MATERIALS AND METHODS
CHAPTER 13

13. MATERIALS AND METHODS

13.1 Bacterial isolates:

The study was conducted in 860 bedded JIPMER hospital, Pondicherry, for about 21 months (October 1996 to July 1998). It included 66 representative Acinetobacter isolates from both clinical and environmental sources. The isolates obtained were mainly from patients admitted to Respiratory intensive care unit (RICU; six beds), pediatric ward and other medical wards. The major reasons for admission to the RICU are mechanical ventilation for respiratory failure, postoperative critical care and organ support following multiple trauma. Such admissions are usually for 2-4 days. The period of collection of samples was from October 1996 to July 1998, and the isolates included were mainly from blood, endotracheal aspirate, pus and other body fluids. A total of ninety-eight isolates were obtained during the study period from 43 patients admitted in various wards. Fifty-five isolates were obtained from eighteen patients of RICU. Remaining 43 isolates were from 25 patients admitted in pediatric ward and other medical wards. In cases, where multiple isolates were obtained from same patient, after performing preliminary biochemical testing and antibiotyping a representative strain/strains were selected for further study. The majority consisted of isolates from clinical sources (49) and the rest (17) originating from the environment. Respiratory intensive care unit (RICU) was monitored for Acinetobacter infections, as it was found to record the
highest incidence of infection in the hospital. Over all 98 isolates of *Acinetobacter* spp. were obtained from a total of 785 (12.4%) nonfermenting gram-negative bacteria encountered from different wards during the study period. The medical records were reviewed of all patients involved in the study.

Twenty-two reference strains belonging to 18 different DNA groups (assigned by DNA hybridization methods) and two strains that could not be unambiguously assigned to any DNA group (one strain is in between DNA groups 1 and 3; other strain was closely related to TU 13) were obtained from Gerner-Smidt (Serum-institut, Copenhagen, Denmark) and Bouvet (Institut Pasteur, Paris) were also included in the study as controls (Table-13.1). All strains were preserved in glycerol broth at −70°C until tested.

The study included over all 90 *Acinetobacter* strains that were investigated by using simplified and extended phenotypic identification schemes (Bouvet and Grimont, 1987; Gerner-Smidt et al 1991). Biotyping of strains belonging to Acb-complex was performed later (Bouvet and Grimont, 1987 & 1989). These strains were also analyzed by their protein profiles obtained by extracting their cell envelope protein fractions. Selected representative significant strains were fingerprinted by Random amplification of polymorphic DNA technique (RAPD) using arbitrary primers (AP-PCR) and Pulse Field Gel Electrophoresis (PFGE).
Table 13.1 List of *Acinetobacter* reference strains included in the study

<table>
<thead>
<tr>
<th>DNA group</th>
<th>Strains and Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ATCC 23055(^T) (<em>A. calcoaceticus</em>), CIP 81.08(^T)</td>
</tr>
<tr>
<td>2</td>
<td>ATCC 19606(^T) (<em>A.baumannii</em>), CIP 70.34(^T)</td>
</tr>
<tr>
<td>3</td>
<td>ATCC 17922, CIP 70.29 (=ATCC19004)</td>
</tr>
<tr>
<td>4</td>
<td>ATCC 17906(^T) (<em>A. haemolyticus</em>)</td>
</tr>
<tr>
<td>5</td>
<td>ATCC 17908(^T) (<em>A. johnsonii</em>)</td>
</tr>
<tr>
<td>6</td>
<td>ATCC 17979</td>
</tr>
<tr>
<td>7</td>
<td>ATCC 17909(^T) (<em>A. johnsonii</em>)</td>
</tr>
<tr>
<td>8/9</td>
<td>NCTC 5866 (<em>A. lwaffii</em>)</td>
</tr>
<tr>
<td>10</td>
<td>ATCC 17924</td>
</tr>
<tr>
<td>11</td>
<td>ATCC 11171</td>
</tr>
<tr>
<td>12</td>
<td>IAM 13186(^T) (<em>A. radioresistans</em>)</td>
</tr>
<tr>
<td>13</td>
<td>ATCC 17903 (TU13)</td>
</tr>
<tr>
<td>14</td>
<td>ATCC 17905 TU14 (BJ=13), CIP 64.2 (=ATCC17905)</td>
</tr>
<tr>
<td>15</td>
<td>M151 a TU 15</td>
</tr>
</tbody>
</table>

