1. INTRODUCTION

1.1 General features of the genus Pseudomonas

The genus *Pseudomonas* is one of the most diverse and ecologically significant groups of bacteria. This organism belongs to the family *Pseudomonodaceae*. Based on 16S rRNA sequence analysis, the analysis of cellular fatty acids, differentiating classical physiological and biochemical tests, 202 species of this genus have been identified so far. They inhabit a wide range of environmental niches, such as terrestrial and marine environments, as well as in association with plants and animals, hence, they are well known for having enormous metabolic versatility (Ozen and Ussery, 2012). The universal distribution of *Pseudomonas* is responsible for its genomic diversity and genetic adaptability. The genus consists of a group of medically and biotechnologically important bacteria. *Pseudomonas aeruginosa* differs from other members of genus *Pseudomonas* because of its potential pathogenicity for human beings and other mammals. *Pseudomonads* are gram negative bacteria, usually 1.5μm - 5μm in length and 0.5μm to 1.0μm in width and appear as straight or slightly curved rods that occur singly or in pairs or short chains. These organisms are non-sporulating, motile due to the presence of polar flagella and aerobic that requires oxygen as the major constraining factor for its growth although they can also respire anaerobically on nitrate or other alternative electron acceptors. The optimum temperature for the growth of *P. aeruginosa* is 37°C although it has the ability to grow at 42°C. The inability to ferment lactose, a positive oxidase reaction, its fruity odour due to production of aminoacetophenone and ability to grow at 42°C help in identification of this organism. Colonies of *P. aeruginosa* are circular, raised with undulate margins, mucoid in appearance due to production of alginate capsule in some strains (Moore et al., 2006). One of the characteristics of this organism is that it produces a number of pigments such as pyocyanin (blue and non fluorescent), pyoverdin (yellow and fluorescent), pyorubrin (red) and pyomelanin (dark brown). These pigments contribute to the pathogenicity of this organism (Oqunnariwo and Hamilton Miller, 1975). *P. aeruginosa* has genome composed of 6.3 million base pairs (Mbp) and contains 5570 open reading frames (Fick and Boca Raton, 1993). So far as nutritional requirements of this organism are concerned, they are simple so much so that it can grow
in distilled water. *P. aeruginosa* requires acetate as a source of carbon and ammonium sulfate as a source of nitrogen for their growth (Todar, 2009). The organism however is physiologically versatile and flourishes as a saprophyte in multiple environments, including sinks, drains, respirators, humidifiers and disinfectant solutions (Collee *et al.*, 1996).

**1.2 Disease conditions caused by *Pseudomonas aeruginosa***

*P. aeruginosa* infections are generally seen in patients with impaired immunity, such as patients suffering from AIDS, cancer, burn wounds, cystic fibrosis, acute leukemia, organ transplants and intravenous-drug addiction (Bodey *et al.*, 1983). *P. aeruginosa* is the most common gram-negative bacterium found in patients who have been hospitalized longer than one week, and frequently associated with nosocomial infections. Data from the National Nosocomial Infections Surveillance system from 1986-2003 reveal that *P. aeruginosa* is the second most common cause of pneumonia (18.1%), the third most common cause of urinary tract infection (16.3%) and the eighth most frequently isolated pathogen from the bloodstream (3.4%) (Winn *et al.*, 2006). *P. aeruginosa* infections of humans range from minor infections of skin to serious systemic infections with the involvement of various organs of the body (Pollack *et al.*, 2000). Several virulence factors such as lipopolysaccharide (LPS), flagellum, type IV pili, exotoxins, type III secretion system, hemolysins, gelatinases, lipases, proteases and biofilm production contribute to the pathogenesis of *P. aeruginosa* infections. These factors lead to increased tissue damage and also protect the *P. aeruginosa* against the recognition by the immune system and the action of antibiotics (Cevahir *et al.*, 2008). This organism is most commonly found in skin and soft tissue infections such as burn patients, hemorrhagic and necrotic lesions, subcutaneous nodules, deep abscesses, cellulitis, and fasciitis and is the most frequent colonizer of medical devices (e.g., catheters). Infections of bones and joints result from direct inoculation or the hematogenous spread of *P. aeruginosa* from other primary sites of infection (Diekema *et al.*, 1997). Colonization of lower respiratory tract of cystic fibrosis patients by mucoid strains of this organism is common and difficult to eradicate (Fine *et al.*, 1996). It is the most prominent pathogen found in some cases of external otitis, including “swimmer’s ear”. *P. aeruginosa* may infects the prosthetic heart.
valves of intravenous (IV) drug users and establishes itself on the endocardium by direct
invasion from the blood stream, resulting in endocarditis. Other disease conditions such
as meningitis and brain abscesses are associated with the invasion of this bacterium from
the inner ear or paranasal sinus to the Central Nervous System. *P. aeruginosa* is one of
the most common cause of bacterial keratitis which involves the ocular epithelium
colonization of 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 loss of the entire eye. Urinary tract infections (UTI)
caused by *P. aeruginosa* are usually hospital-acquired and related to urinary tract
catheterization, instrumentation or surgery. This organism can produce disease in any
part of the gastrointestinal tract from the oropharynx to the rectum and most commonly
implicated in perirectal infections, pediatric diarrhea, typical gastroenteritis, and
necrotizing enterocolitis (Todar, 2009).

