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Dissemination of Aminoglycoside-Modifying Enzymes (AME) in Methicillin-Resistant *Staphylococcus aureus* Among Egyptian Isolates

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Abstract: The multiplex polymerase chain reaction (PCR) was used to identify the genes, encoding the (AME) aminoglycoside-modifying enzymes *viz.,* AAC (6')/APH(2''), APH(3')-IIIa and ANT(4'*)- Ia*, respectively and the methicillin resistance determinant *mecA in m*ethicillin-resistant isolates of *Staphylococcus aureus.* In total, 66 isolates collected in a period between April 2010 and January 2011 were obtained from 2 different teaching hospitals were tested by conventional susceptibility testing using the disk diffusion method 47 (71.2%) were methicillin resistant (MRSA), 5 (7.6%) intermediately resistant and 14 (21.2%) were methicillin sensitive. Thirty two isolates (48.5%) were resistant to gentamicin, 12 (18.2%) of the isolates were resistant to netilmicin, 24 isolates (36.4%) were resistant to neomycin, 20 isolates (30.3%) were resistant to amikacin, 30 (45.5%) were resistant to tobramycin and 35 (53.3%) isolates were resistant to kanamycin. The bifunctional AAC (6')/ APH (2'') was the enzyme encountered most frequently found in 36 (76.5%) of the strains. The prevalence of APH (3')-IIIa was too low present in only 6 (12.7%) of the isolates while ANT (4') - Ia was found in Eighteen (38.2%) of the isolates. The *mecA* gene was detected in all isolates 19 (40.4%) strains carried only the *mecA.* The concordances between gentamicin, kanamycin, tobramycin and amikacin resistance and the presence of *aac(6')/aph(2'')* gene were 100%, 100%, 85% and 42%, respectively. The concordance between the presence of the kanamycin resistance and the presence of *aph(3')-IIIa was 100%,* tobramycin resistance and the presence of *ant(4')-Ia was 80%* and amikacin resistance and the presence of *ant(4')-Ia,* was 60%.

Key words: Antibiotics · Aminoglycoside · Aminoglycoside Modifying Enzyme · Polymerase Chain Reaction · *Staphylococcus aureus* · Methicillin Resistance

INTRODUCTION

Staphylococcus aureus is a versatile human pathogen that continues to be an important cause of nosocomial infection [1]. Methicillin-resistant staph aureus (MRSA) strains showing multiple antibiotic resistances and an enhanced capacity to cause wide spread outbreaks of infections [2]. Such strains seem to have a special capacity to colonize patients and staff [3, 4]. There seems to be a general agreement that infections caused by this type of strain are often associated with a high degree of morbidity and mortality [5, 6]. Moreover, methicillin-resistant staphylococci are also resistant to antibiotics, including ß-lactams, aminoglycosides and macrolide [6]. Considering the importance of antibiotic resistance in treatment, more rapid and reliable diagnostic methods can be used to determine their resistance profiles are required [7]. The main mechanism of aminoglycoside resistance inactivation mediated is drug by aminoglycoside modifying enzymes (AME). The bifunctional enzyme AAC (6')/APH(2'') encoded by the *aac(6')/aph(2'')* gene is the most frequently encountered AME in staphylococcal isolates. Additional enzymes, such as APH (3')-III encoded by *aph(3')- IIIa* gene and the ANT(4')-I by *ant(4')-Ia* gene, are also found in *Staphylococcus* species [8]. The *mecA* gene encoding PBP 2a, has very high levels of homology in methicillin-resistant *S. aureus* (MRSA). Therefore the *mecA* gene is considered a useful molecular marker of methicillin-resistance in all staphylococci [7]. Polymerase chain reaction (PCR) appears to be the more rapid, sensitive and specific assay for such detection than southern blot hybridization, macrorestriction, fingerprinting and determining the minimal inhibitory concentration (MIC) [9, 10, 11]. In particular, multiplex PCR that detects several

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genes simultaneously in the same PCR tube has the advantage of identifying genotypic resistance for several antibiotics more rapidly and reliably [12, 13]. The aims of the present study were: (i) to develop a rapid multiplex PCR assay for the simultaneous detection of genes encoding AME and PBP 2a; (ii) to compare this multiplex PCR assay with standard microbiological methods of susceptibility testing; and (iii) to evaluate the prevalence of these resistance genes in *Staphylococcus* species.

