CHAPTER-I

1. INTRODUCTION

Pseudomonas aeruginosa is a clinically significant pathogen, often causing infections in immuno-compromised and catheterized patients. In fact, *P. aeruginosa* is the epitome of an opportunistic pathogen of humans. The bacterium almost never infects uncompromised tissues, yet there is hardly any tissue that it cannot infect if the tissue defences are compromised in some manner. It causes urinary tract infections, respiratory system infections, infective endocarditis, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections, particularly in patients who are immunosuppressed (CDC, 2004; Chen and Rudoy, 2010; Silby *et al*., 2011). *P. aeruginosa* infection is a serious problem in patients hospitalized with cancer, cystic fibrosis, and burns, the case fatality rate in these patients being nearly 50 percent (Todar, 2011). However, community acquired infections such as otitis externa, varicose ulcers, corneal ulcers resulting from contaminated contact lenses, etc are also associated with *P. aeruginosa* (Greenwood *et al.,* 2002). In addition to causing serious and often life-threatening diseases, these organisms exhibit innate resistance against many antibiotics and can develop new resistance after exposure to antimicrobial agents (Yasar *et al*., 2011).

Pseudomonas aeruginosa is a Gram-negative rod measuring 0.5 to 0.8 µm by 1.5 to 3.0 µm. Almost all strains are motile by means of a single polar flagellum (Victoria *et al*., 2010; Toutain *et al*., 2005). It generally produces bluish-green and yellowish-green colonies, which produce typical grape-like odour because of 2-aminoacetophenone (Moore *et al*., 2011). Its metabolism is respiratory and never fermentative, but it will grow in the absence of O_2 if NO_3 is available as a respiratory electron acceptor. *P. aeruginosa* is oxidase and catalse positive.

Patients admitted to the ICU have been shown to be at particular risk of acquiring infection with a prevalence rate as high as 30% (Craven *et al*., 1988). One of the studies showed that an outbreak of *Pseudomonas aeruginosa* infections was associated with flexible bronchoscopes (Srinivasan *et al*., 2003). The risk of infection in ICU is 5–10

times greater than those acquired in general medical and surgical wards (Vincent *et al*., 1995). Benn *et al* in a review of infective endocarditis from 1984 through 1993 found the increasing incidence of the infective endocarditis due to *P. aeruginosa* (Benn *et al*., 1997). Moreover, long term use of intravenous catheters especially in ICU settings and long term care facilities remain a risk factor and increase the probability of pseudomonas endocarditis (Ahmed, 2001).

Pseudomonas aeruginosa colonization reportedly occurs in more than 50% of humans (Chen and Rudoy, 2010) and is primarily a nosocomial pathogen. Nosocomial infections caused by *P. aeruginosa* are very common; 11-13.8% of all nosocomial infections are due to *P. aeruginosa*, with an even higher rate (13.2-22.6%) of nosocomial infections among patients in an ICU (Driscoll *et* al., 2007). According to the CDC, the overall incidence of *P. aeruginosa* infections in U.S. hospitals averages about 0.4 percent (4 per 1000 discharges), and the bacterium is the fourth most commonly-isolated nosocomial pathogen accounting for 10.1 % of all hospital-acquired infections (Todar, 2011) leading to hospital stay, antibiotics use ,costs and mortality.

Since *P. aeruginosa* is one of the most common flora of the ICUs and due to its long persistence time (on dry inanimate surfaces: 6 hours – 16 months; on dry floor: 5 weeks) (Kramer *et al*., 2006), the chances of infection by this organism increase rapidly. The recurrent patient acquisition of *P. aeruginosa* has been linked to widespread environmental contamination by this bacterium (Fanci *et al*., 2006). *P. aeruginosa* may be acquired through exogenous source, from another person in the hospital (cross infection) or from inanimate objects or substances recently contaminated from another human source (environmental infection).

In the beginning strains of *P. aeruginosa* were sensitive to various classes of antibiotics such as aminoglycosides, cephalosporins, carbapenems and fluoroquinolones but they tend to develop resistance to the antibiotics as time passed (Landman *et al*., 2007). Several studies have documented increasing resistance rates in P. aeruginosa to fluoroquinolones, cephalosporins and carbapenems, particularly among ICU isolates (CDC, 2004; Streit *et al*., 2004; Jones *et al*., 2004). In these reports, carbapenem and

ciprofloxacin resistance has been noted in about 20% and 30% of the isolates, respectively.

Of most immediate concern is the accumulation of mutations in genes of *P. aeruginosa* affecting simultaneously the bacterial sensitivity to different classes of antibiotics resulting in the evolution of MDR bacteria. The prevalence of MDR *P. aeruginosa* has increased in the last years and in some hospitals in USA reaches today up to 30% of the isolates leaving the clinician with few therapeutic alternatives (Bratu *et al*., 2005). The overall prevalence of antibiotic resistant *P. aeruginosa* is increasing, with up to 10% of global isolates found to be multidrug-resistant (Gales *et al*., 2001).

Since *Pseudomonas aeruginosa* is one of the notorious nosocomial pathogens as well as multidrug resistant organism, it should not be under-estimated by any of the hospitals regarding its threatening activities to the patients and health care workers. Despite the increasing significance and frequency of multi-drug resistant *P. aeruginosa* infections, many laboratories as well as clinicians still lack an appreciation of the potential importance of these organisms in hospitals, in part because of the low prevalence rate. The incidence of *P. aeruginosa* in clinical infections and in various natural environments, coupled with the difficulty of diagnosis of this microorganism, has led to an increased interest in its isolation and characterization. The aim of this study was therefore to isolate and characterise the organism from different clinical samples and surface swabs taken from the Intensive Care Units (ICUs) and to assess their prevalence, colony characteristics, biochemical properties and antibiogram so that certain predictions can be made about antibiotics to be prescribed, occurrence of Multidrug resistant *P. aeruginosa* (MDRPA), surface disinfection and the control of pseudomonal infections.

CHAPTER-II

2. OBJECTIVES OF THE STUDY

2.1 General objective

1. To characterize *Pseudomonas aeruginosa* isolated from clinical and surface swab samples taken from ASICU, PSICU and MICU in Shahid Gangalal National Heart Center.

2.2 Specific objectives

1. Isolation and identification of *P. aeruginosa* from different clinical samples collected from patients admitted to the intensive care units of the hospital.
2. To determine the prevalence of *P. aeruginosa* in clinical and surface swab samples in ASICU, PSICU and MICU.
3. To identify the antibiotic susceptibility pattern of the isolated organism.
4. To estimate the burden of the multidrug resistant *P. aeruginosa* in the ICUs.

CHAPTER III

3. LITERATURE REVIEW

3.1 Medical importance of *Pseudomonas aeruginosa*

Bergey's Manual of Systematic Bacteriology (2005) has classified *Pseudomonas aeruginosa* in the phylum Protobacteria and class Gamma Proteobacteria. It is a Gram-negative, aerobic rod belonging to the bacterial family *Pseudomonadaceae.* Like other members of the genus, *P. aeruginosa* is a free-living bacterium, commonly found in soil and water. *P. aeruginosa* has become increasingly recognized as an emerging opportunistic pathogen of clinical relevance. It is now recognized that *Pseudomonas aeruginosa* plays a significant role in the colonization and infection of patients admitted to hospitals.

It is ubiquitous and flourishes as a saprophyte in warm moist situations in the human environment, including sinks, drains, respirators, humidifiers and disinfectant solutions (Gillespie and Hawkey, 2006). Isolation of *P. aeruginosa* from healthy carriers or environmental sites is significant only when there is a risk of transfer to compromised patients, e.g. by nurses' hands or via respirators. Normally, human faecal carrier rate for *P. aeruginosa* is less than 10%; however, carrier rates increase with the length of stay of patients in hospital, reaching 30% after 3 weeks, and this represents a distinct risk of endogenous infection. Colonization is often iatrogenic and associated with prior instrumentation such as catheterization, tracheostomy, etc. Such infections are often extremely difficult for the clinician to treat because of the widespread resistance of these bacteria to the major groups of antibiotics. Various mechanisms of antibiotic resistance have been recognized in these bacteria, and combination therapy is usually required for effective treatment of such infections.

Pseudomonas aeruginosa is a Gram-negative rod measuring 0.5 to 0.8 µm by 1.5 to 3.0 µm. Almost all strains are motile by means of a single polar flagellum (Victoria *et al*., 2010; Toutain *et al*., 2005). The bacterium is ubiquitous in soil and water, and on surfaces in contact with soil or water. Its metabolism is respiratory and never

fermentative, but it will grow in the absence of O_2 if NO_3 is available as a respiratory electron acceptor. Organic growth factors are not required, and it can use more than seventy-five organic compounds for growth. Most strains can grow in a simple medium containing acetate as a source of carbon and ammonium sulfate as a source of nitrogen (Todar, 2011). *P. aeruginosa* can grow in distilled water to greater than 10^6 per ml, which is evidence of its minimal nutritional requirements (Chen, 2005). Growth can occur on simple to complex media between 4°C to 42°C (Lafontaine & Sokol, 1998) with optimum temperature being 38°C without growth factor requirements. It generally produces bluish-green and yellowish-green colonies, which produce typical grape-like odour because of 2-aminoacetophenone (Moore *et al*., 2011). The resistance to antibiotics aspect of this bacterium attributes to the antibiotic resistance plasmids (R factors) contained in them, which are further passed on genetically to the next generation by means of transduction and conjugation. These natural properties of the bacterium undoubtedly contribute to its ecological success as an opportunistic pathogen.

3.2 Epidemiology of *Pseudomonas aeruginosa* in ICUs

Within the hospital, *P. aeruginosa* finds numerous reservoirs: disinfectants, respiratory equipment, food, sinks, taps, toilets, showers and mops. Furthermore, it is constantly reintroduced into the hospital environment on fruits, plants, vegetables, as well by visitors and patients transferred from other facilities. Spread occurs from patient to patient on the hands of hospital personnel, by direct patient contact with contaminated reservoirs, and by the ingestion of contaminated foods and water (Todar, 2011).

Over a period of seven months, 5 (29%) of 17 patients were infected with *P. aeruginosa* genotypes that were also detectable in tap water (Trautmann *et al*., 2001). However, the simple detection of a strain of *P. aeruginosa* in tap water during an outbreak is not, by itself, proof of a causative association. For most of the outbreaks reported in the literature, it is not possible to distinguish between infections of patients from a water source and infections from contamination of the tap from the hands of patients and staff.
Contamination of the hospital environment by *P. aeruginosa* is common, and the bacteria are found in particularly high numbers in drains from sinks and baths (Levin *et al*. 1984; Doring *et al*., 1993). While convincing outbreaks of infection from such sources have

been described, *P. aeruginosa* appears to be more commonly an endogenous rather than an exogenous infection (Gruner *et al*. 1993). Indeed, in one study where 73 isolates were characterized, there appeared to be little similarity between human and environmental strains, supporting the hypothesis that environmental sources of infection are less important than contact with other infected individuals (Orsi *et al.* 1994).

Pseudomonas aeruginosa is one of the most important gram-negative pathogens causing infections in ICUs (Trautmann *et al*., 2005). The last 2 decades have witnessed significant changes in the spectrum of microorganisms causing nosocomial infections. Techniques such as early enteral feeding, use of closed suctioning systems for aspiration of tracheal secretions during mechanical ventilation, and the concept of semi-upright positioning of ventilated patients have contributed to create functional or physical barriers between gastrointestinal flora and the respiratory tract. In spite of these significant changes, *P. aeruginosa* has held a nearly unchanged position in the rank order of pathogens causing ICU-related infections for more than 4 decades. In the newest US National Nosocomial Infections Surveillance (NNIS) system report, *P. aeruginosa* continues to represent the third most frequent organism associated with wound or pulmonary infections, the fourth most frequent organism causing urinary tract infection, and the fifth most frequent organism isolated from blood cultures in cases of septicemia (Richards *et al*., 2000). Studies performed in individual ICUs revealed a significant role of *P. aeruginosa* as a cause of ventilator-associated pneumonia (VAP) (Dupont *et al*., 2001).

A European survey showed that *P. aeruginosa* is one of the most frequent pathogens isolated from ICU-acquired infections (Vincent *et al*., 2000) and data derived from recent multicenter surveillance studies place this microrganism as the first Gram-negative species recovered in ICUs in Belgium (Glupczynski *et al*., 2001) and Italy (Jones *et al*., 2004). Several patient and pathogen-specific risk factors are associated with acquisition of this pathogen in ICUs, such as length of stay, severity of underlying disease and exposure to invasive procedures, on the one hand (Rello *et al*., 2002), and virulence, adherence, and antimicrobial drug resistance on the other (Aloush *et al*., 2006). The hospital environment particularly moist sites are known reservoirs of *P. aeruginosa*

strains, often multidrug resistant (MDR) due to intrinsic and acquired determinants (Deplano *et al*., 2005).

ICUs are generally considered epicenters of multidrug resistant (MDR) organisms. Although one possible explanation of the spread of antibiotic-resistant strains in ICUs is the selection exerted by extensive use of antibiotics, increased spread of MDRPA may be due to transmissions of resistant clones between patients (Meyer *et al*., 2003). Colonization may be the first step of an endogenous infection, while the colonized patients represent a continuous exogenous source of microrganism for colonization/infection of other patients (Bertrand *et al*., 2001).

