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Study on prevalence and genetic discrimination of methicillin-resistant *Staphylococcus aureus* (MRSA) in Egyptian hospitals

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Methicillin-resistant *Staphylococcus aureus* (MRSA) continues to be a global problem in infection control. The highest proportions of MRSA are reported by Jordan, Egypt and Cyprus investigators, where more than 50% of the invasive isolates are methicillin-resistant. The aim of this work was to study the prevalence, antibiotic sensitivity and genetic discrimination of MRSA in Egypt. Microbiological identification was done using Gram stain, catalase, coagulase and mannitol fermentation along with biochemical identification by analytical profile index (API) tests. Molecular identification was conducted by the polymerase chain reaction (PCR) targeting 16S ribosomal RNA and the *nuc* genes. Additionally, identification of methicillin-resistant *S. aureus* (MRSA) was performed by the amplification of 310 bp of the *mec*A gene. Antibiograms were performed for all isolates. Only 73 isolates out of 166 were oxacillin resistant. The percentage of resistant isolates to erythromycin, rifampicin, vancomycin, Ofloxacin, gentamycin, Amoxicillin clavulanic acid, ciprofloxacin, chloramphenicol, trimethoprim sulfamethoxazole, teicoplanin and tetracycline were 58, 32.50, 2.4, 45.18, 37.9, 39.7, 23.5, 21.6, 40.3, 0 and 39.1%, respectively. MRSA isolates were subdivided into eight biotypes according to their resistance pattern. Random amplification of polymorphic DNA (RAPD) and repetitive sequence DNA (REP) were performed on samples representing each biotype.

Key words: Methicillin-resistant *Staphylococcus aureus* (MRSA), nuclease, *mec*A, 16S rRNA, random amplified polymorphic DNA (RAPD), polymerase chain reaction (PCR).

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) continues to be a global problem in infection control. MRSA may be present on human skin (particularly the scalp, armpits and groins) as well as in the nose and throat, and less commonly in the colon and urine, but its presence does not always mean infection (Kresser, 2012). MRSA has the ability to survive from days to weeks on environmental surfaces in healthcare facilities.

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Table 1. Distribution of clinical isolates and its gender from different hospitals.

Hospital		Gender		Total
		Male	Female	
Alhoodalmarsod	Count	26	18	44
	% within Hospital	59.10	40.90	100.00
Banha	Count	13	0	13
	% within Hospital	100.00	0.00	100.00
El Hussain	Count	8	10	18
	% within Hospital	44.40	55.60	100.00
ElsayedGalal	Count	7	4	11
	% within Hospital	63.60	36.40	100.00
KafrElshiekh	Count	3	4	7
	% within Hospital	42.90	57.10	100.00
Kasralaini	Count	31	30	61
	% within Hospital	50.80	49.20	100.00
Sheikh zayed	Count	7	5	12
	% within Hospital	58.30	41.70	100.00
Total	Count	95	71	166
	% within Hospital	57.20	42.80	100.00

It is capable of withstanding a wide range of temperatures, humidity and exposure to sunlight and is resistant to desiccation. These properties make it able to contaminate a large variety of hospital items, e.g. chairs, mattresses, bed frames and computer keyboards (Bhalla et al., 2004; Lu et al., 2008). The primary site of infection is the nasal inner wall opposite the nostril wing otherwise known as the anterior nares or vestibulum nasi (Williams et al., 2000; Wertheim et al., 2005). Most of the countries in the Mediterranean region are experiencing a surge in MRSA infections (Borg, 2007). The primary site of infection is the nasal inner wall opposite the nostril wing otherwise known as the anterior nares (Williams et al., 2000; Wertheim et al., 2005). MRSA infections result in higher mortality, greater lengths of hospital stay and increased cost compared with methicillin sensitive *S. aureus* (MSSA) infections (Engemann et al., 2003). Patients with MRSA bacteremia have a mortality of 1.78%, three times higher than with MSSA bacteremia (Wang et al., 2011). MRSA show resistance to a wide range of antibiotics, thus limiting the treatment options to few agents, such as vancomycin and teicoplanin. It is therefore important to keep the prevalence of MRSA carriage and MRSA infections as low as possible (Wertheim et al., 2005). The aim of this work is to study the prevalence of MRSA in Egyptian hospitals, isolation and identification of MRSA from different infection sites, different hospitals and different hospital units around Egypt and further identification of MRSA using PCR. It also includes studying the antibiotic sensitivity of MRSA and grouping of the isolates according to their resistance.

MATERIALS AND METHODS

Sample collection

Clinical isolates recovered from blood, skin, pus, sputum, urine, nasal swab, throat swab and surgical wound were collected from different Egyptian hospital laboratories: Kasr Al-Aini, Al Sayed Galal, Al Hussain, AlHoud Almarsood, Banha, Al Sheikh Zayed and Kafr Al Sheikh as shown in Table 1. A total of 166 isolates were collected.

Isolation and identification of staphylococci

The swab specimens were inoculated on nutrient agar and incubated at 37°C for 24 to 48 h; thereafter the colonies were streaked with sterilized wire loap on mannitol salt agar so as to obtain discrete colonies. The plates were incubated for 24 h at 37°C under aerobic conditions after which the cultured plates were examined recording the appearance, size, colour, and morphology of the colonies.

Gram stain reaction, catalase test and coagulase test were carried out. Isolates that were Gram-positive cocci, catalase

Table 2. Interpretive standards for *S. aureus* according to CLSI guidelines 2014.

Antimicrobial agent	Zone diameter interpretive (nearest mm)		
	Sensitive (S)	Intermediate (I)	Resistant (R)
Rifampin	S ≥20	17--19	R ≤16
Amoxycillin/clavulinic	S ≥20	-	R≤19
Ciprofloxacin	S ≥21	16-20	R ≤15
Chloramphenicol	S ≥18	13-17	R ≤12
Trimethoprim Sulphamethoxazole	S ≥16	11-15	R≤ 10
Ofluxacin	S ≥18	15-17	R ≤14
Tetracyclin	S ≥19	15-18	R ≤14
Erythromycin	S≥ 23	14-22	R ≤13
Gentamicin	S ≥15	13-14	R ≤12
Vancomycin	S ≥15	-	R ≤14
Teicoplanin	S ≥14	11-13	R ≤10
Oxacillin	S≥13	-	R≤10

positive, and coagulated human plasma were considered *S. aureus* in this study (Chigbu and Ezeronye, 2003).

Preparation and examination of gram stained films of the collected isolate

Gram stain was performed according to Isenberg (1992).

Growth on mannitol salt agar

Isolates which proved to be from *Staphylococcus* species were subcultured on mannitol salt agar plates. *S. aureus* ferments mannitol changing the medium colour from red to yellow (Pumipuntu et al., 2017).

Biochemical tests

Catalase test

The catalase test involves the addition of hydrogen peroxide to a culture sample or agar slant. If the bacteria in question produce catalase, hydrogen peroxide will be hydrolyzed and oxygen gas will be evolved. A positive test was denoted by evolution of gas resulting in bubbles formation (Kloos and Schleifer, 1986).

Coagulase test

Colonies from different samples were inoculated onto 0.5 ml of rabbit plasma diluted by 1/10 using saline and incubated at 37°C for 1 to 4 h. A positive test was denoted by clot formation in the test tube after chosen time intervals (Sperber and Tatini, 1975).

Analytical profile index (API)

API staph (Bioumerieux, Inc., Marcy E'toile, France) was used to confirm identification of *S. aureus* with the test performed as described by Radebold and Essers (1980).