MATERIALS AND METHODS

Bacterial Strains: Over a period between April 2010 and January 2011 a total of 66 *Staphylococcus aureus* samples were taken from inpatients after 48 hours of their admission into two teaching hospitals and clinically diagnosed with respiratory tract infections, urinary tract infections, wounds sepsis. Specimens were taken as urine, pus, sputum, blood according to each type of infection. Clinical samples were cultured on nutrient agar after collection separate colonies were processed for qualitative conventional phenotypic and diagnostic methods of identification according to the standard microbiological techniques. Stock cultures were stored frozen (-70°C) in brain-heart infusion (BHI), containing 10% glycerol.

Antimicrobial Agents and Susceptibility Testing: Antimicrobial susceptibility testing was performed using disk diffusion method on Mueller-Hinton agar plates. The antibacterial agents tested were gentamicin (10 µg), amikacin (30 µg), netilmicin (30 µg), tobramycin (10 µg), kanamycin (30 µg), neomycin (30 µg) and Oxacillin (1µg). All Antibiotic discs included were supplied from Oxoid Basingstoke, Hampshire, England. Susceptibility to aminoglycosides was interpreted according to document M2-A8 of the Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards, NCCLS) [14].

DNA Extraction from Cultured Samples: DNA was extracted using ready PCR DNA column kit (5prime, Hamburg, Germany). DNA was purified from staphylococcal isolates, a single colony of the isolates was inoculated into brain heart infusion broth (Oxoid Unipath Basingstoke, Hampshire, England) and was incubated for 24 hr at 37°C. The bacterial pellet was then obtained by centrifugation at 5000 rpm for 10min, resuspended in 200 µL of lysis buffer (containing 200 µg/ml lysostaphin) and incubated at 37°C for 1 hour to digest the cell wall. Turn on dry block heater to pre heat to 99°C for each sample to be processed assemble 1 ready PCR DNA capture column contained in a blue waste collection tube and 1 clear DNA collection tube and the tubes were labeled. Add 200 µL well mixed sample to ready PCR DNA column kit by gently touching center of matrix with the pipette. Let sample absorb at room temperature for at least 1 min. Add 400 µL ready PCR DNA purify solution 1 and incubate for 1 minute at room temperature. Solution begins to drain into the blue waste collection tube during incubation. Centrifuge 10 seconds at 8,000 rpm. A waste volume of 600 µL will be collected in the blue waste collection tube. Add another 400 µL ready PCR DNA purify solution 1 and incubate for one minute at room temperature then centrifuge at 8,000 rpm for 10 seconds a waste volume of 400 µL is collected. The matrix is white or nearly white then adds 200 µL of the ready PCR DNA Elution solution 2. Centrifuge for 10 seconds at 8000 rpm to collect an additional 200 µL waste. The matrix containing the purified DNA. Transfer ready PCR DNA column kit clear DNA collection tube and discard second blue waste collection tube containing 600 µL waste solutions. Add 200 µL ready PCR DNA elution solution 2 and incubate for 10 minutes in dry block heater preheated to 99°C. Centrifuge for 20 seconds at 8000 rpm immediately following heating step to release purified DNA from PCR DNA capture column 200 µL will appear clear. Discard ready PCR DNA column kit and purified DNA is ready for analysis.

Primers for Multiplex PCR: DNA solution (2 µL) was processed as above and added to a PCR mixture containing 0.2µM of the respective primers, 10µ L of a 10-fold concentrate of PCR buffer, 200 µM of deoxynucleoside triphosphates and 0.5 U of *Taq* polymerase (Roche Diagnostics, Mannheim, Germany). A Genecycler (Bio-Rad Laboratories, Hercules, CA, U.S.A.) was used for DNA amplification. After an initial denaturation (for 5 min at 95°C), 30 cycles of amplification were performed as follows: denaturation at 95°C for 2 min, annealing at 58°C for 30 sec and DNA extension at 72°C for 30 sec. The reaction was achieved with a final extension at 72°C for 7 min. 10 µL of the PCR products were then loaded onto a 2.5% agarose gel and electrophoresis was performed in Tris-borate-EDTA buffer containing 0.5 µg of ethidium bromide per mL. Amplified ethidium bromide-stained DNA fragments were then visualized on UV transilluminator at 300 nm [7].

