Distribution of Class D Carbapenemase and Extended-Spectrum β-Lactamase Genes among Acinetobacter Baumannii Isolated from Burn Wound and Ventilator Associated Pneumonia Infections

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ABSTRACT

Introduction: Resistance to Acinetobacter baumannii is dramatically on the rise in Iran. Therefore, it is important to study resistance pattern among Acinetobacter isolates which is a common cause of nosocomial infections.

Aim: To investigate antibiotic resistance patterns and the role of resistant genes and biofilm formation in the induction of resistance among Acinetobacter baumannii isolated from burn wound and ventilator associated pneumonia infections.

Materials and Methods: Total 103 isolates such as 33 burn samples from Rasool Akram Hospital and 70 isolates from ventilated patients in Shahid Motahhari Hospital were identified with A. baumannii using biochemical method, and then identified to species level with PCR of gyrB and blaOXA-51 gene. Antibiotic sensitivity pattern for β-lactam and carbapenem antibiotics was assessed using Agar disc diffusion test and E-test. The presence of different carbapenemase and metallo-β-lactamase (blaOXA-51-like, blaIMP, blaVIM, extended-spectrum β-lactamases (blaTEM, blaSHV) and two insertion sequences genes (IS1113) was assessed.

INTRODUCTION

Genus Acinetobacter are cause of infections in hospitalised patients and especially those in Intensive Care Unit (ICU) [4]. Due to Acinetobacter spp. biofilm producing ability, they survive longer on dry surfaces or on instruments and disseminate in hospital environments and cause nosocomial infections [2]. This organism is the cause of hospital infections which occur in most disabled ICU patients. Presence of resistance genes among in Acinetobacter spp. make them more prevalent in healthcare settings [3]. The most common Acinetobacter spp. isolated from human samples is Acinetobacter baumannii [4].

This bacterium is resistant to many available antibiotics because it has been in contact with other gram-negative bacteria in hospital environments and also exposed to extensive bombardment with antibiotics, so most strains of A. baumannii are resistant to ampicillin, tetracycline, rifampin, amoxicillin/clavulanic acid, macrolides, anti-staphylococcal penicillin, and wide range cephalosporins except for cefepime, ceftazidine, and chloramphenicol [5]. Therefore, it can acquire resistance mechanisms from plasmids, integrons, transposons, and other gram-negatives, in addition to its inherent tendency to acquire resistance [3].

Today, Multiple Drug Resistance (MDR) and especially Extensively-Drug Resistant (XDR) pathogen are global issues [1]. Usually it is called MDR Acinetobacter when it is resistant to three or more groups of antibiotics or to one key treatment antibiotic, and is Pan Drug-Resistant (PDR) Acinetobacter when it is resistant to all available groups of antibiotics for the experimental treatment of its infections [6]. Emergence of β-lactamases among bacteria has caused resistance in many bacteria responsible for hospital infections, and has therefore caused serious problems in treating bacterial infections [7]. Production of carbapenemase is mostly the mechanism for resistance to carbapenems. Carbapenem resistance in A. baumannii is mediated by presence of blaOXA-23, blaOXA-24, and blaOXA-58 type of class D family of serine β-lactamases and IMP/VIM class B of metallo-β-lactamases [8]. Mostly the isolates of Acinetobacter spp. have multiple copies of Insertion Sequence (IS) [9]. IS1113 and other ISs, are on the upstream of OXA type class D carbapenemases and modulates the expression and transfer of OXA-type carbapenemase genes [10]. Resistance of antimicrobial agents among clinical isolates may add to the burden of treating infections and also negatively affect clinical results and treatment costs [2,11].

Biofilm formation of all isolates was then assessed. Chi-square analysis or Fisher’s-exact tests were used for statistical analysis. A p-value <0.05 was considered statistically significant.

Results: Colistin was the most effective antimicrobial agents, although 10.7% (11/103) of the isolates were resistant. The high rate of resistance to meropenem (93.2%) and imipenem (90.3%) was determined. Also, with exception of ampicillin-sulbactam, surprisingly the resistant rate was 28.2%, the resistance to β-lactam antibiotic was dramatically increased. Co-existence of two and three blaOXA genes was also determined. The blaOXA-58 was detected in only one isolate. The blaTEM and blaOXA-23 was the most prevalent Extended-Spectrum β-Lactamases (ESBL) gene. All isolates were biofilm producers.

