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The role of *Van A* gene in the resistance of *nuc*-gene posi*tive* vancomycin resistant *Staphylococcus aureus* isolated from patients with skin infections.

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ABSTRACT

Vancomycin resistance *Staphylococcus aureus* (VRSA) have become an increasing problem worldwide. This study has been undertaken for detection the role of *VanA* gene in vancomycin resistant *S. aureus* isolated from patients with skin infections.Seventy five specimensobtained from patients admitted to Dermatology Department in Al-Ramadi Teaching Hospitaland outpatient from Private clinics were studied. The suspected *Staphylococcal* colonies were bacteriologically identified and confirmed by biochemical test. Preliminary agar screening plate for detection of vancomycin resistant *S. aureus* which includes Muller Hinton agar containing 6mg/100ml vancomycin was performed. Also, broth dilution technique to detect minimal inhibitory concentration for VRSA was achieved. Further, Amplification of *nuc* and *vanA* genes by polymerase chain reaction was also performed.Out of Fourty five*isolates of Staphylococci*, 38 (84.4%) were diagnosed bacteriologically as *S. aureus*. Of these isolates, 27 (71.1%) isolates were resistant to oxacillin. Vancomycin resistant *S. aureus* were detected in 10 (37%) of them while the remaining, 1728.9%) were vancomycin sensitive. Among 10 (37%) of Vancomycin resistant *S. aureus*, 6 (60%) of them showed positive result for the presence of *VanA* gene.The increase in vancomycin resistance among MRSA and excessive use of antimicrobial agents have worsened the sensitivity. Also, the study suggested that *nuc* gene is cut off molecular diagnostic tool for confirmed diagnosis of *S. aureus*. Further, *VanA* gene play an important role in *nuc* gene positive vancomycin resistant *S. aureus*.

Key Words: Skin infection, ORSA, VRSA, *Nuc* gene, *Van A* gene.

الملخص باللغة العربية

اصبحت مشكلة مقاومة بكتريا العنقوديات الذهبية لمضاد الفانكومايسين في تزايد مستمر في العالم. ان هدف الدراسة هو لتحديد دور جين *VanA* في مقاومة البكتريا اعلاه المعزولة من الاخماج الجلدية لمضاد الفانكومايسين. تم دراسة 75 عينة من المرضى الداخلين لقسم الجلدية في مستشفى الرمادي التعليمي والمرضى المراجعين للعيادات الخاصة. شخصت مستعمرات العنقوديات بكتريولوجياً وتم تأكيد التشخيص باستخدام الفحوص الكيميوحيوية. تم إنجاز الفحص الأولي لكشف عن مقاومة هذا المضاد باستخدام اكارالمولرهينتون الحاوي على مضاد الفانكومايسين بتركيز 6ملغم/100مل . استخدمت طريقة التخافيف في المرق المغذي لتحديد بكتريا ال *VRSA*كما وتم تضخيم الجينات *nuc* وال *vanA* باستخدام تقنية تضاعف البلمرة التسلسلي.

أظهرت النتائج بأنه من مجموع 45 عينة من العنقوديات فأن 38 (84.4 %) بأنها تنتمي لنوع العنقوديات الذهبية منها 27 (71.1%) كانت مقاومة لمضاد الاوكساسلين... حددت بكتريا العنقوديات المضادة للفانكومايسين في 10 (37 %). من مجموع المذكور اعلاه فأن 6(60%) اظهرت نتائج موجبة لوجود الجين *VanA* .

تستنتج الدراسة بأن الزيادة في مقاومة مضاد الفانكومايسين لبكتريا العنقوديات الذهبية لمقاومة للمثيسيلين والاستخدام الزائد للمضادات الميكروبية سبب تقليل الحساسية الدوائية للمضادات اعلاه. كذلك تقترح الدراسة بان جين ال *nuc* هو اداة تشخيصية بيولوجية جزيئية قاطعة للتشخيص المؤكد لبكتريا العنقوديات الذهبية كما وان الجين *VanA* يلعب دوراً مهما في العنقوديات الذهبية المقاومة لمضاد الفانكومايسين والمنتجة لجين *nuc* .

