2. REVIEW OF LITERATURE

2.1 Historical background

The opportunistic bacterial pathogen currently known as *Pseudomonas aeruginosa* has received several names throughout its history based on the characteristic blue-green coloration produced during its culture on suitable bacteriological media. *Pseudomonas* was first isolated by a French military surgeon, Sedillot in 1850 from a blue-green discharge on surgical dressing with associated infection (Poirel *et al.*, 2000). Fordos in 1860 extracted the pigment responsible for the blue coloration and the pigment production was later associated with rod-shaped organism was identified by Lucke in 1862. *P. aeruginosa* was successfully isolated in pure culture by Carle Gessard (1882) in France. He discovered *P. aeruginosa* through an experiment that identified this microbe by its water soluble pigments that turned blue-green when exposed to ultra-violet light. During the period 1889 – 1894, several other workers reported *Bacillus pyocyaneus* as the causative agent of blue-green purulence in the wounds of patients (Lister *et al.*, 2009). This organism is now known as *Pseudomonas aeruginosa*.

2.2 Taxonomy

*Pseudomonas* has been derived from a Greek word ‘Pseudo’ meaning false and ‘monas’ meaning a single unit. Around 202 species of *Pseudomonas* have been identified till now (Ozen and Ussery, 2012). Gilardi *et al.*, 1985 divided the genus *Pseudomonas* into 7 groups based on phenotypic characteristics: fluorescent, stutzeri, alcaligenes, pseudomallei, acidovarans, facilis-delafieldii and diminuta. Taxonomy and nomenclature of the genus *Pseudomonas* has been changed on the basis of 16SrRNA sequence analysis. Strains formerly classified in the genera *Chryseomonas* and *Flavimonas* are now classified in the genus *Pseudomonas* and the strains which were previously classified in the genus *Pseudomonas* are now classified in the genera *Burkholderia* and *Ralstonia* (Winn *et al.*, 2006). Based on rRNA-DNA homology studies, Palleroni *et al.*, 1973 separated the Pseudomonads into five ribosomal RNA homology groups. The taxonomic status of *Pseudomonas aeruginosa* is as follows: this organism belongs to the Kingdom: *Bacteria*, Phylum: *Proteobacteria*, Class: *Gammaproteobacteria*, Order: *Pseudomonadales*, Family: *Pseudomonodaceae* and Genus: *Pseudomonas* (Todar, 2009).
2.3 Characteristics of *Pseudomonas aeruginosa*

*P. aeruginosa* is a motile, gram negative, straight or slightly curved rod-shaped bacterium measuring 0.5 to 1.0μm by 1.5 to 5.0μm in length. This organism is aerobic, non-spore forming, motile by one or more polar flagella. It preferably grows in the moist environments. In hospitals, *P. aeruginosa* can be isolated from nebulizers, dialysate fluids, saline, catheters and other devices. This organism is rarely part of the normal flora but has the capability of easily colonizing hospitalized patients particularly the immunocompromised ones. Most strains of *P. aeruginosa* can ferment carbohydrates rather than utilizing it as a source of energy by oxidative means. Various biochemical characteristics of *P. aeruginosa* are: it is oxidase and catalase positive, glucose is oxidatively utilized, reduces nitrates to nitrites. This organism is unable to produce indole. Methyl-red and Voges-Proskauer tests are negative and do not decarboxylate lysine and ornithine and do not hydrolyze urea and aesculin but it can liquefy gelatin. Citrate is utilized as source of carbon and energy by *P. aeruginosa*. It utilizes acetamide producing grape-like odour due to the presence of 2- aminoacetophenone (Brenner and Tramontano, 2005). *P. aeruginosa* can be grown on different bacteriological media. On nutrient agar, its colonies are large and pigmented (bluish green due to pyocyanin and fluorescein pigments) and on 5% sheep blood agar, it produces β-type of hemolysis. Non-lactose fermenting colonies with green pigmentation and metallic sheen are produced on MacConkey agar. Colonies recovered from respiratory tract infection produce large amounts of alginate which is an exopolysaccharide. This substance consists of mannuronic and guluronic acids. These compounds help in the formation of mucoid colonies (Collee et al., 1996). *P. aeruginosa* secretes a variety of pigments such as pyocyanin, pyoverdine, pyomelanin and pyorubin (Stanier et al., 1966). This organism is armed with a wide variety of virulence factors such as flagellum, type-IV pili, pyocyanin, type-III secretion system, endotoxin (lipopolysaccharide) and various exotoxins (proteases, hemolysins, lecithinase, elastase and DNase). Besides, biofilm formation can also be regarded as one of the important virulence factor which permits the survival of this organism in a variety of unexpected surfaces (Borriello et al., 2004). *P. aeruginosa* secretes an exopolysaccharide, also known as slime layer which helps in prevention from phagocytosis by mammalian leucocytes.
2.4 Genome Organization

Genome of *P. aeruginosa* has been sequenced completely. The genome is 6.3Mbp and contains 5570 open reading frames. *P. aeruginosa* strains are found in various environmental habitats as well as in animal and human hosts, where they can act as opportunistic pathogens (Ramos, 2004). A major factor in its prominence as a pathogen is its intrinsic resistance to antibiotics and disinfectants (Stover *et al*., 2000). The genome of *P. aeruginosa* consists of two components: a core genome and an accessory genome. The core genome of *P. aeruginosa* is defined as the genes that are present in nearly all strains of this bacterium and encodes a set of metabolic and pathogenic factors shared by all strains, irrespective of its origin (environmental, clinical, or laboratory). The core genome constitutes approximately 90% of the total genome and is highly conserved among strains. In contrast, accessory genome consists of DNA elements consisting of a few hundred bases to more than 200 kbp that are not conserved among *P. aeruginosa* strains (Mathee *et al*., 2008). These segments are not scattered randomly throughout the core genome; rather, they tend to cluster in certain loci and such loci are known as “regions of genomic plasticity (RGPs).” The genetic sequences occupying many RGPs are often referred to as genomic islands (>10 kb) or islets (<10 kb). Genetic elements within the accessory genome may encode properties that contribute to the niche-based adaptation of the particular strains that harbor those (Kung *et al*., 2010). The first complete genome sequencing was performed for strain PAO1 (NC_002516), which originated from an Australian wound isolate of *P. aeruginosa*. The PAO1 genome consists of a 6.264-Mbp circular chromosome encoding 5,570 predicted protein coding sequences. More than 500 regulatory genes were identified in the genome of strain PAO1 (Stover *et al*., 2000). The genomes of *P. aeruginosa* strains are larger than those of most sequenced bacteria. Within the species, the genome size varies between 5.5 and 7 Mbp (Schmidt *et al*., 1996). The second *P. aeruginosa* genome sequence was published for the ExoU-positive strain PA14 (NC_008463), a clinical isolate displaying higher virulence than PAO1 (Lee *et al*., 2006). LESB58, a so-called “Liverpool epidemic strain,” was another *P. aeruginosa* genome (NC_011770). The genome of this strain contains previously unknown accessory genome elements (Winstanley *et al*., 2009). PA7 (NC_009656) is a clinical isolate from Argentina with unusual antimicrobial resistance pattern and shares only 93.5% nucleotide
identity in the core genome with the other sequenced strains (Roy et al., 2010). As shown in the Fig. 2.1 below, the accessory genome of \textit{P. aeruginosa} consists of: (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)**

Burrus et al. (2002) coined the term ICE to describe self-transmissible genetic elements that must integrate into an existing replicon to accomplish replication. ICEs possess a combination of both plasmid and phage-associated DNA properties. They can exist as circular extrachromosomal elements like plasmids, transferred by self-mediated conjugation and become chromosomally integrated through RecA-dependent homologous recombination. They can also undergo phage integrase-mediated chromosomal integration which occurs via site-specific recombination between an ICE recombination site (\textit{attP}) and a recombination site on the bacterial chromosome (\textit{attB}). ICEs of \textit{P. aeruginosa} range in size from 81 to 108 kb and share a syntenic set of 72 ORFs with >75% sequence identity (Klockgether et al., 2004). Several \textit{P. aeruginosa} specific ICEs like PAGI-2, PAGI-3, PAGI-4, LESGI-1, LESGI-3, LESGI-5 and PAPI-1 have been identified and partially characterized (Larbig et al., 2002 and Klockgether et al., 2004).

**Replacement Islands**

Replacement Island is defined as the cluster of genes responsible for the synthesis of lipopolysaccharide (LPS) O antigen, pyoverdine, pili, flagella and post-translational modification of each of these macromolecules. These macromolecules are the surface exposed critical determinants of \textit{P. aeruginosa} that mediate fundamental processes such as bacterium-bacterium interaction, iron acquisition, adhesion and motility. The term replacement island was coined by Smith et al. (2005) to illustrate genetic loci similar to genomic islands which are responsible for O antigen biosynthesis, pyoverdine, pilin, and flagellar glycosylation. Each of these gene clusters contain horizontally acquired components and is highly divergent between different strains. There are a number of \textit{P. aeruginosa} specific replacement islands like 20 different \textit{P. aeruginosa} O antigen
serotypes such as O15 and O17 (Liu and Wang, 1990), three structurally different pyoverdine types (type I, II and III) (Meyer, 2000) siderovars (Meyer et al., 2002), five distinct groups of *P. aeruginosa* pilin genes (groups I to V) (Kus et al., 2004) and two different types (type a or b) of flagellin protein subunits (Schirm et al., 2004).

