3.0 RESEARCH METHODOLOGY

The composition of media for the growth of *P. aeruginosa*, chemicals and reagents, glassware, equipment etc used in the study are given in Annexure-I, II, III respectively.

3.1 Collection of isolates of *P. aeruginosa* and their processing

A total of 153 isolates of *P. aeruginosa* used in the study were obtained from the Indira Gandhi Medical College (IGMC), Shimla, Himachal Pradesh. Majority of the isolates originated from pus of the patients. The isolates were collected on stock culture agar medium during 15 months period i.e from October 2011 to February 2013, transported to the laboratory on ice. All the cultures enriched in trypticase soy broth were streaked on non-selective nutrient agar. A single, well isolated colony was then sub-cultured on Pseudomonas isolation agar (PIA), Muller-Hinton agar (MHA) and Nutrient agar. After overnight incubation at 37°C, the cultures were observed for pigment production. The confirmation was done on the basis of colony characteristics, Gram’s staining and biochemical tests. Purified colonies were maintained on nutrient agar slants at 4°C and stored in 50% glycerol at -80°C until used in the experiment. The proposed research project (No. SUIEC/12/05) was cleared by the Institute Ethics Committee (IEC) of the Shoolini University, Solan through letter no.SUBMS/IEC/12/46 dated March 19, 2012.

3.2 Reference strains

*P. aeruginosa* strain ATCC 27853 was included as positive control in antibiotic susceptibility test and negative control for phenotypic tests of MBL detection. *Stenotrophomonas maltophilia* ATCC 13636 was included as positive control for phenotypic detection of MBL. These strains were obtained from Himedia Laboratories Pvt Ltd (Mumbai, India).

3.3 Confirmation of the isolates

All the isolates included in the studies were confirmed on the basis of colony morphology, pigment production, growth at 42°C, motility in nutrient agar, Gram’s staining and biochemical test as described below.
3.3.1 Morphological identification

A. Gross morphology (Colony characteristics, pigment production, motility)

Colony characteristics
The colony characteristics (Colour, shape, edge, elevation etc) of the isolates were observed following growth of the organisms on nutrient agar.

Pigment Production
King’s medium A and B are recommended for non-selective isolation, cultivation and pigment production of Pseudomonas spp. P. aeruginosa is known to produce two types of pigments, pyocyanin and fluorescein, a characteristic feature that aids in isolation and identification of Pseudomonas recovered from samples obtained from clinical cases. An additional pigment called pyorubin was reported by King (1954). Pyocyanin is green while fluorescein is fluorescent yellow and pyorubin is reddish brown. Some strains produce all these pigments while others produce one or two pigments. King’s medium A is particularly suited for pyocyanin and pyorubin whereas King’s medium B is suited for fluorescein. For visualising pigment production King’s medium A and B were prepared seperately and autoclaved at 121°C for 15 minutes. The media were poured into petri plates and solidified medium was streaked with fresh bacterial culture and incubated at 37°C for 18-24 hrs. Green coloured pigmentation is produced in King’s medium A while fluorescent yellow pigmentation is observed in King’s medium B by the isolates of P. aeruginosa.

Growth at 42°C

P. aeruginosa has the ability to grow at wide range of temperatures; it can grow in nutrient agar at temperature as high as 42°C. Growth at this temperature is also advocated to differentiate species of Pseudomonas group as well as to differ other non-fermentative bacteria.

Motility Test
Motility has long been recognized as an important taxonomic tool and biological characteristic of microorganisms. Motility in bacteria can be provided by a variety of mechanisms including flagellar movement. For observing motility of P. aeruginosa,
motility test medium (Annexure I) in 5ml volume was dispensed in each tube and autoclaved at 121°C for 15 minutes, kept upright and allowed to cool. A well isolated colony was picked up with sterile platinum loop and stabbed in the medium so as to reach a distance 1cm from the bottom of the tube, incubated at 37°C for 18-24 hours. A positive test was indicated by a diffuse cloud of growth away from the line of inoculation, suggesting motility.

B. Microscopic examination of the Gram’s stained preparation

Gram’s staining

Gram’s staining is an extremely important technique by which bacteria can be differentiated into two different groups: Gram positive and Gram negative bacteria (Gram, 1884). Precisely, a freshly grown culture of P. aeruginosa isolate was smeared on a glass slide with a drop of normal saline and then heat fixed. The smear was then flooded with crystal violet strain for 1 min, washed with running tap water for 10 sec. Iodine solution was then added and allowed to act for 30 sec. The slides were washed again under the tap water for 5 sec and decolourised with acetone for two sec. The cells were then washed with water and counterstained with safranin for 30 sec. The excess of the stain was drained off and smear examined under high power of light microscope.

