academicJournals

Vol. 7(16), pp. 1598-1604, 16 April, 2013
DOI: 10.5897/AJMR12.2333
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Detection of high levels of methicillin and multi-drug resistance among clinical isolates of *Staphylococcus aureus*

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Accepted 29 March, 2013

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become a frequent cause of serious infections worldwide. We identified clinical isolates of *S. aureus* using conventional methods based on morphological and biochemical characteristics. Resistance of isolates to oxacillin was tested by growth on Oxacillin Resistance Screening Agar Base (ORSAB) and disc diffusion method. Oxacillin MICs were determined by agar dilution method. Sensitivity of isolates to a range of antibiotics was also tested by disc diffusion method. We further confirmed methicillin resistance using a PCR-based molecular approach. Data revealed that among 120 clinical bacterial samples tested 81 were confirmed as *S. aureus*. Out of these 81 isolates, 72 were MRSA (88.9%). The distribution of resistance among MRSA isolates was alarming. Twenty (20) MRSA isolates (27.8%) showed the highest level of resistance detected in this study with oxacillin MIC >6400 µg/ml. Most isolates were also resistant to multiple antibiotics. PCR results revealed the detection of *mecA* gene responsible for resistance in all tested isolates and therefore confirmed the conventional identification of MRSA isolates. The present study provides additional evidence that the rate of emergence of MRSA is in a continuous increase.

Key words: *Staphylococcus aureus*, MRSA, *mecA* gene, methicillin resistance, multi-drug resistance.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a Gram-positive bacterium that has developed antibiotic resistance to all penicillins and has become a major health problem since it was first identified (Barrett et al., 1968). Emergence of new MRSA strains as public health threat has also recently been reported (Grundmann et al., 2006; Yamamoto et al., 2010). This bacterium can cause nosocomial and community-acquired infections ranging from minor skin infections to serious life-threatening diseases such as bacteraemia and invasive endocarditis (Chambers, 2001; Enright et al., 2002; Wisplinghoff et al., 2004).

Methicillin was the first penicillinase-resistant penicillin to be used in the treatment of *S. aureus* infections. It was recognized as the most reliable agent for routine susceptibility testing and hence resistant strains were termed methicillin-resistant *S. aureus* (MRSA). Methicillin was then replaced by oxacillin and the term oxacillin- resistant *S.*

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aureus (ORSA) was used interchangeably with MRSA. Methicillin resistance is conferred by the *mecA* gene, which encodes a penicillin-binding protein PBP2a or PBP2' with decreased affinity for β-lactam antibiotics (Hartman and Tomasz, 1984; Hiramatsu et al., 2001; Reynolds and Brown, 1985; Wu et al., 1996). The *mecA* gene allows the synthesis of cell wall in MRSA strains regardless of the presence of β-lactam antibiotics. *mecA* is carried on a large mobile genetic element integrated into the chromosome of MRSA isolates. This genetic element is called "staphylococcal cassette chromosome *mec*" (*SCCmec*). Community-acquired MRSA normally contain *SCCmecIV* or *SCCmecV* (Diep and Otto, 2008; Yamamoto et al., 2010; Zetola et al., 2005). *SCCmecIV* likely contributes to the spread in the community because it is smaller in size compared to other *SCCmec* elements (IWG-SCC 2009). It is a characteristic therefore of all MRSA isolates to carry *mecA (*Ito et al., 2001). Other mobile genetic elements may contribute to methicillin and multi-drug resistance in *S. aureus*. A recent study used a comparative genomics approach and identified a novel mobile resistance structure in a community-acquired MRSA strain in Taiwan (Hung et al., 2012). This structure is the enterococcal insertion sequence (IS*1216V*) that originated in enterococci and was rarely found in *S. aureus*.

The aim of the present study was to detect methicillin and multi-drug resistance among *S. aureus* isolates collected from two famous laboratories in Egypt. These laboratories normally have large numbers of clinical samples from different sites of infections that are representative of different locations. We used conventional and molecular methods to identify MRSA isolates among clinical *S. aureus* isolates studied here.

