2. REVIEW OF LITERATURE

Although a voluminous literature on *Pseudomonas aeruginosa* is available but the literature pertinent only to the proposed research project has been reviewed in this chapter.

2.1 Historical background

*Pseudomonas* is a genus of gamma proteobacteria, belonging to the larger family of Pseudomonads. *Pseudomonas* was first isolated by a French military surgeon Sedillot in 1850 from a blue-green discharge on surgical dressing with associated infection. The pigment responsible for the blue colouration was extracted by Fordos in 1860. Lucke in 1862 was the first to relate this pigment with the rod shaped organisms (Pitt, 1998). *P. aeruginosa* was not successfully isolated in pure culture until 1882. In several reports during the period 1889 and 1894, *P. aeruginosa* (*Bacillus pyocyaneus*) was described as the causative agent of blue-green purulence in the wounds of patients (Villavicencio, 1998). *Pseudomonas* is the most important genus in the order *Pseudomonadales* of the family *Pseudomonaceae*. The genus contains around 150 species and 11 subspecies with valid published names (Parte, 2013).

2.2 Morphology, cultural characteristics, biochemical characterizations of *P. aeruginosa*

*P. aeruginosa* is a Gram negative, uniformly stained, straight or slightly curved rods, measuring 0.5 to 1.0 μm by 1.5 to 5.0 μm in length. This organism is aerobic, although nitrate can be used to permit anaerobic growth, nonspore forming, motile by one or more polar flagella. *P. aeruginosa* is non capsulated but mucoid strains have extracellular polysaccharide capsule composed of alginate polymers and the organism is often pilated. Many strains have mucoid slime layer (Ananthanarayan and Paniker, 2009). Temperature range for growth is 5-42°C, optimum being 37°C. Unlike most other Pseudomonads, *P. aeruginosa* can grow well at 42°C at the optimum pH range is 7.4-7.6 (Ananthanarayan and Paniker 2009). *P. aeruginosa* is either incapable of utilizing carbohydrates as source of energy or degrade them “oxidatively” rather than fermentative pathway without gas
formation. On nutrient agar, colonies are large and pigmented. The pigments, pyocyanin / fluorescein are often produced on this medium. On 5% sheep blood agar, the organism produces β-hemolytic, large flat spreading, mucoid, rough and pigmented colonies with characteristic metallic sheen. Many strains may produce a fruity, sweety, musty or grape like odor due to the presence of 2-aminoacetophenone. This is a peculiar feature of this organism. On Mac Conkey’s agar, *P. aeruginosa* produces colourless colonies as the organism is non lactose fermenter. It also produces green pigmentation and metallic sheen. In broth, the organism produces a dense turbidity with a surface pellicle. Colonies recovered from respiratory tracts of patients often produce large amounts of exopolysaccharide which is composed of alginate that consists of mannnuronic and guluronic acids. These compounds help in the production of mucoid colonies.

*P. aeruginosa* is oxidase and catalase positive. Glucose is oxidatively utilized in some strains, and nitrate is reduces to nitrite. Indole, methyl red and Voges Proskauer tests are negative. *P. aeruginosa* utilizes citrate, does not decarboxylate lysine and ornithine. This organism does not hydrolyze urea and aesculin, liquefies gelatin and utilizes acetamide (Brenner et al., 2005). Cetrimide agar is a selective and differential medium for the isolation of *P. aeruginosa*. Cetrimide acts as detergent which inhibits most bacteria and enhances the production of the pigments, pyocyanin and pyoverdine by *P. aeruginosa*. However, about 4% of clinical strains of *P. aeruginosa* do not produce pyocyanin.

*P. aeruginosa* is a ubiquitous organism present in many diverse environmental settings and can be isolated from various living sources, including plants, animals, and humans due to its highly adaptable nature as depicted through Figure 2.1.
Figure 2.1 Adaption of *P. aeruginosa* in wide range of abiotic and biotic environments (adapted from Silby *et al.*, 2011).

*P. aeruginosa* prefers moist environments. The ability of *P. aeruginosa* to survive on minimal nutritional requirements and to tolerate a variety of physical conditions has allowed this organism to persist in both community as well as hospital settings. In the latter, *P. aeruginosa* can be isolated from a variety of sources, including nebulizers, dialysate fluids, saline solutions, antiseptics, soap, sinks, mops, catheters and other diagnostic and therapeutic devices. Community reservoirs of this organism include: swimming pools, whirlpools, hot tubs, contact lens solution, home humidifiers, soil and rhizosphere and vegetables. *P. aeruginosa* can even grow in distilled water using dissolved carbon dioxide and residual sulphur, phosphorus, iron and divalent cations as nutritional substrates. It can withstand treatment with chlorohexidine and quaternary
ammonium compounds. *P. aeruginosa* is rarely a constituent of the normal flora but can easily colonize hospitalized patients (Palleroni, 2005). The colonization rates vary with the site of infection in human patients: 0 to 2% for skin, 0 to 3.3% for the nasal mucosa, 0 to 6.6% for the throat and 2.6 to 24% for fecal samples. However, these rates may exceed 50% during hospitalization, especially among immunocompromised patients (Lister et al., 2009).

### 2.3 Genome of *P. aeruginosa*

The genome of *P. aeruginosa* is large and complex. The complete genome of *P. aeruginosa* strain PAO1 was sequenced for its nucleotides. This strain was recovered from wound infection in Australia in 1950s (Stover et al., 2000). The genome is made up of a single circular chromosome having G + C content of 65-67%. The size of the genome is 5.5-7 Mbp, which may encode 5,570 predicted protein coding sequences and a variable number of plasmids (Klockgether et al., 2011). The genome is the sum of two components: a core genome and an accessory genome. The core genome is defined as the genes that are present in nearly all strains of *P. aeruginosa* and encode a set of metabolic and pathogenic factors shared by all *P. aeruginosa* strains, irrespective of their origin (environmental, clinical, or laboratory). The core genome constitutes approximately 90% of the total genome and is highly conserved among strains. In contrast, the accessory genome has genes that are found in some *P. aeruginosa* strains but not in others. These segments are not scattered randomly throughout the core genome but they tend to cluster in certain loci. Elements within the accessory genome of *P. aeruginosa* have been associated with differences in virulence and antibiotic resistance (Ozer et al., 2014). The term “regions of genomic plasticity (RGPs)” has been used to describe such loci (Mathee et al., 2008). The genetic sequences occupying many RGPs are often referred to as genomic islands or islets (size >10 kb - <10 kb). The genetic content of the RGPs forms a large proportion of the accessory genome. The elements of the accessory genome were actually acquired by horizontal gene transfer from different sources including other species or genera. Upon integration into the host chromosome they appear as “foreign” blocks in the core genome. Therefore, a *P. aeruginosa* chromosome is often described as
a mosaic structure of conserved core genome frequently interrupted by the inserted parts of the accessory genome.

*P. aeruginosa* contains the highest proportion of regulatory genes. The size and complexity of the *P. aeruginosa* genome reflect an evolutionary adaptation permitting it to thrive in diverse environments and resist the effects of a variety of antimicrobial substances (Stover *et al.*, 2000).

### 2.3.1 Major components of *P. aeruginosa* accessory genome

The majority of the *P. aeruginosa* accessory genome can be grouped into four broad categories:

(i) Integrative and conjugative elements (ICEs), (ii) replacement islands, (iii) prophages and phage like elements and (iv) transposons, insertion sequences (ISs) and integrons.

#### Integrative and conjugative elements (ICEs)

ICE is a self transmissible genetic element that must integrate into an existing replicon to accomplish replication. ICEs possess properties of both plasmid and phage associated DNA properties (Burrus *et al.*, 2002). *P. aeruginosa* ICEs range in size from 81 kb to 108 kb (Klockgether *et al.*, 2004). A number of *P. aeruginosa* ICEs have been identified and partially characterized.

#### Replacement islands

The loci in the core genome that are under diversifying selection are called as replacement islands. The types of each replacement island were identified by comparative sequencing of the respective gene clusters in *P. aeruginosa* strain collections. Lipopolysaccharide (LPS) O antigen, pyoverdine, pili and flagella are critical determinants of pathogenicity of *P. aeruginosa*. These macromolecules mediate fundamental processes such as bacterium-bacterium interaction, iron acquisition, adhesion, and motility. Moreover, these macromolecules are surface exposed and are under selective pressure because they are targets of phage predation and immune recognition. The genes responsible for the synthesis and post translational modification of
each of these macromolecules are grouped together in gene clusters known as “replacement islands” (Kung et al., 2010). Smith et al. (2005) coined this term to describe the genetic loci which are associated with O antigen biosynthesis, pyoverdine, pilin, and flagellar glycosylation because each of these gene clusters contains horizontally acquired components and is highly divergent between different strains.

**Prophages and phage like elements**

Once the bacteriophage is integrated into a bacterial chromosome it is termed as “prophage” which may develop mutations or undergo recombination events with other prophages in the bacterial host chromosome. In this process, they may become permanently fixed in the chromosome. However, in the case of lysogenic conversion, integrated phages may provide their bacterial host’s novel phenotypic properties, such as toxin production. The transduction process can specifically be either generalized (bacterial DNA by itself may be packaged into phage particles) or specialized (phage DNA along with adjacent bacterial DNA is packaged into phage particles). Generalized transduction in particular is a significant contributor to the horizontal transfer of virulence and antibiotic resistance genes. There are 60 different temperate *P. aeruginosa* phages, and the majority of *P. aeruginosa* isolates are thought to be lysogenized by at least one phage. Double stranded DNA tailed phages mainly constitute the majority of phages infecting *P. aeruginosa*. The phages infecting *P. aeruginosa* are divided into three families based on tail morphology: *Siphoviridae, Myoviridae* and *Podoviridae* (Kung et al., 2010).