**Fig: 2.1 Genome of *P. aeruginosa***

The outermost circle indicates the chromosomal location in base pairs (each tick is 100 kb). The distribution of genes is depicted by coloured boxes according to functional category and direction of transcription (outer band is the plus strand; inner band is the minus strand). Red arrows indicate the locations and direction of transcription of ribosomal RNA genes; green arrow indicates the inverted region that resulted from a homologous recombination event between *rrnA* and *rrnB* genes; blue arrows represent location of two regions containing probable bacteriophages. The black plot in the centre is percentage G+C content plotted as the average for non-overlapping 1-kb windows spanning one strand for the entire *P. aeruginosa* genome. Yellow bars represent regions
of ≥ 3.0 kb with G+C content of two standard deviations (< 58.8%) below the mean (66.6%).

Adapted from: Stover et al., 2000

**Prophages and Phage-like elements**

Bacteriophages are the viruses that kill bacteria and composed of a single- or double-stranded DNA or RNA genome surrounded by a protein coat. It contains minimum essential genes encoding proteins that allow the phage to parasitize the bacterial replication machinery. Bacteriophages may be either virulent or temperate. Virulent bacteriophages lyses their bacterial host cells while temperate bacteriophages are lysogenic i.e. their genome is integrated into the bacterial host chromosome in a site-specific recombinase-dependent manner. After integration, it may develop mutations or undergo several recombination events with other prophages in the bacterial host chromosomes. Consequently, prophages may deteriorate and become permanently fixed in the chromosome and induce novel phenotypic properties, such as toxin production to their bacterial hosts. If these properties enhance fitness, lysogenized bacterial strains are at a competitive advantage compared to non-lysogenized strains. Under the influence of DNA damaging stimuli, necessary genes can be retained by the prophages and may be transferred from bacterial chromosome through horizontal gene transfer (Hertveldt et al., 2008). The study of *P. aeruginosa* phages has not only limited to epidemiological typing but these phages also contribute to inter-strain differences in virulence factors. There are at least 60 different temperate *P. aeruginosa* specific phages such as ϕCTX, D3, Pf1, Pf4, PAGI-6 and LES prophage 2 and many more. (Akhverdian et al., 1984). Majority of *P. aeruginosa* isolates are thought to be lysogenized by at least one phage (Holloway, 1955).

**Transposons, Insertion Sequences and Integrons**

Transposons (Tn) are defined as the larger transposable elements that generally encode functions in addition to transposition (Bennett, 2004). Transposable elements are genetic entities that mediate their own translocation from one site to another, usually unrelated site on the same or a different DNA molecule. They are ubiquitous in *P. aeruginosa* and
other bacteria (Bennett, 1999). All functional transposable elements contain a gene or a
group of genes encoding a transposase or transposase complex, which mediates
transposition by binding to short (15 to 40 bp) inverted repeat sequences at the borders of
the transposable element. Most \textit{P. aeruginosa} transposable elements can transpose into
many different sites on a DNA molecule (Peters and Craig, 2001). These elements
generally produce a staggered cut at their insertion site; inserted sequences then become
flanked by short direct repeats upon transposition.

Integrons (In) 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 (\textit{attI}) located downstream of the promoter, and (iii) a gene
encoding a tyrosine recombinase family integrase (Mazel, 2006). They are not mobile by
themselves rather, they achieve mobility only when linked to an existing mobile genetic
element (Recchia and Hall, 1995). Common mobile genetic element carriers on which
integrons can be found are conjugative plasmids and transposons. On the basis of
integrase sequences, at least five classes of integrons have been described (Mazel, 2006).
In \textit{P. aeruginosa}, majority of the integrons belong to class 1, in which antibiotic
resistance gene cassettes are particularly common. Other classes of integrons are quite
rare in \textit{P. aeruginosa} and there has been only one report of class 2 integrons found in \textit{P.
aeruginosa} from Southern China and no published reports of class 3, 4, or 5 integrons
(Xu et al., 2009). A number of transposons and integrons have been identified
specifically in \textit{P. aeruginosa} such as Tn6061 (Coyne et al., 2010), Tn1213 (Empel et al.,
2007), Tn4401b (Naas et al., 2008), Tn6001 (Tseng et al., 2009), In59.2 and In59.3
(Siarkou et al., 2009), In70.2 (Riccio et al., 2005), In113 (Sekiguchi et al., 2005), In120
(Poirel et al., 2005) and In163 (Carvalho et al., 2006).

\section*{2.5 Disease conditions caused by \textit{P. aeruginosa}}

\textit{Pseudomonas} spp. often associated with opportunistic infections and known to cause
disease in humans include: \textit{P. aeruginosa}, \textit{P. fluorescens}, \textit{P. putida}, \textit{P. cepacia}, \textit{P.
stutzeri}, \textit{P. maltophilia}, and \textit{P. putrefaciens}. Approximately 80 percent of pseudomonads
recovered from clinical specimens are identified as \textit{P. aeruginosa} and \textit{P. maltophilia}.
Among all the Pseudomonads, \textit{P. aeruginosa} has received the most attention because it is
the most frequently isolated pathogen from human clinical cases. It is the leading opportunistic pathogen causing nosocomial infections, including pneumonia (ventilator associated pneumonia), urinary tract infections and bacteremia (Elkhatib et al., 2008). Apaka et al. (2002) reported P. aeruginosa as the second most common isolate among patients in ICU. A study from India had shown 32% prevalence rate of P. aeruginosa in wound infections (Anupurba et al., 2006). 48.22% prevalence of P. aeruginosa in neonatal septicemia had been reported by Sharma et al., 2010. P. aeruginosa is responsible for 3-7% bloodstream infections and high mortality rates of 27-48% in critically ill patients (Navneeth et al., 2002). P. aeruginosa is the most common pathogen among diabetic foot infections (Ramakant et al., 2011, Sivanmaliappan et al., 2011, Bansal et al., 2008, Sharma et al., 2006, Shankar et al., 2006). About 80% of adults with cystic fibrosis lung infection due to P. aeruginosa resulting in increased morbidity and mortality (Ramakant et al., 2011). P. aeruginosa causes infections commonly at moist sites such as tracheostomies, indwelling catheters, burns, swimmer’s ear and weeping cutaneous wounds. It is a major threat to hospitalized individuals, particularly patients with impaired immunity such as cancer and burn cases. The high mortality rate associated with these infections is due to a combination of weakened host defenses, bacterial resistance to antibiotics and the production of extracellular bacterial enzymes and toxins. Wide ranges of clinical conditions caused by P. aeruginosa in humans as well as animals are discussed below:

Primary pneumonia is observed in patients with chronic lung disease and congestive heart failure. It may be acquired nosocomially in the intensive care unit (ICU) setting and is associated with positive-pressure ventilation and endotracheal tubes. Bacteremic pneumonia commonly occurs in neutropenic cancer patients undergoing chemotherapy and patients with AIDS. Symptoms of pneumonia may include fever, chills, severe dyspnea, cyanosis, productive cough, confusion, and other signs of a systemic inflammatory response (Cunha, 2006). Lower respiratory tract colonization of cystic fibrosis patients by mucoid strains of P. aeruginosa is common and difficult to eradicate. Such patients may experience chronic productive cough, anorexia, weight loss, wheezing, and tachypnea. (Ratjen et al., 2010).
Chapter 2

Review of Literature

Urinary tract infections (UTI) caused by *P. aeruginosa* are usually hospital-acquired and generally involves urinary tract catheterization, instrumentation or surgery (Todar, 2009). *P. aeruginosa* is the third leading cause of hospital-acquired UTIs. This bacterium adheres itself to the uroepithelium and invades the bloodstream from the urinary tract, and accounts for 40 percent of *Pseudomonas* bacteremias (Bitsori et al., 2012).

In the gastrointestinal (GI) tract *P. aeruginosa* can produce disease in any part from the oropharynx to the rectum, and implicated in a number of conditions such as perirectal infections, pediatric diarrhea, typical gastroenteritis, and necrotizing enterocolitis. Colonization of the GI tract is an important portal of entry for pseudomonal bacteremia and septicemia. The infection can cause enteritis, headache, fever (Shanghai fever), and diarrhea (Pollack et al., 2000).

*P. aeruginosa* can cause a variety of skin infections, both localized and diffuse. The main predisposing factor responsible for the pathogenesis is the breakdown of the integument which may result from burns, trauma or dermatitis. It does not grow on dry skin but flourishes itself on moist skin (Illgner et al., 2013). Green nail syndrome is a paronychial infection that can develop in individuals whose hands are frequently submerged in water. Jacuzzi syndrome is the pseudomonal skin infection associated with frequent use of a hot tub and patients may present with pruritic follicular, maculopapular, vesicular, or pustular lesions on any part of the body that was immersed in water (Yu et al., 2007).

*Pseudomonas* keratitis, is the infection of eye which involves the colonization of ocular epithelium by this organism by means of a fimbrial attachment to sialic acid receptors and through where it can proliferate rapidly and can cause a serious disease known as endophthalmitis that can lead to permanent loss of vision (Abuqaddom et al., 2003).

*P. aeruginosa* infects heart valves of intravenous drug users and may infect prosthetic heart valves. The organism establishes itself on the endocardium by direct invasion from the blood stream. Left-sided endocarditis typically presents with symptoms of congestive heart failure (Gavin et al., 2003).

