Detection of Aminoglycoside Modifying Enzymes and β -lactamases in *Acinetobacter baumannii* Isolated from Hospitalized Iraqi Patients

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ABSTRACT

Background/aim: *Acinetobacter baumannii* is one of the most commonly encountered as multiresistance microorganism. This study was designated for investigating of β -lactamases resistance genes and aminoglycosides modifying enzymes (AMEs) in *Acinetobacter baumannii* isolated from Iraqi patients.

Materials and methods: A total of 36 *A. baumannii* isolates were recovered from hospitalized Iraqi patients during May to September 2012. Antibiotic susceptibility test was performed against aminoglycosides antibiotics and phenotypic detection of β -lactamases was done using Extended Spectrum Beta Lactamases (ES β L) strip. Polymerase Chain Reaction (PCR) amplification was achieved for investigating β -lactamases genes (*bla_{SHV}*, *bla_{TEM}*, and *bla_{CTX}*) and aminoglycosides modifying enzymes (*aac(6)-Ib*, *ant(4')-IIb*, *aa(c) 3'* and *aph(3')-VI*).

Results: Out of 36 *A. baumannii* isolates, 15 (41.66%) were resistant to aminoglycosides antibiotics according to disk diffusion method. 17 (47.22%) isolates gave positive results in the preliminary screening of β -lactamases, the ES β Ls type and AmpC enzyme, while 13 (36.11%) of the isolates gave a positive result in Metallo- β -lactamases production test. PCR analysis demonstrated that *bla_{TEM}* was detected in 3 (20%) of the resistant isolates, *bla_{SHV}* was detected in 9 (60%), *bla_{CTX-M}* was detected in 11 (73.33%), *aac(6)-Ib* was detected in 7 (46.66%), *ant(4')-IIb* was detected in 5 (33.33%), *aph(3')-VI* in 2 (13.33%), and *aa(c) 3'* was found in 10 (66.66%) of the resistant isolates.

Conclusion: Local *A. baumannii* isolates with AMEs and β -lactamases genes represent a powerful nosocomial pathogen that threat the antibiotic era and life of immunocompromised and hospitalizes patients and this should be taken into account to find a new ways for the restriction of these powerful pathogens.

INTRODUCTION

Acinetobacter baumannii is an emerging pathogen responsible for causing a wide range of nosocomial infections, including pneumonia, urinary tract infections, and septicemia particularly in immunocompromised patients (Karlowsky et al., 2003). Due to its outstanding ability to upregulate or attain resistance determinants and its environmental flexibility, it's considered one of the most microbial threat to the antibiotic treatment strategy (Nordmann, 2004).

Recently multi-drug resistant *A. baumannii* strains have now been reported, signifying a sentinel event that should be acted on promptly by the international health care community (Hussein et al., 2013). Extensive use of antimicrobial chemotherapy within hospitals has contributed to the emergence and procreation of *A. baumannii* strains which are resistant to a wide range of antibiotics, including broad spectrum β -lactams, aminoglycosides, and fluoroquinolones (Asadollahi et al., 2012). Resistance to β -lactams appears to be primarily caused by β -lactamase production, including the extended spectrum β -lactamases (*bla*_{TEM}, *bla*_{SHV}, *bla*_{VEB}, *bla*_{PER}), metallo- β -lactamases (*bla*_{IMP}, *bla*_{VIM}, *bla*_{SIM}, *bla*_{GIM}), and most commonly, oxacillinases (*bla*_{OXA51}, 23, 24 and 58), while the resistance to aminoglycosides may be through many known mechanisms but the enzymatic modification is the most prevalent in the clinical setting (Asadollahi et al., 2012).

