Molecular epidemiology of carbapenem resistance in *Acinetobacter baumannii* isolates in Shahid Mohammadi Hospital, Bandar Abbas, Iran

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(Received 22 Dec, 2013 Accepted 18 May, 2014)

Original Article

Abstract

Introduction: *Acinetobacter baumannii* is a major cause of nosocomial infections which affect mainly disabled patients in intensive care units. The bacteria may acquire resistance to antibiotics and hence can seriously endanger antibiotic therapy. The most important problem facing treatment of *A. baumannii* is increasing reports of resistance to a wide range of antibiotics, including carbapenems, as the treatment of choice for this bacterial infection. Oxacillinase-type carbapenemases belonging to Class D beta-lactamases (OXA-type) are among the main mechanisms of reasons for resistance to carbapenems. The present study aimed to evaluate the patterns of antibiotic resistance and the prevalence of carbapenemase genes of oxacillinases in clinical isolates of *A. baumannii* in a hospital in Bandar Abbas, Iran.

Methods: A total of 69 isolates of *Acinetobacter* were collected within two years from different samples of patients' bodies in the Shahid Mohammadi Hospital of Bandar Abbas. The isolates genus was identified using biochemical methods and *A. baumannii* species using PCR. Antibiotic resistance to imipenem and meropenem was identified through disk diffusion method. OXA-type carbapenem resistance genes were identified by multiplex PCR. The data were statistically analyzed through the chi-square test using SPSS 17, and the graphs were plotted using Excel.

Results: Out of 69 *Acinetobacter* isolates, 57 (82.6%) had bla$_{OXA-51}$ gene and were identified as *A. baumannii*. Antibiogram showed a significant resistance to beta-lactams and other antibiotics studied. The resistance percentage of the isolates to imipenem and meropenem antibiotics were 29.8% and 70.2%, respectively. Although all isolates were susceptible to colistin and polymyxin B, 78.9% of isolates had bla$_{oxa-23}$, 8.8% bla$_{OXA-24}$, and 1.7% bla$_{OXA-58}$ genes.

Conclusion: This study showed that beta-lactamase OXA-23 gene is the common identified known carbapenemase among carbapenem-resistant *A. baumannii* in Bandar Abbas Hospital. Evaluation of antibiotic-resistant genes in *A. baumannii* is necessary to further control dissemination of antibiotic resistance genes.

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Key words: A*cinetobacter baumannii* – Carbapenemase – OXA-58 beta-lactmase

Citation: Najar Peerayeh S, Karmostaji A, Ansari M, Javadpour S, Davoodian P, Moradi N, Vahdani M. Molecular epidemiology of carbapenem resistance in *Acinetobacter baumannii* isolates in Shahid Mohammadi Hospital, Bandar Abbas, Iran. Hormozgan Medical Journal 2015;19(1):14-21.

Introduction:

Acinetobacter is a Gram-negative, obligate aerobe, non-motile, oxidase-negative, non-fermenting, indole-negative, catalase-positive, and hemolytic bacterium which can use a variety of food sources (1). Laboratory methods based on biochemical and phenotypic properties can identify the genus of *Acinetobacter*; however, they cannot differentiate its various species. The most common type of nosocomial infections are caused by *A. baumannii*, *A. calcoaceticus*, *A. nosocomialis*, and genomic species 3. Bouvet and Grimut (1986) proposed 28 phenotypic tests for determination of the species (2); however, they were not able to differentiate the two species of *A. baumannii* and *A. nosocomialis* frequently isolated from clinical samples. As a result, the molecular methods of bla$_{OXA-51}$ gene identification were used to identify the dominant species in nosocomial infections, *i.e. A. baumannii* (1,3,4).

