Original Article

Assessment of antibiotic resistance pattern in Acinetobacter baumannii carrying bla oxA type genes isolated from hospitalized patients

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Abstract

Background & Objective: *Acinetobacter baumannii* is a Gram-negative coccobacillus and one of the most opportunistic pathogens responsible for serious infections in hospitalized patients.
Materials and Methods: During a 12 months study, 221 clinical isolates and 22 environmental *Acinetobacter baumannii* isolates were collected. In vitro susceptibility of *Acinetobacter baumannii* isolates to 13 antimicrobial agents: amikacin; cefepime; ceftazidime; ciprofloxacin; meropenem; piperacillin/tazobactam; sulfamethoxazole/ trimethoprim; imipenem; tigecycline; colistin; gentamycin; ceftriaxone; levofloxacin was performed by the disk diffusion method. Also Minimum Inhibitory Concentration (MICs) of imipenem; levofloxacin and cefepime was performed by the E-test according to Clinical and Laboratory Standards Institute (CLSI) criteria. bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-58}, bla_{OXA-51} genes were detected by polymerase chain reaction and sequencing.
Results: The result of antimicrobial susceptibility test of clinical isolates by the disk diffusion method revealed that all strains of *Acinetobacter baumannii* were resistant to piperacillin/tazobactam. The rates of resistance to the majority of antibiotics tested varied between 69% and 100 %, with the exception of tigecycline and colistin. Of 221 isolates tested 99(44.8%) were XDR. All strains carried a bla_{OXA-51}-like gene. bla_{OXA-23} gene was the most prevalent among bla_{OXA}-*types*
Conclusion: Colistin and tigecycline can be effective drugs for treatment of *Acinetobacter baumannii* infections. Continuous Surveillance for *Acinetobacter baumannii* multidrug-resistant strains is necessary to prevent the further spread of resistant isolates.
Keywords: *Acinetobacter baumannii*, Antibiotic resistance, MIC

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Introduction

A. baumannii is a Gram-negative coccobacillus and one of the most opportunistic pathogens responsible for serious infections. This organism is found frequently as a skin and throat commensal in humans and also in various environmental sources such as soil and foods, including vegetables, meat, and fish [1]. *A. baumannii* is responsible for a spectrum of infections that can range from urinary or wound infections to peritonitis, endocarditis, cerebrospinal meningitis and septicaemia [2]. *A. baumannii* is a fastidious organism able to grow at various temperatures and pH conditions. It can survive in the hospital environment and have a remarkable

propensity to develop resistance to antimicrobial agents [3]. During the past decade, *A. baumannii* exhibited a remarkable ability to rapidly develop antibiotic resistance. Resistance to antimicrobial agents among *A. baumannii* clinical isolates is higher than community isolates [4]. *A. baumannii* possesses mechanisms of resistance to most of antibiotic classes, as well as a great propensity for developing mechanisms of drug resistance rapidly. To date, some strains of *A. baumannii* have become almost resistant to all currently available antibacterial agents and thus, empirical treatment choices are extremely limited [5]. The most prevalent resistance-related determinants in multi-drug resistant *A. baumannii* include genes for AmpC cephalosporinases, OXA-type carbapenemases, metallo-_-lactamases (MBLs), efflux pumps and integrons. Carbapenems are the first choice in the treatment of severe *A. baumannii* infections. Unfortunately, resistance to Carbapenems among *A. baumannii* clinical and environmental isolates is increasingly reported [6, 7].

The resistance to carbapenems is due to carbapenem-hydrolysing β-lactamase enzymes of Ambler molecular class B (metallo-β-lactamases) and D (oxacillinases). The OXA-type carbapenemases have emerged globally as the main mechanism responsible for this resistance. Currently, OXA-type carbapenemases are classified into eight subgroups and four of them were identified in *A. baumannii*: OXA-23, OXA-24, OXA-58, and OXA-51–like enzymes. Recently it has been suggested that enzymes belonging to the OXA-51-like subgroup are intrinsic to *A. baumannii*. $bla_{OXA\text{-}51\text{-}like}$ type genes are intrinsically harbored by *A. baumannii* and exhibit presence of a direct reservoir of β-lactam-resistance genes. Detection of bla_{OXA} can be used as a simple and reliable method to differentiate *A. baumannii* strains from other species [8, 9, 10, 11]. The study of resistance to this group of antimicrobials to treatment of infected patients with *A.baumannii* is therefore essential. The aim of this study was to investigate the antimicrobial susceptibility patterns of *A. baumannii* carrying bla_{oxA} type genes isolated from hospitalized patients and environment.