3.3 Virulence Factors

According to Kipnis *et al* (2006), the types of virulence factors that assist *P. aeruginosa* to initiate colonization and progress into infection are mentioned as follows.

3.3.1 Bacterial cell surface

Flagella: Flagella have a critical role in pathogenesis by tethering and adhering to epithelial cells through binding with a common membrane component, asialoGM1 (Feldman *et al*., 1998). However, flagella are also very immunogenic, rendering their presence a liability for *P. aeruginosa* after successful colonization. This is why *P. aeruginosa* is capable of adapting by selecting aflagellar mutants to evade host response in chronic infections.

Pili: The twitching motility due to pilli facilitates the rapid colonization of the airway. Like flagella, pili are crucial to the adhesion phase of colonization through binding to asialoGM1 of the epithelial cell membrane.

LPS: LPS associates a hydrophobic domain, Lipid A inserted into the phospholipid bilayer to a hydrophilic tail composed of the core polysaccharide and the O-specific polysaccharide. The variable O-specific polysaccharide chains are the basis of antigenic identification of *P. aeruginosa* serotypes. This immunogenicity makes them obvious targets for immunotherapy.

3.3.2 Secreted virulence factors

Pyocyanin: Pyocyanin is a blue pigment metabolite of *P. aeruginosa* that has been shown to have numerous pathogenic effects such as increasing IL-8, depressing host-response, and inducing apoptosis in neutrophils.

Pyoverdin: Pyoverdin is a siderophore, a small molecule chelating iron from the environment for use in *P. aeruginosa* metabolism. Pyoverdin regulates the secretion of other *P. aeruginosa* virulence factors, exotoxin A and an endoprotease and its own secretion.

Alkaline protease

Protease IV

Elastase

Phospholipase C

Exotoxin A

3.4 Pathogenesis

For an opportunistic pathogen such as *P. aeruginosa*, the disease process begins with some alteration or circumvention of normal host defences. The pathogenesis of *Pseudomonas* infections is multi-factorial, as suggested by the number and wide array of virulence determinants possessed by the bacterium (Driscoll *et al.*, 2007; Lodise, *et al.*, 2007; Mcphee, *et al.,* 2003; Mittal, *et al.*, 2006). Multiple and diverse determinants of virulence are expected in the wide range of diseases caused, which include septicaemia, urinary tract infections, pneumonia, chronic lung infections, endocarditis, dermatitis, and osteochondritis.

The different stages of pathogenesis are as follows.

Colonization: Although colonization usually precedes infections by *Pseudomonas aeruginosa*, the exact source and mode of transmission of the pathogen are often unclear because of its ubiquitous presence in the environment. It is sometimes present as part of the normal flora of humans, although the prevalence of colonization of healthy individuals outside the hospital is relatively low (estimates range from 0 to 24 percent depending on the anatomical locale) (MCC, 2004).

Flagella have a critical role in pathogenesis by tethering and adhering to epithelial cells through binding with a common membrane component, asialoGM1 (Feldman *et al*., 1998). The pili of *P. aeruginosa* will adhere to the epithelial cells of the upper respiratory tract and, by inference, to other epithelial cells as well (Beatson *et al.*, 2002). Tissue injury may also play a role in colonization of the respiratory tract, 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). This has been called opportunistic adherence, and it may be an important step in *Pseudomonas* keratitis and urinary tract infections, as well as infections of the respiratory tract.

The mucoid exopolysaccharide produced by *P. aeruginosa* is a repeating polymer of mannuronic and glucuronic acid referred to as alginate. Alginate slime forms the matrix of the *Pseudomonas* biofilm which anchors the cells to their environment and in medical situations it protects the bacteria from the host defenses such as lymphocytes, phagocytes, the ciliary action of the respiratory tract, antibodies and complement. Biofilm mucoid strains of *Pseudomonas* are also less susceptible to antibiotics than their planktonic counterparts (Leid, 2009).

Invasion: The ability of *P. aeruginosa* to invade tissues depends upon production of extracellular enzymes and toxins that break down physical barriers and damage host cells, as well as resistance to phagocytosis and the host immune defenses.

Two extracellular proteases have been associated with virulence that exert their activity at the invasive stage: elastase and alkaline protease. Elastase has several activities that relate to virulence. The enzyme cleaves collagen, IgG, IgA, and complement. It also lyses fibronectin to expose receptors for bacterial attachment on the mucosa of the lung. Elastase disrupts the respiratory epithelium and interferes with ciliary function. Alkaline protease interferes with fibrin formation and will lyse fibrin. Elastase and alkaline protease together are also reported to cause the inactivation of gamma interferon (IFN) and tumor necrosis factor (TNF).

The *Pseudomonas* blue pigment, pyocyanin, impairs the normal function of human nasal cilia, disrupts the respiratory epithelium, and exerts a proinflammatory effect on

phagocytes (Salyers & Whitt, 1994). A derivative of pyocyanin, pyochelin, is a siderophore that is produced under low-iron conditions to sequester iron from the environment for growth of the pathogen (Davis *et al.*, 1996). It could play a role in invasion if it extracts iron from the host to permit bacterial growth in a relatively iron-limited environment.

Dissemination: Blood stream invasion and dissemination of *Pseudomonas* from local sites of infection is probably mediated by the same cell-associated and extracellular products responsible for the localized disease, although it is not entirely clear how the bacterium produces systemic illness. *P. aeruginosa* is resistant to phagocytosis (Beatson *et al.*, 2002) and the serum bactericidal response due to its mucoid capsule and possibly LPS (Ohno *et al.*, 1995). The proteases inactivate complement, cleave IgG antibodies, and inactivate IFN, TNF and probably other cytokines. The Lipid A moiety of *Pseudomonas* LPS (endotoxin) mediates the usual pathologic aspects of Gram-negative septicemia, e.g. fever, hypotension, intravascular coagulation, etc. It is also assumed that *Pseudomonas* Exotoxin A exerts some pathologic activity during the dissemination stage.

3.5 *Pseudomonas aeruginosa* related nosocomial infections

P. aeruginosa is the commonest cause of most of the NIs in the hospital. It accounts for about 11% of all nosocomial infections (Bennett, 1974). Since it causes disease primarily in persons whose health is compromised in some manner, it is considered an opportunistic pathogen. Mechanical ventilation, for instance, predisposes patients to pneumonia caused by *P aeruginosa*. Likewise, the presence of a urinary catheter is associated with an increased risk of urinary tract infections. Patients with cancer who have neutropenia resulting from chemotherapy or hematologic malignancies are prone to bacteremia, and burn patients often experience wound infections (Hauser and Sriram, 2005).This non-fermentative multidrug resistant bacterium can survive for long periods in equipments around the patients therefore is responsible for causation of many device associated infections in the hospitalized patients especially in the intensive care unit. It is the commonest pathogen causing VAP as well as bacteraemia, UTIs, etc in the ICU patients (Pollack, 2000). Rello *et al.*, 1996 reported that about 40-50% of the mortality of intubated patients in hospital is caused mainly due to infection by *P. aeruginosa*.

Infections due to *P. aeruginosa* are seldom encountered in healthy adults but in last two decades the organism has become increasingly recognized as the etiological agent in a variety of serious infection in hospitalized patients with impaired immune defense (Harris *et al.*, 2002). Despite advance in medical and surgical care and introduction of wide variety of antimicrobial agents against anti-pseudomonal activities, life threatening infection caused by *P. aeruginosa* continue to cause complications in hospital acquired infections (Mayhall, 1996).

3.5.1 Respiratory infections: *P. aeruginosa* is a major causative microorganism of nosocomial respiratory infections. Importantly, immunocompromised patients are at increased risk for *P. aeruginosa* infection, and it is the predominant cause of morbidity and mortality in patients with cystic fibrosis (Burns *et al*., 2001; Emerson *et al*., 2002; West *et al*., 2002). *P. aeruginosa* is a frequently identified pathogen in patients with ventilator- associated pneumonia (a severe complication of intensive care), and has a high mortality rate compared with other pathogens (34–48%) (Cook *et al*., 1998). According to Trouillet *et* al., 2002, ventilator-associated pneumonia (VAP) is defined as any lower respiratory tract infection that developed after a patient received mechanical ventilation for 2 days. VAP caused by *P. aeruginosa* remains a severe and dreaded complication (Fagon *et al*., 1989; Crouch *et al*., 1996). Patients with VAP due to *Pseudomonas aeruginosa* have an attributable mortality exceeding 40% (Fagon *et al*., 1993). Additionally, *P. aeruginosa* is associated with exacerbations of chronic obstructive pulmonary disease (Murphy *et al*., 2008).

3.5.2 Urinary tract infections: *P. aeruginosa*, an opportunistic pathogen, is the third most common pathogen associated with nosocomial urinary tract infections and the virulence of this organism is due to its ability to produce quorum-sensing (QS) signal molecules and form biofilms, which are usually resistant to conventional antibiotics and host immune responses (Bala *et al*., 2011). Primary infection with *P. aeruginosa* is uncommon (Siegel *et al*., 1973). This organism may easily enter the urinary tract when catheters are introduced for diagnostic or therapeutic reasons or as a consequence of inappropriate antibiotic prophylaxis (Kunin, 1979). Urinary tract infections (UTI) caused by *P. aeruginosa* are related to urinary tract catheterization, instrumentation or surgery.

Pseudomonal infection accounts for about 12 % of all infections of this type (Todar, 2011).

3.5.3 Wound infections: *P. aeruginosa* is an opportunistic pathogen found as part of the normal flora of the human skin (Larson *et al*., 2002). When the host is immunocompromised, as in the case of a thermal burn or surgical wound, this opportunistic bacterium can quickly colonize and infect the burn and wound sites. In case studies of burn patients who developed *P. aeruginosa* septicemia, the mortality rate was >75% (Holder, 1985; Wurtz *et al*., 1995). A recent study carried out in a state of India revealed that *P. aeruginosa* accounted the highest prevalence rate among the total wound infections (29.6%) (Ranjan *et al*., 2010).

3.5.4 Heart and blood stream infections: Nosocomial bloodstream infections have become a common occurrence in modern medicine. Over 250,000 of these infections occur annually in the United States at a rate of 1.5 to 6.8 events per 1000 central line days (Pittet *et al*., 1997; Edwards *et al*., 2007). In this setting, *P. aeruginosa* is a frequently identified pathogen, accounting for approximately 20% of nosocomial bloodstream infections caused by gram-negative bacteria (Wisplinghoff *et al*., 2004). In addition to being common, *P. aeruginosa* bloodstream infections (PABSIs) portend dire outcomes for patients. In one of the studies conducted in the Western part of Nepal the BSI rate was found out to be 8.3% (Easow *et al*., 2010). In a recent large multicenter study, PABSI was associated with crude mortality rates of 39% in all patients and 48% in intensive care unit patients (Wisplinghoff *et al*., 2004). Bacterial attributes predisposing to mortality include a high degree of intrinsic virulence (Roy-Burman *et al*., 2001; Hauser *et al*., 2002), widespread antibiotic resistance (NNIS, 2004), and the fact that the organisms incur minimal fitness costs with multiple resistance mutations (Hocquet *et al*., 2007). Infective endocarditis (IE) is associated with high morbidity and mortality rates. The in-hospital mortality rate report for patients with IE is 15% to 20% with a 1-year mortality rate approaching 40% (Cabell *et al*., 2002). *P. aeruginosa* is one of the most common organisms causing such disease (Morpeth *et al*., 2007).

3.5.5 Endocarditis: *P. aeruginosa* infects heart valves of IV drug users and prosthetic heart valves. The organism establishes itself on the endocardium by direct invasion from the blood stream. Pseudomonas endocarditis may produce intractable congestive heart failure, large systemic arterial amboli, cardiac valve ring abscesses and high grade conduction disturbance. Brain abscess, cerebrities and mycotic ancurysm sometimes occur in *P. aeruginosa* endocarditis.

3.5.6 Central nervous system infections: *P. aeruginosa* can cause inflammation of the tissues covering the brain and spinal cord (meningitis) and brain abscesses. These infections may result from brain injury or surgery, the spread of infection from other parts of the body, or bacteremia. Clinical signs of pseudomonal meningitis are indistinguishable from bacterial meningitis; except for the hallmark EG (ecthyma gangrenosum) skin lesions (Chen and Rudoy, 2010).

3.5.7 Gastro-intestinal infections: *P. aeruginosa* can produce disease in any part of the gastrointestinal tract from the oropharynx to the rectum. As in other forms of *Pseudomonas* disease, those involving the GI tract occur primarily in immunocompromised individuals. The organism has been implicated in peri-rectal infections, pediatric diarrhea, typical gastroenteritis, and necrotizing enterocolitis. The GI tract is also an important portal of entry in *Pseudomonas* septicemia and bacteremia (Todar, 2011).

