CHAPTER-4

MATERIALS AND METHODS

4.1. DURATION OF SAMPLE COLLECTION

December 2009-December 2011

4.2. INCLUSION AND EXCLUSION CRITERIA

Inclusion criteria:

➢ Samples with pure and predominant growth of Acinetobacter
➢ Presence of pus cells

Exclusion criteria:

➢ Insignificant or mixed growth of bacteria
➢ No valid clinical history

During this period all isolates of genus Acinetobacter satisfying inclusion criteria were included in the study. The isolates were obtained from various specimens like urine, pus, blood, and sputum from the patients admitted to various wards of SRM hospital.

A detailed clinical history and clinical data were recorded from patients whose samples grew Acinetobacter. The proforma included the person’s name, age, sex, admitted ward, clinical diagnosis and previous antibiotic therapy. To check the prevalence of Acinetobacter in hospital environments, and to trace out the source of infection, environmental swabs were collected from various wards which included ICU, postoperative, and surgical wards.
4.3. COLLECTION OF SPECIMEN

Clinical specimens: Pus, sputum, aspirates, blood, urine.

Environmental samples: Swabs collected from various wards (Intensive care unit, postoperative, and surgical wards)

Processing of specimens:

i. Performance of Gram staining. (Direct Smear)

ii. Specimens inoculated onto MacConkey agar, 5% Sheep Blood agar, CLED agar. Incubated overnight at 37°C aerobically (MacConkey agar and CLED agar), and in 5% CO₂ (Blood agar).

iii. Performance of Motility Test.

iv. Isolates fully identified using Conventional Biochemical methods according to Standard microbiological techniques.

v. The strains were tested for susceptibility to the following antibiotic agents by Kirby Bauer techniques on Mueller Hinton agar as per CLSI guidelines (2012): Amikacin (30µg), Gentamycin (10µg), Cefuroxime (30µg), Cephotaxime (30µg), Ceftriazone (30µg), Ceftazidime (30µg), Imipenem (10µg), Polymyxin B (300 U), Ofloxacin (5µg).

vi. Imipenem resistance by disk diffusion) were further tested and confirmed by minimum Inhibitory concentration (MIC) by Agar Dilution technique as per CLSI guidelines.

4.4. ANTIMICROBIAL SUSCEPTIBILITY TESTING

i. Four or five identical colonies picked from overnight growth in Nutrient agar, were inoculated into 4 to 5ml of peptone water and incubated at 37°C until turbid and compared with 0.5 McFarland standard.
ii. After standardization, a sterile cotton swab was immersed in the suspension and a lawn culture was made on the surface of Mueller Hinton agar (MHA) supplemented with 4% NaCl.

iii. Each disc was made to adhere perfectly by gently pressing using a sterile forceps and placed 20mm apart.

4.5. AGAR DILUTION METHOD (MIC)

i. For agar dilution, the antimicrobial concentrations and microorganisms to be tested were brought together on an agar based medium rather than in broth.

ii. Each doubling dilution of an antimicrobial agent was incorporated into single Mueller Hinton agar plate.

iii. The surface of each plate inoculated with $1 \times 10^6$/spot.

iv. Incubated at $37^\circ$C aerobically for 24hrs.

v. Plates were examined for growth and compared with positive growth control plate without the antibiotic agent.

vi. By this method, one or more bacterial isolates were tested per plate. MIC is the lowest concentration of an antimicrobial agent in agar that completely inhibits visible growth.

