

Original Research Article

Characterization of Vancomycin Resistant *Staphylococcus aureus* in Tanta University Hospital

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ABSTRACT

Keywords

Staphylococcus aureus
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resistance-
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Staphylococcus aureus is a major cause of potentially life threatening infections acquired in health care and community settings. It has developed resistance to most classes of antimicrobial agents with dramatic increase in the number of health care associated infections due to vancomycin resistant *S. aureus* (VRSA). In our study, 437 staphylococci strains were isolated from different wards of Tanta university hospital. Agar diffusion method revealed that 88 (20.13%) isolates were VRSA. Two mechanisms of staphylococci resistance to vancomycin were studied. The first one, by having the *van* genes which was detected in resistant isolates by polymerase chain reaction (PCR). The only gene found in the selected VRSA isolates was *vanA* gene. The second type, by the increase in cell wall thickness of resistant isolates relative to sensitive isolates and it was studied by transmission electron microscope. Plasmid curing experiment was applied to *vanA* positive isolates using ethidium bromide at 42°C. Treated isolates were tested again for detection of *vanA* gene using traditional PCR. Loss of bands with a size of 1032 bp was observed in all treated isolates which indicates successful curing and loss of plasmid mediated *vanA* gene. There was also an increase in autolytic activity in VRSA isolates.

Introduction

S. aureus has been recognized as an important cause of human disease for more than 100 years. It is recognized as a cause of a wide range of infections. These infections range from minor skin infections and chronic bone infections to devastating septicemia and endocarditis (Howden *et al.*, 2010).

Before the discovery of antibiotics, mortality rate of *S. aureus* strains was more than 75 % (Van *et al.*, 2012). After the initial success of penicillin in treating *S. aureus* infections, resistance to this drug began to emerge (Atkinson and Lorian, 1984). Now, more than 90% of staphylococcus strains are resistant to penicillin (Chambers, 2001), followed by increasing resistance to

methicillin, aminoglycosides, macrolides and lincosamide (Levin *et al.*, 2005). The glycopeptide antibiotic vancomycin was first released in 1958. Subsequently, vancomycin has been the best treatment for serious infections caused by staphylococci, which are becoming increasingly common globally (Hiramatsu *et al.*, 1997).

For many years there was no indication that vancomycin resistance in *S. aureus* was likely to be a problem. Therefore, initial reports of vancomycin resistance in clinical isolates of *S. aureus* from Japan in 1997 generated a significant concern (Hiramatsu *et al.*, 1997). In addition to Japan, VRSA strains have been isolated from different countries all over the world, indicating that the problem is a global one (Trakulsomboon *et al.*, 2001).

Two forms of staphylococci resistance to vancomycin have been identified. The first type of resistance is due to *van* genes (Perichon and Courvalin, 2009). The second type is due to increased cell wall synthesis and increase autolytic activity leading to thicker and more disorganized cell wall (Hanaki *et al.*, 1998).

In this study, we aimed to show the emergence of vancomycin resistance in Tanta university hospital and to study the mechanisms of resistance.

Materials and Methods

Bacterial strains

A total of 437 staphylococci isolates were recovered randomly from clinical samples (blood, urine, sputum, surgical wound and nasal swabs) from patients admitted to different departments of Tanta university hospital during the period from October 2011 to August 2012. *S. aureus* was

identified biochemically according to Cheesbrough (2000).

Screening of vancomycin-resistant *S. aureus*

It was performed using the disk diffusion method according to clinical and laboratory standard institute (CLSI, 2012) standards. The antibiotic disk used was vancomycin (30µg) (Sigma, USA). All staphylococci isolates were cultured in Mueller-Hinton agar (Oxoid, UK) to determine MICs of vancomycin using agar dilution method according to CLSI (2012).

DNA extraction

Total DNA was extracted by freeze and thaw (F and T) method as described by Mozioglu *et al.* (2014). Briefly, bacterial pellet was resuspended in 100 µL of TE buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0), and the tubes were kept at –80 °C for 5 min and incubated at 60 °C for 10 min. These 2 steps (F and T) were repeated 5 times. The tubes were then centrifuged at 5000 × g at 4 °C for 10 min, and the supernatant was transferred to a clean tube and stored at –20 °C until used as total DNA extract.