3.6 Laboratory Identification of *Pseudomonas aeruginosa*

The clinical specimens include almost all the possible samples (like sputum, urine, catheters, blood, etc.) that are requested for the diagnosis of a disease in a hospital. *Pseudomonas aeruginosa* can be grown readily on common laboratory media such as nutrient agar, Mac Conkey agar and blood agar. However, for direct isolation from clinical specimens it is more useful to use a selective medium that suppresses the growth of other microorganisms. For this purpose, a selective medium containing cetrimide (cetyl trimethyl ammonium bromide) is available commercially as Cetrimide agar, Pseudosel agar, Pseudomonas isolation agar, etc. Cetrimide Agar is used for the selective

isolation and presumptive identification of *P. aeruginosa* from clinical and nonclinical specimens.

3.6.1 Morphological, Cultural, and Metabolic Characteristics

P. aeruginosa is a Gram-negative rod measuring 0.5 to 0.8 µm by 1.5 to 3.0 µm. Almost all strains are motile by means of a single polar flagellum. Some strains can produce a viscous extracellular slime layer. These mucoid strains are frequently isolated in material from cystic fibrosis patients (Doggett *et al.*, 1971). *P. aeruginosa* can only be grown in culture mediums containing free O_2 as a terminal electron acceptor. Its metabolism is respiratory and never fermentative, but it will grow in the absence of O_2 if NO_3 is available as a respiratory electron acceptor (Vasil, 1986). In nutrient broth, the organism therefore grows at the surface to form a so-called pellicle. Colonies on nutrient agar often have a metallic sheen. Its optimum temperature for growth is 37°C, and it is able to grow at temperatures as high as 42°C (Lafontaine & Sokol, 1998). *P. aeruginosa* isolates may produce mucoid as well as soft colonies. The smooth and mucoid colonies are presumed to play a role in colonization and virulence. But isolation of non-mucoid colonies from clinical specimens (blood culture and urine culture) has also been reported (Anastassiou *et al.*, 1987). Given suitable conditions, *P. aeruginosa* can produce two pigments, pyoverdin, water-soluble yellowish green fluorescent pigment and pyocyanin, choloform- and water- soluble bluish green pigment. Occasionally production of red pigment, pyorubrin and brown pigment pyomelanin has also been recorded (Ogunnariwo and Hamilton-Miller, 1975).

3.6.2 Biochemical Properties

Pseudomonas aeruginosa shows various biochemical properties that aid in its identification in the laboratory. Pseudosel agar (PSA) contains cetrimide which inhibits most bacteria other than *P. aeruginosa*. It also stimulates *P. aeruginosa* to produce the pigment pyocyanin as well as fluorescein, a fluorescent product. *P. aeruginosa* will typically produce a green to blue, water-soluble pigment and will also fluoresce when the plate is placed under a short wavelength (254 nm) ultraviolet light. Unlike the *Enterobacteriaceae, Pseudomonas* does not ferment glucose. If the phenol red remains red or orange then acid was not produced indicating no glucose fermentation. *P.*

aeruginosa changes the color of the tube containing OF medium without oil into yellow color while the other tube remains colorless indicative of oxidative nature of the organism. In Citrate Utilization Test due to the utilization of citrate, the indicator bromothymol blue changes to blue color indicative of positive test. In Sulphur Indole Motility (SIM) Test, absence of blackening of the medium and no formation of pink ring in the interface of the medium after the addition of Kovac's reagent suggests negative sulphur and indole reaction. Hazy growth indicates the motile nature of the organism.

3.7 Antibiotic resistance in *Pseudomonas aeruginosa*

Given the number of compounds active against *P. aeruginosa,* it might be expected that treatment of infections caused by this bacterium would be straightforward. The situation, however, is complicated by the predilection of *P. aeruginosa* to develop resistance to nearly any antimicrobial agent. Resistance is problematic at three levels: intrinsic resistance, acquired resistance, and emergence of resistance during therapy. Each of these must be considered when choosing an antibiotic regimen for patients infected with *P. aeruginosa* (Hauser and Sriram, 2005).

3.7.1 Intrinsic resistance

In general, *P. aeruginosa* is naturally less susceptible than other gram-negative bacilli to many antibiotics, such as ampicillin, most cephalosporins, and the macrolides. This is because of its relatively impermeable outer membrane and its ability to actively transport some antibiotics out of the cell, preventing accumulation. Many studies have concluded that the susceptibility of *P. aeruginosa* to some β-lactams (e.g. ceftazidime, cefepime, piperacillin, aztreonam) is more strongly influenced by efflux, whereas susceptibility to others (imipenem, panipenem) is more strongly affected by the presence of β-lactamase, while a third group (ceftriaxone, meropenem, moxalactam) is influenced only by knockout of both efflux and β-lactamase.

3.7.2 Acquired resistance

In addition to its intrinsic resistance, *P aeruginosa* has the ability, through mutational changes or acquisition of exogenous genetic material, to develop resistance to each of the antipseudomonal antibiotics. For example, the chromosomally encoded AmpC b-

lactamase is capable of degrading β-lactams, such as piperacillin and ceftazidime, when mutations result in production of large amounts of this enzyme.

3.7.3 Emergence of resistance

Even when antimicrobial agents to which an isolate is susceptible are chosen, a successful therapeutic outcome is not ensured. One reason for this uncertainty is that *P. aeruginosa* has shown a regrettable propensity to develop resistance to antibiotics during therapy. Resistance develops because of the natural occurrence of mutations essential for antibiotic penetration or activity. Within infected tissue, selection for individual bacteria that harbor these mutations occurs because the antibiotic is present, and eventually these organisms compose the majority of the bacterial population. As would be expected, antimicrobial agents for whom a single mutation is sufficient to compromise activity are most prone to the emergence of resistance during therapy. These include imipenem and the fluoroquinolones. For example, resistance emerged in 25% to 50% of *P. aeruginosa*-infected patients who received imipenem monotherapy and 33% to 58% of such patients who received ciprofloxacin monotherapy (Lode *et al*., 1987).

3.7.4 Multidrug resistant *Pseudomonas aeruginosa*

Multidrug resistance in *P. aeruginosa* can be defined as diminished susceptibility to >1 of the following drug classes—antipseudomonal cephalosporins, antipseudomonal carbapenems, β-lactam– β-lactamase inhibitor combinations, antipseudomonal fluoroquinolones, and aminoglycosides (Paterson, 2006; Ortega *et al.*, 2004). Multi-Drug Resistant *P. aeruginosa* (MDRPA) strains were first reported in patients with cystic fibrosis (Aris *et al.,* 1997), and dissemination of these resistant organisms has since been reported among hospitalised patients (Bert *et al*., 1998; Pitten *et al*., 2001; Harris *et al*., 1999). Nowadays, prevalence of MDR strains of *P.aeruginosa* are seen mainly in hospital acquired infections due to the selective pressure exerted on the bacteria by over usage of broad-spectrum antibiotics. Among the tracheal aspirates pathogens, 88.8% of *P. aeruginosa* were found to be Multi-Drug Resistant (MDR) in Nepal (Koirala *et al*., 2010).

Resistance to β-lactams

Multi drug efflux pumps in the inner and outer membrane of *Pseudomonas aeruginosa* may protect the bacterium from to β-lactam agents (Poole, 2001). All *P. aeruginosa* strains have a chromosomal AmpC b-lactamase that is normally inducible but may be derepressed by mutation, or can be induced by certain β-lactams. Such inducers include clavulanate, normally used as a β-lactamase inhibitor (but not against class C, AmpC-like β-lactamases) and the antibiotic imipenem. Mutations resulting in b-lactamase derepression are the most common clinical cause of β-lactam resistance in *P. aeruginosa*.

The national nosocomial infections surveillance system (NNIS) of USA showed increases of 17.7%, 27.3% and 26.4% in resistance to imipenem, quinolone and third-generation cephalosporin, respectively (Gaynes and Edwards, 2005). In various studies, it was reported that increased resistance rates have been detected against to carbapenems, and third-generation cephalosporins for *P. aeruginosa* worldwide (Hancock *et al.,* 1998).

Carbapenem antibiotics are considered agents of choice to treat serious infections caused by *P. aeruginosa*. Progressive antimicrobial resistance to most classes of antibiotics, including carbapenems, has made treatment of infection caused by this bacteria particurlarly difficult, even with combination therapy. One growing factor leading to carbapenem resistance is the presence of carbapenemases. The most important clinically-significant carbapenemases in *P. aeruginosa* are class B metallo-β-lactamases such as VIM and IMP-type (Zhiyong *et al*., 2003). In the literature, it was reported that resistance to imipenem was 14% in Spain (Bouza *et al*., 1999), 19.3% in Italy (Bonfiglio *et al*., 1998), and 68% in Saudi Arabia (Rotimi *et al*., 1998). The National Nosocomial Infections Surveillance (NNIS) system reported the incidence of imipenem resistance as 18.5% among isolates of *P. aeruginosa* from ICU patients (CDC, 1999).

According to different reports, resistance to ceftazidime was 15%-22% in the world. Resistance to piperacillin was higher, similar to ceftazidime. While piperacillin resistance rate was 10% in Spain (Bouza *et al*., 1999), 12% in Italy (Bonfiglio *et al*., 1998), 14% in Latin America (Jones, 2001), it was found as 28.7% in the study done by Savas *et al*., 2005.

Resitance to aminoglycosides

One of the significant resistant groups detected against aminoglycosides was *P. aeruginosa*. Reports of the susceptibility of *P. aeruginosa* to gentamicin and tobramycin have ranged from as low as 49.8% and 77.7%, in Greece, to as high as 96.6% and 99.2%, respectively, in the United Kingdom (Van Landuyt *et al*., 1986). Previous studies reported that antipseudomonal effects of amikacin were greater than those of gentamicin (Pourshafie *et al*., 2007).

Most studies have indicated that around 10% of *P. aeruginosa* isolates are aminoglycoside resistant, although higher levels of resistance occur in some studies for specific aminoglycosides (Hancock and Speert, 2000). Mutations in different genes appear to correlate with this phenotype involves up glycosides resistance (Westbrock-Wadman *et al*., 1999; Aires *et al*., 1999). Another relevant form of aminoglycoside resistance is adaptive resistance (Karlowsky *et al*., 1996) which is reversible, after a post-antibiotic effect, upon removal of selective pressure. It was shown to occur in an artificial biofilm (Brooun *et al*., 2000).

Resistance to quinolones

Quinolone resistance in *P. aeruginosa* strains is due to missense mutation in the quinolone target (the *gyrA* subunit of DNA gyrase) (Takenouchi *et al*., 1999). However, higher levels of resistance may involve additional mutations in *gyrB* (DNA gyrase B subunit) or *parC* (topoisomerase IV). The resistance of *Pseudomonas* to the antibiotics in the quinolone group is variable in different centers (Snydman, 1991). In a prospective study, resistance to ciprofloxacin in ICU was reported as 8-31% (Tassios *et al*., 1988). In the study done by Savas *et al.,* 2005, resistance rates against ciprofloxacin and norfloxacin were found as 27.4%, 25.5%, respectively. Ciprofloxacin resistance rate was 23% in Spain (Bouza *et al*., 1999), 31.9% in Italy (Bonfiglio *et al*., 1988) and 26.8% in Latin America (Jones, 2001).

3.8 Treatment and control

3.8.1 Antibiotic options

Despite the fact that *P. aeruginosa* has high intrinsic resistance to antimicrobial agents, a number of drugs are available for treatment of pseudomonal infections. The major classes

that have been used with success include aminoglycosides (such as gentamicin and amikacin), combined antibiotics (such as piperacillin/tazobactam and cepoparazone/sublactam), third generation cephalosporins (including ceftazidime, cefixime and ceftriaxone), quinolones (such as ciprofloxacin), carbapenems (including meropenem) and colistins (such as polymixin-B). In addition to these antibiotics, newer agents are being developed to counter the problem of antimicrobial resistance.

3.8.2 Combination therapy

Therapy of serious infections caused by *P. aeruginosa* usually consists of a combination of semisynthetic penicillin, such as ticarcillin or piperacillin (with or without a β-lactamase inhibitor) and an aminoglycoside, such as tobramycin. These drugs have been shown to be synergistic in vitro against *P. aeruginosa*. β-lactams β-lactamase inhibitors combinations (such as cefoparazone/sublactam and piperacillin/tazobactam) can be effective against serious infections and help to reduce the resistance against antibiotics.

Since cross-resistance between major classes of anti-pseudomonal antibiotics is unlikely to develop, it is common practice to treat serious infections with a combination of a β-lactam and an aminoglycoside. Not only do the drugs appear to exert a synergistic antibacterial effect, but they may also delay or prevent the emergence of resistance during therapy. There are a few novel approaches under development in which the combination of agents would include an antibacterial agent and an inhibitor of a major resistance mechanism. The best example of this would be the use of a combination of β-lactamase inhibitor and β-lactam (Darwaz and Bonomo, 2010; Mohanty *et al*., 2005).

