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Phenotypic and Genotypic Study of ESBL and MBL Encoding Genes and Molecular Typing in *Acinetobacter Baumannii* Strains Isolated from Hospitalized Patients

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Abstract

Background: *Acinetobacter baumannii,* as an opportunistic pathogen, is responsible for several nosocomial infections. The aim of this study was to investigate the prevalence of ESBLs and MBLs encoding genes and the molecular typing of *Acinetobacter baumannii* isolates by RAPD-PCR.

Methods: In the present study, 48 *A.baumannii* isolates were collected from various clinical specimens. Phenotypic tests and also detection of bla_{TEM}, bla_{SHV}, bla_{VIM} family, $bla_{OXA23\text{-}like}$, $bla_{OXA51\text{-}like}$, bla_{KPC} and bla_{IMP} genes were evaluated by PCR and finally the molecular typing of *A. baumannii* isolates was performed by using random amplification of polymorphic DNA (RAPD-PCR) analysis.

Results: According to the results, 9 (18.7%), 28 (58.3%) and 2 (4.1%) of the isolates were identified as Modified Hodge Test (MHT) positive, Metallo-β-lactamases (MBL) and Extended-spectrum β-lactamases (ESBL) producer respectively. In this study 46 (95.8%) of isolates were detected as multidrug resistant (MDR) isolates. Moreover, $bla_{OXA\text{-}51\text{-}like}$, $bla_{OXA\text{-}23\text{-}like}$ enzymes had the highest frequency amongst our studied isolates. The frequency of bla_{VIM}, bla_{TEM} and bla_{SHV} were 23(47.9%), 44 (91.6%) and 27(56.2%) and none of the isolates harbored bla_{IMP} and bla_{KPC} genes. By RAPD-PCR method31 different RAPD types were distinguished.

Conclusion: The frequency of drug resistance among our *A. baumannii* isolated was very high.Therefore, systematic surveillance to detect MBL and ESBL producing bacteria, rational prescription and use of carbapenems and cephalosporins could be helpful to prevent the spread of carbapenem and cephalosporins resistance isolates.

Keyword: *Acinetobacter baumannii;* Antibiotic Resistance; Molecular Typing

Introduction

Acinetobacter baumannii is known as opportunistic Gram-negative coccobacilli which is resistant to a wide range of antibiotics and may lead to several infections such as skin and soft tissue, urinary tract infection and sepsis especially in hospitals and health care centers. Neutropenic, cystic fibrosis and immunocompromised patients are regarded as the main cases of *A. baumannii* infections [1-3]. The emergence and widespread distribution of beta-lactam-resistant isolates, in particular to third-generation cephalosporins and carbapenems, have been led to a significant global challenge in the last two decades [4]. *A. baumannii* isolates by producing β-lactamase enzymes can hydrolysis the central nucleus of beta-lactam antibiotics, so the resistance to antimicrobial agents may occur [5,6]. Metallo-β-lactamases (MBLs) and Extended-spectrum β-lactamases (ESBL) are the most significant enzymes, which are responsible for resistance to different antibiotic family. Among mentioned groups, different genes such as bla_{VIM}, bla_{IMP}, bla_{KPC}, bla_{TEM} and bla_{SHV} have been studied in *A. baumannii* isolates which can play an important role in resistance to broad-spectrum cephalosporins such as cefepime, cefotaxime, ceftazidime and etc. [2]. Due to various plasmids and chromosomal sources of these enzymes, the emergence of multi-drug resistant isolates is not limited to medical centers, hence evaluating the incidence of these genes and preventing the nosocomial outbreaks of *A. baumannii* infections is one of the main health policies for controlling the community acquired infections [7].

Among the PCR based methods, random amplified polymorphic DNA (RAPD) is a rapid technique for evaluating DNA profiles in comparison to other molecular typing methods such as Pulsed-field Gel Electrophoresis)PFGE) and Multilocus sequence typing

(MLST) in genetic fingerprinting methods [8]. Therefore, RAPD-PCR can be applied for molecular epidemiological studies on a wide variety of organisms.

The aim of this study was investigating the presence of a number of significant resistance genes and molecular typing method among *A. baumannii* clinical isolates.

Methods

Bacterial Isolates

In this descriptive cross-sectional study from November 2015 to March 2017, 48 non-duplicates *A. baumannii* isolates were recovered from laboratory ward of Babol's educational hospitals and referred to the microbiology department of Babol University of medical sciences. It should be noted that, the isolates were collected from clinical specimens such as blood stream, wound, urinary tract infection and etc. from different wards (ICU, NICU, Infectious disease, Surgery and Neurology). Thereafter, confirmation of isolates were performed by using microbiological tests and finally stored at -20 °C on BHI broth containing 15% glycerol [9,10].

