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Original Article

Microbiology Section

Molecular Methods for Identification of *Acinetobacter* Species by Partial Sequencing of the *rpoB* and 16S rRNA Genes

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ABSTRACT

Background: *Acinetobacter* spp. is a diverse group of Gram-negative bacteria which are ubiquitous in soil and water, and an important cause of nosocomial infections. The purpose of this study was to identify a collection of *Acinetobacter* spp. clinical isolates accurately and to investigate their antibiotic susceptibility patterns.

Materials and Methods: A total of 197 non-duplicate clinical isolates of *Acinetobacter* spp. isolates identified using conventional biochemical tests. The molecular technique of PCR-RFLP and sequence analysis of *rpoB* and 16S rRNA genes was applied for species identification. Antimicrobial susceptibility test was performed with a disk diffusion assay.

Results: Based on 16S rRNA and *rpoB* genes analysis separately, most of clinical isolates can be identified with high bootstrap values. However, the identity of the isolate 555T was uncertain due to high similarity of *A. grimontii* and *A. junii*.

Identification by concatenation of 16S rRNA and *rpoB* confirmed the identity of clinical isolates of *Acenitobacer* to species level confidently. Accordingly, the isolate 555T assigned as *A. grimontii* due to 100% similarity to *A. grimontii.* Moreover, this isolate showed 98.64% to *A. junii*. Besides, the identity of the isolates 218T and 364T was confirmed as Genomic species 3 and *A. calcoaceticus* respectively. So, the majority of *Acinetobacter* spp. isolates, were identified as: *A. baumannii* (131 isolates, 66%), *A. calcoaceticus* (9 isolates, 4.5%), and *A. genomosp* 16 (8 isolates, 4%). The rest of identified species showed the lower frequencies. In susceptibility test, 105 isolates (53%), presented high antibiotic resistance of 90% to ceftriaxone, piperacillin, piperacillin tazobactam, amikacin, and 81% to ciprofloxacin.

Conclusion: Sequence analysis of the 16S rRNA and *rpoB* spacer simultaneously was able to do identification of *Acinetobacter* spp. to species level. *A.baumannii* was identified as the most prevalent species with high antibiotic resistance. Other species showed lower frequencies ranged from 4 to 9 strains.

Keywords: Nosocomial infections,PCR-RFLP, Phenotypic tests, Susceptibility testing

INTRODUCTION

Acinetobacter spp. is Gram-negative coccobacilli with wide distribution in environmental sources such as soil and water. They are also common organisms found in the hospital environment [1]. Recent molecular studies have shown 31 distinct species with valid names among the genus *Acinetobacter*. Besides, the genus comprises a number of taxa including species with published names [2,3]. Of these, *A. calcoaceticus, A. baumannii, A. pilli*, and *A. nosocomialis* (genomic species 1, 2, 3 and 13TU, respectively) are genetically and phenotypically very similar [4].

During the last 20 years, *Acinetobacter* species have emerged as opportunistic and important nosocomial pathogens that are associated with hospital acquired infections [5]. *A. baumannii* is the most important species is responsible for a significant proportion of nosocomial infections, including urinary tract infections, endocarditis, surgical-site infections, meningitis, septicemia, and ventilator-associated pneumonia among patients in intensive care units [6]. *A. baumannii* has more recently become a cause for major concern in clinical practice due to its high level of antimicrobial resistance. In particular, the worldwide emergence of the resistance to carbapenems [7], which have been accounted as the most effective antimicrobial agents for the treatment of infections caused by multidrug resistant (MDR), is reported increasingly [8]. Outbreaks of carbapenem-resistant *A. baumannii* were recently reported from Pakistan, Korea and China [9-11]. Due to growing importance of *Acinetobacter* species in hospital infections and particularly those with multidrug resistance capacity, the precise identification of the species is important to elucidate the ecology, epidemiology, and pathology of these species. Conventional phenotypic tests are proved to be unable to identify the *Acinetobacter* isolates to the species level [4]. However, in recent years, species identification has been made possible by using the developed molecular techniques [12]. These methods include the currently identification methods based on 16S rRNA and RNA polymerase B subunit (*rpoB*) genes sequences, for the description of *Acinetobacter* species. Both methods have been reported to be useful for molecular characterization of bacteria including *Acinetobacters* [13,14].

