

Original Research Article

Phenotypic and Genotypic Characterization of Nosocomial Isolates of *Staphylococcus aureus* with Reference to Methicillin Resistance

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Received: 13 September 2013

Revised accepted: 22 June 2014

Abstract

Purpose: To investigate the occurrence, species prevalence, antibacterial resistance, and molecular characteristics of *S. aureus* isolates from different wards located in a university hospital.

Methods: A total of one hundred and fifty *S. aureus* isolates were recovered from various clinical specimens. The isolates were tested phenotypically by conventional methods and genotypically by polymerase chain reaction (PCR) for direct detection of *femB* and *mecA* genes.

Results: Thirty one isolates (20.7 %) of these were identified as methicillin-resistant *Staphylococcus aureus* (MRSA) by oxacillin agar screen test and 124 (82.7 %) of the isolates were β -lactamase producers. The prevalence of MRSA strains among *S. aureus* isolates was 20.7 %. The overall resistance of MRSA to a variety of antibiotics tested was linezolid, 48.7 %; ciprofloxacin, 15.3 %; sulfamethoxazole/trimethoprim (TMS), 14.0 %; gentamicin, 12.7 %; and rifampicin, 6.7 %. All MRSA isolates were positive for *femB* and *mecA* genes; one MSSA carried *mecA* gene.

Conclusion: Since *S. aureus* isolates are commonly associated with wound infections, skin and soft tissue infections and blood stream infections, glycopeptides, mupirocin, and quinupristin/dalfopristin (Q/D) would be the most effective antibiotics for the treatment of MRSA infections.

Keywords: *Staphylococcus aureus*, *mecA*, *femB*, Antibiotics, Staphylococcal infections, Methicillin resistance

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

S. aureus is a major cause of serious hospital and community-acquired infections associated with morbidity and mortality rates with rapid development of resistance [1]. MRSA is one of the highest ranking pathogens worldwide and represents a real challenge to the clinical practice with significant public health concern [2]. The global problem of increasing trend in antimicrobial resistance is particularly pressing in the developing countries, where the MRSA is

often the severe casual agent in hospital-acquired infections [3]. The main bacterial species has been associated with a variety of diseases, such as skin and soft tissue infections, endovascular infections, pneumonia, endocarditis and septic shock [4]. These strains show resistance to a wide range of antimicrobials including vancomycin, the drug of last resort for MRSA infections [5].

The introduction of methicillin in 1960s had an important impact on the treatment of infections

caused by penicillinase producing *S. aureus* [6]. Shortly later, MRSA strains with a changed penicillin binding protein (PBP) appeared and spread worldwide [7]. Such resistance mechanism is due to production of a modified PBP2 (PBP2a) with low affinity to β -lactam antibiotics as a result of the acquisition of a *mecA* gene [8]. It can be difficult to detect MRSA because of the heterogeneous nature of methicillin resistance. The *mecA* gene is highly conserved among the Staphylococcal species and consequently, the detection of this gene by the polymerase chain reaction (PCR) is considered as the "gold standard" for the detection of methicillin resistance in Staphylococci [9]. The existence of the *mecA* gene in *S. aureus* characterizes methicillin resistance, whereas the absence of the gene indicates methicillin susceptibility [10]. More over different studies have shown that *fem* (factors essential for methicillin resistance) or the auxiliary genes like *fem A/B/X* in addition to *mecA* are also significant in the expression of methicillin resistance [11].

A significant increase of MRSA in Egyptian hospitals has been reported [12,13,14]. However, regional differences in the epidemiologic factors of MRSA appear to be significant. Varying prevalence rates of MRSA were reported from different areas in Egypt, with figures from more than 16 % [15] to as high as 61.45 % [16].

The treatment of severe infections caused by some epidemic strains of MRSA is much more difficult and expensive than with MSSA because most of MRSA strains are resistant to multiple antibiotics [6]. Accurate and rapid identification of MRSA and their antimicrobial susceptibility profile is essential for the selection of appropriate therapy [17]. Therefore, it is clinically crucial to determine quickly whether *S. aureus* isolates are methicillin resistant or not, as this is very significant for both treatment and requires extensive hygienic precautions to limit the spread of such strains. The present study was carried out to determine the prevalence of *S. aureus* and MRSA in a university hospital and to determine the prevalence of β -lactamase production and susceptibility pattern to various antimicrobial agents used in treatment of MRSA infections, including some new drugs.