**Transposons, insertion sequences (ISs) and integrons**

Transposable elements are DNA sequences that can change their position within the genome, sometimes creating or reversing mutations and altering the size of the bacterial cell. Most *P. aeruginosa* transposable elements can transpose into many different sites on a DNA molecule. These elements generally produce a staggered cut at their insertion site and inserted sequences become flanked by short direct repeats upon
transposition. In bacteria, transposons usually carry an additional gene for function other than transposition e.g. often for antibiotic resistance whereas insertion sequence (IS) elements are small transposable elements that encode only the functions needed for transposition and lack additional genes.

Integrons are genetic entities that capture exogenous gene cassettes and ensure their expression. All integrons are composed of three core components: (i) a promoter, (ii) a primary recombination site (attI) located downstream of the promoter and (iii) a gene encoding a tyrosine recombinase family integrase. Integrons are found on common mobile genetic element carriers such as conjugative plasmids and transposons. They achieve mobility only when they are linked to such an existing mobile genetic element so that they can insert gene cassettes containing integrons. On the basis of integrase sequences, at least five classes of integrons have been described. In P. aeruginosa, the majority of integrons belong to class 1, in which antibiotic resistance gene cassettes are particularly common. These integrons are frequently associated with Tn402-derived transposons (Mazel, 2006). Other classes of integrons are quite rare in P. aeruginosa. For example, to date, there has been only one report of class 2 integrons found in P. aeruginosa (Xu et al., 2009) and no published reports of class 3, 4 or 5 integrons.

2.4 Clinical manifestations of P. aeruginosa infections

P. aeruginosa has emerged as an important pathogen during the past two decades. It is the most common pathogen in humans, but infections may also result from other species of Pseudomonas e.g. P. paucimobilis, P. putida, P. fluorescens or P. acidovorans. Other important hospital acquired pathogens previously classified as Pseudomonas include Burkholderia cepacia and Stenotrophomonas maltophilia (Fundukian, 2008). P. aeruginosa is an opportunistic, nosocomial pathogen of immunocompromised individuals, typically infects the airway, urinary tract, burns, wounds and also causes other blood infections (Todar, 2009). Pseudomonas infection is especially prevalent among patients with burn wounds, cystic fibrosis, acute leukemia, organ transplants, trauma to skin or conjunctiva, urinary tract manipulations and intravenous drug addiction (Bodey, 1983). P. aeruginosa isolates resistant to multiple drugs are cause of most nosocomial infections in burn patients.
The site of *P. aeruginosa* infections varies with the portal of entry and the patient's vulnerability. It is the most common pathogen recovered from patients who have been hospitalized for longer than one week. Such infections range between 10% and 20% in most hospitals and occur when the immune system is breached, either locally or systemically. Infection also occurs after therapeutic procedures like catheterization. Previous antibiotic therapy also favours infection with *P. aeruginosa*. This bacterium is found on the skin (axilla and anogenital areas) of some healthy persons and has been isolated from the throat and stool of 5% and 3% of nonhospitalized patients, respectively. The gastrointestinal carriage rates among hospitalized patients increases to 20% within 72 hours of admission. In addition to its pathogenicity, this bacterium has minimal nutritional requirements and can tolerate a wide variety of physical conditions. The pathogenesis of *P. aeruginosa* infections is multifactorial and complex. Pseudomonas species are both invasive and toxigenic. Infections due to *P. aeruginosa* take place in three stages: i bacterial attachment and colonization, ii local infection and iii bloodstream dissemination and systemic disease (Pollack, 2000). It produces a wide range of clinical conditions in humans as described below:

**Urinary tract infections**

*P. aeruginosa* is common cause of nosocomial urinary tract infections (UTIs) and accounts for about 7% of such infections in North America and Europe. Nosocomial infections are associated with an indwelling catheter, instrumentation of the urinary system, chronic prostatitis, nephrolithiasis, as well as prior antibiotic therapy. Community acquired UTIs are rarely caused by *P. aeruginosa* (Gupta et al., 2001).

**Respiratory tract infections**

*P. aeruginosa* is a rare cause of community acquired pneumonia. It mainly occurs in HIV infected patients, solid organ or bone marrow transplant recipients or patients with neutropenia (Hatchette et al., 2000). The bacterium is the second most common cause of nosocomial pneumonia and the most common cause of intensive care unit (ICU) pneumonia. Patients with immunosuppression and chronic lung disease often develop
pneumonia due to *P. aeruginosa*. In the intensive care unit (ICU) setting, the infection is associated with positive pressure ventilation and endotracheal tubes. The pneumonia may be primary, following aspiration of the organism from the upper respiratory tract, particularly in patients on mechanical ventilation. Alternatively, it may occur as a result of bacteremic spread to the lungs. Symptoms of pneumonia include: fever, chills, severe dyspnea, cyanosis, productive cough, confusion, and other signs of a systemic inflammatory response. Chronic infection of the lower respiratory tract with *P. aeruginosa* leads to a ventilator associated pneumonia (VAP), accounting for nearly 21% of cases (Richards *et al*., 1999) and it is prevalent among patients who have chronic disease, require respiratory/ventilatory assistance, have cystic fibrosis or are immunocompromised (El-Solh *et al*., 2001). These patients may present with chronic severe productive cough, anorexia, weight loss, wheezing, and tachypnea (Ratjen *et al*., 2010).

**Endocarditis**

*P. aeruginosa* may infect native heart valves in individuals who abuse intravenous drugs and may infect prosthetic heart valves. Nonspecific symptoms include: fever and malaise, with more specific symptoms depending on which cardiac valve is involved. Heart murmurs are heard in approximately 85% of patients. It may lead to intractable congestive heart failure and myocardial abscesses. If untreated, infective endocarditis is generally fatal.

**Meningitis**

Central nervous system (CNS) infection due to *P. aeruginosa* is extremely rare, but may result from brain injury or surgery and the spread of infection from other parts of the body or through bacteremia. *P. aeruginosa* can cause inflammation of the tissues covering the brain and spinal cord (meningitis) and may cause brain abscesses. Pseudomonas meningitis is manifested by symptoms like fever, headache, irritability and clouded consciousness.
Ocular infections

*P. aeruginosa* cause corneal ulcerations, keratitis and endophthalmitis. It causes orbital cellulitis and endophthalmitis as a complication of sepsis in neonates, patients with hematologic malignancy and HIV infected patients. Risk factors associated with *P. aeruginosa* eye infections are: eye trauma and recent ophthalmic surgery, as well as use of contact lens and with contaminated contact lens solutions (Boyle *et al.*, 2001, Reedy and Wood, 2000).

Ear infections

There are three major ear infections caused by *P. aeruginosa*: Perichondritis of the ear, otitis externa, otitis media. Perichondritis of the auricle due to *P. aeruginosa* infections has become more common with the recent fashion of ear piercing. The pinna may be markedly swollen, red and tender, with infection progressing to necrosis of the cartilage. Otitis externa is infection of the external ear canal, popularly known as swimmer's ear, patients present with pain, pruritis and ear discharge. The pain is worsened by traction on the pinna. Simple otitis media externa is associated with warm, humid atmospheric conditions, aural water exposure and ear canal trauma which may occur with increased water exposure due to frequent swimming or when trauma occurs to the ear canal epithelium. Pain is recorded in 97.2% cases (Battikhi and Ammar, 2004).

Malignant otitis media externa is a subset of osteomyelitis caused by *P. aeruginosa* in which the temporal bone and skull base is involved (Green *et al.*, 1992). Patients with diabetes mellitus and advanced age are at risk for necrotizing otitis externa. It has also been reported in HIV infected patients. The condition commences as ordinary otitis externa that fails to respond to antibiotic therapy. Symptoms such as persistent pain, edema and tenderness of the soft tissues of the ear, with a purulent discharge are observed in the infected patients. Fever is uncommon and some patients present with a facial nerve palsy. Extension of the infection to the temporal bone can result in osteomyelitis and further extension can create cranial nerve palsies and possibly a CNS infection.
Skin infections

*P. aeruginosa* can grow on moist skin causing symptoms ranging from mild rash to large bleeding ulcers of the skin. This organism can cause folliculitis which is evident as red itchy rash, headache, dizziness, earache, sore eyes, nose and throat, breast tenderness and stomach pain. *P. aeruginosa* is the second most common cause of burn wound infections in hospitalized patients. The wound infections may secrete a blue green coloured fluid and have a fruity smell. Burn wound infections usually occur one to two weeks after the burn resulting into discoloration of the burn scab, destruction of the tissue below the scab, early scab loss, bleeding, swelling and a blue-green drainage. Secondary wound infections occur in patients with eczema and tinea pedis (Fundukian, 2008).

