Detection of vanA and vanB Genetic Determinants in Vancomycin Resistant Enterococci in Kashmir Region of North India-A Hospital Based Study

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Authors’ contributions

This work was carried out in collaboration between all authors. Author MM designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AB and JA managed literature searches. Authors DKK, NKB, SR and AJ managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Introduction: Enterococci have emerged as one of the most important multidrug resistant microorganisms over the past few decades and have been reported to be the third most important hospital-acquired pathogens. Indiscriminate use of vancomycin and extended spectrum cephalosporins in hospitals has significantly contributed to the emergence of vancomycin resistance in Enterococci. Transferable vancomycin resistance in Enterococci is predominantly encoded for by vanA and vanB gene clusters. Polymerase Chain Reaction (PCR) allows rapid detection of these genes which is very important for infection control and prevention of nosocomial spread.

Aims: To estimate prevalence of VRE infections in our hospital and identify genetic determinants of vancomycin resistance in these isolates.

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Methodology: This prospective study was carried out in the department of Microbiology, Sher-i-Kashmir Institute of Medical Sciences, Srinagar J&K over a period of one year from 1st August 2013 to 30th July 2014. VRE isolated from clinical samples taken from patients admitted in the hospital or attending OPD were subject to DNA extraction and genotyping by PCR using vanA and vanB specific primers. PCR amplicons were then analyzed on 2% agarose gel by electrophoresis using ethidium bromide stain.

Results: Out of total 498 isolated Enterococcus spp., 35 (7.0%) were found to be vancomycin resistant after MIC testing. All the VRE were isolated from inpatients particularly from ICU (34.3%; p<0.001). Majority of VRE were isolated from blood samples (34.3%; p<0.001). Prior use of vancomycin was noted in 54.3% patients from whom VRE were isolated (p=0.003). All the 35 VRE isolates were positive for presence of vanA gene, whereas none of the VRE in our study harbored vanB gene which was in accordance with phenotypic resistance pattern of the isolates.

Conclusion: VRE have emerged as important pathogens in our hospital with a prevalence of 7.0% and vanA is the predominant genotype of the resistant strains.

Keywords: vanA and vanB genes; vancomycin resistant enterococci; hospital pathogens; Kashmir Region of North India.

1. INTRODUCTION

Associated with both community and hospital acquired infections, Enterococci have emerged as the most important multidrug resistant microorganisms in the last two decades [1]. There are at present, a total of 25 species of Enterococcus. However, the most common isolated species are E. faecalis (80-90%) and E. faecium (10-15%) [2]. The most frequent infections reported to be caused by Enterococci are those of urinary tract followed by intra-abdominal and intra-pelvic abscesses or post surgery wound infections[3]. The third most frequent infection caused by these organisms is blood stream infection (BSI) [4]. Other less frequent infections include CNS and neonatal infections.

Resistance to several commonly used antibiotics is a remarkable feature of Enterococci. The emergence of vancomycin resistance as a problem in enterococcal strains was first documented during 1980’s in Western Europe and in the United States [5-7]. However, as of now VRE has been reported from diverse geographic locations and its prevalence has increased dramatically worldwide [8]. The reported prevalence from tertiary care hospitals across India ranges from 1.7% to 20% [9-12]. The indiscriminate use of antimicrobial agents and the rising colonization pressure are the largest contributors to selection of vancomycin resistance in Enterococci [13].

