Detection of vancomycin resistance among clinical isolates of Staphylococcus aureus in a tertiary hospital, tirupati
N.Ramakrishna 1, B.Kailasanatha Reddy 2, DS Murthy 3

Abstract
Aim: The present study was undertaken to determine the sensitivity of Staphylococcus aureus isolated from infected patients to common antibiotics and to evaluate the possible presence of VISA (vancomycin intermediate Staphylococcus aureus) and VRSA (vancomycin resistant staphylococcus aureus) in Sri Venkateswara Ramnarayan Ruia Government General Hospital (S.V.R.R.R.G.G. H), Tirupati. Materials and methods: A total of 120 isolates of S.aureus which were isolated from various clinical samples were tested for methicillin resistance using the oxacillin disc diffusion test(1µg) and cefoxitin disc diffusion test(30µg). All isolates were subjected to minimum inhibitory concentration(MIC) testing with agar dilution method according to the CLSI(Clinical Laboratory Standards Institutes) guidelines. Disc diffusion method was also used to determine the susceptibility of strains to common antibiotics. Results: 64(53.3%) isolates were found to be MRSA by the cefoxitin disc diffusion method and 63(52.5%) isolates were found to be MRSA by oxacillin disc diffusion method. No VISA&VRSA isolates were detected by using the MIC agar dilution method. Conclusion: No VISA and VRSA was isolated. High prevalence of resistance among Staphylococcus aureus to commonly used antimicrobial agents was also noted.

Key words: Staphylococcus aureus, Minimum inhibitory concentration, Vancomycin Resistance

Introduction
Staphylococcus aureus is one of the most important pathogen and forms the commonest cause of localized suppurative lesions. Staphylococcus aureus causes a variety of infections ranging from relatively benign skin infections like folliculitis, furuncles, impetigo, abscesses and carbuncles to life threatening systemic illnesses like toxic shock syndrome, bronchopneumonia, septicemia, endocarditis, and meningitis etc. Staphylococcus aureus has long been recognized as a major pathogen of hospital acquired infections.

With in 4 years of introduction of Penicillin G into clinical practice in 1941, beta lactamase
mediated resistance was reported. Methicillin, the first beta lactamase stable semi synthetic penicillin was introduced in 1960. But within a year of its introduction, resistance was detected even for that. These methicillin resistant Staphylococcus aureus are resistant to all beta lactam antibiotics.

Over the last decade, methicillin resistant Staphylococcus aureus (MRSA) strains have become endemic in hospitals worldwide. In addition, it is now incipient community pathogen in many geographical regions. The relentless spread of antibiotic resistance among strains of Staphylococcus aureus is one of the greatest challenges faced by clinicians today.

MRSA is important because, in addition to being methicillin resistant, most strains are also resistant to other β-lactam antibiotics, with the exception of glycopeptide antibiotics. In 1980s, because of widespread occurrence of MRSA, empiric therapy for staphylococcal infections (particularly nosocomial sepsis) was changed to vancomycin in many health care institutions.

Vancomycin use in United States also increased during this period because of the growing numbers of infections with Clostridium difficile and Coagulase negative Staphylococci (CoNS) in health care institutions. Thus, the early 1990s have shown a discernible increase in vancomycin use. As a consequence, 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.

In 1997, the first strain of Staphylococcus aureus with reduced susceptibility to vancomycin and teicoplanin was reported from Japan. Shortly after, two additional cases were reported from United States. However, first clinical isolate of vancomycin resistant Staphylococcus aureus (VRSA) was reported from United States in 2002. More recently some workers have reported vancomycin resistant staphylococcal strains from Brazil and Jordan. Strains of vancomycin Intermediate Staphylococcus aureus (VISA) with vancomycin MIC of 8μg/ml have been reported from Japan, United States, France, United Kingdom and Germany. Most of these isolates appear to have developed from preexisting MRSA infections.

Such resistance results in serious clinical and public health consequences because currently few licensed alternatives to vancomycin are available to treat serious resistant staphylococcus aureus infections.

NCCLS (National Committee for Clinical Laboratory Safety) guidelines define staphylococci for which the MIC of vancomycin is ≤4μg/ml to be sensitive while isolates for which the MIC is 8 to 16μg/ml are intermediate and those for which the MIC is ≥32μg/ml are resistant.

