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Aminoglycosides Resistance among *Acinetobacter baumannii* Complex Isolated from Hospital Acquired Blood Stream Infections

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A B S T R A C T

Resistant *Acinetobacter baumannii* complex associated with bloodstream infections is a major concern in hospitalized patients. Aminoglycosides resistance in *Acinetobacter baumannii* complex is mainly caused by enzymatic modification of aminoglycosides. This study was designed to identify the prevalence of aminoglycosides resistance and its association with aminoglycosides modifying enzymes (AMEs) genes and *armA* gene for 16S rRNA methylase among *Acinetobacter baumannii* complex causing bloodstream infections in patients of Mansoura University Hospitals (MUHs). Also, to evaluate the effect of aminglycosides combinations on extensively resistant strains to all tested agents. Blood culture samples were collected from patients with signs and symptoms of blood stream infections. *Acinetobacter baumannii* complex were identified by API 20 NE. Antibiotic susceptibilities testing were performed by disc diffusion method. Activity of amikacin and gentamicin was evaluated *in vitro* in combination with ciprofloxacin and imipenem against resistant strains of *Acinetobacter baumannii* complex to all tested antibiotics using checkerboard titration method and the fractional inhibitory concentration index (FICI) was calculated for each combination. Resistant *Acinetobacter baumannii* complex were tested for presence of *aac(3)-I*, *ant(2'')-Ia*, *aac(6')-I h*, *aac(6')-I b*, *aph (3')-V1* AME genes and *armA* gene for 16S rRNA methylase using PCR. A total of 67 *Acinetobacter baumannii* complex isolates were collected during the period of the study. Forty-one isolates (61.2%) were resistant to gentamicin and/ or amikacin. There were 15 isolates resistant to all tested antibiotics agents. Amikacin combination with ciprofloxacin and imipenem showed the highest synergistic effect against 40%, 33.3% respectively of these extensively drug resistant *A. baumannii* complex isolates. Combination of gentamicin-ciprofloxacin and gentamicin-imipenem each showed synergistic effect against 26.7% of these isolates. The most commonly encountered AME genes were *aac(6')-I b* and *aac(3)-I* (70.7% and 65.8% respectively). *ArmA* 16S rRNA methylase gene was encountered in 56.1% of resistant strains. The most prevalent gene profile was *aac(3)-I + aac(6')-Ib + ant(2'')-Ia + armA* detected in 31.7% of resistant isolates. The extensively resistant strains mostly express the gene pattern *aac(3)-I + aac(6')-Ib + ant(2'')-Ia +armA* (6/15). This study concluded that aminoglycosides modifying enzymes are widespread in aminoglycosides resistant *A. baumannii* complex causing bloodstream infections. Combination therapy including amikacin and gentamicin with ciprofloxacin and imipenem can be used as for treatment of severe resistant *A. baumannii* blood stream infection.

Introduction

Acinetobacter baumannii is an important hospital acquired pathogen. Multi and extensively antibiotic resistance stains emerged and widely distributed all over the world with extensive use of broad spectrum antibiotics (Hou *et al.,* 2012). *Acinetobacter baumannii* complex *(Acinetobacter calcoaceticus- Acinetobacter baumannii)* complex composed of *A. baumannii* (genospecies 2), *A. pittii* (formerly known as *Acinetobacter* genospecies 3), and *A. nosocomialis* (formerly known as *Acinetobacter* genospecies 13TU). They are genetically and phenotypically similar to *A. calcoaceticu* (*Acinetobacter* genospecies 1) and hence are grouped in the so called *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex (Ko *et al.,* 2008; Nemec *et al.,* 2011).

Aminoglycosides is a bactericidal group of antibiotics that have a vital role in treatment of serious Gram negative bacterial infections like blood stream infections (BSIs). However, many studies reported development of aminoglycoside resistance in *Acinetobacter baumannii* (Potron *et al.,* 2015; Poole, 2005). Resistance to aminoglycosides is mediated by several mechanisms including aminoglycoside modifying enzymes (AMEs), efflux pump, 16S rRNA methylation and restricted bacterial membrane permeability to aminoglycosides agents. The production of AMEs and 16S rRNA methylase are the major mechanisms responsible for clinical resistance of *Acinetobacter baumannii* complex to aminoglycosides (Yadegar *et al.,* 2009; Shaw *et al.,* 1993).