EXPERIMENTAL

Clinical specimens

Clinical specimens were collected from 18083 patients admitted in various departments during February to September 2012 at Tanta University

hospital (TUH) (1100-bed tertiary care teaching hospital). The samples represented; wound discharge, blood, body fluids aspirates, urine, faeces, sputum, swabs from nose, throat, ear and genital areas. In some patients, MRSA was isolated from multiple culture sites but a single representative isolate was analyzed. Samples were inserted in Stuart's transport medium (SSI) and delivered to the laboratory within one hour.

Identification of *S. aureus*

The specimen of each patient were cultured into a 10 ml nutrient broth and incubated overnight at 37 °C. Following overnight incubation, 10 μ l of the broth was plated on agar plates containing horse blood 5 % v/v and mannitol salt agar (MSA) media then incubated at 37 °C for 24 h. The agar plates were examined after incubation for 2 days for typical staphylococcal colonies. Identification of the developed colonies was depended on morphology and biochemical reactions according to standard laboratory criteria [18]. Phenotypic characterization to the species level was performed using the API system for the identification of *S. aureus* (Biomerieux, France).

Antibacterial susceptibility studies

The *S. aureus* isolates were subjected to susceptibility testing using disc diffusion technique according to the Clinical and Laboratory Standards Institute (CLSI) [19] guidelines with quality controls. The antimicrobials tested included trimethoprim-sulphamethoxazole TMS (25 μ g); gentamycin (10 μ g); rifampicin (30 μ g); ciprofloxacin (5 μ g); vancomycin (30 μ g); teicoplanin (30 μ g); Q/D (15 μ g); mupirocin (200 μ g) and linezolid (30 μ g).

Detection of MRSA

The MRSA detection was carried out according to CLSI guidelines with Oxacillin agar screen test [19] using Mueller-Hinton agar MHA (Difco Laboratories, Detroit, Mich.) containing 4 % NaCl and 6 μ g/ml oxacillin. Standard inocula were prepared from overnight culture in Mueller Hinton Broth MHB (Difco Laboratories, Detroit, Mich.) after matching the 0.5 McFarland (10^5 CFU/ml) and further dilution so as final inoculum is approximately 10^4 CFU/ml. The oxacillin agar plates were inoculated 10 μ L of the broth. The plates were incubated at 35 °C for 48 h. The MRSA positive and negative standard reference controls were concurrently used. The growth of one or more colonies per spot indicates positive test. Indistinct results were confirmed by the MIC determined with oxacillin by agar dilution method.

All isolates were confirmed by PCR for detection of the *mecA* - *femB* gene.

Microorganisms

The reference strains *S. aureus* ATCC 29213 (methicillin sensitive), *S. aureus* ATCC 23591 (MRSA), *Haemophilus influenzae* R368 (β -lactamase positive), and *H. influenzae* ATCC 49247 (β -lactamase negative) were used in the study. All the test organisms were maintained at -70 °C in nutrient broth medium.

Minimum inhibitory concentration (MIC)

The agar dilution method was used according to the CLSI recommendations [19]. Two-fold serial dilutions of antimicrobial agent were prepared in MHA medium. Standardized suspensions of the test organisms (equivalent to the 0.5 McFarland) were prepared from overnight cultures in MHB. The test organisms were inoculated in approximately 10^4 CFU/ml. The plates were incubated at 37 °C for 24 h. Reference quality control organisms were used. The following antibacterial agents were used with the respective concentration range in μ g/ml: rifampicin (0.00125 - 64), trimethoprim-sulphamethoxazole TMS (0.124 - 512), gentamicin (0.06 - 512), ciprofloxacin (0.124 - 512), vancomycin (0.125 - 64), linezolid (0.25 - 32), Q/D (0.03 - 16), and mupirocin (0.03 - 64).

Beta-lactamase detection

The ability of the isolates to produce β -lactamase was detected with nitrocefin (Oxoid) according to the manufacturer's instructions. The test organisms were first inoculated onto tryptic soy agar containing 0.5 μ g/ml ampicillin as inducer. Nitrocefin solution (5 μ l) was dropped onto a loopful of pure overnight growth placed on a filter membrane. The development of a red colour within 60s indicated a positive result.

