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Detection of Methicillin/Oxacillin Resistant *Staphylococcus aureus* Isolated from Some Clinical Hospitals in Cairo Using *Meca/Nuc* Genes and Antibiotic Susceptibility Profile

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ABSTRACT

This study reports the detection of methicillin/oxacillin-resistant *Staphylococcus aureus* (MRSA/ORSA) strains and generation of antibiogram profile of *S. aureus* clinical isolates from three Egyptian hospitals. PCR amplification, spot inoculation and oxacillin disc diffusion tests were applied to compare their MRSA/ORSA detection abilities. From 200 bacterial isolates tested, 83 (41.5%) were confirmed as *S. aureus* of which 51 (61.45%) were oxacillin resistant (ORSA). Out of 51 isolates 26 had single resistance (oxacillin resistance), while 25 had double resistance (oxacillin & methicillin) resistance (MRSA/ORSA). Antibiotic susceptibility, of all isolates, to seven different antibiotic groups was tested. Quinolones and aminoglycosides were the most effective groups, 45.1% of the isolates were susceptible to vancomycin. However, 27.5% of the isolates were multidrug resistant, against many of the available antibiotics and thus there is an urgent need for alternative antibiotics. Using the PCR assay, 26 ORSA isolates were found to have *nuc* gene and 25 MRSA/ORSA had *mec* A and *nuc* genes. Their amplification on agarose gel revealed the presence of *nuc* gene at 276 bp and for *mec* A gene at 533 bp. The sequencing of both genes was done and sequence allignment of both genes showed 99% and 97% homology between *mec* A and *nuc* genes, respectively.

Key Words: *S. aureus*; MRSA/ORSA; Methicillin resistant; *mec* A/*nuc* genes; Antibiotic-susceptibility

INTRODUCTION

Staphylococcus aureus is the causal agent of most of the staphylococcal diseases and is currently a versatile microbial pathogen that has evolved resistance to all antibiotic classes (Jomaa *et al.,* 2006; Cockfield, 2007). It is associated with serious community-acquired and nosocomial infections (Day *et al.,* 2001; Berger-Bachi, 2002). Its high level of adaptation to hospital environments has been deeply facilitated by the acquisition of methicillin resistance, an evolutionary step that converted *S. aureus* to one of the most common nosocomial pathogen nowadays (Oluwatuyi *et al*., 2004; Saxena & Panhotara, 2005).

The global spread of methicillin-resistant *S. aureus* (MRSA) constitutes one of the most serious contemporary challenges to the treatment of hospital-acquired infections (Szczepanik *et al.,* 2007). MRSA carries a uniquely effective antibiotic resistance mechanism that can protect the microorganisms against all members of ß-lactam antibiotics. Compounding the problem is the striking ability of MRSA to acquire resistance to other groups of antimicrobial agents, which makes infections caused by these pathogens very difficult to manage and costly to treat (Crisóstomo *et al.,* 2001; Hiramatsu *et al.,* 2002; Aires De Sousa & De Lencastre, 2004).

Currently, vancomycin is accepted worldwide as the last armament against MRSA infections (Hiramatsu 2001; Robinson & Enright, 2004). Un-fortunately, clinical isolates of vancomycin-resistant *S. aureus* have been reported recently (Centers for Disease Control & Prevention, 2004). The emergence of *S. aureus* isolates resistant to vancomycin and other wide range of structurally un-related antibiotics have elevated MRSA into a multidrug-resistant 'Superbug", making it more dangerous than ever in a hospital environment and also recently, in the healthy community (Norazah *et al.,* 2003; Lu *et al.,* 2005).

The objective of the present study was to detect and identify the multidrug and non-multidrug methicillin/ oxacillin resistant *S. aureus* (MRSA & ORSA) from three hospitals in Egypt using antibiotic disc diffusion method. The study also aimed at the identification of specific genes responsible for resistance to oxacillin and to methicillin using the PCR technique.

