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Detection of Different Levels of Resistance to Vancomycin among *Staphylococcus aureus* Isolated from Surgical Departments of Tanta University Hospitals

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A B S T R A C T

Antibiotic resistance is the most worrisome problem in treating bacterial infections. It is very important to maintain the clinical efficacy of vancomycin because of their critical role in preventing and treating life-threatening healthcare associated infections (HAIs). Therefore, this study aimed to determine the existence of different levels of vancomycin resistance among *Staphylococcus aureus* (*S. aureus*) strains in Surgical Departments of Tanta University Hospitals. This study was carried out on 200 patients who had evidence of infections. All specimens were cultured on blood and mannitol salt agarsand their antimicrobial susceptibility were investigated by modified Kirby-Bauer method and VITEK 2 system. Vancomycin susceptibility was assessed by E-test and vancomycin screen agar. *mecA*, *vanA* and *vanB* genes were investigated by polymerase chain reaction (PCR). Methicillin resistance was detected in 70% of *S. aureus* isolates and *mecA*gene was detected in 68% of these isolates. The total percentage of *S. aureus* with reduced vancomycin susceptibility (SA-RVS) was 20%. All isolates were negative for *vanA* and *vanB* genes. VITEK 2 system failed to detect any isolates of SA-RVS.

Introduction

Staphylococcus aureus is a major bacterial human pathogen causing a wide variety of clinical manifestations ranging from minor skin infection to life-threatening disease. Infections are both community-acquired and hospital-acquired **(**Piewngam *et al.*, 2020). The mortality of *S. aureus* invasive infections has fallen from 80% in the pre-antibiotic era to 16%–30% over the past two decades. Further reductions in mortality below 20% have remained far-fetched despite the introduction of new antibiotics, rapid diagnostic and

susceptibility testing, widespread antibiotic stewardship programs and improvements in therapeutic supportive care (Miller *et al.,* 2020).

Treatment options remains challenging due to the emergence of multi-drug resistant (MDR) strains such as methicillin-resistant *S. aureus* (MRSA). In 2014 global report, the World Health Organization (WHO) listed MRSA as one of the seven pathogens of international concern and that has been associated with a high number of mortality and septic shock cases compared tomethicillin-sensitive *S. aureus* (MSSA) (Che Hamzah *et al.,* 2019).

Poor infection control measures as well as continued indiscriminate exposure of humans and animals to antibiotics have resulted in this huge problem of acquisition and dissemination of MRSA. This in turn limits the choice of treatment for MRSA infections and leads to enormous increases in vancomycin use for therapeutic, prophylactic, and empirical strategies (Lakhundi *et al.,* 2018).

Two main forms of *S. aureus* with reduced vancomycin susceptibility (SA-RVS) have been identified. One form is vancomycin intermediate *S. aureus* (VISA) strains, which have minimum inhibitory concentrations (MICs) to vancomycin of 4–8μg/ml. It is mainly result from changes in peptidoglycan biosynthesis and thickened cell walls. The second form of vancomycin resistance called vancomycin resistant *S. aureus* (VRSA)which have MICs to vancomycin of≥16μg/ml and usually resulted from the conjugal transfer of the *vanA* operon from a vancomycin-resistant *Enterococcus faecalis* (Lowy, 2003).

In 1997, the first VISA strain (Mu50) with MIC of 8μg/ml was reported from Japan (Hiramatsu *et al.,*1997). In 2002, the first case of VRSA was reported in a diabetic patient in the USA (Goldrick, 2002). The VISA strains are generally believed to be initiated from heterogenous vancomycin intermediate *S. aureus* (hVISA), which is defined as an *S. aureus* strain with a vancomycin MIC within the susceptible range (≤2μg/ml) by conventional methods, while a cell subpopulation is in the vancomycin-intermediate range (≥4μg/ml) (Alam *et al.,* 2014). The VISA and its precursor hVISA were discovered almost 20 years ago and have continued to be a stumbling block in the chemotherapy of MRSA (Hiramatsu *et al.,* 2014).

