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Research Article

Methicillin Resistant *Staphylococcus aureus* Antibiotic Profile and Genotypes in Critically Ill Neurosurgery and Medical Oncology Patients

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Received: February 25, 2016; **Published:** April 02, 2016

Abstract

Background: Methicillin resistant *staphylococcus aureus* (MRSA) is a serious health problem. Infections caused with such microorganisms increase hospital stay, cost of treatment and incidence of complication. Epidemiological studies are an important tool help in preventing and controlling infection. Both antibiotic susceptibility tests and genotyping are important pillars to perform epidemiological studies.

The aim of this study was to detect antibiotic susceptibility patterns of MRSA strains common in two groups of critically ill patients in neurosurgery intensive care unit and medical oncology unit, Faculty of Medicine Zagazig University.

Methods and materials: 36 MRSA isolates were isolated from either blood or CVCs. MRSA identification was done using both conventional methods and detection of mecA gene as the gold standard for identification of MRSA strains. Antibiotic sensitivity testing was done using Vitek2, genotyping of coa gene and spa gene was done using PCR and PCR-RFLP respectively. One vancomycin resistant strain was found and was genotyped using sequence specific primers.

Results: MRSA strains showed 100% resistance to benzyl penicillin, 80%resistannt to erythromycin, 50% resistant to tetracycline, 40% resistant to gentamycin and 20% resistant to clindamycin. However, they were 100% sensitive to ciprofloxacin, levofloxacin, moxifloxacin, Quanuprisitin/dalfopristin, nitrofurantoin, trimethoprim/sulphamethoxazole and rifampicin. One strain was resistant to vancomycin MIC ≥32 and Linozolid MIC ≥ 8. 27 isolates showed coa band at 570bp and 7 showed 630bp, spa genotyping by PCR-RFLP yielded three different genotypes. Van A and Van B was found to mediated vancomycin resistant in the detected VRSA strain.

Conclusion: Multi-resistant MRSA is a progressively increasing problem, emergence of vancomycin and linozolid resistance in our hospitals is an alarming sign that need rapid and prompt intervention. We did not detect any statistically significant relationship between antibiotic sensitivity pattern and studied genotypes.

Keywords: *MRSA; Antibiotic sensitivity testing; genotyping; Vitek2*

Abbreviations: MRSA: methicillin resistant *Staphylococcus aureus*; coa gene: coagulase gene; spa gene: staphylococcus protein A gene; van: Vacomycin; MIC: minimal inhibitory concentration

Introduction

Methicillin resistant *staphylococcus aureus* (MRSA) is one of the most important nosocomial infections especially in critically ill patients. MRSA infections are associated with prolonged hospitalization, increased economic cost, increased morbidity and mortality [1,2].

Antibiotic resistance is a progressively growing problem worldwide. For those infected with MRSA, antibiotic choices are limited, the primary option is vancomycin. However, the increased usage of glycopeptides for treating MRSA, highlight the fact that resistance against this group of drug will soon emerge. New antibiotic regimens are thus needed [3].

Genotyping is an essential element in epidemiological analyses that help in designing prevention and control programs for outbreaks [2].

The aim of this study is to detect the most common MRSA genotypes in Neurosurgery intensive care unit and Medical Oncology Department, Zagazig University hospitals and to detect their antibiotic susceptibility patterns.

Methods and Materials

We collected blood samples from peripheral blood and central venous catheters (CVCs) specimens from patients suffering from clinical signs of bacteraemia attending Neurosurgery Intensive Care Unit and Medical oncology department, Zagazig University Hospitals for at least three days in the period from January 2014 to March 2015.

Identification of MRSA isolates

All isolates were identified as *Staphylococcus aureus*, according to the standard microbiology techniques.

mecA Screening: *S. aureus* resistance to methicillin was determined on the base of presence of *mecA* gene, using specific primers (Table 1); the amplicon size was 310 bp *ATCC 33591* was used as a reference strain. Strains were also tested against both oxacillin and cefoxitine using Vitek2 system.

