CHAPTER I
INTRODUCTION AND OBJECTIVES

1.1 BACKGROUND

Pseudomonas aeruginosa are widespread throughout nature, inhabiting soil, water, plants and animals including human (Baltimore, 2007). *P. aeruginosa* is also a clinically significant pathogen, often causing infection in immunocompromised and catheterized patients. *Pseudomonas aeruginosa* has become an important cause of infection, especially in patients with compromised host defense mechanisms. It is the most common pathogen isolated from patients who have been hospitalized longer than 1 week, and it is a frequent cause of nosocomial infections (Qarah *et al.,* 2009). Most of the human infections due to these species are opportunistic and occurs among low birth weight infants, older infant, children and adults with impaired host defense, such as those with traumatic wounds, cystic fibrosis, malignancies, extreme burns, malnutrition, primary immunodeficiency and people under immune suppressive therapy (Baltimore, 2007; Ghorashi *et al.*, 2010).

The various infection caused by this organisms are endocarditis, respiratory infections, bacteremia and septicemia, central nervous system infection, bone and joint infection, eye infection, ear infection including external otitis media, urinary tract infection, gastrointestinal infection, skin and soft tissue infection, wound infection pyoderma and dermatidis (Rashid *et al.,* 2007)..

P. aeruginosa is seldom a member of the normal microbial flora in humans. Representative colonization rates for specific sites in humans are 0 to 2% for skin, 0 to 3.3% for the nasal mucosa, 0 to 6.6% for the throat, and 2.6 to 24% for fecal samples (Lister *et al.,* 2009). However, colonization rates may exceed 50% during hospitalization, especially among patients who have experienced trauma to or a breach in cutaneous or mucosal barriers by mechanical ventilation, tracheostomy,

catheters, surgery, or severe burns (Ohara and Itoh, 2003; Thuong *et al.,* 2003). Patients with impaired immunity have higher risks for colonization by this organism (Moya *et al.,*2009) and disruption in the normal microbial flora as a result of antimicrobial therapy has also been shown to increase colonization by *P. aeruginosa* (Takesue *et al.,* 2002; Lister *et al.,* 2009).

Nosocomial infections caused by *P. aeruginosa* are very common; 11-13.8% of all nosocomial infections are due to *P. aeruginosa*, with an even of higher rate (13.2-22.6%) nosocomial infection among an ICU (Driscol *et al.,* 2007). It is the fifth common pathogen among hospital microorganisms which cause hospital acquired infections (Rashid *et al.,* 2007). *P. aeruginosa* was also the second leading cause of nosocomial pneumonia (14 to 16%), third most common cause of urinary tract infections (7 to 11%), fourth most frequently isolated pathogen in surgical site infections (8%), and seventh leading contributor to bloodstream infections (2 to 6%) (Lister *et al.,* 2009).

The beta-lactam antibiotics are the largest and most commonly used group of antibiotics globally. Bacteria often develop resistance to β-lactam antibiotics by synthesizing β- lactamases, an enzyme that attacks the β-lactam ring. There are four classes of beta -lactamases (A–D) based upon DNA sequence similarity. Classes A, C, and D utilize serine on the active site, while the class-B enzymes (MBLs) usually utilize zinc (Ambler, 1980). The updated classification system includes group 1 (class C) cephalosporinases; group 2 (classes A and D) broad-spectrum, inhibitor-resistant beta lactamases, ESBLs and serine carbapenemases; and group 3 (class B) MBLs (Bush *et al.,* 1995). Metallo β-lactamases belong to Amber class B type of β- lactamase (Pitout *et al.,* 2005) and act on a broad spectrum of substrates including penicillins, cephalosporins, and carbapenems (Herzberg and Fitzgerald, 2011). *P. aeruginosa* isolates producing metallo β-lactamases were first reported in Japan in 1991, since then it has been described from various parts of world, including Asia, Europe, Australia, South America and North America (Pitout *et al.,* 2005). In some countries, *P. aeruginosa*

possessing MBLs constitute nearly 20% of all nosocomial isolates (Walsh *et al.*, 2005).

Excessive use of broad spectrum antibiotics in hospitals has led to the emergence of resistance strain of *P. aeruginosa*. To reduce the selection pressure for resistance, it is important to determine antibiotic susceptibility pattern of organism which helps in appropriate treatment with narrow spectrum and target specific antibiotics (Aoki *et al.,* 2004). Carbapenems have been the predominant antibiotic class for treatment of *P. aeruginosa* infection because of their stability against most β-lactamases. However, metallo β-lactamases able to hydrolyze carbapenem as well and *P. aeruginosa* strains carrying metallo-β-lactamase genes are a recent clinical threat (Aoki *et al.,* 2004). Therefore it is important to know the status of metallo β- lactamase producing *P. aeruginosa* in our hospital to prevent the transmission of resistant bacteria. Antibiotic susceptibility pattern and reporting of MBL producing organism would help in appropriate selection of treatment regimes and helps to decrease the overuse and misuse of antibiotics. The purpose of this present is to find the prevalence of *P. aeruginosa* in different samples along with their antimicrobial susceptibility profile, find out the burden of MDR among *P. aeruginosa* and the production of metallo β- lactamases among those isolates.

1.1 Objectives

1.1.1 General objective

To assess metallo β-lactamase producing *Pseudomonas aeruginosa* from different clinical samples and to know their antibiotic susceptibility pattern.

1.1.2 Specific objectives

a) To assess the prevalence of *P. aeruginosa* in different clinical samples.
b) To find out the burden of MDR among *P. aeruginosa*.
c) To detect metello β-lactamase producing *P. aeruginosa*
d) To assess the antibiotic susceptibility pattern of isolates..

CHAPTER -II
LITERATURE REVIEW

2.1 *Pseudomons aeruginosa*

Bergey's Manual of Systematic Bacteriology (2005) has classified *Pseudomonas aeruginosa* in the phylum Protobacteria and class Gamma Protobacteria. *Pseudomonas* is the most important genus in the order *Pseudomonadales* and the family *Pseudomonaceae*. It was first isolated by a French military surgeon Sedillot in 1850 from a blue-green discharge on surgical dressing with associated infections (Lister *et al.,* 2009). Typical biochemical features of *P. aeruginosa* isolates are: positive oxidase test, growth at 42 °C, hydrolysis of arginine and gelatine, and nitrate reduction. *P. aeruginosa* strains produce two types of soluble pigments, pyoverdin and pyocyanin (Gilligan, 1991). The latter blue pigment is produced abundantly in media of low-iron content and functions in iron metabolism in the bacterium. Pyocyanin (from "pyocyaneus") refers to "blue pus", which is characteristic for suppurative infections caused by *P. aeruginosa* (Gilligan, 1991).

P. aeruginosa normally lives in moist environments, and uses a wide range of organic compounds for growth, thus giving it an exceptional ability to colonize in different ecological condition where nutrients are limited, from water and soil to plant and animal tissues (Lister *et al.*, 2009). The ability of *P. aeruginosa* to survive on minimal nutritional requirements and to tolerate a variety of physical conditions has allowed this organism to persist in both community and hospital setting. In the hospital, *P. aeruginosa* can be isolated from a variety of sources, including respiratory therapy equipment, antiseptics, soap, sinks, mops, medicines, and physiotherapy and hydrotherapy pools (Poirel *et al.*, 2001). Isolation of *P. aeruginosa* from healthy carriers or environmental sites is significant only when there is significant risk of transfer to immunocompromised patients, e.g. by nurses' hands or via respirators. Normally, human fecal carriage rate for *P. aeruginosa* is less than 10%; however, carrier rate increases with the

increase in the length of hospital stay of the patient, reaching upto 30% after 3 weeks (Anzai *et al.,* 2000). This represents a distinct risk of endogenous infection.

P. aeruginosa mainly is an opportunistic pathogen. Infections due to *P. aeruginosa* are seldom encountered in healthy adults (Bonomo and Szabo, 2006). Infection begins with some alteration in the host immune defense system. The bacterium does not infect uncompromised tissue. But there is hardly any tissue that organism cannot infects if the host immune system is suppressed in some manner (Rashid *et al.,* 2007). In the last few decades the organism has become increasingly recognized as the aetiological agent in a variety of serious infections in hospitalized patients with impaired immune defenses (Bonomo and Szabo, 2006). *P. aeruginosa* is one of the main organisms responsible for drug-resistant nosocomial infections, and is one of the leading causes of bacteraemia and pneumonia in hospitalized patients. In addition being intrinsically resistant to several antimicrobial agents, *P. aeruginosa* can easily develop resistance to all conventional antipseudomonal antibiotics via different mechanisms (Farrel *et al.,* 2007). The resistance to antibiotics by this organism is govern by the antibiotic resistant plasmid (R factors) contained in them, which are further passed on genetically to the next generation (Bagde *et al.,* 2012). These natural properties of the bacterium contributed to its success as an opportunistic pathogen.

2.2 Characteristic of *P. aeruginosa*

Pseudomonas aeruginosa is a gram negative, uniformly stained, straight or slightly curved rods, measuring 0.5 to 1.0µm by 1.5 to 5.0µm in length. They are aerobic, nonspore forming, motile by one or more polar flagella. They are either incapable of utilizing carbohydrates as source of energy or degrade them "oxidatively" rather than fermentative pathway without gas formation Few species of *P. aeruginosa* are asaccharolytic (De Freitas and Barth, 2002).

On nutrient agar, colonies are large and pigmented (pyocyanin/Fluorescence) and on 5% sheep blood agar, they produce β-hemolytic, large flat spreading, mucoid,

rough, pigmented colonies. Many strains may produce a fruity, sweaty, musty or grape like odor due to the presence of 2-aminoacetophenone (Moore *et al.*, 2011). On MacConkey agar, they produce non lactose fermenting colonies with green pigmentation. Colonies from respiratory tract infection samples produce large amounts of alginate, an exopolysaccharide consisting of mannuronic and guluronic acids which aids in forming mucoid colonies on agar medium (Ryder *et al.*, 2007).

Biochemically, they are oxidase and catalase positive, motile by means of flagella, grows well at 42˚C. They utilize glucose oxidatively, reduces nitrate to nitrite, gives Methyl red/Voges Proskauer test negative, do not decarboxylate lysine and ornithine, but dihydrolyze agrinine, mannitol not fermented, motile, produce alkaline slant/alkaline butt with no gas and no H_2S in TSI, indole negative, utilize citrate, do not hydrolyze urea, do not produce phenyl pyruvic acid, liquefies gelatin, do not hydrolyze aesculin and utilize acetamide (Cheesbrough, 2000)

Cetrimide agar is a selective and differential medium for the identification of *Pseudomonas aeruginosa*. In this medium, Cetrimide acts as detergent which inhibits most other bacteria and enhances the growth of *P. aeruginosa* (Brown and Lowbury, 1965). Nutrient agar with 0.03% cetrimide allows the growth of *Pseudomonas aeruginosa* with the production of fluorescein if it contains added phosphate and magnesium, while inhibiting the growth of many other bacteria (Goto and Enomoto, 1970). However, some of clinical strains of *P. aeruginosa* do not produce pyocyanin (Finlayson and Brown, 2011).

2.3 Virulence factor

Virulence of *P. aeruginosa* depends on a large number of cell-associated and extracellular factors. The virulence factors play an important pathological role in the colonization, the survival of the bacteria and the invasion of tissues (Khalifa *et al.*, 2011). The importance of these virulence factors for the pathogenesis of

human *P. aeruginosa* infections is dependent on the type of infection (Doring, 1987). The major virulence factors that assist *P. aeruginosa* to colonize and progress into infection are as follows.

2.3.1 Bacterial cell surface virulence factor

Flagella: The *P. aeruginosa* flagellum is an important virulence factor and a potent activator of host immunity (Zhang *et al.*,2007) The single polar flagellum is a polymer composed of flagellin, the product of the *fliC* gene, although a large number of gene products are required for flagellar assembly and function (Dasgupta *et al.*,2003). Flagella are required for adhesion to cells, swimming motility, and biofilm formation (Bucior *et al.,* 2012). Flagella have a critical role in pathogenesis by adhering to epithelial cells through binding with common membrane component glycosphingolipid asialo-GM1 (aGM1) (Feldman *et al.,* 1998). A number of studies have shown that *P. aeruginosa* strains lacking flagella are much less readily phagocytosed by alveolar macrophages and polymorphonuclear leukocytes. Thus, the loss of flagellar expression may represent an adaptive response that allows *P. aeruginosa* to evade detection and clearance by host defense mechanisms during the chronic phase of Cystic Fibrosis lung infection (Zhang *et al.,* 2007).

Pili: *P. aeruginosa* pili are polar, flexible organelles that are composed of a single protein subunit, PilA (Comolli *et al.,* 1999). Type IV pili are polarly localized appendages that undergo reversible assembly and disassembly, allowing the bacteria to move over a solid surface in a process termed twitching motility (Bucior *et al.,* 2012). Like flagella, pili are crucial to the adhesion phase of colonization through binding with glycosphingolipid asialo-GM1 of epithelial cell (Feldman *et al.,* 1998).

Liopopolysaccharides (LPS): LPS contains a hydrophobic domain, Lipid A, inserted into the phospholipid bilayer to a hydrophobic tail composed of core polysaccharide and the O- specific polysaccharide The O-antigen portion of the *P.*

aeruginosa LPS is responsible for conferring serogroup specificity, which is defined by antibodies specific to the different variants of this antigen (Pier, 2007). The presence of O antigen is critical for bacterial virulence. O-antigen-deficient mutants appear to be more attenuated than wild-type organisms. They disseminate less readily from the initial site of infection, are more serum sensitive than wild-type strain and also exhibit decreased adherence to epithelial cells (Ivanov *et al.*, 2011)

2.3.2 Secreted virulence factor

Pyocynin: Pyocyanin is a blue redox-active secondary metabolite that is produced by *Pseudomonas aeruginosa* (Lau *et al.*, 2004). Pyocyanin interferes with the regulation of ion transport, ciliary beat frequency, and mucus secretion in airway epithelial cells by altering the cytosolic concentration of calcium. It may interact with endothelium-derived relaxing factor or with nitric oxide (which plays a central role in the control of blood pressure, blood flow, and immune function) through the formation of a complex, or it may act by inhibition of nitric oxide synthase (Mavrodi *et al.*, 2001). Pyocyanin can also stimulate alveolar macrophages to produce two neutrophil chemotaxins, IL-8 and leukotriene B_4, that attract neutrophils into airways, causing an inflammatory response and neutrophil-mediated tissue damage (Mavrodi *et al.*, 2001).

Pyoverdine: Pyoverdines are a group of structurally related siderophores produced by *P. aeruginosa*. The siderophore is a primary virulence factor of the human pathogenic strain, acting as the primary iron source. In addition to its role in nutrition, pyoverdine is implicated in biofilm control, cell-to-cell communication, and virulence regulation (Visca *et al.*, 2007). By a mechanism known as surface signaling, pyoverdine tunes the expression of virulence factors, including exotoxin A, exoprotease PrpL, and pyoverdine itself (Imperi *et al.*, 2009).

