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Prevalence of ESBLs in *Acinetobacter baumannii* isolated from intensive care unit (ICU) of Ghaem hospital, Mashhad, Iran

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Acinetobacter baumannii **is an important opportunistic pathogen that mainly infects critically patients in intensive care units (ICU). The production of plasmid-mediated extended-spectrum β-lactamases (ESBLs) is one of the most important mechanisms of resistance against β-lactam antibiotics. This study aimed to evaluate the prevalence of ESBLs in *A. baumannii* isolated from ICU of Ghaem hospital, Mashhad, Iran. A total of 140 *A. baumannii* isolates recovered from hospitalized patients in ICU of Ghaem hospital in Mashhad city from December 2014 to March 2015. Identification of *A. baumannii* isolates carried out using biochemical laboratory methods and then confirmed by OXA-51 PCR screening. Susceptibility testing performed using disk diffusion (Kirby-Bauer) method as recommended by CLSI guidelines. *A. baumannii* isolates screened for production of ESBLs using combination disk test. bla*PER, blaGES, blaTEM, blaSHV*, *blaCTX*, *blaVEB* and *blaOXA-10* beta-lactamase genes detected using conventional PCR. The most antibacterial resistance was against cefuroxime (99.3%) and colistin was the most effective antibiotic. None of the isolates were ESBL producer by combination disk test. However, results of PCR revealed that the prevalence of *blaPER*, *blaGES* and *blaTEM* genes were 7.1%, 4.3% and 27.1%, respectively. *blaCTX*, *blaVEB*, and *blaOXA-10* were not found in any of isolates. According to the results, the high resistance was seen against selected antibiotics and the phenotypic tests are not sufficient alone for determination of ESBLs producer of *A. baumannii* isolates. So, molecular tests are also necessary for detection of these enzymes.**

Keywords: *A. baumannii*, ESBLs, ICU, Iran.

Bacteria which constitute the *Acinetobacter* genus were originally identified in the first decade of the 20th century. *Acinetobacter* is a genus of gram-negative bacteria belonging to the *gammaproteobacterial*[1]. *Acinetobacter* are rode-shape during rapid growth and coco-bacillary in the stationary phase. They are generally encapsulated, nonmotile, aerobic, gram-negative organisms with tendency to retain crystal violet and therefore to be incorrectly identified as gram-positive cocci[2].

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Frequent misidentification of *Acinetobacter* as *Neisseria* or *Moraxella* on gram staining is readily clarified by the negative oxidase reaction of *Acinetobacter*. Additionally, *Acinetobacter* are catalase-positive. Hemolysis of red blood cells, acidification of glucose, growth at 44°C, and variability in carbon source uptake are few of the phenotypic characteristics applied to distinguish *Acinetobacter* strains[3], *A. baumannii* isolates are more likely caused disease in patients with immunosuppression, serious underlying disease and people who are exposed to invasive procedures accompanying treatment with broad-spectrum antibiotics. Therefore, the spread of these species in ICU and burn wards is more. *A. baumannii* is an important cause of nosocomial infection, such as ventilator associated pneumonia(VAP), urinary tract infections, wound infections, and septicemia[4]. *A. baumannii* is a significant opportunistic pathogen that mainly infects critically ill patients in ICU[5].

As known the ability of *A. baumannii* to achieve different mechanisms of resistance, also, resistance to all available common antibiotics as well as lack of new effective antimicrobial drugs are the most important causes of risk about this organism. *A. baumannii* isolates which are resistant to three or more classes of antibiotics are called multi-drug resistant strains (MDR). Increasing antibiotic resistance in *Acinetobacte*r inhibits from appropriate management in antibiotic therapy[6].

A. baumannii has several innate resistance mechanisms to a number of antibiotics, such as aminopenicillins, first-and second- generation cephalosporins and chloramphenicol. Besides this, it has a considerable capacity to acquire mechanisms conferring resistance to broad-spectrum β-lactams, carbapenems, aminoglycosides and fluoroquinolones. Beta lactam antibiotics (mainly carbapenems) are now the first drug of choice to treat this microorganism; however, in the last decade, resistance to carbapenems has appeared in hospitals worldwide owing to the production of beta-lactamase, change in permeability, increase in efflux, and modification of the affinity of penicillin-binding proteins (PBP_S) in these bacteria[7]. Production of plasmid-mediated extended-spectrum β-lactamases (ESBLs) is one of the most important mechanisms of resistance against beta-lactam antibiotics. Many of these enzymes have evolved from TEM and SHV -lactamases, but recently a large number of ESBLs are related to TEM and SHV, such as GES and VEB, have been described[8]. Plasmid is accounted for distribution of the most beta lactamases; however, the gene encoding these enzymes may also be on the chromosome or transposable elements and integrons.

