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VANCOMYCIN RESISTANCE IN *STAPHYLOCOCCUS EPIDERMIDIS* CLINICAL ISOLATES

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ABSTRACT

Minimum inhibitory concentrations of vancomycin to thirty *Staphylococcus epidermidis* isolates were determined . The results revealed that (12) *S.epidermidis* isolates (40 %) were vancomycin resistant, the MIC of them were between 256 µg\ml and 32 µg\ml , (4) *S.epidermidis* isolates (13.3%) were intermediate resistance , the MIC to 3 of them were 16 µg\ml and the last was 8 µg\ml. The isolates were subjected to polymerase chain reaction (PCR) technique in monoplex pattern to amplify resistant incoding the *vanA* and *vanB* genes. The results by this study showed that 12 (40%) *S. epidermidis* isolates gave the implicone size (1030 base pair) of the *vanA* gene. However the results of MIC and PCR were similar but no any isolates gave product for presence of *vanB* gene.

KEYWORDS: *Staphylococcus epidermidis*, vancomycin resistant, *vanA* gene, *vanB* gene.

INTRODUCTION

Coagulase-negative staphylococci (CoNS) have become increasingly recognized as important agents of nosocomial infection. One of the characteristics of Coagulase-negative staphylococci is their resistance to multiple antimicrobial agents commonly used for the treatment of staphylococcal infections.[1,2]

The frequency of oxacillin resistance in Coagulase-negative staphylococci strains has increased substantially over recent decades[3], oxacillin resistance and methicillin resistance are found in over 70% of strains.[4] This has led to the frequent use of glycopeptide antibiotics for treatment of Coagulase-negative staphylococci infections.[5,6,7] As a result, the emergence of strains with decreased levels of susceptibility to vancomycin and teicoplanin has been

reported.[8,9] Since 1990s, a worldwide increase in the number of glycopeptide-resistant observation among Coagulase-negative staphylococci has been described.[10] The appearance of resistance mechanisms against glycopeptide antibiotics among clinical isolates of enterococci and the laboratory demonstration of the transfer of the *vanA* gene complex to *Staphylococcus aureus* have raised concern about the occurrence of such a genetic transfer in clinical isolates of methicillin-resistant staphylococci, against which the most frequently used chemotherapy is vancomycin based.[11,12] While clinical isolates of staphylococci with the enterococcal glycopeptide resistance mechanism have not been reported so far, clinical failure of teicoplanin and vancomycin treatment in Coagulase-negative staphylococci [12], in methicillin-resistant *S. aureus* (MRSA) infections in Japan[13] and the United States[14] have been reported.In Iraq two clinical *S. aureus* isolates gave the implicone size (1032 base pair) of the vanA gene , these two isolates were multi-drug resistant.[15] This study describes clinical isolate community–acquired Vancomycin resistant *S. epidermidis* from patients in Baghdad and aims to Investing the Vancomycin resistance Staphylococci distribution among patients in Baghdad community and Detecting the presence of *vanA* and *van B* genes by PCR technique.

MATERIALS AND METHODS

Bacterial isolates

Thirty *Staphylococcus epidermidis* isolates were isolated from clinical specimens of patients attending baghdad teaching Hospital, Laboratery teaching of Madienat AL- Teb and AL-kindy teaching Hospital for the period from August to December 2013. The isolates were identified depending on the morphological features on culture media and biochemical tests according to Bergey's Manual. *S. epidermidis* was identified according to the morphological features on culture medium and biochemical test with the use of API Staph system.[16,17]

Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations of vancomycin to thirty *Staphylococcus epidermidis* isolates were determined .This test was achieved according to Morello *et al.*[18]

Plasmid DNA extraction from *S.epidermidis* by using AccuPrep® Plasmid Mini Extraction Kit.

1. Single well-isolated colony from fresh mannitol salt agar was inoculated into 5 ml of brain heart infusion broth and incubated in 37ºC for 16 hr. in shaker incubator.

