Phenotypic and Genotypic Patterns of Aminoglycosides-Resistant *Staphylococcus aureus* Clinical Isolates

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ABSTRACT

Aminoglycosides still play an important role in anti-staphylococcal therapies, although emerging resistance amongst Staphylococcus aureus is widespread. The aim of this work is to study the different phenotypic patterns of aminoglycosides-resistant S. aureus in relation to the results of a multiplex PCR assay for the genes encoding aminoglycoside-modifying enzymes (AMEs). This study was carried out in Microbiology and Immunology and Surgical Departments, Faculty of Medicine, Zagazig University during the period from August, 2008 to June, 2009. One hundred and seventy clinical samples were collected and cultured. S. aureus isolates were identified using the standard microbiology laboratory techniques. Antimicrobial susceptibility testing was performed using disc diffusion method with 5 aminoglycoside antibiotics. A multiplex PCR assay was used to identify AMEs-encoding genes. A total of 81 (47.6%) S. aureus isolates were collected during this work and 43 (53.1%) isolates of which were methicillin resistant (MRSA). In this study, 31 (38.3%) isolates were resistant to at least one of the tested aminoglycosides, and the highest resistance was to kanamycin (38.3%), followed by tobramycin (30.9%), gentamicin (29.6%), amikacin (9.9%) and netilmicin (8.6%). As regard the results of the multiplex PCR assay, the aac(6')-Ie+ aph(2") gene encoding the bifunctional enzyme was the most common, followed by the ant(4')-Ia gene encoding the ANT(4')-Ia enzyme and the aph(3')-IIIa gene encoding the APH(3')-IIIa enzyme. The results of this study showed statistically significant agreement between phenotypic and genotypic aminoglycoside resistance and methicillin resistance. In conclusion, this study has increased knowledge of the distribution of AMEs in S. aureus isolated in our hospitals. Continued surveillance at both the phenotypic and genotypic levels is recommended for monitoring the presence of other variants of the genes or new aminoglycoside resistant genes that may be produced within the S. aureus population and detecting early emergence of resistant organisms to establish effective antibiotic therapies and prevent nosocomial as well as environmental spread of resistant isolates.

INTRODUCTION

Staphylococcus aureus is a major cause of hospital- and community- acquired infections, and can result in serious consequences. Hospital infections caused by *S. aureus* include those affecting the blood stream, lower respiratory tract, skin and soft tissues, as well as ventilator associated pneumonia and central venous catheter associated bacteraemia[(1)]. The importance of *S. aureus* as a human pathogen, apart from its ability to cause a diverse range of life threatening infections, is its extra-ordinary potential to develop antimicrobial resistance[(2)].

Although emerging antimicrobial resistance amongst staphylococci is widespread, aminoglycosides still play an important role in the therapy of serious staphylococcal infections[(3)]. Gentamicin and tobramycin are the most active against staphylococci and are often used in combination with either a β-lactam or a glycopeptide, especially in the treatment of staphylococcal endocarditis, as these drugs act synergically[(4)].

Clinical isolates of *S. aureus* exhibit resistance to aminoglycosides by producing three types of aminoglycoside-modifying enzymes (AMEs). These enzymes are of particular significance among staphylococci since they modify and thereby inactivate the traditional aminoglycosides of particular importance. The genes encoding such modifying enzymes include *aac (6')-Ie+ aph(2")*, *aph(3')-IIIa* and *ant(4')-Ia*[(5)].

Susceptibility testing for individual aminoglycoside antibiotics is usually performed in clinical microbiology laboratories by disc diffusion or by measurement of the minimum inhibitory concentration (MIC) of the antibiotic[(6)]. While these methods provide relevant data concerning the susceptibility of a particular organism to the tested aminoglycosides, they may not provide data for untested but related antibiotics which may be susceptible to the modifying activity of an AME that modifies the tested antibiotics. Moreover, resistance testing does not discriminate between the types and combination of enzymes, which

might be present in a bacterial isolate[7]. This limitation has been addressed by the use of genotypic methods including dot blot hybridization and polymerase chain reaction (PCR) for detecting the presence of genes encoding AMEs and specifying their types from Staphylococci[3 & 8].

