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Original Article

Antibiogram and genetic relatedness of clinical isolates of *Enterococcus* spp. in Mangalore, India

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Abstract

Introduction: Drug-resistant *Enterococcus* species is a persisting clinical problem and may serve as a reservoir of resistant genes. The present study was undertaken in Mangalore, India to know the antibiogram and genetic relatedness of *Enterococcus* spp. isolated from clinical samples.
Methodology: A total of 150 non–repetitive *Enterococcus* spp. isolated from clinical samples were subjected to antimicrobial susceptibility testing by Kirby Bauer disk diffusion method. Molecular typing of the isolates was done by Random amplification of polymorphic DNA (RAPD).
Results: Among the 150 isolates, 79 were from urine, 68 from pus and three from blood samples. Of this 58.7 % were *E. faecalis* and the remaining were *E. faecium*. Urinary isolates of *E. faecium* showed a higher percentage of antibiotic resistance when compared to *E. faecium* isolates from pus ($p < 0.001$). *E. faecium* from blood samples were resistant to ampicillin, penicillin, ciprofloxacin and were sensitive to vancomycin and teicoplanin. *E. faecalis* blood isolates were resistant to ciprofloxacin, penicillin, and erythromycin. 73% of *Enterococcus* isolates from pus were resistant to erythromycin. All the *Enterococcus* spp. were sensitive to vancomycin. Among the total *Enterococcus* isolates 44 were high-level aminoglycoside resistant (HLAR) by disc diffusion method which corresponded to MIC of > 500 µg/mL for gentamicin and > 1000 µg/mL for streptomycin. These isolates were subjected to RAPD, which showed similarity and differences in the banding patterns.
Conclusions: Our study showed a baseline resistance among *Enterococcus* spp. in our area, which poses a challenge to the treating physicians and a reservoir for transmission of antibiotic resistant genes.

Key words: *Enterococcus*; multidrug resistance; RAPD; HLAR; molecular typing.

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Introduction

Enterococci, being part of commensal flora, are increasingly recognized as a cause of nosocomial infections, due to their inherited and acquired resistance to several antimicrobial agents [1]. *E. faecalis* and *E. faecium* are responsible for 80–90% of human enterococcal infections [2]. Most frequent infections caused by enterococci include urinary tract infections (UTIs) followed by intra-abdominal and intra-pelvic abscesses, post-surgery wound infections and bloodstream infections [3]. Epidemiological data suggest enterococci as an essential reservoir for the transmission of antibiotic resistance genes among different species of bacteria [4].

Enterococci show two types of resistance to aminoglycosides. A low to moderate level intrinsic resistance (MIC 60-500µg/mL) which is due to low permeability and low uptake of the drug which can be overcome by using a cell wall active drug like β-lactams as they increase the uptake of aminoglycosides by altering the cell wall. The other type of resistance is a high-level aminoglycoside resistance (HLAR) with MIC of > 500 µg/mL for gentamicin and > 1000 µg/mL for streptomycin. This resistance is either ribosomally mediated or due to the aminoglycoside inactivating enzymes [5]. Gentamicin resistance is associated with a bifunctional enzyme, 2'-phosphotransferase 6'-acetyltransferase, and high-level streptomycin resistance is due to the enzyme streptomycin adenyltransferase. Hence, resistance to high-level gentamicin does not indicate resistance to high-level streptomycin and vice versa [6].

Further, the emergence of enterococci with β-lactam resistance, HLAR, glycopeptide resistance pose a therapeutic challenge to physicians as they easily acquire and transfer drug resistance. [7,8]. As per CLSI

guidelines severe enterococcal systemic infections can be treated with a combination of penicillin and aminoglycosides if the isolate is susceptible to high-level aminoglycosides and exhibits low-level resistance to penicillins (MIC16-64) or ampicillin (MIC16-32) [9]. Several Indian studies report the rate of HLAR to be varying from 15 to 80% [10-13] because of which combination therapy of ampicillin and gentamycin could not be used for severe systemic infections.

