Genotyping of MRSA by Coa and *Spa* Gene Polymorphism among Nasal Carriage of Health Care Workers and Patients Clinical Isolates at Ismailia General hospital

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Abstract
Staphylococcus aureus is a frequent cause of infections in both the community and hospital. Methicillin-resistant *Staphylococcus aureus* is a common nosocomial pathogen that causes the infections in different department of the hospital. Present study was conducted in Ismailia General Hospital to screen the nasal carriage of *Methicilin resistant Staphylococcus aureus* among health care worker who have contact with patients to help in control the spread of this pathogen from the health care workers to the patients. A total of 400 samples: 230 nasal swabs from healthy medical staff and worker and 150 patients' clinical specimens and 20 environmental swabs were subjected to bacteriological investigation following standard protocol. Sixty two isolates belong to *S. aureus* from 400 isolates. Out of twenty eight isolates were Methicillin-resistant *Staphylococcus aureus*. Antimicrobial susceptibility test was performed by disk diffusion method as per the CLSI guidelines. *MecA* gene was identified for isolates at 310bp. Genotyping by PCR-RFLP of *Coa* and *Spa* gene was identified for MRSA isolates has a special role in assessing the relatedness of MRSA isolates and its control.

Introdution

S. aureus has been recognized as an epidemiologically important pathogen.*S. aureus* infections in hospitalized patients have severe consequences ranging from benign superficial skin infections to life threatening infections like Endocarditis, Pneumonia, Meningitis and Septicemia.(1) Methicillin was introduced to treat *S. aureus* infections, but in 1961, *S. aureus* isolates that had acquired resistance to methicillin (methiciliin-resistant *S.aureus*) were reported.(2) *Meticillin-resistant Staphylococcus aureus* (MRSA) infections, are a long-lasting problem of health care associated infection. A widespread of strains and therapeutically difficult infections are associated with increased mortality and morbidity.

The association between *S aureus* nasal carriage and staphylococcal disease was first reported by

Danbolt in1931, who studied furunculosis. [3] Colonization of *S. aureus* at multiple body sites occurs; anterior nares are the most frequent carriage site. Nasal colonization can be an indicator of high risk for subsequent infection as MRSA is a well-known risk factor whenever *S. aureus* colonization is present. Health care workers are an important reservoir of *S. aureus* and several studies has observed the rate of nasal carriage amongst HCWs ranging from 16.8% to 56.1%.[4] Colonised HCWs may develop infection or may remain asymptomatic, but they are a potential source to the patients and other HCWs.[5] So identification of health care workers (in outbreak settings) colonized with MRSA, combined with hand hygiene and other precautions have been shown to be effective in reducing the transmission and controlling the spread of MRSA.

Typing techniques have been developed to discriminate between the related and unrelated MRSA strains.[6] Traditionally, phenotypic methods including antibiotic susceptibility testing and bacteriophage typing were used to distinguish between MRSA isolates. [7]

Genotyping methods by PCR - restriction fragment length polymorphism (RFLP) for two species-specific proteins, coagulase *(coa*) and protein A (*spa*), was support epidemiological typing. The coagulase protein is an important virulence factor of MRSA. *Coa* has a polymorphic repeat region that can be used for typing of MRSA isolates.[8] Its discriminatory power depends on the variability of the region containing the 81 bp tandem repeats at the 3' coding region of the gene. It differs both in the number of tandem repeats and the restriction sites among different isolates.[9] Protein A is a surface protein known to carry polymorphic regions. The *spa* gene harbors a number of functionally distinctive regions, called X region,[10] which is polymorphism widely used as a base for genotyping methods, the discriminatory power of which allows the recognition of small differences among genetically related strains and enables effective epidemiological investigation.[1] It includes a varying number of highly polymorphic 24-b prepeats. The aim of this study was to evaluate PCR-RFLP as a molecular typing technique for MRSA strains on the basis of protein A and coagulase gene polymorphisms and to verify their ability in assessing the relatedness of MRSA isolates between nasal carrier of healthy care worker and clinical specimens of patients.

