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ORIGINAL ARTICLE

Reduced Vancomycin Susceptibility in *Staphylococcus aureus*; Laboratory Detection and Genomic Characterization

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ABSTRACT

Background*: The emergence of resistance to methicillin resistant Staphylococcus aureus (S.aurus) (MRSA), followed by Vancomycin resistant S.aurus has turned the therapy of staphylococcal infections into a worldwide challenge. Three classes of vancomycin-resistance have emerged that differ in vancomycin susceptibility; vancomycin resistant S.aureus (VRSA), Vancomycin intermediate S.aureus (VISA) and heterogenous vancomycin-Intermediate S.aureus (hVISA).* ***Objectives****: The present study aimed to detect S.aureus with reduced susceptibility to vancomycin in different types of clinical samples and their genomic characterizations.* ***Methodology:*** *The study was carried out on 250 S.aureus isolates from different types of clinical samples collected from patients admitted to various departments in the Alexandria University Hospitals, Egypt from May 2014 to April 2015.* ***Results:*** *We detected 22 S.aureus isolates with reduced sensitivity to vancomycin out of the 250 S.aureus test isolates by PAP-AUC and agar dilution methods. Three of them were VISA and 19 were hVISA; mainly isolated from pyogenic infections. Molecular typing of VISA and hVISA exhibited dominance of agr group Type I.* ***Conclusion:*** *Strict infection control measures and antibiotic policy should be adopted to control the problem of VISA and hVISA.*

INTRODUCTION

Infections caused by antibiotic-resistant bacteria are a growing problem worldwide.[1] *Staphylococcus aureus* (*S.aureus*) is a leading cause of hospital and community acquired infections.[2]

Several years ago, the problem of antibiotic resistance of *Staphylococcus aureus* (*S.aureus*) was accentuated by the appearance of clinical infections caused by glycopeptides resistant strains. [3-5]

Whereas, vancomycin-resistant *S. aureus* is a result of the acquisition of the vanA gene from vancomycin-resistant enterococcus.[6] This gene is integrated into a *S. aureus* conjugative plasmid. Vancomycin resistance is only expressed on drug exposure. [7] Such strains are potentially associated with the clinical failure of vancomycin treatment.[8]

A series of studies has shown that glycopeptides resistance of *S.aureus* is not a "yes-no" phenomenon, but includes intermediate levels of resistance (VISA) as well as heteroresistant (*hVISA*) strains. Thus, posing challenges in therapy.[9]

Vancomycin heteroresistance among *S.aureus* isolates was the reason that Tenover and Moellering cited for the Clinical and Laboratory Standards Institute decision in 2006 to lower the vancomycin MIC breakpoints for *S. aureus* from 4 mg/liter to 2 mg/liter for susceptible, from 8 - 16 mg/liter to 4 - 8 mg/liter for intermediate, and from 32 mg/liter to 16 mg/liter for resistant.[10]

Accessory gene regulator (agr) is a global regulator gene of *S.aureus* that controls the expression of major virulence factors.[11, 12] The analysis of the polymorphism in agr genes, have allowed molecular typing of isolates generating epidemic outbreaks. According to this gene's polymorphism, four variants have been identified; groups I to IV.[13]

The aim of the current study was the detection of *S.aureus* with reduced susceptibility to vancomycin in different types of clinical samples and their genomic characterizations.

METHODOLOGY

Two hundred and fifty *S.aureus* isolates were collected from different types of clinical samples from Inpatient Departments at Alexandria University Hospitals, Egypt from May 2014 to April 2015.

- **Culture and Identification:**

All samples were inoculated on blood, MacConkey, and Mannitol salt agar plates and identified as *S.aureus*

according to standard biochemical tests.[14, 15] The reference strain *S.aureus ATCC 29213* was used as a quality control strain.

