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ORIGINAL ARTICLE

Prevalence of ESBL and MBL encoding genes in *Acinetobacter baumannii* strains isolated from patients of intensive care units (ICU)

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KEYWORDS

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Abstract The aim of this study was to investigate the prevalence of ESBL and MBL encoding genes among *A. baumannii* isolates. In this cross sectional study, 100 *A. baumannii* strains were isolated from ICU wards of 3 educational hospitals of Hamadan City, Iran in 2011. Phenotypic identification of the production of ESBLs and MBLs has been carried out by using E-test and DDST methods, respectively. PCR technique was used for amplification of the ESBL and MBL encoding genes, namely: CTX-M, SHV, TEM, OXA-51, VIM-Family, IMP-Family, SPM-1, SIM-1, and GIM-1. Eighty seven (87%), 95 (95%), 98 (98%) and 95 (95%) out of 100 *A. baumannii* isolates were resistant to imipenem, meropenem, ceftazidime and cefotaxime, respectively. Also, 99% and 7% of the isolates were MBLs and ESBLs produced phenotypically. Thirty (30%), 20 (20%) and 58 (58%) out of 100 *A. baumannii* isolates have been confirmed to harbor the bla_{VIM}-family, TEM and SHV genes, respectively. Our results show no significant relationship between the detected gens with production of MBLs and ESBLs in spite of high prevalence of MBL encoding

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and drug resistant *A. baumannii*. Probably some other genes rather than what we studied are involved in phenotypic production of MBLs and ESBLs and subsequent drug resistance in Hamadan area, Iran.

1. Introduction

Acinetobacter baumannii is an opportunistic pathogen, with the following characteristics of being Gram-negative, oxidase-negative, non-fermentative, nonmotile coccobacilli and is an unknown natural reservoir and has broad range of antibiotic resistance. The bacterium is prevalent in most places especially in hospitals and other health care institutes (Perez et al., 2007; Yeom et al., 2013; Poirel et al., 2011). Over the last 30 years, *Acinetobacter* genus has been faced with considerable changes in its taxonomic place (Peleg et al., 2008). High morbidity and mortality are the characteristics of nosocomial infections caused by *Acinetobacter* spp., which included urinary tract, skin and soft tissue infections, pneumonia and bacteremia especially in patients with severe health conditions (Karageorgopoulos et al., 2008; Safari et al., 2013; Kuo et al., 2012). Most studies conducted by researchers on this bacterium have been carried out concentrating on its drug resistant aspects, which is a major factor limiting the treatment of nosocomial infections (Poirel et al., 2011; Cerqueira and Peleg, 2011). During the last two decades, the advent and widespread dissemination of bacterial infections resistant to beta-lactams, especially to 3rd generation of cephalosporins and carbapenems, has become a globally significant problem (Pfeifer et al., 2010). Metallo beta lactamases (MBLs) are sorts of powerful enzymes called carbapenemases responsible for antibiotic resistance (Bush, 2001). Four groups of these enzymes have been described in *A. baumannii*, including IMP-like, SIM-1, NDM-type and VIM-like carbapenemases (Poirel et al., 2011, 2010). Genetic characteristic of MBLs-encoding *A. baumannii* isolates revealed the presence of bla_{SIM}, bla_{IMP} and bla_{VIM} genes (Poirel et al., 2011). Extended-spectrum ß-lactamases (ESBLs) are encoded by TEM-type, SHV-type and CTX-M-type genes, which are subjected to phonotypical resistance to penicillins and 3rd generation cephalosporins (Pfeifer et al., 2010). ESBLs are mostly plasmid-mediated and most are parts of the TEM and SHV families of enzymes (Mehrgan and Rahbar, 2008).

One of the notable carbapenem-resistance mechanisms is caused by carbapenem-hydrolyzing β-lactamases, carbapenemases. In addition, the metallo-β-lactamases (MβLs) paly a crucial role in drug resistance against carbapenems (Poirel and Nordmann, 2006). Also, the extended-spectrum β-lactamases, ESBLs, play an important role in resistance against later generation cephalosporins such as cefepime, cefotaxime and ceftazidime (Zhanel et al., 2013).

The aim of this study was to investigate the prevalence of ESBLs and MBLs encoding genes and drug resistance against meropenem, imipenem, ceftazidime and cefotaxime among *A. baumannii* isolates.