*P. aeruginosa* causes bacteremia primarily in immunocompromised patients. Predisposing conditions include, hematologic malignancies, immunodeficiency relating to AIDS, neutropenia, diabetes mellitus, and severe burns. Most *Pseudomonas* bacteremia is acquired in hospitals and nursing

*P. aeruginosa* is also one of the causes of meningitis and brain abscesses. The organism invades the CNS from a contiguous structure such as the inner ear or paranasal sinus, or is inoculated directly by means of head trauma, surgery or invasive diagnostic procedures, or spreads from a distant site of infection such as the urinary tract and patient may experience fever, headache and confusion (Taneja *et al.*, 2009).

*Pseudomonas* infections of bones and joints result from direct inoculation of the bacteria or the hematogenous spread of the bacteria from other primary sites of infection. *P. aeruginosa* has a particular tropism for fibrocartilagenous joints of the axial skeleton and causes chronic contiguous osteomyelitis and septic arthritis (Finkelstein *et al.*, 1989).

### 2.6 Virulence factors of *P. aeruginosa*

*P. aeruginosa* possesses a variety of virulence factors. These factors may be cell-associated as well as extracellular products. The virulence factors play an important role in the colonization, survival, invasion and spread of this bacterium to different organs. The pathogenesis of *P. aeruginosa* infections is multifactorial because it has wide array of virulence determinants (Todar, 2009). The powerful exotoxins and endotoxins released by *P. aeruginosa* during bacteremia continue to infect the host even after this bacterium has been killed by antibiotics (Irvin and Randall, 1993). This bacterium adheres itself to the tissue surfaces and replicates in the host tissue to increase bacterial load and finally damages the tissue using its virulence factors. Various virulence factors that are involved in the pathogenesis of *P. aeruginosa* infections are discussed below:

#### 2.6.1 Adhesion factors

**Flagella**

*P. aeruginosa* is a motile organism due to presence of one or more polar flagella at one end. Three types of motility of *P. aeruginosa*: swimming, swarming and twitching motility are observed. Outside a host, the type of motility depends upon agar concentration: it performs swimming motility on aqueous surfaces containing 0.3% agar, swarming motility on semi-solid surfaces containing 0.5% agar and twitching motility on
solid surfaces containing 1.0% agar (Overhage et al., 2007; Rashid and Kornberg, 2000). Apart from motility, flagellum of *P. aeruginosa* also plays an important role in biofilm dispersal and adhesion to the surface of host cells (Veessenmeyer et al., 2009). Flagellum is basically composed of flagellin protein encoded by *fliC* gene. It is recognized by toll-like receptor 5 (TLR-5) present on the surface of host epithelial cells. TLR-5 is used as a surveillance mechanism by host cell to detect invading *P. aeruginosa* and in turn trigger the immune response by inducing the synthesis of cytokines such as TNF, IL-6 and IL-8 (Zhang et al., 2003). Flagellum is used as a virulence factor only in the pathogenesis of acute infection. *P. aeruginosa* down regulates the expression of flagellin over the course of chronic infection especially in cystic fibrosis patients to evade the host immune response (Palmer et al., 2005). About 40% isolates of *P. aeruginosa* from cystic fibrosis patients do not produce flagella, although, this virulence factor is thought to be necessary for the initial infection of these patients (Mahenthiralingam et al., 1994).

**Type IV Pili**

Type IV pili (T4P) are very strong flexible surface appendages measuring up to 4 µm in length and 50-80Å in diameter. They are composed of helical polymer of a single protein, *PilA*. T4P are polar and mediate adhesive properties to *P. aeruginosa* (Hahn, 1997). During *P. aeruginosa* infection, they are involved in many processes such as adhesion to biotic and abiotic surfaces, bacteriophage adsorption, twitching motility, initiation and development of biofilms and DNA uptake (Watson et al., 1996). Twitching motility is a unique form of surface-associated movement in the absence of flagella. In this type of motility, *P. aeruginosa* pull them rapidly towards or along a surface by retracting their T4P. Hence, adhesion and twitching motility play important roles in pathogenesis of *P. aeruginosa* infections (Burrows, 2012). *P. aeruginosa* type IV pili promote infection of cystic fibrosis and immunocompromised patients. The absence of T4P impairs colonization and makes the bacteria avirulent. Hence, T4P are striking targets for the design of novel therapeutics (Tammam et al., 2013).

**Alginate capsule**

*P. aeruginosa* is a noncapsulated organism but some strains appear mucoid due to production of extracellular polysaccharide known as alginate. It is a simple, unbranched
polysaccharide composed of two uronic acids: β-d-mannuronic acid and its C5 epimer α-l-guluronic acid (Jain and Ohman, 2005). It forms a prominent capsule on the bacterial surface and protects this organism from phagocytosis and antibiotic killing. The genes controlling production of alginate polysaccharide can be activated in patients, such as those with cystic fibrosis or other chronic respiratory disease (Murray et al., 2012). About 80% of the *P. aeruginosa* isolates recovered from cystic fibrosis patients undergo mucoid conversion in vivo, whereas only about 1% of clinical *P. aeruginosa* isolates from other types of infections produce alginate (Doggett et al., 1966). Thus, alginate strongly influences the ability of mucoid strains to persist and establish chronic infections in the cystic fibrosis lung. Alginate is anionic in nature and it acts as a polyanionic barrier which binds the cationic peptide antibiotics and prevents them to enter into the bacterial cell (Pedersen, 1992). Alginate is also required for biofilm formation. However, it has been observed that alginate is not the major component of the biofilm matrix formed by non-mucoid *P. aeruginosa* strains such as PAO1 and PA14 (Wozniak et al., 2003). *AlgD* mutant (defective gene in alginate biosynthesis) has been shown to play a role in initial biofilm development (Nivens et al., 2001).

### 2.6.2 Invasins

#### Proteases

Proteases are the hydrolytic enzymes which can degrade peptide bonds and can therefore, degrade proteins and peptides. These enzymes can be divided into six different catalytic types based on their active residues involved in the catalysis such as aspartic-proteases, metallo-proteases, serine-proteases, cysteine-proteases, threonine-proteases and glumatic-proteases. (Barrett et al., 2004). Elastase is one of the major virulence factors of *P. aeruginosa* regulated by the quorum sensing cascade (Williams and Camara, 2009). Elastase B (LasB) is a metalloprotease encoded by *lasB* gene (Thayer et al., 1991). It has been shown to degrade human and bovine elastin (protein forming a biopolymer in organs and tissues of vertebrates that gives them elastic properties) and collagen (a protein found in different human tissues) which in turn disrupt the integrity of the host basement membrane (Saulnier et al., 1989; Hamdaoui et al., 1987). It is known to
perform various functions in the pathogenesis of different acute as well as chronic infections caused by *P. aeruginosa* such as inhibition of fibroblast growth (Schmidtchen *et al*., 2003), degradation of immunoglobulin A (IgA) (Heck *et al*., 1990), immunoglobulin G (IgG), prevention of opsonophagocytosis (Bainbridge and Fick, 1989) and inactivation of key components of complement system such as C1, C3, C5, C8 and C9 (Schultz and Miller, 1974).

Elastase A (LasA) is a zinc metalloprotease encoded by *lasA* gene (Kessler *et al*., 1997). It is also known as staphylolysin and is one among the four most abundant secreted endopeptidases by *P. aeruginosa* (Ohman *et al*., 1980). This enzyme cleaves glycine containing proteins such as tropoelastin-derived pentapeptides, glycine-rich synthetic peptides and specific sequences present in elastin (Kessler *et al*., 1997). LasA also enhances the virulence activity of LasB and several other host elastolytic proteases, including human leukocyte elastase, human neutrophil elastase and other proteases (Peters and Galloway, 1990). It also possesses staphylolytic activity in addition to elastolytic activity (Kessler *et al*., 1993). The staphylolytic activity causes rapid lysis of *Staphylococcus aureus* cells by cleaving the pentaglycine bridges of their cell wall peptidoglycan. The staphylolytic activity of *P. aeruginosa* is purely LasA dependent and may represent a defense strategy to outcompete *S. aureus* during colonization of the cystic fibrosis lung (Smith *et al*., 2000).

Protease IV is a serine protease encoded by *piv* gene. It is also known as lysyl endopeptidase (Elliott and Cohen, 1986). This enzyme is known to cleave bovine fibrinogen (a large biopolymer and part of the blood clotting system) which results in hemorrhage (Walsh and Ahmad, 2002). It also degrades other host proteins such as plasminogen, immunoglobulin G (IgG) and the complement components C3 and C1q (Engel *et al*., 1998).

Alkaline protease (AprA) is another zinc-dependent metalloendopeptidase secreted by *P. aeruginosa* (Guzzo *et al*., 1990) which is encoded by *aprA* gene (Duong *et al*., 1992). It is also known as aeruginolysin. AprA has been shown to cleave a large number of physiological substrates such as laminin (an important and biologically active part of the basal lamina) (Matsumoto, 2004) and directly involved in invasion and hemorrhagic tissue necrosis in infections caused by *P. aeruginosa* (Heck *et al*., 1986). Both AprA and
LasB of *P. aeruginosa* inactivate human γ-interferon, human tumor necrosis factor-α (Horvat and Parmely, 1988), interleukin-6 (IL-6) (Matheson *et al.*, 2006), neutrophils (Kharazmi *et al.*, 1984) and natural killer (NK) cells (Pedersen and Kharazmi, 1987) and hence, impairs the host immune response.