Different types of genes that impart resistance to vancomycin have been reported in Enterococci. Out of nine recognized genotypes of vancomycin resistance in Enterococci; vanA-E, vanG, vanL, vanM and vanN, transferable vancomycin resistance in clinical isolates of Enterococci is primarily linked to the acquisition of vanA or vanB gene clusters. The vanA cluster is carried on Tn1546-like mobile genetic elements which are typically located on conjugative plasmids [14] and mediates high-level resistance to both vancomycin and teicoplanin (VanA-type) [15]. The vanB cluster is located either on the chromosome or on plasmids and mediates low to high level resistance to vancomycin only (VanB-type) [15]. These genes encode an alternate biosynthetic pathway for the production of cell wall precursors that bind vancomycin poorly [16,17]. Unlike the usual peptidoglycan precursors found in Enterococci, which have D-alanyl-D-alanine depsipeptide termini, those with acquired vancomycin resistance end with the depsipeptide D-alanyl-D-Lactate. Enterococci, as reservoirs of antibiotic resistant genes tend to transfer their resistance genes to the other bacteria, including methicillin-resistant Staphylococcus aureus [18].

Polymerase Chain Reaction (PCR) has been used to discriminate between different kinds of genes encoding resistance to glycopeptides in Enterococci. In this instance, primers that specifically hybridize to different conserved DNA sequences in different resistant genotypes are used to amplify particular gene fragments that allow different resistance types to be distinguished [19]. This prospective study was undertaken to find out the prevalence of VRE in a tertiary care hospital in North India and using molecular methods, find out the genetic determinants responsible for glycopeptide resistance in the recovered strains.
2. METHODOLOGY

This prospective study was carried out in the department of Microbiology, Sher-i-Kashmir Institute of Medical Sciences, J&K, India over a period of one year from 1st August 2013 to 30th July 2014. Patients of all age groups admitted or attending the OPD were included in this study.

2.1 Isolate Collection and Identification

Samples like blood, sputum, urine, pus and other body fluids were processed for the recovery of bacterial pathogens as per standard microbiological techniques [20]. Gram positive cocci that were catalase negative were classified as Enterococcii on the basis of growth in the presence of 40% bile and subsequent hydrolysis of esculin, and growth in 6.5% NaCl. Tryplicase Soy Broth was used as culture medium. Species identification was done on the basis of acid production from arabinose, mannitol, raffinose, sorbitol, and ADH positivity. In addition, motility and pigment production were also noted [21].

2.2 MIC Determination

Antimicrobial susceptibility of the Enterococcus isolates was performed on cation adjusted Muller Hinton agar plates by Kirby-Bauer disc diffusion method according to CLSI guidelines [22]. The minimum inhibitory concentration (MIC) of vancomycin and teicoplanin for isolates that were resistant to these glycopeptides by disk diffusion was done by microbroth dilution method as per CLSI guidelines. All strains found to be resistant by disc diffusion method were also resistant on microbroth dilution method.

2.3 PCR Amplification and Genotyping

Strains of Enterococcci, with MIC in the resistant range (n=35) were subjected to polymerase chain reaction (PCR) to identify the genes encoding the vancomycin resistance determinants (vanA, and vanB) using specific primers. Clinical isolates of Enterococci were preserved in a solution of brain heart infusion broth with 10% saline and stored at -74°C. Fresh cultures were prepared from those stock cultures whenever required. Enterococci were grown overnight at 37°C in Todd-Hewitt broth, and then 1 ml volumes were taken and DNA was extracted using DNA Extraction kit (Gene Jet, Genomic DNA purification kit; Thermo Scientific). The DNA was used as a template for vanA and vanB gene amplification by PCR. The following sets of primers were used:

- vanA- F 5’-GGG AAA ACG ACA ATT GC-3’
- vanA- R 5’-GTA CAA TGC GGC GTT TA-3’
- vanB- F 5’-ATG GGA AGC CGA TAG TC-3’
- vanB- R 5’-GAT TTC GTT CCT CGA CC-3’

PCR reaction was performed in a 25 µl volume consisting of PCR buffer 2.5 µl, dNTP’s 0.5 µl, 2.0 µl each of the forward and reverse primer, Taq polymerase 0.2 µl, MgCl2 1.5 µl, and distilled water 14.3 µl. The PCR conditions consisted of a pre-denaturation step at 94°C for 5 minutes, followed by 35 cycles of 1 min denaturation at 94°C, 2 min annealing at 60°C, 2 min elongation at 70°C, and 5 min final extension at 72°C. Amplified products were analysed by electrophoresis on 2% agarose gel. Enterococcus faecium ATCC 15559 and E. faecium ATCC 29212 were used as positive control strains for vanA and vanB genes respectively. DNA bands were visualised by staining with ethidium bromide and photographed under UV illumination (Fig. 1).