2. Materials and methods

2.1. Sampling and isolation of bacteria

The study was a cross sectional study initiated in June 2011 by collecting 100 non-duplicate *A. baumannii* isolates from clinical specimens from ICU ward patients hospitalized in three educational hospitals of Hamadan City in Iran. The sampling was conducted for 17 months and the isolates were almost from 74 tracheal aspirate, 16 blood, 5 urine, 4 sputum and 1 wound samples. The identification of the isolates has been accomplished by biochemical tests and confirmed by tracking the bla-OXA-51 -like carbapenemase gene, which is intrinsic to this species, using single PCR (Turton et al., 2006). The confirmed *A. baumannii* isolates were kept frozen at −70 °C for further tests.

2.2. Antibiogram

Susceptibility to meropenem, imipenem, ceftazidime and cefotaxime (Mast CO, UK) was tested by Kirby-Bauer disk diffusion method. The antibiogram procedure was performed as the manufacturer constructed. In brief, 1.5×10^8 CFU of bacterial suspension, equivalent to McFarland Turbidity Standard No. 0.5, was transferred on Muller-Hinton agar medium (Merck, Germany) and antibiogram disks containing meropenem (10 μg), imipenem (10 μg), ceftazidime (30 μg) and cefotaxime (30 μg) were placed on the medium. Then, the media were incubated for 18 h at 35 °C. The results were interpreted according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2007). A control strain of *Pseudomonas aeruginosa* ATCC 27853 was used for quality control of susceptibility testing.

2.3. Phenotypic MBL detection

For determination of phenotypic MβL production among the bacterial isolates, MIC Test Strips (Lioflichem® Italy) containing imipenem and imipenem plus EDTA were used. The E-test procedure was performed according to the manufacturer's manual. Reduction in the MIC of imipenem of $\geqslant 3$ dilutions in the presence of EDTA is regarded as a positive result. Also, *A. baumannii* strain was considered MβL producer if a phantom zone or deformation of the ellipse was obviously observed.

2.4. Phenotypic identification of ESBL producing isolates

Phenotypic identification of ESBL producing isolates have been carried out using DDST screening method. Antibiogram disks containing ceftazidime (30 μg), cefotaxime (30 μg), ceftazidime (30 μg) + clavulanic acid (10 μg) and cfotaxime (30 μg) + clavulanic (10 μg) were used. Pairs of disks (ceftazidime with ceftazidime/clavulanic acid and cefotaxime

Table 1 Primers used for PCR amplification of the studied genes.

Primer name	Primer sequence (5′ to 3′)	Annealing temp (°C)	Product size (bp)	Reference
CTX-M	F:TCTTCCAGAATAAGGAATCCC R:CCGTTTCCGCTATTACAAAC	51	909	Kalai Blagui et al. (2007)
SHV	F: CTTTACTCGCTTTATCG R: TCCCGCAGATAAATCAC	53	868	Kolar et al. (2010)
TEM	F:ATGAGTATTCAACATTTCCG R:CCAATGCTTAATCAGTGAGC	53	931	Kalai Blagui et al. (2007)
OXA-51	F:TAATGCTTTGATCGGCCTTG R:TGG ATTGCACTTCATCTTGG	52	353	Turton et al. (2006)
VIM-Family	F: GATGGTGTTTGGTCGCATA R: CGA ATGCGCAGCACCAG	52	390	Ellington et al. (2007)
IMP-Family	F: GGAATAGAGTGGCTTAAYTCTC R: CCA AACYACTASGTTATCT	52	188	Ellington et al. (2007)
SPM-1	F:AAAATCTGGGTACGCAAACG R:ACATTATCCGCTGGAACAGG	54	271	Ellington et al., 2007
SIM-1	F:TAC AAGGGATTCGGCATCG R: TAATGGCCTGTTCCCATGTG	54	570	Ellington et al. (2007)
GIM-1	F: TCG ACACACCTTGGTCTGAA	54	477	Ellington et al. (2007)

with cfotaxime /clavulanic) were placed on Muller-Hinton agar medium (Merck, Germany) with 20 mm space between them. According to the CLSI criteria and manufacturer instruction, the $\geqslant$5 mm inhibition zone of growth in ceftazidime/clavulanic acid and cfotaxime/clavulanic than ceftazidime and cfotaxime was regarded as an isolate that is producing ESBLs.