3.3.2 Biochemical characterization

Gelatin hydrolysis, nitrate reduction, oxidase test, pigment production, citrate test, β-hemolysis, catalase test were performed as described below for characterizing the isolates biochemically.

Nitrate reduction test

Bacterial species may be differentiated on the basis of their ability to reduce nitrate to nitrite or nitrogenous gases. Reduction of nitrate is generally an anaerobic respiration in which an organism derives its oxygen from nitrate. The nitrate reduction test is based on the detection of nitrite and its ability to form a red compound when it reacts with sulfanilic acid (reagent A) to form a complex (nitrite-sulfanilic acid) which then reacts with α-naphthylamine (Reagent B) to give a red precipitate. For performing this test, a special nitrate broth medium was prepared and dispensed in 2ml vol. in each test tube,
autoclaved for 15 minutes at 121°C. A heavy inoculum of *P. aeruginosa* was added to the medium, incubated at 30°C for 48 h. Six drops each of the sulfanilic acid and $\alpha$-naphthylamine reagents were added to each tube, the contents were mixed well by shaking. If there is no change in colour of the contents, the test is regarded as positive for nitrate reduction. However, the development of pink colour indicates negative test for nitrate reduction. In order to further confirm the positivity of the test, zinc dust is added to the contents as prepared above. If there is no change in colour even on addition of zinc dust i.e. the contents remain colourless, the positivity of the test is confirmed.

**Gelatin hydrolysis test**
The gelatin hydrolysis test is used to detect the ability of microorganisms to produce the enzyme gelatinase. Gelatinases are proteases secreted extracellularly by some bacteria which hydrolyze or digest gelatin. This process takes place in two steps. In the first reaction, gelatinases degrade gelatine to polypeptides and then polypeptides are further converted into amino acids. The test was performed as follows: nutrient gelatin medium was prepared and dispensed in a volume of two ml was dispensed in each tube, autoclaved at 121°C for 15 minutes. The medium was then allowed to cool in an upright position and heavily inoculated with the bacterial isolate, incubated at 37°C for up to one week and observed daily for gelatin liquefaction. Normally the gelatin liquefies at or above 28°C. To confirm that liquefaction was due to gelatinase activity produced by bacteria, the tubes were kept in a refrigerator for 15-30 minutes, and then tilted to observe if gelatin has been hydrolysed. Hydrolysed gelatin resulted in a liquid medium even after exposure to cold temperature which indicated the secretion of gelatinase by the test organism into the medium.

**Oxidase test**
The oxidase test is employed to detect the presence of cytochrome oxidase, an enzyme sometimes called indophenol oxidase. In the presence of an organism that contains the cytochrome oxidase enzyme, the reduced colourless Kovac’s oxidase reagent (1% tetramethyl-$p$-phenylenediamine dihydrochloride) becomes an oxidized coloured product. In this test, a sterile disc is soaked in 1% Kovac’s oxidase reagent and allowed to dry. A well-isolated colony from 18-24 hr old is picked up with a sterile loop and rubbed onto
Kovac’s reagent soaked filter paper. The development of dark purple colour within 5 seconds is indicative of positive oxidase test.

**Catalase test**

This test is utilized for the detection of the enzyme catalase in the bacteria. The catalase enzyme serves to neutralize the bactericidal effects of hydrogen peroxide. Catalase accelerates the breakdown of hydrogen peroxide (H₂O₂) into water and oxygen. This reaction is observable by the development of effervescence/bubble formation. This test is performed as follows: a glass slide was cleaned thoroughly and made grease-free. With the help of a sterile inoculating loop, a small amount of bacterial culture (18-24 hr old) was placed on the glass slide. One drop of 3% H₂O₂ was added to the culture with the help of a dropper. A brisk effervescence/bubble formation indicated positive catalase test.

**Citrate test**

The citrate test is used to determine the ability of bacteria to utilize citrate as its sole source of carbon and inorganic ammonium salt (NH₄H₂PO₄) as the sole fixed nitrogen source. When an organic acid such as citrate is used as a carbon and energy source, alkaline carbonates and bicarbonates are ultimately produced. The visible presence of growth on the medium and the change in colour due to the increased pH are the signs that an organism can import citrate and use it as a sole carbon and energy source; such organisms are considered to be citrate positive. The test was performed as follows: Simmon’s citrate agar medium dispensed in two ml volume in each tube, autoclaved at 121°C for 15 minutes, allowed to cool in a slanting position in the tube, slightly inoculated with the isolate and incubated at 37°C for 18-24 hr. Visible growth and change of the colour of the slant from forest green to intense Prussian blue indicated positive test.

