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Full Length Research Paper

Detection and molecular characterization of vancomycin resistant *Staphylococcus aureus* from clinical isolates

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Until now, few strains of vancomycin resistant *Staphylococcus aureus* (VRSA) have been reported worldwide. The disk diffusion method for determination of vancomycin sensitivity frequently misclassifies intermediately susceptible isolates to fully susceptible. However, non-automated minimum inhibitory concentration (MIC) detection methods are the gold standards. In the present study, 439 Gram positive clinical isolates were collected. Among them, 220 *Staphylococcus aureus* were identified. Multiplex polymerase chain reaction (PCR) detection method for vancomycin resistant *S. aureus* (VRSA) was developed for detection of both vanA gene (for vancomycin resistance) and nuc gene (specific for *S. aureus*) in a single step PCR compared to conventional non-automated disc diffusion and MIC detection methods. Molecular typing of VRSA isolates was performed using randomly amplified polymorphic (RAPD) DNA assay technique. The results show 10 VRSA isolates detected by disc diffusion method and MIC determination. Five out of them harbored vanA gene that were detected by multiplex PCR and most of them showed low clonal diversity.

Key words: Molecular characterization, multiplex polymerase chain reaction (PCR), vancomycin resistant *Staphylococcus aureus* (VRSA), vanA gene, nuc gene, randomly amplified polymorphic DNA (RAPD) assay technique.

INTRODUCTION

It was initially feared that *Staphylococcus aureus* would acquire the *van* genes that code for vancomycin resistance in *Enterococcus* species (Cercenado et al., 1998). Several years prior to the report of the first clinical vancomycin intermediate sensitive *S. aureus* (VISA) isolate, laboratory strains of VISA and vancomycin resistant *S. aureus* (VRSA) that had much thicker cell walls than the sensitive parent strains were produced (May et al., 1998). Subsequent investigators demonstrated that cell wall synthesis and turnover are upregulated in VRSA isolates, leading to thicker and more-disorganized cell walls (Rybak and Akins, 2001). Furthermore, the altered cell walls that appear to have a reduced affinity for vancomycin as soluble targets are able to bind more antibiotics in the presence of vancomycin-resistant isolates (Fahmy and El-Hendy, 2002). Hence, the role of penicillin-binding proteins (PBPs) in vancomycin resistance was unclear (Sieradzki and Tomasz, 1997).

The most common way VRSA are transmitted in the health care setting is from patient to patient via the hands of health care workers (Murray, 2000). The risk of such transmission may increase in colonized health care workers with sinus or upper respiratory infections and in those wearing artificial nails (Tacconelli et al., 2001). The

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administration of more than three antibiotics is associated with the development of VRSA infection amongst patients already colonized with VRSA (Harbarth et al., 2002). Infection rates increased with the prolonged duration of preoperative hospital stay (Livermore, 2000).

Six types of vancomycin resistance have been characterized on both a phenotypic and a genotypic basis in enterococci. Five of these types (VanA, B, D, E, and G) correspond to acquired resistance; one type (VanC) is an intrinsic property of *Enterococcus gallinarum* and *Enterococcus casseliflavus–Enterococcus flavescens.* Classification of glycopeptide resistance is currently based on the primary sequence of the structural genes for the resistance ligases rather than on the levels of resistance to glycopeptides because the minimum inhibitory concentration (MIC) ranges of vancomycin and teicoplanin against the various types overlap. VanA-type strains display high levels of inducible resistance to both vancomycin and teicoplanin, whereas VanB-type strains have variable levels of inducible resistance to vancomycin only (Arthur et al., 1996a; b). VanD-type strains are characterized by constitutive resistance to moderate levels of the 2 glycopeptides (Depardieu et al., 2003b) while VanC-, VanE-, and VanG-type strains are resistant to low levels of vancomycin but remain susceptible to teicoplanin (Reynolds and Courvalin, 2005).

Although the six types of resistance involve related enzymatic functions, they can be distinguished by the location of the corresponding genes and by the mode of regulation of gene expression. The *vanA* and *vanB* operons are located on plasmids or in the chromosome (Arthur et al., 1996a; b), whereas the *vanD* (Depardieu et al., 2003b), *vanC* (Arias et al., 2000), *vanE* (Abadia et al., 2002), and *vanG* (Depardieu et al., 2003a) operons have, thus far, been found only in the chromosome.

MATERIALS AND METHODS

Patient and sampling

The samples were collected from outpatient clinics as well as inpatient departments mainly from intensive care unit and orthopedic department at Zagazig University hospitals, Egypt. These samples harbored mainly from surgical sites by swabbing or from urine, blood and sputum samples.

Bacterial cultivation and isolation

The collected samples were cultivated on nutrient agar, blood agar, deoxyribonuclease, mannitol salt agar and Mueller Hinton Agar (Oxoid).

Bacterial identification

Morphology and microscopy

Colonies were examined for morphology and Gram positive grape like clusters of *Staphylococci* (Kloos and Bannerman, 1995).

Biochemical reactions

Catalase test, tube and slide coagulase test, mannitol salt agar and deoxyribonuclease (Oxoid) test were performed.

Antibiotic susceptibility test

Disc diffusion method

Sensitivity of the isolated strains to methicillin (5 µg), vancomycin (30 µg), penicillin G (10 µg), and oxacillin (30 µg) (antibiotics from Oxoid) was performed on Mueller-Hinton agar plates (Oxoid). VRSA isolates were subjected to further antibiotic sensitivity tests.

