

Antibiotic Resistance Profile and Distribution of Oxacillinase Genes Among Clinical Isolates of *Acinetobacter baumannii* in Shiraz Teaching Hospitals, 2012 - 2013

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Received: May 14, 2014; Revised: June 16, 2014; Accepted: July 26, 2014

Background: The emergence of multidrug-resistant *Acinetobacter baumannii* complicates the therapy of the related infections. Hospital isolates of *A. baumannii* are usually multidrug-resistant. The problem is compounded by increasing resistance to broad-spectrum antibiotics including carbapenems.

Objectives: The aim of this study was to determine antimicrobial susceptibility patterns and distribution of *bla*_{OXA-type} carbapenemases genes among *A. baumannii* isolates from hospitalized patients in Shiraz, Southwest Iran.

Materials and Methods: Two hundred *A. baumannii* isolates were recovered from different clinical specimens in four Shiraz teaching hospitals. Isolates were detected as *A. baumannii* by Microgen kit and PCR with specific primers of *bla*_{OXA-51-like} gene. Antimicrobial susceptibility testing was determined by disk diffusion method for all the isolates. Multiplex PCR assays were performed for detection of *bla*_{OXA-23-like}, *bla*_{OXA-24-like} and *bla*_{OXA-58-like} genes.

Results: All the isolates were susceptible to colistin and polymyxin B. Moreover, all of them were resistant to piperacillin, piperacillin-tazobactam, ampicillin, ceftazidime, cefoxitin and aztreonam. Eighty (40%) isolates had positive results for *bla*_{OXA-23-like}, 14 (7%) for *bla*_{OXA-24-like} and 1 (0.5%) isolate for *bla*_{OXA-58-like}. The co-existence of studied genes was detected for *bla*_{OXA-23-like} plus *bla*_{OXA-24-like} in nine (4.5%) isolates.

Conclusions: The prevalence of carbapenem resistant *A. baumannii* isolates in Shiraz hospitals is high. The *bla*_{OXA-23-like} gene was the most frequent carbapenemase identified among resistant *A. baumannii* isolated in Shiraz hospitals. The increasing incidence of *A. baumannii* is a serious concern, therefore control of this pathogen and taking preventive measures are emphasized.

Keywords: OXA-51 beta-lactamase; Oxacillinase; *Acinetobacter baumannii*

1. Background

Acinetobacter baumannii is a glucose non-fermentative Gram-negative bacillus classified as an opportunistic pathogen responsible for nosocomial infections, especially in Intensive Care Units (ICU) and Burn Therapy Units (BTU) (1, 2). *A. baumannii* is responsible for nosocomial pneumonia, wound infections, bacteremia, urinary tract infection, meningitis, endocarditis, osteomyelitis and keratitis (3). Also infections of respiratory tract, central nervous system, urinary tract and peritonitis can be caused by *A. baumannii* especially in immunocompromised patients (3). In the recent years, the emergence of multidrug-resistant *A. baumannii* has complicated the therapy of *A. baumannii* infections (4). Hospital strains of *Acinetobacter* are usually multidrug-resistant. Multidrug-Resistance (MDR) is defined as resistance to three or more representatives of the following classes of antibiotics: fluoroquinolones, third generation cephalosporins, aminoglycosides and carbapenems (5).

MDR is compounded by increasing resistance to broad-spectrum antibiotics including carbapenems as one of the most effective antibiotics against Gram-negative rods (6, 7). Although, most of *A. baumannii* isolates were susceptible to carbapenems previously and imipenem was the most effective treatment for *A. baumannii* infections, widespread use of carbapenem has caused emergence of resistant strains (8, 9). The emergence of carbapenem resistance in *A. baumannii* is a significant public health concern (3). One of the major mechanisms of carbapenem resistance in this pathogen is production of carbapenem hydrolyzing beta-lactamases mostly Oxacillinase (OXA) types carbapenemases (10). Carbapenem resistance in *A. baumannii* is mediated by acquisition of a class B or class D beta-lactamase genes, such as oxacillinase genes (11). OXA-type carbapenemases have been divided into eight subgroups, four of which have been identified in *A. baumannii*: OXA-23-like, OXA-24-like, OXA-58-like and OXA-

51-like (12). Recently, it has been suggested that enzymes belonging to the OXA-51-like subgroup are intrinsic to *A. baumannii*, occurring in most or all strains, although they are very variably expressed (13).

