Material and Methods:

Study Design:
Prospective study of observational analytical design.

Place of Study:
Rama Medical College and Hospitals

Study Period:
The study will be conducted in duration of two years 6 months.

Study Population:
- All clinically isolated Pseudomonas aeruginosa from sputum, urine, blood, pus, wound swab, otitis media and aspirated fluids will be collected from the IPD and OPD patients (both males and females of all age groups), sent to the microbiology laboratory for the culture identification and sensitivity testing will be included in the study.

Sample Size determination:
Sample size can be calculated by following formula
\[
\text{Sample size} = \frac{2(p)(1-p)(Z_{\text{p}}+Z_{\text{w}})^2}{d^2}
\]
\[Z_{\text{p}} = \text{It is standard normal variate} \ [\text{at 5\% type I error}(p<0.05) \text{it is 1.96}]
\]
\[Z_{\text{w}} = \text{Power of the test. It is 0.84 for 80\% power.}
\]
\[P = \text{expected proportion in population based on previous study. According to previous studies it may not be more than 15\%}
\]
\[d = \text{Expected difference of incidence from various studies. So, its 0.1}
\]

Sample size = \[
\frac{2(0.15)(0.85)(0.84+1.96)^2}{(0.1)^2}
\]
=199.7
So, for this study sample size to be taken should be 200.

So, a total of 200 isolates of Pseudomonas aeruginosa will be isolated from different clinical isolates.

Inclusion criteria
The Patients that are not on Carbepenem drug treatment will be included in the study.

Exclusion criteria
Patients taking Carbepenem drugs previously in treatment will be excluded from the study.
**Ethical consideration**

Ethical clearance will be taken from ethical committee.

**Methodology:**

All samples will be collected and routinely cultured on MacConkey and blood agar plates. The organisms will be identified by their colony characteristics, staining procedures, pigment production, motility and other relevant biochemical reactions as per standard laboratory methods for identification of bacteria[61, 62].

**MBL screening methods:**

- Antimicrobial susceptibility of Pseudomonas aeruginosa will be performed by the disc-diffusion method (Modified- Kirby baur disc diffusion method) as per CLSI guidelines[60].
  
  Isolates resistant to imipenem, meropenem, ertapenem, and third generation cephalosporin will be considered as screening positive. Stock cultures will be maintained.

  Quality control strains that will be used are *Escherichia coli* - American type culture collection(ATCC) 25922 and *Pseudomonas aeruginosa* – ATCC 27853 [60].

**Phenotypic Detection of Metallo β-Lactamase activity:**

1. **Modified Hodge Test:**

   The modified Hodge Test (MHT) detects carbapenemase production in gram negative isolates. An overnight culture suspension of *Escherichia coli* ATCC 25922 adjusted to 0.5 McFarland will be inoculated using a sterile cotton swab on the surface of Muller-Hinton agar(MHA). After drying, 10 µg imipenem disk(Hi-Media, Mumbai, India) will be kept at the centre of the MHA plate and the test strains suspesion will be inoculated by streaking method from the edge of the imipenem disc to the periphery of the petriplate in four different directions. The plates will be incubated overnight at optimum temperature. If the test stain will be carbenemase producing there will be the presence of “cloverleaf shaped” zone of inhibition. The test organsism will be considered as Metallo-beta lactamase (MBL) positive.[63][Fig 1]

2. **Imipenem(IMP)- EDTA Combined disc test:**
The test organisms will be inoculated by lawn culture technique on the plates of Muller-Hinton agar (MHA) as recommended by CLSI[1]. 10 µg Imipenem Disk and 750 µg Imipenem-EDTA Disk (Hi-media SD281) will be placed on the plate. The inhibition zones of the imipenem and imipenem-EDTA disks will be compared after 16 to 18 hours of incubation at 37°C. In the combined disc test, if the increase in inhibition zone with the imipenem and EDTA disc will be $\geq 7$ mm than the imipenem disc alone, it is considered as MBL positive.[64] [Fig 2]

3. **MBL E test:**

The E-test MBL Strip contains a double sided seven-dilution range of IP(Imipenem) (4 to 256 µg/ml) and Imipenem (1 to 64µg/ml) in combination with a fixed concentration of EDTA is considered as the most sensitive method for MBL detection[30]. The E-test will be done according to manufacturer’s instructions. MIC ratio of IP/IP (Imipenem+EDTA) of $>8$ or $>3$ log dilutions indicates MBL production.[65] [Fig 3]

**Phenotypic Detection of Biofilm production:**

- A loopful of bacterial culture of isolates to be screened will be inoculated into separate test tubes containing 3ml of brain heart infusion broth (BHIB).
- 200µl of each bacterial suspension will be transferred to the microtiter plate wells and will be covered and then incubated at 37°C for 24 hours.
- After incubation, the contents of the each wells will be aspirated 3 times with 250µl of PBS.
- The remaining bacteria will be fixed with 200 µl of 99% methanol per well and plate will be emptied and left to dry for 15min.
- Then the wells of plates will be stained for 5min with 0.2ml of 2% crystal violet (Hi-media- Mumbai). Excess stain will be removed by gentle pipetting. Final wash will be given 3 times with PBS (170 µl).
- The plates will be air dried, the dye bound to the adherent cells.
- Optical density (OD) of each wells were measured at 595nm by using Bio-rad model 550 microtiter plate reader.
Fig 1: Modified hodge test

Fig 2: Imipenem (IMP) - EDTA Combined disc test

Fig 3: E-test
Genotypic Detection of Metallo β- Lactamase:

All the Metallo β- lactamase producing strains will be identified for blaNDM genes by PCR assays. The oligonucleotides blaNDM are:

- **blaNDM- Forward** 5'-CTGAGCACCACATGAGCC-3'
  
- **blaNDM- Reverse** 5'- GGCCGTATGAGTATGTTGC-3'[Preifer y. 2011]

All of the steps related to DNA extraction and PCR will be done according to the kit's instructions.

Statistical analysis:

Both descriptive and inferential statistical methods will be used to analyze the data. Specifically sensitivity, specificity, positive predictive value and negative predictive value will be calculated.