

## Vancomycin resistant enterococci: an emerging problem in a tertiary care hospital, Pune, India

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### Abstract

**Background and Objectives:** Vancomycin resistant Enterococci (VRE) have recently emerged as nosocomial pathogen with intrinsic resistance to many antimicrobial agents making them difficult to treat. We investigated the prevalence of vancomycin resistance in enterococci isolated in a tertiary health care set up.

**Method:** 200 stool samples from preoperative patients and 200 enterococci isolates from patients' specimens were screened for VRE. Vancomycin resistance was confirmed phenotypically by macro broth dilution method and genotypically by PCR followed by sequencing. DNA sequencing results were used for genotyping

**Results:** 19(4.75%) VRE were obtained. All VRE isolates showed van A and van M phenotypes. Van A gene was detected in all isolates by PCR. PCR amplified product was subjected to DNA sequencing. Three genotypes were obtained. Genotype I consisted of 16 VRE isolates indicating clonal dissemination. Genotypes II and III were comprised of 2 and 1 VRE isolate respectively indicating mutation or introduction of a new strain.

**Interpretation and Conclusion:** Being an emerging pathogen, VRE acts as a sensitive marker for measuring the effectiveness of infection control programme and the appropriate application of preventive measures. The study resulted in an increased awareness about VRE and implementation of control measures in the hospital to restrict spread of VRE.

**Keywords:** Genotype, VRE, MIC, Nosocomial, PCR, Sequencing.

### Introduction

In the last two decades, the emergence of Vancomycin Resistant Enterococci (VRE) and their increasing prevalence worldwide has made it difficult to treat serious enterococcal infections. VRE can remain viable in the environment for an extended period of time and therefore pose a problem for infection control in hospitals. In addition, VRE have been detected as part of enteric flora in non symptomatic patients. These colonized patients serve as potential source for transfer of these organisms to other patients and medical personnel.

Enterococcus, particularly *E. faecium* is intrinsically resistant to many antibiotics such as cephalosporins, clindamycin and penicillinase resistant penicillins. Later on the organism emerged as having acquired resistance to ampicillin, aminoglycosides and vancomycin.<sup>(1,2,3)</sup> The pathogen thus became untreatable with most available antibiotics. The organism was also noted to transfer the resistance horizontally to other Gram positive cocci. Control of VRE by preventing its colonization and spread at centers where VRE is endemic was observed to be unsuccessful.<sup>(4,5)</sup>

Nine types of vancomycin resistance genes have been described in *Enterococci*. Knowledge of type of resistance is important and critical for infection control purposes. Van A and van B genes are transferable and can spread from organism to organism leading to major outbreaks in hospitals. Van C gene is non transferable and less commonly associated with outbreaks.<sup>(6)</sup>

The present study was aimed to know the prevalence of vancomycin resistance in enterococci isolated as colonizers and pathogen. 'VAN' phenotype and genotype of the isolated VRE strains was determined and genotyping was done to study the epidemiology.

### Material and Methods

**Study design:** The study was prospective, laboratory based and approved by Ethical Committee. Total of 400 samples were collected from three different categories of patients over a period of one year -

Category A-the colonizers- A1] 100 stool samples collected from preoperative patients from surgery ward. A2] 100 stool samples from patients with predisposition for acquisition of VRE. Patients with immunosuppression like malignancy, solid organ transplant, patients with Diabetes Mellitus, renal failure, chronic kidney disease, patients with prolonged hospitalization for more than 7 days and patients with previous or current antibiotic exposure within one month were included in this category.

Category B-the pathogens-B1] 200 isolates of enterococci obtained from clinical samples like blood, urine, pus, CSF, peritoneal fluid, plural fluid were included in this category.

2.2- Isolation and identification of enterococci- Stool samples were inoculated on bile esculin azide agar (Hi media Lab Pvt. Ltd, India) plus 6 µg/ml vancomycin (Hi media Lab Pvt. Ltd, India). The isolate

obtained was identified till species level by routine laboratory tests.<sup>(7)</sup>

Enterococci isolated from clinical samples were screened for vancomycin resistance by using brain heart infusion agar (Hi media Lab Pvt. Ltd, India) with 6µg/ml vancomycin (Hi media Lab Pvt. Ltd, India).

2.3- Antibiotic susceptibility by disc diffusion method was performed as per CLSI guidelines.<sup>(8)</sup> Along with routine antibiotics, vancomycin, teicoplanin, linezolid, high level gentamycin and high level streptomycin were also tested.