Determination of minimum inhibitory concentration (MIC)

Broth dilution method was done in tubes for different concentration of vancomycin (4, 8, 16, 32, 64 mg/L). Broth dilution was performed in Muller-Hinton broth and read after 24 h, as recommended by the National Committee for Clinical Laboratory Standards (NCCLS). MIC for different isolates to vancomycin differentiates the resistant from the sensitive strains. By definition, VRSA shows *in-vitro* MICs of > 4 mg/L (Rybak et al., 2001). However, other workers reported that the most resistant strains bear the vanA gene cluster and these are generally recognizable as MICs of vancomycin are usually found to be in the range 32 to 64 mg/L (Hawkey, 2009). VanA-type strains display high levels of inducible resistance to both vancomycin and teicoplanin, whereas VanB-type strains have variable levels of inducible resistance to vancomycin only (Arthur et al., 1996). VanD-type strains are characterized by constitutive resistance to moderate levels of the 2 glycopeptides (Depardieu et al., 2003). VanC-, VanE-, and VanG-type strains are resistant to low levels of vancomycin but remain susceptible to teicoplanin (Reynolds and Courvalin, 2005).

Multiplex polymerase chain reaction (PCR)

The primers were one pair for (vanA) gene detection “vancomycin resistance gene” (forward primer EA1, 5´-GGGAAAACGACGACAATTGC-3´ and reverse primer EA2, 5´-GTACAATGCGGCCGTTA-3´), and the other pair of primer for (nuc) gene detection “confirmatory gene for *S. aureus*” (forward primer TN1, 5´-GACTATTATTGGTTGATCCACCTG-3´ and reverse primer TN2, 5´- GCCTTGACGAACTAAAGCTTCG-3´) (Depardieu et al., 2004)

Bacterial isolates

From an overnight shaken culture, 1.5 ml of staphylococci in brain heart infusion broth (Oxoid) was centrifugated at 15,000 *g*, for 5 min.

DNA extraction

The bacterial cells were suspended in 150 µl of a solution containing 50 mM Tris hydrochloride (pH 8.0), 10 mM EDTA, and 7% sucrose with lysozyme lysostaphin (5 mg/ml; AMBI, Trowbridge, UK) and incubated at 37°C for 10 min. The resulting protoplasts were lysed with sodium dodecyl sulfate (1.25%) for 10 min on ice, and after two phenol-chloroform extractions, total DNA was

Table 1. Distribution of MRSA and VRSA isolated from different specimens.

Types of specimen	MRSA isolates	VRSA isolates
	Number/Total	Number/Total
Pus	38/94*	6/94**
Abscess	15/35	1/35
Blood	16/71	1/71
Urine	3/9	1/9
Sinusitis	1/1	0/1
Catheters	1/ 2	1/2
Sputum	1/7	0/7
Peritoneal	1/1	0/1

*P < 0.05 compared to blood, P > 0.05 compared to abscess, sputum, urine, sinusitis, v. catheter and peritoneal fluid and **P > 0.05 compared to all other specimen types.

recovered in the supernatant after centrifugation (15,000 *g,* 5 min).

PCR amplification

In brief, 1 to 3 µL of total DNA was subjected to multiplex PCR amplification in a 100 µL reaction mixture containing PCR buffer [10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 Mm $MgCl_2$, 0.1% Triton X-100, 0.2 mg/ml of bovine serum albumin, 50 µM each deoxynucleoside triphosphate, of each of the 2 primer pairs, and 2 U of Taq polymerase (QBIOgene, Montreal, Quebec, Canada)]. Amplification was carried out with the following thermal cycling profile: 3 min at 94°C and 30 cycles of amplification consisting of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C, and 7 min at 72°C for the final extension.

Detection of PCR product

DNA fragments were analyzed by electrophoresis in 0.5X TBE (Tris-borate-EDTA) on a 1% agarose (Sigma-Aldrich) gel stained with ethidium bromide (Sigma-Aldrich).

Molecular typing

Molecular typing was performed using randomly amplified polymorphic (RAPD) DNA assay (Tambic et al., 1997). The arbitrary primers (From Neest) that were used for RAPD typing are: OPA-05 5′- AGGGGTCTTG -3′; OPB-12 5′ - CCTTGACGCA -3′; OPC-09 5′ -CTCACCGTCC - 3′; OPD-05 5′ - TGAGCGGACA - 3′. The four primers that were used after a good ability to discriminate *S. aureus* strains for typing purposes are shown in the pilot experiments. Each reaction mixture included 200 µM nucleotide (each) (Sigma), 5 µL of 10× reaction buffer, 500 nM primer, 50 ng template DNA, and 2U of Taq XL DNA polymerase (Northum-bria Biochemicals Ltd., Cramlington, United Kingdom), and the mixture was made up to 50 µL with molecular biology-grade water. This mixture was overlaid with 50 µL of mineral oil (Sigma). RAPD cycling parameters were 94°C for 5 s, 34°C for 30 s, and 72°C for 1 min for 35 cycles, with a final extension of 72°C for 5 min. Reactions were carried out in a Hybaid Thermal Reactor (Perkin Elmer). The products were electrophoresed on 2% agarose gels in 0.5X TBE (Tris, borate, EDTA) buffer. Synthetic PCR molecular size markers were included in each gel. The gels were run for 5 to 6 h at 60 V to allow the complete separation of the bands.