3. RESULTS

A total of 498 non-duplicate Enterococcus strains were recovered from patients during the study period. Overall, maximum number of isolates were from urine specimens (n=253, 50.8%), followed by pus and exudates (n=176, 35.3%). Only 69 isolates (13.8%) were from blood samples. A higher number of VRE isolates, however, were from blood samples (12/35, 34.3%) followed by pus (7/35, 20%) and urine (6/35, 17.1%). Three hundred and eleven (62.44%) of the 498 Enterococci were obtained from inpatients and the rest from outpatients. Majority of isolated strains were E. faecalis (65.4%) followed by E. faecium (33.5%). Thirty five of 498 isolates (7%) were vancomycin resistant. Of the VRE isolates, majority were E. faecium (60%) followed by E. faecalis (40%).

3.1 Antimicrobial Resistance among Enterococcus Isolates

Out of the 498 isolates tested, 35 (7.0%) were resistant to vancomycin and there was a variable sensitivity to other antimicrobials. All the E. faecium VRE and 57.1% of E. faecalis VRE isolates had high level vancomycin resistance (MIC ≥64 µg/ml). Out of the isolates recovered from urine, 188 (76.1%) were resistant to norfloxacin and 119 (48.2%) to nitrofurantoin. VRE isolates exhibited higher resistance pattern (Table 1).
MIC by microbroth dilution for vancomycin and teicoplanin was done on all the 35 VRE isolates. For 14 (40%) isolates the MIC for vancomycin was 32 µg/ml, whereas for 21 (60%) isolates it was 64 µg/ml. MIC for teicoplanin was 32 µg/ml in 23 (65.7%) isolates and 64 µg/ml in 12 (34.3%) isolates. All the \textit{E. faecium} isolates 21 (100%) and 8 (57.1%) \textit{E. faecalis} isolates had high level vancomycin resistance (MIC \geq 64 µg/ml) (Table 2).

All the 35 VRE isolates were recovered from inpatients and frequently from patients in ICU 12 (34.3%), (p<0.001). VRE isolates were more likely to be obtained from inpatients (n=35, 100%) rather than outpatients as compared to vancomycin sensitive \textit{Enterococcus} (VSE) isolates (p<0.001). Higher number of \textit{Enterococci} were vancomycin resistant when isolated from blood (12/69, 17.39%), followed by those isolated from pus (7/55, 7.85%) and urine (6/253, 2.37%). Prolonged hospital stay, presence of IV line catheters, prior use of β-lactam antibiotics (p<0.001 respectively) and vancomycin (p<0.003) were found to be significantly higher in patients from whom VRE were isolated. All the 35 VRE isolates were positive for presence of \textit{vanA} gene on analysis of PCR amplicons, whereas none of the VRE in our study harbored \textit{vanB} gene which was in accordance with phenotypic resistance pattern of the isolates (Fig. 1).

4. DISCUSSION

The first reports of vancomycin resistant \textit{Enterococci} (VRE) appeared in 1987 in France [23] and the United Kingdom [24]. In 1989, North America reported their first incidence. Mathur et al. [25] from New Delhi were the first to report VRE from India in 1999. Although the prevalence of VRE infections in India is much lower than in the western world, it has been increasing over the past decade. Recent studies have shown that vancomycin resistance in \textit{Enterococci} can vary between 1.7-20% in tertiary care hospitals across India [10-13]. According to the CLSI guidelines the strains are considered susceptible for vancomycin at MIC level \leq 4 µg/ml intermediate at MIC level 8-16 µg/ml and resistant at MIC level > 32 µg/ml. In our study 35 isolates (7.0%) were found to be vancomycin resistant with majority (60%) of these having high level vancomycin resistance (MIC > 64 µg/ml). These results were similar to those seen by Praharaj et al. [26], who in their study found vancomycin resistance among 8.7% (32/367) of \textit{Enterococcus} isolates with 96.8% exhibiting high level resistance to it.