RESULTS

Of total 66 *S. aureus* isolates included in this study, 47 (71.2%) were methicillin resistant (MRSA), 5 (7.6%) intermediately resistant and 14 (21.2%) were methicillin sensitive. Thirty two isolates (48.5%) were resistant to gentamicin, 12 (18.2%) of the isolates were resistant to netilmicin, 24 isolates (36.4%) were resistant to neomycin, 20 isolates (30.3%) were resistant to amikacin, 30 (45.5%) were resistant to tobramycin and 35 (53.3%) isolates were resistant to kanamycin. With regard to resistance phenotypes 6 (12.7%) isolates showed resistance to all aminoglycosides tested. 6 (12.7%) isolates showed resistance to all antibiotics tested except netilmicin. Another six (12.7%) showed only resistance to gentamicin, tobramycin, kanamycin, neomycin and 5 (10.6%) isolates showed resistance to gentamicin, tobramycin and kanamycin only. One isolate showed resistance to gentamicin, netilmicin, amikacin and kanamycin.

Distribution of Resistance Gene as Detected by Multiplex PCR: Amplified DNA fragments of four different sizes (135, 242, 314 and 491 bp) were detected on agarose gel electrophoresis (Fig. 1). The most prevalent resistance gene was *aac(6')/aph(2") was* found in 36 (76.5%) of the strains. Eighteen (38.2%) of the isolates carried *ant (4')-Ia* gene, whilst *aph (3')-IIIa* gene was detected in only 6 (12.7%) of the isolates. All strains harbored the *mecA gene* and 19 (40.4%) of the strains carried only the *mecA*.

DISCUSSION

Beginning in the late 1970s and continuing for the last 20 years, MRSA have been isolated in connection with outbreaks of nosocomial infections in many countries around the world [15, 16]. MRSA typically are resistant to various antimicrobial agents such as penicillins, cephalosporins, macrolides, aminoglycosides, tetracyclines and fluoroquinolones [17]. Because of this multidrug resistance and tendency to spread in hospital populations, MRSA have a special clinical significance, requiring epidemiologic monitoring as a measure for control of nosocomial infection. Aminoglycoside resistance is common in *S. aureus* isolated from different hospitals and especially gentamicin resistance, is of clinical importance because it can compromise the therapeutic effectiveness of these antibacterial agents [10]. Although culture-based methods are generally reliable for detecting methicillin-resistant staphylococci, the detection of *mecA* gene by PCR assay is now considered as the gold standard. In particular, multiplex PCR assay that simultaneously detects several genes in a single reaction has the advantage of identifying genotypic resistance for several antibiotics more rapidly and reliably. Several studies have used multiplex PCR for the detection of genes encoding methicillin-resistance and/ or genes for species identification in cases of staphylococcal infection [8, 11, 18]. We designed 4 sets of primers, which were specific for *aac (6')/aph(2"), aph(3')-IIIa* and *ant(4')-Ia.* The genes encoding the most clinically relevant AME and *mecA* gene. A rapid multiplex PCR method was performed in less than 6 hour. The accuracy of this method was reliable, compared with conventional testing. Aminoglycosides play an important role in serious staphylococcal infections despite reports of increased resistance to these drugs. Gentamicin and tobramycin are the most active against staphylococci and are often used in combination with either a ß-lactam or a glycopeptides. Drug inactivation by AME is the main mechanism of aminoglycoside resistance. The bifunctional enzyme, AAC (6')/APH (2"), encoded by the *aac(6')/ aph(2")* gene, is the most frequently encountered AME in staphylococcal isolates and mediates resistance to gentamicin, tobramycin, netilmicin, amikacin [8]. In the present study, the concordances between gentamicin, kanamycin, tobramycin and amikacin resistance and the presence of *aac(6')/aph(2")* gene were 100%, 100%, 85% and 42%, respectively. An ANT(4")-I enzyme encoded by *ant(4')-Ia* is known to mediate resistance to neomycin, kanamycin, tobramycin and amikacin in staphylococci and resistance to neomycin and kanamycin is conferred by an APH(3')-III enzyme encoded by *aph(3')-IIIa* [7,8]. However, the concordance between kanamycin resistance and the presence of *aph(3')-IIIa was 100%,* tobramycin resistance and the presence of *ant(4')-Ia 80%* and amikacin resistance and the presence of *ant(4')-Ia,* was 60%. Several reports have stated that aminoglycoside resistance is closely related to methicillin-resistance [7, 8, 19, 20]. This study also shows a significant correlation between aminoglycoside and methicillin-resistance. This is presumed to be due to the adjacent locations of *mecA* gene and of the AME encoding gene [21]. The mechanisms of aminoglycoside resistance have become more complex with the increased usage of aminoglycosides over time. Moreover, there is no rapid and reliable method for detecting aminoglycoside resistance. PCR assays allow faster establishment of