These isolates are difficult to identify by routine laboratory methods as the vancomycin MIC level is within the susceptible range. It is determined by a plating-based, population analysis profile-area under the curve (PAP-AUC) method. Unfortunately, PAP-AUC method is time-consuming, labor-intensive, and costly which limits its use in routine clinical laboratories. A variety of alternative methods for detection of the heteroresistant phenotype have been evaluated with varying success(Satola *et al.,*2011).

The clinical isolates of SA-RVS have emerged within the past two decades becoming a serious public health concern. These infections lead to higher rates of vancomycin treatment failure and are associated with extended hospitalization, higher risk of persistent infection and elevated treatment costs (Shariati *et al.,* 2020).

Materials and Methods

The present study was carried out in Medical Microbiology and Immunology Department, Faculty of Medicine and Central Research Laboratory, Tanta University, on 200 patients admitted to Surgical Departments in Tanta University Hospitals during the period of research from October 2018 to October 2020.The study included in patients admitted at these departments for more than 3 days and patients received antibiotics for more than 10 days without improvement.

Written informed consents were obtained from all participants in this research. Ethical approval for this study was provided by Ethics and Research Committee, Faculty of Medicine, Tanta University.

Collection of samples

Specimens were collected under complete aseptic precautions. The samples included wound and burn swabs, sputum samples, endotracheal aspirates as well as urine samples. Specimens were labeled and delivered as soon as possible to the laboratory. When immediate delivery to the laboratory was not possible, the specimen was refrigerated at 4–6 °C (Cheesbrough, 2006)

All specimens were cultured on blood, nutrient and mannitol salt agars. The plates were incubated at 37°C for 24 hours and then the isolates in the primary plates were identified by colony morphology, Gram film and various biochemical reactions according to traditional microbiological methods such as catalase test, coagulase test.

These biochemical reactions were done from colonies on nutrient agar (Adesoji *et al.*, 2019).

Antibiotic susceptibility testing

Disc diffusion method

All the isolates of *S. aureus* from blood agar were tested for antibiotic susceptibility by modified Kirby- Bauer disc diffusion method according to CLSI guidelines (CLSI, 2020).

The following antibiotics (Oxoid, UK) were used Penicillin (10 µg), Clindamycin (2µg), Trimethoprim-sulfamethoxazole (25µg), Erythromycin (15µg), Tetracycline (30µg), Ciprofloxacin (5µg), Gentamicin (10µg), Cefoxitin (30 µg) and Rifampin (5µg).

Vancomycin E-test

The MIC for vancomycin was detected by E-test strips according to the manufacturer's instructions(Liofilchem, Italy). Using sterile forceps, the E- test strips were applied to the inoculated agar surface with the MIC scale facing upwards, followed by incubation at 35ºC for 24 hours. The MIC was read directly from the scale in terms of µg/ml at the point where the edge of the inhibition ellipse intersects with the MIC test strip.

Antibiotic susceptibility by VITEK 2 system

The steps done according to the manufacturer's instructions(BioMérieux SA, France). The results were automatically analyzed by VITEK system and interpreted as sensitive, intermediate, or resistant according to the MIC of each tested drug.

Vancomycin screen agar

A stock solution of vancomycin was prepared by dissolving 500 mg of vancomycin powder in 10 ml of sterile distilled water (final concentration was 50 mg/ml). Further dilution of 1:10 was done twice to produce a working solution of 0.5 mg/ml vancomycin. For the final preparation of vancomycin screening agar; six ml of 500 ml prepared media were removed under complete aseptic precautions and replaced by 6 ml of working solution of vancomycin to prepare vancomycin screening agar with 6µg/ml. A drop from 0.5 McFarland *S. aureus* suspension was inoculated using a micropipette to spot a 10µl drop on the surface of vancomycin screening agars. Alternatively, a swab was dipped in the McFarland suspension, the excess liquid expressed, and used to inoculate the vancomycin agar screen plates. Plates were examined carefully at 24 and 48 hours with transmitted light.