Material and Methods

The present study was carried out in the Department of Microbiology, Sri Venkateswara Medical College, Tirupati. 120 Staphylococcus aureus isolates, were included in this study which were isolated from clinical samples of patients who were admitted in S.V.R.R.G.G. Hospital, Tirupati.

The samples were inoculated on Nutrient agar, Blood agar and Mac Conkey agar. The inoculated plates were incubated at 37°C for overnight. If any growth was seen on the plates, it was processed according to the standard bacteriological techniques. The colonial appearance and morphological characters of the isolated bacteria was noted. On nutrient agar colonies were large, convex, smooth, shiny, opaque and most of the strains produce yellow pigment. On blood agar colonies were smooth, low convex, glistening, opaque and sometimes surrounded by a narrow zone of beta haemolysis. On Mac Conkey agar small, pink colonies were observed. The isolated colonies were subjected to preliminary tests like Grams staining, Catalase test and Oxidase test. These preliminary tests were followed by coagulase test and biochemical reactions for the identification of Staphylococcus aureus.

Antibiotic Sensitivity

The antibiotic susceptibility pattern of isolated Staphylococcus aureus was done by Kirby-Bauer disc diffusion method. Mueller-Hinton (MH) agar plates were used. Commercially available Hi Media discs were used. The S. aureus suspension was made by inoculating 4-5 isolated identical colonies in peptone water. After 2 hours of incubation, the turbidity was standardized by using 0.5 McFarland standards. By using sterile swab, a lawn culture was made on the MH plates. The 6-8 antibiotic discs per plate were placed and inoculated plates were incubated at 37°C. The results were read after overnight incubation and compared with the standard chart. The Oxacillin disc was kept on separate MH plate and was incubated at 35°C for 24 hrs.

The following antibiotic discs were used:

1) Amoxyclav (30μg)
2) Cefoxitin (30µg)
3) Clindamycin (2µg)
4) Erythromycin (15µg)
5) Gentamycin (10µg)
6) Imipenem (10µg)
7) Oxacillin (1µg)
8) Penicillin (10 units)
9) Vancomycin (30µg)

**Minimum Inhibitory Concentration**
The MIC is defined as the lowest concentration of antibiotic at which there is no visible growth.

Vancomycin sensitivity of *S. aureus* was further tested by using standard Minimum Inhibitory Concentration by agar dilution method. This was done by using MH agar and vancomycin powder with potency of 950mcg/1mg. Vancomycin was obtained from Himedia laboratories Ltd, Mumbai.

Stock solution of antibiotic was prepared based on the following:

\[ W = 1000 \times V \times C / P \]

W= Weight of antibiotic required 
V=Volume required (in ml) 
C=Final concentration of solution in multiples of 1000 
P=Potency of the antibiotic stock solution was prepared by dissolving 256mg of vancomycin powder in 100ml of sterile distil water. [ie., 1ml master stock solution = 2560µg ].

Vancomycin solutions were prepared in different dilutions so that their final concentrations in media were 0.25µg, 0.5µg, 1µg, 2µg, 4µg, 8µg, 16µg, 32µg, 64µg and 128µg. Mueller-Hinton agar was sterilized by autoclaving and the media was cooled down to 45-50°C.

Each plate was labeled according to the final concentration of the drug after pouring. After drying of the plates, 1µl of spot inoculation of the *S. aureus* was done on the plate. The *S. aureus* suspension was made by inoculating 4-5 isolated identical colonies on blood agar plate after 18-24 hrs incubation and the inoculum was standardized with 0.5 McFarland standards. The inoculated plates were incubated at 35°C for 24hours and the results were read after 24hours and compared with CLSI standards.

**Results:**
The present study was done in the Department of Microbiology, S.V.Medical College, Tirupati. 120 isolates of *Staphylococcus aureus* isolated from various clinical samples from different age groups of patients admitted in S.V.R.R.G.G. Hospital, Tirupati, were included in this study.

All 120 isolates of *Staphylococcus aureus* were tested for vancomycin sensitivity by both disc diffusion method and MIC. All strains were found to be vancomycin sensitive by both methods. None of them were resistant to vancomycin. No vancomycin intermediate *S.aureus* strains were isolated.