Conclusion: Antibiotic resistance is increasing among A. baumannii isolates which is due to excessive use of antibiotics and also acquired resistant genes and biofilm production. Resistance to nearly all antimicrobial agents especially colistin as end choice for treatment of multiple drug resistance A. baumannii is a big concern.

Keywords: β-lactams, Insertion elements, Nosocomial infections
In addition to the ability to acquire resistance indicators by strains of A. baumannii, another issue adding to the clinical importance of these bacteria in the last 15 years which threatens antibiotic therapy is their ability to form biofilm [12]. In fact, biofilm formation ability is an important strategy in their survival, and increases their resistance to antimicrobial compounds under stress such as host defense or antibiotic use [13].

The present study was conducted in Rasool Akram and Shahid Motahhari Hospitals in order to determine the frequency of resistance to β-lactam antibiotics; the prevalence of different β-lactamases genes and relationship between expression of antibiotic resistance and biofilm formation in strains of A. baumannii isolated from burn wounds and Ventilator Associated Pneumonia (VAP) infections.

**MATERIALS AND METHODS**

**Studied Population, Phenotypic and Genotypic Confirmation of Isolates**

This prospective study was conducted from April 2015 to March 2016. Total 103 Acinetobacter isolates consisting of 33 burn samples (swab) from Rasool Akram Hospital (Tehran city) and 70 isolates from ventilated patients in Shahid Motahhari Hospital (Tehran city) were collected. After culturing isolates on nutrient media (Conda, Spain), routine tests such as growth in 45°C and 37°C and producing acid in Oxidative/Fermentation glucose (OF glucose) (Conda, Spain) were conducted to identify A. baumannii species.

Inner genes of Acinetobacter strains including bla_oxa, 51-like and gyrB were tested using PCR for genotypic confirmation of isolates. After genotypic and phenotypic confirmation of samples, isolates were moved to an environment of 15% glycerol and 5% liquid Brain-Heart Infusion (BHI) medium (Conda, Spain) for storage and preservation at -70°C.

**Antimicrobial Susceptibility Testing**

Antibiotic sensitivity pattern with antibiotic disks (Mast, UK) imipenem (10 µg), meropenem (10 µg), cefepime (30 µg), piperacillin/tazobactam (100/10 µg), ampicillin/sublactam (10/10 µg), piperacillin (100 µg), ticarcillin/clavulanic acid (75/10 µg), cefazidime (30 µg) and ceftriaxone (30 µg) was assessed using agar disc diffusion test according to the recommendations and definitions of the manufacturers and CLSI 2015 guidelines [14]. Minimum Inhibitory Concentration (MIC) against Colistin (MGC) against Colistin was determined using E-test (AB BIODISK, Sweden). Escherichia coli ATCC® 25922™ and ATCC® 35218™ and Pseudomonas aeruginosa ATCC® 27853™ were used as quality controls in each susceptibility determination.

**Determining of Antibiotic Resistant Genes and Insertion Elements**

PCR for determining the presence of 15 different β-lactamases, bla_oxa, 51-like, gyrB, IS, 6, bla_oxa, 23-like, IS, 4, bla_oxa, 48-like, bla_TEM, bla_CBC, bla_PER, bla_eglA, bla_eriA, bla_MIP, IS, 1113 genes were carried out. Primers used to identify genes were listed in [Table/Fig-1]. A. baumannii NCTC 12156, NCTC 13302, NCTC 13303, NCTC 13304 were used as standard control for bla_oxa, 51, bla_oxa, 23, bla_oxa, 48 and bla_oxa, 6 genes respectively. For IS, 1113 gene was repeated twice. For all other PCR amplification, the product obtained was considered positive based on amplification size and direct sequencing of selected amplicons. In negative result, PCR amplification was repeated at least twice for these genes.