Thus the occurrence of HLAR and vancomycin resistance enterococci (VRE) is a persisting clinical problem leaving the clinicians with very few drugs for treatment. Moreover, these drug-resistant strains may disseminate within the healthcare facility and warrants continued surveillance. Since there are no available data on the drug resistance and molecular typing of clinical enterococcal isolates in this part of the country, the present study was undertaken in Mangalore, India to know antibiogram and genetic relatedness of *Enterococcus* spp. isolated from different clinical samples.

Methodology

Isolation and Identification of Enterococcus spp.

Clinical samples like blood, urine, and pus, (n = 150) received for routine culture at the Department of Microbiology, Kasturba Medical College (KMC) Mangalore, from June 2015 to December 2015 was included in the study. All the media, antibiotic discs and chemicals used in the study was procured from Hi-Media Laboratories Pvt Ltd. Mumbai, India. Gram-positive cocci isolated from these clinical samples were identified by colony characteristics and common biochemical reactions [14].

Antibiotic susceptibility test

Antimicrobial susceptibility to ampicillin, penicillin, erythromycin, vancomycin, teicoplanin, ciprofloxacin, nitrofurantoin, High-level gentamicin (HLG), and High-level streptomycin (HLS), was determined by Kirby Bauer disk diffusion and interpreted as per CLSI guidelines [9,15]. *E. faecalis* ATCC 29212 was used as quality control strains.

High-level aminoglycoside resistance

Detection of HLAR was performed by disk diffusion method using gentamicin (120 µg) and streptomycin (300 µg). Results were read after incubation at 35^0C for 24 hours for gentamycin, and after 48 hours for streptomycin. A zone diameter of 6 mm indicates resistance, 7-9 mm shows the results are inconclusive, and more than 10 mm suggests that the isolates are sensitive to aminoglycosides [9,16]. Resistance by disc diffusion to gentamicin corresponds to MIC of >500 µg/mL, and susceptible corresponds to MIC of < 500 µg/mL. However, for high-level streptomycin MIC of >1000 µg/mL by broth dilution and >2000 µg/mL by agar dilution method corresponds to a zone diameter of 6 mm by disk diffusion. MIC of ≤ 500 µg/mL by broth and ≤ 1000 µg/mL by agar dilution corresponds to 10 mm diameter by disk diffusion method [16].

Molecular typing of the Enterococcus isolates

Molecular typing of the enterococcal isolates was done as per the protocol of Ratnayake *et al*. [17] *E. faecalis* and *E. faecium* isolates were grown on Brain heart infusion (BHI) agar plates for 24 hours at 37°C to obtain isolated colonies. Five colonies were emulsified in 100 µL of PCR grade water heated for 15 minutes in a dry bath at 100°C, centrifuged and one µL of the supernatant was used as DNA for polymerase chain reaction (PCR).

Amplifications were performed in 25 µL reaction mixture consisting of genomic DNA (96 ng/reaction); 1X reaction buffer; 100 mM each of dATP, dCTP, dGTP, and dTTP; 0.2 mM random primer; 2.5 mM $MgCl_2$ and 1U of Taq polymerase (Hi-Media Laboratories Pvt Ltd. Mumbai, India). A single random primer (5'CCGCAGCCAA3') was used in the reaction. PCR reaction was carried for 35 cycles. The reaction conditions were: initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 1 minute, annealing at 36°C for 1 minute and extension at 72°C for 2 minutes and the final extension at 72°C for 10 minutes.

The amplified product was resolved by agarose gel electrophoresis using 2% agarose in 1X TAE buffer containing 0.5 mg/mL ethidium bromide. Gels were visualized under UV transilluminator, and gel pictures were photographed. Banding patterns were analyzed, and a dendrogram was constructed by using the software GelJ V.2.0. (Jonathan Heras, University of La Rioja, Spain).

Results

In our study, a total of 150 enterococcal isolates were obtained from clinical samples, like urine, blood, and pus (Table 1). Among the urinary isolates, 80% of *E. faecium* (n = 28) strains were resistant to ampicillin and penicillin each (Table 2).

