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Original Article

Microbiology Section

Random Amplified Polymorphic DNA (RAPD) Typing of Multidrug Resistant *Enterococcus faecium* Urinary Isolates from a Tertiary Care Centre, Northern India

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ABSTRACT

Background: Enterococci, though they are a part of commensal flora, are becoming increasingly important as nosocomial pathogens, due to their inherited and acquired resistances to several antimicrobial agents. In this context, *Enterococcus faecium* (*E.faecium*) requires a special mention due to its characteristic of Multidrug Resistance (MDR) and its ability to disseminate.

Aim: This study was undertaken to phenotypically characterize and determine clonal relatedness amongst the indoor isolates of *Enterococcus faecium* (*E. faecium*) which were isolated from patients with urinary tract infections (UTIs).

Settings and design: This study was carried out prospectively in a tertiary care university hospital and in Department of Microbiology, Varanasi, India.

Material and Methods: Urine samples were collected from patients who were admitted in different departments of the hospital with a clinical diagnosis of UTIs and they were processed for a period of one year. *Enterococcal* species were identified by doing extensive biochemical tests. Anti–microbial susceptibility testing was done by disc diffusion and agar dilution methods. Molecular typing of the isolates was done by Random Amplified Polymorphic DNA (RAPD) typing method.

Results: A total of 48 *Enterococcal* urinary isolates were identified in indoor patients, among which a majority (46, 95.83%) were *E.faecium* isolates. These isolates exhibited high resistance to fluoroquinolones (91.3%) and to ampicillin (60.86%) in particular. Two isolates were found to be resistant to vancomycin on screen agar. RAPD typing showed two major clusters, one of which had ten strains of 100% similarity, all of which were isolated from a common source.

Conclusion: This study showed dissemination of multidrug resistant *E. faecium* isolates within the hospital. Being a quick and cost effective method, RAPD typing can be used to show clonal relatedness and to trace possible sources of organisms for epidemiological purposes.

Keywords: RAPD typing, *E.faecium*, Clonal cluster

INTRODUCTION

Enterococci are ubiquitous and they are normal inhabitants of the Gastrointestinal Tracts (GITs) of humans and animals. However, besides having roles as commensals, they are increasingly being reported worldwide as the leading causes of nosocomial and community acquired bacteraemia, urinary tract infections, surgical wound infections, pelvic infections, intra-abdominal infections and rarely, of meningitis [1]. Enterococci are the second leading cause of nosocomial infections, as was reported by CDC survey [2]. Recently, there has been a change in the ratio of infections caused by different *Enterococcal* species, due to the emergence of MDR *E.faecium* [3].

E. faecium has emerged as an important nosocomial pathogen worldwide, particularly being intrinsically resistant to different classes of antibiotics, along with its immense ability to acquire high levels of drug resistance through horizontal gene transfer. These isolates have often been reported to be Multidrug Resistant (MDR), with varying resistances against β lactam agents like ampicillin and glycopeptides like vancomycin, along with High Level Aminoglycoside Resistance (HLAR) and fluoroquinolone resistance. This trend has been associated with dissemination of a genetic lineage designated clonal complex-17 (CC17) all over the world, which is correlated with ampicillin and quinolone resistance in particular [4].

Effective typing of microorganisms is a prerequisite for establishing epidemiological or phylogenetic links between corresponding isolates. Various methods have been successfully used to type and differentiate bacterial strains and clonal groups from each other [5].

Methods used for molecular typing of *Enterococcus spp* include macrorestriction analysis using Pulsed-Field Gel Electrophoresis (PFGE), ribotyping, Random Amplification of Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), the latest being, Multi Locus Sequence Typing (MLST). Among the different methods, RAPD typing is perhaps the simplest one. Results of recent studies which have been done have shown that AFLP and RAPD may be used for epidemiological surveillances of distantly related *Enterococcal* isolates and other gram-positive bacteria [6], at least in low resource laboratories.

