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Original Article

Antimicrobial Susceptibility and AP-PCR Typing of *Acinetobacter* Spp. Strains

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Abstract

Background: *Acinetobacter* spp., as important opportunistic pathogens, have been found to be responsible for an increasing number of nosocomial infections. This study was undertaken to investigate the antimicrobial susceptibility and molecular typing of Iranian isolates of *A. baumannii*.

Methods: The study was conducted over a period of 19 months in three hospitals in Tehran, Iran. *Acinetobacter* spp. were isolated from different clinical specimens using standard bacteriological methods. Antimicrobial susceptibility test was performed according to the standard CLSI guideline using 17 antibiotic disks. The AP-PCR fingerprinting was carried out using ARB11 primer. The PCR product was run and visualized in 2% agarose gels and stained with ethidium bromide. The AP-PCR profiles were grouped depending on the patterns of the amplified bands.

Results: Sixty seven strains of *Acinetobacter* spp. (including 21 *A. baumannii* and 46 non- *A. baumannii*) were isolated. The sources of these isolates were blood, urine, wound, and respiratory tract. *A. baumannii* isolates were further studied. Results showed that all *A. baumannii* isolates were resistant to at least 11 antibiotics tested. AP-PCR analysis of *A. baumannii* strains resulted in 7 different patterns. The dominant AP-PCR pattern was E (57.1%).

Conclusion: *Acinetobacter* spp. are still important nosocomial pathogens in the region studied and most of isolates were multi-drug resistant. Our results also indicate that the AP-PCR technique represents a rapid and simple means for typing of *A. baumannii.*

Keywords: Acinetobacter baumannii, *Antimicrobial agents, PCR*

Introduction

Acinetobacter baumannii is a gram-negative, coccobacillus found in water and is a significant nosocomial pathogen in hospitals (1). The organism is usually commensal, however, because of rapidly evolving towards multi-drug resistance, is emerging as important opportunistic pathogen and involving in various nosocomial infections such as bacteremia, meningitis, or pneumonia (1-3). In general, in recent years, emerging gram-negative organisms such as *A. baumannii* have provided the same challenge with regard to multiple-antibiotic resistance (4-9).

Although many outbreaks of *A. baumannii* infection or colonization in medical, surgical, neonatal, and burn intensive care units have been reported, the epidemiology of these infections remains unclear, because *A. baumannii* is ubiquitous and infections may occur on either a sporadic or an epidemic basis (10-12). Studies on genetic relationships within the genus *Acinetobacter* have resulted in the description of 21 DNA homology groups (13). Different species of the genus predominate in different environment; however *A. baumannii* is the species most frequently isolated from patients and hospital environment (14-16).

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Extensive use of antimicrobial chemotherapy has contributed to emergence and increase in the number of *A. baumannii* strains resistant to a wide range of antibiotics, including broad-spectrum beta-lactams, aminoglycosides, and fluoroquinolones (17-19). Because of the multiple antibiotic resistance exhibited by *A. baumannii*, nosocomial infections caused by this organism are difficult to treat. These therapeutic difficulties are coupled with the fact that these bacteria have a significant capacity for long-term survival in the hospital environment, thus favoring the transmission between patients, either via human reservoirs or via inanimate materials (14, 15, 17, 18).

The development of molecular typing methods has given the clinical microbiology laboratory powerful tools, thus providing the means for a better knowledge of the epidemiology of bacterial infections. Many traditional and molecular typing methods such as antibiotic resistant typing, biotyping, phage typing, serotyping, cell envelope protein typing, plasmid typing, ribotyping, restriction fragment length polymorphisms and arbitrarily primed PCR (AP-PCR) typing have been employed for the epidemiological investigation of outbreaks caused by *Acinetobacter* spp. (3, 20). AP-PCR can be applied to detect polymorphism in a wide variety of organisms using a variety of different primers. This method is very rapid and simple and generates a fingerprint of PCR products (21). AP-PCR does not require a particular set of primers. Instead, this method uses primers chosen without regard to the sequence of the genome to be fingerprinted. Thus, AP-PCR requires no prior knowledge of the molecular biology of the organisms to be investigated. Each primer gives a different pattern of AP-PCR products, each with the potential of detecting polymorphisms between strains. Thus, the data produced allows the differentiation of even closely related strains of the same species (21).

The aim of this study was to investigate the antimicrobial susceptibility and molecular typing of *A. baumannii* strains isolated from four hospitals in Tehran, Iran in the years 2003-4.

