2.0 REVIEW OF LITERATURE

2.1 Historical background

*Pseudomonas* belongs to the genus gammaproteobacteria, of family *Pseudomonadaceae* and contains around 191 species (Poole, 2004). It was first isolated from pus in 1882 (Stover et al., 2000). The transmissible agent that was responsible for the bluish-green colour of surgical wound bandages was recognized by Se’dillot in 1850. Fordos (1860) extracted the pyocyanin pigment that gives such colouration, while Lucke (1862) associated the pigment with a rod-shaped organism. However, it was not until 1882 when *P. aeruginosa* was first described as a pathogen in France by Carle Gessard and isolated the organism, under its initial name *Bacillus pyocyaneus*, from two patients having cutaneous wounds with bluish-green pus (Gessard, 1984, Lister et al., 2009). This explains the original name for *bacillus* meaning rod, *pyo* refers to pus, and *cyaneous* to blue. The present name, *Pseudomonas aeruginosa*, means that this etiologic unit of infection (*monad*) has resemblance (*pseudo*) to green or copper-like shapes (*aeruginosa*) (Villavicencio, 1998). The genus *Pseudomonas* was described in 1894 and is one of the most diverse and ubiquitous bacterial genera whose species have been isolated worldwide and in all kinds of environments (Forster et al, 1998). Members of this genus are aerobic, non-spore forming, Gram negative rods having one or more polar flagella.

2.2 Characteristics of *P. aeruginosa*

*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. The bacterium is ubiquitous and is present in soil and water. Its metabolism is never fermentative, but it can grow in the absence of O₂ if NO₃ is available as a respiratory electron acceptor. It can oxidise sugars including fructose, galactose, glucose and xylose by utilizing Entner-Doudoroff pathway instead of glycolysis (Palleroni, 1984). The typical *Pseudomonas* bacterium in nature might be found in a biofilm, attached to some surface or substrate, or in a planktonic form, as a unicellular organism, actively swimming by means of its flagellum. *Pseudomonas* is one of the most vigorous, fast-swimming bacteria seen in hay infusions and pond water samples. It exhibits three distinct forms of motility: swimming, twitching and swarming which allow its movement in liquid and solid surfaces. It swims
by a single polar flagellum whereas twitching requires sequential extension and retraction of type IV pili, while swarming requires both flagella as well as type IV pili (Kohler et al, 2000, Merz et al, 2000). In its natural habitat *Pseudomonas aeruginosa* is not particularly distinctive as a pseudomonad, but it does have a combination of physiological traits that are noteworthy and may relate to its pathogenesis (Todar, 2004).

*P. aeruginosa* has simple nutritional requirements. It is often observed "growing in distilled water", which is evidence of its minimal nutritional needs. In the laboratory, the simplest medium for growth of this organism consists of acetate as a source of carbon and ammonium sulfate as a source of nitrogen. It possesses the metabolic versatility and can use more than eighty organic compounds for growth and serve as energy as well as carbon sources (Palleroni, 1984).

*P. aeruginosa* is tolerant to a wide variety of physical conditions, including temperature, high concentrations of salts and dyes, weak antiseptics, and many commonly used antibiotics. This organism has a predilection for growth in moist environments, which is probably a reflection of its natural existence in the soil and water. These natural properties of the bacterium contribute to its ecological success as an opportunistic pathogen and ubiquitous nature of the organism and its prominence as a nosocomial pathogen (Todar, 2004).

*P. aeruginosa* isolates may produce three types of colonies. Natural isolates from the soil or water typically produce small, rough colonies. Clinical samples, in general, yield one or more smooth colony types. One type has a fried-egg appearance which is large, smooth, with flat edges and an elevated appearance. Another type has a mucoid appearance and is frequently obtained from respiratory tract and urinary tract secretions, which is attributed to the production of alginate slime. The smooth and mucoid colonies are presumed to play a role in colonization and virulence. Strains with alterations in colony morphology are produced during biofilm growth, following exposure to environmental and antibiotic stresses and during chronic infections of the human airways (Sheehan et al, 1982, Haussler et al, 1999, Haussler et al, 2003, Deziel et al, 2001, Drenkard and Ausubel, 2002, Boles et al, 2004, Gotz et al, 2004). *P.*
*P. aeruginosa* strains produce two types of soluble pigments, the fluorescent pigment pyoverdin and the blue pigment pyocyanin. The latter is produced abundantly in media of low-iron content and has a role in the iron metabolism of the bacterium. Pyocyanin refers to "blue pus" which is a characteristic of suppurative infections caused by *P. aeruginosa* (Murray et al, 2003). Only a few antibiotics are effective against *Pseudomonas* such as fluoroquinolones, gentamicin and imipenem. However these antibiotics may not be effective against all the strains of *P. aeruginosa*. The futility of treating *Pseudomonas* infections with antibiotics is most dramatically illustrated in cystic fibrosis patients, virtually all of whom eventually become infected with a strain that is resistant and difficult to treat.

*P. aeruginosa* can grow over a wide range of temperatures (5-42°C), with an optimum temperature of 37°C. Typical biochemical features are: positive oxidase test, hydrolysis of arginine, nitrate reduction and grape like odour (Todar, 2004). The organism is capable of producing a wide variety of virulence factors, such as lipopolysaccharides, flagellum, type IV pili, proteases, exotoxins, pyocyanin, exopolysaccharides, and type III secretion. *P. aeruginosa* can gain entry into the human host with the help of type IV pili and flagella. Pili are composed of a helical polymer of the PilA subunit and are thought to mediate adherence to a variety of cultured epithelial cells by binding to the cell glycopolysaccharides and low molecular weight polypeptides. It binds to the carbohydrate moiety of the glycosphingolipids (GSL), asialo-G(M1) and asialoG(M2) and to lactosyl ceramide and ceramide trihexoside. The disaccharide sequence GalNAc beta (1-4) Gal, common in both asialo-G(M1) and asialo-G(M2), likely represents the minimal structural receptor motif recognized by the pili. *P. aeruginosa* pili also bind to surface-localized proteins of human epithelial cells and other cell types, suggesting that non-sialylated GSL and (glyco)proteins function as receptors of pili (Hahn, 1997). Pili are also important in biofilm development, mediating twitching and flagellar motility. Biofilm formation can be regarded as one of the virulence factors. Flagella can bind to cell surface molecules asialo-GM1 and TLR5, activating interleukin-8 production. *P. aeruginosa* uses the type III secretion system for efficient delivery of bacterial toxins that inhibit the actin cytoskeleton and protein synthesis of the host cell, causing cell death and...
inhibit phagocytosis. Expression of type III toxins is tightly regulated by various environmental and genetic factors such as contact dependence, low calcium levels and the presence of serum.