Aminoglycoside-modifying enzymes are classified into three categories according to their action; acetyltransferase (AACs) which cause acetylating of free hydroxyl group. The most important genes encode this enzyme are

include *aac(3)-I*, and *aac(6')-Ib*. Second is phosphotransferase (APH) whose genes include *aph(3')V1*. It causes phosphorylation of the free hydroxyl group. Third, the nucleotide transferase (ANT) which is also known as adenosine transferase (AAD), includes *ant(3")-I* and *ant(2")-I*, which can lead to nucleosidation of free hydroxyl groups. 16S rRNA methylase is another mechanism in aminoglycosides resistance (Nemec *et al.,* 2004).

Empirical combination antibiotic therapy is suggested as a potential option for treatment of severe infections caused by Gram negative bacteria. Combination is mainly of concern in infections caused by bacteria with high rates of resistance like *Acinetobacter baumannii*. Combination therapy should contain bactericidal broad spectrum antibiotics like aminoglycoside, beta-lactam and fluoroquinolone (Paul *et al.,* 2014; Tamma *et al.,* 2012).

The objectives of this study were to identify the prevalence of aminoglycosides resistance among isolates of *Acinetobacter baumannii* complex causing BSIs, to assess the possible synergistic effect of aminoglycosides drugs combinations with imipenem and ciprofloxacin against resistant *Acinetobacter baumannii* complex strains and detection of AMEs and 16S rRNA methylase genes associated with aminoglycosides resistance in these isolates.

Materials and Methods

This study was conducted on patients who have BSIs in Mansoura university hospitals for a period of 2 years extending from March 2015 to March 2017. Blood samples were collected from patients with suspected BSIs according to CDC criteria (Horan *et al.,* 2008). *Acinetobacter baumannii* complex was identified by routine microbiological methods

and API 20 NE (bioMérieux) which was performed according to the manufacture instructions (Koneman *et al.,* 1997).

Antibiotic sensitivity testing was done by disc diffusion method according to the guidelines proposed by the Clinical Laboratory Standard Institute (CLSI, 2015) for *Acinetobacter baumannii*.

Synergy testing

Evaluation of the effect of aminoglycoside agents (gentamicin and amikacin) combinations with ciprofloxacin and imipenem on extensively resistant strains to all tested antibiotics was estimated by the checkerboard titration method (Lorian, 2005).

The MICs of individual drugs (imipenem, amikacin, gentamicin and ciprofloxacin) were estimated using broth microdilution method.

The MICs of aminoglycosides antibiotics (gentamicin and amikacin), imipenem and ciprofloxacin in combinations were estimated. Determination of MICs was done using the broth microdilution technique as recommended by the CLSI (2015).

The fractional inhibitory concentration index (FICI) was calculated for each antibiotic in each combination by using the following formula:

FICA=MIC of drug A in combination/MIC of drug A alone

FICB=MIC of drug B in combination/MIC of drug B alone

FICI=FICA+FICB

FICI were interpreted as follows: ≤0.5 Synergy, >0.5-1 (Additive effect), >1<4 (Indifferent), and ≥ 4 Antagonism.

Molecular identification of aminoglycosides resistance genes

Bacterial DNA was extracted using QIAamp DNA extraction kits according to the manufacture instructions. PCR was performed according to the protocol described before (Van de Klundert *et al.,* 1993; Shaw *et al.,* 1993; Maynard *et al.,* 2003; Díaz *et al.,* 2004; Nie *et al.,* 2014). Primers used are described in table 1.

Statistical methods

Descriptive data were presented in the form of number and parentages.

Results and Discussion

Aminoglycosides are important class of broad spectrum antibiotics that enter in treatment of severe life threatening infections as BSIs. They are used mainly in treatment of Gram negative bacterial infections. They are used also in treatment of Gram positive bacterial infections and *Mycobacterium tuberculosis* in combination with other antibacterial agents (Yao *et al.,* 2007; Brossier *et al.,* 2010). The growing problem of antibiotic resistance in *A. baumannii* complex has been worldwide concerns as it causes failure in treatment of severe infections (Hou *et al.,* 2012; Potron *et al.,* 2015).

In the present study, a total of 67 isolates of *Acinetobacter baumannii* complex were detected from bloodstream infections during the period of study. Most of the collected isolates were from neonatal and pediatric ICUs figure (1). Antibiotic susceptibilities to different antibiotics were determined by disc diffusion method according to CLSI recommendations (2015). All isolates were resistant to 2nd generation cephalosprine agent (cefuraxime). Resistance to cefotaxime and imipenem were (92.5%) and (71.5%)

respectively. Resistance to ciprofloxacin was (74.6%). A total of 41 (61.2%) isolates were resistant to one or more aminoglycosides agents. Thirty one (46.3%) isolates were resistant to gentamicin and 24 isolates (35.8) were resistant to amikacin figure (2). Fifteen isolates (22.4%) isolates showed co-resistance to amikacin and gentamicin. These isolates were resistant to all tested antibiotic agents

This high resistance to 3rd generations cepahlosprins, imipenem and ciprofloxacin agrees with most of the previous studies like Lee *et al.,* (2011) and Gao *et al.,* (2017). However their findings regarding aminoglycosides resistance is lower than that of the present study. Also, aminoglycosides resistance in the current study is lower than finding of Kishii *et al.,* (2014).