Aminoglycoside modifying enzymes (AMEs) catalyze the modification at different –OH or –NH₂ groups of the 2-deoxystreptamine nucleus or the sugar moieties and can be nucleotidyltransferases, phosphotransferases, or acetyltransferases (Ramirez and Tolmasky, 2010). Thus, there are three types of these enzymes: Aminoglycoside N-Acetyltransferases (AACs), Aminoglycoside O-Nucleotidyltransferases (ANTs), Aminoglycoside O-Phosphotransferases (APHs). The AAC family of enzymes is composed of four major subclasses based on region specificity of aminoglycoside acetyltransfer: AAC(6'), AAC(2'), AAC(1), and AAC(3), while there are five members of the ANT family of enzymes, ANT(2''), ANT(4'), ANT(3'), ANT(6') and ANT(9) (Arya, 2007). The third mechanism of aminoglycoside modification is ATP-dependent phosphorylation of key hydroxyl groups, a reaction catalyzed by the APH family of kinases including APH(2''), APH(3'), APH(3''), APH(7''), APH(4), APH(6), and APH(9) (Arya, 2007). Aminoglycoside-modifying enzymes have become widespread throughout bacterial communities because many of the encoding genes are found on mobile genetic elements such as integrons, transposons, and plasmids, thus facilitating horizontal gene transfer (Arya, 2007). The emergence of aminoglycoside-inactivating enzymes has contributed to the diminished use of several aminoglycoside antibiotics and the presence of these types of enzymes have been

found in *A. baumannii*, often occurring in combination (Arya, 2007, Seward et al., 1998).

Some reports referred to the presence of these types of enzymes in Iraq, especially the studies that were done on the military and civilian casualties from the Iraqi conflict and some genes responsible for Aminoglycoside modifying enzymes were detected, e.g: *aacC1*, *aadA1a*, *aadB*, *aacA4*, and *aadA1* (Turton et al., 2005). So this study aimed for the detection of other types of AMEs genes besides some of β -lactamases resistance genes in some of *A. baumannii* that have been isolated from hospitalized patients in Iraq.

MATERIAL AND METHODS

Bacterial strains and identification

A total of 36 *A. baumannii* isolates were recovered from hospitalized patients during the period from May to September 2012. The isolates were collected from different sites including: sputum (one isolate), cerebrospinal fluid (one isolate), urine (17 isolates), wounds (17 isolates), and blood (6 isolates). All these isolates were identified according to morphological features and biochemical tests. What's more, identification was confirmed using the api20E system.

Antibiotic susceptibility test

Antibiotic susceptibility for *A. baumannii* isolates was performed against aminoglycosides antibiotics including: amikacin (30 μ g), gentamicin (30 μ g), netilmicin (30 μ g), tobramycin (10 μ g), and sisomicin (10 μ g) (Bioanalyse, Turkey) using disc diffusion method in accordance with the guidelines established by the Clinical and Laboratory Standard Institute (CLSI, 2013). *Escherichia coli* (*E. coli* ATCC 25922) was used as a quality control in susceptibility determination.

Rapid detection of ES β L production via ES β L strip:

The detection of β -lactamases production was performed using Rapid ES β L Detection kit (MAST Group (UK). This kit includes four tests: Preliminary screening kit, Metallo β lactamases, ES β Ls confirmation and AmpC detection. The test was performed according to the procedure suggested by the manufacturing company: first, primary screening test to detect β -lactams resistance was performed by culturing bacterial isolates on appropriate medium with cefotaxime 30 μ g/disc. The resisted isolates were submitted to ES β L production test. One drop of test substrate (approximately 20 μ l) was dispensed on the filter pad of the strip. The test substrate was added immediately to the strip before testing. Using a loop, several identical colonies were picked up and spread on the filter pad of the test strip. Any change in color (from yellow to red)

observed around the streaked line was considered as a positive result. The tested strips were observed after 2 to 15 minutes at room temperature, and the result was read after 15 minutes.