Alkaline protease: The alkaline protease of *Pseudomonas aeruginosa* is associated with bacterial virulence and is known to interfere with complement-mediated lysis of bacteria by interfering with complement activation and complement-dependent neutrophil functions. Alkaline protease potently blocked phagocytosis and killing of *Pseudomonas* by human neutrophils (Laarman *et al.*, 2012).

Exotoxin A: Exotoxin A binds to a specific receptor on host cells, allowing endocytosis of the toxin. Once in endosomes, the exotoxin can be processed. The toxin then blocks protein synthesis by ADP ribosylation of elongation factor 2, thereby triggering cell death (Guyot *et al.,* 2009).

Phospholipase c: Phospholipase c is selectively toxic to endothelial cells through its ability to bind a cell receptor. It causes vascular lesions and inhibits the healing of wounds during septic infection (Vasil *et al.,* 2009)

Biofilm: Growth of *P. aeruginosa* begins in the form of microcolonies, which later coalesce together to form biofilms. Alginate, which is an acetylated polymer of beta- D -mannouronic acid and alpha-L-guluronic acids, is the most important component of *P. aeruginosa* biofilms. However, some other exopolysaccharides like psl and pel have also been shown to play an important role in biofilm forming ability of non-alginate producing strains of *P. aeruginosa* (Ryder *et al.,* 2007). Biofilms are resistant to antimicrobial agents as well as to host defense mechanisms and hence are difficult to eradicate. Biofilms contribute towards pathogenicity of *P. aeruginosa* as these often lead to persistent and recurrent infections (Mittal *et al.,* 2009).Once an opportunistic pathogen like *P. aeruginosa* enters the host, its ability to cause infection has been correlated with its tendency to form biofilms (Hall-Stoodley and Stoodley, 2005).

Quorum-sensing: In *Pseudomonas aeruginosa*, it have been reported that the organism monitor their cell density as well as expression of virulence factors through chemical signals. These signals known as quorum-sensing signals are

mainly operative through autoinducers generally acylhomoserine Lactones. In *P. aeruginosa* two types of quorum-sensing systems, *las* and *rhl* have been reported (Mittal *et al.,* 2009). Quorum sensing in *P. aeruginosa* is involved in regulating expression of a number of virulence factors, and as such, this regulation is believed to play an important role in the pathogenicity of this organism (De Kievit and Iglewski, 2000).

2.4 Pathogenicity of *P. aeruginosa*

Pseudomonas aeruginosa is responsible for severe nosocomial infections, life-threatening infections in immunocompromised persons, and chronic infections in cystic fibrosis patients (Khalifa *et al.,* 2011). The bacterium does not infect uncompromised tissue. But there is hardly any tissue that organism cannot infects if the host immune system is suppressed in some manner (Rashid *et al.,* 2007). To cause infection, *P. aeruginosa* should first overcome the primary mechanisms of defense including the cell elements and humoral factors of the skin, as well as non-specific responses-phagocytosis, inflammation, acute phase response (Andonova and Urumova, 2013). The pathogenesis of *P. aeruginosa* infection is multifactorial, due to presence of numerous toxins, or virulence factors, it produces and the variety of diseases it causes. *P. aeruginosa* is invasive and toxigenic. Infections appear to occur in stages: bacterial adherence, colonization, invasion and dissemination, and systemic or toxemic disease (Vasil, 1986).

Adherence of *P. aeruginosa* is mediated by flagella and pili. They bind with the specific glycosphingolipid asialo-GM1 component of the host epithelial cell surface (Feldman *et al.,* 1998). Colonization by adherence to the surface may be aided by the production of proteases enzymes that degrades mucin in order to expose the receptors in the cell surfaces. Mucin degradation by bacterial proteases may also be necessary for bacteria to use mucin as a nutrient source (Aristoteli and Willcox, 2003). Tissue injury may also play role in the colonization of organism, since *P. aeruginosa* will adhere to tracheal epithelial cells of mice

infected with influenza virus but not to normal tracheal epithelium (Ramphal *et al.,* 1980).

The ability of *P. aeruginosa* to invade tissue depends upon production of extracellular enzymes and toxins that break down physical barriers and damage host cells, as well as resistance to phagocytes and the host immune defences (Todar, 2013). Exotoxin A is produced by most *P. aeruginosa* strains that cause clinical infections. Like diphtheria toxin, *P. aeruginosa* exotoxin A catalyzes ADP-ribosylation and inactivation of elongation factor 2, leading to inhibition of protein biosynthesis and cell death. Exotoxin A is responsible for local tissue damage, bacterial invasion and immunosuppression(Van and Iglewski, 1998). Phospholipase C is a thermolabile haemolysin. The pathogenic role of exoenzyme S is attributable to the disruption of normal cytoskeletal organization, the destruction of immunoglobulin G and A, leads to depolymerization of actin filaments and contributes to the resistance to macrophages (Khalifa *et al.,* 2011). Bacterial invasion might result in desquamation of infected cells, intracellular survival of bacteria without killing the host cells, and host cell death through bacteria-mediated cytotoxicity (Byfield *et al.,* 2011).

2.5 Antibiotic resistance in *P. aeruginosa*

Pseudomonas aeruginosa is intrinsically resistant to many structurally unrelated antimicrobial agents (Mesaros *et al.,* 2007). *P. aeruginosa* presents a serious therapeutic challenge for treatment of both community-acquired and nosocomial infections, and selection of the appropriate antibiotic to initiate therapy is essential to optimizing the clinical outcome (Micek *et al.,* 2005). Unfortunately, selection of the most appropriate antibiotic is complicated by the ability of *P. aeruginosa* to develop resistance to multiple classes of antibacterial agents, even during the course of treating an infection (Lister *et al.,* 2009). This is primarily due to active efflux systems that are present in all wild type strains, its membrane permeability properties, mutation in chromosomal genes which regulate resistance genes and acquire additional resistance genes from other organisms via plasmids,

transposons and bacteriophages (Lambert, 2002; Livermore, 2002). With 5570 open reading frames, the *P. aeruginosa* genome is among the largest genomes in the prokaryotic world, and encodes an unusually high proportion of proteins involved in regulation, transport and virulence functions, which may explain the high versatility and adaptive capacity of this species (Mesaros *et al.,* 2007). Antimicrobial resistance in *P. aeruginosa* lies on one hand in the extraordinary capacity of this microorganism to develop resistance to almost any available antibiotic by the selection of mutations in chromosomal genes and, on the other, in the increasing prevalence of transferable resistance determinants, particularly those encoding class B carbapenemases (or metallo-β- lactamases [MBLs]) (Gutierrez *et al.,* 2007)

2.6 Multidrug resistant *Pseudomonas aeruginosa*

Different definitions regarding Multidrug resistant are used in different publications. According to CDC, 2006 Multidrug resistant is defined as resistant to two or more classes of antimicrobial agents. Rs to antimicrobials of esistance the therapeutic management of creat complicatation in different classes has considered multidrug resistant if they show resistance to three einfections and ar or more routinely used antibiotics (Daniel*et al.,* .(2001 The multidrug resistance in *Pseudomonas aeruginosa* is defined as resistance to at least three drugs from a variety of antibiotic classes, mainly aminoglycosides, antipseudomonal penicillins, cephalosporins, carbapenems and fluoroquinolones (Hirsch and Tam, 2010)

A multidrug-resistant phenotype of *P. aeruginosa* can arise through the acquisition of multiple imported resistance mechanisms on mobile genetic elements, a combination of imported and chromosomally encoded resistance mechanisms, accumulation of multiple chromosomal changes over time, and/or a single mutational event leading to the over expression of multidrug resistance mechanism, i.e., efflux pump (Lister *et al.,*2009). Other resistance mechanisms in *P. aeruginosa* include enzyme production and target mutations. Expression of

aminoglycoside-modifying enzymes (acetyltransferases, nucleotidyltransferases and phosphotransferases), mediates aminoglycoside resistance (Kotra *et al.,* 2000). Mutations in DNA gyrase and topoisomerase IV (encoded by the *gyrA* and *parC* genes), mediate resistance to the fluoroquinolones (Jalal *et al.,* 2000). Furthermore, a wide variety of β-lactamases are produced by *P. aeruginosa*. PSE-1 and PSE-4 are those most frequently acquired but confer resistance only to the penicillins; activity of the antipseudomonal cephalosporins, carbapenems and monobactam is retained. The overexpression of chromosomally encoded cephalosporinase, AmpC, is prevalent in *P. aeruginosa* (Tam *et al.,* 2007). Broad spectrum β-lactam resistance attributed to β-lactamases is generally due to the metallo-β-lactamases (MBLs): IMP, VIM, SPM and GIM (Bonomo *et al.,* 2006). These MBLs hydrolyze antipseudomonal cephalosporins and carbapenems effectively and their activity is not suppressed by the β-lactamase inhibitors that are currently commercially available (Hirsch and Tam, 2010). The carriage of these genes on plasmids is responsible for their worldwide dissemination and transfer amongst various genera and species (Galimand *et al.,* 2005).

2.7 β - lactams and β- lactamases

2.7.1 β - lactams

β-lactam antibiotics are the most commonly used antibiotics accounting approximately 50% of global antibiotic consumption (Livermore, 1998). These antibiotics act by inhibiting a set of transpeptidase enzymes (also called penicillin binding proteins or PBPs) that are essential for the synthesis of the peptidoglycan layer of the bacterial cell wall (Sauvage *et al.,* 2008). The inhibition of peptidoglycan synthesis results in the death of growing bacteria and accounts for the antimicrobial effect of β-lactam antibiotics (Palzkill, 2013; Moya2009 ,.et al). β-lactam antibioticslactam -βmembered -includes the penicillins, where the four membered sulfur ring system, and the cephalosporins, -ring, is fused to a five containing ring expanded system. Some -lactam is fused to sulfur-βwhere the roup antibiotics include Carbapenems; lactam g-βother important members of the monocyclic lactam antibiotics, Monobactams; the-βcomprising a family of fused

Walsh) lactam antibiotics-β*et al.*, lactam -βowever, the clinical utility of H .(2003 at due toantibiotics is under threresistance arising via (i) modification of the normal penicillin-binding proteins (PBPs), (ii) bypassing of the normal PBPs, (iii) impermeability of the Gram-negative organism outer membrane and (iv) production of β- lactamases (Livermore, 1998).

Carbapenems are important therapeutic agents for the treatment of infection with multidrug-resistant gram negative bacteria, particularly those carrying genes for extended-spectrum and derepressed AmpC β -lactamases (Peleg *et al.,* 2005). Of the lactams, carbapenems have the broadest spectrum and greatest stability against hydrolysis by β-lactamases. However, the clinical utility of these antimicrobials is under threat with the emergence of acquired genes for carbapenemases, particularly those coding for metallo-b-lactamases (Peleg *et al.,* 2005).

2.7.2 β - lactamases

Lactamases are a heterogenous group of proteins with structural similarities, -β pleated sheets and are the members of a superfamily -βhelices and -αcomposed of of active site serine proteases (Knox*et al.,*.(1998 Plasmids are responsible for the spread of most of the β-lactamases, but the genes encoding these enzymes may also be located on the bacterial chromosome Jacoby)*et al.,* .(2005 Some of the enzyme genes are on transposons, facilitating dissemination among different plasmids and organisms Livermore), .(1995 Genes for many of the new β-lactamases are found in integrons, which often include genes conferring resistance to other antibiotics Jacoby)*et al.,* .(2005

2.7.3 Classification β – lactamases

Lactamases, many -βBecause of the diversity of the enzymatic characteristics of attempts have been made to categorize these enzymes by using several attributes like their hydrolytic spectrum, susceptibility to inhibitors, and whether they are classification schemes the most frequently .encoded by plasmids or chromosome

lassification scheme of Ambler proposed in 1980 and functional c Molecular are ,classification scheme proposed by Bush in 1989expanded in 1995Bush, by .ated in 2010 by Bush and Jcobyand upd Medeiros Jacoby and

lactamases into four distinct classes -βThe Ambler classification scheme separates Classes A, C, and D .A,B,C and D based on similarities in amino acid sequence lactamases that require zinc-βare metallo b lactamases, whereas class-βare serine Jacoby classification system its activity (Ambler, 1980). Bush and for ion inhibitor -lactamases according to functional similarities i.e. substrate-βclassifies .profilesThe updated system includes group 1 (class C) cephalosporinases; group 2 (classes A and D) broad-spectrum, inhibitor-resistant, and extended-spectrum β-lactamases and serine carbapenemases; and group 3 metallo-β-lactamases (Bush .(and Jcoby, 2010

Group 1 enzymes are cephalosporinases belonging to molecular class C that are encoded on the chromosomes of many *Enterobacteriaceae* and a few other organisms (Jacoby, 2009). They are more active on cephalosporins than benzylpenicillin and are usually resistant to inhibition by clavulanic acid and active on cephamycins, such as cefoxitin. Functional group 2 β-lactamases are serine β- lactamases, including molecular classes A and D, represent the largest group of β-lactamases, due primarily to the increasing identification of ESBLs. Group 3 are Metallo-β-lactamases (MBLs). They are unique group of β-lactamases both structurally and functionally and are usually produced in combination other β –lactamases in clinical isolates. They differ structurally from the other β -lactamases by their requirement for a zinc ion at the active site (Bush .(and Jcoby, 2010

2.8 Metallo β – lactamases (MBLs)

Jacoby Bush and roup 3 ofg lactamases that are included in-βMBLs are molecular classification of roup B in theg classification whereas members of .Ambler They use a metal ion, usually zinc at an active site for the hydrolysis of

β-lactam ring (Hall *et al.,* 2003). Class B metallo-β-lactamases (MBLs) have a broad substrate spectrum and can catalyze the hydrolysis of virtually all β-lactam antibiotics with the exception of monobactams. They are not inhibited by mechanism-based inhibitors such as clavulanate, sulbactam, or tazobactam that are effective against serine-based, class A β–lactamases (Drawz. and Bonomo, 2010; Perez-Llarena and Bou, 2009). But they possess the characteristics of being universally inhibited by EDTA and other chelating agent of $Zn^{++}$and other divalent cations (Queenan and Bush, 2007).

The first metallo-β-lactamases detected and studied were chromosomal enzymes present in environmental and opportunistic pathogenic bacteria such as *Bacillus cereus*, *Aeromonas* spp. and *Stenotrophomonas maltophilia* (Queenan and Bush, 2007). These chromosomal enzymes were usually found in bacteria and are not easily transferred (Walsh *et al.,* 2005). Fortunately, with the exception of *S. maltophilia*, these bacteria have not been frequently associated with serious nosocomial infections, as they are generally opportunistic pathogens, and the chromosomal metallo-β-lactamase genes (Queenan and Bush, 2007). However, concerns have been raised that acquired MBL genes are located on integron structure that reside on mobile genetic elements such as plasmids or transposons thus enabling widespread dissemination (Franklin*et al.,* .(2006 These MBL genes are often carried along with other resistance genes resulting in multi-drug resistance limiting treatment options (Walsh *et al.,* 2005).