To control and prevent dissemination of resistant isolates, molecular method for typing of MDR *A. baumannii* is essential (14). The significant contribution of oxacillinase genes to carbapenem resistance has been emphasized, particularly when they are accompanied by ISAb_{a1} and ISAb_{a3} in the naturally occurring plasmids (2, 15). Several studies on these genes have been performed in the world and in some parts of Iran; however, there is no data on the distribution of *bla*_{OXA-type} genes in *A. baumannii* isolates in Shiraz. Therefore, this study was performed to provide information in this regard. This study can help us to know the prevalence of *bla*_{OXA-type} genes in *A. baumannii* isolates in our hospital settings for better infection control and treatment in hospitals in Shiraz.

2. Objectives

The aim of this study was to determine antimicrobial susceptibility patterns and distribution of *bla*_{OXA-type} carbapenemase genes among *A. baumannii* isolates from hospitalized patients in four teaching hospitals, in Shiraz, Southwest Iran.

3. Materials and Methods

3.1. Bacterial Strains

From December 2013 to May 2013, 200 *A. baumannii* were isolated from different clinical specimens, including urine, wound, blood, sputum, endotracheal tube (ETT), body fluid, nose, throat and eye from four Shiraz teaching hospitals (Faghihi, Aliasghar, Ghotebedin and Nemazee) in Shiraz, southwest Iran. Some relevant information including sex, sample type and ward name was recorded. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences. The specimens were cultured on MacConkey agar (Merck, Germany) and blood agar (Merck, Germany), then incubated at 37°C for 24 - 48 hours. These isolates were identified using standard biochemical tests, including Oxidase test, TSI (Triple Sugar Iron agar) medium (Merck, Germany) and SIM (Sulfide, Indole, Motility) medium (Merck, Germany). Negative result for oxidase test, no motility and non-fermentation and growth in temperature of 42 - 44°C was considered as the elementary criteria for *A. baumannii* detection. They were confirmed by Microgen kits (Mast, UK) according to the manufacturer instructions (www.microgenbioproducts.com).

3.2. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed by disk diffusion method according to the Clinical and Lab-

oratory Standards Institute (CLSI) (4, 16). Disk diffusion method was performed on Muller-Hinton agar (Merck, Germany), using an inoculum of 10⁵ CFU. Antibiotic disks (Mast, UK) containing ampicillin (10 µg), ampicillin-sulbactam (10 µg), piperacillin (100 µg), piperacillin-tazobactam (110 µg), amikacin (30 µg), gentamicin (120 µg), imipenem (10 µg), meropenem (10 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), ceftazidime (30 µg), cefoxitin (30 µg), aztreonam (30 µg), colistin (25 µg), polymyxin B (300 unit) and tigecycline (15 µg). These antibiotic disks were then placed on agar plates and incubated at 37°C for 24 hours. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality controls strain in each susceptibility determination (6).

3.3. DNA Extraction

2 - 3 grown bacterial colonies on Muller-Hinton agar plates were suspended in 180 µL PBS. Then DNA of the bacteria was extracted by phenol-chloroform (CinnaGen, Iran) method with some modifications as described before (14). They were used as template in Polymerase Chain Reaction (PCR) assay to amplify *bla*_{OXA} carbapenemase genes (17).

3.4. Detection of *bla*_{OXA-51-like} Gene

Identification of *A. baumannii* was confirmed using *bla*_{OXA-51-like} PCR assay by specific primers (Gene Fanavaran, Iran) (18). To amplify the *bla*_{OXA-51-like} gene, each reaction final volume of 25 µL was considered, containing 1x PCR buffer (CinnaGen, Iran), 1 U Taq polymerase (CinnaGen, Iran), 1.5 mM MgCl₂ (CinnaGen, Iran), 200 µM dNTP (CinnaGen, Iran), 10 pmol of each primer and 1 µL of extracted DNA. PCR conditions were programmed in an Eppendorf Mastercycler as follows: Initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 45 s, 57°C for 45 s, 72°C for 1 min and 5 minute final extension of 72°C. PCR products were electrophoresed (Bio Rad, USA) on 1.2% agarose gel (CinnaGen, Iran), stained with ethidium bromide solution (1 mg/mL) (CinnaGen, Iran) and placed under UV gel transilluminator. *A. baumannii* reference strain ATCC 19606 was used as positive control for *bla*_{OXA-51-like}. Negative control was also included in each PCR reaction, containing all components except the DNA template which was replaced by distilled water (6, 14).

