Aminoglycoside and chlorhexidine resistance genes in *Staphylococcus aureus* isolated from surgical wound infections

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Abstract

Objective: *Staphylococcus aureus* especially the methicillin-resistant population (MRSA) is a major human pathogen resistant to many antimicrobial agents. This study aims to investigate the prevalence of the aminoglycoside-modifying enzymes (AMEs); genes *aac(6')* *aph(2')*, *aph(3')-IIIa*, *Ant(4')-Ia* and the biocide (quaternary ammonium compounds) resistance genes (*qacA/qacB*, *qacC*) in *S. aureus* isolated from surgical site infections.

Methods: Swabs from 280 infected surgical sites were collected from different surgical wards at Mansoura University Hospitals between January 2014 and December 2014). Sixty-six staphylococcal strains were isolated and included in this study. Verification of the presence of methicillin resistance gene (*mecA*), aminoglycoside resistance genes [*aac(6')*/*aph(2')*, *ant(4')-Ia* and *aph(3')-IIIa*] and *qac* resistance genes (*qacA/qacB*, *qacC*) in *S. aureus* was carried out by PCR. Chlorhexidine MIC was also determined.

Results: Out of the 66 *S. aureus* isolates included in this study, 17/66 isolates (25.8%) were phenotypically characterized as MRSA and *mecA* gene was detected in 19 *S. aureus* isolates (28.7 %) by PCR. Aminoglycoside resistant *S. aureus* accounted for 21/66 (31.8%) of the isolates. AME genes were detected in all aminoglycoside-resistant *S. aureus*; *aac(6')*/*aph(2')* and was the most frequently detected 11/21(52.4%), followed by *aph(3')-IIIa* 6/21 (28.6%) and the least frequent was *ant(4')-Ia* 4/21(19%). Aminoglycoside resistance in 9/21 (42.9%) of aminoglycoside resistant *S. aureus* isolates was found to be plasmid mediated as proved by plasmid curing experiment. A total of 14/21 aminoglycoside-resistant *S. aureus* isolates (66.7 %) carried the *mecA* gene. Nine of the 21 aminoglycoside-resistant *S. aureus*

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isolates (42.9%) were positive for *qacA/qacB* genes and in all of them *mecA* gene co-existed.

Five aminoglycoside resistant *S. aureus* isolates were *qacC* positive (23.8%). Among the 45 aminoglycoside-sensitive *S. aureus* isolates, 5 (11.1%) were *mecA* positive.

Conclusion: This study shows a high proportion of *S. aureus* isolated from infected surgical sites carried aminoglycoside and methicillin-resistance genes together with quaternary ammonium compounds resistance genes.

Keywords: Staphylococcal Infections, Surgical Site Infections, Disinfectant Resistance, Drug Resistance.

Introduction

Staphylococcus aureus is a potentially serious opportunistic human pathogen that colonizes over 30% of individuals. MRSA is a major nosocomial pathogen that can easily circulate among patients and personnel in hospitals [1].

An important tool in controlling nosocomial MRSA infection is hand hygiene and decontamination of potentially contaminated rooms, utensils and colonized patients. Many antiseptic agents are in use for this purpose but overuse and sub-lethal concentrations of antiseptic agents have led to the emergence of MRSA with decreased antiseptic susceptibility [2].

Multidrug efflux pumps are membrane proteins that form channels to transport toxic compounds such as biocides and disinfectants out of the cell. Their substrates are diverse including quaternary ammonium compounds (Qac) for which they are named [3]. These *qac* genes, confer resistance to organic cations by means of a proton motive force-dependent multidrug efflux [4].

Within *Staphylococcus* species, six different plasmid-encoded Qac efflux pumps were known:

QacA and QacB are members of the Major Facilitator Superfamily (MFS) and are highly related and conserved in *Staphylococcus* species. Whereas, QacC, QacG, QacH, and QacJ belong to the Small Multidrug Resistance (SMR) family. Genes encoding these proteins are generally carried on plasmids [5,6]. Some of these plasmids (pST6, pSK4, and pSK41) contain antibiotic resistance genes encoding resistance to gentamicin, penicillin, kanamycin and tobramycin [7].