INTRODUCTION

With the increase of staphylococcal resistance to methicillin, vancomycin (or another glycopeptide antibiotic, teicoplanin) is often a treatment of choice in infections with methicillin-resistant *S. aureus* (MRSA). Three classes of vancomycin-resistant *S. aureus* have emerged that differ in vancomycin susceptibilities: vancomycin-intermediate *S. aureus* (VISA), heterogenous vancomycin-intermediate *S. aureus* (hVISA), and high-level vancomycin-resistant *S. aureus* (VRSA) (1). High-level vancomycin resistance in *S. aureus* has been rarely reported (2). However, these strains may also be resistant to meropenem and imipenem, two other antibiotics that can be used in sensitive *Staphylococcus* strains.

Clinical experience suggests that barriers exist to the evolution of endogenous vancomycin resistance in *S. aureus*, given occasional reports of low-level vancomycin resistance. However*, in vitro* and *in vivo*experiments reported in 1992 demonstrated that vancomycin resistance genes from *Enterococcus faecalis* could be transferred by horizontal gene transfer to *S. aureus*, conferring high-level vancomycin resistance to *S. aureus (3)***.** Until 2002 such a genetic transfer was not reported for wild *S.aureus* strains.

In 2002, a VRSA strain was isolated from the catheter tip of a diabetic, renal dialysis patient in Michigan (4). The isolate contained the *mecA* gene for methicillin resistance. Vancomycin MICs of the VRSA isolate were consistent with the *VanA* phenotype of *Enterococcus*species, and the presence of the *vanA* gene was confirmed by polymerase chain reaction. The DNA sequence of the VRSA *vanA* gene was identical to that of a vancomycin-resistant strain of *Enterococcus faecalis* recovered from the same catheter tip. The *vanA* gene was later found to be encoded within a transposon located on a plasmid carried by the VRSA isolate (5). This transposon, Tn1546, confers vanA-type vancomycin resistance in enterococci (6).

This study has been undertaken for molecular detection of *Staphylococcus aureus* via nuc gene amplification and detection the role of *van A* gene in the resistance of *Staphylococcus aureus*to vancomycin.

PATIENTS AND METHODS

The current study includes Seventy five clinical specimens obtained from patients infected with skin infection admitted to Dermatology Department in Al-Ramadi Teaching Hospital and outpatient department during the period from May to September, 2011. The patients were of different sex and the mean of age was 21 ±13.7. Out of these specimens, 38(50.7%)isolates were bacteriologically identified as *Staphylococcus aureus.*

Detection of *Staphylococcus aureus* and*Staph. epidermidis*: Baron et al.(7) identified the suspected Staphylococcal colonies bacteriologically according to the following confirmatory methods which include staining with gram stain, morphology of colony, MIC, agar screening which including(mannitol salt agar, Muller Hinton agar with 6mg/100ml oxacillin, coagulase test (8,9).

1-preliminary screening agar plates:

A-Screening agar plates for Oxacillin resistance

Mueller-Hinton agar plates with 4% NaCl and 6 mg of oxacillin per ml (MHOX; Prepared Media Laboratories, Tualatin, Oreg.) were inoculated as a streak in three directions by using a cotton swab dipped into a direct colony suspension equivalent to a 0.5 McFarland standard in Tryptic soy broth (10). As a control, the same medium containing 4% NaCl without oxacillin was inoculated first. Plates were incubated in ambient air at 358C and were read at 24 and 48 h. Any growth was considered a positive test result (11).

B-Screening agar plates for Vancomycin resistance

Muller Hinton agar (Hi-Media, India) screen plates containing 6 µg/ml vancomycin (Lilly Pharma, Giessen, Germany) were prepared. Inoculum suspensions were prepared by selecting colonies from overnight growth on nutrient agar plates. The colonies were transferred to sterile saline to produce a suspension that matches the turbidity of a 0.5 McFarland standard. The final inoculum concentration of 10^5 to 10^6 CFU per ml was prepared by adding the sterile saline to the

bacterial suspension. These suspensions were inoculated onto Muller Hinton agar plates and were incubated for 24 h at 35°C in ambient air. Any visible growth indicated the vancomycin resistance (12).

