Original Research Article

Aminoglycoside and carbapenem resistance genes in *Pseudomonas aeruginosa*

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A B S T R A C T

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The purpose of this study was to assess the co-existence of four of the most commonly detected aminoglycoside modifying enzyme (AME) genes [*aac (6′)-I*, *aac (6′)-II*, *ant (2′)-I* and *aph (3′)-VI*] in association with two types of metallo-β-lactamase (MBL) genes [IMP and VIM] among *Pseudomonas aeruginosa* (*P. aeruginosa*) isolates obtained from patients admitted in different wards of Zagazig University Hospitals, Egypt. Among 85 *P. aeruginosa* isolates examined in this study, MBL genes were detected in 92.9% of them and were more prevalent than AME genes that were detected in 69.4% of isolates. Both types of genes were detected together in 69.4% of isolates with a high significant association (P<0.001). Six different genetic combinations of AME and MBL genes were detected. The most prevalent one (detected in 25.9% of isolates) was that of *ant (2′)-I* and VIM type of MBL genes. The emergence of antibiotic resistance in *P. aeruginosa* isolates is inevitable which emphasizes the implementation of proper infection control measures and calls for a more restricted use of carbapenems in hospital infections.

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) remains one of the leading causes of nosocomial infections. In addition to its natural intrinsic resistance, this organism has high capacity to acquire resistance to a wide spectrum of antimicrobials via multiple mechanisms (Hocquet*et al.* 2007). This forms a serious challenge for antimicrobial therapy and in most cases can result in high morbidity and mortality rates (Hirsch & Tam 2010).

Both aminoglycosides and carbapenems represent an important therapeutic option for serious *Pseudomonas* infections and are often used in combination due to their synergistic bactericidal activity(Nakamura *et al.* 2000). However, resistance to aminoglycosides and even carbapenems among *P. aeruginosa* strains continues to be reported with an increasing frequency in virtually all areas of the world (Edson & Terrell 1999; Andrade *et al.* 2003; Saderi*et*

al. 2010; Arunagiri*et al*. 2012; Liakopoulos*et al*. 2013).

Among different mechanisms contributing for aminoglycoside resistance, the production of aminoglycoside modifying enzymes (AME) is considered the most common mechanism of aminoglycoside resistance in *P. aeruginosa*. These enzymes can phosphorylate (aminoglycoside phosphoryltransferases [APH]), acetylate (aminoglycoside acetyltransferases [ACC]) or adenylate (aminoglycoside nucleotidyltransferases [ANT]) aminoglycosides rendering them inactive (Smith & Baker 2002; Poole 2005).

On the other hand, the production of metallo-β-lactamases (MBL) in *P. aeruginosa* is of particular concern as these enzymes, including the imipenemases (IMP) and the Verona integorn-encoded β-lactamases (VIM), can hydrolyze almost all clinically available β-lactams, including carbapenems, except monobactams. Furthermore, they are hardly blocked by suicide β-lactamase inhibitors such as clavulanate, sulbactam and tazobactam(Drawz&Bonomo 2010).

In general, the genes coding for MBL are often carried in integrons along with other resistance determinants especially the AME genes. In the majority of these cases, the AME genes appear to be functional and confer significant resistance (Mendes *et al*. 2004).

In this study, we aimed to assess the co-existence of four of the most commonly detected AME genes [*aac (6')-I*, *aac (6')-II*, *ant (2″)-I* and *aph (3')-VI*] in association with two types of MBL genes (IMP and VIM) among *P. aeruginosa* isolates obtained from patients admitted in different wards of Zagazig University Hospitals (ZUHs), Egypt.

Materials and Methods

Bacterial isolates

This study was conducted on a total of 85 non-duplicate *P. aeruginosa* clinical isolates that were collected from different clinical specimens obtained from patients (n=85) admitted in different wards of ZUHs in the period from June 2013 to January 2014. Isolates were from sputum (n= 8), endotracheal aspirates (n= 15), urine (n= 30), wound discharge (n= 25), and blood (n= 7). Isolates were identified as *P. aeruginosa* by conventional biochemical tests and confirmed using API 20NE (bio-Mérieux, France).