Close to TU13: 10090

Close between 1 & 3: 10095

BJ14: K.Irino 105/85 (Brazil)

BJ15: M.M.Adam Ac606 180:40 va (Hungary)

BJ16: CIP 70.18 (=ATCC17988)

BJ17: SEIP Ac87.314

ATCC - American Type Culture Collection, Rockville, Md.; CIP - Collection de l' Institut Pasteur, Paris, France. NCTC - National Collection of Type Cultures, London, United Kingdom; IAM - Culture Collection of The Institute Of Applied Microbiology, University Of Tokyo, Tokyo, Japan; SEIP - collection du Service des Enterobacteries, Institut Pasteur, Paris, France.

The reference strains provided by Dr. P.J.M Bouvet were underlined. Rest of the strains were provided by Dr. P. Gerner-Smidt.

13.2 Phenotyping:

A total of 66 *Acinetobacter* isolates, were identified using a phenotyping system involving 21-test system (acid production from glucose, gelatin hydrolysis, haemolysis and utilization of 18 carbon sources) (Bouvet and Grimont, 1987; Gerner-Smidt et al., 1991). Twenty-four reference strains belonging to different DNA groups...
were also tested simultaneously. Identification of 90 *Acinetobacter* isolates was done using a matrix constructed of phenotypical data available elsewhere (Bouvet and Grimont, 1986; Bouvet and Jeanjean, 1989; Gerner-Smidt et al., 1991). Glucose oxidation was tested in Hugh and Leifson's medium containing 1% glucose. Gelatin liquefaction was tested by the classical gelatin stab method (Barrow et al. 1993). Tests for hemolysis were done on 5% sheep blood agar plates.

### 13.2.1 Carbon Assimilation Test:

Assimilation tests were performed in a fluid medium containing a mineral base as described by Stainer et al. (1966) with the addition of an appropriate carbon source in a 0.1% (wt/vol) concentration. The following carbon sources were used: DL-lactate, Glutarate, L-phenylalanine, phenylacetate, Malonate, L-Histidine, Azelate, D-Malate, L-Aspartate, L-Leucine, Histamine, L-Tyrosine, β-Alanine, Ethanol, 2-3 Butanediol, L-Arginine, L-Ornithine and DL-4-Aminobutyrate. For biotyping of *A. baumannii* and related groups (*Acb*-complex), further four more carbon sources were used along with phenylalanine viz Levulinate, Citraconate, 4-hydroxybenzoate and L-tartarate. Substrates were tested using 4ml quantities in test tubes of 12mm diameter. The pH of the all solutions were maintained at 7.0.

A suspension of the test strain was made by mixing a loopful of a plate culture incubated overnight in 10ml of 0.9% NaCl. From this suspension 10μl was taken by a pipette and was added to the assimilation media. Solid tube media were inoculated from the same suspension with a straight inoculation wire. The incubation
temperature was 30°C for all tests except the for gelatinase production, which was performed at 22°C. All reactions were read visually after 24 and 48 hours of incubation.

In addition, the assimilation tests and gelatinase liquification reactions were read until 6 days after the beginning of incubation. The assimilation tests were scored as positive when the medium was turbid or when an abundant sediment was seen. Randomly selected tubes were subcultured on to blood agar to cross check the test results. Reactions with unclear results were repeated. Inoculated negative controls having pure mineral base without a C source were used in the assimilation studies.

13.2.2 Growth at different temperatures:

Tests for growth at 30°C (as control), 37°C, 41°C, and 44°C were performed in brain heart infusion (BHI) in water bath (Gerner-Smidt et al 1991). Tubes containing 4ml of BHI broth were inoculated with a loopful of an overnight broth culture, which was a modification over methods used by earlier workers (Bouvet and Grimont, 1986).