Unfortunately, there is still a lack of information on the incidence and types of AMEs in many countries worldwide especially in the Middle East and Arab countries[9].

The aim of this work is to study the different phenotypic patterns of aminoglycosides-resistant *S. aureus* in relation to the results of a multiplex PCR assay for the genes encoding AMEs.

SUBJECTS, MATERIALS & METHODS

This study was carried out in Microbiology and Immunology and Surgical Departments, Faculty of Medicine, Zagazig University during the period from August, 2008 to June, 2009.

Subjects:

The study was conducted on 170 patients from surgical departments and ICUs. They included 96 males and 74 females with age range of 1 to 85 years (Mean 39.9±17.5).

Clinical specimens:

One hundred and seventy clinical samples were collected from all patients including wound/pus swabs, sputum, endotracheal aspirate, urine and urinary catheters samples. All specimens were inoculated directly without delay on nutrient and blood agar media (Oxoid, UK) and incubated at 37°C for 24 hours. The colonies were identified as being *S. aurues* by colony morphology, microscopic appearance and catalase, coagulase and DNase tests[10].

Antimicrobial susceptibility testing:

Antimicrobial susceptibility testing was performed by disc diffusion method as recommended by the Clinical and Laboratory Standards Institute; CLSI[11] on Muller-Henton agar (Oxoid) with commercial antibiotic discs (Oxoid). The aminoglycoside antibiotic discs used in this study were: gentamicin (10µg), amikacin (30µg), netilmicin (30µg), tobramycin (10µg) and kanamycin (30µg). Susceptibility to antimicrobial agents was interpreted according to the guidelines of CLSI[11].

Screening for methicillin resistance among all *S. aureus* isolates was done by 1 µg oxacillin (Oxoid) disc diffusion testing on Muller-Hinton agar (Oxoid) and incubated for 24 hours at 30°C as recommended by CLSI (11). Intermediate resistance (disc zone diameter between 11 and 12 mm) was confirmed by the MIC determined with oxacillin E-test strips (AB Biodisk, Slona, Sweden).

Genotypic detection of staphylococcal resistance to aminoglycosides:

The staphylococcal genes; *aac(6')-Ie+aph(2")*, *ant(4')-Ia* and *aph(3)-IIIa* were detected by multiplex PCR amplification using specific primers (Quiagen, USA) as previously reported (3). The sequences of these primers were: aac(6')-Ie+ aph(2")-F 5'-CCAAGAG AATAAGGGCATACC-3' and aac(6')-Ie+aph(2")-R 5'-GCCACTACCAATC ATCCA -3' for *aac(6')-Ie+aph(2")*, ant(4')-Ia-F 5'-CTGCTAAATCGGTAGAAGC-3' and ant(4')-Ia-R 5'-CCACGGTACAACTAACCAGAC-3' for *ant(4')-Ia* and aph(3')-IIIa-F 5'-CTGAT GAATAC GCTGC-3' and aph(3')-IIIa-R 5'-GGAAACGA CCTTCTCATACT-3' for *aph(3)-IIIa*.

DNA extraction was performed using E.Z.N.A commercial kit (Omega Bioteck, Doraville, USA), according to the manufacturer's instructions.

The PCR amplification mixture consisted of pure Taq ready-to-go PCR bead (Amersham Bioscience, Buckinghamshire, UK), 0.4 mM of each primer and 1 µl of staphylococcal DNA extract in a total volume of 25 µl. Two drops of mineral oil (Sigma, USA) were added to overlay each sample. The amplification was performed in a thermal cycler (Cyclogene Techne, UK). The reaction mixtures were heated to 94°C for 10 minutes, followed by 35 amplification cycles, each consisting of 20 seconds at 94°C, 60 seconds at 55°C and 50 seconds at 72°C. A final extension cycle of 72°C for 10 minutes was included[3]. The amplified products were electrophoresed in 1.5% agarose gel. A DNA molecular weight marker (100 to 1000 bp) (Bioron, Germany) was run in parallel. The gels were stained with ethidium bromide and visualized under ultraviolet trans-illuminator (Cole-Parmer, USA). The AMEs encoding genes were determined by the size of the amplified products: 326 bp DNA amplicon for *aac(6')-Ie+ aph(2")*, 248 bp amplicon for *ant(4')-Ia* and 154 bp amplicon for *aph(3)-IIIa* (Figure 1).