ESBLs are also able to hydrolyze three and four generation cephalosporins and monobactams. ESBLs producer isolates are inhibited by β-lactamase inhibitors (clavulanic acid, sulbactam and tazobactam), At present, there are more than 300 different ESBL variants, and these have been clustered into nine different structures and these evolutionary families based on amino acid sequence[9]. TEM, CTX, SHV, GES, VEB, OXA-10 and PER are the major types.

In according to the information on the prevalence of these enzymes and antibacterial resistance pattern, control, prevention and treatment of this bacterium is important, thus, this study aimed to evaluate the prevalence of ESBLs in *A. baumannii* isolated from ICU of Ghaem hospital, Mashhad, Iran.

MATERIALS AND METHODS

Bacterial sources

A total of 140 *A. baumannii* isolates were recovered from hospitalized patients in ICU of Ghaem hospital in Mashhad city from December 2014 to March 2015. All nonlactose fermenting members were subjected to microbiologic and biochemical tests such as; gram staining, oxidase, catalase, O/F, and growth at 42 °C on nutrient agar medium. For confirmation of *A. baumannii* isolates, API20NE kit (version 6.0, bio-Merieux, Marcy L'Etoile, France) was applied. Then until use, clinical isolates were stored in nutrient broth containing 20% glycerol at -80 °C.

Antibiotic Susceptibility Testing

Antibiotic Susceptibility Testing performed using modified Kirby-Bauer disk diffusion method based on CLSI guidelines[6, 10]. The potency of antibiotics disks was checked by reference strains *Pseudomonas aeruginosa* ATCC 27853. After incubation for a period of 24 h, results were reported as sensitive, intermediate, or resistant according to the zone diameters. The antibiotics

used were imipenem (10 µg), meropenem (10 µg), colistin (25 µg), amikacin (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), cefuroxime (30 µg), ceftriaxone (30 µg), cefepime (30 µg), ertapenem (10 µg) and ampicillin/sulbactam (10 µg).

Phenotypic Detection of Beta-Lactamase

In phenotypic confirmation of ESBLs producers on Muller Hinton agar, the combination disc test (CDT) was applied as previously defined[11]. Cefotaxime (30 µ) or ceftazidime disks (30 µ) with or without clavulanate (10 µ) were used. After incubation of plates for 24 h at 37 °C, if the diameter of inhibition zone for each of these antibiotics in combination with clavulanic acid compared to antibiotics alone, increased by more than 5 mm, they defined as the ESBL-producing isolates, if no, isolates were reported as ESBL negative. *P. aeruginosa* ATCC 27853 was applied for quality control of isolates.

DNA extraction and PCR

For DNA extraction and template preparation, the boiling method was used as previously described[12]and lastly samples stored at -20 °C, till use. The primer pair sequences designed by primer premier software for detection of ESBL genes in clinical isolates of *A. baumannii* using PCR technique are shown in Table 1. Of note, PCR of $bla_{\text{OXA-51}}$-like gene was also used for confirmation of isolates identification. The PCR program for bla_{GES}, bla_{CTX} and bla_{PER} genes was composed of an initial denaturation step (94 °C, 5 min) followed by 30 cycles of denaturation step (94 °C, 1 min), annealing step (60 °C, 1 min), and extension step (72 °C, 1 min) with final extension (72 °C, 7 min). The DNA amplification program for $bla_{oxa\text{-}10}$, bla_{SHV}, bla_{VEB} *and* bla_{TEM} genes was similar to previous genes except that the annealing temperature was 51 °C. Components of PCR master mix (Amplicon, Denmark) were as follows; 1.5 mM $Mgcl_2$, 10 pmol/µl of each primer, 0.2 mM dNTPs, 1U Taq DNA polymerase, 1X PCR buffer and 50 ng/µl DNA. PCR products were analyzed using 2% agarose gel electrophoresis (Cinaagen, IRAN). And 50bp DNA ladder (Fermentas company product) was used to detect the specific PCR products related to the bla genes. Then, results were observed under UV light gel documentation system.