Green nail syndrome is a paronychial infection that can develop in individuals whose hands are frequently submerged in water. The green coloration of nail is due to the pigment pyocyanin adhering to the undersurface of the nail plate with accumulated debris below the nail (Greenberg, 1975). *P. aeruginosa* is a common cause of hot tub or swimming pool folliculitis. Hot tub folliculitis is an infection involving the hair follicles and can occur as a result of bathing in a contaminated tub. It is associated with the development of a maculopapular, pustular rash due to *P. aeruginosa*, serotype 0:4. Pruritus, malaise and low grade fever are some of the main symptoms (Chandrasekar, 1984). Ecthyma gangrenosum is a skin lesion that occurs in neutropenic patients usually associated with bacteremia and sepsis and is usually caused by *P. aeruginosa*. It is characterized by erythematous, centrally ulcerated, purple black areas about one cm in diameter occurring most often in the axillary, inguinal or anogenital areas. The infection may lead to secondary ischemic necrosis.

Bones and joint infections

Pseudomonas infections in bone and joints can result from injury and by the spread of infection from other body tissues or through bacteremia. Bone infections are marked by swelling, redness and pain at the infected site and possibly fever. Persons at risk for Pseudomonas infections of the bones and joints include: diabetics, intravenous drug abusers and bone surgery patients. The most common sites of involvement are the
vertebral column, pelvis and sternoclavicular joint. Vertebral osteomyelitis may involve the cervical spine and patients present with neck or back pain lasting for weeks to months. Occasionally, patients with complicated UTI may develop lumbosacral vertebral osteomyelitis. Patients with pyoarthrosis present with swelling and pain in the affected joint. Patients are persistently febrile.

**Gastrointestinal infections (GI)**

Pseudomonal infections can affect every portion of the GI tract. The disease is often underestimated but usually affects very young children and adults with hematologic malignancies and chemotherapy induced neutropenia. The spectrum of disease can range from very mild symptoms to severe necrotizing enterocolitis with significant morbidity and mortality. Young infants may present with irritability, vomiting, diarrhea and dehydration. The infection of GIT can cause enteritis, with patients presenting with prostration, headache, fever and diarrhea (Shanghai fever). Pseudomonas typhlitis most often occurs in patients with neutropenia resulting from acute leukemia and presents with a sudden onset of fever, abdominal distension and worsening abdominal pain.

**Bacteremia**

Bacteremia may be acquired from contaminated medical devices in hospitals and nursing homes, accounting for about 6% of all bacteremias, 75% of nosocomial bacteremias (Elkhatab et al., 2008) and *P. aeruginosa* is the third most common cause of bacteremia in Gram negative pathogens, producing fever, tiredness, muscle pains, joint pains and productive cough, difficult breathing, chills and blue-tinted skin etc (Al-Hasan, 2008). Bacteremia is common in patients with blood cancer and patients who have Pseudomonas infections elsewhere in the body.

**Infections of plants and invertebrates**

Besides causing the above mentioned clinical conditions, *P. aeruginosa* induces symptoms of soft rot, for example in *Arabidopsis thaliana* (Thale cress) and *Ocimum basilicum* (Sweet basil) plants (Walker et al., 2004). It is also pathogenic to invertebrate animals, including the nematode *Caenorhabditis elegans* (Mahajan-Miklos et al., 2004).
1999), the fruit fly, *Drosophila* (D'Argenio *et al*., 2001) and the moth *Galleria mellonella* (Miyata *et al*., 2003).

### 2.5 Virulence factors

The pathogenesis of *P. aeruginosa* infections is due to a large number of cell associated and extracellular factors of this organism. These virulence factors play an important role in the colonization and survival of the bacteria and their invasion in the tissues (Khalifa *et al*., 2011). Several metabolites produced by *P. aeruginosa* are thought to enhance the colonization and infection of the host tissue. Various virulence factors include: capsule, flagella and pilli and several exotoxins - proteases, haemolysins, lecithinase, elastase and DNase and endotoxins which are described below:

**Alginate capsule**

Alginate capsule is possessed by only some strains of *P. aeruginosa*. The capsule is formed by a polymer of manuronic acid and glucuronic acids. Pseudomonas producing alginate capsules have a mucoid appearance of their colonies. The capsule enables the bacteria to survive in aquatic environment. It protects the organism from phagocytosis and also functions as an adhesion factor. Alginate is a layer of the anionic polysaccharide which surrounds the cells and binds them together in aggregates. The capsule has the capacity to bind cationic antibiotics such as the aminoglycosides and restrict their diffusion (Nichols *et al*., 1988).

**Flagella and type 4 pilli**

*P. aeruginosa* cell have a single polar flagellum and several small type 4 pilli localized at a cell pole. The latter can extend and retract like grappling hooks to pull the cell along solid surfaces by a process termed as ‘twitching motility’ (Kipnis *et al*., 2006). These proteinaceous appendages function both as adhesins and as a means of motility. Flagella and pilli can also initiate an inflammatory response. During an infection, the bacterium can adhere to host epithelial cells through the binding of its flagellum and can elicit a strong inflammatory response via signaling through TLR5 (toll like receptor 5) and a caspase-1-mediated response through the nod like receptor (nucleotide binding
oligomerization domain receptors), Ipaf (Miao et al., 2007). Type 4 pili are the most important adhesins of *P. aeruginosa*. These also help in the formation of biofilms. Pili can also lead to aggregation of bacteria on target tissues, causing them to form microcolonies, effectively concentrating the bacteria in one location and potentially offering protection from the host immune system and from antibiotics (Craig et al., 2004; Sriramulu et al., 2005).

**Type 3 secretion system (T3SS)**

Type 3 secretion system (T3SS) is major virulence factor among many pathogenic Gram-negative bacteria as a means of injecting toxins directly into host cells. *P. aeruginosa* T3SS is a major determinant of virulence and its expression is frequently associated with acute invasive infections (Sadikot et al., 2005; Hauser, 2009). The needle like appendage of the T3SS is evolutionarily related to flagella and it allows the translocation of effector proteins from the bacterium into the host cell through a pore formed in the host cell membrane. Only four effectors have been identified in *P. aeruginosa*, namely, ExoY, ExoS, ExoT, and ExoU. All strains of *P. aeruginosa* express one of the two major exotoxins (exoU or exoS) but strains possess both type of exotoxins very rare, while most strains express exoY and exoT, which have minor roles (Hauser, 2009).

**Biofilm formation**

A significant number of *Pseudomonas* spp. can produce exopolysaccharides that are known as slime layers due to which it becomes difficult for the mammalian white blood cells to phagocytose them. Slime production also contributes to the formation of surface colonizing biofilms which are difficult to remove from surfaces. Due to the ability of biofilm formation, they are thus able to survive in a variety of unexpected surfaces (Borriello et al., 2004). Their formation is linked to quorum sensing (Bjarnsholt et al., 2010). Biofilm producing *P. aeruginosa* strains are highly resistant to most antimicrobials due to impermeability mediates by biofilm formation (Donlan, 2000).
Proteases
Several proteases are secreted by *P. aeruginosa*, LasA elastase (serine protease), LasB elastase (zinc metalloprotease) and alkaline protease have main role in pathogenesis. These proteases have roles in ocular infections and in sepsis, where they can degrade immunoglobulins and fibrin and disrupt epithelial tight junctions (Kipnis *et al.*, 2006). Proteases have been shown to contribute to tissue damage in respiratory infections, including the degradation of host lung surfactant (Fleiszig and Evans, 2002; Hobden, 2002; Kipnis *et al.*, 2006). Alkaline protease is a type -1 secreted zinc metalloprotease which helps in the degradation of host complement proteins and fibronectin (Laarman *et al.*, 2012). The ability of *P. aeruginosa* to destroy the protein elastin is a major virulence determinant during acute infection. Elastin is a major part of human lung tissue and is responsible for lung expansion and contraction. Moreover, elastin is an important component of blood vessels, which rely on it for their resilience. The concerted activity of two enzymes, LasB elastase and LasA elastase, is responsible for elastolytic activity. Elastolytic activity is believed to destroy elastin containing human lung tissue and cause the pulmonary hemorrhages of invasive *P. aeruginosa* infections. LasB elastase degrades not only elastin but also fibrin and collagen.

Lipopolysaccharide (endotoxin)
Lipopolysaccharide is an outer glycolipid membrane of Gram-negative bacteria composed of a membrane anchored lipid A, polysaccharide core region and a highly variable O-specific polysaccharide (O-antigen or O-polysaccharide). It has roles in antigenicity, inflammatory response and exclusion of external molecules. This component of the bacterium mediates interactions with antibiotics (King *et al.*, 2009).

Exotoxins
Exotoxin A is produced by most *P. aeruginosa* strains that cause clinical infections. *P. aeruginosa* exotoxin A catalyzes ADP (adenosine diphosphate) ribosylation that inhibits host elongation factor 2, leading to inhibition of protein biosynthesis and cell death. Exotoxin A is responsible for local tissue damage, bacterial invasion and immunosuppression.
Purified exotoxin A supports its role as a major systemic virulence factor of *P. aeruginosa*. Exotoxin S and T are also adenosine diphosphate (ADP) ribosyl transferases, but they preferentially ribosylates GTP binding proteins. This exoproduct is responsible for direct tissue destruction in lung infection and may be important for bacterial dissemination.