Macrobroth tube dilution method was employed to detect MIC of vancomycin and teichoplanin.<sup>(9,10)</sup> The results were interpreted as per 2013 CLSI guidelines<sup>8</sup> as vancomycin susceptible  $\leq 4\mu\text{g/ml}$ , intermediate 8-16µg/ml, resistant  $\geq 32\mu\text{g/ml}$ . For teicoplanin, susceptible  $\leq 1\mu\text{g/ml}$ , intermediate 16µg/ml, resistant  $\geq 32\mu\text{g/ml}$ . Based on MIC values of vancomycin and teicoplanin, VRE phenotype were determined.

2.4-Vancomycin resistance was confirmed genotypically by PCR followed by DNA sequencing.

DNA was extracted from the isolated VRE by silica gel method<sup>(11,12)</sup> and was measured using spectrophotometer (Eppendorf, Germany). Extracted DNA from all VRE isolates were first subjected to PCR for van A gene detection. Primers used were van A forward-GTAGGCTGCGATATTCAAAGC and van A reverse- CGATTCAATTGCGTAGTCCAA. Reaction mixture containing PCR master mix (Bangalore Genie), primers and VRE DNA was subjected to 35 PCR cycles (94°C/2min, 60°C/2min, final extension 72°C/2min).<sup>(13)</sup> 231 bp amplified product was analyzed by electrophoresis using 2% agarose gel and was observed using UV transilluminator (Ultra-violet products, USA). VRE strains negative for vanA were subjected to van B PCR. Primers used were van B forward GTAGGCTGCGATATTCAAAGC van B reverse GCCGACAATCAAATCATCCTC.<sup>(13)</sup>

Amplified PCR product was subjected to sequencing using BIG DYE termination kit (Applied Biosystems, Foster city C.A.). Results of DNA sequencing were analyzed in 3730xl DNA analyzer (Applied biosystem, Foster city, C.A.). Nucleotide sequences obtained were subjected to BLAST analysis in NCBI database to determine the genotype.

2.5-Genotyping was done by using DNA sequencing data. To prepare a phylogenetic tree, 19 VRE isolates were numbered as D2 to D19 and D 28F. Nineteen reference strains, one for each test strain, were used and numbered as DR2 to DR19 and DR\_28. Phylogenetic tree of all isolates was prepared to see the genetic relatedness of the strains on the basis of nucleotide sequences using software DAMBE<sup>(14)</sup> and MEGA.<sup>(15)</sup>

## Results

In category A, which includes colonizers, 6% VRE were isolated (12 from 200 samples) (Table 1). In

category B (the pathogens), among enterococcal isolates obtained from various clinical samples (n=100), 3.5% VRE were isolated (7 from 200 samples). Overall prevalence of VRE was found to be 4.75% (19 from 400 samples)

**Table 1: VRE isolates obtained from various samples**

Group	Type of sample	No. of samples	No. of VRE identified	Van Genotype
A1- Preoperative patients	Stool	100	8	Van A
A2- patients with predisposition for acquisition	Stool	100	4	Van A
B1- Clinical samples	urine	94	5	Van A
	pus	58	1	Van A
	blood	20	1	Van A
	Body fluids	28	0	----
Total		400	19	

Overall prevalence of VRE was found to be 4.75% (19 from 400 samples) and all showed van A genotype.

All the 19 VRE isolates were *E.faecium* by phenotypic method. All showed 100% sensitivity to linezolid followed by nitrofurantoin (42.1%).

The MIC value for vancomycin and teicoplanin was 64µg/ml-256µg/ml and 16µg/ml-256µg/ml respectively. All VRE isolates showed Van A and van M phenotypes in the present study. Four showed van D phenotype in addition to van A and van M.

PCR showed all the 19 isolates of VRE to harbor van A gene. Sequencing analysis of the amplified PCR product confirmed the species of VRE to be *E.faecium*. Majority of the VRE isolates (52.63%) were found to have 100% identity with partial sequences of van A ligase gene of four *E.faecium* strains. (Gen Bank Accession no. KF 181100, JN 207928, JN 207929, JN 207930). The remaining 47.36% isolates showed 99-100% identity to partial sequences of van A gene of *E.faecium* (Gen Bank Accession no. KF 052036) and partial sequence of plasmid Tn 1546 of *E.faecium* (Gen Bank Accession no. JN 982328).

Phylogenetic tree revealed three genotypes, designated as I, II and III which contained 16, 2 and one VRE isolates respectively.