**Hemolysis test**

Blood agar is referred to an enriched base medium to which defibrinated blood is added. Base medium used is tryptone soy agar. It is used to grow fastidious organisms and to differentiate bacteria based on their haemolytic properties. The test was performed as
follows: Tryptone Soy Agar was prepared and autoclaved at 121°C for 15 minutes (Annexure I), cooled to 45 to 50°C, 5% (w/v) sterile defibrinated blood was added to it. The flask was swirled thoroughly to mix and the contents dispensed into sterile petri plates. The plates were inoculated with the bacteria and incubated at 37°C for 24 hrs. Hemolytic reaction was read by holding the plate to a light source and observed with the light coming from behind (transmitted light). The type of haemolytic reaction was recorded as α, β or γ.

3.3.3 Identification of isolates by amplification and sequencing of 16s rRNA region

The DNA was extracted (as described in 3.6.1) was used as a template for the amplification of 16s rRNA gene by polymerase chain reaction (PCR) employing the following primers: Forward: 5'-AACGATGCATAGCCGACCTG-3' and Reverse: 5'-GTGGACTACCAGGGTATCTAATC-3'. The amplification was carried out in 50µl reaction volume containing 10x PCR buffer (1.5µl), genomic DNA (50µg/µl), 2mmol/L of each dNTP (5 µl), 20 pmol of each primer (1 µl) and enzyme Taq polymerase 5U/ µl and sterile grade water (Himedia) to a final volume of 50 µl. Amplification was performed in thermal cycler (AB Biosystems). The reaction conditions were as follows: The initial denaturation was done at 95°C for 4 min, followed by 35 cycles, each consisting of denaturation at 95°C for 40 sec, annealing of primers at 58°C for 30 sec and extension at 72°C for 45 sec. After completion of 35 cycles the final extension was carried out at 72°C for 7 min. The PCR products were electrophoresed in 0.8% agarose gel (containing 0.5µg/ml of ethidium bromide) and visualized under gel documentation system for the band of amplicon.

3.4. In vitro antibiotic culture sensitivity assay

The in vitro antibiotic culture sensitivity of P. aeruginosa was determined by disc diffusion method (Bauer et al, 1966) and modified and updated by Clinical and Laboratory Standards Institute guidelines (2012). The following antibiotics were used in the assay: amkacin, gentamicin, cefepime, ceftazidime, ciprofloxacin, piperacillin, piperacillin/tazobactum, imipenem, meropenem, doripenem, ertapenem, levofloxacin, aztreonam, ceferapone, colistin, tobramycin, carbenicillin, polymixin B, gatifloxacin, cefazolin, azthromycin, tigecycline, ticarcillin/clavulanic acid, ofloxacin, netillin,
cefotaxime (Himedia Laboratories Pvt Ltd, Mumbai, India). The results of sensitivity/resistance of an isolate to a particular antibiotic were interpreted according to the standard zone diameter interpretive tables (Annexure IV).

### 3.5. Determination of minimum inhibitory concentration (MIC)

MIC values of the carbapenems (imipenem, meropenem, doripenem and ertapenem) were obtained by using MIC test strip (Himedia). Test was performed according to manufacturer’s instructions.

Muller-Hinton agar medium was prepared from dehydrated powder and autoclaved at 121°C for 15 minutes. The medium was allowed to cool to 45-50°C and poured in sterile petri plates to a depth of 4mm and allowed to solidify. For preparation of the inoculum, 4-5 bacterial colonies were transferred with the help of a loop to tryptone soya broth in 5ml volume and incubate at 35-37°C for 18-24 hrs. The turbidity of the inoculums was compared with that of standard 0.5 McFarland. A sterile cotton swab was taken and dipped in the inoculum. The agar surface was swabbed three times with the cotton swab and left in the laminar flow for 15 min. Ezy MIC strip was placed on agar surface with the help of applicator. The plates were incubated at 37°C for 18 hrs.