Material and methods

1. Sample Collection and Bacterial Species Identification

Atotal of 400 isolates : 150 patients clinical specimens were collected fr om different department in Ismaillia

General Hospital and 230 nose swab specimens from the mentioned hospitals staff were collected during the period from first of January till the end of March 2011. Using microbiological standard met hods including, catalase, coagulase and manitol fermentation on manitt ol salt agar, the sixty two isolated S. aureus was confirmed and tested for oxacillin resistance by oxacillin disk diffusion susceptibility methods according to CLSI 2012 . Out twenty eight isolates were considered MRSA as presented.

Susceptibility testing:

The antibiotic sensitivity of 28 MRSA isolates to the antimicrobial agents was carried out by the disk diffusion method according to CLSI2012 on Muller-Hinton agar were used Ampicillin (AMP,10 µg), Amoxicillin-Clavulanic acid (AMC, 20/10 µg), Vancomycin (VA, 30 µg), Gentamicin (GN, 10 µg), Erythromycin (E, 15 µg), Cefotaxime (CTX, 30 µg), Clindamycin (DA, 2 µg), Sulfamethoxazole -Trimethoprim (SXT, 1.25/23.75 µg), Imipenem (IPM, 10 µg), Rifampicin (RD, 5 µg), Linezolid (LZ, 2 µg) and chloramphenicol (C, 30 µg) were obtained from Oxoid, Hamphsire England.

2- Multiplex PCR to detect *mecA* gene and IS432[11]

Twenty eight MRSA isolates were tested for the presence of the 310 base pair (bp) PCR product of mecA gene, using the following primers:

Forward (5′-TGGCTATCGTGTCACAATCG -3′), reverse (5′-CTGGAACTTGTTGAGCAGAG -3′). And presence of the 444 base pair (bp) PCR product of IS432 gene as a positive control for the PCR, using the following primers: forward (5′-AGGATGTTATCACTGTAGCC -3′),

Reverse (5′-GATGTACAATGACAGTCAGG -3′). Ten micro liters of DNA samples was added to 90 µL of PCR mixture each deoxynucleoside triphosphates, 100 pmol of each primer, and 1.25 U of Taq DNA polymerase. After an initial denaturation step (3 min at 92^0C), 30 cycles of amplification were performed as follows: denaturation at 92^0C for 1 min, annealing at 56^0C for 1 min, and DNA extension at 72^0C for 1 min with an increment of 2 s per cycle. The reaction was achieved with a final extension at 72^0C for 3 min. Amplification was carried out in a Gene E thermal cycler (Techne, Cambridge, United Kingdom).

2. Polymerase Chain Reaction for De tection of *Coa* and *Spa* Genes

(A)- PCR for *Coa* Gene Detection. [9] Using the following primers:

Forward (5′-CGA GAC CAA GAT TCA ACA AG-3′), reverse (5′-AAA GAA AAC CAC TCA CAT CA-3′). Which were designed to amplify the 3′ end hyper variable

region containing 81bp tandem repeats of *Coa* gene. The amplification reaction consisted of :- Initial denaturation step at 94°C for 5min, followed by 30cycles of denaturation at 95°C for 30sec, annealing at 55°C for 45sec, extension at 72°C for 2min, followed by final extension at 72°C for 7min.

-RFLP of *Coa* Gene PCR Products.[9]

Depending on the number of 81bp repeats, a strain analysis of PCR RFLP products was performed with *HaeIII* restriction enzyme (New England BioLabs, Frankfurt, Germany), where 10µL of PCR product of *Coa* gene was incubated with 6U of the enzyme at 37°C for 1h 45min in a water bath.