Out of about a dozen \textit{Enterococcus} species, only two are responsible for majority of human infections, \textit{i.e.}, \textit{E. faecalis} and \textit{E. faecium}. Whereas \textit{E. faecalis} is considered more virulent, \textit{E. faecium} is more likely to be antibiotic resistant. Ghoshal U et al. [27], in their study found 67% of VRE isolates to be \textit{E. faecalis} and 33% to be \textit{E. faecium}. Of the 35 VRE isolates in our study also, majority were \textit{E. faecalis} (60%) followed by \textit{E. faecium} (40%). All the \textit{E. faecium} isolates and 57.1% of \textit{E. faecalis} isolates had high level vancomycin resistance (MIC \geq 64 µg/ml). A significant proportion of vancomycin resistant isolates in our study were found to be \textit{E. faecium} in contrast to vancomycin sensitive isolates that were mostly \textit{E. faecalis} (p=0.001).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Penicillin</th>
<th>Amoxicillin</th>
<th>Piperacillin</th>
<th>Piperacillin + Tazobactam</th>
<th>Amoxicillin + Sulbactam</th>
<th>Amoxicillin + Clauvulanate</th>
<th>Chloramphenicol</th>
<th>Tetracycline</th>
<th>Nitrofurantoin</th>
<th>Norfloxacine</th>
<th>Vancomycin</th>
<th>Teicoplanin</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Tested (VRE)</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>6</td>
<td>6</td>
<td>35</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>VRE (n=35) Sensitive</td>
<td>N 0</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>8</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>% 0</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>22.9</td>
<td>50</td>
<td>16.7</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Resistant</td>
<td>N 35</td>
<td>28</td>
<td>28</td>
<td>28</td>
<td>28</td>
<td>28</td>
<td>27</td>
<td>3</td>
<td>5</td>
<td>35</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>% 100</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>77.1</td>
<td>50</td>
<td>83.3</td>
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<td>100</td>
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</tr>
<tr>
<td>No. Tested (VSE)</td>
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<td>463</td>
<td>463</td>
<td>463</td>
<td>463</td>
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<td>247</td>
<td>247</td>
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<td>463</td>
<td></td>
</tr>
<tr>
<td>VSE (n=463) Sensitive</td>
<td>N 9</td>
<td>125</td>
<td>125</td>
<td>125</td>
<td>125</td>
<td>125</td>
<td>125</td>
<td>128</td>
<td>128</td>
<td>463</td>
<td>463</td>
<td></td>
</tr>
<tr>
<td></td>
<td>% 1.9</td>
<td>27</td>
<td>27</td>
<td>27</td>
<td>27</td>
<td>27</td>
<td>35.8</td>
<td>51.8</td>
<td>23.9</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Resistant</td>
<td>N 454</td>
<td>338</td>
<td>338</td>
<td>338</td>
<td>338</td>
<td>338</td>
<td>338</td>
<td>297</td>
<td>119</td>
<td>188</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>% 98.1</td>
<td>73</td>
<td>73</td>
<td>73</td>
<td>73</td>
<td>73</td>
<td>64.2</td>
<td>48.2</td>
<td>76.1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
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</table>
Table 2. Vancomycin resistance in *Enterococcus* species