The higher rate of resistance to imipenem compared to aminoglycoside agents in this study may be due to the wide use of imipenem as an empirical therapy in bloodstream infections compared to aminoglycosides agents. In addition, the current study did not differentiate the susceptibilities of the different species of *A. baumannii complex. A. baumannii* (genospecies 2) is of higher resistance compared to other members of the complex.

Effects of aminoglycosides combinations

In the present study, the checkerboard titration method was used to evaluate the possible synergistic effect of aminoglycosides combinations with ciprofloxacin and imipenem to overcome resistance in *A. baumannii* complex.

Fifteen (15/41) (36.6%) isolates of extensively drug resistant *Acinetobacter baumannii* complex (Magiorakos *et al.,* 2012) were resistant to both aminoglycosides agents and to all tested antibiotics. The checkerboard

titration method was performed against these resistant isolates. The MICs of gentamicin and amikacin were determined before and after combination with ciprofloxacin and imipenem and the FIC was calculated for each drug. The FICI was calculated for each drug combination (gentamicin with ciprofloxacin, gentamicin with imipenem, amikacin with ciprofloxacin and amikacin with imipenem).

The most effective aminglycoside combinations were amikacin-ciprofloxacin followed by amikacin-imipenem, they showed synergistic effect against 40% (6\15) and 33.3% (5\15) respectively of extensively drug resistant *A. baumannii* complex isolates. They showed additive effect against 46.7 % (7\15) and 53.3% (8\15) of the isolates respectively. Each of gentamicin-ciprofloxacin and gentamicin-imipenem showed synergistic effect against 26.7% (4\15) of extensively drug resistant *A. baumannii* isolates table (2). Antagonistic effect was not detected in any of the tested combinations.

These results are matched with previous reports (Yadav *et al.,* 2015; Yadav *et al.,* 2016). The exact mechanism for synergism is not known. However, synergism of imipenem could be explained by increased cell permeability and bacterial aminoglycosides uptake by cell wall synthesis inhibitors increasing their intracellular concentrations (Vakulenko and Mobashery, 2003). However the use of these combinations is limited by increased side effects.

So, these antibiotic combinations should used only in resistant cases when its benefits mostly outweigh the risks of increased side effects. Application of infection control policies is critical to reduce the problem of bacterial resistance, especially in countries where empirical antibiotic use is wide without prescription or culture sensitivity testing.

Table.1 Primers used for detection of AMEs genes and 16S rRNA methylase

Gene	Primer	Amplicone size
Aminoglycoside modifying enzymes		
aac(3)-I	F: 5′ GACATAAGCCTGTTCGGTT3′ R: 5′-CTCCGAACTCACGACCGA3′	372 bp
ant(2″)-Ia	F: 5′-ATCTGCCGCTCTGGAT3′ R: 5′-CGAGCCTGTAGGACT3′	404 bp
aac(6′)-I h	F: 5′-TGCCGATATCTGAATC3′ R: 5′-ACACCACACGTTCAG3′	407 bp
aac(6′)-I b	F: 5′-TATGAGTGGCTAAATCGAT3′ R: 5′-CCCGCTTTCTCGTAGCA3′	395 bp
aph (3')-V1	F: 5′-CGGAAACAGCGTTTTAGA3′ R: 5′-TTCCTTTTGTCAGGTC3′	716 bp
16S rRNA methylase gene		
armA	F: 5′-ATTCTGCCTATCCTAATTGG-3′ R: 5′-ACCTATACTTTATCGTCGTC-3′	315 bp

Table.2 Effects of gentamicin and amikacin drugs combinations on extensively drug resistant *Acinetobacter baumannii* complex

	Gentamicin-ciprofloxacin	Gentamicin-imipenem	Amikacin-ciprofloxacin	Amikacin--imipenem
≤0.5 Synergism	4 (26.7)	4 (26.7)	6 (40)	5 (33.3)
> 0.5-1 (Additive)	6 (40)	8 (53.3)	7 (46.7)	8 (53.3)
>1<4 (Indifferent)	5 (33.3)	3 (20)	2 (13.3)	2 (13.3)
≥ 4 Antagonism	0	0	0	0