Materials and Methods

Bacterial isolates

A total of 221 clinical isolates of *A.baumannii* which were recovered from specimens of patients suspected with *A.baumannii* infection and 22 environmental isolates which were obtained from patients' surroundings, medical equipment and hands of staff during November 2010 to Oct 2011 were included in this study. A questionnaire containing different clinical and personal data i.e. clinical symptoms, antibiotic usages and underlying conditions was completed for all persons. All the patient and environment samples were transported to the Microbiology Research Laboratory in the Department of Microbiology and were processed immediately. Samples were streaked across MacConkey and blood agar plates for all specimens as routine, Trypticase Soy Broth (TSB), and sub-cultured on chocolate agar for blood specimens, and chocolate agar for specimens other than urine. Presumptive identification was done based on culture characteristics and gram stain. According to Conventional biochemical tests, typical reaction of *A. baumanni* to glucose is positive and to oxidase, mannitol, maltose, Esculin, Indole, and H2S are negative. *A. baumannii* has also ALK/ALK reaction on Triple sugar iron (TSI) agar [12]. Standard identification, confirmation and complete method was conducted including using API® 20E (bioMérieux, France) the commercial identification system. Non *A. baumannii* isolates were excluded from the study. Samples confirmed as a *A.baumannii* were stored in Tryptic Soy Broth (TSB; Merck, Germany) containing 20% glycerol at -70°C and were subjected to further molecular identification.

Antimicrobial susceptibility testing

To evaluate antimicrobial susceptibility of *A.baumannii* isolates, Disk diffusion method was performed according to Clinical Laboratory and Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards) criteria [13]. The following antimicrobial agents were used in this study: amikacin; cefepime; ceftazidime; ciprofloxacin; meropenem; piperacillin/tazobactam; sulfamethoxazole/ trimethoprim; imipenem; tigecycline; colistin; gentamycin; ceftriaxone; levofloxacin. Antibiotic disks used in this research were supplied by MAST Laboratories Ltd (Bootle, Merseyside, UK). Briefly, a bacterial suspension was obtained from overnight cultures. The turbidity of each bacterial suspension was

adjusted equivalent to a no. 0.5 McFarland standard and then inoculated on Mueller-Hinton agar (Oxoid, UK). Diameter of inhibition zones was measured after incubation at 35ºC for 18-24 hours, and data were reported as susceptible, intermediate, and resistant. Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853 were used as reference strains for susceptibility testing

Minimum Inhibitory Concentration (MIC)

The antimicrobial susceptibility profile for all isolates was determined by estimating MIC of 3 antibiotics using E test method according CLSI; interpretive standards [13]. The MIC was defined as the lowest concentration of each antimicrobial agent that inhibited visible growth of the tested isolate. The following antimicrobial agents were used in this study: imipenem; levofloxacin and cefepime (AB BIODISK, Solna, Sweden). The ranges of MIC value used for antimicrobial agents included: imipenem 0.38 to 32 µg/ml; levofloxacin 3 to 256 µg/ml and cefepime 0.064 to 32 µg/ml. Briefly, ta bacterial suspension was obtained from overnight cultures. The turbidity of each of them was adjusted equivalent to a no. 0.5 McFarland standard inoculated on Mueller-Hinton plates, then E test strips were placed on inoculated plates. Plates were incubated at 37° C for 24 h. All plates were monitored daily. The MIC value was read where the ellipse of growth inhibition intersects the strip. Antibiotic resistance was defined as follows: MIC≥32 µg/ml for imipenem, MIC≥ 16 µg/ml for imipenem, MIC≥8 µg/ml for levofloxacin, according to the Clinical and Laboratory Standard Institute (CLSI) recommendations.

DNA extraction and PCR of blaOXA genes:

DNA was extracted from bacteria on nutrient agar medium by Using QIAamp DNA isolation columns (Qiagen, Hilden, Germany) according to the manufacturer's procedure. The presence of OXA-type genes was detected by PCR as described previously by Turton et al. and Niel et al [14, 15]. Primer sequences used for detection of blaOXA-51-like, blaOXA -23- like, blaOXA -24- like and blaOXA-58-like and their fragment size are presented in Table 1. The PCR reactions for detection blaOXA types genes were done on a total volume of 25 µL. The reaction mixture contained 1x buffer (10 mM Tris-HCl, 50 mM KCl), 1.5 mM MgCl2, 0.2µM of each deoxynucleoside triphosphate, 0.5µM of primers and 1.5 U of Takara Taq (Takara Shuzo Co., Ltd., Shiga, Japan). For amplification of 412 bp fragment of the tcdB gene the following time-temperature profile was used: 5 min at 94ºC for initial denaturation, 35 cycles of 1min at 94 ºC, 1 min at 51 ºC, and 80s at 72 ºC ; and a final extension cycle of 5 min at 72 ºC. Amplified fragments were separated by 1.2% agarose gel electrophoresis at 80V for 2h [16]. Finally, fragments were stained by ethidium bromide and detected under UV light.