**Table 1: Sample-wise distribution of the isolates of *Staphylococcus aureus***

<table>
<thead>
<tr>
<th>SI No</th>
<th>Nature of the specimen</th>
<th>No of isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pus(wounds, postoperative wound infections, burns, osteomyelitis)</td>
<td>88 (73.3%)</td>
</tr>
<tr>
<td>2</td>
<td>Urine (urinary tract infections)</td>
<td>4 (3.3%)</td>
</tr>
<tr>
<td>3</td>
<td>Sputum (respiratory infections)</td>
<td>3 (2.5%)</td>
</tr>
<tr>
<td>4</td>
<td>Blood (septicemias)</td>
<td>7 (5.8%)</td>
</tr>
<tr>
<td>5</td>
<td>Other body fluids (pleural fluid, cerebro spinal fluid etc)</td>
<td>18 (15%)</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>120</strong></td>
</tr>
</tbody>
</table>

Most of the isolates were from the pus samples (n=88, 73.3%), followed by other body fluids (n=18,15%), blood (n=7,5.8%), urine (n=4,3.3%) and sputum (n=3,2.5%).

**Table 2: The resistance pattern among MRSA (n= 63)**

<table>
<thead>
<tr>
<th>Name of the antibiotic tested</th>
<th>No of MRSA strains resistant</th>
<th>No of MRSA strains sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin</td>
<td>49 (77.8%)</td>
<td>14 (22.2%)</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>26 (41.3%)</td>
<td>37 (58.7%)</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>19 (30.2%)</td>
<td>44 (69.8%)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>29 (46%)</td>
<td>34 (53.9%)</td>
</tr>
<tr>
<td>Penicillin</td>
<td>63 (100%)</td>
<td>NIL</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>63 (100%)</td>
<td>NIL</td>
</tr>
<tr>
<td>Cefoxitine</td>
<td>63 (100%)</td>
<td>NIL</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>NIL</td>
<td>63 (100%)</td>
</tr>
<tr>
<td>Amoxyclav</td>
<td>44 (69.8%)</td>
<td>19 (30.2%)</td>
</tr>
</tbody>
</table>

All MRSA strains were resistant to penicillin and cefoxitine. All MRSA strains were sensitive to vancomycin.
Table 3: Detection of MRSA by both oxacillin and cefoxitine by disc diffusion method

<table>
<thead>
<tr>
<th>Name of the antibiotic</th>
<th>No of S.aureus strains resistant</th>
<th>No of S.aureus strains sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxacillin</td>
<td>63 (52.5%)</td>
<td>57 (47.5%)</td>
</tr>
<tr>
<td>Cefoxitine</td>
<td>64 (53.3%)</td>
<td>56 (46.6%)</td>
</tr>
</tbody>
</table>

Out of the 120 isolates, 63 (52.5%) were found to be methicillin resistant by the oxacillin disc diffusion method and 64 (53.3%) isolates were resistant by cefoxitine disc diffusion method.

Table 4: Vancomycin sensitivity pattern of S.aureus

<table>
<thead>
<tr>
<th>Test method</th>
<th>No of isolates sensitive to vancomycin (VSSA)</th>
<th>No of isolates intermedia sensitive to vancomycin (VISA)</th>
<th>No of isolates resistant to vancomycin (VRSA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disc diffusion method</td>
<td>120</td>
<td>NIL</td>
<td>NIL</td>
</tr>
<tr>
<td>Minimum Inhibitory Concentration</td>
<td>120</td>
<td>NIL</td>
<td>NIL</td>
</tr>
</tbody>
</table>

All 120 isolates of *Staphylococcus aureus* were tested for vancomycin sensitivity by both disc diffusion method and MIC. All strains were found to be vancomycin sensitive by both methods. None of them were resistant to vancomycin. No VISA strains were isolated.

Discussion

*Staphylococcus aureus* is one of the most important pathogen and forms the commonest cause of localized suppurative lesions and it has long been recognized as a major pathogen of hospital acquired infections.

When Penicillin was first used in early 1940s, virtually all staphylococci were susceptible. But within 4 years betalactamase mediated resistance was reported. In 1960, betalactamase resistant drugs - methicillin, nafcillin, oxacillin etc., were introduced. Methicillin resistant strains of staphylococci emerged by late 1970s and have become increasingly more prevalent as nosocomial pathogens. Empiric therapy for MRSA infections was changed to vancomycin. Thus, the early 1990s have shown a discernible increase in vancomycin use. As a consequence, 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.

A total of 120 isolates of *Staphylococcus aureus* were included in this study. They were isolated from various clinical samples like pus from wounds, postoperative wound infections, osteomyelitis, burns, sputum from respiratory infections, blood from septicemias, urine from urinary tract infections and conjunctival swabs.