Antibiotic Susceptibility Testing using Vitek2

Identification was performed with the Vitek 2 (bioMérieux Inc., Durham, NC) system using GP ID cards. The quality control (QC) strains tested with each run were *S. aureus* ATCC 29213.

Genotyping of isolates

Detection of coa gene and RFLP for spa genes: All isolates were tested for coa and spa gene then further PFLP was done for spa PCR product.

Van genes: One isolate was vancomycin resistant; this strain only, was tested for the presence of vancomycin resistant genes (vanA, vanB, vanC1, vanC2/C3, van X, vanS, vanH and vanR).

For all PCR reactions, oligonucleotide Primers used were supplied from (Metabion-Germany) are listed in table (1).

PCR amplification reaction: Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

Analysis of the PCR Products

The products of PCR were separated by electrophoresis on 1% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. 100 bp DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

RFLP of spa gene: Preparation of restriction Master Mix according to Thermo FastDigest® HindIII Cat. No. FD0504. In 20 ul reaction volume the following were added, 1ul of HindIII enzyme, 2ul of 10X FastDigest Green buffer, 10 ul of PCR product and 1 ul of water. The reaction was done at 37°C for 20 min. Then thermal inactivation of the enzyme was done at 80oC for 10 min. Then Product was electrophoresed using 1% agarose.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
vanA	CATGAATAGAATA-AAAGTTGCAATA	1030	94°C 5 min.	94°C 30 sec.	55°C 45 sec.	72°C 1 min.	72°C 10 min.	[4]
	CCCCTTTAACGCTA-ATACGATCAA							
vanB	GTGACAAACCGGAG-GCGAGGA	433	94°C 5 min.	94°C 30 sec.	55°C 45 sec.	72°C 45 sec.	72°C 10 min.	
	CCGCCATCCTCCTG-CAAAAAA							
vanC1	GGTAT-CAAGGAAACCTC	822	94°C 5 min.	94°C 30 sec.	54°C 45 sec.	72°C 45 sec.	72°C 10 min.	
	CTTCCGCCATCATAGCT							
vanC2/C3	CGGGGAAGATGGCAG-TAT	484	94°C 5 min.	94°C 30 sec.	54°C 45 sec.	72°C 45 sec.	72°C 10 min.	
	CGCAGGGACGGT-GATTTT							
vanX	ATGGAAATAGGATT-TACTTT	600	94°C 5 min.	94°C 30 sec.	50°C 45 sec.	72°C 45 sec.	72°C 10 min.	[5]
	TTATTTAAC-GGGGAAATC							
vanS	AACGACTATTCCAAAC-TAGAAC	1094	94°C 5 min.	94°C 30 sec.	60°C 45 sec.	72°C 1 min.	72°C 10 min.	[6]
	GCTGGAAGCTC-TACCCTAAA							
vanH	ATCGGCATTACTGTT-TATGGAT	943	94°C 5 min.	94°C 30 sec.	62°C 45 sec.	72°C 45 sec.	72°C 10 min.	
	TCCTTTCAAAATC-CAAACAGTTT							

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
vanR	AGCGATAAAATACT-TATTGTGGA CGGATTATCAATGGT-GTCGTT	645	94°C 5 min.	94°C 30 sec.	62°C 45 sec.	72°C 1 min.	72°C 10 min.	[6]
coa	ATA GAG ATG CTG GTA CAG G GCT TCC GAT TGT TCG ATG C	Four different types of bands may be detected 350 bp 430 bp 570 bp	94°C 5 min.	94°C 30 sec.	55°C 45 sec.	72°C 45 sec.	72°C 10 min.	[7]

Table 1: *Primers sequences, target genes, amplicon sizes and cycling conditions.*

Statistical analysis: Was performed using the Statistical Package for the Social Sciences for Windows (version 17.0; SPSS, Chicago, IL, USA). Data were expressed using number and percentage. Data was analyzed using chi-square and P value was detected at <0.05 significant level.