Table 1. Primers sequence used for amplification *bla*$_{OXA}$ genes [16]

Gene	Nucleotide sequence	Fragment length(bp)
bla$_{OXA-51-like}$	5´-TAA TGC TTT GAT CGG CCT TG-3´ 5´-TGG ATT GCA CTT CAT CTT GG-3´	353
bla$_{OXA-23-like}$	5´GAT CGG ATT GGA GAA CCA GA-3´ 5´ ATT TCT GAC CGC ATT TCC AT-3´	501
bla$_{OXA-24-like}$	5´-GGT TAG TTG GCC CCC TTA AA-3´ 5´AGT TGA GCG AAA AGG GGA TT-3´	246
bla$_{OXA-58-like}$	5´-AAG TAT TGG GGC TTGTGC TG-3´ 5´-CCC CTC TGC GCT CTA CATAC-3´	599

Results

Overall, 221 strains of *A. baumannii* were isolated from hospitalized patients in 3 hospitals. The patients were distributed in 10 hospital departments. Sixty percent (n=108) of the isolates were from males. The median age was 55.1 years old and patient age ranged from 1month to 90 years old. Most *A. baumannii* were isolated from the ICU (75.1%). Isolates were most frequently recovered from respiratory secretions (n=129) followed by wound (n=25), blood (n=24), urine (n=19), catheter (n=11), cerebrospinal fluid (n=4), eye (n=2) and other body fluid (n=7).

The result of antimicrobial susceptibility test of clinical isolates by the disk diffusion method revealed that all strains of *A. baumannii* were resistant to piperacillin/tazobactam. The majority of isolates were resistant to Cefepime (99%), ceftazidime (99%), ciprofloxacin (98%), meropenem (99%), sulfamethoxazole/ trimethoprim (99%), imipenem (91.5%), ceftriaxone (99%), levofloxacin (96.5%), amikacin (70%) and gentamycin (85%). The rates of resistance to the majority of antibiotics tested varied

between 69% and 100 %, with the exception of tigecycline (4%) and colistin (0%). Antimicrobial susceptibilities of 221 *A. baumannii* isolates to 13 antimicrobial are shown in figure 1. MDR *A. baumannii* was defined as those resistant to 3 or more different classes of antibiotics while extensively drug-resistant (XDR) *A. baumannii* was defined as the isolates that were resistant to all tested antibiotics except colistin and tigecycline [17]. Of 221 isolates tested 99(44.8%) were XDR. XDR strains to three or more tested antibiotics were isolates from hospitalized patients in ICU, Surgery, internal medicine and infectious wards.

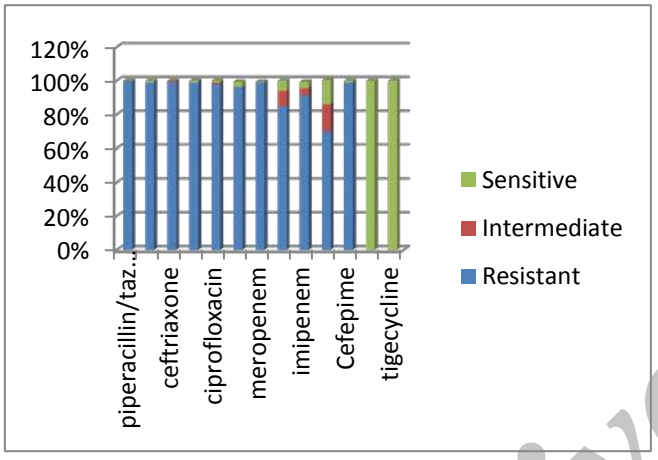

Figure1. Antimicrobial susceptibilities of 221 *A. baumannii* isolates to 13 antimicrobial

In vitro susceptibility of the *A.baumannii* isolates to 3 antibiotics tested and the range of Minimum Inhibitory Concentration required to inhibit the growth of 50% of organisms (MIC50) and Minimum Inhibitory Concentration required to inhibit the growth of 90% of organisms (MIC90) are summarized in Table 2.