Antibiotic sensitivity test

166 staphylococci strains were examined *in vitro* against 12 different antibiotics. The antibiotic discs were obtained from Oxoid, UK including: Rifampicin, Amoxicillin clavulanic, Chloramphenicol, trimethoprim Sulfamethoxazole, Ofloxacin, Tetracycline, Erythromycin, Gentamicin, Vancomycin, Teicoplanin and Oxacillin. Approximately 10^8cfu/ml bacterial inoculums were prepared in 5 ml Muller-Hinton broth and inoculated on nutrient agar plates after incubation at 37°C overnight. 3 to 5 isolated colonies were picked from the plate containing the test organism. The turbidity was adjusted the same as the McFarland No. 0.5 standard. Thereafter, swab was streaked onto the surface of the Mueller-Hinton agar (3 times in 3 quadrants) and left for 5 to 10 min to dry the surface of agar. The chosen drug-impregnated discs were placed onto the surface of the inoculated agar plate which was then inverted and incubated at 35°C for 18 to 24 h (Drew et al., 1972).

Interpretation of antibiogram

According to CLSI, the results of antibiogram were interrelated as shown in Table 2.

PCR amplification

Extraction of chromosomal DNA

This was carried out using the traditional method. A total of 5 ml in 24 h broth culture isolates was centrifuged for 10 min at 12000 rpm. The pellet was washed twice by 50 µl distilled water. The cell suspension was heated directly at 100°C for 10 min in a heat block to break the cell membranes and then cooled in the refrigerator for 5 min. Finally, the cell suspension was centrifuged for 5 min at 12000 rpm and the supernatant containing chromosomal DNA was collected.

Amplification of 16SrRNA, nuc, mecA, RAPD, REP1R and REP2 genes

The reaction mixtures were prepared following the protocols described in the Mastermix pcr kits. Thereafter, PCR was performed in a thermal cycler using the following settings; Stage 1 (x1): initial denaturation at 94°C for 5 min; Stage 2 (x35): secondary denaturation at 94°C for 30 sec, annealing at 55°C for 1 min and extension at 72°C for 1 min; Stage 3 (x1): a final extension at 72°C for 10 min. Other changes made for amplification of specific genes

Table 3. Temperature and time conditions of the primers during PCR for each of the tested genes.

Gene	Primary denaturation (5 min)	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension (min)
16SrRNA	94°C	94°C 30 s	55°C 1 min	72°C 1 min	35	72°C 10 min
nuc	94°C	94°C 30 s	55°C 45 s	72°C 45 s	35	72°C 10 min
mecA	94°C	94°C 30 s	50°C 30 s	72°C 40 s	35	72°C 10 min
RAPD	94°C	94°C 1 min	32°C 1 min	72°C 2 min	35	72°C 12 min
REP1R	94°C	94°C 1 min	38°C 1 min	72°C 2 min	35	72°C 12 min
REP2	94°C	94°C 1 min	46°C 1 min	72°C 2 min	35	72°C 12 min

are as stated in Table 3.

Statistical methods

Statistical analysis was done using IBM© SPSS© Statistics version 22 (IBM© Corp., Armonk, NY, USA). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test or Fisher's exact test was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Student's t-test. All tests were two-tailed. P-value <0.05 was considered significant.

RESULTS

Isolation and identification of staphylococcal isolates

A total of 166 clinical isolates were recovered from blood (12 isolates), throat swabs (3 isolates), nasal swabs (2 isolates), wound (53 isolates), pus (9 isolates), sputum (15 isolates), urine (16 isolates) and skin (56 isolates).

The number of clinical isolates from each gender (male and female), collected from each infection site (blood, throat swabs, pus, skin, surgical wound, urine, nasal swab and sputum) is shown in Table 4.

Table 4. Distribution of clinical specimens.

Clinical specimen	No. of isolates		Total
	Male	Female	
Blood	6	6	12
Skin	31	25	56
Surgical wound	35	18	53
Pus	5	4	9
Sputum	9	6	15
Urine	4	12	16
Throat swab	2	1	3
Nasal swab	2	0	2
Total	-	-	166

Morphological characteristics

Gram staining and microscopical examination of the recovered isolates revealed that 166 isolates were Gram positive cocci arranged in bunch. *Staphylococci* found as gram-positive bacteria occur in microscopic clusters resembling grapes. They form large round golden-yellow colonies.

Growth on mannitol salt agar (MSA)

All isolates grown on MSA were mannitol fermenters and are presumed to be *S. aureus*.

Biochemical tests

Catalase and coagulase tests showed that all recovered isolates were catalase and coagulase positive.

Identification of *Staphylococcus aureus* using API staph

Based on the above tests, all clinical isolates were presumptively identified as *S. aureus.* The identity of these isolates was confirmed using API Staph kits.. All tested isolates were identified as *S. aureus* and gave positive results with GLU, FRU, MNE, MAL, LAC, TRE, MAN, NIT, PAL, VP, SAC, NAG, ADH and URE, however showing negative results with XLT, MEL, RAF, XYL and MDG. Identification was made using the Analytic Profile Index with API Staph (bioMerieux) system. The pattern of the reactions are obtained and coded into 7-digit numerical profile. All 166 *S. aureus* isolates gave analytic profile index of 6736153. Upon performing API on isolates, they were all *S. aureus.* All API numerical profiles for 166 isolates showed very good identity of *S. aureus* similar to each other in the test results.

Antimicrobial susceptibility of the isolates to different antibiotics

Antibiogram for isolates

Patterns of susceptibility to antimicrobial agents are used

Table 5. Susceptibility profile of *Staphylococcus aureus* isolates to various antibiotics.

Antimicrobial agent	Concentration (µg/disc)	*Staphylococcus aureus* (166 isolates)					
		Sensitive (S)		Intermediate (I)		Resistant (R)	
		NO.	%	NO.	%	NO.	%
Rifampin	5	107	64.4	5	3.01	54	32.5
Amoxycillin/clavulinic	20/10	100	60.24	Zero	0	66	39.7
Ciprofloxacin	5	121	72.89	6	3.6	39	23.49
Chloramphenicol	30	121	72.89	9	5.42	36	21.6
Trimethoprim sulphamethoxazole	1.25+ 23.75	93	56.02	6	3.6	67	40.3
Ofluxacin	5	88	53.01	3	1.8	75	45.18
Tetracyclin	30	98	59.03	3	1.8	65	39.1
Erythromycin	15	96	57.83	6	3.6	64	38.55
Gentamicin	10	101	60.84	2	1.2	63	37.9
Vancomycin	30	162	97.5	Zero	0	4	2.4
Teicoplanin	30	166	100	Zero	0	Zero	0
Oxacillin	5	93	56.02	Zero	0	73	43.9

for typing because staphylococci isolates can be broken down into separate groups. The susceptibility of *S. aureus* isolates to different antibiotics commonly used for *S. aureus* infections, namely, Rifampicin, Amoxicillin clavulanic ciprofloxacin, Chloramphenicol, Trimethoprim sulfamethoxazole, Ofloxacin, Tetracycline, Erythromycin, Gentamycin, Vancomycin, Teicoplanin and Oxacillin was determined.

An antibiogram was performed for all isolates which showed the characteristics of *S. aureus*. The percentage resistance (% R) shows the degree of multiresistance of the isolate. The distribution of antibiotic resistance within *S. aureus,* either sensitive or resistant to Oxacillin was shown on Table 5. The isolates which showed resistance to oxacillin were considered MRSA as shown in Table 7, while isolates sensitive to oxacillin were considered MSSA as shown in Table 8. In many cases, the resistance to certain antibiotics exceeded 40% of the isolates Table 6. The percentage resistance to Erythromycin was 58%, Rifampicin (32.50%), Vancomycin (2.4%), Ofloxacin (45.18%), Gentamycin (37.9%), Amoxicillin clavulanic acid (39.7%), Ciprofloxacin (23.5%), Chloramphenicol (21.6%), Trimethoprim sulfamethoxazole (40.3%), Teicoplanin (0%), and Tetracycline (39.1%) as previously shown in Table 5.