PCR was carried out with 50 ng of the template DNA, 10 pmol of each primer, 1 X PCR buffer, 2.5 mM MgCl₂, 0.2 mM dNTP mix, and 1U of Taq DNA polymerase (Fermentas, Lithuania) in a total volume of 25 µl. PCR amplification was carried out under the following conditions: 30 cycles of denaturation at 95°C for 30 seconds, annealing at primer set specific temperatures for one minute, and extension at 72°C for one minute followed by a final extension cycle at 72°C for 10 minute. PCR products were resolved on 1.0% agarose gels (Roche, Switzerland), stained with ethidium bromide and photographed with UV illumination.

**Biofilm Formation Assay**

First, an 18-24 hour colony was added to a tube with Lysogeny Broth medium (LB medium) (Conda, Spain). After 18-24 hour of incubation, its concentration was regulated to use to spectrophotometer at 650 nm, 0.1-0.08. About 190 µl of LB medium and 10 µl of microbial suspension were added into each well of a 96-well microplate and incubated in 37°C for 24-48 hour.

Biofilm formation assay was conducted three times for each bacterium. Negative control for each bacterium had 200 µl of LB medium. After rinsing microplates with distilled water, each well was stained using 0.1% crystal violet for 10 minute at room temperature, and then rinsed three more times with distilled water.

In the last step, 200 µl of 95% ethanol was added to each well, and light absorption at 492 nm was assessed using ELISA reader. Light absorption values were considered as indicators of bacteria’s link to the surface and formation of biofilm. For quantitative analysis of biofilm formation, mean light absorption of three wells (A) was calculated, compared with that of a control well (Ac), and then assessed as: no biofilm formation, A ≤ Ac; weak biofilm formation, Ac < A ≤ (2×Ac); average biofilm formation, (2×Ac) < A ≤ (4×Ac); and strong biofilm formation, (4×Ac) < A.

**STATISTICAL ANALYSIS**

Chi-square analysis and Fisher’s exact test using SPSS, version 21.0, were employed for statistical analyses. A p-value < 0.05 were employed as statistically significant.

**RESULTS**

**Antimicrobial Susceptibility Test**

All 103 samples had inherent gyrB and bla_oxa, 51-like genes, identified as A. baumannii. These isolates were 93.2% resistant to meropenem, 90.3% to imipenem, 88.3% to cefepime, 87.4% to ceftazidime, 82.4% to ceftriaxone. The highest resistance in clinical isolates of A. baumannii was against meropenem (93.2%), and the lowest resistance was against ampicillin/sublactam (28.2%). MIC range for colistin was 0.064 µg/ml to 1024 µg/ml of concentration, and MIC50 for ventilated patients and MIC90 for burn patients were 0.125 µg / ml and 0.5 µg /ml for this antibiotic respectively [Table/Fig-2].

**Frequency of Antibiotic-resistant and Insertion Elements Genes**

Frequencies of bla_oxa, 23, bla_oxa, 48, bla_TEM, bla_CBC, bla_MIP and bla_eriA genes were 90.3%, 38.9%, 1%, 60.2%, and 18.5% respectively. The bla_TEM and bla_oxa, 23 genes had the highest frequencies. In this study, bla_CBC, bla_MIP, bla_CBC, bla_SM, bla_CBC, bla_SM, bla_CBC, bla_SM and bla_CBC, bla_SM genes were not identified [Table/Fig-3].

Based on statistical analysis, there is a significant relationship between the presence of bla_TEM gene and resistance to ceftriaxone and ceftazidime (p=0.01). A high level of resistance to these two antibiotics was observed in strains where this gene was present. Statistical analysis show a significant relationship between the presence of bla_oxa, 23 and resistance to imipenem (p=0.04) and meropenem (p=0.01). Analysis also show a significant relationship between the presence of bla_oxa, 23 and resistance to Imipenem (p=0.02), while no significant relationship was observed between the presence of this gene and resistance to Meropenem (p=0.08). A 69.9% and 56.3% of strains had IS, 6, and IS, 1113 insertion elements respectively. Statistical analysis demonstrated a significant relationship between the presence of IS, 6 insertion elements and resistance to imipenem, meropenem, and ceftazidime (p=0.01).
Investigating the frequency of oxacillinase genes in A. baumannii strains resistant to carbapenem showed there were only three samples with bla<sub>OXA-23</sub> gene and without other carbapenemase genes which did not show any resistance to carbapenem. Here, 57.3% of strains with only bla<sub>OXA-23</sub> oxacillinase gene and without other oxacillinase genes were resistant to both imipenem or meropenem and one of them indicates the prominent role of this gene in resistance to carbapenem in A. baumannii. In 32%, both bla<sub> bla</sub> and bla<sub>OXA-24</sub> were present; 31.1% of these strains were resistant to both or one of carbapenems. The simultaneous presence of bla<sub>bla</sub> and bla<sub>OXA-24</sub> was seen in 1 strain which was resistant to both carbapenems. No simultaneous presence of bla<sub>bla</sub> and bla<sub>OXA-51</sub> was observed.