MATERIALS AND METHODS

Clinical specimens. Two hundred swab samples were collected in 2003 on mannitol salt broth medium from different hospital departments; 126 swab samples from Intensive Care Units of Neurosurgery, Chest and Cardiac Surgery and Cardiovascular department of Al-Hussein University Hospital, 54 samples from Orthopedic department of Sayed Galall-University Hospital and 20 from the medical laboratory of Dar El-Shefa Hospital. Samples were taken from, patients, nurses and workers and indoor environment of the three hospitals.

Sterile gloves were worn, while collecting samples to prevent skin bacterial contamination. Samples were transported in an ice box (at 4ºC). Microbiological examination was performed within 24 h.

Bacterial identification. The samples collected on mannitol salt broth media were incubated at 37ºC for 24 h and then inoculated onto mannitol salt agar media and incubated at 37ºC for 24 h. The media turn yellow if they were positive for the growth of *S. aureus* as it can grow at high concentration of salt, ferment mannitol and form an acid, which changes the colour of the media to yellow colour, while *S. epidermidis* forms red colour, a distinguishing test between the two strains.

All isolates were identified by conventional methods (gram-positive cocci, catalase positive & mannitol fermenting) (Kohner *et al.,* 1999) and were confirmed as *S. aureus* by their ability to coagulate rabbit plasma (bioMerieux, Marcy I'Etoile, France) and to produce clumping factor (Staphyslide test, bioMerieux). The biotype was determined by API-20 Staph. (bioMerieux, Marcy I'Etoile, France).

Antibiotic susceptibility tests (disc diffusion). The disc diffusion assay was performed according to the Clinical and Laboratory Standards Institute [CLSI] [formerly known as National Committee for Clinical Laboratory Standards (NCCLS)] guidelines. Antimicrobial drugs tested included different groups of antibiotics (Table I).

Antibiotic discs were placed on Muller-Hinton agar plates inoculated with 0.5 mL overnight cultures. Gently each disc was pressed down with sterile forceps to insure complete contact with agar. Within 15 min of applying discs, the inverted plates were aerobically incubated at 37ºC for 24 h. Experiments were done in triplicate and the inhibition zone diameters were measured. The mean of inhibition zone diameters, were then compared to specified criteria (NCCLS, 2005).

MRSA screening. Culture swabs from different hospital departments were processed as described previously (Que *et al.*, 2003). A broth enrichment step (mannitol-salt medium, Oxoid) was used, followed by plating onto oxacillin (6 μ mL^{-1}) blood and mannitol salt agar. Resistance to methicillin was determined by the methicillin disk susceptibility test and confirmed by *mec A* by PCR (Ho, 2003; NCCLS, 2005). Oxacillin resistance was confirmed by the detection of *nuc* gene by PCR.

Preparation of genomic DNA and PCR procedures (genotypic identification). Total bacterial DNA was extracted using the boiling approach. The precipitate of the 5 mL nutrient broth culture was resuspended in 150 μL TE buffer and lysed by 10 μL lysozyme (10 mg mL^{-1}) then incubated at 37°C for 30 min. The suspension was boiled for 10 min., then centrifuged for 5 min and the supernatant was transferred to another clean tube. Five μL of protinase K (20 mg mL^{-1}) was mixed with the supernatant by vortex and then incubated at 37°C for 30 Min. The suspension was boiled for 15 min to stop the enzyme action. Extracted DNA was stored at -20ºC until PCR was performed. The crude cell lysate was used directly for PCR according to Perez-Roth *et al*. (2001) with some modifications. PCR was performed using the following primers that would detect the *nuc* gene, specific in *S. aureus* and *mec A* gene, un-ambiguous to MRSA isolates. The *nuc* primers were 5′-GCG ATT GAT GGT GAT ACG GTT-3′ and 5′-AGC CAA GCC TTG ACG AAC TAA AGC-3′, while the *mecA* primers were 5′-AAA ATC GAT GGT AAA GGT TGG C-3′ and 5′-AGT TCT GCA GTA CCG GAT TTG C-3′. Temperature was decreased 0.5°C in each cycle (touch down PCR).

