1.0 INTRODUCTION

The genus *Pseudomonas* is one of the most diverse and ecologically significant groups of bacteria. Members of this genus are found in large numbers in a wide range of environmental niches. *P. aeruginosa* is ubiquitous in soil, aqueous solutions including disinfectants, soaps, eye drops, as well as sinks and respiratory equipment. This organism is a highly adaptable and has a large genome of 6.26 Mbp (5567 genes) compared to 4.64 Mbp (4279 genes) in *Escherichia coli* (Lambert, 2002). The number of genes needed for growth of the cell and division in a minimal medium is normally estimated at around 1500. *P. aeruginosa*, therefore, has considerable additional genetic capacity. It is for this reason that this organism has highly adaptable, including the ability to develop resistance against antibiotics. This organism normally lives in moist environments, and uses a wide range of organic compounds for growth, thus giving it an exceptional ability to colonize ecological niches where nutrients are limited, such as water, soil, plant and animal tissues.

*P. aeruginosa* is an aerobic gram negative rod, usually 1.5-5µm in length and 0.5 to 1.0 µm in width and is motile due to the presence of flagella. It can tolerate low oxygen conditions, can survive with low levels of nutrients and grow in temperatures ranging from 4-42°C (Stover et al, 2000). Pyocyanin (the word has been derived from "pyocyaneus") which means "blue pus", which is characteristic for suppurative infections caused by *P. aeruginosa*. This blue pigment is produced abundantly in medium of low-iron content and functions in iron metabolism of the bacterium (Murray et al, 2003).

Other biochemical features are: positive oxidase test, growth at 42°C, hydrolysis of arginine and gelatin, positive catalase test, citrate utilization and nitrate reduction. Most isolates produce the water-soluble pigments pyoverdine and pyocyanin, which is responsible for the bright green colour characteristic of the organism. *P. aeruginosa* differs from other members of the *Pseudomonas* genus because of its potential pathogenicity for human beings and other mammals. This organism is rarely a constituent of normal microflora of healthy individuals. However, it can colonise human body sites occasionally, with a preference for moist areas, such as the perineum, axilla, ear, nasal mucosa and throat, as well as gastrointestinal tract. The frequency of
colonisation in healthy individuals is usually low. Higher colonisation rates can be encountered following hospitalization, especially among patients treated with broad spectrum antibiotics.

Normally, for an infection to occur, some disruption of the physical barriers (skin or mucous membrane), or by-passing of them (invasive devices), and/or an underlying dysfunction of the immune defense mechanisms is necessary. *P. aeruginosa* is an opportunistic, nosocomial pathogen of immune-compromised individuals, patients suffering from Acquired immuno deficiency syndrome (AIDS), cancer, burn wounds and cystic fibrosis. Infections associated with this bacterium are nosocomial, respiratory tract infections including ventilator-associated pneumonia (VAP), dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections, particularly in immunosuppressed patients, HIV patients and individuals with severe burns or cancer. Community acquired infections caused by *P. aeruginosa* are uncommon. The most frequent ones are: urinary tract infections, otitis externa, folliculitis acquired in swimming pools, keratitis due to wearing contact lenses. The mucoid phenotype of *P. aeruginosa* frequently chronically colonizes and infects patients with cystic fibrosis having decreased pulmonary function.

*P. aeruginosa* is responsible for 11-13.8% of the hospital acquired infections (Driscoll et al, 2007) but in ICU’s this percentage is even higher i.e 13.8-22.6% (Gaynes et al, 2005, Kim et al, 2000, Erbay et al, 2003). This organism has been identified as the second most frequent organism causing Ventilator-associated pneumonia (VAP) (Mansour et al, 2013). It is the third leading cause of hospital acquired urinary tract infections (Obritsch et al, 2005, Mesaros et al, 2007), the fifth cause of surgical site infections and seventh cause of central-line-associated bloodstream infections (Hidron et al, 2008). Mortality rates for mechanically ventilated patients with *P. aeruginosa* pneumonia have been estimated at about 40%-60% (Kung et al, 2010, Rassolini and Mantegoli, 2005). In cancer patients, *P. aeruginosa* can be responsible for upto 30% of culture proven cases of bacteraemia, with mortality rates ranging from 5-50% (Maschmeyer et al, 2000). High mortality and morbidity rates are associated with cystic fibrosis patients, where *P.
P. aeruginosa causes respiratory tract infections. Infections occur through childhood, ultimately affecting about 80% of adult cystic fibrosis patients (Spilker et al, 2004).

The virulence mechanisms of P. aeruginosa include; the constituents of the organism such as flagella, pili, lipopolysaccharide, alginate, secreted virulence factors (pyocyanin, pyoverdine, alkaline protease, protease IV, elastase, phospholipase C, exotoxin A), Type III secretion system (Exotoxins S, T, E, Y, U), Quorum sensing (las, rhl). In addition, this organism produces a number of toxic proteins which not only cause extensive tissue damage, but also interfere with the host defense mechanisms (Kipnis, 2006 & Matsumoto, 2004).

