3. MATERIALS AND METHODS

This chapter deals with the materials used and research methodologies employed to achieve the specified objectives of the study. All the materials, chemicals, glasswares and equipments used in the experiments along with the detail of manufactures are mentioned in annexure-1.

The study was conducted during period December, 2012 to December, 2015 at the department of Microbiology of Shoolini University of Biotechnology and Management Sciences, Solan in the state of Himachal Pradesh. The research proposal was approved by the Institute Ethics Committee (IEC) of the university as communicated through its letter no. SUIEC/13/29, dated 10th April, 2013.

3.1 Pseudomonas aeruginosa (isolates / strains)

3.1.1 Procurement of clinical isolates

A total of 100 isolates of *P. aeruginosa* recovered from human patients at Indira Gandhi Medical College (IGMC), Shimla, Himachal Pradesh were collected during one year period (December, 2012 to December, 2013) and characterized in the Microbiology laboratory of the university.

3.1.2 Standard / reference strains

*P. aeruginosa* strain ATCC 27853 (Hi- media, Mumbai) was used as quality control strain whereas *E. coli* ATCC 25922 (Hi- media, Mumbai) and *K. pneumoniae* ATCC 700603(Hi- media, Mumbai) were used as quality control strains in the assay for extended spectrum beta - lactamase (ESBL) detection.

3.2 Confirmation of the isolates

Based on the cultural characteristics of the isolates on different media, microscopic examination of their Gram stained preparations and biochemical tests; they were confirmed as *P. aeruginosa*. Subculturing of the isolates was done on the following media and their compositions are given in Annexure-II;

3.2.1 Nutrient agar

The medium was prepared and then poured aseptically in petri dishes in a biological safety cabinet. The plates were inoculated with bacterial isolates and a plate without
inoculum was kept as a control in order to detect the environmental contamination, if any. The plates were incubated at 37°C overnight and the cultural characteristics recorded.

### 3.2.2 Blood agar

Blood agar medium was prepared according to manufacturer’s instructions. The petri plates containing this medium were inoculated with bacterial isolates and a control plate was placed to check the environmental contamination, if any. The plates were incubated overnight at 37 °C and observed for hemolysis.

### 3.2.3 MacConkey agar

The bacterial isolates were inoculated in petri plates containing this medium along with a control to detect environmental contamination and incubated overnight at 37 °C and observed for the colour of the colonies.

### 3.2.4 Pseudomonas isolation agar

Pseudomonas isolation agar base is a selective medium for the isolation of *P. aeruginosa* from clinical and non-clinical specimens. This medium was inoculated with bacterial isolates. One control plate was kept as control for detecting environmental contamination, incubated at 37°C. The plates were observed for colony characteristics the following day.

### 3.3 Gram’s staining

Gram’s staining is a common technique which differentiates Gram positive and Gram negative bacteria on the basis of the stain the bacterial cells take up and the morphology of the bacterial cells. The procedure is described below:

A thin smear of bacterial suspension was prepared on a clean, grease-free slide. The smear was heat fixed on a flame by passing the slide over a bunsen burner 3-4 times. After fixation, the smear was flooded with the primary stain (crystal violet) and incubated for one minute. The excessive stain was drained off and smear washed gently under tap water. Gram's iodine solution was then laid over the smear for one minute followed by washing under tap water until excess of the iodine solution was removed. The smear was then decolorized by adding acetone or alcohol. It was again washed gently with a stream of water. The slide was flooded with secondary stain...
(safranin) and incubated for one minute. The smear was again washed in a gentle stream of water followed by blot drying. The stained smear was examined under the oil immersion lens of the microscope. Gram positive bacteria retain the primary stain (crystal violet), as a result the bacteria appear violet/purple under a microscope while Gram negative bacteria lose the primary stain and take the secondary stain which imparts red or pink colour to the bacteria.