The PCR products were subsequently loaded onto 1.5% agarose gel and electrophoresis was performed in 1 X TBE buffer at 100 V for about 30 min. The gels were then stained with 1 μL mL^{-1} ethidium bromide (Sigma). DNA bands were visualized using UV transeleminator (UVP-dual-intensity transeleminator, model: TM-20) with wave length 312 Nm and photographed using UVP-gel documentation system.

Sequencing of DNA fragment. Sequencing was done using "3130 Sequence Instrument" with (Sequence Analysis Software V.5.2) in Genetic Engineering and Biotechnology, Research Center of Ain-Shams University. The sequences of the two genes were exposed to the Basic Local Alignment Search Tool (BLAST) homology search and motif analysis using BLAST nucleotide (BLASTN 2.2.13) computer software and compared to all GenBank, European Molecular Biology Laboratory (EMBL), DNA Data Bank of Japan (DDBJ) and Protein Data Bank (PDB) sequence database.

RESULTS

Eighty-three samples out of the 200 isolates (41.5%) collected from three hospitals were Gram-positive, cluster-forming coccus, non-motile, non-spore forming, catalase-positive and coagulase positive. They fermented mannitol and formed β-hemolysis on blood agar. These isolates were identified as *S. aureus* and were screened for their sensitivity to oxacillin (Table II).

From 52 *S. aureus* isolates of Al-Hussein University Hospital 36.5% were single resistant ORSA and 38.5% isolates were double resistant *S. aureus*, while 19 *S. aureus* isolates of Sayed Galall University Hospital had 31.6% ORSA and 21.1% MRSA/ORSA. On the other hand, 12 isolates of Dar El-Shefa Hospital had 8.3% ORSA and 8.3% MRSA/ORSA (Table II). It was found that 51 (61.45%) out of the 83 *S. aureus* isolates were oxacillin resistant. Further investigation of their susceptibility to methicillin showed that 25 out of 51 oxacillin resistant isolates were resistant to both oxacillin and methicillin (double resistance M/ORSA), while remaining 26 were resistant only to oxacillin (single resistant, ORSA).

In Al-Hussein University Hospital, a total of twenty three patient swab samples showed single and double

Table I. Antibiotics used in antibiotic susceptibility test

Penicillins	Cephalosporins	Aminoglycosides	Lincosamides
Penicillin (10 µg)	Cefotaxime (30 µg)	Amikacin (30 µg)	Clindamycin(2 µg)
Oxacillin(1mcg)	Cefadroxil (30 µg)	Gentamycin (10 µg)	
Methicillin(5 mcg)			
Ampicillin(30 µg)	Glycopeptides	Quinolones	Rifampin
Amoxicillin(10 mcg)	Vancomycin(30 µg)	Ciprofloxacin(5 µg)	Rifampicin(30 µg)
Claviolenate (30 µg)			

Table II. *S. aureus* isolates (sensitive & resistant) in the three hospitals

Hospitals	Total number	No. of S.aureus (%)	No. of OSSA (%)	No. of MSSA (%)	No. of ORSA (%)	No. of M/ORSA (%)
Al-Hussein University Hospital	126	52 (41.3)	13 (25)	32 (61.5)	19 (36.5)	20 (38.5)
Sayed Galall University Hospital	54	19 (35.2)	9 (47.4)	15 (78.9)	6 (31.6)	4 (21.1)
Dar El-Shefa Hospital	20	12 (6)	10 (83.3)	11 (91.7)	1 (8.3)	1 (8.3)
Total	200	83 (41.5)	32 (38.6)	58 (69.9)	26 (31.3)	25 (30.1)

MSSA: Methicillin susceptible *S.aureus*; M/ORSA: Methicillin and oxacillin resistant *S. aureus*.
ORSA: Oxacillin resistant *S.aureus*. OSSA: Oxacillin susceptible *S. aureus*

resistant *S. aureus*; 65.2% were ORSA and 34.8% were both MRSA/ORSA. (Table III). Thirteen isolates were from males with age ranging between 6 - 38 years old were infected with *S. aureus*, 69.2% ORSA (single resistance) and 30.8% both ORSA/MRSA (double resistance). Ten females with age ranging between 15 - 65 years were infected with *S. aureus*; 60% of them had ORSA and 40% showed double resistance.