2.5. Amplification of bla_{OXA-51}-like gene

The bacterial DNA was extracted by the alkaline lysis method (Kheyrodin and Ghazvinian, 2012). The pair of bla_{OXA-51} primers (Bioneer® Korea); **OXA**-F 5′-TAATGCTTTGATCGGC CTTG-3′, and **OXA**-R 5′-TGGATTGCACTTCATCTTGG-3′ were used for PCR detection of the genes (Turton et al., 2006). PCR amplification procedure was performed using 25 µl of master mix containing 0.2 µl of Taq polymerase 5 U/µl, 2.5 µl of 10XPCR buffer along with MgCl2, 1 µl of 10 pM from each reverse and forward primers, 2.5 µl of dNTPs MIX (2 Mm), 3 µl of DNA template, 14.8 µl of DNase-Free and RNase-Free Distilled Water. PCR amplification was done in the thermal cycler device. Agarose gel electrophoresis of the amplified DNA product with 100 bp size marker (Fermentas®, Korea) was carried out in a 2% agarose gel for 2 h at 80 V and stained with ethidium bromide to manifest and detect the 353 bp band. Also, PCR reaction contained positive and negative control. The *A. baumannii* carrying bla_{OXA-51}-like gene (obtained from Dr. Bahador, Tehran University of Medical Sciences, Tehran, Iran) was used as the positive control.

2.6. Amplification of ESBL genes

The specific primers (Bioneer® Korea) including CTX-M, SHV and TEM (Table 1) were used for PCR amplification of the genes (Ellington et al., 2007; Kalai Blagui et al., 2007; Kolar et al., 2010; Turton et al., 2006). PCR amplification procedure was performed with 25 µl of master mix containing 0.2 µl of Taq polymerase 5 U/µl, 2.5 µl of 10× PCR buffer along with $MgCl_2$, 1 µl of 10 pM from each reverse and forward primers, 2.5 µl of dNTPs MIX (2 Mm), 3 µl of DNA template,14.8 µl of DNase-Free and RNase-Free Distilled Water. PCR amplification was done in the thermal cycler device. Agarose gel electrophoresis of the amplified DNA product with 100 bp size marker (Fermentas®, Korea) was carried out in a 2% agarose gel for 2 h at 80 V and stained with ethidium bromide.

2.7. Amplification of MBL genes

The specific primers (Bioneer® Korea) including VIM-Family, IMP-Family, SPM-1, SIM-1 and GIM-1 (Table 1) were used for PCR amplification of the genes (Ellington et al., 2007). PCR amplification procedure was performed with 25 µl of master mix containing 0.2 µl of Taq polymerase 5 U/µl, 2.5 µl of 10X PCR buffer along with $MgCl_2$, 1 µl of 10 pM from each reverse and forward primers, 2.5 µl of dNTPs MIX (2 Mm), 3 µl of DNA template,14.8 µl of DNase-Free and RNase-Free Distilled Water. PCR amplification was done in the thermal cycler device. Agarose gel electrophoresis of the amplified DNA product with 100 bp size marker (Fermentas®, Korea) was carried out in a 2% agarose gel for 2 h at 80 V and stained with ethidium bromide.

2.8. Statistical analysis

Statistical results were calculated by the Statistical Package for the Social Sciences (SPSS Inc., USA) version 16.0 for windows and also, using McNemar and Chi square tests regarding $P \leqslant 0.05$ as significance level.

3. Results

A. baumannii isolates were confirmed with microbiological and PCR (bla-$_{OXA-51}$) methods and entered to the study (Fig. 1).

3.1. Antibiogram

Susceptibility to meropenem, imipenem, ceftazidime and cefotaxime was evaluated by the Kirby-Bauer disk diffusion