MATERIALS AND METHODS

Bacterial isolates, culture media and antibiotics

A total of 120 clinical isolates preliminary identified as *Staphylococcus sp.* recovered from blood, throat swabs, pus, vaginal smears, semen, and stool were collected from two different laboratories in Cairo, Egypt; the central laboratories of the Ministry of Health and El Mokhtabar (Moe'mena Kamel). Glycerol stocks of all isolates were stored at -70°C prior to identification steps using standard methods. Isolates were manipulated on nutrient agar, Mueller-Hinton agar and mannitol salts agar media (Oxoid, UK). The antibiotic oxacillin was used to identify MRSA isolates in this study. Antibiotics representing different classes were also used to detect multi-drug resistance among clinical isolates of *S. aureus*. Antibiotic discs were obtained from Oxoid, UK and oxacillin powders were purchased from Sigma pharmaceuticals, Egypt.

Identification of *S. aureus* isolates

Identification of isolates was carried out based on morphological and biochemical characteristics of *S. aureus* as follows; Isolates from glycerol stocks were grown on nutrient agar plates and incubited at 37°C for 24 h under aerobic conditions. After incubation, the cultured plates were examined for the appearance, size, colour, and morphology of colonies. Gram stain reaction was performed to test morphological characteristics of isolates. The ability of isolates to ferment mannitol was tested by growing the isolates on mannitol salts agar media. Catalase test was carried out according to a published method (Kloos and Schleifer, 1986). Coagulase-producing isolates were identified by inoculating bacterial colonies onto 0.5 ml of rabbit plasma diluted 1/10 in saline. After incubation at 37°C for 4 h, a positive reaction was denoted by a clot formation (Sperber and Tatini, 1975). Isolates that were Gram-positive cocci, catalase positive and coagulated human plasma were considered *S. aureus* in this study.

Detection of oxacillin and multi-drug resistance among *S. aureus* isolates

All isolates identified as *S. aureus* were tested for oxacillin resistance. Isolates were grown on Oxacillin Resistance Screening Agar Base (ORSAB) which contains polymyxin B (50000 IU/L) and oxacillin (2 mg/L) (Oxoid, UK). This medium inhibits the growth of non-staphylococcal organisms and select for MRSA **(**Becker et al., 2002). Microbial growth and blue coloration of the media are considered a positive result of the test which indicates the presence of MRSA. Oxacillin resistance of isolates was also tested by Kirby-Bauer disc diffusion method. Oxacillin discs (1 µg) were placed on the surface of Muller-Hinton agar media supplemented with 4% NaCl and seeded with the test organism (Churcher, 1968). Susceptibilities of isolates to different antibiotics were also studied by disc diffusion test. 24 h post incubation at 37°C, inhibition zones around antibiotic discs were measured and isolates were classified as susceptible, intermediate-resistant or resistant according to the guidelines of CLSI M07-A8 (2009). A control strain of MRSA (ATCC 12692) was included in this test.

Determination of oxacillin MIC

The minimum inhibitory concentrations (MICs) of oxacillin against identified isolates were determined by agar dilution method according to the guidelines of CLSI M07-A8 (2009). Three identical colonies of the test organisms were selected after overnight incubation on nutrient agar plates and suspended in 0.9% NaCl to a density equivalent to a 0.5 McFarland standard. The suspension was further diluted to obtain a final inoculum of about 10^4 CFU/spot. Inocula were then applied onto Muller-Hinton dilution plates with 2% NaCl previously prepared with a wide range of oxacillin concentrations (3-6400 µg/ml), the antibiotic concentrations used for determining MICs were 3, 6, 12.5, 25, 50, 100, 200, 400, 800, 1600, 3200, 6400 µg/ml. Antibiotic-free plates were used as growth controls. Plates were incubated at 35°C for 18-20 h. After incubation, growth of organisms on the antibiotic-free control plates was ensured first and then the MIC was defined as the lowest concentration of oxacillin at which there was no visible growth of the test organism. Isolates with oxacillin MIC > 6 µg/ml were considered methicillin resistant in this study (Brown et al., 2005).

DNA manipulation

Chromosomal DNAs were prepared (Sambrook et al., 1989) from four selected MRSA isolates (numbered 10, 26, 59, and 90 in Table 2, which showed highest oxacillin MICs) for the detection of *mecA* gene by PCR. DNAs from MRSA strain ATCC 12692 and isolate number 68 were also prepared to be used as a positive and a negative control, respectively. Overnight cultures grown in 10 ml Brain Heart Infusion (BHI) were centrifuged at 5,000 ×g at room temperature for 15 min, resuspended in 1-ml of extraction buffer (10 mM Tris pH 8.0, 100 mM EDTA pH 8.0, 20 µg/ml RNAseA, 0.5% SDS w/vol) and incubated for one hour at 37°C. Proteinase K (Sigma-Aldrich, UK) was then added to a final concentration of 100

Table 1. Susceptibility of *S. aureus* isolates to 1-µg oxacillin discs.