3.8.3 Restriction of use

Good antibiotic stewardship plays an important role in limiting the emergence of antibiotic resistance. Some antimicrobial agents should be reserved for therapy of infections when all other agents have failed. For instance, it is inadvisable to use a drug such as meropenem for the initial infection with *P. aeruginosa* in a patient with cystic fibrosis; its use should be reserved for infections when the infecting strain is resistant to the first-line drugs: semisynthetic penicillin and an aminoglycoside. Some hospitals impose restrictions on certain antimicrobial agents to maximize the likelihood that they

will be effective when a legitimate indication for their use arises (Hancock and Speert, 2000).

3.8.4 Prevention of infection

Infection control plays an exceedingly important role in preventing the spread of antimicrobial-resistant bacteria within hospitals. *P. aeruginosa* can be transiently carried on the hands of medical and nursing personnel resulting in the spread of infection among patients. Principles of good infection control such as careful hand washing and barrier precautions should be utilized when dealing with patients with antibiotic- resistant bacteria. Immunization against *P. aeruginosa* would be a strategy for preventing acquisition and spread of antibiotic-resistant strains. Whereas several different vaccine strategies have been considered and tested, none has yet entered clinical use (Hancock and Speert, 2000).

CHAPTER IV

4. MATERIALS AND METHODS

A cross-sectional study was conducted during a six month period from November, 2010 to May, 2011 in ASICU, PSICU and MICU at Shahid Gangalal National Heart Center, a refferal heart hospital in the country.

A total of 1060 samples were processed out of which 700 were clinical samples directly taken from the admitted patients while 360 were the surface swab samples. Among 700 clinical samples there were 160 ET tube tips, 142 Suction tube tips, 151 Foley's tube tips 160 CVP tube tips, 30 sputum, 20 wound swab, 8 pus, 25 blood and 4 tracheal secretion samples. Among 360 surface swab samples, 90 samples from each floor, bed lining, working tables and aprons of the HCWs were taken in the study.

126 samples yielded the growth of *Pseudomonas aeruginosa* including 66 from the clinical samples and 60 from the surface swab samples.

4.1 Sample collection and processing

The clinical samples were collected in a sterile, leak proof and well capped container with the help of medical staffs working in the wards. As soon as the samples were collected they were transported to the laboratory immediately and microbiological processing was carried out.

Swabs from the floor, bed linings, working table and apron of the HCWs who were working in the ICUs were taken. Sterilized cotton plugged swabs dipped into normal saline were used to collect sample for investigation from inanimate objects. Using a sterile cotton swab dipped in normal saline the surfaces were swabbed revolving the swab in 'S' shaped fashion so as to collect the remains of microbial load (Sehulster, 2008). For the purpose of quality control each batch of swab was tested for sterility by streaking on culture medium and incubation at 37°C for 24 hours to observe colonies if growth occurs. For proper collection of samples it was made sure that the swab was revolved during the procedure. All the samples were collected aseptically.

4.2 Culture

As soon as the samples were transported into the laboratory, they were inoculated into the culture media like Mac Conkey agar (MA) and Blood agar (BA) plates with proper labels. Subculture was done in Cetrimide agar for selective isolation and Nutrient agar for Gram's staining and other biochemical tests. Then the plates were incubated at 37°C for 24 hours. However, for the swab samples enrichment was done in BHI broth (incubation at 37°C for 24 hours) before inoculating into the plates.

4.3 Examination of plates

After proper incubation, the plates were examined for the appearance of the colonies. Haemolysis in BA and lactose fermentation in MA was also observed. Pigmentation was also observed in the culture plates. To obtain a pure culture a single colony was picked up and sub-cultured in Nutrient agar for the biochemical and other tests.

4.4 Characterization of isolates

Standard protocol provided by Cheesbrough (2000) and Bergey's Mannual (2005) was followed for the identification and characterization of isolates.

Those isolates which developed colonies in the cetrimide agar were confirmed as *P. aeruginosa*, along with Gram's staining and other biochemical tests (catalase test, oxidase test, indole test, TSI, MR/VP, citrate utilization test, O/F test).

On the basis of consistency strains of *P. aeruginosa* were determined as mucoid and non-mucoid strains. Capsule stain was done to identify the mucoid strains (Breakwell *et al*., 2009). Pigmented colonies were identified from the non-pigmented colonies by the color observation as the latter ones were colorless. On the basis of the antibiogram, the organism was categorized into sensitive and resistant strains towards various classes of antibiotics. Moreover MDRPA and PDRPA strains were also identified.

The Gram staining and capsule staining procedures are mentioned in the Appendix IV. The composition and preparation of biochemical media and reagents used in the tests are mentioned in the Appendix III.

4.5 Purity Plate

Purity plate culture of each biochemical tests is performed to observe whether the tests were preceded in an aseptic condition or not. The 4 hours incubated broth culture prepared for biochemical tests was inoculated on one half of the NA plate just before preceding the biochemical tests. The other half of the same plate was inoculated immediately after completing the biochemical test. The plate was incubated at 37°C for overnight. The pure growth of same organism in both the pre and post inoculated portion of the plate is the indication of maintenance of aseptic condition throughout the experiment.

4.6 Antibiotic susceptibility testing of isolates

Antibiotic susceptibility test of all isolates were performed by Kirby Bauer disc diffusion method. In this technique the antimicrobial agent diffuses from the disc to the medium. Following overnight incubation, the culture was examined for areas of no growth around the disc. Bacterial strains sensitive to the antimicrobial are inhibited from the disc where as resistant strain grows up to the edge of the disc. Procedure for antibiotic susceptibility test is mentioned in Appendix V.

Multi-drug Resistant (MDR) isolates were defined as those which showed resistance to >1 of the following 5 drug classes—antipseudomonal cephalosporins, antipseudomonal carbapenems, β-lactam–β-lactamase inhibitor combinations, antipseudomonal fluoroquinolones, polymixins and aminoglycosides.

The preparation and composition of Mueller-Hinton Agar medium is mentioned in the Appendix III. The detail about antibiotic discs used and their interpretative chart are mentioned in the Appendix V.

4.7 Quality control

Quality control is considered as one of the important factors for the correct result interpretation. During this study quality control was applied in various areas. During sample collection, aseptic technique was followed for collecting samples from objects in order to avoid contamination. During sample processing, all the tests were carried out appropriately in aseptic conditions. While using readymade dehydrated media, the manufacturer’s instructions for preparation, sterilization and storage were followed to

prevent the alteration of the nutritional, selective, inhibitory and biochemical properties of the media. The performances of newly prepared media were tested using control species of bacteria (i.e., known organism giving positive and negative reactions). For stains and reagents, wherever a new batch of them was prepared, a control smear was stained to ensure correct staining reaction. Control strain of *Pseudomonas aeruginosa* (ATCC 27853) was used for the standardization of the Kirby-Bauer test and also for the correct interpretation of zone of inhibition.

CHAPTER-V

5. RESULTS

Both the clinical and inanimate surface swab samples were collected from three different ICUs: ASICU, PSICU and MICU in SGNHC. A total of 1060 samples were processed. Out of 700 clinical samples 66 yielded *Pseudomonas aeruginosa*. Likewise, among 360 inanimate surface swab samples, 60 yielded *P. aeruginosa*. Thus the prevalence of *P. aeruginosa* was 9.43% in the clinical samples while in case of inanimate surface swab samples it was 16.67%. The median age of the patients admitted in the ICUs was 28.0±24.3yrs (median age± SD), range from 1 month to 72 yrs.

5.1 Distribution of *P. aeruginosa*

The highest percentage of *P. aeruginosa* was found in ASICU, 60.6% in case of clinical samples. However, in case of inanimate surface swab samples, the result seemed to be highest in PSICU, 48.3%. Although, the organism was not isolated in MICU from clinical samples, the surface swab samples yielded lowest percentage, 6.7%.

Table 1: Ward wise Distribution of *P. aeruginosa*

Ward	Clinical Sample		Surface Sample	
	Number	Percent	Number	Percent
ASICU	40	60.6	27	45.0
PSICU	26	39.4	29	48.3
MICU	0	0.0	4	6.7
Total	66	100.0	60	100.0

Of total of 54 floor swab isolates, the maximum number of *P. aeruginosa* was isolated from PSICU, 26 (48.1%) followed by ASICU, 25 (46.3%). Floor swabs from MICU showed least growth. Apron swabs showed very less growth while bed lining showed no growth.

Table 2: Sample wise Distribution of *P. aeruginosa* (Surface sample)

Sample	Ward					
	ASICU		PSICU		MICU	
	Number	Percent	Number	Percent	Number	Percent
Floor (n=54)	25	46.3	26	48.1	3	5.6
Bed Lining (n=0)	0	0.0	0	0.0	0	0.0
Working Table (n=4)	1	25.0	2	50.0	1	25.0
Apron (n=2)	1	50.0	1	50.0	0	0.0

Among the clinical samples, ET tube tip yielded the maximum number of *P. aeruginosa*, 29 (43.9%) followed by CVP tube tip, 15 (22.7%). The least count was observed in pus and tracheal secretion samples, 1 (1.5%).

Table 3: Sample wise Distribution of *P. aeruginosa* (Clinical sample)

Sample Type	Number (n=66)	Percent
ET Tube tip	29	43.9
Suction Tube tip	7	10.6
Foley's Tube tip	6	9.1
CVP Tube tip	15	22.7
Wound Swab	2	3.0
Pus	1	1.5
Sputum	3	4.5
Blood	2	3.0
Tracheal Secretion	1	1.5

5.2 Consistency of *P. aeruginosa*

Among the surface swab samples, the highest percentage of both mucoid and soft or non-mucoid strains of *P. aeruginosa* were isolated from the floor swabs as 21 (87.5%) and 33 (91.6%) respectively. Colonies were not isolated from bed lining swabs.

Table 4: Consistency of *P. aeruginosa* from Surface Sample

Sample	Consistency			
	Mucoid (n=24)		Soft (n=36)	
	Number	Percent	Number	Percent
Floor	21	87.5	33	91.6
Bed Lining	0	0.0	0	0.0
Working Table	2	8.3	2	5.6
Apron	1	4.2	1	2.8

ET tube tip samples showed highest number of mucoid colonies of *P. aeruginosa*, 23 (47.9%) followed by CVP tube tip samples, 12 (25.0%). Mucoid colonies were not observed from wound swab and pus samples while soft colonies were not observed from tracheal secretion samples.

Table 5: Consistency of *P. aeruginosa* from Clinical Sample

Sample Type	Consistency			
	Mucoid (n=48)		Soft (n=18)	
	Number	Percent	Number	Percent
ET Tube tip	23	47.9	6	33.3
Suction Tube tip	5	10.4	2	11.1
Foley's Tube tip	4	8.3	2	11.1
CVP Tube tip	12	25.0	3	16.7
Wound swab	0	0.0	2	11.1
Pus	0	0.0	1	5.6
Sputum	2	4.2	1	5.6
Blood	1	2.1	1	5.6
Tracheal Secretion	1	2.1	0	0.0

The mucoid strains of *P. aeruginosa* were isolated from the clinical samples in higher amount, 48 (72.7%) than that from the surface swab samples 24 (40.0%). On the contrary, non-mucoid or soft strains of *P. aeruginosa* were isolated from the surface swab samples 36 (60.0%) in higher amount than that from the clinical samples 18 (27.3%). The data are statistically significant (p=0.0002).

Table 6: Colony's consistency of *P. aeruginosa*

Consistency	Clinical Sample (n=66)		Surface Sample (n=60)	
	Number	Percent	Number	Percent
Mucoid	48	72.7	24	40.0
Soft	18	27.3	36	60.0
p- value	0.0002			

5.2 Pigmentation of *P. aeruginosa*

Among the inanimate surface swab samples, 45 (75.0%) were pigment producing strains of *P. aeruginosa* (the pigment could be pyocyanin or pyoverdin) while 15 (25.0%) were non-pigmented strains. Of the pigment producing strains most i.e., 23 (51.1%) were found in ASICU, 20 (44.4%) were found in PSICU and 2 (4.5%) were found in MICU. The highest number, 9 (60.0%) of non-pigmented strains were isolated from the samples of PSICU.

Table 7: Pigment wise Distribution of *P. aeruginosa* from Surface Sample

Ward	Type of pigment			
	Pigmented (n=45)		Non-pigmented (n=15)	
	Number	Percent	Number	Percent
ASICU	23	51.1	4	26.7
PSICU	20	44.4	9	60.0
MICU	2	4.5	2	13.3

When accounted for the pigment production among clinical samples, 61 (92.4%) were pigment producing strains while 5 (7.6%) were non-pigmented strains. and sample type among clinical samples, most pigment producing strains were isolated from ET tube tip sample, 27 (44.3%) followed by CVP tube tip sample, 14 (23.0%). However, of the total clinical samples only 5 (7.6%) yielded non-pigmented strains of *P. aeruginosa*.