Sequencing of PCR products

The PCR products of three samples for each mentioned gene were subjected to direct sequencing and the nucleotide sequences were evaluated and analyzed with CLUSTAL W2 and BLAST softwares.

Data analysis

SPSS software (version 22, Chicago, IL, USA) was used for performing the statistical analysis using chi-squire and Fisher's exact tests. Also, *P*-value<0.05 was considered as significant statistically.

RESULTS

In this study, a total of 140 isolates *A. baumannii* collected from ICU of Ghaem hospital in Mashhad, Iran, from December 2014 to March

Table 1. Primer sequences of ESBL genes amplified by PCR

Primer Name	5'- primer sequence - 3'	Size, bp
F- GES	CGCTTCATTCACGCACTATTACTG	682
R- GES	TCTCTCCAACAACCCAATCTTTAGG	
F- VEB	GCCAGAATAGGAGTAGCAAT	547
R- VEB	GGTTACTTCCTGTTGTTGTT	
F-TEM	CAGTGCTGCCATAACCAT	271
R-TEM	CGCCTCCATCCAGTCTAT	
F- SHV	TATTCGCCTGTGTATTATCTCC	378
R-SHV	CTGTTATCGCTCATGGTAATG	
F-OXA	GCGGCACCTGAATATCTA	165
R-OXA	TCTTAGCGGCAACTTACTT	
F-PER	GCAATACTCGGTCTCGCACAG	461
R-PER	TTCGGCTTGACTCGGCTGAG	
F-CTX	GCGACAATACTGCCATGAATAAGC	349
R-CTX	ATATCGTTGGTGGTGCCATAATCTC	

2015. The sources of isolates were as follows; Trashes 71 (50.7%), Urine 50 (35.7%), Wound 10 (7.2%) and Blood Culture 9 (6.4%). Overall, 51.4%, 48.6% of the hospitalized patients were female and male, respectively. The most frequent isolates of *A. baumannii* (with prevalence of 40%) were in the age group 31-50 years. Also, the most rates of isolates (with prevalence of 51.4%) were seen in female than male.

As shown in Table 2, results of antibacterial susceptibility pattern revealed that in *A. baumannii* the high resistance was to all antibiotics except colistin, as resistance rates to imipenem, meropenem, ceftazidime, cefotaxime, cefuroxime, ceftriaxone, Cefepime, ertapenem and ampicillin/sulbactam were 97.9%, 98.1%, 96.4%, 97.9%, 99.3%, 97.9%, 97.9%,98.6% and 97.1%, respectively. The most effective antibiotic against *A. baumannii* was colistin with susceptibility 97.9% followed by amikacin with sensitivity 27.1 % (Table 2).

It was also presented that none of the isolates were ESBL producers by Combination disk method. Although *A. baumannii* isolates exhibited a high degree of resistance to third-generation cephalosporins but they did not produce ESBL.

Table 2. Antimicrobial susceptibility pattern of *A. baumannii* isolates

Antimicrobials	Resistance, No. (%)	Intermediate, No. (%)	Sensitivity, No. (%)
imipenem	137 (97.9)	0	3 (2.1)
meropenem	138 (98.6)	0	2 (1.4)
colistin	3 (2.1)	0	137 (97.9)
amikacin	94 (67.1)	8 (5.7)	38 (27.1)
ceftazidime	135 (96.4)	0	5 (3.6)
cefotaxime	137 (97.9)	1 (0.7)	2 (1.4)
cefuroxime	139 (99.3)	0	1 (0.7)
ceftriaxone	137 (97.9)	1 (0.7)	2 (1.4)
cefepime	137 (97.9)	0	3 (2.1)
ertapenem	138 (98.6)	0	2 (1.4)
ampicillin/sulbactam	136 (97.1)	2 (1.4)	2 (1.4)

Table 3. Frequency distribution of ESBLs genes in clinical *A. baumannii* isolates

Genes	Number	Genes	Number (%)
bla_{TEM}	38(27.1%)	bla_{CTX}	000
bla_{SHV}	9(6.4%)	bla_{OXA-10}	
bla_{PER}	10(7.1%)	bla_{VEB}	
bla_{GES}	6(4.2%)		

PCR revealed that the prevalence of *blaPER*, *blaGES*, *blaTEM* genes were 7.1%, 4.3% and 27.1%, respectively. *blaCTX*, *blaVEB*, and *blaOXA-10* were not found in any of isolates (Table 3 and Figures 1-3).