Results

According to our results, 147 blood and CVP samples were collected, 82 samples were collected from Neurosurgery intensive care unit and 65 were collected from Medical Oncology Department. Out of those 147 specimen 36 (24.5%), were found to contain MRSA as diagnosed by conventional methods and mecA gene. PCR detection of MRSA strains showed a band of 310bp (Figure 1). When these strains were tested for oxacillin sensitivity, 2 (6%) showed sensitivity to oxacillin while all strains were resistant to cefoxitine.

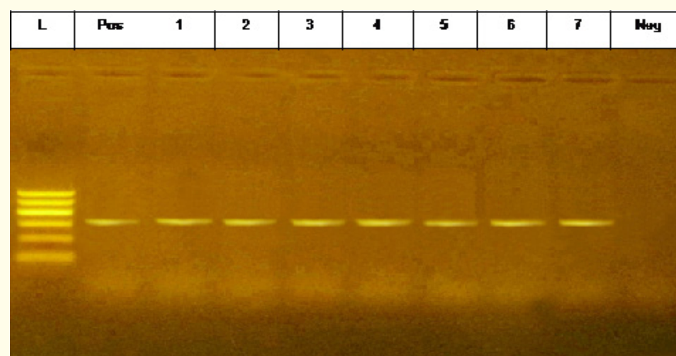

Figure 1: *PCR based detection of mecA gene positive showing bands at 310 bp.*
- L=100 bpDNA ladder. - Pos. = mecA positive control (ATCC 33591). - Lanes from 1 to 3: Products of mecA genes from isolates.

Antibiotic sensitivity results: The result of antibiotic sensitivity testing as detected with Vitek 2 system is shown in table 2 The result of antibiotic sensitivity testing as detected with Vitek 2 system is shown in table 2.

Antibiotic	*S (no (%))	***R (no (%))	MIC range	MIC 50	MIC 90
Benzylpenicillin	----------	36(100%)	0.25 ->0.5	≥ 0.5	≥ 0.5
Gentamycin	22 (61%)	15(40%)	<0.5->16	≤ 1	16
Ciprofloxacin	36 (100%)	------	≤ 0.5	≤ 0.5	≤ 0.5
Levofloxacin	36 (100%)	-------	≤ 0.12-0.25	≤ 0.12	0.25
Moxifloxacin	36 (100%)	-------	≤ 0.25	≤ 0.25	≤ 0.25
Erythromycin	7 (20%)	29(80%)	≤ 0.25-16	<4	16
Clindamycin	29(80%)	7 (20%)	≤ 0.25-1	≤ 0.5	≤ 1
Quinupristin/Dalfopristin	36 (100%)	--------	≤ 0.25	≤ 0.25	≤ 0.25
Linezolid	35(97%)	1(3%)	1-≥8	1	2
Vancomycin	35(97%)	1(3%)	0.5 -≥32	≤ 0.5	1
Tetracyclin	18 (50%)	18 (50%)	≤1 - ≥ 16	≤1	8
Nitrofurantoin	36 (100%)	-------	≤16	≤ 16	≤16
Rifampicin	36 (100%)	-------	≤ 0.5	≤ 0.5	≤ 0.5
Trimthoprim/Sulfamethoxazole	36 (100%)	-------	≤10-20	10	20

Table 2: *Antibiotic sensitivity test as detected by the Vitek.*
**S susceptible, **I intermediate, ***R Resistant.*

Inducible clindamycin resistance were also tested, 22 isolates were found to be clindamycin sensitive and in the same time were Erythromycin sensitive, 100% of these isolates showed inducible clindamycin resistance.

***Coa* gene genotyping:** When we genotyped MRSA isolates according to the coa gene, two different genotypes were obtained. 27 (75%) isolates showed a band of 570 bp and the 9(25%) isolates showed the band of 630 bp (Figure 2).