DNA Preparation and Polymerase Chain Reaction (PCR) amplification:

Total genomic DNA was extracted by boiling according to the method described by Ruppé *et al.* (2009). Briefly, few isolated colonies of overnight growth bacteria were suspended thoroughly in 1 ml distilled water and boiled in a water bath for 10 min. After centrifugation, supernatant was used as template DNA for the PCR (Ruppé *et al.*, 2009). PCR was performed using primers specific for β -lactamases enzymes families (*bla_{SHV}*, *bla_{TEM}*, *bla_{CTX}*) and aminoglycosides modifying enzymes (*aac(6)-Ib*, *ant(4)-IIb*, *aa(c) 3'* and *aph(3')-VI*). The oligonucleotide PCR primers specific for the target

genes, annealing temperature and sizes of the expected amplification product were listed in Table 1.

The PCR reaction mixture was accomplished according to the procedure suggested by the manufacture company (KAPA, south Africa) within a total volume of 25 μ l containing 5 μ l of DNA template, 12.5 μ l of Go Taq® Green Master Mix, 2X (KAPA, south Africa), 0.5 μ l (0.6 pmol) of each of the forward and the reverse of the specific primers. The final volume (25 μ l) was completed with 6.5 μ l of nuclease free water. The conditions of PCR amplification steps was carried out using the thermal cycler (C 1000 thermal cycler, BIO-RAD, USA) and the amplified PCR products were submitted to electrophoresis using 1% agarose gel with ethidium bromide (0.5 μ g/ml) for 7 V/cm for 90 min. DNA ladder (100bp) was employed for assessing PCR product size. Subsequently, PCR products were visualized by UV light at 336 nm, and photographs were taken by an aid of digital camera.

Table (1): The oligonucleotide PCR primers specific for the target genes, annealing temperature and sizes of the expected amplification product

Gene	Sequence of forward and reverse Primer (5' → 3')	Annealing temperature(°C)	Size product	Reference
<i>bla_{SHV}</i>	F- AAGATCCACTATCGCCAGCAG R- ATTCAGTTCGTTTCCCAGCGG	59	200bp	[Oliveira et al., 2011]
<i>bla_{CTX-M}</i>	F -CGCTTTGCGATGTGAAG R-ACCGCGATATCGTTGGT	55	550bp	[Oliveira et al., 2011]
<i>bla_{TEM}</i>	F- GAGTATTCAACATTTCCGTGTC R-TAATCAGAGGCACCTATCTC	48	800bp	[Oliveira et al., 2011]
<i>aac(3)-I</i>	F- AGCCCGCATGGATTTGA R- GGCATACGGGAAGAAGT	43	227	[Ndegwa et al., 2012]
<i>aac(6)-Ib</i>	F- TTG CGA TGC TCT ATG AGT GGC TA R- CTC GAA TGC CTG GCG TGT TT	56	482	[Haldorsen , 2011]
<i>ant(4')IIb</i>	F- GACGACGACAAGGATATGGAATTGCCCAAT ATTATT R- GGAACAAGACCCGTTCAATTCAATTCATC AAGTTT	57	364	[Haldorsen , 2011]
<i>aph(3')V I</i>	F- TAT CTC GGC GGC GGT CGA GT R- CAC GCG GGG AAA CGC GAG AA	55	800	[Vaziri et al., 2011]

RESULTS

Morphological and biochemical characterization

All clinical isolates obtained from various clinical specimens identified as *A. baumannii* based on their morphological and biochemical characterization.

Antibiotic resistance pattern

The aminoglycosides susceptibility results are depicted in Figure 1. Accordingly, 15 (41.66%) *A. baumannii* isolates were found to be resistant to the tested antibiotics.

Regarding to the source of the infection, high resistance rate was observed among the wound isolates which were completely resistance (100%) to gentamicin and sisomicin. Furthermore, they showed high resistance rate (72.72%) to tobramycin and intermediate resistance to netilmicin (54.54%) and amikacin

(45.45%). On contrary, all urine isolates were sensitive to all antibiotic under investigation. For the blood isolates, results revealed a high sensitivity to aminoglycosides; amikacin and netilmicin (100%) and 83.44% for gentamicin, tobramycin, and

sisomycine. While the sputum and the Cerebrospinal fluid isolates were completely sensitive to all the antibiotics except for the amikacin in which the sputum isolates were sensitive to this antibiotic.

