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ANTIMICROBIAL RESISTANCE TRENDS WITH SPECIAL REFERENCE TO VANCOMYCIN RESISTANCE AMONG DIFFERENT SPECIES OF ENTEROCOCCI

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ABSTRACT

Enterococci present a therapeutic challenge because of their resistance to a vast array of antimicrobial drugs and the propensity of *Enterococci* to acquire resistance. Thousand clinical specimens were collected from two tertiary care hospitals in Kerala. *Enterococci* were isolated and identified from clinical specimens by conventional methods. Antibiotic resistance and MIC were detected. Vancomycin resistant genes were confirmed by PCR and broth mating was performed to detect resistance gene transfer. Of the isolates *E.faecalis* was the predominant species followed by *E.faecium*, *E.gallinarum*, *E.avium*, *E.mundtii* and *E.raffinosus* .Multidrug resistance was demonstrated by most of the isolates. Among the six vancomycin resistant *E.faecium* isolates, three were of vanA and three vanB phenotypes. VanB resistant genes were located on plasmid and were transferred to recipients through conjugation. The present study reveals the emergence of transferrable vancomycin resistance from this part of the country.

KEY WORDS: Enterococci, vancomycin resistant enterococci, antibiotics, conjugation.

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INTRODUCTION

Enterococci are an important part of the normal intestinal flora of humans and animals. Many surveys point that *Enterococci* remain the most common pathogen that cause nosocomial infection and serious community acquired infections. Though *E.faecalis* and *E.faecium* are responsible for majority of the infections, other *Enterococcus* species known to cause human infections include *E.avium, E.gallinarum, E.casseliflavus, E.durans, E.raffinosus* and *E.mundtii.* The common nosocomial infections produced by these organisms are urinary tract infections, intra abdominal and pelvic infections, surgical and other wound infections, bacteremia, endocarditis, neonatal sepsis and meningitis [1]. Their emergence in the past decades has occurred mostly due to their resistance to a wide variety of commonly used antibiotic agents rather than from their virulence factors [2]. *Enterococci* have intrinsic resistance to many antimicrobial agents and have developed resistance to the drugs used in clinical practice. They have even shown resistance to vancomycin, which is considered to be an ultimate resource drug against infection with multi resistant gram positive bacteria. Vancomycin resistant enterococci [VRE] have emerged as an important nosocomial pathogen and the speed with which vancomycin resistance has spread through hospitals is already causing a crisis. Acquired glycopeptide resistance in *Enterococci* is phenotypically and genotypically heterogenous. To date six gene clusters associated with glycopeptides have been identified in Enterococcus species. They are vanA, vanB, vanC, vanD, vanE and vanG[3]. *Enterococci* possess potent and unique abilities to exchange genetic materials among them and with other genera [4]. Conjugative transposons, pheromone responsive plasmids and broad host range plasmids play a major role in the acquisition and dissemination of drug resistance in enterococci. In *E.faecium,* drug resistance especially vancomycin resistance is encoded on a mobile genetic element or plasmid. Reports from most parts of the world regarding the antibiotic resistance have been increasing over the years, but reports of similar nature were less frequent from South India. We are witnessing a change in this trend, recently with more instances of resistance being reported from different parts of the country [5]. So in view of the emerging importance of enterococci a study was conducted to investigate the recent antibiotic resistance trends among enterococcal isolates from Kerala, a southern state in India, with special reference to vancomycin resistance and their transfer.

MATERIALS AND METHODS

(i) Isolation and identification of Enterococci

Enterococci were isolated from clinical specimens like urine, exudates and blood collected from two tertiary care hospitals in Kerala. Specimens were processed by routine methods and the enterococci were identified, studying the cultural, morphological and physiological and biochemical properties. The species level identification was done according to Facklam and Collins identification scheme [6].

(ii) Antibiotic Susceptibility test

Antibiotic susceptibility testing of the clinical isolates were performed on Muller Hinton Agar by disc diffusion method (For the antibiotics mentioned in Table 2) and interpreted as per NCCLS guidelines [7].