**Hemolysins**

*P. aeruginosa* produces two types of hemolysins. One is phospholipase C, which is a heat labile hemolysin (Vasil *et al.*, 1982). This toxin is encoded by *plcH* gene (Kida *et al.*, 2011). It catalyzes the hydrolysis of phosphatidylcholine to phosphorylcholine and diacylglycerol. Phospholipid surfactants reduce the surface tension of the alveolar wall so that the alveoli do not collapse completely when air leaves them during breathing. Phospholipase C degrades the phospholipid surfactants and hence plays a vital role in the pathogenesis of pulmonary infections caused by *P. aeruginosa*. It also appears to be necrotic or cytolytic toxin like the phospholipase C (alpha toxin) of *Clostridium perfringens* (Songer, 1997).

Another type of hemolysin produced by *P. aeruginosa* is rhamnolipid, which is a heat stable hemolysin (Vasil *et al.*, 1982). Rhamnolipids are the glycolipid-type biosurfactants composed of one or two rhamnose sugar subunits (Chen *et al.*, 2007). They can disrupt the respiratory epithelium and promote the invasion of rhamnolipid-deficient strains of *P. aeruginosa* (Zulianello *et al.*, 2006). In *P. aeruginosa*, rhamnolipids are involved in swarming motility and biofilm formation (Boles *et al.*, 2005). Both hemolysins are produced during stationary phase in low-phosphate media (Gray *et al.*, 1982) and act synergistically with alkaline phosphatase to liberate inorganic phosphates (P$_i$) from the phospholipids present in the eukaryotic cells.

**Siderophores and siderophore uptake systems**

Siderophores are the iron chelators which can bind iron efficiently and then taken up by the bacteria through specific cell surface receptors. Iron is essential component for the growth of all bacterial species (Tsuda *et al.*, 1995). In the host tissue, iron is tightly bound to transferrin or lactoferrin (iron transport proteins) and is not freely available to acquire by pathogen (Xiao and Kisaalita, 1997). *P. aeruginosa* compete with transferrin and
lactoferrin for iron by developing a complex regulatory system known as siderophore uptake system (Takase et al., 2000). Siderophores are the important virulence factors not only for providing iron to support bacterial metabolic processes but also for controlling the expression of other P. aeruginosa virulence factors, such as exotoxin A (ExoA), endoprotease, and pyoverdin (Lamont et al., 2002). P. aeruginosa produce two types of siderophores i.e. pyochelin and pyoverdin to accumulate iron (Poole and McKay, 2003).

Pyoverdin is the main siderophore of P. aeruginosa whose production is mainly regulated by the pvd operon (Cunliffe et al., 1995). It consists of a dihydroxyquinoline fluorescence chromophore to which an acyl side-chain and a peptide chain are attached. It can obtain iron from the host’s transferrin and lactoferrin (Xiao and Kisaalita, 1997) and pyoverdine-dependent iron transport is also essential for biofilm development (Visca et al., 2007).

Pyochelin is the second important siderophore produced by P. aeruginosa. It is a low molecular weight thiazoline derivative [2(2-o-hydroxy-phenyl-2-thiazolin-4-yl)-3-methylthiazolidine-4-carboxylic acid] (Cox et al., 1981). Pyochelin synthesis is regulated by two operons pchDCBA and pchEFGHI (Poole and McKay, 2003). This siderophore plays a minor role in the pathogenicity of P. aeruginosa because it has low affinity to Fe\(^{3+}\) (Meyer et al., 1996). Moreover, pyochelin is capable of chelating other transition metals and is responsible for the uptake of Co\(^{2+}\) and Mo\(^{4+}\) into P. aeruginosa cells (Visca et al., 1992).

**Pyocyanin diffusible pigment**

Most strains of P. aeruginosa secrete pyocyanin (N-methyl-1-hydroxyphenazine). It is a diffusible pigment that gives blue green coloration to the bacterial colonies (Sadikot et al., 2005). Pyocyanin is a redox-active compound that is unique for P. aeruginosa and is permeable to all biological membranes (Lau et al., 2004). A high concentration of pyocyanin has been detected in pulmonary secretions of patients with cystic fibrosis. This pigment disrupts the bronchial epithelium, impairs ciliary function and exerts a proinflammatory effect. It interferes with the antioxidant defenses in the lung and facilitates oxidative damage to the lung epithelium through inhibition of catalase activity (O'Malley et al., 2003).
2.6.3 Toxins

Type III secretion system

*P. aeruginosa* possesses a type III secretion system that allows this bacterium to inject its toxins into the host cell. It is a major virulence and cytotoxicity determinant of *P. aeruginosa* (Holder *et al.*, 2001). This system consists of three components: the secretion apparatus, the translocation or targeting apparatus and the secreted toxins (effector proteins) (Gauthier *et al.*, 2003). *P. aeruginosa* secretes four known effector proteins via type III secretion system: ExoS, ExoT, ExoU, and ExoY (Frank, 1997). PcrV, PopB and PopD are three other proteins secreted by type III secretion system in addition to these effector proteins, as described in Fig. 2.2. These proteins are thought to make channel through which the effector proteins are injected into the host cells (Vallis *et al.*, 1999).

Exotoxins

Exotoxin T (ExoT) and exotoxin S (ExoS) are the most potent cytotoxins secreted by *P. aeruginosa*. They are 76% identical and encode an N-terminal GTPase-activating protein (GAP) domain and a C-terminal adenosine diphosphate (ADP)-riboyltransferase (ADPRT) domain (Balachandran *et al.*, 2007). Both the cytotoxins work together to modulate many processes: disruption of actin cytoskeletal rearrangement, focal adhesions, inhibition of wound repair by affecting cell migration and cell proliferation, disruption of signal transduction cascades particularly those that regulate the innate immune response and modulation of bacterial internalization by epithelial cells (Barbieri and Sun, 2004; Kipnis *et al.*, 2006). Exotoxin Y (ExoY) and Exotoxin U (ExoU) are two another exotoxins secreted by type III secretion system of *P. aeruginosa*. ExoY has adenylate cyclase activity while ExoU shows phospholipase type of activity. ExoU has been shown to cause irreversible damage to cellular membranes and rapid necrotic death (Sato and Frank, 2004).
Fig. 2.2 Structure of the type three secretion system injectisome in *P. aeruginosa*

Adapted from: Mota, 2006

Type III Secretion System injectisome is composed of a basal body, which is usually topped by a needle-like structure that protrudes from the bacterial surface. The needle is thought to function as the channel for protein translocation. *P. aeruginosa* delivers four toxins known as ExoS, ExoT, ExoY and ExoU along the injectisome directly into the host cell. Delivery of these toxins also requires a set of type III secreted proteins called translocators (PopB and PopD). These proteins assemble into a translocation pore in the target-cell membrane. PcrV at the needle tip is required for the membrane insertion of PopD.
Chapter 2

Review of Literature

Exotoxin A (ExoA) is a major virulence factor of *P. aeruginosa* (Morlon-Guyot *et al.*, 2009). It has exactly the same mechanism of action as the diphtheria toxin (Todar, 2009). It enters the eukaryotic cell by receptor-mediated endocytosis and undergoes a proteolytic cleavage in the endosome. After that, it reaches the cytosol and catalyzes the ADP-ribosylation of eukaryotic elongation factor-2 (EF-2) which is required for protein synthesis (Zdanovsky *et al.*, 1993). Thus, it blocks the protein synthesis of host cell which ultimately leads to cell death (Yates and Merrill, 2001). ExoA appears to mediate both local and systemic disease processes caused by *P. aeruginosa*. This toxin has necrotizing activity at the site of bacterial colonization thereby contribute to colonization process of this bacterium. It also facilitates the dissemination of infection caused by this bacterium. Increased titers of anti–exotoxin A antibodies in serum from patients with *P. aeruginosa* sepsis have been shown to be associated with better survival in some human studies (Kurahashi *et al.*, 2004).

Endotoxin (Lipopolysaccharide)

Lipopolysaccharide (LPS) is an important component of *P. aeruginosa* and other gram negative bacteria. It is basically composed of three parts: O side chain, core oligosaccharide and lipid A. Environmental isolates of *P. aeruginosa* typically express smooth LPS with long O-side chains but rough strains (unable to produce O-side chains) predominate in the lungs of chronically infected cystic fibrosis patients (Sadikot *et al.*, 2005). Rough LPS harbored isolates of *P. aeruginosa* can also be obtained from patients with pneumonia (Hirakata *et al.*, 2000). Apart from this, LPS also plays a key role in the inhibition of phagocytosis, adhesion to cell surfaces as well as inanimate objects like glass and polystyrene (Makin and Beveridge, 1996).

2.6.4 Biofilm formation in *P. aeruginosa*

Biofilm is a thin but robust layer of extracellular polymeric substances (EPS) which adheres to a solid surface and contains a community of bacteria and other microorganisms (Hoiby *et al.*, 2001). It represents a protective mode of growth that allows microorganisms to survive under unfavorable environments and disperse seeding cells to colonize new niches in desirable conditions. The biofilm is responsible for many
persistent and chronic infections due to their inherent resistance to antimicrobial agents. The biofilm matrix consists of bacterial cells, secreted cell products, proteins, polysaccharides, DNA, and water (Sutherland, 2001). The formation of biofilm includes five steps: (i) attachment of bacterial cells (ii) irreversible attachment and production of exopolysaccharides (iii) early development of biofilms (iv) maturation of biofilms and (v) dispersal of biofilms (Fig 2.3). Swimming bacteria attach to an abiotic surfaces and aggregate to form microcolonies. As a result, they lose the flagella and produce the extracellular polymeric substances (EPS). In mature biofilms, mushroom like structures are separated by water-filled channels. Finally, the biofilm is dispersed by death of subpopulations of cells and detachment of planktonic bacterial cells from the biofilm.