The physiology, biochemistry and resistance to antibiotics and disinfectants explain *P. aeruginosa*’s comprehensive adaptability which allows it to survive and multiply easily in many different habitats including human host (Stainer et al, 1966). *P. aeruginosa* consists of O and H antigens. Somatic or O antigens are group-specific and *P. aeruginosa* possesses 19 of these, which are heat stable and can be extracted with acid or formamide. Serogroups O6 and O11 are isolated from majority of the clinical specimens. Flagellar or H antigen found in the flagella is heat labile.

### 2.3 Genome of *P. aeruginosa*

The genome of *P. aeruginosa* strain PaO1 was sequenced by Stover et al (2000). The determination of the entire genome sequence of *P. aeruginosa* and the application of powerful DNA array techniques to reveal microbial gene expression *in vivo* should provide us with a clearer insight into the mechanisms involved. It is the largest bacterial genome sequenced so far and contains 6.26 Mbp (encoding 5567 genes) compared to 4.64 Mbp (4279 genes) in *Eschericia coli* K12, 2.81 Mbp (2594 genes) in *Staphylococcus aureus* N315 and 1.83 Mbp (1714 genes) in *Haemophilus influenzae* Rd. It contains the highest proportion of regulatory genes. Large numbers of genes are involved in the catabolism, transport and efflux of organic compounds as well as chemosensory systems with four loci that encode probable chemotaxis signal-transduction pathways. In general the number of genes needed for cell growth and division in a minimal salts medium, including all enzymes needed for metabolism and structural proteins is estimated around 1500. *P. aeruginosa* therefore, possesses considerable additional genetic capacity compared with other organisms. This explains its highly adaptable and versatile nature, including the ability to develop resistance where antibiotics are used extensively. (Lambert et al, 2002)

### 2.4 Clinical manifestations

*P. aeruginosa* is involved in several clinical conditions such as urinary tract infections, respiratory tract infections, cystic fibrosis, ophthalmic infections, burns, surgical wounds,
otitis etc. This organism infects healthy tissues rarely, but, in immunocompromised individuals, it can infect all tissues. This explains why most infections are nosocomial (Morrison & Wenzel, 1984). These infections should be considered as severe, and even life-threatening in specific situations, with the highest rates of mortality recorded for cases of bacteraemia in neutropenic patients (30–50%) (Maschmeyer et al, 2000) and cases of nosocomial pneumonia (45–70%) (Crouch et al, 1996, Rello et al, 1997). P. aeruginosa is well-adapted to the respiratory tract environment, especially in patients with chronic obstructive bronchopulmonary disease, who are immunocompromised, or hospitalised in intensive care units (Ferrara et al, 2006, Shaw, 2005). P. aeruginosa is the predominant cause of nosocomial pneumonia in ventilated patients (Chastre et al, 2002) and of lung infections of cystic fibrosis cases (Ratjen et al, 2006). It also causes chronic colonisation of the airways of patients suffering from bronchiestasis, chronic obstructive bronchopulmonary disease or cystic fibrosis (Nicotra et al, 1995). Cancer patients undergoing chemotherapy which are having neutropenia, bacteraemia with P. aeruginosa is a common complication (Kremery et al, 2006). Bacteraemia and septicaemia can also occur in cases of immunodeficiency related diseases such as AIDS, diabetes mellitus or severe burns (Marra et al, 2006, Sligl et al, 2006, Milstone et al, 2005). Most of these infections are acquired in hospitals and nursing homes (Dubois et al, 2005). This organism is also the third leading cause (12%) of hospital-acquired urinary tract infections (Obritsch et al, 2005). It is the predominant causal agent of ‘swimmer’s ear’ (a form of external otitis) (Wang et al, 2005) and of malignant otitis in diabetics (Rubin et al, 1988). It can also cause the ophthalmic infections (e.g., bacterial keratitis in individuals with contact lenses (Stern et al,1990), or neonatal ophthalmia), meningitis and brain abscesses (Wise et al,1969), and endocarditis in intravenous drug users (Vrochides et al,2003). Skin and bone infections are rare, but can occur after puncture wounds (Pier et al, 2005). P. aeruginosa rarely causes true infections of the digestive tract, although peri-rectal infections, typical gastroenteritis and necrotising enterocolitis have been reported, but colonization by P. aeruginosa favours the development of invasive infections in individuals at risk (Marshall et al, 1993, Mesaros et al, 2007).
In a study carried out in intensive care unit (ICU) of a university hospital of the West Indies, Apaka et al in 2002 found *P. aeruginosa* to be the second most common isolate among ICU patients. A study from India has revealed 32% prevalence rate of *P. aeruginosa* in patients of wound infections (Anupurba et al, 2006). A study conducted by Sharma et al, 2010 revealed the prevalence rate of 48.22% of *P. aeruginosa* in neonatal septicemia. *P. aeruginosa* is responsible for 3-7% bloodstream infections and high mortality rates of 27-48% in critically ill patients in India (Navneeth et al, 2002). Several studies conducted in India demonstrated that *P. aeruginosa* is the most common pathogen among diabetic-foot infections (Ramakant et al, 2011, Sivanmaliappan et al, 2011, Bansal et al, 2008, Sharma, 2006, Shankar et al, 2006). About 80% of adults with cystic fibrosis have chronic lung infections due to *P. aeruginosa* which is responsible for high morbidity and mortality rates (Ramakant et al, 2011).

2.5 Epidemiology

*P. aeruginosa* is an aerobic Gram-negative opportunistic pathogen which is one of the pathogens implicated in both community-acquired and hospital-acquired infections. Around 11-13.8% nosocomial infections are caused by *P. aeruginosa*. These include pneumonias, urinary tract infections (UTI’s), bloodstream infections, surgical site infections and skin infections (Driscoll et al, 2007). Community infections are acquired from places other than a healthcare facility. Some of the community acquired infections include ulcerative keratitis, otitis externa, osteomyelitis of the calcaneus in children, endocarditis in intravenous drug users and skin and soft tissue infections. *P. aeruginosa* is the major pathogen in Cystic fibrosis patients. Infections caused by *P. aeruginosa* are not only common (Rello et al, 2002, Kollef et al, 2005) but they have also been associated with high morbidity and mortality (Rossolini and Mantengoli, 2005, Driscoll et al, 2007). This organism been identified as a common pathogen in burn-patients in numerous studies worldwide and believed to be responsible for approximately 6% of surgical site infections, 9.5% in ICU’s according to a study reported to the National Nosocomial Infections Surveillance (NNIS) System from 1986-2003. In another study, *P. aeruginosa* was reported to be responsible for 16% surgical site infections (Richards et al, 1999). Around 9% of nosocomial urinary tract infections are caused by *P. aeruginosa*.
and 16.3% of UTI's in ICU patients. *P. aeruginosa* is also responsible for 10.5% of UTI’s in patients with indwelling urinary catheters and 4.1% in those without these devices. Nosocomial bloodstream infections due to *P. aeruginosa* account for 4.6% cases. Occurrence rates ranging between 14-20% has been reported in ICU’s. *P. aeruginosa* is an important pathogen in both the primary and acquired immunodeficiency cases. This organism also plays an important role is patients with cystic fibrosis, 97.5% of cystic fibrosis patients infected with *P. aeruginosa* have been reported in the children of three years of age (Burns et al, 2001).