Antibiotic susceptibility testing

Antibiotic susceptibility tests were performed using disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2010). The following antimicrobial discs were used, ceftazidime (30 µg), imipenem (10 µg), meropenem (10 µg), and three aminoglycosides which are gentamycin (10 µg), amikacin (30 µg) and tobramycin (10 µg). All discs were supplied from Oxoid (Oxoid, England). *P. aeruginosa* ATCC 27853 served as a control strain.

Phenotypic detection of MBL

Combined disc diffusion test (CDDT) was used to detect the presence of MBL in ceftazidime and carbapenems resistant isolates (n=40). Two discs of imipenem (10 µg) were placed on the plate and 5 µl of 0.5 M EDTA (930 µg) were applied to one of them. Plates were left for 16-18 h at 35˚C. An increase of 7 mm or more in zone diameter in the presence of EDTA compared to that with imipenem alone was considered to be a positive test (Pitout*et al*., 2005).

PCR reactions

DNA was extracted from all isolates using QIAamp DNA Mini and Blood Minikit (Qiagen, Germany) according to the manufacturer's instructions. For all isolates, two duplex PCR reactions were performed as described previously by Kim *et al*. (2008). The first reaction was targeting both *aac (6□)-I* and *aac (6□)-II* genes, while the other reaction was targeting both *ant (2□□)-I* and *aph (3□)-VI* genes. In addition, another duplex PCR reaction was also performed for all isolates targeting both VIM and IMP types of MBL as described previously by Manohara*net al*. (2010).Universal primers (Louie *et al*., 2002) that target the 16S rRNA were used as internal control to exclude false negative results. PCR reactions were performed in a total volume of 20 µl using ready to go Maxime PCR PreMix Kit (*i*-Taq) (iNtRON Biotechnology, Korea). For each reaction, 20 pM of each primer and 5 µl of template DNA (50-100 ng) were added. Reactions were performed in Biometra T gradient DNA thermal cycler (Germany). Primers were supplied from The Midland Certified Reagent Company Inc., Texas. Amplified PCR products were visualized using a UV transilluminator (Fisher, USA) after agarose gel (1%) electrophoresis.

Analysis of data. Data were checked, entered and analyzed using EPI-INFO 6 for data processing and statistics. Data were expressed as numbers and percentages.The comparison was done using Chi- square test (X^2). P value < 0.05 was considered significant.

Results and Discussion

Results of antibiotic susceptibility tests and phenotypic detection of MBL

Among the 85 *P. aeruginosa* isolates examined in this study, 47 isolates (55.3%) were resistant to one or more aminoglycosides. All 47 isolates (55.3%) were resistant to both gentamycin and tobramycin, while 41 isolates among them (48.2%) were resistant to amikacin as well as to the other two aminoglycosides. Forty six isolates (54.1%) were resistant to meropemen and 40 isolates (47.1 %) were resistant to both carbapenems as well as to ceftazidime. Out of these 40 isolates, CDDT test was positive in 32 isolates (80%) while 8 isolates (20%) had negative test.

Results of PCR reactions

The prevalence of the examined genes is presented in Fig. 1. In general, the genes coding for AME genes were detected in 59 isolates (69.4%). Among the aminoglycoside resistant isolates (n=47), 37 isolates (78.7%) had one or more of the examined genes. Twenty two isolates (46.8%) had only one gene. *ant (2□□)-I* was the most prevalent gene being detected in 26 isolates among the 47 resistant isolates (55.3%) followed by *aac(6□)-I* (20/47, 42.5%). Both *ant (2□□)-I* and *aac (6□)-I* were detected in 9 isolates (19.1%) while both *aac (6□)-I* and *aac (6□)-II* were detected in 6 isolates (12.8%). Among aminoglycoside susceptible isolates (n=38), 22 isolates had one or more genes. Seventeen isolates (44.7%) had one gene [*ant (2□□)-I*] while 5 isolates (13.1%) harbored both *ant (2□□)-I* and *aph (3□)-VI*.