- **Antibiotic susceptibility test:**

Sensitivity of *S.aureus* isolates to different classes of antibiotics in addition to preliminary screening for their sensitivity to vancomycin was done using Kirby-bauer method according to the Clinical and Laboratory Standards Institute guidelines (CLSI).[16, 17]

- **Detection of Reduced Vancomycin Susceptibility in *S.aureus*:**

S. aureus isolates were screened for their sensitivity to vancomycin by the following methods: Agar screen test, broth microdilution method as a gold standard for *VRSA* and *VISA* and by population analysis profile/area under curve (PAP/AUC) as a gold standard for *hVISA*.[18]

- **Vancomycin Agar screen test:**

All *S.aureus* isolates were screened for *VRSA*, *VISA* and *hVISA* isolates on brain heart infusion agar vancomycin (BHIA-V2, 4 and 6 µg/ml plates as described[19,20] and confirmed by using broth microdilution method. The culture was considered positive if there was a growth of one or more colonies after 24h or 48h. All positive isolates were further confirmed by BHIA-V 8 and 16µg/ml for (*VRSA*). Isolates with *hVISA* or *VISA* profile on the BHIV screening plates were further confirmed by PAP-AUC approach using the technique of Wootton *et al*, [18] in addition to subculture on (BHIA-V3 & BHIA-V4 µg/ml) agar.[18] The reference strains *S.aureus ATCC 29213* (VSSA), Vancomycin resistance *Enterococcus faecalis ATCC51299*, *S.aureus Mu3 (ATCC700698) (hVISA)* were used as controls.

- **Population analysis profile/area under curve (PAP/AUC):**

This method has been proposed as the most precise method of determining hetero-resistance. One hundred microliters of a bacterial suspension adjusted to 2.0 McFarland standard was spread on BHI agar plates supplemented with 0,1,2,4,6 or 8 µg/ml of vancomycin. Plates were incubated and growth observed after 48 hours.[21] An isolate was considered *hVISA* if the ratio of the AUC of the test strain to that of *Mu3* was ≥0.9; an isolate with a ratio of <0.9 was defined as *VSSA*. The *VSSA* strain *ATCC 29213* and the *hVISA* strain *Mu3 ATCC 700698* were used as negative and positive controls respectively. The results from each experiment were recorded only when positive and negative controls were confirmed. [21]

- **BHI-V3 and BHI-V4 for diagnosis *hVISA*:**

Isolates were screened on BHIA-V3 and BHIA-V4 using 10-µl volumes of bacterial suspensions with densities equivalent to a 0.5 McFarland turbidity standard. Four isolates were inoculated onto each plate, and the screening tests were performed in duplicate. The plates were incubated for 48h at 35°C. An isolate was considered positive *hVISA* if one or more colonies had grown after 48 h. *S.aureus Mu3 ATCC 700698* was used as control positive to *hVISA* strains.

- **Molecular Detection of *VRSA*:**

Screening for *VRSA* among *S.aureus* isolates was done by detection of vanA gene using PCR. Primers *vanA* Forward: F5' ATGAATAGAATAAAAGTTGC 3' and vanA Reverse:
R5' TCACCCCTTTAACGCTAATA3' according to Saha *et al*.[22] Positive isolates show an amplified product of 1032 bp.

- **Molecular Typing of *VISA* and *hVISA* isolates:**

The agr specificity groups were determined in all *S.aureus* isolates with reduced sensitivity to vancomycin by PCR according to Wenjia Sun *et al*,.[23] Five primers were used; one agr pan-forward primer and 4 reverse primers specific for each gene table (1).

Table 1: Agr primers

Primer	Oligonucleotide sequence(5¯-3¯)	Amplicon size (bP)	Specificity
Agr pan F	ATGCACATGGTGCACATGC		
Agr1 R	GTCACAAGTACTATAAGCTGCGAT	439	*agrI*
Agr2 R	GTATTACTAATTGAAAAGTGCCATAGC	572	*agrII*
Agr3 R	CTGTTGAAAAAGTCAACTAAAAGCTC	406	*agrIII*
Agr4 R	CGATAATGCCGTAATACCCG	657	*agrIV*

RESULTS

A total of 250 *S.aureus* isolates were collected from different types of clinical samples. Most of *S.aureus* isolates (69.2%) were from pus followed by sputum (10.4%), blood (9.6%), nasal swab (4.8%), central venous catheter (C.V.C) (1.6%), urine (1.6%), pleural fluid (1.2%) and ascetic and synovial fluid (0.8%each) (table 2).