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility pattern was carried out by using disc diffusion method according to Clinical and Laboratory Standards Institute 2016 guidelines for following antibiotics: Imipenem (10μg), Meropenem (10μg), Ceftazidime (30μg), Cefepime (30μg), Cefotaxime (30μg), Aztreonam (30μg), Amikacin (30μg), Ciprofloxacin (5μg), Colistin (10μg), Amoxicillin (30μg), Ticarcillin (75μg), Gentamicin(10μg), Ampicillin (10μg), Piperacillin (100μg) and Tetracycline (30μg) (Rosco, Denmark) [10,11]. *Escherichia coli* ATCC25922 was used as a quality control (Babol University of medical sciences, Iran)

Phenotypic Detection of MBL

For identification of MBL-producing isolates the disc potentiation test (DPT) was performed by using a bacterial suspension equivalent to a 0.5 McFarland standard, Muller-Hinton agar medium, antibiotic discs (Imipenem (10μg) & Meropenem (10μg)) and 0.5 M Ethylenediaminetetraacetic acid (EDTA) solution. First, the bacterial suspension was swabbed onto Mueller Hinton agar plate and thereafter two Imipenem (10μg) and Meropenem discs (10μg) were placed on inoculated plate and EDTA solution (5μL) was added to one of Meropenem and Imipenem discs. Finally, an increase in zone size of at least 7 mm around the Imipenem-EDTA and Meropenem-EDTA discs after 18 h at 37 °C was considered as positive result [12].

Phenotypic Detection of ESBL

According to the CLSI 2016 guidelines, Combination Disc Test (CDT) screening was carried out to determine the ESBL-producing isolates. A bacterial suspension equivalent to a 0.5 McFarland standard was inoculated on Muller-Hinton agar medium and two discs of ceftazidime (30μg) and ceftazidime/clavulanic acid (30/10μg) was placed in an appropriate spacing from each other's. An increase of the inhibition zone diameter ≥ 5mm around the combined disk after 18 hours incubating at 37 °C in comparison to the ceftazidime disc alone was regarded to be ESBL-producing isolates [13].

Phenotypic Detection of Carbapenemases

At first, a bacterial suspension equivalent to a 0.5 McFarland standard of *E. coli* ATCC25922 was inoculated on Muller-Hinton agar medium. The Ertapenem (10μg) antibiotic disc was placed in the center of the culture medium. The test organism, positive and negative controls were cultured in a straight line from the disc edge to the edge of the plate and incubated for 24 hours at 37 °C. A cloverleaf-like indentation of the *E. coli* ATCC25922 growing along the test organism growth streak within the disc diffusion zone was considered as a positive MHT [14-19].

Amplification of MBL and ESBL Genes

In order to perform the molecular reaction of PCR, bacterial DNA was first extracted by using commercial kits (Roche, Germany). Each PCR reaction was carried out in a final volume of 25μL using a 12.5μL Super PCR Master Mix 2X (Gene-Fanavaran, Iran),3 μL DNA template, 1 μL from each primer, 7.5 μL of DNase-Free and RNase-Free Distilled Water.

Amplification was carried out with the following thermal cycling conditions: 4 minutes at 94 °C and 36 cycles of amplification consisting of 40 second at 94 °C, 40 second at 52-56 °C, and 45 second at 72 °C, with 4 minutes at 72 °C for the final extension. Finally, the PCR product was evaluated by 1.5% agarose gel. The following primers were used for PCR test (Table 1).

Primer name	Primer sequence (5´ to 3)	Annealing Temp (°C)	Product Size (bp)	Reference
OXA51	F: TAATGCTTTGATCGGCCTTG R: TGGATTGCACTTCATCTTGG	53	353	[2]
OXA23	F: GATCGGATTGGAGAACCAGA R: ATT TCTGACCGCATTTCCA	56	501	[15]

Primer name	Primer sequence (5´ to 3)	Annealing Temp (°C)	Product Size (bp)	Reference
SHV	F: CGCCTGTGTATTATCTCCCT R:CGAGTAGTCCACCAGATCCT	56	293	[16]
TEM	F:TTTCGTGTCGCCCTTATTCC R:ATCGTTGTCAGAAGTTGG	56	401	[16]
VIM	F: CAGATTGCCGATGGTGTTTGG R: AGGTGGGCCATTCAGCCAGA	56	523	[17]
IMP	F:GGAATAGAGTGGCTTAATTCTC R: CCAAACCACTAGGTTATCT	52	188	[18]
KPC	F: GTATCGCCGTCTAGTTCTGC R: GGTCGTGTTTCCCTTTAGCC	56	636	[19]

Table 1: The sequence of primers, annealing temperature and amplification size of studied genes

RAPD-PCR Fingerprinting

RAPD-PCR assay was described previously by 5-GCTTGTGAAC-3 primer for *A. baumannii* isolates [20]. In brief, PCR amplification was performed in a 25 µL total volume containing 12.5µL Super PCR Master Mix 2X (Gene-Fanavaran, Iran), 7.5 µL Distilled water, 2µL of primer, and 3µL DNA templates. After PCR procedure, PCR products were electrophoresed on1% agarose gel and visualized. To determine the similarity rate among the isolates, they were analyzed by the unweighted pair-group method with arithmetic averages (UPGMA) using GelClust software.