### 2.6 Pathogenesis

The disease process in opportunistic pathogen such as *Pseudomonas aeruginosa*, begins with some alteration or circumvention of normal host defenses. Wide array of virulence determinants are possessed by this bacterium. Multiple and diverse determinants of virulence are expected in the wide range of diseases caused by *P. aeruginosa* which include: septicemia, urinary tract infections, pneumonia, chronic lung infections, endocarditis, dermatitis, and osteochondritis. A single flagellum that is responsible for motility and multiple cell surface pilus of *P. aeruginosa* are responsible for adherence to cell membranes and other surfaces (O’Toole et al, 1998). Some isolates of *P. aeruginosa* over produce the extracellular polysaccharide alginate, that has been found to have a number of effects that may delay bacterial killing by the host, including scavenging of free radicals released by macrophages, providing a physical barrier that weakens phagocytosis, and inhibiting neutrophil chemotaxis and complement activation (Ramsay et al, 2005). Alginate appears to be important for the formation of *P. aeruginosa* biofilms. Such biofilms are present in the airways of patients with cystic fibrosis (Singh et al, 2000). The biofilms are believed to arise in the respiratory tract of cystic fibrosis patients through a series of steps beginning with the attachment of planktonic *P. aeruginosa* to epithelial cells of the respiratory tract or debris within the airways (Ramsay et al, 2005). Groups of these planktonic bacteria are able to communicate via intercellular signals in a process called ‘quorum sensing’ (Smith et al, 2003). Additionally, when the organism binds to an epithelial cell, the type III secretion system may be activated which enables *P. aeruginosa* to inject certain effector proteins

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directly into the epithelial cells, which results in injury and death of the cells as well as altered immune responses. Four exoenzymes (ExoS, ExoT, ExoU and ExoY) are variably expressed in different strains which have different activities. ExoU may be responsible for the greatest virulence (Sadikot et al, 2005, Hauser et al, 2002, Schulert et al,2003). Expression of type III secretion system in *P. aeruginosa* isolates has been associated with increased mortality in the cases of pneumonia including ventilator associated pneumonia, sepsis and respiratory failure. Other secreted virulence factors like exotoxin A inhibits elongation factor 2 resulting in the slowing of protein synthesis and finally host cell death (Lau et al, 2005). Alkaline proteases, elastases and protease IV are secreted enzymes capable of degrading multiple immunoregulatory proteins, including surfactant proteins A and D, complement, immunoglobulin and antibacterial peptides (Mariencheck et al, 2003, Schmidtchen et al, 2003). The phenazines (e.g. pyocyanin) are secreted metabolites that cause ciliary dysfunction in the respiratory tract and exert proinflammatory and oxidative effects damaging the host cells (Lau et al, 2005).

### 2.7 Multiple drug Resistance to antibiotics

The ability of bacteria to modify their genomes is the main reason behind the bacterial resistance to antimicrobial agents. The Gram negative bacteria are showing slow but steady upward trend of resistance as compared to Gram positive bacteria. The main problem with the Gram negative bacterial infections is their effective treatment. The beta lactam group of antibiotics includes: penicillins (ticarcillin, piperacillin), cephalosporins (ceftazidime, cefepime), monobactams (aztreonam) and carbapenems (imipenem, meropenem). These antibiotics are commonly used in the treatment of *P. aeruginosa* infections (Paul et al, 2010) and resistance to these agents is increasing progressively. This resistance is mediated by a variety of mechanisms, most commonly antibiotic cleavage by beta lactamase enzymes, antibiotic expulsion by chromosomally encoded efflux mechanisms and reduced drug uptake owing to loss of outer membrane porin proteins (Poole, 2004, Pfeifer et al, 2010). Acquired metallo-β-lactamases have emerged as the resistance mechanism owing to their capacity to hydrolyze all the beta lactams including carbapenems (Walsh, 2008). In a recent report, the Infectious Diseases Society of America addressed three categories of emerging multiple drug resistant Gram negative
bacilli: carbapenem-resistant organisms like *Acinetobacter* species, *Pseudomonas* and *Klebsiella* (Talbot et al, 2006). The occurrence of multidrug resistant *P.aeruginosa* strains is increasing worldwide and limiting our therapeutic options. In a study conducted in Taiwan, 80% clinical isolates were found resistant to beta lactam antibiotics (Yang et al, 2008). Muller-Premru et al (2000) observed 2.6, 9.1, 9.6, 45.7, 39.9 and 51.0 percent of *P. aeruginosa* isolates resistant respectively to piperacillin, ceftazidime, imipenem, ciprofloxacin, amikacin and gentamicin. Resistance to the tune of 40% to imipenem, meropenem and gentamicin has been reported from Europe (Chinou et al, 2006). Multidrug resistant isolates have been reported by Shahid et al (2003) of which 83.3% was resistant to seven or more antibiotics with markedly high resistance (56.7%) to amikacin. Mehta et al, 2001, from Chandigarh reported 11, 12, 13.5, 30, 20 percent resistant isolates recovered from blood and 10, 10, 10, 28, 16 percent resistant isolates recovered from pus against amikacin, ciprofloxacin, piperacillin, gentamicin, ceftazidime. Sharma et al (2010) reported 63.6% MDR isolates of *P. aeruginosa* out of which 62% were sensitive to imipenem and 37.9% resistant to this antibiotic and 92.3, 91.2, 88.8 and 76.2 percent were resistant to gentamicin, amikacin, piperacillin and ciprofloxacin respectively. In various studies around the world, varying resistance (4-60%) has been observed towards imipenem and meropenem (Forster et al, 1998). Navneeth et al (2002) reported that 12% of *Pseudomonas* isolates from Bangalore were resistant to imipenem and were MBL producers. In another study conducted at tertiary health care center of North India in Chandigarh 42% of *Pseudomonas* spp. and 18.5% of *Acinetobacters* were resistant to imipenem (Taneja et al, 2003). Jesudasen et al (2005) reported 28/48 (58.3%) were *P. aeruginosa*. Another study from Chennai showed 16% *P. aeruginosa* strains resistant to imipenem (Shankar et al, 2006). Higher proportion of resistance to imipenem (17.32%) and meropenem (2.2 – 16%) has been observed among *P. aeruginosa*, *Acinetobacter* species and other Gram negative bacteria at All India Institute of Medical Sciences (AIIMS), New Delhi (Gupta et al, 2006), 25% of the isolates were resistant to both meropenem and imipenem. 20.8% were MBL producers at Mumbai (Varaiya et al, 2008). *P. aeruginosa* is a highly adaptable organism. It can grow on a wide variety of substrates and alter its properties in response to changes in the environment.
2.8 Carbapenems