<table>
<thead>
<tr>
<th>Organism</th>
<th>VSE (n=463)</th>
<th>VRE (n=35)</th>
<th>Total (n=498)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. faecalis</em></td>
<td>312 (67.4%)</td>
<td>14 (40%)</td>
<td>326</td>
<td>0.001 (S)</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>146 (31.5%)</td>
<td>21 (60%)</td>
<td>167</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Agarose Gel Electrophoresis showing positive amplification of 732 base fragments specific for *vanA* of VRE
Lane L: Size marker (100-bp DNA ladder); Lane C: *E. faecium* ATCC 15559 (Positive Control – 732 bp);
Lanes 1 to 6: VRE test strains showing positive result for *vanA*

Majority of VRE isolates in our study were recovered from blood samples (34.3%), followed by pus (20%) and urine (17.1%). The higher isolation of VRE from blood was found to be statistically significant (p<0.001). Results similar to ours were seen in many other studies [27-29] that reported a higher isolation of VRE from blood samples. Shah L, et al. [30], however, reported a higher isolation of VRE strains from urine (62.5%) followed by blood (25%). Although many studies have demonstrated a higher isolation of *Enterococci* from urine samples, VRE have been isolated from blood and urine specimens with variable frequency. In our study a higher isolation of VRE was seen from blood samples.

Prolonged hospitalization especially in intensive care units (ICUs) is a known risk factor for enterococcal infections [26,31,32]. In this study, history of prolonged hospital stay (>10 days) was noted in 68.6% of the patients from whom VRE were recovered, as against 36.1% of patients whose samples yielded VSE (p<0.05). All the VRE were recovered from inpatients, most frequently from ICU (34.3%) followed by general medicine (20%) and cardiology (17.2%). Higher isolation of VRE from ICU as compared to VSE isolates was found to be statistically significant (p<0.001).

Intensive use of antibiotics particularly vancomycin is known to be associated with vancomycin resistance in *Enterococci* [33,34]. Deivid William da Fonseca Batistão et al. [35], in their study found that use of vancomycin and carbapenems was related to an increased incidence of VRE colonization (p<0.05). In our study, prior use of vancomycin was noted in 54.3% of the patients from whom VRE were isolated as compared to only 30% from whom VSE were recovered and the difference was found to be statistically significant (p<0.05).

The *vanA* is the predominant gene encoding vancomycin resistance in *Enterococci* [26,36,37]. Studies have shown that *Enterococci* that harbour *vanA* gene but are teicoplanin sensitive can assume VanA phenotype once exposed to glycopeptides [38]. All the 35 VRE isolates in our study were positive for presence of *vanA* gene on PCR analysis, whereas none of the isolates harboured *vanB* gene. Results of PCR in our study correlated well with phenotypic resistance pattern (*vanA*: resistant to vancomycin and teicoplanin). Similar results were also shown by Emaneini M, et al. [39], who reported that 12% of *Enterococci* in their study were VRE and all had presence of *vanA* genotype with *vanA* phenotype while none of the VRE isolates harboured *vanB* gene. Likewise Fasih N, et al. [40], demonstrated in their study that 91.8% of VRE isolates harboured *vanA* gene and none had *vanB* gene.
5. CONCLUSION

Vancomycin resistant Enterococci are a significant problem in our hospital with a prevalence of 7.0% and vanA is the predominant resistance determinant in VRE isolates of our hospital. All the VRE isolates in our study exhibited vanA phenotype with majority showing high level vancomycin resistance (MIC ≥ 64 µg/ml). The emergence of VRE is a matter of concern as the treatment options are limited. Furthermore, nosocomial spread of these pathogens may create a reservoir of mobile resistance genes for other, more virulent nosocomial organisms like Staphylococcus aureus. As these strains are resistant to nearly all the available antimicrobial agents, their dissemination may lead to treatment failures with increased morbidity and mortality.

ETHICAL APPROVAL

This study was approved by Institute Ethical Committee (IEC) of the Sher-I-Kashmir Institute of Medical Sciences, J&K, India held on 28-09-2013 with study reference protocol no. 110/2013.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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