(B)- PCR for *Spa* Gene Detection[12]

Using the following primers:

Forward (5′-ATC TGG TGG CGT AAC ACC TG-3′), Reverse (5′-CGC TGC ACC TAA CGC TAA TG-3′). Which were designed to amplify the polymorphic X region that contains a variable number of 24bp tandem repeats of the *Spa* gene coding for protein A. Amplification reaction consisted of: Initial denaturation step at 94°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, extension at 72°C for 3 minutes, followed by final extension at 72°C for 5 minutes.

-RFLP of spa Gene PCR Products.[9,12]

Five µL of each *Spa* gene amplicon and 10 units of *HaeII* restriction enzyme (New England BioLabs, Frankfurt, Germany) were incubated at 37°C for 3 hours. The PCR products and restriction digest fragments were detected by electrophoresis in 2% agarose gel.

The interpretation criteria for identifying different strains were a single band difference. Unique PCR-RFLP patterns were assigned a genotype.

Results:

Identification of isolates

1-The Results of identification of isolates from Patients

Among 150 patients clinical specimens from different hospital department were 26 positive *S. aureus* (17.3% of total sample) isolates and out 13 isolates were considered MRSA (50% of *S. aureus*). The higher percent of patients with positive MRSA in NICU department (100%), then Orthopedic , ICU and burn unit (50%) , MRSA in surgery (44.4%). There was high number of sample type with positive MRSA in blood samples (66.6%), then urine (50%), after that pus and sputum (40%).

2. The Results of identification of isolates from Hospitals Staff

Among hospitals staff (doctors, nurses and servants) 230 specimens was collected, were 34 positive *S. aureus* isolates (14.8% of total sample) and out 14 isolates were considered MRSA (41.1% of *S. aureus*). The highest number of

workers from total department with positive nasal carriage MRSA in the Anesthesia (25%), Surgery (17%), ICU (13.3%) and Kitchen staff (10%).

3. The Results of identification of isolates from Environmental swab

From 20 environmental swabs was collected, only 2 isolates positive *S. aureus* (10% of total sample) and out one isolate was considered MRSA (45% of *S. aureus*).

Susceptibility testing

The twenty eight phenotypically identified for the MRSA isolates were tested for their susceptibility to 12 antimicrobial chemotherapeutic agents by disk diffusion method according to CLSI (2012) are shown in table (1) .

All isolates were resistant to Ampicillin, Cefotaxime and AMC. However, all MRSA isolates were susceptible to Linezolide (100%) and Rifampin (100%) then Imipenem (92%) followed by Vancomycin (89%) and Clindamycin (82%) SXT (61%), Erythromycin and Gentamicin (50%), Chloramphenicol (46%) .

Detection of *mecA* Gene for confirmation of MRSA

Multiplex PCR which used for amplification and detection of *mecA* genes of MRSA and *IS*431 , (92.8%) give band at 310bp of mecA gene from total 28 MRSA isolates. IS431 gene give band at 444bp, shown in figure(1).

Genotyping of MRSA isolates

A-Coagulase Gene Typing

The isolates were primarily classified according to the number and size of *coa* amplicon and secondarily on the number and sizes of the restriction fragment.

PCR amplification of the *coa* gene yielded products of 7 sizes, ranging from 567 to1134 bp (567, 648, 729, 810, 891, 972, 1134). Electrophoresis analysis generated 2 different band types (coI, coII) and 6 types of pattern . The majority of MRSA strains showed single band: (18/28=64%). The most common PCR *coa* gene product shown was the 810bp band size product (Table 2, Figure2).

• *Coa*-RFLP Typing Using HaeIII Restriction Enzyme

Restriction digestion was performed on the amplified coagulase PCR product with *HaeIII* . The bands produced were multiples of 81, divided into 5 band classes of *Coa*I. Five distinct RFLP banding pattern (digested as a, b, c, d, e) and *Coa*II , 12 sub types designated as (*Coa*Ia1, 2/*Coa*Ib1, 2, 3, 4, 5/ *Coa*Ic/ *Coa*Id1, 2/*Coa*Ie and *Coa*II) were obtained. The majority of strains (11/28=39%) belonged to RFLP banding pattern CoII (Table 2, Figure2). DI value of *Coa*-RFLP typing was 0.81.