Multiplex PCR

DNA extraction: Bacterial DNA was extracted from overnight cultures of *S. aureus* at 37 °C in tryptic soy broth according to (Hexadecyl trimethylammonium bromide- Sodium Chloride) CTAB-NaCl method [20]. The quality of isolated DNA was determined visually by horizontal gel electrophoresis in 1 % (w/v) agarose gel and met the requirements for experimental use.

PCR amplification: According to the basis of the DNA sequences of the *mecA* and *femB* genes, the oligonucleotides in Table 1 were employed for the detection of susceptible genes. The

primers used for amplification of the genes were designed as published by [21].

PCR amplification was carried out on purified genomic DNA extracted from clinical isolates. Each DNA sample (10 μ l) was added to 90 μ l of the PCR mixture consisting of 10x Buffer, 25 mM of MgCl₂, 2.5 U of Taq polymerase, 0.25 mM of dNTP and 0.5 μ M of each primer; *mecA* and *femB*. Initial denaturation was carried out for 3 mins at 92 °C followed by 30 cycles of amplification (denaturation at 92 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min with an increment of 2 secs per cycle). The final extension was performed at 72 °C for 3 min.

Amplification was carried out in a Bio-Rad thermal cycler. After amplification, 17 μ l of PCR sample was loaded on a 1 % (w/v) agarose gel (Bioline, London, UK) containing 0.5 g/ml ethidium bromide and run in a horizontal gel electrophoresis unit (Mini-Sub DNA cell, BioRad). The running buffer was TAE [40 mM Tris, 20 mM acetic acid, 1 mM ethylene diamine tetra-acetic acid (EDTA), pH 8.0]. Electrophoresis was carried out at 100 V for 2 h on an Amersham-Pharmacia Biotech (Uppsala, Sweden) power supplier unit ECPS3000/150. The stained bands were visualized with UV light (309 nm) using a trans-illuminator and gels were recorded as digital TIFF images using a gel documentation system (UVI-Tech).

Statistical analysis

Data from patients infected with MRSA strains were saved in an EXCEL 5.0 program (Microsoft) and analyzed lately with version 6.04 of the Epi Info 6 software. Differences were considered significant at $p < 0.05$.

RESULTS

Of the 18083 clinical specimens included in the present study, only 2764 (15.3 %) were positively cultured and 150 (5.4 %) were identified as *S. aureus*. The number of isolates and their frequency of isolation from each type of specimen are shown in Table 2. The distribution of isolates and MRSA strains according to specimen type and patient site are shown in Table 3. Out of the 150 *S. aureus* isolates, 31 MRSA (20.7 %) were identified by the oxacillin agar screen test.

The majority of isolated MRSA was mainly from in-patients (80.6 %) while the remaining (19.4 %) were from specimens from outpatients. β -

lactamase production was high among the isolates, thus, 124 (82.7 %) out of the 150 *S. aureus* isolates were β -lactamase positive isolates comprised 31 MRSA and 93 MSSA isolates. Thus, the rate of β -lactamase production among MRSA isolates was 98.4 % and among MSSA was 78.6 %.

The susceptibility of the 150 isolates was determined for nine anti-staphylococcal chemotherapeutic agents including TMS, gentamicin, rifampicin, ciprofloxacin, mupirocin, vancomycin, teicoplanin, Q/D, and linezolid. The susceptibility results for individual drugs were interpreted according to CLSI criteria [19]. The breakpoints of susceptibility and resistance and the overall and differential susceptibility profiles of MRSA and MSSA isolates are shown in Table 4. The MIC₅₀ and MIC₉₀ for MRSA and MSSA are shown in Table 5. The data reflect the remarkable differences between the two groups for gentamicin, TMS, and ciprofloxacin. However, rifampicin, mupirocin, vancomycin, and Q/D demonstrated normal and high activity against both groups. The pattern of susceptibility was

almost similar for linezolid, which showed relatively low activity.

The *S. aureus* isolates were tested genotypically using multiplex PCR for direct detection of *femB* and *mecA* genes. Figure 1 shows an agarose gel stained with ethidium bromide to demonstrate the results by the *femB* and *mecA* duplex PCR. It revealed the presence of *femB* and *mecA* in all of the MRSA isolates. However, one isolate of MSSA have shown the presence of *mecA* gene having the potential to become MRSA in prospect. PCR product of *mecA* gene 310 bp for the resistant isolates was applied on lane 3-8 which showed clear bands confirmed that, all the tested isolates were MRSA.

