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VANCOMYCIN RESISTANT ENTEROCOCCAL INFECTION IN CLINICAL ISOLATES FROM KOLKATA WITH SPECIAL REFERENCE TO DISTRIBUTION OF *vanA* AND *vanB* GENES

Microbiology

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ABSTRACT

Vancomycin resistant enterococcai (VRE) is gradually becoming an important nosocomial infection with limited therapeutic options. This study was undertaken to find out the rate of VRE infection and distribution of *vanA* and *vanB* genes among them. Samples were collected from indoor and outdoor patients attending a tertiary care hospital. *Enterococcus* sp. was isolated and characterised by Disc Diffusion Test (DDT), VITEK 2 system, multiplex PCR and DNA sequencing. Enterococcal infection was detected in 3.37% samples. *Enterococcus faecalis* and *Enterococcus faecium* were the two predominant species. Vancomycin resistance was recorded among 13.46% of *E. faecalis* and 29.2% *E. faecium* and all were carrying *vanA* gene. Multiplex PCR was found to be more sensitive for detecting VRE than other two methods. Observed higher rate of VRE is quite alarming. Such study with a greater sample size in other health care set-up is highly suggestive to ascertain the prevalence of VRE in India.

KEYWORDS

Enterococcus, Vancomycin Resistance, *vanA, vanB*

INTRODUCTION

Enterococci are facultative anaerobic, Gram-positive commensals of healthy human intestine and may also colonise in skin, genitourinary tract, oral cavity which may persist from months to years without any clinical manifestations [1]. Asymptomatic carriers are the source of nosocomial infection in any health care facilities via shedding into the surrounding environment and onto health care workers. *Enterococci* are opportunistic environmental inhabitants with a remarkable adaptive capacity to evolve and transmit antimicrobial resistance [2]. Several species of *Enterococcus* have been identified, while *Enterococcus faecalis* and *Enterococcus faecium* are the two major species being strongly associated with most of the infections in human. It has been reported that *E. faecalis* accounts for 80- 90% of all clinical isolates, while *E. faecium* for only 5-15% worldwide [3]. *E. gallinarum, E. casseliflavus, E. durans, E. avium* and *E. raffinasus* are other *Enterococcus* species that are found less frequently (<5%) in clinical isolates [3]. *Enterococci* are important pathogen causing urinary tract infections and are sometimes responsible for infective endocarditis (IE), intra-abdominal abscess, bed sore, pelvic infections, and central nervous system (CNS) involvement [4]. IE and CNS involvement are the serious manifestations associated with enterococcal infection. β-lactam or glycopeptides (vancomycin) targeting cell wall along with an aminoglycoside with synergistic effect are the antimicrobial agents of choice for the treatment of severe enterococcal infections. The development and spread of resistance against these antibiotics are the major concern for the management of such cases.

Enterococci are intrinsically resistant to low levels of aminoglycoside due to decreased cellular permeability, which can be overcome by addition of a cell-wall-active agent like a β-lactam, which increases the entry of the aminoglycoside into the cell [5]. Glycopeptides (vancomycin, teicoplanin) are cell-wall-active agents that assist in inhibition of the synthesis of peptidoglycan by binding with high affinity to the D-Ala-D-Ala termini of pentapeptide precursors [6]. Binding of glycopeptides blocks transpeptide linkage of cell wall components, resulting in reduced integrity and, ultimately, cell death.

Resistance to glycopeptides in *Enterococcus sp.* is mediated through *Van* operon. The *Van* operon consists of a response regulator (*vanS-vanR*), D-lactate dehydrogenase gene (*vanH*), D-Ala-D-Ala dipeptidase gene (*vanX*) and a variable ligase in which 9 variant genes have been identified (*vanA, vanB, vanC, vanD, vanE, vanG, vanL, vanM,* and *vanN*). The variable ligase genes are the determining factor of the level of resistance against vancomycin [6, 7]. *Enterococcus sp.* carrying *vanA* are highly resistant to glycopeptides and are the dominant VRE variants of *E. faecium* and *E. faecalis* globally. The *vanA* / *vanB* gene and other genes (*vanR, vanS, vanH, vanX,* and *vanZ*) are located on a transposon which is located on a plasmid [8].