Figure 1: Antibiotic resistance of the *A. baumannii* isolates towards aminoglycoside antibiotic

Detection of ESβL Production

The investigation of β -lactamases production was carried out using ESβL strips. According to this test, 17 (47.22%) isolates gave positive results in the preliminary screening of β -lactamases, the ESβLs type and AmpC enzyme while 13 (36.11%) isolates showed a positive result in Metallo- β -lactamases production test. Figure 2 illustrates the results for one isolates, *A. baumannii* (no. 10) in which red color appeared with the first strip (A no.1) for preliminary screening after addition of specific

inhibitors, while the second stripe (A no. 2) represents a positive result for ESβLs after the addition of Clavulanic Acid, the third positive result (A no.3) for MβL strip with red color appeared after the addition of EDTA and Mercaptoacetic acid and finally the 4th strip (A no.4) signifies AmpC strip which appeared red color after the addition of Boronic Acid. No change occurred with the negative control represented by the standard strain *E. coli* ATCC 25922 in the Figure (2) which clearly appeared as a yellow color.

Figure 2: Detection of β -lactemase types using easy strips test. A-1: positive result for primary screening test of *E. coli* no. 10. A-2: positive result for ESβLs of *A. baumannii* no.10. A-3: positive result for MβL of *A. baumannii* no.10. A-4: positive result for AmpC of *A. baumannii* no.10. B 1-4: negative result for all above tests of the standard strain *E. coli* ATCC 25922

PCR analysis

The prevalence of β -lactamas and aminoglycoside resistance genes in 15 resistant *A. baumannii* isolates (as determined by the disk diffusion method) was as follows: *bla*_{TEM} was detected in 3 (20%) isolates, *bla*_{SHV} was detected in 9 (60%) isolates, *bla*_{CTX-M} was detected in 11 (73.33%) isolates, *aac* (6)-*lb* in 7 (46.66%) isolates,

ant(4')-*IIb* in 5 (33.33%) isolates, *aph*(3')-VI in 2 (13.33%) isolates, and *aa*(c) 3' was found in 10 (66.66%) isolates (Figure 3,4,5,6,7,8,9). Interestingly, individual aminoglycoside-resistant isolates carried multiple (two to six) β -lactamases and modifying enzyme genes. Table 2 demonstrates the distribution of these genes in the *A. baumannii* isolates.

Table (2): the distribution of these genes among the *A. baumannii* isolates

<i>A. baumannii</i> isolates	Resistance profile	Resistance genotype
Aw10	GEN + SIS	SHV + <i>aa</i> (c) 3'
Aw11	GEN + SIS	<i>aa</i> (c) 3'
Ab12	GEN + SIS	CTX + <i>aa</i> (c) 3'
Aw 14	GEN + SIS	SHV + <i>aa</i> (c) 3'
Ab15	TOB	SHV + CTX + <i>aac</i> (6)- <i>lb</i> + <i>ant</i> (4')- <i>IIb</i> + <i>aa</i> (c) 3'
As19	GEN + TOB + NTil + SIS	CTX + <i>ant</i> (4')- <i>IIb</i> + <i>aa</i> (c) 3'
Aw 20	GEN + TOB + SIS	SHV + CTX + <i>ant</i> (4')- <i>IIb</i> + <i>aa</i> (c) 3'
Aw 21	AKN + GEN + TOB + NTil + SIS	CTX + <i>aac</i> (6)- <i>lb</i>
Aw 22	AKN + GEN + TOB + NTil + SIS	SHV + CTX + <i>aac</i> (6)- <i>lb</i>
Aw 23	AKN + GEN + TOB + NTil + SIS	SHV + CTX + <i>aac</i> (6)- <i>lb</i> + <i>ant</i> (4')- <i>IIb</i> + <i>aph</i> (3')-VI + <i>aa</i> (c) 3'
Aw24	AKN + GEN + TOB + NTil + SIS	TEM + CTX
Aw25	GEN + TOB + NTil + SIS	TEM + SHV + CTX + <i>aac</i> (6)- <i>lb</i>
Aw 32	AKN + GEN + TOB + NTil + SIS	SHV + CTX + <i>aac</i> (6)- <i>lb</i> + <i>aph</i> (3')-VI + <i>aa</i> (c) 3'
Aw 33	GEN + TOB + NTil + SIS	TEM + SHV
Ac 36	AKN + GEN + TOB + NTil + SIS	CTX + <i>aac</i> (6)- <i>lb</i> + <i>ant</i> (4')- <i>IIb</i> + <i>aa</i> (c) 3'