1.3 Emergence of multi drug resistant *Pseudomonas aeruginosa* (MDRPA) strains

Nosocomial bacterial pathogens have emerged as, “Superbugs” with acquired resistance
to almost all available antimicrobial agents and have severely endangered therapeutic
choices in the last few decades (Neu, 1983). MDRPA was defined as *P. aeruginosa* with
combined decreased susceptibility to piperacillin, ceftazidime, imipenem, and
ciprofloxacin (Paramythiotou *et al.*, 2004). Although the definition of multi drug
resistance is not standardized in many of the studies published on such resistance.
Different agents from different antimicrobial classes are selected as standards for
resistance and the number of agents required for a strain to be classified as MDRPA is
not always specified within these studies. The major risk factors for MDRPA infection
include: prolonged hospitalization, indiscriminate use of antibiotics and immune status of
the patient. Emergence of MDRPA isolates during therapy was reported in 27-72% of
patients with initially susceptible *P. aeruginosa* isolates (Obritsch *et al.*, 2005). A report
from the Infectious Diseases Society of America revealed three categories of emerging
multiple drug resistant gram negative bacilli which included carbapenem-resistant species
of *Acinetobacter*, *Pseudomonas* and *Klebsiella* (Talbot *et al.*, 2006). In this study, an
MDRPA strain was selected on the basis of its resistance to at least three drugs of the
following classes: ureidopenicillins (piperacillin and piperacillin-tazobactam), cephalosporins (ceftazidime, cefepime, cefoperazone), carbapenems (imipenem and meropenem), aminoglycosides (gentamicin and amikacin), fluoroquinolones (ciprofloxacin and levofloxacin) and monobactams (aztreonam). Ventilator-associated pneumonia is one of the main MDRPA infections in the intensive care unit (ICU) (Trouillet *et al.*, 2002). There are several mechanisms which can contribute to acquire drug resistance in *P. aeruginosa*, including β-lactamase production, the up-regulation of efflux systems and decreased outer membrane permeability. However, acquired extended spectrum β-lactamases (ESBLs), ampicillin class C β-lactamases (AmpCs) and metallo β-lactamases (MBLs) mediated resistance is important emerging resistance mechanisms in *P. aeruginosa* (Hirsch and Tam, 2010). Despite of various improvements in the antibiotic therapy, *Pseudomonas aeruginosa* is intrinsically resistant to a number of antimicrobial agents and is, therefore, a particularly dangerous and dreaded pathogen and hence gains public health significance. β-lactamases are hydrolytic enzymes which cleave the β-lactam ring of the β-lactam antibiotics and are the primary mechanism of conferring bacterial resistance to this group of antibiotics. β-lactam antibiotics are commonly used to treat bacterial infections. The groups of antibiotics in this category include penicillins, cephalosporins, carbapenems and monobactams (Upadhayay *et al.*, 2010). Increased use of antibiotics, particularly the third generation of cephalosporins has been associated with the emergence of β-lactamases mediated bacterial resistance. Such resistance is of two types: intrinsic as well as acquired. Acquired resistance has been reported by production of plasmid mediated ampicillin class C (AmpC) β-lactamases, extended spectrum β-lactamases (ESBLs) and metallo β-lactamases (MBLs). Genetic control of β-lactamase production resides either on plasmids or on the chromosome, with the potential to move between bacterial populations.