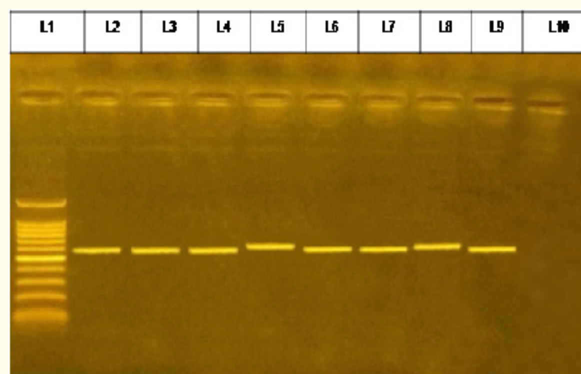

Figure 2: *Agarose gel electrophoresis of coa gene PCR products. Bands were observed at 570 and 630 bp size.*
L1: (1500-100) bp DNA ladder. L2, 3,4,6,7and 9 show bands at 570bp. L5 and 8 show bands at 630bp. L10: Negative control.

***Spa* gene PCR:** When MRSA isolates were subjected to spa gene detection, spa gene was detected in 34/36 (94.4 %). However, we did not find the spa gene band in two isolates (5.6%). Absence of spa gene PCR products in 2 isolates is considered a separate type. The size of the detected band was 1164 bp in all tested isolates (Figure 3).

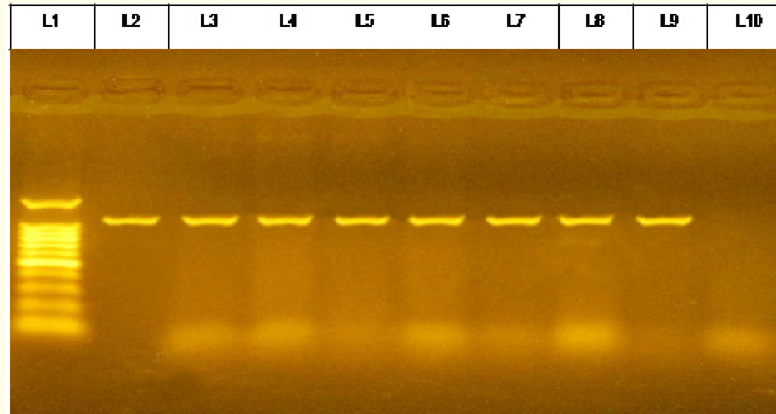

Figure 3: *Agarose gel electrophoresis of spa gene PCR products.*
L1: 1500-100bp DNA ladder. L2- 9: spa gen bands at 1164bp. L10: Negative control.

***Spa* gene RFLP:** When amplicon of 34 spa gene were subjected to digestion by Hea II digestion enzyme. Thirty three showed bands at 240 and 690 bp (Figure 4), one PCR product of spa gene were not digested by Hae II.

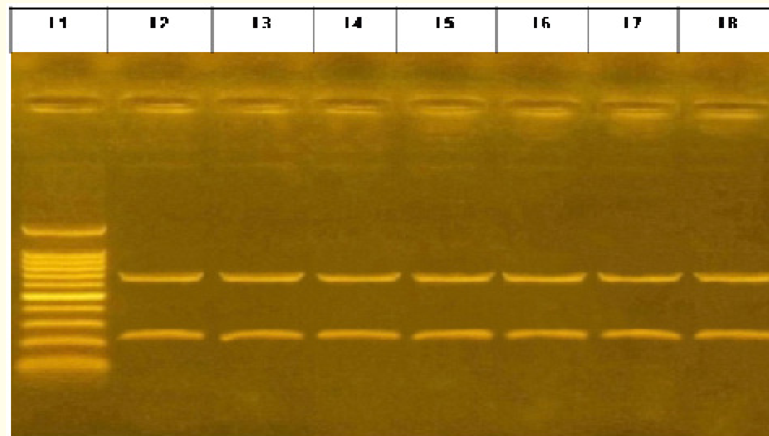

Figure 4: *Agrose gel electrophoresis of spa gene Hea II restriction enzyme digestion.*
L1: 100-1500 bp DNA ladder, L2-8: bands of spa gene digestion of 240 and 690bp.

***Van* gene genotyping:** According to the result of antibiotic sensitivity, one strain was found to be vancomycin resistant. When we genotyped the van gene using specific primer sequences we found that this isolate contain both vanA and VanB (Figure 5).