2. Bacterial growth 1.5 ml was transferred to a 2ml microcentrifuge tubes and centrifuged at 14000g for 2 min to pellet the cells. The supernatant was removed.
3. The pellet cells was resuspended in 480µl of 50mM EDTA, 120 µl of lysozyme was added to the resuspended cells and gently the pipetting to mix, after incubation at 37ºC for 2hr. in water bath, the microcentrifuge tubes were centrifuged for 2 minutes at 14000g and the supernatant was removed.
4. Buffer① 250 µl was added to the collected cells and completely resuspended by vortexing to make high lysis efficiency.
5. Buffer② 250 µl was added and mix by inverting 3-4 times gently.
6. Buffer③ 350 µl was added to the cell lysate and immediately mix by inverting 4 times gently.
7. The tubes were centrifuged at 13000 rpm for 10 min. in microfuge, after centrifugation white protein aggregation will appear at the bottom of the tube.
8. The cleared lysate was transferred to the DNA binding column tube and centrifuged at 13000 rpm for 1 min. Pour off the flow-through and re-assemble the DNA binding filter column with the 2 ml collection tube.
9. Buffer④ 700 µl was added to the DNA binding column tube and centrifuge at 13000 rpm for 1 min. Pour off the flow-through and re-assemble the DNA binding filter column with the 2 ml collection tube. This removes salts and soluble debris. The amount of plasmid washed away in 80 % ethanol is negligible.
10. Centrifugation at 13000 rpm for 1 min. to remove the residual ethanol.
11. DNA binding filter column was transferred to the new 2 ml microcentrifuge tube, Buffer⑤ 50-100 µl was added to the DNA binding filter column, and waited for at least 1 min. for elution
12. The plasmid DNA was eluted by centrifugation at 13000 rpm for 1 min.

The DNA concentration and purity were determined by using Nano drop instrument from ACT gene (China). The principle of this instrument is measuring the absorbency of nucleic acid in the wave length 260/280. A 1µL sample was pipetted onto the end of a fiber optic cable (the receiving fiber). A second fiber optic cable (the source fiber) was then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends. The gap was controlled to a 1mm path. A pulsed xenon flash lamp provides the light source and a spectrometer utilizing a linear array was used to analyze the light after passing

through the sample. The instrument was controlled by special software run from a PC, and the data were logged in an archive file on the PC.

Buffers often absorbed in the UV range; therefore, it was critical to blank the instrument with exactly the same material that the sample was suspended in clean all surfaces with de-ionized water after the final sample measurement. After selecting nucleic acid button from Nano drop program on PC, the user can display the entries comprising the current sample report by selecting the Show Report button. Descriptive parameters specific for the individual application modules were populated for each individual sample ID. The quality of the extracted DNA was checked by 0.8 % agarose gel electrophoresis.

Polymerase Chain Reaction (PCR) Technique

The polymerase chain reaction (PCR) is an *in vitro* amplification of target DNA with a pair of primers and a DNA polymerase, resulting in several million fold amplification of the target sequence within few hr.[19]. PCR assay was performed in a monoplex.

The primers listed in table (1) were selected for this study; these primers were provided in a lyophilized form, dissolved in sterile distilled water to give a final concentration of 100 pmol ⁄µL and stored in deep freezer until used in PCR amplification.

Table 1: The primers and their sequences used in conventional PCR for detection of *van A* and *van B Staphylococcus epidermidis*:

Gene	Primer Name	Sequence 5' ⟶ 3'	Size (bp)	Length	References
vanA	VAF	CAT GAA TAG AAT AAA AGT TGC AAT A	**25**	**1,030**	Clark *et al.*[20] Gazzola and Cocconcelli[21]
vanA	VAR	CCC CTT TAA CGC TAA TAC GAT CAA	24	1,030	
vanB	VBF	GTG ACA AAC CGG AGG CGA GGA	21	433	Clark *et al* .[20] Gazzola and Cocconcelli [21]
vanB	VBR	CCG CCA TCC TCC TGC AAA AAA	21	433	

PCR Amplification

The extracted DNA, primers and PCR premix (Bionner), were thawed at 4°C, vortex and centrifuged briefly to bring the contents to the bottom of the tubes.PCR mixture was set up in a total volume of 20 µL included 5µL of PCR premix 2 µL of each primer, 5 µL of template DNA have been used and 1.5 µL DMSO. The rest volume was completed with sterile de-ionized distilled water, then vortexed and finally 5 µL of template DNA was added. Negative control contained all material except template DNA, so instead that distilled water was added.