Statistical methods:

Data were coded, entered and analyzed using Statistical Package for the Social Sciences program; SPSS, version 10. (Chicago, USA).

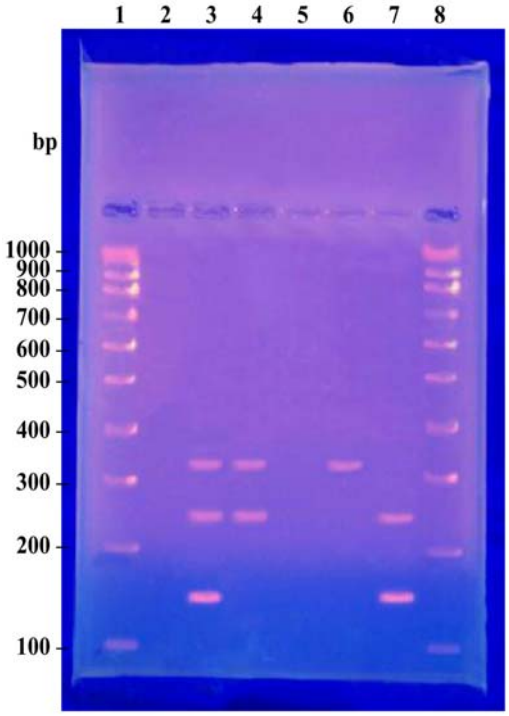

Figure (I): Agarose gel showing PCR amplified products of the *aac(6')-Ie+ aph(2")*, *aph(3')-IIIa* and *ant(4')-Ia*. Lane 1 and 8 show a DNA ladder. Lane 2 is the negative control showing no bands. Lane 3 shows the three amplification product (*aac(6')-Ie+ aph(2")* at 326 bp, *aph(3')-IIIa* at 248 bp and *ant(4')-Ia* at 154 bp). Lane 4 shows the amplification product of the *aac(6')-Ie+aph(2")* and the *aph(3')-IIIa* genes. Lane 5 shows no amplification product. Lane 6 shows the amplification product of the *aac(6')-Ie+ aph(2")* gene. Lane 7 shows the amplification product of the *aph(3')-IIIa* and the *ant(4')-Ia* genes.

RESULTS

A total of 81 (47.6%) *S. aureus* isolates were collected during this work and 43 (53.1%) isolates of which were methicillin resistant (MRSA).

In this study, 31 (38.3%) isolates were resistant to at least one of the tested aminoglycosides, while the remaining 50 (61.7%) isolates were fully susceptible to all of the tested aminoglycosides. The highest staphylococcal resistance was to kanamycin (38.3%), followed by tobramycin (30.9%), gentamicin (29.6%), amikacin (9.9%) and netilmicin (8.6%). There was a statistically high significant agreement between gentamicin, kanamycin and tobramycin resistance and methicillin resistance; P value = 0.000 (Table 1).

Table (1): Aminoglycosides resistance in *S. aureus* clinical isolates

Individual aminoglycosides	**MRSA (43 isolates) No (%)**	**MSSA (38 isolates) No (%)**	**Total (81 isolates) No (%)**
Kanamycin	28 (65.1%)	3 (7.9%)	31 (38.3%)
Tobramycin	24 (55.8%)	1 (2.6%)	25 (30.9%)
gentamicin	24 (55.8%)	0(0%)	24 (29.6)
Amikacin	8 (18.6%)	0 (0%)	8 (9.9%)
Netilmicin	7 (16.3%)	0 (0%)	7 (8.6%)

Of the 31 aminoglycoside-resistant *S. aureus* isolates, 21 (67.7%) harbored *aac(6')-Ie+aph(2")*, 18 (58.1%) harbored *ant(4')-Ia* and 17 (54.8%) harbored *aph(3')-IIIa*, while the remaining 8 (25.8%) and the 50 fully aminoglycosides-sensitive *S. aureus* isolates did not harbor any of the amplified genes (Table 2).