Materials and Methods

Settings and study period The study was conducted over a period of 19 mo from April 2003 to November 2004 in three hospitals (Imam Khomayni, Shariati, and Sina) in Tehran, Iran.

Bacterial isolation and antimicrobial* susceptibility *testing Bacterial isolation and identification were performed using standard bacteriological methods (15, 16). Antimicrobial susceptibility testing was performed according to the standard CLSI guideline (4, 22, 23) using 17 antimicrobial agents (Hi Media Laboratories, Mumbai, India): ticarcillin (Ti 75µg), ceftriaxone (Ci 30µg), cefixime (Cfx 5µg), meropenem (Mr 10µg), aztreonam (Ao 30µg), cephotaxime (Ce 30µg), ticarcillin/clavulanic acid (Tc 75/10µg), ceftazidime (Ca 30µg), cefoprazone (Cs 75µg), ceftizoxime (Ck 30µg), colistin (C 110µg), polymixinB (Pb 300µg), amikacin (Ak 30µg), piperacillin/tazobactam (Pt 100/10µg), carbenicillin (Cb 10µg), tobramycin (Tb 10µg), and netillmicin (Nt 30µg). For this purpose, Mueller-Hinton agar (bio Merieux, France) plates were inoculated with a bacterial suspension in saline of standardized density (optical density of 0.5 Mc Farland) that has been prepared from a 24-h culture on blood agar. Plates were incubated for 24 h at 37° C, after which inhibition zones were measured.

DNA Extraction Bacterial strains were cultured aerobically in LB broth at 37° C. Two ml of overnight culture was centrifuged at 4,000 rmp for 20 min. The pellet was resuspended in 620 ml of lysis buffer (10 mM Tris-HCl, 50 mM EDTA, 100 mM NaCl, pH 8) containing 1% SDS and 0.4 mg/ml of proteinase K. The mixture was incubated for 1 h at 56° C and then at 100° C for 1 h. Then, an equal volume of phenol/chloroform/isoamylalcohol was added to mixture and centrifuged at 10,000 rpm for 10 min. The supernatant was added to an equal volume of chloroform and after centrifuging at 10000 rmp, the top layer was collected and DNA was precipitated with two volumes of cold isopropanol at -20° C for 10 min. The pellet was obtained by centrifugation for 20 min and washed with

1.5 ml of 70% cold ethanol. Finally the pellet was resuspended in 100 ml of TE 1X buffer (10 mM Tris-Cl,1 mM Na2 EDTA, pH 8) and 1 µl was used for AP- PCR mixture (24).

AP-PCR The AP-PCR fingerprinting was carried out using ARB11 primer (AP-ARB11; 5'-CTAGGACCGC-3') (25) and under the following conditions: Initial denaturation at 94° C for 5 min, 40 cycles of denaturation at 94° C for 30 sec, annealing at 40° C for 1 min, and extension at 72° C for 1 min, plus a final extension of 10 min at 72° C. The PCR product was run and visualized on 2% agarose gels then were stained with ethidium bromide (25).

Results

A total of 67 *Acinetobacter* spp. isolates including 21 *A. baumannii* and 46 non-*A. baumannii* strains were recovered. Among these, 36 isolates (54%) belonged to Imam Khomayni, 16 isolates (24%) to Shariati, and 15 isolates (22%) to Sina Hospital. Table 1 shows distribution of the isolates in terms of the sample sources. The strains were frequently isolated from urine samples. Analysis of antibiotic resistance patterns showed that All *Acinetobacter* spp. were multi-drug resistant. As shown in Table 2, all *A. baumannii* isolates were resistant to at least 11 antibiotic. Antibiotic resistance of *A. baumannii* isolates revealed nine different patterns designated arbitrarily A to I. Pattern A accounted for seven (33%) of *A. baumannii* isolates that were resistant to all antibiotics but susceptible to tobramycin, polymixinB, piperacillin/tazobactam and colistin. Pattern B accounted for four (19%) of *A. baumannii* isolates that were resistant to all antibiotics but susceptible to tobramycin, netilmicin, polymixinB, piperacillin/tazobactam and colistin. Two *A. baumannii* isolates showed Pattern C that were resistant to all antibiotics but susceptible to amikacin, piperacillin/tazobactam and colistin. Patterns D-I were represented only by single isolate. Pattern D represented resistance to all antibiotics but susceptible to amikacin, piperacillin/tazobactam, colistin and polymixin B. The strain with pattern E was resistant to all of antibiotics but susceptible to polymixin B and colistin. Pattern F showed resistance to all of antibiotics but susceptible to amikacin, polymixin B and colistin. The strain with pattern G was resistant to all of antibiotics but susceptible to colistin. Pattern H found to be resistant to all of antibiotics but susceptible to amikacin and colistin and finally pattern I represented resistance to all antibiotics but susceptible to tobramycin, netilmicin, piperacillin/tazobactam, amikacin and colistin.