All these tests were selected after a detailed analysis of works carried out by the different research groups. Identification of the isolates were done using numerical approach based on the matrix constructed of phenotypical data by these earlier workers (Bouvet and Grimont, 1986, 1987; Bouvet and Jeanjean, 1989; Gerner-Smidt et al 1991; Tjernberg, 1990).
13.3 Antibiotyping:

Antibiogram typing is probably the most widely used typing method for typing Acinetobacter spp. It is possible to recognize an outbreak on the basis of constant monitoring of the resistance profiles of Acinetobacter isolates (Biendo et al., 1999). Numerous studies have used antibiotic susceptibility patterns to detect emerging resistance and to group similar isolates (Allen and Green, 1987; Bergogne-Berezin E and Joly-Guillou ML, 1985; Vila et al., 1989; Joly-Guillou et al., 1991; Struelens et al., 1993; Thurm and Ritter, 1993). Often this clustering is on the basis of MICs, breakpoints or zone diffusion sizes. In the present study, distribution of the MICs and inhibition zones of the each isolate was studied by means of histograms (Gerner-Smidt, 1994) and interpreted. Only five second line antibiotics such as cefotaxime, ceftazidime, amikacin, ciprofloxacin and ofloxacin were tested for MICs. For these antibiotics the inhibition zones by disk diffusion method were also noted. These results were used for clustering of isolates. Antibiotyping was then correlated with other typing methods.

13.4 Cell envelope protein analysis (CEPA):

The isolation of cell-envelope protein fractions and the preparation of samples for sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were performed according to the method described by Dijkshoorn et al (1987). Briefly, cells from overnight cultures were suspended in 5ml of 50mM Tris(hydroxymethyl aminomethane)Hcl, 2mM EDTA pH 8.5. This was centrifuged
at 500g. The cell pellet obtained was resuspended in 5ml of 50mM Tris(hydroxymethyl aminomethane)HCl, 2mM EDTA pH 8.5. The cells were disintegrated by ultrasonic treatments of 20s for 5 times under cold condition (Vibra cell™, Sonics & materials Inc., USA). The cell debris was centrifuged for 20 minutes at 900g. The supernatant obtained was centrifuged for one hour at 12,300g. The pellet of the cell envelope fractions obtained were resuspended in 100μl of 2mM Tris-HCl (pH 7.7). The amount of total protein in these fractions were estimated by lowry’s method (Appendix-I). Uniform amounts of protein from the samples were calibrated and used for SDS-PAGE. SDS-PAGE was performed at constant currents 30mA and 35mA for the stacking and the running gel respectively for finer separation of protein fractions. The molecular weight markers (Sigma chemicals, St Louis USA) were used in each run. The protein patterns obtained from all the isolates were scanned using densitometry and analyzed using quantity one Gel doc 2000 software system. Cluster analysis was performed using dice coefficient calculated using unweighted pair group of arithmetic method for averages (UPGAMA).

UPGAMA is based on the algorithm described below (Sneath and Sokal, 1973):

1. Begin with n clusters (one cluster for each isolate or sample)

2. Compute the similarity matrix (SM) for the samples.

3. Convert the SM into a distance matrix d using the appropriate distance formula.

4. Join the two clusters with the minimum distance into one cluster. Derive the similarity value for this cluster.

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5. Recompute the distance matrix $d$ using the cluster that was formed previously.

This is repeated until there is only one cluster.

UPGAMA - unweighted pair group method using arithmetic averages, also referred as weighted average linkage calculated using the following formula:

$$d_{ki} = \left( \frac{n_p}{n} \right) d_{pi} + \left( \frac{n_q}{n} \right) d_{qi}$$

Where $p$ and $q$ being indices indicating two clusters those are to be joined into a single cluster.

$k$ is the index of the cluster formed by joining clusters $p$ and $q$.

$i$ is the index of any remaining clusters other than cluster $p, q, \text{or} k$.

$n_p$ is the number of samples in the $p$'th cluster.

$n_q$ is the number of samples in the $q$'th cluster.

$n$ is the number of clusters in the $k$'th cluster formed by joining the $p$'th and $q$'th cluster. $n = n_p + n_q$

$d_{pq}$ is the distance between cluster $p$ and cluster $q$.

