

Drug Resistance in Clinical Isolates of Enterococci with Special Reference to Vancomycin, from North India

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Over the last decades, infections due to multi-drug resistant enterococci have been increasingly reported worldwide. This study was done so as to determine the prevalence of enterococcal infections and various species, to detect drug resistance and epidemiological pattern of the resistant isolates. The study was conducted in a tertiary care hospital in North India. Isolates of enterococci were collected from various clinical specimens and speciated using extensive phenotypic and physiological tests. Antimicrobial susceptibility testing was performed by disc diffusion and agar screening method as per CLSI guidelines. RAPD typing was done to characterize the vancomycin resistant isolates. In our study we found *E. faecium* as the major isolated species from the specimens from enterococcal infections. The antimicrobial susceptibility testing depicted multi-drug resistant isolates. The RAPD typing of VRE revealed heterogeneous patterns without any dominant clone. Our study showed prevalence of multi-drug resistant enterococcal isolates, with notable resistance to ampicillin, fluoroquinolones and high level aminoglycosides. Epidemiological pattern of VRE revealed acquisition of resistance determinants independently.

Key Words: Clinical isolates, Enterococci, Vancomycin, North India.

Enterococci, generally regarded as normal flora of gastrointestinal and genitourinary tract of humans, have emerged as the etiogen of several nosocomial as well community acquired infections since last two decades. Globally, many studies have revealed that enterococci tend to be one of the leading causes of several nosocomial infections, with the emergence and spread of multi drug resistance among isolates¹. Enterococci are organisms with a remarkable ability to adapt to the

environment and acquire antibiotic resistance determinants. The evolution of antimicrobial resistance in these organisms poses enormous challenges for clinicians when faced with patients affected with severe infections. The increased prevalence and dissemination of multidrug-resistant *Enterococcus faecium* worldwide has resulted in a major decrease in therapeutic options because the majority of *E. faecium* isolates are now resistant to ampicillin and vancomycin, and exhibit high-level resistance to aminoglycosides, which are three of the traditionally most useful anti-enterococcal antibiotics². Clonal spread of vancomycin resistant enterococci (VRE) has been documented, but polyclonal outbreaks associated with antimicrobial use are also common³.

The present study was undertaken with the following objectives as to determine the prevalence of enterococci from clinical specimens

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in a tertiary care hospital in North India, to detect the antimicrobial resistance among enterococci and to document the epidemiological pattern of enterococci by molecular typing of the vancomycin resistant isolates.

MATERIALS & METHODS

The study was conducted in Sir Sunderlal Hospital, an 1150 bedded tertiary care hospital at Varanasi, North India, over a period of one year. Isolates of enterococci were collected during the study period January 2008 to January 2009, from various clinical specimens sent to the Microbiology laboratory such as blood, urine and exudates including wound swabs and pus, intravenous catheters, wound tissues, ascitic fluid, synovial fluid and other body fluids. The clinical specimens were plated on cystine lactose electrolyte deficient (CLED) agar, blood agar and Mac Conkey agar, as per nature of the specimen and incubated overnight at 37°C. The morphology of the colonies was observed and gram stain performed. The unknown gram positive cocci occurring in pairs and short chains were presumptively identified as enterococcus and subjected to bile aesculin hydrolysis and 6.5% salt tolerance test. Those that were positive for both above were subjected to a series of biochemical and physiological tests for species identification devised by Facklam and Collins as per standard procedures⁴.

All isolates identified as enterococci were tested for their antibiotic susceptibility pattern using standard procedures and interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines⁵. Antibiotic sensitivity testing was performed by Kirby Bauer disc diffusion method on Mueller Hinton agar. The

following discs (HiMedia, India) were used ampicillin (10µg), ciprofloxacin (5µg), high strength gentamicin (120µg), vancomycin (30µg), teicoplanin (30µg), linezolid (30µg), nitrofurantoin (300µg) for urinary isolates and pristinamycin (15µg) for *E.faecium* isolates.