**Hemolysins**

Two hemolysins, phospholipase C and rhamnolipid are produced by *P. aeruginosa* which act synergistically to break down lipids and lecithin (Johnson and Marrazzo, 1980). Both the hemolysins may contribute to tissue invasion by their cytotoxic effects. Rhamnolipid is a rhamnose containing glycolipid biosurfactant. It has a detergent like structure and is believed to solubilize the phospholipids of lung surfactant, making them more accessible to cleavage by phospholipase C. The resulting loss of lung surfactant may be responsible for the atelectasis (collapse or closure of the lung resulting in reduced or absent gas exchange) associated with chronic and acute *P. aeruginosa* lung infection. Rhamnolipid also inhibits the mucociliary transport and ciliary function of human respiratory epithelium. However, the relative role of rhamnolipid in acute or chronic infection is not clearly understood.

**Other virulence factors**

In addition to the virulence factors mentioned above, other factors such as lipases, pyocyanin and siderophores also contribute to virulence. Phospholipases break down surfactant lipids and the phospholipids of host cell membranes (Kipnis *et al.*, 2006). The blue green pigment pyocyanin produced by *P. aeruginosa* causes oxidative stress to the host and disrupts host catalase and mitochondrial electron transport system (Lau *et al.*, 2004). This pigment imparts blue green color to colonies. Siderophore possess iron gathering capacity. Iron is sequestered by iron transport proteins (e.g. transferrin and lactoferrin) in the biological fluids of the hosts and it is a limiting factor for the growth of *P. aeruginosa* in host cell. To overcome this limiting factor, siderophores are produced. The pyoverdin and Pyochelin are the main siderophores produced by *P. aeruginosa*.
(Poole and McKay, 2003). Pyoverdin competes for iron with the human iron-binding proteins transferrin and lactoferrin (Xiao and Kisaalita, 1997).

### 2.6 Drug resistance in *P. aeruginosa*

The successful use of any therapeutic agent is compromised by the potential development of resistance to that agent from the time it is first employed. A wide range of biochemical and physiological mechanisms may be responsible for resistance to antibiotics in *P. aeruginosa*. The indiscriminate use (under as well as overuse) of antimicrobial agents is associated with the emergence of resistance. As a result, it is not possible to achieve the desired therapeutic outcomes. The predominance of microbial resistance to several antimicrobial agents varies between geographical regions but with extent of time resistance emerges against each and every antimicrobial agent (WHO, 2001). Antibiotic resistance is of two types i. it can be intrinsic or ii. can be acquired genetically. Most of the resistant microbes which are now difficult to treat are of genetic origin. The resistance is transferable between species and genera of bacteria. *P. aeruginosa* is highly adaptable microorganism that can rapidly develop resistance to different types of broad spectrum antibiotics. *P. aeruginosa* is intrinsically resistant to many antimicrobial agents, including most β-lactams, older quinolones, chloramphenicol, tetracyclines, macrolides, trimethoprim–sulfamethoxazole and rifampin. The resistance to multiple antibiotics can be relatively common amongst nosocomial isolates of *P. aeruginosa*, which represent the large majority of clinical isolates (Dundar and Otkun, 2010; Rossolini and Mantengoli, 2005). To survive antibiotic treatment, the following basic mechanisms of resistance are employed by *P. aeruginosa*:

#### 2.6.1 Permeability of cell membrane

The innate resistance of *P. aeruginosa* to all classes of antibiotics has generally been attributed to the low permeability of its cell wall. Another mechanism present in *P. aeruginosa* is the formation of permeability barriers (Kadry, 2003). Impaired penetration of different substances through the membrane is due to diminished expression of specific outer membrane protein. The outer membrane of Gram negative bacteria acts as a barrier to both hydrophobic and hydrophilic compounds. Small hydrophilic antibiotics such as
the β-lactams and quinolones can only cross the outer membrane by passing through the aqueous channels provided by porin proteins, thus outer membrane acts as a selective barrier. The permeability properties of this barrier have a major impact on the susceptibility of the microorganism to antibiotics. *P. aeruginosa* produces several different porins, for example lack of OprD protein in outer member leads to a reduction of active antibiotic molecules capable of reaching the target penicillin-binding-proteins and it is frequently associated with resistance to imipenem which requires this porin to cross the outer membrane (Livermore, 2001).

### 2.6.2 Efflux of antibiotics from bacteria

Efflux systems are energy dependent mechanism which pumps out unwanted toxic substances through specific efflux pumps. Efflux pumps play a major role in antibiotic resistance. It allows the uptake of essential nutrients and ions, excretion of metabolic end products and deleterious substances as well as the communication between cells and environment (Li and Nikaido, 2004).

Efflux pumps are transporter proteins which are found both in Gram positive and Gram negative bacteria as well as in eukaryotic organisms. Bacterial drug efflux transporters are classified into five families: i major facilitator superfamily (MFS), ii adenosine triphosphate (ATP) binding cassette (ABC) superfamily, iii small multidrug resistance (SMR) family, iv resistance nodulation cell division (RND) superfamily and v multidrug and toxic compound extrusion (MATE) family. Efflux transporters can be further classified into single or multicomponent pumps. Multicomponent pumps are found in Gram-negative organisms which function in association with a periplasmic membrane fusion protein (MFP) component and an outer membrane protein (OMP) component (Dzidic *et al.*, 2008). *P. aeruginosa* exhibits several efflux pump systems that allow it to be resistant to several antimicrobial agents. Four different RND-MFP-OMF type MDR efflux systems have been described in *P. aeruginosa*: mexAB-oprM, mexXY-oprM, mexCD-oprJ and mexEF-oprN (Poole, 2001) and distribution of their components are presented through Table 2.1. All classes of antibiotics except the polymyxins are susceptible to extrusion by one or more of the efflux systems. MexAB-oprM is responsible for extrusion of β-lactams, quinolones and a range of disinfectants. MexXY-
oprM extrudes aminoglycosides and mexEF-oprN extrudes carbapenems and quinolones. MexCD-oprJ extrudes beta-lactams, fluoroquinolones. Apart from antibiotics, mex efflux systems also extrude organic solvents, biocids, dyes and cell signaling molecules.

<table>
<thead>
<tr>
<th>Efflux systems</th>
<th>Components</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>mexAB-oprM</td>
<td>mexA</td>
<td>mexB</td>
</tr>
<tr>
<td>mexXY-oprM</td>
<td>mexX</td>
<td>mexY</td>
</tr>
<tr>
<td>mexCD-oprJ</td>
<td>mexC</td>
<td>mexD</td>
</tr>
<tr>
<td>mexEF-oprN</td>
<td>mexE</td>
<td>mexF</td>
</tr>
</tbody>
</table>

* MFP: membrane fusion protein, RND: resistance nodulation cell division and OMF: outer membrane protein (now called outer-membrane factor)

2.6.3 Target modifications

This mechanism is based on modifications of target sites and thus, preventing the antibiotic from binding to its site of action. For example, fluoroquinolone resistance is attributed to mutations within the drugs targets (DNA gyrase and topoisomerase) (Livermore, 2003). In *P. aeruginosa*, quinolones resistance is encountered through mutation in the gyrA gene which encodes the A subunit of the target enzyme, DNA gyrase (Akasaka et al., 2001).

2.6.4 Enzymatic modification of the antibiotics

Enzymes that modify antibiotics are divided into two general classes: i. beta-lactamases that degrades antibiotics and ii. others (including the macrolide and aminoglycoside...
modifying proteins). These enzymes perform chemical transformations to render the antibiotic inefficient (Livermore, 2003). At least 14 different aminoglycoside modifying enzymes can be produced by *P. aeruginosa*, the most common of which is acetyltransferase (Sabath, 1984). Among β-lactamases, extended-spectrum β-lactamases (ESBLs) and carbapenemases (mainly metallo-betalactamases) have spread widely in recent years. ESBLs usually confer resistance to all β-lactams except carbapenems (Weldhagen et al., 2003). Over production of the ampC β-lactamase poses a particular threat to cephalosporins (Vahaboglu et al., 2001). Various mechanisms of antibiotic resistance are depicted through Figure 2.2

![Figure 2.2 Mechanism of resistance to antibiotics](image)

2.7 Acquisition and spread of antibiotic resistance
Antibiotic resistance may be an inherent (natural) trait of the organism or it may be acquired by means of mutation in its own DNA or acquisition of resistance conferring DNA from another source (Toder, 2009). Several mechanisms are developed by bacteria in order to acquire resistance to antibiotics. All require either the modification of existing genetic material or the acquisition of new genetic material from another source. Antibiotic resistance can be transferred through vertical gene transfer or by horizontal gene transfer.
2.7.1 Vertical gene transfer
Vertical gene transfer is the transmission of genetic material from mother cell to daughter cell during cell division. Once the resistance genes have developed, they are transferred directly to the progenies of all the bacteria during DNA replication. This is known as vertical gene transfer or vertical evolution.

2.7.2 Horizontal gene transfer
Horizontal gene transfer (HGT) is the movement of genetic material between individual bacteria of the same species or even between different species. There are three mechanisms of HGT: transduction, transformation or conjugation. Conjugation is the process of transfer of genetic material (plasmid) between two bacterial cells in which direct cell to cell contact occurs. This is believed to be the main mechanism of HGT. Transformation is a process where parts of DNA are taken up by the bacteria from the external environment. This DNA is normally present in the external environment due to the death and lysis of another bacterium. Transduction occurs when bacteria specific viruses i.e. bacteriophages transfer DNA between two closely related bacteria (Toder, 2009).