3.5. Multiplex PCR Assay for Detection of Oxacillinase Genes

A Multiplex PCR targeting *bla*_{OXA-23-like}, *bla*_{OXA-24-like} and *bla*_{OXA-58-like} genes was used to screen the isolates as previously described (17). All the isolates were subjected to Multiplex PCR performed to detect *bla*_{OXA-23-like}, *bla*_{OXA-24-like} and *bla*_{OXA-58-like} using specific primers (Gene Fanavaran, Iran), as listed in Table 1 (19). Final volume of 25 µL included 1x PCR buffer, 1 U Taq polymerase, 2 mM MgCl₂, 200 µM of dNTP, 0.2 µM of each primer and

1 μ L of template DNA. Amplification protocol was initial denaturation at 94°C for 5 min, 94°C for 30 s, 53°C for 40 s and 72°C for 50 s and final extension at 72°C for 6 min. The PCR products were run in 2.5% agarose gel electrophoresis, stained with ethidium bromide (CinnaGene, Iran) and visualized under UV gel transilluminator. *A. baumannii* reference strains NCTC 13304, NCTC 13302 and NCTC 13305 were used as positive control for *bla*_{OXA-23-like}, *bla*_{OXA-24-like} and *bla*_{OXA-58-like}, respectively (6, 14).

3.6. Statistical Analyses

To determine the association between variables, data was analyzed by Chi-square and Fisher's exact test using SPSS, version 21 (SPSS Inc. Chicago, IL, USA). P values < 0.05 were considered as statistically significant.

4. Results

4.1. Bacterial Isolates

Of two hundred isolates, 109 (54.5%) were from male and 91 (45.5%) from female patients. Isolates were collected from four hospitals; 82 (41%) from Nemazee hospital, 53 (26.5%) from Aliasghar hospital, 48 (24%) from Faghihi hospital and 17 (8.5%) from Ghotbedin Hospital. The rates of isolation from different wards were as follows; ICU 143 (71.5%), surgeries ward 16 (8%), neurosurgical ICU 14 (7%), neonates 8 (4%), female internal ward 7 (3.5%), male internal ward 7 (3.5%) and organ transplantation ward 5 (2.5%). Of two hundred isolates, 81 (40.5%) were from sputum, 43 (21.5%) from endotracheal tube, 22 (11%) from wound, 16 (8%) from urine, 12 (6%) from blood, 12 (6%) from body fluids, 4 (2%) from nose, 3 (1.5%) from throat, 2 (1%) from CSF, 1 (0.5%) from eye specimens and 4 (2%) other samples. All *A. baumannii* isolates (n = 200) in this study had positive results for *bla*_{OXA-51-like} by PCR (Figure 1).

4.2. Antimicrobial Susceptibility Testing

All the isolates were susceptible to colistin and polymyxin B and all were MDR as defined before, they were totally resistant to piperacillin, piperacillin-tazobactam, ampicillin, ceftazidime, cefoxitin and aztreonam (Table 2). The association between source of isolates and antibiotic resistance pattern was not statistically significant (P > 0.05).

