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Epidemiological investigation of nosocomial *Acinetobacter* infections using arbitrarily primed PCR & pulse field gel electrophoresis

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***Background & objectives*: Nosocomial infections caused by *Acinetobacter* spp. are a significant problem worldwide. Information on epidemiological investigation of outbreaks caused by *Acinetobacter* species in India is lacking. The present investigation was carried out to elucidate molecular epidemiology of *Acinetobacter* species isolated from nosocomial infections in a tertiary care hospital in south India using two DNA-based typing methods.**

***Methods*: The medical records of 43 patients with *Acinetobacter* infection during a period of 24 months were reviewed and *Acinetobacter* isolates obtained from these patients were characterized phenotypically by assimilation tests and genotypically by arbitrarily primed PCR (AP-PCR) and pulse field gel electrophoresis (PFGE). Susceptibility testing results of the *Acinetobacter* isolates were also analysed.**

***Results*: Most of the infections were nosocomial, and the majority of these were acquired in intensive care units (ICUs). *A. baumannii* accounted for 41.8 per cent (n=18) of all pneumonia acquired in the ICU. AP-PCR with M13 primer distinguished 8 different PCR patterns comprising of 2 to 6 DNA fragments of 0.1 to 1.0 kb. PFGE identified 9 distinct profiles with five subvarients. By AP-PCR, epidemiologically unrelated strains could not be differentiated and often differences within biotypes of *A. baumannii* were not detectable. *Apa*I macrorestriction (PFGE) identified at least 4 outbreaks caused by 3 clones of *A. baumannii* and one clone of DNA group 13TU, one replacing the other in a well-defined temporal order.**

***Interpretation & conclusion*: Most of *A. baumannii* isolates were multidrug resistant. PFGE was more discriminatory [Discriminatory Index (DI)=0.96 than AP-PCR fingerprinting (DI=0.88)] in the present study. However, AP-PCR fingerprinting is more useful as a simple and rapid identification technique for epidemiological investigation of nosocomial *Acinetobacter* infections.**

Key words *Acinetobacter baumannii* - molecular methods - multiple drug resistance - nosocomial infection

Bacteria of the genus *Acinetobacter* are increasingly being implicated in numerous outbreaks and have become a growing concern in hospitals[1-4]. Resistance to multiple antibiotics is a frequent finding with these organisms[1,4,5]. *Acinetobacter baumannii* is now recognized to be the *Acinetobacter* genomic species of great clinical importance. However, many other *Acinetobacter* species are also responsible for nosocomial infections[6-9]. Numerous reports implicates *A. baumannii* as a major pathogen involved in nosocomial infections causing epidemic outbreaks or endemic occurrence with a documented high mortality rates[1,9,10].

According to recent taxonomy, there are 24 DNA groups identified by DNA-DNA hybridization methods[11,12]. Four closely related DNA groups have been grouped under *A. calcoaceticus - A. baumannii* - complex (*Acb*-complex), as they are phenotypically very similar and often difficult to differentiate from each other by conventional phenotypic methods. Many traditional and molecular typing methods have been employed for the epidemiological investigation of outbreaks caused by *Acinetobacter* spp[1-4]. However, traditional methods often lack sufficient reproducibility, typeability and discriminatory power (DP)[13]. Reports of outbreaks of *A. baumannii* infection in Indian hospitals have been published[14,15]. However, knowledge regarding species, strains and clones of *Acinetobacter* circulating in Indian hospital environments is lacking. The present study was undertaken to elucidate the molecular epidemiology of *Acinetobacter* species using two most widely applied DNA-based typing methods namely arbitrarily primed-polymerase chain reaction (AP-PCR) and pulse field gel electrophoresis (PFGE). In addition, the present study also attempted to determine feasibility, affordability and the discriminatory powers of these typing methods.

Material & Methods

The study was conducted in Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER) hospital, Pondicherry, India. In this retrospective study, case sheets of patients (43) admitted to the hospital during a 24 month period (October1998-September 2000) who developed *Acinetobacter* infection were studied. True case of infection was recorded from notes of physician in charge who has evaluated on the basis of the patient's history, clinical findings, microbiological results, and number of positive cultures. The *Acinetobacter* isolates obtained were mainly from patients admitted to 6-bedded Respiratory Intensive Care Unit (RICU). Other cases of *Acinetobacter* infection were from paediatric and medical wards. Clinical specimens included blood, CSF, endotracheal aspirate, pus and other body fluids.