PCR amplification

PCR was performed on *S. aureus* isolates with different vancomycin MICs to detect vancomycin resistance genes *vanA*, *vanB*, *vanC*. The sequence of these primers and the expected product size is listed in table 1. The PCR reaction tube contained 12.5 µl 2X PCR master mix (Fermentas, USA), 2µl DNA solution, 1 µl of each primer synthesized by a custom primer service (Fermentas, USA) and 8.5 µl nuclease free water. The recommended PCR conditions are presented in table 2. PCR reaction tubes

were centrifuged briefly to mix and bring the contents to the bottom of the tubes, and placed into PCR thermal cycler (Thermo SCIENTIFIC, USA) where DNA was amplified. PCR products were analysed by 1.5% agarose gel (Pharmacia, Sweden) electrophoresis and made visible by ethidium bromide (15 mg/ml) staining and UV transillumination (Kowell, Spain).

Plasmid curing

It was performed as described by Rubin and Rosenblum (1971) to determine if the resistance gene is encoded by a plasmid. Ethidium bromide was used to eliminate plasmid of the selected VRS isolates at elevated temperature 42 °C. Two fold serial dilutions of EtBr were prepared in LB broth. Aliquots of 100 µl of 10⁷ CFU/ml suspension of each isolate were inoculated into the serial EtBr dilutions. The tubes were gently shaken and incubated at 42 °C for 24 hrs. After overnight incubation, tubes were inspected for the presence of turbidity. Hundred microliters from subinhibitory concentration for each isolate were subcultured on LB agar plates. After overnight incubation at 37 °C, each of the resultant colonies was toothpicked and subcultured on LB agar plates containing the breakpoints of the tested antimicrobials. Control plates containing LB agar without antimicrobial agent was simultaneously subcultured. Colonies which grew on control plates but failed to grow on antimicrobial agent containing plate were recorded as the cured cells.

Transmission electron microscope

The procedures were carried out according to McDowell and Trump (1976). Cells in logarithmic phase were fixed in 2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 2 h, and treated with 1%

osmium tetroxide for 2 hat 4 °C. Cells were dehydrated with graded concentrations of ethanol, and embedded in EPOK812 (Ohken, Tokyo, Japan). Ultra-thin sections were stained with uranyl acetate and lead citrate, and then examined with a transmission electron microscope TEM-1200EX (Jeol, Tokyo, Japan).

Autolysis assay

Cells were grown to an OD₆₀₀ of 0.7 in TSB broth at 37°C, and were pelleted by centrifugation at 3700 rpm for 5 minutes. The cells were washed once with saline, and resuspended in 0.01 M sodium phosphate buffer (pH 7.0) to about 0.8 at OD₆₀₀. The cell suspension was incubated at 37 °C with continuous gentle shaking. Decrease in optical density was monitored every 1 hr with UV-Vis spectrophotometer (SCHIMADZU, Japan) as described by Hanaki *et al.* (1998).

Result and Discussion

Screening of vancomycin-resistant *S. aureus*

Among the 437 clinical isolates of *S. aureus*, 88 (20.13%) were identified as VRSA by disc diffusion method. Vancomycin MICs against staphylococci isolates are presented in table 3.

Polymerase chain reaction

Nine staphylococci isolates were selected for PCR studies. The selected isolates were representatives from each vancomycin MICs ranging from 2 to 512 µg/ml. Traditional PCR was performed on total DNA of each selected isolate for detection of *vanA*, *vanB* and *vanC* genes.

The amplified products were

electrophoresed and the amplicons were visualized with approximate size of 1032 base pairs for *vanA* gene and of 647, 815 for *vanB* and *vanC* genes respectively.