![Fig 2.3 Stages of the biofilm development in P. aeruginosa](adapted from: McDougald et al., 2008)

The biofilm formation in *P. aeruginosa* depends on many factors such as quorum sensing (QS) (Diggle et al., 2006), iron concentration (Bollinger et al., 2001) and nutritional conditions (Sauer et al., 2004). This organism has been shown to form biofilms on different surfaces such as lungs of patients suffering from cystic fibrosis (Costerton et al., 1999), contact lenses (Sankaridurg et al., 2000), urinary catheters and prosthetic heart valves (Donlan and Costerton, 2002) and plants (Attila et al., 2008; Walker et al., 2004). The formation of biofilms makes conditions more favorable for bacterial persistence in the lungs. Bacteria in biofilms are inherently more difficult to eradicate than those in the planktonic form (Lesprit et al., 2003). The polysaccharides of biofilm matrix of *P. aeruginosa* (PAO1 strain) is encoded by *psl* gene cluster and consists of glucose,
ketodeoxyoctulosonate, mannose, rhamnose, xylose, N-acetyl fucosamine, N-acetyl galactosamine, N-acetyl glucosamine and other unknown sugars (Wozniak et al., 2003). Mannose is one of the major polysaccharide component required for biofilm formation because it plays a vital role in biofilm initiation (Ma et al., 2006). Biofilm formation is one of the important virulence factors of *P. aeruginosa* because it protects this bacterium against innate immune response and restricts the diffusion of antimicrobial compounds into the cell (Kokare et al., 2009).

### 2.6.5 Quorum sensing in *P. aeruginosa*

Quorum sensing (QS) is defined as a mechanism by which bacterial cells are able to communicate with each other. It is also known as intercellular signaling or cell to cell signaling (Joint et al., 2007). This communication mechanism is mediated by the production of self generated diffusible signal molecules known as autoinducers or pheromones. QS permits bacteria to monitor their population density by sensing extracellular concentration of autoinducers (Lazdunski et al., 2004). The concentration of the autoinducers increases along with bacterial cell density and once a certain concentration threshold is reached, the autoinducers bind to receptors (regulators) and as a result of which expression of the target genes is induced or repressed (Keller and Surette, 2006). *P. aeruginosa* possesses three QS systems: the *las* system with signal molecule N-3-oxo-dodycanoyl homoserine lactone (3-oxo-C12-HSL), the *rhl* system with signal molecule N-butanoyl-homoserine lactone (C4-HSL) and the *Pseudomonas* quinolone system (PQS) with signal molecule 2-heptyl-3-hydroxy-4-quinolone (Fuqua and Greenberg, 2002; Latifi et al., 1996). These three QS systems are intertwined and regulate the production of various secreted virulence factors such as proteases, elastases, rhamnolipids, pyocyanin and biofilm formation by altering the genes responsible for the production of these virulence traits (de Kievit, 2009). Furanone, indole, 7-hydroxy indole and 5-fluorouracil are some quorum sensing inhibitors which interfere with the functioning of signaling molecules (Cegelski et al., 2008).

### 2.7 Drug Resistance

Drug resistance is defined as the ability of a pathogen to resist the action of an antimicrobial agent. A pathogen continues to grow normally and multiply in the presence
of a drug to which it is resistant and spread with the same rate to different sites. The drug resistance is thus, reduction in the effectiveness of a particular drug to a pathogen. Microbes adapt to their surroundings and continue to evolve over time and can change their genetic makeup through mutations and acquire traits that are fit for survival under harsh environments. There are mainly four mechanisms by which gram negative bacteria exhibit resistance to various antibiotics: i. efflux of the antibiotics from bacterial cell ii. alteration in the permeability of outer membrane which leads to impermeability of antibiotics across the cell surface iii. enzymatic modification of the target antibiotic which either degrade the antibiotics (β-lactamase production) or chemically transform the antibiotic (macrolide and aminoglycoside modifying proteins) that renders the antibiotic ineffective and (iv) alterations in the target site of the antibiotics which reduce the binding capacity of antibiotics to their respective targets (Schmieder and Edwards, 2012).

The genes encoding these resistance mechanisms may be inherent part of the bacterial genome or it may develop by mutating the existing genes i.e. vertical evolution (Martinez and Baquero, 2000) or it may be acquired from other strains through horizontal gene transfer (Palmer et al., 2010). Certain elements mediate transfer of resistance genes between bacteria by horizontal gene transfer such as integrative and conjugative elements (ICEs), replacement islands, bacteriophages, plasmids, transposons, insertion sequences and integrons. The presence of low levels of the antibiotic in the environment is the key signal that promotes gene transfer (Jeters et al., 2009). The horizontal gene transfer takes place through conjugation, transformation, and transduction. The gene can be transferred between different strains of the same species or even between different bacterial species.
Fig. 2.4 Mechanisms of transfer of resistance genes through Horizontal gene transfer adapted from: Furuya and Lowy, 2006

2.7.1 Multidrug Resistant *P. aeruginosa* (MDRPA)

The primary mechanisms involved in resistance of *P. aeruginosa* to different antibiotics include: reduced cell permeability, use of efflux pumps, alteration in the target site for antibiotics and inactivation of the antibiotics (Lambert, 2002; Matsuo et al., 2004). Resistance is associated with adverse clinical outcomes (Carmeli et al., 1999). *P. aeruginosa* may have a multidrug resistant (MDR) phenotype which may possess one or more than one mechanisms of drug resistance. The MDR strains emerge with high frequency due to selective pressure of antimicrobial therapy. The selection of the resistant mutants and their subsequent spread in the community is confirmed through genetic analysis. With the widespread use of third-generation cephalosporins or carbapenems against multidrug resistant *P. aeruginosa* (MDRPA), the resistance to multiple antibiotics may develop (Wroblewska, 2006). In majority of the published reports, MDR phenotype has been defined as a bacterial phenotype which possesses resistance to at least three classes of antibiotics, such as aminoglycosides, penicillins, cephalosporins, carbapenems and fluoroquinolones (Hirsch and Tam, 2010). Prolonged hospitalization, immune status of the patient, prior exposure to *P. aeruginosa* infections and inappropriate use of anti-
Pseudomonas antibiotics specifically fluoroquinolones and carbapenems, are some of the risk factors for MDRPA.

MDRPA is a leading public health problem that affects many countries of the world resulting in high morbidity and mortality rates. It also increases length of hospital stay and elevates hospital costs. Resistance patterns of MDRPA have been increased from 10% during the period 1986 to 2004 in the United States (National Nosocomial Infection Surveillance System, 2004). Similar patterns of increased resistance to different antibiotics have been recorded world over. For example, Europe, Japan, North America and Latin America have reported a 10% or more increase in antibiotic resistance over the last decade, with some areas reaching 50% resistance to common antibacterial agents (Sader et al., 2004). This shows resistance to antibiotics is increasing at an alarming pace, therefore, the knowledge of frequency and susceptibility pattern of the MDRPA is very important for clinicians for better management of the patients.

2.8 Beta-lactam antibiotics and their mechanism of action

Beta-lactams belong to the family of antimicrobial agents that contain a β-lactam ring in their molecular structures (Fig 2.5). This ring is essential for their activity. This group of antibiotics includes four major classes: penicillins, cephalosporins, monobactum, and carbapenems (Kotra et al, 2002) which are commonly used for treating P. aeruginosa infections (Paul et al., 2010). Piperacillin and ticarcillin (penicillins), ceftazidime (3rd generation cephalosporin), cefepime (4th generation cephalosporin), aztreonam (monobactam), imipenem, meropenem, ertapenem and doripenem (carbapenems) are some of the most active β-lactams against P. aeruginosa. The mechanism of action of these antibiotics includes inhibition of bacterial cell wall synthesis. As illustrated in Fig 2.6, these antibiotics bind to penicillin-binding proteins (PBPs) that are located in the bacterial cell walls and inactivate them. Consequently, the transpeptidation reaction is ceased and ultimately leading to the inhibition of synthesis of peptidoglycan. (Tipper, 1985).
Fig 2.5 The arrangement of β-lactam rings in different classes of β-lactam antibiotics and sites of action of β-lactamases
The antibiotics bind to the penicillin binding protein located on the cell walls and inactivate them inhibiting thereby the synthesis of cell wall

2.8.1 The enzymes, β-lactamases and their production by *P. aeruginosa*

Resistance to β-lactam antibiotics is multi-factorial but is mediated mainly by inactivating enzymes called β-lactamases (Fig 2.5). These enzymes cleave the amide (CO-NH) bond of the β-lactam ring leading to inactivation of the antibiotic. Based upon their amino acid sequences, four classes (A, B, C and D) of these enzymes have been identified (Ambler, 1980). These fall into four groups I, II, III and IV on the basis of substrate (e.g. imipenem) and inhibitor (e.g. clavulanic acid) activity (Bush *et al.*, 1995). There are more than 1000 β-lactamases exist in gram-negative bacteria (Marsik and Nambiar, 2011). The genes encoding β-lactamases (*bla* genes) can be located on the bacterial chromosome, on plasmids, or transposons. Some *bla* genes have been found on plasmids and part of transmissible genetic elements called integrons (Boucher *et al.*, 2007). 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. These integrons are responsible for transfer of bla genes among divergent species of gram-negative bacteria. An increasing number of MBL genes (IMP-type or VIM-type) are mobilized by integrons and transposons (Weldhagen, 2004). Mobile genetic elements that contain integrons are important source for spread of bla genes and for the dissemination of other determinants. Integrons are not mobile but their location in mobile genetic elements (plasmids, transposons) enables their movement (Bennett, 1999). These integrons could be carried by large plasmids or be located on the chromosome (Walsh et al., 2005, Pournaras et al., 2002). Many multidrug-resistant bacteria produce multiple β-lactamases including combinations of ESBLs and carbapenemases. Newly acquired enzymes are emerging resistance mechanism against beta-lactam antibiotics in *P. aeruginosa*. These include: extended spectrum beta-lactamases (ESBLs), ambler class C beta-lactamases (AmpC) and metallo beta-lactamases (MBLs).