### 3.6. Phenotypic detection of metallo-β-lactamases (MBLs)

Metallo-β-lactamases were detected by two phenotypic methods: combined disc test (CDT) (Lee et al, 2003) and IMP + IMP-EDTA Ezy MIC Test (Himedia)

#### 3.6.1. Combined disc test

An overnight broth culture of the isolates of *P. aeruginosa* (opacity adjusted to 0.5 Mcfarland standards) was used to inoculate the plates of Mueller-Hinton agar (Himedia). An imipenem disc 10µg was initially placed on MHA. Another disc containing imipenem/EDTA in the ratio 10µg/750µg was placed at a distance of 24mm from the first disc. After an incubation of 24h at 37°C, an increased zone of IMP/EDTA by 7mm or more as compared to IMP disc alone was considered to be due to MBL producing isolate of *P. aeruginosa.*
3.6.2 Ezy MIC test
An inoculum (0.5 Mcfarland densities) was prepared from a 24 hr culture of the test strain. It was then inoculated on a MHA plate with cotton swabs. The Ezy MIC strips (Hi-Media) containing concentrations of this antibiotic at one end and similar concentrations of imipenem at the other end along with a fixed concentration of EDTA was then applied, and the plates incubated at 37°C for 18 to 24 hrs. The results were interpreted according to the interpretive criteria for MBL detection as given in Table 3.4.

3.7. Molecular characterization of the multidrug resistant and phenotypically MBL positive isolates
The isolates were characterized at the molecular level for detection of integron class each MBL positive isolate had and detection of bla\textsubscript{IMP1} and bla\textsubscript{VIM2} gene by PCR assays and nucleotide sequencing of the amplicons as discussed below:

3.7.1 DNA extraction
DNA of all the 27 MBL positive isolates was extracted according to the protocol given by Wilson (1997). Nutrient broth in 5ml volume in each tube was inoculated with \textit{P. aeruginosa} isolates individually and incubated for 24h. Bacterial culture in 2ml volume was centrifuged for 10 min to get a compact pellet. The pellet was resuspended in 567μl TE buffer by repeated pipetting. 10% SDS in 30 μl volume and 3 μl of 20mg/ml proteinase K were then added to give a final concentration of 100μg/ml proteinase K in 0.5% SDS. The contents were thoroughly mixed and incubated for 1 h at 37°C. 5M NaCl in a volume of 100μl was then added and mixed thoroughly. Cetyl trimethyl ammonium bromide (CTAB)/NaCl) solution in 80μl volume was added mixed thoroughly and incubated at 65°C for 30 min. To this equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) mixture was added and mixed thoroughly, centrifuged for 10 minutes. Aqueous supernatant was removed to a fresh microcentrifuge tube, leaving behind the interface. An equal volume of phenol/chloroform/isoamyl alcohol was again added and the contents were centrifuged for 10 minutes at 10,000 rpm. The supernatant was transferred to a fresh tube. In order to precipitate the DNA, 600μl of 70% ethanol was added, the tube was shaken back and forth until a stringy white DNA precipitate was
clearly visible. The precipitate was pelleted by spinning briefly at room temperature. The pellet was re-dissolved in 100 μl of TE buffer and kept at -20°C until used.

3.7.2 Quality assessment of DNA
The DNA stock samples were quantified using spectrophotometer at 260nm and 280 nm using the convention that one absorbance unit at 260 nm wavelength equaled 50 μg DNA per ml. Purity of DNA was determined on the basis of optical density ratio at 260:280 nm. The DNA having ratio between 1.8 to 2.0 values was considered to be of good purity. Concentration of DNA was estimated using the formula

\[
\text{Concentration of DNA (mg/ml) = OD}_{260} \times 50 \times \text{Dilution factor}
\]

Quality and purity of DNA were assessed by agarose gel electrophoresis. Agarose 0.8% (w/v) in 0.5X TAE (pH 8.0) buffer was used for submarine gel electrophoresis (Sambrook et al., 2001). Ethidium bromide (1%) was added for staining the DNA band. The wells were charged with 5 μl of DNA preparations mixed with 1 μl gel loading dye (Bromophenol blue 6x). Electrophoresis was carried out at 80V for 30 minute at room temperature. The DNA was visualized under UV transilluminator.