2.8 Emergence of multidrug resistance among P. aeruginosa strains
The evolution and spread of resistance are relatively recent and have occurred mainly during past few decades. The problem of antibiotic resistance is also on increase in P. aeruginosa (National Nosocomial Infection Surveillance System, 2004) as this organism can harbor several mechanisms of resistance which generate multi drug resistant isolates (MDR). The first case of MDR P. aeruginosa strain was isolated in the hematologic unit in 1992 (Tacconelli, 2002). Since then they have been isolated from clinical specimens with increasing frequency all over the world (Deptula and Gospodarek, 2010). Emergence of MDR strains is due to selective pressure of antimicrobial therapy. Surveillance regarding these resistant pathogens is therefore, quite essential in order to provide efficient healthcare services to the community (Nadeem et al., 2009). According to surveillance program conducted during 1997-1999 the occurrence of multiple drug resistant P. aeruginosa strains were reported to the extent of 8.2% in Latin America,
4.7% in Europe, 1.6% in Asia-Pacific, 1.2% in the United States, and 0.9% in Canada (Gales et al., 2001).

There is no standard definition of MDR *P. aeruginosa* till now (Gill et al., 2011). Obritsch et al., 2005 defined MDR *P. aeruginosa* as those isolates which were resistant to three agents like β-lactam, carbapenems, aminoglycosides and fluoroquinolone. However, some workers have used different criteria for defining an isolate as MDR e.g. Irfan et al. (2008) and Saderi et al. (2010) defined MDR isolate as the one which was resistant to two or more drugs or drug classes of therapeutic relevance. Other researchers regard those which are resistant to at least four classes of antibiotics of therapeutic importance (Karlowsky et al., 2003; Chauhan and Sharma, 2013). Besides MDR, some isolates which are resistant to most antibiotics used for treatment of *P. aeruginosa* infections are referred to as “extensively drug resistant” (XDR) isolates and those which are resistant to all approved antimicrobial agents are considered as “pan-drug resistant (PDR)” (Tavajjohi and Moniri, 2011, Tam et al., 2010).

### 2.9 Resistance of *P. aeruginosa* to Beta lactams

Antibiotics of beta lactam group are routinely used for the treatment of Gram positive, Gram negative and anaerobic bacterial infections (Ambler 1980; Holten and Onusko, 2000). These antibiotics belong to a family of antimicrobial agents that consists of four major groups: penicillins, cephalosporins, monobactum, and carbapenems. They all have a beta lactam ring, which can be hydrolyzed by beta lactamases. The groups differ from each other by additional rings e.g. thiazolidine ring in penicillin, cephem nucleus in cephalosporin, none in monobactum, double ring structure in carbapenems (Levinson, 2010). These antibiotics act on bacteria by two different mechanisms: i. they act on bacterial cell wall and inhibit the action of transpeptidase, responsible for completion of cell wall, ii. they attach to the penicillin binding proteins (PBPs) that normally suppress cell wall hydrolases, thus freeing these hydrolases, which in turn result in hydrolysis of the bacterial cell wall. Bacteria can produce beta lactam inactivating enzymes (beta-lactamases) to degrade the beta lactam antibiotics (Samaha-Kfoury and Araj, 2003). This
mechanism is shown below in the Figure 2.3.

**Figure 2.3** Inactivation of penicillin by the action of β-lactamases

Beta-lactamases are most commonly classified according to two general schemes: the Ambler molecular classification scheme and the Bush-Jacoby-Mediero functional classification system. The Ambler scheme categorizes β-lactamases into four major classes (A to D). This classification is based upon protein homology. In the Ambler classification scheme, β-lactamases of classes A, C, and D are serine β-lactamases whereas the class B enzymes are zinc based or metallo-β-lactamases. The Bush-Jacoby-Medeiros classification scheme classified β-lactamases according to functional similarities (substrate and inhibitor profile). There are four main groups and multiple subgroups in this system. This classification scheme is of more importance to the physician or microbiologist in a diagnostic laboratory because it considers β-lactamase inhibitors and β-lactam substrates that are clinically relevant (Drawz et al., 2010, Bush et al., 1995). The functional and molecular classification of beta-lactamases is presented in Table 2.2.

**Table 2.2** Functional and molecular classification of beta-lactamases

<table>
<thead>
<tr>
<th>Bush group</th>
<th>Enzyme type</th>
<th>Inhibition by clavulunate</th>
<th>Ambler molecular class</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cephalosporinase</td>
<td>No</td>
<td>C</td>
</tr>
<tr>
<td>2a</td>
<td>Penicillínase</td>
<td>Yes</td>
<td>A</td>
</tr>
</tbody>
</table>
2.9.1 Role of beta-lactamases in drug resistance

Beta-lactamases play very important role in widespread β-lactam resistance. These enzymes catalyze the hydrolysis of amide bond of beta lactam ring present in the antibiotic. More than 500 different β-lactamases have been reported so far (CLSI, 2010). These versatile enzymes are present in both Gram positive as well as Gram negative bacteria (Holten and Onusko, 2000). β-lactamases were naturally present in bacteria long before the introduction of penicillins to treat bacterial infections (Woodruff and Foster, 1945). The genes encoding these enzymes were originally located on the bacterial chromosome (Hanson et al., 1999; Yusha’u et al., 2010). Furthermore, these enzymes are inducible and constitutively expressed in low quantities. First report of a plasmid encoded β-lactamase in a Gram negative bacterium appeared from Greece in 1965 (Datta and Kontomichalou, 1965). Since then β-lactamase producing bacteria are increasing in number and causing more severe infections (Shobha et al., 2007). ESBL and MBL are the main mechanisms of resistance in MDR P. aeruginosa against β-lactam drugs (Aggarwal et al., 2008). Carbapenems have stability against most serine β-lactamases (ESBL) and high

| 2b  | Broad spectrum | Yes | A |
| 2be | Extended spectrum | Yes | A |
| 2br | Inhibitor resistant | Diminished | A |
| 2c  | Carbenicillinase | Yes | A |
| 2d  | Cloxacillinase | Yes | D or A |
| 2e  | Cephalosporinase | Yes | A |
| 2f  | Carbapenamase | Yes | A |
| 3   | Metalloenzyme | No | B |
| 4   | Pencillinase | No | |
permeability across the outer membrane (Livermore, 1995). However, intensive use of carbapenems facilitated the emergence of carbapenem resistant *P. aeruginosa*.

### 2.10 Extended spectrum beta lactamases (ESBLs)

Extended spectrum beta-lactamases are beta-lactamases that are capable of hydrolyzing oxyimino cephalosporins. These enzymes are encoded by different genes located on either chromosomes or plasmids and inhibited by beta-lactamase inhibitors (Ghafourian *et al.*, 2011). ESBL belongs to class A of Ambler molecular classification scheme and Group 2be of Jacoby-Medieros functional classification system where letter “e” for extended spectrum of activity, represents the ESBLs. There are three main types of ESBLs: Temoneira (TEM), sulfhydryl variable (SHV) and cefotaximase (CTX-M).

ESBL producing *P. aeruginosa* was first detected in Western Europe in the mid-1980s and later reported from different countries all over the world (Vahdani *et al.*, 2012). ESBLs conferring resistance to extended spectrum cephalosporins were first reported in the members of the family *Enterobacteriaceae* and later in *P. aeruginosa* (Nordmann and Guibert, 1998).

An increasing number of ESBLs have recently been described. The total number of ESBL genes mutated and characterized till date exceeds 300 (Bokaeian *et al.*, 2015). These enzymes are typically derived from TEM or SHV. The mutations in these genes result in alteration of the amino acid configuration around the active site of these enzymes. Also, the mutations extend the spectrum of β-lactam antibiotics. The first report of plasmid encoded β-lactamase capable of hydrolyzing the extended spectrum cephalosporins was published in 1983 (Knothe *et al.*, 1983). ESBL producing gram negative organisms are typically resistant to penicillin, first and second generation cephalosporins and also to third generation oxyimino cephalosporins and monobactams. Various ESBLs that have been reported in *P. aeruginosa* include TEM (Temoneira), SHV (sulphhydryl variable) and CTX-M (cefotaximase), PER (Pseudomonas extended spectrum beta-lactamase), VEB (Vietnamese extended spectrum beta-lactamase) and IBS (integron-borne cephalosporinase)/GES (Guiana extended spectrum) types. PER types were mostly originated from Turkish isolates and VEB type from Southeast Asia and GES and IBC types have been reported from France, Greece, and South Africa. The presence of ESBLs is of
great clinical significance as therapeutic options in the treatment of ESBL producing organisms are extremely limited. Although ESBL producing organisms may appear susceptible to some extended spectrum cephalosporins, treatment with such antibiotics has been associated with higher rates of failure (Paterson and Bonomo, 2005).