(Ш) Determination of MIC of antibiotics

The isolates were tested by the E-test (HiMedia) for detecting the MIC. HiComb MIC strips were applied on the inoculated agar

plates and incubated. The MIC values were detected from the concentration of antibiotic at which the zone intersects the test strip [8].

(i□) Beta lactamase test

Production of beta lactamase was determined by using cefinase discs (BD diagnostic systems) which were impregnated with chromogenic cephalosporin, nitrocefin. Colonies were smeared onto the surface of moistened disc and observed for colour change [9].

(□) Detection of vancomycin resistance determinants

Vancomycin resistance determinants, vanA and vanB were detected by using polymerase chain reaction (PCR). Van gene fragment from genomic DNA/plasmid was amplified by PCR using vancomycin specific primers:

Details of primers used for PCR:

Van A Forward primer GGGAAAACGACAATTGC
Van A Reverse primer GTACAATGCGGCCGTTA
Van B Forward primer ACCTACCCTGTCTTTGTGAA
Van B Reverse primer AATGTCTGCTGGAACGATA

PCR amplifications were performed in 200 µl reaction tubes each with 25 µl of mixtures composed the primers and the following reagents *TAQ Buffer 9,* 3 mM of magnesium fluoride, 0.25 mM of each dNTP, and 2 U of TAQ DNA polymerase and diluted DNA. The PCR tubes with all the components were transferred to thermal cycler. 30 cycles of amplification and final extension at 72°C for 5 minutes were carried out. The PCR products were subjected to electrophoresis through a 1.5% agarose gel stained with, ethidium bromide for 40 minutes at 60 V and photographed under UV light. and were added to 5 ml of Luria Bertani broth [11].The mixture was incubated at 37° C for 4 hours and the transconjugants were selected on agar plates containing 16µg/ml vancomycin and 20 µg/ml of rifampicin. The presence of trasconjugants was confirmed by determining the phenotypic properties and plasmid detection. The conjugation efficiency was determined based on the number of transconjugants per donor cells.

(□ i) Conjugation Experiments

E.faecium with van B plasmid was selected as donors based on their resistance to vancomycin and sensitivity to rifampicin. This vancomycin resistant rifampicin sensitive strain was mixed with *E.faecium* which was Van^s Rif^r. Broth mating was done as described by Ike *et al* [10] with a modification. 0.5 ml of donor culture were mixed with 0.5 ml of the recipient culture

RESULTS

Species distribution of enterococcal isolates from various specimens is given in Table 1. In the present study out of the 1000 clinical specimens tested 210 enterococcal isolates were obtained. The predominant species isolated was *E.faecalis* followed by *E.faecium, E.gallinarum, E.mundtii, E.avium and E.raffinosus*. The resistance to different antibiotics shown by the isolates of different enterococci is summarized in Table 2. Majority of the isolates have shown resistance to the tested antibiotics. Penicillin resistance was found to be high in the isolates. Six of the isolates were vancomycin resistant. Three of them were of vanA phenotype and three were of vanB. Linezolid resistance was not exhibited by the isolates. The minimum inhibitory concentrations of the selected antibiotics with respect to different *Enterococcus* species are shown in Table 2. None of the enterococcal isolates showed detectable β-lactamase production.

Table 1
Distribution of Enterococcus Species in different clinical specimens

Specimens	No. of specimen tested	No. & % of +ve specimen	No. & *percentage of isolates belonging to each species					
			E.avium	*E.faecalis*	*E.faecium*	*E.gallinarum*	*E.mundtii*	*E.raffinosus*
Blood	250	22(8.80)	-	14(63.63)	8(36.36)	-	-	-
Pus	300	38(12.67)	2(5.26)	26(68.42)	10(26.32)	0	0	0
Urine	450	150(33.33)	-	108(72.00)	34(22.67)	4(2.67)	2(1.33)	2(1.33)
Total	1000	210(21)	2(0.95)	148(70.48)	52(24.76)	4(1.90)	2(0.95)	2(0.95)

Percentage is represented in the brackets

As represented in Table 3, there was no significant difference among the enterococcal species regarding the MIC of antibiotics towards penicillin and ampicillin, but significant difference was observed in the MIC towards vancomycin and gentamicin.