Table2. Antimicrobial susceptibilities of 221 *A.baumannii* isolates to 3 antimicrobial agents

Agent	MIC(µg/ml)			No.(%)of isolates			MIC Interpretive Breakpoints[a] (S/R)
	Range	50 %	90 %	S	I	R	≤8/32 ≥ ≤8/32 ≥ ≤2/8 ≥
Imipenem	0.38-32	32	32	7(3.1)	0(0)	214(96.8)	
Cefepime	3-256	128	256	2(0.9)	0(0)	219(99.1)	
Levofloxacin	0.064-32	6	32	2(0.9)	0(0)	219(99.1)	

[a] MIC breakpoints applied were those recommended for anaerobes by the Clinical and Laboratory Standards Institute [13]

PCR results for detection of 4 types of bla$_{OXA}$ genes showed that all strains carry a blaOXA-51-like gene, confirming the strain identification (Figure 2). The presence of *bla$_{OXA-23}$* was confirmed in 123 (55.7%) (Figure 3), blaoxa-58 in 28 (12.7%) (Figure 4), *bla$_{OXA-24}$* in 3 (1/4%) strains (Figure 5). The co-existence of *bla$_{OXA-58}$* and *bla$_{OXA-23}$* were detected in 15 (6.8%) isolates and blaOXA-23-like and *bla$_{OXA-24}$* in 1 (0.5%). There is no co-existence of *bla$_{OXA-24}$*-like and *bla$_{OXA-58}$* among isolates. The frequency of *bla$_{OXA}$*-types among *A.baumannii* isolates are summarized in Table 3.

Table3. The frequency of blaOXA-types among *A.baumannii* isolates

OXA-type	***A. baumannii* isolates**	
	No.(%)of isolates positive	No.(%)of isolates negative
bla$_{OXA}$-23-like	123(55.7)	98(44.3)
bla$_{OXA}$ -58-like	28(12.7)	193(87.3)
bla$_{OXA}$ -24-like	3(1.4)	218(98.6)
bla$_{OXA}$ -58/ *bla$_{OXA}$* -23-like	15(6.8)	206(93.2)
bla$_{OXA}$ -23/ *bla$_{OXA}$* -24-like	1(0.5)	220(99.5)
bla$_{OXA}$ -58/ *bla$_{OXA}$* -24-like	0(0)	221(100)

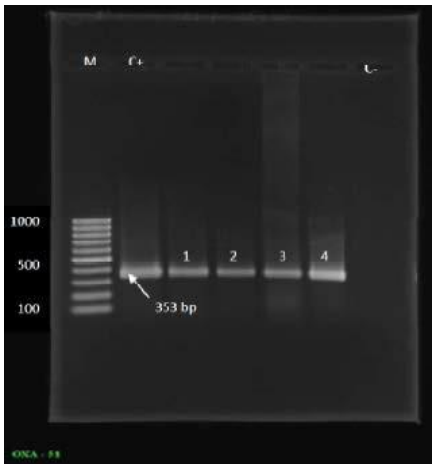

Figure 2. Detection of genes encoding *bla$_{OXA}$* -51 in *A. baumannii* by PCR. M, 100 pb DNA ladder (Fermentas); G+, positive control; 1-4, representative strains tested and G-, negative control.

Figure 3. Detection of genes encoding *bla*$_{OXA}$ -23 in A. baumannii by PCR. M, 100 pb DNA ladder (Fermentas); G+, positive control; 1-4, representative strains tested and G-, negative control.

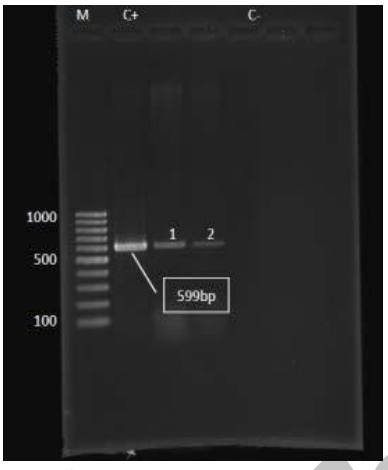

Figure 4. Detection of genes encoding *bla*$_{OXA}$ -58 in *A. baumannii* by PCR. M, 100 pb DNA ladder (Fermentas); G+, positive control; 1and 2, representative strains tested and G-, negative control.