Microbiological investigation: A total of 55 isolates were obtained from 18 patients of RICU. In addition, 43 isolates were from 25 patients admitted in paediatric ward and other medical wards. In cases, where multiple isolates were obtained from the same patient, based on initial screenings by biochemical testing and comparing antibiograms (by testing for 10 first and second line antibiotics), a representative strain was selected for further typing. The method of comparing antibiograms in the clinical laboratory as a screening technique also helped in recognizing potentially epidemic *A. baumannii* strains. This kind of screening helped in final selection of 49 isolates from 98 isolates obtained from 43 patients. Of these, two different isolates were obtained from six patients (5 from RICU and one from paediatric ward). In all, 49 representative *Acinetobacter* isolates were characterized both phenotypically using carbon assimilation tests and genotypically by AP-PCR and PFGE techniques[13,16-19]. After phenotypic characterization of these isolates using different carbon assimilation schemes, four outbreaks were suspected. Environmental isolates (n=5) recovered during the study period that were closely related to outbreak isolates were also characterized genotypically. Six standard reference strains belonging to different DNA groups namely 1, 2, 3, 4, 13 TU and 9, were also analysed by AP-PCR and PFGE. Minimum inhibitory concentrations (MIC) were determined using agar dilution method for important broad-spectrum antibiotics such as cefotaxime, ceftazidime, amikacin, ciprofloxacin and ofloxacin that are commonly used in the hospital[20].

Molecular characterization

AP-PCR - AP-PCR was performed with M13 universal primers. A loopful of bacterial growth containing 2 to 3 small representative colonies were suspended in 100 µl of sterile distilled water in a 0.5 ml microcentrifuge tubes. These were heated for 10 min at 95ºC, cooled on ice, and centrifued at 12,000 *g* for 20 seconds to remove all cell debris. These crude DNA extracts were either forzen at -20ºC or were kept on ice for immediate use, 2 µl of these extracts were used in the 25 µl PCR mixtures without any further purification.

For PCR, 18 µl of sterile distilled water, 5 µl of a primer (2 µM), 200 µM dNTP's together with lU of *Taq* polymerase (Boehringer Mannheim, Germany) was used. M13 primers were obtained from Pharmacia Biotech, Sweden. The primers sequences were 5' - GTA AAA CGA CGG CCA GTG AA- 3' (forward amplification primer) and 5' -GGA AAC AGC TAT GAC CAT GA- 3' (reverse amplification primer). The PCR conditions were as follows: 94ºC for 2 min followed by 35 cycles of 94ºC for 30 seconds, 45ºC for 1 min, and 72ºC for 40 seconds followed by a final extension at 72ºC for 5 min PCR end products from all isolates were analyzed by electrophoresis on 2 per cent (wt/vol) agarose gels prepared in Tris-borate electrophoresis buffer (TBE 89 m*M* Tris, 89 m*M* boric acid, 2 m*M* EDTA). The gels were stained with ethidium bromide for 20 min, destained and examined on a UV transilluminator. The AP-PCR fingerprints were photographed for further analysis[21].

PFGE - For PFGE analysis, bacterial DNA was prepared directly in a solid plug or insert as described by Schwartz and Cantor[22] with minor modifications. Briefly, low melting point agarose (1.2% LMP, Gibco BRL, USA) in phosphate buffered saline (PBS) *p*H7.0was prepared as described earlier[23]. Equal volumes of agarose and a bacterial suspension of 10^9 cfu/ml were mixed in PBS and dispensed in a plug mould. The plugs/inserts were then incubated with a mixture of 0.5 *M* EDTA, 1 per cent SDS, and 1 mg of pronase (Sigma, USA) per ml for 48 h at 37ºC. Protein digestion products were removed by washing the plugs twice for 1 h at 37ºC in 10 m*M* Tris-0.1 m*M* EDTA (*p*H 7.5) - 1 m*M* phenyl methyl sulphonyl fluoride (PMSF) and then three times in Tris-EDTA alone for 1 h at room temperature. Restriction enzyme digestion was performed by incubating a single plug of DNA for 6 h with 30U of *Sma*I (Fermentas) or *Apa*I (Pharmacia, Sweden) in a total volume of 100 µl. DNA fragments obtained were separated by PFGE, using the pulsephor apparatus (Pharmacia-LKB Bromma, Uppasala, Sweden). The 15x15 cm agarose gels were prepared by dissolving 1.3 g agarose in 110 ml 0.5 X TBE [100 m*M* Tris (*p*H8.0), 100 m*M* boric acid and 0.2 m*M* EDTA] buffer, *p*H 8.5. Plugs were placed into the slots of a 1.2 per cent agarose gel and run in 0.5 X TBE buffer for 12 h at 150 V with a pulsing time of 5 seconds and another 24 h at 150 V with a pulsing time of 10 seconds. A ladder of bacteriophage lambda concatamers (Bangalore Genei, Bangalore) was used as molecular weight markers. Gels were stained with ethidium bromide and photographed under UV light. Reproducibility of PFGE fingerprinting was examined by repeated testing of the same isolates. The patterns of AP-PCR and PFGE gels were captured and scanned by digital imaging system (Bio-Rad) and analyzed.