	Susceptibility to oxacillin	
	Resistant	**sensitive**
Number of isolates	72	9
Percentage	88.9%	11.1%

µg/ml and the mixture was further incubated for 3 hours at 50°C. Equal volume of phenol/chloroform/isoamylalcohol (25:24:1 vol/vol, BDH and Fisher Scientific UK. Ltd.) was then added and the mixture was gently mixed. Following centrifugation at 13,000 ×g for 3 min (Eppendorf centrifuge 5417C, Germany), the aqueous layer was removed and DNA was precipitated by adding 0.2 volume of 10 M ammonium acetate (Sigma-Aldrich, UK) and 5 volumes of 100% ethanol. The mixture was then centrifuged at 13,000 ×g for 30 min and DNA pellet was left to air dry for 30 - 60 min. DNA was resuspended in TE buffer (Qiagen) pH 7.4 and stored at – 20°C.

PCR amplification of *mecA* gene

Genomic DNA from selected MRSA isolates was prepared as described above. Specific primers; primer *mecA* forward (5'-AAAATCGATGGTAAAGGTTGGC-3') and *mecA* reverse (5'-AGTTCTGCAGTACCGGATTTGC-3') were used to amplify a 533-bp DNA fragment of *mecA* gene (Murakami et al., 1991). PCR reactions (100 µl) were set up as follows; 5 µl (~500 ng) genomic DNA, 1 µM of each primer (Clinilab, Egypt), 10 µl of thermopol buffer, 2 µl of $MgSO_4$, 2 units of Vent® polymerase (all purchased from New England Biolabs, UK) and 50 µM of dNTPs (Promega). The reaction volume was made up to 100 µl using dH_2O. The PCR reactions were heated to 95°C for 5 min and run through 35 cycles of (95°C for 30 s, 55°C for 30s, 72°C for 1 min) and then heated to 72°C for 5 min. Annealing and extension temperatures were variable and were optimized using a gradient program (Hybaid Omnigene PCR machine). PCR products were visualized by running on 1% agarose gel containing 0.5 µg/ml ethidium bromide (Sigma-Aldrich, UK) in TAE buffer. DNA was visualized using an UV transilluminator and images were taken using a UVP GelDoc system (UVP Laboratories).

RESULTS

Phenotypic and biochemical characteristics of isolates

Clinical isolates in this study were identified based on their morphological and biochemical characteristics. Examination of isolates grown on nutrient agar plates revealed the characteristic appearance of staphylococcal colonies. Bacterial colonies were golden yellow, circular, smooth and glistening. Analysis of Gram-stained cultures of all 120 isolates showed that they were Gram-positive cocci arranged in bunches, a characteristic morphology of staphylococci. Eighty-one isolates were able to ferment mannitol when grown on mannitol salts agar (MSA) medium. The other 39 isolates were non-mannitol fer-menters and therefore, they were excluded from further identification steps.

As *S. aureus* is presumed to produce catalase and coagulase enzymes, we tested the ability of isolates which have grown on MSA to produce both enzymes. Results show that the 81 isolates were catalase and coagulase positive (data not shown).

Methicillin resistance among isolates

To identify methicillin-resistant *S. aureus* isolates, we tested the susceptibility of the 81 isolates proved to be *S. aureus* to the antibiotic oxacillin by growing isolates on ORSAB medium and also by disc diffusion method. Growth on ORSAB is a confirmation test for the identification of MRSA. Seventy-two isolates were able to grow on ORSAB giving the characteristic blue colonies and causing the blue coloration of the medium. Only 9 isolates were unable to grow on ORSAB suggesting that they were sensitive to oxacillin and therefore presumed as methicillin-sensitive *S. aureus* (MSSA). Results of the disc diffusion assay were consistent with that obtained from ORSAB plates and are shown in Table 1.