DISCUSSION

The present study was conducted to determine the prevalence of MRSA in clinical specimens recorded in Delta area through the studying of the clinical specimens received in routine bacteriology laboratory of the TUH over a limited

Table 1: Nucleotide sequences of multiplex PCR primers used for identification of MRSA

Name of primer	Sequence 5'-3'	Product size bp	Reference
<i>mecA</i> -F	GTA GAA ATG ACT GAA CGT CCG ATA A	310 bp	[21]
<i>mecA</i> -R	CCA ATT CCA CAT TGT TTC GGT CTA A		
<i>femB</i> -F	TTA CAG AGT TAA CTG TTA CC	651 bp	[21]
<i>femB</i> -R	ATA CAA ATC CAG CAC GCT CT		

Table 2: Specimen source, prevalence of *S. aureus* and distribution of MRSA in clinical specimens

Specimen	No. of specimens	No. of positive culture	<i>S. aureus</i> isolates/per positive specimens, N (%)	MRSA per <i>S. aureus</i> isolates, N (%)
Urine	9611	869	3 (0.3)	1 (33.3)
Wound discharge	3879	941	98 (10.4)	21 (21.4)
Blood	1796	189	11 (5.8)	1 (9.1)
Faeces	1170	47	0 (0)	0 (0)
Sputum	543	153	3 (2.0)	1 (33.3)
Aspirate	309	134	7 (5.2)	1 (14.3)
Body fluid	277	24	2 (9.1)	0 (0)
Genital swab	197	196	0 (0)	0 (0)
Tissue biopsies	143	57	4 (7.0)	2 (50.0)
Nasal swabs	52	52	14 (26.9)	2 (14.3)
Eye swab	41	36	4 (11.1)	1 (25.0)
Throat swab	35	36	3 (8.3)	1 (33.3)
Ear swab	30	30	1 (3.3)	0 (0)
Total	18083	2764 (15.3%)	150 (5.4)	31 (20.7)

Table 3: Distribution of MRSA in different clinical specimens

Specimen	<i>S. aureus</i> isolates (N)	Isolates (N)		MRSA isolates, N (%)	MRSA isolates, N (%)	
		Outpatient	In-patients		Outpatient	In-patients
Wound discharge	98	43	55	21 (21.4)	5 (11.6)	16 (29.1)
Nasal swabs	14	3	11	2 (14.3)	0.0	2 (18.9)
Blood	11	0	11	1 (9.1)	0.0	1 (9.1)
Body aspirate	7	1	6	1 (14.3)	0.0	1 (16.7)
Eye swabs	4	3	1	1 (25.0)	0.0	1 (100)
Tissue biopsies	4	0	4	2 (50.0)	0.0	2 (50)
Throat swabs	3	2	1	1 (33.3)	0.0	1 (100)
Urine	3	2	1	1 (33.3)	0.0	1 (100)
Sputum	3	0	3	1 (33.3)	1 (100)	0.0
Body fluids	2	1	1	0 (0)	0.0	0.0
Ear swabs	1	1	0	0 (0)	0.0	0.0
Total	150	56	94	31 (20.7)	6 (10.7)	25 (26.6)

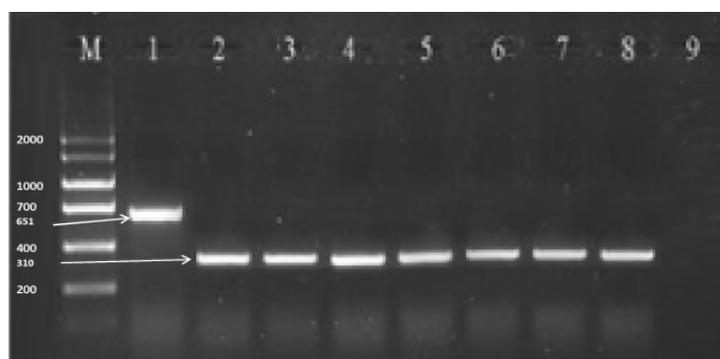
Table 4: Total and differential antibiotic susceptibility pattern of *S. aureus* isolates