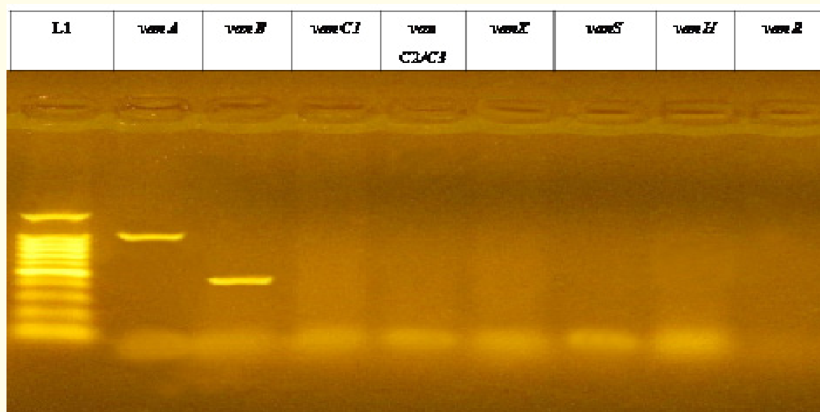

Figure 5: *van gene genotyping.*
L1: 100-1500 bp DNA ladder, bands of vanA (1030 bp) and vanB (433) were shown.

When we tried to find out the relation between different *coa gene* genotypes and antibiotic sensitivity, we found no statistically significant relationship between *coa* genotype and any of the studied antibiotic sensitivity.

Antibiotic	Susceptible isolates expressing 570bp	Susceptible isolates expressing 630pb	X2	P value
Benzylpenicillin	Zero	Zero	------	------
Gentamycin	18 (66%)	4 (44%)	1.40	0.23
Ciprofloxacin	27 (100%)	9 (100%)	-----	------
Levofloxacin	27 (100%)	9 (100%)	------	-----
Moxifloxacin	27 (100%)	9 (100%)	------	-----
Erythromycin	6 (22%)	1 (11%)	0.53	0.46
Clindamycin	25 (93%)	4 (44%)	0.059	0.80
Quinupristin/Dalfopristin	27 (100%)	9 (100%)	-------	--------
Linezolid	26 (96%)	9 (100%)	0.34	0.55
Vancomycin	26 (96%)	9 (100%)	0.34	0.55
Tetracyclin	15 (55%)	3 (33%)	2.41	0.12
Nitrofurantoin	27 (100%)	9 (100%)	-------	------
Rifampicin	27 (100%)	9 (100%)	-------	------
Trimthoprim/Sulfamethoxazole	27 (100%)	9 (100%)	-----	-------
Total	27 (100%)	9 (100%)		

Table 3: *Relation between coa genotype and antibiotic sensitivity patterns.*

Distribution of different isolates of spa gene revealed three subtypes, The first subtype contains two isolate in which spa gene could not detected, the other subtype contains one isolates where spa gene was detected, however, it resist the action of restriction enzyme. The third subtype contained and 33 isolates. Statistical relationship between spa genotyping and antibiotic profile could not thus be established.

Discussion

Antimicrobial resistance

Antimicrobial resistance is a continuously increasing problem worldwide. It increases mortality, morbidity and hospital cost. MRSA is one of the most important causes of hospital acquired infections. It affects seriously ill patients and significantly increases the mortality of patients suffering from bacteriemia [10].

Unfortunately the number of multidrug resistant MRSA is progressively increasing worldwide, which limits the availability of effective antibiotic [2] As a result, applying strict infection control measures is of increasing importance. Genotyping provides essential information needed for the epidemiologic studies [10,11].

This study was conducted in Zagazig University Hospitals. MRSA was isolated from severely ill patients suffering from bacteriemia. The Aim of the study was to detect antibiotic sensitivity patterns and to correlate these patterns with genotypes of MRSA isolates, this may provide useful information to Zagazig University Hospitals Infection Control Unit to guide current policy of infection control and to support epidemiological studies.

mecA gene is considered to be the gold standard for MRSA diagnosis [12]. This study included only isolates that had mecA gene as shown with PCR.