Twenty two reference strains belonging to 18 different DNA groups (assigned by DNA hybridization methods) obtained from Dr Gerner-Smidt (Serum-institut, Copenhagen, Denmark) and Dr Bouvet (Institut Pasteur, Paris) were also included as controls for phenotypic assimilation. There is some confusion and overlapping in numbering of few DNA groups as the two research groups followed different ways of numbering. When we referred to those DNA groups of Bouvet and Jeanjean, and Tjernberg and Ursing, the DNA group number is followed by designation BJ or TU[13,16,17].

Statistical analysis: All the compiled data was analyzed at JIPMER initially and later at CDFD. The images of PFGE and PCR gels were scanned by digital imaging system (Bio-Rad) and analyzed using *Quantity one*™ software (Bio-Rad). A tolerance in the band position of 2 per cent was applied during the comparison of PFGE and AP-PCR patterns. The patterns were analyzed and compared by calculating 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[24]. A dendrogram representing the similarity coefficients were constructed using neighbour joining method[25]. The discriminatory index (DI) of each typing system was calculated on an IBM compatible PC using a software programme developed by Dr P. Gerner-Smidt (Serum institut, Copenhagen, Denmark). DI was devised based on Simpon's index of diversity to describe the discriminatory power (DP) of a typing system[13].

Results

A total of 43 patients (25 males, 18 females) admitted to the hospital who developed *Acinetobacter* infection were in the age range from 6 months to 87 yr (mean age ± SD, 32.7 ± 22.9 yr; median age, 30 yr). *Acinetobacter* was isolated from various types of nosocomial infections such as respiratory tract infections (n=21, 48.8%), blood stream infections (BSI's) (n=7, 16.27%) (mainly from RICU), secondary meningitis (n=6, 14%), urinary tract infections (n=4, 9.3%), peritonitis (n=2, 4.65%), and one each from corneal infection, narcotizing faciitis and osteomyletis. Two cases of community acquired infections were also diagnosed.

Microbiological investigations

Carbon assimilation testing - Of the 49 isolates of *Acinetobacter* spp., *A. baumannii* constituted majority of isolates (n=35) through assimilation test identification. One isolate belonged to DNA group 3, and three to DNA group 13TU. Another isolate, which was haemolytic, belonged to DNA group 13 BJ (14TU). There were 5 isolates of *A. lwoffii*. Other species encountered were, *A. johnsonii* (1), *A. haemolyticus* (2) and *A. junii* (1).

Antibiotyping - Six antibiotypes were observed among the *Acinetobacter* isolates tested. Antibiotype A had resistance for all the five broad spectrum antimicrobials tested. Antibiotype B showed resistance to only cefotaxime. Isolates of antibiotype C were resistant to ceftazidime, amikacin, cefotaxime and susceptible only to quinolones. Antibiotype D isolates were susceptible to ceftazidime, amikacin, cefotaxime and showed resistance to both the quinolones. Group E isolates were susceptible to all the antibiotics tested. Isolates of antibiotype F had resistance to amikacin and ciprofloxacin only. Antibiotypes A, C, D were common among the out break isolates (Table I). Six *A. baumannii* biotype 6 isolates showed the antibiotype A pattern and one belonged to antibiotype D. Those isolates classified either as *A. baumannii* biotype 9 or as DNA group 13TU, exhibited antibiotypes A and C. All isolates of biotype 10 were of antibiotype A. Two isolates of *A. baumannii* biotype 15 showed antibiotype E. These 2 isolates slightly differed from rest of the group E isolates by exhibiting intermediate susceptibility to cefotaxime (Tables I & II).

Genotyping investigations: AP-PCR and PFGE clearly generated DNA group specific profiles. From all the strains tested, eight different banding patterns were distinguished, comprising 2 to 6 DNA fragments of 0.1 to 1.0 kb in size from AP-PCR. These banding patterns were designated using small case alphabets as a-h. Testing all the strains twice ensured the reproducibility of the technique. The results from one assay to another were reproducible. However, the intensity of the bands varied occasionally. All DNA 13TU strains showed profile 'a', *A. baumannii* biotype 6 showed pattern 'g', *A. baumannii* biotype 10 strains showed pattern 'd' and biotype 15 showed profile 'h'. However, on few occasions the differences within the biotypes of *A. baumannii* could not be detected by AP-PCR technique (*A. baumannii* biotype 7 was identified as biotype 6 *i.e.*, pattern 'g'). Representative PCR profiles of different strains obtained from the hospitalized patients belonging to different genomic groups of *Acinetobacter* are shown in Fig.1.

