ISSN NO. 2320-5407

*Journal homepage: http://www.journalijar.com*

*INTERNATIONAL JOURNAL OF ADVANCED RESEARCH*

**RESEARCH ARTICLE**

# Detection of van A gene of Vancomycin resistant Staphylococcus aureus by PCR technique

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## *Manuscript Info*

***Manuscript History:***

Received: 15 May 2014
Final Accepted: 19 June 2014
Published Online: July 2014

***Key words:***
DNA extraction, Estimation of DNA, PCR.

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## *Abstract*

The pattern of minimum inhibitory concentration of S.aureus isolates to vancomycin , was determined; The results revealed that 2 S.aureus isolates(33,56) were vancomycin resistant, the MIC was 32-64 µg\ml respectively ,these two isolates were multi-drug resistant , while MIC of 8 S.aureus isolates was vancomycin-intermediate between (8-16 µg\ml).
Deoxyribo nucleic acid (DNA) extraction from S.ureus isolates was done manually, the isolates were subjected to polymerase chain reaction (PCR) technique in monoplex pattern to amplify resistant incoding gene : the vanA gene ; results by this study showed that 2 S. aureus isolated gave the implicone size (1032 base pair) of the vanA gene , the results of MIC &PCR was similar .
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## INTRODUCTION

Staphylococcus aureus is known as one of the most frequent pathogens , and it can cause septicemia, endocarditis, osteomyelitis, abscesses,pneumonia ,wound infection, impetigo, cutaneous rash, in addition to various toxin-mediated disease, the variety of such spectrum of clinical manifestations is mostly dependent on the numerous virulence factors produced by each strain (Le Loir et al., 2003).Approximately 30-50 % of the human population carriers S. aureus , and its main habitat is the nasopharynx , a site where strains can persist as transitory or persistent member of the normal microbiota without causing any symptomatology (Partida et al., 2010). Because of the spread of multi-drug resistant Gram-positive bacteria as well as methicilin resistant S. aureus (MRSA), glycopeptides antibiotics, vancomycin and teicoplanin were used to treat severe staphylococcal infections(Pêrichon et al.,2004). The first clinical Vancomycin-resistance S.aureus (MIC ≥ 32 µg/mL) was reported from Michigan, USA in 2002 (Dezfulian et al., 2011). The first vancomycin-resistant strain was isolated in 2002. This strain was shown to carry a plasmid which contains, among other resistance genes, the vanA gene plus several additional genes required for Vancomycin resistance. The proteins encoded by these genes are responsible for lowering the cell wall affinity for Vancomycin (Sibbald et al., 2006).With the increased isolation of clinically significant of coagulase-negative Staphylococci (CONS), interest in their susceptibility to various antimicrobial agents and the establishment of resistance to various agents has also increased. It seems that the development of Vancomycin-resistant Enterococci (VRE) in 1988 led to the emergence of VRSA through acquisition of the vanA gene cluster from Entercoccus spp. (Saha et al., 2008).The first detection of the VRSA in iran was in 2007 (Emaneini et al., 2007) and this report describes clinical isolate of community–acquired Vancomycin resistant S. aureus from a diabetic patient in Iran with the Vancomycin MIC 512 ≥ µg/ml.

## Material & Methods

### DNA Extraction

**DNA Extraction from Bacterial Isolates**

DNA extraction method described by De Baere et al. (2002) was modified by (Al-khaffaji,2013) to be suitable for the DNA extraction from staphylococcal isolates
: five average sized pure isolated colonies from a fresh overnight culture plate (Brain heart infusion agar) were picked up, placed in an eppendorf tube containing 200 µL of distilled water, the tube was vortexed, then incubated in a dry bath at 85°C for 20 min, then immediately frozen(-18°C) for 10 min. There after it had been centrifuged at 10000 rpm for 5 min. The concentration of DNA and their purity were estimated(Stephenson, 2003).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.(Al-khafaji, 2013). PCR assay was performed in a monoplex patterns in order to amplify different fragments of genes under study in a single tube for detecting S. aureus (van A) .