VanA gene was detected in 5 isolates out of 9 isolates with vancomycin MICs ranging from 32 to 512 µg/ml as illustrated in figure 1 where *vanB* and *vanC* genes were not found in any isolate.

Plasmid curing experiment was applied to *vanA* positive isolates using ethidium bromide. Treated isolate were tested again for detection of *vanA* gene using traditional PCR. Loss of bands with a size of 1032 bp was observed in all treated isolates which indicates successful curing and loss of plasmid mediated *vanA* gene as shown in figure 2.

Table.1 PCR primers and products for detection of *van* genes among the selected clinical isolates

Genes	Primer *	Sequence (5' to 3')	Amplicon size (bps)	Reference
<i>vanA</i>	Fw	5'-CATGAATAGAATAAAAAGTTGCAATA-3'	1032	Abd El-Baky <i>et al.</i> (2014)
	Rv	5'-CCCCTTTAACGCTAATACGACGATCAA-3'		
<i>vanB</i>	Fw	5'-ACGGAATGGGAAGCCGA-3'	647	Bhatt <i>et al.</i> (2014)
	Rv	5'-TGCACCCGATTTTCGTTC-3'		
<i>vanC</i>	Fw	5'-ATGGATTGGTACTGGTAT-3'	815	
	Rv	5'-TAGCGGGAGTGACCAGTAA-3'		

Table.2 PCR conditions for *van* genes

Genes	Initial denaturation		Denaturation		Annealing		Extension		No. of cycles	Final extension	
	Temp. (°C)	Time (min)	Temp. (°C)	Time (min)	Temp. (°C)	Time (min)	Temp. (°C)	Time (min)		Temp. (°C)	Time (min)
<i>vanA</i>	95	5	94	1	45	1	72	1	35	72	10
<i>vanB</i>	95	5	94	1	50	1	72	1	35	72	10
<i>vanC</i>	95	5	94	1	50	1	72	1	35	72	10

Table.3 Vanomycin MIC values against staphylococci isolates

Vancomycin MIC* (µg/ml)	≤ 2	2	4	8	16	32	64	128	256	512
No. of Staphylococci Isolates	88	75	68	63	55	30	20	17	16	5

*Vanomycin breakpoint ≥ 32 µg/ml.

Figure.1 Electrophoregram of *vanA* gene amplicon Lane M was 1kb DNA ladder

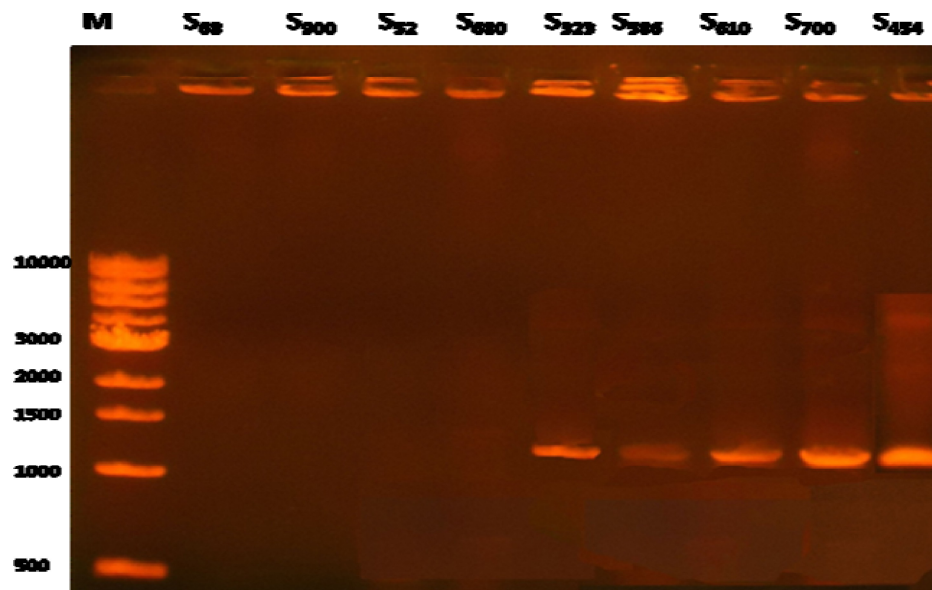


Figure.2 Electrophoregram of *vanA* positive isolates after curing
Lane M was 1kb DNA ladder