Statistical analysis

Data analysis was performed using Chi-squared test, Fisher's exact test and T-test. Polymorphism analysis was performed and illustrated in dendrogram and similarity index.

RESULTS

Two hundred and twenty (220) *S. aureus* strains were defined from 439 Gram positive specimens (50.1%). Methicillin resistant *S. aureus* were 76 isolates (34.5%), and vancomycin resistant was 10 isolates (4.5%). MRSA isolates among *S. aureus* from intensive care unit (ICU) were 29.0% (27/93), 22.2% (10/45) from orthopedic department and 47.6% (39/82) from outpatient clinics. Only statistically significant difference was observed regarding MRSA isolates between outpatient and each of ICU (P < 0.05) and orthopedic ward (P < 0.01), while no statistical difference between ICU and orthopedic ward were detected (P > 0.05). VRSA isolates from ICU, orthopedic unit and outpatient clinics were 6.5% (6/93), 6.7% (3/45) and 1.2% (1/82), respectively. VRSA isolates distribution to different wards showed no statistical significance (P> 0.05).

Table 1 shows that MRSA isolates from pus were 40.4% (38/94) with statistical significant difference compared to blood (16/71, 22.5%), P < 0.05. However, there were no significant differences when compared with pus with other types of specimens (P > 0.05). On the other hand, for VRSA isolates, there were no statistically significant differences when pus was compared with other specimens (P > 0.05), and there was no statistical significance for patients' age or gender on the distribution of VRSA and MRSA. Moreover, all VRSA isolates were sensitive to cefoperazone, trimethoprime, amikin and rifampin and resistant to clavulinic acid, cefoxtin, cefazolin, oxacillin, tetracyclin, vancomycin and cefexime. Table 2 shows that five isolates were identified as VRSA by multiplex PCR out of 10 isolates by conventional

Table 2. Distribution of the conventional and molecular detected VRSA isolates.

Specimen	Ward	Multiplex PCR	Conventional method	Isolate
wound	Orthopedic	-	+	1
wound	Orthopedic	-	+	2
wound	Orthopedic	-	+	3
Ven. Catheter	ICU	-	+	4
Abscess	ICU	-	+	5
Blood	ICU	+	+	6
Pus	ICU	+	+	7
Urine	ICU	+	+	8
Pus	ICU	+	+	9
Pus	outpatient	+	+	10

*The 10 isolates identified as VRSA by conventional methods (MIC = 32 mg/dl)

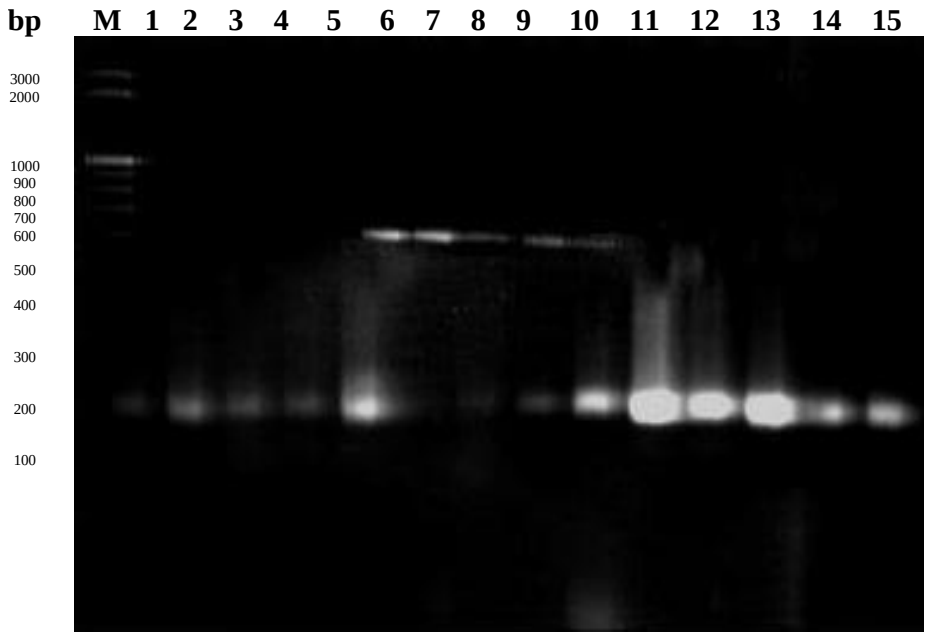

Figure 1. Multiplex PCR for detection of vanA and nuc genes. M, Molecular size marker; lanes 1 to 10 (isolates 1 to 10), the 10 tested VRSA strains (5 of them “isolates 1 to 5” are negative for vanA gene, while 5 strains “isolates 6 to 10” are positive for vanA gene at molecular size of 732 bp); lanes 11 to 15 (isolates 11 to 15), methicillin and vancomycin sensitive *S. aureus* strains “negative controls”. All the tested isolates “1 to 15” were confirmed as *S. aureus* as they are positive for nuc gene with PCR product molecular size of 218 bp.

methods (50%).

