

Speciation of Enterococcal Isolates in a Tertiary Care Hospital and Molecular Characterisation of Vancomycin Resistant Enterococci (VRE)

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Abstract

Background: Enterococci are gram positive cocci which reside as commensal microbial flora in gastrointestinal tract, vagina, biliary tract and male urethra. *E.faecalis* is the predominant species followed by *E.faecium*. Resistance against glycopeptides and aminoglycosides are being increasingly demonstrated and that is conferred upon largely by van A gene.

Aims: To isolate and speciate Enterococci from various clinical samples, antibiogram pattern determination, followed by genotyping of vancomycin resistant Enterococci. Settings and Design: Prospective study.

Materials and Methods: 200 Enterococcal isolates were collected and processed for speciation based on battery of phenotypic characteristics. Antibiotic sensitivity testing was performed as per CLSI guidelines 2014 for glycopeptide and aminoglycoside antibiotics. Enterococci which were resistant to vancomycin were subjected to polymerase chain reaction for van A gene detection. Statistical analysis used: Nil.

Results: Of the 200 isolates 5 were resistant to vancomycin and teicoplanin (2.5%), 30 were resistant to high level gentamicin (15%). van A gene was detected in 3 of the 5 vancomycin resistant isolates. *E.faecium* was observed to be more resistant to the antimicrobials.

Conclusions: If the vancomycin resistance in Enterococci gets dispersed throughout the world, it may pose a challenge in the treatment of Gram positive infections and may lead to treatment failure. Further treatment options may not be available. Hence these infections have to be prevented and the sequence of development of resistance to higher drugs has to be controlled by active surveillance and prevention.

Key-words: *E.faecalis*, *E.faecium*, High-level Gentamicin resistance, Vancomycin-resistance, Van A gene

Key Messages: Implementation of vigorous surveillance and provision of timely data

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Introduction

Enterococci are Gram positive cocci, belonging to group-D streptococci and comprise of 37 species; they usually inhabit the gastrointestinal as part of the commensal microbial flora. The predominant species are *Enterococcus faecalis* (85-90%) and *Enterococcus faecium* (5-10%)^[1]. *E.avium*, *E.casseliflavus*, *E.durans*, *E.cecorum*, *E.gallinarum*, *E.malodoratus*, *E.raffinosis*, *E.hirae*, *E.dispar* and *E.mundtii* are rarely isolated in humans^[2]. Enterococcus isolated from nosocomial infections acquires resistance particularly against glycopeptides^[3]. Enterococci cause urinary tract infection, wound infection, biliary tract infection and occasionally meningitis, bacteraemia and endocarditis^[1].

This organism resides in biliary tract, vagina and male urethra. The most worrisome issue is that it shows intrinsic resistance to most of the beta-lactam antibiotics, low level resistance to aminoglycosides and intermediate-resistance to fluoroquinolones. The resistance was more with *E.faecium* than *E.faecalis*^[1]. Avoparcin use in animal feeds has led to the emergence of vancomycin-resistant enterococci and vancomycin-resistance has been transferred to methicillin-resistant *Staphylococcus aureus*^[4]. *Enterococcus faecium* isolates of hospital origin holds a subsidiary genetic expression that may play a role in virulence^[5].

Nine genes have been identified that ascertain vancomycin resistance namely: *van A, B, C, D, E, G, L, M* and *N*. *van A* type takes the lead globally and imparts a high level of resistance to glycopeptide antibiotics seen particularly in vancomycin-resistant *Enterococcus faecium*^[6]. Genotype *van A* is prevalent in *E.faecium* while *van B* prevalent in *E.faecalis*^[6].

The study was conducted to isolate and identify Enterococci from the clinical samples followed by speciation. Further antibiotic sensitivity testing was performed for determining the sensitivity pattern and

for the detection of glycopeptide and high level gentamicin resistance along with genotyping of resistant Enterococcal species.

Materials and Methods

The study was performed in the department of Microbiology, Chettinad Hospital and Research Institute, Kelambakkam, Chennai, India. A total of 200 isolates were collected from both inpatients and outpatients treated in the hospital from various clinical departments during the period of February 2013 to January 2014 (1 year).