## Discussion

During 1990's, vancomycin resistant enterococcus (VRE) emerged as a nosocomial pathogen. VRE isolated in the hospital scenario, may represent acquisition of resistance genes or represent preexisting but undetected colonization following exposure to long antimicrobial therapy.<sup>(1)</sup>

In the present study, overall prevalence (colonization+infection) of VRE was 4.75% (19 VRE

isolated from 400 samples). Salem-Bakhit et al<sup>(16)</sup> showed similar prevalence (3.9%) of VRE colonization plus infection.

The prevalence of VRE colonization was 6%. The prevalence of VRE colonization varies from 1.9 to 37% in different geographical area. The prevalence of VRE colonization in the present study was comparable with study conducted by Gikas et al<sup>(17)</sup> in Greece (7.5%). Higher prevalence shown by Gambarotto et al<sup>(18)</sup> was due to selection of patients who were from agricultural area where vancomycin related antibiotic was used recently in animal husbandry. In the study by Rathore et al<sup>(19)</sup> the patients were from lower socioeconomic group which were referred from other hospitals and were on long term antibiotic treatment. Variable antibiotic use in different hospital settings and lack of hospital infection policy may have contributed to high prevalence in the study by Rathore et al.

Previous treatment with antibiotics results in the acquisition of resistant genes. Prevalence of VRE infection in the present study was 3.5%. Antibiotic exposure within previous one month was present in 12 patients with VRE colonization and 7 patients with VRE infection. Similar finding was also documented in several studies.<sup>(20,21,22,23)</sup>

All 19 VRE isolates (12 from colonizers and 7 from infection) were *E.faecium* and sensitive to linezolid followed by 42.1% and 26.31% sensitive to nitrofurantoin and tetracyclin respectively. Modi et al<sup>(24)</sup> Praharaj et al<sup>(25)</sup> and Rahangdale et al<sup>(26)</sup> also reported similar finding.

It is important to know the phenotype of VRE because only van A and van B phenotype can transfer resistance to other organisms. All 19 (100%) VRE isolates showed van A phenotype. Recent Indian studies by Modi et al<sup>(24)</sup> and Praharaj et al<sup>(25)</sup> also showed van A phenotype. Taneja et al<sup>(23)</sup> documented that confirmation of results at genetic level by PCR is required.

In the present study, all 19 (100%) VRE isolates were shown to carry van A gene. So the genotype of all VRE isolate was van A. Most of the studies<sup>(16,20,25,27)</sup> showed van A genotype. Other transferable genes are van G, van M and van N which are not reported from India till date. Van C, van E and van L gene mediate low level resistance and are non-transferable.<sup>(28)</sup>

Transposon (Tn)1546 was originally detected on plasmid pIP816 from *E.faecium* BM 4147. Tn 1546 consist of 10,851 bps and encodes nine polypeptides that can be assigned to four functional groups: transposition function(open reading frame ORF1 and ORF 2), Regulation of vancomycin resistance genes(van R, van S), resistance to glycopeptides by production of depsipeptides (van H, van A, van X) and accessory proteins that may be involved in peptidoglycan synthesis. Three important proteins are secreted by van A gene namely van A ligase protein, van H protein and vanX protein. Van A ligase protein is

an essential component for expressing vancomycin resistance.<sup>(29,30)</sup>

Tn 1546 like element was detected in the present study by DNA sequence analysis, which may reflect the unique characteristic of VRE in the present study. The predominance of Tn 1546 element suggest that the main contributory factor for the prevalence of VRE in the present study could be the horizontal dissemination of Tn 1546 like element in the hospital.

Analysis of phylogenetic tree obtained after DNA sequencing revealed 3 patterns. Pattern I was containing 16 VRE isolates. Such major *E.faecium* genotype pattern by phylogenetic analysis indicates clonal dissemination. In addition to genotype I pattern, genotype pattern II and III were obtained suggesting either mutation or introduction of a new strain. Clonal dissemination of VRE suggests the need for implementation of vigorous control measures for prevention of spread of VRE in the hospital setting.

Being an emerging pathogen, VRE acts as a sensitive marker for measuring the effectiveness of infection control programs and the appropriate application of preventive measures.<sup>(27)</sup> In the present study, all measures recommended in CDC guideline were discussed with hospital staff and recommendations were put up in the wards. VRE isolation was seen to be reduced following control measures in the wards. The study resulted in increased awareness about VRE and implementation of control measures to prevent the spread of VRE in the hospital. However periodic re-enforcement needs to be done to monitor the spread of VRE.

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