**Primers selection**
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.

**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).

**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(Stephenson, 2003).

## Results & Discussion

**Table (1): The primers and their sequences used in conventional PCR for detection of Staphylococcus aureus .**

| Gene | Primer name | Sequence 5`⟶3` | length | Expected size of amplicon | References |
|---|---|---|---|---|---|
| van A | VF | ATGAATAGAATAAAAGTTGC | 20 | 1032 bp | Saha et al. (2008), Chakrabory et al.(2011) |
| van A | VR | TCACCCCTTTAACGCTAATA | 20 | 1032 bp | |

**Table (2): Program used to amplify the vanA gene.**

| Stage | Temperature (time) | |
|---|---|---|
| Initial denaturation | 95°C (2 min) | |
| Denaturation | 95°C (1 min) | |
| Annealing | 56°C (1 min) | 40 cycles |

| | |
|---|---|
| Extension | 72°C (1 min) |
| Final extension | 72°C (5 min) |

**Molecular Studies**

Results of antibiotic susceptibility obtained by this study confirmed that 32 S.aureus isolates (86.48%) found to be ampicillin resistant,37 isolates (100%) were amoxycillin resistant, 20 isolates (54.05%) cephotaxime-erythromycin – tetracycline resistant, 3 isolates (8.10%) amikacin resistant.

The least resistance (highest susceptibility) was displayed by Amoxicillin which is another penicillin. Penicillins are known to exert their antimicrobial effect by inhibition of the synthesis of peptidoglycan, which is a heteropolymeric component of the cell wall, which provides a rigid mechanical stability by virtue of its highly cross-linked lattice wall structure (18-20), and the result of this inhibition is loss of bacteria cell rigidity and subsequent rupture or lysis of the bacteria cells (Hugo&Russel, 2004). Hence it is very plausible to envisage quite uniform pattern of susceptibility by the test microorganisms to the members of the penicillins family, albeit with only slightly varying differences. Moreover, the inherent weakness associated with this antimicrobial class is resident in their β-lactam chemical ring nucleus which has been subject to attack by β-lactamase enzymes produced by certain microorganisms including some S. aureus strains (Esimone &Adikwu, 2002).

It is quite possible that the Amoxicillin-Clavulanic acid complex, which is a larger molecule than Amoxicillin, may experience greater difficulty in permeability and overall transport across the microbial cell wall/ membrane barrier. Thus only relatively limited quantity may be available to exert an antimicrobial effect since antibiotics must first penetrate the bacteria cells before they can be mobilized to produce their antimicrobial effect.

The results for isolates of Staphylococcus to test ability to susceptibility to antibiotics Ampicillin (100%) resistant , (55%) tetracycline were resistant (Abdulbari and Adil ,2011).

Resistance to tetracycline occurs by three mechanisms efflux, ribosomal protection, and chemical modification (Brooks, 2007).

Results of antibiotic susceptibility obtained by this study confined that 8 S.aureus isolates (21.62%) found to be methicillin resistant (MRSA), 20 (54.05%) methicillin sensitive (MSSA). From total isolates the 4 isolates (10.81%) were vancomycin resistant (VRSA) , 26 (70.27%) vancomycin sensitive .

Al-Hossainy (2007) showed that VRSA were 20% among S. aureus. While Al-Geobory (2011)revealed that the rate of resistant to vancomycin was 2.27%. Where the VRSA isolate among S. aureus is isolates 4 out of 50 (8%) (Mohammed, 2011).

CLSI (2011) guidelines define staphylococci for which the MIC of vancomycin is ≤ 4 μg⁄mL to be susceptible, while isolates for which the MIC is 8 to 16 μg⁄mL are intermediate and those for which the MIC is ≥ 32 μg⁄mL are resistant .

Using minimum inhibitory concentration (MIC) broth dilution method to determine the susceptibility of all isolates of staphylococci to vancomycin . Given that, the disk test does not differentiate vancomycin-susceptible isolates of S. aureus from vancomycin-intermediate isolates, all of which give similar size zones of inhibition. MIC tests should be performed to determine the susceptibility of staphylococcal isolates to vancomycin.

Of the 139 S. aureus isolates, 134 (96.4%) were considered susceptible and 5 (3.6%) resistant to vancomycin (NCCLS, 2003).