### 2.8.1.1 Extended spectrum beta-lactamases (ESBLs)

Extended-spectrum beta-lactamases (ESBLs) are rapidly evolving group of β-lactamase enzymes that contribute to resistance against beta-lactam antibiotics. These enzymes have the ability to hydrolyze and cause resistance to extended spectrum cephalosporins such as oxyiminocephalosporins (cefotaxime, ceftazidime, ceftriaxone, cefuroxime and cefepime) and monobactams (aztreonam), but not the cephamycins (cefoxitin and cefotetan) or carbapenems (imipenem, meropenem and ertapenem). ESBLs belong to class A of Ambler classification system that includes enzymes acted by serine based mechanism and inhibits by clavulanic acid (Ambler, 1980). This enzyme was first detected in 1979 in the family *Enterobacteriaceae* (Sanders and Sanders, 1979). ESBLs have been reported worldwide in many different genera of *Enterobacteriaceae* and also increasingly found in *P. aeruginosa* (Friedman et al., 2005). However, these are most commonly found in *Klebsiella pneumoniae* and *Escherichia coli* (Agrawal et al. 2008). ESBL producing organisms are often resistant to several other classes of antibiotics because the genetic elements carrying gene encoding ESBLs often carry other resistance
determinants. Horizontal gene transfer is considered to be responsible for the high frequency transmission of ESBL genes among different genera including *P. aeruginosa* (Jiang *et al.*, 2006).

### 2.8.1.2 Metallo beta-lactamases (MBLs)

MBLs constitute molecularly diverse group of broad spectrum beta-lactamases. A metallo β-lactamase enzyme was first reported in *Bacillus cereus* in mid-1960’s (Sabath *et al.*, 1966). MBLs belong to class B of Ambler classification system. It can be further subdivided into three subclasses 'B1', 'B2' and 'B3' (Hall *et al.*, 2004). These enzymes require divalent cations of zinc (Zn$^{2+}$) as co-factors for enzymatic activity and are universally inhibited by ethylenediamine tetra-acetic acid (EDTA) and other chelating agents of divalent cations (Sharma *et al.*, 2010). MBLs have the ability to hydrolyze not only carbapenems but also other beta-lactam antibiotics. They are widespread in *P. aeruginosa*, *Acinetobacter* spp. and more recently detected in the members of *Enterobacteriaceae* (Walsh, 2008). The most effective antimicrobial agents used for the treatment of MDR *P. aeruginosa* infections are carbapenems, mainly imipenem, meropenem and panipenem. However, the prevalence of carbapenem resistant *P. aeruginosa* has been on the rise very recently and such strains have been implicated as important cause of nosocomial infections associated with clonal spread.

#### Epidemiology of Metallo beta-lactamase (MBL) enzyme

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). In some countries, MBLs possessing strains of *P. aeruginosa* constitute nearly 20% of all nosocomial isolates (Walsh *et al.*, 2005) while Behera *et al.* (2008) from India reported prevalence of MBLs in the range of 7-65%. Castanheira *et al.* (2009) reported 34% occurrence. Several outbreaks of infections due to MBL producing strains of *P. aeruginosa* had been reported from different 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 et al., 2005). In a study from Mumbai (India), prevalence of MBL producing *P. aeruginosa* were recovered from intensive care areas to a tune of 33.33% where overall mortality rates in MBL positive cases were as high as 46.15% (De et al., 2010). In some studies from India 8.05%, 20.8, 20%, and 42.6% strains of *P. aeruginosa* were found to be MBL producers (Agrawal et al., 2008, Varaiya et al., 2008, Singh et al., 2009, Manoharan et al., 2010). Sharma et al. (2010) reported 69.5% isolates of *P. aeruginosa* as MBL producers in Haryana. A very high percentage of 96.97% isolates of *P. aeruginosa* were found to be positive for MBL production in Chandigarh (Gupta et al., 2008). Various types of MBLs have been reported in *P. aeruginosa* and other gram negative bacilli such as Imipenemase (IMP), Verona imipenemase (VIM), German imipenemase (GIM), Sao Paulo metallo beta-lactamase (SPM), Seoul imipenemase (SIM), New Delhi metallo beta-lactamase (NDM), Adelaide imipenemase (AIM), Florence imipenemase (FIM).

**Types of MBL genes**

**Imipenemase (IMP)**

IMP type beta lactamases were among the first acquired MBLs identified and detected in *Pseudomonas* spp., *Acinetobacter* spp., and *Enterobacteriaceae* (Zhao et al., 2011). This enzyme was first reported in *P. aeruginosa* strain GN17203 in Japan in 1988 as a consequence of heavy carbapenem usage. This strain showed resistance to imipenem with minimum inhibitory concentration (MIC) of 50μg/ml. The resistance to extended-spectrum cephalosporins such as ceftazidime, the MIC of >400 μg/ml was also observed (Watanabe et al., 1991). Genes encoding IMP type MBL (*blaIMP*) are usually found as gene cassettes in class 1 integrons that could be readily mobilized to other *Pseudomonas* strains. These genes have also been found on class 3 integrons (Walsh et al., 2005). Among *Enterobacteriaceae*, *blaIMP-1* was found in *Serratia marcescens* isolate in Japan in 1991 (Ito et al., 1995). A new IMP variant, *blaIMP-33* has been detected in *P. aeruginosa* from Italy (Deshpande et al., 2013). *blaIMP-35*, a phylogenetically distinct IMP-type metallo β-lactamase has been detected in *P. aeruginosa* as a gene cassette in the class-I integron from the Dutch–German border region (Pournaras et al., 2013). So far, 48 IMP
variants have been discovered and IMP type MBL producers have spread worldwide (Nordmann and Poirel, 2014).

**Verona imipenemase (VIM)**

VIM type beta-lactamase is the second dominant group of MBL which was first reported from Verona in Italy from a *P. aeruginosa* isolate in 1997 (Lauretti *et al*., 1999). This isolate was resistant to piperacillin, ceftazidime, imipenem and aztreonam, with MIC of imipenem >128μg/ml. The *bla*\textsubscript{VIM-1} gene was integrated as a gene cassette into a class-1 integron located on the chromosome of *P. aeruginosa* (Arakawa *et al*., 1995). Among several types of MBL enzymes identified, VIM type enzymes appears to be most prevalent. The *bla*\textsubscript{VIM-2} gene was first identified in southern France from *P. aeruginosa* isolate recovered from blood culture of neutropenic patients. Similarly, VIM-2 producing *P. aeruginosa* was isolated from Italy and Greece and is the most common MBL identified worldwide (Navaneeth *et al*., 2002). VIM-2 has got 90% amino acid identity with VIM-1. Some novel variants of the VIM series, VIM-3 and VIM-4 has also been identified in Taiwan (Yan *et al*., 2001) and Greece (Pourmaras *et al*., 2002). VIM-2 producing MDRPA isolates was recently recovered from a hospital in Spain (Viedma *et al*., 2012). A total of 11 variants of VIM have been identified so far (Sedighi *et al*., 2015). Liakopoulos *et al*. (2013) from Greece revealed 14.1% isolates of *P. aeruginosa* as VIM producers. Recently, *bla*\textsubscript{VIM} gene was reported in 33% strains of *P. aeruginosa* recovered from different hospitals in Tehran, Iran (Aghamiri *et al*., 2014). *bla*\textsubscript{IMP} and *bla*\textsubscript{VIM} mediated carbapenem resistance has also been detected in the species of *Pseudomonas* and *Acinetobacter* at Chennai, India (Amudhan *et al*., 2012).

**New Delhi metallo beta-lactamase (NDM)**

The NDM-1 enzyme was named after New Delhi, India, as it was first detected in a *Klebsiella pneumoniae* isolate from a Swedish patient who fell ill with an antibiotic-resistant bacterial infection that he acquired in India in 2008 (Yong *et al*., 2009). It was later reported from several countries, India, Pakistan, the United Kingdom, the United States, Canada, and Japan. It may be chromosome encoded or plasmid mediated. Genes encoding NDM-1 (*bla*\textsubscript{NDM-1}) can spread from one strain of bacteria to another by horizontal gene transfer. There are 10 variants of *bla*\textsubscript{NDM} (NDM-1 to NDM-9, NDM-12)
have been reported so far which differ from each other by one or two amino acid residues (Khan, 2015). At Mumbai, most carbapenem-resistant bacteria isolated from patients carried the \( \text{bla}_{\text{NDM-1}} \) gene (Deshpande et al., 2010). The clinical isolates of \( P. \text{aeruginosa} \) from Serbia have been shown to carry \( \text{bla}_{\text{NDM-1}} \) gene (Jovcic et al., 2011). Plasmid bearing this gene was recovered from clinical \( \text{Acinetobacter lwofii} \) strains in China (Hu et al., 2012). NDM-1 has been detected in \( P. \text{aeruginosa} \) in a single centre in southern India (Shanthi et al., 2014). The prevalence of NDM-1 producing gram negative bacilli including \( P. \text{aeruginosa} \) has been reported from Jammu and Kashmir State of India (Fomda et al., 2014). Recently, a high prevalence (53.4\%) of \( \text{bla}_{\text{NDM-1}} \) gene was reported from PGIMER, Chandigarh among different gram negative bacilli by Mohan et al., 2015.