Statistical Analysis

Statistical analysis was done by SPSS version 22.

Results

Out of 48 *A. baumannii* strains, 42% and 58% were isolated from male and female respectively. The most clinical samples were belonged to ICU 22 (45.8%) and Infectious disease 9 (18.7%) wards.

Antimicrobial Susceptibility Testing

Antimicrobial test illustrated that the resistance rate of isolate included as Cefotaxime 48 (100%), Aztreonam 48 (100%), Cefepime 47 (97.9%), Ceftazidime 46 (95.8%), Amoxicillin 39 (81.2%), Ticarcillin 37 (77%), Ciprofloxacin 36 (75%), Ampicillin 36 (75%), Piperacillin 35 (72.9%), Gentamicin 34 (70.8%),Imipenem 34 (70.8%) ,Amikacin 33 (68.7%), Tetracycline 32 (66.6%), Meropenem 31 (64.5%), and Colistin 14 (29%).

Due to the most publications, *A. baumannii* MDR strains were defined as resistant to at least three of the following five drug classes: Cephalosporins, carbapenems, ampicillin-sulbactam, fluoroquinolones and aminoglycosides [20]. Consequently, 46(95.8%) of isolates were distinguished as MDR isolates .The tested isolates were distributed into 14 antimicrobial resistance patterns.

MBL Producing Isolates

The phenotypic results showed that 28 (58.3%) of *A. baumannii* isolates were distinguished as MBL producing strains. On the other hand, PCR experiments revealed that bla_{VIM} gene in 23 (47.9%) of isolates and bla_{IMP} gene was not detected in this study.

ESBL Producing Isolates

Based on phenotypic results 2 (4.2%) of studied isolates were identified as ESBL producing isolates, while by molecular PCR test 48 (100%) of *A. baumannii* isolates harbored $bla_{OXA51\text{-}like}$ and $bla_{OXA23\text{-}like}$ genes.

In addition, the frequency of bla_{TEM} and bla_{SHV} genes was determined 44 (91.6%) and 27 (56.2%) respectively

Detection of Carbapenemase-Producing *A. baumannii*

Among 48 collected *A. baumannii* isolates from clinical specimens 9 (18.7%) were identified as MHT positive, whereas the bla_{KPC} gene was not detected in this study.

RAPD-PCR Fingerprinting

According to RAPD analysis based on the unweighted pair-group method with averages (UPGMA) by 80% similarity cut-off point 31 different RAPD types were determined. In addition, four major clusters were observed. Cluster A, B and D with three and cluster C with four members (Figure 1).