2-Confirmatory antibiogram:-

Broth dilution method (Minimum Inhibitory Concentration):

The antimicrobial agents used were pure powder of oxacillinand vancomycin, which were purchased from Himedia Company, India. In Bacterial standardization, Twenty-four isolates of different bacteria were included. The bacterial standardization was performed according to 0.5 McFarland turbidity standards (13). The result of minimal inhibitory concentration (MIC) was interpreted as the lowest concentration of antimicrobial agents, which inhibits visible bacterial growth after overnight incubation (14).

Molecular detection:-

DNA extraction:-

The procedure of DNA extraction was done according to the instructions laid down by Promega Company (10).

DNA quantitation:-

DNA samples, which were prepared from bacterial growth, were quantified by Ultraviolet spectrophotometer (Unico, USA) reading at 260 and 280 nm (10). All samples were stored at -20 ^{o}C until use.Reading were taken at wavelengths of 260 nm (OD 260) for DNA of sample and 280 nm (OD 280) for protein concentration of sample, the spectrophotometer was rezeroid between each wavelength reading. The ratio between the readings (OD260/OD280) provided an estimation of sample purity, which should be between (1-2). Valuesof (OD260/OD280) of less than 1 indicate contamination of the DNA by protein (10).

The concentration of DNA (µg/µl) was estimated by using the following equation:Concentration (µg/µI) = (reading in OD 260) X (total/sample volume) X constant (50 µg/1000 µI) = 50 ng/µI.

Polymerase chain reaction:-

All primers were supplied by Alpha DNA Co., Canadaas a lyophilized product of different picomols concentrations and resuspendedusing deionized water to reach a final concentration for 10 picomols /µl of suspension.

All the samples of bacterial culture examined for DNA extraction, which were assayed by PCR amplification process. The specific primers were synthesized from Alpha DNA (Alpha DNA Co., Canada), which were designed on the basis sequence information of the gene repeated unit that amplifies a highly repeated sequence of *Staphylococcus aureus* DNA.

Sequences of nuc gene and Van A gene,

Gene name nucleotide sequence (5" to 3") base pair

VanA- R
CCCCTTTAACGCTAATACGACGATCAA1
030 bp

VanA-F
CATGAATAGAATAAAAGTTGCAATA

Sequences' of *nuc* gene

Gene name Nucleotide sequence (5'-3' (base pairs)

nuc gene AGCCAAGCCTTGACGAACTAAAGC447 bp

Table (1) below shows the original PCR reagents and final concentrations, which were used in the procedure

Table (1): The original PCR reagents and final concentrations, which were used in procedure

Component	Volume	Final concentration
Go Taq Green Master Mix 2x	12.5 µl	1 X
Forward primer	2.5µl	10 µM
Reverse primer	2.5 µl	10 µ
DNA template	5µl	250 ng
Nuclease free water	2.5µl	
Final volume	25 µl	

The thermal cycler (ESCO, USA) was used with the following thermal profile for *VanA* and *Nuc* gene: tables (2,3)

Table (2): The PCR program that used in the amplification of *Nuc* gene

PCR Program			
Initial Denaturation	10 min.	94 ^{o}C	
Denaturation	30 sec.	94 ^{o}C	
Annealing	1 min	50^{o}C	
Extension	1:30 min	72 ^{o}C	30 cycles
Final Extension	10 min.	72 ^{o}C	

Table (3): The PCR program which used in the amplification of *Van A* gene

PCR Program			
Initial Denaturation	10 min.	94 ^{o}C	
Denaturation	30 sec.	94 ^{o}C	30
Annealing	45 sec.	50 ^{o}C	cycles
Extension	30 sec	72 ^{o}C	
Final Extension	10 min.	72 ^{o}C	

Loading DNA sample:

DNA ladder (Promega, 50-1000 bp , USA) was transferred onto the gel well by micropipette.12 µl of the amplification DNA samples were loaded to the wells of the gel. the gel with tray was laid into the chamber with 1x TBE, and assured that the gel was completely covered with TBE, Electrophoresis condition was set up at 100 volts for 1 hour for small tank and 150 volts for large tank with same time. The gel for DNA fragments were observed by examining the gel under UV light of transilluminator with protective glasses and photographed (10).

Agarose gel electrophoresis:-

After genomic DNA extraction, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA[10].In addition, the same process was used after amplification the genes by PCR to detect the target bands.