Regarding the presence of MBL genes, 79 isolates (92.9%) had one or more genes. Among carbapenem resistant isolates (n=40), VIM type of MBL was detected alone in 15 isolates (37.5%) while both VIM and IMP types were detected in the remaining 25 isolates (62.5%). Among carbapenem susceptible isolates, that were susceptible to both types of carbapenems (n=39), VIM type of MBL was detected

alone in 29 isolates (74.3%) while both VIM and IMP types were detected in 4 isolates (10.2%). Among the isolates that were susceptible to imipenem but resistant to meropenem (n=6), three isolates had VIM alone, while the other three isolates had both VIM and IMP. Among all examined isolates, no isolate harbored only IMP type of MBL and 6/85 isolates (7.05%) were negative for both types of MBL genes.

All the isolates that were positive in CDDT test (n=32) had VIM or VIM and IMP types of MBL. Both types were present in 23 isolates (71.9%) while VIM type was detected alone in 9 isolates (28.1%). Interestingly, 3 isolates among those which had negative CDDT (n=8) had both types of MBL genes and 5 isolates had only VIM type of MBL.

The co-existence of individual genes in *P. aeruginosa* isolates is presented in Table 1. Among six different genetic combinations detected in our study, the highest ratio of *P. aeruginosa* isolates (25.9%) co-harbored *ant (2″)-I* and VIM type of MBL. Twelve isolates (14.1%) had *ant (2″)-I* and both VIM and IMP. Other combinations are presented in Table 1. The association of the examined AME genes and MBL genes is presented in Table 2 & Fig 2 where a highly significant (P < 0.001) association was found between both types of genes.

The aminoglycoside resistance ratio detected among *P. aeruginosa* - isolates in our study comes higher than that detected in previous studies (Kim *et al*. 2008; Vaziri*et al*. 2011) where the resistance ratio to amikacin was 22% and 24% compared to 48.2% in our study. Vaziri*et al*. recorded 43% and 38% resistance ratios to gentamycin and tobramycin, respectively which comes also lower than that detected in our study (55.3% for each antibiotic). On the other hand, the

carbapenem resistance ratio detected in our study comes lower than what detected in India(Arunagiri*et al*. 2012) and in Greece (Liakopoulos*et al*. 2013) where the ratio was 59.7% and 50% respectively compared to 47.1% to both carbapenems in our study

Among the different techniques used for the phenotypic detection of MBL, the CDDT using EDTA and imipenem suggested by CLSI is simple to perform and interpret. Furthermore, it had specificity and sensitivity close to 100% as recorded previously(Picão*et al*. 2008). In our study, 80% of the examined carpabenem-resistant *P. aeruginosa* isolates were found to be MBL producers in CDDT. This comes higher than that recorded in previous studies(32.8% in Manoharan*et al*. 2010, 28% in Liakopoulos*et al*. 2013 and 70.1% in Arunagiri*et al*. 2012). Our result comes, on the other hand, lower than that detected by Saderi*et al*. (2010) where 65 isolates among 69 carbapenem-resistant isolates (94.2%) were MBL producers. The variability between our results and those of previous studies could be due to the difference in carbapenem prescription patterns or the difference in the method used to detect MBLs phenotypically.

Regarding the prevalence of AME genes, our study has revealed that *ant (2″)-I* was the most prevalent gene among the aminoglycoside-resistant isolates (55.3%) followed by *aac (6″)-I* (42.5%). *aac (6″)-II* had lower prevalence (12.8%) among resistant isolates and *aph (3″)-VI* was not detected in any aminoglycoside-resistant isolate. Furthermore, 31.9% of resistant isolates (15/47) co-harbored more than one AME gene. In a European study as well as in an Iranian study, the *aac (6″)-II* gene was the most prevalent gene being detected in 32.5% and 36% of aminoglycoside-resistant isolates, respectively (Miller *et al*. 1997; Vaziri*et al*. 2011)

In another study (Kim *et al*. 2008), *ant (2⬚)-I* and *aph (3⬚)-VI* were together the most prevalent (43.6%) among resistant isolates. In the same study, 75% of resistant isolates co-harbored more than one gene which is much higher than the ratio detected in our study (31.9%).