The PFGE fingerprints generated by macrorestriction with either *Sma*I or *Apa*I comprised approximately 15 to 25 bands with sizes approximately 5 to 300 kb. Isolates with identical restriction profiles were assigned to same type. Isolates that differed by one or two bands were considered as epidemiologically related. According to the interpretive criteria of Tenovar *et al*[26] eight and nine major distinct PFGE patterns were

Table I. Origin, site of isolation of representative *Acinetobacter* isolates and results of antibiotyping, AP-PCR and PFGE

Patient no.[a]	Age (yr)/ sex	Diagnosis	Out-come[c]	Sites positive	Phenotypic identification & biotype[b]	Anti-biotype	AP-PCR profiles	PFGE profiles generated by 2 enzymes	
								*Apa*I	*Sma*I
1	40/F	Post-surgery (Oesophagostomy), septicaemia	D	Bl*, PI, Sp, TA, PS	*A. baumannii*, biotype 9 or DNA group 13TU	C	a	I[d]	I
2	30/M	Intracranial space-occupying lesion	E	B1*	*A. baumannii*, biotype 6	A	g	VII	VII
3	20/F	Aspiration pneumonia	E	Bl, TA	*A. baumannii*, biotype 6	A	g	VII	VII
4	47/M	Peritonitis	D	TA, Bl	*A. baumannii*, biotype 6	A	g	VII	VII
5	20/F	Cardiac arrest	D	Bl, TA	DNA group 13TU	A	a	I	I
6	11/F	Demyelinating disease, pneumonia	T	Bl*, TA, Sp	*A. baumannii*, biotype 6	A	g	VII[d]	VII
7	74/M	Multiple organ dysfunction, VAP	E	TA, b, Bl*	*A. baumannii*, biotype 16	A	b	II	II
8	63/M	Aspiration pneumonia	D	Bl*, TA	DNA group 13TU	A	a	I	I
9	20/M	Septicaemia, pneumonia	D	TA, Bl*	*A. baumannii*, biotype 10	A	d	IV[d]	IV
10	45/F	Trauma, ARDS	T	TA, Bl*	*A. baumannii*, biotype 7	D	g	IX	VII
11	35/M	D.U. perforation, septicaemia	E	Bl*, PF	*A. baumannii*, biotype 10	A	d	IV[d]	IV[d]
12	43/M	Obesity, VAP	E	Bl*, TA	*A. baumannii*, biotype 6	D	g	VII	VII
13	4mo/F	Meningitis	E	CSF*	*A. baumannii*, biotype 15	E	h	VIII	VIII
14	4/F	Empyema	D	Bl*, Ps	*A. baumannii*, biotype 19	A	f	VI	VI
15	10/F	UTI, acute renal failure	D	Urine, Cath.tip	*A. baumannii*, biotype 16	A	b	II	II
16	6mo/M	LRI	T	Bl*, Pl, TA	*A. baumannii*, biotype 15	E	h	VIII	VIII
17	45/M	Cellulitis, wound infection	D	Ps, Bl	*A. lwoffii*	E	e	V	V
18	34/F	Meningitis	D	CSF*	*A. lwoffii*	E	e	V	V
19	13/F	LRI, meningitis	D	TA, CSF	*A. lwoffii*	E	e	V	V
20	18/F	Septicaemia	D	Bl*	*A. lwoffii*	E	e	V	V
21	45/M	Peritonitis	D	Bl	DNA group 3	B	c	III	III

[a] Patient 1-12 - from RICU, P13-16-paediatric ward PICU and P17-21 from other medical wards; [b] phenotypic identification using the identification schemes of Bouvet & Grimont[16] and Gerner-Smidt[13]; [e] D, discharged; E, expired; T, transferred; [d] sub variants were also seen in these profiles. ARDS, adult respiratory distress syndrome; LRI, lower respiratory infection; UTI, urinary tract infection; VAP, ventilator associated pneumonia; Bl, blood; TA, tracheal aspirate; Pl, pleural fluid; Sp, sputum; Ps, pus; b, bile; PF, peritoneal fluid; * From two sites the organism was isolated more than twice.