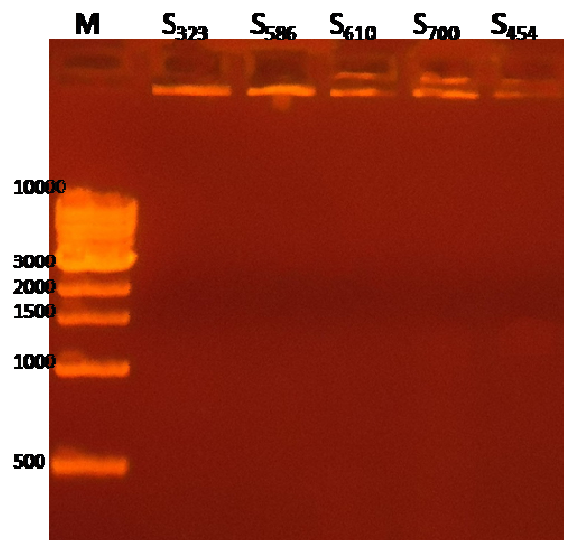
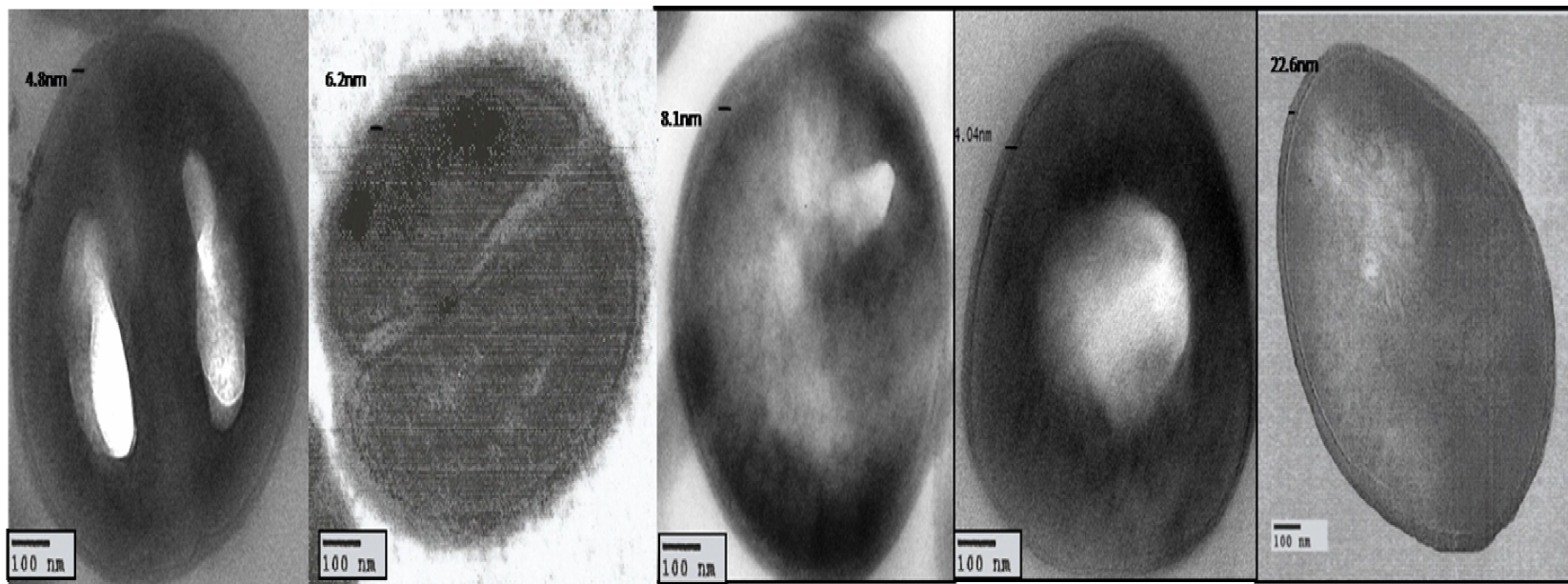