The percentage resistance of MRSA against each antibiotic from different classes which in many cases exceeded 70% resistance to Oxacillin was found to be 100% while resistance to Teicoplanin was 0% and Vancomycin was 5.5%. All isolates were sensitive to Teicoplanin with R% equal to 0% while 43.7% isolates remained resistant to Oxacillin and were considered as MRSA. These results are summarized in Table 6. On the other hand, 56.3% of the *S. aureus* isolates were oxacillin sensitive (MSSA) and their antibiogram results are summarized in Table 8. Based on the results of antibiotic sensitivity, 73 isolates were assumed to be MRSA (43.7% of the *S. aureus* isolates). Total number of MRSA isolates versus total number of MSSA isolates collected from each clinical specimen (blood, throat swabs, pus, skin, surgical wound, urine, nasal swab and sputum) is shown in Table 9. All isolates obtained from both nasal and throat swab were Oxacillin resistant according to the results of antibiogram.

Table 6. Percentage resistance (R %) of *S. aureus* against each antibiotic.

Antibiotic	*S. aureus* R% (No. of isolates)
Rifampin	32.50 (54)
Amoxycillin/Clavulinic	39.7(66)
Ciprofloxacin	23.5 (39)
Chloramphenicol	21.6 (36)
Trimethoprim sulphamethoxazole	40.3 (67)
Ofluxacin	45.18 (75)
Tetracyclin	39.1 (65)
Erythromycin	58 (96)
Gentamicin	37.9 (63)
Vancomycin	2.4 (4)
Teicoplanin	0
Oxacillin	43.7 (73)

Antibiotypes

MRSA isolates were subdivided into eight biotypes according to the resistance pattern as shown in Table 13. One sample from each biotype was taken as representative as shown in Table 14.

Biotype 1 includes isolates resistant to Oxacillin, Gentamicin, Erythromycin, Tetracycline, Ofloxacin,

Table 7. Percentage resistance (R%) of MRSA against each antibiotic.

Antibiotic	MRSA R% (No. of isolates)
Rifampin	76 (56)
Amoxycillin/Clavulinic	84.70 (62)
Ciprofloxacin	52.70 (39)
Chloramphenicol	52.70 (38.4)
Trimethoprim sulphamethoxazole	81.90 (60)
Ofluxacin	97.22 (71)
Tetracyclin	83.33 (61)
Erythromycin	76.30 (56)
Gentamicin	83.33 (61)
Vancomycin	5.50 (4)
Teicoplanin	0
Oxacillin	100 (73)

Table 8. Percentage resistance (R %) of MSSA against each antibiotic.

Antibiotic	MRSA (R %)
Rifampin	1 (1)
Amoxycillin/Clavulinic	5.37 (5)
Ciprofloxacin	1.00 (1)
Chloramphenicol	1.00 (1)
Trimethoprim sulphamethoxazole	9.60 (9)
Ofluxacin	5.37 (5)
Tetracyclin	5.44 (6)
Erythromycin	9.60 (9)
Gentamicin	3.22 (3)
Vancomycin	0.00
Teicoplanin	0
Oxacillin	0

Trimethoprim sulfamethoxazole and Amoxicillin clavulanic.

Biotype 2 includes isolates resistant to Gentamicin, Tetracycline, Ofloxacin, Trimethoprim sulfamethoxazole, Chloramphenicol, Ciprofloxacin, Rifampin and Oxacillin.

Biotype 3 includes isolates resistant to Gentamicin, Erythromycin, Tetracycline, Ofloxacin, Trimethoprim sulfamethoxazole, Amoxicillin clavulanic, Rifampin and Oxacillin.

Biotype 4 includes isolates resistant to Oxacillin, Gentamicin, Erythromycin, Tetracycline, Ofloxacin, Chloramphenicol, Ciprofloxacin, Amoxicillin clavulanic and Rifampin.

Biotype 5 includes isolates resistant to Oxacillin, Erythromycin, Ofloxacin, Trimethoprim sulfamethoxazole, Chloramphenicol, Ciprofloxacin, Amoxicillin clavulanic and Rifampin.

Biotype 6 includes isolates resistant to Oxacillin, Gentamicin, Tetracycline, Ofloxacin, Trimethoprim sulfamethoxazole, Amoxicillin clavulanic and Rifampin.

Table 9. Distribution in clinical specimens.

Sample	No. of isolates		Total	%MRSA
	MRSA	MSSA		
Blood	7	5	12	58.33
Skin	19	38	57	33.9
Surgical wound	28	25	53	52.8
Pus	5	4	9	55.5
Sputum	8	6	14	53.5
Urine	1	15	16	6.25
Throat swab	3	0	3	100
Nasal swab	2	0	2	100
Total	73 43.90%	93 56.10%	166	

Biotype 7 includes isolates resistant to Oxacillin, Gentamicin, Erythromycin, Tetracycline, Trimethoprim sulfamethoxazole, Chloramphenicol, Ciprofloxacin and Amoxicillin clavulanic.

Biotype 8 that includes isolates resistant to Oxacillin, Vancomycin, Gentamicin, Erythromycin, Tetracycline, Ofloxacin, Trimethoprim sulfamethoxazole, Amoxicillin clavulanic and Rifampin.

Polymerase chain reaction (PCR)

The 166 *S. aureus* isolates were subjected to genotyping (*S. aureus* identity confirmation and detection of antibiotic resistance genes).

16s RNA

Identification of the 166 isolates as *Staphylococcus* was confirmed by PCR amplification of *16S rRNA*. The results of amplification of 791bp of *16SrRNA* using the forward primer (CCTATAAGACTGGGATAACTTCGGG) and reverse primer (CTTTGAGTTTCAACCTTGCGGTCG) are shown in Figure 1. All isolates produced a single band at 791 bp size. The 166 isolates were therefore confirmed to be *S. aureus* (Mason et al., 2001) as shown in Figure 1.

Nuc gene

The presence of *Nuc* gene in 166 isolates was confirmed by PCR amplification. Amplification of 395 bp of *NUC* gene were done for all 166 isolates aimed at further confirmation of *S. aureus* using forward primer (ATATGTATGGCAATCGTTTCAAT) and reverse primer

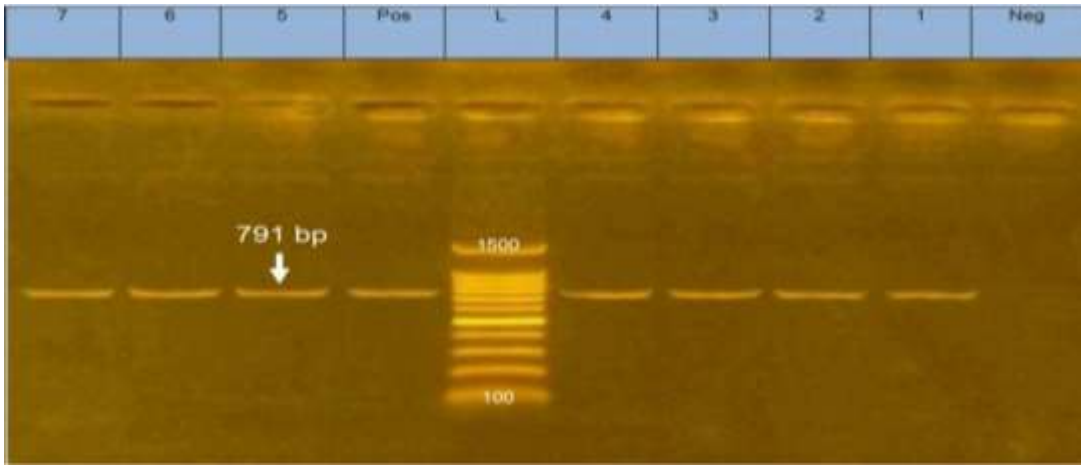

Figure 1. Agarose gel photo documentation of conventional PCR on genetic material extracted from *S. aureus* strains as a molecular typing for detection of 16Sr RNA gene using forward primer: CCTATAAGACTGGGATAACTTCGGG and reverse primer CTTTGAGTTTCAACCTTGCGGTCG. Lane L: molecular weight marker (100 –1500 bp). Lanes 1-7: positive samples *S.aureus*16Sr RNA gene with amplicon size of 791 bp. Lane Neg: negative control. Lane Pos.: positive control.