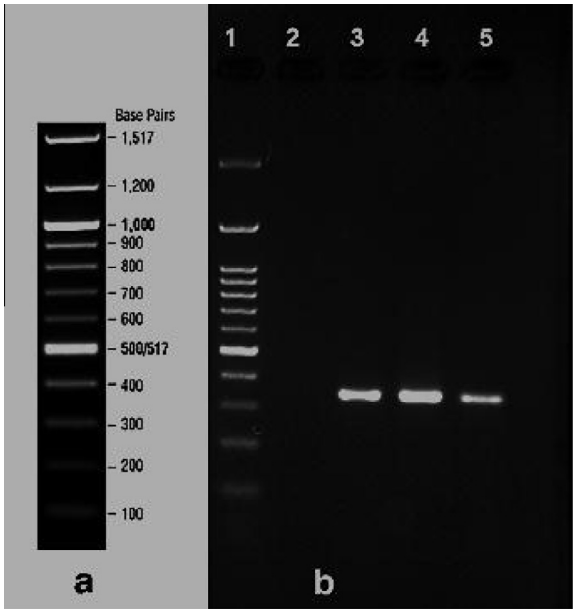

Figure 1 PCR results for amplification of bla_{OXA}-51 gene in *A. baumannii* isolates; Lane 1:100 bp DNA ladder; Lane 2: negative control; Lane 3: Positive control; Lanes 4, 5 different isolates harboring bla_{OXA}-51-like gene.

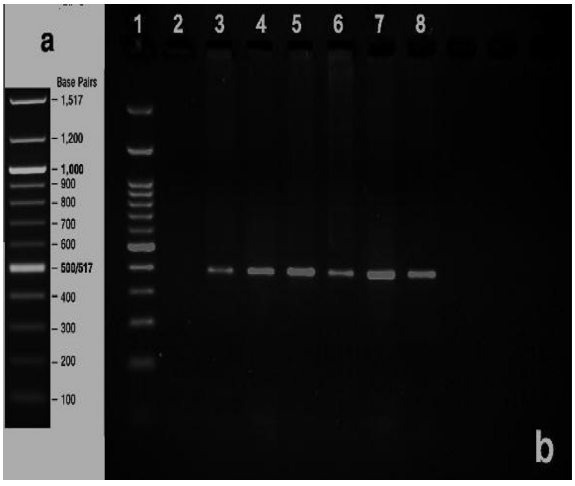

Figure 2 PCR product gel electrophoresis; a: 100 bp DNA ladder template of the company; b: PCR results for amplification of bla_{VIM}-family genes in *A. baumannii* isolates; Lane 1:100 bp DNA ladder; Lane 2: negative control; Lane 3, Positive control; Lanes 4, 8 different isolates harboring bla_{VIM}-family gene.

method. Eighty seven percent, 95%, 98% and 95% out of 100 *A. baumannii* isolates were resistant to imipenem, meropenem, ceftazidime and cefotaxime, respectively.

3.2. Phenotypic MBL detection

For determination of phenotypic MβL production among isolates, MIC Test strips containing imipenem and imipenem along with EDTA were used. Ninety nine percent out of 100 *A. baumannii* isolates were MBL producing.

3.3. Phenotypic identification of ESBL producing isolates

Phenotypic identification of ESBL producing isolates have been carried out using DDST screening method. From total of 100 samples, 7% *A. baumannii* isolates identified to be produce ESBL enzymes.

3.4. Detection of ESBL–SHV, -CTX-M and -TEM genes

Of all 100 *A. baumannii* isolates, 58%, and 20% isolates were harboring HSV, and CTX-M genes, respectively. The TEM gene was not found in the studied strains. There was no statistically significant relationship between the cefotaxime and ceftazidime resistance and presence of SHV, CTX-M genes in the isolates.

3.5. Detection of VIM-Family, IMP-Family, SPM-1, SIM-1 and GIM-1genes

Thirty percent out of 100 *A. baumannii* isolates has been confirmed to harbor the bla_{VIM}-family genes (Fig. 2), but the other genes including IMP-Family, SPM-1, SIM-1 and GIM-1 have not been detected (Table 2). No significant relationship was observed between the presence and absence of bla_{VIM}-family genes in the isolates' resistance to imipenem and meropenem.

Table 2 Frequency of the studied genes among 100 *A. baumannii* isolates.

Gene	PCR results	
	Positive *N* (%)	Negative *N* (%)
SHV	58 (58)	42 (42)
TEM	20 (20)	80 (80)
CTX-M	0 (0)	100 (100)
VIM-Family	30 (30)	70 (70)
IM-Family	0 (0)	100 (100)
SIM	0 (0)	100 (100)
SPM	0 (0)	100 (100)
GIM	0 (0)	100 (100)

4. Discussion

Production of carbapenem-hydrolyzing β-lactamases, also called carbapenemases, is one of the significant mechanisms of carbapenem resistance, in which Methalo β-lactamases (MβLs) possess the principal role in drug resistance against carbapenems (Poirel and Nordmann, 2006). Also, the extended-spectrum β-lactamases, ESBLs, play an important role in resistance against later generation cephalosporins such as cefotaxime, ceftazidime, and cefepime (Zhanel et al., 2013). In this study, the prevalence of ESBLs and MBLs encoding genes and drug resistance against meropenem, imipenem, ceftazidime and cefotaxime among *A. baumannii* isolates has been investigated showing a high resistance rate among the antibiotics.