### Biofilm Formation

Results of biofilm formation study showed that all (100%) isolated strains tended to form biofilm. Frequency of ability of strong biofilm, intermediate biofilm and weak biofilm formation in clinical strains tended to form biofilm. Frequency of strong biofilm, intermediate biofilm and weak biofilm formation in clinical strains were 37.8%, 37.9% and 24.3% respectively. In this study, biofilm formation was investigated in resistant, intermediate and sensitive strains; based on results and on statistical analysis, there was no significant relationship between the amount of biofilm formation using microtiter plate and resistance to studied antibiotics (p=0.13). Nevertheless, the amount of biofilm formation was higher in resistant strains compared with sensitive strains.

### DISCUSSION

A. baumannii shows resistance to most β-lactam antibiotics and quinolones due to its ability to survive in hospital environments.
create resistance mechanism, and cause severe infections in patients. Its resistance to aminoglycosides is also emerging [19,20]. Extensive use of antibiotics has increased antibiotic resistance in this bacterium, so it is reported to be resistant to β-lactamases all over the world [21]. Wide range β-lactamases have different prevalence rates not only among different countries, but also in various geographical regions of one country [22]. Results of bla\textsubscript{OXA-51} gene proliferation in this study showed that it existed in all isolates. Prevalence of bla\textsubscript{OXA-51} among strains of A. baumannii has been reported by various studies to be 84.37% in Iran [23], 50% in Taiwan [24], 82.94% in UK [25] and 100% in a study from Iran [26]. In addition to the bla\textsubscript{OXA-51} gene PCR, results of gyrB gene PCR were also used in this study for genotypic confirmation. Results of gyrB gene proliferation demonstrated that it was present in all isolates, perfectly compatible with Higgins P et al., [16]. According to our results, PCR detection of gyrB gene can be a cheaper, easier, and more accurate method compared to other genotypic-based diagnosis methods.

The highest antibiotic resistance observed in this study was against Imipenem (90.3%) and Meropenem (93.2%) which increased over time. Moradi J et al., in their review reported resistance to Imipenem and Meropenem in Iran in 2012-2014 to be 76.5% and 81.5% respectively which was increased over time [27]. Their study showed that many species of A. baumannii have at least two carbapenemase genes simultaneously; bla\textsubscript{OXA-51} which is inherent to this bacterium; bla\textsubscript{OXA-24} and bla\textsubscript{OXA-23} which are acquired.

In the present study, bla\textsubscript{OXA-23} gene was present in 90.3% of A. baumannii strains. In another study done by Sohrabi N et al., bla\textsubscript{OXA-23} was reported in 88.7% of imipenem resistant strains, which was closer to our results [28]. The high prevalence of bla\textsubscript{OXA-23} is compatible with global reports which estimate it to be 70-100% [26,29,30].

In our study, 38.9% of strains had bla\textsubscript{OXA-24}, and 1% had bla\textsubscript{OXA-58}; 32% carried both bla\textsubscript{OXA-23} and bla\textsubscript{OXA-24}, and 1% carried bla\textsubscript{OXA-58} and bla\textsubscript{OXA-23}. All resistant or sensitive strains carried bla\textsubscript{OXA-51}. In another study, none of the strains had bla\textsubscript{OXA-24} and IS\textsubscript{Ab1-OXA-23} was found in four strains without any bla\textsubscript{OXA-51} or bla\textsubscript{OXA-23} genes [31]. Different results of studies can be attributed to assessment of different hospitals. Studies from all over the world show that numerous geographical differences have been observed in molecular epidemiology of carbapenemase genes. In the present study, 54.4% of strains with only bla\textsubscript{OXA-23} oxacillinase gene and without other oxacillinase genes were resistant to both imipenem or meropenem or one of them indicating the prominent role of this gene in resistance or decreasing sensitivity of A. baumannii against carbapenemases. Of course, we must consider three strains which were sensitive to both carbapenems in spite of carrying carbapenemase gene, similar to a study from China in which, 14 carbapenem sensitive A. baumannii strains harboring the bla\textsubscript{OXA-51} like gene showed no resistance to carbapenem drugs [32]. Since resistance to Ceftazidime, Imipenem, and Meropenem was also high in this study strains were investigated for the presence of insertion elements as well. Results showed that 69.9% and 58.3% of strains had IS\textsubscript{spal} and IS\textsubscript{11133} insertion elements respectively.