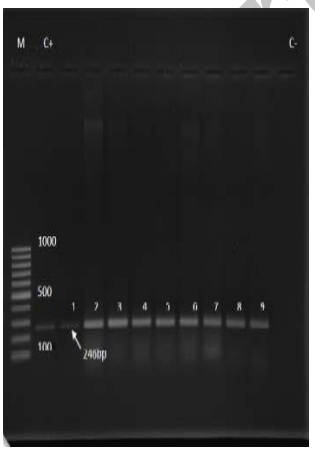

Figure 5. Detection of genes encoding *bla*$_{OXA}$ -24 in *A. baumannii* by PCR. M, 100 pb DNA ladder (Fermentas); G+, positive control; 1-9, representative strains tested and G-, negative control.

Discussion

A. baumannii has become an important pathogen in recent years and has been shown to increase morbidity and mortality. *A. baumannii* are a cause of healthcare-associated and nosocomial infections. They are widely distributed in nature. Control of *A. baumannii* infections because of resistance to antimicrobial agents is always difficult [1-3].

Studies performed by several investigators exhibited that resistance patterns among nosocomial bacterial pathogens is increasing and varies from one geographic region to another.

In this study we studied susceptibility pattern of the 221 clinical and 22 environmental isolates of *A. baumannii* to 13 different antibiotics as common therapeutic drugs in hospitalized patients.

Our findings showed that all strains were resistant to piperacillin/tazobactam. The rates of resistance to the majority of antibiotics (Cefepime, ceftazidime, ciprofloxacin, meropenem, sulfamethoxazole/ trimethoprime, imipenem, ceftriaxone, levofloxacin) were more than 90%. This data is consistent with some earlier reports [18-20]. In 2012, Yan et al. showed that antimocrobial resistance of *A.baumannii* isolates increased from 2001 to 2009. The resistance rates to aztreonam, ceftazidime, cefotaxim; piperacillin/tazobactam, levofloxacin, cefepime were all above 75%. Several investigators showed that Less than 20% of the isolates were sensitive to the majority of antibiotics. Decreased susceptibility to the majority of antibiotics has been reported previously [18-20].

Studies conducted in other countries, has shown that all isolates were susceptible to colistin and tigecycline [19, 21, 22]. Our findings about colistin and tigecycline are in accordance with recent data. Although decreased susceptibility to colistin and tigecycline among *A.baumannii* isolates has been reported but they can be used as effective drugs for treatment of *A.baumannii* infections, but dissemination of *A. baumannii* resistant to colistin is worrying [21].

The carbapenems have been the drug of choice against this pathogen, but the number of isolates resistant to these antimicrobial agents has considerably increased [23, 24].

The MIC values for imipenem have been reported differently by several researchers. In 2009, Boroumand et al. showed that the MIC_{50} results of imipenem, ciprofloxacin, and ceftazidime in 191 clinical isolates of *A.baumannii* were 1.5, 0.5, and >256, whereas and MIC_{90} results of these antibiotics were >32, >32, and > 256, respectively in Iran [25]. In this study the percentages of *A. baumannii* isolates resistant to imipenem, ciprofloxacin and ceftazidime were 24.6%, 53.4% and 41.4%, respectively [25].

Another study that were done on 108 *A.baumannii* isolates in Iran by Feizabadi et al. showed that 50.9% isolates were resistant to imipenem and rate of MIC50 and MIC90 for imipenem were 16 µg/ml and 64 µg/ml respectively [16].

In a study conducted in Iraq by Shali et al. the highest resistant rate was against ampicillin (100%) while the lowest rate was against imipenem (57.1%). The MICs of imipenem for the resistant isolates were ≥16 and all isolates show multi drug resistance to different antibiotics used. The present study revealed that imipenem resistant isolates (91.5%) is increasing [26].

Amazian et al., 2006 showed that the percentage of imipenem-resistant *A. baumannii* strains differed among countries and ranged from 5.2% in Algeria to 28.8% in Tunisia [27]. In 2013, Ramoul et al reported a high prevalence of imipenem-resistant (91.30%) among *A.baumannii* isolated from two intensive care units (ICUs) of two Algerian University hospitals [28]. Yan et al showed that the rate of resistance of *A. baumannii* to imipenem was high (87.8%) in china 2010 [18]. Carvalho et al. reported an increased rate of resistant to imipenem with MIC50 and MIC90 values 32 µg/ml.