The carbapenems are a class of broad spectrum β-lactam antibiotics that is considered to be the most potent with a very broad spectrum of antimicrobial activity. Their spectrum of antimicrobial activity includes Gram-positive, Gram-negative and anaerobic pathogens (Shah, 2008 and Chan, 2008). Unfortunately, the emergence of multidrug-resistant pathogens seriously affects this class of β-lactam antibiotics. As described earlier the resistance to carbapenems is increasing throughout the world. Various carbapenems that are used as therapeutic options to treat *P. aeruginosa* infections are imipenem, meropenem, ertapenem, doripenem. Carbapenems contain a four-member lactam ring fused to a five member thiazolidinic secondary ring through the nitrogen and adjacent tetrahedral carbon atom. The side chains (encircled) attached to the basic two ring structure differentiate the carbapenems from each other and influence antimicrobial activity and provide significant activity against β-lactamase producing bacteria. The structures of these four carbapenems are shown through Fig. 2.1.

**Imipenem**

Imipenem is a semisynthetic carbapenem (C₁₂H₁₇N₃O₄S) that has a wide spectrum of antibacterial activity against Gram-negative and Gram-positive aerobic and anaerobic bacteria, including many multidrug resistant strains. It is stable to beta-lactamases. It is chemically (5R,6S)-3-[2-(aminomethylideneamino)ethylsulfanyl]-6-(1-hydroxyethyl)-7-oxo-1-azabicyclo [3.2.0] hept-2-ene-2-carboxylic acid with a molecular weight equal to 299.3460. It is important for its activity against *P. aeruginosa* and *Enterococcus* species. Imipenem is rapidly degraded by the renal enzyme dehydropeptidase when administered alone, that’s why it is always co-administered with cilastin to prevent its inactivation. It acts through inhibiting cell wall synthesis of various Gram-positive and Gram-negative bacteria.

**Meropenem**

Meropenem is a broad spectrum antibiotic (C₁₇H₂₅N₃O₅S) effective against both Gram-positive and Gram-negative bacteria. It exerts its action by penetrating bacterial cells readily and interfering with the synthesis of cell wall components, which leads to cell
death. It is chemically (4R,5S,6S)-3-[(2S,5S)-5-(dimethylcarbamoyl)pyrrolidin-2-yl]sulfanyl-6-(1-hydroxyethyl)-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid with a molecular weight equal to 383.4630. It reaches the penicillin-binding-protein (PBP) targets by penetrating the cell wall.

![Fig 2.1 Structures of various carbapenems](image)

**Doripenem**

It is a new synthetic 1β-methylcarbapenem (C_{15}H_{24}N_{4}O_{6}S_{2}). Its structure is very similar to meropenem. It is chemically (4R,5S,6S)-3-[(3S,5S)-5-(sulfamoylaminomethyl)pyrrolidin-3-yl] thiol-6-[(1R)-1-hydroxyethyl]-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid with a molecular weight equal to 420.50426. It inactivates multiple essential PBPs resulting in cell wall synthesis with subsequent cell death.
Ertapenem
Meropenem (C_{22}H_{25}N_{3}O_{7}S) is very similar to meropenem in that it possesses a 1-beta-methyl group. It is chemically (4R,5S,6S)-3-[(3S,5S)-5-(3-carboxyphenyl) carbamoyl] pyrrolidin-3-yl]sulfanyl-6-(1-hydroxyethyl)-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2 carboxylic acid with a molecular weight equal to 475.5150. Its bactericidal activity results from the inhibition of cell wall synthesis and is mediated through ertapenem binding to PBPs. It is less active against P. aeruginosa.

2.8.1 Prevalence of carbapenem resistance in P. aeruginosa
The increasing prevalence of nosocomial infections produced by carbapenem resistant P. aeruginosa strains severely compromises the selection of appropriate therapy and is therefore, associated with significant morbidity and mortality. In Spain, 18.8% strains were reported resistant to imipenem and/or meropenem (Gutierrez et al, 2007). In France 87.5% strains were reported resistant to imipenem while 78.1% were resistant to meropenem (Rodríguez-Martínez, 2009). Overall percentages of strains resistant to meropenem and imipenem have been reported as 22.16 and 17.32 respectively was seen in various bacterial species at Delhi (Gupta et al, 2006). Karthika et al, 2008 reported all the strains tested resistant to imipenem whereas 89% strains were found resistant to meropenem. Manoharan (2010) reported 42.6% resistance to imipenem and meropenem including ceftazidime. 100% resistance to imipenem and 87% resistance to meropenem have been reported from Mexico (Garza-Ramos et al, 2008). 31% isolates were resistant to imipenem in Serbia (Jovcic et al, 2011). Khan et al (2008) reported 26.7% imipenem and 28% meropenem resistance in P. aeruginosa in Pakistan. In Iran, 38.28% P. aeruginosa strains were resistant to imipenem (Saderi et al, 2008). Strateva et al (2007) reported 42.3% & 45.5% resistance towards imipenem and meropenem respectively. In Greece, Tsakris et al (2000) reported 16.5% resistance for imipenem and meropenem in P. aeruginosa during 1996 to 1998.

2.8.2 Mechanisms of carbapenem resistance
Resistance to carbapenems is mediated by a variety of mechanisms e.g reduced drug uptake owing to loss of outer membrane porin proteins, target-site modifications by Penicillin binding proteins (PBPs), antibiotic expulsion by chromosomally encoded efflux mechanisms and most commonly by antibiotic cleavage by carbapenemases.
2.8.2.1 Outer membrane proteins

The outer membrane of *P. aeruginosa* presents a significant barrier to the penetration of antibiotics, restricting the rate of penetration of small hydrophilic molecules and excluding others such as the β-lactams and quinolones which can only cross the outer membrane by passing through the aqueous channels provided by porin proteins (Lambert, 2002). Porins are barrel-shaped molecules which span the outer membrane, usually associated as trimers. Three large families of porins, the OprD family of specific porins (19 members), the OprM family of efflux porins (18 members) and the TonB-interacting family of gated porins (35 members), have been reported in *P. aeruginosa* (Zavascki et al., 2010).

