Molecular Detection of Class-D OXA Carbapenemase Genes in Biofilm and Non-Biofilm Forming Clinical Isolates of Acinetobacter baumannii

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1. Background

Acinetobacter baumannii has increasingly allocated an important situation as a hospital-acquired pathogen worldwide. The contribution of A. baumannii to nosocomial infections has increasingly risen over the past three decades, and many outbreaks of hospital infections involving this microorganism are reported worldwide (1-3). One of the important therapeutic features of this organism is resistance to a wide range of antibiotics including the third generation cephalosporins, carbapenems, aminoglycosides, monobactams, and fluoroquinolones (4). This property, along with the ability to form biofilm, as documented by recurring outbreaks could not be found in any isolates (12). The blaOXA-23 gene was found in all the isolates; while, blaOXA-24/40 like gene was only detected in 29 imipenem-resistant strains (P ≤ 0.05). The blaOXA-58 like gene was not detected among the isolated strains. Quantification of biofilm introduced 23 isolates of MDR-AB strains particularly with indiscriminate use of imipenem, complicated treatment of the patients infected with these bacteria in the hospitals understudy.

Keywords: Acinetobacter baumannii; Biofilms; Carbapenemase

1. Background

Acinetobacter baumannii has increasingly allocated an important situation as a hospital-acquired pathogen worldwide. The contribution of A. baumannii to nosocomial infections has increasingly risen over the past three decades, and many outbreaks of hospital infections involving this microorganism are reported worldwide (1-3). One of the important therapeutic features of this organism is resistance to a wide range of antibiotics including the third generation cephalosporins, carbapenems, aminoglycosides, monobactams, and fluoroquinolones (4). This property, along with the ability to form biofilm, as documented by recurring outbreaks contributed significantly to the infections caused by this microorganism (5, 6).

Carbapenem hydrolyzing β-lactamases (CHDLs) belonging to molecular class-D (OXA enzymes) is one of the main enzymes contributing to the inactivation of imipenem and meropenem in A. baumannii (7, 8). The OXA carbapenemases in Acinetobacter spp. are divided into four phylogenetic subgroups: OXA-23, OXA-24, OXA-51, and OXA-58 (9), but recently some researchers have reported the presence of blaOXA-23 and blaOXA-51 in non A. baumannii species. The blaOXA-23 gene was found in A. pittii (10). Of the 174 imipenem-resistant A. baumannii isolated from clinical specimens of the ICU ward in a tertiary care hospital in Greece, from 2010 to 2011 (11), 95% carried blaOXA-23 gene. Distribution of OXA subgroups in A. baumannii strains isolated from different regions of Turkey revealed that all the isolates carried blaOXA-51; however, blaOXA-24 gene could not be found in any isolates (12). The blaOXA-23 gene was first identified in Scotland and then was increasingly reported worldwide (13, 14).

In a study carried out on 104 carbapenem-resistant A. baumannii isolated from ICU of the hospitals in Krakow, Poland, blaOXA-51 like gene was detected in all the isolates; while 46 isolates also carried blaOXA-23 gene (15). Similarly,
antimicrobial susceptibility patterns and the prevalence of OXA-type carbapenemases among the clinical isolates of A. baumannii in Tehran hospitals, Iran (16), introduced 123 isolates, 100 (81.3 %) had OXA-23, 10 (8.1%) possessed OXA-24, and one (0.81%) carried OXA-58 carbapenemase.

Several studies reported the relationship between biofilm and antibiotic resistance biofilm-based infections. In this regard, A. baumannii is among the leading nosocomial pathogens capable of producing severe biofilm-related infections such as colonization on venous catheters (CVCs) and lower respiratory tract infections (due to contaminated ventilators) (17). Furthermore, biofilm formation is important in the chronic infections, and colonizing bacteria can resist phagocytosis and evade the immune system.

2. Objectives

Due to overwhelming increase in carbapenem-resistant A. baumannii (CR-AB) in ICUs of the hospitals in Kerman, Iran, the present study was carried-out to evaluate the prevalence of carbapenem-resistant genes belonging to molecular class-D (OXA) in biofilm and non-biofilm forming A. baumannii by duplex-polymerase chain reaction.