MIC of oxacillin against *S. aureus* isolates

The MIC of oxacillin was determined for the 81 clinical isolates which have shown the characteristics of *S. aureus*. 72 *S. aureus* isolates were oxacillin resistant that is MIC > 6 µg/ml. Out of these 72 isolates, 20 (27.8%) showed the highest level of resistance (>6400 µg/ml) whereas, only 3 isolates (4.2%) showed the least resistance (12.5 µg/ml). Nine (9) out of 81 studied isolates showed oxacillin MIC ≤ 6 µg/ml and therefore considered MSSA isolates. The MICs of oxacillin for all tested isolates are summarized in Table 2. The 9 MSSA isolates are numbered (50, 53, 54, 56, 68, 74, 75, 76 and 82) and their MIC results are shown in Table 2. The distribu-tion of oxacillin resistance among all *S. aureus* isolates is shown in Figure 1. Results reveal that with MIC (>6400 µg/ml, large number of isolates showed the highest degree of resistance to oxacillin (20 isolates), 5 isolates were highly resistant (MIC 3200 µg/ml), 11 isolates showed MIC of 1600 µg/ml, and the rest of isolates were less resistant (Figure 1).

Multi-drug resistance among *S. aureus* isolates

The susceptibilities of 81 *S. aureus* isolates to different antibiotics were studied by disc diffusion test. The

Table 2. Oxacillin MICs against 81 *S. aureus* isolates.

Isolate number	MIC in µg/ml	Isolate number	MIC in µg/ml	Isolate number	MIC in µg/ml
2	3200	**45**	**> 6400**	79	800
3	100	46	200	82	< 3
4	100	47	1600	84	1600
5	200	48	400	**85**	**> 6400**
7	3200	50	6	86	12.5
10	**> 6400**	51	200	**87**	**> 6400**
11	**> 6400**	53	6	88	1600
14	25	54	6	**89**	**> 6400**
15	50	56	< 3	**90**	**> 6400**
16	50	58	50	**91**	**> 6400**
17	**> 6400**	**59**	**> 6400**	**92**	**> 6400**
21	400	62	200	93	100
22	**> 6400**	64	400	94	100
25	3200	65	25	**96**	**> 6400**
26	**> 6400**	66	50	97	1600
27	1600	67	800	98	800
28	3200	68	< 3	102	800
29	25	69	25	103	100
30	1600	**70**	**> 6400**	104	1600
31	12.5	71	400	105	1600
31'	1600	72	400	106	1600
32	12.5	**73**	**> 6400**	**109**	**> 6400**
40	**> 6400**	74	< 3	110	50
41	50	75	< 3	112	1600
42	**> 6400**	76	6	**114**	**> 6400**
43	3200	77	25	115	50
44	100	78	800	118	50

The MIC determination was carried out on Muller-Hinton dilution plates. The 20 highest methicillin-resistant *S. aureus* (MRSA) isolates with oxacillin MIC of > 6400 µg/ml are shown in bold.

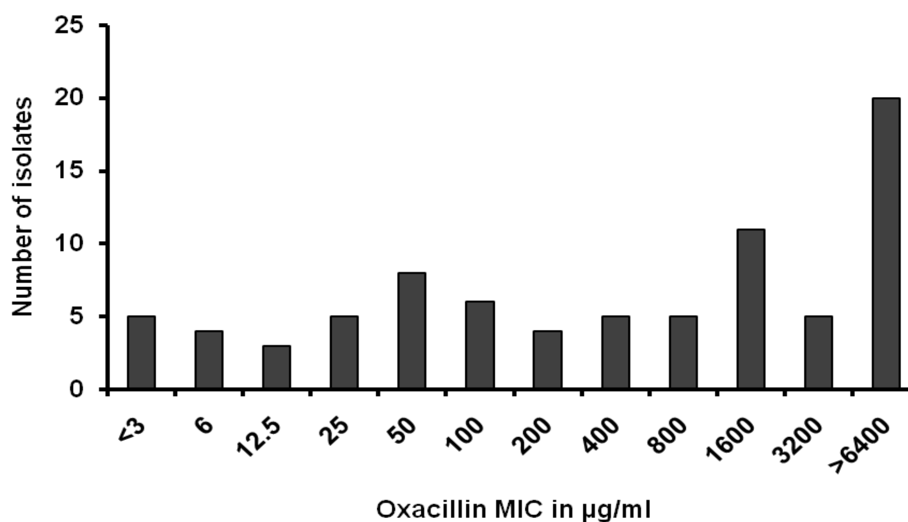

Figure 1. Distribution of oxacillin resistance among *S. aureus* isolates. 20 out of 72 isolates showed the highest resistance pattern (>6400 µg/ml). 5 isolates were highly resistant (MIC 3200 µg/ml), 11 isolates showed MIC of 1600 µg/ml, and the rest of isolates were less resistant or sensitive to oxacillin.