High resistance to antimicrobials such as β-lactams among *P. aeruginosa* isolates is well documented. It is due to the low outer-membrane permeability to hydrophilic compounds. Most of the porin channels are non-specific, therefore, the entry of other small hydrophilic agents, such as tetracyclines, aminoglycosides and chloramphenicol can be affected by porin loss (Zavascki et al., 2010).

### Table 2.1 Details of outer membrane proteins (OMPs)

<table>
<thead>
<tr>
<th>OMP</th>
<th>OMP family</th>
<th>Molecular weight (kDa)</th>
<th>Substrates/observation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>OprF</td>
<td>OprF</td>
<td>38</td>
<td>Controversial</td>
<td>Hancock, 2002</td>
</tr>
<tr>
<td>OprM</td>
<td>OprM</td>
<td>50</td>
<td>Component of an efflux system</td>
<td>Hancock, 2002, Masuda et al., 2000</td>
</tr>
<tr>
<td>OprN</td>
<td>OprM</td>
<td>50</td>
<td>Component of an efflux system</td>
<td>Masuda et al., 2000</td>
</tr>
<tr>
<td>OprJ</td>
<td>OprM</td>
<td>54</td>
<td>Component of an efflux system</td>
<td>Masuda et al., 2000, Masuda et al., 1995, Gotoh et al., 1998</td>
</tr>
<tr>
<td>TonB</td>
<td>TonB</td>
<td>37</td>
<td>ND</td>
<td>Zhao et al., 1998, Poole et al., 1996</td>
</tr>
</tbody>
</table>

AMG: Aminoglycoside; BL: β-lactam, CHL: Chloramphenicol; CIP: Ciprofloxacin; IMI: Imipenem; ND: Not determined; TET: Tetracycline.

2.8.2.2 Multi drug efflux pumps

The multidrug efflux systems are composed of three protein components, an energy-dependent pump located in the cytoplasmic membrane, an outer membrane porin and a linker protein which couples the two membrane components together (Lambert, 2002).
Table 2.2 The major efflux pumps identified in *P.aeruginosa*:

<table>
<thead>
<tr>
<th>Efflux system</th>
<th>Efflux pump family</th>
<th>Gene location</th>
<th>Substrates</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MexAB-OprM</td>
<td>RND</td>
<td>Chromosome</td>
<td>FQ, AMG, BL (MEM*, TIC*), TET, TGC, CHL</td>
<td>Poole, 2005</td>
</tr>
<tr>
<td>MexCD-OprJ</td>
<td>RND</td>
<td>Plasmid</td>
<td>FQ, BL (MEM*, TIC*), TET, TGC, CHL, ERY, ROX</td>
<td>Poole, 2005</td>
</tr>
<tr>
<td>MexEF-OprN</td>
<td>RND</td>
<td>Chromosome</td>
<td>FQ, BL (MEM*, TIC*), TET, TGC, CHL</td>
<td>Poole, 2005, Kohler <em>et al</em>, 2001</td>
</tr>
<tr>
<td>MexXY-OprM</td>
<td>RND</td>
<td>Chromosome</td>
<td>FQ, AMG, BL (MEM*, TIC*, FEP*), TET, TGC, CHL</td>
<td>Poole, 2005</td>
</tr>
<tr>
<td>AmrAB-OprA</td>
<td>RND</td>
<td>Chromosome</td>
<td>AMG</td>
<td>Westbrock-Wadman <em>et al</em>, 2004</td>
</tr>
<tr>
<td>PmpM</td>
<td>MATE</td>
<td>ND</td>
<td>FQ</td>
<td>Poole, 2005</td>
</tr>
<tr>
<td>Mef(A)</td>
<td>MFS</td>
<td>Chromosome</td>
<td>MC</td>
<td>Pozzi <em>et al</em>, 2004</td>
</tr>
<tr>
<td>ErmE*full</td>
<td>SMR</td>
<td>ND</td>
<td>AMG</td>
<td>Li <em>et al</em>, 2003</td>
</tr>
</tbody>
</table>

*Preferred substrates.

AMG: Aminoglycoside; BL: β-lactam; CHL: Chloramphenicol; ERY: Erythromycin; FAC: Fusidic acid; FEP: Cefepime; FQ: Fluoroquinolone; LIN: Lincosamide; MATE: Multidrug and toxic compound extrusion; MC: Macrolides; MEM: Meropenem; MFS: Major facilitator superfamily; D: Not determined; NOV: Novobiocin; RIF: Rifampicin; RND: Resistance-nodulation division; ROX: Roxithromycin; SMR: Small multidrug resistance; TET: Tetracycline; TGC: Tigecycline; TIC: Ticarcillin; TMP: Trimethoprim.

Resistance to many antibiotics in *P. aeruginosa* has been identified to be related to hyperexpression of efflux systems acting by themselves or in conjugation with a loss or decreased expression of porins. It may be innate feature or result of a mutation in the efflux system regulatory genes. Many plasmid and chromosomally encoded efflux mechanisms specific for an antimicrobial have been categorized into five classes: multidrug and toxic compound extrusion family, major facilitator superfamily, small multidrug resistance family, and ATP-binding cassette family (Zavaski *et al*, 2010).

### 2.8.2.3 Penicillin binding proteins (PBP s)

The role of penicillin binding proteins in alterations on β-lactam resistance in *P. aeruginosa* is not exactly defined as there are conflicting reports in this regard. There are seven different *Pseudomonas* PBPs (1a, 1b, 2, 3, 4, 6 and 7). PBP1a, -1b, -2, -3 carry essential functions and others are not essential for cell viability. It has been demonstrated that anti-pseudomonal cephalosporins appear to bind preferentially to PBP3, while carbapenems bind to PBP2. Apart from carbapenem binding, PBP2 is the binding site for several β-lactam antimicrobials and is responsible for the rod-shaped morphology of the cells.
2.8.2.4 Carbapenemases

The Gram-negative bacteria that produce carbapenemases show resistance mainly to carbapenems, penicillins and cephalosporins. They usually exhibit resistance to aminoglycosides and quinolones. These enzymes are encoded by genes on chromosomes and mobile genetic elements such as plasmids. Carbapenamases belong to three molecular classes of β-lactamases, Ambler class A, B and D (Ambler, 1980, Bush et al, 1995). Molecular structure and classifications of these enzymes were first of all proposed by Ambler (1980) where β-lactamases were divided into four classes (A, B, C and D) based upon their amino acid sequences. In 1995, Bush-Jacoby-Medeiros developed a β-lactamase classification scheme based on functional characteristics and have incorporated the Ambler classification. The correlation of the Bush-Jacoby-Medeiros classification with that proposed by Ambler, is shown in Table 2.3.