3. Materials and Methods

3.1. Patients and Bacterial Isolates

A total of 65 non-duplicated MDR-AB strains were isolated from 266 patients hospitalized in the ICU wards of two main hospitals affiliated to the Kerman University of Medical Sciences (Afzali Pour and Shahid-Bahonar) in Kerman, Iran, from February to August 2013. From these isolates, 42% (n = 28) were collected from the lung of the patients assisted by ventilator, 27.5% (n = 18) from UTI patients, and the remaining were collected from blood and soft tissue of burnt patients (P ≤ 0.05). The length of hospitalization among these patients was between 15 and 20 days. Samples were collected by an expert laboratory technician and inoculated into 5 mL sterile Stuart Transport (ST) medium (Hi-Media, India) broth and transferred to the department of microbiology laboratory within 24 hours of the collection. Prior to collection of the samples, demographic information including gender, age, clinical conditions and previous antibiotic exposure were considered.

3.2. Bacterial Identification

The isolates were inoculated into blood and Luria-Bertani (LB) agar (Merck, Germany) medium and incubated overnight at 37°C. The individual colonies were identified by conventional microbiological tests such as colony characteristic on cysteine lactose electrolyte-deficient agar (CLED) (Hi-Media, India), Gram staining, oxidation/fermentation of various sugars (OF), motility, oxidase and catalase tests (18), and further the species were confirmed by API 20NE assay (BioMerieux, Marcy l’Etoile, France). The reference strains of A. baumannii 19606 and Escherichia coli K12 DH5α were used as positive and negative controls.

3.3. Antibiotic Susceptibility Testing

Antimicrobial susceptibility of the isolates to the following antibiotics were carried out by the Kirby-Bauer (KB) disk diffusion method according to the Clinical Laboratory Standards Institute (CLSI 2012) guide lines (19); tetracycline (TE) [30 μg], amikacin (AN) [30 μg], tigecycline (TIG) [15 μg], colistin (CL) [10 μg], gentamicin (GM) [10 μg], piperacillin (PIP) [100 μg], ciprofloxacin (CIP) [5 μg], ceftazidime (CAZ) [30 μg], cefotaxime (CTX) [30 μg], tobramycin (TOB) [10 μg], amoxicillin + clavulanic acid (AMC) [30 μg], rifampin (Rif) [30 μg], cefixime (CFM) [5 μg], nalidixic acid (NA) [30 μg], imipenem (IMP) [10 μg] and meropenem (MEM) [10 μg] (Hi-media-India). Susceptibility to tigecycline was classified based on EUCAST criteria (20) (MIC ≤ 0.5 μg/mL; inhibition zone ≤ 17 mm). Minimum inhibitory concentrations (MICs) of the IMP and MEM were determined by E-test (Hi-Media, India) as described by the manufacturer’s instructions and interpreted according to CLSI guidelines (19). E. coli ATCC 25922 was used as the quality control strain.

3.4. Genomic DNA Isolation

Genomic DNA was isolated from individual colonies grown on TSA medium with the Genomic isolation kit (Thermo scientific, Lithuania) (21). DNA quantification was performed by spectrophotometry at 260 nm. The purity of DNA was evaluated by the ratio of the absorbance at 260 and 280 nm (A260/A280).

3.5. Detection of Carbapenemase (OXA) Genes by Duplex-PCR

Presence of blaOXA- like genes in A. baumannii strains were detected by duplex-PCR. The primers sequence (forward and reverse) specific to the above genes are shown in Table 1. PCR conditions were as follows: 10 μl master mix containing, 0.9 μl primer for each blaOXA-like gene, OXA-51 (324 base pair), OXA-23 (501 bp), OXA-24 (246 bp), OXA-58 (599 bp), 2 μl genomic DNA, 5.5 μl DDW, 1U Taq polymerase, 1 × PCR buffer containing, 1,5 μM MgCl2 (25 mM) (QIAGEN Inc., Valencia, CA, USA) and 0.7 μM of each deoxynucleoside triphosphate (dNTPs). The duplex-PCR amplification was conducted in the gradient thermal cycler (Biometra-T gradient, Australia) and the initial denaturation temperature was 94°C for five minutes, 30 cycles of 94°C for 35 seconds, annealing 60°C for 35 seconds followed by 72°C for 40 seconds with the final extension step at 72°C for six minutes (21). DNA ladder consisted of a plasmid double digest with the size range 100-1200 bp purchased from Cinnagen Co. (Tehran, Iran). Simultaneously, positive and negative controls (E. coli K12 DH5α provided from institute pasture, Iran) were run along with the tested samples.
3.6. Primary Attachment Assay