Table 3. Antibiogram of *S. aureus* isolates.

Antibiotic	Number of *S. aureus* isolate		
	Resistant	Intermediate	Sensitive
Ciprofloxacin	34	25	22
Gentamycin	36	7	38
Rifampicin	51	4	26
Chloramphenicol	31	21	29
Oxacillin	72	0	9
Erythromycin	46	26	9
Ofloxacin	39	8	34
Amoxycillin/Clavulanic acid	62	0	19
Tetracycline	62	11	8
Ampicillin/Sulbactam	46	18	17
Vancomycin	53	0	28*
Imipenem	68	4	9
Piperacillin/Tazobactam	49	0	32
Sulphamethoxazole/Trimethoprim	43	24	14

*Disc diffusion method does not differentiate Vancomycin-sensetive from Vancomycin-intermediate *S. aureus* isolates that is the 28 isolates may be considered either sensitive or intermediate.

antibiogram of all isolates is shown in Table 3. Results reveal that in many cases the resistance to certain antibiotics exceeded 50% of the isolates. Other than Oxacillin, the resistance to Imipenem was the greatest among isolates (84%), although it was only shortly introduced. The percentage of resistant isolates to Erythromycin was 57% which was less than that of Rifampicin (63%), Vancomycin (65%) and Tetracycline (77%).

Four antibiotic combinations were also tested. Resistance of *S. aureus* isolates to all four combinations was observed. The least resistance to antibiotic combinations tested was observed in case of Sulphamethoxazole / Trimethoprim (53%). 57% of the *S. aureus* isolates were resistant to Ampicillin/Sulbactam, 60% were resistant to Piperacillin/Tazobactam and 77% to Amoxicillin/Clavulonic acid (Table 3).

Molecular confirmation of MRSA isolates

To confirm methicillin resistance in MRSA isolates on the molecular level, we tested the presence of *mecA* gene in isolates which gave the highest level of oxacillin resistance. Results show that a 533-bp fragment of *mecA* was amplified from the DNA of all MRSA isolates (the PCR results for only 4 isolates are represented in Figure 2). A similar DNA band was also amplified from the positive control MRSA strain (ATCC 12692) while *mecA* was never detected in case of the negative control MSSA isolate 68 (Figure 2). PCR results are therefore consis-tent with those obtained from disc diffusion assay and MICs determinations at least for the tested isolates.

DISCUSSION

The aim of the present study was to investigate the proportions of methicillin and multidrug resistance among clinical *Staphylococcus aureus* isolates. *S. aureus* are Gram-positive bacteria whose spherical cells arranged in clusters or grape like form. They are catalase and coagulase producers and can ferment mannitol (Bannerman, 2003). In this study, we first identified clinical *S. aureus* isolates based on their morphological and biochemical characteristics. Then we detected MRSA isolates by the ability to grow on ORSAB medium and by disc diffusion assay. Oxacillin was the official antibiotic used for the identification of MRSA, since Methicillin is no more clinically used. ORSAB medium is a commercially available selective medium that is used for the detection of methicillin resistance in *S. aureus* (Cesur et al., 2010) and other streptococci (Horstmann et al., 2012). Minimum inhibitory concentrations (MICs) are considered the ‘gold standard’ for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing. We combined a number of conventional identification methods in the present study to overcome the sensitivity problems with individual methods.

Most molecular methods for identification of *S. aureus* have been PCR based. PCR methods are now used extensively to confirm the presence of the genetic determinants of methicillin resistance (*mecA*) in *S. aureus* (Bignardi et al., 1996; Oliveira and de Lencastre, 2002; Vannuffel et al., 1995). PCR methods were found 94% sensitive and 100% specific in detecting MRSA isolates in a