The *qacA* and *qacB* genes are closely related and confer resistance to a range of structurally disparate organic cations, including divalent cations like chlorhexidine. They differ only in the amino acid residue 323 that is (Asp) in QacA and (Ala) in QacB. Both are located on a variety of plasmids and *qacA* exists also on chromosomes [8].

Chlorhexidine gluconate is a water-soluble cationic bisbiguanide widely used as an antiseptic agent since 1954. It has been approved by the U.S. Food and Drug Administration for infection control in various applications, including general skin cleaning, central venous catheter site preparation, surgical hand and preoperative scrub as well as vascular catheter dressings [9]. Chlorhexidine destroys the

cell membrane and causes coagulation of intracellular contents of a variety of microorganisms, including Gram-positive and Gram-negative bacteria, lipophilic virus, protozoa, and fungi [10].

The main mechanism of aminoglycoside resistance in staphylococci is the drug inactivation by aminoglycoside-modifying enzymes (AMEs). Among staphylococci, the most common AME is 6'-N-acetyltransferase-2''-O-phosphotransferase [aac(6')-aph(2'')] that is encoded by *aac(6')-aph(2'')* gene. This enzyme inactivates amikacin, gentamicin, kanamycin, tobramycin and neomycin. Another AME is 4'-O adenylyltransferase I [ant(4')-I] encoded by *ant(4')-Ia* gene, and it inactivates amikacin, kanamycin, neomycin, and tobramycin. The third AME is 3'-O-III[aph(3')-III] that inactivates amikacin and kanamycin and is encoded by *aph(3')-IIIa* gene [11-13].

The purpose of the present study was to investigate the prevalence of the antiseptic-resistant genes *qacA*, *qacB* and *qacC* and aminoglycoside resistance genes in *S. aureus* clinical isolates from surgical site infections at Mansoura University Hospitals.

Materials and Methods

Sample collection, culture and identification

This study was approved by the ethical committee of the Faculty of Medicine, Mansoura University. Specimens from infected surgical sites were collected using sterile saline moistened cotton swabs and immediately transported to the laboratory in sterile screw-capped tubes containing 5 ml saline. Specimens were processed at the

Microbiology Diagnostic and Infection Control Unit (MDICU) in the department of Medical Microbiology and Immunology, Mansoura faculty of medicine. Specimens were inoculated within 1–2 h onto mannitol salt agar and sub-cultured to Baird-Parker agar medium (Merck, Germany). Plates were incubated aerobically at 37°C for 48 hours. Colo-

nies with typical black appearance surrounded by a clear zone were considered Staphylococci and when they grow in blood agar they were identified as *S. aureus*. Further identification of isolates included Gram-stain as well as catalase, and coagulase tests. *S. aureus* ATCC 12600 was used as positive control and *S. epidermidis* as a negative control [14].

Antimicrobial susceptibility testing/Disc diffusion method

Susceptibility tests were performed using the Kirby–Bauer disc diffusion method on Muller-Hinton agar plates which included the following antibiotics: ampicillin/sulbactam (20 µg), oxacillin (1µg), gentamicin (10µg), amikacin (30µg), erythromycin (15µg), clindamycin (2 µg), chloramphenicol (30µg), ceftazidime (30 µg), cefpime (30 µg), aztreonam (10 µg), imipenem (10µg) trimethoprim-sulfamethoxazole (25µg), fusidic acid (10µg) and vancomycin (30µg) according to Clinical Laboratory Standards Institute guidelines (CLSI) [15]. Antibiotic discs were obtained from (Oxoid, UK). Plates were incubated aerobically at 35 (for oxacillin) and 37°C for 24 hours and *S. aureus* ATCC 12600 was used as quality control strain. Amikacin/kanamycin double resistant strains were used to represent aminoglycosides resistance.