Sample collection: The samples collected were pus, wound swabs, blood, urine, endotracheal aspirates, sputum and other body fluids. Pus and wound discharge from the infected area were collected using sterile cotton swabs (Himedia). Blood samples were collected and transported to the lab in brain heart infusion broth. Clean catch midstream urine samples were collected in sterile wide mouthed containers and transported to the lab immediately after collection.

Processing: Samples were processed as per the standard protocol. All the samples were inoculated onto three culture media (Nutrient agar plate, Blood agar plate and MacConkey agar). The next step in the processing was to identify Enterococcus from the isolates. This was done on the basis of Gram staining, colony morphology, catalase test and esculin hydrolysis with appropriate ATCC controls. The other tests included were growth at 45°C and 10°C, growth in 6.5% NaCl, Arginine deamination test, Pyrrolidonyl arylamidase test, Voges Proskauer test, Pyruvate Fermentation Test and Sugar fermentation tests.

Antibiotic Susceptibility Testing (Kirby – Bauer Disk Diffusion Method)

All the Enterococcal isolates were subjected to antimicrobial susceptibility testing on Mueller Hinton agar by Kirby-Bauer disc diffusion method as per the CLSI guidelines 2014, for determining the antibiogram pattern and for the detection of Vancomycin Resistance. The discs used were: Ampicillin (10 µg), Penicillin (10 µg), Tetracycline (30 µg), Ciprofloxacin (5 µg), Linezolid (30 µg) vancomycin (30 µg) and teicoplanin (30 µg) and high level gentamicin (120 µg). The antibiotic discs were provided by Himedia laboratories. Zone size interpretation for each antibiotic was done based on CLSI guidelines. Enterococcus isolates with vancomycin zone size ≤ 17 mm and teicoplanin ≤ 14 mm were taken as VRE. This was confirmed by using the Vancomycin E strip. MIC for Vancomycin greater than 32µg/ml was considered as resistant, MIC range of 8-16µg/ml was considered as intermediate and MIC < 4 µg/ml was considered as sensitive

Polymerase Chain Reaction for van A gene from vancomycin resistant Enterococci

Isolation of Genomic DNA was performed by phenol: chloroform method and the DNA pellets were dried and re suspended in 20µl of TE buffer. **PCR mixture:** Includes 1.5µl of Taq polymerase, 10µl of PCR amplification mix, 10pmol of primer, 200mmol of dNTPS and distilled water – final volume is 50µl. **PCR amplification:** PCR amplification was then done by adding 1µl of the extracted DNA to the PCR mixture followed by repeated cycles of DNA denaturation, primer annealing and primer extension.

Primer used for van A gene detection

Forward	Primer:	5'-
TGCGCGGAATGGGAAAACGACA-3'		
Reverse	Primer:	5'-
CAGCCCGAAACAGCCTGCTCAA-3'		

PCR Product size: 473bp.

The PCR product was then subjected to electrophoresis to confirm the presence of the expected amplicon fragments in comparison with suitable DNA ladder. The visualization of DNA fragments was enabled by adding Ethidium bromide to agarose gel and isolation of bands was determined by examination of the gel under the UV transilluminator.

Results

A total of 200 Enterococcal isolates were selected for the study. Table 1 shows the species distribution of the Enterococci. Among the species of Enterococci, *Enterococcus faecalis* was the most common isolate (55%). Table 2 shows the clinical distribution of the Enterococci. Most of Enterococcal isolates were reported from medical ward (55%), followed by surgical ward (26%), gynaecology ward (8%), ICU (3%), paediatric ward (3%) and other wards (5%).

The maximum number of Enterococci infected patients were in the age group of 11yrs to 30yrs. The second largest age group affected was between 31yrs to 50yrs followed by 51yrs to 70yrs. The pediatric age group was least affected.