Table II. Antimicrobial susceptibility of *A. baumannii* and DNA group 13 TU outbreak isolates

Patient no.	Biotype	MICs (μg/ml)					Antibiotype
		CA	CX	AK	CIP	OF	
2, 3, 4, 6	6	32	64	>256	16-256	16-32	A
12	6	4	8	4	128	32	D
9, 11	10	128	>256	>256	128	16	A
5, 8	9[b]	32	32-64	32->256	>256	32	A
1	9[c]	128	128	64	0.125	0.25	C
13, 16	15	2	16	2	0.25	0.25	E

CA, ceftazidime; CX, cefotaxime; AK, amikacin; CIP, ciprofloxacin; OF, ofloxacin

[b] DNA group 13TU strain; [c] phenotypically classified as either *A. baumannii* biotype 9 or DNA group 13TU

MIC, Minimum inhibitory concentration

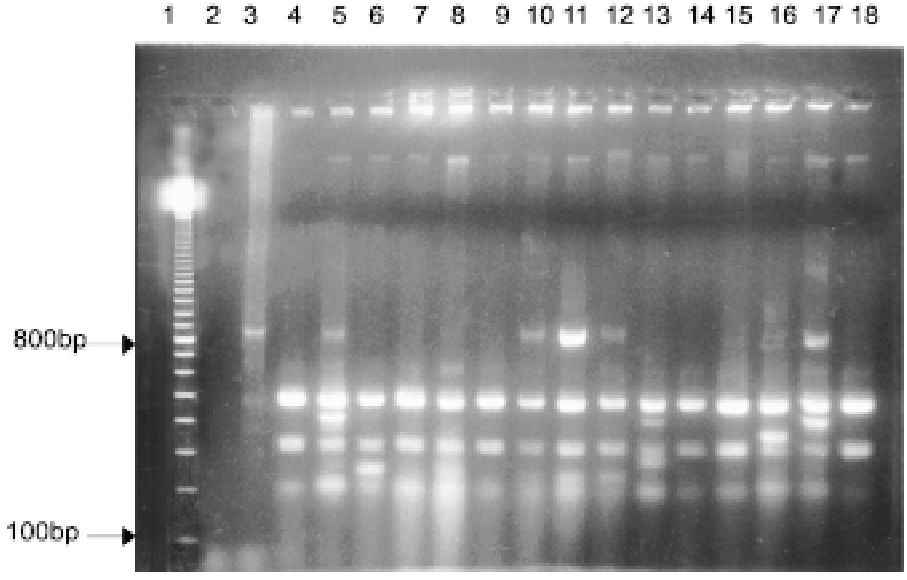

Fig. 1. AP-PCR fingerprinting of representative clinical strains of *A. baumannii*, DNA group13TU & *A. lwoffii* with forward and reverse M13 primer. Lane 1 - 100 bp ladder molecular marker; Lane 2 - Negative control; Lane 3 - Positive control (plasmid blue script); Lanes 5 & 17 - endemic *A. baumannii* biotype 16 isolates (pattern 'b'); Lanes 10,11,12 - epidemic *A. baumannii* biotype 10 outbreak isolates (pattern 'd'); Lanes 4, 7, 8, & 9 representative epidemic isolates of DNA group13TU (pattern 'a'); Lanes 15 & 18 - *A. lwoffii* (pattern 'e'); Lanes 6, 13, 14 & 16 - other *Acinetobacter* species. (Note: DNA fragment sizes are expressed in base pairs in left margin; last band is 100 bp; high intensity bright band is 800 bp).

distinguished among the isolates of *Acinetobacter* by using *Sma*I and *Apa*I, respectively. There were marked restriction polymorphisms among all these profiles. Roman numerals were used to designate this restriction profiles (I to IX). By using *Sma*I, 8 patterns (I to VIII) could be detected and with *Apa*I, one more additional pattern (IX) could be detected. Five subtypes within major patterns could be identified. Among *A. baumannii* isolates, 6 and 5 major restriction patterns could be distinguished using *Apa*I and *Sma*I, respectively.

*Sma*I restriction showed identical pattern for two isolates belonging to different biotype (biotype 6 and 7), however, *Apa*I restriction was able to differentiate them. One distinct pattern (III) was found for *Acinetobacter* DNA group 3 isolates. Two DNA group 13 isolates showed difference in one band shift and were regarded as only one PFGE pattern (I). Among *A. lwoffii* all the strains were exhibiting an identical pattern V. All outbreak-related strains were correctly identified (I, IV, VII & VIII). One to two band differences were noticed among isolates of *A. baumannii* biotype 10. However, they were considered as subvarients of the same profile IV. Outbreak strains could be clearly distinguished from epidemiologically unrelated strains by PFGE, which showed distinct PFGE types (II & III). Restriction profile VII was obtained from all *A. baumannii* biotype 6 tested. Fourteen PFGE patterns were observed from both *Apa*I and *Sma*I macrorestriction including variants. Unique PFGE patterns of outbreak related strains of DNA group 2 (*A. baumannii*) biotype 10, DNA group 13TU and DNA group 2 biotype 6 are shown in Figs 2 and 3.