Broth microdilution test

The minimum inhibitory concentrations (MICs) of chlorhexidine were determined by the reference broth microdilution procedure recommended by the CLSI using 96-well microtiter plates with serial 2-fold dilutions. Dilutions ranged from 32ug/ml to 0.25ug/ml. The MIC was defined as the lowest concentration of antimicrobial agent that produced no visible growth after 20-h incubation in Mueller-Hinton broth at 37°C [15].

DNA manipulation

Total DNA extraction

A single bacterial colony was grown overnight on L-broth with shaking at 37°C to stationary phase

Table 1. Specific PCR primers used for amplification of examined genes

Primer's name	5' to 3' Sequence	Expected amplicon size	Reference
16S rDNA forward 16S rDNA reverse	CAG CTC GTG TCG TGA GAT GT AAT CAT TTG TCC CAC CTT CG	420bp	GenBank: Y15856
<i>mecA</i> forward <i>mecA</i> reverse	GTA GAA ATG ACT GAA CGT CCG ATA A CCA ATT CCA CAT TGT TTC GGT CTA A	310bp	[18]
<i>qacA/B</i> : forward <i>qacA/B</i> : reverse	CTA TGG CAA TAG GAG ATA TGG TGT CCA CTA CAG ATT CTT CAG CTA CAT G	321bp	[19]
<i>qacC</i> : forward <i>qacC</i> : reverse	GGC TTT TCA AAA TTT ATA CCA TCC TAT GCG ATG TTC CGA AAA TGT	249bp	[20]
<i>aac(6')/aph (2'')</i> : forward <i>aac(6')/aph (2'')</i> : reverse	GAA GTA CGC AGA AGA GA ACA TGG CAA GCT CTA GGA	491bp	[21]
<i>aph(3)-IIIa</i> forward <i>aph(3)-IIIa</i> reverse	CGA TGT GGA TTG CGA AAA CT CAC CGA AAT AAC TAG AAC CC	175bp	[22]
<i>ant (4')-1a</i> forward <i>ant (4')-1a</i> reverse	ATG GCT CTC TTG GTC GTC AG TAA GCA CAC GTT CCT GGC TG	367bp	[23]

and 1.5 ml culture was harvested by centrifugation at 11600xg for 2 min in a microfuge. The manufacturer's recommendations (Sigma) using Thermo Scientific DNA extraction method were used. The extracted DNA was used for PCR amplification of the studied genes including the house-keeping rDNA genes.

Polymerase Chain Reaction (PCR)

PCR was performed in a 25µl reaction volume containing 1µl (50 ng) of extracted DNA, 1µl of each pair of primers 12.5µl of 2x *Taq* premix Mastermix (Sigma) and 9.5µl sterile double-distilled water. The specific PCR primers and the expected products' sizes are listed in (Table 1). The PCR program involved an initial denaturation step at 95°C for 5 min followed by 35 cycles of a denaturation step at 95°C for 30 seconds, a primer annealing step at 54- 60°C for 30 seconds, an extension step at 72°C for 30 seconds, and a final step at 72°C for 10 min. In each reaction a positive and a negative control were included as appropriate. The negative control

contained all the reagents without template DNA whereas the positive control was the staphylococcal 16S rDNA. All PCR products were analyzed by agarose gel electrophoresis using 1.5 % (w/v) agarose, performed at 100v in TAE buffer (0.5M Tris acetate, 5.7% acetic acid, 10mM EDTA pH 8.0) containing 0.5µg/ml ethidium bromide.

Plasmid curing

S. aureus isolates were sequentially passed in LB (approximately 100 cells into 100 ml) at 43°C with shaking for about 30 generations to eliminate the plasmid. Cured strains were diluted and plated on LA plates to obtain single colonies. Replica plating at 37°C was used to screen for loss of resistance [16]. Loss of the plasmid was confirmed by loss of unselected phenotypic traits (drug resistance) and by PCR of relevant genes [17]. All statistical analyses were performed using SPSS version 16.0 (SPAA Inc., Chicago, IL, USA), using Chi square and Independent sample T tests. p- value <0.05 is considered significant.

