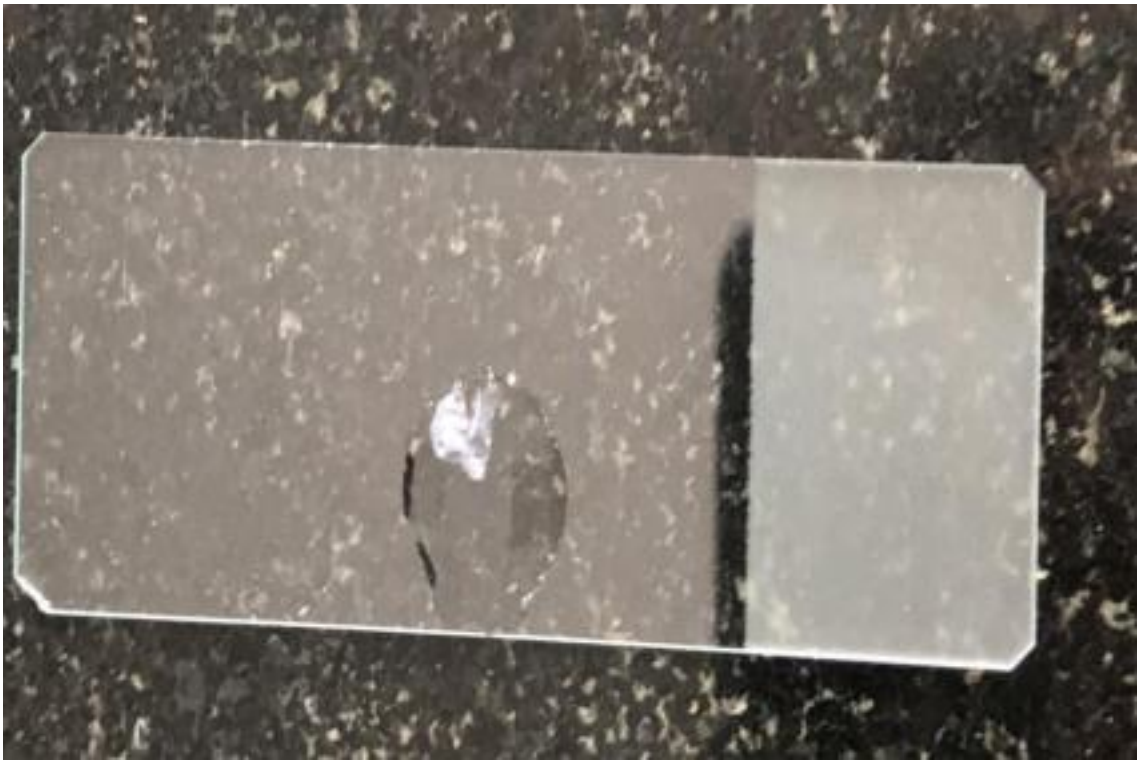
	Resistant to		**Resistant to**		
Antibiotics	**MBL**	**%**	**Non Mbl**	**%**	**Total**
Amikacin(30mcg)	14	36.84	24	63.16	38
Aztreonam(30mcg)	7	33.33	14	66.67	21
Cefepime(30mcg)	19	51.35	18	48.65	37
Cefoperazone sulbactam(75/10mcg)	19	65.52	10	34.48	29
Ceftazidime(30mcg)	20	48.78	21	51.22	41
Meropenem(10mcg)	21	80.77	5	19.23	26
Levofloxacin(5mcg)	10	66.67	5	33.33	15
Piperacillin Tazobactam(100/10mcg)	11	32.35	23	67.65	34
Nitrofurantion(300mcg) (ForUrine Isolates)	21	56.76	16	43.24	37
Norfloxacin(10mcg) (For urine isolates)	10	66.67	5	33.33	15
Azithromycin(15mcg) (for sputum isolates)	13	61.9	8	38.10	21



Photograph 1: *Pseudomonas aeruginosa* colony on NA.