P. aeruginosa isolates producing metallo β-lactamases were first reported in Japan in 1991, since then it has been described from various parts of world, including Asia, Europe, Australia, South America and North America (Pitout *et al.,* 2005). In India, the prevalence of *P. aeruginosa* producing MBL is 7.5% to 75% (Kumar *et al.,* 2012). A nationwide study carried out in France, prevalence of MBL pruducers is 9.5% among the Ceftazidime resistant isolates and 0.6% of the total isolates (Hocquet *et al.,* 2010). In context of Nepal, a study carried out in TUTH, 3.3% of the isolates were MBL producer (Mishra *et al.,* 2012).

2.8.1 Types of MBLs

Five major clinically important groups of MBLs have been identified: IMP, VIM, SPM, GIM and the recently described SIM (Lee *et al.,* 2002). The most common and widespread acquired MBLs are those of the IMP and VIM types, which exhibit a worldwide distribution and for which several allelic variants are known Their genetic determinants are carried on mobile gene cassettes inserted into chromosome or plasmid-borne integrons and can rapidly disseminate in the clinical setting via the integron system and the associated mobile DNA elements (Lagatolla *et al.,* 2006).

IMP Type:

IMP (Imipenemase) was the first mobile MBL discovered from *P. aeruginosa* in Japan during 1988. The resistance allele was found on a transferable conjugative plasmid that could be readily mobilized to other *Pseudomonas* strains (Zavascki *et al.,* 2006). They are most commonly found in *P. aeruginosa*, *Acinetobacter baumanii, Klebsiella pneumonia, E. coli, Citrobactor* etc (Walsh *et al.,* 2005).

VIM Type:

Second dominant group of acquired MBL is VIM (Verona Integron encoded Metallo β-lactamases) (Veronese Imipenemase) type enzyme, which was first described in Verona, Italy from a *Pseudomonas aeruginosa* which was resistant to Piperacillin, Ceftazidime, Imipenem and Aztreonam . Among several types of MBL enzymes identified, VIM type enzymes appears to be most prevalent (Poirel *et al.,* 2001).

2.8.2 Detection of MBLs

The emergence of metallo-β-lactamase (MBL)-producing isolates is a challenge to routine microbiology laboratories, Unfortunately, there are no standardized phenotypic methods available and the testing criteria are likely to depend on whether the gene is carried by *P. aeruginosa* (Walsh *et al.,* 2005). Given the fact that all the MBLs are inhibited by the removal of the zinc from the active site, in forward, and studies have focused on this principle, their detection is straight

lactam combinations to detect strains -β-inhibitor principle and used a variety of possessing these clinically important enzymes (Yan*et al.,* Several .(2004 phenotypic methods have been developed for MBL detection, such as the MBL rodilution, and the cassay, mi ynergy tests, combined diskdisk s-Etest, double Hodge test(Walsh *et al.,* 2005).

Although PCR-based genotyping remains as the golden standard for MBL detection and classification, its use is mainly restricted to research purposes. As genotyping information is necessary, diagnostic centers and laboratories still rely mostly on culture-based phenotypic test as a means for rapid detection of MBL activity (Khosravi *et al.,* 2012). So far, many variations of phenotypic assays for MBLs detection have been reported, and these assays are not standardized. It has been well documented that the activity of MBLs is dependent on zinc or cadmium (Franklin *et al.,* 2006). Several screening methods incorporating the use of metal chelating agents, such as ethylenediaminetetraacetic acid (EDTA) and thiol-based compounds like 2-mercaptopropionic acid , which are capable of blocking MBL activity, have been developed to detect MBL-producing organisms (Arakawa *et al.,* 2000; Livermore and Woodford, 2000; Migliavacca *et al.,* 2002; Walsh *et al.,* 2002 and Yong *et al.,* 2002). A double-disk synergy test using Imipenem or ceftazidime disk and a 2-MPA disk, designed by Arakawa *et al.,* (2000) was able to indicate the presence of MBLs through the display of an enhanced zone of inhibition around the CAZ disk toward the 2-MPA disk. The combined IPM-EDTA disk test (CDT) works by comparing the zones of inhibition obtained with IPM disks with and without EDTA (Yong *et al.,* 2002). In contrast, the microdilution method compares the minimal inhibitory concentrations of IPM with and without EDTA (Migliavacca *et al.,* 2002).

CHAPTER III
MATERIALS AND METHODS

MATERIALS 3.1

The materials, equipment and various reagents used in different stages of this .A study are listed in Appendix

METHODOLOGY 3.2

Shree Birendra Hospital, Chhauni, prospectively at as conductedThe study w Kathmandu fromJanuary 2013 to July 2013 to assess the prevance of *Pseudomonas aeruginosa*their antibiotic suceptibility pattern and MBL , f all age groups o production from all clinical samples. This study included paients sent for routine culture were visiting hospital, from whom samples and both sex and antibiotic susceptibility testing. The demographic parameters, clinical history, .(Appendix C) prior antibiotic use were recorded

mple typesSample size and sa 3.2.1

under study. The specimens included A total of 5833 different samples were included sputum, pus, tracheal secretion, bronchial secretion, urine, blood and to different body fluids like CSF, peritoneal fluid, acetic fliud which were sent for routine culture and antibiotic susceptibility testing were processed rylaborato obtained in a clean, leak proof container that are during the study period. Samples with no visible signs of contamination and labeled properly with demographic .ents were accepted, otherwise a second sample was requestedinformation of pati

Collection and transportation of specimen 3.2.2

Urine samples 3.2.2.1

ml of clean voided (clean catch) first 20-Patients were asked to collect 10 The .necked, leakproof container demorning mid stream urine in a sterile, dry, wi container was then labelled properly and immediately delivered to the laboratory Forbes) with the request form as soon as possible for further processing*et al.,* .(2007

Sputum samples 3.2.2.2

collected in a sterile, leakproof, disposable container. The The sputum sample was sterile water or -patient was asked not to rinse or gargle the mouth with non mouthwash prior to sample collection and also instructed to collect specimen nasal discharge. The container -or post resulting from deep cough not the saliva was then labelled properly and immediately delivered to the laboratory as soon as Forbes) possible for further processing*et al.,* .(2007

roncheal secretionB Tracheal secretion and 3.2.2.3

The tracheal secretion and bronchial secretion were collected in a sterile, leakproof, disposable container. The container was then labelled properly and immediately delivered to the laboratory as soon as possible for further processing Forbes)*et al.,* .(2007

Pus samples 3.2.2.4

from wounds or abscesses that are clinically infected or taken Pus samples were deteriorating or that fail to heal over a long period. For closed wounds and aspirates, 2% Cholorohexidine followed by an iodine solution was used for disinfection whereas for open wounds, it was debrided then rinsed thoroughly with sterile saline prior to collection of pus sample. Pus samples should be such that it contains the deepest portion of the lesion or exudate rather than superficial b collection should be avoided as long as aspirates or biopsy samples debris. Swa can be obtained. If swab is the only option, then sterile cotton wool swab was gently rolled over the surface of the wound approximately five times, focusing on Forbes) nce of pus or inflamed tissuearea where there is evide*et al.,* .(2007

Blood sample 3.2.2.5

After proper sterilization of skin, 5 ml of blood was withdrawn by sterile syringe. The blood was transferred immediately in sterile containing 50 ml of Brain-Heart Infusion broth aseptically. The bottles were taken to laboratory as soon as possible for culture Forbes)*et al.,* (2007.

(Body fluid specimens (CSF, Pleural, Peritoneal and synovial fluid 3.2.2.6

puncture site The needle .collected by trained physician These speimens were was cleaned with alcohol and disinfected with iodine solution to prevent ml of 5-introduction of specimen contamination or infection of patient. About 1 the sample was drawn and transported to laboratory, properly labelled, in a sterile .or vial for further processing tube

Culture of Specimens 3.2.3

Urine culture 3.2.3.1

The urine samples were cultured onto the CLED agar plates by the semi-quantitative culture technique using a standardcalibrated loop.

1. -vertically just below the surface of well A calibrated loop was immersed .mixed uncentrifuged urine specimen
2. A loopful of urine was then streaked on the plate to make straight line inoculum down the center of the plate and the urine was streaked by making .hroughout the inoculumangle t ^{0}series of passes at 90
3. .C overnight^{0}incubated at 37 nThe plates were the
4. Colony count was performed so as to calculate the number of CFU per ml :of urine and the bacterial count was reported as
    i. Less than 10^4/ml organisms: not significant
    ii. 10^4-10^5/ml organisms: doubtful significance (suggest repeat specimen).
    iii. More than 10^5/ml organisms: significant bacteriuria

If the culture indicates presence of two uropathogens both showing significant y testing of both growth, definitive identification and antimicrobial susceptibilit were performed whereas in cases of ≥3 pathogens, it was reported as multiple bacterial morphotypes and asked for appropriate recollection with timely delivery .(to laboratory (Isenberg, 2004

Sputum 3.2.3.2

The sputum samples w ereinoculated into the Blood agar and MacConkey agar plates. Theand lood agarB chocolate agar and MacConkey agar plates were incubated at 37^0C for overnight agar plate w asincubatedC^0at 37 in an aerobic condition Forbes)*et al.,* (2007.

Fluids Pus and Body 3.2.3.3

These samples were inoculated intoBlood agar and MacConkey agar platesThe . Blood agar for 48 hours $c^0$and Macconkey plates were incubated aerobically at 37 Forbes)*et al.,* .(2007

3.2.3.3 Blood Culture

^{0}at 37 incubated Blood culture bottles after receving wereC for overnight. Next day it was subcultured in Blood agar and Mac Conkey agar plates. The plates were 37^0C for 24 hours. The negative bottles were incubated and subcultured till 7^{th} days. Culture bottles showing negative growth until the 7^{th} day were discarded Forbes)*et al.,* (2007.

Tracheal and Broncheal Secreation 3.2.3.4

The tracheal secretion and bronchial secretion were inoculated on the Blood agar and MacConkey agar plates. The plates were incubated for 37^0C for 48 hours Forbes)*et al.,* (2007.

Identificaion of the isolates 3.2.4

The identification of bacterial isolates was done by using standard microbiological techniques as described in Bergey's Manual of systemic morphology, staining bacteriology which comprises of studying the colonial reactions and various biochemical properties. Isolated colonies from the pure culture with oxidase positivity were identified by performing staining the standard .conventional biochemical tests

ngIdentification with gram staini 3.2.4.1

Gram staining was performed for the presumptive identification of the bacteria .according to standard technique*P. aeruginosa*was identified as a gram negative .rod

Identification with Biochemical Tests 3.2.4.2

and agar Typical colonies of bacterial isolates were sub cultured on Nutrient C for 4 hours. After incubation, fresh culture of test organism ^{0}incubated at 37 .was inoculated into different biochemical media*P. aeruginosa*were characterise oloy morphology, Gram stain sing a combination of cand identified u teristics and different biochemical tests.Catalase Test, Oxidase Test, Indole charac Test, Methyl Red Test, Voges Progeskaur Test, Citrate utilisation Test, Triple performed for identification of Sugar Iron Agar Test and Urease Test were*P. aeruginosa*Result interpritation was dome based on identifying characteristics of . Forbes) the isolates*et al.,* .(2007

susceptibility testing bioticAnti 3.2.5

Antibiotic susceptibility tests of all the clinical isolates towards various antibiotics were performed by Kirby-Bauer disk diffusion method as recommended by Clinical Laboratory Standard Institute (CLSI). In this method, the broth culture of test organism (comparable to McFarland tube no.0.5; inoculums density 1.5×10^{8}organisms/ml) were uniformly carpeted on the surface of Mueller Hinton agar. Then, antibiotics discs were placed over the lawn culture of the test organism by sterile foreceps. The inoculated and seeded MHA plates then were incubated at 37° C for 18 hours (or overnight). After incubation the zone diameter of each antibiotic were interpreted using the interpretative chart and the organism was reported as “Resistant”, “Intermediate” or “Susceptible”. Control strains of *P. aeruginosa* (ATCC 27853) were used in parallel as a part of quality control when using new batch of media or antibiotics (Cheesbrough, 2000).

3.2.6Detection of Metallo-β-lactamase producing strain

were isolates showing resistance to Imipenem and Ceftazidime his studyIn t .for the detection of possible MBL production subjectedTest organisms were inoculated onto plates of Mueller-Hinton agar. Two 10 µg imipenem disks were placed on the plate, and 750µg of 0.5 M EDTA solution of pH 8 were added to one of them. The inhibition zones of the imipenem and imipenem-EDTA disks were compared after 16 to 18 h of incubation in aerobic condition at 35°C. All of the MBL-positive isolates were well separated from MBL-negative isolates by the criterion of a ≥7-mm increase of inhibition zone with the disks to which 750 µg of EDTA was added (Yong *et al.*, 2002).

Quality Control 3.3

a**ediM Monitoring of 3.3.1**

lot for The quality of media prepared was checked by incubating one plate of each .sterility and using standard control strains for performance testing

3.3.2Purity Plate

The purity plate was used to ensure that the inoculation used for the biochemical tests was pure culture and also to see whether the biochemical tests were performed in an aseptic condition or not. Thus, while performing biochemical tests, the same inoculum was subcultured in respective medium and incubated. The media were then checked for the appearance of pure growth of organisms.

antimicrobial susceptibility testing Quality control during 3.3.3

Mueller Hinton agar and the antibiotic discs were checked for their lot number, manufacture and expiry date, and proper storage. For the standardization of MHA, control Bauer test and for performance testing of antibiotics and-Kirby strains of*Pseudomonas aeruginosa* (ATCC 27853 .were tested primarily (Quality of sensitivity tests was maintained by maintaining the thickness of Mueller-Hinton agar at 4 mm and the pH at 7.2-7.4.

3.4 Data Analysis

All the results will be entered in the worksheet of Statistical Package for Social Science (SPSS) software. Chi-square test will be used to determine significant association of dependent variable to different independent variables.

CHAPTER IV

RESULTS

During six month period, a total of 5833 clinical specimens were collected and processed in the Microbiology Laboratory of hospital. The clinical specimens included were sputum, pus, tracheal secretion, bronchial secretion, urine, blood .fluids and different body Out of the 5833 specimens, 942 showed positive growth. Among the bacterial isolates, *Escherichia coli* was found to be most predominent consituting 261/942 (27.7%). Other most frequently isolated organisams are *Staphylococcus aureus* (12.8%), *Salmonella* Typhi (12.6%), *Pseudomonas aeruginosa* (12.1%) and Coagulase negetive staphylococcci (11.0%).