The objectives of the present study were to apply the molecular methods for the species identification of the *Acinetobacter spp*. clinical isolates and to determine their antibiotic susceptibility patterns.

MATERIALS AND METHODS

Bacterial isolates

A total of 197 non-duplicate isolates from a wide range of clinical samples were collected from Golestan and Imam Khomeini teaching Hospitals in Ahvaz and Sina Hospital in Tehran from November 2011 to January 2013. Clinical sources of the isolates were blood, endotracheal, urine, wound, sputum, exudates, percutaneous endoscopic gastrostomy (PEG), pleural fluid, cerebrospinal fluid (CSF), and soft tissue, though the majority of strains were isolated from endotracheal tube and blood. The isolates were kept in Trypticase Soy Broth (Merck, Germany) containing 20% glycerol at -80°C until use. Conventional phenotypic tests including growth on MacConkey agar (Merck, Germany), sugar fermentation, motility, catalase, oxidase, and other standard recommended tests were used to identify the genus of the isolates [15].

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by agar disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [16]. The following antimicrobial agents were tested: ciprofloxacin (30µg), piperacillin (100µg), gentamycin (10µg), amikacin (30µg), trimethoprim sulfamethoxazole (25µg), meropenem (10µg), piperacillin tazobactam (100/10µg), ceftazidime (30µg), ceftriaxone (30µg), polymyxinB (20µg) and tetracycline (30µg) {MAST Co., UK}. *A. baumannii* ATCC 16906 was used as control strain. Determination of MDR and extensively drug resistance (XDR) was based on the CLSI criteria reported elsewhere [17].

Molecular identification

DNA extraction

All the isolates were grown on Muller Hinton Agar (Merck, Germany) and were incubated for 48 hours at 37°C under aerobic conditions and the bacterial colonies were then used for DNA extraction. The boiling method described by Higgins et al., [18], was used to extract the DNA from the bacterial colonies.

Fingerprinting of the 16S rRNA gene by PCR-RFLP analysis for strain clustering

Amplification of the 16S rRNA gene (1500 bp) was performed by using universal primers 27F (5'-AGAGTTTGATYMTGGCTCAG-3') and 1525R (5'-AGAAAGGAGGTGATCCAGCC-3') and PCR protocol as described elsewhere [14,19]. The PCR products were electrophoresed on a 2% agarose gel containing 0.5 µg/ml ethidium bromide (Qiagen, USA) to determine the size of the product. Both negative and reagent controls were included in each PCR run. The product was photographed by using the gel documentation system (UV Tech, UK). All PCR products were then digested by *Hae III* and *Tag I* restriction enzymes (Fermentas, Canada). Enzymatic digestion of the PCR products was performed according to the manufacturer's instructions. In brief, 10 µl of the amplicon was treated separately with 2 units of *TaqI* or *Hae III* enzymes plus 10 µl corresponding buffer and were overnight incubated at 37°C. The digested reactions were then placed at 65°C for 30 minutes to avoid further enzymatic digestion. Ten microliters of digestion products were separated by electrophoresis on a 3% agarose gel containing 0.5 µg/ml ethidium bromides. *A. baumannii* ATCC16906 was used as a positive control. Restriction patterns were analysed visually comparing with a 1000 bp DNA size marker, to group the patterns according to a similarity coefficient using the UPGMA method, allowing a 5% level of discrimination between the bands.

Nucleotide sequence determination of *rpoB* and 16S rDNA genes and phylogenetic analysis

One isolate from each cluster was selected randomly for further identification by sequencing of 16S rDNA and *rpoB*. The PCR products were sequenced using 27F, 1525R and 907R (5'-CCGTCAATTCMTTTRAGTTT-3') with an ABI 3100 genetic analyser. To determine the sequencing of PCR products, commercial kit of Dye Terminator Cycle Sequencing Genmeo Lab TM (Biometra Co., Germany), according to the manufacturer's instructions was used.