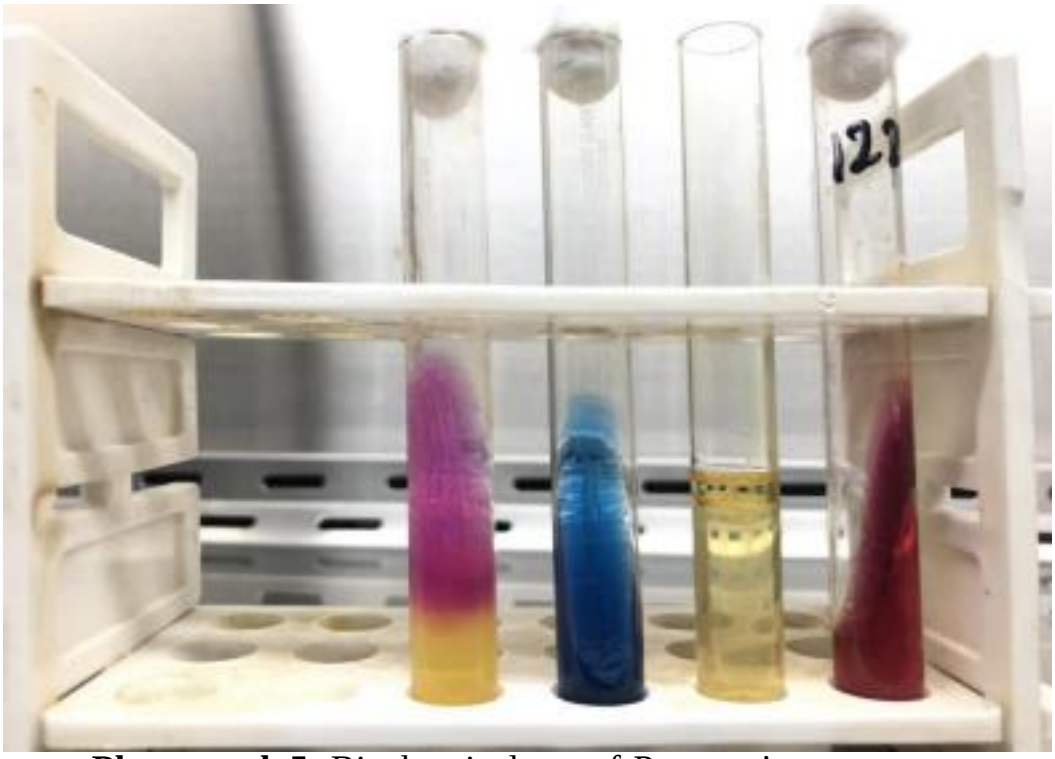


Photograph 2: Showing gram negative rods microscope

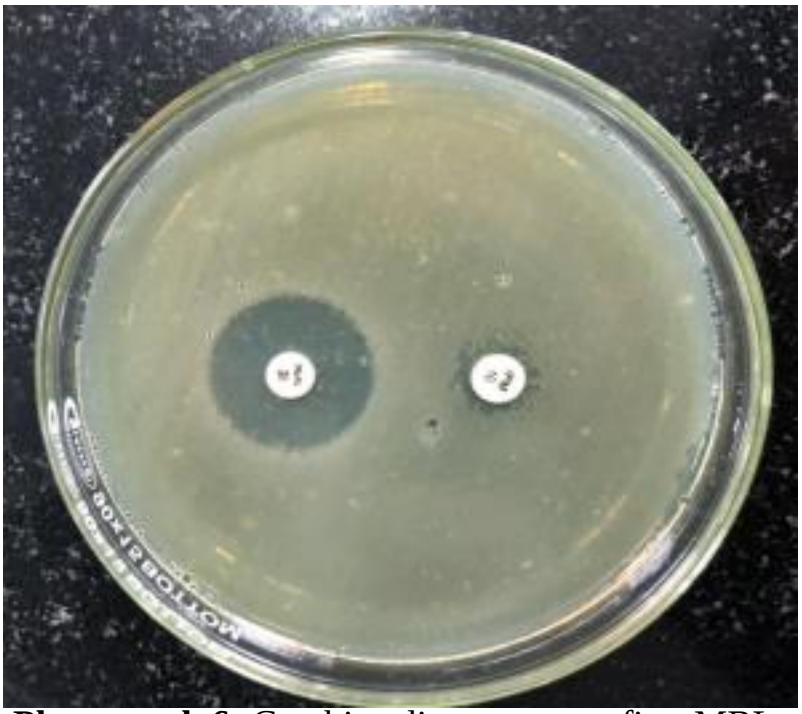


Photograph 3: Catalase test

Photograph 4: Oxidase test



Photograph 5: Biochemical test of *P. aeruginosa*



Photograph 6: Combine disc test to confirm MBL producer

CHAPTER V

DISCUSSION

A total of 1500 samples were processed in about six months. All types of clinical samples were accepted and processed. About 126 (8.2%) of *P. aeruginosa* were isolated from different samples. Antibiotic testing showed 34% Multidrug resistant among the bacterial isolates. About 16% of isolates were detected as MBL producer by combined disc method where the positive isolates showed Imipenem impregned with EDTA having greater than 5mm of zone of inhibition form the imipenem.

P. aeruginosa is one of the leading causes of infection in the hospital settings. With the presence of different virulence factor and ability to produce biofilm and quorum sensing, it has ability to colonize the prosthetic devices in hospitals. Through these devices and other sources, it has successfully established as one of the predominant nosocomial infection causing agent. A bigger threat comes when the organism comes to be the metallo beta lactamase producer just narrowing the use of antibiotics for the treatment. So it is important to screen the metallo beta lactamase producing *P. aeruginosa* in these settings.

MBL producing organisms are linked with the high mortality and morbidity in hospital settings. Treating these organism has always been a problem in hospital settings. Moreover, it is a problem for infection control management to control the spreading of these isolates that might cause a disaster in therapeutic administration. MBL often have capacity to hydrolyze most of the classes of beta lactam drugs, choice of antibiotics for the treatment is not more. There are still no mechanism or drugs to alter or cleave the metallo beta lactamase enzymes their spread in the environment is a challenging phase to achieve.