Although vancomycin resistant in S.aureus remains rare, there is widespread concern that vancomycin-resistant S.aureus poses, by far, the greatest risk to patients, given the virulence of the organism(Srinivasan, 2002). The presence of vanA genes in VRSA suggests that the resistance determinate was acquired from a vancomycin-resistant Enterococcus .

In this study , a simple method modified(Al-khafaji,2013) from that described by De Baere et al. (2002) was depended . Thus , using boiling method , DNA extracted simultaneously , as shown in(figure 1). The DNA was extracted by physical cell wall disruption substituted the enzymatic cell wall digestion, no incubation periods needed , only heating at 85°C for 20 min , and immediate freezing for 10 min, so after 30 min only. While according Montanaro et al. ( 1999 ) , lysostaphin was added to the cell suspension with an incubation at 37°C for 30 min , then proteinase K with Tris-HCl (pH 7.5) were added , a further incubation period at 37°C for 20 min, finally; heating at 95°C for 10 min (to inactivate proteinase K).

The protocol described in this study is suitable for DNA extraction from bacterial growth from solid media. It was preferred to use growths from solid media as this enhanced biosafety by reducing the chance of aerosol generation and laboratory spillover(Al-kafaji, 2013).
Results from agarose gel electrophoresis figure (2) showed that the sensitive isolates don't have any plasmid but one plasmid found in isolate resistant to vancomycin and other antibiotics.

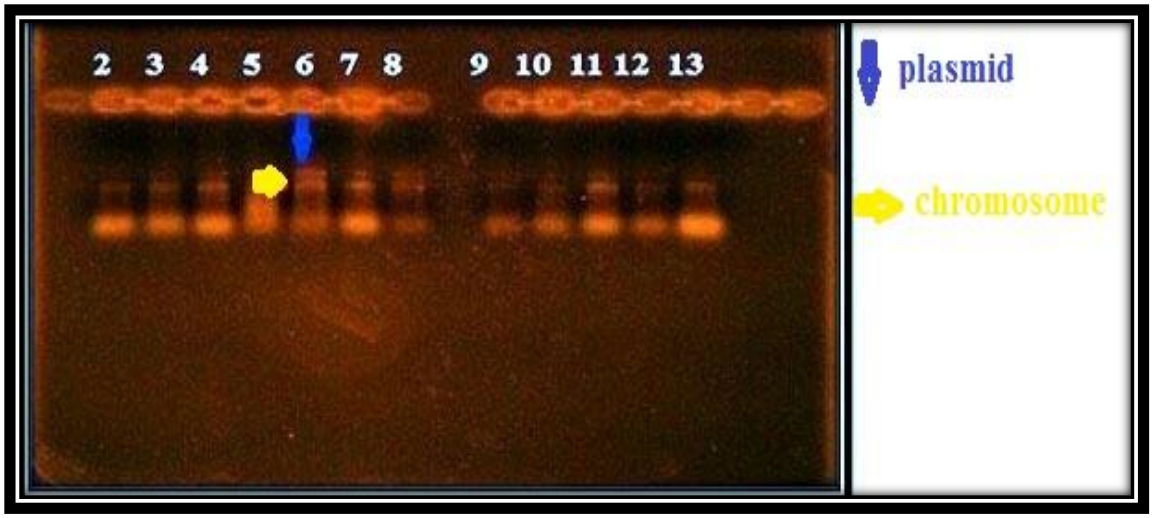


**Figure 1**:DNA bands extracted from staphylococcal isolates Gel electrophoresis :agarose (0.8%), TBE buffer (1x), 70 volt for 1 hr. stained with ethidium bromide. Lane 1: DNA extracted from the isolate 27(vssa); Lane 2: DNA extracted from the isolate 36(visa); Lane 3: DNA extracted from the isolate 9(vssa); Lane 4: DNA extracted from the isolate 7(vssa); Lane 5: DNA extracted from the isolate 56(vssa); Lane 6: DNA extracted from the isolate 33(vrsa); Lane 7: DNA extracted from the isolate 50(vssa); Lane 8: DNA extracted from the isolate 49(vssa); Lane 10: DNA extracted from the isolate 38(vssa); Lane 11: DNA extracted from the isolate 3(vssa); Lane 12: DNA extracted from the isolate 13(vssa); Lane 14: DNA extracted from the isolate 31(vssa); Lane 15:DNA extracted from the isolate 5(vssa);Lane 16: DNA extracted from the isolate 34(vssa); Lane 17: DNA extracted from the isolate 52(vssa); Lane 18: DNA extracted from the isolate 54(vssa); Lane 19: DNA extracted from the isolate 1(vssa); Lane 20: DNA extracted from the isolate 4(vssa); Lane 21: DNA extracted from the isolate 6(vssa); Lane 22: DNA extracted from the isolate 10(vssa); Lane 23: DNA extracted from the isolate 43(vssa); Lane 24: DNA extracted from the isolate 41(vssa); Lane 25: DNA extracted from the isolate 42(vssa).