RESULTS

Out of Fourty five isolates of Staphylococci, 38 (84.4%) were diagnosed bacteriologically as S. *aureus*. Of these isolates, 27 (71.1%) isolates were resistant to oxacillin. Vancomycin resistant *S. aureus* were detected in 10 (37%) of them while the remaining, 17 28.9%) were vancomycin sensitive. Table (4).

Table (4): Distribution Oxacillin resistance *Staphylococci aureus* according to type of infection.

	Clinical type of infection	No. of strain(%)
Staphylococcus aureus	Impetigo	8 (29.6%)
	Superficial bacterial infection	12 (44.5%)
	Furunclosis	7 (25.9%)

Antimicrobial susceptibility test to choose MRSA from Staphylococcal isolates were detectedaccording to the criteria laid down by National Committee for Clinical Laboratory Standard (NCCLS, 2001) by using an international quality isolate of *Staphylococcus aureus* ATCC 25923. our results showed that MICs of oxacillin against *S. aureus* wereas follow:- Two (7.4%) was appeared with MICs eight µg/ml, Six (22.2%) with MIC 16 µg/ml, 10 (37.1%) with MIC 32µg/ml, while six (22.2%) were produced MIC value of 64 µg/ml and 3 (11.1%) were appeared with MIC 128µg/ml.

On the other hand, Vancomycin resistant *S. aureus* were detected in 10 (37%) of them while the remaining, 17 (28.9%) were vancomycin sensitive as represented in the following table (5).

Table (5): Detailed description of vancomycin resistant *Staphylococcus aureus*in three study groups.

Type of isolates	No. of isolates	MIC (µg/ml) of Vancomycin (Mean ± SD)
Oxacillin and vancomycin resistant *S. aureus* (VRSA)	10 (37%)	42.5± 10.7
Oxacillin resistant and Vancomycin sensitive *S. aureus (*VSSA) positive	17 (28.9%)	1.2 ± 6.2
Oxacillin and Vancomycin sensitive *S. aureus*	11 (28.9%)	0.78 ± 3.7

In molecular part of this study, DNA was extracted from all study isolates of *S. aureus* with mean of purity of 1.57 ± 4.6 (figure 1)

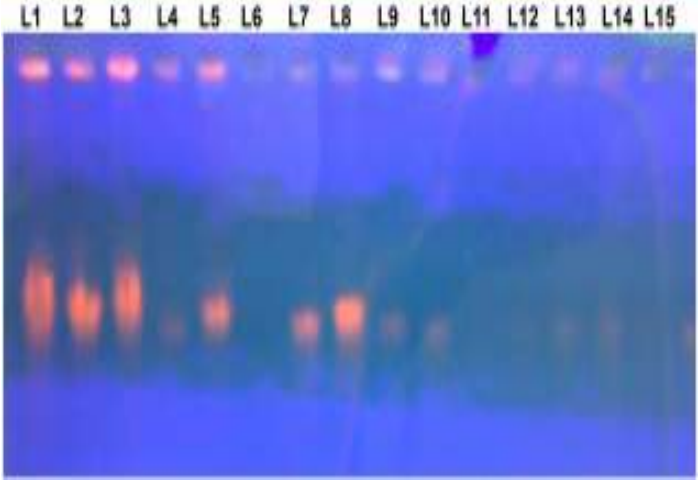

Figure (1): The result of agarose gel electrophoresis (0.7%) with ethidium bromide of DNA (pre-Polymerase chain reaction) from *Staphylococcal* genome.

Amplification of *nuc* gene target by polymerase chain reaction that is highly specific for S. aureus was performed. After that, electrophoresis by agarose gel electrophoresis for PCR product was done. The study result showed that *nuc* gene was observed in all the study isolates of *Staphylococcus aureus* while they were not appears in *S. epidermidis* as represented in figure (2).