The total number of ORSA and MRSA isolates detected from nurses and workers (personnel) in the hospital departments were 7, the single resistant 28.6%, while 71.4% were double resistant *S. aureus*. Two isolates were collected from males with age 22 and 35 years old; 50% of them were ORSA and the other 50% were both ORSA and MRSA. Five samples were taken from females with age ranging between 18 - 25 years. About 20% ORSA and another 80% showed double resistance. In Sayed Galall University Hospital 66.7% of patient isolates were single resistant *S. aureus* and 33.3% were double resistant *S. aureus* (Table IV). Two isolates were taken from males (aged 33 & 53 years) and one from female aged 22 years. Two males were infected with ORSA and only one female was infected with ORSA and MRSA (double resistant). Seven resistant isolates were detected; 57.1% of these showed single resistance and 42.9% double resistant *S. aureus*.

Samples were taken from medical laboratory of Dar El-Shefa Hospital (Table V). Two samples were collected from a male patient aged 50 years infected with double resistant *S. aureus* and a female aged 42 years was infected with single resistant *S. aureus*. None of the other personnel or environmental samples collected from this hospital was infected with either ORSA or MRSA.

Further antibiotic susceptibility tests were done for both single and double resistant *S. aureus* using fourteen antibiotics of seven different antibiotic groups. The number and percentage of isolates susceptible to the screened antibiotics showed that ciprofloxacin and amikacin were the most powerful antibiotics used (Table VI) About 96% of the 51 tested isolates were susceptible to both and 94% of the isolated ORSA were susceptible to gentamycin and cefadroxil. Testing susceptibility to other antibiotics showed that 86.3% were susceptible to ampicillin sulbactam, 82.4% to rifampicin, 80.4% to clindamycin, 68.6% to cefotaxime, 64.7 % to amoxicillin claviolenate, while only 45.1% were susceptible to vancomycin. PCR analysis showed that 26 isolates that were resistant to oxacillin were positive for the presence of (*nuc*) gene (Fig. 1) and 25 isolates resistant to oxacillin and methicillin were positive for both *mec* A and *nuc* genes (Fig. 2).

The PCR copies of both *mec A* and *nuc* genes were subjected to DNA sequencing to reveal their internal structure. DNA sequences of these genes revealed GC content of 32.2% and 42.4%, respectively. Sequence alignment of *mec A* gene from Egyptian isolate of *S. aureus* with similar sequence stored on four gene banks database on the world wide web revealed a homology of 98 - 99%. In case of *nuc* gene sequence alignment revealed DNA homology of 97%.

DISCUSSION

Recent developments suggest that antimicrobial resistance may be intimately related to the possession of various virulence factors (Martinez & Baquero, 2002; Sakoulas *et al*., 2003; Gill *et al*., 2005) and *S. aureus* provides an example of this phenomenon. MRSA is a major human pathogen responsible for a wide spectrum of diseases (Ito *et al.,* 2001; Cockfield, 2007). In the past, MRSA was confined to nosocomial setting, but recently community associated MRSA has become a significant concern worldwide (Liassine *et al.,* 2004). Our results showed the distribution of resistant bacterial isolates from 200 samples collected from patients, personnel and indoor environment of the three studied hospitals.

Table III. Comparison between single and double resistance in *S. aureus* strains isolated from different sources in relation to age and gender in Al-Hussein University Hospital

Source	Total No. of resistant S.aureus	Gender and Age group								Total No. (%) of single resistance	Total No. (%) of double resistance
		Male				Female					
		No. of isolates	Age	No. (%) of S*	No (%) of D+	No. of isolates	Age	No. (%) of S*	No (%) of D+		
Patient	23	13	6-38	9 (69.2)	4 (30.8)	10	15-65	6 (60)	4 (40)	15 (65.22)	8 (34.8)
Personnel	7	2	22-35	1 (50)	1 (50)	5	18-25	1 (20)	4 (80)	2 (28.6)	5 (71.4)
Environment	9	-								2 (22.2)	7 (77.8)