A. baumannii isolates which at one time had two ESBL genes were: bla_{PER}/bla_{GES} 2(1.4%),

Table 4. Frequency distribution of ESBLs genes in *A. baumannii* isolates based on types of clinical samples

Genes	Trashes, N=71	Urine, N=50	Wound, N=10	Blood culture, N=9	*P- value*
blaTEM	18(25.4%)	15(30%)	2(20%)	3(33.3%)	0.86
blaSHV	5(7%)	3(6%)	0	1(11.1%)	0.78
blaPER	3(4.2%)	6(12%)	1(10%)	0	0.32
blaGES	2(2.8%)	4(8%)	0	0	0.40
blaCTX	0	0	0	0	0.00
blaOXA-10	0	0	0	0	0.00
blaVEB	0	0	0	0	0.00

bla_{SHV}/bla_{GES} 2(1.4%), bla_{SHV}/bla_{TEM} 2(1.4%), bla_{GES}/bla_{TEM} 1(0.7%).

In addition, results showed that there no correlation was found between prevalence of ESBLs genes and types of clinical samples (p>0.05), as per Table 4.

DISCUSSION

A. baumannii is mostly found in hospital settings and is nowadays noticed more than ever due to its survival ability in such environments and causing nosocomial infections. In this study, among the aminoglycosides, amikacin was resistant in 67.1% of cases and in other groups; cephalosporins, carbapenems, and penicillin were almost 100% resistant, indicating multiple drug resistance in these isolates. Colistin was the most effective antibiotic (97.9%). Our results demonstrated that the rate of resistance was significantly lower in colistin compared to other antibiotics. The possibly reason for low resistance to this antibiotic may be owing to its infrequent prescription during the recent period. In a study conducted by Shahcheraghi et al (2009) on *A. baumannii* isolated from patients hospitalized in Tehran hospitals showed a large proportion of isolates were resistant to ceftriaxone (96.8%), cefotaxime (95.7%), ceftazidime (78.9%), and cefexime (100%), while 95.8% of isolates were susceptible to colistin, which their findings were consistent with current study. This study, like the studies carried out by Tseng et al in China in 2007 [13] and Smolyakov et al in 2000[14], confirmed that most isolates were resistant to ceftazidime and cefepime. Regarding the present results and similar studies, due to over-administration of the third generation cephalosporins and lack of observing the hygienic principles in the community, a

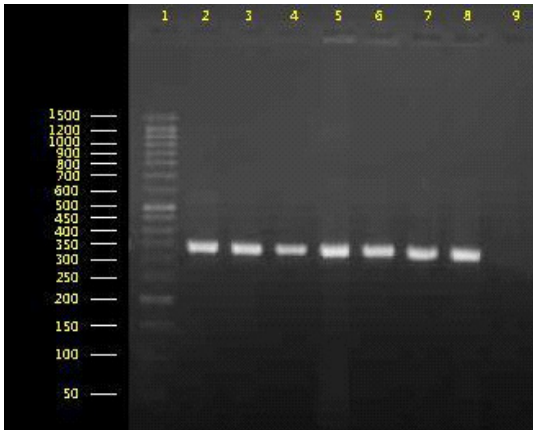

Fig. 1. Agarose gel electrophoresis for analyzing of bla_{oxA-51} gene amplification. Lane 1: 50bp DNA Ladder, lanes 2-8: Isolates containing the fragment of 353bp bla_{oxA-51} gene, lane 9: negative control