Antimicrobial agent	MSSA (n = 119)			MRSA (n = 31)			Total (n=150)		
	S	I	R	S	I	R	S	I	R
Rifampicin	116 (97.5)	0 (0)	3 (2.5)	24 (77.4)	0 (0)	7 (22.6)	140 (93.3)	0 (0)	10 (6.7)
Gentamicin	116 (97.5)	0 (0)	3 (2.5)	15 (48.4)	0 (0)	16 (51.6)	131 (87.3)	0 (0)	19 (12.7)
TMS	115 (96.6)	1 (0.8)	3 (2.5)	12 (38.7)	1 (3.2)	18 (58.1)	127 (84.7)	2 (1.3)	21 (14.0)
Ciprofloxacin	113 (95.0)	3 (2.5)	3 (2.5)	10 (32.3)	2 (6.5)	19 (61.3)	123 (81.3)	5 (3.3)	22 (15.3)
Mupirocin	118 (99.2)	1 (0.8)	0 (0)	30 (96.8)	1 (3.2)	0 (0)	148 (98.7)	2 (1.3)	0 (0)
Linezolid	56 (47.1)	0 (0)	63 (53.0)	21 (67.7)	0 (0)	10 (32.3)	77 (51.3)	0 (0)	73 (48.7)
Vancomycin	119 (100)	0 (0)	0 (0)	31 (100)	0 (0)	0 (0)	150 (100)	0 (0)	0 (0)
Q/D	118 (99.2)	0 (0)	1 (0.8)	31 (100)	0 (0)	0 (0)	149 (99.3)	0 (0)	1 (0.7)
Teicoplanin	119 (100)	0 (0)	0 (0)	31 (100)	0 (0)	0 (0)	150 (100)	0 (0)	0 (0)

S = susceptibility; I = Intermediate; R = resistance

Table 5: Distribution of MIC, MIC₅₀, MIC₉₀ (µg/ml) for *S. aureus* isolates to different antimicrobial agents

Antimicrobial agent	MIC range µg/ml	MSSA (n=119)		MRSA (n=31)	
		MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀
Gentamicin	0.06-512	0.25	1	64	256
Rifampicin	0.00125-64	0.01	0.01	0.01	32
TMS	0.25-512	1	4	128	512
Ciprofloxacin	0.06-64	0.5	1	16	32
Mupirocin	0.03-64	0.25	0.25	0.25	0.25
Linezolid	0.25-32	4	4	2	4
Vancomycin	0.125-64	1	2	1	2
Q/D	0.03-16	0.25	1	0.5	1

**Figure 1:** Agarose gel electrophoresis showing the PCR patterns of 651-bp and 310 bp amplified genes of *femB* and *mecA* respectively for detection of *S. aureus* from clinical isolates. Lane 1: MSSA; lane 2: MRSA; lanes 3–8: MRSA strains; lane 9: blank; lanes M: marker

period of 8 months from February to September. One hundred and fifty *S. aureus* isolates were recovered from 18083 specimens, representing 5.4 % of all bacterial-positive cultures. Sixty five percent of *S. aureus* isolates were recovered from pus or wound discharge with isolation rate of 10.4 % of such specimens. MRSA represented 21.4 % of the *S. aureus* isolates as determined by the oxacillin agar plate screen. Though high, this figure is still lower than those reported in some studies from South Europe, USA and the Far East [6,7,29].

Concerning Egypt, prevalence rates of MRSA among *S. aureus* isolates ranged from less than 17 % (16.3 %) in a University Hospital in Cairo [15], through 26.6 – 28 % in two tertiary care hospitals in Mansoura and Cairo cities [13, 22] to as high as 61 % in three hospitals in Cairo city, two are tertiary care hospitals and one public hospital [16]. Varying prevalence rates of MRSA were obtained from different hospitals in the same country and even different sites in the same hospital [23]. High percentage of *S. aureus* isolates (82.7 %) were β -lactamase producer with higher predominance among MRSA (98.4 %) compared with MSSA (78.6 %). The MRSA prevalence was higher among the inpatients specimens (62.6 %) than those of outpatients (10.7 %). MRSA represented 20.5 % of *S. aureus* pus isolates (195) and again with more predominance among those from inpatients (26.6 %) than from outpatients (12.8 %). This may be attributed to the presence of hospital related strains.