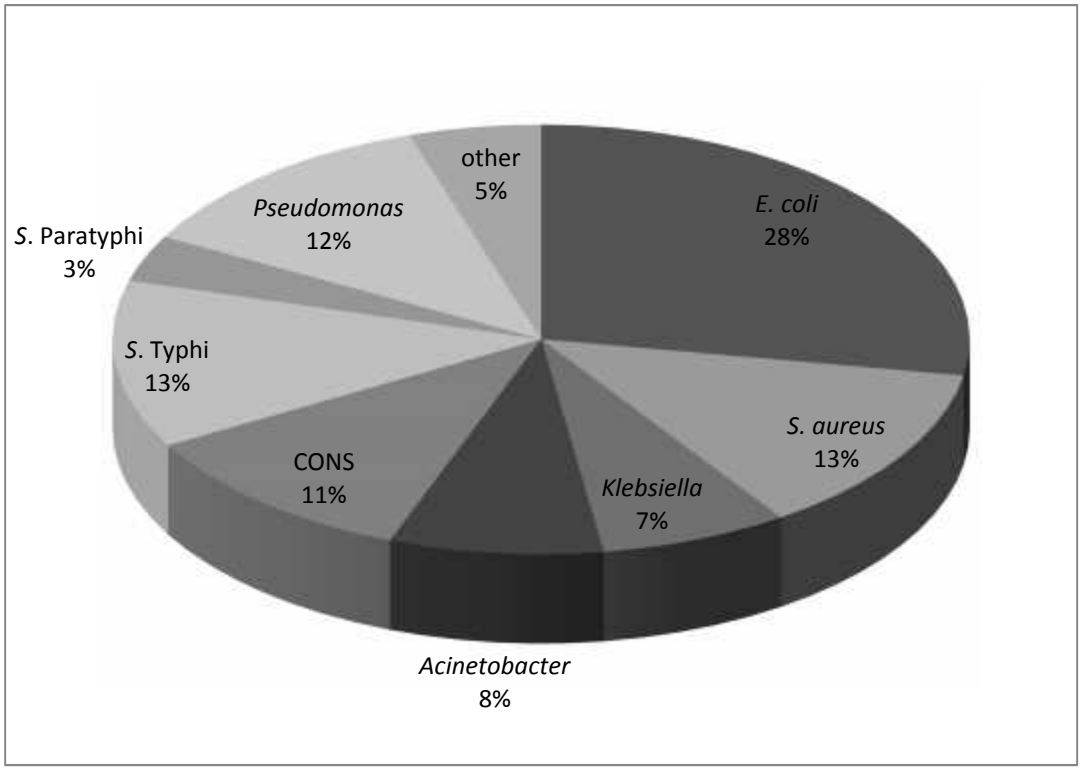


Fig 2: Different types of bacterial organisms grown

4.1 Distribution pattern of *Pseudomonas aeruginosa* according to gender and age

Among the 114 *Pseudomonas aeruginosa*, 60.5% were isolated from male patients and remaining 39.5% were from female patients. Highest growth was obtained from the age group 61-70 (27.2%), of whom 58.1% (n=18) were male and 41.9% (n= 13) were female. The least percentage of positive growth was obtained from age group 11-20 (2.6%). In total, 60.5% (n=69) of the *P. aeruginosa* was isolated from male patient and 39.5% (n=45) of the isolates from female patients. The highest number of growth in male patients (n=18) was obtained from age group 61-70 and 71- 80 and that in female patients (n=13) was obtained from age group 61-70.

Table 1: Distribution pattern of *Pseudomonas aeruginosa* according to gender and age

Age Category	Male		Female		Total	
	Number (n)	%	Number (n)	%	Number (n)	%
<10	1	20.0	4	80.0	5	4.4
11-20	1	33.3	2	66.7	3	2.6
21-30	6	75.0	2	25.0	8	7.0
31-40	3	60.0	2	40.0	5	4.4
41-50	8	50.0	8	50.0	16	14.0
51-60	9	60.0	6	40.0	15	13.2
61-70	18	58.1	13	41.9	30	27.2
71-80	18	72.0	7	28.0	25	21.9
>80	5	83.3	1	16.7	6	5.3
Total	69	60.5	45	39.5	114	100

4.2 Distribution of *P. aeruginosa* from various samples

Out of total 114 *P. aeruginosa* isolates, maximum number of organisms were isolated from pus (41.2%) followed by sputum (27.2%), tracheal secretion (9.8%)

and urine (9.8%). Among them 44.7% of isolates were isolated from samples from lower respiratory tract. Only 1.8% of organisms were isolated from the blood sample.

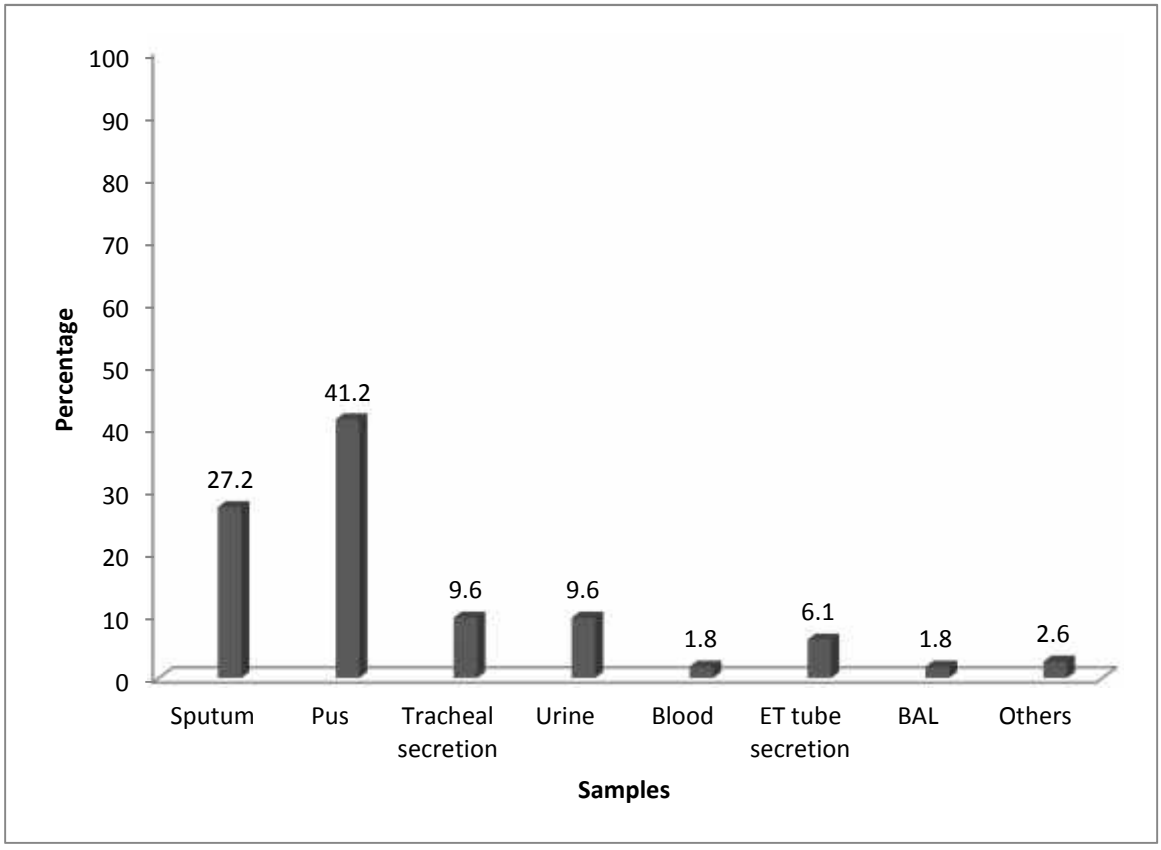


Fig 3: Distribution of *P. aeruginosa* from various samples

4.3 Distribution of *P. aeruginosa* according to different wards

Among 114 positive cases, 42.1% (n=48) of specimens were from Intensive Care Unit and 57.9% (n=66) of the specimens were from other than ICU units (General wards and OPD). Among ICU patient, 27.2% of the isolates were from general ICU and 14.9% of patients were from Intensive Trauma Care Unit. Among Other than ICU units, highest numbers of *P. aeruginosa* were isolated from surgical ward (28.9%) which was followed by medical ward (13.3%). Only 7% of the specimens isolated were from the samples from OPD department.

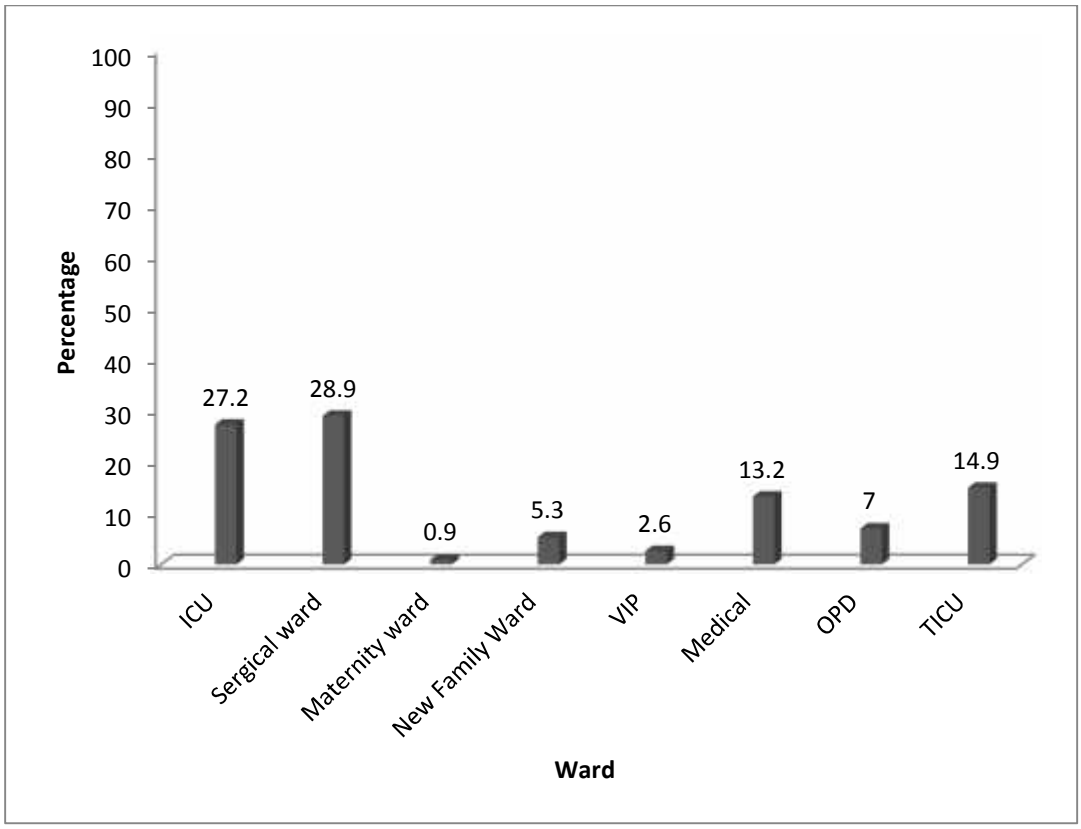


Fig 4: Distribution of *P. aeruginosa* according to different wards

4.4 Growth status of the *P. aeruginosa*

Out of 114 *P. aeruginosa*, 72.8% had monomicrobial growth where as 27.2% had polymicrobial growth. Among monomicrobial growth, 39.9% of isolates were from ICU and 60.2% were from other than ICU wards. Among polymicrobial growth, 48.8% of isolates were from ICU and 51.6% were from other than ICU wards. There was no significant association (p-value > 0.05) between wards and growth of organisms.

Table 2: Growth status of the *P. aeruginosa*

		Ward		Total	p-value
		ICU	Other than ICU		
Growth obtain	single	33 (39.9%)	50 (60.2%)	83 (72.8%)	P= 0.406
	polymicrobial	15 (48.4%)	16 (51.6%)	31 (27.2%)	
Total		48 (42.1%)	66 (57.9%)	114 (100%)	

4.5 Antibiotic sensitivity pattern of *P. aeruginosa* isolated from different growth positive specimen

The *P. aeruginosa* isolates exhibited maximum resistance towards Ceftazidime (57.0%) followed by Ofloxacin (50.9%), Cefepime (47.4%) and Ciprofloxacin (44.7%). Only 4.4% of organisms show resistance towards Imipenam. Most of the isolates were susceptible towards Imipenam (95.6%) followed by Amikacin (71.9%), Gentamicin (56.1%) and Piepracin + Tazobactam (54.4%). Organism show least susceptibility towards ceftazidime (28.1%).

Table 3: Antibiotic sensitivity pattern of *P. aeruginosa* isolated from different growth positive specimen

Antibiotics	Sensitive		Intermediate		Resistant	
	n	%	n	%	n	%
Amikacin	82	71.9	8	7.0	24	21.1
Cefepime	47	41.2	13	11.4	54	47.4
Piepracin+ Tazobactam	62	54.4	15	13.2	37	32.5
Ciprofloxacin	49	43.0	14	12.3	51	44.7
Ofloxacin	45	39.5	11	9.6	58	50.9
Ceftazidime	32	28.1	17	14.9	65	57.0
Gentamicin	64	56.1	2	1.8	48	42.1
Imipenam	109	95.6	0	0	5	4.4

4.5.1 Antibiotic resistant pattern of *P. aeruginosa* with reference to ward

P. aeruginosa isolated from ICU ward showed more resistance towards antibiotic agent used than those are isolated from other than ICU wards. Among organism those are isolated from ICU, 10.3% showed resistance to Imipenem whereas only 1.3% isolates were resistant from other than ICU wards. Similarly, 28.2% are resistant to Amikacin, 61.5% to Cefepime, 51.3% to Ciprofloxacin in ICU and

whereas only 17.3% are resistant to Amikacin, 40% to Cefepime, 41.3% to Ciprofloxacin from other than ICU wards.

Table 4: Antibiotic resistant pattern of *P. aeruginosa* with reference to ward

Antibiotics	ICU Ward		Other than ICU	
	Resistant	%	Resistant	%
Amikacin	11	28.2	13	17.3
Cefepime	24	61.5	30	40
Piepracin+ Tazobactam	14	35.9	23	30.7
Ciprofloxacin	20	51.3	31	41.3
Ofloxacin	24	61.5	34	45.3
Ceftazidime	22	56.4	43	57.3
Gentamicin	14	46.2	30	40
Imipenem	4	10.3	1	1.3

4.6 Distribution and analysis of Multidrug Resistant *P. aeruginosa* in various specimens

Among the total 114 *P. aeruginosa* isolates, only 33.3% were multidrug resistant. Isolates from pus sample showed maximum percentage of multidrug resistant isolates (57.4%) followed by tracheal secretion (36.4%) and ET Tube secretion (28.6%). Among 31 organisms isolated from sputum only 9.7% are multidrug resistant isolates.

Table 5: Disribution and analysis of Multidrug Resistant *P. aeruginosa* in various specimens

Type of specimen	Total *P. aeruginosa* isolated	Multidrug Resistant	Percentage
Sputum	31	3	9.7
Pus	47	27	57.4
Tracheal secretion	11	4	36.4
Urine	11	1	9.1
Blood	2	0	0
ET Tube Secretion	7	2	28.6
BAL	2	0	0
Others	3	1	33.3
Total	114	38	33.3

4.7 Distribution of Multidrug Resistant *P. aeruginosa* in different wards

Out of total 48 isolates from the ICU ward, 37.5% of the isolates were MDR and among other than ICU ward, only 33.3% of the isolates were multidrug resistant *P. aeruginosa.* There was no significant association (p-value > 0.05) between wards and multidrug resistant organisams.