Diagnosis and detection of *Enterococcus sp.* is generally done by three major methods. In the routine laboratory method, *Enterococcus* allowed to grow in ordinary media like nutrient agar media, MacConkey's agar media showing tiny magenta coloured colonies. The organism is Gram positive cocci arranged in pair mostly or in short chains. They are catalase negative or weakly positive in some cases, grows in brain-heart infusion broth containing 6.5% NaCl and survived heating to 60°C for 30 min. Vancomycin resistant strain were identified by disc-diffusion method, using Muller Hinton agar medium. Criteria for inclusion into the genus *Enterococcus* are based on a combination of results of DNA–DNA re-association experiments, 16S rRNA gene sequencing, analysis of whole-cell protein profiles, and conventional phenotypic tests [9]. VITEK 2 system is another automated system which is designed to provide rapid and accurate identification and susceptibility testing results for most clinical isolates including *Enterococcus* species. Identification is made on the

basis of biochemical reactions, and MIC determinations are made by applying an algorithm to the growth kinetics monitored by the VITEK 2 system [10]. Molecular characterization by multiplex PCR of *vanA* and *vanB* genes in VRE strain accurately detect the genotype of the resistant strain because of amplification of the target genes.

Vancomycin resistant *Enterococcus* (VRE) was first reported from England in 1988. Thereafter, VRE was reported from United Kingdom and France [11, 12]. Gradually VRE spread in many other countries like, Australia, Belgium, Canada, Denmark, Germany, Italy, Malaysia, Netherlands, Spain and Sweden [13]. VRE have been reported from different parts of India [14, 15] but such data from West Bengal is very rare [16].

The study was designed to determine the prevalence of enterococcal infection among the patients attended OPD or admitted at Calcutta School of Tropical Medicine and characterization of isolated strains by three different methods.

MATERIALS AND METHODS

Enterococcal strains were isolated from the clinical samples received by the Microbiology department of Calcutta School of Tropical Medicine.

2.1. Conventional phenotypic method

The isolates were identified primarily up to genus level with the help of conventional phenotypic methods. On MacConkey agar enterococcus shows tiny lactose fermenting colony. On Gram staining, enterococci are paired Gram-positive cocci. Catalase test was performed by placing one drop of 30% H_2O_2 into a clean grease free glass slide followed by adding of small inoculum onto it. Absence of bubbles (nascent oxygen) indicates presence of *Enterococcus.* Enterococci are capable to grow in the presence of 4% bile and able to hydrolyze esculin to esculetin that differentiates enterococci and group D streptococci from non–group D *Streptococcus viridans.* One / two colonies from culture were inoculated into bile esculin agar and incubated at 37°C for overnight and the slant became black/ brown in colour. Following overnight incubation at 37°C in brain heart infusion broth containing 6.5% NaCl, growth of the organism indicate tolerance to high salt content.

2.2. Vancomycin susceptibility testing by disc diffusion method

Susceptibility to Vancomycin was carried out by Kirby–Bauer disc diffusion method (DDT) using vancomycin disc (30 µg, HiMedia) and the results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines. The broth culture was spread in a Muller Hinton agar plate, to obtain a semi confluent growth. Then 30 µg vancomycin disc was placed in it. After overnight incubation the plate was examined for the inhibitory zone surrounding the disc. Inhibitory zone size <14mm was considered as resistant and zone size >17 mm considered as sensitive for vancomycin. All the tests were performed comparing it with a standard strain, *Enterococcus faecalis* ATCC 29212 (vancomycin sensitive) and *Enterococcus faecalis* ATCC 51299 (vancomycin resistant).

2.3. Automated VITEK® 2 System for Species identification &minimal inhibitory concentration (MIC) determination

VITEK® 2 System, (bioMérieux, Inc., USA) was employed to identify the isolates up to species level. In brief, suspension was prepared from the growth of pure cultures of bacteria cultivated for 18 to 24 hour on nutrient agar. Suspensions were prepared in sterile saline (0.45% NaCl) to a turbidity equivalent to that of a 0.5 McFarland standard. Within 30 minutes these suspensions were used for the inoculation of both cards (ID-GP and AST-P628). The cards were manually placed in the VITEK 2 system's reader-incubator module (incubation temperature, 35.5°C). The results were interpreted by the ID-GPC database after an incubation period of 4 hours, and final results were obtained automatically after a minimum of 4 hour and a maximum of 15 hour of incubation.