A panel of 9 commonly used anti-staphylococcal agents including two new drugs (Q/D and linezolid) and one used topically for control of nasal MRSA carrier (mupirocin) were tested for their efficacy against the isolates. As the case with β -lactamase production, the antibiotic susceptibility profiles reflected differences between the MRSA and MSSA isolates. While both groups showed high and comparable susceptibility to vancomycin, teicoplanin and mupirocin, MRSA were particularly more resistant to ciprofloxacin, TMS, gentamicin, and rifampicin. MIC₅₀s and MIC₉₀s demonstrated such differences between the MRSA and MSSA isolates for ciprofloxacin, TMS, and gentamicin. Although rifampicin showed similar MIC₅₀s values, different MIC₉₀s values were obtained for both groups with the resistant fractions representing 25 % and only 1.7 % of MRSA and MSSA, respectively. On the contrary, MSSA isolates were more resistant to linezolid that showed an overall modest activity. These

susceptibility differences may reflect the frequent exposure of the MRSA clones resident in hospital environment due to the wide use of these drugs, as these clones are more likely to acquire resistance to many antibiotics [7].

Resistance to ciprofloxacin was reported to show considerable increase among MRSA after being used at large scale. For vancomycin, teicoplanin, Q/D and mupirocin both groups of isolates demonstrated comparable susceptibility patterns. This is expected for the drug Q/D, which is a streptogramin antibiotic combination and for mupirocin which is not used therapeutically and used only for control of nasal carriage in some countries [5] but not in Egypt. The present results are in favor of proposing Q/D as alternative therapy for MRSA infection [24]. The retained susceptibility to vancomycin and teicoplanin correlates with the restricted use of the drug and indicates its usefulness [25] and extending its role in being the mainstay in treatment of MRSA. On the other hand, linezolid which is one of a new class of antimicrobial chemotherapeutic agents, oxazolidinones, demonstrated low activity against MRSA and could not be proposed as an alternative therapy for MRSA infections, as previously reported [7]. Mupirocin has shown excellent anti-staphylococcal activity and proved effectiveness in eradication of MRSA carriage from patients and health care workers. However, reports of mupirocin resistance [26] undermine this role.

Accurate and rapid detection of methicillin resistance in *S. aureus* is imperative for the use of appropriate antimicrobial therapy and for the control of nosocomial spread of MRSA strains. Early diagnosis and appropriate treatment can significantly limit the duration and outcome of infection. The detection of *femB* and *mecA* genes in *S. aureus* may also be helpful in explaining the severity of the infection. The PCR of *femB* and *mecA* genes were used as the gold standard for the evaluation of the traditional methods. The isolates were tested genotypically using multiplex PCR for direct detection of *femB* and *mecA* genes. In this study, *mecA* has been detected in all of the MRSA isolates and one isolate of MSSA showed the presence of *mecA* gene having the potential to become MRSA in prospect.

Several studies have reported the use of *mecA* as marker for MRSA detection and *fem* genes for recognition of *S. aureus* species. Positive correlation of phenotype with genotype tests were reported for MRSA isolates, which

harboured *mecA* and *femB* genes [27]. Kobayashi *et al* reported that *mecA* was detected in 100 % of MRSA, 16.7 % of MSSA isolates [28]. In Egypt, only few studies have reported the use of *femA* along with *mecA* as molecular targets for identification of *S. aureus* and characterisation of MRSA [12,22] and there were no reports which specifically investigated the prognostic value of *femB* gene for the detection of *S. aureus*.

CONCLUSION

This study demonstrates the relatively high prevalence of MRSA in *S. aureus* from the hospital specimens and high resistance rates among these strains to ciprofloxacin, TMS, gentamicin and rifampicin. High susceptibility rates were found to vancomycin, teicoplanin, Q/D and mupirocin but unexpected modest activity was found for linezolid. Further, *femB* along with *mecA* appears the most accurate method for detection of MRSA and the *mecA* PCR in this study showed absolute positive correlation to MRSA phenotype.

ACKNOWLEDGEMENT

The Author extends his appreciation to the Deanship of Scientific Research at King Saud University for funding the work through the research group project No. RGP-VPP-202.

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