Table 8: Pigment wise distribution of *P. aeruginosa* from Clinical Sample

Sample	Type of Pigment	
	Pigmented (%) (n=61)	Non-pigmented (%) (n=5)
ET Tube tip	27 (44.3)	2(40.0)
Suction Tube tip	5 (8.2)	2(40.0)
Foley's Tube tip	6 (9.8)	0(0.0)
CVP Tube tip	14 (23.0)	1(20.0)
Wound swab	2 (3.3)	0(0.0)
Pus	1 (1.6)	0(0.0)
Sputum	3 (4.9)	0(0.0)
Blood	2 (3.3)	0(0.0)
Tracheal Secretion	1 (1.6)	0(0.0)

5.3 Antibiotic susceptibility test of *P. aeruginosa*

Altogether 126 strains of *P. aeruginosa* were tested for commonly used antibiotics. Among the clinical samples, most of the antibiotics were highly resistant. The least sensitive antibiotic was CFM for accounting resistance of 65 (98.5%) followed by CTR and CAZ both accounting for 62 (93.9%). Among the antibiotics used the most sensitive one was CSL, 56 (84.8%) followed by PT, 36 (54.5%) and MEM, 34 (51.5%).

Table 9: Antibiogram for Isolated *P. aeruginosa* (Clinical Samples)

S.N.	Sample code	PT	CSL	MEM	CIP	GEN	CTR	POL	CFM	CAZ	AK
1	M819/15	S	S	S	R	R	R	R	R	R	I S
2	M833/17	S	S	S	R	R	I S	R	R	R	R
3	M833/18	S	S	I S	R	R	R	R	R	R	R
4	M833/19	S	S	S	R	R	R	R	R	R	R
5	M833/20	S	S	S	R	R	R	R	R	R	I S
6	M833/21	S	S	S	R	R	R	R	R	R	I S
7	M833/22	S	S	S	R	R	R	R	R	R	R
8	M833/23	S	S	S	R	R	R	R	R	R	R
9	M833/24	I S	S	S	R	R	R	R	R	R	I S
10	M833/25	I S	S	S	R	R	R	R	R	R	I S
11	M833/26	S	S	I S	R	R	R	R	R	R	R
12	M833/27	R	S	S	I S	R	R	S	R	R	I S
13	M833/28	I S	S	R	S	R	R	S	R	R	I S
14	M833/29	R	S	R	I S	R	R	S	R	R	I S

15	M833/30	S	S	S	R	R	R	S	R	R	R
16	M833/31	R	S	R	R	R	R	S	R	R	R
17	M833/32	R	S	R	I S	R	R	S	R	R	R
18	M833/33	S	S	S	S	S	S	S	R	I S	S
19	M833/34	I S	S	R	I S	R	R	S	R	R	S
20	M833/35	S	S	S	S	S	S	S	R	S	S
21	M833/36	R	S	R	R	R	R	R	R	R	I S
22	M833/37	S	S	S	S	R	R	S	I S	I S	S
23	M833/38	S	S	R	R	S	R	I S	R	R	R
24	M833/39	S	S	S	S	S	S	S	R	S	S
25	M833/40	S	S	S	R	R	R	R	R	R	R
26	M833/41	I S	S	R	I S	S	R	I S	R	R	R
27	M833/42	S	S	S	R	R	R	R	R	R	S
28	M833/43	S	S	S	R	R	R	R	R	R	S
29	M833/44	S	S	S	R	R	R	R	R	R	R
30	M833/45	S	S	R	I S	R	R	I S	R	R	R
31	M833/46	I S	S	I S	S	R	R	S	R	R	R
32	M833/47	S	I S	S	I S	R	R	S	R	R	R
33	M833/48	R	S	S	I S	R	R	S	R	R	R
34	M833/49	I S	R	S	I S	R	R	S	R	R	R
35	M833/50	S	S	S	R	R	R	R	R	R	R
36	M833/51	I S	R	S	R	R	R	S	R	R	R
37	M833/52	S	R	S	R	R	R	S	R	R	R
38	M833/53	R	R	S	R	R	R	S	R	R	R
39	M833/54	I S	S	S	R	R	R	S	R	R	R
40	M833/55	S	S	I S	S	R	R	S	R	R	R
41	M833/56	I S	S	I S	I S	R	R	I S	R	R	R
42	M833/57	R	I S	S	S	R	R	S	R	R	I S
43	M833/58	R	S	R	I S	R	R	S	R	R	R
44	M833/59	I S	S	S	I S	R	R	S	R	R	R
45	M833/60	S	R	R	R	R	R	S	R	R	R
46	M833/61	R	S	S	R	R	R	S	R	R	R
47	M833/62	S	S	I S	R	R	R	S	R	R	R
48	M833/63	I S	S	I S	S	R	R	S	R	R	R
49	M833/64	I S	S	R	R	R	R	S	R	R	R
50	M833/65	I S	S	R	R	R	R	S	R	R	R
51	M833/66	S	S	S	R	R	R	R	R	R	R
52	M833/67	S	S	R	R	R	R	S	R	R	R
53	M833/68	S	S	R	I S	R	R	S	R	R	R
54	M833/69	S	S	R	S	R	R	S	R	R	R
55	M833/70	I S	S	R	R	R	R	S	R	R	R
56	M833/71	I S	S	I S	S	R	R	S	R	R	R
57	M833/72	I S	I S	R	R	R	R	S	R	R	R
58	M833/73	I S	S	R	I S	R	R	S	R	R	R
59	M833/74	S	S	R	S	R	R	S	R	R	R
60	M833/75	R	S	I S	S	R	R	S	R	R	R

61	M833/76	S	S	R	S	R	R	I S	R	R	R
62	M833/77	S	S	S	I S	R	R	S	R	R	R
63	M833/78	S	S	S	I S	R	R	S	R	R	R
64	M833/79	S	I S	R	R	R	R	S	R	R	R
65	M833/80	S	I S	S	R	R	R	S	R	R	R
66	M833/81	I S	S	R	S	R	R	R	R	R	R

Note: PT= Piperacillin-tazobactam, CSL= cefoparazone-sulbactam, MEM= meropenem, CIP= ciprofloxacin, GEN= gentamicin, CTR= ceftriaxone, POL= polymixin-B, CFM= cefixime, CAZ= ceftazidime, AK= amikacin, S= sensitive, IS= intermediate sensitive, R= resistant

Table 10: Antibiotic Susceptibility Test of *P. aeruginosa* from Clinical Sample

Antibiotics	Sensitive		Intermediate Sensitive		Resistant	
	Number	Percent	Number	Percent	Number	Percent
PT	36	54.5	19	28.8	11	16.7
CSL	56	84.8	5	7.6	5	7.6
MEM	34	51.5	9	13.6	23	34.8
CIP	15	22.7	16	24.2	35	53.0
GEN	5	7.6	0	0.0	61	92.4
CTR	3	4.5	1	1.5	62	93.9
POL	42	63.6	5	7.6	19	28.8
CFM	0	0.0	1	1.5	65	98.5
CAZ	2	3.0	2	3.0	62	93.9
AK	7	10.6	10	15.2	49	74.2

Among the inanimate surface swab samples, most of the antibiotics were highly sensitive. The most sensitive antibiotics were CFM, CAZ and POL for accounting 60 (100.0%) sensitivity. Very less percentage of antibiotics was resistant.

Table 11: Antibiogram for Isolated *P. aeruginosa* (Surface Swab Samples)

S.N.	Sample code	PT	CSL	MEM	CIP	GEN	CTR	POL	CFM	CAZ	AK
1	1	S	I S	S	S	S	I S	S	S	S	S
2	1b	R	I S	S	S	S	I S	S	S	S	S
3	2	S	S	S	S	S	S	S	S	S	S
4	2b	R	I S	S	S	S	I S	S	S	S	I S
5	3	S	S	S	S	S	S	S	S	S	S

6	3b	S	S	S	S	S	S	S	S	S	S
7	4	S	S	S	S	S	S	S	S	S	S
8	5	S	S	S	S	S	I S	S	S	S	S
9	5b	S	S	S	S	S	S	S	S	S	S
10	6	S	S	S	S	S	I S	S	S	S	S
11	6b	S	S	S	S	S	I S	S	S	S	S
12	7	R	S	S	S	S	I S	S	S	S	S
13	8	S	S	S	S	S	S	S	S	S	S
14	8b	S	S	S	S	S	S	S	S	S	I S
15	9	S	S	S	S	S	S	S	S	S	S
16	9b	S	S	S	S	S	S	S	S	S	S
17	10	S	S	S	I S	S	S	S	S	S	S
18	10b	S	S	S	S	S	S	S	S	S	S
19	11	S	S	S	S	S	S	S	S	S	S
20	11b	S	S	S	S	S	S	S	S	S	S
21	12	S	S	S	S	S	S	S	S	S	S
22	12b	S	S	S	S	S	S	S	S	S	S
23	13	S	S	S	S	S	S	S	S	S	S
24	14	S	S	S	S	S	S	S	S	S	S
25	14b	S	S	S	S	S	S	S	S	S	S
26	15	S	S	S	S	S	I S	S	S	S	S
27	15b	S	S	S	I S	S	S	S	S	S	S
28	16	S	S	S	S	S	S	S	S	S	S
29	16b	S	S	S	S	S	S	S	S	S	S
30	17	S	S	S	S	S	R	S	S	S	S
31	17b	S	S	R	S	S	S	S	S	S	S
32	17c	R	I S	R	I S	S	I S	S	S	S	S
33	17d	S	S	S	S	S	I S	S	S	S	S
34	18	S	S	S	S	S	I S	S	S	S	S
35	18b	S	S	S	S	S	S	S	S	S	S
36	19	S	S	S	S	S	S	S	S	S	S
37	19b	S	S	S	S	S	S	S	S	S	S
38	20	S	S	S	S	S	S	S	S	S	S
39	20b	S	S	S	S	I S	S	S	S	S	S
40	21	S	S	S	S	S	S	S	S	S	S
41	21b	S	S	S	S	S	S	S	S	S	S
42	22	S	S	S	S	R	R	S	S	S	S
43	22b	S	S	S	S	S	S	S	S	S	S
44	22c	S	S	S	S	I S	S	S	S	S	S
45	23	S	S	S	S	S	S	S	S	S	S
46	24	S	S	I S	S	S	S	S	S	S	S
47	24b	S	S	S	S	S	S	S	S	S	S
48	24c	S	S	S	S	S	S	S	S	S	S
49	25	S	S	S	S	R	R	S	S	S	S
50	25b	S	S	S	S	S	S	S	S	S	S
51	26	S	S	S	S	S	S	S	S	S	S

52	26b	S	S	S	S	S	S	S	S	S	S
53	27	S	S	S	S	S	S	S	S	S	I S
54	27b	S	S	S	S	S	S	S	S	S	S
55	28	S	S	S	S	S	S	S	S	S	S
56	28b	S	S	S	S	S	I S	S	S	S	S
57	29	S	S	S	S	S	S	S	S	S	S
58	29b	S	S	S	S	S	S	S	S	S	S
59	30	S	S	S	S	S	S	S	S	S	S
60	30b	S	S	S	S	S	S	S	S	S	S

Table 12: Antibiotic Susceptibility Test of *P. aeruginosa* from Surface Sample

Antibiotics	Sensitive		Intermediate Sensitive		Resistant	
	Number	Percent	Number	Percent	Number	Percent
PT	56	93.3	0	0.0	4	6.7
CSL	56	93.3	4	6.7	0	0.0
MEM	57	95.0	1	1.7	2	3.3
CIP	57	95.0	3	5.0	0	0.0
GEN	56	93.3	2	3.3	2	3.3
CTR	45	75.0	12	20.0	3	5.0
POL	60	100.0	0	0.0	0	0.0
CFM	60	100.0	0	0.0	0	0.0
CAZ	60	100.0	0	0.0	0	0.0
AK	57	95.0	3	5.0	0	0.0

In clinical samples, both the mucoid and non-mucoid strains were mostly sensitive to CSL. Mucoid strains were more resistant to CIP, 27 (40.9%) than non-mucoid strains, 8 (12.1%). Both types of strains were not sensitive to CFM at all. However, in surface samples CFM was effective to both types of strains. Overall, soft strains were more sensitive to the antibiotics than the mucoid strains.