Regarding the sensitivity to the different classes of antibiotics used, a high rate of resistance was observed to oxacillin (72%), cefoxitin (69.2%), amoxaicillin/clavulanic (65.2%), and ceftriaxone (59.6%). While moderate resistance was observed to levofloxacin (40.4%), amikacin (32%) and imipenem

(32.4%). Resistance to clindamycin was observed in 90 isolates of them 20(22.2%) had positive D-test. The lowest resistance was observed to vancomycin (5.2%) and teicoplanin. While, all (100%) *S.aureus* isolates were Linzeolid sensitive.

Among the 250 *S.aureus* isolates no *VRSA* was detected. The MIC of Vancomycin was ≤ 1 µg/ml in 228 isolates (*VSSA*) and 2-4 µg/ml in 22 isolates. Screening of these isolates with reduced sensitivity to vancomycin for presence of vanA gene was negative (Figure 1).

Fig. 1: Detection of amplified *vanA* gene by PCR. Ethidium bromide stained agarose gel showing negative 11 *S.aureus* isolates with reduced susceptibility to vancomycin for vanA gene (lane 2-12). Positive control *Enterococcus feacalis* (*VRE*) show the amplified band *of* vanA gene at 1032bp in (lane 13) while lane 1 shows 100bp DNA ladder. The gel was visualized at 302 nm by UV transillumination.

The result of vancomycin agar screening method for detection of *S.aureus* with reduced sensitivity to vancomycin shows that 22(8.8%) *S.aureus* isolates grew on 2µg/ml vancomycin agar compared to only 3(1.2%) isolates grew on 4µg/ml Vancomycin agar and no growth was found on the 6 µg/ml vancomycin agar (Figure 2,3). According to CLSI; These 3 isolates suspected to be *VISA*.

The PAP/AUC ratio was determined in 22 isolates with vancomycin MIC ≥2 µg/ml. PAP/AUC revealed 3 *VISA* and 19 *hVISA* isolates (Figure 4).

The feasibility of using BHI-V3&V4 for screening of *hVISA* and *VISA* was confirmed when compared with the results of PAP/AUC ratio.

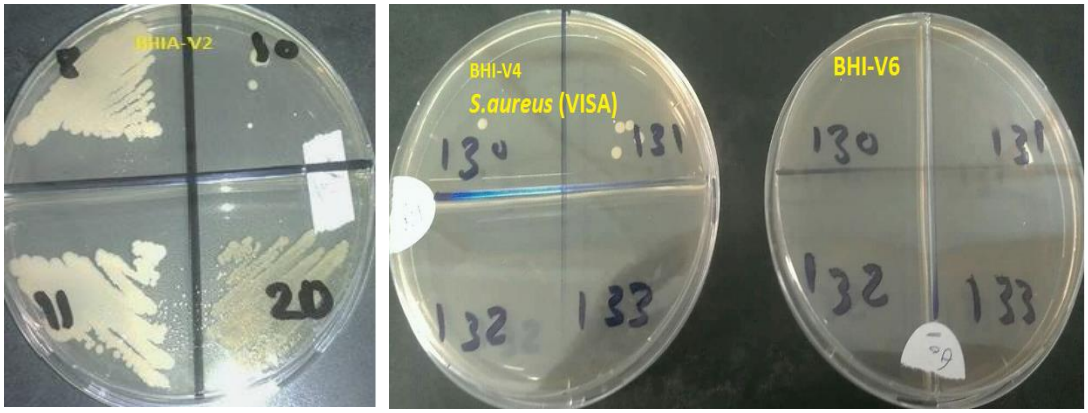

Fig. 2,3: *S.aureus* growth on BHIA with vancomycin 2, 4 and 6 µg/ml .