These study revealed that 57.9% of aminoglycoside-susceptible *P. aeruginosa* isolates had AME genes (22/38), a result supported by a previous study(Kim *et al*. 2008), though higher ratio was detected in our study (57.9% compared to 16.4% in Kim *et al*.).

The prevalence of different MBL genes among *P. aeruginosa* isolates has been studied extensively in previous works (Arunagiri *et al*. 2012; Saderi*et al*. 2010; Franco *et al*. 2010; Sepehriseresht*et al*. 2012; Liakopoulos*etal*. 2013). It becomes apparent that different MBL genes and enzymes have different prevalence in different areas of the world and even within the same country(Doosti*et al*. 2013). In general, of the different six types of MBL, currently identified, the IMP and VIM types occur most frequently (Senda*et al*. 1996). In an Indian study, 3% only of imipenem-resistant *P. aeruginosa* isolates harbored IMP compared to 61% having VIM (Arunagiri*et al*. 2012).

In another Chinese study, IMP-1 predominates at 89.7% while the VIM-2 has a low frequency (10.3%) among carbapenem-resistant *P. aeruginosa* isolates in a pediatric clinic (Dong *et al*. 2008). In our study, 28.1% of carbapenem-resistant isolates had VIM only and 71.9% had both VIM and IMP types. All isolates that were positive in CDDT had MBL genes. The prevalence of MBL genes in our study comes higher than that recorded in Brazilwhere 30.4% of those having positive phenotypic test for MBL production had MBL genes (Franco *et al*. 2010). Our result is supported, on the other hand, by a previous study carried in Greece where 100% of those having positive phenotypic test for MBL had VIM type of MBL (Liakopoulos*et al*. 2013). Interestingly, all isolates that had negative CDDT, in our study, harbored MBL genes. This comes much higher than that recorded by Arunagiri*et al*.(2012) where only 3 isolates among 20 with negative CDDT had VIM type of MBL.

In general, the prevalence of MBL genes in our study (92.9%) was higher than that of AME genes (69.4%) which could be explained by the increase in carbapenems usage in our hospital that can select for carbapenemase producing strains (Rasmussen & Bush 1997). Nevertheless, we could not generalize this result as only 4 types of AME genes, which are known to be the most common AME genes in *P. aeruginosa*, were examined in this study.

The co-existence of genes accounting for resistance to carbapenems and aminoglycosides in *P.aeruginosa* isolates has been reported previously, mostly as part of class I integrons (Mendes *et al*. 2004; Odumosu*et al*. 2013). Furthermore, this association has also been detected recentlyin *Acinetobacterbaumannii*, a closely related organism to *P. aeruginosa* (Nowak *et al*. 2014). In our study, six different genetic combinations of AME genes and MBL genes have been detected. The most prevalent among them was the association of *ant (2⬚)-I* and VIM (22/85, 25.9%), followed by the association of *ant (2⬚)-I* and both VIM and IMP types of MBL (12/85, 14.1%). Among all studied *P. aeruginosa* isolates, 69.4% (59/85) co-harbored AME genes and MBL genes which could be considered a relatively high ratio.