Figure 1 shows the multiplex PCR for specific and rapid identification of VRSA in one step using a pair of primers. The first one is specific for detection of vancomycin resistant gene (vanA) with product molecular size of 732 bp. The second for nuc gene for identification of *S. aureus* with product molecular size of 218 bp. PCR results show that strains of isolates 6, 7, 8, 9 and 10 were positive for both vanA gene and nuc gene. Figures 2 to 5 show the random amplified polymorphism (RAPD) of 15 isolates. Polymorphism analysis was performed using statistical analysis illustrated in dendrogram and similarity index (Figure 6; Table 3). It was observed that the dendrogram divided the isolates at the value of 15 on the scale into four groups; the first group included the isolates 5, 8, 7, 4, 2, 3, 9, 11, 10, 6 (which include all the vancomycin resistant strains except strain 1), the second group included isolates 13, 14, 15 (which includes all the vancomycin sensitive isolates except 11, 12), the third group included isolate 1 and the fourth group included

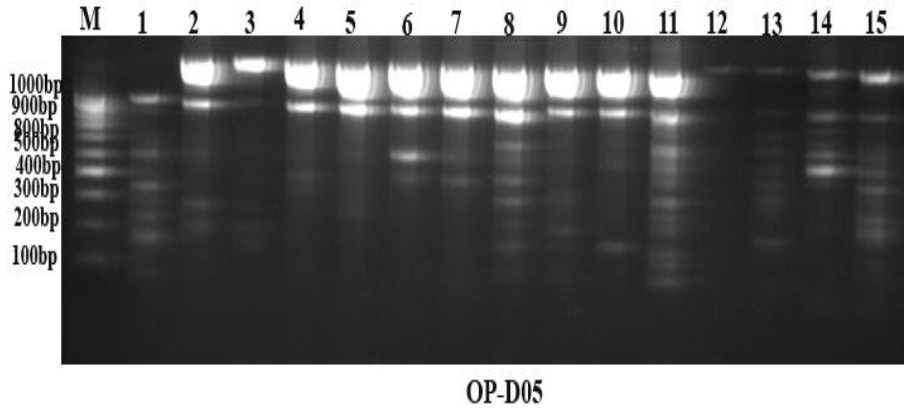

Figure 2. Pattern of RAPD using operon D05 primer for the 15 isolates previously tested by PCR for vanA and nuc genes. M, Molecular size marker; lanes 1 to 10 (isolates 1 to 10), the 10 tested VRSA strains (5 of them "isolates 1 to 5" are negative for vanA gene, 5 strains "isolates 6 to 10" are positive for vanA gene); lanes 11 to 15 (isolates 11 to 15), methicillin and vancomycin sensitive *S. aureus* strains "negative controls". All the tested isolates "1 to 15" were confirmed as *S. aureus* as they are positive for nuc gene.

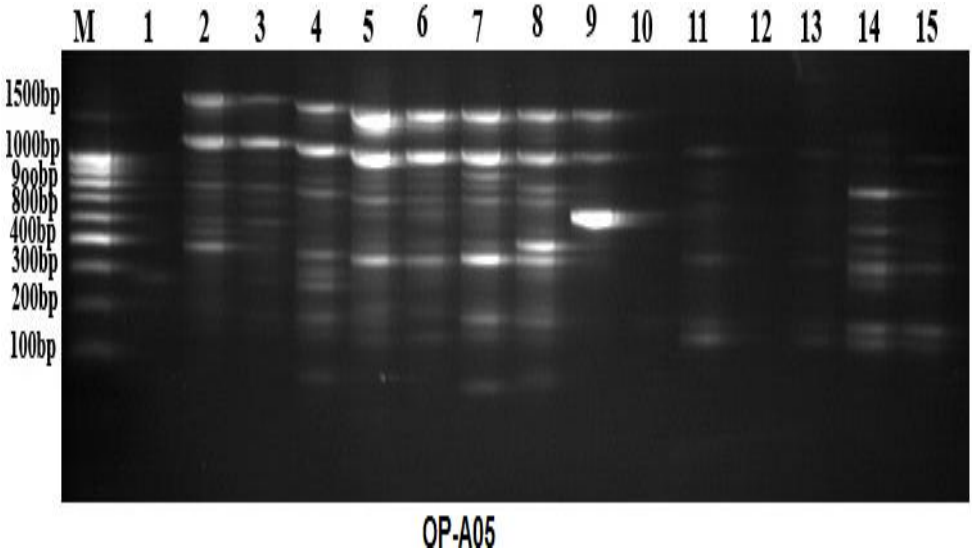

Figure 3. Pattern of RAPD using operon A 05 primer for the 15 isolates previously tested by PCR for vanA and nuc genes. M: Molecular size marker, Lanes 1 to10 (isolates 1 to 10), the 10 tested VRSA strains (5 of them "isolates 1 to 5" are negative for vanA gene, while 5 strains "isolates 6 to 10" are positive for vanA gene); lanes 11 to 15 (isolates 11 to 15), methicillin and vancomycin sensitive *S. aureus* strains "negative controls". All the tested isolates "1 to 15" were confirmed as *S. aureus* as they are positive for nuc gene.

isolate 12 (Figure 6). The 5 VRSA isolates "vanA gene positive" (6, 7, 8, 9, 10) showed level of similarity with range between 0.4 to 1.0. On the other hand, the similarities between 6, 1 and 9, 1 were 0.0 and 0.3, respectively.