Presence of more than one colony of the strain or light film of growth is interpreted as reduced susceptibility to vancomycin. For quality control purposes, vancomycin-resistant *Enterococcus faecalis* ATCC 51299 and *S. aureus* ATCC 25923 were used as positive and negative controls, respectively (Othman *et al.,* 2018).

Conventional PCR

DNA extraction

According to the manufacturer's instructions (Qiagen-Germany), the bacteria from culture plate were removed by the inoculation loop and suspended in 180 µl of lysis buffer ATL by vigorous stirring.20 µl of proteinase K were added, mixed by vortexing and incubated at 56 ºC for 30 minutes. The samples were centrifuged to remove drops from inside the lid (8000 rpm for 1 minute).200 µl of lysis buffer AL were added to the sample, mixed by pulse vortexing for 15 seconds, and incubated at 70 ºC for 10 minutes. 200 µl of ethanol (96-100%) were added to the sample, mixed by pulse vortexing for 15 seconds, and incubated at 70 ºC for 10 minutes.

The mixture from the previous step was carefully applied to the QIAamp Mini spin column (in a 2 ml collection tube), the cap was closed and centrifuged at 8000 rpm for 1 minute. 500 µl of buffer AW1 were added to the sample and centrifuged at 8000 rpm for 1 minute. 500 µl of buffer AW2 were added and centrifuged at 14000 rpm for 3 minutes.

The QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube, 200 µl of buffer AE were added and centrifuged at 8000 rpm for 1 minute. The centrifuged elutes were taken for gene detection.

Nucleic acid amplification

According to the manufacture instructions (Applied Biosystems-USA), primers in table 1 were prepared by concentration 0.4 µm. The reaction was performed at a defined volume of 25µl and included 10 µl of sample, 12.5 µl of TaqMan Universal Master Mix, 1 µl of forward primer and 1 µl of reverse primer. All components were mixed well and centrifuged. The tubes were placed in the thermal cycler to be amplified. For amplification of *mecA* gene, initial denaturation was at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 30 sec and elongation at 72 °C for 1 min, and a final extension at 72 °C for 3 min. For amplification of *vanA* gene, initial denaturation occurred at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, elongation at 72°C for 1 min, and a final extension at 72°C for 10 min. For the amplification of *vanB* gene, initial denaturation occurred at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 25 s, annealing at 52°C for 40 s, elongation at 72°C for 1 min, and final extension at 72°C for 6 min (Bamigboye *et al.,* 2018).

Agarose gel electrophoresis of the amplified DNA

The PCR products were visualized and photographed under UV light after electrophoresis for 45 minutes at 100 V through 1% agarose gel containing ethidium bromide (1µg/ml).

Statistical analysis

Statistical presentation and analysis of the results of the present study was conducted and the data were presented as mean ± standard deviation (SD) or number and percentage. Comparison between groups were calculated by ANOVA and chi-square tests using computer program Statistical Package for the Social Sciences (SPSS) V. 20. P- value less than 0.05 was considered significant.

Results and Discussion

Prevalence and distribution of bacterial isolates

Out of the 200 samples, 155 (77.5%) showed mono-microbial growth, 15 (7.5%) had poly-microbial growth with 2 organisms while 30 (15%) displayed no growth. Gram negative organisms were the most prevalent organisms isolated from studied patients (63.2%), followed by Gram positive organisms (32.5%). Of notice, *S. aureus* represented 27% of total isolates.