13.5 **Random Amplified Polymorphic DNA fingerprinting (RAPD)**

*or Arbitrary Primed-PCR (AP-PCR):*

Polymerase chain reaction - based DNA fingerprinting is a well described method for taxonomic and epidemiological investigations of bacterial pathogens (van Belkum, 1994). In contrast to target specific PCR, AP-PCR and RAPD analysis
generally use a single, short arbitrarily chosen primer which anneals to multiple sites on the bacterial chromosome (Welsh et al., 1990; Cactano-Anolles et al., 1991). These primers anneal sufficiently close together and in the correct orientation, intervening sequences are amplified in a PCR to give multiple products. Electrophoretic separation of these products yields patterns, or fingerprints, that may allow discrimination of strains at the subspecies level (Grundmann et al., 1995). Random amplified polymorphic DNA fingerprinting was performed for representative strains in the present study.

Phenotypically characterized significant strains were selected for RAPD analysis. A loopful of growth containing 2 to 3 small representative colonies were suspended in 100 μL of sterile distilled water in a 0.5ml microcentrifuge tubes. These microcentrifuge tubes were heated for 10 minutes at 95°C, cooled on ice, and centrifuged at 12,000g for 20 secs to remove all the cell debris. These crude DNA extracts were either frozen at -20°C or were kept on ice for immediate use. 2μL of these extracts were used in the 25μL PCR mixtures without any further purification.

For PCR, 18μL of sterile distilled water, 5μL of a primer (2μM), 200μM dNTPs together with 1U of Taq polymerase (Boehringer mannheim) was used. The primers used were: 5' -GTA AAA CGA CGG CCA GTG AA- 3' (M13 forward amplification primer) and 5' -GGA AAC AGC TAT GAC CAT GA- 3' (M13 reverse amplification primer), obtained from Pharmacia biotech, Sweden. The PCR conditions were as follows: 94°C for 2 minutes followed by 35 cycles of 94°C for 30s, 45°C for 1 min, and 72°C for 40s, followed by a final extension at 72°C for 5min. PCR end products from all isolates were analyzed by electrophoresis on 2%
(wt/vol) agarose gels prepared in Tris-borate electrophoresis buffer (TBE 89mM Tris, 89mM boric acid, 2mM EDTA). Each gel contained molecular markers in first and last lane and also had positive control (plasmid blue script PBS 250bp) and negative control. Electrophoresis was performed until the bromophenol dye had migrated 10cm. The gels were then stained with ethidium bromide for 20 min, destained and examined on a UV transilluminator. The PCR fingerprints were photographed, and the isolates were grouped visually (Grundmann et al 1997). DNA fingerprints were examined visually for similarity and fingerprints were considered highly similar if all visible bands of each isolate had the same migration distance. Heterogeneity with respect to the intensity and shape of bands was not considered to be a difference. The images of DNA gels obtained from selected isolates were captured and scanned by digital imaging system (Biorad) and analyzed using Quantity one Gel doc 2000 computer software. Similarity coefficient was determined using neighbour joining method and unweighted pair group of arithmetic method for averages (UPGAMA). Neighbour joining method is a type of phylogenetic tree is calculated based on minimizing the total branch length at each stage of clustering (Saito and Nei, 1987). The method also finds branch lengths between nodes. The approximate distance between any two samples in this tree can be found by adding the branch lengths that connect the samples. UPGAMA is based on the algorithm described by Sneath and Sokal, 1973.
13.6 *Pulse field Gel Electrophoresis (PFGE)*:

Pulse field gel electrophoresis (PFGE) is a method of choice for the epidemiological analysis of most pathogens of clinical concern. It is also regarded as 'gold standard' and a technique of third-generation molecular epidemiology (Arbeit, 1999; Goering, 2000). The chromosomal macro-restriction patterns resulting from PFGE analysis is based on the spatial distribution of rare, repeated, restriction sequences around the bacterial chromosome (Goering, 2000). Thus, in the intervening years PFGE proved itself as a powerful epidemiological tool by providing a sense of global chromosomal comparison (Gouby et al., 1992; Struelens et al., 1993; Tenover et al., 1995; Arbeit, 1999; Goering, 2000). Macro-restriction DNA fragment analysis by PFGE has reported to yield a very high discrimination of strains in *Acinetobacter* spp. (Gouby et al., 1992; Struelens et al., 1993). Phenotypically characterized significant strains were selected for PFGE analysis in our investigations. Bacterial DNA was prepared directly in a solid plug or insert as described by Schwartz et al (1984) with minor modifications as under. Briefly, low melting point agarose (1.2%, Gibco BRL) in phosphate buffered saline was prepared as described by Jackson and Cook (1985). Equal volumes of agarose and a bacterial suspension of $10^9$ cfu/ml were mixed in phosphate buffered saline and dispensed in a plug mould. The plugs /inserts were then incubated with a mixture of 0.5M EDTA, 1% wt/vol SDS, and 1 mg of pronase (Sigma) per ml for 48 hours at 37°C. Protein digestion products were removed by washing the plugs twice for 1 hour at 37°C in 10mM Tris- 0.1mM EDTA (pH 7.5)- 1mM phenylmethyl sulfonyl fluoride (PMSF) and then three times in Tris-EDTA alone for 1 hour at room temperature.
13.6.1 *Restriction enzyme digestion and electrophoresis:*

One plug of DNA was incubated for 6 hours with 30U of *SmaI* (Fermentas) or *ApaI* (Pharmacia biotech) in a total volume of 100uL, following the recommendations of the manufacturer.

13.6.2 *Preparation of electrophoresis tank for PFGE:*

Approximately 3 litres of 0.5x TBE (0.5M) buffer was used for electrophoresis. This was prepared from the stock of 5x TBE buffer containing Tris base, boric acid and EDTA (see Appendix-I).

13.6.3 *Preparation of gel:*

The gels were made from the same buffer that was used for electrophoresis. 15x15 cm gels were prepared by dissolving 1.3g agarose in 110ml 0.5x TBE buffer with pH 8.5.

13.6.4 *Casting of gels:*

HEX electrode with 15 x 15 cm frame was used. The rubber frame was placed in a square position, with its sides parallel to the east and west walls of the Pulsephor unit. Comb position was marked by colored tape and then agarose was poured. After pouring the agarose the comb was placed such that teeth of comb should touch the bottom of the tray. After solidification, by removing the comb well formed slots or wells were obtained.

DNA fragments obtained were separated by PFGE, with use of the Pulsephor apparatus (Pharmacia-LKB Bromma, Uppasala, Sweden). Plugs were placed into the slots of a 1.2% agarose gel and run in 0.5X TBE buffer (100mMTris (pH8.0),
100 mM boric acid and 0.2 mM EDTA) for 12 hours at 150 V with a pulsing time of 5 seconds and another 24 hours at 150 V with a pulsing time of 10 seconds. A ladder of bacteriophage lambda concatamers (Bangalore genei, India) was used as molecular weight markers. Gels were stained with ethidium bromide and photographed under UV light. Reproducibility of PFGE finger printing was examined by repeated testing of the same isolates. The PFGE patterns were analyzed and compared using dice coefficient. The relationship between two given isolates was estimated by the calculation of dice coefficient of similarity: 2 X the number of matching bands/ total number of bands in both strains (Tenover et al., 1995; Liu et al., 1997). The macro-restriction patterns obtained from selected isolates analyzed using Quantity one Gel doc 2000 computer software (Bio-rad), after the autoradiographic images were captured by Biorad digital imaging system. Similarity coefficient was also determined using neighbour joining method (Saito and Nei, 1987) and unweighted pair group of arithmetic method for averages (UPGAMA). A tolerance in the band position of 2% was applied during the comparison of PFGE fingerprinting patterns.