DISCUSSION

Vancomycin resistance in *S. aureus* has emerged over the last ten years. The most resistant strains (fortunately rare) bear the vanA gene cluster (Hawkey, 2009). The molecular target of glycopeptide antibiotics is the D-alanyl-D-alanine (D-Ala-D-Ala) terminus of growing peptidoglycan, the rigid polymer which protects bacterial cells from osmotic lysis. By binding to this terminal dipeptide, glycopeptides antibiotics interfere with proper cell wall formation, which results in an eventual cell death (Barna and Williams, 1984). Nevertheless, glycopeptide-resistant organisms avoid such a fate by modifying the

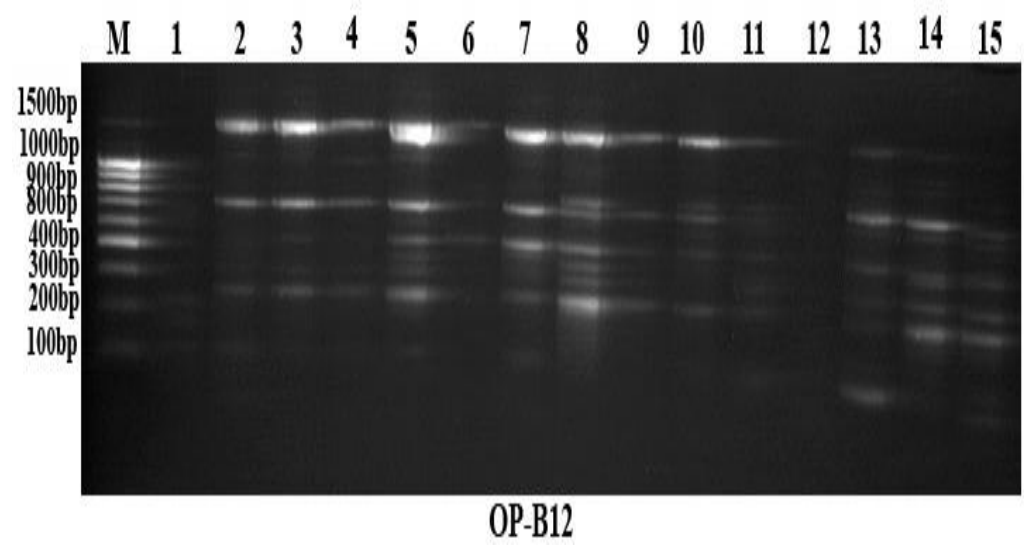

Figure 4. Pattern of RAPD using operon B12 primer for the 15 isolates previously tested by PCR for vanA and nuc genes. M, Molecular size marker; lanes 1 to 10 (isolates 1 to 10), the 10 tested VRSA strains (5 of them "isolates 1 to 5" are negative for vanA gene, while 5 strains "isolates 6 to 10" are positive for vanA gene); lanes 11 to 15 (isolates 11 to 15), methicillin and vancomycin sensitive *S. aureus* strains "negative controls". All the tested isolates "1 to 15" were confirmed as *S. aureus* as they are positive for nuc gene.

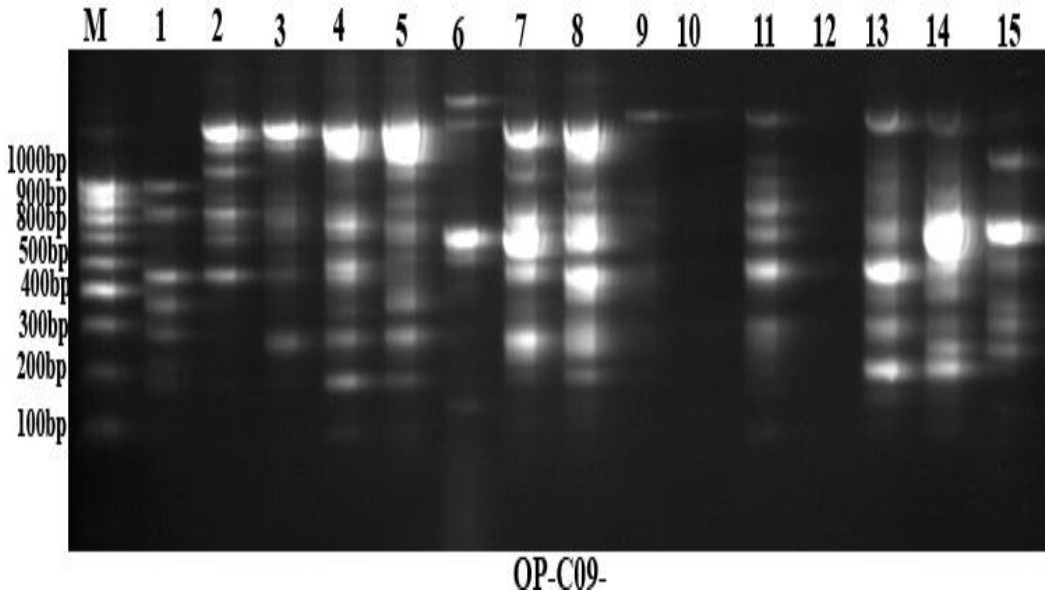

Figure 5. Pattern of RAPD using operon C 09 primer for the 15 isolates previously tested by PCR for vanA and nuc genes. M, Molecular size marker; lanes 1 to 10 (isolates 1 to 10), the 10 tested VRSA strains (5 of them "isolates 1 to 5" are negative for vanA gene, while 5 strains "isolates 6 to 10" are positive for vanA gene); lanes 11 to 15 (isolates 11 to 15), methicillin and vancomycin sensitive *S. aureus* strains "negative controls". All the tested isolates "1 to 15" were confirmed as *S. aureus* as they are positive for nuc gene.