Table 1: Oligonucleotides used in this study

Primers	Sequence (5'-3')	Size
Aph(3')-IIIa	F AAATACCGCTGCGTA	242 bp
	R CATACTCTTCCGAGCAA	
Ant(4')-Ia	F AATCGGTAGAAGCCCAA	135 bp
	R GCACCTGCCATTGCTA	
Aac(6') /aph(2")	F GAAGTACGCAGAAGAGA	491 bp
	R ACATGGCAAGCTCTAGGA	
Mec A	F CCTAGTAAAGCTCCGGAA	314 bp
	R CTAGTCCATTCGGTCCA	

Table 2: Aminoglycoside resistance genes in Methicillin resistant *S. aureus with* different aminoglycoside resistance phenotypes

Resistance phenotypes	AMEs	No of isolates
CN,NET,N,AK,TOB, K	*aac(6')/aph(2")+ ant (4')-Ia + aph(3')-IIIa*	6
CN,N,AK,TOB,K	*aac(6')/aph(2") + ant (4')-Ia*	6
CN,N,TOB,K	*aac(6')/aph(2") + ant (4')-Ia*	1
CN,N,TOB,K	*aac(6')/aph(2")*	5
CN,TOB, K	*aac(6')/aph(2")*	1
CN,TOB, K	*aac(6')/aph(2") + ant (4')-Ia*	4
CN, NET,AK,K	*aac(6')/aph(2") + ant (4')-Ia*	1
CN,AK,TOB,K	-	2
CN,N,K	-	1
CN	-	1

Abbreviations: Gn, gentamicin; Ak, amikacin; Tob, tobramycin; K, kanamycin; N, neomycin; Net, netilmicin

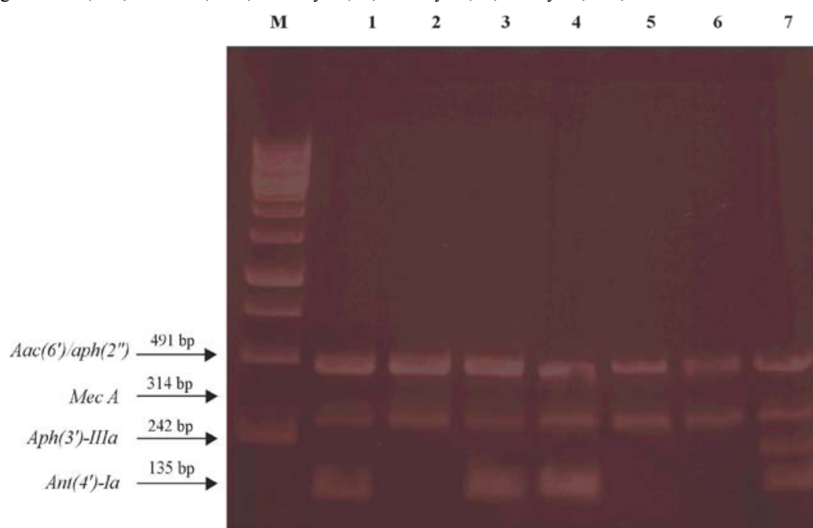

Fig. 1: Gel electrophoresis of DNA fragments amplified by multiplex PCR Lane M 10kb DNA Ladder. Lane 1, 3 and 4 are clinical MRSA isolates possessing the (*aac(6')/aph(2"), ant (4')-Ia and the mec A) genes. Lane 2, 5 and 6 are isolates* possessing only the (*aac(6')/aph(2") and the mec A) genes and lane 7 was isolate possessing the (aac(6')/aph(2"), ant (4')-Ia, aph(3')-IIIa and the mec A) gene.*

effective antibiotic therapies and leads to improved therapeutic success and reduced empirical treatments with broad-spectrum antibiotics, which are costly and have high toxicities and eventually slow potential development of antibiotic resistant organisms. In terms of infection control programs, such rapid detection of resistance could be used to prevent nosocomial spread of MRSA in advance.

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