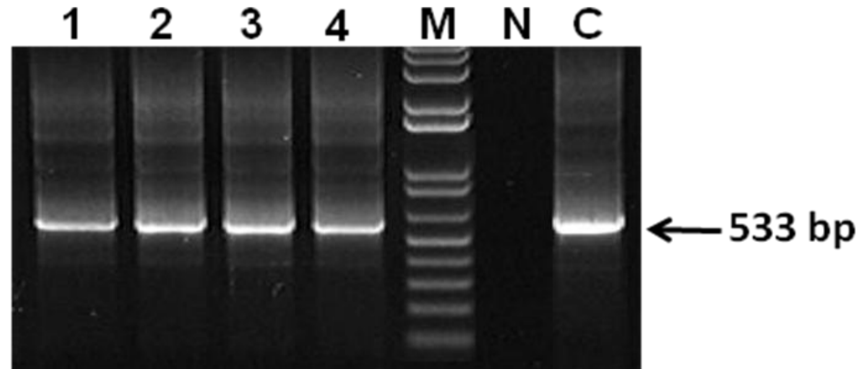

Figure 2. Agarose gel electrophoresis of PCR products amplified by *mecA* forward and *mecA* reverse primers. The picture shows 1 Kb DNA ladder in lane M (the sizes of bands are 100, 200, 300 bp, …etc), a negative control MSSA isolate 68 (lane N), a positive control MRSA strain ATCC 12692 (lane C), and four selected MRSA isolates numbered 10, 26, 59, and 90 in Table 2, which showed highest oxacillin MICs (lanes 1-4). The arrow points at the 533-bp fragment of *mecA* gene.

recent study (El-Sharif and Ashour, 2008). We further confirmed methicillin resistance in MRSA isolates by PCR amplification of a 533-bp fragment of *mecA* gene in four PCR is a rapid and reliable method for identifying MRSA isolates.

In recent years, MRSA has become widespread worldwide. In the United States, the proportion of MRSA isolates increased from 2.4% in 1970s to 55% in 1990s (Diekema et al., 2001; Panlilio et al., 1992). Similar increase in the resistance rate and detection of new MRSA strains were also reported in Europe (Durand et al., 2006; Voss et al., 1994) and in other parts of the world (Diekema et al., 2001). Other studies conducted in Turkey reported higher proportions of MRSA in nosocomial *S. aureus* strains isolated from bloodstream infections (Aygen et al., 2004; Esel et al., 2003). Data in the present study was alarming and revealed even higher proportions of MRSA isolates among identified *S. aureus* isolates. Identification tests revealed that 81 clinical isolates (67.5% of the total number of isolates) showed the known characteristics of *S. aureus* out of which 72 isolates were confirmed to be MRSA (88.9%). This is a surprisingly high percentage knowing that most of these isolates were taken from patients not hospitalized at the time of sampling. The prevalence of MRSA in Egypt in the years 2003, 2004, and 2005 was studied. Percentages of MRSA isolates were 33%, 50%, and 63% of the studied invasive *S. aureus* isolates respectively (Borg et al., 2007). In a recent study, Bassyouni and co-workers reported the detection of 53% of MRSA among the studied *S. aureus* clinical isolates (Bassyouni et al., 2012). The percentage of MRSA isolates in our study is much higher and indicates the rapid increase in the prevalence of MRSA over years. These results are also in agreement with the increased rate of drug resistance reported all over the world (Aygen et al., 2004; Diekema et al., 2001; Esel et al., 2003; Panlilio et al., 1992; Schito,

selected isolates. Results from PCR in the present study were totally consistent with those obtained from conventional methods of identification suggesting that 2006; Voss et al., 1994). Data in the present study also revealed that most MRSA isolates were resistant to multiple antibiotics. These high proportions of multi-drug resistant isolates could be attributed in part to the antibiotic misuse as exposure to antimicrobial agents is considered a risk factor for the acquisition and transmission of MRSA (Muller et al., 2003). Other factors may also contribute to the increasing antibiotic resistance such as age and low immunity (Cookson, 2000).

In conclusion, the present study reports the detection of high prevalence of methicillin and multi-drug resistance among clinical *S. aureus* isolates. Percentage of resistant isolates was remarkably higher than that reported in previous studies. This study therefore provides additional evidence that the rate of emergence of MRSA is in a continuous increase over years. Unfortunately, one can speculate that in the near future all *S. aureus* isolates would become drug resistant. This necessitates the need for combined international efforts to control the spread of antibiotic resistance among bacterial pathogens.

ACKNOWLEDGEMENTS

This work is a part of M.Sc. thesis of the first author and was supported by the National Organization for Drug Control and Research (NODCAR), Egypt.

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