drug's peptide target, specifically by modifying it to the depsipeptide D-alanyl-D-lactate (D-Ala-D-Lac) (Walsh et al., 1996). The biosynthetic machinery required to effect this transformation in vancomycin-resistant *Enterococcus faecium* is found on a transposable element, Tn*1546*, which incorporates five genes necessary and sufficient to

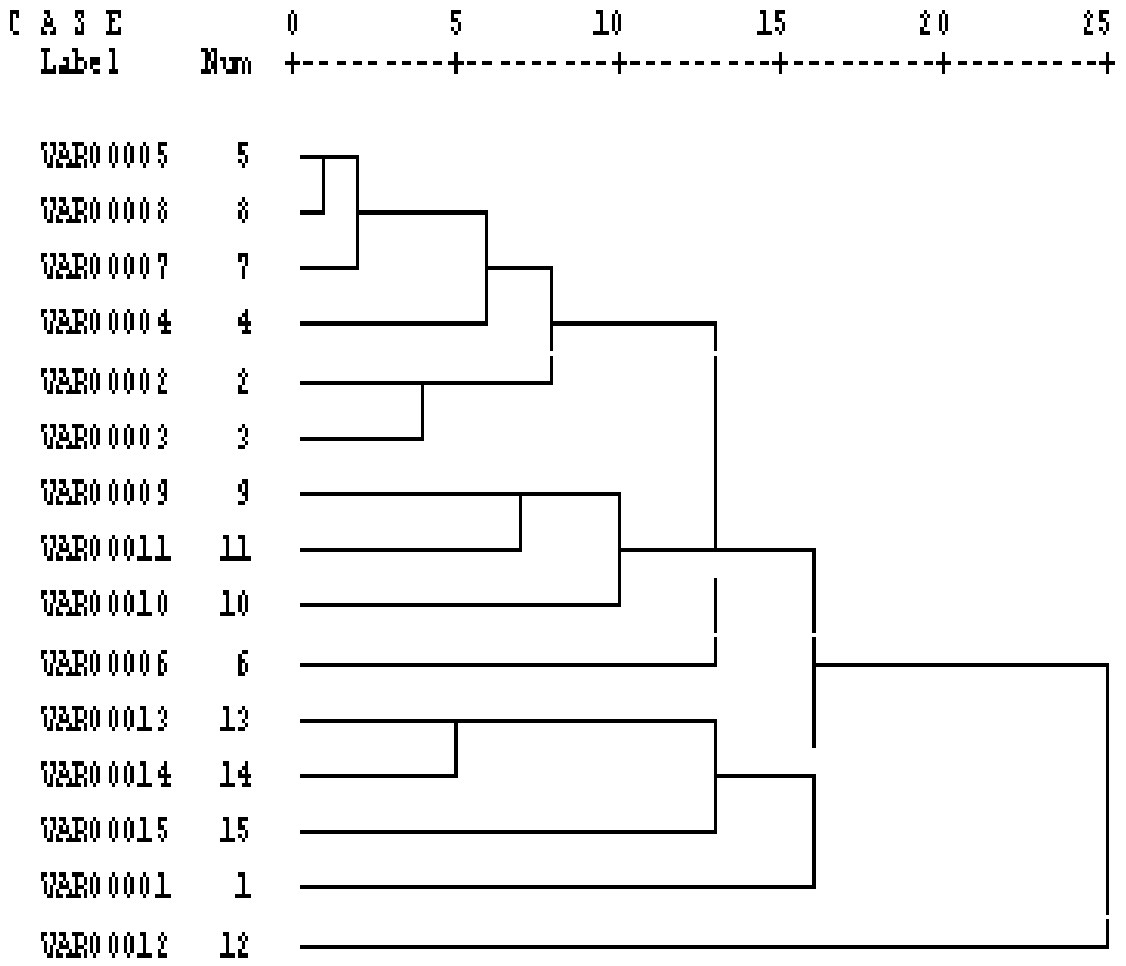

Figure 6. Similarity dendrogram for the 15 strains tested by RAPD. Isolates 1 to 10, The 10 tested VRSA strains (5 of them "isolates 1 to 5" are negative for vanA gene, while 5 strains "isolates 6 to 10" are positive for vanA gene); Isolates 11 to15, methicillin and vancomycin sensitive *S. aureus* strains "negative controls". All the tested isolates "1 to 15" were confirmed as *S. aureus* as they are positive for nuc gene.

confer high-level inducible glycopeptide resistance (Arthur et al., 1993). Two of these gene products, VanR and VanS, are required for the vancomycin-induced resistance response and are members of a two component regulatory system directing the transcription of *vanH*, *vanA*, and *vanX*. These genes encode the three proteins that are necessary for D-Ala-D-Lac synthesis and are thus essential for glycopeptide resistance (Arthur et al., 1992).