Table (2): The frequency of aminoglycoside resistance genes among *S. aureus* isolates

Types of genes	Aminoglycoside-resistant *S. aureus* (No. = 31)		Aminoglycoside-sensitive *S. aureus* (No. = 50)	
	No	**(%)**	**No**	**(%)**
aac(6')-Ie+aph(2")	21	(67.7)	0	(0)
ant(4')-Ia	18	(58.1)	0	(0)
aph(3')-IIIa	17	(54.8)	0	(0)
No detected genes	8	(25.8)	50	(100)

The 31 aminoglycoside-resistant *S. aureus* isolates showed 5 different phenotypic patterns. The highest resistance pattern was of gentamicin, kanamycin and tobramycin (51.6%), followed by gentamicin, amikacin, kanamycin, netilmicin and tobramycin (22.6%). Whereas, the lowest resistance pattern was of kanamycin and tobramycin as well as gentamicin, amikacin, kanamycin and tobramycin (3.2% for each). The frequency of AMEs encoding genes among different aminoglycoside resistance phenotypic patterns are summarized in table (3).

Table (3): Aminoglycoside resistance genes in *S. aureus* isolates in relation to the different aminoglycoside resistance phenotypes

Resistance phenotypes (No.)	AMEs	No. of isolates
CN, K and TOB (16)	*aac(6')-Ie+ aph(2"), ant(4')-Ia* and *aph(3')-IIIa*	5
	ant(4')-Ia and *aph(3')-IIIa*	1
	aac(6')-Ie+ aph(2")	4
	aac(6')-Ie+ aph(2") and *ant(4')-Ia*	1
	ant(4')-Ia	1
	-	4
CN, Ak, K, NET and TOB (7)	*aac(6')-Ie+ aph(2")* and *ant(4')-Ia, aph(3')-IIIa*	6
	aac(6')-Ie+ aph(2") and *aph(3')-IIIa*	1
K (6)	*aac(6')-Ie+ aph(2"), ant(4')-Ia* and *aph(3')-IIIa*	3
	-	3
CN, AK, K and TOB (1)	*aac(6')-Ie+ aph(2"), ant(4')-Ia* and *aph(3')-IIIa*	1
K and TOB (1)	-	1

CN: gentamicin, AK: Amikacin, K: Kanamycin, NET: Netilmicin, TOB: Tobramycin, AMEs: Aminoglycoside modifying enzymes, -: No AMEs detected

Table (4) shows the distribution of AMEs encoding genes determined by multiplex PCR assay in *S. aureus* isolates in relation to methicillin resistance. There was a statistically high significant agreement (P value = <0.001) between methicillin resistance and *aac(6')-Ie+ aph(2")* gene followed by *ant(4')-Ia* and *aph(3')-IIIa*.

Table (4): The distribution of AMEs encoding genes in S. aureus isolates in relation to methicillin susceptibility

AMEs encoding genes	Multiplex PCR results	MRSA	MSSA	Kappa	P value
ant(4')-Ia	Positive	17	1	0.355	0.000
	Negative	26	37		
aph(3')-IIIa	Positive	16	1	0.333	0.000
	Negative	27	37		
aac(6')-Ie+ aph(2")	Positive	20	1	0.425	0.000
	Negative	23	37		

There was a statistically high significant agreement (P value = <0.001) between phenotypic aminoglycoside resistance and genotypic multiplex PCR results for AMEs encoding genes (Table 5). However, 88.9% concordance was observed between gentamicin and *aac(6')-Ie+ aph(2")* gene and between amikacin and *aph(3')-IIIa* gene, while it was 85.2% between gentamicin and *ant(4')-Ia* gene.