Due to overzealous and indiscriminate use of antibiotics for treating P. aeruginosa infections, multidrug resistant strains of this organism have emerged particularly in hospitals. Multidrug resistance has been defined as resistance to more than two classes, more than three classes and more than four classes of antibiotics by different research groups (Engel, 2009, Giske, 2008). P. aeruginosa is one of the main organisms responsible for drug-resistant nosocomial infections, and is one of the leading causes of bacteremia and pneumonia in hospitalised patients. This organism can easily develop resistance to all conventional anti-pseudomonal antibiotics through different intrinsic and acquired resistance mechanisms. The aminoglycosides for example, inhibit protein synthesis by binding to the 30S subunit of the ribosome while quinolones bind to the A subunit of DNA gyrase enzyme, which maintains the ordered structure of the chromosome inside the cells. The β-lactams inhibit the peptidoglycan assembling transpeptidases located on the outer face of the cytoplasmic membrane. The polymyxins bind to phospholipids in the cytoplasmic membrane, destroying its barrier function. There are three basic mechanisms by which P. aeruginosa resists the action of the above mentioned antimicrobial agents: restricted uptake and efflux; enzymatic drug inactivation and mutations on the targets of antibiotics.

The carbapenems are the most common beta-lactam antibiotics which are used for treating P. aeruginosa infections. The beta-lactam antibiotics are structurally related to
the penicillins and cephalosporins. These antibiotics have a β-lactam ring which is fused to a five-membered ring with variable side chains (Fig 1.1).

![β-lactam ring]

The five-membered ring differs from the thiazolidine ring of penicillin in two ways: A methylene group replaces sulphur and the ring contains a double bond. The structure of carbapenems results in three properties that account for their broad spectrum. First, these molecules are quite small and have charge characteristics that allow them to utilize special porins in the outer membrane of gram-negative bacteria to gain access to the penicillin binding proteins (PBPs). Second, the structures of the carbapenems make them resistant to cleavage by most β-lactamases. Third, the carbapenems have an affinity for a broad range of PBPs from many different kinds of bacteria. Due to these three properties, the carbapenems are proficient at gaining access to periplasm, resisting destruction by β-lactamases that reside there, and binding to PBPs to cause bacterial cell death. Carbapenems are broad-spectrum antibiotics and act against many aerobic gram-positive, most aerobic gram-negative bacteria and most anaerobes (Bonfiglio et al, 2002, Gupta et al, 2006). Imipenem, meropenem, doripenem and ertapenem are commercially available antibiotics of this class. *P. aeruginosa* is considered as poor target for ertapenem (Parakh et al, 2009).

Although, carbapenems have a very broad spectrum of activity, some bacteria have acquired the ability to produce extremely powerful β-lactamases that are capable of cleaving carbapenems. Extended spectrum beta lactamases (ESBLs), and AmpC β-lactamases and metallo-β-lactamases (MBLs) are the types of these enzymes. Production
of MBLs is probably the major defense mechanism of *P. aeruginosa* against β-lactam antibiotics which degrade this group of antibiotics (Lambert et al, 2002, Gupta et al, 2008). MBLs or class B beta lactamases are gaining clinical significance because these enzymes hydrolyze almost all β-lactams, with the exception of monobactams and no clinically available inhibitor is known till now (De et al, 2010). The common feature of MBLs is the principal zinc binding motif, histidine-X-histidine-X-aspartic acid in the active site, which coordinates the arrangement of two H₂O molecules that are important in the hydrolysis. Hence, chelation of zinc by EDTA or mercaptopropionic acid, impairs β-lactam hydrolysis and restores susceptibility to carbapenems. MBLs have a wide and plastic active site, which allows all β-lactams to accommodate in there, except aztreonam β-lactamase inhibitors such as clavulanic acid, tazobactam and sulbactam are also hydrolysed by MBLs.

Genes encoding MBLs are located as cassettes in integrons that provide them with the potential for expression and dissemination. To date, five major types of MBLs have been described throughout the world namely, imipenamase (IMP), Verona imipenamase (VIM), Sao Paulo metallo-β-lactamase (SPM), German imipenamase (GIM) and Seoul imipenamase (SIM). Of these IMP and VIM are the most dominant MBLs worldwide. These have been subgrouped as VIM-7, VIM-2, VIM-1 and IMP-12, IMP-1, IMP-2, IMP-1 groups. Several enzyme variants have been identified. IMP has 44 and VIM has 38 variants which have been identified in various gram negative species (Tada et al, 2013, Meini et al, 2014). While GIM has been reported from Germany and SPM from Brazil (Wendel et al, 2013), the IMP and VIM enzymes have been disseminated throughout the world. The IMP enzymes were originally detected in Asia, but later spread to Europe, United States and Australia, while the VIM gene was first found in Europe, and shortly after emerged in other continents also. Despite the worldwide dissemination of these two groups of enzymes, the tendency of the dominance of the IMP enzyme in Asia, and the VIM enzyme in Europe prevails.