%: percentage from total. S*: Single resistant *S.aureus* (ORSA). D+: Double resistant *S. aureus* (MRSA & ORSA

Table IV. Comparison between single and double resistance in *S. aureus* strains isolated from different sources in relation to age and gender in Sayed Galall University Hospital

Source	Total No. of resistant S.aureus	Gender and Age group								Total No. (%) of single resistance	Total No. (%) of double resistance
		Male				Female					
		No. of isolates	Age	No. (%) of S*	No (%) of D+	No. of isolates	Age	No. (%) of S*	No (%) of D+		
Patient	3	2	33&53	2 (100)	0 (0.0)	1	22	0 (0.0)	1 (100)	2 (66.7)	1 (33.3)
Personnel	7	3	22-35	2 (66.7)	1 (33.3)	4	20-38	2 (50)	2 (50)	4 (57.1)	3 (42.9)
Environment	0	-								0	0

%: percentage from total. S*: Single resistant *S.aureus* (ORSA). D+: Double resistant *S. aureus* (MRSA & ORSA

Table V. Comparison between single and double resistance in *S. aureus* strains isolated from different sources in relation to age and gender in Dar El-Shefa Hospital

Source	Total No. of resistant S.aureus	Gender and Age group								Total No. (%) of single resistance	Total No. (%) of double resistance
		Male				Female					
		No. of isolates	Age	No. (%) of S*	No (%) of D+	No. of isolates	Age	No. (%) of S*	No (%) of D+		
Patient	2	1	50	0(0.0)	1 (100)	1	42	1 (100)	0	1 (50)	1 (50)
Personnel	0	0	-	-	-	0	-	-	-	0	0
Environment	0	-								0	0

%: percentage from total. S*: Single resistant *S.aureus* (ORSA). D+: Double resistant *S. aureus* (MRSA &ORSA

Table VI. Effect of antibiotics used on both single and double resistant *S. aureus* strains.

Antibiotics susceptibility		Susceptible isolates		Resistant isolates	
		No	%	No	%
	Penicillin	0.0	0	51	100
	Oxacillin	0.0	0	51	100
Penicillin	Methicillin	25	49	25	49
	Unasyn	44	86.3	5	9.8
	Amoxil	10	19.6	41	80.4
	Augmentin	33	64.7	18	35.3
Cephalosporins	Clafuran	35	68.6	14	27.5
	Duricef	48	94	3	5.9
Glycopeptides	Vancocin	23	45.1	21	41.2
Aminoglycosides	Amikin	49	96.1	2	3.9
	Garamycin	48	94	3	5.9
Lincosamides	Dalacin	41	80.4	9	17.6
Qunolones	Ciprocin	49	96.1	0.0	0
Rifampin	Rifadin	42	82.4	8	15.7

N.B: The intermediate susceptible isolates are not included in this table

Several studies have found differences between MRSA and MSSA hospital isolates concerning possible virulence markers (Soriano *et al*., 2000; Cosgrove *et al*., 2003; Kim *et al*., 2003). Most clinical studies have not generally considered the roles of microbiologic factors, such as virulence genes, in the pathogenicity of antibiotic-resistant *S. aureus*. In the present study MRSA/ORSA were identified by biochemical and antibiotics sensitivity tests. PCR technique used to detect *mec* A and *nuc* genes responsible for resistance in MRSA/ORSA provided rapid, specific and sensitive results. In the current study it was found that the number of single resistant *S. aureus* (ORSA) isolates taken from patients was almost equal to the number of double resistant ones (MRSA/ORSA). On the contrary, MRSA/ORSA isolates outnumbered ORSA in the personnel 71.4% and the environment 77.8% at Al-Hussein University hospital. This indicated that cross infection might have occurred between the environment and the personnel, but to be sure a DNA finger print study must be done.