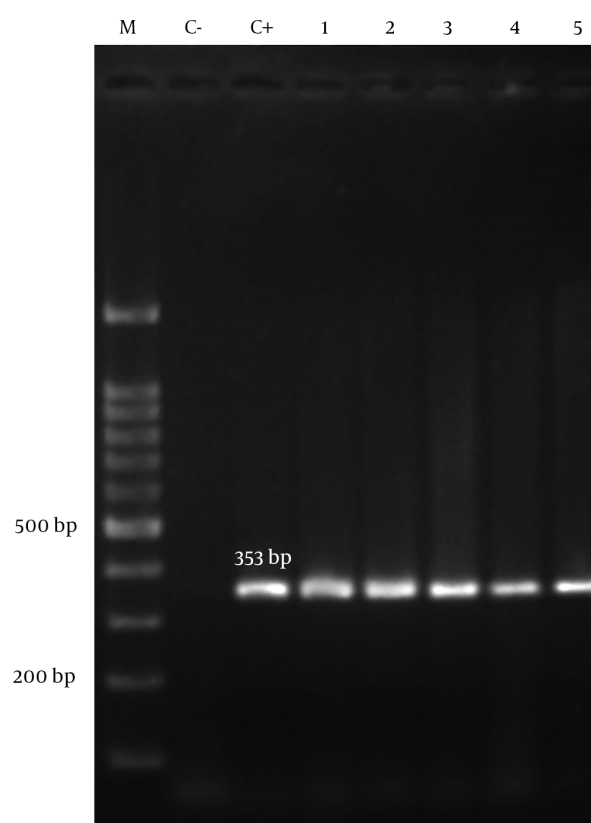
4.3. Detection of Oxacillinase Genes

All isolates of *A. baumannii* were tested by Multiplex PCR for the presence of oxacillinase genes. Eighty (40%) of 200 isolates had positive results for *bla*_{OXA-23-like}, 14 (7%) for *bla*_{OXA-24-like} and 1 (0.5%) isolate for *bla*_{OXA-58-like}. The co-existence of two different *bla*_{OXA} genes in the samples was detected for *bla*_{OXA-23-like} plus *bla*_{OXA-24-like} in nine (4.5%) isolates (Figure 2). The association between source of isolates and presence of genes was not statistically significant.

Table 1. Primer Sequences Used in This Study to Detect Oxacillinase Genes

Primer Sequence (5' - 3')	Amplicon Size, bp
<i>bla</i>_{OXA-23-like}	501
GATCGGATTGGAGAACCAGA	
ATTTCTGACCGCATTCCAT	
<i>bla</i>_{OXA-24-like}	246
GGTTAGTTGGCCCCCTAAAA	
AGTTGAGCGAAAAGGGGATT	
<i>bla</i>_{OXA-51-like}	353
TAATGCTTTGATCGGCCTTG	
TGGATTGCACTTCATCTTGG	
<i>bla</i>_{OXA-58-like}	599
AAGTATTGGGGCTTGCTG	
CCCCTCTGCGCTCTACATAC	

Figure 1. Agarose Gel Electrophoresis of *bla*_{OXA-51-like} Gene in *Acinetobacter baumannii*

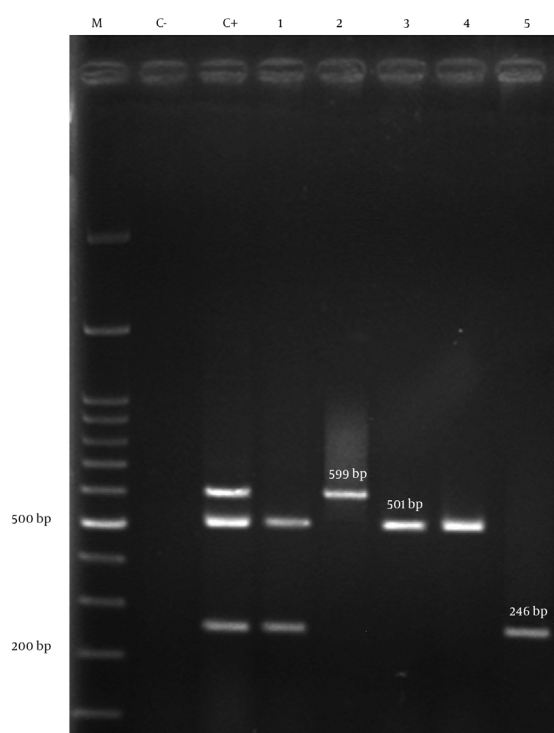


1, 2, 3, 4, 5, clinical isolates of *A. baumannii* with *bla*_{OXA-51-like} gene; C-, negative control; C+, positive control; M, 100 bp DNA ladder.

Table 2. The Results of Antibiogram for Studied *A. baumannii* Isolates^a

Antibiotics	Sensitive	Intermediate	Resistant
Imipenem	2 (1)	1 (0.5)	197 (98.5)
Meropenem	1 (0.5)	-	199 (99.5)
Piperacillin	-	-	200 (100)
Piperacillin-tazobactam	-	-	200 (100)
Ampicillin	-	-	200 (100)
Ampicillin-sulbactam	6 (3)	3 (1.5)	191 (95.5)
Ceftazidime	-	1 (0.5)	199 (99.5)
Cefoxitine	-	-	200 (100)
Gentamicin	27 (13.5)	4 (2)	169 (84.5)
Amikacin	17 (8.5)	10 (5)	173 (86.5)
Ciprofloxacin	1 (0.5)	-	199 (99.5)
Levofloxacin	1 (0.5)	-	199 (99.5)
Aztreonam	-	-	200 (100)
Colistin	200 (100)	-	-
Polymyxin B	200 (100)	-	-
Tigecycline	175 (87.5)	21 (10.5)	4 (2)

^a Values are presented as No (%).