Table 13: Colony wise verification of AST of *P. aeruginosa*

		Clinical Samples				Surface Samples			
		Mucoid		Soft		Mucoid		Soft	
		No.	Percent	No.	Percent	No.	Percent	No.	Percent
PT	S	28	42.4	8	12.1	21	35.0	35	58.3
	IS	14	21.2	5	7.6	0	0.0	0	0.0
	R	6	9.1	5	7.6	3	5.0	1	1.7
CSL	S	43	65.2	13	19.7	21	35.0	35	58.3

	IS	4	6.1	1	1.5	3	5.0	1	1.7
	R	1	1.5	4	6.1	0	0.0	0	0.0
MEM	S	24	36.4	10	15.2	24	40.0	33	55.0
	IS	7	10.6	2	3.0	0	0.0	1	1.7
	R	17	25.8	6	9.1	0	0.0	2	3.3
CIP	S	10	15.2	5	7.6	22	36.7	35	58.3
	IS	11	16.7	5	7.6	2	3.3	1	1.7
	R	27	40.9	8	12.1	0	0.0	0	0.0
GEN	S	4	6.1	1	1.5	22	36.7	34	56.7
	IS	0	0.0	0	0.0	1	1.7	1	1.7
	R	44	66.7	17	25.8	1	1.7	1	1.7
CTR	S	2	3.0	1	1.5	13	21.7	32	53.3
	IS	0	0.0	1	1.5	9	15.0	3	5.0
	R	46	69.7	16	24.2	2	3.3	1	1.7
POL	S	27	40.9	15	22.7	24	40.0	36	60.0
	IS	5	7.6	0	0.0	0	0.0	0	0.0
	R	16	24.2	3	4.5	0	0.0	0	0.0
CFM	S	0	0.0	0	0.0	24	40.0	36	60.0
	IS	1	1.5	0	0.0	0	0.0	0	0.0
	R	47	71.2	18	27.3	0	0.0	0	0.0
CAZ	S	1	1.5	1	1.5	24	40.0	36	60.0
	IS	2	3.0	0	0.0	0	0.0	0	0.0
	R	45	68.2	17	25.8	0	0.0	0	0.0
AK	S	6	9.1	1	1.5	23	38.3	34	56.7
	IS	7	10.6	3	4.5	1	1.7	2	3.3
	R	35	53.0	14	21.2	0	0.0	0	0.0

Among the various classes of antibiotics used, *P. aeruginosa* against cephalosporins registered highest resistance rate (100.0%) followed by aminoglycosides (95.5%) in case of isolation from clinical samples. We obtained similar results in case of surface samples as well; cephalosporins (25.0%) and aminoglycosides (11.7%) ineffective. *P. aeruginosa* isolated from surface samples showed no resistance against polymixins (0.0%) while that isolated from clinical samples showed 36.4% resistance rate.

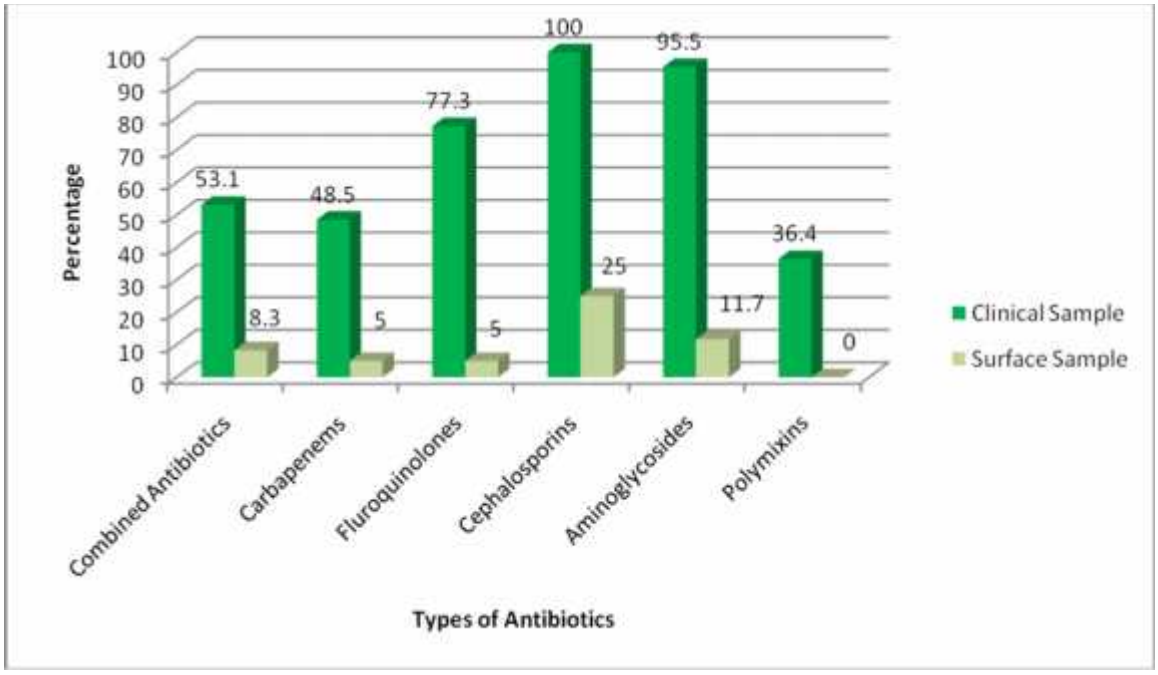

Fig 1: Resistivity pattern of *P. aeruginosa* against various antibiotics

When we studied the MDR pattern of the organism, we got drastic results when we compared the isolates obtained from clinical samples to that from the surface samples. 59 (89.4%) were MDRPA isolates from the clinical samples while only 7 (11.7%) were MDRPA isolates from surface samples.

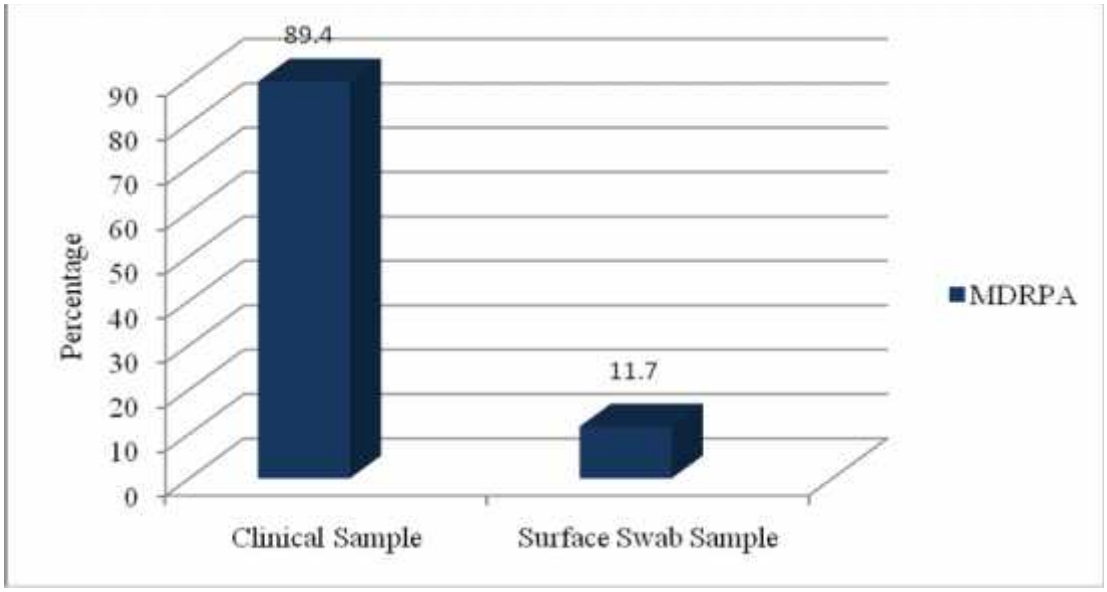

Fig 2: MDRPA isolates

CHAPTER VI

6. DISCUSSION AND CONCLUSION

6.1 DISCUSSION

In the three Intensive Care Units (ICUs), special facility was provided dedicating to treat patients who were critically ill. So it had different environment from other areas of the hospital. Because of the presence of debilitated, immunocompromised patients, greater age group patients or children and the use of indwelling medical devices, invasive diagnostic procedures and profound use of antibiotics, the patients were at an increased risk of acquiring infections at the hospital.

Pseudomonas aeruginosa is one of the most common pathogens that have posed threat to the ICU patients in the hospital. The environment in which patients were kept harbored *P. aeruginosa*. In our study, the aprons of HCWs, working tables, bed linings, floor, all harbored *P. aeruginosa*, the sources of which can be exogenous or endogenous. Different studies (Karki *et al*., 2011 and Sharma, 2006) revealed that the organism as an opportunistic environment pathogen. In addition, the persistence of *P. aeruginosa* in the inanimate surfaces of the hospital environment (6 hours to 16 months) (Kramer *et al.*, 2006) increases the chances of infections. However, irrespective of the site and due to immediate vicinity of the patients in any ICU, there is high chance of acquiring the organisms by the patients. Although, microorganisms are present in air, ultimately they get settled on the surfaces like floor, working table, bed lining, etc. and these surfaces become important sources for the infection. Therefore, proper disinfection should be done regularly.

Three different wards, ASICU, PSICU and MICU accounted for at least some percentage of growth of *P. aeruginosa*. The environment might be favorable and the floors were damp for their growth. Dwivedi *et al.*, 2009 and Jamaati *et al.*, 2010 also showed the existence of *P. aeruginosa* in the ICU environment. It infers that there has been a type of established relationship between the organism and the ICU environment. So, the ICU environment and pseudomonal infections should be monitored periodically.

There was a significant growth of *P. aeruginosa* from the clinical samples obtained from the immuno-compromised patients in both ASICU and PSICU. When *P. aeruginosa* is present in the ICU environment, it can easily colonize such patients and commence infections. However, growth was not observed in case of MICU samples. Such kind of result was due to absence of immuno-compromised and catheterized patients in the ward. Furthermore, the highest load of *P. aeruginosa* was found in the ET tube tip followed by the CVP tube tip samples. However, less isolates were obtained from non-catheter samples. Thus, immunocompromised patients and the use of indwelling catheters proved to be the cause of pseudomonal infections and therefore they should be of particular concern.

Among the surface swab samples, floor swabs yielded the higher growth of *P. aeruginosa* in PSICU and ASICU while only 5.6% of growth was obtained from floor swabs of MICU. The difference in results is due to different types of flooring carpets used in the wards. ASICU and PSICU had a bit rough type of polyvinyl chloride (PVC) carpet than MICU. Due to the nature of the floors, different strains of *P. aeruginosa* were able to thrive on the floor surfaces. There are equal chances of isolating the organism from the working tables and aprons irrespective of the type of ICUs. So, for the disinfection of the surfaces many kinds of disinfectants, including 70% ethanol, 50% isopropanol and 3% saponated cresol are effective (NIH, 1994).

Out of 36 soft strains, 91.6% were isolated from floor alone. Similarly, from the same site out of 24 mucoid strains, 87.5% were isolated. Even in the natural habitat, *P. aeruginosa* produced alginate slime despite it did not have to cause infection. Grobe *et al*., 1995 mentioned that mucoid strains of *P. aeruginosa* are found even outside the patients. Irrespective of the type of strains, they could be found anywhere in ICU. So, we should not ignore any type of strains isolated from anywhere as they could be equally significant.

Occurrence of *P. aeruginosa* in indwelling catheters has significant role in the pathogenesis of the organism especially in ICU patients. Our data revealed that out of 66 clinical samples, 86.4% were catheter related that showed growth of *P. aeruginosa*.

Although 91.6% mucoid producing strains were from catheter samples, only 8.4% were from non-catheter samples. Due to the production of alginate slime the organism attaches itself to the surfaces of the catheters so that simple mechanical forces cannot remove them from the surfaces. The prevalence of mucoid strains in ICU patients has also been mentioned by various workers; Doggett, 1969 and Hoiby, 1975. So, there are high chances of pseudomonal infections with the use of indwelling catheters. Therefore, catheters should be used under highly asceptic conditions and changed at proper intervals.

The samples taken from ET tubes, suction tubes, sputum and tracheal secretions were samples taken from thoracic site and others were from other sites. Higher occurrence of mucoid strains of *P. aeruginosa* was seen from the clinical samples taken from thoracic sites (64.6%) in comparison to samples from other sites (65.4%). The mucoid strains of *P. aeruginosa* can be isolated from thoracic site in patients infected with cystic fibrosis (Lam *et al*., 1980 and Boucher *et al*., 1997).

In both the clinical and inanimate surface samples, the maximum number of isolates produced pigmentation, which could be pyocyanin or pyoverdin. However, non-pigmented strains were found to be higher in case of surface samples. Non-pigmented strains were also isolated in significant amount from PSICU. Although there has not been any established relation between various wards and the type of pigment produced, the production of these secondary metabolites seems to be dependent on environmental cues (Jensen *et al*., 2006). This is supported by the fact that there is impact of the *rhl* QS (Quorum Sensing) on the biosynthesis of the secondary metabolites pyocyanin and rhamnolipids in *P. aeruginosa* (Pearson *et* al., 1997).

A total of 126 isolates of *P. aeruginosa* were tested against different classes of antibiotics. Among them, the most sensitive antibiotic was CSL accounting for 88.9% whereas the most resistive antibiotic was cefixime and cefttriaxone both accounting for 51.6%. CSL is a combined antibiotic that is used to treat multi-drug resistance *P. aeruginosa*. Since this was the most recently used antibiotic in the hospital where the study was carried out, the organism developed less resistance against it. Although

combined antibiotics are prescribed in order to combat multi-drug resistance trend, we observed resistance markedly with the use of PT, 11.9%.

What we observed was, the organism isolated from inanimate surface swabs became sensitive to almost all of the antibiotics used. POL, CFM and CAZ showed cent percent effectiveness while rest of others showed more than 90% effectiveness except CTR, which was 75% effective. In its natural habitat, *P. aeruginosa* did not receive any type of antibiotics. So it was not necessary for the organism to change its mechanism in order to elude the effects of the antibiotics. The organism had not undergone changes in any of the systems to combat the effects of the drugs. There had not been mutations, changes in the efflux pumps, changes in the targets and inactivation and modification of antibiotics. In their natural habitats, most of them did not produce slime layer of alginate polysaccharide (non-mucoid) and it leads to the reduction of the biofilm production rendering vulnerable to antibiotics. There is increased resistance of organism due to biofilm production (Stewart and Costerton, 2001). Biofilm is one of the ways to develop resistance. However, due to lack of biofilm production, we observed almost all of the isolates were sensitive to all the antibiotics used.