Table.3 Distribution of aminoglycoside resistance genes in resistant *Acinetobacter baumannii* complex

Aminoglycoside resistance genes	Number	Percentages
aac(3)-I	27	65.8
ant(2″)-Ia	18	43.9
aac(6′)-I h	2	4.9
aac(6′)-I b	29	70.7
aph (3')-V1	6	14.6
armA	23	56.1

Table.4 Aminoglycoside resistance gene profile in resistant *Acinetobacter baumannii* complex

Resistance gene pattern (Total number)	Aminoglycoside resistance phenotype	Number	Total Number (%)
aac(3)-I	AK	3	7
	GM	4	(17.2)
ant(2″)-Ia	AK	1	1
			(2.4)
aac(6′)-I h	GM	2	2
			(4.9)
aac(6′)-I b+ armA	GM	4	8
	AK	3	(19.5)
	GM+AK	1[a]	
aph(3′)-I+armA	GM+AK	2[a]	2
			(4.9)
aac(6′)-I b+ aac(3)-I	GM	2	4
	GM+AK	2[a]	(9.7)
aac(3)-I + aac(6′)-Ib + ant(2″)-Ia + aph (3')-V1	GM+AK	4[a]	4
			(9.7)
aac(3)-I + aac(6′)-Ib + ant(2″)-Ia + armA	GM	5	13
	AK	2	(31.7)
	GM+AK	6[a]	

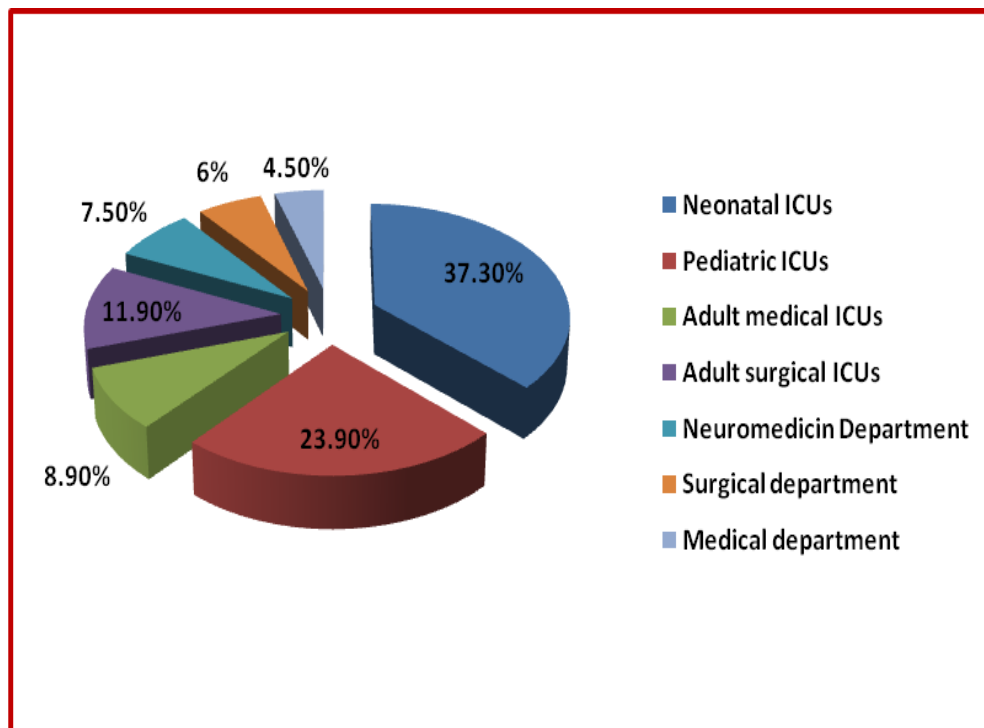GM: Gentamicin AK: Amikacin [a] Extensively resistant isolates to all tested antibiotics