Table 6: Distribution of Multidrug Resistant *P. aeruginosa* ing different wards

	MDR		Non MDR		Total	p- Value
	Number	%	Number	%		
ICU ward	18	37.5	30	62.5	48	P=0.421
Other than ICU Ward	20	30.3	46	69.7	66	
Total	38	33.3	76	66.7	114	

4.8 MBL Production in *P. aeruginosa*

Out of total 114 *P. aeruginosa* isolates, only 4.4% (n=5) were Metallo β-lactamases producer. Other 95.6% of isolates did not produce the enzyme. All of the MBL producing isolates were obtained from ICU ward. Maximum number of MBL producing *P. aeruginosa* were isolated from Pus sample (60%) followed by Tracheal secretion (20%) and ET Tube secretion (20%). All of the isolates which were resistant to Imipenam were found to be MBL producer. Among Ceftadizime resistant isolates, only 7.7% isolates produce MBL.

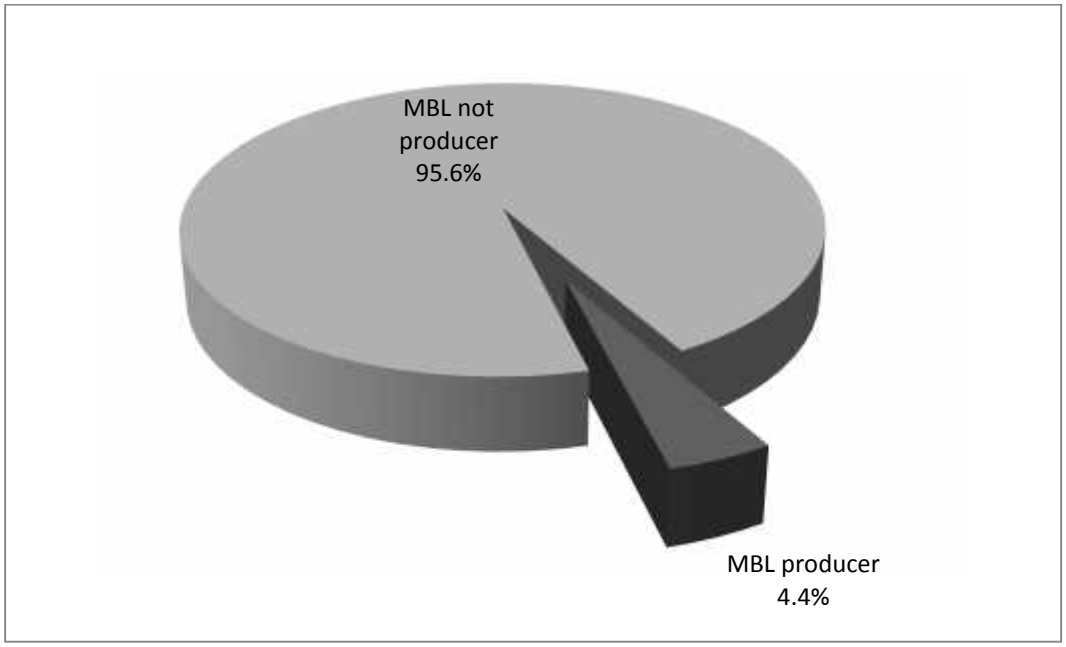


Fig 5: Metallo β- lactamases production in *P. aeruginosa*

CHAPTER V
DISCUSSION

Nosocomial infections constitute an important worldwide health problem resulting in high morbility and mortality as well as economic concequences. *Pseudomonas aeruginosa* is one of the leading causes of nosocomial infections. *P. aeruginosa* exhibits intrinsic resistance to several antimicrobial agents. However, acquired resistance to anti pseudomonal β-lactams can be a major challenge in managing infection caused by *P. aeruginosa*. Since carbapenems are used as the last resort for treatment of MDR gram-negative bacterial infection, the acquired resistance to this life saving drug has been increasingly reported in *Pseudomonas.* This resistance is mainly mediated by MBLs. Therefore this present study was conducted in the Microbiology Laboratory of Shree Birendra Hospital, Chhauni to find the prevalence of *P. aeruginosa* in different samples along with their antimicrobial susceptibility profile, find out the burden of MDR among *P. aeruginosa* and the production of metallo β- lactamases among those isolates.

Out of the 5833 specimens, 16.2% of the sample showed positive growth. Among 942 positive isolates, 12.1% were *Pseudomonas aeruginosa*. The proportion of isolation of *P. aeruginosa* among various bacterial pathogens isolated ranges from 5% to 29.6% in various studies (Olayinka *et al.,* 2004; Khan *et al.,* 2008; Ranjan *et al.,* 2010; Mohanasoundaram, 2011; De Francesco *et al.,* 2013). The difference in the result may be due to the other variables that influence the outcome of results such as, clinical specimens received for examination, studied population, type of hospitals and geographical locations. In other tertiary care hospital of Nepal, the proportion of isolation of *P. aeruginosa* was 13% (Gupta *et al.,* 2012), which was similar to our finding. Which suggest that *P. aeruginosa* was the constantly isolated pathogen in our hospital settings and regular intervention and monitoring is require for the control of infection caused by this organism.

In our study the highest percentage of *Pseudomonas aeruginosa* isolates were observed in pus sample which yields 41.2% of the total isolates. *P. aeruginosa* is the one of the important organism that causes wound infection in the hospital setting. The virulence and invasive capability of this organisms influence the infection of the wound, but the physiological state of the tissue in the wound and immunological integrity of the host also play important role in infection (Ranjan *et al.,* 2010). The higher incidence of *P. aeruginosa* in post operative wound infection may be due to relaxation in general hygienic measures, mass production of low quality antiseptic and medical solutions for treatment, difficulties in proper definition of the responsibility among the hospital staff (Bertrand *et al.,* 2002; Masaadeh and Jaran, 2009). Khan *et al.*, (2008), isolate 57.64% of the organism in pus sample. In a similar type of study Mohanasoundaram (2011) also isolated maximum number of *P. aeruginosa* (36%) from Pus specimen. In study conducted by Rakesh *et al.,* (2012), the maximum clinical isolates of *P. aeruginosa* were isolated from pus/swab (71%). Since *P. aeruginosa* was the opportunistic environmental bacteria, it can easily invade the open wound and cause infection. So, proper hygienic measure and sanitation should be maintained at the hospital setting.

In this study 42.1% of the total Pseudomonal isolates were obtain from ICU. In other report, the prevalence of *P. aeruginosa* was found to be 28.4% in ICU (Pal *et al.,* 2010). A European survey on the prevalence of nosocomial infection in ICU patients showed that *P. aeruginosa* was one of the most frequent pathogens isolated from ICU accounting 29.0% of ICU acquired infections (Vincent, 2000). The most important risk factors are excessive consumption of broad spectrum antibiotics exerting selective pressure on bacteria, the frequent use of invasive device and relative density of a susceptible patient population with severe underlying diseases (Kumar, 2006).

On the other hand isolation of *P. aeruginosa* from other other than ICU units was higher than ICU unit (57.9%). Among them maximum number of isolates were

from surgical ward (28.9%). According to different reports, high percentage of *Pseudomonas* was isolated from the postoperative wound infection in surgical ward (Masaadeh and Jaran, 2009; Bertrand *et al.,* 2002). The higher incidence of *P. aeruginosa* in surgical ward may be due immunocompromised state of the patient due to surgery, by use of invasive device and use of immunosupresive drug which facilitate the infection by *P. aeruginosa*.

The Antibiotic sensitivity test of *P. aeruginosa* isolated in this study, in overall showed higher sensitivity towards Imipenem (95.6%) followed by Amikacin (71.9%) whereas highest resistance was observed towards Ceftadizime (57.0%) followed by Ofloxacin (50.9%) and Cefepime (47.4%). Resistance to Ciprofloxacin (44.7%), Gentamicin (42.1%) and Pipracilin+ Tazobactam (32.5%) were shown by the isolates. Carbapemem antibiotics are considered agents of choice to treat serious infection caused by *P. aeruginosa*. Our study also showed Imipenem was the most effective agent. Tripathi *et al.*, (2011) also found highest sensitivity of *P. aeruginosa* to Imipenem (87.26%). Imipenem was consistently the most active agent (90.6%) against most of the Pseudomonal isolates (Rijal, 2012). Since *P. aeruginosa* constantly develop resistance against first and second line drugs, Imipenem could be the drug of choice for the treatment of infection caused multidrug resistant *P. aeruginosa*.

Pseudomonas exhibited high resistance to Cephalosporin groups in comparision to other group of β- lactams antibiotics in our study. The increased prevalence of ceftazidime resistant *P. aeruginosa* is related to the increased use of β-lactam antibiotics such as amoxicillin and ceftazidime (Mohanasoundaram, 2011). *P. aeruginosa* became resistance to third generation cephalosporins by production of β-lactamases which are chromosomal β-lactamases and plasmid associated extended spectrum β-lactamases (ESBLs) (Livermore, 1995). Selective pressure from the use of antimicrobial agents is also a major determinant for the emergence of resistant strains (Mohanasoundaram, 2011).So, judicial use of

antibiotics should be emphasized in order to prevent the development of drug resistant bacteria.

In our study, resistance to Quinolones was higher than the study done by Tripathi *et al.,* (2011) and Mishra *et al.,* (2012). Increase in the levels of resistance to the fluoroquinolones among nosocomial isolates like *P. aeruginosa* has also been also reported by Prakash *et al.,* (2012). Principal mechanisms of bacterial resistance to quinolones are modification of target enzymes, DNA gyrase (gyr A) and topoisomerase IV, or reduction of intracellular concentration due to mutations in the regulatory genes for efflux systems, such as mexR and nfxB (Shah and Wretlind, 1998). Our study showed ciprofloxacin as an effective agent than ofloxacin against *P. aeruginosa.* Prakash *et al.,* (2012) and Rijal (2012) also found ciprofloxacin as more effective agent. Higher resistance to quinolones in our study may be due to the overuse of these drugs.

Development of multidrug resistance by *P. aeruginosa* isolates requires several different genetic events such as acquisition of different mutations and/or horizontal transfer of antibiotic resistance genes. In the present study 33.3% of the isolates were multi drug resistant. In our study, most of the multi drug resistance isolates were isolated from pus sample. Aloush *et al.,* (2006) also found that the common site of isolation of MDR *P. aeruginosa* was pus (39%). The multidrug-resistant (MDR) phenotype in *P. aeruginosa* could be mediated by several mechanisms including multidrug efflux systems, enzyme production, outer membrane protein (porin) loss and target mutations (Hirsch and Tam, 2010). The number of multidrug resistant *P. aeruginosa* ranges from 20% to 85.4% in various studies throughout the world (Shankar *et al.,* 2005; Kohanteb *et al.,* 2007; Jayakumar *et al.,* 2007; Francesco *et al.,* 2013; Rijal, 2012). In a study done in other hospitals of Nepal, higher prevalence of MDR *P. aeruginosa* was observed. Gupta *et al.,* (2012) found 65.9% MDR *P. aeruginosa* and Rijal (2012) isolated 85.4% MDR isolates. Our study showed lower prevalence of MDR *P. aeruginosa* but continuous monitoring and the restriction of the inappropriate use of

antimicrobial agents should be emphasized in order to prevent the emergence MDR *P.aeruginosa*.

Comparing the multidrug resistant pattern of the *P. aeruginosa* isolates from the ICU and Other than ICU wards, it was observed that the isolates from ICU wards exhibited higher percentage of MDR isolates (37.5%) than from Other than ICU ward (30.3%). Studies have demonstrated that rates of antimicrobial resistance are greater in bacterial isolates from ICUs compared to other hospital wards and outpatient clinics (Archibald *et al.,* 1997). Our finding correlate to the findings of Slamma *et al.,* (2011), where ICU *P. aeruginosa* isolates were highly MDR compared to other wards. Intensive care units are generally considered epicenters of antibiotic resistance and principal sources of outbreak of multidrug resistant bacteria. However, statically there was no association between wards and MDR Pattern of the isolates in our study.

Several phenotypic methods are available for the detection of MBL-producing bacteria. All of these methods are based on the ability of metal chelators, such as EDTA and thiol-based compounds, to inhibit the activity of MBLs. The combined disk test using EDTA with imipenem is simple to perform and interpret and can be easily introduced into the workflow of a clinical laboratory. This test has been used in several studies where it produced excellent sensitivity and specificity for detecting MBL-producing *P. aeruginosa* strains (Walsh *et al.,* 2002; Yong *et al.,* 2002 and Lee *et al.,* 2002). By this method, we demonstrated that the prevalence of MBL producing *P. aeruginosa* strains is 4.4%. In another study conducted by Jones *et al.,* (2005), 6.5% of *P. aeruginosa* isolates carried MBLs. Higher rate of MBL producing isolates were observed by Kumar *et al.,* (2012), in which Prevalence of MBLs was found to be 26.9%. In another study done by Varaiya *et al.,* (2007), showed 20.8% of *P. aeruginosa* were MBL producer. In a study conducted at Tribhuvan University Teaching Hospital (TUTH) by Mishra *et al.,* (2012), prevalence of MBLs producing *P. aeruginosa* to be 3.3%. Finding of our study was slightly higher than the previous study done in Nepal showing

increasing trend of the MBL producing isolates. So, rigorous monitoring of the MBL producing *P.aeruginosa* should be done in order to prevent infection and transmission of these isolates.

All of metallo β- lactamase producing in our study were MDR and resistant to most of the antibiotic used. This is due to the fact that the MBL genes are often carried along with other resistance genes resulting in multi-drug resistance limiting treatment options (Walsh *et al.,* 2005). Similar result was obtained by Anuradha *et al.,* (2010), Mishra *et al.,* (2012). All of the MBL producing isolates were isolated from ICU ward. Out of 65 Ceftazidime resistant *P. aeruginosa* only 7.7% of isolates were MBL producer which closely matches with the finding of Gupta *et al.,* (7.5%), Mishra *et al.,* (2012) (6.5%) and Agrawal *et al.,* (8.05%).

CHAPTER VI

CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

P. aeruginosa was fourth most common organism causing bacterial infection. The rate of isolation of *P. aeruginosa* is higher in other than ICU wards than in ICU ward. Most of the organisms were isolated from surgical ward. Pus sample yields maximum number of *Pseudomonas aeruginosa* followed by sputum.

Imipenem was the drug showing high sensitivity to most of the *P. aeruginosa* isolates followed by Amikacin and Piperacilin/Tazobactam. The isolates showed maximum resistance towards Ceftazidime. Among the aminoglycosides, Amikacin has the highest sensitivity against *P. aeruginosa*. Among quinolones, ciprofloxacin was more effective agent than ofloxacin. *Pseudomonas* exhibited high resistance to Cephalosporin groups in comparison to other group of β-lactams antibiotics.