Comparing antibiotic susceptibilities in patients and

Fig. 1. Agarose gel (1.5%) electrophoresis of amplified 276 bp DNA fragment (*nuc*) gene of *S. aureus*

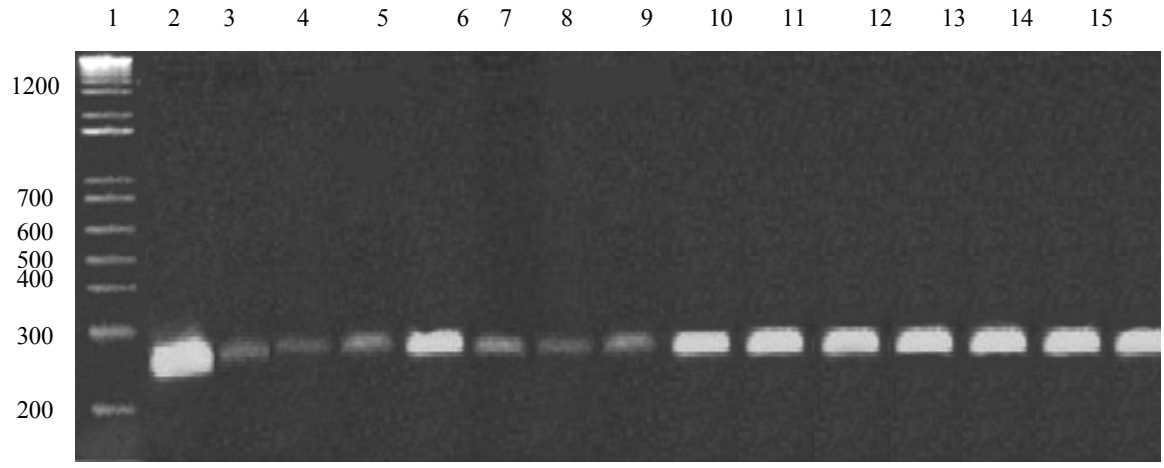
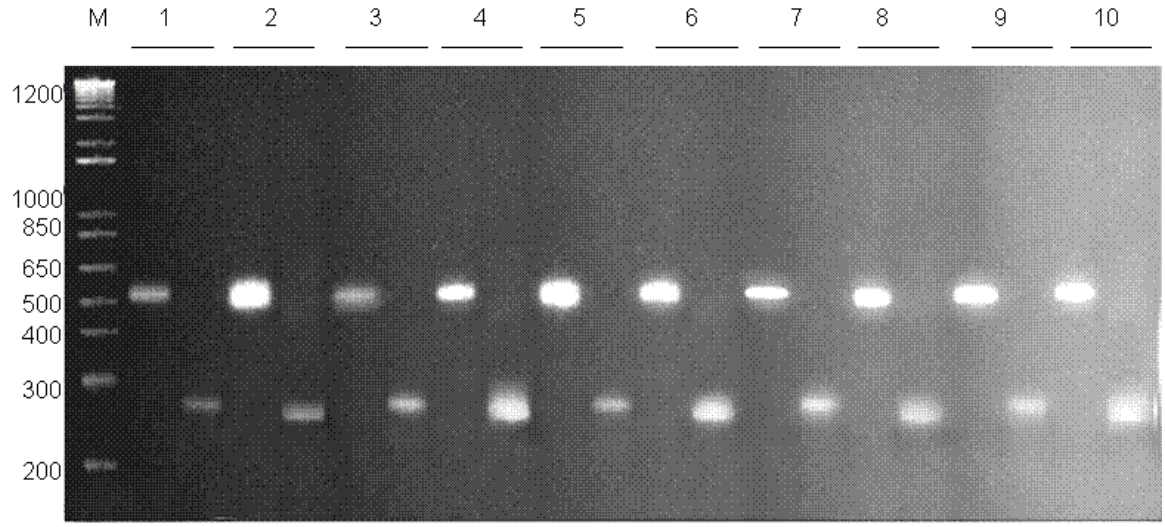


Fig. 2. Samples containing both *mec*A and *nuc* genes (533 & 276 bp, respectively) of *S. aureus*



personnel samples at Al-Hussein and Sayed Galall Hospitals showed that the *S. aureus* strains in both hospitals were resistant to penicillin, oxacillin and amoxicillin and susceptible to the other screened groups except for rifampin in patient samples at Sayed Galall Hospital. Similarities in multidrug resistance observed in both patients and personnel may support the possibility of cross contamination. In accordance with these data Nur *et al.* (1996) found no differences in antibiotic susceptibilities when isolates from staff personnel were compared with those of patients.