While comparing two DNA typing methods, all distinct PCR patterns had distinct PFGE types. PCR patterns of outbreak strains a, d, g and h corresponded well with PFGE profiles I, IV, VII and VIII. In addition, PCR showed single pattern for both biotype 6 and 7 isolates of *A. baumannii*. A dendrogram of *Apa*I profile that is constructed by calculating dice similarity coefficient generated 9 clusters from all the isolates of 9 DNA groups encountered in the study. Dendrogram analysis of AP-PCR and *Sma*I PFGE profiles had 8 clusters that represented 8 distinct patterns. PFGE identified 4 outbreaks in the RICU caused by clones of *A. baumannii* and DNA group 13 TU (Fig. 4).

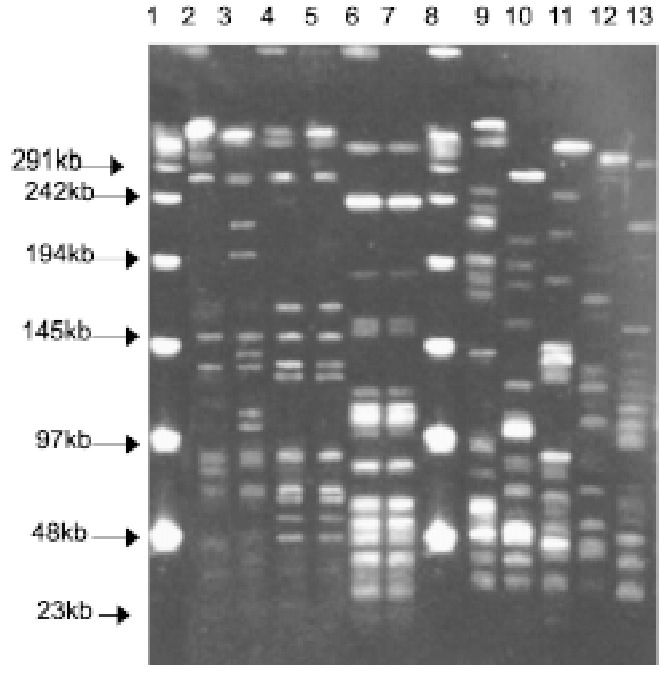

Fig. 2. Representative PFGE patterns of strains belonging to *A. baumannii*, DNA group 3 and DNA group13TU after macro-restriction with *Apa*I restriction enzyme. Lanes 1 & 8 - Molecular marker Lambda DNA concatemers; Lane 2 - isolates of DNA group 3 (pattern III); Lane 3 - endemic *A. baumannii* biotype 19 isolates (profile VI); Lanes 4 & 5 - isolates of *A. baumannii* biotype 10 strains (profile IV); Lanes 6 & 7 - representative outbreak isolates of DNA group13TU (profile I); Lane 9 - *A. baumannii* environmental isolate; Lanes 10 to 13 - Epidemiologically unrelated isolates of *A. baumannii*.
(Note: DNA band sizes are expressed in kb in left margin; Lambda DNA concatemer sizes: 291, 242, 194, 145, 97, 48 & 23; 23 kb fragment not visible).