Different types of methods are there for the detection of MBL producers in laboratory. Phenotypically MBL can be detected by combined disc method by using imipenem and imipenem with EDTA, double disc synergy test by using imipenem and EDTA, E test, modified Hodge test and PCR. PCR can detected the different gene responsible for causing MBL.

In this study, the prevalence of *P. aeruginosa* was found to be 8.6 % from all clinical isolates. There are similar cases of prevalence from the studies performed before in Nepal. Studies done by(Maharjan, 2022) shows more or less similar prevalence with the study above which is 6.4%. Similarly, the study done by (Chand et al., 2021) shows some lower prevalence of 4.6%. As these studies were from earlier time period, increase in prevalence in our study might suggest that the *P. aeruginosa* infection might have been increasing over the year. Similarly, the number of samples and prevalence in the area of study in those study might also play a factor in variable results with our study.

A study done in Nepal by (Pathak et al., 2017.) shows the 14.2% of infection by *P. aeruginosa.*(Mahaseth et al., 2020a), (Maharjan et al., 2020)(Ansari, Aryal, et al., 2021), (Raza et al., 2018) found the prevalence of *P. aeruginosa* as 11.29%, 16.2% and 17% respectively in their study done in different year. A study done in Iraq by(Al-Khudhairy & Al-Shammari, 2020), study done in India by (Rajput et al., 2012) shows the prevalence of 12.4% and 14% which is similar to the study done by , (Maharjan et al., 2020)(Ansari, Aryal, et al., 2021), (Raza et al., 2018). A higher prevalence of *P.aeruginoa* is found by (Kunwar et al., 2021) of 21.8% in Nepal. These studies show higher prevalence of *P. aeruginosa* than our study. This might be due to the longer study period, larger volume of sample size and higher prevalence in that study area. Increase in prevalence might also be due to the higher infection ratio in those area in past.

Similarly, infection is found more in Urine and Sputum sample viz. 44 (34.9%)and 40 (31.7%)respectively. 24(19%) isolates were found in Blood samples and 6(4.8%) samples from wound swab was isolated. Least was found in pus sample of 0.8%. Prevalence of infection in Urine and sputum sample might be higher compared to other sample may be due to the frequently contact to those area with environment. In hospitals, patients might have to put catheters or who have to intubated might acquire the infection through those devices via nosocomial infection. The study also show that more male population are prone to the infection than females.61.1% of Male are found to be infected by *P. aeruginosa* whereas 38.9% of female are found to be infected. This study is similar to the study done by (Chand et al., 2020). (Chand et al., 2020) also found the male population are more susceptible to infection. About 64.36% of male

are found to be infection in their study which is more or less similar with our study. Males may be more susceptible to infection since they work outside on a regular basis and are more likely to come in contact with infected environment.

In this study, *P. aeruginosa* infection is more prevalent more in age group above 70. The number of isolates in age group above 70 is about 42(33.3%), from age group 60-70 the isolates are 24(19%), 20(15.9%) from age group of 50-60 and least isolates 5 (4%) were obtained from age group 20- 30. The data from this study suggests that old age group are more susceptible in causing the infection of *P. aeruginosa*. This may be due to old age people are more often immunocompromised compared to other age groups. Most of the old aged people suffers from chronic obstructive pulmonary disease and has to be hospitalized. On the other hand, old age people might not be able to pay proper attention to hygiene. This may make them frequent contact with the contaminated environment. This might be another reason on acquiring the *P. aeruginosa* infection. Moreover, the catheter associated infection are also common in old age.

In this study, Levofloxacin (80%) is found to be more sensitive, followed by Meropenem (79.4%) and Piperacillin Tazobactam (73%). The least sensitive antibiotics is Nitrofurantoin with susceptible percent of 9.8%. Azithromycin (51.2%) is also less sensitive compared to other antibiotics. Ceftazidime, a third-generation cephalosporin, is found susceptible to only 66% of isolates which is of the potent drug used for the treatment of *P. aeruginosa*. The result of the study done by (Shrestha et al., 2019) also displays the increase resistant in Ceftazidime. In their study, 75% of Ceftazidime is found to be resistant. Decrease in sensitivity patterns may indicate that the organism might have develop resistant mechanism with these therapies resulted by overuse of these antibiotics in treatment. However, (Ansari, Aryal, et al., 2021)and (Shrestha et al., 2019) shows Amikacin as most effective drug and (Acharya et al., 2017) found Aztreonam to be more effective which are contract to our study.

In our study 34.1 % is found to be Multidrug resistant isolates and 7.9% are extremely drug resistant isolates. This result is similar to the study done by (Shrestha et al., 2019) and (Maharjan, 2022), where they found the MDR

isolates to be 32% and 31% respectively. On the other hand, study done by (Mahaseth et al., 2020a) and (Chand et al., 2020) found higher multidrug resistant isolates which are 42% and 43% respectively.