The strains of *S.aureus* isolated were in majority from pus samples (73.3%), followed by body fluids (15%), blood (5.8%), urine (3.3%) and sputum (2.5%).

Penicillin resistant strains of *S. aureus* began emerging shortly after the introduction of penicillin in medicine in the early 1940s. The percentage of penicillin resistant strains had risen to 20-100% with the highest percentage found in hospital strains. In the present study all the strains of *S. aureus* showed resistance to penicillin. Most of the strains were found to be resistant to penicillin in the studies conducted by Dhanalakshmi et al [1], Horiph Saderi et al [2].

Resistance to methicillin, a semisynthetic penicillin which is beta lactamase resistant, was observed in *S. aureus* soon after its introduction in Britain in 1961. Emergence of strains resistant to methicillin were also reported to be increasing steadily year after year.

Over the last decade, Methicillin resistant *Staphylococcus aureus* (MRSA) strains have become endemic in hospitals worldwide. In addition to being methicillin resistant, most strains are also resistant to other β-lactam antibiotics.

In the present study isolation of MRSA is 52.5%, which correlates with the study of Benu Dhawan et al (2004) [3].

In the present study out of 120 isolates 64 were found to be methicillin resistant by cefoxitine disc diffusion method and 63 were found to be resistant by the oxacillin disc diffusion method which is correlated with the study of Dhanalakshmi et al [1] where out of 250, 80 were found to be MRSA by cefoxitine disc diffusion method, and 77 were found to be oxacillin resistant by disc diffusion method.
There is no much variation of resistance by these two methods.

These MRSA isolates were resistant to several other antibiotics, including Cefoxitine (100%), Erythromycin (77.8%), Clindamycin (41.3%), Gentamycin (30.2%), Amoxyclov (70%), Imipenem (31.7%)

The empiric therapy for MRSA was changed to vancomycin. Vancomycin is the main antibacterial agent available to treat serious infections with MRSA. Widespread use of vancomycin to treat infections caused by MRSA has led to the emergence of vancomycin resistance. The large scale of development and subsequent spread of resistance to vancomycin has been perceived fearsome threat to the already challenging therapy of MRSA.

In Indian hospitals, MRSA is one of the common cause of hospital acquired infections and 30-80 per cent MRSA has been reported from different hospitals. High prevalence of MRSA causes a major threat for the occurrence of VISA or VRSA infections.

The major determinant of VRSA was acquired from vancomycin resistant enterococcal species and is due to modification of the D-Ala-D-Ala binding site of peptidoglycan building block in which the terminal D-Ala is replaced by D-Lactate. This results in loss of critical hydrogen bond that facilitates high affinity binding of vancomycin to its target and loss of activity.

The underlying mechanism for reduced vancomycin susceptibility in vancomycin intermediate staphylococcus aureus is not known. However these strains have altered cell metabolism that results in a thickened cell wall with increased numbers of D-Ala-D-Ala residues, which serve as dead-end binding sites for vancomycin. Vancomycin is sequestered within the cell wall by these false targets and is unable to reach its site of action.

In the present study vancomycin susceptibility was tested by both disc diffusion method (Kirby- Baure), and MIC by Agar Dilution Method. All strains showed sensitivity in disc diffusion method and all were sensitive (<4µg/ml) by MIC- agar dilution method. Wide spread use of vancomycin among MRSA has been reported to result in reduced susceptibility to vancomycin. Vancomycin was not used clinically in treating infections in our hospital during the study period. This could be the possible reason for not detecting vancomycin resistance among S.aureus isolates in the present study.

Jae-hoon et al. [4], Bhatia P et al.[5], Dhanalakshmi et al.[1] , Benu Dhavan et al.[3], Sandra M. Tallent et al.[6] , have also reported 100% sensitivity to vancomycin by both disc diffusion and by MIC.

Isolates of vancomycin resistant S.aureus have emerged in many parts of the world. These isolates appear to achieve clinically relevant levels of resistance to vancomycin that leads to treatment failure. At present, the proportion of MRSA with reduced susceptibility to vancomycin is well known.

CDC have reported first VRSA isolate from United States in June 2002 and second VRSA isolate from Pennsylvania in September 2002. Both contain mec A and van A genes. But some studies like Venubabu Thati et al.(10) , Tiwari et al [7] have showed van A negative VRSA.