PCR reaction tubes were centrifuged briefly to mix and bring the contents to the bottom of the tubes, and placed into thermocycler PCR instrument where DNA was amplified as indicating in the table (2).

Table (2): Program used to amplify the *vanA and Van B* genes according to Clark *et al.* (1993).

Stage	Temperature	Time	Cycle
Initial denaturation	95 ºC	10 min	30
Denaturation	94 ºC	30sec	
Annealing	58 ºC	30sec	
Extension	72 ºC	30sec	
Final Extension	72 ºC	10 min	

Determination of PCR Specificity

The extracted DNA from staphylococcal isolates were checked for their concentration and purity, and thereafter were analyzed by PCR and the results confirmed by using 1.5 % agarose gel electrophoresis.[22]

RESULTS AND DISCUSSION

Determination of minimum inhibitory concentration (MIC)

Vancomycin susceptibility was determined by the minimum inhibitory concentration (MIC) for all *S.epidermidis* isolates, according to Clinical and Laboratory Standards Institute[23], if the MIC ≤ 4 µg / ml then the isolate is sensitive, MIC 8–16 µg/mL the isolate have intermediate resistance and if the MIC ≥ 32 µg/ml the isolate is resistant to vancomycin.

Results of Vancomycin sensitivity test obtained by this study showed that from 30 isolates, 12 isolates (40%) were resistant to Vancomycin, 4 isolates (13.3%) were intermediate resistant and 14 isolates (46.6%) were sensitive (Table 3).

Table (3): Vancomycin susceptibility percentages of *S.epidermidis* isolates from each source.

Specimens	VSSE	VISE	VRSE
Blood	-	2	8
Catheter	-	1	3
Burn	1	-	1
Wound	3	1	-
Nasal S.	10	-	-
Total	14	4	12

VSSE: Vancomycin Sensitive *S.epidermidis*, **VISE**: Vancomycin Intermediate *S.epidermidis*, **VRSE**: Vancomycin Resistant *S.epidermidis.*

The Coagulase-negative staphylococci population is clearly shifting toward greater resistance to glycopeptides, probably as a result of pressure due to the increase in use of these molecules.[6]

Plasmid Extraction from some *S.epidermidis* isolates

The plasmid DNA was extracted from 30 isolates of *S.epidermidis* 21 isolates have plasmid DNA and 9 didn't as shown in figure (1) , by using Accuprep ® PCR plasmid mini extraction kit.The purpose of plasmidic DNA extraction is for detection of presence VRSE gene(*van A* and *B*).