B- *Spa* Gene Typing

The isolates were primarily classified according to the number and size of *Spa* amplicon and secondarily on the number and sizes of the restriction fragment.

The size of the PCR product ranged from 210 to 1512 bp (216, 360, 840, 984, 1272 , 1296, 1320,1464 and 1512). These PCR product generated 3 major type (SI- SII- SIII) based on number of band and 11 subtypes based on molecular size (SI a ,b ,c ,d ,e/SII a ,b ,c ,d /SIII a ,b) as shown in (Table3, Figure 3).

Spa-RFLP Typing Using *HaeII* Restriction Enzyme

Restriction digestion was performed on the amplified *spa*-PCR product with *HaeII* .The bands observed to be multiples of 24. Thirteen distinct banding patterns as (SI a, b, c, d, e / SII a (a1, a2), b (b1, b2), c, d/ SIII a, b). Most strains belonged to pattern $SIIa_1$ then pattern SIb (Table3, Figure 3). DI value of spa-RFLP typing was 0.86.

Correlation between Antibiotype, *Coa*-RFLP typing and *Spa*-RFLP typing:

The studied isolates could be distinguished from each other based on 3 typing methods (antibiotyping, *coa* – RFLP pattern and *spa*-RFLP pattern).

Calculating the discrimination index for the 3 typing methods used in this study, revealed that it was 0.85, 0.81, and 0.86 for antibiotyping , *coa*– RFLP typing, and *spa*– RFLP typing respectively. Highest discriminatory index was provided by combination of all methods (0.86). Combination of all typing methods could differentiate MRSAisolates into 17 groups, the commonest group1: (5 isolates), of type (antibiotype 2- *coa* pattern CoII -*spa* pattern SIb) followed by group 2 :(4 isolates), of type (antibiotype 3-*coa* pattern CoII - *spa* pattern$SIIa_1$) and group 3 :(2 isolates), of type (antibiotype 6-*coa* pattern CoIb1 - *spa* pattern SId) . These groups considered as epidemic, (epidemiologically related i.e. association found in terms of time & location). Seventeen isolates were classified as sporadic (epidemiologically unrelated i.e. no association found in terms of time & location).

Correlations between various typing methods are presented in tables (12 and1 3).

Studying the 5 isolated epidemiologically related strains from an outbreak in Surjury department (2 doctors staff and 3 patients (wound sample), they were found to be homogeneous using the 3typing methods, all isolates were of (antibiotype 1- *coa* pattern F - *spa* pattern II).

The 4isolated epidemiologically related strains from ICU: 1 nasal swap of ICU doctor, 3 deffrint sample of patient: 1 sputum, 2 blood.

The 2 isolated epidemiologically related strains from NICU: 2 blood sample of patient from NICU.

Table (1) *Antibiotic susceptibility of MRSA isolates to different antimicrobial chemotherapeutic agents*

ANTIBIOTIC DISC	Number and percentage of isolates					
	R		I		S	
	NO.	%	NO.	%	NO.	%
Oxacillin (ox)	28	100%	0	0	0	0
Ampicillin (AMP)	28	100%	0	0	0	0
Gentamicin (CN)	8	28%	8	28%	14	50%
Clindamycin (DA)	4	15%	1	3%	23	82%
Erythromycin (E)	14	50%	-	-	14	50%
Trimethoprim/ Sulphamethoxazol (SXT)	7	25%	4	14%	17	61%
Amoxycillin/ Clavulanate (AMC)	24	86%	4	14%	-	-
Imipenem (IPM)	2	7%	0	0	26	92%
Rifampicin (RD)	0	0	0	0	28	100%
Linezolide (LZ)	0	0	0	0	28	100%
Cefotaxime (CTX)	20	75%	8	28%	-	-
Vancomycin (VA)	0	0	3	11%	25	89%
Chloramphenicol (C)	7	25%	8	29%	13	46%