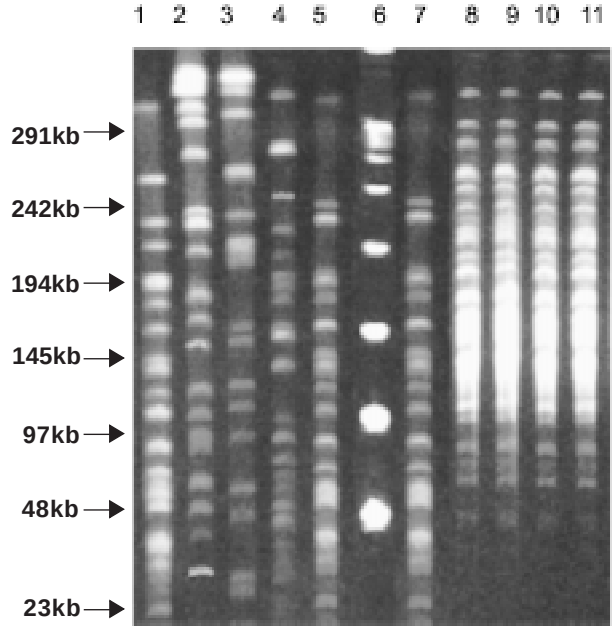

Fig. 3. Representative PFGE patterns of strains belonging to *A. baumannii*, *A. lwoffii*, DNA group 3, and DNA group13TU after macrorestriction with *Sma*I restriction enzyme. Lane 6 - Molecular marker Lambda DNA concatemers; Lane 1 - isolates of DNA group 3 (pattern III); Lane 2 - *A. lwoffii* (DNA group 9) (profile V); Lanes 3 & 4 - epidemiologically unrelated isolates of *A. baumannii*; Lanes 5 & 7 - outbreak isolates of DNA group13TU (profile I); Lanes 8-10 - representative outbreak isolates of *A. baumannii* biotype 6 (profile VII); Lane 11 - Environmental isolate profile (61E) that was identical of *A. baumannii* biotype 6 isolates (profile-VII).

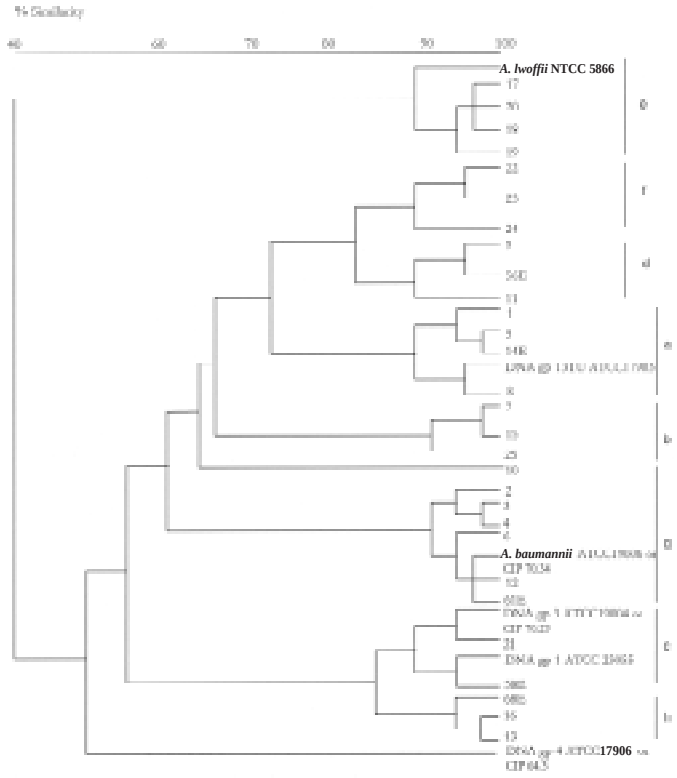

Fig. 4. Dendrogram depicting 35 representative isolates of *Acinetobacter* species obtained mainly from RICU. Paediatric ward and other medical wards. Six reference strains corresponding to 6 DNA groups were also included in this analysis. The tree was inferred from AP-PCR profile's similarity coefficient data drawn using neighbour joining method. Numbers 1-21 correspond to strains obtained from 21 patients described in Table I. Numbers 22-25 represent endemic isolates of *Acinetobacter*. E- represents environmental isolates.
Reference strains: NTCC5866 - *A. lwoffii*, ATCC17903 - DNA group TU13, ATCC19606 or CIP70.34 - *A. baumannii*, ATCC19004 or CIP 70.29 - DNA group 3, ATCC23055 - *A. calcoaceticus*, ATCC17906 or CIP 64.2 - *A. haemolyticus*.

Discussion

Phenotypic identification of *Acinetobacter* to the species or DNA group level is very difficult. Bouvet & Grimont[16] used it taxonomically for typing by using large panel of 28 tests that is tedious and time consuming. The reliability of this identification scheme was evaluated by Gerner-Smidt *et al* using a numerical approach[17]. All except seven strains in our study could be assigned to various DNA groups by phenotyping. The results of phenotyping were more meaningful and accurate when analyzed in conjunction with antibiotyping results. There was difficulty in the identification mainly between DNA groups 2 and 13 as well between 8, 9 and 15. Biotyping was helpful in differentiating the biotypes within the *A. calcoaceticus- A. baumannii* complex (*Acb*)[13]. An earlier Indian study showed that identification of isolates of *Acb*-complex based upon growth at 44, 41 and 37^0 C along with acid production from glucose could be very useful[27].

Our findings revealed that the multiple drug resistant (MDR) *A. baumannii* isolates were associated with infection. Almost all the patients who died, were essentially infected with MDR antibiotypes A, C and D. Particularly, antibiotype A strains were associated with high mortality. High mortality rates associated with MDR strains were also noted in other hospitals of India[28, 29]. The possible explanation for this could be extensive indiscriminate use of antibiotics in ICU. Broad spectrum antibiotics should be used intravenously with caution.