3.4 Biochemical characterization of isolates of *P. aeruginosa*
For further characterization, certain standard biochemical reactions such as oxidase, catalase, motility, carbohydrate fermentation, nitrate reduction, methyl red/Voges-Proskauer, indole production, urease and citrate utilization test etc. were performed. These tests are discussed below:

3.4.1 Oxidase test
Oxidase discs are used for the detection of oxidase production by microorganisms. *Neisseria, Alcaligenes, Aeromonas, Vibrios, Campylobacter* and *Pseudomonas* give positive reactions and *Enterobacteriaceae* is negative for oxidase production. Oxidase reaction was carried out by touching and spreading a well isolated colony on the oxidase disc (Hi-media, Mumbai). The reaction was observed within 5-10 seconds. Development of purple or violet colour indicated oxidase positive organism. A change later than 10 seconds or no change at all is considered negative reaction.

3.4.2 Catalase test
This test was done to determine the presence of catalase enzymes. In this test, one drop of hydrogen peroxide (3%) reagent was placed on slide with pasture pipette and a pure colony of bacterial culture picked up with wooden stick was placed over it. The formation of effervescence or bubbles indicated positive result.

3.4.3 Motility test
The ability of an organism to move by itself is called motility. This test is done to determine if an organism is motile or non motile. Semi solid medium was inoculated with a straight wire about 8-10mm deep into the medium only once and incubated at
37°C for 24 h. Diffused growth from the stab line showed positive motility test whereas growth restricted to stab line reflects the bacteria species is non motile.

3.4.4 Carbohydrate fermentation test

This test determines the ability of an organism to degrade and ferment carbohydrates (glucose, sucrose, lactose, maltose and xylose etc.) with production of an acid or acid and gas. Four tubes containing four different kind of broth namely glucose, lactose, sucrose and mannitol which consist of 1% sugar, 1% peptone water and 1% Andrade’s reagent were taken. Inverted Durham tube placed in each tube was in inverted position. Autoclaving was done at 10 lb pressure. Each tube was inoculated with bacterial culture and incubated at 37°C for 24 h. Pink colour formation indicated acid production while cream or yellow colour with slight pink tinge showed no fermentation of carbohydrates. Production of pink colour and accumulation of gas in Durham tube indicated generation of both acid and gas. *P. aeruginosa* is a non-fermenter while certain other bacteria *Escherichia coli*, *Proteus mirabilis*, *Shigella dysenteriae* are capable of fermenting glucose.

3.4.5 Nitrate reduction test

This test differentiates bacteria on the basis of their ability or inability to reduce nitrate (NO$_3^-$) to nitrite (NO$_2^-$) or other nitrogenous compounds via the action of the enzyme nitrate reductase. This test is important in the identification of both Gram-positive and Gram-negative bacteria.

For performing this test, nitrate broth was prepared by suspending 3.9 grams in 100 ml of distilled water, dispensed in tubes and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The medium was inoculated and incubated at 37°C for 24 h. Test reagents (sulfanilic acid and alpha-naphthylamine) in a volume of 0.5 ml were added to the 5 ml incubated culture. Pink to deep red colour indicated positive test i.e. nitrates were reduced to nitrites. If no red color forms upon addition of test reagent, this indicated that either the nitrate has not been converted to nitrite, or nitrate was converted to nitrite and then immediately reduced to some other form of nitrogen. Zinc dust was therefore, added to the broth to further confirm this. No colour development indicated absence of nitrate in the medium which was a positive result while development of pink to deep red colour showed negative test which means nitrate was present in the medium but not reduced by the organism.
3.4.6 Indole production test
This test determines the ability of an organism to split indole from amino acid tryptophan and is generally performed to differentiate species of the family *Enterobacteriaceae*. In this test, 24 h cultures in peptone water were incubated at 37°C. Kovac’s reagent (4-5 drops) was added to it. The production of red colour indicates the production of indole from tryptophan in a positive case.

3.4.7 Methyl red and Voges-Proskauer test
Methyl red (MR) and Voges-Proskauer (VP) tests are generally used in the biochemical identification of bacterial species. These tests are based on the detection of specific breakdown products of carbohydrate metabolism. MR/VP broth in a volume of 5 ml taken in test tubes and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min. Test isolates were inoculated in test tubes containing MR/VP broth and incubated 37°C for 24 h. Four to five drops of MR/VP reagents were added to 24 h old MR/VP culture. The appearance of red or pink colour indicated a positive reaction.