VRSA and VISA isolates have been reported by other researchers like Hare Krishna Tiwari et al [7]., G.A.Menezes et al [8], Horieh Saderi et al [2], Rajendra Goud et al. [9], Venubabu Thati et al. [10], Biswajit Saha et al. [11], who stated that it was mainly due to excessive use of antibiotics in intensive care units and in other health care sectors.

Studies have shown that vancomycin and linezolide have good efficacy against MRSA infections. Treatment for VISA or VRSA are drugs like Teicoplanin, Quinupristin, Dalfopristin.

The development of antibiotic resistance in developing countries like ours seems to be very much related to the irrational antibiotic usage due to its easy availability at the drug stores without prescription, injudicious use in hospitals and uncontrolled use in agriculture, animal husbandry and fisheries.

Vancomycin resistance can be difficult to detect in clinical microbiology laboratory. Disk diffusion sensitivity testing by standard 30µg vancomycin frequently misclassifies intermediately susceptible isolates as fully susceptible. Presently MIC determinations by broth or agar dilution or by E test are the gold standard for determining vancomycin susceptibility but these methods are not suitable for routine use in the diagnostic laboratories.

It is recommended that diagnostic laboratories screen their S.aureus isolates by the CDC method and submit strains to reference laboratories for confirmation of vancomycin resistance by determining the MIC.

Clinicians should continue to exercise caution in their use of vancomycin in order to preserve this useful antibiotic and prolong its therapeutic usefulness.
Table 5: Comparative analysis

<table>
<thead>
<tr>
<th>Study</th>
<th>Period</th>
<th>Place</th>
<th>No of isolates</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sandra M. Tallent et al</td>
<td>1997-2000</td>
<td>VIRGIN EA</td>
<td>619 ORSA</td>
<td>No VISA or VRSA</td>
</tr>
<tr>
<td>Jae Hoon Song et al</td>
<td>2002-2004</td>
<td>Japan</td>
<td>1,357</td>
<td>No VISA or VRSA</td>
</tr>
<tr>
<td>Bhaveja P et al</td>
<td>2005</td>
<td>AIIMS, INDIA</td>
<td>160</td>
<td>No VISA or VRSA</td>
</tr>
<tr>
<td>Benu Dhavan et al</td>
<td>2004-08</td>
<td>Bangalore</td>
<td>250</td>
<td>No VISA or VRSA</td>
</tr>
<tr>
<td>Present study</td>
<td>2011-2012</td>
<td>Tirupati</td>
<td>120</td>
<td>No VISA or VRSA</td>
</tr>
</tbody>
</table>

Conclusion
- The present study was carried out at Sri Venkateswara Medical College, Tirupati.
- 120 Staphylococcus aureus isolates were included in this study.
- 74(61.6%) of S.aureus were isolated from male patients and 46(38.3%) were from female patients and it showed a male predominance.
- Most of the isolates were from pus samples (73.3%) followed by body fluids (15%), blood (5.8%), urine (3.3%) and sputum (2.5%).
- All strains were resistant to penicillin, 52.5% of strains were resistant to oxacillin and 53.3% strains were resistant to cefoxitin.
- Significant differences in the drug susceptibility pattern of methicillin resistant and methicillin sensitive S.aureus was observed.
- All methicillin resistant S.aureus were resistant to penicillin and cefoxitin.
- Among MRSA isolates 77.8% were resistant to erythromycin, 69.8% isolates were resistant to amoxyclav, 41.3% resistant to clindamycin, 30.2% were resistant to gentamycin, 46% resistant to imipenem.
- All 120 S. aureus strains were sensitive to vancomycin by disc diffusion method.
- All strains were tested by MIC, most strains were sensitive to vancomycin at the concentration of 0.5µg/ml (35%) to 1µg/ml (58.3%). Only 6 strains were sensitive to vancomycin at 2µg/ml and 2 strains were sensitive at 0.25µg/ml concentration.
- All S.aureus isolates had MIC <4µg/ml. Thus all S.aureus isolates were sensitive to vancomycin.
- S.aureus isolates were sensitive to vancomycin by both disc diffusion method and minimum inhibitory concentration method (MIC).
- High prevalence of resistance among S.aureus to commonly used antimicrobial agents is noted. Hence proper precautions should be taken to control this super bug by following strict antibiotic policy.

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References