Table 3 displays the resistance pattern of the Enterococci. Of the 200 Enterococci isolates studied, 30 (15%) isolates were High Level Gentamicin Resistant and 170 isolates were High Level Gentamicin Sensitive. Among the 30 isolates of HLAR Enterococci 18 were found to be *E.faecium*. The antibiotic sensitivity revealed 73% resistance to Erythromycin, 47% resistance to Ciprofloxacin and Tetracycline, 32% resistance to Norfloxacin, 26% resistance to Penicillin, 16% resistance to Ampicillin, 15% resistance to High Level Gentamicin, 5% resistance to Linezolid and 4% resistance to Nitrofurantoin (Urine isolates). Enterococci are intrinsically resistant to Cephalosporins and Cotrimoxazole. 5 isolates were Vancomycin Resistant Enterococci (VRE-2.5%) and 195 were

Vancomycin Sensitive Enterococci (VSE-97.5%). The prevalence of VRE in our hospital was found to be 2.5%. Fig. 1 and 2 show the strains resistant to Vancomycin and high level gentamicin detected by the MIC method using E strip.

Fig. 3 shows the Gel electrophoresis picture for van A gene detection by PCR. Highest number of VRE

isolates was obtained from pus and exudates (80%) followed by 1 isolate (20%) which was from urine sample. Van A gene detection by PCR revealed its presence in 3 (60%) of the 5 Vancomycin Resistant Enterococci isolates tested. All the three vancomycin resistant isolates were found to be *E.faecium*.

Table 1: Number of Enterococcal species identified

S. No	Enterococci isolated	No. of isolates	Percentage
1	<i>E.faecalis</i>	110	55%
2	<i>E.faecium</i>	58	29%
3	<i>E.avium</i>	21	10.5%
4	<i>E.hirae</i>	6	3%
5	<i>E.casseliflavus</i>	2	1%
6	<i>E.durans</i>	2	1%
7	<i>E.gallinarum</i>	1	0.5%

Table 2: Clinical distribution of Enterococci

S. No	Ward	No. of samples	Percentage
1	Medical ward	110	55%
2	Surgical ward	52	26%
3	Gynaecology	16	8%
4	Paediatrics	6	3%
5	ICU	6	3%
6	Other	10	5%

Table 3: Resistance pattern of the Enterococci

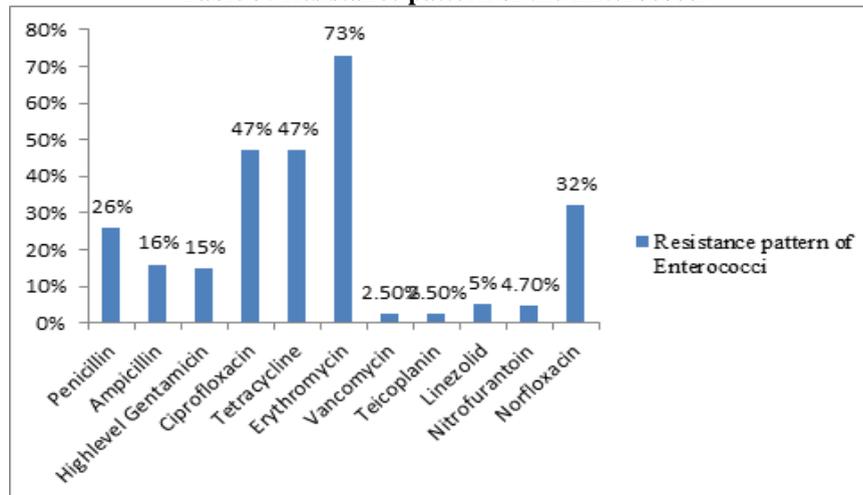




Fig. 1: Vancomycin and teicoplanin resistant isolate (MIC \geq 64 μ g/ml)