2.4. Molecular diagnosis

Genomic DNA of *Enterococcus sp.* from the clinical isolates was extracted using "DNeasy Blood & Tissue Kit" (Qiagen, Hilden, Germany), following the manufacturer's instructions. Extracted DNA of all the samples was preserved at (-) 20°C and aliquot was used as the DNA source for further study.

2.5. PCR detection of vancomycin resistant (*vanA* and *vanB*) genes

Two vancomycin resistance associated genes, *vanA* and *vanB* were targeted by multiplex PCR using primers as described earlier [17, 18]. The used primers and PCR conditions are given in (**Table 1**). The PCR products were ascertained by 1.5 % agarose gel electrophoresis following ethidium bromide staining. The required amplicon size for *vanA* was 732 bp while that for *vanB* is 647 bp.

Table 1: Primer and multiplex PCR conditions used for amplification of *vanA* and *vanB* gene

Primer name	PCR Primers (5'-3')	PCR condition	Reference
Van A (+)	5'-GGGAAAACGACAATTGC-3'	Initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing 54°C for 30 sec, extension at 72°C for 45 sec, followed by further extension at 72°C for 7 min.	Dutka-Malen *et al*, 1995; Jayaratne and Rutherford, 1999 [17]
Van A (-)	5'-GTACAATGCGGCCGTTA-3'		
Van BF	5'-ATGGGAAGCCGATAGTC-3'		
Van BR	5'-GATTTCGTTCCTCGACC-3'		

2.6. DNA sequencing and Analysis of sequences

PCR product was gel purified using Qiagen gel extraction kit and sequenced using ABI sequencing platform in both the directions using forward and reverse primers. The sequences were analyzed using the software BioEdit Sequence Alignment Editor version 7.0.9.0. The sequences were then aligned using the online multiple sequence alignment tool ClustalW2 (http://www.ebi.ac.uk/clustalw) with reference sequence GenBank **KR047792.1** and **AB247327.1.**

2.7. Ethical aspects

The study protocol was approved by the Institutional Scientific Advisory Committee and Institutional Ethics Committee of the Calcutta School of Tropical Medicine.

3. Results

3.1. Prevalence of *Enterococcus sp.*

A total of 3049 clinical samples were collected during August 2017 - July 2018. *Enterococcus* was detected in 3.37% (103/3049) of the samples collected with 50.48%, 46.60%, 1.94%, 0.97% occurrence of *E. faecalis, E. faecium, E. gallinarum* and *E. durans* respectively among the positive samples. Occurrence of *Enterococcus sp.* was highest in urine sample (93, 4.94%) of which 48 (51.61%) were *E. faecalis* and 42 (45.16%) were *E. faecium*. Rest of specimens were blood, wound swab, pus and endotracheal tube with Enterococcal infection of 0.19%, 0.03%, 0.06% and 0.03% respectively (**Table 2**). Only *E. faecalis* and *E. faecium* were considered for this study. Identification of the genus *Enterococcus* was confirmed phenotypically by Catalase test, bile esculin test and growth of the isolate in brain-heart infusion broth containing 6.5% NaCl, and tolerance to temperature at 60 C for 30 minutes.

Table 2: Prevalence of *Enterococcus sp.* among clinical samples collected for diagnosis

Sample type	n	*E.faecalis*	*E. faecium*	*E. gallinarum*	*E. dunans*	*Enterococcus sp.*
Urine	1880	48 (51.61%)	42 (45.16%)	2 (2.15%)	1 (1.07%)	93 (4.94%)
Blood	633	2 (33.33%)	4 (66.66%)	0 (0%)	0 (0%)	6 (0.19%)
Endotracheal tube	48	0 (0%)	1 (100%)	0 (0%)	0 (0%)	1 (0.03%)
Wound swab	70	1 (100%)	0 (0%)	0 (0%)	0 (0%)	1 (0.03%)
Pus	90	1 (50%)	1 (50%)	0 (0%)	0 (0%)	2 (0.06%)
Total	**3049**	**52 (50.48%)**	**48 (46.60%**	**2 (1.94%)**	**1 (0.97%**	**103 (3.37%)**