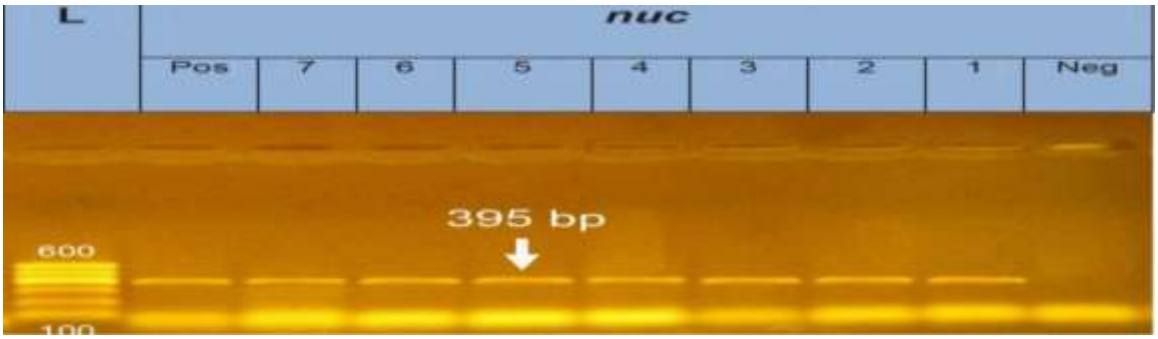

Figure 2. Agarose gel photo documentation of conventional PCR on genetic material extracted from *S. aureus* strains as a molecular typing for detection of *nuc* gene using forward primer: ATATGTATGGCAATCGTTTCAAT and reverse primer: GTAAATGCACTTGCTTCAGGAC . Lane L: molecular weight marker (100 – 600 bp). Lanes 1-7: positive samples nuc gene with amplicon size of 395 bp. Lane Neg: negative control. Lane Pos.: positive control.

(GTAAATGCACTTGCTTCAGGAC).166 clinical isolates contain the *NUC* gene and single band at 600 bp (Gao et al., 2011) as shown in Figure 2.

mecA gene

The presence of antibiotic resistance *mecA* gene in 166 clinical isolates was confirmed by PCR amplification. Amplification of 310bp *mec*A gene was carried out using forward primer (GTA GAA ATG ACT GAA CGT CCG ATA A) and reverse primer (CCA ATT CCA CAT TGT TTC GGT CTA A) (McClure et al., 2006). Only 73 MRSA samples shows positive results for *mec*A single band at 310 bp as shown in Figures 3, 4 and 5.

Random amplification of polymorphic DNA (RAPD)

RAPD was carried out for only one clinical isolate from each biotype as shown in Table 14. Each representing sample was randomly amplified against primers A1, A2, A3, A4, A5 and A6. The results obtained are shown in Figures 6, 7, 8, 9, 10 and 11.

i. RAPD using primers A1 and A2 are shown in Figure 6.
ii. RAPD using primers A3 is shown in Figure 7.
iii. RAPD using primers A4 is shown in Figure 8.
iv. RAPD using primers A5 is shown in Figure 9.
v. RAPD using primers A6 is shown in Figure 10.
vi. RAPD using primers: A1, A2, A3, A4, A5 and A6 against sample 30 (antibiotype 8) are shown in Figure 11.

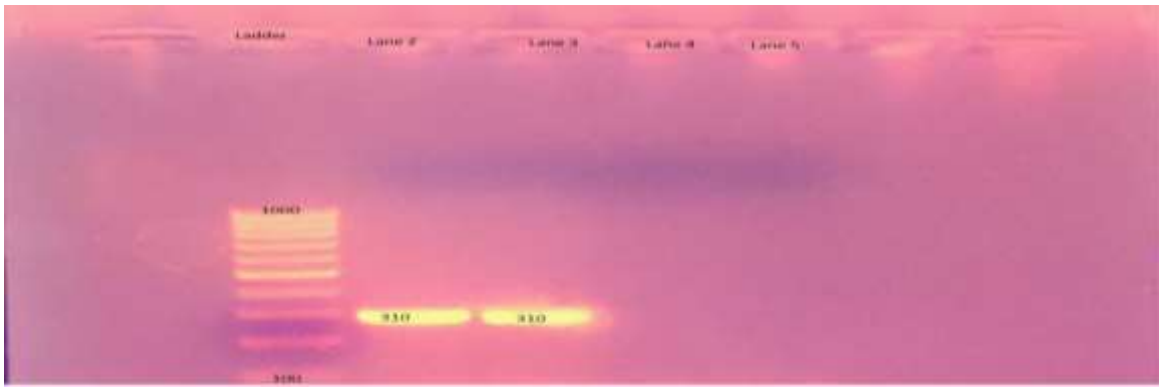

Figure 3. Agarose gel photo documentation of conventional PCR on genetic material extracted from *S. aureus* strains as a molecular typing for detection of *mecA* gene using forward primer : GTA GAA ATG ACT GAA CGT CCG ATA A and reverse primer CCA ATT CCA CAT TGT TTC GGT CTA A . Lane L: molecular weight marker (100 – 1000 bp). Lanes 2, 3: positive samples MRSA mecA gene with amplicon size of 310 bp., Lane 3,4: negative samples MSSA *mec*A.

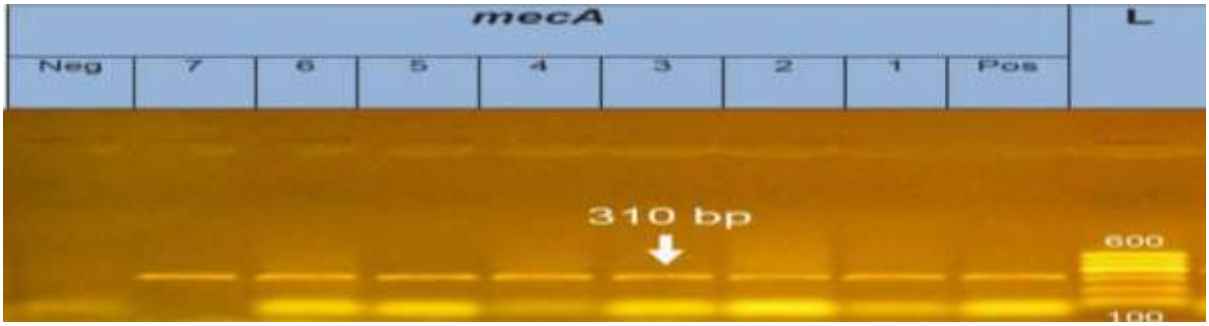

Figure 4. Agarose gel photo documentation of conventional PCR on genetic material extracted from *S. aureus* strains as a molecular typing for detection of *mec*A gene using forward primer: GTA GAA ATG ACT GAA CGT CCG ATA A and reverse primer CCA ATT CCA CAT TGT TTC GGT CTA A . Lane L: molecular weight marker (100 – 600 bp). Lanes 1-7: positive samples MRSA mec A gene with amplicon size of 310 bp. Lane Neg: negative control. Lane Pos.: positive control.