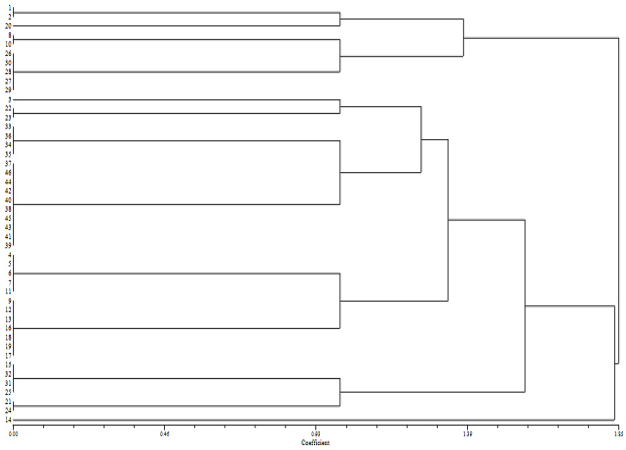
The aim of the study were to phenotypically and genotypically characterize the *Enterococcus faecium* isolates by RAPD fingerprinting, to check their clonal relatedness and to trace the sources of the isolates which were implicated in urinary tract infections, in order to study the dissemination of similar strains from common sources within the hospital environment.

MATERIAL AND METHODS

Mid-stream clean catch urine samples were collected for a period of one year after obtaining prior consents of hospitalized patients with a clinical diagnosis of UTIs at a tertiary care hospital, Varanasi, north India. The samples were plated on Cystine Lactose Electrolyte Deficient (CLED) agar, immediately after their collection. Following incubation of the plates at 37^{0}C overnight, *Enterococcal* isolates were identified by gram staining, bile esculin hydrolysis, growth in 6.5% sodium chloride and growth at pH 9.6 [7]. All *Enterococcal* isolates were tested for their phenotypic characteristics, by performing an array of conventional biochemical and physiological

Antibiotics	Resistance (%) shown by all the isolates (n=46)	Resistance (%) shown by the isolates of cluster I (n=10)
ampicillin	60.86 (28)	80 (8)
ciprofloxacin	91.3 (42)	100 (10)
norfloxacin	93.4 (43)	100 (10)
nitrofurantoin	13.1 (6)	60 (6)
High strength gentamicin	56.52 (26)	60 (6)
High strength streptomycin	43.47 (20)	50 (5)
vancomycin	4.3 (2)	10 (1)
teicoplanin	4.3 (2)	10 (1)
linezolid	0 (0)	0 (0)

[Table/Fig-1]: Antimicrobial resistance profile of the urinary *E.faecium* indoor isolates



[Table/Fig-2]: Dendrogram of *Enterococcal* isolates based on RAPD typing

tests which were devised by Facklam and Collins, as per standard procedures [8]. Briefly, the carbohydrate fermentation tests which were used were those which were done for mannitol, sorbitol, sorbose, arabinose, raffinose, lactose, sucrose, pyruvate (Hi Media, India) in a 1% broth base. Deaminaton of arginine was detected in Moeller's decarboxylase media, while motility was seen in sulphide-indole-motility media. Pigment formation was seen on Trypticase soy agar. All the isolates were further tested for their antibiotic susceptibility patterns against the following drugs; ampicillin (30µg), ciprofloxacin (5 µg), norfloxacin (5 µg), nitrofurantoin (300 µg), vancomycin (30 µg), teicoplanin (30 µg) and linezolid (30 µg) (Hi Media, India) by using standard procedures like disc diffusion and agar dilution methods for gentamicin (500 µg/ml), streptomycin (2000 µg/ml) and vancomycin (6 µg/ml) and they were interpreted according to CLSI guidelines [9].

RAPD typing was performed by using primers, AP4 (5 TCA CGC TGC A 3) and ERIC1R (5 ATG TAA GCT CCT GGG GAT TCA C 3), as previous work on this primer combination had proved to be satisfactory in discriminating the strains [10]. DNA was extracted by following the protocol for DNA isolation in lactic acid bacteria and Enterococci [11] and PCR amplification was done, based on protocol which was given elsewhere [12]. The amplified products were electrophoresed on a 1.2% agarose gel with ethidium bromide (Genei, Bangalore, India), at a constant of 60 volts for 60 min with Tris Acetate EDTA (TAE) buffer. The products were visualized under UV illumination and images were saved by using a multi Image Light Cabinet (Alpha Innotech Corporation, USA). Band sizes, band attributes and standard molecular weights were assigned according to molecular weight markers. Numerical index of the discriminatory ability of RAPD typing methods was calculated by applying Simpson's Index of Diversity equation [13]. Dendrograms for cluster analyses of all the isolates were constructed by using NTSYS pc2.0 programme of Unweighted Pair-Group Method and Arithmetic Mean (UPGMA).