The initial attachment to surfaces is an important step in biofilm formation, and the subsequent pathogenesis of biofilm-associated infections. Therefore, the current study tested the capability of the strains to attach to a polystyrene surface. Briefly, overnight growth of *A. baumannii* cultures were adjusted to an OD 600 nm of 0.5 (1 × 10⁸ CFU/mL) and diluted to 10³ CFU/mL. Aliquots (100 μL) of the suspension were inoculated on microtiter plate wells. After 30 minutes of incubation at 37°C, the plate was gently rinsed three times with sterile 0.1 M phosphate-buffered saline (PBS) and then covered with 150 μL of Tryptic Soy Broth (TSB) (Merck, Germany). Primary attachment was expressed as the mean percentage of CFU (± SD) based on attachment to microtiter plate using the formula given by Stepanovic et al. (21). Simultaneously, CFU/mL of each isolate in the wells were determined and subtracted from CFU/mL control. All the mentioned experiments were performed in triplicate and the most potent biofilm producer isolates were selected for further investigations.

3.7. Biofilm Formation Under Static Condition

Formation of the biofilm in each *A. baumannii* strain was quantified by microtiter plate method (22) with some modifications. Briefly, one loopful from each colony grown on LB-agar was inoculated into a sterile TSB medium (2 mL) containing glucose (1% W/V) to optimize biofilm production. The optical density (OD 650 nm) was then adjusted to 0.13 to reach 0.5 McFarland standard (1.5 × 10⁸ CFU/mL) followed by further dilution of the prepared bacterial suspension to reach ~10⁶ CFU/mL. One hundred microliter of each prepared inoculum was added into 96-well polystyrene flat bottom tissue culture microplate. Similarly, 100 μL of the TSB medium free of bacteria (negative control) was added to the related well and the microtiter plate was then incubated at 37°C under static condition. The TSB medium free of bacteria was used as the no biofilm-forming control. After 24 hours, non-adherent cell suspensions were aseptically aspirated, washed and replaced with 10 μL of sterile phosphate buffered solution (pH = 7.2) to remove any remaining suspended cells. In order to fix the biofilm, 150 μL of methanol was added to each well and kept at room temperature (25°C) for 20 minutes. The methanol was then removed and replaced with 200 μL of crystal violet solution (1% W/V). The wells containing biofilm matrix were slowly washed with sterile deionized water and kept at room temperature till dried. Thereafter, 200 μL of glacial acetic acid (33% V/V) was added to each well and the optical density was measured at OD570 nm by S2 multi-mode microplate reader (BioTek, USA). The isolates were classified into strong, moderate, weak, and no biofilm based on attachment to microtiter plate using the formula as level of significance for the two-tailed tests. Statistical analyses were performed using SPSS 17.0 (SPSS, Chicago, IL, USA); P ≤ 0.05 was considered as level of significance for the two-tailed tests.

3.8. Statistical Analyses

Pearson χ² or Fisher’s exact tests were performed to compare the number of isolates resistant and sensitive to antibiotics. Statistical analyses were performed using SPSS 17.0 (SPSS, Chicago, IL, USA); P ≤ 0.05 was considered as level of significance for the two-tailed tests.

### 4. Results

#### 4.1. Susceptibility to Antibiotics

The susceptibility of the *A. baumannii* isolates to various antibiotics is shown in Figure 1. Approximately, all of the isolates were fully resistant to CIP, LE, CAZ, CTX, PI, TE, followed by AN (78.5%; n = 51) and TOB (48%; n = 31); whereas, 93% (n = 61) of the isolates were susceptible to tigecycline, a derivative of minocycline (Figure 1). In case of colistin, 13% of the population was resistant to this antibiotic. The isolates exhibited high MIC to the imipenem and meropenem as illustrated in Table 2. Eighty percent of the *A. baumannii* exhibited an MIC of 256 μg/mL and 15.4% showed an MIC of 128 μg/mL to both the IMP and MEM (P ≤ 0.05).
Table 2. Minimum Inhibitory Concentrations of Imipenem and Meropenem for *Acinetobacter baumannii*

| Antibiotic | MIC, μg/mL | MIC 50 | MIC 90 |
|------------|------------|--------|--------|
| IMP        | 0 (0) 3.1 (2) 0 (0) 0 (0) 0 (0) 1.5 (1) 3.1 (2) 15.4 (10) 76.9 (50) 64 256 |
| MEM        | 0 (0) 1.5 (1) 0 (0) 0 (0) 0 (0) 1.5 (1) 1.5 (1) 4.2 (4) 12.1 (8) 76.7 (50) 64 256 |