Aw: *A. baumannii* isolated from wound , Ab: *A. baumannii* isolated from blood , As: *A. baumannii* isolated from sputum Ac : *A. baumannii* isolated from , AKN : amikacin, GEN : gentamicin, TOB : tobramycin, NTil : netilmicin, SIS: sisomicine.

Figure 3: Gel electrophoresis (1% agarose, 7 V/cm for 90 min) for *bla* TEM gene for some *A. baumannii* isolates. Line M 100bp DNA ladder, lines (1, 4) positive results with 800bp amplicon

Figure 4: Gel electrophoresis (1% agarose, 7 V/cm for 90 min) for bla SHV gene for some *A. baumannii* isolates. line M 100bp DNA ladder, lines (1,2,3,4,5,7,8,9) positive results with 200bp amplicon

Figure 5: Gel electrophoresis (1% agarose, 7 V/cm for 90 min) for blaCTX-M gene for some *A. baumannii* isolates. Line M 100bp DNA ladder, lines (1, 2,3,4 ,5,6,7,8) positive results with 550bp amplicon

Figure 6: Gel electrophoresis (1% agarose, 7 V/cm for 90 min) for aa(c) 3' gene for some *A. baumannii* isolates. Line M 100bp DNA ladder, lines (1, 2, 4 ,5,6,7) positive results with 227 bp amplicon

Figure 7: Gel electrophoresis (1% agarose, 7 V/cm for 90 min) for ant(4')-IIb gene for some *A. baumannii* isolates. Line M 100bp DNA ladder, lines (3,5,6,7) positive results with 364 bp amplicon

Figure 8: Gel electrophoresis (1% agarose, 7 V/cm for 90 min) for aph(3')-VI gene for some *A. baumannii* isolates. Line M 100bp DNA ladder, lines (1, 3,4) positive results with 800bp amplicon

DISCUSSION

The present study describes the emergence and spread of β -lactamases and aminoglycoside resistant *A. baumannii* isolates in Baghdad, Iraq. Antibiotic resistance and genetic features were investigated. Remarkably, most isolates of *A. baumannii* were obtained from urine and wound specimens. The result of susceptibility for all *A. baumannii* isolates to aminoglycosides group revealed that Amikacin had the

best efficacy against these isolates, given that only 16.66% of total isolates developed resistance. Dent *et al.* (2010) and Xu *et al.* (2012) found that resistance rate to Amikacin reached 56%, 58% and 53.7%, respectively. The resistance rate of gentamicin antibiotic was 38.88%, this results was in accordance with the result of Wei-feug *et al.* (2005) in which they found that 48.6% of the isolates were resistance. Hassan *et al.* (2010) reported that 28% of *A. baumannii* Pakistani isolates were resistant to tobramycin. Such finding goes with

ours. Nonetheless, Moniri *et al.* (2010) stated that the resistance rate to tobramycin was 68.3%. Whereas, the resistance rate of netilmicin reached 22.22% which is lower than what Jaggi *et al.* (2012) indicated, as they recorded 90.3% of the isolates were resistance.