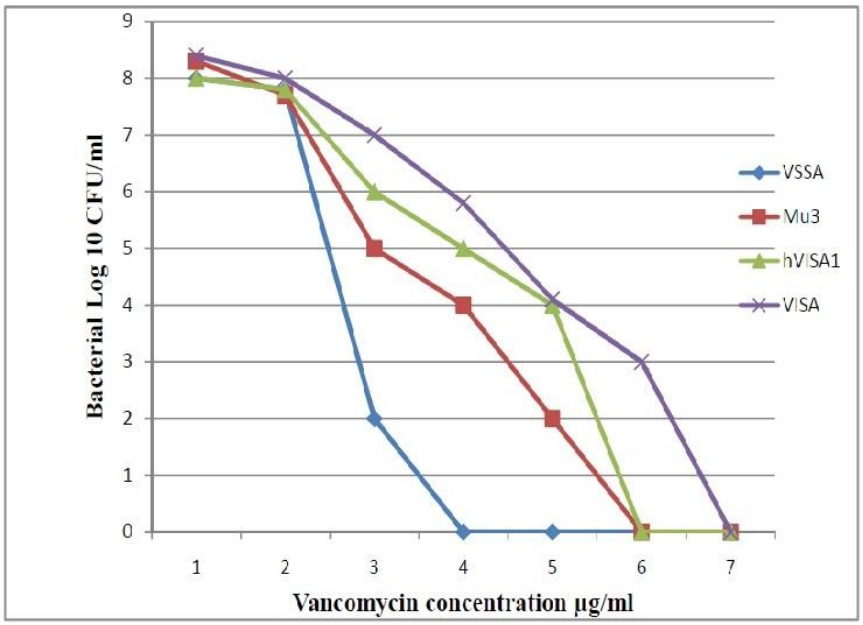

Fig. 4: Population analysis profile curves (PAP) for detection of *hVISA.*

Among the 250 *S.aureus* isolates; 173 (69.2%) were *MRSA*. On the other hand, 22/250 isolates (8.8%) were *VISA* and *hVISA*. All of them were *MRSA* and 19 out of them were sensitive to linezolid (Table 2).

Table 2: Distribution of reduced susceptibility to vancomycin and resistance to methicillin among the 250 *S.aureus* isolates.

	VRSA	VISA	hVISA	VSSA	Total
MRSA	0	3 (1.2%)	19 (7.6%)	151 (60.4%)	173 (69.2%)
MSSA	0	0	0	77 (30.8%)	77 (30.8%)
Total	0	3 (1.2%)	19 (7.6%)	228 (91.2)	250 (100%)

Molecular typing of S.aureus with reduced susceptibility to vancomycin according to agr typing (Figure 5 & 6).