Among the *E. faecalis* and *E. faecium* isolated from pus, 73% were resistant to erythromycin (Table 3).

Table 1. Frequency and distribution of the *Enterococcus* spp. in various clinical samples.

Sample (n = 150)	*E. faecalis* (n = 88)	*E. faecium* (n = 62)	Total (%)
Urine (n = 79)	44	35	52.7
Pus (68)	42	26	45.3
Blood (3)	2	1	2.0
Total (%)	**58.7**	**41.3**	**100**

Table 2. Antibiogram of Enterococcal isolates from urine samples.

Antibiotics tested	*E. faecalis* (n = 44)			*E. faecium* (n = 35)		
	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Ampicillin	26(59.1)	2(4.5)	16(36.4)	7(20.0)	0(0)	28(80.0)
Penicillin	26(59.1)	0(0)	18(40.9)	7(20.0)	0(0)	28(80.0)
Nitrofurantoin	40(90.9)	0(0)	4(9.1)	26(74.3)	2(5.7)	7(20.0)
High level Gentamicin	26(59.1)	0(0)	18(40.9)	8(22.9)	0(0)	27(77.1)
High level Streptomycin	28(63.6)	0(0)	16(36.4)	14(40.0)	0(0)	21(60.0)
Teicoplanin	43(97.7)	1(2.3)	0(0)	34(97.1)	0(0)	1(2.9)
Vancomycin	44(100)	0(0)	0(0)	35(100)	0(0)	0(0)

S: Sensitive; I: Intermediate; R: Resistant.

Table 3. Antibiogram of Enterococcus isolates from pus.

Antibiotic tested	*E. faecalis* (n = 42)			*E. faecium* (n = 26)		
	S(%)	I(%)	R(%)	S(%)	I(%)	R(%)
Ampicillin	35(83.3)	0(0)	7(16.7)	22(84.6)	0(0)	4(15.4)
Penicillin	26(61.9)	0(0)	16(38.1)	19(73.1)	0(0)	7(26.9)
Ciprofloxacin	9(21.4)	3(7.1)	30(71.4)	11(42.3)	0(0)	15(57.7)
Erythromycin	7(16.6)	4(9.5)	31(73.8)	6(23.1)	1(3.8)	19(73.1)
High level Gentamicin	32(76.2)	0(0)	10(23.8)	23(88.5)	0(0)	3(11.5)
High level Streptomycin	30(71.4)	1(2.4)	11(26.2)	23(88.5)	0(0)	3(11.5)
Teicoplanin	41(97.6)	0(0)	1(2.4)	25(96.2)	0(0)	1(3.8)
Vancomycin	42(100)	0(0)	0(0)	26(100)	0(0)	0(0)

S: Sensitive; I: Intermediate; R: Resistant.

Table 4. Comparison of Antibiotic resistance of *E. faecalis* & *E. faecium* from urine & pus samples.

Antibiotics tested	*E. faecalis* (n = 44) Urine	*E. faecalis* (n = 42) Pus	Chi square test	*E. faecium* (n = 35) Urine	*E. faecium* (n = 26) Pus	Chi square test
	R (%)	R (%)	*p* value	R (%)	R (%)	*p* value
Ampicillin	16(36.4)	7(16.7)	*0.033*	28(80.0)	4(15.4)	*≤ 0.001*
Penicillin	18(40.9)	16(38.1)	*0.79*	28(80.0)	7(26.9)	*≤ 0.001*
High level gentamicin	18(40.9)	10(23.8)	*0.091*	27(77.1)	3(11.5)	*≤ 0.001*
High level streptomycin	16(36.4)	11(26.2)	*0.377*	21(60.0)	3(11.5)	*≤ 0.001*
Teicoplanin	0(0)	1(2.4)	*0.367*	1(2.9)	1(3.8)	*≤ 0.001*

S: Sensitive; I: Intermediate; R: Resistant; P value ≤ 0.001 is significant.