Table (2): *Typing of MRSA isolates based on PCR-RFLP of Coa gene*

Coa band types	*Coa* gene types (6)	*Coa subtype*	Size of PCR product (approximate bp)	Size of HaeIII fragments (approximate bp)	Isolate serial No.	Total Isolate No.
CoI(1band)	CoIa	$CoIa_1$	648	(567, 81)	12	1 (3%)
		$CoIa_2$	648	(486, -)	24	1
	CoIb	$CoIb_1$	810	405, 324	5,6,7,17,18	5 (17%)
		$CoIb_2$	810	567, 243	9,10,20	3 (10%)
		$CoIb_3$	810	(405, 243, 81)	15	1
		$CoIb_4$	810	(no band)	16	1 1
		$CoIb_5$	810	(405, 162, (81))	2	
	CoIc	CoIc	891	(729, 81), (567, 324)	4	1
	CoId	$CoId_1$	972	(567, 405)	3, 14	2
		$CoId_2$	972	(648, 324)	11	1
	CoIe	CoIe	1134	567, 324, 243	13	1
CoII(2 band)	CoII	CoII	810+ 567	(405, 324) + 567(un cut)	8, 19, 22, 23 , 25, 26, 27, 28, 29, 30, 31	11 (39%)

Table (3): Typing of isolates based on PCR-RFLP of *spa* gene

Spa band type (3)	*Spa* gene (11) types	(13) subtypes	Size of PCR Product (approximat bp)	Size of HaeII fragments (approximate bp)	Total isolate no.(%)	Isolate serial no.
S1 (1 band)	S1a	S1a	984	(960) (696, 288)	1	20
	S1b	S1b	1296	(1224) (767, 456)	5	13, 29, 30, 27 26, 28
	S1c	S1c	1320	(1224) (770, 550)	1	22
	S1d	S1d	1464	(888, 456)	2	17, 18
	S1e	S1e	1512	(Partial digestion) -	1	3
SII (2 bands)	SIIa	$SIIa_1$	1296 360	1224 744, 456 288	9	2, 5, 9, 16, 19, 23, 25, 31
		$SIIa_2$	1296 360	768, 456 288.	1	14
	SIIb	$SIIb_1$	1296 840	744, 528 456, 288	2	4, 6
		$SIIb_2$	1296 840	888, 456 528, 288	3	7, 8, 15
	SIIc	SIIc	1272 216	744, 528 210	1	12
	SIId	SIId	1320 360	1224 888, 456 768, 528 288	1	24
SIII (3 bands)	SIIIa	SIIIa	1080 936 360	888, 288 936 360	1	10
	SIIIb	SIIIb	1296 984 360	840, 456 528, 360 288	1	11

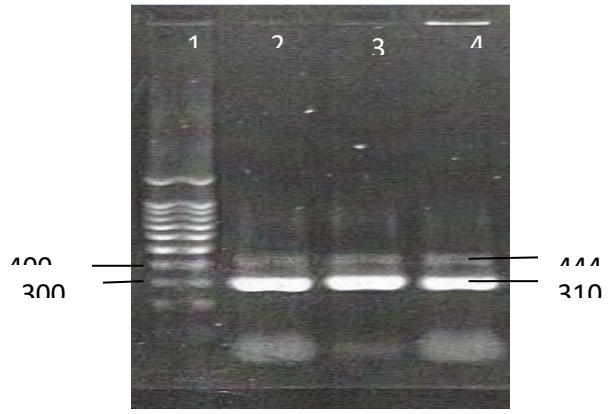

Figure 1: 2% agarose gel electrophoresis analysis of Multiplex- PCR amplification products of mecA gene of 310 bp and IS431gene of 444 bp, extracted from *S. aureus*. Lane1: lanes 2,3: methicillin-resistant *S. aureus* (MRSA); lane 1: DNA molecular size marker (100 bp ladder).