Table.1 Co-existence of individual genes in *P. aeruginosa* isolates (n=85)

	IMP only No. (%)	VIM only No. (%)	Both VIM + IMP No. (%)	None No. (%)	Total
ant (2")-I	- (0.0)	22 (64.7)	12 (35.3)	- (0.0)	34
aac (6")-I	- (0.0)	5 (100)	- (0.0)	- (0.0)	5
aac (6")-II	- (0.0)	- (0.0)	- (0.0)	- (0.0)	0
aph (3")-VI	- (0.0)	- (0.0)	- (0.0)	- (0.0)	0
ant (2")-I + aac (6")-I	- (0.0)	- (0.0)	9 (100)	- (0.0)	9
aac (6")-I + aac (6")-II	- (0.0)	- (0.0)	6 (100)	- (0.0)	6
ant (2")-I + aph (3")-VI	- (0.0)	5 (100)	- (0.0)	- (0.0)	5
None	- (0.0)	15 (57.7)	5 (19.2)	6 (23.1)	26

Table.2 Co-existence of the examined AME genes and MBLs genes in *P. aeruginosa* isolates (n=85)

	Isolates that had AME genes (n=59) No. (%)	Isolates that did not have AME genes (n=26) No. (%)	X^2	P
Isolates that had VIM (n=47)	32 (54.2)	15 (57.7)	12.3	<0.001 **
Isolates that had both VIM and IMP (n=32)	27 (45.8)	5 (19.2)	30.25	<0.001 **
Isolates that did not have either VIM or IMP (n=6)	-(0.0)	6 (23.1)	12.0	<0.001 **
X^2	17.03			
P	<0.001**			