In comparison to studies performed in Colombia, Brazil, Iraq, Algeria, Thailand and china [17, 18, 19, 23, 26], a high resistance to imipenem was seen in our study. This high resistance to imipenem in Iran can be caused by indiscriminate use of imipenem in the treatment *A.baumannii* infections and acquired OXA-type β-lactamases.

In this study all of isolates were simultaneously resistant to at least 3 antibiotics. Our study showed that, 99(44.8%) isolates were XDR. According to a study conducted in Thailand 21.1% of isolates were resistant to at least three antibiotics. The frequency of MDR among isolates of *A. baumannii* is increasing. Studies performed by several investigators exhibited that resistance to all beta-lactam antibiotics (including carbapenems), all fluoroquinolones, trimethoprim sulfamethoxazole, and most, if not all, aminoglycosides increased among *A. baumannii* isolates [29]. A high incidence of MDR strains was found in ICU and internal medicine wards in our study. It could be attributable to high usage of antimicrobials agents in ICU. Continued use of antibiotic for treatment of infection should be supported by monitoring of antimicrobial susceptibility to prevent the spread of resistant isolates and also eliminate the need of antibiotics for a prolonged period [30].

The $bla_{OXA\text{-}51}$ gene, considered as a natural component of the species chromosome has been used to identify *A. baumannii* [28]. In accordance to past researches $bla_{OXA\text{-}51}$ genes are present in the vast majority of isolates of *A. baumannii and* may be associated with resistance to carbapenems [31]. In our study, all strains carried a $bla_{OXA\text{-}51}$-like gene and were identified as *A. baumannii*.

Most studies showed that percentage of bla_{OXA} types varies from one geographic region to another [28]. In this study, all of isolates with $bla_{OXA\ 23}$ gene were resistant to carbapenem. This result agrees with studies done by Ben et al. and Kempf et al. [2, 23].

A study conducted in Taiwan showed that all isolates possessed $bla_{OXA\ 51}$ genes. None of the strains carried $bla_{OXA\ 23}$. The coexistences of $bla_{OXA\ 51}$/ $bla_{OXA\ 23}$ and $bla_{OXA\ 51}$/ $bla_{OXA\ 24}$ genes detected in hospitals B and C were 26% (9/34), 12% (4/34), 58% (18/31) and 3% (1/31), respectively [23]. In China, the frequency of $bla_{OXA\ 23}$, $bla_{OXA\ 51}$, $bla_{OXA\ 58\ were}$ reported to be 73%, 12.2% and 2% of clinical strains of multidrug-resistant *A. baumannii* [18].

In comparison to studies performed in Spain, Belgium, Portugal, the Czech Republic, Georgia, France and the USA [2], a similar frequency of $bla_{OXA\ 24}$ enzymes were seen in our study. The existence of $bla_{OXA\ 58}$ gene was certified in France, Spain, Belgium, Italy, Australia, USA [2, 15, 16, 21]. The co-existence of $bla_{OXA\ 58}$ and $bla_{OXA\ 23}$ was detected in 15 (6.8%) isolates and $bla_{OXA\ 23}$ and $bla_{OXA\ 24}$ in 1 (0.5%). There is no co-existence of $bla_{OXA\ 24}$ and $bla_{OXA\ 58}$ among isolates.

Isolates with $bla_{OXA\ 58}$ and $bla_{OXA\ 23}$ genes were highly resistant to imipenem. This result is in accordance with that of Raffaele *et al.* [12]. Usually, OXA-type enzymes exhibit a weak hydrolysis of carbapenems and may not

always show resistance profile, but, they may have an increase in its expression and show resistance to carbapenems when they are associated with insertion elements [31].

Conclusion

In conclusion, this study has shown that resistance to the majority of antibiotics in the population of Acinetobacter strains is high with most of isolates showing multidrug resistance. Although resistance to carbapenem has been seen among our isolates but it seems that carbapenem, colistin and tigecycline can be effective drugs for treatment of *A.baumannii* infections. According to our findings, piperacillin/tazobactam, Cefepime, ceftazidime, ciprofloxacin, meropenem, sulfamethoxazole/trimethoprim, imipenem, ceftriaxone, levofloxacin, with resistance more than 90%, are not effective drugs for treatment of *A.baumannii* infections. Progressive increase in resistance to the majority of antibiotics and multiple resistances in the present study may be related to increased usage of these antibiotics for treatment of CDI and ability of strains in acquisition of resistance genes. Continuous surveillance of *A.baumannii* multidrug-resistant strains is necessary to prevent the further spread of resistant isolates.

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