Different MBL groups differ in their hydrolytic capacities significantly. SPM-1 is very efficient, while GIM-1 is rather a weak carbapenemase. Within the IMP group, IMP-6 and IMP-3 hydrolyse imipenem but to a lesser extent. Within the VIM group, VIM-1 hydrolyses meropenem, ceftazidime and piperacillin more efficiently than VIM-2.
However majority of the MBL producers still remain highly resistant to carbapenems and cephalosporins. Because of the ability of *P. aeruginosa* to spread through horizontal gene transfer, therefore carbapenem resistance related to MBL production has become a serious concern.

Horizontal gene transfer is one of the most important mechanisms for widespread distribution of antibiotic resistance genes. This process is mediated by conjugation, transduction or transformation. Conjugation is a process of gene transfer by means of mobile genetic elements, called plasmids, which are extra-chromosomal DNA molecules that are transferred from the donor to the recipient through sex pili. Second type of mobile genetic elements is transposons, which are also capable of mediating the transfer of DNA by site-specific insertions and excisions. An integrin is another class of mobile genetic elements which can integrate resistant gene cassettes at specific site, *attI* (Fig. 2.1). Transposons and integrons are frequently carried on plasmids, but can also have a chromosomal location.

The genes encoding the MBLs are almost always located on class 1 integrons. Integrons are genetic elements that although unable to move themselves, contain gene cassettes that can be mobilized to other integrons or to secondary sites in the bacterial genome. The majority of integrons described till date belong to integron class 1. These integrons consist of two conserved segments (5'CS and 3'CS) and a variable region, where different gene cassettes can be inserted. The 5'CS part contains an integrase gene (*intI*), an adjacent recombination site (*attI*) and a common promoter. The 3'CS region usually consist of a partially deleted gene, encoding a quaternary ammonium compound efflux pump (*qacEAl*) fused with a sulphonamide resistance gene (*sulI*). The structure of integron and integron mediated gene capture is presented through Fig. 2.1 given under section 2.11.

Different β-lactamases, usually MBLs, and/or aminoglycoside-resistance genes are found between the genes recombination sites (also known as 59-base element) in the variable region. The 59-base elements are not highly conserved, and contain inverted repeats. Integration of new gene cassette, which is mediated by the integrase, can take place either between the gene cassettes and *attI*, or between two gene cassettes. The integrase
excises the gene cassettes. The recombination takes place close to one end of the 59-base element. Due to the integration of gene cassette, part of the 59-base element ends up at the 5' side of the coding sequence of the gene cassette to which it belongs. Integrons are usually located on transposons, although chromosomal fixation of integrons can also occur. They can also be found on conjugative plasmids, conferring horizontal dissemination. Integrons encoding MBLs often harbor other resistance genes, most frequently the gene responsible for resistance to aminoglycosides, thus increasing the likelihood of dissemination of multiple resistances.

The present study has been planned with a view to assess the antimicrobial susceptibility patterns of clinical isolates of *P. aeruginosa* recovered from human patients at Indira Gandhi Medical College, Shimla, Himachal Pradesh. These isolates shall be further screened for the presence of MBLs. For treating infections due to *P. aeruginosa*, carbapenems are considered to be therapeutic option. The resistance to this class of antibiotics is an emerging threat. There is an urgent need therefore, to investigate carbapenem resistance in *P. aeruginosa*. In this study, highly resistant phenotypes of *P. aeruginosa* shall be screened for the presence of different metallo-β-lactamase types such as (imipenamase (IMP) and Verona imipenamase (VIM) in clinical isolates of *P. aeruginosa*. Also, the presence of different classes of integrons (Class 1, 2 & 3) in *P. aeruginosa* isolates of this region shall be determined. The integrons play an important role in the dissemination of MDR strains of *P. aeruginosa*. Since scanty or very little data are available on the status of MDR and MBL producing *P. aeruginosa* strains in the context of Himachal Pradesh, the clinical isolates shall be characterized in order to obtain the useful epidemiological data. In order to achieve this aim, the following study has been planned with the objectives mentioned below.

1. Confirmation of clinical isolates of *P. aeruginosa* and selection of multi drug resistant (MDR) isolates by antibiotyping.
2. Identification of metallo-β-lactamase producing MDR isolates of *P. aeruginosa*.
3. Molecular characterization based on PCR amplification of selective drug resistance genes of metallo-β-lactamase (IMP, VIM) producing *P. aeruginosa*.
4. Detection of types of integron class present in IMP and VIM classes.