Zone 1 of the *rpoB* gene (350bp) was amplified from randomly selected isolate of each group using the primers Ac696F (5'-TAYCGYAAAGAYTTGAAAGAAG-3') and Ac1093R (5'-CMACACCYTTGTTMCCRTGA-3') as described elsewhere [14]. The PCR products were sequenced using Ac696F and Ac1093R.

Multiple alignments, sequence similarities of the 16S rDNA and *rpoB* gene were determined with all existing sequences of type strain of *Acinetobacter* retrieved from GenBank™ database using the jPhydit program [20].

The phylogenetic relationship between sequences of the 16S rDNA and *rpoB* genes was estimated by the neighbor-joining method. After the construction of phylogenetic trees, bootstrap replicates were performed to estimate their node reliabilities using the MEGA 4 program.

The GenBank accession numbers for the sequences of the 16S rRNA and *rpoB* genes of the representative isolates are KM281495-KM281506 and KM668180-KM668191 respectively.

RESULTS

The clinical isolates of *Acinetobacter* belonged to 130 male (66%) and 67 female (34%) patients with an age range of 2 to 84 years. The majority of the isolates were from endotracheal tubes (95 isolates, 48.2%), followed by ulcer discharge (26 isolates, 13.2%) and urine (27 isolates,13.7%). The results from susceptibility testing showed that 159 (81%) *Acinetobacter* isolates were MDR and 26 (13%) were XDR. The rate of antibiotic susceptibility was only 6% (12 isolates). The highest antibiotic resistance among *Acinetobacter* isolates were equally (96%) seen against ceftriaxone, piperacillin and piperacillin tazobactam, while polymyxin B was the most sensitive antibiotic (49.2%) in present study [Table/Fig-1].

The isolates showed several RFLP patterns which were grouped into 13 clusters designated from A to M. The majority of isolates were classified in A (131 isolates, 66.5%) [Table/Fig-2,3]. Most of clinical isolates can be confidently identified with high bootstrap values using 16S rRNA gene sequencing. However based on 16S rRNA gene, the identity of the isolate 555T was uncertain due to high similarity of *A. grimontii* and *A. junii.* Similarly on the basis of 16S rRNA gene, the isolates 218T and 364T were unidentifiable due to identical 16S rRNA sequence of genomic species (Gen.sp.) 3 and *A. calcoaceticus*.

Sequence analysis of zone 1 of the *rpoB* gene for representative isolates from each cluster in RFLP grouping, identified most of the isolates to species level with high similarity. Based on *rpoB* gene, clinical isolate 555T showed 100% and 99% similarity to those of *A. grimontii.* and Gen. sp. 5 (*A. junii*) respectively. Based on *rpoB* gene, the isolates 218T and 364T were identifiable to species level confidently of Gen. sp.3 *A. calcoaceticus*.

Identification by concatenation of 16S rRNA and *rpoB* confirmed the identity of clinical isolates of *Acenitobacer* to species level. Accordingly, the isolate 555T assigned as *A. grimontii* due to 100% similarity to *A. grimontii* [Table/Fig-2]. Moreover, this isolate showed 98.64% (24 nucleotide mismathes) to *A. junii*. On the other hand, the identity of the isolates 218T and 364T was confirmed as Gen. sp. 3 and *A. calcoaceticus* respectively [Table/Fig-4].

DISCUSSION

Acinetobacter spp. and mainly *A. baumannii* are associated with hospital outbreaks worldwide. These organisms are particularly problematic due to the large number of MDR strains that have become endemic in hospital settings which is a growing concern in many countries [21,22].

In the present study, *A. baumannii* was the most prevalent (66.5%) *Acinetobacter* species. Several studies have investigated the distribution of *Acinetobacters* in clinical specimens at the species level, and considerable differences in outcome have been reported. In a recent report by Lee et al., [23], about 80% of their *Acinetobacter* isolates were identified as *A. baumannii*, which was higher than our findings. This rate was much lower in the study of Karah et al., [12], as 24% and in the study of Boo et al., [24], as 22%. In the latter study, *A. pittii* was the most frequent species found, representing 39% of their 114 isolates, whereas only 4 isolates in our study were identified as *A. pittii*, which the difference is considerable. This inconsistency may be explained either by the use of different interval surveillance cultures in their study or by the use of different methods for species identification. However, whether these differences are due to geographic, methodological, or epidemiological differences between the studies is hard to say.