According to the Centers for Disease Control (CDC) in USA in 2004, *A. baumannii* was the cause of about 80% of *Acinetobacter* infections. The ability to acquire resistance by this bacterium has seriously endangered antibiotic therapy, especially in recent years (5). Although nosocomial pneumonia is the most common infection caused by the microorganism, infections of nervous system, skin, soft tissue, and bone have also been reported in some hospitals. This bacterium is the cause of more than 1% of nosocomial infections which mostly occur in patients in ICU and burn unit, immunocompromised patients, and those with underground diseases such as diabetes and chronic lung disease (6). The most important problem threatening the health system and hospitals about this bacterium is the occurrence of multi-drug resistant strains. *Acinetobacter* resistant to three or more antibiotics or resistant to a key antibiotic is known as multi-drug resistant bacteria (7). The bacterium is resistant to most commonly used antibiotics for treating infections such as aminoglycosides, quinolones, and broad spectrum beta-lactamases. Carbapenems are the treatment of choice for infections caused by this bacterium. The most important mechanism of resistance of *A. baumannii* against beta-lactam antibiotics is the production of beta-lactams, coded in the bacterial chromosome or plasmid, which are categorized as Class D in the Ambler classification and are known as oxacillinases (8). Oxacillinases exert carbapenem hydrolyzing activity in *Acinetobacter* species and can hydrolyze imipenem and meropenem, and are classified in the 2df group in a new published classification (2010) (9). Five phylogenetic subtypes of beta-lactamases Class D have been identified so far in *A. baumannii* including the bla$_{OXA-51/69}$ groups and four branches of acquired enzymes including bla$_{OXA-23}$, bla$_{OXA-24/40}$, bla$_{OXA-58}$, and bla$_{OXA-143}$, each one possessing various enzymes with different sequences. Oxacillinase bla$_{OXA-51}$ naturally occurs merely in *A. bumannii* (10) and weakly hydrolyzes only the beta-lactam substrate of penicillin and carbapenem (11), unless additional elements of ISAbaI or ISAba9 located at Bla$_{OXA-51-like}$ genes upstream enhance the expression of these genes (12). Over 68 varieties of different sequences of bla$_{OXA-51}$ have been categorized as Class D enzymes (13). Bla$_{oxa-23}$ enzyme is the first reported (1993) oxacillinase with carbapenemase activity which was initially called ARI-1 and was detected in *A. baumannii* plasmid in Scotland (14). Then, bla$_{OXA-23}$ gene was identified on chromosome and plasmid throughout the world; it was apparently unique to the genus *Acinetobacter*. One exception was reported in a *Proteus mirabilis* isolate in France (15). The second group of Class D enzymes was named bla$_{OXA-24/40}$ which was isolated from carbapenem resistant *A. baumannii* isolates in Spain (16). The third group of Class D carbapenemases detected in *A. baumannii* is characterized with bla$_{oxa-58}$ (17). Bla$_{OXA-58}$ gene has only been found so far in *Acinetobacter* species, including *A. junii* in Romania and Australia (18,19) and *A. nosocomialis* in Taiwan (2010) (20). Bla$_{OXA-58}$ is often a plasmid gene and most likely is responsible for its worldwide distribution. This gene is common in Italy and Greece (21,22). Carbapenem-resistant, bla$_{OXA-58}$-producing *A. baumannii* species were responsible for several occurrences in neonatal intensive care units (23,24). Since updating information about changes in patterns of antibiotic resistance will improve experimental treatment in local hospitals, this study was carried out to detect the species of *A. baumannii* and to identify carbapenem-resistant strains and carbapenemase oxacillinase genes in *A. baumannii*.

Methods:

A total of 69 *Acinetobacter* isolates were collected from Shahid Mohammadi Hospital of Bandar Abbas for this descriptive, cross-sectional study from October 2010 to March 2011. The bacteria were isolated from clinical specimens such as wounds, sputum, endotracheal aspirates, urine, blood, cerebrospinal fluid, and other body secretions.

An overnight culture in Mueller Hinton agar medium was prepared for each isolate. Gram staining was performed in order to see the Gram variable coccobacilli. To identify the genus of *Acinetobacter*, different biochemical tests were performed including oxidase (negative), indole (negative), motility in the SIM medium (negative), sugars fermentation in TSI (negative), growth at 42 °C, and melting the gelatin (25).

After identification, the *Acinetobacter* isolates were cultured in trypticase soy broth containing 15% glycerol, and stored at -70 °C on glass pearls.

To extract DNA, about six colonies of 24-hour grown bacteria were transferred into 1.5 mL Eppendorf tubes containing 200 mL TE buffer and vortexed to obtain a uniform suspension. These samples were then centrifuged at 8000 rpm for 8 min and the supernatant was discarded; 200 mL distilled water was added to the sample and mixed, homogenized, and centrifuged in the same way. The bacterial suspension was then placed 10 min in a boiling water bath and 10 min on ice. Freezing and thawing process was repeated 2 times. The suspension was centrifuged at 8000 rpm for 4 min and the supernatant was aliquoted in 0.5 mL sterile Eppendorf tubes and stored at -20 °C until PCR (26).