Among these, pattern G (the most resistant pattern) was found in urinary samples and pattern H and I (most sensitive patterns) were found in respiratory tract and blood samples. Furthermore, pattern A, was the most frequent pattern between the isolates. In contrast to *A. baumannii* strains, non-*A. baumanni* isolates had lower resistance to used antibiotics. These 46 isolates showed the high diversity in antibiotic resistance patterns (data not shown).

Seven different PCR patterns (designated arbitrary A-G) were revealed among the isolates of *A. baumannii* (Fig.1). Of 21 isolates, 12 strains belonged to pattern E, 2 to pattern C or D. Other patterns (A, B, F and G) were represented only by single isolate.

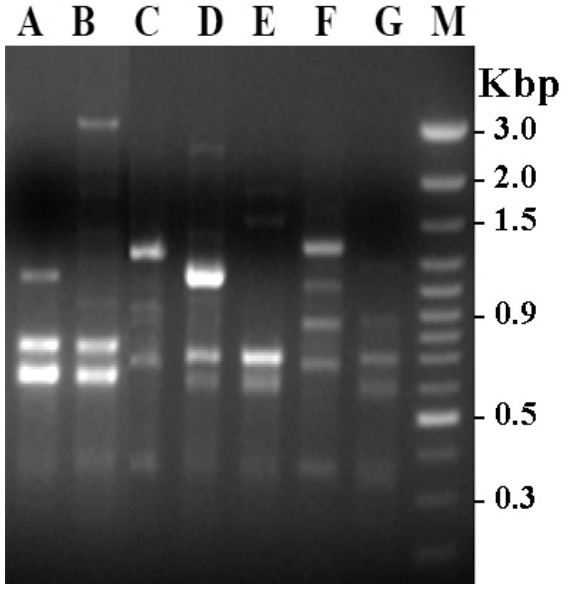

Fig. 1: AP-PCR typing of the *Acinetobcter baumannii* isolates, lane M: molecular marker (100 bp), lanes 1-7: AP-PCR patterns (A-G).

Table 1: Distribution of the isolates in terms of the source of isolation

Source	*Acinetobacter* spp.		Total n (%)
	***A. baumannii* n (%)**	**Non-*A. baumannii* n (%)**	
Blood	8 (38)	8 (17)	16 (24)
Urine	10 (48)	19 (42)	29 (43)
Wound	2 (9)	8 (17)	10 (15)
Respiratory tract	1 (5)	11 (24)	12 (18)
Total	21 (100)	46 (100)	67 (100)

Table 2: The results of antimicrobial susceptibility of 67 *Acinetobacter* spp. Strains

Antibiotic	*Acinetobacter* spp.					
	***A. baumannii* isolates**			**Non-*A. baumannii* isolates**		
	R n (%)	**I n (%)**	**S n (%)**	**R n (%)**	**I n (%)**	**S n (%)**
Cefoprazone	21 (100)	0	0	27 (60)	9(19)	10 (21)
Ceftazidime	21 (100)	0	0	15 (32)	10 (21)	21 (45)
Ticarcillin/Clavulanic acid	21 (100)	0	0	18 (40)	11 (23)	17 (37)
Aztreonam	21 (100)	0	0	23 (50)	20 (43)	3 (7)
Meropenem	21 (100)	0	0	45 (97)	0	1 (3)
Piperacillin/Ta-obactam	6 (29)	1(5)	14 (66)	32 (70)	5 (10)	9 (20)
Ceftizoxime	21 (100)	0	0	42 (90)	2 (5)	2 (5)
Carbenicillin	21 (100)	0	0	43 (93)	0	3 (7)
Netillmicin	16 (76)	0	5 (24)	5 (10)	0	41 (90)
Cefixime	21 (100)	0	0	46 (100)	0	0
Tobramycin	9 (43)	0	12 (57)	9 (20)	5 (10)	32 (70)
Ticarcillin	21(100)	0	0	37 (79)	2 (6)	7 (15)
Ceftriaxone	21(100)	0	0	42 (91)	0	4 (9)
Cephotaxime	21 (100)	0	0	43 (93)	0	3 (7)
Polymixin B	0	3(14)	18 (86)	0	0	46 (100)
Amikacin	11 (52)	0	10 (48)	11 (23)	10 (21)	25 (57)
Colistin	0	0	21 (100)	1 (3)	0	45 (97)

