Molecular characterization of Van a gene from vancomycin resistant Staphylococcus aureus isolated from clinical samples

Prasad. M.P.

Abstract
Vancomycin is commonly used for patients with Staphylococcus aureus infections, and reduced susceptibility to vancomycin is becoming increasingly common. Therefore the treatment of Staphylococcus aureus infections is becoming increasingly more complicated due to the emergence of various types of antibiotic resistance. The resistance in these organisms is conferred by several vancomycin resistance genes (Van) like VanA, VanB, VanD, VanC and VanM. Knowledge of presence of Van genes in various organisms was felt necessary prior to molecular investigation. The present study was aimed at focusing on the prevalence of vancomycin intermediate and vancomycin resistant Staphylococcus aureus (VISA and VRSA respectively) by amplifying the presence of Van-A gene isolated from various clinical samples from Bangalore. Results indicated that out of 18 Staphylococcus aureus strains isolated from clinical samples, 5 strains of Staphylococcus aureus showed presence of Van-A gene after amplification. The present findings provides the data about emergence of vancomycin resistance Staphylococcus aureus which instigates the scientists to further investigate on the significance of these genes and emphasis is placed on using molecular and bioinformatics tools to help minimize acquired resistance in antibiotics.

Keywords: Vancomycin, Drug Resistance, Staphylococcus aureus, Van A gene.

1. Introduction:
Staphylococcus aureus, a Gram positive cocci, is major human pathogen causing large variety of infections worldwide and predominates in surgical wound infections with prevalence rate ranging from 4.6% - 54.4% [1]. Staphylococcus aureus is one of the most common causes of nosocomial infection like surgical wound infection, blood stream infection, pneumonia etc. The treatment for this bacterium is a problem with the emergence and spread of methicillin resistance gene. Vancomycin is commonly used for treatment of methicillin resistant Staphylococcus aureus (MRSA) [2]. But the incidence of vancomycin-intermediate S. aureus (VISA) and vancomycin-resistant S. aureus (VRSA) has been increasing in various parts of the world [3].
Vancomycin is a glycopeptide antibiotic effective against majority of gram positive bacteria, particularly against multiple drug resistant Enterococci and Staphylococci which are resistant to β-lactum antibiotics [4]. These pathogens have acquired resistance to this compound by virtue of their intrinsic property especially in clinical isolates. This leads to severe complications in immuno compromised as well as surgical patients. vancomycin resistance enterococci (VRE) was first reported in 1988 in Europe [5] and USA [6]. In Asia nosocomial infections in hospitals have been reported from China [7], Japan [8] and South Korea [9]. In India emergence of vancomycin resistance S.aureus was analysed [10] and the Vancomycin resistant gene in S.aureus was detected first time [11].
Vancomycin binds with the C-terminal D-alanyl-D-alanine (D-Ala-D-Ala) residue of pentapeptide and blocks the addition of precursors by transglycosylation to peptidoglycan chain and inhibits cross linking of cell wall by transpeptidation [12]. Resistance to vancomycin is caused by synthesis of precursors with low affinity for these antibiotics conferred by operons present on Van gene clusters that encode enzymes which synthesize low affinity precursors wherein C-terminal D-Ala residue is replaced by D-lactate (D-Lac) or D-serine (D-Ser) that modify vancomycin binding site. These genes confer high or low level resistance to vancomycin and teicoplanin which may be inducible or constitutive.
VanA type gene confers inducible high levels of resistance to vancomycin and teicoplanin. Inducible various levels of resistance to vancomycin and susceptibility to teicoplanin are conferred by type VanB. VanC confined to Enterococcus gallinarum is intrinsically resistant to low levels of vancomycin but susceptible to teicoplanin. VanC resistance expressed constitutively or inducibly by production of peptidoglycan precursor ending in D-Ser [14]. VanD type confers resistance to intermediate levels of vancomycin and low levels of teicoplanin that is expressed constitutively. VanD is not transferable to other Enterococci and that distinguishes it from VanA and VanB.

2. Materials and Methods

2.1 Sample Collection
Blood and pus samples were collected from various hospitals and diagnostic centers in and around Bangalore. The samples were aseptically collected in sterile glass vials and sterile swabs and immediately transported to the laboratory for analysis. The samples were appropriately labeled and kept in a refrigerator at 4 °C until they were used for experimental analysis.

2.2 Isolation of Organism
The samples were spread on nutrient agar plates using sterile cotton swabs and incubated at 37 ºC for 24 hrs. Morphological appearances of the inoculated plates were observed after 24 hours incubation and distinct colonies were sub-cultured to obtain pure isolates which were then subcultured on Nutrient Agar plates. The Colonies which appeared as Gram-positive cocci arranged in clusters upon microscopic observations were further subcultured on Nutrient agar plates and preserved at 4 ºC in the refrigerator until use. Isolates suspected to be S. aureus were further subjected to biochemical tests, after Gram-staining had confirmed them to be Gram-positive cocci arranged in clusters. Specific Identification of the isolates was performed by growth on Mannitol salt agar which is a selective media for Staphylococcus aureus. S.aureus grows as yellow white colonies surrounded by yellow zone.