Metallo-β lactamase producing *Pseudomonasaeruginosa* is found to be 16.6 % in our study. This data is similar to the study done by (Maharjan et al., 2020) and (Maharjan et al., 2019). They show MBL prevalence by 16% and 17% respectively. A lower prevalence data is shown by the study done by (Maharjan, 2022). They found 8.82% MBL producer *P. aeruginosa* in their study. A similar data was found by (Rajput et al., 2012) with 11% of MBL *P. aeruginosa*. This suggests that the MBL producing *P. aeruginosa* is increasing and is a matter of concern. Nonetheless, higher prevalence has been found in Nepal from 23%-68%. (Pathak et al., 2017.) found 25%, (Mahaseth et al., 2020b) found 28%,(Takahashi et al., 2021) found 39.5%, (Chand et al., 2020) found 43% and (Acharya et al., 2017) found 68.6% of MBL producing *P. aeruginosa* from different studies in Nepal. A study done in Brazil by (Franco et al., 2010) found 32% MBL positive and 55.2% by (Wang & Wang, 2020) in China. MBL producing *P. aeruginosa* has been found prevailed upto 96% of the total *P. aeruginosa* isolates from Iraq as well. A study done by (Saffari et al., 2016) found 144 MBL producing *P. aeruginosa* from 150 isolates of *P. aeruginosa.* These study shows the prevalence of the MBL higher than our study. Low prevalence in our study might be due to the less study period, less positive isolates and covers wide range of clinical isolates. Prevalence of MBL might be less in our study area. However, no previous data has been found about MBL producer *P. aeruginosa* in our study area.

In our study, most of the isolates of MBL were from urine sample. A total of 47.6% positive MBL producer were found in urine, 28.6% samples were found in sputum, 14.3% in blood and 4.8% each in pus and tissue sample. In our study, MBL is more predominant in Urine sample. The prevalence in Urine might be more due to the catheterization in patients for a prolonged period of time.

In present study, Piperacillin-Tazobactam and Aztreonam were found to be more effective for metallo-β lactamase producing *P. aeruginosa*. They are resistant about 32% and 33% only. Amikacin also have less resistance of 36%

in compared to other antibiotics. Higher resistant is found for quinolones; Levofloxacin and Norfloxacin which are about 66% resistant to MBL producer followed by Cefoperazone sulbactam 61%. Our study suggests that Piperacillin-Tazobactam and macrolides like Aztreonam might be somehow useful in MBL *P. aeruginosa*.

Pseudomonas aeruginosa is one of the notorious organisms that cause nosocomial infection. Every year infection cause by *P. aeruginosa* is increasing every year globally. More concern has been because of multidrug resistant isolates causing difficult in antimicrobial therapies. Development of metallo β lactamase isolates has even become a nuisance in the medical field resulting in lower choices and use of more dangerous and side effect drugs. Therefore, implicating a proper way to control these isolates is a major need in medical emergencies.

CHAPTER VI

CONCLUSION AND RECOMMENDATION

6.1 Conclusion:

From processing various clinical samples during the study time period, the prevalence of *Pseudomonas aeruginosa* was found to be 8.2%. More bacterial isolates were found in Urine sample(34.9%)followed by sputum(31.7%) while least bacterial isolates were isolated from pus(1.6%) and tissue(0.8%) samples.Similarly 61.1% of male and 38.9% of female patients were found to be infected with *P. aeruginosa.* Likewise, age group of more than 70 years had higher infection percentage of 33.3%, age group of 60-70 years had 19.04% and 20-30 years of age group had 3.9% infection percentage. Among the isolated bacteria, on performing AST, Levofloxacin, Piperacillin Tazobactam and Meropenem were more sensitive with 82.4%, 79.4% and 73% respectively and Nitrofurantoin had more resistant 90.2% followed by Azithromycin with 48.8%. Multidrug resistant bacteria was found to be 34.1% and extreme drug resistant was found to be 7.9%. A total of the metallo beta lactamase producing *P. aeruginosa* was found to be 16.6%. More MBL producing isolates were from ICU(47.6%), 33.3% from general wards and 19.1% isolates were from outpatient. Similarly, on clinical samples, higher MBL producers were from Urine(47.6%), 28.6% from Sputum and least were found in pus and tissue samples(4.8%). From MBL isolates, least resistant antibiotics was Aztreaonam(33.3%) and higher resistant (65.52% and 66.67 %) isolates were resistant to Cefoperazone sulbactam and Levofloxacin respectively.

Antibiotics like Amikacin, Pepiperacillin Tazobactam, Aztreonam might be used to treat the MBL producing isolates. However without antibiotic susceptibility testing, it is not recommended to administer the antibiotics. This prevalence of MBL producer isolates is lower than the previous studies done in different settings. Even our studies show lower prevalence rate, it is still a concern of topic in the hospital isolates. If the MBL isolates are not controlled and surveillance if not done properly it can result in acquiring nosocomial infection in no time. Similarly, practicing hand hygiene, frequently monitoring

the quality of the hospital settings, rooms and devices should be done to ensure the control of the strains. Infection by *P. aeruginosa* can cause a medical threat. A multidrug resistant isolates can even be more difficult to treat. Increased MBL isolates has been a nuisance in hospital settings. Therefore, routine evaluation should be done to ensure the control of these isolates.