Figure 2. Agarose Gel Electrophoresis of *bla*_{OXA-type} Carbapenemases by Multiplex PCR

1, clinical isolate of *A. baumannii* containing *bla*_{OXA-23-like} and *bla*_{OXA-24-like} genes; 2, clinical isolate of *A. baumannii* having *bla*_{OXA-58-like} gene; 3,4, clinical isolates of *A. baumannii* having *bla*_{OXA-23-like} genes; 5, clinical isolate of *A. baumannii* with *bla*_{OXA-24-like} gene; C-, negative control; C+, positive control (*A. baumannii* NCTC 13304, NCTC 13302, NCTC 13305 for *bla*_{OXA-23-like}, *bla*_{OXA-24-like}, *bla*_{OXA-58-like}); M, 100 bp DNA ladder.

5. Discussion

Acinetobacter baumannii is an emerging nosocomial pathogen which is in part due to its capacity of acquiring resistance to multiple antimicrobial agents (20). Occurrence of multidrug-resistance and Pandrug-Resistant (PDR) in *A. baumannii* is a growing concern (7). All of the isolates in this study were multidrug-resistant and resistant to most of the antibiotics (Table 2). This pathogen is an important Gram-negative bacterium involved in nosocomial infections, especially in ICU wards (18). As shown in our study, 71.5% of the isolates were obtained from hospitalized patients in ICU. This result confirms the fact that *A. baumannii* is often an important cause of infection in hospitalized patients in ICU.

To detect antibiotic resistant patterns, the isolates were investigated primarily by phenotypic methods (21). Our results revealed that 98.5% and 99.5% of the isolates were resistant to imipenem and meropenem, respectively. In some studies in Tehran, 50.9%, 52.5%, 62% and 67.5% of the isolates were resistant to imipenem and 51.8%, 52.5%, 62% and 84.5% to meropenem in 2008, 2009, 2011 and 2013, respectively (6, 12, 18, 20). In another study in Tehran in 2013, resistance rates of 99% and 91.5% were reported to imipenem and meropenem, respectively (22). Mirnejad and Vafaei, reported 76% resistance to imipenem and 69% resistance to meropenem among tested isolates in Tehran in 2013 (23). In two separate studies, one in Ahvaz in 2013, 96.1% resistance to imipenem and meropenem (14), and another in Kermanshah in 2013, 79.8% resistance to imipenem and 75% resistance to meropenem were reported (15). Therefore, our results showed that resistance to carbapenems has an increasing trend which is proba-

bly because of dissemination of highly resistant lineages of *A. baumannii* in our area. Increased resistance to carbapenem causes a real concern over an imminent threat of untreatable *A. baumannii* infections (3).

The current study showed low susceptibility rates to most of available antimicrobial agents for the treatment of infections caused by *A. baumannii*, except for polymyxin B and colistin. In our study, all of the isolates were sensitive to colistin and polymyxin B, while other studies conducted in Tehran demonstrated 12% resistance to colistin and 3% resistance to polymyxin B in 2011 (17). Moreover, in two studies in Tehran and Ahvaz in 2011, all of the isolates were sensitive to these antibiotics (12, 14). In a study in Kermanshah in 2013, all isolates were sensitive to polymyxin B and colistin (15). Sensitivity of all *A. baumannii* isolates to colistin was also reported in Tehran in 2013 (22). Also in a study in Tehran in 2013, among all antibiotic tested, the lowest resistance rate to polymyxin B (3%) was observed (23). The current study also described the important role of class D carbapenem hydrolyzing beta-lactamases. Production of class D oxacillinase by *A. baumannii* distributed worldwide is the main mechanism of resistance to carbapenems in this organism. The major carbapenemase genes involved in carbapenem resistance in *A. baumannii* are *bla*_{OXA-23-like}, *bla*_{OXA-24-like} and *bla*_{OXA-58-like} (8, 14). Alleles encoding OXA-23-like, OXA-24-like and OXA-58-like enzymes were consistently associated with resistance or at least reduced susceptibility to carbapenemases (24).

