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Antimicrobial resistance pattern in *Enterococcus* species and detection of *vanA* gene among vancomycin resistant isolates in a tertiary care hospital

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Abstract
Background: Since late 1980s, emergence of vancomycin (a glycopeptide) resistance has been observed among Enterococcal isolates worldwide. Glycopeptide resistance in Enterococci has been dealt with in a few studies in India.
Aims and Objectives: (1) Studying antimicrobial resistance pattern among *Enterococcus* species and (2) Detection of *vanA* gene in vancomycin resistant Enterococcal isolates.
Materials and Methods: A total of 145 consecutive Enterococcal isolates identified by conventional methods were included in the study. Antibiotic susceptibility testing was done by disk diffusion and MIC testing method. Vancomycin resistant *Enterococcus* (VRE) isolates were subjected to PCR amplification targeting *vanA* gene. Statistical analysis was done in Microsoft Office Excel 2007.
Results and Conclusion: Penicillin resistance was found in most number of *Enterococcus* isolates. Vancomycin resistance was found in 6.6% of isolates, most (90%) of which were from hospitalized patients. Most of the VRE isolates were multi drug resistant. Among VRE isolates, 70% were *Enterococcus faecium*. Eighty (80) % of VRE isolates had *vanA* gene.

Keywords: *Enterococcus*, vancomycin resistance, PCR, *vanA* gene

1. Introduction
In the last few decades, Enterococci have emerged as a leading cause of nosocomial infection especially because of increased antimicrobial resistance observed in these organisms [1]. It has been implicated in conditions like urinary tract infections, bacteraemias, surgical site infections etc. worldwide. It is the third leading cause of nosocomial blood stream infection [2] and second most common pathogen causing morbidity and mortality [3].
Since late 1980s, there has been increasing occurrence of *Enterococcus* species worldwide which is of particular concern due to the emergence of vancomycin resistance [4]. Vancomycin resistance in Enterococci leaves fewer therapeutic options and there is also potential risk of vancomycin resistance gene transfer from *Enterococcus* to *Staphylococcus aureus* [5].
There are three glycopeptide resistance phenotypes in Enterococci on the basis of the level and inducibility of resistance to vancomycin and teicoplanin [6]. In *van*A type of glycopeptide resistance, there is acquired inducible resistance to both vancomycin and teicoplanin and in VanB phenotype, there is variable level of resistance to vancomycin but susceptibility to teicoplanin [7, 8]. VanC phenotype is characterized by constitutive low level resistance to vancomycin which is an intrinsic property of motile Enterococci, *E. gallinarum*, *E. casseliflavus* and *E. flavescens* [6].
Glycopeptide resistance in Enterococci is mediated by *van* gene clusters that alters drug target from D-alanine-D-alanine to D-alanine-D-lactate [9]. Eight genes of glycopeptide resistance have been described so far for Enterococci. Five of the van genes viz. *vanA, B, D, E & G* are acquired and three viz. *vanC, C2, C3* are intrinsic. The most prevalent gene is *vanA* which is predominantly found in *E. faecium* and *E. faecalis* [10].
The present study was undertaken with an aim to study antimicrobial resistance pattern among *Enterococcus* isolates and detect *vanA* gene in vancomycin resistant isolates.

2. Materials and Methods
2.1 Study Setting
The study was carried out in the Department of Microbiology & Multidisciplinary Research Laboratory, Assam Medical College and Hospital, Assam, India from June, 2015 to June, 2016.

2.2 Study design and population
This was a hospital based observational study. All the isolates of *Enterococcus* species obtained from different clinical specimens over the study period were included in the study.

2.3 Identification of *Enterococcus* species
Enterococcus species were identified by standard procedure [9].

2.4 Antimicrobial susceptibility testing
All the isolates of *Enterococcus* were tested for their antimicrobial susceptibility pattern using Clinical Laboratories Standards Institute (CLSI) guidelines. Two methods were used for studying the antimicrobial susceptibility patterns in *Enterococcus* isolates- Kirby-Bauer disk diffusion method and minimum inhibitory concentration (MIC) testing by Vitek 2

Compact system, version 7.01. Kirby-Bauer disk diffusion technique was used for determining the susceptibility of the isolates to the antibiotics ampicillin (10 µg), penicillin (10 U), ciprofloxacin (5 µg), levofloxacin (5 µg), tetracycline (30 µg), vancomycin (30 µg) and linezolid (30 µg). Susceptibility testing of norfloxacin (10 µg) and nitrofurantoin (300 µg) were also done for urine isolates. Isolates found to be resistant or intermediate to vancomycin by disk diffusion method were also tested by Vitek 2 Compact system for minimum inhibitory concentration (MIC) of vancomycin, teicoplanin, high level gentamicin etc. Any Enterococcal isolate with vacomycin MIC ≥16 µg/ml was considered vancomycin resistant *Enterococcus* (VRE). Vitek confirmed VRE isolates were selected for genotypic characterization and were stored in peptone water with 25% Glycerol at -80°C for further processing.