2.10.1 Types of ESBL genes in *P. aeruginosa*

**BlaSHV**

*BlaSHV* refers to sulphhydryl variable and hence named as *blaSHV*. These types of ESBLs may be more frequently found in clinical isolates than any other type of ESBLs (Jacoby, 1997). More than 100 *blaSHV* variants have been identified (Achour *et al*., 2014). *SHV-1* is a β-lactamase having activity against penicillins and narrow spectrum cephalosporins such as cephalothin and cephaloridine (Harada *et al*., 2008). *BlaSHV-2* was first detected in France in 1995 (Naas *et al*., 1999) and later in Thailand and Poland (Chanawong *et al*., 2001). *BlaSHV-2* differs from *blaSHV-1* by substitution of glycine by serine at 238 position by point mutation. This enzyme vigorously hydrolyses fourth generation cephalosporins (Weldhagen *et al*., 2003). *BlaSHV-5* and *blaSHV-12* producing *P. aeruginosa* strains have also been reported from Thailand (Chanawong *et al*., 2001) and Greece (Neonakis *et al*., 2003). *BlaSHV-5* determines elevated level of resistance to monobactams and ceftazidime (Poirel *et al*., 2004). This type of ESBL efficiently hydrolyzes cefotaxime and to a lesser extent ceftazidime.

**BlaTEM**

The *blaTEM* derives its name from “Temoneira” and was isolated first from an *E. coli* isolate from a patient in Athens, Greece in 1965. *BlaTEM-1* variant of this gene hydrolyzes ampicillin at a higher rate than carbenicillin, oxacillin or cephalothin and has negligible activity against extended spectrum cephalosporins and they are inhibited by clavulanic acid (Upadhyay *et al*., 2010). At present, more than 100 *blaTEM* variant have been described (Ghafourian *et al*., 2014). However, complex mutants of the *blaTEM* derivatives (called *blaCMT-1*, *blaCMT-2*, *blaCMT-3* and *blaCMT-4*) have been identified that have the ability to hydrolyse both third generation cephalosporins and β-lactamase inhibitors (Fiett *et al*., 2000; Neuwirth *et al*., 2001). *BlaTEM* type ESBL gene is found mainly in *E. coli* and *K. pneumoniae*. However, they could appear in other Gram negative
bacteria including *P. aeruginosa* and also in different genera of *enterobacteriaceae* (Ghafourian *et al.*, 2014).

**Bla\(_{\text{CTX-M}}\)**

A new family of plasmid mediated ESBL genes, called *bla\(_{\text{CTX-M}}\)* has come up which shows strong hydrolytic activity against cefotaxime. These genes are derived from the mobilization of chromosomal enzymes to the plasmid of the same or other genera. The first clinical isolate with a cefotaximase property was reported from an *E. coli* isolate of ear discharge of four month old child in Munich, Germany in 1989 and it was named as *bla\(_{\text{CTX-M-1}}\)* (*blaCTX* for cefotaximase and M for Munich). These enzymes are predominantly produced by the strains of *Salmonella enterica* serovar *Typhimurium* and *E. coli*, but have also been described in other species of *Enterobacteriaceae*. *Bla\(_{\text{CTX-M}}\)* type ESBLs typically hydrolyzes cefotaxime and ceftriaxone more efficiently than ceftazidime (Pitout, 2010).

*Bla\(_{\text{CTX-M}}\)* ESBLs were predominantly found in three geographic areas initially: South America, the Far East, and Eastern Europe but they are now widespread all over the globe (Paterson and Bonomo, 2005). More than 130 allelic variants of *bla\(_{\text{CTX-M}}\)* have been described (Wang *et al.*, 2013). CTX-M-15 is the most commonly reported ESBL enzyme in Europe (Pitout and Laupland, 2008). This enzyme is derived from *bla\(_{\text{CTX-M-3}}\)* gene variant by replacement of asparagine by glycine at 240 position by point mutation. This substitution confers an increased catalytic activity against ceftazidime (Peirano and Pitout, 2010).

**Bla\(_{\text{PER}}\)**

The Pseudomonas extended spectrum beta-lactamase (*bla\(_{\text{PER}}\)*) was first identified and fully characterized ESBL gene of *P. aeruginosa*. It was first reported in France in 1991 from a Turkish patient in Paris (Nordmann and Naas, 1994) and this enzyme was found chromosome encoded in the isolate. Later, plasmid encoded PER-1 were also reported (Nordmann and Guibert, 1998) which shares only 18 to 20% amino acid identity with the TEM and SHV types of ESBLs. *Bla\(_{\text{PER-1}}\)* gene was identified in up to 38% of ceftazidime resistant *P. aeruginosa* isolates. *Bla\(_{\text{PER-1}}\)* among nosocomial *P. aeruginosa* isolates in Turkey is now widely disseminated (Vahaboglu *et al.*, 2001; Kolayli *et al.*, 2005). Other geographical regions where PER-1 enzyme
producing *P. aeruginosa* strains are prevalent include: Italy, Belgium, France, Spain, Romania, Hungary, Serbia, Korea, Japan, China, Europe and Poland (Luzzaro et al., 2001; Pagani et al., 2004; Claeys et al., 2000; Empel et al., 2007). *Bla*<sub>PER-1</sub> β-lactamase gene efficiently hydrolyses penicillins and cephalosporins and it is moderately inhibited by β-lactamase inhibitors and imipenem (Weldhagen et al., 2003).

**Bla<sub>VEB</sub>**

VEB is a recently discovered class A enzyme which is either plasmid mediated or integron associated. Apart from β-lactam antibiotics, this enzyme confers resistance to non β-lactam antibiotics also. VEB-1 β-lactamase was first isolated in *Escherichia coli* and *Klebsiella* isolates in France in 1998 from a four month old Vietnamese child and subsequently detected in *P. aeruginosa* strains from Thailand, Kuwait and China (Naas et al., 1999). Later, Girlich et al. (2002) found a high prevalence of *bla<sub>VEB</sub>* like genes (93%) in ceftazidime resistant clinical isolates of *P. aeruginosa* in the university hospital in Thailand and reported a new variant named *bla<sub>VEB-2</sub>*. High dissemination (56.8%) of *bla<sub>VEB-1</sub>* gene of ESBL among ceftazidime resistant nosocomial *P. aeruginosa* isolates of Bulgaria was later on reported (Strateva et al., 2007). The substrate profile of VEB enzymes was found identical with that of PER-1 (Weldhagen et al., 2003). A total of eight variants of *bla<sub>VEB</sub>* gene have been identified: *bla<sub>VEB-1</sub>, bla<sub>VEB-1a</sub>, bla<sub>VEB-1b</sub>, bla<sub>VEB-2</sub>, bla<sub>VEB-3</sub>, bla<sub>VEB-4</sub>, bla<sub>VEB-5</sub> and bla<sub>VEB-6</sub> (Zong et al., 2009).

**Bla<sub>OXA</sub>**

Oxacillinases (OXA) were discovered which belong to the molecular class D of Ambler’s classification scheme (Prashanth et al., 2010). Because of their oxacillin hydrolyzing abilities they have been named as oxacillinases. *Bla<sub>OXA</sub>* type β-lactamase genes are predominant in *P. aeruginosa* as well as other Gram negative bacteria. Oxacillinases (OXA) are not inhibited by clavulanic acid, but OXA-18 and OXA-45 were reported to be inhibited by this acid (Naas et al., 2008). In *E. coli*, *bla<sub>OXA</sub>* type ESBL genes provide weak resistance to oxyimino-cephalosporins whereas high level of resistance has been observed in *P. aeruginosa*. More than eighty types of *bla<sub>OXA</sub>* gene have been identified (Zander et al., 2014).
Other genes

A novel family of ESBL genes referred as Guiana extended spectrum (\textit{bla}\textsubscript{GES}) has been described, which originated from French Guiana. \textit{Bla}\textsubscript{GES-1} and \textit{bla}\textsubscript{GES-2} variants were found in France, Brazil (Dubois \textit{et al.}, 2002; Castanheira \textit{et al.}, 2004) and South Africa (Poirel \textit{et al.}, 2002) respectively. \textit{Bla}\textsubscript{GES-1} variant has unusually low level of catalytic activity, low affinity to the most of the substrates, and an unusual inhibition profile that includes clavulanic acid and imipenem. Unlike most of class A ESBLs, GES-1 enzyme has strong affinity to the second generation cephalosporin cefoxitin (Weldhagen \textit{et al.}, 2003). GES-2 and GES-4 possess carbapenemase activity (Weldhagen and Prinsloo, 2004; Wachino \textit{et al.}, 2004) and these enzymes originated through a point mutation of GES-1 and GES-3 respectively. GES-2 possesses 100 times higher catalytic activity towards imipenem as compared to GES-1 (Nordmann and Poirel, 2002). New variants of GES-1 enzyme have been reported viz GES-5 and GES-9 (Poirel \textit{et al.}, 2005). In comparison with GES-1 identified in 1999 (in France) the newly discovered GES-5 enzyme hydrolyses penicillins as well as extended-spectrum cephalosporins and aztreonam to a greater extent. The ESBL \textit{bla}\textsubscript{IBC-2} (integron-borne cephalosporinase) (now called \textit{bla}\textsubscript{GES-8}), a variant of \textit{bla}\textsubscript{GES-1} gene was discovered in a \textit{P. aeruginosa} isolate in Greece (Mavroidi \textit{et al.}, 2001). This enzyme has a single base substitution and possesses hydrolytic activity similar to class A ESBLs. It is inhibited by β-lactamase inhibitors but generally classified into ESBLs (Poirel \textit{et al.}, 2000).