3.4.8 Citrate utilization test
Citrate utilization test determines capability of an organism to utilize citrate as the sole source of carbon for their metabolism. Simmon’s citrate medium was prepared by dissolving 2.42 grams of powder in 100ml of distilled water and autoclaving was done at 121°C for 15 minutes. Slants were prepared and test bacterial colonies were streaked over the surface of the slants. Incubation was done at 37°C for 24 h. The production of blue colour indicated positive test.

3.4.9 Urease test
Urease test was done to determine the ability of an organism to hydrolyze urea by the action of urease enzyme. Urea agar base (2.40 grams) (Hi-media, Mumbai) was added in 95 ml of distilled water. The medium was heated to dissolve the medium completely and sterilized by autoclaving at 10 lbs pressure for 20 minutes followed by cooling to 50°C. Sterile 40% urea (5 ml) solution was added and mixed well. Sterile tubes were dispensed with medium and allowed to set in the slanting position. Overheating or reheating of the medium was avoided as urea decomposed very easily.
3.5 Preservation of the isolates
The isolates of *P. aeruginosa* were maintained on slants of Pseudomonas isolation agar (Hi-media, Mumbai) and preserved in 50% glycerol at -80°C. Subculturing was done on regular basis in order to maintain fresh cultures for the phenotypic tests. Frequent subculturing was avoided for preventing changes in nucleotides due to mutation/deletion or substitution which might affect the results of molecular characterization and for this purpose the subculturing was done from the stock culture.

3.6 *In vitro* cultural antibiotic sensitive assay

3.6.1 Standard antibiotic discs used in the assay
The standard antibiotic discs (Hi-media, Mumbai) used were as follows: aminoglycosides: amikacin (30µg) and gentamicin (10 µg); cephalosporins: cefepime (30 µg), ceftazidime (30 µg) and cefoperazone (75 µg); quinolones: ciprofloxacin (5 µg) and levofloxacin (5 µg); ureidopenicillins: piperacillin/tazobactum (100/10 µg) and piperacillin (100 µg); carbapenems: imipenem (10 µg) and meropenem (10 µg); monobactam: aztreonam (30 µg).

3.6.2 Disc diffusion method
The disc diffusion method of Kirby Bauer (1966) was followed. Isolated colonies (3-5) were picked up from 24 h Pseudomonas isolation agar plates and emulsified in normal saline. The turbidity of saline was measured by using spectrophotometer at 625nm. The optical density (OD) of the suspension was adjusted to 0.08 to 0.10 which is equivalent to 0.5 McFarland’s standards (Chapin and Lauderdale, 2003). The suspension at this OD value contained approximately $1 \times 10^8$ cfu/ml to $2 \times 10^8$ cfu/ml. A lawn culture of bacterial isolates was made onto the surface of Muller-Hinton agar (MHA) using a sterile cotton swab soaked with the inoculum. The inoculated plates were left at room temperature for 5-10 min. Later, antimicrobial discs as mentioned under section 3.5.1 were aseptically placed onto the inoculated MHA plates with the help of sterile forceps. Plates were incubated at 37°C for 18-24 h. The diameter of zones of inhibition were recorded and interpreted as sensitive, intermediate and resistant as per the CLSI zone interpretative criterion (M100-S22). *P. aeruginosa* strain ATCC 27853 was included as reference strain in the assay.
3.7 Detection of ESBL producing *P. aeruginosa*

3.7.1 Preliminary screening

For preliminary screening, a total of 180 isolates were used, the breakup of which is as follows: 93 isolates confirmed in the present study plus 87 isolates confirmed as *P. aeruginosa* in the laboratory in another study (Minhas et al., 2015). These isolates (n=180) were studied for ESBL production by *in vitro* antibiotic culture sensitivity assay using the following discs of cephalosporins: ceftazidime (30μg), cefotaxime (30μg), ceftriaxone (30μg), cefuroxime (30μg), aztreonam (30μg) and cefpodoxime (10μg) (Hi-Media, Mumbai, India) following the disc diffusion method of Kirby Bauer (1966). The diameters of zones of inhibition were measured after 24h of incubation at 37°C. The results were interpreted according to recommended ESBL screening criteria as specified in clinical laboratory standard institute (CLSI) protocol M100-S22.