**Sao Paulo metallo beta-lactamase (SPM)**

SPM is a new family of MBL with 35.5\% amino acid identity to IMP-1. This was originally identified in \( P. \text{aeruginosa} \) strain 48-1997A in Sao Paulo, Brazil in 1997 (Toleman et al., 2002). Single clones of SPM-1 containing \( P. \text{aeruginosa} \) caused multiple hospital outbreaks with high mortality in Brazil (Marra et al., 2006). This enzyme is mainly plasmid encoded. Genetic analysis revealed that it was not part of an integron but instead was associated with a new type of transposable structure with potential recombinase and promoter sequences (Datta and Wattal, 2010). SPM-1 has a broad hydrolytic profile across a wide range of \( \beta \)-lactam antibiotics which includes cephalosporins, penicillins and carbapenems as demonstrated by kinetic analysis (Murphy et al., 2003). \( P. \text{aeruginosa} \) strains of blood origin in Sao Paulo, Brazil were found to be positive for the production of \( \text{bla}_{\text{SPM-1}} \) (Franco et al., 2010). Another study from Brazil reported 32\% isolates of carbapenem resistant \( P. \text{aeruginosa} \) were found to be positive for the production of \( \text{bla}_{\text{SPM-1}} \) (Rizek et al., 2014).

**German imipenemase (GIM)**

\( P. \text{aeruginosa} \) isolates from Germany were shown to possess a novel class-B \( \beta \)-lactamase designated GIM-1 (Castanheira et al., 2004). This enzyme had 30\% homology to VIM, 43\% homology to IMP-1, and 29\% and 28\% homology to NDM and SPM respectively.
The \( \text{bla}_{\text{GIM-1}} \) gene was located on a class 1 integron which was carried on relatively 45 kb plasmid. This plasmid also carried aminoglycoside resistance genes (Borra et al., 2013). The emergence of \( \text{bla}_{\text{GIM-1}} \) has been reported in MDRPA isolates of Germany (Rieber et al., 2012).

**Seoul imipenemase (SIM)**

SIM is the latest member of the MBL family originally isolated from clinical isolates of Acinetobacter baumannii from Seoul, Korea. This enzyme had 64 to 69% amino acid identity with the IMP family of MBLs. All SIM-1 producing isolates exhibited relatively low imipenem and meropenem MICs (8 to 16μg/ml) and had a multidrug resistance phenotype. SIM is capable of hydrolyzing a broad array of β-lactams, including penicillins, narrow to expanded-spectrum cephalosporins, and carbapenems. The \( \text{bla}_{\text{SIM-1}} \) gene was present on a gene cassette inserted into a class 1 integron on plasmid (Lee et al., 2005).

**Adelaide imipenemase (AIM)**

MBLs belong to three different subgroups namely B1, B2, and B3. Mobile MBLs are mainly confined to subgroup B1 but MBLs constituting the subgroup B3 are predominantly chromosomally encoded enzymes. AIM-1 was the first mobile subgroup B3 MBL detected (Leiros et al., 2012). This enzyme was originally isolated from clinical P. aeruginosa isolates in Adelaide, Australia (Yong et al., 2012).

**Florence imipenemase (FIM)**

This enzyme was identified and characterized from a multidrug-resistant P. aeruginosa clinical isolate recovered from a patient with a vascular graft infection in Italy. FIM is a member of subclass B1 and falls among acquired MBLs. It has 40% amino acid similarity with NDM-type MBLs. FIM-1 has broad substrate specificity, with a preference for penicillins (except the 6α-methoxy derivative temocillin) and carbapenems. Aztreonam was not hydrolyzed by this enzyme (Pollini et al., 2013).
2.8.1.3 Ampicillin class C beta-lactamases (AmpC-BLs)

AmpC enzymes constitute another large group of broad-spectrum β-lactamases and belong to class C of the Ambler classification scheme and 1 β-lactamases group of the Bush- Jacoby-Medeiros classification scheme. These enzymes can hydrolyze penicillins, cephalosporins (including the third-generation but usually not the fourth-generation cephalosporins), monobactams as well as cephemycins (cefoxitin, cephalothin, cefotetan, cefmetazole). AmpC type enzymes are generally poorly inhibited by β-lactamase inhibitors, especially clavulanic acid (Jacoby, 2009). The features that discriminate AmpC β-lactamases (AmpC-BLs) from ESBLs include: i. hydrolysis of cephemycins and ii. resistance to β-lactamase inhibitors. Genes encoding AmpC-BLs are usually present on the bacterial chromosomes or plasmids of many gram-negative bacteria. P. aeruginosa, Citrobacter, Serratia and Enterobacter species are the important ones (Hanson et al., 1999). Those AmpC genes which are present on the bacterial chromosome are usually inducible in nature. These genes are generally repressed and produce low levels of AmpC-BLs. They become de-repressed (induced) by a number of β-lactam antibiotics e.g., benzyl penicillin and narrow spectrum cephalosporins such as cefoxitin and hyperproduce AmpC-BLs. Thus, strains carrying inducible AmpC genes are intrinsically resistant to those β-lactam antibiotics which induce them. In contrast, AmpC genes which are located on plasmids are commonly non-inducible. They constitutively produce AmpC enzymes (Marsik and Nambiar, 2011). However, some plasmid mediated AmpC enzymes have been reported to be inducible. AmpC-BLs has minimum activity against carbapenems and monobactam (aztreonam). Although carbapenems (imipenem) are excellent inducers of AmpC genes but their rapid bactericidal activity and stability to hydrolysis renders them effective against AmpC producing P. aeruginosa (Livermore and Woodford, 2000). In P. aeruginosa, mutational de-repression of AmpC genes is the most common mechanism of resistance to β-lactams including extended spectrum cephalosporins (ceftazidime) and penicillin (ticarcillin) (Livermore, 1995). The production of AmpC variants with improved activity against oxyiminocephalosporins and carbapenems have also been reported from P. aeruginosa strains (Rodriguez-Martinez et al., 2009).
Some of the common AmpC enzymes which are either chromosome or plasmid mediated are: CMY (cephamycins) having 43 variants, FOX (cefoxitin) having 7 variants, MOX (moxalactam) 3, LAT (latamoxef) 4 variants, ACC (Ambler class C) 4 variants, ACT (AmpC type) have 3 variants, MIR (Miriam Hospital in Providence) or the DHA (Dhahran Hospital in Saudi Arabia) have 2 variants. Another AmpC-type enzyme derived from \textit{P. aeruginosa} is known as Pseudomonas derived cephalosporinase (PDC). AmpC enzyme, BIL was named after the patient (Bilal) who provided the original sample (Philippon et al., 2002).

**Clinical and epidemiological importance**

The initial isolates producing acquired AmpCs were identified at the end of 1980s, and since then they have been observed globally as a result of clonal spread, horizontal transfer of AmpC genes, and their emergence \textit{de novo}. The most prevalent and most widely disseminated of these are: the CMY-2- like enzymes, although the inducible DHA-like β-lactamases and some others have also spread extensively. \textit{E. coli, K. pneumoniae, Klebsiella oxytoca, Salmonella enterica} and \textit{Proteus mirabilis} are some of the main bacterial spp. producing acquired AmpCs. Isolates with these enzymes have been recovered from both hospitalized and community patients. Most of the clinical laboratories are not looking for plasmid mediated AmpC β-lactamases routinely. The treatment options for infections caused by organisms expressing AmpC β-lactamases are limited. Thus, there is a need to detect AmpC β-lactamases in order to avoid therapeutic failures (Gupta et al., 2012). The most important clinical problem associated with beta-lactamases is the emergence of multiple resistant strains which are responsible for therapeutic failures. The reports of β-lactamases from Himachal Pradesh are scanty. We, therefore, undertook this study to look for Metallo β-lactamases and AmpC β-lactamases in \textit{Pseudomonas aeruginosa} isolates recovered from patients suffering from different clinical conditions due to this organism.

**2.9 Laboratory Identification of \textit{P. aeruginosa}**

Due to frequent spread in hospitals and the high level of drug resistance in \textit{P. aeruginosa} infections, diagnostic procedures should not only aim at identifying the pathogen, but also to determine its susceptibility to different antibiotics. This would help the clinicians
for better management of *P. aeruginosa* infections. Both phenotypic and genotypic methods are employed for the diagnosis. The conventional methods utilize the culturing of the sample obtained from the patients in the laboratory on suitable media and followed by identification of the same organism by conventional methods. Biotyping, serotyping, pyocin typing, phage typing and antibiograms are traditional phenotypic methods of typing which are used to type unrelated strains. However, the discriminatory power of these methods is much lower than that obtained by molecular typing methods. Also, phenotypic tests are time and labour consuming and very often their results are ambiguous to interpret (Czekajlo-Kolodziej et al., 2006). Further, since these methods are based on the presence or absence of expressed and strain characteristic features, the instability of such features in various environmental conditions is the main disadvantage of these tests e.g. types of *P. aeruginosa* growth on cetrimide agar medium differed significantly with in strains belonging to same genotype as well as among strains of different genotypes.