Methicilin resistance is mediated by mecA gene, this gene is located in Staphylococcus Chromosomal Cassette (SCC) and it is a site-specific transposon-like element that is present only in staphylococcal species [13]. It codes for PBP2 which is present in the cell wall and has low affinity for beta lactam antibiotics [14].

According to our results, 2 (6%) of isolates showed oxacillin sensitivity despite containing mecA gene. This can be explained on the bases of heteroresistance where all cells in the tested population have mecA gene, but not all of them express resistance to oxacillin [15]. Those isolates, however, were cefoxitin resistant. Cefoxitin is a better predictor of methicillin resistant than oxacillin because it is a stronger inducer of PBP2. Efficiency of Cefoxitin in detecting heteroresistant MRSA population was confirmed by previous studies cefoxitin disks had high sensitivities (97.0 to 100.0%) and specificities (99.0 to 100.0%) [16, 17].

According to our results, MRSA strains were 100% resistant to Benzlpenicillin. These results are similar to results obtained from other studies [18, 19].

According to our hospital antibiotic policy, Vancomycin is the first choice for treating MRSA. When Vancomycin MIC is below 2mg/ml, strains are identified as vancomycin sensitive. The MIC of 4mg/ml or 8mg/ml identifies strain as being vancomycin intermediate resistant (VISA). Vancomycin resistant strains are those strains with MIC ≥16µg/ml. Mechanism of this resistance includes acquisition of Van transposon from Vancomycin resistant enterococci VRE.

According to our result, there were one strain of VRSA MIC>32 mg/ml while all other isolates were vancomycin sensitive. When we genotyped this isolate using primer specific PCR, we found that this isolate genotype was vanA/vanB. These genotypes are The comments across VRSA strains [20]. Previous study in our hospitals had shown that Van A and Van B were the most common genotype across enterococci, suggesting horizontal transfer of resistance genes [21].

Unfortunately, the same isolate was resistant to linozolid MIC >8 mg/ml, which limits the treatment choices. Resistance to vancomycin and linozolid although rare, have been reported earlier in other studies [22-24]. 80% of isolates tested against erythromycin in this study were resistant the tested antibiotic MIC 50 4mg/ml, MIC 16 mg/ml. The incidence of resistance to erythromycin detected by Yildiz *et al.* 2014 was 56.7%, MIC 50 16mg/ml, MIC 90 >128 mg/ml respectively. However Kaddora 2010 found that 100% of the tested isolates were resistant [14,19].

Clindamycin is primarily used for treating soft tissue infections caused by community acquired MRSA [25] Our results showed that 20 % were resistant to clindamycin, while, %80 were clindamycin sensitive. Reported resistant to clindamycin is widely variable across different centers. In a multicenter study in USA the incidence of resistant strain ranges from 12-75% [26], while it was 100% in another study in India [19].

When 22 clindamycin sensitive/erythromycin resistant isolates were specifically tested for inducible clindamycin resistance (ICR) we found that all isolates 100% showed inducible resistant [25]. In a multicenter study in Japan, the incidence of inducible clindamycin resistance was found to be 91% [27].

Isolates tested in this study were 60% sensitive to Gentamycin MIC 50 ≤ 1mg/ml and MIC 90 16 mg/ml and 40 % were sensitive. 100% were sensitive to Amikacin. Different results have been reported in different studies. Kaddora, 2010, and Randrianiria et al., reported 100% of isolates sensitive to Gentamycin [19,28]. However, this was quite different from the result obtained in Turkey by Yildiz *et al.* 2014 where Gentamycin Resistant strains 90.2%, resistant 3.1%, MIC 50 64 mg/ml, MIC 90 >128 mg/ml and Saravanan et al., 2013 where gentamycin resistance was 100% [14, 18]. 50% of tested isolates were sensitive to tetracycline, again this was different from that detected by Kaddora, 2010 where 100% of isolates were sensitive to Tetracyclin and from the result obtained in Turkey by Yildiz *et al.* 2014 where resistant strains were 88.2% [14]. The difference in results can be explained by different antibiotic policy followed in different places.