Figure (3): Transimission electron micrograph of VSS isolates.



a- Reference strain with vancomycin MIC=0.5 µg/ml

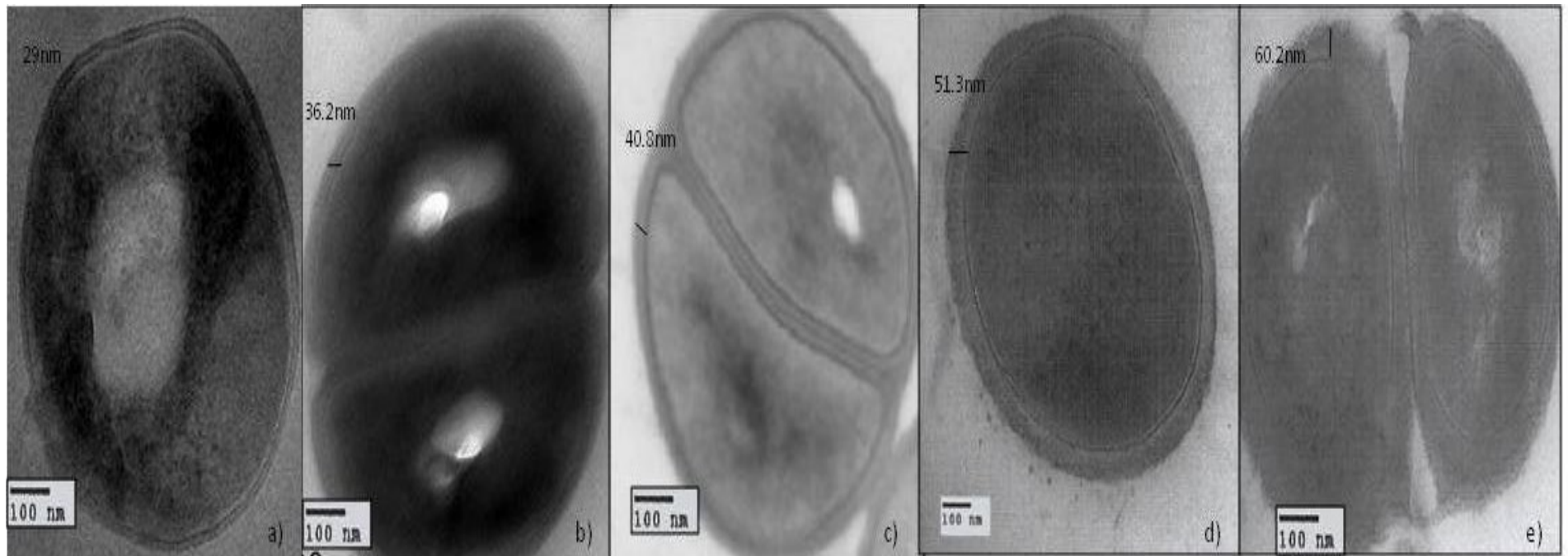
b- S₆₈ with vancomycin MIC=2 µg/ml

c- S₉₀₀ with vancomycin MIC=4 µg/ml

d- S₅₂ with vancomycin MIC=8 µg/ml

e- S₆₈₀ with vancomycin MIC=16 µg/ml

Figure (4): Transimission electron micrograph of VRS isolates



a-S₄₅₄ with vancomycin MIC=32 µg/ml

b-S₁₆₁ with vancomycin MIC=64 µg/ml

c-S₆₁₀ with vancomycin MIC=128 µg/ml

d-S₅₈₆ with vancomycin MIC=256 µg/ml

e-S₃₂₃ with vancomycin MIC=512 µg/ml

Figure.5 Relation between vancomycin MICs of staphylococci isolates and their cell wall thicknesses