6.2 Recommendation

From this study, following suggestions could be done for better

- MIC could be done for confirming right dosage amount for antibiotic administration.
- Similary, molecular techniques can be done to detect the prevalence of specific gene responsible for metallo beta lactam producer.

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APPENDICES

APPENDIX-A

LIST OF EQUIPMENT AND MATERIALS USED DURING THIS STUDY

1. EQUIPMENTS

Autoclave Hot air oven Incubator Weighing Machine Refrigerator Microscope Centrifuge Distilled waterBunsen burner

2. MICROBIOLOGICAL MEDIA

Mueller Hinton broth (Hi-Media) MacConkey agar (Hi-Media) MR-VP medium (Hi-Media) Triple Sugar Iron agar (Hi Media) Simmons Citrate Agar (Hi-Media) Nutrient agar (Hi-Media) Sulphur Indole Motility agar (Hi-Media)

3. CHEMICALS AND REAGENTS

3% Hydrogen peroxide Barrit's reagent Crystal violet Kovac's Reagent Gram's Iodine Barium chloride Absolute 95% alcohol Sulphuric acid Safranin Oxidase reagent

4. ANTIBIOTICS DISCS

· Amikacin (30µg)

· Azthromycin (30µg)

· Cefoperazone/Subactam (30µg)

· Aztreonam (30µg)

· Levofloxacin (5µg)

· Meropenem (10µg)

· Nitrofurantoin (300µg)

Norfloxacin

· Piperacillin Tazobactam (100/10µg)

5.MISCELLANEOUS

Conical flasks, Cotton, Distilled water, Droppers, Forceps, Glass slides and coverslips, Immersion oil, Inoculating loop, Inoculating wire, Lysol, Measuring cylinder, Petri dishes, Pipettes, Plastic containers, Spatula, Test tubes, Wooden applicator sticks.

APPENDIX B

Sample size

Sample size will be calculated using the

formula: $N = Z^2P\ (1-P)/d^2$ where , n= 200

N= Sample size

Z = Static level for confidence of 95% which value corresponds to 1.96 p = percentage picking a choice, expressed as decimal (16%) d = precision = 0.05

Sample size = 200 (Odari and Dawadi 2022)

APPENDIX-C

COMPOSITION AND PREPARATION OF DIFFERENT CULTURE MEDIA

The culture media used were from HI-Media Laboratories Pvt. Ltd, Bombay, India.

All compositions are given in grams per liters and at 25℃ temperature.

1. Cystine Lactose Electrolyte Deficient Agar

Ingredients gm/liter

Peptone 4.0

Lactose 10.0

Tryptone 4.0

Beef Extract 3.0

L-Cystine 0.128

Bromothymol Blue 0.02

Agar 15.0

Final pH (at 25℃) 7.5 ± 0.2

Direction:36.25 grams of media was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121℃ (15 lbs. pressure) for 15 minutes and poured into sterile Petri plates.

3. MacConkey Agar (MA)

Without sodium taurocholate, without salt and crystal violet

Ingredients gm/liter

Peptone 20.0

Lactose 10.0

Sodium taurocholate 5.0

Sodium chloride 5.0

Neutral Red 0.04

Agar 20.0

Final pH (at 25℃) 7.4 ± 0.2

55 grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121℃ (15 lbs. pressure) for 15 minutes and poured into sterile Petri plates.

4. Mueller Hinton Agar

Ingredients gm/liter

Beef, Infusion form 300.0

Casein Hydrolysate 17.0

Starch 1.5

Agar 17.0

Final pH (at 25℃) 7.4 ± 0.2

Directions: 38 grams of the medium was suspected in 1000 ml distilled water and the medium was warmed to ease the dissolvent completely. It was sterilized by autoclaving at 121℃ (15 lbs. pressure) for 15 minutes and poured into sterile Petri plates.

5. Nutrient Agar

Ingredients gm/liter

Peptone 10.0

Sodium chloride 5.0

Beef extract 10.0

Yeast extract 1.5

Agar 12.0

Final pH (at 25℃) 7.4 ± 0.2

Direction: 37 grams was suspended in 1000 ml distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121℃ (15 lbs. pressure) for 15 minutes and poured into sterile Petri plates.

6. Nutrient Broth

Ingredients gm/liter

Peptone 5.0

Sodium chloride 5.0

Beef 1.5

Yeast extract 1.5

Agar 20.0

Final pH (at 25℃) 7.4 ± 0.2

Direction: 13 grams of the medium was dissolved in 1000 ml distilled water, boiled, and dispensed into small containers. It was then autoclaved at 121℃ (15 lbs. pressure) for 15 minutes.

7. Luria-Bertani broth

Ingredients gm/liter

Tryptone 10.0

Sodium chloride 5

Yeast extract 5

Final pH (at 25℃) 7.0 ± 0.2

Direction: 20 grams of the medium was dissolved in 1000 ml distilled water, boiled, and dispensed into small containers. It was then autoclaved at 121℃ (15 lbs. pressure) for 15 minutes.