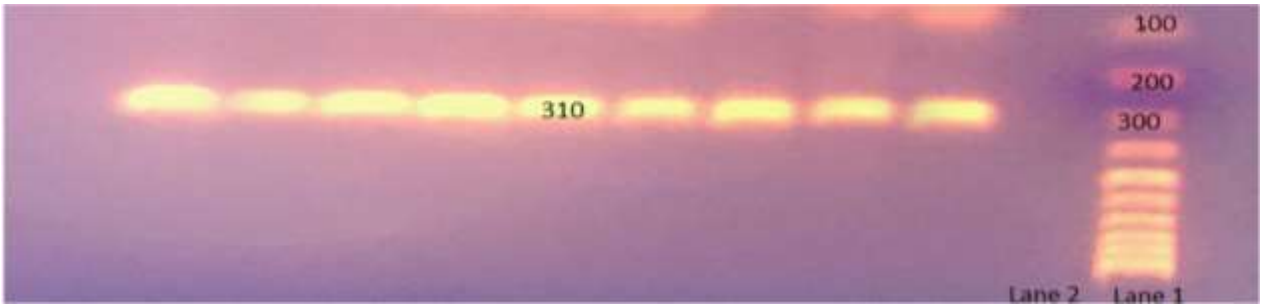

Figure 5. Agarose gel photo documentation of conventional PCR on genetic material extracted from *S. aureus* strains as a molecular typing for detection of *mecA* gene using forward primer : GTA GAA ATG ACT GAA CGT CCG ATA A and reverse primer CCA ATT CCA CAT TGT TTC GGT CTA A . Lane L: molecular weight marker (100 – 600 BP). Lanes 1-7: positive samples MRSA *mecA* gene with amplicon size of 310 bp. Lane Neg: negative control. Lane Pos.: positive control.

Repetitive sequence DNA (REP)

REP was performed for isolates 69, 23, 164, 83, 74, 56 and 88 using REP primers REP1 and REP2.

i. REP using primer REP1 and REP2 with 69, 23, 164, 74, 56 and 88 and representing biotypes 1, 2, 3, 4, 5, 6

Figure 6. Agarose gel electrophoresis of RAPD PCR with A1 and A2.

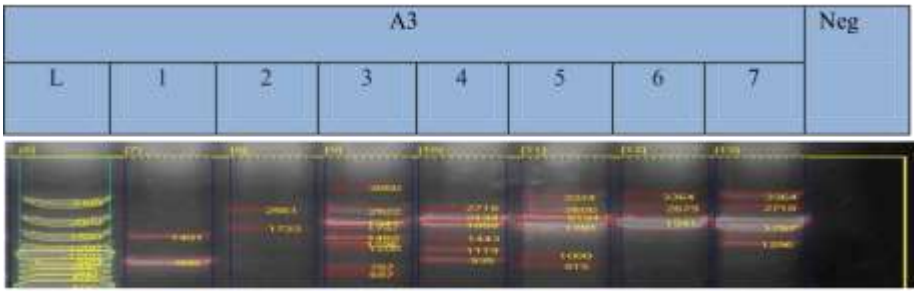

Figure 7. Agarose gel electrophoresis of RAPD PCR with A3.

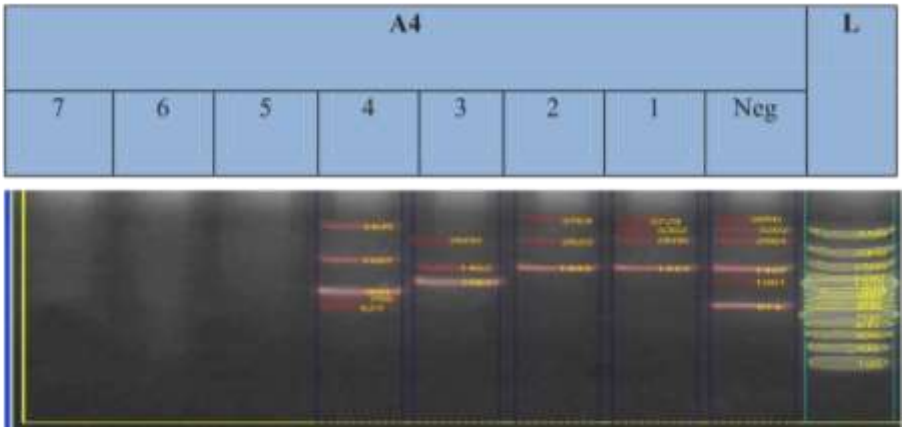

Figure 8. Agarose gel electrophoresis of RAPD PCR with A4.

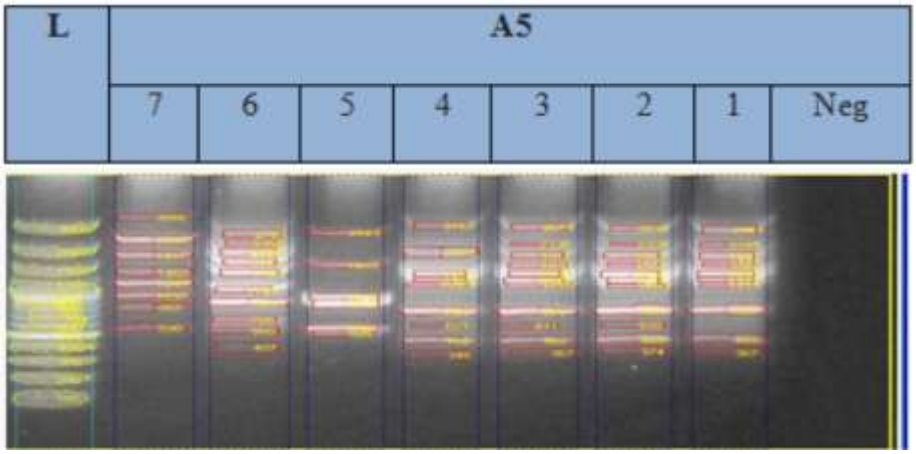

Figure 9. Agarose gel electrophoresis of RAPD PCR with A5.

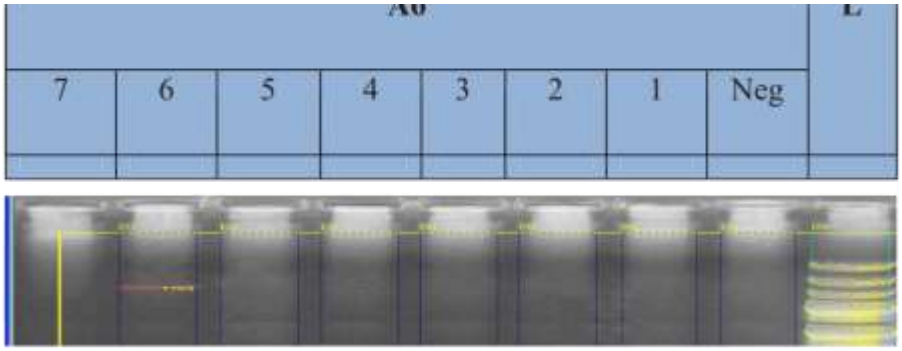

Figure 10. Agarose gel electrophoresis of RAPD PCR with A6.

Figure 11. Sample 30 with primers A1 to A6.

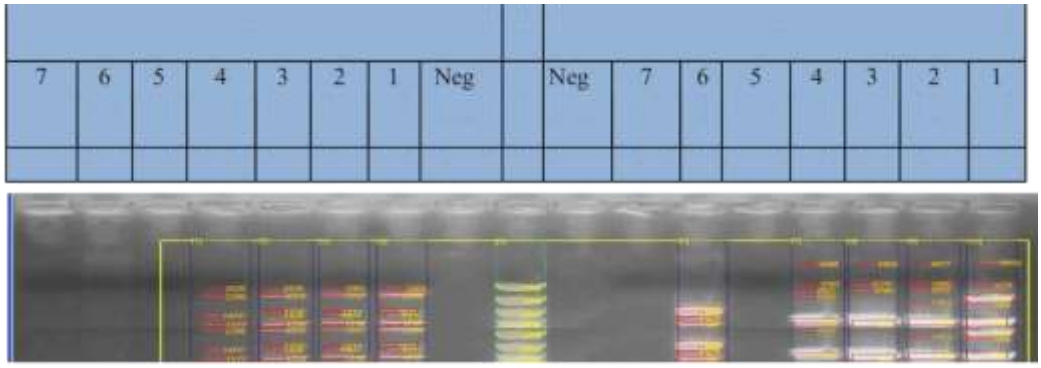

Figure 12. Agarose gel electrophoresis of Repetitive sequence DNA was done for isolate 69, 23, 164, 83, 74, 56 and 88 using primers REP1and REP2.