3.2. Vancomycin resistant status as measured by three different methods

One hundred *Enterococcus* isolates were tested for vancomycin resistance following Kirby–Bauer disc diffusion test. The outcome of the test was determined by measuring the inhibitory zone size. Out of hundred, 10 isolates were diagnosed as vancomycin resistant with an inhibitory zone size of <14 mm and rest 90 isolates were diagnosed as sensitive to vancomycin with inhibitory zone size >17 mm.

By using automated VITEK 2 system (bioMérieux, Inc., USA) 85 isolates were detected as sensitive to vancomycin and 15 as vancomycin resistant. Species identification was also made by this system. Among 100 isolates 52 were diagnosed as *E. faecalis* and 48 *E. faecium*. The rate of vancomycin resistant *E. faecium* was 22.9% (11/48) and for *E. faecalis* 7.69% (4/52). The only vanB like resistance was noted in *E. faecium.* The MIC of sensitive strains were ≤ 0.5 whereas for resistant strains was 2- ≥32 (**Table 3)**.

Table 3: Vancomycin sensitivity pattern and molecular characterization of *Enterococcus sp.*

Enterococcus Sp.	Pheno type CULTURE	Geno type PCR& VITEK	N(%)	MIC [0.5-1]	MIC [1-2]	MIC [2->=32]
***E. faecium* (n=48)**	vancomycin sensitive	Without *vanA/vanB* gene	33 (68.75%)	28	5	0
	vancomycin sensitive	With *vanA* gene	4 (8.33%)	2	2	0
	vancomycin resistant	With *vanA*	10 (20.83%)	0	2	8
	vancomycin resistant	With *vanB*	1 (2.08%)	0	0	1
***E. faecalis* (n=52)**	vancomycin sensitive	Without *vanA/vanB* gene	45 (86.53%)	8	37	0
	vancomycin sensitive	With*vanA* gene	3 (5.76%)	1	2	0
	vancomycin resistant	With *vanA*	4 (7.69%)	0	4	0
	vancomycin resistant	With *vanB*	0 (0%)	0	0	0

3.3. Result of multiplex PCR and sequencing

By the multiplex PCR a band size of 732 bp corresponding to *vanA* gene was detected in 21% (21/100) isolates of which 14 in *E. faecium* (66.66, 14/21) and 7 (33.33%, 7/21) *E. faecalis* **(Table 4).** Interestingly no *vanB* gene was identified in any of the isolates. Nineteen out of 21 PCR positive isolates were sequenced successfully. It was observed that all sequences were perfectly aligned with reference sequences of *vanA* gene. The sequence *vanA* genes of *E. faecalis* and *E. faecium* have been submitted to GenBank under accession Nos.**MH837621** and **MH837622** respectively.

3.4. Comparison of three methods

In this study three diagnostic methods were used for detection of vancomycin resistance among 100 isolates of *Enterococcus sp.* Vancomycin resistance was detected in 22 isolates by at least any one of the diagnostic methods. By disc diffusion test, only 10% were resistant, while using VITEK, 15% was detected as resistant. By PCR, 21% were diagnosed as vancomycin resistant that was highest rate of resistance detected among these three methods.

Nine strains diagnosed as vancomycin resistant by all three methods whereas 7 strains showed vancomycin resistance by PCR only. Five strains were detected as vancomycin resistance by both VITEK and PCR method. It is evident that the sensitivity of PCR for detection of vancomycin resistance is significantly superior over VITEK (Z=1.9, p= 0.0302) and disc-diffusion (Z=2.6, p= 0.005) method. No such difference was noted between VITEK and disc-diffusion (Z= 0.9, p=0.209) method, though a higher rate of vancomycin resistance was observed by VITEK (15%) than disc-diffusion test (10%).