When we observed the other part of the study i.e. sensitivity pattern with the clinical samples, the results were very different. It means that *P. aeruginosa*, with the profound use of various antibiotics, had changed itself into a stubborn organism, resistant to almost all the antibiotics. It showed resistance to CFM (98.5%), CTR and CAZ (93.9%), GEN (92.4%), MEM (34.8%), CIP (53.0%) and POL (28.8%). Resistance was observed even in case of combined antibiotics, PT (16.7%) and CSL (7.6%). A ten year study conducted by Obritsch *et al.*, 2004 also suggest about the increasing pattern of the antibiotic resistance to various drugs against *P. aeruginosa*. In the MYSTIC 2006 results, among 1,012 *P. aeruginosa* isolates collected from 40 European centers, resistance to PT was the lowest (15%), followed by meropenem (22%), amikacin (23%), ceftazidime (25%), gentamicin (29%) and ciprofloxacin (33%) (Turner, 2008). Thus, the organism is sensitive towards combined antibiotics to some extent while single antibiotics are being futile in the treatment of pseudomonal infections. Thus emphasis should be given towards combined antibiotics in the treatment of pseudomonal infections.

The organism possesses an outer membrane with a low permeability and is thereby intrinsically resistant to a wide variety of commonly used antibiotics (Angus *et al*., 1982). What nevertheless makes *P. aeruginosa* uniquely problematic is a combination of the following: the species' inherent resistance to many drug classes; its ability to acquire resistance, via mutations, to all relevant treatments; its high and increasing rates of resistance locally; and its frequent role in serious infections. A few isolates of *P. aeruginosa* are resistant to all reliable antibiotics, and this problem seems likely to grow with the emergence of integrins that carry gene cassettes encoding both carbapenemases and amikacin acetyltransferases (Livermore, 2002).

Multi-Drug Resistant *P. aeruginosa* (MDRPA) strains are the most challenging organisms for the clinicians to be overcome. In our study 89.4% of the total isolates from the clinical samples were MDRPA which is similar to a study carried out by Koirala *et al*., 2010 in Nepal. In a recent study carried out by Karki *et al*., 2011 in a tertiary care hospital in Nepal, MDRPA were also isolated from the clinical samples. According to the MYSTIC program conducted from 1997 to 2000, the incidence of MDR *P. aeruginosa* isolates in Europe (nosocomial infections) was 4.7% while in the ICU setting (33 European ICUs) it ranged from 50% in Turkey to ≤3% in Spain, UK, Germany, Bulgaria and Malta (Goossens, 2003). This indicates the alarming situation of MDR pseudomonal infections in ICUs of various parts of the world including Nepal. So, habit of rational use of antibiotics should be developed.

De-repression of the chromosomal AmpC β-lactamases is mainly responsible for reduction in susceptibility to β-lactam antibiotics (Livermore, 1995). The loss of OprD, an outer membrane porin that forms narrow transmembrane channels accessible to carbapenems, is associated with resistance to imipenem and reduced susceptibility to meropenem (Studemeister and Quinn, 1988). Mutations to topoisomerase II and IV enzymes confer resistance to fluoroquinolones (Jalal and Wretlind, 1998). Permeability mutations, amino-glycoside-modifying enzymes, and decreased membrane penetration are blamed for aminoglycoside resistance (Poole, 2005). Finally, resistance to the polymyxins is postulated to be induced by a gene (*PmrA*) that, in the presence of low magnesium concentrations, modifies the lipopolysaccharide, resulting in reduced binding

affinity of colistin and perhaps related antimicrobial peptides to the outer membrane (Evans *et al*., 1999; Groisman *et al*., 1997). When there is combination of these mechanisms, there is high possibility that the organism develop MDR.

We have observed that out of 66 clinical isolates of *P. aeruginosa*, 28.8% were resistant to polymyxin-B while 7.6% were intermediate sensitive even when this organism was not exposed to the antibiotic in the hospital. *P. aeruginosa* develops resistance to polymyxins through mutation or adaptation mechanisms (Falagas and Kasiakou, 2005). Mutation is inherited, low-level, and independent of the continuous presence of the antibiotic, whereas adaptation is the opposite (non-inherited, high level, and requires the continuous presence of the antibiotic). It has been suggested that a basic mechanism of resistance in *Pseudomonas aeruginosa* strains is that the outer membrane protein OprH blocks the self-promoted uptake pathway of polymyxins by replacing Mg^{2+} on the LPS molecule. Thus, the overexpression of OprH caused by mutation or as a result of adaptation to an Mg^{2+}-deficient medium can be associated with resistance to polymyxins (Moore and Hancock, 1986). Specifically, the absence of 2-hydroxylaurate, the presence of 4-aminoarabinose and increase in the palmitate content of lipid A has been associated with resistance of *Pseudomonas aeruginosa* to polymyxins (Denton *et al*., 2002; Gunn *et al*., 1998).

If targeting at the mechanisms of drug resistance, combined antibiotic therapy (including carbapenems, ciprofloxacin, macrolide antibiotics and β-lactamase inhibitors) could effectively control infection by MDRPA. Some clinicians try to use polymyxin to combat MDRPA infection (Kallel *et al*., 2007). However, polymyxin is not usually incorporated into the routine treatment protocol because of its severe toxicity. Elimination of MDRPA infection through antibiotic use is not realistic if the granulating wound is large and the patient has reduced immunity. However, appropriate use of antibiotics inhibits infection, and "buys" time for wound recovery. Once the overall wound is treated, the physiological condition and immunity will improve and MDRPA infection can be controlled.

The emergence of MDRPA may be a harbinger of the so-called post-antibiotic era. A stringent antibiotic control policy should be exercised as part of efforts to control the emergence and spread of these multiresistant organisms, and strict compliance with

infection control measures is essential to reduce the likelihood of nosocomial spread of infection.

The organism which was sensitive to various drugs outside the host acquired multiple resistance mechanisms when once it entered the host. It is obvious that still the organism is the same, its genes and other structures have been changed for the adaptability inside the host. This multiple resistance developed in the organism has led a kind of difficulty for clinicians to adopt a definitive way of treatment. Not only this, when the organism shows resistance to all the available drugs, it will be a matter of serious concern not only for the clinicians but to the whole health department of nation.

We have seen that there is no close association between the clinical isolates of *P. aeruginosa* and inanimate surface swabs in terms of sensitivity pattern and other factors as well. In a study of the genetic diversity of clinical and environmental *P. aeruginosa* isolates from a Spanish hospital, Ruiz and colleagues concluded, based on analyses of whole-cell proteins, outer membrane proteins, antibiotic susceptibility patterns, pulsed-field gel electrophoresis, and randomly amplified polymorphic DNA, that there was not a close relationship between clinical and environmental isolates (Ruiz *et al*., 2004). Supporting our study, it infers that the strains of *P. aeruginosa* on the inanimate surface change themselves into different strains when they infect immuno-compromised patients. Furthermore, rational use of antibiotics should be made in order to combat with the emergence of MDRPA.

6.2 CONCLUSION

Pseudomonal infection is still one of the most common nosocomial infections in the ICUs of Nepal, where floors serve as the important source of infections. Close association between the strains isolated from the clinical and inanimate surface samples cannot be confirmed as indicated by their different antibiograms. Mucoid strains have developed higher resistivity than the soft strains. Irrespective of the nature of strains, *P. aeruginosa* has shown the reduced susceptibility towards carbapenems, fluoroquinolones, aminoglycosides and cephalosporins. Since polymixins are toxic to human cells, combined antibiotics like CSL and PT remain the choice of treatment. Moreover, the upsurge of MDRPA can be best controlled by the rational use of antibiotics.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATION

7.1 SUMMARY

1. Intensive Care Units are specialized environment in hospitals where critically ill patients are treated and contain special hospital settings. Moreover they are like ecological niches that harbor one of the most notorious nosocomial pathogen, *Pseudomonas aeruginosa*. So, the study was conducted in order to deal with the source of infections to the patients from the environment.
2. Since infections are caused from the close surroundings, the immediate vicinity of the patients like floor, bed linings, working table and aprons of the HCWs from where swabs were collected as the inanimate surface samples.
3. Clinical specimens and surface swab samples from these ICUs were collected in order to isolate *P. aeruginosa* and characterize them.
4. Pigmentation produced, consistency of the colonies and AST pattern of the organism were studied.
5. A total of 360 surface swabs were processed out of which 60 samples yielded *P. aeruginosa*. Similarly, out of 700 clinical samples the organism was isolated from only 66 samples.
6. The antibiotic discs were selected according to their mode of actions so as to include all the possible ways to affect the organism.
7. Maximum number of antibiotics proved to be ineffective against *P. aeruginosa* isolated from clinical samples. However, *P. aeruginosa* isolated from the surface swabs were highly sensitive to almost all the antibiotics used.
8. Significant number of MDRPA was isolated from clinical samples.

7.2 RECOMMENDATION

1. Whenever multiple resistance is observed with cephalosporins, fluoroquinolones, carbapenems and aminoglycosides, *P. aeruginosa* should be tested for resistance against combined antibiotics as well as polymixins. So it is always prudent to treat infections based on infecting organism's antibiograms obtained from microbiological analysis.

2. Since higher rates of MDRPA were identified, rational use of antibiotics should be made which helps to combat with the alarming rate.

3. Many mucoid strains isolated from clinical samples are reluctant towards different antibiotics. So further investigation should be done when they are isolated.

4. Since the organism is present in inanimate surfaces, regular disinfection of the floors, working tables and aprons must be done and its effectiveness must be tested.
5. Hospital staffs should wear aprons prior to be in contact with the patients infected with *P. aeruginosa* and should not handle other patients with the same attire as the organism persists on them.

CHAPTER VIII

8. REFERENCES

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APPENDIX-I

Sample information and record keeping

[A] Clinical Samples

Ward: ASICU ☐ PSICU ☐ MICU ☐

Date: ……

Name of Patient: ……. Age: …… Sex: …….

Bed No.: ……

Sample Type: ……..

[B] Surface Swab Samples

Ward: ASICU ☐ PSICU ☐ MICU ☐

Date: ……

Swab Type: ……

Day 1

1. Collection of samples
2. Inoculation into BHI broth (for inanimate surface swabs) for enrichment
3. Inoculation into MA and BA (for clinical samples)

Day 2

1. If turbidity present in BHI broth, inoculation into MA and BA
2. Observation for the NLF colonies on MA
3. Subculture on cetrimide agar

Day 3

1. Observation for the NLF colonies on MA and subculture on cetrimide agar
2. If pigmentation seen on cetrimide agar subculture on NA
3. If no pigmentation seen on cetrimide agar, further incubation for 24 hours

Day 4

1. Gram staining
2. Catalase and Oxidase tests and their results
3. Biochemical tests

Day 5

1. Results of biochemical tests
2. Antibiotic sensitivity testing

Day 6

1. Results of AST

S.N.	Antibiotics Used	Results
1	Piperacillin/Tazobactam	
2	Cefoparazone/sulbactam	
3	Meropenem	
4	Ciprofloxacin	
5	Gentamicin	
6	Amikacin	
7	Ceftiaxone	
8	Cefixime	
9	Ceftazidime	
10	Polymyxin-B	

APPENDIX-II

Equipments and materials used during the study

Equipments

Autoclave: Sakura, Japan
Distillation plant: Elix 5 UV Millipore, France
Refrigerator: LG, Korea
Hot Air Oven: SKDO-1, Korea
Incubator: Clayson IM 1000, Australia
Weighing balance: Ohaus, USA
Microscope: Olympus, Japan
Heater: India

Media used

Brain Heart Infusion broth
Mac Conkey agar
Nutrient agar
Blood agar base
Mueller Hinton agar
Simmon's citrate agar
Urea broth
Triple sugar iron agar
Sulfide indole motility agar
MR-VP medium

The entire media used were from Hi-media Laboratory

Reagents

Crystal violet
Gram's iodine
Absolute alcohol
Safranin
3% H_2O_2
Paraffin oil
Normal saline
Barritt's reagent
Kovac's reagent

All of these reagents were brought from the local suppliers.

Antibiotic discs

Piperacillin/Tazobactam (100/10 µg)
Cefoparazone/sulbactam (75/30 µg)

Meropenem (10 µg)
Ciprofloxacin (5 µg)
Gentamicin (10 µg)
Amikacin (30 µg)
Ceftriaxone (30 µg)
Cefixime (5 µg)
Ceftazidime (30 µg)
Polymyxin-B (300 units)

These entire antibiotic discs used were brought from Hi-media Laboratory except for the cefoparazone/sulbactam, which was from Becton, Dickinson and Company.

APPENDIX III

A. Composition and preparation of different culture media

All the culture media used were from the Hi-media Laboratory.