All types of transferable carbapenemases, except Seoul imipenamase (SIM-1), have been detected in *P. aeruginosa* isolates around the world (Lee et al, 2005). Among them, the metallo-β-lactamases are considered as the most clinically important for *P. aeruginosa*. Most genes encoding MBLs are found as gene cassettes in integrons and are transferable (Walsh et al, 2005). Furthermore, more resistance genes for other antibiotic classes can be present in the same integrons contributing to the development of multidrug resistant phenotypes (Meletis et al, 2012, Sianou et al, 2012).

Imipenamase (IMP) (active on imipenem) and Verona imipenamase (VIM) (Verona integrin-encoded metallo-β-lactamase) type MBLs were first reported from Japan (Watanabe et al, 1991) and Italy (Lauretta et al, 1999) respectively which have now spread through all continents while other metallo-enzymes have been detected sporadically. SPM-1 (Sao Paulo metallo-β-lactamase) has caused serious outbreaks in Brazil and has also been recently found in Basel, Switzerland (Salabi et al, 2010) in an isolate of *P. aeruginosa* recovered by a patient previously hospitalized in Brazil. GIM-1 (German imipenemase) and AIM-1 (Australian imipenemase) have been reported from Germany (Castanheira et al, 2004) and Australia (Yong et al, 2007) respectively. These MBLs did not spread elsewhere. The first and to date only report for New Delhi metallo-
Table 2.3 Classification of β-lactamas (Sacha et al, 2008)

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

β-lactamase (NDM-1) in *P. aeruginosa* was made from Serbia (Jovcic et al, 2011).
Beta-lactamases of *P. aeruginosa* with carbapenemase activity are classified- apart from class B- in the remaining three enzymatic classes as well. Ambler class A carbapenemase KPC (*Klebsiella pneumoniae* carbapenemase) was first reported in *P. aeruginosa* isolates in Colombia (Villegas et al, 2007) and unlikely to KPC-producing *Klebsiella pneumoniae*, KPC-producing *P. aeruginosa* did not reach other continents except Latin America. Due to their high rates of carbapenem hydrolysis, KPCs make powerful
resistance determinants that do not need additional mechanisms such as efflux or impermeability. Enzymes GES/IBC (Guiana extended spectrum) / (integron-borne cefalosporinase) of the same enzymatic class possess carbapenemase activity which may become clinically important when combined with diminished outer membrane permeability or efflux over-expression. For *P. aeruginosa*, GES-2 has been reported from South Africa (Poirel et al, 2001) and IBC-2 from Greece (Mavroidi et al, 2001).

Class C beta-lactamases are not carbapenemases. However, they possess a low potential of carbapenem hydrolysis and their overproduction combined with efflux systems over-expression and/or diminished outer membrane permeability has been proven to lead to resistance to carbapenem (Quale et al, 2011).

Class D carbapenemases like OXA-198 (El Garcg et al, 2011) are rare for *P. aeruginosa* and do not have the same clinical impact as for *Acinetobacter baumannii* (Brown and Amyes, 2006).

Carbapenemases may be detected by PCR amplification using specific primers for each carbapenemase-encoding gene. Phenotypic assays are also used for detecting class A and B enzymes. Ethylene-diamine-tetraacetic acid (EDTA) is a polyamino carboxylic acid that binds metal ions like Zn$^{++}$ and can inactivate the metallo-beta-lactamases. EDTA is used for the detection of class B enzymes by a carbapenem-EDTA disc synergy test or the comparison of the inhibition zone of a carbapenem and a carbapenem+EDTA disc. For the detection of class A carbapenemases, a double disc synergy test may be performed using clavulanic acid with carbapenems, aztreonam and third generation cephalosporins. For *P. aeruginosa*, cloxacillin (200μg cloxacillin/1ml) is dissolved into Mueller-Hinton agar to eliminate any possible AmpC beta-lactamase interference (Meletis et al, 2012, Jiang et al, 2006).

2.9 Metallo-β-lactamase (MBL) enzyme

The categorization of β-lactamase enzymes involves the use of two classification schemes. The enzymes fall into four classes on the basis of their sequence homology, or on the basis of their substrate spectrum and responses to inhibitors into a larger number of functional groups. In the Ambler classification, class A, C and D enzymes employ serine as the reactive site to attack the β-lactam bond of penicillins, cephalosporins and
carbapenems while class B (metallo-β-lactamases) require zinc ions for their catalytic activity. The class B β-lactamases are completely distinct from the serine β-lactamases in terms of amino acid sequence, fold and resistance mechanisms (Heinz et al, 2004). MBL was first reported as zinc dependent enzyme in *Bacillus cereus* in mid-1960’s (Sabath et al, 1966). Accordingly, this classification further segregated these enzymes into subgroups primarily on the basis of imipenem and hydrolysis of other β-lactam antibiotics (Rasmussen et al, 1997). Imipenem hydrolyzing metalloenzymes were found in *Aeromonas hydrophila* (Shannon et al, 1986) and *Bacteroides fragilis* (Cuchural et al, 1986). Essentially, group 3a enzymes essentially possess a broad spectrum of activity; group 3b enzymes possess a preferential avidity for carbapenems; and group 3c enzymes hydrolyze carbapenems poorly compared to other β-lactam substrates. MBLs were first formally categorized from serine β-lactamases in 1980 in the classification scheme proposed by Ambler (1980).