Table 2
Antibiotic resistance in isolates of enterococci

Antibiotics §	*E.faecalis*	%†	*E.faecium*	%†	Others	%†
Penicillin	120	(81.0)	38	(73.0)	3	(60.0)
Ampicillin	20	(13.5)	14	(26.9)	3	(60.0)
Gentamicin	104	(70.2)	34	(65.3)	0	(0.00)
Streptomycin	88	(59.4)	18	(34.6)	1	(20.0)
Gentamicin 120	60	(40.5)	32	(61.5)	0	(0.00)
Amoxyclav	20	(13.5)	14	(26.9)	2	(40.0)
Tetracyclin	54	(36.4)	14	(26.9)	2	(40.0)
Erithromycin	98	(66.2)	28	(53.85)	0	(0.00)
Ciprofloxacin	60	(40.5)	18	(34.6)	1	(20.0)
Chloramphenicol	8	(5.41)	6	(11.5)	0	(0.00)
Nitrofurantoin	10	(6.76)	10	(19.2)	0	(0.00)
Clindamycin	148	(100)	52	(100.)	5	(100.)
Vancomycin	0	(0.0)	6	(11.5)	0	(0.00)
Teicoplanin	0	(0.0)	3	(5.7)	0	(0.00)

† % is given in brackets and is represented based on the total number of isolates tested
§ Antibiotics in µg/ml, Total number of E.faecalis isolates tested was 148, Total number of E.faecium isolates tested was 52, Total number of other isolates tested was 5.

The vanA and vanB genes were detected by PCR in the two vancomycin resistant isolates tested. MIC of the vancomycin and teicoplanin towards one group of the resistant strains was 128µg/ml and 64µg/ml respectively indicating that their phenotype was vanA. MIC of vancomycin and teicoplanin shown by other strains were 16-32µg/ml and 0.05µg/ml respectively and they belonged to vanB phenotype.

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Table 3
Minimum Inhibitory Concentration

MIC range in µg/ml shown by the isolates of different enterococcal species			
Antibiotics	***E.faecalis* (30) ‡**	***E.faecium* (20) ‡**	**Others¶ (5) ‡**
Penicillin	8 to 16	8 to 16	8 to 16
Streptomycin	0.1 to >240	0.1 to >240	0.1-32
Teicoplanin	.01 to 0.05	0.05 to 64	0.01 to 4
Vancomycin	0.5 to 2	0.5 to 128	0.5 to 8
Gentamicin	0.128 - >1024	0.512 - >1024	0.128 - .512
Ampicillin	0.5 – 32	0.5 – 32	0.5 – 32

These results were confirmed by PCR analysis. One isolate corresponding to the vanA phenotype generated amplification products with vanA specific primers. And an isolate with vanB phenotype produced amplification products with vanB specific primers.

Figure A **Figure B**

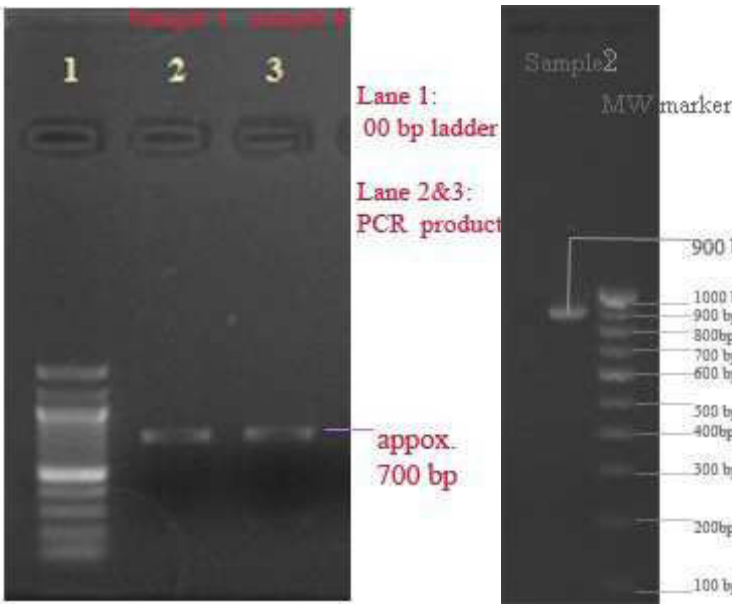

Figure A represents the VRE harboring vanB genes, amplified with the primer. Lane 2 and lane 3 are the samples 1 and 4 respectively with their corresponding PCR product. Figure B represents the vanA gene product.