Our finding represented extremely high rate of MDR as 81% and lower rate of XDR as 13%. This was in line with a report by Joshi et al., with more than 75% MDR among their *Acinetobacter* isolates

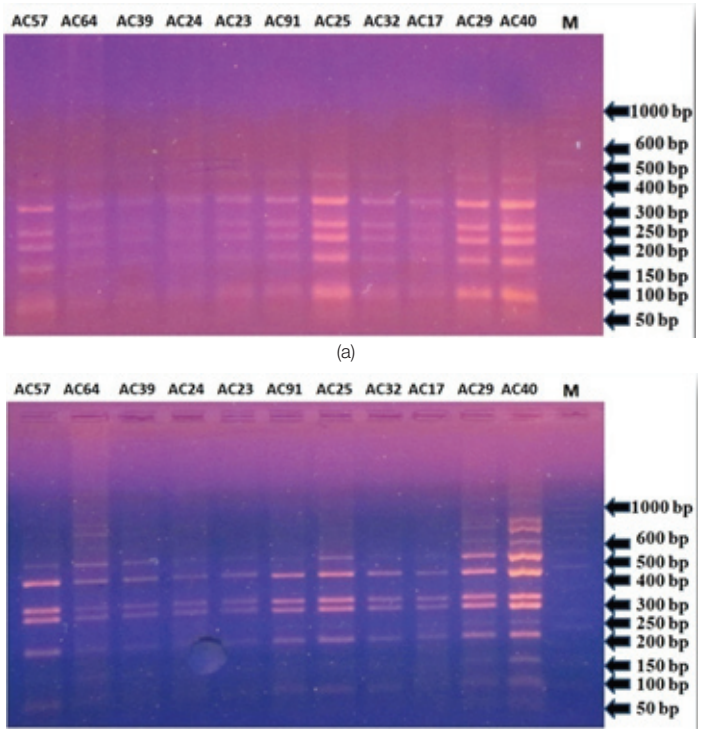
Number *(Acinetobacter)*	Isolates	AMK	CIP	CTZ	PMB	MRP	TMP-SMX	CTX	TZP	PIP	GEN	TCN	MDR Status
57,136,113, 82	*A.pittii*	S	S	I	S	S	S	I	S	I	R	S	_
25,34,85,148	*A.schindleri*	R	R	R	S	I	R	R	R	R	R	R	XDR
AC99,2, 17,28	*A.calcoaceticus*	S	S	S	S	R	S	R	R	R	S	S	MDR
70,104,117,129	*A.gen.sp. 16*	R	R	R	S	R	R	R	R	R	R	I	MDR
79,96,108,120	*A.gen.sp. 9*	R	R	R	I	R	R	R	R	R	R	I	MDR
35,12,20,22,23,52,48,49	*A.baumanii*	S	S	S	S	S	S	S	S	S	S	S	_
60,65,95*161,66, 67,69,80,81	*A.baumanii*	R	R	R	S	R	R	R	R	R	R	S	XDR
51,55,58,36,37,54,61,62,46,162	*A.baumanii*	R	R	R	S	R	I	R	R	R	S	I	MDR
41, 45 ,50,9 ,14,15, 32,123,71	*A.baumanii*	R	R	R	R	R	R	R	R	R	R	I	MDR
26,29,40,53,64,76,89,101,125,152,4,18,6,59,24 ,72,73,74,84,86,98,19,30,31,42 ,44	*A.baumanii*	R	R	R	S	I	R	R	R	R	R	I	MDR
1,8,11,16,27,38,75,88,100,112, 124,135 151,9 1,93,94,103,105,115,118,127,130,138,139,14 0,156,7,158,168,107,109,110,166,119,121,12 2,131,132,15,133,134,143,144,149,159,160,1 62,170,171,163,77,90,102,114,153,165,116,1 67,126,137	*A.baumanii*	R	R	R	R	R	I	R	R	R	R	R	MDR
313,470,79,415,553 527,27,475,545	*A.baumanii*	R	R	R	S	R	R	R	R	R	S	R	XDR
99,384,169,339,379	*A.lwoffii*	R	R	I	I	R	R	R	R	R	I	R	MDR
36,505,25,262	*A.ursingii*	R	S	S	S	R	R	R	R	R	R	R	MDR
20,476,420,546,550,670	*A.johnsonii*	R	R	R	S	R	I	R	R	R	R	I	MDR
227,106,233,406	*A.parvus*	S	R	R	S	I	R	R	R	R	I	I	MDR
218,49,56,121	*A.gen.sp. 3*	R	R	R	R	R	R	R	R	R	R	R	XDR
544,59,502,409	*A.gen.sp.16*	S	R	R	S	I	R	R	R	R	R	I	MDR
544,59,502,409	*A.genompsp.16*	S	R	R	S	I	R	R	R	R	R	I	MDR
555,67,127,552	*A.girmontii*	S	R	R	I	R	I	R	R	R	S	I	MDR
364,255,259,319,336	*A.calcoaceticus*	S	I	S	S	R	I	R	R	R	I	I	MDR
383,26,41,76,469,509	*A.gen.sp. 13 TU*	S	R	R	R	I	R	R	R	R	S	I	MDR