Results

A total of 280 swabs were collected from infected surgical sites at different surgical departments of Mansoura University Hospitals. *S. aureus* was isolated from 66 (23.6%) samples. *S. aureus* isolates resistant to both amikacin and kanamycin were used to represent aminoglycosides resistance. The genes for aminoglycoside modifying enzymes (*aac(6')*/*aph(2'')*, *aph(3')*-*IIIa* and *Ant(4')*-*Ia*) were carried by 21/66 (31.8 %) *S. aureus* isolates. Among these genes *aac(6')*/*aph(2'')* was the most

frequent being detected in 11/21 (52.4%) of aminoglycoside resistant *S. aureus* followed by *aph(3')*-

IIIa that was positive in 6/21 (28.6%) while *ant(4')*-*Ia* was the least common, only detected in 4/21(19%) of aminoglycoside resistant *S. aureus* (Table 2). Genes for quaternary ammonium compounds resistance were detected in 14/21 aminoglycoside resistant *S. aureus* (66.7%); *qacA/qacB* was detected in 9/21 (42.9%) and *qacC* was detected by PCR in 5/21 (23.8%). The antimicrobial susceptibility patterns of the 66 *S. aureus* isolates by disc diffusion revealed 17 oxacillin resistant isolates (MRSA) (25.8%). The *mecA* gene was detected by PCR in the genomic DNA of 19 *S. aureus* isolates (28.7%). *mecA* gene was detected in 14/21 (66.7%) aminoglycosides resistant isolates as shown in (Table 3). Genes for re-

Table 2. The relationship between aminoglycoside resistance pattern and the presence of aminoglycoside-modifying enzymes genes in *S. aureus* isolates.

<i>S. aureus</i> isolates	Aminoglycoside susceptibility	Aminoglycoside-modifying enzymes genes			PCR negative isolates
		<i>aac(6')</i> / <i>aph(2'')</i>	<i>aph(3')</i> - <i>IIIa</i>	<i>Ant(4')</i> - <i>Ia</i>	
	N (%)	N (%)	N (%)	N (%)	N (%)
Resistant	21 (31.8%)	11 (52.4%)	6 (28.6%)	4 (19%)	0
Sensitive	45 (68.2%)	6 (13.3%)	2 (4.4%)	1 (2.2%)	36 (80%)
Total	66	17 (25.8%)	8 (12.1%)	5 (7.6%)	36 (54.5%)

p- value < 0.001 for the test of homogeneity of the 3 types of AME genes in *S. aureus*

Table 3. Frequencies of methicillin and quaternary ammonium compounds resistance genes among aminoglycoside resistant *S. aureus*

Isolates	Aminoglycoside resistance		Methicillin resistance gene		Quaternary ammonium resistance genes		
					Positive		Negative
			<i>mecA</i> (+ve)	<i>mecA</i> (-ve)	<i>qacA/qacB</i>	<i>qacC</i>	
		N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
<i>S. aureus</i> (66)	Resistant N/ %	21 (31.8)	14 (66.7)	7 (33.3)	9 (47.4)	5 (28.9)	7 (23.7)
	Sensitive N/ %	45 (68.2)	5 (11.1)	40 (88.9)	-----	-----	45 (100)

p- value < 0.001

Table 4. The frequencies of quaternary ammonium compounds resistance genes among methicillin resistant *S. aureus*

<i>S. aureus</i> isolates	Methicillin resistance gene (<i>mecA</i>)		Quaternary ammonium resistance genes		
			Positive/ N (%)		Negative-/N (%)
			<i>qacA/qacB</i>	<i>qacC</i>	
(66)	(+ve) N (%)	19 (28.7)	9 (47.4)	5 (26.3)	5 (26.3)
	(-ve) N (%)	47 (61.3)	-	-	47 (100)

sistance to quaternary ammonium compounds were found in 14/19 (73.7%) methicillin resistant *S. aureus*. *qacA/qacB* genes was detected in 9/19 (47.4%) and *qacC* gene was detected in 5/19 (26.3%) of MRSA isolates (**Table 4**).