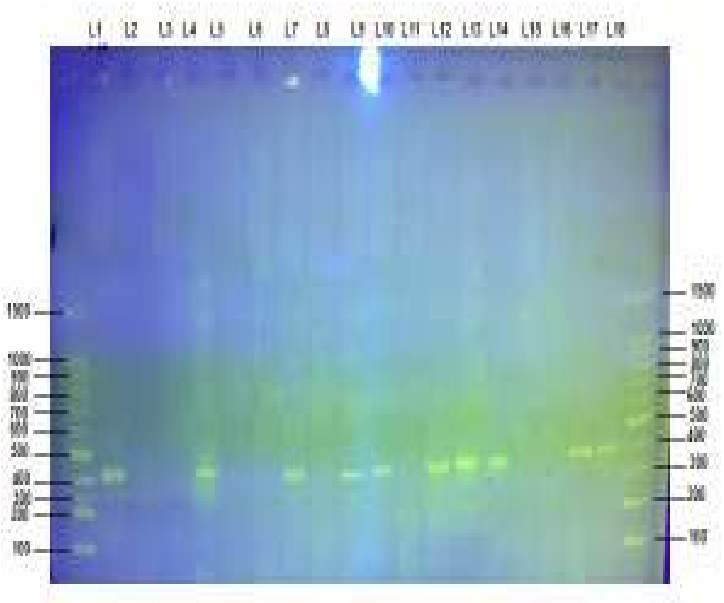

Figure (2): The results of agarose gel electrophoresis (2%) with Red safe stain, bands with amplified *nuc* gene obtained from Staphylococcal isolates which showed that positive results for nuc gen of *Staphylococcal* isolates were (L1, L4, L7, L9, L10, L12, L13, L14, L17, L18) while (L2, L3, L5, L6, L8, L11, L15, L16) were negative results, ladder with (100-1500 pb) on the right and left were used as DNA molecular weight marker.

Among 10 (37%) of Vancomycin resistant *S. aureus*, 6 (60%) of them showed positive result for the presence of *vanA* gene while the other 4 (40%) VRSA were negative for *vanA* gene (figure 3).

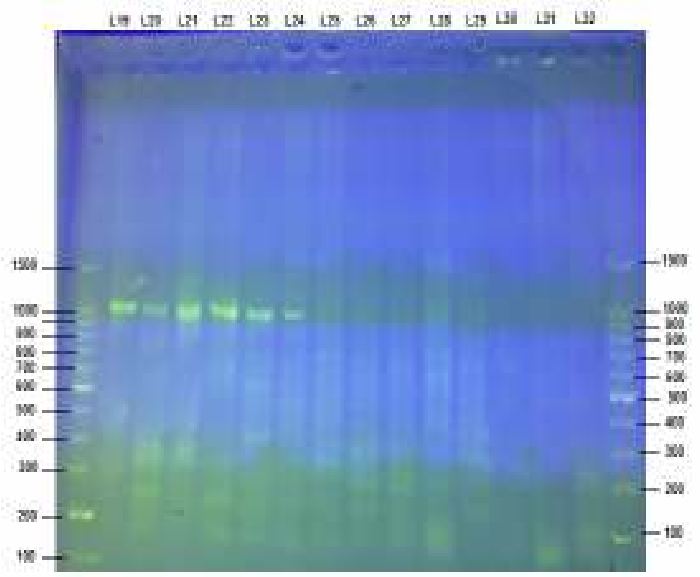

Figure (3): The results of agarose gel electrophoresis (2%) with Redsafe stain, bands with amplified *VanA* geneobtained from *Staphylococcal* isolates which showed that positive results for *VanA gene* of*Staphylococcal* isolates were (L19, L20, L21, L22, L23, L24) while (L25, L26, L27, L28, L29, L30, L31, L32) were negative results, ladder with (100-1500 pb) on the right and left were used as DNA molecular weight marker.

DISCUSSION

It is well realized that glycopeptides such as vancomycin are frequently the antibiotics of choice for the treatment of infections caused by methicillin resistant *Staphylococcus aureus* (MRSA) (Tiwariand Sen, 2006).The early 1990s have shown a discernible increase in vancomycin use. Consequently, selective pressure was established that eventually lead to the emergence of strains of *S. aureus* and other species of staphylococci with decreased susceptibility to vancomycin and other glycopeptides (15). In 1997, the first strain of *S. aureus* with reduced susceptibility to vancomycin and teicoplanin was reported from Japan (16).