RESULTS

A total of 48 *Enterococcal* isolates were collected during the study period, which comprised of 46 (95.83%) *E.faecium* and 2 (4.17%) *E.faecalis* isolates. Further characterization of the *E.faecium* isolates revealed that all the isolates exhibited high antibiotic resistances, especially to ampicillin (60.86%) and fluoroquinolones (91.3%) by disc diffusion method and to high level aminoglycosides, namely gentamicin (56.52%) and streptomycin (43.47%), by screen agar methods. Two isolates were found to be resistant to vancomycin on screen agar. Moreover, ten isolates from the Urology Indoor Ward showed still higher resistances to ampicillin (80%), ciprofloxacin (100%), vancomycin (10%), gentamicin (60%), streptomycin (50%), nitrofurantoin (60%). However, no resistance to linezolid was reported in any of the isolates [Table/Fig-1].

In RAPD PCR, the presence of bands of molecular weights 3.2kb, 3kb, 1.5kb, 1kb, 756p, 600bp and 510bp were seen in most of the isolates. RAPD patterns of the tested isolates were all reproducible, with a discriminatory index of 0.86.

When dendrograms were constructed from the RAPD results, the isolates were classified into twelve groups, based on RAPD fingerprints. The dendrograms showed [Table/Fig-2] two major clusters. A majority of the strains belonged to the second cluster. Ten strains in the second cluster had 100% similarity (cluster I), whereas seven strains in the other cluster had 100% similarity. Other clusters were smaller than these. All the ten strains in the cluster were isolated from Urology Indoor Ward, from different patients at different points of time. These strains were much more resistant than the others, as has been mentioned. The other seven strains from the second cluster were distributed among the paediatric indoor ward (3), urology indoor ward (2) and gynaecology ward (1) and nephrology ward (1).

DISCUSSION

This study showed widespread dissemination of the *Enterococcal* isolates within the hospital environment. The heterogeneity which was observed might have been caused by the diversity of the sources. However, a single clone which circulated in the urology ward was seen. We did not find clustering of the similar isolates at any one point of time, which could suggest an outbreak. But the clustering of isolates of the same type from the same indoor location or connected wards in the same isolation time interval could suggest a direct patient to patient transmission [14,15]. We found strains with same typing profiles, but with different resistant patterns, as was suggested by their antibiograms. Therefore, it was quite possible that these resistant determinants were being frequently exchanged amongst the circulating strains via mobility of the resistance genes on plasmids and transposons and via chromosomal exchange. However, such conclusions require further confirmation by other methods of typing.

Recently, it was shown that *E. faecium* isolates which belonged to the clonal complex-17 were epidemic-virulent, hospital-adapted strains that often caused outbreaks of infections, but which were also found in sporadic infections. The characteristics of this complex are besides others, ampicillin and quinolone resistance and genetic clustering of the isolates by MLST. Additionally, such bacteria are reservoirs of mobile resistance genes for other, more virulent pathogens, namely, *S. aureus* [16]. In our study, we could not assign particular clonal complexes by RAPD, their clustering at least hints towards locally dispersed clonal lineage of multidrug resistant *E.faecium* isolates in the hospital environment. Our study showed that RAPD could also be used as a tool for epidemiological

investigations by coupling laboratory information with adequate epidata [12]. In this regards, MLST provides an excellent tool for epidemiology, population structure and genetic evolution of Enterococci.

Lastly, we conclude that the RAPD method with AP4 plus ERIC1R primers, which has been described in this study, revealed that this molecular typing method was a cheap, affordable and a powerful means for microbiologists to find out relatedness of multidrug resistant *E. faecium* isolates in urinary infections.

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