Table (5): The agreement between phenotypic aminoglycosides resistance and genotypic multiplex PCR results for AMEs encoding genes

Resistant isolates by disc diffusion (No)	Multiplex PCR results								
	aac(6')-Ie+ aph(2")			*aph(3')-IIIa*			*ant(4')-Ia*		
	Positive	Negative	Kappa	Positive	Negative	Kappa	Positive	Negative	Kappa
CN-resistant (24)	18	6	0.724	14	10	0.580	15	9	0.617
AK-resistant (8)	8	0	0.477	8	0	0.584	7	1	0.465
K-resistant (31)	21	10	0.722	17	14	0.600	18	13	0.631
NET-resistant (7)	7	0	0.426	7	0	0.525	6	1	0.406
TOB-resistant (25)	18	7	0.697	14	11	0.556	15	10	0.592

CN: gentamicin, AK: Amikacin, K: Kanamycin, NET: Netilmicin, TOB: Tobramycin

DISCUSSION

Multi-drug resistance is the role among staphylococci, which is perhaps the pathogen of greatest concern because of its intrinsic virulence, its ability to cause a diverse array of life-threatening infections and its capacity to adapt to different environmental conditions[1]. In this study, 43 (53.1 %) of the 81 *S. aureus* isolates were MRSA. This result conformed to the results of Choi *et al.*[12], who found that MRSA represent 53% of *S. aureus* isolates. On the other hand, higher figures (64%) were obtained by Kim *et al.*[13], whereas, lower figures (27.7%) were reported by Schmitz *et al.*[3]. These different rates of MRSA from different studies may be attributed to variations in patient populations, the biological characteristics of the *S. aureus* strains and/or infection control practices.

Aminoglycoside resistance is common in *S. aureus* isolated from different countries, and especially gentamicin resistance, is of clinical importance because it can compromise the therapeutic effectiveness of these antibacterial agents[14]. In the present study, 38.3 % of the 81 *S.aureus* isolates were resistant to at least one of the tested aminoglycoside antibiotics. In agreement, Hauschild *et al.*[15] found that 38.1% of *S. aureus* isolates were resistant to at least one of the tested aminoglycoside antibiotics. However, higher results (71 %) were reported in the study conducted by Yadegar *et al.*[16].

In this study, the highest staphylococcal resistance was to kanamycin, while the lowest resistance was to netilmicin. This result may be explained by the fact that netilmicin is seldom prescribed in our hospitals. Similar results were reported by Hauschild *et al.*[15], who found that 97.8 % of the 45 staphylococcal isolates were resistant to kanamycin while all isolates were sensitive to netilmicin.

Regarding the frequency of the genes encoding AMEs, the *aac(6')-Ie+ aph(2")* gene encoding the bifunctional enzyme AAC(6')-Ie + APH(2") was the most common, followed by the *ant(4')-Ia* gene encoding the ANT(4')-Ia enzyme and the *aph(3')-IIIa* gene encoding the

APH(3')-IIIa enzyme. Similar results were reported in other previous studies[12&17]. This may be explained by the fact that the gene for this bifunctional enzyme is part of a composite transposon Tn*4001,* which is widely distributed in both *S.aureus* and coagulase negative staphylococci that facilitated its rapid dissemination in the presence of selective antibiotic pressure[18]. In a study carried out by Schmitz *et al.*[3], the *aac(6')-Ie+ aph(2")*, *ant(4')-Ia* and *aph(3')-IIIa* were found in a prevalence of 68%, 48% and 14%, respectively of aminoglycoside-resistant staphylococci throughout Europe.

In this work there was a statistically significant agreement between aminoglycoside and methicillin resistance and there was a statistically high significant agreement between methicillin resistance and the distribution of genes encoding AMEs detected by multiplex PCR assay. Similarly, several reports have stated that aminoglycoside resistance is closely related to methicillin resistance[3,12,13&16]. This might be due to the adjacent locations of methicillin-resistance encoding *mec*A gene and of the AME encoding genes.

It is noteworthy to mention that in 8 isolates demonstrating phenotypic resistance to one of the tested aminoglycosides in this work, none of the three AMEs-encoding genes was detected in these isolates. Similar results were reported by Hauschild *et al.*[15], who found that from a total of 45 isolates demonstrating phenotypic resistance to one of tested aminoglycosides, 10 isolates did not harbor the tested genes encoding AMEs. This finding may be attributed to the presence of a variant gene that cannot be detected within the primer sets or that new aminoglycoside resistance genes are produced within the *S. aureus* population[15]. Another explanation is perhaps the presence of other resistance mechanisms like modification of the target by mutation in genes encoding ribosomal proteins, alteration of membrane permeability or active efflux of the drug[19].