Antimicrobial susceptibility testing

By disc diffusion method, all studied isolates (100%) were resistant to penicillin. Of notice, 35 cases (70%) from *S. aureus* isolated were cefoxitin-resistant. The lowest resistance was to rifampin (8%) and clindamycin including inducible clindamycin resistance (ICR) (34%). On the other hand, all cases (100%) were sensitive to linezolid. By using VITEK 2 system, the studied isolates showed the highest resistance (100%) to penicillin and fusidic acid. The lowest resistance was to rifampin (8%) and clindamycin (36%).

On the other hand, all cases (100%) showed sensitivity to linezolid, tigecycline, teicoplanin and vancomycin. Table 2 shows comparison between resistance pattern of isolated *S. aureus* to different antimicrobial agents by disc diffusion method and VITEK 2 system. 70% of isolated *S. aureus* were resistant, while 30% of these isolates were sensitive to cefoxitin.

Susceptibility pattern of isolated *S. aureus* to vancomycin by E-test

In this study, 86% of *S. aureus* isolates were sensitive to vancomycin (MICs $\leq$ 2 µg/ml), while 8% showed intermediate resistance to vancomycin (MICs 4-8 µg/ml) by E-test (figure 1). No VRSA strains were detected (MICs 8-16 µg/ml). Of notice, 6%of *S. aureus* isolates had MIC value of 3 µg/ml (query hVISA).

Growth on vancomycin screen agar

In this study, 9 (18%) of *S. aureus* isolates grew on vancomycin screen agar with more than one colony. Three of them had MIC $\leq$ 2 ug/ml (VSSA) by vancomycin E-test and 3 isolates had MIC of 4-8 ug/ml (VISA). The remaining 3 isolates had MIC of 3 ug/ml, that were query hVISA. Of notice, 3 out of 4 VISA strains grew on vancomycin screen agar with 75% sensitivity in detection of VISA. 82% of isolates showed no growth on vancomycin screen agar.

Out of the 50 isolates of *S. aureus*, 10 (20%) were SA-RVS; 4 isolates (8%) were VISA and 6 isolates (12%) were hVISA as diagnosed by E-test and vancomycin screen agar.

Conventional PCR results

Out of 50 *S. aureus* isolates, 34 (68 %) were *mecA* positive, while 16 (32%) were *mecA* negative (figure 2).*van A* and *van B* genes were not detected in any of *S. aureus* isolates.

Antimicrobial resistance is a growing problem in medicine, resulting in limitations in the antimicrobial choices available for treatment of severe infections and increasing the risk of morbidity and mortality in patients (Weissman *et al.*, 2013).There is no need of a crystal ball to predict that vancomycin resistance is going to represent the most serious therapeutical challenge in *S. aureus* infections. It is more difficult to predict, on the other hand, how long linezolid and daptomycin will maintain their full activity against MRSA (Livermore, 2016).

In the present study, when the susceptibility of *S. aureus* was studied by disc diffusion

method, it was found that the highest resistance rate was detected to penicillin (100%), cefoxitin (70%), gatifloxacin (56%), erythromycin and gentamicin (54%, each).

Also, *S. aureus* isolates showed resistance to ciprofloxacin and ofloxacin (46%, each), tetracycline (44%), doxycycline (42%), trimethoprim-sulfamethoxazole (40%) and clindamycin (34%). However, these isolates exhibited highest sensitivity rates to linezolid (100%) and rifampin (92%).

Supportive to results of the current study, Kishk*et al.,* (2020) reported that erythromycin and clindamycin resistance by disc diffusion which detected in *S. aureus* isolates was 54.5% and 38.6%, respectively. Additionally**,** Li *et al.,* (2019) reported 100% sensitivity to linezolid in *S. aureus* isolates by disc diffusion method. Resistance rate to erythromycin, tetracycline and trimethoprim-sulfamethoxazole was 53, 40 and 35%, respectively.