In this study, burden of MDR *P. aeruginosa* was lower than the previous report of other hospitals of Nepal. However, it is higher than the reports of some other parts of world. Most of the MDR isolates of *P. aeruginosa* were isolated from the ICU ward. Highest numbers of MDR isolates were isolated from pus sample.

Prevalence metallo β-lactamases producing *P. aeruginosa* was slightly higher than the previous study done in Nepal showing increasing trend of the MBL producing isolates. All of the MBL producing isolates were MDR and are isolated from ICU.

6.2 RECOMMENDATIONS

1. Imipenem was found to be most effective drug for the treatment of the infection caused by *P. aeruginosa* and can be useful for the treatment of the infection by multi drug resistant isolates in hospital setings.
2. Continuous monitoring of the MBL producing *P.aeruginosa* should be done in order to prevent infection and transmission of these isolates.

REFERENCES

Agrawal G, Lodhi RB, Kamalakar UP, Khadse RK and Jalgaonkar SV (2008). Study of metallo-β-lactamase production in clinical isolates of *Pseudomonas aeruginosa*. *Ind J Med Microbiol*. **26**:349-51.

Aloush V, Venezia SN, Igra YS, Cabili S, and Carmeli Y (2006). Multidrug-resistant *Pseudomonas aeruginosa*: risk factors and clinical impact. *Antimicrob. Agents Chemother*. **50:**43–48.

Ambler RP (1980). The structure of β- lactamases. Phiosophilical Transcation of Royal Society of London 289; 321-331.

Andonova M and Urumova V (2013). Immune surveillance mechanisms of the skin against the stealth infection strategy of *Pseudomonas aeruginosa*-Review. *Comp Immunol Microbiol Infect Dis*.

Anjum F and Mir A (2012). Susceptibility pattern of *Pseudomonas aeruginosa* against various antibiotics. *Afr. J. Microbiol. Res.* **4**:1005-1012.

Anzai Y, Kim H, Park JY, Wakabayashi H and Oyaizu H (2000). Phylogenetic affiliation of the *Pseudomonads* based on 16SrRNA sequence. *Int J Syst Evol Microbiology*. **50**:1563-1589.

Aoki S, Hirakata Y, Gotoh N, Yanagihara K, Kondoh A, Miyazaki Y, Tomono K, Yamada Y, Kohno S and Kamihira S (2004). Virulence of Metallo-β-Lactamase-Producing *Pseudomonas aeruginosa* In Vitro and In Vivo. *Antimicrob. Agent. Chemother* **48**: 1876-1878.

Arakawa Y, Shibata N, Shibayama K Kurokawa H, Yagi T, Fujiwara H and Goto M (2000). Convenient test for screening metallo-β-lactamase-producing gram- negative bacteria by using thiol compounds. *J. Clin. Microbiol* **38**:40–43.

Archibald L, Phillips L, Monnet D, McGowan JE Jr, Tenover F and Gaynes R (1997). Antimicrobial resistance in isolates from inpatients and outpatients in the United States: increasing importance of the intensive care unit. *Clin Infect Dis*.**24**: 211-215.

Aristoteli LP and Willcox MDP (2003). MDP Mucin Degradation Mechanisms by Distinct *Pseudomonas aeruginosa* Isolates In Vitro. *Infect. Immun.* **71**:5565-5575.

Bagde S, Tiwari N, Patidar RK, Khare M and Singh V (2012). Antibiogram assessment against Upper Respiratory Tract infection causing Bacteria. *Int J Pharm Sci Res*. **3**: 5024-5028.

Baltimore RS (2007). *Pseudomonas, Bhurkholderia* and *Stenotrophomonas* in Nelson Textbook of Pediatrics, Kliegman RM, Behrman RE, Jenson HB and Staton BF(Eds) 18th Edition, Sounders, Philadelphia:1208-1212.

Begum S, Ahmed I, Alam F, Zaman S, Hassan P, Absar N and Haq JA (2007). Characterization of an intracellular protease from *Pseudomonas aeruginosa. Pak. J. Med. Sci* **23**: 227-232.

Bertrand X, Thouverez M, Patry C, Balvay P and Talon D (2002). *Pseudomonas aeruginosa*: antibiotic susceptibility and genotypic characterization of strains isolated in the intensive care unit. *Clin Microbiol Infect* **7**: 706–708.

Bonomo RA and Szabo D (2006). Mechanisms of multidrug resistance in *Acinetobacter* species and *Pseudomonas aeruginosa*. Clin. *Infect. Dis.***43**:S49–S56.

Brown VI and Lowbury JL (1965). Use of an improved cetrimide agar medium and other culture methods for *Pseudomonas aeruginosa*. *J. clin. Path*. **18**: 752-56.

Bucior I, Pielage JF and Engel JN (2012). *Pseudomonas aeruginosa* Pili and Flagella Mediate Distinct Binding and Signaling Events at the Apical and Basolateral Surface of Airway Epithelium. *PLoS Pathog* **8**: e1002616.

Bush K and Jacoby GA (2010). Updated Functional Classification of β-Lactamases. *Antimicrob Agents Chemother*. **54**: 969–976.

Bush K, Jacoby GA and Medeiros AA (1995). A functional classification scheme for β-lactamases and its correlation with molecular structure. *Antimicrob Agents Chemother*. **39**: 1211-1233.

Byfield FJ, Kowalski M, Cruz K, Leszczynska K, Namiot A and Savage PB (2011). Cathelicidin LL-37 increases lung epithelial cell stiffness, decreases transepithelial permeability, and prevents epithelial invasion by *Pseudomonas aeruginosa*. *J Immunol* **187**: 6402-6409.

District laboratory Practice in tropical Countries Part 2 .(Cheesbrough M (2000 .194 Page .Cambridge University Press

Comolli JC, Waite LL, Mostov KEM and Engel JN (1999). Pili Binding to Asialo-GM1 on Epithelial Cells Can Mediate Cytotoxicity or Bacterial Internalization by *Pseudomonas aeruginosa. Infect. Immun.* **67**:3207-3214.

.(nes ME, and Karlowsky JA (2001Daniel FS, Thornsberry C, Mayfield DC, Jo prevalence :*Escherichia coli* Resistant urinary tract isolates of-Multidrug .and patient demographics in the united states in 2000*J Antimicrob Chemother*. **65**.1406-1402:

Dasgupta N, Wolfgang MC, Goodman AL, Arora SK, Jyot J, Lory S and Ramphal R(2003). A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in *Pseudomonas aeruginosa*. *Molecular Microbiology*. **50**:809–824.

De Francesco MA, Ravizzolz G, Peroni L, Bonfanti C and Manca N (2013). Prevalence of multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* in an Italian hospital. *Journal of Infection and Public Health.* **6**:179-185.

De Freitas AL and Barth AL **(** 2002). Antibiotic resistance and molecular typing of *Pseudomonas aeruginosa*: focus on imipenem. *Braz. J. Infect. Dis*. **6:**1-7.

De Kievit TR and Iglewski BH (2000). Bacterial quorum sensing in pathogenic relationships. *Infect Immun*.**68**:4839–4849.

Doring G (1987). Significance of *Pseudomonas aeruginosa* virulence factors in acute and chronic *Pseudomonas aeruginosa* infections. *Infection*.**15**:47–50.

Drawz SM and Bonomo RA (2010). Three decades of β- lactamase inhibitors. *Clin. Microbiol. Rev.* **23:** 160–201.

Driscoll JA, Brody SL and Kollef MH (2007). The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. *Drugs* **67**: 351-368.

Farrel P, Joffe S, Foley L, Canny GJ, Mayne P and Rosenberg M (2007). Diagnosis of cystic fibrosis in the Republic of Ireland: epidemiology and costs. *Ir Med J* **100**:557-560.

Feldman M, Bryan R, Rajan S, Scheffler L, Brunnert S and Tang H (1998). Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. *Infect Immun* **66**:43-51.

Finlayson EA and Brown PD (2011). Comparison of antibiotic resistance and virulence factors in pigmented and Non-pigmented *Pseudomonas aeruginosa. West Indian med. J*. **60**: 24-32.

Bailey and Scott's Diagnostic .(Forbes BA, Sahm DF and Weissfeld AS (2007 -Page 150 edition. Mosby Elsevier Publication, USA thMicrobiology 12 .187

Franklin C, Liolios L and Peleg AY (2006) Phenotypic detection of carbapenem-susceptible metallo-β-lactamase- producing gram-negative bacilli in the clinical laboratory. *J. Clin. Microbiol*. **44:** 3139–3144.

Galimand M, Sabtcheva S, Courvalin P and Lambert T (2005). Worldwide disseminated armA aminoglycoside resistance methylase gene is borne by composite transposone Tn1548. *Antimicrob. Agents Chemother*. **49**:2949–2953.

Ghorashi Z, Nezami N, Ghotaslou R and Ghorashi S (2010). Pattern of *Pseudomonas aeruginosa* drug resistance in Tabriz Children Hospital. *Pak. J. Bio. Sci.* **13**; 400-404.

Gilligan PH (1991). Microbiology of airway disease in patients with cystic fibrosis. *Clin Microbiol Rev*. **4**:35-51.

Goto S and Enomoto S (1970). Nalidixic acid cetrimide agar. A new selective plating medium for the selective isolation of *Pseudomonas aeruginosa*. *Japanese J Microbiol*.**14**: 65-72.

Gupta V, Datta P and Chander J (2006). Prevalence of metallo -b lactamase (MBL) producing *Pseudomonas* spp. and *Acinetobacter* spp. in a tertiary care hospital in India. *J Infect.* **52**:311-314.

Gutierrez O, Juan C, Cercenado E, Navarro F, Bouza E, Coll P, Pérez JL and Oliver A (2007). Molecular Epidemiology and Mechanisms of Carbapenem Resistance in *Pseudomonas aeruginosa* Isolates from Spanish Hospitals. *Antimicrob. Agents Chemother*. 51: 4329–4335.

Guyot JM, Mere J, Bonhoure A and Beaumelle B (2009). Processing of *Pseudomonas aeruginosa* Exotoxin A Is Dispensable for Cell Intoxication. *Infect. Immun.*. **77:**3090-3099.

Hall BG, Salipante SJ and Barlow M (2003). The Metallo-b-Lactamases Fall into Two Distinct Phylogenetic Groups. *J. Mol. Evol*. **57**:249–254.

Hall-Stoodley L and Stoodley P (2005). Biofilm formation and dispersal and the transmission of human pathogens. *Trends Microbiol*. **13**:7-10.

Herzberg O and Fitzgerald PM (2011). Metallo β-Lactamases. Encyclopedia of Inorganic and Bioinorganic Chemistry.

Hirsch EB and Tam VH (2010). Impact of multidrug-resistant *Pseudomonas aeruginosa* infection on patient outcomes. *Expert Rev Pharmacoecon Outcomes Res* **10**: 441-451.

Hocquet D, Plesiat P, Dehecq B, Mariotte P Talon D and Bertrand X (2010). Nationwide investigation of extended-spectrum beta-lactamases, metallo-beta-lactamases, and extended-spectrum oxacillinases produced by ceftazidime-resistant *Pseudomonas aeruginosa* strains in France. *Antimicrob Agents Chemother* **54**: 3512-3515.

Imperi F, Tiburzi F and Visca P (2009). Molecular basis of pyoverdine siderophore recycling in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **106**:20440–20445.

Ivanov IE, Kintz EN, Porter LA, Goldberg JB, Burnham NA and Terri A (2011). Relating the Physical Properties of *Pseudomonas aeruginosa* Lipopolysaccharides to Virulence by Atomic Force Microscopy. *J Bacteriol.* **193**: 1259–1266.

Jacoby GA (2009). AmpC β -lactamases. *Clin. Microbiol. Rev*. **22:**161–182.

Jacoby GA and Munoz-Price LS (2005). The New β–Lactamases. *N Engl J Med*.**352**:380-391.

Jalal S, Ciofu O, Hoiby N, Gotoh N and Wretlind B (2000). Molecular mechanisms of fluoroquinolone resistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Antimicrob. Agents Chemother.* **44**:710–712.

Javiya VA, Ghatak SB, Patel KR and Patel JA (2008). Antibiotic susceptibility patterns of *Pseudomonas aeruginosa* at a tertiary care hospital in Gujarat, India. *Indian J Pharmacol*. **40**:230-234.

Jayakumar S and Appalaraju B (2007). Prevalance of multi and pan drug resistant *Pseudomonas aeruginosa* with respect to ESBL and MBL in a tertiary care hospital. *Indian Journal of pathology and Microbiology*.**50**:922-925.

Jones RN, Biedenbach DJ, Sader HS, Fritsche TR, Toleman MA and Walsh TR (2005). Emerging epidemic of metallo-b-lactamase mediated resistances. *Diagn Microbiol Infect Dis*. **5**:77–84.

Khalifa BHA, Moissenet D, Thien VH and Khedher M (2011). Virulence factors in *Pseudomonas aeruginosa*: mechanisms and modes of regulation. *Ann Biol Clin (Paris)* **69**: 393-403.

Khan JA, Iqbal Z, Rahman SU, Farzana K and Khan A (2008). Prevalence and resistance pattern of *Pseudomonas aeruginosa* against various antibiotics. *Pak J Pharm Sci*. **21**:311–315.

Khosravi Y, Loke MF, Chua EG, Tay ST and Vadivelu J (2012). Phenotypic Detection of Metallo-β-Lactamase in Imipenem-Resistant *Pseudomonas aeruginosa*. *The Scientific World Journal:* **7.**

-type beta-Extended Spectrum and Inhibitor resistant TEM .(Knox JR (1995 .lactamases: mutation, specificity, and three dimensional structure *Antimicrob. Agents Chemother* **39**.2601-2593:

Kohanteb J, Dayaghi M and Motazedian M (2007). Comparsion of biotyping and antibiotyping of *Pseudomonas aeruginosa* isolated from patients with burn wound infection and nosocomial pneumonia in Shiraz, Iran. *Pak J Biol Sci.* **10**:1817-1822.

Kotra LP, Haddad J and Mobashery S (2000). Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance. *Antimicrob. Agents Chemother*. **44**:3249–3256.

Kumar A (2006). Multi Drug Resistance: Another Fatel ICU Concern. *Pulmon* **8**: 84-88.

Kumar SH, De AS, Baveja SM and Gore MA (2012). Prevalence and Risk Factors of Metallo β-lactamase Producing *Pseudomonas aeruginosa* and *Acinetobacter species* in Burns and Surgical Wards in a Tertiary Care Hospital. *J Lab Physicians*. **4**: 39–42.

Laarman AJ, Bardoel BW, Ruyken M, Fernie J, Milder FJ, Strijp JAV and Rooijakkers SH (2012). *Pseudomonas aeruginosa* alkaline protease blocks complement activation via the classical and lectin pathways. *J Immunol.* **188**:386-393.