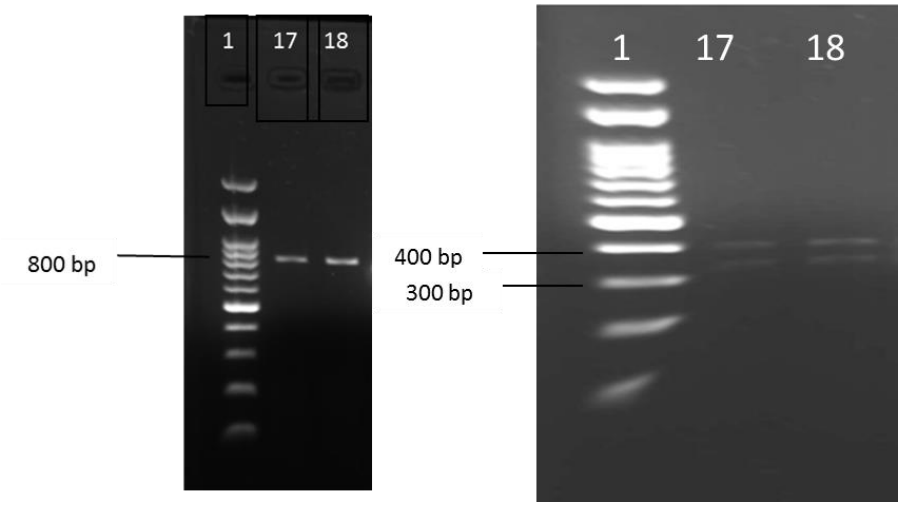

Figure 2: Representative 2% agarose gel electrophoresis of *Coa* gene *HaeIII* restriction enzyme digestion PCR products, where lane 1 is DNA molecular size marker (100 bp ladder), (a) isolate 17, 18 showing single band *Coa* gene PCR products and (b) after cutting with *HaeIII* restriction enzyme, isolates 17, 18 give two bands of PCR-RFLP product.

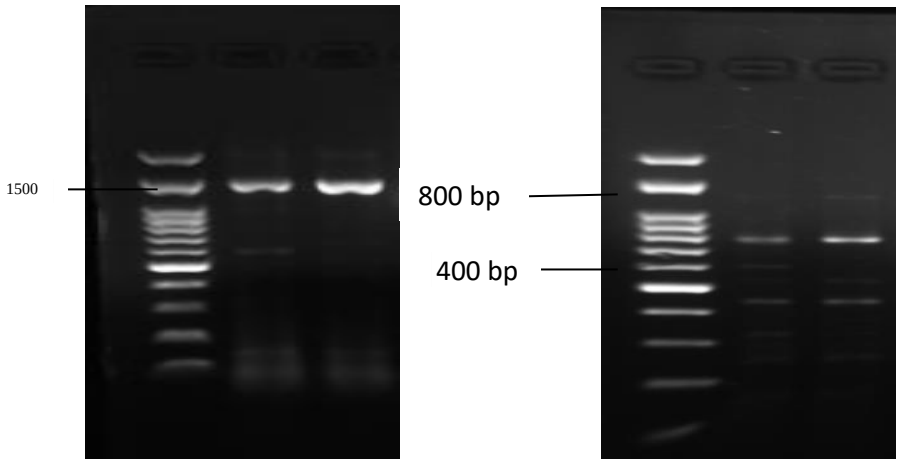

Figure 3: Representative 2% agarose gel electrophoresis of *Spa* gene PCR products where lane1 is DNA molecular size marker (100 bp ladder). (a) Isolates 2 and 3 showing single band PCR products, (b) after *HaeII* restriction enzyme digestion PCR products and its corresponding 2 bands *HaeII* restriction digestion products.

Discussion

The prevalence of methicillin resistant *S. aureus* and inhibition of these infections and determination of spreading center in hospitals are definitely important subjects, the carriers of methicilln resistant strains have the original role in bacteria transmission.[13] The current study showed methicillin resistance in 50% of *S.aureus* clinical isolates and 41.1% of *S. aureus* hospitals Staff`s.