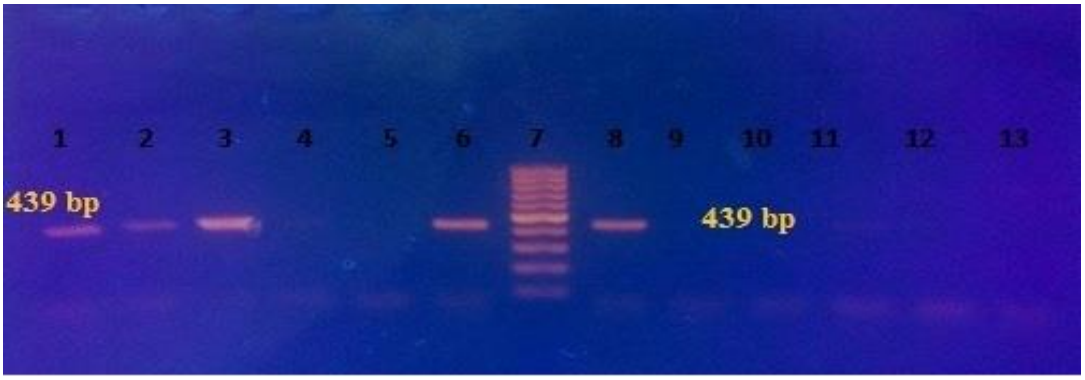

Fig. 5: Ethidium bromide stained agarose gel showing the expected band of the amplified *agrI* genes at 439bp in lanes (1,2,3,6,8) while lane 4,5 ,9,10,11,12 and 13 show no band. Lane 7 shows 100bp DNA ladder. The gel was visualized at 302 nm by UV transillumination

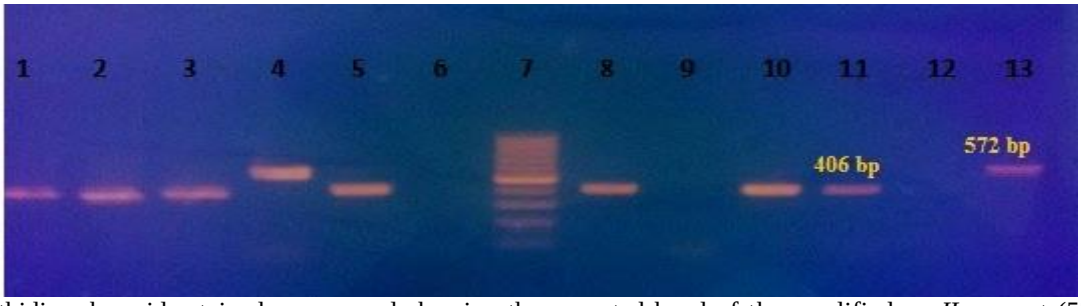

Fig. 6: Ethidium bromide stained agarose gel showing the expected band of the amplified *agrII* gene at (572 bp) in lanes 4,13 while lanes (1,2,3,5,8,10 and 11) show the expected band of the *agrIII* gene at (406bp). Lane 7 shows 100bp DNA ladder. The gel was visualized at 302 nm by UV transillumination**.**

The dominant *agr* group among the 22 VISA and hVISA *S.aureus* isolates was *agr* group I (45.5%) followed by *agr* group III (36.3%) then *agr* group II (9.1%) . Two isolates (9.1%) were non-typable and none of the isolate was *agr* group IV (table 3).

Table 3: Molecular typing of *S.aureus* with reduced susceptibility to vancomycin according to agr typing.

	***Agr*I**	***Agr*II**	***Agr*III**	***Agr*IV**	***Agr*NT**
VISA	2 (9.1%)	0	1(4.5%)	0	0
hVISA	8(36.4%)	2(9.1%)	7(31.8%)	0	2(9.1%)
Total	10(45.5)	2(9.1)	8(36.4%)	0	2(9.1)

DISCUSSION

In the current study, 250 *S.aureus* isolates were collected from different types of clinical samples mainly from pus and demonstrated high prevalence (69.2%) of *MRSA*. Several studies also reported a high prevalence of *MRSA* isolated from pus specimens (43.8%, 71.2%, 43.1% and 40.4%).[24-27]

The prevalence of *MRSA* varies among countries continues to increase approaching 70 percent in Japan,[28] and 45% in the United Kingdom (England and Wales only). In Egypt , *MRSA* was found to be 34.5% at Ein Shams Hospital, [27] and 54% at medical Research Institute, Alexandria University Hospital. [29]

The infections caused by *MRSA* are problematic because of the limited antimicrobial drugs choice for therapy and the concomitant high mortality. [30]

In this work, detection of *S.aureus* with reduced susceptibility to vancomycin was done using the standardized reference methods for susceptibility testing, namely; CLSI broth microdilution as gold standard and agar dilution method. In addition, the screening method previously reported as a first-line detection system (disc diffusion) was also evaluated. The sensitivities and specificities for the disc diffusion and agar dilution methods were (13.6%, 95.6%) and (86.4%, 100%) respectively. Vancomycin agar screening method was superior to the disc diffusion method in detection of *hVISA* and *VISA* phenotypes. A poor level of sensitivity (13.6 %) of the disk diffusion method was reported previously [31, 32] and was attributed to the high number of false negative results.