Recently, a new type of ESBL gene (Belgium extended spectrum β-lactamase, \textit{bla}\textsubscript{BEL}) was discovered in Belgium in a clinical strain of \textit{P. aeruginosa} isolated from a hospital setting (Poirel \textit{et al.}, 2005). Later on \textit{bla}\textsubscript{BEL-1} producing \textit{P. aeruginosa} isolates were reported from several hospitals located in different geographical areas of Belgium (Bogaerts \textit{et al.}, 2007). Other minor ESBL genes are \textit{bla}\textsubscript{BES} (Brazilian extended spectrum beta-lactamase), \textit{bla}\textsubscript{TLA} (named after Tlauhucas Indians), \textit{bla}\textsubscript{SF0} (Serratia fonticola) and \textit{P. aeruginosa} extended spectrum beta-lactamase (\textit{bla}\textsubscript{PME}).
2.11 Diagnosis of *P. aeruginosa*

A number of phenotypic and genotypic methods are in use for identifying *P. aeruginosa* from different clinical specimens: pus, wound swab, mid-stream specimen of urine, cerebrospinal fluid (CSF), sputum or blood. *P. aeruginosa* recovered from the samples can be identified on the basis of Gram’s staining, colony morphology on solid media and biochemical tests. The conventional phenotypic methods are simple, easy to perform and cost effective but less specific enough to discriminate among species and strains (Vijayakumar *et al*., 2011). Genotypic methods are more discriminatory but technically more demanding and costly. It is also possible to classify microorganism up to the species level with the help of genotypic methods. Therefore, there is a need to develop and perform a rapid procedure to detect *P. aeruginosa*. Various phenotypic and genotypic methods used for identifying the organism are discussed below:

2.11.1 Phenotypic methods

Selective culture media like Pseudomonas isolation agar and cetrimide agar have been used for recovering *P. aeruginosa* for the clinical samples. These media contain components such as magnesium chloride, potassium sulphate, protein hydrolysate and agar which are helpful in selective isolation of this organism. (Brown and Lowbury, 1965; Fujita *et al*., 1992; Laine *et al*., 2009). Additional characteristics such as production of pigments viz pyocyanin, proteolytic activity, nitrate utilisation, lipolytic activity and glutamate utilisation need to be performed which aid in identification up to the species level. (Franzetti and Scarpellini, 2007). In order to type the unrelated strains, other conventional methods have been followed such as serotyping, biotyping, phage-typing and determination of antibiograms.

Some phenotypic and genotypic methods are also used to detect ESBL producing *P. aeruginosa*. The phenotypic tests are easy to perform and cost effective. Clinical diagnostic laboratories therefore, employ phenotypic methods mostly to detect ESBL producing *P. aeruginosa*, which include an initial screening test, a disc diffusion method using any one of the following beta-lactam antibiotics: cefpodoxime, ceftazidime, aztreonam, cefotaxime, or ceftriaxone. Further confirmation is however done with other recommended methods such as: double disc synergy test (Jarlier *et al*., 1988), three dimensional test, modified double disc diffusion test, disc
replacement method, combination disc test, vitek ESBL cards, BD phonex automated microbiology system and E test method (Epsilon test) (CLSI, 2013). Phenotypic ESBL confirmation tests which are routinely being used are based on in vitro inhibition of ESBL by clavulanic acid (CA). Recently, chromogenic media (e.g. Brilliance ESBL agar and ChromID ESBL agar) designed specifically for screening and identification of ESBLs producing *Enterobacteriaceae* are commercially available (Huang et al., 2010).

### 2.11.2 Genotypic methods

Molecular methods have been extensively used in bacterial identification as well as for determining the phylogenetic relationships among the organisms during past few years. These methods include DNA and RNA based techniques. DNA based technologies include PCR, and its variations, and DNA microarrays while RNA based technologies include reverse transcriptase PCR (RT-PCR), transcription mediated amplification (TMA) and 16S rRNA gene sequencing etc. (Malik et al., 2008). These methods are rapid and have higher sensitivity as compared to culture methods (Singh et al., 2012). However, some of limitations of genotypic methods for routine use are: higher cost for processing the samples and the requirement of specialized equipment as well as expertise. Some of the important molecular methods employed for typing of *P. aeruginosa* strains are discussed below:

**Multilocus sequence analysis**

Multilocus sequence analysis (MLSA) has been considered as excellent alternative to DNA-DNA hybridisation and 16S rRNA (Martens et al., 2008). Multilocus sequence typing (MLST) is a technique for the typing of multiple loci. This technique utilizes the DNA sequences of internal fragments of multiple housekeeping genes to characterize the isolates of bacterial species. In case of *P. aeruginosa* seven such genes (*acsA, aroE, guaA, mutL, nuoD, ppsA and trpE*) are used to discriminate *P. aeruginosa* isolates (Curran et al., 2004). However, Frapolli et al. (2007) utilized ten such genes in order to determine the phylogenetic relationship among the species of Pseudomonas. This technique can be used as a better tool for differentiating *P. aeruginosa* strains.


**Oligotyping**

It was the first molecular method developed for the identification of β-lactamases. Ouellette et al. (1988) used this method to discriminate \( bla_{TEM-1} \) and \( bla_{TEM-2} \) genes. This method utilizes oligonucleotide probes that were designed to detect point mutations under stringent hybridization conditions. Using this method, several new \( bla_{TEM} \) variants have been identified within a set of clinical isolates using radioisotope or biotin as labels.

**Pulsed field gel electrophoresis**

Developed in 1984, this method is now considered as the “gold standard” among molecular typing methods. Clinically, it is an invaluable tool in detecting the occurrence of an outbreak and tracing its possible source (Tenover et al., 1995). PFGE of chromosomal DNA is probably the most widely used method of genotyping ESBL producing organisms (Paterson and Bonomo, 2005). PFGE is based on the digestion of bacterial DNA with restriction enzymes which recognize few sites along the chromosome, larger DNA fragments (30-800 Kb) are generated in the process. These fragments can be separated on agarose gel by pulsed-field electrophoresis in which the orientation of the electric field across the gel is changed periodically. The separated DNA fragments can be visualized as bands in the gel, producing a particular pattern, the PFGE pattern. This method is one of the most reproducible and most reliable technique for analysis of variety of pathogens including \( P. aeruginosa \) (Grundmann et al., 1995).

**Polymerase chain reaction (PCR) - based methods**

PCR based methods have played an important role in the diagnosis and epidemiology of infectious diseases. Three basic procedures are: i. polymerase chain reaction (PCR), ii. the PCR-RFLP and iii. sequencing of nucleotides of the amplicons. PCR methods are simple and rapid for discriminating \( P. aeruginosa \) strains. PCR amplification followed by nucleotide sequencing remains the gold standard for the identification of specific point mutation of ESBL genes. There are many types of PCR based methods; some of them are described below:
**PCR-RFLP**

Restriction fragment length polymorphism (RFLP) analysis involves characterization of DNA after digestion with restriction endonucleases and subsequent separation of DNA fragments in agarose gel. Only the genomic DNA fragments that hybridize to the probes are visible in RFLP analysis (Olive and Bean, 1999). Restriction fragment length polymorphism among various DNAs can be analyzed by this technique. The polymorphism occurs due to the presence or absence of restriction sites (Walker and Rapley, 2005). PCR-RFLP allows differentiation of strains. This method is simple, rapid, discriminatory and reproducible. Arlet et al. (1995) used this method to detect single base mutations within $bla_{TEM}$ gene of *P. aeruginosa*.

**Random amplification of polymorphic DNA (RAPD) and arbitrarily primed polymerase chain reaction**

Random amplification of polymorphic DNA (RAPD) is based on the parallel amplification of a set of fragments by using short sequences as primers (usually 8-12 base pairs) that target several unspecified genomic sequences. RAPD amplicons can be analysed by agarose gel electrophoresis or by DNA sequencing depending on the labeling of primers with appropriate fluorescent dyes such as fluorescein, 4-chloro-7-nitrobenzo-2-1-diazole (NBD), tetramethylrhodamine, and texas red. It detects differences along the whole bacterial genome, not only in particular sequences. RAPD-PCR is a method of genotypic identification and characterization. This method has great specificity and sensitivity to define a wide range of bacterial isolates. RAPD typing has been used effectively for typing *P. aeruginosa* strains also (Mahenhiralingam et al., 1996). Eftekhari and co-workers (2003) used RAPD-PCR for fingerprinting *P. aeruginosa* obtained from patients with cystic fibrosis and found 13 different genotypes. A RAPD-PCR method was also used to discriminate between isolates from different patients and to compare genotype with morphology and antibiograms of the *P. aeruginosa* isolates (Abou-Dobara et al., 2010).

Arbitrarily primed PCR (AP-PCR) is a variant of the original RAPD method. This method is based on the use of small oligonucleotide primers (8-12 oligomers) with an arbitrary sequence
under less stringent PCR conditions (Welsh and McClelland, 1990). This is used for the quick screening of outbreak strains.

**PCR amplification of 16S rRNA and its sequencing of the amplicon**

16S rRNA has been used for species identification (Wagner et al., 2008) as well as to know the phylogenetic relationship among the species of the genus Pseudomonas (Anzai et al., 2000; Purohit et al., 2003). Since the 1980s, sequencing of the 16S rRNA gene has been used as an important tool for phylogenetic analysis and classification of bacteria. The 16S rRNA gene has well conserved regions in all organisms (Cai et al., 2003). Selective amplification of Pseudomonas 16S rDNA gene by PCR followed by restriction fragment length polymorphism analysis or denaturing gradient gel electrophoresis has been used to detect and differentiate Pseudomonas species from clinical and environmental samples (Mohammed et al., 2014). 16S rDNA based PCR assays provide rapid, simple, and reliable identification of *P. aeruginosa* and its differentiation from other phylogenetically closely related Pseudomonas species.