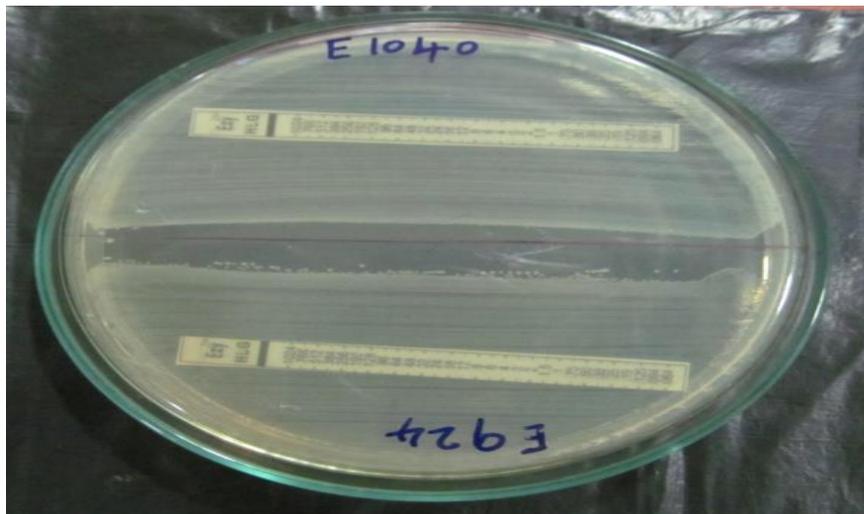


Fig. 2: High Level Gentamicin Resistant isolate (MIC \geq 2000 mgm/ml)

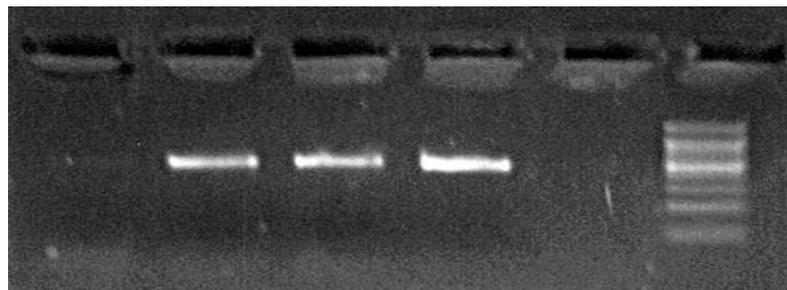


Fig. 3: Gel electrophoretic picture of van A gene from VRE
PCR Product size: 473bp

Discussion

In our study the prevalence of vancomycin-Resistant Enterococci among the clinical samples was 2.5% which is similar to the study findings of Sievert et al, an epidemiological study conducted by CDC, Atlanta, Georgia, which showed 3% prevalence of vancomycin-resistant enterococci^[7]. Another study by Saroj Golia et al showed 7% prevalence of Vancomycin

resistance^[8]. The second study had a slightly increased percentage of Vancomycin resistance as when compared to this study.

Out of 5 isolates, 3 VRE isolates showed the presence of vanA genotype, which is the predominant one as similar to Mathur et al, in which out of 5 VRE four were vanA type and one was vanB type of resistance^[9]. *Enterococcus faecium* showed more

resistance than *E. faecalis*, which correlated well with other studies (Saroj Golia et al)⁸.

Regarding High-level aminoglycoside resistance the prevalence was 15%, quite different from the study in a rural hospital in central India, where it was as high as 46% in a study undertaken by Mendiratta et al to determine the status of HLAR enterococci in rural setup^[10]. This may be due to the differences in the distribution of resistant isolates between the urban and rural setup.

The prevalent age group affected were between 11-30yrs (38%) and followed by 31-50yrs (37%), however the average age group were 20-40yrs which is similar to Srivastava et al, a study conducted in Jaipur, North India^[11].

Vancomycin-Resistant Enterococci may lead to serious consequences as they are associated with increased mortality due to their recurrence and reinfection even after the administration of higher drugs like linezolid and daptomycin^[12].

Most of the infected individuals were hospitalized patients in our study and this is similar to the observation by Marothi et al (Enterococcal resistance- an overview)^[13] and more number of Enterococcal infections were reported from medical ward (55%), followed by surgical ward (26%), gynaecology ward (8%), ICU (3%), paediatric ward (3%) and other ward (5%).

If these VRE isolates gets dispersed throughout the world, it may pose treatment failure to newer drugs and there may not be a treatment option. Hence these infections have to be prevented and the cycle of resistance formation to newer drugs by active surveillance and prevention.

Conflict of Interest: None

Source of Support: Nil

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