Various studies have shown different results of bacterial resistance and carriers which may be related to various bacterial detecting methods. Rashwan *et al*. (2006)[14] showed 55% methicillin resistant among 110 *S. aureus* clinical isolates .

Mounir *et al*. (2013)[15] showed 20% MRSA among 150 *S. aureus* clinical isolates.

Our study of MRSA nasal carrier not far away from Saroj *et al*. (2013)[16] study who showed 21 (53.8%) MRSA among 39 *S. aureus* nasal carrier Staff.

Partha *et al*. (2015)[17] study of 183 were nasal carriage of *S. aureus* in their anterior nares. Out of these, 39 (21.47%) HCWs were detected as MRSA nasal carriers.

Kogekar *et al*. (2015)[18] showed 16(53.33%) nasal carriage of MRSA among 30 S. *aureus* staff carriage at various hospital department.

Genotyping by PCR-RFLP is a preliminary screening method for the epidemiological study of nosocomial infection caused by MRSA.

In the present study PCR-RFLP of *Coa* using *HaeIII* was conducted to 12 distinct RFLP banding pattern, this result not far away from Walker *et al.* (1998)[19] showed the *HaeIII* digestion of the *coa* gene PCR products yielded 13 different RFLP patterns and also Lawrence *et al*. (1998)[20] study that showed after digestion with *Haelll*, 17 RFLP patterns could be distinguished .

Montesinos *et al*. (2002)[21] study in four patterns of amplified *Coa* gene (co1to4) detected by using both *AluI* and *HaeIII* restriction enzymes.

Typing based on RFLP-PCR product of *Spa* gene using *HaeII* restriction enzyme was used in our study generated 13 distinct banding pattern. This result not far away from study of Omar *et al.* (2014)[22] showed typing and *HaeII* Restriction digestion give12 subtypes designated

In Shakeri *et al*. (2010)[23] study after digestion by *HaeII*, three patterns with 2, 3 and 4 fragments were observed.

The outcome of this study and the other similar researches, show that spa and coa typing are suitable methods for MRSA isolates typing because it is easy to use and interpret them, and that these methods can be useful in infection source detection and its control especially in epidemic situations.

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الملخص العربي

لمكورات العنقودية الذهبية هي سبب متكرر للعدوى في كل من المجتمع والمستشفى. المكورات العنقودية المقاومة للميثيسيلين المكورات العنقودية الذهبية هي مسببة للأمراض المشيمية الشائعة التي تسبب الالتهابات في أقسام مختلفة من المستشفى. أجريت الدراسة الحالية في مستشفى الإسماعيلية العام لفحص نقل الأنف من ميثيسيلين مقاومة المكورات العنقودية الذهبية بين العاملين في مجال الرعاية الصحية الذين لديهم اتصال مع المرضى للمساعدة في السيطرة على انتشار هذا الممرض من العاملين في مجال الرعاية الصحية للمرضى. ما مجموعه 400 عينة: 230 مسحات الأنف من الموظفين الطبيين الصحيين والعاملين و 150 مريضا العينات السريرية و 20 مسحات البيئية تعرضت للتحقيق البكتريولوجي بعد بروتوكول القياسية. وهناك ستون عزلة تنتمي إلى S. أوريوس من 400 عزلة. من بين ثمان وعشرين عزلة كانت مقاومة للميثيسيلين المكورات العنقودية الذهبية. تم إجراء اختبار الحساسية المضادة للميكروبات بطريقة نشر القرص وفقا للمبادئ التوجيهية كلسي. تم التعرف على الجين الميكا لعزل في bp310. تم التعرف على التنميط الجيني بواسطة ير-رفلب لجين كوا وسبا لعقاقير مرسا لها دور خاص في تقييم علاقة عزلات مرسا ومكافحتها.