Lagatolla C, Edalucci E, Dolzani L, Riccio ML, De Luca F, Medessi E, Rossolini GM and Tonin AE (2006). Molecular Evolution of Metallo-β-Lactamase-Producing *Pseudomonas aeruginosa* in a Nosocomial Setting of High-Level Endemicity. *J. Clin. Microbiol.* **44**:2348-2353.

Lau GW, Ran H, Kong F, Hassett DJ and Mavrodi D (2004). *Pseudomonas aeruginosa* pyocyanin is critical for lung infection in mice. *Infect Immun* **72**: 4275–4278.

Lee K, Lim JB, Yum JH, Yong D, Chong Y,Kim JM and Livermore DM (2002). *bla*VIM-2 Cassette-Containing Novel Integrons in Metallo-β-Lactamase Producing *Pseudomonas aeruginosa* and *Pseudomonas putida* Isolates Disseminated in a Korean Hospital *Antimicrob. Agents Chemother.* **46**:1053-1058 .

Lee YK, Yum JH, Shin HB, Rossolini GM and Chong Y (2002). Imipenem-EDTA disk method for differentiation of metallo-β-lactamase-producing clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp. *J. Clin. Microbiol.* **40**:3798–3801.

Lister PD, Wolter DJ and Hanson ND (2009). Antibacterial-Resistant *Pseudomonas aeruginosa*: Clinical Impact and Complex Regulation of Chromosomally Encoded Resistance Mechanisms. *Clin Microbiol Rev* **22:** 582-610.

Livermore DM (2002). Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? *Clin. Infect. Dis*. **34**:634–640.

.lactamases in laboratory and clinical resistance-β .(Livermore DM (1995*Clin Microbiol Rev* **8**.584-557:

Livermore DM (1998). β-Lactamase-mediated resistance and opportunities for its control. *J. Antimicrob.Chemother*.**41**: 25–41.

Livermore DM and Woodford N (2000). Carbapenemases: a problem in waiting?" *Current Opinion in Microbiology*. **3**:489–495.

Masaadeh HA and Jaran AS (2009). Incident of *Pseudomonas aeruginosa* in Post-Operative Wound Infection. *Am. J. Infect. Dis*. **5**: 1-6.

Mavrodi DV, Bonsall RF, Delaney SM, Soule MJ, Phillips G and Thomashow LS (2001). Functional Analysis of Genes for Biosynthesis of Pyocyanin and Phenazine-1-Carboxamide from *Pseudomonas aeruginosa* PAO1. *J Bacteriol*. **183**: 6454–6465.

Mesaros N, Nordmann P, Plesiat P, Roussel-Delvallez M, Van Eldere J, Glupczynski Y, VanLaethemY, Jacobs F, Lebecque P, Malfroot A, Tulkens PM and Van Bambeke F (2007). *Pseudomonas aeruginosa*: resistance and therapeutic options at the turn of the new millennium. *Clin Microbiol Infect* **13**: 560–578.

Micek ST, Lloyd AE, Ritchie DJ, Reichley RM, Fraser VJ and Kollef MH (2005). *Pseudomonas aeruginosa* bloodstream infection: importance of appropriate initial antimicrobial treatment. *Antimicrob. Agents Chemother.* **49:**1306–1311.

Migliavacca R, Docquier JD, C. Mugnaioli C, Amicosante G, Daturi R, Lee K, Rossolini GM and Pagani L (2002). Simple microdilution test for detection of metallo-β-lactamase production in *Pseudomonas aeruginosa*. *J. Clin. Microbiol*. **40:**4388-4390.

Mishra SK, Acharya J, Kattel HP, Koirala J, Rijal BP and Pokhrel BM (2012). Metallo-beta lactamase Producing Gram-negative Bacterial isolates. *J Nepal Health Res Counc* **10**:208-213.

Mittal R, Aggarwal S, Sharma S, Chhibber S and Harjai K (2009). Urinary tract infections caused by *Pseudomonas aeruginosa*: a minireview. *J Infect Public Health* **2**: 101–111.

Mohanasoundaram KM (2011). The Antimicrobial Resistance Pattern in the Clinical Isolates of *Pseudomonas aeruginosa* in a Tertiary Care Hospital; 2008–2010 (A 3 Year Study) *Journal of Clinical and Diagnostic Research*. **5**: 491-494.

Moya B, Dotsch A, Juan C, Blazquez J, Zamorano L, Haussler S and Oliver A **(**2009). Beta lactam resistance response triggered by inactivation of a nonessential penicillin-binding protein. *PLoS Pathog*. **5:**e1000353.

Ogundipeju OO and Nwobu RAU (2004). Occurrence of *Pseudomonas aeruginosa* in post-operative wound infection. *Pak. J. Med.***20**: 187-191.

Ohara T and Itoh K (2003). Significance of *Pseudomonas aeruginosa* colonization of the gastrointestinal tract. *Intern. Med*. **42:**1072–1076.

Olayinka AT, Onile BA and Olayinka BO (2004). Prevalence of Multi-Drug Resistant (Mdr) *Pseudomonas Aeruginosa* Isolates In Surgical Units Of Ahmadu Bello University Teaching Hospital, Zaria, Nigeria: An Indication For Effective Control Measures *Annals of African Medicine* **3**: 13-16.

Pal RB, Rodrigues M and Datta S (2010). Role of *Pseudomonas* in nosocomial infections and biological characterization of local strains. *J Biosci Tech.* **1**:170-179.

Palzkill T (2013). Metallo-β-lactamase structure and function. *Ann. N. Y. Acad. Sci.* **1277***:* 91–104.

Peleg AY, Franklin C, Bell JM and Spelman D (2005). Dissemination of the Metallo-b-Lactamase Gene *bla*IMP-4 among Gram-Negative Pathogens in

a Clinical Setting in Australia Clinical Infectious Diseases. *Clin Infect Dis.***41**:1549–1556.

Perez-Llarena FJ and Bou G (2009). β-lactamase inhibitors: the story so far. *Curr. Med. Chem.* **16:** 3740–3765.

Picao RC, Andrade SS, Nicoletti AG, Campana EH, Moraes GC, Mendes RE and Gales AC (2008) Metallo-β-Lactamase Detection: Comparative Evaluation of Double Disk Synergy versus Combined Disk Tests for IMP, GIM-, SIM-, SPM-, or VIM- Producing Isolates. *J. Clin. Microbiol* **46**: 2028- 2037.

Pier GB (2007). *Pseudomonas aeruginosa* lipopolysaccharide: a major virulence factor, initiator of inflammation and target for effective immunity. *Int J Med Microbiol.* **297**: 277-295.

Pitout JDD, Gregson DB, McClure JA, Le P and Church DL (2005). Detection of *Pseudomonas aeruginosa* Producing Metallo-β-Lactamases in a Large Centralized Laboratory. *J Clin Microbio* **43**: 3129–3135.

Poirel L, Rotimi VO and Mokaddas E M **(**2001). VEB-1-like extended spectrum beta-lactamases in *Pseudomonas aeruginosa*, Kuwait. *Emerg. Infect.Dis.* **17:**468–470.

Prakash HP, Belodu R, Karangate N, Sonth S, Anitha MR and Vijayanath V (2012). Antimicrobial susceptibility pattern of *Pseudomonas aeruginosa* strains isolated from clinical sources. *Journal of Pharmaceutical and Biomedical Sciences* **14**:1065-1069.

Qarah S, Cunha BA, Dua P and Lessnau K (2009). *Pseudomonas aeruginosa* infections. http://emedicine.medscape.com/article/226748-overview

Queenan AM and Bush K (2007). Carbapenemases: the Versatile -β-Lactamases. *Clin Microbiol Rev* **20**: 440-458.

Rakesh MR, Govind NL, Kalpesh M, Rosy P, Kanu P and Vegad MM (2012). Antibiotic Resistance Pattern in *Pseudomonas aeruginosa* Species Isolated at a Tertiary Care Hospital, Ahmadabad. *Natl J Med Res*, **2**: 156-159.

Ramana BV and Chaudhury A (2012). Antibiotic resistance pattern of *Pseudomonas aureuginosa* isolated from healthcare associated infections at a tertiary care hospital. *J Sci Soc.* **39**:78-80.

Ramphal R, Small PM, Shands JW, Fischlschweiger W and Small PM (1980). Adherence of *Pseudomonas aeruginosa* to Tracheal Cells Injured by Influenza Infection or by endotracheal Intubation. *Infect. Immun.***27**:614-619.

Ranjan KP, Ranjan N, Bansal S and Arora DR (2010). Prevalence of *Pseudomonas aeruginosa* in Post-operative Wound Infection in a Referral Hospital in Haryana. *India.J Lab Physicians*. **2**: 74–77.

Rashid A, Chowdhury A, Rahman S, Begum S and Muazzam N (2007). Infections by *Pseudomonas aeruginosa* and Antobiotic Resistance pattern of Isolates from Dhaka Medical College Hospital Bangladesh. *J Med Microbiol* **1**: 48-51.

Rijal D (2012). Prevalanve of *Pseudomonas aeruginosa* among the indoor patients of national institute of neurological and allied sciences and its antibiotic susceptibility profile.M.Sc. Dissertation submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu, .50-age 45P .Nepal

Ryder C, Byrd M and Wozniak DJ (2007). Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. *Curr Opin Microbiol.* **10**:644-648.

Sauvage E, Kerff F, Terrak M,Ayala JA and Charlier P (2008). The penicillin binding proteins: structure and role in peptidoglycan biosynthesis. *FEMS Microbiol. Rev.* **32:** 234–258.

Shah J and Wretlind B (1998). Mechanism of Quinolone resistance in Clinical strains of *Pseudomonas aeruginosa. Microb. Drug Resist.* **4**:257-261.

Shankar EM, Mohan V and Premalatha G (2005). Bacterial etiology of diabetic foot infections in South India. *Eur J Intern Med.* **16**:567-570.

Slama KB, Gharbi S, Jouini A, Maarouf M, Fendri C, Boudabous A and Gtari M (2011). Epidemiology of *Pseudomonas aeruginosa* in Intensive Care Unit and Otolaryngology Department of a Tunisian Hospital. *Afr. J. Microbiol. Res.* **5**:3005-3011.

Smitha S, Lalitha P, Prajna VN and Srinivasan M (2005). Susceptibility trends of *Pseudomonas* species from corneal ulcers. *Indian J Med Microbiol* **23**:168-171.

Takesue Y, Yokoyama T, Akagi S, Ohge H, Imamura Y, Murakami Y and Sueda T **(**2002). Changes in the intestinal flora after the administration of prophylactic antibiotics to patients undergoing a gastrectomy. *Surg. Today* **32:**581–586.

Tam VH, Schilling AN, LaRocco MT, Gentry LO, Lolans K, Quinn JP and Garey KW (2007). Prevalence of AmpC over-expression in bloodstream isolates of *Pseudomonas aeruginosa*. *Clin. Microbiol. Infect*. **13**:413–418.

Thuong M, Arvaniti K, Ruimy R, Salmoniere P, Scanvic-Hameg A, Lucet JC and Regnier B **(**2003). Epidemiology of *Pseudomonas aeruginosa* and risk factors for carriage acquisition in an intensive care unit. *J. Hosp. Infect*. **53**:274–282.

Todar K (2012). Todar's online Textbook of Bacteriology.

Toutain CM, Zegans ME and O'Toole GA (2005). Evidence for two flagellar stators and their role in the motility of *Pseudomonas aeruginosa*. *J Bacteriol* **187**: 771-777.

Tripathi P, Banerjee G, Saxena S Gupta MK and Ramteke PW (2011). Antibiotic resistance pattern of *Pseudomonas aeruginosa* isolated from patients of lower respiratory tract infection. *Afr. J. Microbiol. Res*. **5**:2955-2959.

Van DC and Iglewski BH (1998). Cell-to-Cell Signaling and *Pseudomonas aeruginosa* Infections. *Emerg Infect Dis* **4**:551-560.

Varaiya A, Kulkarni N, Kulkarni M, Bhalekar P and Dogra J (2008). Incidence of metallo beta lactamase producing *Pseudomona aeruginosa* in ICU patients. *Ind J Med Res*. **127**:398-402.

Vasil ML (1986). *Pseudomonas aeruginosa*: biology, mechanisms of virulence, epidemiology. *J Pediatr.***108**:800-805.

Vasil ML, Stonehouse MJ, Vasil AI, Wadsworth SJ, Goldfine H, Bolcome RE and Chan J (2009). A Complex Extracellular Sphingomyelinase of *Pseudomonas aeruginosa* Inhibits Angiogenesis by Selective Cytotoxicity to Endothelial Cells. *PLoS Pathog* **5**: e1000420.

Vincent JL (2000). Microbial resistance: lesson from the EPIC study. European Prevalence of Infection. *Intensive Care Med*. **26**: S3-8.

Visca P, Imperi F and Lamont IL (2007). Pyoverdine siderophores: from biogenesis to biosignificance. *Trends Microbiol* **15**:22–30.

Antibiotics: Action, Origins, Resistance. ASM press, .(Walsh C (2003 .Washington, D.C

Walsh TR, Bolmstrom A, Qwarnstrom A and Gales A (2002). Evaluation of a new Etest for detecting metallo-β-lactamases in routine clinical testing. *J. Clin. Microbiol.* **40**: 2755–2759.

Walsh TR, Toleman ML, Poiral L and Nordmann P (2005). Metallo-β-lactamases: The quiet before storm? *Clin Microbiol. Rev.* **18**: 306-325.

Zavascki AP, Barth AL, Gaspareto PB, Goncalves ALS, Moro ALD, Fernandes JF and Goldani LZ (2006). Risk factors for nosocomial infections due to *Pseudomonas aeruginosa* producing metallo-b-lactamase in two tertiary-care teaching hospitals. *J Antimicrob Chemother*. **58**:882–885.

Zhang S, McCormack FX, Levesque RC, O'Toole GA and Lau GW (2007) The Flagellum of *Pseudomonas aeruginosa* Is Required for Resistance to Clearance by Surfactant Protein A. PLoS ONE **2**: e564. doi:10.1371/journal.pone.0000564.