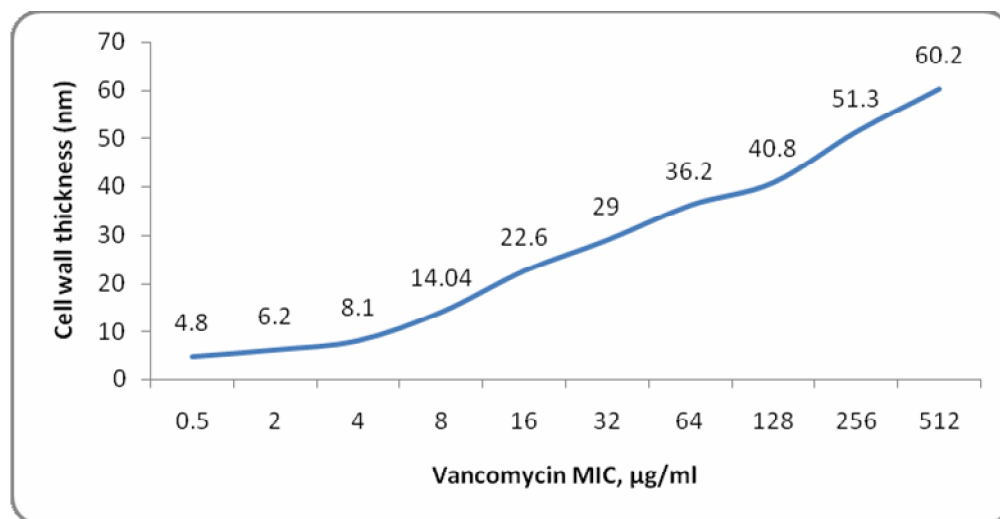


Figure.6 Autolytic activity of S₄₅₄

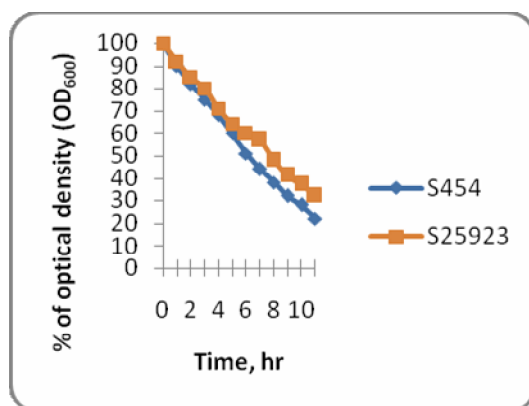
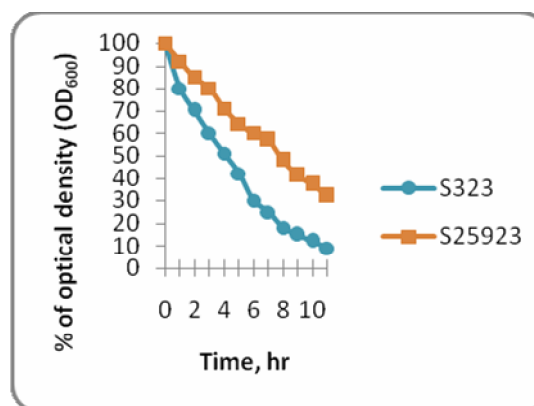


Figure.7 Autolytic activity of S₃₂₃



Transmission electron microscope

Transmission electron microscopy of nine isolates of staphylococci with vancomycin MIC values ranging from 2 to 512 µg/ml plus *S. aureus* reference with vancomycin MIC=0.5 µg/ml was performed and their pictures are shown in figure 3 and figure 4. Relation between vancomycin MICs of staphylococci isolates and their cell wall thicknesses is shown in figure 5.

Transmission electron microscopy of the five cured VRS isolates was also performed

and it was found that their cell wall thicknesses were the same as the isolates before curing.

Autolysis assay

Five VRSA with different vancomycin MICs plus *Staphylococcus aureus* reference strain were selected for this test. Autolysis activity of isolates S₄₅₄ with vancomycin MIC=32 µg/ml and S₃₂₃ with vancomycin MIC=512 µg/ml are shown in figure 6 and figure 7.