Metallo-β-lactamases (MBLs) are metallo enzymes of Ambler class B and are clavulanic acid-resistant enzymes. They require divalent cations of zinc as co-factors for enzymatic activity and are universally inhibited by ethylenediamine tetra-acetic acid (EDTA), as well as other chelating agents of divalent cations (Sharma et al, 2010). Metallo-beta-lactamase (MBL) producing *P. aeruginosa* was first reported in Japan in 1988 (Watanabe et al, 1991) and since then its prevalence has been reported from various parts of the world including East Asia (Tahiry et al, 2010), Europe (Struelens et al, 2010), Australia (Poirel et al, 2010) and South America (Villegas, 2006). The first plasmid-mediated MBL was reported in *P. aeruginosa* in Japan in 1991. Since then many countries including India reported the prevalence of MBLs (Sharma et al, 2010). In some countries, *P. aeruginosa* possessing MBLs constitute nearly 20% of all nosocomial isolates (Walsh et al, 2005) while in India some reports indicate the prevalence of MBLs in the range of 7-65% (Behera et al, 2008) while one study reports occurrence of MBLs to the tune of 34% (Castanheira et al, 2009). Outbreaks of infections caused by *P. aeruginosa* producing MBLs had occurred in several countries such as Brazil (Zavascki et al, 2005), Colombia (Crespo et al, 2004), Italy (Cornaglia et al, 2000, Pagani, 2005), Greece (Tsakris, 2000) and Korea (Kim, 2005). Mainly five types of MBLs have so far been reported in *P. aeruginosa*, namely IMP (imipenemase), VIM (Verona imipenemase),
GIM (German imipenemase), SPM (Sao Paulo metallo beta lactamase) and SIM (Seoul imipenemase) which originated from Japan, Italy, Germany, Sao Paulo and Seoul respectively. Among all these, IMP and VIM have emerged as predominant MBLs worldwide. New MBLs AIM-1 was reported in \textit{P. aeruginosa} originated in Australia (Gupta, 2008) and NDM-1 (New Delhi metallo-beta lactamase-1) reported in \textit{K. pneumoniae}. A study from Lithuania revealed that the percentage of strains resistant to carbapenems increased from 53.3 to 87.8 within a five year period (Vitkauskiene et al, 2011). In a study from Mumbai, prevalence of MBL producing \textit{P. aeruginosa} was seen in isolates recovered from intensive care areas with a prevalence of 33.33%. In this study an overall mortality rate in MBL-positive cases was as high as 46.15% (De et al, 2010). In a study from Chandigarh, 41.7% isolates of \textit{P. aeruginosa} were MBL producers. Studies from India reported 8.05, 20.8, 20, and 42.6% strains of \textit{P. aeruginosa} as MBL producer (Agrawal, 2008, Varaiya, 2008, Singh et al, 2009, Manoharan et al, 2010). Sharma et al (2010) found 69.5% isolates as MBL producer. A very high percentage to the tune of 96.97% isolates of \textit{P. aeruginosa} was found to be positive for MBL production in Chandigarh (Gupta et al, 2008). Similarly, a study from Delhi showed that 20 imipenem resistant isolates out of 21 examined produced MBLs. In a study from Gujarat the prevalence of MBL production was 11.42% (Ahir et al, 2012). In another study conducted in Pune, out of 125 clinical isolates of \textit{P. aeruginosa}, 21.6% were resistant to imipenem and 6 out of 27 i.e. 22.2% were positive for metallo beta lactamase production (Angadi et al, 2012). VIM was detected in a study from Saudi Arabia where out of 254 \textit{P. aeruginosa} isolates, only 5 were VIM positive (Asaad et al, 2009).

MBL activity of an organism can be inhibited by the action of various chelating agents. Modified Hodge test and double disc synergy test have been used to screen metallo beta lactamase producing \textit{P. aeruginosa}. CuCl\textsubscript{2}, FeCl\textsubscript{2}, EDTA and thiol compounds including mercaptoacetic acid, 2-mercaptopropionic acid and mercaptoethanol have been used for evaluating IMP-1 inhibition as these agents block metallo-beta lactamase activity (Lee et al, 2003). The commercially available E-test strip is also used for MBL detection. This test strip showed a sensitivity of 94% and a specificity of 95% (Walsh et al, 2002). Two substrates commonly used for screening MBLs are ceftazidime and imipenem. The problem encountered in the detection of MBLs carrying organisms is that there are no

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specific CLSI guidelines available for testing of MBLs which can yield false positive results. All isolates found positive by phenotypic methods should be validated by appropriate genetic techniques like PCR.

2.10 Genetic basis of MBL producing strains of \textit{P. aeruginosa}

MBLs constitute a group of heterogeneous proteins which can be divided into three subclasses 'B1', 'B2' and 'B3' (Wommer et al, 2002, Garau et al, 2004, Hall et al, 2004). Subclass B1 exhibits a broad substrate spectrum profile and is characterized by zinc binding site 1 composed of three histidine (His) residues (His-116, His-118, His-196) and zinc binding site 2, composed of one his-263, one cys-221 and one asp-120. In subclass B2, the zinc ligands on site 2 are conserved whereas his-116 in site 1 is replaced by aspartic acid. Representatives of subclass B2 efficiently hydrolyze only carbapenems (Galleni et al, 2001) and are active as mono-zinc enzymes whereas binding of a second zinc ion causes non-competitive inhibition (Hernandez et al, 1997). Subclass B3 has the same ligands in zinc binding site 1 as subclass B1, but the cysteine ligand of subclasses B1 and B2 in binding site 2 is replaced by histidine. These enzymes exhibit a broad-spectrum activity profile with a putative preference for cephalosporins and carbapenems (only subclasses B2) (Boschi et al, 2000). The genes encoding β-lactamases can be located on the bacterial chromosome, on plasmids, or transposons. The genetic environment of β- lactamase (bla) gene dictates whether the β-lactamases are produced in constitutive or inducible manner. Some enzymes of subclass B1 (metallo-β-lactamases) have been found on plasmids and part of transmissible genetic elements called integrons (Boucher et al, 2007).

2.11 Integrons

Integrons were first described by Hall and Collis in 1995. An integron is a specialized group of gene cassettes each of which encode an antibiotic resistance gene. Each gene cassette is composed of a resistance gene bounded at the 5' end by ribosomal binding site and downstream by a 59-base element that is a recombination site common to the cassette. The integron normally encodes its own integrase (int) that facilitates insertion of the gene cassette into integrations site (attl) of the integron (as depicted in Fig 2.1). An integron can be located on the bacterial chromosome or on a plasmid. It has the genes for
integrase site (*int*) and for an adjacent recombination site (*attI*). Gene cassettes become part of an integron when they are integrated. Two types of integrons have been described: resistance integrons and larger super integrons. The latter integrons are always located on bacterial chromosomes. Resistance integrons can be transferred between bacterial cells of the same or different species and can also be transferred between the chromosome and plasmids. They are subdivided into classes with distinctly different *int* genes but only 5 classes have been described. However, only the first three have been involved in antibiotic-resistance determinants (Mazel, 2006). Class I integrons are most common and widespread and clinically very important (Norrby et al, 2005, Cambray et al, 2010). They have been detected in 22-59% of Gram-negative clinical isolates (Labbate et al, 2009) as well as identified in Gram positive bacteria (Nandi et al, 2004, Nesvera et al, 1998). A study from China reported 40.8% prevalence of Class 1 integrons (Gu et al, 2007). Class 2 integrons are exclusively associated with transposon, Tn7 derivatives.

The integrase gene of class 2 integrons, *intI2* generally contains a nonsense mutation in codon 179 that yields a non-functional protein, which can be rescued by a single mutation (Hansson et al, 2002). Class 3 integrons are also thought to be located in transposon (Arakawa et al, 1995, Collis et al, 2002) and are less prevalent than class 2. The class 4 integron is embedded in a subset of the integrative and conjugative element SXT found in *Vibrio cholerae* (Hochhut et al, 2001) whereas class 5 integron is located in a compound transposon carried on the pRSV1 plasmid.