*a* MIC was determined by E-strip test and the result was interpreted after 24 hours of incubation at 37°C. Figures in the brackets indicate the number of isolates and the ones out of brackets indicate the percent in the population. The inoculum concentration was $10 \times 10^8$. The break point for each antibiotic was calculated according to the CLSI procedure.

*b* IMP, imipenem; MEM, meropenem.

Figure 2. A) Duplex-PCR for *bla*OXA-23 and *bla*OXA-24/40 Like Genes; B) Multiplex-PCR for *bla*OXA-51 and *bla*OXA-58 genes for *A. baumannii* isolated from ICU patients; OXA-58 was absent in all the tested isolates.

4.2. Detection of *bla*OXA Like Genes by Duplex-PCR

The results of OXA genes detection among the isolates by duplex-PCR are presented in Figure 2 A and Figure 2 B. All of the isolates carried *bla*OXA-23 as well as *bla*OXA-51 like genes both in the imipenem resistant and imipenem sensitive strains, while *bla*OXA-24/40 was detected in 29 isolates with high MIC to imipenem and meropenem (256 μg/mL) ($P \leq 0.5$). The *bla*OXA-58 like gene was absent in the entire population.

4.3. Primary Attachment Assay

Primary attachment assays revealed significant initial binding differences between the isolates with strong biofilm, and moderate or weak biofilm forming *A. baumannii* to polystyrene surface. Those with both *bla*OXA-23 and *bla*OXA-24 genes had stronger initial attachment to microtiter wells compared to imipenem sensitive isolates.

4.4. Biofilm Assay Under Static Condition

Quantification of biofilm formation by microtiter plate method introduced 35.3% (n = 23) of the isolates as strong biofilm producer, furthermore these isolates had stronger initial attachment ability to microtiter wells. About 16.9% (n = 11) exhibited moderate biofilm formation, and also showed initial binding activity; while, in 23% (n = 15) no biofilm was observed. Those isolates with both *bla*OXA-23 and *bla*OXA-24 genes had strong biofilm capability.

5. Discussion

*Acinetobacter baumannii* is one of the six most important multidrug-resistant microorganisms in hospitals worldwide. *A. baumannii* antimicrobial resistance has progressively increased since the 1970s, when the vast majority of strains were sensitive to the commonly used antibiotics. By 2007, up to 70% of the isolates in certain settings were MDR, including resistance to carbapenems, which were once considered the mainstay against MDR *A. baumannii* infections. The current study found 65 strains of *A. baumannii* recovered from the ICU of two main hospitals in Kerman, Iran, which were highly resistant to different classes of antibiotics. These clinical isolates formed various degrees of attachments and biofilms in microtiter plates contributed to their outstanding antibiotic resistance properties to a wide range of antibiotics (23). Thirty percent of the isolates were resistant to colistin. This is very interesting, since the majority of the evaluated isolates in the present study showed high MICs to imipenem. Resistance to colistin in non-fermenters such as *A. baumannii* is rarely reported. In general, few studies have shown a relationship between possession of distinct antibiotic resistant genes and the resulted biofilm formation in the treatment failure infected patients with such strains in ICU (24). Experimental data in the current case showed three different subtypes of OXA genes in the strains showing strong biofilm. The study found that *bla*OXA-23 along with *bla*OXA-23 were the sole carbapenemase genes detected in all the isolates (including imipenem...
wards under study were resistant to high concentrations. This bl bla genes; however, further research should be conducted on OXA-23, and colistin. The current research was limited to OXA

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\[\text{Authors’ Contributions}\]

Dr. Shakibaie supervised the project, wrote the manuscript and helped in analysis and interpretation of data: Azizi and Modarresi performed experimental data and contributed to the development of the protocol; Dr. Shahcherghi contributed to the Critical reading of the manuscript.

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