2. COMPOSITION AND PREPARATION OFDIFFERENTBIOCHEMICAL MEDIA

A. MR-VP Medium

Ingredients gm/liter

Peptone 5.0

Dextrose 5.0

Dipotassium phosphate 5.0

Final pH (at 25℃) 7.4 ± 0.2

Direction: 15 grams of powder was dissolved in 1000 ml distilled water and mixed well 3 ml of medium was distributed in each test tube and then autoclaved at 121℃ (15 lbs. pressure) for 15 minutes.

B. Sulphide Indole Motility (SIM) Medium

Ingredients gm/liter

Tripeptone 20.0

Peptone 6.1

Ferrous ammonium sulphate 0.2

Sodium thiosulphate 0.2

Agar 3.5

Final pH (at 25℃) 7.4 ± 0.2

Directions: 30 grams of the medium was suspended in 1000 ml of distilled water and dissolved completely. Then it was distributed in tubes to a depth of about 3 inches and sterilized by autoclaving at 121℃ (15 lbs. pressure) for 15 minutes.

C. Simmons Citrate Agar

Ingredients gm/liter

Magnesium sulphate 0.2

Ammonium dihydrogen phosphate 0.2

Sodium ammonium phosphate 1.0

Sodium citrate, tribasic 2.0

Agar 15.0

Bromothymol blue 0.08

Final pH (at 25℃) 7.4 ± 0.2

Direction: 23 grams medium was dissolved in 1000 ml of distilled water. 3 ml of medium was distributed in each test tube and sterilized by autoclaving at 121℃ (15 lbs. pressure) for 15 minutes. After autoclaving, tubes containing medium were tilted to form a slant.

D.Triple Sugar Iron agar (TSI)

Ingredients gm/lit

Lab-lemco powder 3.0

Yeast extract 3.0

Peptone 20.0

Lactose 10.0

Sucrose 10.0

Glucose 1.0

Ferric citrate 0.3

Sodium chloride 5.0

Sodium thiosulphate 0.3

Phenol red 0.025

Agar 12.0

Final pH (at 25℃) 7.4 ± 0.2

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

E.Urease agar

Ingredients gm/lit Monopotassium phosphate 9.1

Dipotassium phosphate 9.5

Yeast extracts 0.1

Phenol red 0.01 Sterile 40 urea solution 0.005 Final pH (at 25℃) 7.4 ± 0.2

Direction: As directed by the manufacturing company, 1.87 grams of the medium was suspended in 95 ml of distilled water and sterilized by autoclaving at 121℃ for 15 minutes. After cooling to about 55℃, 5 ml of sterile urea solution was added aseptically, mixed well, and distributed 5 ml amount in sterile test tubes.

F.Hugh and Leifson's agar

Ingredients gm/lit

Peptone 2.0

Sodium chloride 5.0

Dipotassium phosphate 0.3

Glucose (Dextrose) 10.0

Bromothymol blue 0.03

Agar 3.0

Final pH (at 25℃) 7.1 ± 0.2

Direction: As directed by the manufacturing company, 9.4 grams of the medium was suspended in 1000 ml of distilled water and then heated to boiling to dissolve the media completely. The medium was distributed in 100 ml amounts and sterilized by autoclaving at 121℃ for 15 minutes. After cooling to about 55℃, 10 ml of sterile dextrose solution was added aseptically, mixed well, and distributed 5 ml amount in sterile test tubes.

3. COMPOSITION AND PREPARATION OF DIFFERENT STAINING AND REAGENTS

For Gram's Stain

a. Crystal Violet Solution:

Ingredients gm/lit Crystal violet 20.0 gm

Ammonium oxalate 9.0 gm Ethanol or Methanol 95 ml

Distilled water D/W to make 1 ml

Direction: In a clean piece of paper, 20 grams of crystal violet was weighed and transferred to a clean brown bottle. Then, 95 ml of ethanol was added and mixed until the dye was completely dissolved. To the mixture, 9 grams of ammonium oxalate dissolved in 20 ml of D/W was added. The final volume was made 1 liter by adding D/W.

b. Lugol's Iodine

Potassium Iodine 20.0 gm

Iodine 10.0 gm

Distilled Water 100 ml

Direction: To 250 ml of D/W, 20 grams of potassium iodide was dissolved. Then 10 grams of iodine were mixed to it until it was dissolved completely. The final volume was made 1 liter by adding D/W.

c. Acetone-Alcohol Decolorizer

Acetone 500 ml

Ethanol (Absolute) 475ml

Distilled water 25 ml

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

d. Safranin (Counter strain)

Safranin 10 g

Distilled Water 1000 ml

Direction: In a clean piece of paper, 10 grams of Safranin was weighed and transferred to a clean bottle. Then 1-liter D/W was added to the bottle and mixed well until safranin dissolved completely.

e. Normal Saline

Sodium chloride 0.85 g

Distilled Water 100 ml

Direction: The sodium chloride was weighed and transferred to a leak proof bottle pre-marked to hold 100 ml. Distilled water was added to the 100 ml jar, and mixed well until the salt was fully dissolved. The bottle was labeled and stored at room temperature.