Primers used to identify genes bla$_{OXA-58}$, bla$_{OXA-24}$, bla$_{OXA-23}$, and bla$_{OXA-51}$ are shown in Table 1 (27).

Table 1. Primers used to identify genes bla$_{OXA-58}$, bla$_{OXA-24}$, bla$_{OXA-23}$, and bla$_{OXA-51}$

Gene	Nucleotide sequence	Amplicon size (bp)
***bla*$_{OXA-23}$-F**	GAT CGG ATT GGA GAA CCA GA	501
***bla*$_{OXA-23}$-R**	ATT TCT GAC CGC ATT TCC AT	
***bla*$_{OXA-24}$-F**	GGT TAG TTG GCC CCC TTA AA	246
***bla*$_{OXA-24}$-R**	AGT TGA GCG AAA AGG GGA TT	
***bla*$_{OXA-58}$-F**	AAG TAT TGG GGC TTG TGC TG	599
***bla*$_{OXA-58}$-R**	CCC CTC TGC GCT CTA CAT AC	
***bla*$_{OXA-51}$-F**	TAA TGC TTT GAT CGG CCT TG	353
***bla*$_{OXA-51}$-R**	TGG ATT GCA CTT CAT CTT GG	

Multiplex PCR was performed for oxacillinase genes according to the method described by Woodford et al. (27), at annealing temperature of 53 °C for one min.

The susceptibility of bacteria to antibiotics was evaluated through the disk diffusion method. Since the US Food and Drug Administration (FDA) has not provided a standard as a cutoff for sensitivity and resistance of *A. baumannii* to tigecycline, the standards proposed for the *Enterobacteriaceae* by FDA were used for interpreting the resistance to this antibiotic; i.e. an inhibition zone of equal to or greater than 19 mm as sensitive, 15-18 mm as intermediate resistance, and equal to or less than 14 mm as resistant (28).

Antibiotic discs containing imipenem (IPM, 10 µg), meropenem (MEN, 10 µg), gentamicin (GM, 10µg), ciprofloxacin (CIP, 5µg), amikacin (AN, 30µg), cotrimoxazole (SXT, 25µg), cefepime (CPM, 30µg), cefotaxime (CTX, 30µg), aztreonam (ATM, 30µg), ceftazidime (CAZ, 30µg), polymixin B (PB, 300 U), tigecycline (TIG, 15µg), and colistin (CO, 10µg) were purchased from MAST Company. The standard strain of *E. coli*, ATCC25922 was used as a quality control disc.

The data were statistically analyzed through the chi-square test using SPSS 17, and the graphs were plotted using Excel.

Results:

Out of 69 *Acinetobacter* isolates, 57 (82.6%) had bla$_{OXA-51}$ gene and were identified as *A. baumannii*; 35 isolates were from patients in ICU, 3 from emergency department, 12 from internal, 2 from neurology, 4 from general surgery, and 1 from neurosurgery wards; 25 isolates were from patients' trachea, 11 from burn wounds, 8 from surgical wounds, 7 from sputum, and 6 from urine; 45 isolates of *A. baumannii* (78.9%) had bla$_{oxa-23}$, 5 (8.8%) bla$_{OXA-24}$, and one (1.7%) bla$_{OXA-58}$ genes. Figure 1 depicts multiplex PCR reactions for genes bla$_{OXA-51}$, bla$_{OXA-24}$, bla$_{OXA-58}$, and bla$_{OXA-23}$.

Figure 1: Multiplex PCR reaction for the genes bla$_{OXA-51}$, bla$_{OXA-24}$, bla$_{OXA-58}$, and bla$_{OXA-23}$. Wells 1 and 6: bla$_{OXA-51}$ (353 bp) and bla$_{OXA-24}$ (246 bp); wells 2-4: bla$_{OXA-51}$ (353 bp) and bla$_{OXA-23}$ (501 bp); well 5: bla$_{OXA-51}$ (353 bp), bla$_{OXA-23}$ (501 bp), and bla$_{OXA-58}$ (599 bp); well 7: 100 bp ladder.