Though *A. baumannii* was the main species responsible for most of the infections, non-

A. baumannii species were also frequently encountered in our study. DNA group 13TU isolates were responsible for the outbreaks in our study. DNA group 13TU has been implicated in many outbreaks earlier[1, 10]. Other species such as *A. lwoffii*, *A. junnii*, *A. haemolyticus* were encountered as opportunistic pathogens among immunosuppressed patients. All *A. lwoffii* isolates exhibited a single pattern. Probably this may be due to presence of distinct clone of *A. lwoffii* as a commensal in this region and their typical prevalence might be specific for this given locality. Only one isolate of DNA group 3 causing peritonitis was observed in our study that showed a distinct PFGE pattern. This might be due to very few clones of this group existing in the hospital. The occurrence of DNA group 3 seems to be less frequent in this geographical region as compared to other regions where it is one of the important pathogen[30,31]. Tjenberg and Ursing[30] found that DNA group 3 was the predominant species among the clinical isolates in Sweden.

Though the most widely used PCR-based epidemiological method to date is AP-PCR, improved newer PCR based methods has also been evolved recently. AP-PCR still enjoys more acceptability owing to its simplicity and feasibility. This is a simple and rapid method with a high DP, which can be used as a complementary technique for typing of clinical isolates of *Acinetobacter*[32]. We used M13 primer for AP-PCR, which had been used earlier by many workers showing good results with more distinct fingerprints[22,33].

Epidemiologically related groups (a, d, g, h) showed identical PCR patterns and were clearly distinct from that of unrelated isolates (b, c, e, f). Similar findings have been reported elsewhere[34]. The DI for different primers used in PCR fingerprinting has been investigated earlier[25]. However, M13 primer had a better DI of 0.87 in another study[32]. Similar results were obtained in the present study where DI was 0.88.

Few studies have already established the diversity of PFGE-generated *Apa*I profiles[20, 35]. Similarly, in the present study more profiles by using *Apa*I than *Sma*I were generated. DNA group specific PFGE profiles could be established in our study, quite contrary to the earlier observations[1]. In our study, the results of PFGE analysis confirmed four distinct group of strains, designated I, IV, VII and VII that were involved in the outbreaks. Outbreak strains were clearly distinguished from epidemiologically unrelated strains by PFGE. An identical strain recovered from ventilator monitor surface in RICU environment, confirmed the survival capacity of *A. baumannii* for prolong periods. Long-term environmental survival provides enhanced opportunities for the transmission of *Acinetobacter* between and among patients.

Epidemiologically unrelated strains showed highly distinct polymorphic PFGE profiles and they were easily distinguishable from one another and from the outbreak isolates. These results were similar to that of Christie *et al*[36]. In this study[36], PFGE typing showed that 59 (83%) of 71 typed isolates had distinct patterns showing the high degree of diversity. Four small clusters of isolates with the same PFGE patterns were observed in our hospital, suggesting cross-transmission in ICU. This is in contrast with results of a study from Vellore[33]. PFGE typing thus can be useful in directing infection control efforts.

Marcos *et al*[37] compared PFGE with other typing methods and observed that the DP of PFGE is best with DI of 0.96. Our investigation showed PFGE having a very high DP with DI of 0.9623 while AP-PCR showed DI of 0.88. In addition, PCR was unable to differentiate biotypes of *A. baumannii* on few occasions. Similar findings from other workers emphasize the limitations of AP-PCR[32, 37]. PFGE technique was shown to be most suitable method for differentiating strains from hospital outbreaks[35]. However, PFGE is laborious and very expensive. PFGE may be reserved for situations in which clinical and other typing methods data (biotyping, antibiotyping, plasmid profiling & PCR) are inconclusive.

In conclusion, molecular typing in the present study detected at least four outbreaks caused by *A. baumannii* and DNA group 13TU, one replacing the other in a well-defined temporal order. MDR *A. baumannii* was the species responsible for majority of the *Acinetobacter* outbreaks in our hospital that

was also associated with high mortality. The use of broad-spectrum antimicrobials was associated with an increased risk of nosocomial pneumonia with these resistant strains. Although the PCR approach is quicker, simpler, cost-effective and less labour intensive than PFGE, the latter is more discriminative especially for differentiating genetically related clonal subtypes. However, AP-PCR is more useful as a simple technique with an adequate DP when compared with that of PFGE. AP-PCR can be effectively used as a supplementary technique along with biotyping for the epidemiological investigations of *Acinetobacter* isolates in least endowed clinical laboratories of developing countries.

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