Our PCR results revealed that all *A. baumannii* isolates had *bla*_{OXA-51-like} gene. The *bla*_{OXA-51-like} genes are located intrinsically in chromosome of all *A. baumannii* strains (8). This result provides evidence that detection of *bla*_{OXA-51-like} can be used as a simple and reliable way of identifying *A. baumannii* (13). GenBank submissions describing variants from isolates of *A. baumannii* from many different countries distributed over four continents clearly suggest that *bla*_{OXA-51-like} is ubiquitous in *A. baumannii* (25). Distribution of other *bla*_{OXA-type} genes in *A. baumannii* is variable. OXA-23-like was the first OXA-type beta-lactamase identified in *A. baumannii* (26). The results obtained in this study indicated that most of our isolates (40%) carried the *bla*_{OXA-23-like} gene. In other studies investigating outbreaks of OXA-23-producing *A. baumannii* strains, the rate of *bla*_{OXA-23-like} ranged 31% to 94% in different parts of the world (14). So our results for *bla*_{OXA-23-like} gene are in the reported ranges. In both separate studies in Tehran, in 2008 and 2009, 25% of the isolates had positive results for *bla*_{OXA-23-like} gene (6, 20). Moreover, in other two studies by Karmostaji et al. (12) and Goudarzi et al. (22), in Tehran in 2013, 81.3% and 55.7% had positive findings for this gene, respectively. 85% and 55.7% positivity rates were reported for *bla*_{OXA-23-like} gene in studied *A. baumannii* isolates in Ahvaz and Kermanshah, respectively (14, 15).

In our study, 7% of the *A. baumannii* isolates had positive results for *bla*_{OXA-24-like} gene. The reported fre-

quency rate of this gene has been previously reported as 0 - 85.4% in different parts of the world (14). In a study in Tehran in 2008, 17.9% of isolates contained *bla*_{OXA-24-like} gene (6); In addition, in another study in Tehran in 2009, 15% had positive results for this gene (20). Three studies in 2013 in Tehran, Ahvaz and Kermanshah showed frequencies of 8.13%, 8.7% and 19.2% for this gene in the studied isolates, respectively (12, 14, 15). Some authors reported *bla*_{OXA-58-like} frequency as 2 - 84.9% in *A. baumannii* isolates in different parts of the world (14). In several studies in Tehran in 2008, 2009 and 2013, 9%, 21.2% and 0.8% of the isolates had positive results for *bla*_{OXA-58-like}, respectively (6, 12, 20). Moreover, a study conducted in Ahvaz and Kermanshah, in 2013, revealed that *bla*_{OXA-58-like} gene was not detected (14, 15). In our study, we identified *bla*_{OXA-58-like} gene only in one *A. baumannii* isolate (0.5%). This frequency was similar to the rate previously reported from central Iran.

We identified nine isolates (4.5%) with co-existence of two different *bla*_{OXA-23-like} plus *bla*_{OXA-24-like}. In two studies in 2013, in Tehran and Kermanshah, 5.7% and 16.4% of *A. baumannii* isolates had such a co-existence (12, 15). In another study in 2013 in Ahvaz, no co-existence between these genes was reported (14). Of note, 105 *A. baumannii* isolates resistant to imipenem and meropenem in our study possessed only the intrinsic *bla*_{OXA-51-like}, but they had negative result for other investigated genes. Resistance to carbapenems in those isolates may be due to other mechanisms other than oxacillinase production (14, 27).

In conclusion, our study confirmed that MDR *A. baumannii* strains were disseminated in Shiraz, Iran. Such *A. baumannii* with different *bla*_{OXA}-carbapenemase genes were isolated from hospitalized patients at Shiraz teaching hospitals (Nemazee, Faghihi, Aliasghar, Ghotbedin) in different wards. The *bla*_{OXA-51-like} genes were the most prevalent subgroup, as they are intrinsic to *A. baumannii*. Moreover, *bla*_{OXA-23-like} gene is another most prevalent resistance gene among MDR *A. baumannii* isolates. The distribution of *bla*_{OXA-58-like} gene was low in our study. Controlling infections of MDR *A. baumannii* in hospitals needs a common strategy issued by decision makers and health-care authorities to make hospitals a safer place for patients.

Acknowledgements

The authors would like to express their sincere appreciation to Dr. Saeed Shoja from Ahvaz Jundishapur University of Medical Sciences for his provision of positive controls for PCR.

Authors' Contributions

Study idea, design and the protocol: Mohammad Motamedifar and Sara Kooti; sample collection, experiments, possession of raw data: Sara Kooti; data analysis, critical revision of the manuscript for important intellectual

content: Mohammad Motamedifar and Jamal Sarvari; final approval of the entire study: Mohammad Motamedifar and Jamal Sarvari. Sara Kooti, and Mohammad Motamedifar wrote the manuscript.

Funding/Support

This study was financially supported by Shiraz University of Medical Sciences, grant No.92-6488. This paper was extracted from MSc thesis of Sara Kooti, supervised by Mohammad Motamedifar.

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