One the other hand, the observed resistance for clinical *S. aureus* isolates in the study of Adesoji *et al.,* (2019) demonstrated the highest resistance (100%) to erythromycin and the lowest resistance (27.5%) to ofloxacin and gentamycin (20%).Also, higher resistance rate to fluoroquinolone class (76.3%) was reported among tested *S. aureus* isolates in the study done by Kashef *et al.,* (2020). The characteristics and antimicrobial resistance profiles of *Staphylococci* differs according to geographical regions and in relation to antibiotic usage. The prevalence of MRSA in the present study was 70% according to both cefoxitin disc diffusion and VITEK 2 MICs for oxacillin and cefoxitin. These results matched with the results obtained by Abdel-Maksoud *et al.,* (2016) from 12 hospitals in Egypt from 2005 to 2013,they reported that MRSA isolates constituted 76% of staphylococcal infections by disc diffusion method. Also, Amr and Gammal, (2017) in Zagazig University Hospitals detected the prevalence of MRSA by VITEK 2 system which was about 78% of *S. aureus* isolates.

On the other hand, the prevalence of MRSA in the present study was higher than that obtained in Egypt by Taha *et al.,* (2019) reported lower prevalence rate of MRSA (20%). Naimi *et al.,* (2017) in Afghanistan, as they reported 56% of isolated *S. aureus* were MRSA by disc diffusion method. Also, Wangai *et al.*,(2019) reported between 2014 and 2016 the overall MRSA prevalence in East Africa was 53.4% by VITEK 2 system. In India, Raut *et al.,* (2017) reported that the prevalence of MRSA was 43.6% by cefoxitin disc diffusion.

Regarding the prevalence of SA-RVS, Othman *et al.,* (2018) revealed that out of their 100 *S. aureus* isolates, 22% were VISA. There were 9 isolates (9%) grew on screening agar 6 µg/ml and were designated as hVISA. All their *S. aureus* isolates were susceptible for both vancomycin and linezolid by VITEK 2 system. Abdel-Maksoud *et al.,* (2016) reported that susceptibility to vancomycin was detected in 80.2% of MRSA isolates by E-test and VISA was detected in 1.2% of their isolates.

However, 18.6% of the isolates showed MIC 3µg/ml. VRSA was not detected among staphylococcal isolates.

Table.1 The primers used and their product size [88]

Primer name	Primer sequence (5´-3´)	Product size (pb)
***mecA*F**	GTAGAAATGACTGAACGTCCGATAA	585
***mecA*R**	CCAATTCCACATTGTTTCGGTCTAA	
***van A* F**	GGCAAGTCAGGTGAAGATG	713
***van A* R**	ATCAAGCGGTCAATCAGTTC	
***van B* F**	GTGACAAACCGGAGGCGAGGA	430
***van B* R**	CCGCCATCCTCCTGCAAAAAA	

Fig.1 Vancomycin E-test showing intermediate isolate of *S. aureus* (MIC= 6µg/ml)