Resistance pattern of *E. faecalis* form urine and pus did not differ much, and the difference was not statistically significant ($p > 0.001$). On the contrary *E. faecium* isolates from urine showed significantly higher resistance rate ($p < 0.001$) to ampicillin, penicillin, teicoplanin, HLG, and HLS when compared to *E. faecium* isolates from pus (Table 4).

Out of the three blood culture isolates, two strains were *E. faecalis,* and one was *E. faecium*. One of the *E. faecalis* isolates showed resistance to penicillin, erythromycin, ciprofloxacin, and HLS, whereas it was sensitive to the other antibiotics tested. The *E. faecium* isolate from blood sample was resistant to all the antibiotics except vancomycin and teicoplanin.

Among the 150 isolates, 44 showed resistance to both HLG and HLS. These HLAR isolates included 13 *E. faecalis* from urine and eight from pus, and 21 *E. faecium* from urine and one each from pus and blood. All the 150 isolates were found to be sensitive to vancomycin.

In the present study, all the 44 HLAR enterococcus isolates were typed by random amplification of polymorphic DNA (RAPD) using a random primer (5'CCGCAGCCAA3'). Percentages of similarity were determined using the Jaccard coefficient and

Figure 1. UPGMA-based dendrogram generated from RAPD-PCR profiles of high level aminoglycoside resistant *Enterococcus faecium* isolates from clinical samples.

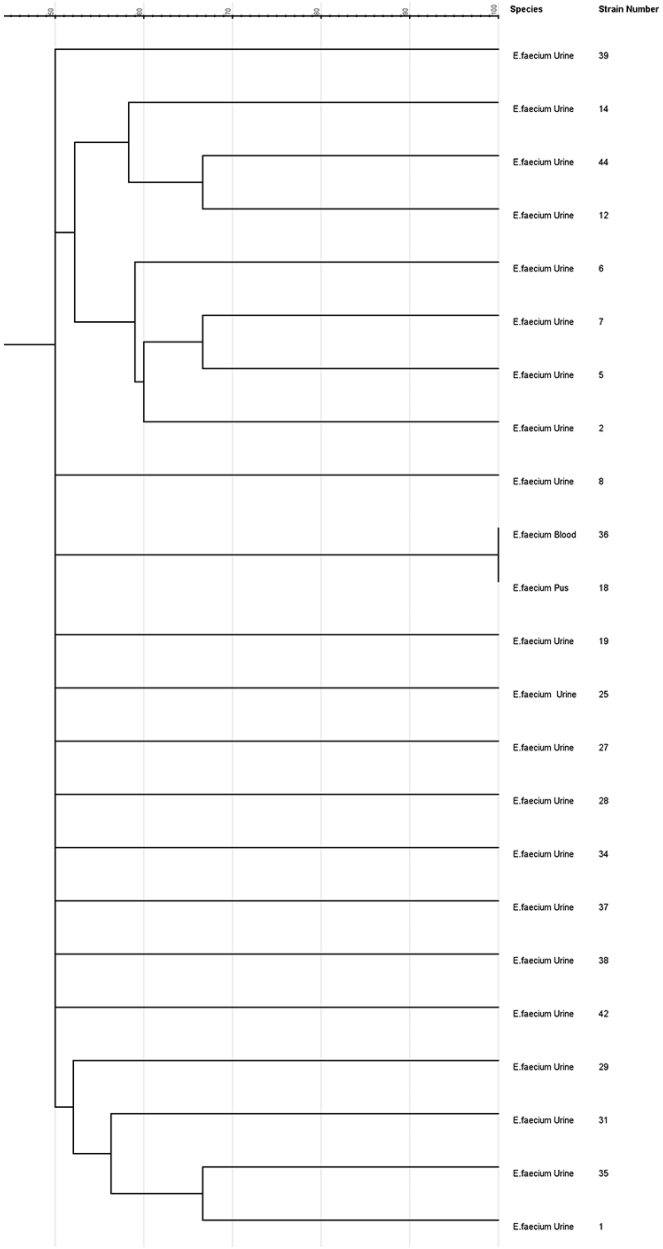
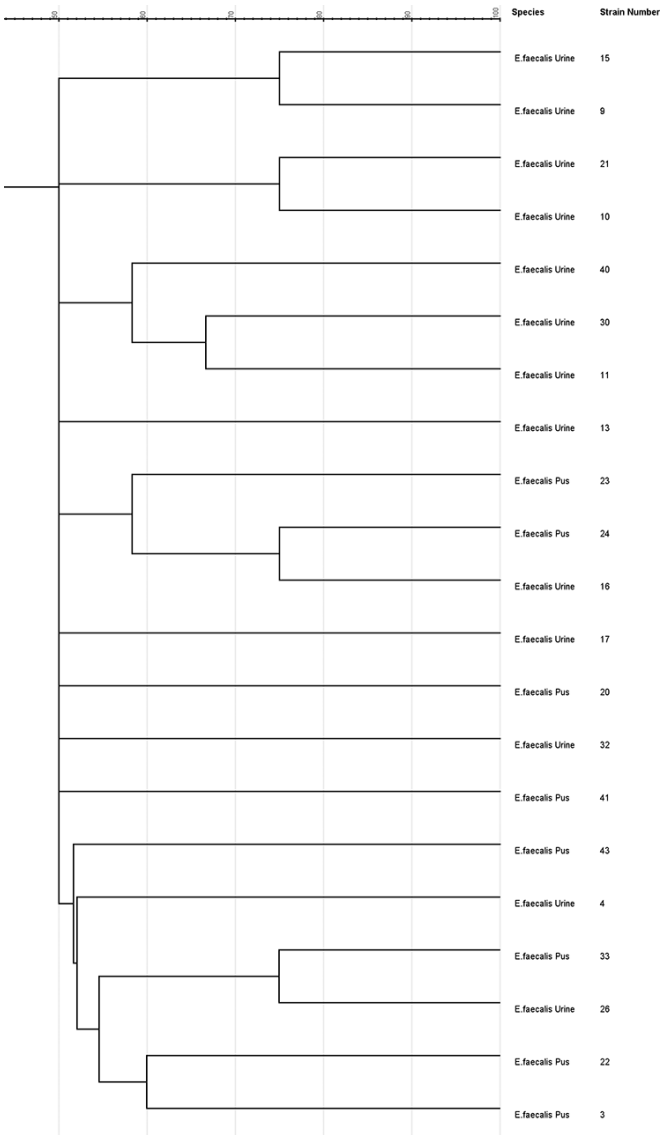