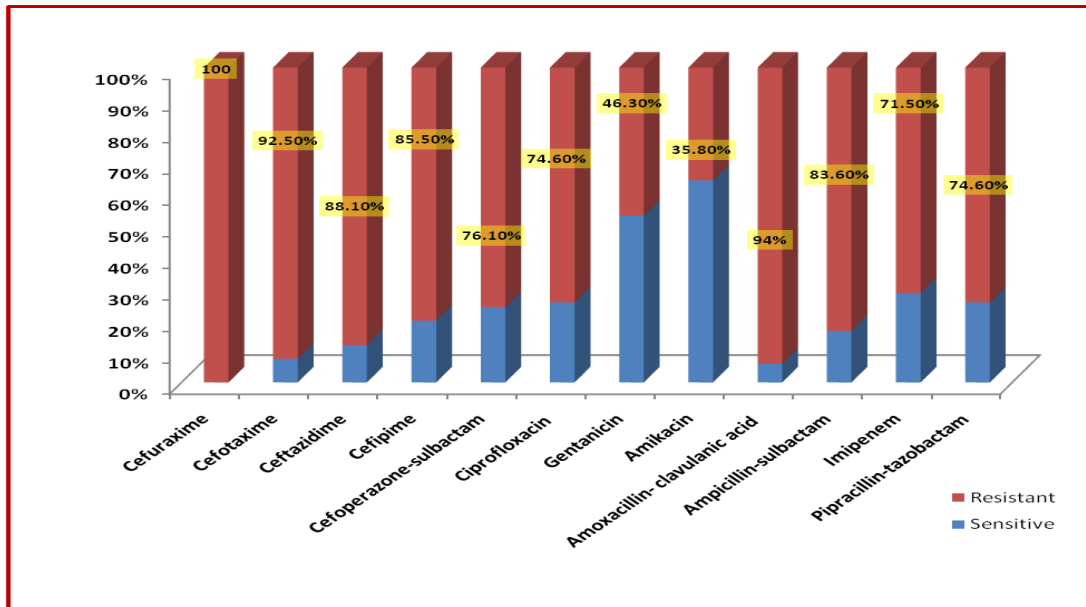**Fig.1** Distribution of *Acinetobacter baumannii* complex in clinical departments

Fig.2 Antibiotic susceptibility pattern of isolated *Acinetobacter baumannii* complex strains

Aminoglycosides resistance genes in aminoglycosides resistant *A. baumannii* complex isolates

Several mechanisms are implicated in bacterial resistance to aminoglycosides. The most prevalent mechanisms described in species of *A. baumannii* complex are enzymatic modification of antibiotic molecule and 16S rRNA methylation (Yadegar *et al.,* 2009; Nemec *et al.,* 2004).

A total of 41 isolates that showed resistance to gentamicin and/or amikacin were tested for the presence of aminoglycoside modifying enzymes genes and 16 S RNA methylase armA *gene* by PCR using sets of primers as described in table (1).

All these resistant strains (41) were harboring at least one type of AME genes. Thirty-one (75.6%) isolates carried more than one type of AMEs genes. The most frequent AMEs genes were N-acetyltransferases (AACs); *aac(6')-Ib* (70.7%) and *aac(3)-I* (65.8%). The least prevalent AME gene was *aac(6')-I h* (4.9%).

Gene of 16S rRNA methylase *armA* was detected in 56.1% table (3). The most prevalent gene profile was *aac(3)- I + aac(6')-Ib + ant(2'')-Ia + armA* detected in 31.7% (13/41). Co-resistance to amikacin and gentamicin were detected in 36.6% (15/41) of aminoglycoside resistant isolates. These isolates were resistant to all tested antibiotic agents. The most prevalent gene profile in these extensively resistant strains were *aac(3)-I + aac(6')-Ib + ant(2'')-Ia + armA* detected in (6/15) isolates and *aac(3)-I + aac(6')-Ib + ant(2'')-Ia +aph (3')-V1* detected in (4/15) isolates table (4).

This result agrees with previous results; Nemec *et al.,* (2004), Wen *et al.,* (2014), Sheikhalizadeh *et al.,* (2017) and Heidary *et al.,* (2017). Different results were obtained by *Zhou et al.,* (2010) they found higher rate of resistance were due to 16S rRNA methylase genes. This difference may be related to difference of the geographic distribution of resistant determinants.

This study has some limitation. First this

study tested the effect of combinations *in vitro* only. More future studies are recommended to assess the outcome of these combinations *in vivo*. Second, all aminoglycosides agents were not tested. Only systemic agents recommended by CLSI (2015) for treatment of *A. baumannii* complex were tested. Lastly, other mechanisms for aminoglycosides resistance like efflux mechanism and the possible association with AMEs were not investigated. Other studies are recommended to cover these points.

In conclusion, there is an alarming increase of aminoglycosides resistance in *A. baumannii* complex isolated from patients with hospital acquired blood stream infections. Aminoglycosides modifying enzymes are a main mechanism associated with aminoglycosides resistance. More than one gene of aminoglycosides enzymes are present in single isolate. Combination of aminoglycosides especially amikacin with ciprofloxacin and imipenem can be used for treatment of infections with resistant isolates.

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