According to CLSI standard the expected breakpoints are follows:

Imipenem:

- $\leq 4 \mu g/l$: Sensitive.
- $8 \mu g/l$: Intermediate.
- $\geq 16 \mu g/l$: Resistance.
4.6. METALLO- β-LACTAMASE DETECTION

Imipenem EDTA Disk synergy test.

i. Preparation of 0.5M EDTA solution: 186.1g of disodium EDTA was dissolved in 1000ml of distilled water and pH adjusted to 8.0 using NaOH. The mixture was then sterilized by autoclaving.

ii. Preparation of EDTA imipenem disks: EDTA solution was added to 10µg Imipenem disks to obtain a concentration of 750µg. Disks were dried immediately in an incubator and stored at 4°C in an air tight vial without desiccant.

iii. Test strains were adjusted to the 0.5Mcfarland standard and were inoculated to Mueller Hinton agar.

iv. 10µg Imipenem disk and Imipenem plus 750µg EDTA were placed 15mm apart on Mueller Hinton agar, Zone diameter difference of ≥ 7mm between Imipenem and Imipenem plus EDTA was interpreted as EDTA synergy positive.

4.7. MODIFIED HODGE’S TEST (MHT)

i. Modified Hodge’s test detects carbapenamase production in isolates of Enterobacteriaceae. Carbapenemase production is detected by the MHT when the test isolate produces the enzymes and allows growth of carbapenem susceptible strains (E.coli ATCC 25922) towards a carbapenem disk.

ii. 0.5 Mc farland dilute of E.coli was prepared in 5ml saline.

iii. A lawn culture of E.coli was prepared on a MHT plate and allowed to dry 3-5mins.

iv. A 10µg Imipenem disk was placed in center of the test area.

v. In a straight line, test organism was streaked from the edge of the disk to edge of the plate. Upto four organisms can be tested on the same plate with one drug.
vi. Plate was incubated overnight at 35°C and examined for a clover leaf type indentation at the intersection of the test organism and the E.coli, within the zone of inhibition of the carbapenem susceptibility disk.

4.8. POLYMERASE CHAIN REACTION (PCR)

Primer Sequence:

OXA23-F-AGTATTGGGGCTTGTGCT
OXA23-R-AACTTCCGTGCTATTTG

OXA58-F-ATGCAAAGTGGAATTGCAACG
OXA58-R-CCCCAGCCACTTTTAGCATA

NDM1-F-GCGCAACACACGCCTGACTTT
NDM1-R-CAGCCACCAAAAGCGATGTC

PCR Procedure:

1. **Table 4.1.** Reactions set up as follows;

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Master mix</td>
<td>25µl</td>
</tr>
<tr>
<td>OXA-23 Primer Mix</td>
<td>1µl</td>
</tr>
<tr>
<td>(10pmoles/µl)</td>
<td></td>
</tr>
<tr>
<td>OXA-58 Primer Mix</td>
<td>1µl</td>
</tr>
<tr>
<td>(10pmoles/µl)</td>
<td></td>
</tr>
<tr>
<td>NDM Primer Mix</td>
<td>1µl</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>1µl</td>
</tr>
<tr>
<td>Water, nuclease free</td>
<td>21µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>50µl</td>
</tr>
</tbody>
</table>
2. Mixed gently and spin down briefly.

3. Placed into PCR machine and programmed as follows;

Initial Denaturation: 94ºC for 3 min

Denaturation: 94ºC for 1 min

Annealing: 55ºC for 1 min

Extension: 72ºC for 1 min

Final extension: 72ºC for 5 min

**Agarose gel electrophoresis:**

2% agarose was prepared. (2gm agarose in 100ml of 1X TAE buffer and melted using micro oven). When the agarose gel temperature was around 60ºc, 5µl of ethidium bromide was added. Warm agarose solution slowly was poured into the gel platform. The gel was set undisturbed till the agarose solidified. 1X TAE buffer was poured into submarine gel tank. Carefully the gel platform was placed into tank. The tank buffer level was maintained 0.5cm above the gel. PCR samples were loaded after mixing with gel loading dye along with 10µl HELINI QuickRef 250 bp DNA Ladder. Electrophoresis was run at 50V till the dye reached three fourth distance of the gel. Gel viewed in UV Transilluminator and observed the bands pattern.

PCR Product size:

OXA58 – 233bp,

OXA23 – 453bp

NDM-1 – 1000bp

QuickRef® 250bp DNA Ladder [100bp, 250bp, 500bp, 750bp, 1000bp, 2000bp, 3000bp, and 4000bp]