Figure 13. Agarose gel electrophoresis for isolate 30 using REP1 and REP2 primers.

and 7 respectively results are shown in Figure 12.
ii. Repetitive sequence PCR for isolate 30 representing antibiotype 8 is shown in Figure 13.

Statistical analysis

Relationships between different factors with MRSA were

Table 10. Relation between MRSA and site of infection.

Variable			Site of infection								Total
			Blood	Nasal swab	Pus	Skin	Sputum	Throat swab	Urine	Wound	
MRSA	S	Count	5	0	4	39	6	0	15	25	94
		% Site of infection	41.7	0.0	44.4	68.4	42.9	0.0	93.8	47.2	56.6
	R	Count	7	2	5	18	8	3	1	28	72
		% Site of infection	58.3	100.0	55.6	31.6	57.1	100.0	6.3	52.8	43.4
Total		Count	12	2	9	57	14	3	16	53	166
		% Site of infection	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

No p-value because of small number of cases within subgroups so we combined both throat and nasal swabs in one group.

Table 11. Relation between MRSA and site of infection.

Variable			Site of infection							Total
			Blood	Pus	Skin	Sputum	Throat and nasal swab	Urine	Wound	
MRSA	S	Count	5	4	39	6	0	15	25	94
		% within infection site 1	41.7	44.4	68.4	42.9	0.0	93.8	47.2	56.6
	R	Count	7	5	18	8	5	1	28	72
		% within infection site 1	58.3	55.6	31.6	57.1	100.0	6.3	52.8	43.4
Total		Count	12	9	57	14	**5**	16	53	166
		% within infection site 1	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Fisher's Exact Test with p-value<0.001, no p-value because of small number of cases within subgroups.

Table 12. Relation between MRSA and hospitals.

Variable			Hospital							Total
			Alhoodalmarsod	Banha	El Hussain	ElsayedGalal	KafrElshiekh	Kasralaini	Sheikh zayed	
S. aureus	S	Count	32	7	11	5	2	29	8	94
		% within Hospital	72.7	53.8	61.1	45.5	28.6	47.5	66.7	56.6
	R	Count	12	6	7	6	5	32	4	72
		% within Hospital	27.3	46.2	38.9	54.5	71.4	52.5	33.3	43.4
Total		Count	44	13	18	11	7	61	12	166
		% within Hospital	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

presented as follows: MRSA and site of infection (Tables 10 and 11); MRSA and gender (Table 15), MRSA and hospitals (Table 12) and relation of hospitals with infection site (Table 16).

DISCUSSION

S. aureus is a ubiquitous bacterial species capable of colonizing and cause infections in a wide range of hosts. It is the cause of serious infections in humans and number one cause of hospital-associated infections. A high proportion of these infections are caused by Methicillin-resistant *S. aureus* (MRSA) which became highly prevalent in hospitals worldwide.

The evolution of MRSA has paralleled penicillin-resistant *S. aureus* from 1940s. MRSA is now pandemic, with dissemination of HA-MRSA clones from 1960s, CA-MRSA clones from 1990s, and LA-MRSA clones from 2000s. Although epidemiological data from separate

Table 13. Subdivision of MRSA into eight biotypes.

Antibiotype	Teicoplanin	Rifampin	Amoxycillin/ Clavulinic	Ciprofloxacin	Chloramphenicol	Trimethoprim Sulphamethoxazole	Ofluxacin	Tetracyclin	Erythromycin	Gentamicin	Vancomycin	Oxacillin
1	S	S	R	S	S	R	R	R	R	R	S	R
2	S	R	S	R	R	R	R	R	S	R	S	R
3	S	R	R	S	S	R	R	R	R	R	S	R
4	S	R	R	R	R	S	R	R	R	R	S	R
5	S	R	R	R	R	R	R	S	R	S	S	R
6	S	R	R	S	S	R	R	R	S	R	S	R
7	S	S	R	R	R	R	S	R	R	R	S	R
8	S	R	R	S	S	R	R	R	R	R	R	R

Isolates within the same group showed the same resistance pattern against different antibiotics.

Table 14. The representing sample for each biotype.

Biotype	Representing isolate	Age	Clinical specimen	Hospital	Gender
1	69	30	Urine	Kasr Elaini	M
2	23	29	Wound	Kasr El-Aini	M
3	164	15	Wound	Kasr Elaini	M
4	83	33	Skin	Kasr Elaini	F
5	74	13	Wound	Kasr Elaini	F
6	56	59	Blood	Elhhod Elmarsod	F
7	88	40	Blood	Sayed Galal	M
8	30	25	Wound	Sayed Galal	M

studies are often not comparable owing to differences in study design and populations sampled, with the highest rates (>50%) reported in North and South America, Asia and Malta, intermediate rates (25 to 50%) are reported in China, Australia, Africa and some European countries [e.g. Portugal (49%), Greece (40%), Italy (37%) and Romania (34%)]. Other European countries have generally low prevalence rates (for example, Netherlands and Scandinavia). High morbidity and mortality rates are associated with MRSA because of the development of multidrug resistance. *S. aureus* strains with the tendency to accumulate additional resistance determinants have resulted in the formation of multiple-antibiotic resistant MRSA strains. These strains show resistance to a wide range of antibiotics, thus limiting the treatment options to few agents, such as Vancomycin and Teicoplanin (Peacock et al., 2005; Perez-Roth et al., 2001). To compound this problem further, *S. aureus* has ability to form biofilms making it difficult to be eradicated from the infected host. They are reported to display susceptibilities towards antibiotics and biocides which are 10 to 1000 times, less than the equivalent populations of free-floating planktonic bacteria (Sasirekha et al., 2012).

Table 15. Relation between MRSA and gender.

Variables			Gender		Total
			Male	Female	
S. aureus	S	Count	53	41	94
		% within gender	55.8	57.7	56.6
	R	Count	42	30	72
		% within gender	44.2	42.3	43.4
Total		Count	95	71	166
		% within gender	100.0	100.0	100.0

Pearson Chi-square test (p-value = 0.801) = 0.063

Table 16. Relation between hospitals and site of infection.

Parameter			Hospital							Total
			Alhood almarsod	Banha	El Hussain	Elsayed Galal	Kafr Elshiekh	Kasr alaini	Sheikh zayed	
Infection.site1	Blood	Count	4	2	2	1	0	2	1	12
		% Hospital	9.10	15.40	11.10	9.10	0.00	3.30	8.30	7.20
	Pus	Count	1	2	1	0	2	3	0	9
		% Hospital	2.30	15.40	5.60	0.00	28.60	4.90	0.00	5.40
	Skin	Count	15	3	7	4	2	24	2	57
		% Hospital	34.10	23.10	38.90	36.40	28.60	39.30	16.70	34.30
	Sputum	Count	3	0	1	0	1	7	2	14
		% Hospital	6.80	0.00	5.60	0.00	14.30	11.50	16.70	8.40
	Throat and Nasal swab	Count	1	0	0	2	0	2	0	5
		% Hospital	2.30	0.00	0.00	18.20	0.00	3.30	0.00	3.00
	Urine	Count	5	1	1	1	0	6	2	16
		% Hospital	11.40	7.70	5.60	9.10	0.00	9.80	16.70	9.60
	Wound	Count	15	5	6	3	2	17	5	53
		% Hospital	34.10	38.50	33.30	27.30	28.60	27.90	41.70	31.90
Total		Count	44	13	18	11	7	61	12	166
		% Hospital	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

No p-value because of small number of cases within subgroups.