It is well know that MIC is the lowest concentration of an antimicrobial agents which inhibits the visible growth of a microorganism after overnight incubation, In this study MICs for oxacillin against logarithmic phase cells were determined by using macro broth dilution technique. It is well known that the minimal inhibitory concentration (MICs) are considered the gold standard for determining the susceptibility of organisms to antimicrobial and therefore used to judge the performance of all other methods of susceptibility testing (17). It is well know that minimal inhibitory concentrations are important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and to monitor the activity of new antimicrobial agents. A lower MIC is an indication of a better antimicrobial agent. An MIC is generally regarded as the most basic laboratory measurement of the activity of an antimicrobial agent against an organism. the minimal inhibitory concentrations are used not only to determine the amount of antibiotic that the patient will receive but also the type of antibiotic used, which in turn lowers the opportunity for microbial resistance to specific antimicrobial agents. Currently, there are a few web-based, freely accessible MIC databases, our study with MICs not for pharmacokinetic reasons but to study role of *VanA* gene in the resistance of *nuc* gene positive *Staphylococcus aureus* to vancomycin.

Our results revealed that vancomycin resistant S. aureus were detected in 10 (37%) of them while the remaining, 17 (28.9%) were vancomycin sensitive. This result is inconsistent with those observed by Tiwari and Sen (2006) who documented that there is a significant rise of reduced susceptibility of oxacillin, vancomycin and teicoplanin and the emergence of the glycopeptide resistance is of great concern and became observed. This may be due to thickening of cell wall of VRSA strains that become thinner with the loss of vancomycin resistance during the drug free passage and again become thick in resistant mutants. Pallazo and associates (18) have also demonstrated the thickening of cell wall in vancomycin resistant staphylococci. This could be the possible mechanism behind the vancomycin resistant staphylococcal isolates.

On the other hand, biochemical and transmission electron microscopy (TEM) examination of the Mu50 cell, suggested that it produces increased amounts of peptidoglycan. More murein monomers and more layers (probably 30–40 layers as judged by cell-wall thickness observed with TEM) of peptidoglycan are considered to be present in the cell wall. As a result, more vancomycin molecules are trapped in the peptidoglycan layers before reaching the cytoplasmic membrane where peptidoglycan synthesis occurs. Moreover, a higher concentration of vancomycin would be required to saturate all the murein monomers that are supplied at an increased rate in Mu50. Besides the vancomycin-trapping mechanism, designated "affinity trapping (19, 20).

With regard to *VanA* gene results, our study revealed the presence of *vanA* gene in Six VRSA isolates while the other 4 (40%) VRSA isolates were negative for *vanA* gene. This study result was in agreement with those observed byThati *et al*. (21).The experimental transfer of the vanA gene cluster from *E. faecalis* to *S. aureus* has raised fears about the occurrence of such genetic transfer in clinical isolates of methicillin resistant *S. aureus* (22).The level of MICs and mode of expression of antibiotic resistance is not controlled only through the transcription and translation of *VanA* gene but can also be profoundly influenced by a variety of environmental factors. These differences in results may be due to epidemiological reason like our study which is done in Iraq because variety of environmental factors that can cause deletion of gene or can drain new features (23). Resistance in bacteria can be intrinsic or acquired, intrinsic resistance is a naturally occurring trait arising from the biology of the organism. Acquired resistance occurs when a bacterium that has been sensitive to antibiotics develops resistance. This may happen by

mutation or by acquisition of new DNA. Mutation is spontaneous event that occurs regardless of whether antibiotic is present. A bacterium carrying such a mutation is at a huge advantage as the susceptible cells are rapidly killed by the antibiotic, leaving a resistant subpopulation (24).

On the other hand, Martineau, and associates (25), showed that the heterogeneous nature of methicillin resistances suggests that numerous factors could explain the sensitive phenotype MRSA;such factors include (i) the regulation of the expression of mecA and (ii) the absence of host factors associated with the phenotypic expression of methicillin resistance. The fact that ability to select resistant cells from originally susceptible strains demonstrates that upon in vitro selection in the presence of increasing gradients of the antimicrobial agent, it is possible to select for resistance. Furthermore, once induced, the resistance phenotype was shown to be stable (25).

The study suggested that the increase in vancomycin resistance among MRSA and excessive use of antimicrobial agents have worsened the sensitivity. In addition, the study suggested that *nuc* gene is cut off molecular diagnostic tool for confirmed diagnosis of *S. aureus*. Further, *VanA*gene plays an important role in *nuc* gene positive vancomycin resistant*S. aureus*.

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