** Highly significant

Fig. 1 Prevalence of examined genes among *P. aeruginosa* isolates (n=85)
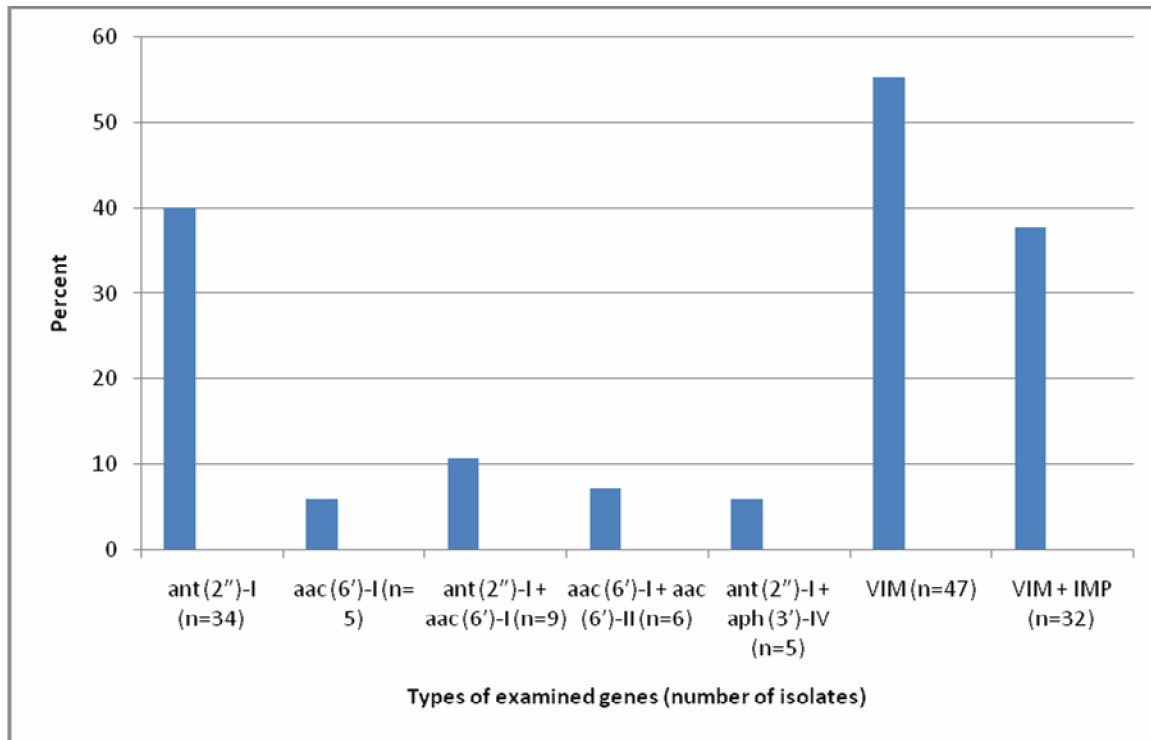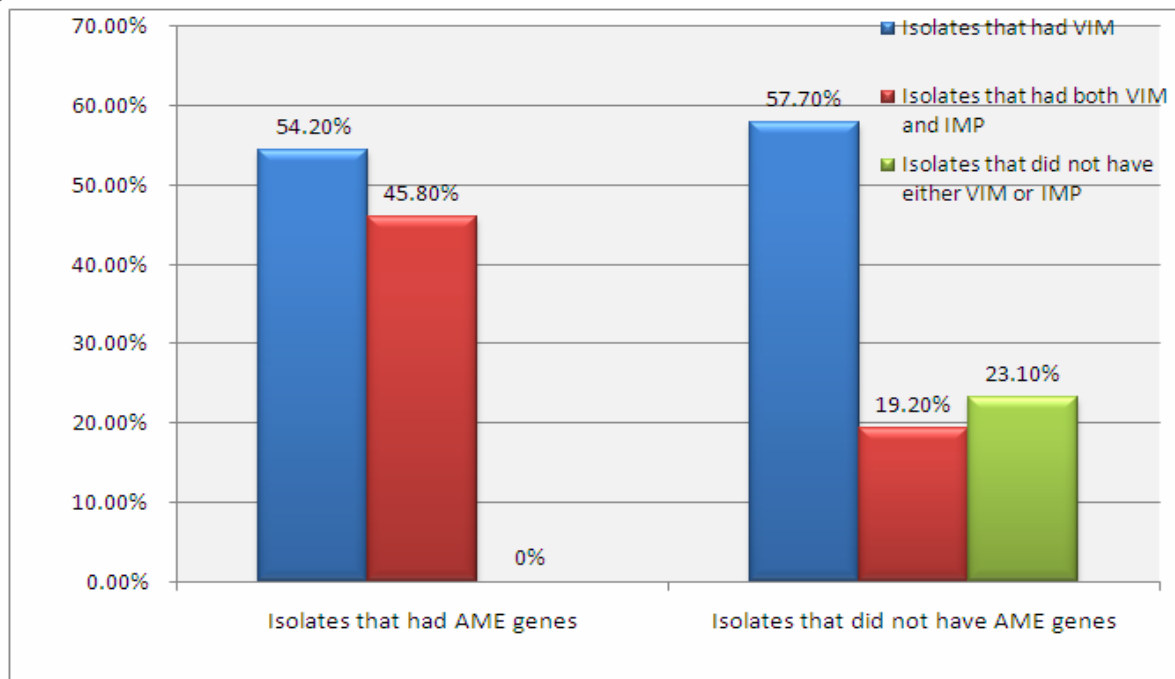

Fig. 2 Co-existence of examined AME and MBLs genes among *P.aeruginosa*isolates

Among these isolates, 22 isolates were susceptible to aminoglycosides and 33 isolates were susceptible to imipenem phenotypically. This probably could be explained by the absence of expression of these genes. Nevertheless, the presence of these genes demonstrates a hidden risk that could explode whenever these genes undergo expression; an event which may possibly occur as a result of prolonged exposure to aminoglycosides or carbapenems which may induce mutations in the promoter regions of these genes. Moreover, being carried on transferrable genetic elements (i.e. class I integrons), these genes have the ability to disseminate further, conferring a great threat in hospital settings with adverse implications on treatment(Walsh *et al.* 2005).

In conclusion, the emergence of antibiotic resistance in *P. aeruginosa* isolates is inevitable. This study tried to highlight the wide prevalence of AME as well as MBL genes among *P. aeruginosa* clinical isolates obtained from Zagazig University Hospitals which emphasizes the implementation of proper infection control measures and calls for a more restricted use of carbapenems in hospital infections.

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