VanA is the pivotal enzyme, producing the ester D-Ala-D-Lac instead of the usual D-Ala-D-Ala dipeptide for incorporation into peptidoglycan precursors (Bugg et al., 1991). VanH is an aldo-keto acid reductase (D-lactate dehydrogenase [D-LDH]) that supplies VanA with substrate by converting pyruvate into D-lactate (Bugg et al., 1991). Courvalin (2006) reported that *vanA* is the most frequently encountered type of glycopeptides resistance in *Enterococci* and was reported as the only one detected in *S. aureus.* The prototype Tn*1546* VanA-type resistance element, which was originally detected on a plasmid in an *E. faecium* clinical isolate, is an 11-kb transposon. It encodes nine polypeptides that can be assigned to various functional groups: transposition (ORF1 and ORF2), regulation of resistance gene expression (VanR and VanS), synthesis of the d-Ala-d-Lac depsipeptide (VanH and VanA), and hydrolysis of peptidoglycan precursors (VanX and VanY); the function of VanZ remains unknown.

In this study carried on patients in Zagazig University Hospital, VRSA strains were isolated from 4.5% of patients infected with *S. aureus* according to conventional methods while only half of this percentage (2.3%) by multiplex PCR for vanA and nuc genes (vanA for vancomycin resistance and nuc for *S. aureus*). This relatively high percentage of VRSA in the present study may be explained by administration of multiple prophylactic and post-operative antibiotics with prolonged hospitalization. The hospital is an ideal environment for

Table 3. Similarity index for the 15 tested isolates according to RAPD.

Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1														
2	0.5													
3	0.4	0.8												
4	0.4	0.8	0.6											
5	0.5	0.7	0.7	0.8										
6	0.0	0.4	0.4	0.5	0.6									
7	0.5	0.7	0.7	0.8	0.9	0.5								
8	0.5	0.6	0.6	0.7	1.0	0.6	0.8							
9	0.3	0.5	0.4	0.4	0.6	0.6	0.5	0.7						
10	0.2	0.4	0.2	0.4	0.4	0.4	0.4	0.4	0.6					
11	0.5	0.5	0.5	0.7	0.7	0.5	0.7	0.7	0.7	0.6				
12	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.3	0.1			
13	0.5	0.5	0.4	0.6	0.6	0.3	0.7	0.7	0.4	0.4	0.6	0.3		
14	0.4	0.3	0.2	0.5	0.5	0.3	0.5	0.5	0.4	0.3	0.5	0.2	0.8	
15	0.4	0.3	0.3	0.3	0.3	0.2	0.4	0.5	0.2	0.3	0.5	0.3	0.5	0.5

Isolates 1 to 10: the 10 tested VRSA strains (5 of them “isolates 1 to 5” were negative for vanA gene, while 5 strains “isolates 6-10” were positive for vanA gene. Isolates 11 to 15, Methicillin and vancomycin sensitive *S. aureus* strains “negative controls”. All the tested isolates “1 to 15” were confirmed as *S. aureus* as they are positive for nuc gene.

the transmission of pathogens, because patients with similar disease and susceptibilities are housed in an enclosed community and also because of the duration of stay in hospital (Kariyama and Kumon, 2001). This emergence was significantly marked in hospitals that had been endemic with strains of methicillin-resistant staphylococci and followed the policy of empirical use of glycopeptides. Extensive use of glycopeptides (vancomycin or teicoplanin) allowed selection of resistant strains of *S. aureus* (Rybak and Akins, 2001). Glycopeptide-resistant mutants of *S. aureus* have been experimentally selected by gradually increasing the levels of vancomycin present during *in-vitro* growth (Shonekan et al., 1992).

The presence of vancomycin hetero-resistance phenomenon in *staphylococci* may increase the need to modify the methods used for detection of vancomycin resistance in the clinical microbiology laboratory (Singh et al., 2003). There was no statistical significance for the distribution of VRSA or MRSA among different patient’s age or gender in the current study. It was reported that patients in the ICU and other critically ill patients are at high risk for co-colonization with MRSA and possibly, VRSA (Merrer et al., 2000). On the contrary, results in the present study show high level of MRSA in inpatient wards. However, the highest percentage of MRSA was observed in outpatient isolates (47.6%) compared to inpatients wards of ICU (29%) and orthopedic unit (22.2%) with statistically significant P values of < 0.05 and < 0.01 respectively. On the other hand, no statistically significant difference was observed between different wards regarding VRSA distribution. MRSA and VRSA isolates from pus (or wound) compared to other types of specimens also had no significant difference except when compared with MRSA isolates from blood. MRSA isolates from pus (40.4%) was significantly higher than that from blood (22.5%), ($P < 0.05$).

Furthermore, all VRSA isolates were sensitive to cefoperazone, trimethoprime, amikin and rifampin and resistant to clavulinic acid, cefoxtin, cefazolin, oxacillin, tetracyclin, vancomycin and Cefexime. This result agrees with Phillips et al. (2003), who reported that all isolates resistant to glycopeptides are resistant to methicillin and susceptible to rifampin, trimethoprime, piperacillin and cefoperazone. Ten vancomycin-resistant *S. aureus* isolates were reported in the present study by conventional methods (disc diffusion method and MIC determination). Only five of them were positive for vanA gene by multiplex PCR, although all were confirmed as *S. aureus* by multiplex PCR (nuc gene positive).