### 1.4 Metallo β-lactamases (MBLs) and Ampicillin class C (AmpC) β-lactamases

Resistance to broad-spectrum β-lactam antibiotics, mediated by extended-spectrum β-lactamase (ESBL), ampicillin class C β-lactamase (AmpC) and metallo β-lactamase (MBL) enzymes, is an increasing problem worldwide (Batchelor *et al.*, 2005). ESBLs confer resistance to most β-lactam antibiotics, but are not active against cephamycins and
carbapenems and are inactivated by β-lactamase inhibitors such as clavulanic acid, tazobactam and sulbactam. This is in contrast to AmpC β-lactamases, which are not inhibited by β-lactamase inhibitors and usually confer resistance to all β-lactams, with the exception of methoxy-imino-cephalosporins, such as cefepime and the carbapenems (Bradford, 2005). Metallo β-lactamases have recently emerged as one of the most worrisome resistance mechanism due to their capacity to hydrolyze all the β-lactam antibiotics including carbapenems with the exception of monobactams e.g., aztreonam (Walsh, 2008). Pseudomonas aeruginosa producing metallo β-lactamases was first reported from Japan in 1991 and then resistance spread to other species (Shobha et al., 2009). Metallo β-lactamases are carbapenemases which require divalent zinc ions (Zn²⁺) at the active site and are predominantly produced by P. aeruginosa. They belong to class B of the Ambler’s classification scheme and Bush-Jacoby Mederios Group 3 (Behera et al., 2008). Acquired MBL genes are located on integron structures that reside on mobile genetic elements such as plasmids or transposons, thus enabling widespread dissemination. In India, only blaVIM and blaNDM-1 have been reported in P. aeruginosa (Buchunde et al., 2012). Another large group of broad-spectrum β-lactamases are the AmpC enzymes. AmpC-type cephalosporinases belong to Ambler class C β-lactamases. They hydrolyze penicillins, cephalosporins (only third-generation but usually not the fourth-generation compounds) and monobactams. In general, AmpC-type enzymes are poorly inhibited by β-lactamase inhibitors, such as clavulanic acid. They are typically encoded on the chromosome of many gram-negative bacteria, including Citrobacter, Serratia and Enterobacter species, where its expression is usually inducible (Jacoby, 2009). AmpC type β-lactamases may be carried on plasmids of bacterial species lacking the chromosomal AmpC gene. Plasmid mediated AmpC β-lactamases differ from chromosomal AmpCs in being uninducible and are typically associated with broad multidrug resistance. Plasmid-mediated AmpC β-lactamases were first reported in 1988 (Bauernfeind et al., 1989). The present study has been designed to phenotypic and genotypic characterization of MBL and AmpC producing Pseudomonas aeruginosa strains recovered from different clinical cases from Indira Gandhi Medical College (IGMC), Shimla in Himachal Pradesh. The proposed study has been planned with the following objectives.
1.5 Objectives of the research:

- Confirmation of clinical isolates of *P. aeruginosa*.
- Selection of multidrug resistant *P. aeruginosa* (MDRPA) strains and *in vitro* studies on their virulence traits.
- Phenotypic characterization of Metallo β-lactamases producing *P. aeruginosa* isolates.
- Phenotypic characterization of AmpC β-lactamases producing *P. aeruginosa* isolates.
- PCR amplification of β-lactamase (*bla*) genes (MBL genes: *bla*<sub>NDM</sub>, *bla*<sub>GIM</sub> & AmpC-type genes: *bla*<sub>PDC</sub>, *bla*<sub>CMY</sub>) for molecular characterization of these isolates.
- Nucleotide sequencing of the PCR amplicons of selective isolates.