The isolates were screened for high level gentamicin resistance (HLGR), high level streptomycin resistance (HLSR) and vancomycin resistance and interpreted using 500 µg/ml, 2000 µg/ml and 6 µg/ml of gentamicin, streptomycin and vancomycin respectively, incorporated in BHI agar for the screening method.

Random amplified polymorphic DNA (RAPD) typing was performed for molecular characterization of the vancomycin resistant isolates.⁶ The amplified products were electrophoresed by 1.2% agarose gel with ethidium bromide (Genei, India), at constant 60 volts for 60 min with Tris Acetate EDTA (TAE) buffer. The products were visualized under UV illumination and image saved using a multi Image Light Cabinet (Alpha Innotech Corporation, USA).

RESULTS

A total of 120 enterococci were isolated from various clinical samples submitted to the Microbiology laboratory, IMS, BHU. The two major species were 90 isolates of *E. faecium* (75%) and 27 *E. faecalis* (22.5%) contributing to 97.5% of all enterococcal species isolated. Remaining 2.5% of enterococci comprised of 2 *E. avium* (1.6%) and 1 *E. casseliflavus* (0.83%).

The overall prevalence of enterococci among various clinical specimens tested routinely during the study period was 0.69% as depicted in Table 1 and the 95% confidence interval (CI) was 0.56%-0.81%. This study showed that urinary

Table 1. Prevalence of enterococcal isolates among various clinical specimens

Specimen type	No. of specimen	No. of enterococci	Prevalence Rate %
Urine	11660	96	80
Blood	1284	10	8.3
Exudate	4208	14	11.7
Total	17152	120(0.69)	
95% confidence interval		0.56%-0.81%	

specimens had a higher prevalence rate of 80%, while blood and exudates specimens showed a prevalence rate of 8.3% and 11.7% respectively.

We investigated the prevalence of resistance among enterococci to various antibiotics as shown in Table 2. A very high resistance to ciprofloxacin (93.3%) was noted, while the urinary isolates exhibited still higher resistance of

95.83% (data not shown). Our results showed ampicillin resistance of 41.6%, with *E. faecium* showing greater resistance (59.25%) than *E. faecalis* (37.7%). The present study showed that 56.66% and 41.6% of all the enterococci tested were resistant to high level gentamicin and streptomycin respectively by agar screening method (Table 3).

Table 2. Antibiotic susceptibility pattern of *E. faecium* and *E. faecalis*

Antibiotic tested	Enterococci (120)No (%)	<i>E. faecium</i> (90)No (%)	<i>E. faecalis</i> (27)No (%)
ampicillin (10 µg)	50(41.6)	16(59.25)	34(37.7)
high strength gentamicin (120 µg)	59(49.16)	44(48.8)	15(55.5)
ciprofloxacin(5 µg)	112(93.3)	86(95.5)	26(96.3)
vancomycin (30 µg)	3(2.5)	2(2.2)	1 (3.7)
teicoplanin (30 µg)	2(1.6)	2(2.2)	0
linezolid (30 µg)	1(0.83)	1(1.1)	0
pristinamycin (15 µg)	56(62.2)	56(62.2)	NA
nitrofurantoin (for urinary isolates only) (300 µg)	17(17.7)	17(20.98)	0

Table 3. MIC testing results of enterococci by agar screening method

Enterococci (no of isolates)	Antibiotic tested	% Susceptible	% Resistant
Enterococci (120)	Vancomycin (6µg/ml)	95	5
	High strength gentamicin (500µg/ml)	43.33	56.66
	High strength streptomycin (2000µg/ml)	58.33	41.66

In our study, 5% of the isolates were resistant to vancomycin by the agar screening method as per CLSI guidelines.⁽⁵⁾ But our disc diffusion results showed only 2.5% of the enterococci as resistant to vancomycin. Disc diffusion testing results depicted that all the 120 isolates tested were highly susceptible to teicoplanin and linezolid. Susceptibility of the urinary isolates to nitrofurantoin was 82.29%. Our results also showed a notable resistance to pristinamycin (62.2%), amongst the *E. faecium* isolates by the disc diffusion method.