1. **Nutrient agar**

Composition	gm/ltr
Peptic digest of animal tissue	5.00
Beef extract	1.50
Yeast extract	1.50
Sodium chloride	5.00
Agar	15.00
Final pH at 25ºC	7.4±0.2

28 gram of medium was suspended in 1000 ml of the distilled water and boiled to dissolve completely. Then medium was autoclaved at 121 ºC (15lbs pressure) for 15 min. the sterilized medium was then poured in to sterilized petridishes and then was allowed to cool.

2. **Blood agar base (Infusion agar)**

Composition	gm/ltr
Beef heart infusion form	500
Tryptose	10.00
Sodium chloride	5.00
Agar	15.00
Final pH at 25ºC	7.3±0.2

42.5 gram of the medium was suspended in 1000 ml of distilled water, dissolved by boiling and sterilized by autoclaved at 121ºC for 15 mins. After cooling to

about 50-55 ºC, 5% v/v defibrinated sheep blood was added aseptically, then mixed with gentle rotation and poured in to sterilized petridishes and was allowed to cool.

3. **MacConkey agar**

Composition	gm/ltr
Peptic digest of animal tissue	17.00
Proteose peptone	3.00
Lactose	10.00
Bile salt	1.50
Sodium chloride	5.00
Neutral red	0.03
Agar	15.00
Final pH at 25ºC	7.1±0.2

51.3 gram of the medium was dissolved in 1000 ml of distilled water and then boiled to dissolve completely. The media was autoclaved at 121ºC for 15 min. Sterilized medium was then poured in to sterile petridishes and was allowed to cool.

4. **Mueller Hinton agar (MHA)**

Composition	gm/ltr
Beef Infusion	300.00
Casein Acid Hydrolysate	17.50
Starch	1.50
Agar	17.00
Final pH at 25ºC	7.3±0.2

38 gram of the medium was dissolved in 1000ml of distilled water and then boiled to dissolve completely. The medium was autoclaved at 121ºC for 15 mins. The sterilized medium was then poured in sterilized petridishes and was allowed to cool.

B. Composition and preparation of different biochemical test media

1. **Simon citrate agar**

Composition	gm/ltr
Magnesium sulfate	0.20
Mono ammonium dihydrogen phosphate	1.00

Dipotassium phosphate	1.00
Sodium citrate	2.00
Sodium chloride	5.00
Bromothymol blue	0.08
Agar	15.00
Final pH at 25°C	6.8±0.5

24.2 gram of the medium was dissolved in 1000 ml of distilled water and boiled to dissolve completely. 3 ml of medium was dispensed in each tube and autoclaved at 121°C or 15 minutes. The sterilized mediums are allowed to settle at slant forming position.

2. **Urea agar base (Christensen urea agar)**

Composition	gm/ltr
Peptic digest of animal tissues	1.00
Dextrose	1.00
Monopotassium phosphate	0.8
Dipotassium phosphate	1.20
Sodium chloride	5.00
Agar	15.00
Phenol red	0.012
Final pH at 25°C	6.8±0.2

24 gram of the medium was suspended in 950 ml of water and dissolved by boiling and autoclaved at 121°C for 15 minutes. After cooling to 50 °C, 50 ml of sterile 40% urea solution was poured in to the medium and mixed with gentle rotation. Then 5 ml of the medium was dispensed in each tube and slant was prepared.

3. **Sulfide indole motility (SIM) agar**

Composition	gm/ltr
Peptic digest of animal	30.00
Beef extract	3.00
Peptonized iron	0.20
Sodium thiosulfate	0.025
Agar	3.00
Final pH at 25ºC	7.3±0.2

36.23 gram of the medium was dissolved in 1000 ml of distilled water and boiled to dissolve completely. Then it was dispensed in the test tube about 4 ml and autoclaved at 121 ºC for 15 minutes. Then it was cool down.

4. MR-VP medium

Composition	gm/ltr
Buffered peptone	7.00
Dextrose	5.00
Dipotassium phosphate	5.00
Final pH at 25ºC	6.9±0.2

17 gram of medium was dissolved in 1000 ml of distilled water and boiled to dissolve completely. 3 ml of medium was dispensed in each tube and autoclaved at 121ºC for 15 minutes.

5. Triple sugar iron (TSI) agar

Composition	gm/ltr
Peptic digest of animal tissue	10.00
Casein Enzymatic Hydrolysate	10.00
Yeast extract	3.00
Beef extract	3.00
Lactose	10.00
Sucrose	10.00
Dextrose	1.00
Sodium chloride	5.00
Ferrous sulphate	0.20
Sodium thiosulfate	0.30
Agar	12.00
Phenol red	0.024
Final pH at 25ºC	7.4±0.2

65 gram of the medium was dissolved in 1000ml of distilled water and dissolved completely. Then it was dispensed in to the tubes and autoclaved at 121ºC for 15 minutes. The sterilized medium in the test tube was then allowed to set in slant with a butt of 1inch thickness.

6. Brain Heart Infusion Broth

Composition	gm/ltr
Peptic digest of animal tissue	10.00
Calf brain, infusion (solids)	12.50
Beef heart, infusion (solids)	5.00
Dextrose	2.00

Sodium chloride	5.00
Disodium phosphate	2.50
Final pH at 25ºC	7.4±0.2

37 gram of medium was dissolved in 1000 ml of distilled water. The medium was dispensed into bottles or tubes and autoclaved at 15 lbs pressure (121ºC) for 15 minutes. For best results, the medium should be used on the day it is prepared, otherwise, it should be boiled or steamed for a few minutes and then cooled before use.

7. **Cetrimide Agar Base**

Composition	gm/ltr
Peptic digest of gelatin	20.00
Magnesium chloride	1.40
Potassium sulphate	10.00
Cetrimide	0.30
Agar	15.00
Final pH at 25ºC	7.4±0.2

46.7 gram of medium was dissolved in 1000 ml of distilled water containing 10 ml glycerol and boiled to dissolve completely. The medium was autoclaved at 15 lbs pressure (121ºC) for 15 minutes.

C. Composition and preparation of different staining reagent

1. **Gram stain**

   a. **Crystal violet solution**

Crystal violet	20.00
Ammonium oxalate	9.00
Ethanol or Methanol	95.00ml
Distilled water	1000ml

Preparation: 20 grams of crystal violet was weighed in a clean piece of paper and transferred to a clean brown bottle. Then 95 ml of ethanol was added and mixed until the dye is completely dissolved. To the mixture, 9 grams of ammonium oxalate dissolved in 200ml of distilled water was added. Finally the volume was made 1000ml by addition of distilled water.

   b. **Lugol's Iodine**

Potassium iodide	20 gm
Iodine	10gm

Distilled water	1000 ml

Preparation: to 250 ml of distilled water, 20 gm of potassium iodide was dissolved and 10 gm of iodine was mixed to it until it was dissolved completely. Finally the volume was made 1000mlby addition of distilled water.

c. Acetone alcohol decolorizer

Acetone	500ml
Ethanol (Absolute)	475ml
Distilled water	25ml

Preparation: 475 ml of ethanol was added to 25 ml of distilled water and mixed and kept in a clean bottle. Then immediately 500ml of acetone was added to the bottle and mixed well.

d. Safranin (Counter stain)

Safranin (2.5% in 95% ethanol)	10.00 ml
Distilled water	100 ml

Preparation: 2.5% of Safranin solution was prepared in 95% ethanol and 10 ml of prepared suspension was mixed in 100 ml of distilled water.

2. Normal saline

Sodium chloride	0.85gm
Distilled water	100ml

Preparation: o.85 gram of sodium chloride was weighed and added to a bottle containing 100ml of distilled water and mixed well to dissolve the salt completely and autoclaved. Then it was stored.

3. Biochemical Test Reagents

a. For catalase test

Catalase reagent (3% H_2O_2)

Hydrogen peroxide	1ml
Distilled water	9ml

Preparation: To the 9ml of distilled water, 1ml of hydrogen peroxide was added and mixed well so as to make 3% solution of hydrogen peroxide.

b. For oxidase test

Oxidase strip soaked in oxidase reagent

Tetra methyl para-phenylene diamine dihydrochloride(TPD)	1gm
Distilled water	100ml

Preparation: 1 gram of TPD was dissolved in 100 ml of distilled water and strips of Whatmann no. 1 paper was soaked and drained for anout 30seconds. Then the strip was freeze dried and stored in dark bottle tightly.

c. For indole test

Kovac's indole reagent

Para Dimethyl amino benzaldehyde	2.00gm
Iso-amyl alcohol	30.00ml
Concentrated hydrochloric acid	10.00ml

Preparation: in 30 ml of isoamyl alcohol, 2 gram of para amino bemzaldehyde was dissolved and transferred to clean brown bottle. Then to this solution,10 ml of concentrated hydrochloric acid was added and mixed well.

d. For methyl red test

Methyl red solution

Methyl red	0.05gm
Ethyl alcohol	28.0ml
Distilled water	22.0ml

Preparation: 0.05 gm of methyl red was dissolved in 28 ml of ethanol and transferred to a clean brown bottle. To this, 22 ml of distilled water was added and mixed well.

e. For Voges Proskauer test

Barritt's reagent

Solution A

Alpha-Napthol	5.0gm
Ethyl alcohol	100ml

Preparation: 5gm of α-Napthol was dissolved in 25 ml ethanol and transferred in to clean bottle. Then final volume was made 100ml by adding ethanol.

Solution B

Potassium hydroxide (KOH)	40.0gm
Distilled water	100ml

Preparation: 40 gram of KOH was dissolved in 25 ml of distilled water and transferred in to the clean bottle and final volume was made 100ml by adding distilled water.

4. Turbidity standard equivalent to McFarland 0.5

1% V/V solution of sulphuric acid was prepared by adding 1ml of concentrated sulphuric acid to 99 ml of distilled water. 1% W/V solution of barium chloride was prepared by dissolving 0.5 gram of dehydrate barium chloride in 50 ml of distilled water. Then to the 99.5ml of 1% sulphuric acid solution, 0.5 ml of barium chloride solution was mixed and stirred continuously. Then the solution was transferred in to the clean screw capped tube and stored at dark place until use. The test tube for the broth preparation should be of same size as of McFarland tube. The tubes can be stored and used for six months.

APPENDIX IV

A. Gram staining procedure (Forbes et al., 2007)

Gram staining is differential staining that differentiates all the bacterial species in to two large groups: gram positive and gram negative. Following steps are involved during gram staining.

1. A thin film of material to be examined was prepared and dried.
2. The material on the slide was heat fixed and allowed to cool before staining.
3. The slide was flooded with crystal violet stain and allowed to remain without drying for 10-30 second.
4. The slide was rinsed with tap water, shaking off excess.
5. Then the slide was flooded with iodine solution and allowed to remain on the surface without drying for twice as long as the crystal violet was in contact with the slide surface.
6. The slide was rinsed with tap water, shaking off excess.
7. The slide was flooded with acetone alcohol decolorizer for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolorizer. Thicker smear require more aggressive decolorization.
8. The slide was flooded with counter stain Safranin for 30 seconds and washed off with tap water.
9. The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 100X.

B. Capsule staining procedure

Gin's Method was followed for capsule staining that uses india ink to color the background and crystal violet to stain the bacterial cell "body".

1. A loop was used to mix a drop of water, a drop of india ink and an isolated colony of *P. aeruginosa* together at the end of a slide.
2. Another slide was used to spread the smear like a blood smear. The smear was allowed to air dry. Heat fixation should not be done.
3. The smear was flooded with crystal violet for 1 minute. It was washed with water, blotted, dried and observed.

C. Procedure for Antibiotic Sensitivity Testing (AST) by Disc Diffusion Method.

In the treatment and control of infectious disease, AST is done to select effective antimicrobial drugs against suspected organisms. Disc diffusion method is widely used technique for susceptibility testing and done by Kirby Bauer disc diffusion method.

The following steps are involved in AST by Kirby Bauer disc diffusion method.

1. An isolated colony of organism was suspended in the nutrient broth and incubated at 37ºC for 4 hours. The turbidity was matched with 0.5 McFarland turbidity standards.
2. A sterile cotton swab was taken and introduced in to the tube taken out the organism and swabbed uniformly on the surface of Mueller Hinton agar medium.
3. The plate was allowed to dry and antibiotic disc were placed on the agar surface and incubated for 18-24 hours.
4. After incubation the zone size was measured and results were interpreted according to the standard guidelines.

APPENDIX V

Zone size of interpretation chart of antibiotics (CLSI interpretation, 2009)

Antibiotic used	Concentration (µg)	Diameter of zone size(m		
		Resistant	intermediate	Sensiti

Piperacillin/Tazobactam	100/10	≤ 17		≥ 18
Cefoparazone/sulbactam	75/30	≤ 15	16-20	≥ 21
Meropenem	10	≤ 13	14-15	≥ 16
Ciprofloxacin	5	≤ 15	16-20	≥ 21
Gentamicin	10	≤ 12	13-14	≥ 15
Amikacin	30	≤ 14	15-16	≥ 17
Ceftiaxone	30	≤ 13	14-20	≥ 21
Cefixime	5	≤15	16-18	≥19
Ceftazidime	30	≤14	15-17	≥18
Polymyxin-B	300 units	≤ 11		≥ 12

(Note: CLSI- Clinical Laboratory Standard Institute)