VanA-type strains display high levels of inducible resistance to both vancomycin and teicoplanin, whereas VanB-type strains have variable levels of inducible resistance to vancomycin only (Arthur et al., 1996). VanD-type strains are characterized by constitutive resistance to moderate levels of the two glycopeptides (Depardieu et al., 2003b). VanC-, VanE-, and VanG-type strains are resistant to low levels of vancomycin but remain susceptible to teicoplanin (Reynolds and Courvalin, 2005). Some studies have reported the emergence of VRSA in clinical isolates that, by definition, show *in-vitro* MICs of > 4 mg/L (Rybak et al., 2001). The present study depended on the same criteria for detection of the ten vancomycin resistant isolates. However, other workers reported that the most resistant strains bear the vanA gene cluster and these are

generally recognizable as MICs of vancomycin are usually found to be in the range 32-64 mg/L (Hawkey, 2009). This may explain why not all vancomycin resistant strains by conventional methods were PCR positive for vanA gene in the present study. All our VRSA isolates showed MIC for vancomycin of 32 mg/L, which means that at this level of MIC, the VRSA isolate may have vanA gene or not (Hawkey, 2009). So, this PCR method is confirmatory for the VRSA isolate if it gave positive result (confirm nuc gene for *S. aureus* and vanA gene for vancomycin resistance), but if positive for nuc gene and negative for vanA gene, it cannot exclude the possibility to be VRSA. It should be regarded that there are van resistance genes other than vanA (which was concerned in the current study); these genes include vanB, vanC, vanD and vanE and vanG (Depardieu et al., 2004).

The transfer of the vanA gene cluster from *Enterococci* to *S. aureus* has been demonstrated in laboratory by *in vitro* experiment and also on the skin of mice. This finding has raised concern about the possibility of occurrence of such mechanism of genetic transfer in clinical isolates of *Staphylococci*, which often co-colonize wound infection sites with *Enterococci*. Until now, few studies have shown the presence of any of van genes clusters in clinical isolates of staphylococci and so such mechanism could not be proved (Fridkin, 2001). Our current study, in addition to other reports (Depardieu et al., 2004) support this mechanism, as the vanA gene was detected in clinical isolates of VRSA. The resistance of *Staphylococci* to vancomycin has been found to be reversible under laboratory conditions (Rybak and Akins, 2001). One study indicated that vancomycin resistance in *S. aureus* may occur under the selective pressure of prolonged vancomycin use and that the resistance to vancomycin in *S. aureus* is reversible on removal of the drug. Moreover, thickening of the bacterial cell wall may be the underlying mechanism for vancomycin resistance in these bacteria (Cui et al., 2003). Actually, thickening of the cell wall of vancomycin-resistant staphylococci has been found to be associated with complex re-organization of cell wall metabolism with extra cell wall material showing reduced peptidoglycan cross-linking of D-Ala-D-Ala termini of side chains (Walsh and Howe, 2002). Cui et al. (2003) reported that in view of low level vancomycin resistance and the absence of van genes or any alteration in the terminal D-alanyl-D-alanine residues of peptidoglycan, it would be reasonable to consider the cell wall thickening as the major contributor to vancomycin resistance of *S. aureus* clinical strains. However, in the current study, the level of vancomycin resistance was not low and the presence of van genes other than vanA was not excluded.

Randomly amplified polymorphic DNA was previously used for typing MRSA (Tambic et al., 1997). The present study provided a molecular typing by RAPD for the reported VRSA strains using four different pairs of primers. Certain degree of discrimination was obtained by this method, and at the genetic distance value of 15, the resultant dendrogram divides the isolates into four groups. With the exception of isolate 1, all the ten vancomycin resistant *S. aureus* strains were found at the first group. Additionally, all VRSA isolates had the same vancomycin MIC and antibiotic sensitivity pattern. Four out of five VRSA isolates that had vanA gene (the same vancomycin MIC and antibiotic sensitivity pattern) were also isolated from ICU (isolates 6, 7, 8, 9). They have similarity index according to RAPD of 0.5, 0.6, 0.6, 0.8, 0.5, 0.7 for isolates 6, 7 and 6, 8 and 6, 9 and 7, 8 and 7, 9 and 8, 9, respectively. This means that some of them may be related strains transmitted from one patient to another or derived from a common origin strain (especially the very close strains 7, 8). Additionally, isolate 5 (from ICU, same vancomycin MIC and antibiotic sensitivity pattern) showed the same RAPD pattern of isolate 8 (similarity index1.0), which may mean that they are the same strain except that isolate 5 lack vanA gene as tested by multiplex PCR. This may be explained if isolate 5 lost the gene carrying plasmid or enough number of the gene copies that it become undetectable by PCR, but still vancomycin resistant as it may be multifactorial process (Wong et al., 1999).

Meanwhile, it is not clear if vanA gene presence, its copy number or expression is related also to the reported reversible mechanism of vancomycin resistance (Cui et al., 2003). Isolates 2, 3 were also very close (RAPD similarity index 0.8, no vanA gene, same vancomycin MIC and antibiotic sensitivity, from orthopedic ward). The general observation is that the clonal diversity among VRSA isolates in the current study was small (RAPD, MIC, antibiotic sensitivity). This may indicate that most of them were derived from common origin strains. Therefore, more studies are still needed to explore the definite mechanisms by which these strains acquire resistance to vancomycin which may open the door to overcome this problem.

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