The VRE strains were typed by RAPD. The presence of bands of molecular weight 1.5kb, 1kb, 756p, 600bp, 510bp and 350bp were seen. Out of 6 total VRE strains, majority were of *E. faecium*

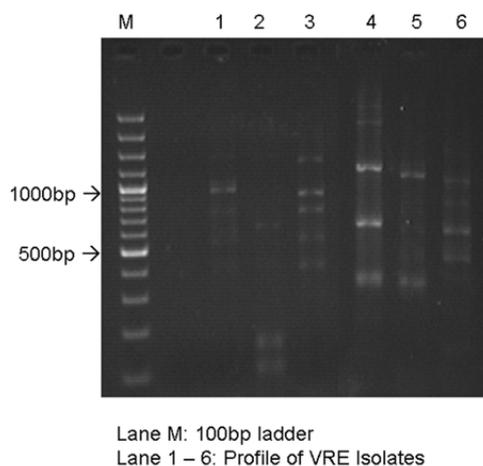


Fig. 1. RAPD Profile of the VRE Isolates

(5 out of 6 strains), 4 were isolated from patients attending outpatient departments. None of the isolates had identical RAPD pattern as shown in Fig. 1.

DISCUSSION

We found *E. faecium* was the major isolated species from clinical specimens. Historically, the ratio of infections due to *E. faecalis* to those due to all other *Enterococcus* species was approximately 10:1. In recent years, there has been a progressive decline in this ratio of enterococcal bacteremia. This microbiologic shift is likely to be explained in part by the emergence of VRE, in particular, the predominance of the species *E. faecium* among this subset of enterococcal isolates⁷. Likewise, few other Indian studies have also reported *E. faecium* as the more prevalent species^{8,9}. Remaining 2.5% of enterococci comprised of *E. avium* (1.6%) and *E. casseliflavus* (0.83%). Previously these isolates were infrequently isolated from India, though there has been a recent emergence of unusual species.⁽¹⁾ This study showed that urinary specimens had a higher prevalence rate of 80%. This is in concordance with several studies carried in different parts of the world which show that urinary tract remains the most common site of isolation for enterococci^{10,11}. Moreover, these organisms are the second most common cause of healthcare acquired urinary tract infections (UTIs)¹⁰.

We investigated the prevalence of resistance among enterococci to various antibiotics. A very high resistance to ciprofloxacin (93.3%) was noted. Comparable susceptibility reports have previously been reported in a study from New Delhi showing only 12% of *E. faecalis* susceptible to ciprofloxacin¹². Studies have shown an increase in the isolation rate of enterococci from urine samples in the hospital correlated with the mounting consumption of fluoroquinolones¹³. Recent exposure to beta lactamase inhibitors, extended spectrum cephalosporins, fluoroquinolones and clindamycin, were among the independent risk factors for fluoroquinolones resistance in UTIs caused by enterococci¹⁰.

Our results were concordant with many Indian studies depicting a gradual increase in the resistance rates of penicillin over the years¹⁴. From

the perspective of *E. faecium* antimicrobial resistance, there is an association between ampicillin and vancomycin resistance. Ampicillin-resistant *E. faecium* isolates are most often detected before vancomycin resistance is detected. Together, the genetic linkage in *E. faecium* between ampicillin, penicillin-binding protein, and vancomycin and clinical studies that have shown prior β -lactam use as a leading predisposing factor suggest that antimicrobial agents such as cephalosporins contribute to the emergence of vancomycin-resistant *E. faecium*. The linkage between a β -lactam resistant penicillin-binding protein and vancomycin resistance does not appear to have occurred yet in *E. faecalis*, which may account for the sporadic detection of vancomycin-resistant *E. faecalis*⁷.