3.7.3 PCR assay for the detection of MBL genes $bla_{IMP1}, bla_{VIM2}$
All the 27 isolates, having MBL phenotype were subjected to PCR amplification. The genomic DNA was used as a template for the amplification of MBL genes. The amplification was carried out in 50μl reaction volume containing 10x PCR buffer (1.5μl), genomic DNA (5 μl) , 2mmol/L of each dNTP (5 μl), 20 pmol of each primer (1 μl) and 5U/ μl and sterile PCR grade water (Himedia) to a final volume to 50 μl. Amplification was performed in thermal cycler (AB Biosystems). The reaction conditions were as follows: one cycle of initial denaturation at 95°C for 4 min, followed by 35 cycles of amplification each consisting of denaturation at 95°C for 40 sec, annealing at 53°C for 30 sec, extention at 72°C for 45 sec. Final extension of the strand was allowed at 72°C for five min. The primers employed were: $bla_{IMP-1}$ Forward: 5’-ATTGCTACCGCAGCAG AGTC-3’ and Reverse -5’-TTTCAAGAGTGATGCTCC-3’ and $bla_{VIM-2}$ Forward: 5’-
TTTGACCGCGTCTATCATGGC-3' and Reverse: 5'-CAACGACTGAGCGATTTGTG-3'.

3.7.4 PCR assay for detection of integron classes (int1, int2 and int3) in the *P. aeruginosa* isolates

The DNAs of all the multidrug-resistant isolates as described earlier were subjected to PCR amplification. The DNA extracted in above manner was used as a template for the amplification of integrase (int) genes by polymerase chain reaction. The amplification was carried out in 50μl reaction volume containing 10x PCR buffer (1.5μl), genomic DNA (50μg/1μl), 2mmol/L of each dNTP (5 μl), 20 pmol of each primer (1 μl) and 0.5 μl of Taq polymerase (5U/ μl) and sterile grade water (Himedia) was added to make final volume to 50 μl. Amplification was performed in thermal cycler (AB Biosystems). The reaction conditions used were as follows: one cycle of initial denaturation at 95°C for 4 min, followed 35 cycles of amplification. Each cycle consisted of denaturation at 95°C for 40 sec, annealing at 58°C (int1), 53°C (int2), 55°C (int3), for 30 sec and extension at 72°C for 45 sec. Final extension of the strands was done at 72°C for 7 min. The primers employed were: *int1* Forward: 5'-CCTACCTCTCATACTTAGG-3' and *int1* Reverse: 5'-GTGCCTTCCATCCGTTTCCAC-3'; *int2* Forward: 5'-CACGGATATGCGACAAAGGTTCATTTTGAG-3' and *int2*-Reverse: 5'-GTAGCAAACGAGTGACGAAATG-3'; *int3* Forward: 5'-GCCTGCAGCGACTTTCAG-3' and *int3*- reverse: 5'-ACGGATCTGCCAACCTGACT-3'.

3.7.5 PCR assay for the detection of sul1 gene

The DNA was extracted as discussed above and used as a template for the amplification of sul1 gene employing the following primers: Forward: 5'-TGGTGACGGTGTTGCAGATTTGATC-3' and Reverse: 5'-GTTTCCGAGAAGGTTGCAGATTTGAG-3'. The amplification was carried out in 50μl reaction volume containing 10x PCR buffer (1.5μl), genomic DNA (50μg/1μl), 2mmol/L of each dNTP (5 μl), 20 pmol of each primer (1 μl) and 5U/ μl and sterile grade water (Himedia) to a final volume to 50 μl. Amplification was performed in thermal cycler (AB Biosystems). The reaction conditions were as follows: one cycle of initial denaturation was done at 95°C for 4 min, followed by 35 cycles, each consisting of
denaturation at 95°C for 40 sec, annealing at 56°C for 30 sec and extension at 72°C for 45 sec. Final extension was carried out at 72°C for 7 min.

3.7.6 Visualization of PCR products
To confirm the expected size of amplified target DNA, the PCR products from each tube were mixed with one μl of 6x gel loading dye were electrophoresed on 1.2% agarose gel containing ethidium bromide at constant 5v/cm for 30 minute in 0.5 X TAE buffer. The amplified products were visualized under UV transilluminator documented by gel documentation system.

3.7.7 Nucleotide sequencing of amplicons
The amplicons of different targeted genes were sequenced for their nucleotides, which was carried out by a commercial sequencing facility, Molecular Diagnostics and Research Laboratories (MDRL) Chandigarh (India). The nucleotide sequence variability in int1, sul1 and blavIM2 of selective P. aeruginosa isolates was detected by multiple sequence alignment of nucleotide sequences using CLUSTAL OMEGA with the homologous gene sequences obtained by BLAST analysis of the sequences with sequences published by National Centre for Biotecnological Information (NCBI).