[Table/Fig-1]: Antibiotic susceptibility patterns of clinical isolates of *Acinetobacter* spp.
Isolates were designated susceptible (S), intermediate (I), or resistant (R) according to antibiotic breakpoint guidelines of the CLSI for *Acinetobacter* spp. Antibiotic abbreviations: AMK: Amikacin, CIP: Ciprofloxacin, CTZ: Ceftazidime , PMB: Polymyxin B , MRP: Meropenem , TMP-SMX: Trimethoprim Sulfamethoxaxole, CTX: Ceftriaxone , TZP: Piperacillin tazobactam , PIP: Piperacillin, GEN: gentamicin, TCN: Tetracycline.

Number of isolates	*Hae* III	*Tag* I	RFLP type
131	100/170/220/250/300/400	100/220/300/320/480	A
4	100/220/250/350/400	220/350/370/470	B
4	50/120/250/300/400	200/310/400/600	C
8	100/170/220/250/300/400	120/250/320/480/600	D
4	100/170/220/250/300/400	70/180/420/610	E
9	100/170/220/250/300/400	70/180/320/610	F
10	70/180/230/400	90/160/400/520	G
5	80/170/200/250/300/390	220/300/320/450	H
4	90/170/220/250/470	100/290/300/320/550	I
6	100/220/250/300/550	50/280/300/320/450	J
4	170/210/250/400/630	90/230/300/320/400	K
4	170/320/450/510	80/120/300/320/390	L
4	170/210/250/420/680	90/210/300/320/400	M

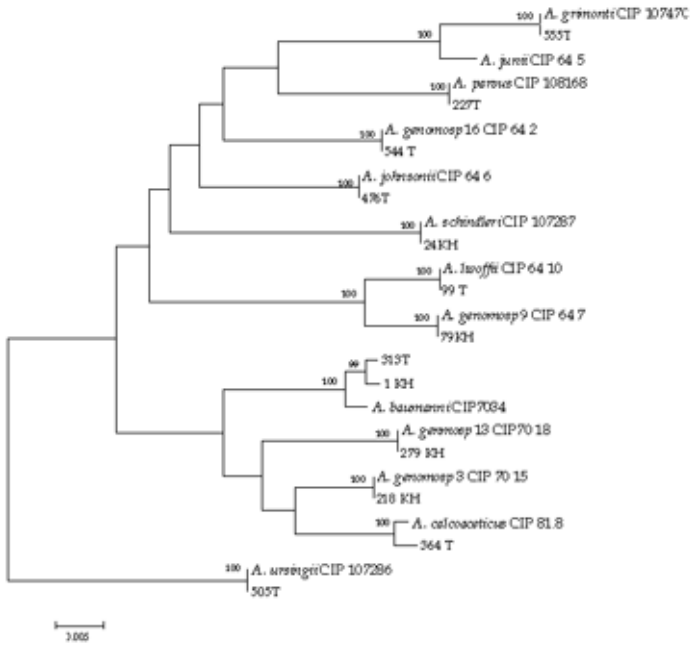
[Table/Fig-2]: Restriction patterns and clustering of clinical isolates of *Acinetobacter* spp. by 16S rRNA RFLP.