Although the bifunctional enzyme encodes resistance to virtually all aminoglycosides, including gentamicin, amikacin, tobramycin, netilmicin and kanamycin[5], the *aac(6')-Ie+ aph(2")* gene encoding this enzyme was detected in three gentamicin susceptible isolates in this study. Similar finding had been reported by Hauschild *et al.*[15]. The detection of resistance genes in antibiotic susceptible isolates may be due to amplification of repressed antibiotic resistance gene[14] or AME of these strains display lower enzymatic activity[20].

This work revealed that the gene for the bifunctional enzyme was absent in 6 gentamicin-resistant isolates. In a similar study, Udo and Dashti[9] reported that this gene was detected in all except two gentamicin-resistant isolates. In both isolates this gene could not be detected by PCR and dot blot hybridization. Such observation may be explained by the presence of another variant of the enzyme that could not be detected or that new aminoglycoside resistant genes are produced within the *S.aureus* population[15].

In this study, there was a statistically significant agreement between gentamicin resistance and the presence of the *aac(6')-Ie+ aph(2")* gene and between kanamycin, tobramycin, amikacin resistance and the presence of the *aac(6')-*I*e+ aph(2")* gene. These findings had been reported in many previous studies[12,15&16]. However, the concordance between gentamicin and *aac(6')-Ie+ aph(2")* gene and between amikacin and *aph(3')-IIIa* gene was 88.9 %. These values were 100% and 83% respectively in the study of Choi *et al.*[12], who explained the discordant results by the presence of other mechanisms, such as loss of permeability and ribosomal alteration that may mediate resistance.

In conclusion, this study has increased knowledge of the distribution of AMEs in *S. aureus* isolated in our hospitals. Moreover, Since the type of the AMEs correlated with aminoglycoside usage in either the geographical regions or in individual hospitals, the frequent and empirical use of aminoglycosides in our hospitals and in the community may lead to wide spread of the genes encoding the AMEs or the appearance of new variants of the enzymes. Therefore, continued surveillance at both the phenotypic and genotypic levels is recommended for monitoring the presence of other variants of the genes or new aminoglycoside resistant genes that may be produced within the *S. aureus* population and detecting early emergence of resistant organisms to establish effective antibiotic therapies and prevent nosocomial as well as environmental spread of resistant isolates.

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الأنماط الظاهرية والجينية للمعزولات الإكلينيكية للمكورات العنقودية الذهبية المقاومة للأمينوجليكوسيدات

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الأمينوجليكوسيدات لا تزال تلعب دورا هاما فى العلاجات المضادة للمكورات العنقودية على الرغم من المقاومة الناشئة بين المكورات العنقودية على نطاق واسع .

يهدف هذا العمل إلى دراسة الأنماط الظاهرية المختلفة للمكورات العنقودية الذهبية المقاومة للأمينوجليكوسيدات وعلاقتها بنتائج تفاعل سلسلة إنزيم البلمرة المتعدد للكشف عن الأنماط الجينية.

إشتمل هذا البحث على ١٧٠ عينة إكلينيكية من أقسام الجراحة ووحدات العناية المركزة بمستشفيات جامعة الزقازيق .وأسفرت نتائج هذا العمل عن فصل 81 معزولة لميكروب المكورات العنقودية الذهبية .أظهرت 43 معزولة منهم مقاومة للميثيسيللين بينما أظهرت 31 معزولة مقاومة للأمينوجليكوسيدات ، وكانت أعلى مقاومة للكاناميسين ثم التوبراميسين والجينتاميسين، أسفرت نتائج هذا البحث عن اتفاق هام إحصائيا بين الأنماط الظاهرية والجينية للمكورات العنقودية الذهبية المقاومة للأمينوجليكوسيدات والميثيسيللين.

من هذا البحث تم استنتاج أن المراقبة المستمرة على المستويين الظاهرى والجينى لمقاومة المكورات العنقودية الذهبية للأمينوجليكوسيدات تعتبر أمرا ضروريا وهاما للكشف المبكر عن المعزولات المقاومة لوضع علاجات فعالة ولمنع انتشار المعزولات المقاومة والمسئولة عن عدوى المستشفيات أو المسئولة عن انتشار العدوى بالبيئة.