2.5 DNA extraction

Isolates resistant to vancomycin were taken for DNA extraction. A single colony was picked up from freshly streaked blood agar and was inoculated into 3 ml of peptone water which was incubated overnight at 37°C. After overnight incubation, DNA extraction was done from the inoculated broth using QIAamp DNA mini kit from Qiagen following manufacturer's protocol [11].

2.6 Polymerase chain reaction for *vanA* gene

PCR amplification for *vanA* gene was done by previously used protocol [6]. Amplification of *vanA* gene was carried out in a thermal cycler by using gene specific primers. The *vanA* gene primer sequence (5'-3') used was: forward primer - A1 GGGAAAACGACAATTGC and reverse primer -A2 GTACAATGCGGCCGTTA [12]. The reaction mixture with final volume of 10 ul contained 1 ul of extracted DNA, 5 ul of master mix (Roche), 0.5 ul of each primer and 3 ul of nuclease free water. PCR conditions were 94°C X 2 min for the first cycle; 94°C X 1 min, 54°C X 1 min and 72°C X 1 min for 30 cycles and final extension at 72°C X 10 min. An in house *vanA* positive *Eneterococcus faecium* isolate was considered as positive control and negative control consisted of *Eneterococcus faecalis* ATCC 29212. PCR product was analyzed by gel electrophoresis using 2% agarose gel and 0.5 mg ethidium bromide. Gel was photographed on an UV light transilluminator by gel documentation system (BIORAD). Molecular marker of 100 bp was used and a final product of 732 bp was considered as *vanA* gene.

2.7 Statistical analysis

Statistical analysis was done on Microsoft Office Excel 2007.

3. Results and Observations

A total of 145 isolates of *Enterococcus* species were isolated from various clinical specimens received in the Department of Microbiology, Assam Medical College over the study period of one year and one month. Maximum number (81.4%) of isolates was from urine specimens followed by isolates from blood (10.3%). Table 1 shows distribution of Enterococcal isolates in various clinical specimens. One hundred thirty nine (96%) of the 145 *Enterococcus* isolates were obtained from hospitalized patients while the rest were from outpatients. Most (51.7%) of the *Enterococcus* isolates were *E. faecium* followed by *E. faecalis* (16.6%) and *E. durans* (2%). Species was not determined in 29.7% of the isolates.

Table 1: Distribution of Enterococcal isolates in various clinical specimens

Clinical samples	No. of Enterococci recovered	Isolation rate (%)
Urine	118	81.4
Blood	15	10.3
Pus	3	2.1
CSF	3	2.1
Sputum	2	1.4
Wound swab	2	1.4
Tracheal swab	1	0.69
High vaginal swab	1	0.69
Total	145	

3.1 Antibiotic resistance among *Enterococcus* isolates

Among 145 Enterococcal isolates, penicillin resistance was observed in highest number of isolates (79.45%) followed by ciprofloxacin (69.9%) and levofloxacin (69.6%). Ampicillin resistance was observed in 67.9% of isolates. Norfloxacin and nitrofurantoin were tested only for urine isolates which showed resistance in 76.5% and 17% of all urine isolates respectively. None of the *Enterococcus* isolates were resistant to linezolid. Resistance to vancomycin was observed in 10 (6.6%) isolates after MIC testing by Vitek2 compact. Table 2 shows percentage antibiotic resistance among Enterococcal isolates.

Table 2: Antibiotic resistance among the Enterococcal isolates

Antibiotic	Resistant (%)
Penicillin	79.4
Ampicillin	67.9
Ciprofloxacin	69.9
Norfloxacin	76.5
Tetracycline	42.7
Levofloxacin	69.6
Nitrofurantoin	17.0
Vancomycin	6.6
Linezolid	0

All Vancomycin resistant isolates were from urine specimens and most of them (90%; 9/10) were isolated from hosptalized

patients. Most vancomycin resistant *Enterococcus* (VRE) isolates were recovered from patients ≥50 yrs of age (Fisher exact two-tailed p value= 0.008).