Mating experiments produced vancomycin resistant transconjugants. Transconjugants showed same MIC as that of the donor *E. faecium* and plasmids were recovered from the transconjugant. The efficiency of conjugation ranged from 10^{-6} to 10^{-7} per donor cell.

DISCUSSION

In this study *E.feacalis* was found to be the most encountered species, followed by *E.faecium, E.gallinarum, E.avium, E.raffinosus* and *E.mundtii*. The percentage of enterococcal isolates was more from urine, followed by pus samples from surgical, burn, and diabetic wounds. The prevalence of various Enterococcus species in clinical specimen observed in this study was similar to that observed in an earlier study conducted in India. In those studies the presence of *E.hirae* and *E.casseliflavus* have also been reported in addition to the above mentioned species [12].*E.faecium* was isolated from 25% of infections and had a high isolation rate from blood samples. These findings, reiterate the significance of the observations, where in *E.faecium* is coming up as an important pathogen. The resistance percentage against penicillin observed in our study was consistent with the findings reported across India and other countries. One finding with a different trend is that of ampicillin resistance. Though ampicillin is the drug of choice for treating enterococcal infections, most studies conducted across India and other parts of the world have reported high ampicillin resistance percentage [13]. But in our study incidence of ampicillin resistance among the isolates of *E.faecalis* and *E.faecium* were much lower. Though a high percentage of the isolates showed antibiotic resistance to penicillin, none expressed detectable beta-lactamase activity when tested with cefinase disks. It is quite possible that the penicillin resistance observed could be due to the altered penicillin binding protein. Many studies conducted in India have reported beta lactamase activity, contrary to our observation [14]. In the present study the enterococcal strains tested showed resistance to amoxyclav. These observations strengthen the reports of other studies from India [15] in which amoxyclav resistance was found to be present, though studies from West Indies and Iran have reported 100% sensitivity for amoxyclav[16.] Aminoglycosides are used because of its synergistic effect with cell wall synthesis inhibitors like penicillin or vancomycin. So aminoglycoside resistance is of great concern in the treatment. In the present study aminoglycoside resistance, especially high level gentamicin resistance (HLGR) was observed as in earlier report [17].

Resistance towards glycopeptides, like vancomycin and teicoplanin was significantly low and was shown only by the isolates of *E.faecium*. Although in many studies conducted in other countries high percentage of glycopeptides resistance was reported, [18,19] the result of this study was in accordance with the situation in northern India where the incidence was low [20,21]. Nevertheless, vancomycin sensitivity was still being reported from some areas[22]. Although nitrofurantoins are used for treating urinary tract infections, many of the isolates in the present study were found to be resistant. None of the isolates in the present study was resistant to linezolid. However linezolid resistance was reported in a study[23]. Vancomycin resistance of vanB type was encoded on plasmids in this study. And this resistant plasmid transfer was achieved by enterococcal broth mating methods. The results showed that vancomycin genes were transferred to recipient *E.faecium*. Vancomycin resistant Enterococci have the ability to spread and cause hospital outbreaks [24]. This study clearly illustrates that vancomycin resistant plasmids of vanB resistance could be transferred by conjugation to other enterococcal strains and gram positive bacteria as documented in previous reports [25]. As these results are alarming and distressing, the discriminated use of above drugs is warranted.

CONCLUSION

This study reveals the emergence of VRE from this part of the country. The van B genes located on the plasmids were transferred to plasmid free enterococci. So this study

suggests its possibility to transfer resistance to other gram positive bacteria. So VRE could be spread with unanticipated rapidity which can be a serious medical problem. This study warrants the implementation of stringent infection control measures and strict adherence to the same. The laboratories should provide accurate antimicrobial resistance patterns so that, effective therapy can be initiated.

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