Rapid detection of ES β L production via ES β L strip

Since there are no standard guidelines for the detection of MBL, different studies have reported the use of various methods (Jaggi *et al.*, 2012). In the current study four types of β -lactamases were detected including: preliminary screening for β -lactemase, ES β LS type, Metallo- β -lactamases and AmpC enzyme. About half of the *A. baumannii* isolates (47.22%) were able to produce β -lactamases, this result was in a good agreement with the result of Trivedi *et al.*, (2012) in which they found that 60% of the isolates were β -lactemase producer. In our study, all of the β -lactemase producing *A. baumannii* isolates were found to be ES β LS and AmpC producer (47.22%). However, a disagreement was noticed, as they found that only 10% of the isolates were ES β LS producer and 20% of the isolates were AmpC producer.

In our study, Metallo- β -lactamases was detected in 36.11% of the *A. baumannii* isolates. Many previously studies reported Metallo- β -lactamases production in *A. baumannii* (Gupta *et al.*, 2006; Ahir *et al.*, 2012; Daef *et al.*, 2012). An Indian study on the *A. baumannii* species stated a significant increase (70.9%) in Metallo- β -lactamases production which is much higher than our findings (Uma *et al.*, 2009), while another study reported from Kerala, India, states that 21% of the *A. baumannii* isolates were found to be metallo- β -lactamase producers which is similar to our finding (Kumar *et al.*, 2011). Another study from India demonstrated that 66.8% of the isolates were harboring Metallo- β -lactamases enzymes (Chaudhary and Payasi, 2012).

Although there are many methods for the phenotypic detection of β -lactamases enzymes (Drieux *et al.*, 2008), we found that ES β L strip is a new advanced method for detecting the types of β -lactemase. It is easy to perform highly specific besides; different types of enzymes could be detected in the same run.

PCR analysis:

In this study two types of resistance genes were investigated in the *A. baumannii* isolates using PCR technique which they are specific for β -lactam and aminoglycoside resistance. Among these resistance genes, the *bla*_{CTX} was the most frequent resistance genes and detected in 13 (73.33%) of the *A. baumannii* isolates, while the *aph*(3')-VI was detected in only 2 (13.33%) isolates.

The other genes (TEM, SHV, *aac* (6)-Ib, *ant*(4)-IIb, and *aa*(c) 3') were detected in 20%, 60%, 46.66%, 33.33% and 66.66% respectively. This finding was not

similar to the finding of Huang *et al.* (2012) in which they found that *aac* (6)-Ib, *aph*(3')-VI and *aa*(c) 3' genes were detected in 20%, 1.25% and 47.50% respectively.

By observing the result in Table 2, we find that most of the resistance *A. baumannii* isolates were harboring more than one gene (40% were harboring 2 genes, 20% were harboring 4 genes, 13.33% were harboring 3 and 5 genes; while 6.66% were harboring 6 and one gene). In 80% of the *A. baumannii* resistance isolates, most of these genes belonging to both β -lactamases and aminoglycoside modifying genes and this indicates the prevalence of multidrug resistance patterns among the *A. baumannii* isolates.

Multidrug resistance patterns in *A. baumannii* have been described previously in many studies done in different hospitals worldwide (Koo *et al.*, 2010), this increase in MDR *A. baumannii* is a frightening reality (Livermore, 2003), and has given rise to significant therapeutic challenges in the treatment of nosocomial infections (Koo *et al.*, 2010). In a recent study which was done by Taitt *et al.* (2014) on clinical *A. baumannii* isolates collected from military treatment facilities (MTFs) they found that 80% of the isolates were found to be MDR and this is similar to our finding. Among these resistance isolates, 2 isolates that were highly resistance to aminoglycoside antibiotic discs were harboring only the β -lactamases resistance genes (TEM, CTX and SHV) and no one of the aminoglycoside modifying enzymes were detected in spite of they were resistance to aminoglycoside, this is maybe due to that these isolates harbor another types of modification enzymes which were out of this study.

As a conclusion, *A. baumannii* isolates with AMEs and β -lactamases resistance genes represent a powerful nosocomial pathogen that threat the antibiotic area and life's of immunocompromised and hospitalizes patients and this should be take in to account to find a new ways for restriction of these powerful pathogens.

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