Plasmids were found in S. aureus isolates that have the ability to mediate the production of drug inactivating enzymes such as β-lactamases (Daini and Akano , 2009 ) . Plasmid mediated β-lactamase conferred high level resistance to the penicillin and cephalosporin antibiotics and the levels of resistance depend on the amount of β-lactamase produced so the mechanism that account for increase production of plasmid-determined β-lactamase include mutation or insertion elements that the promoter strength , multicopy plasmids and gene duplications (Medeiros,1997). Staphylococcal plasmids range from small rolling-circle replicating (RC) plasmids , which are usually cryptic or encode a single resistance determinant , to larger multiresistance plasmids that are capable of conjugation and replicate by a theta-mechanism (Kwong et al., 2004).
Resistance to high levels of antibiotics has been ascribed in most instances to the presence of plasmids . Plasmid profiling analysis distinguished more strains than the antimicrobial susceptibility patterns.

To detect S.aureus isolates with vanA gene, it was subjected to PCR technique in a monoplex pattern. vanA positivity was confirmed by agarose gel electrophoresis in a 1.5% agarose stained with ethidium bromide, electrophoresed in 70 volt for 1hr and photographed under ultraviolet (UV) transilluminator (Figure 2).

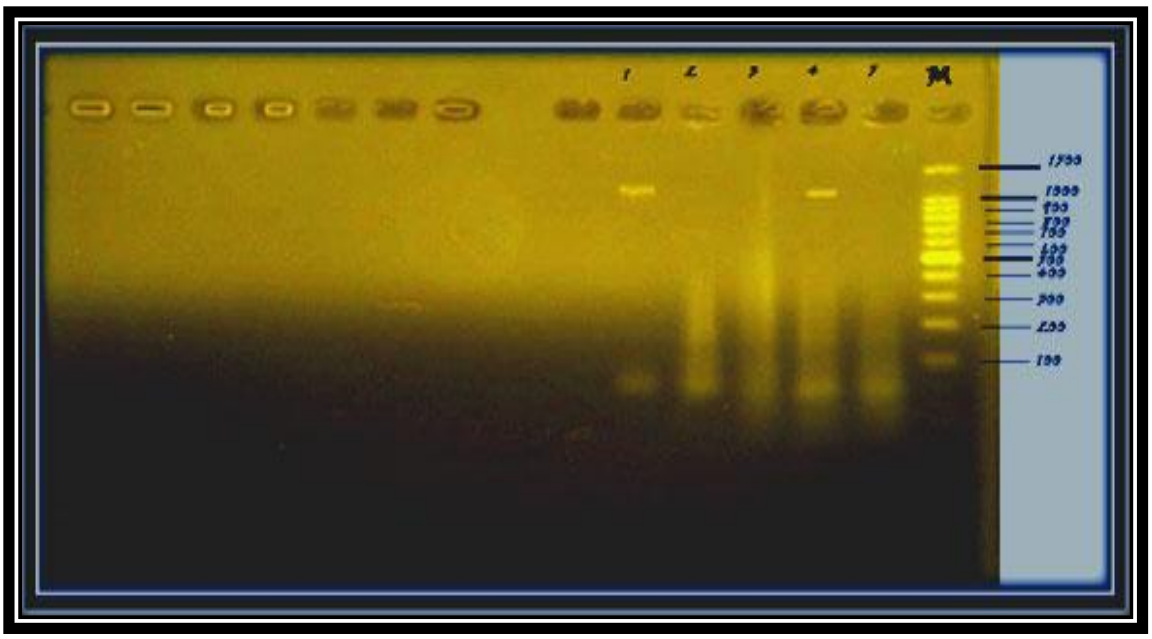


**Figure 2 : Gel electrophoresis of amplified PCR products of vanA gene (1032 bp) of S.aureus isolates in monoplex PCR technique. Agarose (1.5%), TBE buffer (1x), 70volt for 1hr stained with ethidium bromide. M: The DNA molecular weight marker (100 bp ladder); Lane 1, Lane 4: Positive amplification of 1032 bp for vanA gene of isolates 33,56 respectively; Lane 2, Lane 3, Lane 5: Negative for amplification of 1032 bp for vanA gene of isolates 22,11,29 respsctivily.**

Results were showed that 2 S.aureus isolates carried the vanA gene(Table 3).
The development and spread of bacterial strains that are resistant to antibacterial drugs has emerged as a global problem(Cohen, 1992). The appearance of antibiotic resistant bacteria over the past decades has been regarded as an inevitable genetic response to the strong selective pressure imposed by antimicrobial chemotherapy, which plays a crucial role in the evolution of antibiotic resistant bacteria . These bacteria then pass the antibiotic resistance plasmid among other bacterial cells and species (Walsh, 2000).