2.3 Vancomycin Sensitivity Test
Screening of antibiotic sensitivity of Vancomycin intermediate resistant Staphylococcus aureus (VISA) and Vancomycin resistant Staphylococcus aureus(VRSA) is done on Muller Hinton agar plate. Different concentrations of antibiotic Vancomycin was added to the medium ranging from 10mg/ml to 50mg/ml. Vancomycin hydrochloride was used which is widely available in the market as intravenous injections (Vanlid cipla). The samples were incubated at 37 ºC for 24-48 hours. The plates were observed for sensitivity or resistance to the antibiotic. The vancomycin sensitive isolates showed clear zone of inhibition around the colonies and vancomycin resistant isolates showed no zone of inhibition around the colonies.

2.4 Genomic DNA Isolation
Genomic DNA was extracted according to the protocol described by [13]. 2ml of each 24 hrs old culture were taken in a centrifuge tube and centrifuged at 5000 rpm for 5 minutes to settle down the pellet. The pellet was dissolve in 1ml of lysis buffer (10mM tris HCl, 5mM EDTA, 1% SDS, 1M NaCl) and incubate at 45ºC for 10 minutes. After incubation 1ml of equal volume of phenol: chloroform mixture (1:1) was added to the sample tubes and centrifuged at 10000 rpm for 10 minutes. The supernatant was aseptically transferred to a fresh vial. To that supernatant equal volume of chloroform: isoamyl alcohol (24:1) and 1/20th the volume of 3M sodium acetate were added and incubated at room temperature for 5 minutes. After incubation samples were centrifuged at 10000 rpm for 10 minutes. To the aqueous layer double the volume of chilled ethanol was added and kept for incubation at -20 ºC for 20 minutes. These samples were centrifuged again at 12000 rpm for 10 minutes. The supernatant was discarded and the pellet was air dried in airflow and finally resuspended in 50µl of nucleus free sterile distilled water and store at 4 ºC.

2.5 Qualitative and Quantitative Estimation of DNA
DNA quantity was assessed by using Nanodrop Spectrophotometer (Thermoscientific Nanodrop 1000, USA) at the absorbance ratio of 260 and 280 nm providing a value of 1.8 which determines pure DNA preparation. The concentration of DNA in the sample was calculated using the given formula:
Concentration of DNA = A260× 50 µg× dilution factor
Purity of the DNA = A260: A280 ratio = A260/A280
Quality of DNA fragment was electrophoretically analyzed through 0.8% agarose gel using 1X TAE buffer at 50 V for 45 mints. A 500 base pair ladder (Chromas biotech) was loaded into the gel as molecular size marker. The gel was visualized by staining with Ethidium bromide (1µl/10ml) and the bands were seen under UV light by using gel documentation system alpha imager hp (Innotech, USA).

2.6 PCR Amplification
PCR amplification of the Van-A gene was performed by using van-A primer (Forward: ATGAATAGAATAAAAGGTGCG Reverse: TCACCCCTTAACGCTAATA) according to the protocol described by [18]. The primers were purchased from Operon Technologies, USA. PCR was carried out in a 25µl reaction volume containing 2.5µl of 10X Taq buffer, 1.5µl of dNTPs, 1µl of each primer, 1µl of Taq polymerase, 18 µl of water and 1µl of DNA (100 ng/ µl) for each sample in a Corbett Research CG1- 96 PCR Palm Cycler. The thermal cycle profiles for 36 cycles were as follows denaturation at 98 ºC for 10 sec, annealing at 37 ºC for 1 min, extension at 72 ºC for 1 min. There was also an initial denaturation step for 2 min at 98 ºC and, at the end of the 30 cycles, a final extension at 72 ºC for 5 min and finally hold at 4 ºC.

2.7 Visualization of Amplified Products
After amplification, PCR products were electrophoretically analyzed through 1.5% agarose gels, in 1X TBE buffer in a Protein II xi Cell (Bio-Rad, USA) Electrophoresis unit at 100 V for 90 minutes. 10 µl (6 µl of amplified sample and 4 µl of tracking dye) of sample was loaded into each well of the 1.0 mm thick gels. Gels were stained with ethidium bromide and photographed.
under UV light by using gel documentation system alpha imager hp (Innotech, USA). The sizes of the amplified products were determined by comparison with a 500 bp ladder purchased from Chromas Biotech).

3. Result

3.1 Isolation and Identification of the Organism

A total of 8 isolates suspected to be *Staphylococcus aureus* were selected and subjected to gram staining. *Staphylococcus aureus* is gram positive cocci which grow as golden yellow to white colonies in nutrient agar. All the 8 isolates were appeared as gram positive cocci under microscopic examination after gram staining. Further identification of the isolates was done using standard biochemical tests. All the isolates showed indole production negative, MR-VP positive, citrate utilization negative and catalase positive. Confirmation of suspected isolates was done by observe their growth in selective media. The selective media was used here for *S. aureus* is Mannitol salt agar (Fig: 1). The isolates were subculture on Nutrient agar plates and slants, preserved at 4 °C in the refrigerator until use.