The MIC for chlorhexidine ranged from 1 to 8 ug/ml while that for the standard *S. aureus* ATCC 12600 was 2 mg/ml. All aminoglycoside and methicillin sensitive *S. aureus* isolates did not carry any quaternary ammonium compound resistance genes and their MIC for chlorhexidine was <2ug/ml. Plasmid curing experiment eliminated AMEs genes and aminoglycoside resistance in 9/21 (42.9%) of aminoglycoside resistant *S. aureus* isolates.

Discussion

Aminoglycosides have an important role in the treatment of *S. aureus* associated surgical site infections in many countries including Egypt. There is a limited amount of data regarding the prevalence of aminoglycosides resistance genes (AMEs genes) among *S. aureus* clinical isolates in Egypt. The results of this study shows that *S. aureus* isolated from infected surgical wounds were positive at a high rate for presence of aminoglycosides and methicillin-resistance genes (66.7%) in association with quaternary ammonium compounds resistance genes. Two thirds of the aminoglycoside resistant *S. aureus* tested in this study carried the *mecA* gene

together with one of the AME genes. None of the *S. aureus* isolated in this work carried more than one AME gene. Other similar studies reported *aac(6')*/*aph (2'')* as the most frequently detected AME gene (83%) among *S. aureus* isolates [24, 25]. Some studies reported a prevalence of the three AME genes in 21% of isolates [26].

The co-existence of *mecA* gene and AME genes in a relevant number of tested *S. aureus* might suggest a genetic linkage as the *mecA* gene in MRSA is carried on the Staphylococcal Cassette Chromosome *mec* that encodes genes for other antibiotics including aminoglycosides [27]. Plasmid curing experiment eliminated AME genes and aminoglycoside resistance in (42.9%) of aminoglycoside resistance *S. aureus* isolates. This result confirmed the carriage of these genes on plasmids, and it is alarming since the horizontal gene transfer by plasmids is highly increasing the risk of drug resistant strain dissemination [28].

Quaternary ammonium compounds are commonly used disinfectants in hospitals to control hospital-associated infection but resistance to these compounds has lately increased [29]. Moreover, it is frequently linked to resistance to other antibiotics such as methicillin and aminoglycosides. However, inconsistent results were reported regarding the frequencies of antiseptic resistance genes including *qacA/B* and *qacC* genes in *S. aureus* in different countries and hospitals [2, 30].

A study carried out by Noguchi *et al.* [2], reported

an occurrence rate of *qacA/B* and *qacC* genes in Asian isolates to be 41.6% and 1.9%, respectively [2]. In our study *qacA/B* was more frequent than *qacC* and was detected in 47.4% of MRSA isolates, whereas *qacC* was positive only in 28.9% of those isolates. In agreement with this but with lower frequencies, Duran *et al.* [21] reported the isolation of *qacA/B* at a higher rate than *qacC* from MRSA with *qacA/B* (20.8%), while *qacC* gene existed at the frequency of 7.7% [29]. A Malaysian study showed much higher rate of detection for *qacA/B* gene among their MRSA isolates (83.3%), and only 1.9% of these strains were positive for *qacC* [31]. On the other hand, the study of Nakipoğlu *et al.*, [32] reported an isolation rate of (36%) for *qacC* in MRSA isolates, while *qacA/B* was detected only in 4% of those isolates. Along with our results, a recent study reported the presence of antiseptic resistance genes in 68.4% of the aminoglycosides resistant MRSA isolated from surgical site infections, among of these 47.4% carried *qacA/B* and 28.9% were *qacC* positive [29].

In conclusion, the current work demonstrates that 66.7% of the aminoglycoside resistant *S. aureus* isolated from surgical site infections were harboring genes for antiseptic resistance and 42.8% and 23.8% were positive for *qacA/B* and *qacC*, respectively.

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