A number of different classes of antimicrobials have been mentioned and both overall institutional use and individual patients' use of antimicrobials increases the risk of MRSA. Many control measures need to be established to decrease the fast spreading of MRSA infections. MRSA has become a significant clinical pathogen due to three factors:

i. An intrinsic pathogenicity mediated by specified (and often unique) virulence factors.
ii. High frequency of nosocomial dissemination and acquisition within the healthcare environment.
ii. Limited therapeutic options.

Usually, the etiological diagnosis is dependent on isolation of the bacterium from the focus of infection or blood cultures. In some cases, access to the focus may be difficult or dangerous or cultural confirmation may be hampered by ongoing antimicrobial therapy. Also, serological assays for *S. aureus* infections are of limited value because of the insufficient diagnostic sensitivity and specificity.

Detection of MRSA by a variety of procedures has been associated with decreasing levels of infection and prevention of the transmission of these microorganisms (Loureino et al., 2000). It is clear that, the sooner a MRSA infection is diagnosed and the susceptibility to antimicrobial agents established, the sooner the appropriate therapy and control measures will be initiated. Laboratory diagnosis and susceptibility testing are crucial steps in controlling and preventing MRSA infections.

In our study, a total of 166 clinical isolates were recovered from blood (12 isolates), throat swabs (3 isolates), nasal swabs (2 isolates), wound (53 isolates), pus (9 isolates), sputum (15 isolates), urine (16 isolates) and skin (56 isolates) specimens, collected from different hospitals, different units and different infection sites. Isolation and identification were done according to Bergey's Manual of Systematic Bacteriology (Holt et al., 1993). Gram stain, growth on specific media and biochemical tests were the tools used for identification. The 166 isolates showed characteristics commonly known of *S. aureus*.

The antibiogram susceptibility pattern of these isolates was investigated to screen and determine the resistance profile for Oxacillin and other antibiotics. Upon performing antibiogram for staphylococcal isolates against Oxacillin discs, only 73 isolates representing 43.9% of the *S. aureus* isolates were Oxacillin resistant whereas resistance to other antibiotics was variable among the collected isolates and these results agree with results of Borg et al. (2007) who found that overall median MRSA proportion was 39% (interquartile range: 27.1 to 51.1%). This percentage was lower than that found by Idil and Aksöz which was 56.5%.

It was speculated that the prevalence of MRSA infections especially in health care units was rapidly increasing all over the world. Overall, the median MRSA proportion was 39% (interquartile range: 27.1 to 51.1%). The highest proportions of MRSA were reported by Jordan, Egypt and Cyprus, where more than 50% of the invasive isolates were methicillin-resistant (Borg et al., 2007). This was in agreement with the results shown in this study, since 73 out of 166 *S. aureus* isolates collected from different laboratories were methicillin resistant.

The present study shows that the prevalence rate of MRSA is higher in males (44/73), which represent 60.27% than in females (29/73) that represent 39.7%. In 2009, Rahman et al. (2011) have also reported a greater percentage of MRSA in males (58%) than in females (42%) in Peshawar. In Riyadh and Saudi Arabia, Baddour et al. (2006) have also reported a greater frequency of MRSA in males (64.4%) than in females (35.6%). Similarly, Tiemersma et al. (2004) have reported a higher rate of MRSA prevalence in males than females. However, India Sharma and Mall (2011) have reported greater rate of prevalence MRSA in females 14/25 (60.86%) than males 9/25 (39.13%).

MRSA rates were highest among clinical specimens from throat and nasal swabs (100%) followed by blood (58%), pus (55.5%), sputum (53.5%), surgical wound (52.8%) and Skin (33.9%). Our observations suggest that the throat and nose are important port of entry of MRSA. On the other hand, MRSA rates were lowest among clinical specimens from urine and skin. In a previous study by Wuduren et al. (1994), it was reported that the respiratory tract is an important port of entry.

Regarding the age of the patients, the occurrence of *S. aureus* was higher among patients in the age group 21 to 40 years. Mulla et al. (2007) also reported that *S. aureus* was commonly isolated from patients in age group 21 to 30 years.

The antibiotics used in this study are known to be efficacious against bacteria through different mechanisms. Penicillin, cephalosporin and carbapenems belong to the family of antibiotics called beta-lactams. These antibiotics work by disrupting the synthesis of the cell envelope in growing cells, inactivating the penicillin-binding proteins thus inhibiting the synthesis of bacterial cell wall.

Antibiotics such as Gentamicin, Tetracycline, Erythromycin and Clindamycin work by inhibiting protein synthesis in bacteria (Salyer and Whitt, 2005). Rifampin belongs to the family of antibiotics which prevents bacterial growth by inhibiting the RNA polymerase and halting the bacteria protein synthesis (Kohanski et al., 2010).

Vancomycin works much like penicillin as it inhibits the synthesis of bacterial cell wall. Another commonly prescribed antibiotic against MRSA is Trimethoprim sulfamethoxazole. This antibiotic inhibits the necessary cofactors for bacterial DNA synthesis (Salyer and Whitt, 2005; Kohanski et al., 2010). The antibiogram performed on methicillin-resistant *S. aureus* is further evidence on

the multi-resistance of MRSA, where the distribution of antibiotic resistance among *S. aureus* isolates, whether sensitive or resistant to Oxacillin was great.

Vancomycin and Teicoplanin showed excellent therapeutic activity against MRSA. No resistant MRSA strain was detected against Teicoplanin in this study, while 5.5% of the strains showed resistance to Vancomycin. Similar results have been obtained for Teicoplanin in previous studies (Perwaiz et al., 2007; Hussain et al., 2005).

The percentage resistance of the total *S. aureus* isolates (166 isolates) to Oxacillin and Erythromycin was 43.7 and 38.55%, respectively, which was higher than Rifampin (32.5%) and Vancomycin (2.4%), but less than Tetracycline (39%). In our study, the prevalence of multidrug resistance in *S. aureus* isolated from different clinical samples was investigated. The results demonstrated that, sensitivity to most of the antibiotics tested among MSSA was significantly higher than MRSA. Rifampicin resistance percentage was 76 and 1% for MRSA and MSSA, respectively; Amoxicillin clavulanic 84.7 and 5.4%; Ciprofloxacin 52.7 and 1%; Chloramphenicol 52.7 and 1%; Trimethoprim sulfamethoxazole 81.9 and 9.6%; Ofloxacin 97.22 and 5.37%; Tetracycline 83.33 and 5.44%; Erythromycin 76.3 and 6%; Gentamicin 83.33 3 and 22%; Vancomycin 5.5 and 0%; Teicoplanin 0 and 0%; and Oxacillin 100 and 0%. These results are in agreement with those reported by Quinn et al. (2002) who proved that, MRSA either produce potent toxins or resist to a wide range of antibiotics. Karska et al. (2010) also showed that MRSA is a multidrug-resistant microorganism and a principal nosocomial pathogen worldwide.

The research showed that, despite the fact that there was an increase in the rate of incidence of MRSA, 100% susceptibility to Teicoplanin and 94.5% to Vancomycin were revealed. Sensitivity of MRSA to Teicoplanin was the same as found by Abbadi et al. (2013), which was 100% .On the other hand, we found that sensitivity to Vancomycin was 94.5% while in Abbadi et al. (2013) it was 100%.

In the present study, MRSA was grouped into eight biotypes on the basis of antibiotic susceptibility profiles and 58.33% were resistant to at least seven types of the antibiotics. In addition, 20.5% of MRSA belonged to biotype 1 followed by biotype 4(17.8%), 5(16.4%), 2(15%), 3(12.3%), 6(9.5%), 8(5.4%) and 7(2.7%). All the biotypes were resistant to Oxacillin and sensitive to Teicoplanin.