**Ligase chain reaction (LCR)**

This is a recently developed technique of DNA amplification and allows for the discrimination of DNA sequences differing in only a single base pair (Barany, 1991). In this method, two pairs of probes, each pair being complementary to a strand of the denatured target DNA are used. Each probe within a pair is designed to hybridize to adjacent stretches of DNA. LCR uses two enzymes: DNA polymerase and DNA ligase. DNA polymerase initially amplifies the template and then inactivated and DNA ligase ligates two probes. Each cycle results in a doubling of the target DNA segment. LCR has greater specificity as compared to PCR. Ligation is inhibited as a result of imperfect hybridization of the probe with target DNA and thus the absence of a ligated product suggests the presence of at least a single base pair mismatch near the junction (Wiedmann et al., 1994). LCR is thus, suitable for detecting point mutations, such as those occurring within the *bla* genes. Niederhauser et al. (2000) used ligase chain reaction to test different ESBL producing strains and clinical isolates for a specific point mutation in the *bla_{SHV}* gene.
DNA microarray

DNA microarray is a genotyping technique with a high multiplexing ability. This technique allows thousands or tens of thousands of specific DNA sequences to be detected simultaneously on 12 cm square small glass or silica slide. Grimm et al. (2004) developed and validated oligonucleotide microarray for the rapid identification of ESBL in Gram-negative bacteria by simultaneously genotyping \( \texttt{bla}_{\text{TEM}}, \texttt{bla}_{\text{SHV}} \) and \( \texttt{bla}_{\text{CTX-M}} \) gene. This array consisted of 168 probes which cover mutations responsible for 156 amino acid substitutions within assay time of 5 hours.

Whole genome sequencing (WGS)

Sequencing of the entire genome of an organism is becoming a powerful tool for epidemiological investigations which permits accurate identification and characterization of bacterial isolates. Whole genome sequencing reveals the complete DNA make up of an organism. The sequence data is helpful for better understanding of the variations both within and between species. Because of its digital, sharable format and ultra-high resolution, this method is able to discriminate two isolates differing by just a single mutation and has been successfully used to investigate outbreaks due to different bacteria and species e.g. \textit{Staphylococcus aureus}, \textit{Acinetobacter baumannii}, \textit{Klebsiella pneumoniae} and \textit{P. aeruginosa} (Quick et al., 2014).

2.12 Prevention and control of \textit{P. aeruginosa} infections

\textit{P. aeruginosa} infections can result in high morbidity and mortality. It is believed that there is no effective traditional antibiotic therapy for MDR \textit{P. aeruginosa}. The prevention of its transmission is the most important strategy for controlling \textit{P. aeruginosa} infection (Yoshikawa et al., 2009). The practices followed by healthcare workers to check further spread of \textit{P. aeruginosa} fall into two broad groups: i. Good hand hygiene and cleanliness ii. appropriate use of antibiotics. An antimicrobial policy should be in place which should contain detailed antimicrobial prescribing guidelines according to which the prescription may be made.

In community, hot tubs or pools must be avoided that are poorly maintained. The contact lenses, equipment and solutions must be kept free from possible contamination. During \textit{P. aeruginosa} outbreak in a ward, the prescriber should avoid use of cephalosporin in other patients of the same
ward. Private room may be preferred for ESBL patient in order to reduce the chances of its transmission.

2.13 Treatment of *P. aeruginosa* infections

The severity of *P. aeruginosa* infection can be restricted by early detection and immediate antibiotic treatment before the bacteria alter into a mucoid phenotype because it is extremely difficult to eradicate such organism once established. Common anti-pseudomonal drugs that should be used for treating the patients are carbenecillin, ceftazidime, cefoprazone-sulbactum, piperacillin-tazobactum. Two anti-pseudomonal drug combination therapy (e.g. a beta-lactam antibiotic with an aminoglycoside) is usually recommended for the initial empiric treatment of a pseudomonal infection, especially for patients with neutropenia, bacteremia, sepsis, severe upper respiratory infections (URIs) or abscess formation. The choice of antibiotic also depends on the site and extent of infection and on local resistance patterns at a particular geographic location (Giamarellou and Antoniadou, 2001). The carbapenems are currently among the last choice for the treating MDR *P. aeruginosa* infections. Meropenem, ertapenem and imipenem are useful against most ESBL and AmpC producing pathogens. However, resistance to these carbapenems has been developed by way of OprD deletions and spread of carbapenemases (Thomson and Bonomo, 2005).

Polymyxins B and E (colistin) have been used in the clinical settings out of five originally described polymyxins (polymyxins A-E) for treating *P. aeruginosa* infections. Polymyxins are cyclic, positively charged lipopeptide antibiotics derived from various species of *Bacillus polymyxa*. These compounds have a detergent like effect that disrupts membrane integrity and results in the leakage of intracellular components (Hermsen *et al*., 2003; Zavascki *et al*., 2007). Colistin (polymyxin E) is more widely used than polymyxin B in clinical practice (Zavascki *et al*., 2007). This antibiotic has been used increasingly as therapy alone or in combination with one or more antibacterial agents for the treatment of pneumonia with MDR strains (Livermore, 2004). The synergy between colistin and rifampicin has been mainly used in clinical settings (Tascini *et al*., 2004) but other antimicrobial agents have also been used in combination with colistin, including imipenem, meropenem, aztreonam, piperacillin, ceftazidime and ciprofloxacin. Intravenous colistin treatment for serious infections caused by MDR *P. aeruginosa* has high risk of nephrotoxicity and neurotoxicity. Nephrotoxicity rates ranging from
8% to 36% using intravenous colistin have been reported (Falagas et al., 2005). Mentzelopoulos et al., 2007 has been also confirmed colistin resistance in metallo-β-lactamase producing P. aeruginosa. Some of the newer antibiotics used in treatment of P. aeruginosa infections are as discussed below:

**Doripenem**
Doripenem is an ultra broad spectrum carbapenem group of antibiotic with potent in vitro activity against both Gram-positive and Gram-negative bacteria. It inhibits the synthesis of cell wall by binding to penicillin-binding proteins, causing cell wall damage and bacterial death. It is stable against many β-lactamases (including ESBLs), except for the class B metallo-β-lactamases.

**Biapenem**
Biapenem (1-β-methyl-carbapenem) is a carbapenem antibiotic, stable to most β-lactamases, including AmpC and extended-spectrum β-lactamases (ESBLs), with a broad spectrum activity against Gram-positive and Gram-negative aerobic as well as anaerobic bacteria (Aldridge et al., 1994; Bonfiglio et al., 1997). This antibiotic combines with penicillin binding proteins and inhibits bacterial cell wall synthesis. Biapenem is more stable than meropenem against hydrolysis by human renal dehydropeptidase-I (DHP-I) (Thamlikitkul and Tiengrim, 2010). Thus, biapenem could be administrated independently as compared to older carbapenems, which must be compounded with a renal DHP-I inhibitor.

**Tomopenem**
Tomopenem is a novel 1-β-methylcarbapenem with broad spectrum activity against Gram-positive and Gram-negative bacteria, including P. aeruginosa, methicillin-resistant Staphylococcus aureus and penicillin resistant Streptococcus pneumoniae (Koga et al., 2008). Tomopenem has a longer half-life compared with other carbapenems except for ertapenem and have high stability against hydrolysis by human renal dehydropeptidase-I (DHP-I) (Morinaga et al., 2008).
Ceftobiprole
Ceftobiprole is a broad-spectrum cephalosporin having activity against Gram-positive cocci, including methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus epidermidis* (MRSE), penicillin-resistant *Streptococcus pneumoniae*, *Enterococcus faecalis* as well as many Gram-negative bacilli including AmpC producing *E. coli* and *P. aeruginosa* (Zhanel *et al*., 2008). Ceftobiprole is a fifth generation cephalosporin having strong affinity for penicillin binding proteins. It is a water soluble prodrug that is rapidly converted by plasma esterases to the active drug.

Aerosolized antibiotics
Most *P. aeruginosa* infections are found in the respiratory tract. Therefore, colistin has been used as an aerosolized nebulizer antibiotic treatment (Czosnowski *et al*., 2009, Sabuda *et al*., 2008, Kwa *et al*., 2005). These antibiotics have been used for the treatment of pneumonia as well as prophylactically, also used in patients with underlying chronic pseudomonal infections such as cystic fibrosis.

Vaccines for prevention of *P. aeruginosa* infections
There is no vaccine currently available in the market against *P. aeruginosa*, although many attempts have been made in the past. A number of different vaccines and several monoclonal antibodies have been developed in the last decades for active and passive immunization against *P. aeruginosa* (Doring and Pier, 2008). Antigens of *P. aeruginosa*, such as the outer membrane porin proteins (Oprs), LPS, toxins, pili and flagella have been investigated as possible targets for the development of vaccines and outer membrane protein antigens have been found to possess some potential to protect against *P. aeruginosa* infection in mice (Peluso *et al*., 2010).