Although all isolates showed sensitivity to Rifampicin, the use of Rifampicin alone is discouraged due to high rate of treatment failure as a result of spontaneous mutation. All isolates were also sensitive to Trimethoprim/sulfamethoxazole [26]. 100% *in vitro* resistance to Rifampicin was detected by Saravanan *et al.* 2013 who isolated MRSA from blood of septicemic children [18].

All Isolates were sensitive to ciprofloxacin, levofloxacin and moxifloxacin. However, Rajaduraipandi and colleagues reported 87.2% sensitivity to ofloxacin, 82.1 to Ciprofolxacin and 89.7% to Norfoloxacin in their study [29]. Randrianiria., *et al*. reports 92.2% sensitivity to ciprofloxacin [28]. Difference in antibiotic susceptibility patterns can be explained on the base of difference in geographical

The *coa* gene is being used as important tool to characterize and identify pathogenic *S. aureus* isolates [30]. In this study all MRSA isolates are *coa* gene positive, giving coa gene PCR detection 100% sensitivity and specificity in accordance with other study [31].

Coagulase gene has discriminatory power between strains, relies on both number and size of bands in each strain. This is due heterogeneity of the region containing the- 81 pb tandem repeats at the 3' ends as well as the location of Hea II restriction sites in the coa gene amplicons. Amplification of this particular region produces fragments of DNA with different sizes that differentiate between strains 350bp, 430 bp, 570 bp and 630 bp [32]. In this study two different amplicons of coa gene with different band sizes were detected. Twenty seven isolates showed coa gene bands of 570 bp (75%) and the remaining 9 isolates (25%) revealed bands of 630 bp, representing two different strain types this results matches the result of Lyer and Kumosani (2012) [7].

In this study 2 isolates of MRSA had no spa gene and 34/36 (94.4%) isolates express spa gene. This finding is in accordance with [9, 33]. studies where spa gene was absent in 3.8% and 5.3% of their S. aureus isolates respectively.

According to our results, 94.4% of strains has single PCR band. This was in agreement with Omar *et al.* 2014 study where the majority of isolates (94.6%) had one PCR band [9]. However, Shakeri and his colleges (2006) declared that 14% of strains isolated from patients have two spa gene PCR bands [34]. This finding may be attributed to either the relative small number of MRSA isolates in our study or the wide variety of clinical specimens included in their studies comparing to our study where samples were either collected from blood or CVCs only.

The number of repeats in the region X of spa has been related to dissemination potential of MRSA, where higher number of repeats associated with higher epidemic capacity as longer length of protein A help in adherence to the epithelial surfaces [35,36].

In this study all MRSA strains which had been isolated from blood stream infection or CVCs samples revealed spa gene band length of 1164 bp. On the other hand, in Omar *et al.* study (2014) they found that longer spa gene bands (1200-1392bp) were detected in MRSA isolates from respiratory secretion than other sample [9]. Our results agreed with Shakeri *et al.* (2006) who detected shorter spa gene in MRSA isolated from blood sample and wound (1150-1200 bp) than those isolated from urinary tract infections (1350-1400bp). [34]. This observation reflect the role of longer spa protein in adherence to epithelial surfaces as in UTIs and RTIs, which protect the strain from being discharged by coughing or in urine stream [36].

Hea II was the restriction enzyme used to digest spa PCR products in this study. Agarose gel electrophoresis of spa gene enzyme digestion revealed bands at 240bp and 690bp in33/36 (91.7%).This is in accordance with Omar *et al.* (2014) where 89.3% of isolates showed a band at 240 bp [9]. A closer result was declared in Mehndiratta *et al.* (2009) where all spa gene PCR –RFLP patterns showed restriction band at 243b [37].

When we tried to find a relationship between antibiotic susceptibility tests and spa and cao genotypes, there was no statistically significant difference between the different genotypes of either genes.

Conclusion

Finally we conclude that MRSA is a serious health problem. Unfortunately, it is widely spreaded in our hospital environment. No relationship has been found between the antibiotic sensitivity results and the genotypes of coa gene; we recommend better prevention and control programs.

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Volume 3 Issue 2 April 2016
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