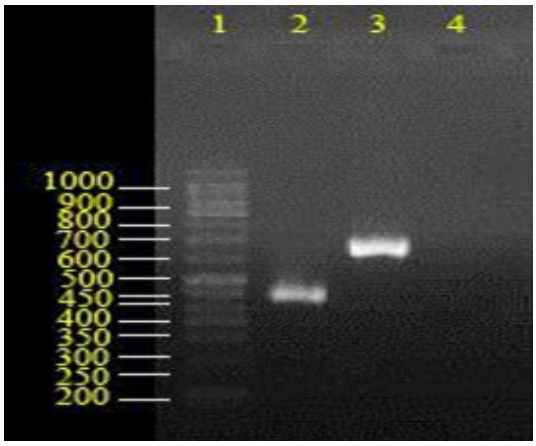

Fig. 2. Agarose gel electrophoresis for bla_{PER} and bla_{GES} genes amplification. Lane 1: 50bp DNA ladder, lane 2: isolate harboring bla_{PER} (461 bp) and lane 3: isolate with 682 bp bla_{GES} gene. 4: negative control

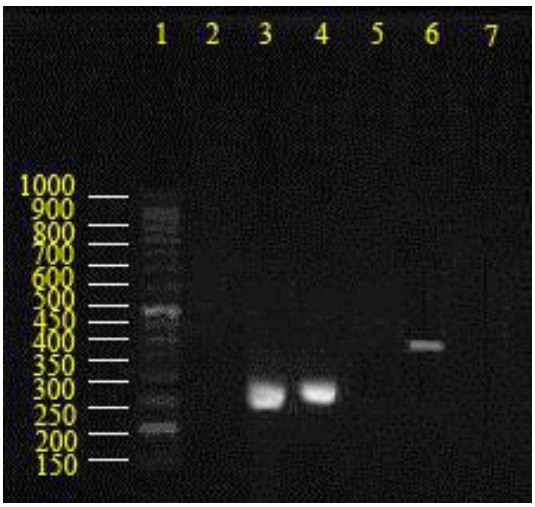

Fig. 3. Agarose gel electrophoresis for amplification analysis of bla_{TEM} and bla_{SHV} genes. Lane 1: 50bp DNA ladder, lanes 2: isolate without bla_{TEM} and bla_{SHV} genes, lanes 3-4: isolates with bla_{TEM}(271 bp), lane 5: negative control for reaction with bla_{TEM} primer, lane 6: isolate with 378 bp bla_{SHV} gene, lane 7: negative control for reaction with bla_{SHV} primer

considerable resistance has been developed against this generation of cephalosporins. So, based these findings, the third generation cephalosporins are not good choice for treatment of infections caused by MDR *A. baumannii* isolates.

The study conducted by Srinivasan and colleagues in Ohio, USA[15], more than 80% of the isolates were resistant to a wide range of cephalosporins and 20% to imipenem, while in our study, resistance to imipenem was more than 90%, which this dissimilarity might be due to unnecessary overuse of antibiotics. An another study conducted by Akan et al in 2002, in Turkey on 277 *A. baumannii* isolates revealed that the resistance rate to imipenem and amikacin was 53.6% and 59.8%, respectively[16], in contrast, this rate was much higher in our study and also in comparable above mentioned ones. All of studies stated here used disk diffusion agar method to evaluate antibiotic susceptibility similar to our study. Therefore, the difference in results could be attributed to diversity in types of isolates, variety in antibiotic disks used, and difference in geographical regions of the studies and policies of infection control[17]. Thus, the regional determination of antibiotic susceptibility of *A. baumannii* can act as a suitable guide for effective use of routine antibiotics. Since in this study and similar ones, the most *A. baumannii* isolates (50.7%) were obtained from pulmonary secretions, it appears that the respiratory tract is the most involved in infections caused by *A. baumannii*. So, disinfection and sterilization of equipment and respiratory devices like respiratory is one of the ways for prevention of infection dissemination. Based on the studies conducted in our country (Iran) and through study reported by Sharif et al in 2013, 51% of *A. baumannii* isolates had the wide-range beta-lactamase-producing phenotype[18]. Also, Owlia et al in 2012, in Tehran reported that 21% of *A. baumannii* isolates had the wide-range beta-lactamase-producing phenotype[19]. Similarly, this rate was reported 28% in a study conducted by Sinha *et al* in India in 2007[8]. Shakibaie et al in 2012 identified only 3 ESBL-producing among 100 *A. baumannii* isolates[20]. The study by Jazani *et al* (2010) reported only 1 ESBL-producing isolate from among 48 *A. baumannii* isolates recovered from clinical samples of patients hospitalized in burn hospital, Tehran[21]. In present study, we used the combination disk method similar to the method used in the mentioned studies, there was no positive test regarding phenotype. One probable reason for lack of production of extended β-lactamase-producing phenotype in the present study, compared to other studies, may be increased expression of AmpC genes and also beta-lactamases and metallo beta-lactamase enzymes. It is also possible that mechanisms other than extended β-lactamase like secretary pumps and variations in porins induce resistance in this organism. Indeed, resistance in *A. baumannii* is associated with a combination of various mechanisms including acquisition of β-lactamases, stable induction of AmpC, reduced permeability, changes in penicillin binding proteins, and somewhat, with an increase in Efflux pumps[21]. Performing of phenotypic tests alone is not able to determine the ESBL-producing isolates in *A. baumannii*. Some molecular tests need be performed to determine the presence of ESBL enzymes.