The current data showed that 94% of MRSA/ORSA isolates were susceptible to gentamycin (one of the aminoglycosides group). Grohs *et al*. (2003) stated that aminoglycosides are bactericidal agents possessing rapid lethal activity on susceptible MRSA strains both *in vitro* and *in vivo*. Moreover, Jacqueline *et al*. (2004) indicated that despite aminoglycosides resistance among clinical MRSA isolates being widespread, gentamycin remains active against most MRSA strains in European countries. The present work also showed that 45.1% of ORSA isolates were susceptible to vancomycin. Moreover, El-Azizi *et al.* (2005) showed that vancomycin and quinupristin/ dalfopristin were capable of killing 99.9% of MRSA. Multidrug-resistant (resist three or more groups of antibiotics) *S. aureus* isolates was identified as a serious cause of nosocomial infections, and more recently, community-acquired MRSA was recognized as an emerging problem in a number of countries (Malik *et al.,* 2006).

In the current study 14 out of 51 isolates (27.5%) of *S. aureus* were multidrug resistant. All were resistant to penicillins; amoxicillin claviolenate and ampicillin/ sulbactam, 64.3% were resistant to vancomycin, 50% resisted each of lincosamides and rifampin, 35.7% resisted cephalosporins and 28.6% resisted amikin and gentamycin. The multi-drug resistance in MRSA/ORSA isolates might be due to the antibiotic's selective exposure. In accordance with the current results, Saderi *et al*. (2005) while studying 87 strains of *S. aureus* isolated from nose of two teaching hospitals personnel against 14 different antibiotics in Tehran, Iran found that a large proportion (90.8%) of the *S.*

aureus isolates was resistant to penicillin. Vancomycin or rifampin resistant strains were not reported. Frequencies of resistance to other antibiotics were: Co-amoxiclav (33.3%), tetracycline (19.8%), erythromycin (8.2%), chloramphenicol (5.9%), clindamycin (2.8), gentamycin (2.3%) and 11% of the isolates were methicillin resistant.

The present investigation provided an evidence for the presence of *mec* A gene at 533 bp in all methicillin resistant isolates. This is supported by the work of Murakami *et al.* (1991), Perez-Roth *et al*. (2001) and Japoni *et al.* (2004) who detected a DNA fragment of 533 bp in all *mec A* gene in positive methicillin resistant *S. aureus,* which was absent in susceptible strains. The current results detected a 276 bp DNA fragment in all *nuc* genes in positive oxacillin resistant *S. aureus*. This is in agreement with Merlino *et al.* (2002), Saiful *et al*. (2006) and Szczepanik *et al.* (2007) who used modified PCR analysis (Multiplex PCR) for detection of *mec A* and *nuc* genes in MDR (multi-drug resistant) and NMDR (nonmulti-drug resistant) resistant MRSA, which gave DNA fragments amplified at 533 and 276 bp, respectively. Nucleotide sequence analyses of several *mec A* genes from different species such as *S. aureus* (DDBJ/EMBL/GenBank) strain N315 (Ito *et al*., 1999) have revealed that the *mec A* gene is more conserved among staphylococcal species of human origin.

CONCLUSION

There was a correlation between the antibiotic sensitivity test and the PCR detection of *mec* A and *nuc* genes resistant to methicillin and oxacillin. Both genes were present in all identified isolates as methicillin and oxacillin resistant. Strict control over the use and distribution of anti-Staphylococcal antibiotics should be immediate policy and search for new antimicrobial agents is a necessity since most of the currently available antibiotics are no longer effective.

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