3.7.2 Confirmatory phenotypic methods

3.7.2.1 Double disc diffusion synergy test (DDST)

ESBL production was confirmed by double disc synergy test as described by Jarlier et al., 1988. This test is a phenotypic confirmatory method. Synergy was determined between a disc of amoxyclav (20 μg amoxycillin and 10 μg clavulanic acid) and a 30μg disc of cefotaxime (3\textsuperscript{rd} generation cephalosporin). Plates were incubated at 37°C for 24 h and the diameters of zones of inhibition were recorded. The isolates that exhibited a distinct shape/size with potentiation towards amoxyclav disc were considered potential ESBL producers as specified in CLSI guidelines of 2013.

3.7.2.2 ESBL E–test

E-test ESBL strips are double ended strips with antibiotic and antibiotic/inhibitor gradients, the upper half of which is impregnated with a mixture of ceftazidime, cefotaxime and ceftepime plus clavulanic acid and tozobactam (mix\textsuperscript{+}) with highest concentration tapering downwards, whereas lower half is similarly coated with mixture of ceftazidime, cefotaxime and ceftepime (mix) in a concentration gradient in reverse direction. We can directly record the minimum inhibitory concentration (MIC) as well as the ratio of the MICs of the mix and mix\textsuperscript{+}, which determines the
presence of an ESBL. The results were interpreted as: if the value of the ratio between 
MIC for mix and MIC for mix’ was equal to 8 or more, the isolate was considered 
ESBL producer, according to the instruction of manufacture.

*E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as negative and 
positive control strains respectively in the E test.

### 3.7.3 Molecular characterization

#### 3.7.3.1 Extraction of genomic DNA of ESBL producers

The genomic DNA was extracted from all the fifty six ESBL producing *P. aeruginosa*
isolates using invitrogen kit (cat. no. K1820-01) as follows:

Overnight cultures of ESBL producing *P. aeruginosa* isolates were used for the 
extraction of genomic DNA using Invitrogen Pure Link DNA kit. The bacterial cells 
were harvested by centrifugation at 10,000 × g for 2 min. The cell pellet was 
suspended in 180μl of pure link genomic digestion buffer. Proteinase K in a volume 
of 20μl was added to the content and briefly vortexed. Tubes were incubated at 55°C 
for 30 min in a water bath with occasional vortexing until lysis was completed. 20μl 
of RNase A was added to the lysate, mixed well by brief vortexing and incubated at 
room temperature for two min. Pure link genomic lysis / binding buffer in a volume of 
200μl was added and mixed well by vortexing again to obtain a homogenous solution. 
Ethanol (96–100%) in a volume of 200μl was then added to the lysate. The mixture 
was vortexed to obtain a homogenous solution. Purification of extracted DNA was 
carried out by adding the lysate to the spin column and centrifuging it at 10,000 × g 
for one min at room temperature. After discarding the filtrate, the collection tube was 
discarded and spin column was placed into another collection tube provided in the kit. 
Washing of DNA was then carried out in two steps. The column was washed with 
500μl of wash buffer 1 containing 95% ethanol; then column was centrifuged at 
10,000 × g for one min at room temperature. The filtrate was discarded and the 
column was placed into a new collection tube and washed with 500μl of wash buffer 
2, centrifuged at 16,000 × g for 3 min at room temperature. The spin column was 
removed and placed in a sterile micro-centrifuge tube. Pure link genomic elution 
buffer (25–200μl) was added to the column and incubated at room temperature for 
one min. The column was centrifuged at 12,000 × g for one min at room temperature.
The tubes containing purified DNA were stored at -20°C for further molecular studies.