β-lactamase (*bla*) genes of MBL producing isolates have been characterized. These genes are inserted into common class I integron (Poirel et al, 2000, Pournaras et al, 2002, Walsh et al, 2003). These integrons are responsible for transfer of *bla* gene among divergent species of Gram-negative bacteria. Recently, an increasing number of *bla* genes are being discovered on integrons (Weldhagen et al, 2004). Mobile genetic elements that contain integrons serve as important vehicle for spread of *bla* genes and for the dissemination of other determinants. Integrons are not mobile but their location in plasmids, transposons etc enables their movement (Bennett, 1999). β-lactamase genes located on integrons are often accompanied by genes encoding resistance to unrelated antibiotics (Boucher et al, 2007). The transferable metallo-β-lactamases are commonly
encoded by genes carried by type 1 or type 3 integrons. These integrons could be carried by large plasmids or be located on the chromosome (Walsh et al, 2005, Pournaras et al, 2002).

![Diagram of integron structure and gene capture](image)

**Fig 2.2** Structure of integron and Integron-mediated gene capture and model for cassette exchange. Outline of the process by which circular gene cassettes are repeatedly inserted at the specific attI site in an integron downstream of the strong promoter Pc.

Majority of the MBL genes (IMP-type or VIM-type) are mobilised by integrons and/or transposons, while a minority appear to be mobilized with mobile common regions (CR) that have also been associated with other mobile elements called SXT regions. The gene encoding SPM-1 enzyme is associated with two different types of CR (ISCR – Insertion
Sequence Common Region) element (Toleman et al, 2002, Toleman et al, 2006). The gene \textit{blaSPM-1} is not part of a gene cassette, nor is it found in the vicinity of class 1 integron as is the case with other metallo-\(\beta\)-lactamase genes. The gene is located besides the ISCR variant ISCR4 (Toleman et al, 2002). ISCR, a new type of genetic element, was recently identified as being closely associated with spread of many antibiotic resistance genes. They can be divided into two groups: ISCRs1 - form complex class 1 integrons and ISCRs 2 to 13 are those associated with other type integrons. Toleman et al (2006) detected ISCR elements in several strains of \textit{P. aeruginosa}. ISCR2 was discovered in one isolate that harboured \textit{blaVIM-1} and ISCR3 was discovered in two of \textit{P. aeruginosa} strains that had \textit{blaVIM-1}.

**2.12 Diagnosis of \textit{P. aeruginosa} infections**

Diagnosis of \textit{P. aeruginosa} infection depends upon recovery on suitable media and laboratory identification of the bacterium. Gram-negative rods are seen in smears. This organism grows well on most laboratory media and is commonly isolated on blood agar, eosin-methylthionine blue agar, MacConkey agar and cetrimide agar. Certimide agar is used for isolation of \textit{P. aeruginosa} from feces or other samples with mixed flora such as wound swabs. The isolates are identified on the basis of its colony morphology (colony size, haemolytic activity, pigmentation, and odour) and biochemical characters like inability to ferment lactose, a positive oxidase reaction and its ability to grow at 42°C. Fluorescence under ultraviolet light is helpful in early identification of \textit{P. aeruginosa} colonies. Fluorescence is also used to suggest the presence of \textit{P. aeruginosa} in wounds. Several typing methods are also used such as phage typing, serologic typing, molecular characterization of DNA or ribosomal RNA, pulse field gel electrophoresis (PFGE). Serology is based upon detection of antibodies specific to \textit{P. aeruginosa} surface antigens in serum by Enzyme-linked immunosorbent assay (ELISA).

Antibodies to \textit{P. aeruginosa} can also be detected by immunoblotting (e.g., Western blot). Another serological method is cross immuno electrophoresis. In Netherlands, Tramper-Standers et al (2006) compared culture techniques to ELISA and reported 79% sensitivity and 89% specificity in cystic fibrosis patients. Polymerase chain reaction (PCR) based methods of bacterial DNA amplification can be useful to detect \textit{P. aeruginosa}. Hasan et
al (2012) reported PCR based methods (16s rRNA and OPRL gene) as quick, more sensitive and specific. Nikbin et al (2012) recommended detection of oprI, oprL and toxA genes by PCR for molecular identification of *P. aeruginosa* whereas in France, Gall et al (2013) reported that oprL qPCR exhibited better sensitivity and gyrB/ecfX qPCR exhibited better specificity for *P. aeruginosa* detection and suggested a molecular protocol combining these two qPCRs. A study from UK suggested that the use of quantitative PCR (qPCR) could increase the detection rate of *P. aeruginosa* when compared to traditional culture techniques. Overall sensitivity in their study was 100% and specificity was 58% when compared to culture as a gold standard (McCulloch et al, 2010). Filipiak et al (2012) suggested gas-chromatography and mass spectrometry (GC-MS) as useful techniques to identify *P. aeruginosa* by analysing the volatile metabolites released by it.

### 2.13 Prevention and control

*P. aeruginosa* is ubiquitous. The spread of *P. aeruginosa* infections can best be controlled by observing strict hand washing, the appropriate use of antiseptics and gloves, proper isolation procedures and use of aseptic techniques. Careful cleaning and monitoring of respirators, catheters, and other instruments is required in order to avoid the horizontal transmission of MDR *P. aeruginosa* isolates. Topical therapy of burn wounds with antibacterial agents such as silver sulfadiazine, coupled with surgical debridement results into remarkable reduction in the incidence of *P. aeruginosa* sepsis in burn patients. For the prevention of nosocomial infections, several procedures and hospital infection control policies may be followed. For example, proper surveillance and monitoring of patients in ICUs, contact isolation precautions for patients carrying MDR bacterial species. Adoption of proper hygienic conditions: hand washing or alcohol-based disinfection before and after every patient contact, proper cleaning and disinfection of the equipments that are generally shared among patients. Maximum sterile barrier precautions when placing central venous, and urinary tract catheters, discontinuation of central venous and urinary tract catheters when not needed in the hospitals (O’Grady et al, 2002, Mangram et al, 1999, Bearman et al, 2006). Availability of molecular typing to determine the diversity of the strains is required in order to chalk out strategies for
prevention of infections due to *P. aeruginosa*. Avoidance of antibiotics could be one of the important prevention strategies as majority of *P. aeruginosa* pneumonias occur in patients already exposed to antibiotics. *Pseudomonas* appears to require the suppression of normal human bacterial flora for its overgrowth which may be prevented by low antibiotic pressure (Vollaard & Clasener, 1994).