**Table 3: The results of the gene amplification for S.aureus isolates .**

| Strain | vanA gene amplification | Strain | vanA gene amplification |
|---|---|---|---|
| 1 | - | 30 | - |
| 2 | - | 31 | - |
| 3 | - | 32 | - |
| 4 | - | 33 | + |
| 5 | - | 34 | - |
| 6 | - | 36 | - |
| 7 | - | 38 | - |
| 9 | - | 39 | - |
| 10 | - | 40 | - |
| 11 | - | 41 | - |
| 13 | - | 42 | - |
| 15 | - | 43 | - |
| 16 | - | 49 | - |
| 20 | - | 50 | - |
| 22 | - | 52 | - |
| 23 | - | 54 | - |
| 24 | - | 55 | - |

| | | | |
|---|---|---|---|
| 25 | - | 56 | + |
| 26 | - | | |

These results in MIC came to be similar to PCR , there are only two strain have vanA gene (Table 4).

**Table 4: Detection of S.aureus Vancomycin resistance by MIC&PCR.**

| Bacterial isolates | Vancomycin MIC (µg/ml) | VanA gene amplification byPCR |
|---|---|---|
| S.aureus 33 | 32(VR) | + |
| S.aureus 56 | 64(VR) | + |
| S.aureus 23 | 8(VI) | _ |
| The rest S.aureus | ≤ 2-4(VS) | |

VR:Vancomycin resistance, VI:Vancomycin intermediate, VS:Vancomycin sensitive.

The distribution of vanA gene amplification according to positive and negative showed in table (5).
These results suggested the presence of a vanA gene cassette in VRSA 33 and 56 .

**Table 5: Distribution of vanA gene amplification according to positive and negative in PCR .**

| Test of gene | No. | Percentage (%) |
|---|---|---|
| Positive | 2 | 5.71 |
| Negative | 35 | 94.29 |
| Total | 37 | 100 % |
| Chi-square | --- | 13.584 ** |
| ** (P<0.01). | | |

Clark et al. concluded that the Vancomycin resistance exhibited by VRSA STM2 is of the vanA type due to the presence of vanHAX analogous to that of the Tn1546-like element. These facts suggest that the VRSA isolate STM2 could have a modified van gene cassette that confers Vancomycin resistance. The occurrence of partial sequence similarity might be due to multiple mutations that occurred during multiple replication (many thousand times) of bacterial genes having species specificity and species diversity, or during inter-species mobilization of resistance genes or gene acquisition. Modification of the van gene complex in the Pennsy-lvania VRSA isolate (the second documented clinical VRSA isolate) has been reported by (Clark et al.,2005); it has a deletion of 3098 bp, and two insertions of 809 and 1499 bp. Modification of the van gene complex in the New York VRSA isolate (the third documented clinicalVRSA isolate) has also been reported by (Weigel et. al., 2007).

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