As VRSA continue to emerge, there is a large need to fully characterize them and conduct well designed research about them. The current VRSA in hospital as well as in community are alarming situation. The development of antibiotic resistance in developing countries like ours is highly related to their rational antibiotic usage due to its easy availability at the drug store without prescription and uncontrolled use in hospitals (Holloway, 2000). Widespread use of vancomycin to treat infections caused by methicillin resistant *S. aureus* (MRSA) and other Gram-positive cocci has led to the emergence of vancomycin resistance. The large scale of spread of resistance to vancomycin has been perceived as a fearsome threat to the already challenging therapy of MRSA (Abd El -Baky *et al.*, 2014).

In this report, we studied the prevalence and mechanisms of vancomycin resistance of VRSA. 88 (20.13%) VRSA isolates with different MICs ranged from 32 µg/ml to 512 µg/ml were detected. Many researchers reported vancomycin resistance; Bataineh (2006) has reported VRSA strains from Jordan. Song *et al.* (2004) have also reported the emergence of VRSA strains from India and its neighboring countries. Alzolibani *et al.* (2012) reported the presence of VRSA in Saudi Arabia.

In this study, polymerase chain reaction was performed for detection of van genes. Nine staphylococci isolates were selected for PCR studies. The selected isolates were representatives from each vancomycin MICs ranging from 2 to 512 µg/ml. Traditional PCR was performed on total DNA of each selected isolate for detection of *vanA*, *vanB* and *vanC* genes. The amplified products were electrophoresed and the amplicons were visualized with approximate size of 1032 base pairs for *vanA* gene and of 647,

815 for *vanB* and *vanC* genes respectively. *VanA* gene was detected in VRSA isolates only and it was not found in VSSA. *VanB* and *vanC* genes were not found in any isolates. Thati *et al.* (2011) reported that out of 7 VRSA 6 isolates (85.7%) contained *vanA* gene only. El-Daker *et al.* (2008) reported that 100% of VRSA isolates possessed the *vanA* gene only. Tiwari and Sen (2006) have reported a van gene-negative VRSA in which vancomycin resistance was a result of increase in cell wall thickness only.

Plasmid curing experiment was applied to *vanA* positive isolates using ethidium bromide. Treated isolate were tested again for detection of *vanA* gene using traditional PCR. Loss of bands with a size of 1032 bp was observed in all treated isolates which indicates successful curing and loss of plasmid mediated *vanA* gene a similar result was reported by Chaudhari and Bajaj (2015).

Transmission electron microscope was used to view the increase in cell wall thickness in VRSA in comparison with VSSA and reference strain. It was found that cell wall thicknesses of VRSA isolates with MICs ranging from 32 to 512 µg/ml was relatively high in comparison with cell wall thicknesses of VSSA with MICs ranging from 2 to 16 µg/ml. These results were also supported by Cui *et al.* (2003). Other studies from different laboratories have shown that the mechanism of resistance to glycopeptide antibiotics in staphylococci isolates is based on a thickened cell wall with decreased cross-linking and increased cell wall metabolism (Geisel *et al.*, 1999; Reipert *et al.*, 2003).

Cured VRS cells showed the same cell wall thickness as non cured cells. This explains why their MICs decreased but not to a large

extent. Vancomycin resistance of the cured cells might be a result of a thickened cell wall which allows the bacteria to inhibit vancomycin molecules from reaching its major target on the cytoplasmic membrane of the cell (Cui *et al.*, 2006). Hanaki *et al.* (1998) observed some features that were present in VRSA and not present in VSSA isolates and these features were high autolytic activity and increase production of autolytic enzymes and this is comparable to our results.

Finally, many new approaches are needed to control vancomycin resistance. The future of VRSA strains is not clear and much research is needed to help further understand all aspects of these organisms including their epidemiology, microbiology, clinical and infection control implications and optimal treatment.

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