BIO-CHEMICAL TEST REAGENTS

1. Catalase Reagent (For catalase test)

Hydrogen peroxide 3 ml

Distilled Water 97 ml

Direction: To 97 ml D/W, 3 ml of hydrogen peroxide was added and mixed well.

2.Oxidase Reagent (Impregnated in a Whatman's No. 1 filter paper)

Tetramethyl p-phenylene diamine dihydrochloride TPD 1 gms Distilled Water 100 ml

Direction: This reagent solution was made by dissolving 1 gm of PTD in 100 ml D/W. To that solution, stripes of Whatman's No.1 filter paper were soaked and drained for about 30 seconds. Then these strips were freeze dried and stored in a dark bottle tightly sealed with a screw cap.

3. Kovac's Indole Reagent for Indole Test

Isoamyl alcohol 30 ml

p-dimethyl amino benzaldehyde 2 grams

Directions: In 30 ml of isoamyl alcohol, 2 grams of p-dimethyl amino benzaldehyde was dissolved and transferred to a clean brown bottle. Then to that, 10 ml of conc. HCL was added and mixed well.

4. Methyl Red Solution for Methyl Red test:

Methyl Red	0.05 g
Ethyl alcohol	28 ml
Distilled Water	22 ml

Direction: To 28 ml ethanol, 0.05 gm of methyl red was dissolved and transferred to a clean brown bottle. Then 22 ml D/W was added to that bottle and mixed well.

5. Barritt's Reagent for Vogues-Proskauer Test:

a. Solution A

α napthal	0.5 g
Ethyl alcohol absolute	100 ml

Direction: To 25 ml ethanol, 5 g of α-naphthol was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding D/W.

b. Solution B

Potassium hydroxide 40.0 g Distilled Water 1000 ml

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

Gram-Staining Procedure

First devised by Hans Christian Gram during the late 19th century, the Gram stain can effectively divide all bacterial species into two large groups: those that take up the basic dye, crystal violet (Gram-positive) and those that allow the crystal dye to was out easily with the decolorize alcohol or acetone (Gram negative). The following steps are involved in Gram-stain:

Bacteria:	*Pseudomonas aeruginosa*
Gram's Stain:	Gram Negative Rods
CLED	Non lactose Fermenting Colony
Mac Conkey	Non Lactose Fermenting Colony
Nutrient Agar	Green Colour Colony
Catalase Test	Positive
Oxidase Test	Positive
Triple Sugar Iron	Alkali/ No change (R/R)
Sulphur Indole Motility	Indole Negative, Microaerophilic
Citrate	Citrate Utilized in blue colour
Urease	Positive, Change into pink colour

1. A thin film of the material to be examined was prepared and dried.
2. The material on the slide was heat fixed and allowed to cool before staining.
3. The slide was flooded with crystal violet stain and allowed to remain dry for 1 minute.
4. The slide was rinsed with tap water, shaking off excess.
5. The slide was flooded with iodine solution and allowed to remain on the surface without drying for twice as long as the crystal violet was in contact with the slide surface.
6. The slide was rinsed with tap water, shaking off excess.

7. The slide was flooded with alcohol acetone decolorizer for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolorizer. Thicker smear requires more aggressive decolorizing.

8. The slide was flooded with counter- strain safranin for 1 minute and washed off with tap water.

9. The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 100X.

APPENDIX D

METHODOLOGY OF BIOCHEMICAL TESTS USED FOR IDENTIFICATION OF BACTERIA

A. Catalase test

This test is performed to demonstrate the presence of catalase, an enzyme that catalysis the release of oxygen from hydrogen peroxide. During aerobic respiration, in the presence of oxygen, microorganisms produce hydrogen peroxide, which is lethal to the cell itself. The enzyme catalase is present in most cytochrome-containing aerobic and facultative anaerobic bacteria, the main exception being *Streptococcus* 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 were put on the surface of the slide. The positive test is inhibited by the formation of active bubbling of the oxygen gas. 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.

B. Oxidase test

This test is performed for the detection of cytochrome oxidase in bacteria which catalyzes the transport of electrons between electron donors. In the presence of redox dye Tetramethyl-p-phenylnene Diamine hydrochloride, cytochrome oxidase oxidizes it into a deep purple-colored end product Indophenol which is detected in the test. The test is used for screening species of *Neisseria Alcaligenes,Aeromonas, Vibrio,Campylobacter* and *Pseudomonas* which give positive reactions and for excluding the Enterobacteriaceae, all species of which give negative reactions.

Procedure: A piece of filter paper was soaked with few drops of oxidase reagent (Whattman's No 1filter paper impregnated with 1% tetramethyl phenylene Diamine di hydrochloride). Then the colony of the test organism

was smeared on the filter paper. The positive test is indicated by the appearance of a blue-purple color within 10 seconds.