Table 4: Comparison Of Three Diagnostic Methods For Detection Of Vancomycin Resistant *Enterococcus Sp.*

Results of three diagnostic methods		INDIVIDUAL TEST METHOD								
		PCR(N=100)			VITEK(N=100)			DISC DIFFUSSION (N=100)		
		Van resistant	Van sensitive	Sensitivity	Van resistant	Van sensitive	Sensitivity	Van resistant	Van sensitive	Sensitivity
PCR (+) Culture (+) VITEK (+)	9	21	79	95.65%	15	85	75.86%	10	90	64.70%
PCR (+) Culture (-) VITEK (-)	7									
PCR (+) Culture (-) VITEK (+)	5									
PCR (-) Culture (+) VITEK (+)	1									
PCR (-) Culture (-) VITEK (-)	78									

DISCUSSION

In the last three decades, the emergence of VRE and their increasing prevalence worldwide has made it difficult to treat serious hospital acquired infections. The magnitude of the problem reported to be higher in western world, but an increasing trend has also been observed in India during last two decades [18].The treatment of bacterial infection depends upon proper diagnosis and antimicrobial susceptibility profile. Genetic information about the bacteria is also important for selection of any antimicrobial agent. There are several methods for identification, determination of antimicrobial resistance. In the present study, we used three methods for this purpose and compare the sensitivity of these methods for detection and molecular characterization of vancomycin resistance among two species of *Enterococcus sp*.

In the present study, we observed an overall 3.37% prevalence of enterococcal infection among 3049 different clinical isolates. *E. faecalis* (52%) and *E. faecium* (48%) were equally distributed. Similar rate of species distribution was reported from different parts of India [16, 19, 20]. In contrast, a higher rate of *E. faecium* or *E. faecalis* [18, 21] has been reported from this country.

We observed a higher rate of vancomycin resistance (22%) among the study isolates. This might be due to use of PCR method for characterization of all 100 isolates. The previous reports showed that most of the studies were designed differently. PCR was used for confirmation of results obtained by DDT or VITEK method [18, 21]. We found that 7 isolates were positive for *vanA* by PCR only. This might be the cause behind higher prevalence of VRE among clinical isolates. The rate of resistance among *E. faecium* (29.2%) was higher than in *E. faecalis* (13.46%). A lower level of resistance in both the species was reported from various parts of the country [18, 19].

Among three diagnostic methods, PCR showed highest levels of sensitivity for detection of VRE, followed by VITEK and DDT. By DDT we found only 10 isolates were resistant to vancomycin which increased to 15 by VITEK 2. VITEK 2 system detected one Van B like resistant with MIC value ≥32 that was negative for both *vanA* as well as *vanB* genes by PCR method. During each set of PCR, we used DNA of known *vanA* and *vanB* (ATCC strains) as positive control. So, VITEK has some limitations for detection of VRE. By PCR we were able to detect *vanA* gene in 21 isolates. It was again confirmed by analysing the DNA sequences of 19 such isolates.

A nucleic acid-based detection system provides a rapid and sensitive method for detection of the presence of resistance genes. The PCR method for detection of glycopeptides resistance was first described in 1995 [17]. Use of PCR-based systems for detection of VRE has increased significantly in clinical microbiology laboratories. Therefore, PCR has advantages over phenotypic methods by reducing the time required for detection of resistance and allowing timely implementation of infection control interventions.

As VRE infection are becoming a serious problem in respect of treatment, Multiplex PCR might be most suitable choice for diagnosis of VRE.

The limitation of the study were total number of isolates (N=100) were low and we detected species of enterococci by VITEK only. No attempt was made for speciation by species specific PCR. Beside this, seven isolates were positive for *vanA* genotypically, but they were phonetically sensitive to vancomycin. An elaborate future study with a larger number of samples is recommended for this purpose.

The observed higher rate of VRE among the clinical isolates is alarming to the health care provider of any hospital. Though VITEK is easier and rapid for diagnosis, but it has some limitations. Multiplex PCR might be used for diagnostic as well as genetic characterization of VRE. Such study with more number of samples in other health care set-up is highly suggestive to ascertain the prevalence of VRE in India.

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Conflict of Interest:
We have no conflicts of interest concerning the work reported in this article.

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