Determination of susceptibility or resistance using phenotypic tests is a gold standard against which newer technologies are compared in terms of performance, cost and ease to use. Unfortunately, these methods have their limitations, which are not discriminating enough, highly dependent on growth conditions and mostly due to phenotypic differences between strains from the same species. For these reasons, methods based on molecular techniques have been developed to stop the spread of MRSA (Giammarinaro et al., 2005).

Early detection of MRSA from clinical specimens enables appropriate antimicrobial therapy with an extensive use of antibiotics over the last 50 years which has led to the emergence of bacterial resistance and the dissemination of resistance genes among pathogenic organisms (Malathi et al., 2009; Méndez et al., 2000).

Molecular methods for detecting resistance would impact more directly on patient care which would be valuable infection-control tools by rapid and accurate identification of staphylococci and their resistant types. Thus, it helps in confirming patients infected by resistant bacteria. Rapid and reliable detection of methicillin-resistant *S. aureus* (MRSA) is a prerequisite for the initiation of effective infection control measurements in order to restrict dissemination of this pathogen. Clearly, rapid detection of a specific resistance mechanism in a molecular test would initially allow clinicians, to avoid potentially inappropriate treatment options (Woodford and Sundsfjord, 2002).

One of the strategies to identify MRSA is multiplex PCR, developed for simultaneous amplification of methicillin resistance gene, *mecA* and one of the *S. aureus*-specific genes, such as coa, gyrA, holB (SA442), femA, femB or *nuc* gene, encoding for *S. aureus* specific thermonuclease. In a comparative study, Brakstad et al. (1989) used a multiplex PCR targeting *mec A* and *nuc* which report a 100% agreement with conventional identification methods. This approach is generally applied for the identification of subcultures of MRSA in routine diagnostic microbiological laboratory.

Other protocols were directed toward the specific detection of *S. aureus* and focused on amplification of genes found only in that species. Specific examples include the genes encoding nuclease (*nuc*) and Staphylococcal 16S rRNA (Mason et al., 2001).

MRSA is primarily mediated over production of PBP2a, an additional altered penicillin-binding protein with low affinity for beta-lactam antibiotics. The *mecA* gene and structural determinant encoding PBP2a is considered a useful molecular marker of putative methicillin resistance in *S. aureus* (Brakstad et al., 1992). Similarly, the *nuc* gene which codes thermonuclease is considered specific for *S. aureus* and was chosen as a target gene for *S. aureus* identification.

In the present study, all *S. aureus* isolates were *nuc* positive, while non *S. aureus* isolates were *nuc* negative, showing that the gene is available and specific which agrees with the results of Gao et al. (2011)

This study thus reveals the isolation and biochemical characterization of MRSA as well as detection of *mec-A gene* using PCR. MRSA sensitivity was checked against different antibiotics from different classes. Similar study was conducted by Abd El-Moez et al. (2011) in Egypt with results that agree with Hudson (1994) and Cookson (1998) who proved that, treatment of *S. aureus* infections

may be complicated by multiple antibiotic resistances and specific virulence factors, causing temporary or long-lasting carriage. The findings is also in line with Quinn et al. (2002) and Abd El-Moez et al. (2011), who branded MRSA of being a critical pathogen responsible for great morbidity and mortality especially among immuno-suppressed cases.

MRSA strains obtained from various clinical samples and collected from different hospitals, were characterized phenotypically by susceptibility testing and genotypically using RAPD-PCR and REP-PCR methods for each biotype. It was found that, there was no significant association between genotypes obtained from RAPD and REP-PCR. This agrees with the results obtained by Idil and Aksöz (2013).

Molecular analysis performed in the present study was necessary for assessing feasibility of the PCR approach for identification of *S. aureus* multiple drug resistant strain. Simultaneous identification of *S. aureus* and detection of methicillin resistance using PCR technique with its reproducibility and discriminating capacity has been shown to be an excellent technique for MRSA detection. PCR was applied for the amplification of a sequence of *mecA* and *nuc* genes using two primers targeted at each gene. All strains of *S. aureus* and MRSA clinical isolates were confirmed for the presence or absence of *mec*A and *nuc* genes by PCR. PCR results revealed that, 43.7% of the tested strains carried *mec-A* gene at 310 bp fragment while all of MRSA (100%) carried *mec*A gene. These results are higher compared to those obtained by Abd El-Moez et al. (2011), who had a percentage of 85.7% and reported that, identification of MRSA by drug susceptibility tests alone presented a serious problem because numbers of clinical *S. aureus* isolates have border line resistant to methicillin. Hence quick and accurate amplification for the detection of *mec-A* gene is necessary.

Additionally, detection of *mec-A* gene by PCR is extremely important for appropriate treatment of MRSA. These results agree with Vannuffel et al. (1995) who indicated that, MRSA has become a major nosocomial pathogen not only in tertiary care hospitals but also in chronic care facilities. The results further agree with those of Anderson and Weese (2006) who found that conventional identification of MRSA requires between 24 to 48 h after sampling and recommended rapid and sensitive method of identification as PCR for detection of *mecA* gene which codes for the drug resistant penicillin-binding protein 2a(PBP2a) or 2(PBP2). Klotz et al. (2005) reported an increase in the frequency of MRSA as an important causative agent of nosocomial infections worldwide, in spite of optimal hygienic conditions.

It was established that, there is no significant association between genotypes obtained from RAPD and REP PCR and antibiotypes profiles vice versa. In the cases of randomly amplified polymorphic DNA (RAPD) assay and a repetitive element sequence-based PCR (rep-PCR) analysis, the major drawback relates to insufficient standardization and low laboratory-to-laboratory reproducibility (Deplano et al., 2000; Van Belkum, 1994).

PCR-based techniques, identified for typing of many bacteria including Staphylococci have contributed significantly to recent advances in tracking the spread of these strains (Stephan et al., 2004). Randomly amplified polymorphic DNA (RAPD)-PCR is one of these methods, based on the use of short oligonucleotide primers with a random sequence which is designed without any prior sequence information concerning the target DNA.

As an alternative to this approach, amplification of highly conserved regions using primers leads to differentiation of the DNA fingerprints (Hyytiä, 1999). A technique called Repetitive element sequence based (REP)-PCR, in which primers derived from REP sequences were used, relies on the amplification of regions between non-coding repetitive sequences (repetitive DNA sequences) and is used for fingerprinting of isolates (Saulnier, 1993). REP PCR for MRSA isolates in this study produced bands with different intensities ranging from 0.15 to 3 kbp.

Another technique used for fingerprinting isolates is RAPD PCR (random amplification of polymorphic DNA) in which knowing the target DNA is not prerequisite. It produces a set of amplification products characteristic for each isolate (Saleki, 2002).

Therefore, implementation of preventive measures is seriously recommended for the control and prevention of increasing MRSA infections. “Cleanliness is next to Godliness”. Environment should be kept clean and hands should be regularly washed with soap and detergents.

Colonized/infected patients should be properly isolated/ treated. Vancomycin and Teicoplanin showed best chemotherapeutic activity against MRSA infections in this study, but their prescription should be kept limited, followed by antibiotic sensitivity tests.

Conclusion

MRSA is a serious problem facing hospitals all over Egypt which needs more attention. So, further studies were recommended as detection of virulence genes and require sequence analysis to methicillin resistance genes which make these strains capable of being multiple multidrug resistances.

Characterization of the antibiotic resistance and sensitivity pattern against different antimicrobials revealed that MRSA was highly sensitive to Teicoplanin (S% was 100%) and Vancomycin (S% was 97.5%), while antibiotic resistance other than Oxacillin was found in 43.9% of the isolates showing multiple drug resistance.

Classification of MRSA samples according to resistance pattern into eight biotypes (then RAPD and REP PCR) were done for a sample from each biotype to

check for genetic relatedness. Molecular techniques were found to be more rapid, highly sensitive and detect non-viable organisms while culture was found to be 100% specific for detection of MRSA.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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