All enterococci have intrinsic low-level resistance to aminoglycosides, with minimal inhibitory concentrations (MICs) ranging from 4 μ g/mL to as high as 256 μ g/mL. The MIC of gentamicin, the most commonly used aminoglycoside against enterococci, typically ranges from 6 to 48 μ g/mL. The facultative anaerobic metabolism of enterococci is thought to produce their low-level resistance to all aminoglycosides by limiting drug uptake, which is associated with the proteins involved in electron transport. The addition of an agent that interferes with cell wall synthesis, such as ampicillin (or vancomycin), markedly increases uptake of the aminoglycoside, greatly enhancing the killing of the enterococcus¹⁵. The present study showed that 56.66% and 41.6% of all the enterococci tested were resistant to high level gentamicin and streptomycin respectively by agar screening method. Our study is consistent with another recent study from India¹⁵, which showed a prevalence of 68% and 43% for HLGR and HLSR respectively, while 66% of high level aminoglycoside resistant (HLAR) isolates were detected in another study¹⁴. The presence of HLAR results in the loss of synergy between cell wall synthesis-inhibiting antibiotics (penicillins and glycopeptides) and aminoglycosides (gentamicin, tobramycin, netilmicin, kanamycin and amikacin), making the treatment of serious infections difficult¹⁷. Since gentamicin is the most widely used aminoglycoside (in combination with a cell wall active agent) for treatment of serious enterococcal infections, an HLAR screen for gentamicin is

usually sufficient. However, if an isolate demonstrates HLAR to gentamicin, screening for HLSR is needed so that streptomycin could be used therapeutically¹⁴. The detection of HLGR is a cause for concern, as it may signify the beginning of a major resistance problem. These isolates are significant because they are also multiply resistant to other antibiotics indicating the use of these antibiotics should be done after susceptibility testing¹⁷.

There has been a steady increase in vancomycin resistance among nosocomial isolates of enterococci worldwide. The prevalence of VRE varies among different countries and continents, which are governed by various factors including the use of glycopeptides in humans and in animals as growth promoters. Although vancomycin resistance among enterococci is quite divergent worldwide, our results were consistent with some studies reporting 8%, 5.5%, 23% VRE from New Delhi, Chandigarh and Mumbai respectively¹⁴. Although the prevalence of glycopeptide resistance was low among the studied isolates, their presence together with HLAR calls for regular surveillance of antibacterial susceptibilities to detect emerging resistance and prevent the establishment and spread of multiply resistant strains¹⁷. In this context, the emergence of vancomycin resistance among enterococci in India is a cause for concern in near future.

We found a notable resistance to pristinamycin (62.2%), amongst the *E. faecium* isolates by the disc diffusion method. Very few studies with pristinamycin have been done in India previously, which report good susceptibility of this compound (92.43%).⁽¹⁸⁾ Pristinamycin is a new streptogramin compound (Quinupristin/Dalfopristin ; w/w 30:70), used against multidrug resistant infections caused by gram positive cocci, including enterococci. Additionally these compounds are also used in animal feed as growth promoters to promote average daily weight gain. *E. faecalis* is resistant to pristinamycin in vitro. Pristinamycin is not a regularly used drug in our setup. Yet the considerable resistance noted could hint at the non human sources as reservoirs for resistant bacteria as well as some pathway of food borne transmission of resistant determinants. Definitely, such inferences require further investigations.

The VRE strains were characterized by RAPD typing. Out of 6 total VRE strains, majority was of *E. faecium* (5 strains), 4 were isolated from patients attending outpatient departments. RAPD typing revealed heterogeneous patterns with no dominant clone. This suggests that the strains acquired vancomycin resistance independently, possibly, by horizontal transfer of the vancomycin-resistance determinants. The absence of a dominant clone among the vancomycin resistant isolates is consistent with the fact that there has been no evidence of an outbreak of VRE in the hospital from where the isolates were obtained¹⁹.

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