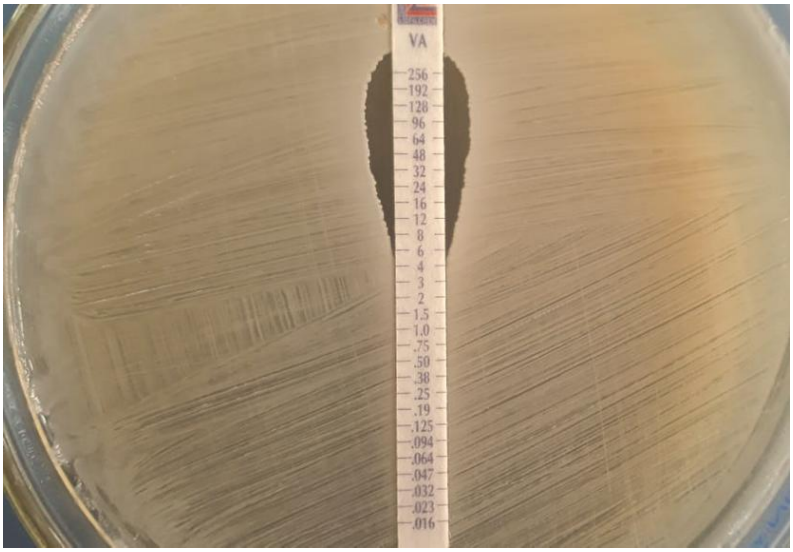
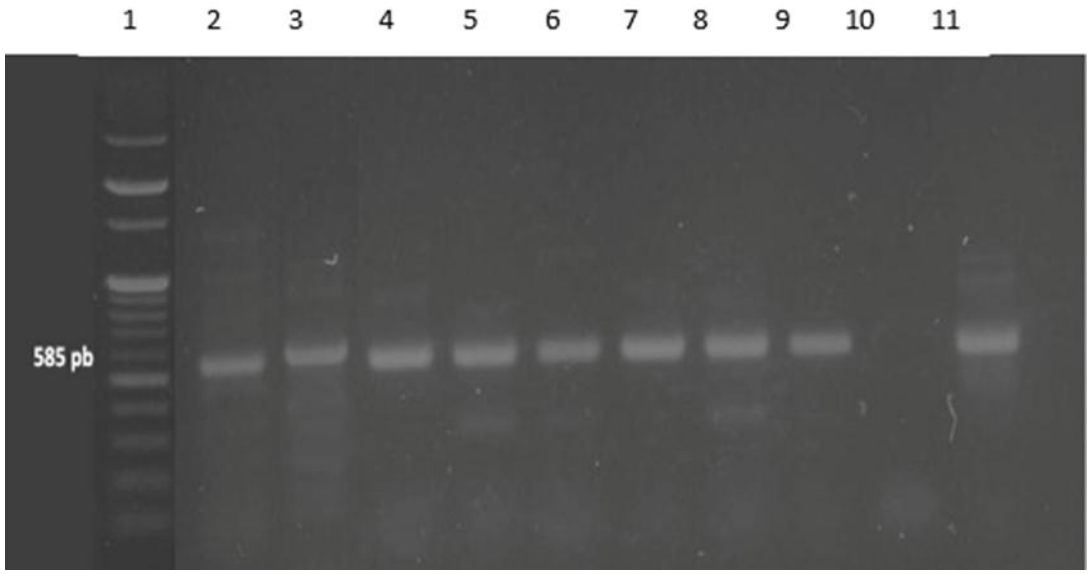


Fig.2 Gel electrophoresis of PCR amplification of *mecA*gene.



Lane 1= marker of 100 pb DNA ladder; Lane 2 = *mecA* positive control (585 pb)
Lane 3,4,5,6,7,8,9 and 11= *mecA* positive isolates
Lane10 = *mecA* negative isolate

Table.2 Comparison between resistance pattern of isolated *S. aureus* to different antimicrobial agents by disc diffusion method and VITEK 2 system

Antibiotic	*S. aureus*isolated (N=50)											
	S				I				R			
	Disc diffusion		VITEK 2		Disc diffusion		VITEK 2		Disc diffusion		VITEK 2	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Penicillin	0	0	0	0	0	0	0	0	50	100	50	100
Cefoxitin	15	30	15	30	0	0	0	0	35	70	35	70
Erythromycin	18	36	20	40	5	10	2	4	27	54	28	56
Gentamicin	19	38	20	40	4	8	2	4	27	54	28	56
Ciprofloxacin	25	50	25	50	2	4	0	0	23	46	25	50
Tetracycline	25	50	27	54	3	6	1	2	22	44	22	44
Trimethoprim-sulfamethoxazole	25	50	25	50	5	10	5	10	20	40	20	40
Clindamycin	30	60	31	62	3	6	1	2	17	34	18	36
Rifampin	46	92	46	92	0	0	0	0	4	8	4	8
Linezolid	50	100	50	100	0	0	0	0	0	0	0	0
X^2	0.153				2.571				0.093			
P- value	0.998				0.765				0.999			

S: susceptible. I: Intermediate. R: Resistant. $P \geq 0.05$ is not significant.
The different results between the 2 tests are highlighted.