Determination of susceptibility of *P. aeruginosa* strains to antibiotics and identification of its resistance mechanisms are some of key points that must be taken care of. Routine procedures include: diffusion methods (disc diffusion and E tests), and dilution methods on solid or liquid media, and automated systems (Pfaller and Segreti, 2006). The susceptibility testing methods are highly dependent on experimental conditions and often require more than one method to be performed in order to obtain an accurate susceptibility profile which can be further enhanced by the resistance profile targeting specific genes can be determined. The development of rapid and sensitive genotypic methods is therefore, an attractive approach for epidemiological typing of *P. aeruginosa* as well as for other infectious agents. Genotypic techniques have the advantage over phenotypic methods in that they are independent of the physiological state of an organism. These methods involve the study of the microbial DNA, the chromosome and plasmid, their composition, nucleotide sequence homology and presence or absence of specific genes. These methods are now being introduced in the diagnostic laboratories also. Due to their complexities and cost involved, they are however, limited to few laboratories only. Polymerase chain reaction (PCR) is the most commonly used technique for nucleic acid amplification for detection of antimicrobial resistance genes. Several
typing methods using PCR based on the microbial genotype or DNA sequence have been developed.

Genotypic methods can be categorized into two: (1) fingerprint or pattern based techniques and (2) sequence based techniques. Fingerprint based techniques utilize restriction enzymes to produce a series of fragments from an organism's chromosomal DNA. These fragments are then electrophoresed and separated according to size to generate a profile or fingerprint that is unique to that organism and its very close relatives.

Sequence based techniques utilize the determination of nucleotide sequence of a specific stretch of DNA, usually associated with a specific gene. The degree of similarity between the two sequences is a measurement of how closely related the two organisms are to one another. A number of computer algorithms have been created that can compare multiple sequences to one another and build a phylogenetic tree based on the results.

**Fingerprinting based methods**

DNA typing methods such as pulse field gel electrophoresis (PFGE), ribotyping, restriction fragment length polymorphic DNA analysis (RFLP), random amplified polymorphic DNA assay (RAPD), arbitrary primed PCR (AP-PCR), amplified fragment length polymorphism (AFLP) and repetitive element based PCR (Rep-PCR) have been frequently used in the molecular epidemiology of *P. aeruginosa*, in order to determine the source of infection and its spread during an epidemic.

**Sequence based methods**

Nucleic acid sequencing is a method for determining the exact order of nucleotides present in a given DNA or RNA molecule. Multilocus sequencing is one of the recent and most powerful methods developed to identify microbial species. This technique is based on the measurement of DNA sequence variation in a set of housekeeping genes. Housekeeping genes encode for proteins that carry out essential cellular processes (Emerson *et al.*, 2008). Housekeeping-gene loci are present in most cells and tend to be conserved among different organisms. As a result, general purpose primers can be designed that can be used to amplify the same genes across multiple genera in the PCR assays. *P. aeruginosa* isolates may be discriminated by multilocus sequence typing.

Whole genome sequencing is another useful tool to determine the complete DNA sequence of an organism's genome at a single time. This is done to understand \textit{P. aeruginosa} population genomics, diversity, mutational mechanisms, genetic adaptation and transmission events. The complete genome sequence of \textit{P. aeruginosa} was determined first in year 2000 (Stover \textit{et al}., 2000). Targeted sequencing of specific genes or genomic regions is preferred in many cases where a suspected disease or condition has been identified, as this method is more affordable, yields much higher coverage of genomic regions of interest, and reduces sequencing cost and time (Grada and Weinbrecht, 2013). DNA microarray is one of the recent methods in which microscopic DNA spots are attached to a solid surface. Each DNA spot contains a specific DNA sequence, known as probe. The probes can be a short section of a gene or other DNA element that are used to hybridize a target sample (cDNA or cRNA) under high stringency conditions. The sample DNA or RNA is labeled with a fluorescent reporter molecule so that samples that hybridize with probes on the microarray can be detected rapidly (Liu \textit{et al}., 2001). The management of infections with multidrug-resistant \textit{P. aeruginosa} needs fast and reliable methods of antibiotic susceptibility testing for immediate therapeutic application by the clinicians. For this purpose, DNA microarray may be used for genotyping antibiotic resistance and few virulence factors (Weile \textit{et al}., 2007).

\subsection*{2.9.1 Diagnosis of MBL and AmpC producing \textit{P. aeruginosa} isolates}

Several phenotypic methods are available for the detection of MBL producing bacteria. All these methods are based on the ability of metal chelators, such as EDTA and thiol based compounds which inhibit the activity of MBLs. These tests include; the double-disc synergy test, Hodge test (Lee \textit{et al}., 2001), combined disc test, the MBL E-test (Yan \textit{et al}., 2004) and a microdilution method (Migliavacca \textit{et al}., 2002). The production of AmpC enzymes is detected by resistance of an organism to cefoxitin (zone size <18mm) (Gupta \textit{et al}., 2012). However, other confirmatory tests such as disc antagonism test, AmpC disc test, modified three dimensional test, cefoxitin agar media, and inhibitor
based methods should be applied for accurate and reliable results (Jacoby, 2009). Molecular methods have played an important role in the diagnosis and epidemiology of infectious diseases. To increase the rapidity and accuracy of phenotypic testing, the molecular methods were employed. Polymerase chain reaction (PCR) is the most commonly used nucleic acid amplification technique for detection of antimicrobial resistance genes. A PCR detection assay was published in 1996 for the detection of gram-negative bacteria producing IMP-1 (Pitout et al., 2005). PCR analysis is always gold standard method for the detection of MBL and AmpC producers. PCR amplification followed by nucleotide sequencing remains gold standard method for the identification of specific point mutation of MBL and AmpC genes. Multiplex PCR can be used to detect a number of genes simultaneously (Woodford, 2010). Isoelectric focusing, multilocus sequence typing (MLST), pulse field gel electrophoresis (PFGE) etc. are other techniques which may be used for the detection of MBL and AmpC genes in P. aeruginosa. But at present, neither PCR nor other molecular methods are available in most clinical microbiology laboratories for daily testing due to their high cost and inconvenience (Galani et al., 2007).

2.10 Control measures

The occurrence of MBL and AmpC producing P. aeruginosa isolates in a hospital setting poses a therapeutic problem as well as a serious concern for infection control management, due to the rapid spread of these multidrug-resistant strains. The risk of selection of resistant mutants are always associated with any antipseudomonal therapy, varies with the type and dosage of antibiotic used and the infection site (Japoni et al., 2009). For the effective control of spread of these isolates certain measures such as effective surveillance, hand hygiene, contact precautions, personal protective equipment, patient care equipment, environmental cleaning, laundry/waste management and antimicrobial stewardship are to be followed.

A large number of antibiotics have been used for treating P. aeruginosa infections. Some of the commonly used drugs to treat infections are; penicillins (ticarcillin and piperacillin), monobactams (aztreonam), cephalosporins (ceftazidime and cefepime) and β-lactam/β-lactamase inhibitor combinations (ticarcillin-clavulanate and piperacillin-
tazobactam). Fluoroquinolones (ciprofloxacin and levofloxacin) have been found effective to treat \textit{P. aeruginosa} infections but the use of levofloxacin might be associated with a higher risk of isolation of quinolone resistant \textit{P. aeruginosa} as compared to ciprofloxacin (Kaye \textit{et al.}, 2006). The carbapenems are currently among the last choice for the treatment of serious MDR \textit{P. aeruginosa} infections. These antimicrobials have different levels of activity against \textit{Pseudomonas} strains. Carbapenems (imipenem, meropenem, doripenem and ertapenem) are useful against most ESBL and AmpC producing pathogens. But MBL producing pathogens show resistance to these novel carbapenems as well (Thomson and Bononomo, 2005). \textit{In vitro} testing of these agents demonstrated that MICs were lowest with doripenem, followed by meropenem, and imipenem (Gimeno \textit{et al.}, 2010).

Colistin (polymixin E) is another choice of antibiotic for treating infections with MBL producing \textit{P. aeruginosa}. Sporadic cases of infections by colistin-resistant \textit{P. aeruginosa} have been reported (Falagas \textit{et al.}, 2005). Colistin resistance has been reported in metallo-\textbeta-lactamase producing \textit{P. aeruginosa}. In addition to this, rifampicin may be an interesting agent for treating multidrug-resistant \textit{P. aeruginosa} infections. Some of the newer antibiotics such as doripenem, ertapenem, biapenem, tomopenem, ceftobiprole and aerosolized antibiotics in selective cases can be used to treat MDR \textit{P. aeruginosa}. Tobramycin has been the most widely used antibiotic in the treatment of \textit{P. aeruginosa} pneumonia. This antibiotic is taken up through inhalation. Colistin has also been successfully used in the management of MDR \textit{P. aeruginosa} pneumonia in a similar manner.

Besides, some effective agents are under the development phase. Sitafloxacin, which is a quinolone having better inhibition activity against gyrA or parC mutants than ciprofloxacin. KB001 is a high affinity antibody fragment that reduces the toxicity and pathogenicity of \textit{P. aeruginosa} and CXA-101 is a novel cephalosporin with potent activity against MDR strains. BLI-489/piperacillin is another \textbeta-lactam/\textbeta-lactamase inhibitor combination and CB-182,804 is a lipopeptide with apparent bactericidal activity against MDR strains (Kanj and Kanafani, 2011).