Figure 2. UPGMA-based dendrogram generated from RAPD-PCR profiles of high level aminoglycoside resistant *Enterococcus faecalis* isolates from clinical samples.



dendrograms (Figure 1 and 2) were constructed via the unweighted pair group method with arithmetic mean clustering (UPGMA). Numerical analysis of RAPD profiles of 23 HLAR *E. faecium* strains revealed four clusters, one at 100% and other three at 68% similarity levels. *E. faecium* isolates from blood and pus samples showed 100% similarities, and the urinary isolates showed similarities below 90%. Among the 21 HLAR *E. faecalis* subjected to RAPD typing, 13strains were from urine and eight strains from pus. None of the isolates were 100% similar. But most had similarities around 75%.

Discussion

In the present study isolation rate of *E. faecalis* (58.7%) was more than that of *E. faecium* (41.3%) which is in line with other studies from India [10,18,19]. In a study from Mangalore in 2013, out of the 150 isolates, 56% was *E. faecalis*, 34% was found to be *E. faecium*, and the remaining 10% were other species [13]. The results indicate no change in isolation rate of *E. faecalis* and *E. faecium* over the last three years. In our study, out of the 150 isolates, maximum isolates were from urine (52.7%), followed by pus (45.3%), and the remaining were from blood (2%) samples. This result was found to be similar to the studies done in Mangalore and Pune [13,19] but was different from a study done in Bangalore where the pus isolates were more [18]. So the distribution of enterococcus varies from place to place and also between the institutions.