APPENDIX A

LIST OF EQUIPMENTS AND MATERIALS

A. EQUIPMENT:

Autoclave, Incubator, Hot air oven, Microscope, Refrigerator, Weighing machine, Water bath, Gas burners, Glass wares, Inoculating wire and loops

B. MICROBIOLOGICAL MEDIA:

Blood Agar	Hugh and Leifson Media
Chocolate Agar	Sulphur Indole Motility Media
Mac conkey Agar	MRVP Broth
Mueller Hinton Agar	Triple Sugar Iron Agar
Mannitol Salt Agar	Urea Agar Base
Mueller Hinton Broth	Simmon's Citrate Agar

C. CHEMICALS AND REAGENTS:

Catalase Reagent (3% H_2O_2), Oxidase Reagent (1% Tetramethyl p-phenylene diamine dihydrochloride), Kovac's Reagent, Barritt's Reagent (40% KOH, 5% α-napthol in a ratio of 1:3), Barium Chloride, Conc. H_2SO_4, Ethylene Diamine Tetra Acetate (EDTA), Glycerol, Gram's reagent

D. ANTIBIOTIC DISKS

The antibiotics that will be required for the susceptibility tests are as follows:

Amikacin (30µg)

Ceftazidime (30µg)

Ciprofloxacin (5µg)

Cefepime (30µg)

Gentamycin (10µg)

Imipenem (10µg)

Ofloxacin (5µg)

Piperacillin/Tazobactam (100/10 µg)

APPENDIX B

METHODOLOGY

1. Disk diffusion method for the antimicrobial susceptibility testing

A. Preparation of 0.5 Mc Farland Standard

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

B. Preparation of inoculum

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

C. Inoculation of Agar plates

a. The agar plates, canister of discs are brought to room temperature before use. It should be made sure that the agar surface doesn't have any moisture, if so should be dried by keeping it in incubator.

b. Using a sterile swab, a plate of Mueller Hinton Agar is inoculated with the bacterial suspension using carpet culture technique. The plate is left for about 5 minutes to let the agar surface dry.

c. Using sterile forceps, appropriate antimicrobial discs (6mm diameter) is placed, evenly distributed on the inoculated plates, not more than 6 discs are placed on a 90 mm diameter Petri plate.

d. After overnight incubation, the plates are examined to ensure confluent growth. Using a measuring scale, the diameter of each zone of inhibition (in mm) is measured and results interpreted accourdingly.

D. Quality Control

a. QC strains

Escherichia coli ATCC 25922, *Staphylococcus aureus* ATCC 25923

b. Monitoring Accuracy

i. Running AST for QC strains side by side with pathogenic bacteria
ii. Monitoring the expiry date of antibiotic discs and MHA
iii. Comparing zone size with CLSI QC table.

2. Combined Disk Assay for MBL detection

Test organisms were inoculated onto plates of Mueller-Hinton agar. Two 10µg-imipenem disks were placed on the plate, and 750µg of 0.5 M EDTA solution of pH 8 was added to one of them. The inhibition zones of the imipenem and imipenem-EDTA disks were compared after 16 to 18 h of incubation in air at 35°C. All of the MBL-positive isolates were well separated from MBL-negative isolates by the criterion of a ≥7-mm increase of inhibition zone with the disks to which 750 µg of EDTA was added.

APPENDIX C

MICROBIOLOGICAL PROFILE OF PATIENT

Day 1 (… …/… …/… … … …)

Specimen: … … … … … … … … …

Time of sample collection: … … … … … … … … …

Mode of collection: … … … … … Receiving time at the Lab: … … …

Direct Microscopic Examination (if necessary):

(1) … … … … … … … … …

Incubation: 1) Aerobic 2) Anaerobic 3) Microaerophilic

Incubation temperature: … … … … … … … Incubation time: … … … … …

Culture on: 1) … … … … 2) … … … … 3) … … … …

Day 2 (… …/… …/… … …. …)

Reading of culture plates:

Media Used	Features	Shape	Size	Color	Texture	Opacity	Consistency

Gram staining result: … … … … … … … … … … … … …

Catalase: … … … … … … … … … … … … Oxidase: … … … … … …

Coagulase: … … … … … … … … … … … Others: … … … … … … …

Provisional Identification of Organism: … … … … … … … … … … … … …

Inoculation on: 1) … … … … … … 2) … … … … … … 3) … … … … … …

Day 3 (… …/… …/… … … …)

BIOCHEMICAL TEST

TSI: … … … … … … SIM: … … … … … …

Citrate: … … … … … … Urea: … … … … … …

Others: … … … … … …

Organism identified as: … … … … … …

Antibiotic sensitivity test method: Kirby-Bauer method

Antibiotics used	***Zone of inhibition (mm)***	***Intrepretation***

Day 4 (… …/… …/… … … …)

Combination disk used	***Increase in size of zone of inhibition (mm)***	***Intrepretation***

Day 5 (… …/… …/ … … … …)

Production of MBL: Positive Negative

APPENDIX D

COMPOSITION AND PREPARATION OF DIFFERENT CULTURE MEDIA

A. Composition and preparation of different culture media

Blood Agar

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

Ingredients	**gm/liter**
Beef heart infusion	500.0
Tryptose	10.0
Sodium chloride	5.0
Agar	15.0

42.5 grams of the blood agar base medium was suspended in 1000 ml distilled water and sterilized by autoclaving at 121^0C (15 lbs pressure) for 15 minutes. After cooling to 40-50^0C, 50 ml of sterile defibrinated sheep blood was added aseptically and mixed well before pouring.

MacConkey Agar

Ingredients	**gm/liter**
Peptone	4.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral Red	0.075
Agar	12.0
Final pH (at 250C)	7.4±0.2

Direction: 52 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 ^{0}C (15 lbs pressure) for 15 minutes. The medium was poured into sterile petriplates.

Nutrient Agar

Ingredients	**gm/l**
Peptone	5
Sodium Chloride	5
Beef extract	1.5
Yeast extract	1.5
Agar	15
Final pH	7.2

Direction: 37 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^{0}C (15 lbs pressure) for 15 minutes.

CLED Agar

Ingredients	**gm/liter**
Peptone	4g/l
'Lab Lemco' powder	3g/l
Tryptone	4g/l
Lactose	10g/l
L-Cysteine	128 mg/l
Bromothymol blue	20 mg/l
Agar	15g/l

Mueller Hinton Agar

Ingredients	gm/liter
Beef, Infusion form	300.0
Casein Hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH (at 250C)	7.4±0.2

Direction: 38 grams of the medium was suspended in 1000 ml distilled water and the medium was warmed to dissolve completely. It was sterilized by autoclaving at 121ºC (15 lbs pressure) for 15 minutes and poured into sterile petriplates.

B. Composition and preparation of different biochemical tests media

MR-VP Medium (Hi-Media laboratories)

Ingredients	gm/litre
Peptone	5.0
Dextrose	5.0
Dipotassium Phosphate	5.0
Final pH (at 250C)	6.9±0.2

Direction: 15 gm powder was dissolved in 1000 ml of distilled water & mixed well. 3 ml of medium was distributed in each test tube and autoclaved at 1210C for 15 minutes.

Simmon's Citrate Agar

Ingredients	gm/l
Ammonium dihydrogen phosphate	1
Dipotassium hydrogen phosphate	1
Sodium chloride	5
Sodium citrate	2
Magnesium sulphate	0.2

Bromothymol blue	0.08
Agar	15
Final pH	6.9

Direction: 23 grams of the medium was dissolved in 1000ml of distilled water. 3ml medium was distributed in test tubes and sterilized by autoclaving at 1210C for 15 minutes. After autoclaving, tubes containing medium were tilted to form slant.

SIM(Sulphide Indole Motility) Agar

Ingredients	**gm/l**
Peptone	30
Beef extract	3
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.025
Agar	3
Final pH	7.3

Direction: 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 121ºC for 15 minutes.

Triple Sugar Iron Agar (TSI)

Ingredients	**gm/litre**
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 250C)	7.4±0.2

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

Urea Broth Base

Ingredients	**gm/litre**
Monopotassium phosphate	9.1
Dipotassium phosphate	9.5
Yeast extract	0.1
Phenol red	0.01
Sterile 40% urea solution	5ml

Direction: As directed by manufacturing company, 1.87 grams of the medium was suspended in 95 ml of distilled water and sterilized by autoclaving at 1210C for 15 minutes. After cooling to about 550C, 5 ml of sterile urea solution was added aseptically, mixed well and distributed 5 ml amount in sterile test tubes.

C. Composition and preparation of different staining and test reagents

For Gram's Stain

(a) Crystal Violet solution

Crystal Violet	20.0 g
Ammonium Oxalate	9.0 g
Ethanol or Methanol	95 ml
Distilled Water (D/W) to make 1 litre	

Direction: In a clean piece of paper, 20 gm 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 gm of ammonium oxalate dissolved in 200 ml of D/W was added. Final volume was made 1 litre by adding D/W.

(b) Lugol's Iodine

Potassium Iodide	20.0 g
Iodine	10.0 g
Distilled Water	1000 ml

Direction: To 250 ml of D/W, 20 gm of potassium iodide was dissolved. Then 10 gm of iodine was mixed to it until it was dissolved completely. Final volume was made 1 litre by adding D/W.

(c) Acetone-Alcohol Decoloriser

Acetone	500 ml
Ethanol (Absolute)	475 ml
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 Stain)

Safranin	10.0 g
Distilled Water	1000 ml

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

Biochemical Test Reagents

(a) Catalase Reagent (For Catalase test)

Hydrogen peroxide	3 ml
Distilled Water	97 ml

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

(b) Oxidase Reagent (impregnated in a Whatman's No. 1 filter paper) (For Oxidase Test)

Tetramethyl *p*-phenylene diamine dihydrochloride (TPD)	1 gm
Distilled Water	100 ml

Direction: This reagent solution was made by dissolving 1 gm of TPD in 100 ml D/W. To that solution strips 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.

(c) Kovac's Indole Reagent (For Indole Test)

Isoamyl alcohol	30 ml
p-dimethyl aminobenzaldehyde	2.0 g
Conc. Hydrochloric acid	10 ml

Direction: In 30 ml of isoamylalcohol, 2 g of *p*-dimethyl aminobenzaldehyde was dissolved and transferred to a clean brown bottle. Then to that, 10 ml of conc. HCl was added and mixed well.

(d) Methyl Red Solution (For Methyl Red Test)

Methyl red	0.05 g
Ethyl alcohol (absolute)	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.

(e) Barritt's Reagent (For Voges-Proskauer Test)

Solution A

α-naphthol	5.0 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.

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 brown bottle. Then the final volume was made 100 ml by adding D/W.

0.5M EDTA solution

186.1 g EDTA was added to ~900 ml dH20 and start stirring. 20 g NaOH was measured and was kept into 50 ml centrifuge tube, add half (10 g) NaOH was added to EDTA solution. Every few hours few more NaOH was added until EDTA goes into solution. The pH of the solution was maintained 8.0 with 10M NaOH.

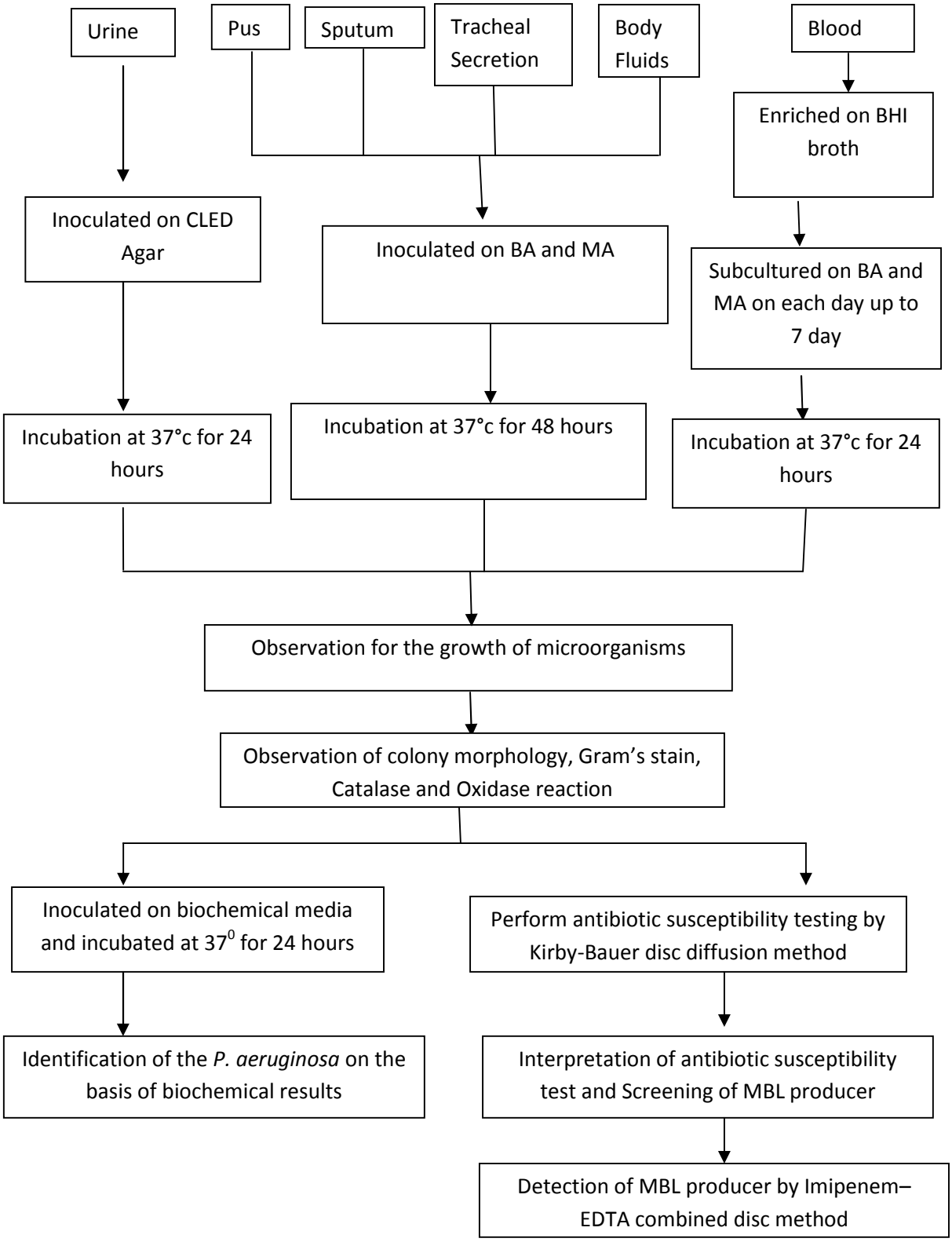
McFarland tube (No. 0.5)

0.5 ml of 0.048 M $BaCl_2$ (1.17% w/v $BaCl_2.H_2O$) was added to 99.5 ml of 0.18 M H_2SO_4 (1% w/v) with constant stirring. The McFarland standard was

thoroughly mixed to ensure that it is evenly suspended. Using matched cuvettes with a 1 cm light path and water as a blank standard, the absorbance was measured in a spectrophotometer at a wavelength of 625 nm. The acceptable range for the turbidity standard is 0.08-0.13. The standard was distributed into screw-cap tubes of the same size and volume as those used to prepare the test inoculum. The tubes were sealed tightly to prevent loss by evaporation and stored protected from light at room temperature. The turbidity standard was then vigorously agitated on a vortex mixer before use. Standards may be stored for up to 6 months, after which time they should be discarded

APPENDIX E

IDENTIFICATION OF *PSEUDOMONAS AERUGINOSA*

Growth at 42^0C	Positive
Gelatin hydrolysis	Positive
Indole	Negative
Methyl Red	Negative
Citrate	Positive
Triple Sugar Iron Agar	Red/Red
Catalase	Positive
Oxidase	Positive
Urease	Negative
Motility	Positive
Gram Stain	Positive
Morphology	Rod
Pigment	Positive
Odour	Positive

Flow chart of Methodology