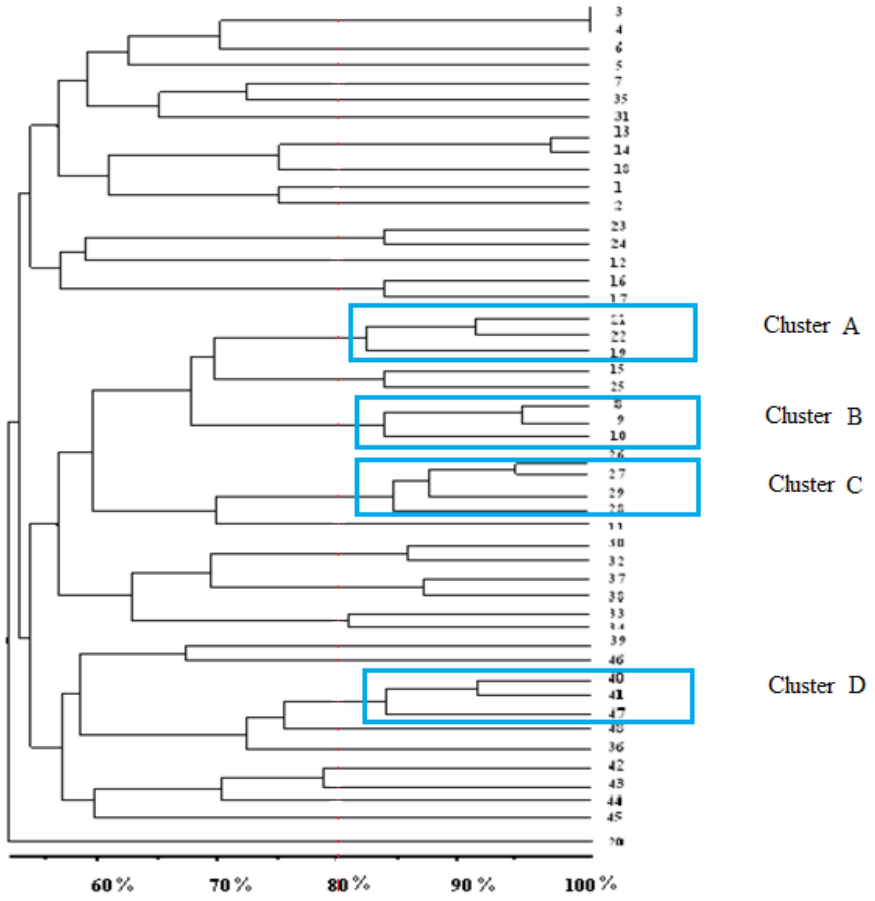

Figure 1: Dendrogram generated with Dice coefficient and the UPGMA clustering method, showing the genetic similarity among *A. baumannii* isolates by RAPD method

Discussion

Nowadays the excessive use of antimicrobial agents, especially broad-spectrum β-lactam antibiotics and also a long-term hospitalization is one of the most important reasons for the prominent prevalence of antibiotic resistance and MBL/ESBL producing bacteria in different regions. The treatment of multidrug-resistant *A. baumannii* and also ESBL producing isolates are regarded as a one of the most important global concerns [21,22]. In the present study, 95.8% of isolates were distinguished as MDR. This issue will create a great ability for Acinetobacter spp. to produce resistance genes and cause serious infections in humans.

Due to our results and conducted studies, *A. baumannii* is considered as one of the main microorganisms in ICU related infections [23]. In this research 45.8% of isolates were collected from ICU ward by phenotypic methods, 18.7%, 4.1% and 58.3% of isolates were identified as MHT, ESBL and MBL respectively. On the other hands, the frequency of bla_{OXA23}, bla_{OXA51}, bla_{TEM}, bla_{VIM} and bla_{SHV} genes was 100%, 100%, 91.6%, 47.9% and 56.2%, and none of the isolates were harbored bla_{IMP} and bla_{KPC} genes.

In Safari *et al.* conducted study, 99% and 7% of *A. baumannii* isolates were MBL and ESBL positive, while the percentage of $bla_{SHV,}$ bla_{TEM} and bla_{VIM} genes were 58%, 20% and 30%, respectively [2]. In Alyamani *et al.* study 94% of isolates were identified as ESBL producing strains, in which the frequency of bla_{TEM}, bla_{OXA51} and bla_{OXA23} genes was reported 71%, 94% and 91% respectively [24]. Carbapenem-resistant *A. baumannii* strains may include other enzymes including bla_{OXA} carbapenemase genes which can hydrolyze penicillins, cephalosporins and carbapenems [25].

One of the powerful tools for molecular epidemiological studies, particularly in hospitalized infections is molecular typing methods. According to available techniques, RAPD-PCR is very quick and affordable for nosocomial studies [26]. In the present research by RAPD-PCR, 31 different RAPD types, in addition four main clusters were distinguished including: A (three members), B (three members), C (four members) and D (three members). It should be noted that the members of cluster A, B and D were isolated from ICU ward. Moreover, three members of cluster C were belonged to ICU, while other member isolated from infectious disease ward.

In a study by Sadeghifard *et al.* by using RAPD_PCR, six different patterns were shown in 66 isolates of *A. baumannii,* while they showed that antibiotic profiles are related to RAPD-PCR [27].

Antimicrobial susceptibility patterns may be appropriate as screening methods in epidemiological studies, but they should be confirmed by accurate and complementary techniques. The analysis of genetic similarity by using RAPD-PCR method showed different patterns among isolates. Due to dendrogram (Figure 1) the existence of various RAPD types reveals the diversity of isolated strains, so the presence of unknown molecular patterns isolates indicates the spread of bacteria among patients. It should be suggested that the discriminatory methods, such as pulsed-field gel electrophoresis (PFGE) or multilocus sequence typing (MLST) are necessary form result confirmation.

Conclusion

In comparison to the mentioned antibiotics, resistance to imipenem, meropenem, and colistin is increasing in our area. The high rate of antibiotic resistance in our study may be due to the excessive use of antibiotics in this region. Despite the importance and emerging the frequency of multiple antibiotic resistances among A. baumannii strains, the therapeutic strategies should be adopted to prevent the spread of MBL, ESBL producing isolates.

Competing interests

The authors declare that they have no competing interests.

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