[25]. Similarly a recent report from Pakistan represented higher multidrug resistance as 96.6% among their tested *Acinetobacter* strains [9]. Although there are some discordant reports with much lower rate of MDR as 5.6% and 7.7% respectively [26,27]. A likely explanation for the relatively low level of resistant *Acinetobacter* isolates in latter studies is probably the restricted use of antibiotics, which is in line with the practice regarding antimicrobial therapy in their countries, the strict isolation of patients with MDR isolates, and the immediate beginning of infection control measures when several patients are infected with the same strain.



[Table/Fig-3a,b]: Enzymatic digestion of 16S rRNA gene PCR products with (a) *Hae* III; (b) *Tag* I restriction enzymes with subsequent separation of the digested products by 3% agarose gel electrophoresis.Lanes: M,1000-bp DNA size marker; 1-11 *Acinetobacter* isolates.

Identification to species level within the genus *Acinetobacter* is often problematic. Currently *Acinetobacter* species are defined on



[Table/Fig-4]: Phylogenetic tree of the genus *Acenitobacter* computed from the concatenation of 16S rRNA and *rpoB* gene sequences by the Neighbor joining method and Kimura 2-parameter (K2P) model as the substitution model. Percentages at nodes represent bootstrap levels supported by 1000 re-sampled datasets. Bar 0.02 substitutions per nucleotide position.
Abbreviations: T: Tehran isolate; KH: Khuzestan isolate

the basis of a battery of phenotypic tests and several genotypic methods [12]. Among the latter, 16S rRNA gene sequencing is one of the most commonly used for bacterial identification [22]. However, the main limitation of 16S rRNA gene sequencing is due to being too conserved and is not polymorphic enough to differentiate all *Acinetobacter* spp. [28]. Other protein-encoding genes, including *recA, rpoB, gyrB,* have recently been used for the identification of *Acinetobacter* isolates. Among them, *rpoB* gene due to variability in its size in different bacterial species, comprises priority for typing subspecies [12,29,30]. In the current study, the isolates were subjected to phenotypic and sequence based identification to access the species spectrum of clinically important species of *Acinetobacter*.

The obtained results revealed that although the 16S rRNA is able to identify most isolates to species level, however as previously stated, because of low polymorphisms, it is not suitable genetic marker for identification. The isolates 555T, 218T and 364T were poorly discriminated by the 16S rRNA gene sequence. Identity of isolate 555T was unclear by *rpoB* sequencing as well. Although, simultaneous application of both 16S rRNA and *rpoB* techniques, was able to identify the randomly selected isolates to species level. Our finding showed 98.64% similarity between *A. grimontii* and *A. junii*. These are the species which previously were shown to be identical based on the complete *rpoB* gene sequences [18]. Other pair species showed less similarities by combination techniques and could not assigned as the identical species in pair wise comparisons.

According to the reports by other investigators, the discriminating power of 16S rRNA gene sequencing is known to be lower than that of *rpoB* gene sequencing [14,31]. Although the *rpoB* significantly improved the resolution of species identification and complemented the phylogeny of genus *Acinetobacter* based on 16S rRNA gene sequences, in our study, using the concatenated sequence analysis of 16S rRNA and *rpoB* of representative isolate of each RFLP pattern, facilitated molecular identification for clinical isolates [Table/Fig-4]. These data clearly showed that identification based on single technique is not suitable for reliable identification and combination of 16S rRNA and *rpoB* genes analysis markedly improved the ability of the sequence based identification.

CONCLUSION

Sequence analysis of the 16S rRNA and *rpoB* spacer simultaneously was able to do identification of *Acinetobacter* spp. to species level. *A. baumannii* was identified as the most prevalent species with high antibiotic resistance. Other species showed lower frequencies ranged from 4 to 9 strains.

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