The bla_{OXA-51} gene is considered as a chromosol component of *A. baumannii* isolates which can used to identify this organism,[22]for this reason in present study we used bla_{OXA-51} gene for confirmation of the *A. baumannii* isolates.

Azhari and et al in 2010 in Tabriz indicated that among 100 isolates of *A. baumannii* from different clinical samples, PER gene was not found in any of samples[23]. The first report of presence of PER gene was detected in a study conducted by Farajnia and et al in 2013 in Tabriz, wherein its prevalence was 51.7% [24] which was higher than over results. But another in 2007 in Argentina presented that among 1 out of 6 isolates was positive for PER gene[25].

Shahcheraghi et al in 2009 in Tehran revealed that of 95 isolates, 12.8% were reported positive for TEM[26] while this rate in study carried out by Sharif and et al in 2012 in Tehran was 56%[18]. In another one in America in 2010, occurrence of TEM was 37%[27], which is partly in line with present study.

GES was first reported in the study by Shahcheraghi et al in 2011 in Tehran, Iran, in which 2 out of 100 *A. baumannii* isolates were resistant to imipenem[26]. Furthermore, in the study carried out by Bogaerts et al in 2009 in Belgium, 9 out of 125 isolates were reported positive for GES gene which is relatively similar to our study with

6 positive isolates[28]. Also VEB was first reported in the study performed by Poirel et al in 2003 in France, in which 7 cases (58.3%) out of 12 *A. baumannii* isolates were positive for *VEB* gene[29]. In the study conducted by Pasteran and colleagues in 2006 in USA, 47.6% of isolates possessed the *VEB* gene[30]. Moreover, in the study carried out by Farajnia et al in 2009 in Tabriz, Iran, 10% out of 100 *A. baumannii* isolates were reported positive for *VEB* gene[24], in contrast none of isolates were positive for *VEB* gene in our study.

Vafaei and et al presented of 130 *A. baumannii* isolates, 19% of those were positive for CTX gene[31]. But in the study conducted by Ramoul and colleagues in 2013, CTX was not found in any of the isolates[32], which is close to our results.

Several studies suggested varying distribution of resistant genes in different geographical regions. Geographical distance and also pattern of antibiotic usage can predispose to emergence of resistant genes in different geographical areas. As Beta-lactamase-producing isolates constitute a lower percentage compared to Beta-lactam-resistant isolates, it seems that in addition to the production of Beta-lactamases, other factors like the presence of Efflux pumps and cellular wall canals or purines also contribute to the creation of resistance. Due to the capacity of these isolates for transmitting resistance genes to other clinical isolates, the exact identification of Beta-lactamases genes contributing resistance is of most importance for care, treatment, and epidemiologic studies on transmission methods in hospitals[33].

The high resistance of isolates to third and fourth generation of cephalosporins compared to the low number of ESBL producing isolates, proposed another resistance mechanisms such as secretory pump, purines, biofilm information involved in development of resistance.

Hence, the development in policies of antibiotic prescription and infection control are more critical to prevent the spreading of such resistant infectious organisms.

CONCLUSION

According to the results, the high resistance was seen against selected antibiotics and the phenotypic tests are not sufficient alone for determination of ESBLs producer of *A. baumannii* isolates. So, molecular tests are also necessary for detection of these enzymes.

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