In a study done in northern India using 46 *E. faecium* urinary isolates, 60.86%, were resistant to ampicillin, 13.1% to nitrofurantoin, 56.52% to HLG, 43.47% to HLS and 4.3% each to vancomycin and teicoplanin [20]. But *E. faecium* urinary isolates from our study showed comparatively more resistance to all the antibiotics (Table 2) except for vancomycin (0%) and teicoplanin (2.9%). Resistance shown by the blood isolates in our study when compared with previous studies done in India was found to be low [10,11]. This is because we could get only three *Enterococcus* spp from blood samples during the study period.

According to the study done in the pediatric hospital, New Delhi, both the Enterococcus species showed 100% resistance to penicillin, whereas in our study highest resistance was seen for erythromycin among isolates from pus, although the resistance to erythromycin in both studies was comparable [11]. This is because Enterococci are intrinsically resistant to macrolides, lincosamides and streptogramin B (MLSB phenotype). Cross-resistance with all macrolides arises from modification of the 23S rRNA target (except linezolid resistance) by a variety of methylase genes, commonly *ermB*. Hence Macrolides and lincosamides are not used to treat enterococcal infections even if *E. faecalis* and *E. faecium* are susceptible to quinupristin-dalfopristin in vitro. Moreover, *E. faecalis* possess a chromosomal gene named *lsa* (lincosamide streptogramin A resistance), and hence intrinsically resistant, whereas *E. faecium* is not. Also, a mutation in the *eatA* (*Enterococcus* ABC transporter) gene was shown to confer resistance to susceptible *E. faecium* strains [21]. There are no previous Indian studies to compare with the antimicrobial resistance demonstrated by our enterococcal isolates from pus samples.

In the present study 21 out of 88 *E. faecalis* (23.86 %) and 23 out of 62 *E. faecium*, (37.09 %) were found to be HLAR and all were sensitive to vancomycin. This result is different from a study conducted in a Paediatric care hospital in India, where 5% of *E. faecalis* and 12% *E. faecium* showed HLAR and all were susceptible to vancomycin [11]. Higher resistance to high-level aminoglycoside in the present study is a cause for concern as vancomycin is the only drug of choice for such clinical isolates and soon these strains may develop resistance to vancomycin. However, our study also showed 100% sensitivity to vancomycin.

RAPD typing of HLAR *E. faecium* (n = 21) isolated from urine showed three clusters with 68% similarities. Since the similarity is below 90%, they were considered genetically unrelated. *E. faecium* isolates from blood and pus are regarded as genetically related as they showed 100% similarities, which indicate their evolution from a single clone, though isolated from different patients. A genetic typing study from North India reported two primary clustering with 100% similarity among urinary *E.faecium* isolates which were HLAR and MDR. [20]. However, all our HLAR isolates were sensitive to vancomycin.

None of our 21 HLAR *E. faecalis* isolates showed 100% similarities in RAPD profiles. Strains 15, 9, 21, 10, 16, 26 from urine and 24 and 33 isolated from pus showed only 75% similarity. Rest of the strains of *E.faecalis* were genetically diverse with similarities below 75%. To the best of our knowledge, there are no previous reports of Indian studies to compare RAPD pattern of our *E. faecalis* isolates. Studies from Lebanon and Saudi Arabia have reported RAPD typing of faecal, urinary and endodontic Enterococcal isolates and proved that RAPD is a tool to study genetic diversity or similarities [22,23]. However, to better understand the clonal relationship of the strains, highly discriminating molecular typing methods needs to be used. Molecular

typing of Enterococcal isolates from patient samples and hospital environment will help to trace the source of infection, which in turn helps to control spread of drug resistance and nosocomial infections.

Conclusion

A high incidence of HLAR in this study poses a challenge to the physicians as vancomycin is the only drug of choice for such isolates. Genetic similarity seen among *E. faecium* isolate from pus and blood indicates the possibility of a common source. Hence the controlled use of antibiotics and periodic surveillance will prevent the dessimination of drug resistant *Enterococcus* spp.

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Authors contribution

DB conceived and designed the experiment. PPN performed the experiments and analyzed the data. DB and PPN wrote the paper. DB and SS edited the article. SK performed the statistical analysis.

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