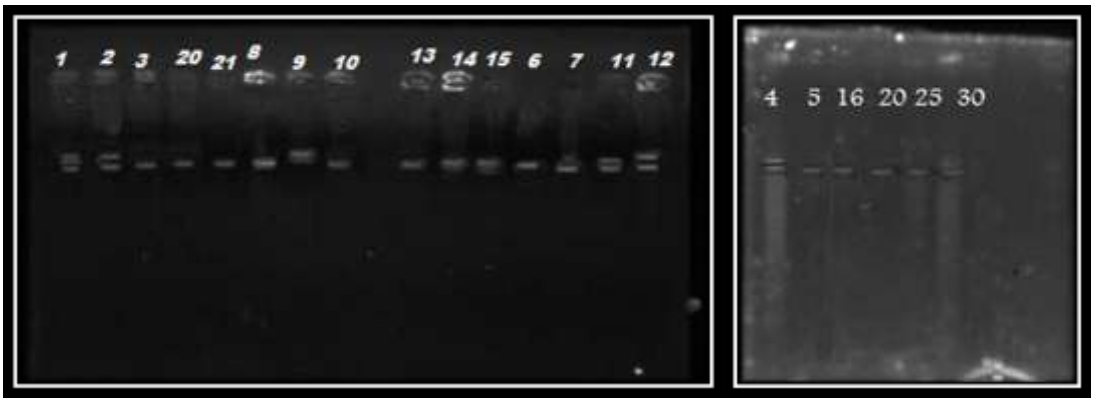

Figure (1): plasmid DNA bands extracted from 15 *S.epidermidis*

Gel electrophoresis: agarose (0.8%), TBE buffer (1x), 60 volt for 2 hr, stained with ethidium bromide.the isolate(1)that isolated from blood was resistant to vancomycin (MIC 256 µg/ml),(2,3,4,5) isolates(from blood) were resistant to vancomycin (MIC 128 µg/ml),(6-8) isolates(from blood) were resistant to vancomycin (MIC 64 µg/ml),(9-12) isolates (from catheter and burn) were resistant to vancomycin (MIC 32 µg/ml), (13-15) isolates (from blood and wound) were intermediate resistant to vancomycin (MIC 16 µg/ml),(20) isolate (from wound) was sensitive (MIC 4 µg/ml),(21) isolate (from nasal) was sensitive (MIC 4 µg/ml),(25,30)isolates (from nasal) were sensitive (MIC 2 µg/ml)**.**

Toh *et al.*[24] proved that staphylococci carried at least one large plasmid large plasmid >20 kb, and other large plasmids were 20–30 kb . These plasmids typically carried multiple antimicrobial , antiseptics, disinfectants, metal resistances , as well as virulence genes, such

as enterotoxins. Multiresistance plasmids between 20 and 30 kb are common in staphylococci from several continents.

The result of this study showed that some isolates contain one large plasmid (mega plasmid) and other contain 2plasmids and 3 plasmids among vancomycin-resistance *S.epidermidis* (VRSE) and vancomycin-intermediate resistance *S.epidermidis* (VISE), one isolate among vancomycin-resistance *S.epidermidis* (VRSE) contain three plasmids , 4 isolates contain two plasmids and 16 isolates have one plasmid , but 9 isolates of vancomycin-sensitive *S.epidermidis* (VSSE) don't contain any plasmid. Abd-Elateef[25] showed that among Methicillin-resistance *S.epidermidis* (MRSE) one isolates has two plasmid and other isolates has three plasmids.

***vanA, vanB* Genes Amplification by Monoplex PCR Technique**

The accurate and rapid diagnosis of antibiotic resistance genes in the treatment of *S.epidermidis* infections is extremely important in preventing the spread of infections. PCR-based molecular methods are often preferred for determination of antibiotics resistant genes. Using PCR technique, the genetic determinants of vancomycin resistant *vanA* and *vanB* were amplified to identify susceptible (lacking *vanA or vanB*) and resistant (*vanA or vanB*) for 30 *S.epidermidis* isolates. The results of the present study showed that, *vanA* gene bands were detected at 1030 bp regions, twelve *S.epidermidis* isolates (40%) were produced 1030 bp band (Figure 2), but non *S.epidermidis* isolates (0%) were produced *vanB* gene bands.

Figure (2): Gel electrophoresis of amplified PCR product of *vanA* gene (1030 bp) of *S.epidermidis isolates* in monoplex pattern, agarose (1.5%), TBE buffer (1x), 65 volt for 1 hrs. stained with ethidium bromide. M: DNA ladder (100 bp);line (1-12)were *van*A positive isolates.

The results of this study similar to study of Gazzola and Cocconcelli[21] were obtain *vanA* gene from PCR product was 1030 bp but also obtain on *vanB* gene. Miele *et al.*[26]; Patel *et al.*[27] and Lim *et al.*[28] ; were reported that molecular weight of *vanA* gene from PCR product was 1029bp, 885 bp, 1114 bp, respectively. These results indicate that *vanA* gene have variation in molecular weight .While Clark *et al.*[20] were reported the molecular weight of *vanA* gene from PCR product was 433 bp. Miele *et al.*[26]; Patel *et al.*[27] were reported that molecular weight of *vanA* gene from PCR product was 457bp, 885 bp, respectively.

Coagulase-negative staphylococci carries a wide variety of multi-drug resistance genes on plasmids which can be exchanged and spread among different species of staphylococci including *S. aureus* and *S. intermedius.*[29] The multi-resistant determinants can be transferred to new bacterial hosts as part of the large conjugative replicons which commonly encode resistance to some major antibiotics ,and that horizontal transmission of vancomycin-resistance genetic determinants between *Enterococcus* and *Staphylococcus* has been demonstrated [11,30], the study looked for the presence of enterococcal vancomycin-resistance determinants in *S. epidermidis* by PCR as same results of Clark *et al.*[20]

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