![Fig 1: Growth of Staphylococcus aureus in Mannitol salt agar plates.](image)

3.2 Screening of Vancomycin Sensitivity

Vancomycin resistance was checked by patching cultures on media containing vancomycin in various concentrations (as 10mg/ml, 30mg/ml and 50 mg/ml). Vancomycin hydrochloride (intravenous injection) was added in to muller hinton agar in various concentraions and checked for the growth after 24-48 hours. After incubation the plates were checked for growth. The isolates which did not grow on MHA plates were sensitive to the vancomycin and the plates which were grown on the plates were considered as Vancomycin resistant. Out of 8 isolates of *Staphylococcus aureus*, 5 isolates showed resistance to vancomycin.

3.3 Qualitative estimation of DNA by Agarose gel Electrophoresis:

The DNA was from the selected isolates by using phenol: chloroform method and the DNA quality was analyzed by staining with Ethidium bromide and the bands were seen under UV light by using gel documentation system alpha imager hp (Innotech, USA). The single sharp bands in all the 9 lanes clearly indicated the presence of DNA in all samples without any RNA contamination (Figure 2).

![Fig 2: Isolated DNA bands on Agarose Gel Electrophoresis.](image)

3.4 Quantification of DNA by using Nanodrop Spectrophotometer

Spectrophotometric analysis of the DNA samples showed the concentration of DNA obtained from different isolated *Staphylococcus aureus* samples in ng/µl were found to be 365.1, 515.1, 462.5, 543.3 and 328.4 by using Nanodrop ND 1000 ((Thermoscientific Nanodrop 1000, USA), (Fig :3). DNA quality was assessed at the absorbance ratio of 260 and 280 nm providing a value of 1.8-1.9 which determines pure DNA preparation.
3.5 Amplification of Van-A Gene using PCR:
After amplification, PCR products were electrophoretically analyzed through 1.5% agarose gels, in 1X TBE buffer. Gels were stained with ethidium bromide and photographed under UV light by using gel documentation system. The bands were seen under the exposure of UV light, indicating the presence of Van-A gene in the samples (Fig: 4).

4. Discussion
Since first being came into light in 1997, the threat of vancomycin resistance in *S. aureus* has been the topic of intensive research and discussion. Although vancomycin resistance in *S. aureus* founds extremely rare, there is widespread concern that vancomycin-resistant *S. aureus* poses, by far, the greatest risk to patients, given the virulence of the organism [14]. The presence of van-A genes in VRSA suggests that the resistance determinate was acquired from a vancomycin resistant *Enterococcus*. In fact, experimental transfer of the van-A genes from enterococci to *S. aureus* has been shown previously [15]. The Centers for Disease Control and Prevention (CDC) recommends contact precautions when caring for patients with VRSA, therefore, clinical microbiology laboratories must ensure that they are using susceptibility testing methods that will detect these organisms and that they are saving potential resistant strains for confirmatory testing. In addition, more systematic surveillance for VRSA will enhance the ability of the public health system to rapidly address this resistant pathogen. Using proper infection-control practices and good antimicrobial agent management will help limit the emergence and spread of antibiotic-resistant microorganisms, including VRSA. The present study describes these 5 isolates were all resistant *in vitro* to vancomycin which was confirmed upon amplification of van-A gene. Vancomycin resistant strains have been isolated in Japan, USA, France, Korea, South Africa, Brazil and Scotland. VRSA strains have been reported from many more countries: hence, the problem of glycopeptides resistance is global.

It is our hope that more surveillance on the susceptibility pattern of *S. aureus* to vancomycin will be carried out. The emergence of VRSA and VISA has been proposed to have been a consequence of building selective pressure of vancomycin [16]. The increased prevalence of MDR-MRSA is due to a lack of sufficient knowledge on the danger of the wrong use of antibiotics, high proximity to a large number of unlicensed drug vendors, high poverty among
the people which hinders them from completing the dosage regimen of the antibiotics, widespread and sometimes, the inappropriate use of broad spectrum antibiotics in the medical and the veterinary practice, antibiotic prophylaxis, high number of immune compromised patients, the increased use of invasive procedures and devices and inadequate infection control measures [17].

5. Conclusion
Vancomycin is a glycopeptide that is reserved for treatment when other antibiotics have failed especially as a result of MRSA and MDR MRSA. Our findings indicate the presence of vancomycin resistance like DNA sequences van-A gene in various S. aureus clinical isolates. Hence the need of molecular characterization and detailed bioinformatics investigation of vancomycin resistance genes in pathogenic, commercially important organisms and animals is emphasized. A continuous surveillance can be done to check the emergence of vancomycin resistance in various organisms. Probably this would facilitate to minimize indiscriminate use of many antibiotics all over the world and pave new ways to find alternative measures to combat the human diseases.

6. References
10. Tiwari HK, Sen MR. Emergence of Vancomycin resistant Staphylococcus aureus (VRSA) from tertiary care hospital from northern part of India. BMC Infectious diseases 2006; 6:156.