C. Indole Production Test

This test detects the ability of the organism to produce an enzyme tryptophanase which oxidizes tryptophan to form indolic metabolites: Indole, skatole (methyl Indole) and indole acetic acid. The enzyme tryptophanase catalyzes the deamination reaction attacking the tryptophan molecule in its side chain and leaving the aromatic ring intact in the form of indole.

Procedure: A smooth bacterial colony was stabbed on SIM (Sulphide Indole Motility) medium by a sterile stab wire and the inoculated media was incubated at 37℃ for 24 hours. After 24 hours of incubation, 2-3 drops of Kovac's reagent were added. The appearance of red color on the top of the media indicated indole positive. Indole if present combines with the aldehyde present in the reagent to give a red color in the alcohol layer. The color reaction is based on the presence of the pyrrole structure present in indole.

D. 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. The medium used for citrate fermentation (Simmon's Citrate medium) also contains inorganic ammonium salts. An organism capable of utilizing citrate as its sole carbon source also utilizes the ammonium salts present in the medium as its sole nitrogen source, the ammonium salts are broken down to ammonia with resulting alkalinity.

Procedure: A loop full of test organism was streaked on the slant area of Simmon's Citrate Agar medium and incubated at 37℃ for 24 hours. A positive test was indicated by growth of the organism and the 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. pH 7.6; a blue color develops due to the alkalinity of the medium.

E. Motility test

This test is done to determine if an organism was motile or non-motile. Bacteria are motile using flagella. Flagella occur primarily among the bacilli; however a few cocci forms are motile. Motile bacteria may contain a single flagella. The motility media used for the motility test are semisolid, making motility interpretations macroscopic.

Procedure: The motility of the organism was tested by handling the drop and cultural method. In the cultural method, the test organism was stabbed in the SIM medium and incubated at 37℃ for 48 hours. Motile organisms migrate from the stab line and diffuse into the medium causing turbidity. Whereas non

motile bacteria slow the growth along the stab line, and the surrounding media remains colorless and clear.

F. Triple Sugar Iron(TSI) Agar Test

The TSI agar is used to determine the ability of an organism to utilize specific carbohydrates incorporated in the medium (glucose, sucrose, and lactose in concentrations of 0.1% 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 the determination of possible hydrogen sulfide production (detected by production of black color in the medium). A pH indicator (phenol red) included in the medium can detect acid production from fermentation of these carbohydrates and it gives a yellow reaction at acidic pH, and a red reaction to indicate an alkaline surrounding.

Procedure: The test organism was streaked and stabbed on the surface of TSI and incubated at 37℃ for 24 hours. Acid production limited only to the butt region of the tube is indicative of glucose utilization, while acid production in the slant and butt indicates sucrose or lactose fermentation. The results are interpreted as follows:

· **Yellow (Acid/Yellow (Acid), Gas, H_2S Lactose/Sucrose fermenter, H_2S producer.**

· **Red (Alkaline/Yellow (Acid), No Gas, No H_2S only Glucose, not Lactose/ Sucrose fermenter, not aerogenic, No H_2S production.**

· **Red (Alkaline)/No change Glucose, Lactose, and Sucrose non fermenter.**

· **Yellow(Acid)/No change Glucose-Oxidizer**

· **No change/No change Non-fermenter**

G. Urea Hydrolysis test

This test demonstrates the urease activity present in certain bacteria which decomposes urea, releasing ammonia and carbon dioxide. Ammonia thus produced changes in the color of the indicator (phenol red) incorporated in the medium.

Procedure: The test organism was inoculated in a medium containing urea and the indicator phenol red. The inoculated medium was incubated at 37℃ overnight. Positive organism shows pink red color to the breakdown of urea to ammonia. With the release of ammonia, the medium becomes alkaline as shown by a change of the indicator to pink.

Table: Biochemical changes of *P. aeruginosa*

Bacteria:	*Pseudomonas aeruginosa*
Gram's Stain:	Gram Negative Rods
CLED	Non lactose Fermenting Colony
Mac Conkey	Non Lactose Fermenting Colony
Nutrient Agar	Green Colour Colony
Catalase Test	Positive
Oxidase Test	Positive
Triple Sugar Iron	Alkali/ No change (R/R)
Sulphur Indole Motility	Indole Negative, Microaerophilic
Citrate	Citrate Utilized in blue colour
Urease	Positive, Change into pink colour

GRANDE INTERNATIONAL HOSPITAL

NABH ACCREDITED

July 28, 2024

TO WHOM IT MAY CONCERN

It is to notify that **Mr. Bonny Shrestha** had interned in Microbiology laboratory in Grande International Hospital, Tokha for his thesis internship entitled "**PHENOTYPIC DETECTION OF METALLO BETA LACTAMASE PRODUCING *Pseudomonas aeruginosa*FROM DIFFERENT CLINICAL SAMPLES.**" We would like to notify that our hospital have **ethical approval broad (IRC)** and his study is under ethical review.

Further, the researcher is directed to strictly abide by the guidelines during the implementation of his research proposal and submit a progress report and a full report upon completion.

If you have any questions, please contact the hospital administration.

Thank you,

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