Totally 100 *A. baumannii* isolates have been examined for 3 ESBLs and 5 MBLs encoding genes. Three out of eight genes have been detected including SHV (58%), TEM (20%) and

VIM (30%). None of the other studied genes has been detected among 100 isolates of *A. baumannii*, which were isolated from ICU wards of 3 educational Hospitals, Hamadan City, Iran, 2011. Also, no significant relationship has been observed about the presence of the detected ESBL and MBL encoding genes with phonotypical resistance against imipenem, meropenem, cefotaxime and ceftazidime. Eighty seven percent, 95%, 98% and 95% out of 100 *A. baumannii* isolates were resistant to imipenem, meropenem, ceftazidime and cefotaxime, respectively.

The results showed that most of the *A. baumannii* isolates were producing MBLs (99%), but not ESBLs (7%). There was not any significant relationship about phonotypic ESBL and MBL producing and the detected genes. Reports from Iran illustrate the high prevalence of drug resistance and multi drug resistance in *A. baumannii* especially against most effective antibiotics such as imipenem and meropenem (Feizabadi et al., 2008; Peymani et al., 2011). Ting et al. (2013) investigated the drug resistance genes in 7 strains of imipenem-resistant *A. baumannii* including TEM, SHV, CTX-M, DHA, CIT, IMP, VIM, KPC, OXA-23. They detected TEM (100%) and OXA-23 (100%) genes among the isolates, but the other genes such as SHV, CTX-M, DHA, CIT, IMP, VIM, KPC could not be detected from 7 strains of imipenem-resistant *A. baumannii*. In the present study, consistent with Ting et al. (2013), just some of the genes have been detected including SHV (58%), TEM (20%) and VIM (30%). In another study by Shahcheraghi et al. (2011) in Tehran, Iran, they showed that the MBL encoding genes included bla VIM-2, bla SPM-1, bla IMP-2, bla GES-1, bla OXA-51, bla OXA-23 genes among 203 *A. baumannii* isolates. They reported that 6 isolates produce MBLs and 94 isolates produce OXA-type carbapenemase. Their finding suggests that in Tehran the prevalence of MBLs producing *A. baumannii* strains is lower than that of the present study from Hamadan City. They detect $bla_{\text{SPM-1}}$, $bla_{\text{GES-1}}$, $bla_{\text{OXA-51}}$, $bla_{\text{OXA-23}}$ genes among 6, 2, 94 and 84 isolates of the bacterium, respectively (Shahcheraghi et al., 2011). The previous research by Rezaee et al. (2013) revealed genes coding for IMP, SPM-1, VIM, PER-1, VEB-1, TEM, SHV, GES-1, and CTX-M among 76 Acinetobacter spp. Also, they reported that 37% of isolates carried at least one of the $bla_{\text{PER-1}}$ or $bla_{\text{TEM-1}}$ genes and 13.15% of their studied isolates reported to harbor $bla_{\text{TEM-1}}$ gene, which is similar to that of the present study (20%). Also, none of their studied *A. baumannii* isolates were harboring for $bla_{\text{VEB-1}}$, $bla_{\text{SHV-}}$, $bla_{\text{CTX-M-2}}$ and $bla_{\text{GES-1}}$ (Rezaee et al., 2013). In our study, none of the following genes, CTX-M, IMP, SIM, SPM, and GIM, has been detected among 100 isolates of *A. baumannii*.

Our results show that there was not any significant relationship between the detected genes with production of MBLs and ESBLs. Probably, some other genes rather than what we studied are involved in phenotypic production of MBLs and ESBLs and the subsequent drug resistance in Hamadan, Iran.

5. Conclusion

Despite the high prevalence of phenotypic MBL production and high resistance rate against imipenem and meropenem *A. baumannii* isolates, lower rates of MBL encoding genes have been detected. Also, the high resistance rate against ceftazidime and cefotaxime was not in relation with phenotypic and genotypic ESBL production. Probably some other genes rather than what we studied are involved in phenotypic production of MBLs and ESBLs and subsequent high drug resistance in Hamadan, Iran.

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