### 3.7.3.2 Amplification of ESBL genes of *P. aeruginosa* isolates

All the 56 isolates of *P. aeruginosa* which were detected ESBL positive by the phenotypic methods were further subjected to molecular characterization. For this purpose, the partial amplification of selective ESBL genes (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{PER}$ and *bla*$_{CTX-M}$) was undertaken. Primer pairs used in the PCR assays and reaction conditions employed are given in Table 3.1 and Table 3.2 respectively.

In the PCR assays, the reference sequences of all the selected genes were downloaded from Gene Bank and primers were designed with the help of Primer3Plus tool. The primer sequences matched with the *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{PER}$ and *bla*$_{CTX-M}$ gene precursors of reported *P. aeruginosa* strains by BLASTn analysis. The forward primer of *bla*$_{TEM}$ gene of *P. aeruginosa* strain (accession no. AY775131.1) was derived from nucleotide no. 215 to 234 while the reverse primer was derived from nucleotide no. 619 to 638 of this strain. The forward primer of *bla*$_{SHV}$ gene of *P. aeruginosa* B2781 strain (accession no. AM988779.1) matched to nucleotide no. 1,147 to 1,166 while the reverse primer matched to nucleotide no. 1,656 to 1,675 of this strain. The forward primer of *bla*$_{PER}$ gene of *P. aeruginosa* strain (accession no. AY953376.2) was derived from nucleotide no. 32 to 51 while the reverse primer was derived from nucleotide no. 319 to 338. The forward primer of *bla*$_{CTX-M}$ gene of *Escherichia coli* strain I-6973 (accession no. KT211409.1) was derived from nucleotide no. 483 to 502 while the reverse primer was derived from nucleotide no. 963 to 981.

### 3.7.3.3 Electrophoresis of amplicons of *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{PER}$ and *bla*$_{CTX-M}$ genes

All the PCR products were electrophoresed in 1.5% agarose gel containing 0.2µg/ml ethidium bromide (EtBr) at constant current of 90mA. The PCR products were loaded in the wells using gel loading buffer containing dye. DNA bands were visualized by gel documentation system.
3.7.3.4 Nucleotide sequencing of the amplicons of \(\text{bla}_{\text{TEM}}, \text{bla}_{\text{SHV}}, \text{bla}_{\text{PER}}\) and \(\text{bla}_{\text{CTX-M}}\) genes of \(P. \text{aeruginosa}\) isolates

The nucleotide sequencing of the amplicons of \(\text{bla}_{\text{TEM}}, \text{bla}_{\text{SHV}}, \text{bla}_{\text{PER}}\) and \(\text{bla}_{\text{CTX-M}}\) genes of \(P. \text{aeruginosa}\) isolates were done by commercial facility. The nucleotide sequences were compared with published nucleotide sequences of standard strains and their homologies were determined. The nucleotide sequences of the amplicons of these genes have been submitted to the National Centre for Biotechnology Information (NCBI) and their accession numbers have been granted.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Product size</th>
<th>Reference</th>
</tr>
</thead>
</table>
| 1.      | \(\text{bla}_{\text{TEM}}\) | F: TTCTGCTATGTGGGTGGTGAAGTA  
R: TTATCGGTCCTCCATGCAGTC | 424bp        | Present study      |
| 2.      | \(\text{bla}_{\text{SHV}}\) | F: AAACGGAACCTGAATGAGGCG  
R: ATACATAGGGGACGACGT | 530bp        | Present study      |
| 3.      | \(\text{bla}_{\text{PER}}\) | F: CAACCTGGAATGACCATCTAGGT  
R: AAAGGTGCTGAGATCTC | 307bp        | Present study      |
| 4.      | \(\text{bla}_{\text{CTX-M}}\) | F: GACGATGTCACTGGCTGAGC  
R: AGCCGGGACGCTAATACA | 499bp        | Present study      |
## Table 3.2 Amplification of \textit{bla}_{TEM}, \textit{bla}_{SHV}, \textit{bla}_{PER} and \textit{bla}_{CTX-M} genes of selective \textit{P. aeruginosa} isolates by PCR

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<th>Reaction conditions</th>
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<td></td>
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<td>Extention</td>
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<td>Primers</td>
<td>Final extension</td>
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