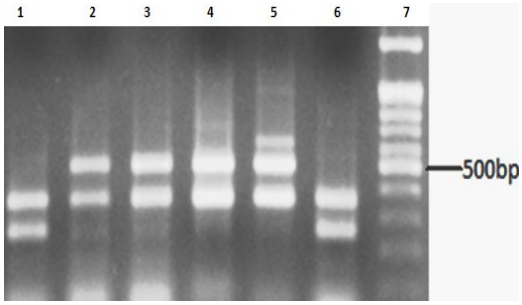

Figure 1. Multiplex PCR reaction for the genes bla$_{OXA-51}$, bla$_{OXA-24}$, bla$_{OXA-58}$, and bla$_{OXA-23}$. Wells 1 and 6: bla$_{OXA-51}$ (353 bp) and bla$_{OXA-24}$ (246 bp); wells 2-4: bla$_{OXA-51}$ (353 bp) and bla$_{OXA-23}$ (501 bp); well 5: bla$_{OXA-51}$ (353 bp), bla$_{OXA-23}$ (501 bp), and bla$_{OXA-58}$ (599 bp); well 7: 100 bp ladder

The resistance level of *A. baumannii* to antibiotics used in this study is shown in Diagram 1. As can be seen, no resistance was found in the case of colistin and polymyxin and the resistance percentage of these isolates to imipenem and meropenem antibiotics was 17 (29.8%) and 40 (70.2%), respectively. All 17 imipenem-resistant isolates carried bla$_{oxa-23}$ gene. In one imipenem-resistant isolate, two bla$_{oxa-23}$ and bla$_{OXA-24}$ genes existed simultaneously, while the two genes bla$_{oxa-23}$ and bla$_{OXA-58}$ existed simultaneously in none of the imipenem-resistant isolates. From a total of 40 meropenem-resistant isolates, 39 (97.5%) carried bla$_{oxa-23}$, 3 (7.5%) had bla$_{oxa-23}$ and bla$_{OXA-24}$ genes simultaneously, and one (2.5%) simultaneously carried three genes of bla$_{oxa-23}$, bla$_{OXA-24}$, and bla$_{OXA-58}$. In one isolate, despite of resistance to meropenem, none of the oxacillinase genes were isolated, except for bla$_{OXA-51}$ which is specific for *A. baumannii*. In 17 isolates that were resistant both to imipenem and meropenem, 16 isolates (94.1%) contained only bla$_{oxa-23}$ and lack other carbapenemase genes.

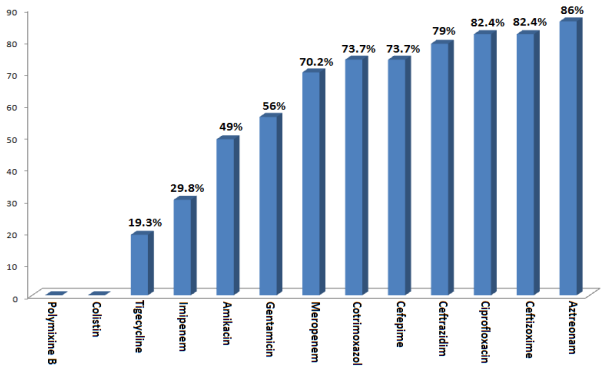

Diagram 1. The Pattern of Antibiotic Resistance of *A. baumannii* to Antibiotics Used in This Study

Conclusion:

Over the past decade, *Acinetobacter baumannii* has been known as the most successful pathogenic bacterium in hospitals throughout the world. Although there is a lot of information about the mechanisms of antibiotic resistance in this microorganism, it is still unstoppable, and today strains with global resistance, even resistant to colistin have been emerged which has seriously limited the treatment. Therefore, it seems necessary to focus on new therapies and to investigate on drugs that not only affect bacterial growth but also interfere with virulence indicators of the bacteria (4). As mentioned, the phenotypic methods are not able to identify the species of *A. baumannii*. Isolation of Bla$_{oxa-51}$ gene is a simple and reliable method for differentiation of *A. baumannii* from other species of *Acinetobacter* (10,30,31). Turton (2006) identified 106 isolates (62.3%) of *A. baumannii* out of 170 isolates of *Acinetobacter via*