In Iran, Asadpour and Ghazanfari(2019) found that of the 110 studied isolates, the vancomycin MIC was > 2 µg/ml in 10% isolates. Of these, three (2.3%) VRSA and eight (7.3%) VISA were identified. In India, Bhattacharya *et al.*,(2015) showed that out of 100 *S. aureus* isolates, the prevalence of VRSA, VISA, and hVISA was found to be 3%, 12% and 6%, respectively by E-test and E test glycopeptide resistance detection strips. Higher rates of vancomycin resistance were detected in a Nigerian study conducted by Olufunmiso *et al.,* (2017)where15% of the isolates were considered VISA and 44.5% of the isolates were considered VRSA.

A lower rate of VISA isolation among *S. aureus* (4%) was reported in 2018byJahanshahi *et al.*, Also, Baseri *et al.*, (2018) revealed that the overall prevalence rate of VISA was 0.09% in Iran between 2010-2017.The relevance of hVISA and its accurate epidemiology will remain controversial until a reliable and practical testing method becomes standardized and adopted for use in clinical laboratories and future studies (Walsh *et al.*, 2001).

Supportive to the results of the current study, Swenson *et al.*, (2009) reported that the VITEK 2 system tended to categorize VISA isolates as susceptible.

This was justified by Edwards *et al.*,(2012) who demonstrated that MICs from automated systems and the E-test were significantly lower after cryopreservation, if compared with those from the E-test analysis at the time of isolation.

In the present study, conventional PCR detected *mecA* gene in 68% of *S. aureus*

isolates. Out of 35 phenotypically diagnosed MRSA isolates, 34 isolates were positive for *mecA* gene (97.1%). Similar results were reported by Bhattacharya *et al.*,(2015) found that out of 47 phenotypically MRSA isolates, 46 was *mecA* positive (97.9%). Sahebnasagh *et al.*, (2014)found among 126 isolates of *S. aureus*, 98 of isolates were determined MRSA using disc diffusion method. Only 87 (69%) isolates harbored the *mecA* gene.

On the other hand, Anand *et al.*, (2009) found that out of the 50 *S. aureus* isolates, 32 were resistant with cefoxitin disc diffusion. For these 32 isolates *mecA* gene was positive. Also, Sadeghi and Mansouri(2014) reported that 56.8% of their isolates were identified as MRSA and all were characterized by the presence of *mecA* gene. The methicillin resistance with negative *mecA*gene could be due to the presence of other resistance mechanisms, such as large amounts of produced beta-lactamase or change in *mecA*gene due to the mutations. Otherwise, it would be due to the lack of optimal PCR conditions(Sahebnasagh *et al.*, 2014).

Regarding detection of *van A* and *van B* genes by PCR in this study, none of *S. aureus* isolates could demonstrate the presence of *vanA* or *vanB* gene by PCR.

Similar results were obtained in 2018 by Bamigboye *et al.*, 61 (83.6%) of the *S. aureus* isolates were VSSA, 11 (15.1%) were VISA, and 1 (1.4%) was VRSA.

All the VISA and VRSA isolates were *vanA* and *vanB* gene negative. Kumar (2016) in India found that among the 47 *S. aureus* isolates, 2 isolates were highly resistant to vancomycin. PCR amplification of both isolates indicated presence of *mecA*and *vanA*genes. Also, Thati *et al.*, (2011) reported among the clinical isolates of *S. aureus*, sixteen isolates were VISA and seven isolates were VRSA. PCR amplification for *vanA* among the seven VRSA showed that six contained *vanA*, whereas all the isolates expressed *mecA*.

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Conflict of interest

There is no conflict of interest between the authors.

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