We determined the PAP for these 22 isolates with suspected reduced susceptibility to vancomycin and stratified cases according to their PAP/AUC ratio. In addition, the feasibility of using BHIA-V3 and BHI-V4 agars for screening for *hVISA* and *VISA* phenotypes was assessed. Our findings showed the complete matching in the results of both methods. These 22 *S.aureus* isolates (3 *VISA* and 19 *hVISA*) were mostly isolated from pyogenic infections (86.4% P<0.001).

In the current series, the feasibility of BHIA-V3 and BHI-V4 agar for screening of *VISA* and *hVISA* was approved as reported previously. [18] Therefore its use in the microbiological laboratory to screen all MRSA isolates for *VISA* and *hVISA* is reliable and more practical than PAP/AUC ratio.

In the current study, no VRSA could be detected among the *S.aureus* isolates. Meanwhile, 22(8.8%) *S.aureus* isolates with reduced susceptibility to vancomycin {3 (1.2%) *VISA* and 19 (7.6%) *hVISA*} were found. All of these isolates were *MRSA* which agrees with the results of the majority of reports which stated that *hVISA* and *VISA* isolates evolved from *MRSA*

strains,[33] On the contrary, Hu J *et al* , [34] reported that; the prevalence rates of *VISA* and *hVISA* among *S.aureus* isolated from different specimens were 0.5% and 10.0% respectively, and the *hVISA* was isolated also from MSSA isolates (4.1%).

There is evidence that *hVISA* and *VISA* are associated with vancomycin treatment failure, increased morbidity and mortality [35] The increase of *hVISA* prevalence rate and reduced vancomycin susceptibility among *MRSA* isolates should raise caution. The increasing prevelance of *hVISA* suggests a high risk for the development of complete vancomycin resistance. [36] Furthermore, it has been shown that such isolates adhere to inanimate surfaces 5- to 20-fold more than does *MRSA*, providing a source of nosocomial infections. [37]

Molecular approaches, like analysis of the polymorphism in agr genes, have permitted typing of isolates generating outbreaks. [38] The agr specific group I had the highest rate of detection (45.5%) among the 22 *S.aureus* isolates with reduced sensitivity to vancomycin.

These findings agree with various studies.[39,40]Abdolmajid Ghasemian [40] reported that the agr specific group I had the highest rate of detection among pathogens isolated from hospitalized children in Tehran. Similar results were reported by Ho CM *et al,*.[39] The relationship between the agr group and type of infections was variable. Although a strong relationship was reported between the agr type and particular infectious syndromes or with hospital acquired *MRSA* as observed by Jarraud *et al.*[13]yet Abdolmajid Ghasemian; reported that there was no such relationship. [40]

Taking in consideration that the all isolates were *MRSA*, it was found that *S.aureus* belonging to *agr* I were more resistant to macrolides (27.3%), aminoglycosides (22.7%), fluoroquinolones (27.3%) and carbapenem (22.7%) than *agr* groups III . Therefore testing for vancomycin susceptibility should include all *MRSA* isolates isolated from different clinical specimens especially pyogenic infections.

CONCLUSION

- Screening all *MRSA* isolates for *VISA* and *hVISA* is recommended.
- Also strict infection control measures and antibiotic policy should be adopted to control the problem of *VISA* and *hVISA*.
- Considering the relation between the age group and the antibiotic resistance in *S.aureus* isolates, further studies are needed to fully elucidate the mechanisms involved in the interface between virulence and antibiotic resistance.

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