Abbreviations: R: Resistant; I: Intermediate; S: Susceptible

Discussion

The control of hospital-acquired infection caused by multiply resistant gram-negative bacilli has proved to be a particular problem over the last 20 yr. An increasing incidence during the 1970s of resistant members of the family *Enterobacteriaceae* involved in nosocomial infections was followed by the therapeutic introduction of newer broad spectrum antibiotics in hospitals and a subsequent increase in the importance of strictly aerobic gram-negative bacilli, including *Pseudomonas aeruginosa*, *Stenotrophomonas* (*Xanthomonas*) *maltophilia*, and *Acinetobacter* spp. (2). *Acinetobacter* spp. do not have fastidious growth requirements and are able to grow at various temperatures and pH conditions. These properties explain the ability of *Acinetobacter* spp. to persist in either moist or dry conditions in the hospital environment, thereby contributing to transmission. This hardiness, combined with its intrinsic resistance to many antimicrobial agents, contributes to the organism's fitness and enables it to spread in the hospital setting (26). Successive papers reported increased resistance in clinical isolates of A. Baumannii, currently resistant to most antibiotics. Although many other *Acinetobacter* species are also responsible for nosocomial infections, but 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 (19, 24).

Despite the increasing significance and frequency of multiply resistant *Acinetobacter* infections, many clinicians still lack an appreciation of the potential importance of these organisms in hospitals, in part because of the confused taxonomic status which, until recently was associated with these organisms. Traditional methods often lack sufficient reproducibility, typeability and discriminatory power. For example antimicrobial susceptibility patterns may be suitable as screening methods in epidemiological investigations but it requires to be confirmed by more precise and complementary techniques. However, new molecular identification and typing methods for members of this genus have now been developed, and these should form a rational scientific foundation for proper epidemiological studies of genotypically related strains involved in outbreaks of hospital infection (2).

All *A. baumannii* strains were resistant to at least 11 antibiotics, presenting potential ability to acquire resistance genes from other bacteria and cause serious infection in humans. As shown in table 2, susceptibility of all *A. baumannii* strains was only to colistin. Similar to our observations, Smolyakov et al. and Das et al. in two separate studies reported that all *Acinetobacter* isolates were sensitive to colistin, but this antibiotic has low clinical value because of its toxicity (27). Wang et al. observed that all *A. baumannii* strains isolated from an outbreak in an ICU setting were resistant to aztreonam, amikacin, cefatzidime, imipenem, meropenem, ticarcillin/clavulanic acid, piperacillin/tazobactam, and were sensitive to polymyxin B (28).

Thirty two tested *A. baumannii* isolates were resistant to ceftazidim, cefexim, ceftriaxone and aztreonam which were the same as our results (29). In a study 52 tested isolates of *A. baumannii* were susceptible to meropenem (30), while in our study all isolates were resistant to meropenem. In current study, minor variations were frequently observed among the isolates tested so interpretation of results was difficult without the help of a complementary typing system.

We demonstrated 7 different AP-PCR patterns among 21 *A. baumannii* isolates. In another study, Prashanth et al. elucidated molecular epidemiology of *Acinetobacter* spp. isolated from nosocomial infections in a tertiary care hospital in south India using AP-PCR method (24). AP-PCR with M13 primer distinguished 8 different PCR patterns in their isolates. They revealed that *A. baumannii* accounted for 18 out of all ICU acquired pneumonia cases.

In conclusion, current study showed that *Acinetobacter* spp. were still important nosocomial pathogens in the region studied and most of isolates were multi-drug resistant. Our results also indi-

cate that the AP-PCR technique represents a rapid and simple means for typing of *A. baumannii.* Continuous studies should be conducted in other parts of this large country in order to investigate the distribution, antibiotic resistance and subtyping of *Acinetobacter* spp. using more powerful molecular methods such as PFGE (31). We hope the results obtained from this study will be useful in epidemiological investigation of *Acinetobacter* spp. in Iran.

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