13.7 Reproducibility:

The reproducibility of a typing system is nothing but the percentage of strains showing the same type upon repeated testing. Sometimes this is also referred as repeatability. The reproducibilities of all the five typing methods adopted in the present study were evaluated by typing the selected strains and few reference strains at least twice. The typing results were read independently by two different persons.
13.8 Antimicrobial susceptibility testing:

Antimicrobial susceptibility of the isolates were determined by both Kirby-Bauer disk diffusion method (Bauer et al., 1966) and agar dilution for minimum inhibitory concentration (MIC) (NCCLS, 1997). *Escherichia coli* ATCC 25922 was included as control. The following antibiotics, which are commonly used in this hospital, were used to determine antimicrobial susceptibility by disk diffusion method: Cefotaxime, ceftazidime, amikacin, ciprofloxacin, gentamicin, ampicillin, piperacillin, netilmicin, norfloxacain, cefazolin, and cephalexin. MIC detection was carried out for five antibiotics viz. Cefotaxime, ceftazidime, amikacin, ciprofloxacin and ofloxacin. All discs and pure antibiotic powders were obtained from Hi-Media, Mumbai. The concentrations of discs are given in table -13.2.

**Table - 13.2** Concentration of drug in each disc:

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Concentration of Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>10μg</td>
</tr>
<tr>
<td>Amikacin</td>
<td>30μg</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>30μg</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>30μg</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>5μg</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>30μg</td>
</tr>
<tr>
<td>Cephalexin.</td>
<td>10μg</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10μg</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>100μg</td>
</tr>
<tr>
<td>Netilmicin</td>
<td>30 μg</td>
</tr>
<tr>
<td>Norfloxacain</td>
<td>10μg</td>
</tr>
</tbody>
</table>
13.8.1 Disk diffusion technique:

Commonly used Kirby- Bauer disk diffusion technique was employed for initial screening of resistance among *Acinetobacter* isolates. Mueller Hinton agar was used as the medium for disk diffusion method (NCCLS, 1997).

13.8.1.1 Mueller Hinton agar:

Mueller Hinton agar was used to test susceptibility of *Acinetobacter* isolates. Mueller Hinton agar preparation includes the following steps:

1. Mueller Hinton agar was prepared from a commercially available dehydrated base according to the manufacturer's instructions (Hi Media, Mumbai).
2. Immediately after autoclaving the media was allowed to cool to 45°C to 50°C using water bath.
3. Freshly prepared cooled medium was poured on to a petridish to give a uniform depth of approximately 4 mm.
4. Agar was allowed to cool to room temperature and the plate was used on the same day.
5. Prior to use, excess surface moisture was removed by placing the plates in incubator (35°C). However the surface moistness was maintained.

13.8.1.2. Preparation of Inoculum and Inoculation:

The growth method of NCCLS was followed and was performed as follows:

1. Three to five well-isolated morphologically similar looking colonies were selected from a plate culture. The top of each colony was touched with a loop and growth was transferred into a tube containing 4 ml of peptone broth.
2. The culture was incubated at 35 °C until it achieves the turbidity of the 0.5 McFarland standard (4 hours). The resulting suspension approximately contained $2 \times 10^8$ CFU/mL.

3. After adjusting turbidity of the inoculum suspension, a sterile cotton swab was dipped into the suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove the excess inoculum.

4. The dried surface of the agar plate was then inoculated by streaking the swab over the entire surface. The procedure was repeated by streaking two or more times, rotating the plate 60 ° each time to ensure uniform distribution of inoculum.

5. Proper application of disks was ensured by pressing down the disk on to the medium, so that there is complete contact between media and disk.

13.8.1.3 Reading and interpretation:

The test was read after overnight incubation at 35° C. After 18 hours of incubation each plate was examined and zones of inhibition were measured to the nearest whole millimeter, using sliding calipers. The diameters of the zones of inhibition were interpreted according to the norms established by NCCLS (NCCLS 1997).

13.8.2 Determination of MIC:

Agar dilution method was used to determine the Minimum inhibitory concentration (MIC). Antibiotic dilutions were made using standard dilution method to include the break points of each antibiotic for the organism. Antibacterial agents in
powder form were obtained from various manufacturers as pure ingredients (Appendix-II). These antibiotic powders were dissolved in the various solvents as instructed by the manufacturer and stock solutions were prepared using phosphate buffer saline pH 7.2. Using the standard procedure, Muellar Hinton agar (MHA) plates were incorporated with different dilutions (range of dilutions used: 0.25µg/mL to 256 µg/mL) of antibiotic solution in the ratio of 1:10 (Antibiotic solution: Medium). All plates were appropriately labelled and kept aside for the inoculation.