3.2 Characterization of vancomycin resistant enterococci (VRE): Among the VRE isolates, 70% were *E. faecium*. All VRE isolates were multidrug resistant and high level Gentamicin resistance was observed in 80% of VRE isolates. Out of 10 VRE isolates, 6 (60%) showed VanA phenotype with teicoplanin MIC ≥ 16 ug/dl. Rest 4 (40%) showed VanB phenotype.

3.3 Detection of *vanA* gene in VRE isolates: Eight (80%) out of 10 VRE isolates showed presence of *vanA* gene (**Fig 1**). Of these 3 (37.5%) showed VanB phenotype while the rest showed resistance to both vancomycin and teicoplanin (VanA phenotype).

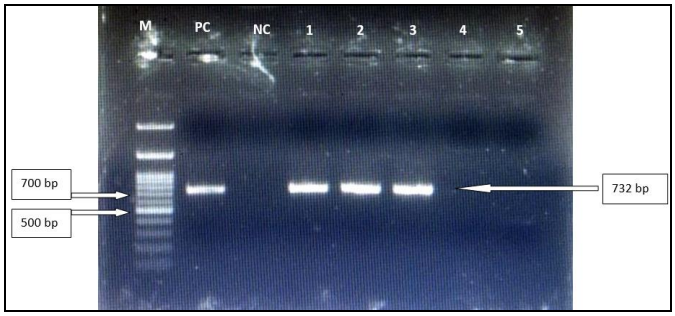

Fig 1: Gel electrophoresis of amplified products by PCR for *vanA* gene. Lane M-100 base pair DNA ladder, PC-positive control for *vanA* gene, NC-negative control for *vanA* gene, lanes 1-5-sample lanes, 1-3 positive for *vanA* gene (732 bp) and 4-5 negative for *vanA* gene (no band seen).

4. Discussion

In the present study, majority of Enterococcal isolates were obtained from urine specimen which is similar to other studies [10, 13, 14]. Most of the isolates were from hospitalized patients as in other studies [10, 13]. Majority of the isolates were found to be *E. faecium* (51.7%) as reported in few studies in India (53-70%) [15, 16]. However, most of the studies from various parts of India had shown *E. faecalis* as the predominant species (55-87%) [5, 17-19]. Resistance to penicillin was observed in maximum number (79.45%) of isolates. Many studies in India have reported resistance to penicillin in the range of 40-80 percent [20-22]. Ampicillin resistance observed in the study (67.9%) was higher as compared to many other studies [21-23] and lower as compared to study by Barman J *et al* [13]. All the isolates were sensitive to linezolid as in other studies [13, 23]. Resistance to nitrofurantoin was seen in 17% of urine isolates which higher than in the study by Barman J *et al* (15%) [13] but was lower as compared to study by Paharaj I *et al* [14]. Vancomycin resistance was observed in 6.6% of the isolates. Mathur P *et al* [5] from New Delhi were the first to report VRE from India in 1999. Studies done in North India have reported vancomycin resistance in 1-8.7% of isolates [5, 14, 17] whereas a study from North-East India [10] found Vancomycin resistance in 24% of isolates. Vancomycin resistance observed in the present study (6.6%) is higher compared to study by Barman J *et al* (1%) [13] (done in the same institute few years back) and few other studies [5,17] though lower than in the study by Phukan C *et al* [10] and studies from Indore (14.29%) [24] and Nagpur (11.38%) [25]. Most of the vancomycin resistant *Enterococcus* were isolated from hospitalized patients as in other studies [14]. VanA phenotype was observed in 60% of VRE isolates whereas in study by Paharaj I *et al* [14], VanA phenotype was seen in 87.5% of VRE isolates. The *vanA* gene was found in 80% of VRE isolates which is higher than in the study by Phukan C *et al* [10]. Paharaj I *et al* [14] found *vanA* gene in 96.87% of VRE isolates. Studies from Taiwan [26] and Brazil [27] reported *vanA* gene in all VRE isolates. VanB phenotype was seen in 3 (37.5%) VRE isolates with *vanA* gene. This type of heterogenous expression of teicoplanin resistance in *vanA* genotype has also been reported in parts of East Asia like China, Japan and South Korea [28]. The term VanB phenotype-*vanA* genotype VRE has been used for such strains by some authors [29]. *vanA* genotype strains showing susceptibility to teicoplanin have also been observed in study by Praharaj I *et al* [14].

5. Conclusion

Vancomycin resistance observed in higher number of isolates in the present study compared to previous study done in the same institute points towards emergence of VRE. This highlights the importance of strict enforcement of antibiotic policies with greater adherence to infection control measures to prevent spread of antibiotic resistant bacteria.

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