ARDRA method; all these isolates had bla_{oxa-51} gene (10). Different frequencies were reported throughout the world for *A. baumannii* identified through bla_{oxa-51} gene, for example, 84.37% in Iran in 2008 (32), 77.8% in Turkey in 2006 (33), and 91.30% in Taiwan (34) and 89.41% in the UK in 2006 (27). The rate of isolation of this microorganism in our study was 82.6%, which is similar to studies in Iran and closer to the rest of the world. Various reports from around the world indicate an increased occurrence of resistance to carbapenem in *A. baumannii*. Several outbreaks of carbapenem-resistant *A. baumannii* have been reported from hospitals in Northern Europe such as Spain, Portugal, France, England, and Ireland, as well as Czech Republic, Poland, Eastern Europe, and Middle East. This rate was 8% in Bulgaria in 2003 but has increased to 52-74% in 2005-2006 (35,36). In Iran, the rate of resistance was reported 49.3% to imipenem and 50% resistance to meropenem in 2008 (32), 52.5% to imipenem and meropenem in 2009 (37), and 49.26% to imipenem in 2011 (38); these are more than the resistance to imipenem observed in this study (29.8%), however, the resistance of strains to meropenem in our study was higher than other Iranian studies (70.2%). Carbapenem-resistant strains with varying degrees of prevalence have been reported from other parts of the world such as Spain, Belgium, Brazil, Cuba, Britain, France, Hong Kong, Kuwait, Singapore, and Argentina (39). In Madagascar, 44% of *A. baumannii* isolated from 5 hospitals during 2006-2009 were carbapenem-resistant (40). In a study in 2006 in Turkey, 26.6% and 7.1% of *Acinetobacter* strains isolated from seven treatment centers were resistant to imipenem and meropenem, respectively (33). Different studies have shown wide dissemination of carbapenem-resistant species carrying oxacillinase genes throughout the world, although significant geographic differences were observed in the molecular epidemiology of Class D carbapenemase or oxacillinase genes. In all studies, same as our research, bla_{oxa-23} gene was highly prevalent among strains resistant to carbapenem. Mendoz et al. found Class D carbapenemase genes in 70% of strains isolated from 41 medical centers in 10 countries during 2006 to 2007; $bla_{oxa-23-like}$ gene was more common and included 95% of genes encoding Class D carbapenemase; and the next genes were bla_{oxa-58} (11.9%) and $bla_{oxa-24/40}$ (5.6%) (41,44). In 2009 in the United States, 13% of imipenem-resistant isolates carried bla_{oxa-23}, and no other carbapenemase genes such as bla_{oxa-24} and bla_{oxa-58} were found (42). In Bulgaria, 72.7% and 27.27% of carbapenem-resistant isolates had $bla_{oxa-23-like}$ and $bla_{oxa-58-like}$ genes, respectively (36). In 2008 in Texas, 11 strains out of 13 carbapenem-resistant strains carried oxacillinase-coding genes, and 6 isolates had bla_{OXA-24} and 5 had bla_{OXA-58} genes; all strains carried bla_{OXA-51} gene which is the gene encoding the constitutive enzyme characteristic of *A. baumannii* (43). In a study in Madagascar during 2006 to 2009, bla_{OXA-23} and bla_{OXA-51} genes were found in all strains of the 44% of carbapenem-resistant isolates of *A. baumannii*, but bla_{oxa-24} and bla_{OXA-58} were absent (40). In our study, despite resistance to meropenem, one strain lacked all oxacillinase genes except bla_{OXA-51} indicating the role of other factors is the resistance which was not addressed in this study. Tigecycline is the antibiotic of choice for treatment of carbapenem-resistant strains, and our study showed that 6 samples (10.5%) of carbapenem-resistant strains were also resistant to tigecycline. Thus, we suggest using this antibiotic for treatment of carbapenem-resistant strains, of course after performing antibiogram test. Out of 17 strains resistant to imipenem and meropenem, 16 isolates (94.1%) carried only bla_{oxa-23} gene. Therefore, resistance to carbapenems can be attributed to the high prevalence of this carbapenemase among the isolates.

The high prevalence of bla_{oxa-23} gene among carbapenem-resistant strains is a warning about the potential distribution of clones carrying this gene in hospital environment. Therefore, conducting extensive epidemiological studies can help increase our knowledge about the epidemiology of this microorganism and its potential transmission paths in hospital environment. The isolates of *Acinetobacter baumannii* in the studied hospital revealed high resistance to commonly used antibiotics and only polymyxin B and colistin are recognized as effective drugs in the treatment of infections caused by this bacterium. Information on changes in resistance patterns of this microorganism in local hospitals can help improve the experimental

treatment in hospitals and the proper management for correct selection of antimicrobial agents.

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