Sohrabi N et al., reported the prevalence of IS\textsubscript{Ab1} gene to be 90% in Iran [28]. Moreover, its prevalence was reported to be 69% by Rezaee MA et al., [33]. In addition, a 2010 study in 10 hospitals in Taiwan showed that 40.2% of 291 A. baumannii isolates included IS\textsubscript{Ab1}, and the prevalence of bla\textsubscript{OXA-51} (IS\textsubscript{Ab1}) insertion element differed between 6.7% to 64.3% in different hospitals [34]. In the present study, bla\textsubscript{GM}, IMP, SM and bla\textsubscript{OA} genes were assessed using PCR in order to investigate the role of metallo-β-lactamases and determine the frequency of productive strains. As expected, none of these genes were identified. In a study from Tehran, Shahrcheraghi F et al., reported that 9% of Acinetobacter produced metallo-β-lactamase (using combined disk method), yet no gene was separated in PCR [35]; no class B metallo-β-lactamase genes, including bla\textsubscript{BIM}, bla\textsubscript{BIM} were observed [36], compatible with our results, showing the low frequency of this class of β-lactamases.

In the present study, biofilm-forming strains had, 93.2% to Meropenem, 89.9% to Imipenem, 88.5% to ceftazidime, 87.4% to ceftriaxone, and 82.5% to tazobactam, and it seems that biofilm-forming strains show a high resistance. Nonetheless, no significant relationship was seen between biofilm formation and level of resistance, in contrast to results of Rodríguez Baño J et al., who had found a low resistance in biofilm-forming strains [37].

A similar study on the relationship between biofilm formation ability and level of resistance was carried out in 2013 in Bangladesh. A total 66% of strains could form biofilm and were 81%, 100%, 100%, and 7% resistant to Imipenem, Ceftriaxone, Ceftazidime, and Colistin respectively [38].

The present study showed a decreased sensitivity to most available antimicrobial agents for the treatment of Acinetobacter infections, except for Colistin and Ampicillin/sulbactam, which can be introduced as choice drugs for treating resistant strains. Among Imipenem- and Meropenem-resistant strains, 54.4% carried bla\textsubscript{OXA-23} therefore, the high prevalence of class D carbapenemases among these isolates may be responsible for resistance to studied carbapenems. Investigating biofilm formation pattern showed that all strains could form biofilm. Since ventilators are highly involved in Acinetobacter infections, disinfection and sterilization of respiratory equipment and devices can be one way to prevent dissemination of these infections.

**LIMITATION**

In present study, the sample size was very small to conclude significant results also, only burn and VAP cases were included which do not reveal much about the resistant genes distribution in other infections caused by Acinetobacter baumannii. Also, pattern of spread of resistance among isolates was not studied in current study which would help in infection control. Thus, further studies are needed to be done to study resistance spread pattern among isolates to control such infections in healthcare settings.

**CONCLUSION**

The present study showed that the presence of insertion elements, co-existence of two or more resistance genes and biofilm producing genes, increases the resistance of isolates. bla\textsubscript{OXA-23} and bla\textsubscript{Ab1} are major resistance gene among Acinetobacter isolates. Colistin and Ampicillin/sulbactam are treatment choices left for such resistant isolates. Finally, due to extension of antibiotic resistance, identified from the current study, performance of a precise and regular national program to control the immethrological consumption of antibiotics is suggested.

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Financial support: This study was financially supported by the Razi Herbal Medicines Research Center, School of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran.

Appendix A