13.8.2.1 Preparation of inoculum: Three to four colonies were picked up from an overnight growth on blood agar medium and inoculated into 4ml of peptone broth, and incubated at 35°C until turbid. This growth suspension was adjusted to McFarlands 0.5 standard (10⁴CFU/ml). By using a calibrated loop, 0.002ml of the 10⁴CFU/ml suspension is delivered to the agar surface, resulting in the final desired inoculum of approximately 10⁴CFU per spot. Inoculated plates were allowed to stand until inoculum was completely absorbed by the medium; then the plates were kept inverted and incubated at 35°C for 16 to 20 hours. Plates without any antibiotic dilutions were used as controls to check for the viability and purity of the organism. Standard reference strains of *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter baumannii* ATCC 19606 and NTCC strain of *Acinetobacter lwoffii* (5866) were tested in each batch as quality control strains.

13.8.2.2 Reading and interpretation of results:

Results obtained with quality control strains were checked to ensure that they were with in the acceptable accuracy ranges just before the reading and recording of
the results obtained with *Acinetobacter* clinical isolates. Antibiotic free control plates were examined for the isolates purity and viability. The MIC of each antibiotic was recorded in µg/milliliter. These quantitative results were read with the appropriate corresponding interpretative categories like susceptible, intermediate and resistant. End point of 90% inhibition of growth was taken as MIC₉₀ and recorded. MIC₉₀ were also noted as the dilutions wherein half the amount of growth was inhibited, when compared with the control plate. MIC interpretative standards recommended by NCCLS (NCCLS, 1997) were followed.

Antimicrobial susceptibility results obtained by both the disc diffusion method and agar dilution method were analysed using the statistical package World Health Organisation networking programme (WHONET4) and results were interpreted.

13.9 STATISTICAL ANALYSIS:

All statistical analysis performed have already been mentioned in the above sections under each typing method described. In brief, cluster analysis was performed using dice coefficient, calculated using unweighted pair group of arithmetic method for averages (UPGAMA), for the analysis of protein and DNA banding patterns (Sneath and Sokal, 1973). Similarity coefficient was determined using neighbour joining method for both PCR and PFGE profiles (Saito and Nei, 1987). Protein and DNA profiles were also scanned using Gel Doc system and analyzed by quantity one statistical package (Bio-rad). Antibiotyping clustering is on the basis of MICs, breakpoints or zone diffusion sizes. In the present study, distribution of the MICs and
inhibition zones of the each isolate was studied by means of histograms (Gerner-Smidt, 1994) and interpreted.

The discriminatory power of each typing method was determined by calculating the discriminatory index. Gaston and Hunter (1989) recently devised a discriminatory index (DI) based on Simpson's index of diversity to describe the discriminatory power of a typing system. The DI states the probability that two unrelated strains will be differently typed by a given typing system or by a combination of typing systems. The discriminatory index is given by the formula:

$$ DI = 1 - \frac{1}{S} \sum \frac{n_j(m_j-1)}{N(N-1)} $$

Where DI is the discriminatory index, N is the number of strains in the study population, S is the number of different types, $n_j$ is the number of strains belonging to the jth type, $m_j$ is the number of strains with types indistinguishable from the jth type. The DI was calculated on an IBM-compatible PC using a software program developed by Dr. Gerner-Smidt, Statens Seruminstitut, Copenhagen, Denmark.

Antimicrobial susceptibility results obtained by both the disc diffusion method and agar dilution method were analysed using the statistical package WHONET4 programme (World Health Organisation networking programme 4). This software enabled us to calculate geometric mean, MIC breakpoints, MIC<sub>∞</sub>, MIC<sub>50</sub> and the percentages of resistant and susceptible isolates.
Contingency tables were calculated with Pearson’s test or Fisher’s exact test by comparing the proportions, when necessary. The odds ratio and its confidence intervals (95%) were calculated. Differences were significant if the $P$ value associated with the test was $< .05$. A multivariate study was performed with use of a backward stepwise logistic regression analysis for the factors influencing prognosis and nosocomial acquisition; a $P$ value of 0.5 was the limit for entering or removing terms.