CIRCULATION OF MULTIPLE PLASMID REPLICON TYPES IN MDR AND HLAR ENTEROCOCCI

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ABSTRACT

Introduction: Enterococci are opportunistic pathogens which expresses antibiotic resistance in hospitalized patients. They acquire antibiotic resistance either by point mutation or by horizontal transfer of mobile genetic elements especially by resistant plasmids or transposons. **Objectives:** To monitor the presence and distribution of resistance mediating plasmids circulating among clinical isolates of enterococci. Further, to better understand the properties carried by each identified plasmid with the phenotypic characteristics and genes distributed in the isolate. **Methods:** Plasmid replicon typing was analyzed by PCR based replicon typing method to characterize plasmids among resistant isolates of enterococci. **Results:** Enterococcal isolates were observed to carry sixteen rep types which were correlated with antibiotic resistant phenotypes and genotypes. We identified the presence of pheromone responsive plasmids, resistance and virulence genes encoding plasmids, cryptic plasmids and small mobilizable plasmids. **Conclusion:** Enterococci by nature are frequently found to carry mobilizable genes which help them to mediate virulence and resistance properties in order to survive the host immune mechanisms. This paper discusses the role of plasmids in understanding the changing epidemiology and spread of antibiotic resistance within species. This study is first to document the presence and distribution of plasmid replicon types in resistant clones of enterococci.

1. INTRODUCTION

Enterococci are prone to acquire antibiotic resistance either by point mutation or by horizontal transfer of mobile genetic elements (especially resistant plasmids or transposons) (Jensen *et al.,* 2010).Plasmids are extra chromosomal circular DNA fragments that replicates autonomously in a host cell. Sex pheromones secreted by recipient strains help in the transfer of plasmids through conjugation. These pheromone responsive plasmids were first described by Dunny *et al.,* in 1978. Novick *et al.,* in 1987 classified plasmids based on their incompatibility typing (Inc). Inc type plasmids are plasmids with same replication controls which are unable to survive in the same cell while, plasmids with different replication controls are termed compatible (Carattoli *et al.,* 2005). PCR based replicon typing method (PBRT) was used in molecular epidemiologic studies of R-plasmids carrying genes with broad spectrum β-lactamases in *Enterobacteriaceae* family (Carattoli *et al.,* 2009; Carattoli *et al.,* 2006). We have employed the PBRT method to analyse and document the distribution of plasmid replicon types with antibiotic resistance in connection with high level resistance among isolates of enterococci collected from this region.

METHODOLOGY

2.1. Study Isolates:

A total of 228 non-duplicate isolates of enterococci collected from tertiary care hospitals and diagnostic centres in Chennai were included in this study. An Institutional human ethical clearance was obtained from University of Madras, Taramani campus, Chennai, to conduct this study (Dr.ALMPGIBMS/Micro/HIEC/Ref No.1168). All the enterococcal isolates were speciated by PCR with primers specific for *E. faeciumddl, E. faecium* (Kariyama *et al.,* 2000) and *E. faecalisddl, E. faecalis* with an annealing temperature (Dutka-Malen *et al.,* 1995) of 54 ˚C for 1 min. Multiplex PCR was carried out to detect *E. gallinarum* (*vanC1*), *E. casseliflavus, E. flavescens* (*vanC2*/*C3*) with annealing temperature of 60 °C for

1min (Bell *et al.*, 1998). The other species such as *E. avium, E. dispar,* and *E. durans*were identified only based on their biochemical characteristics.

2.2. Antibiotic resistance by Minimum inhibitory concentration:

Aminoglycoside resistance for gentamicin, amikacin, kanamycin and streptomycin, glycopeptide resistance for vancomycin and teicoplanin and macrolide resistance for erythromycin were determined by Minimum inhibitory concentration by using standard agar dilution method. The overnight bacterial cultures were adjusted to 0.5 McFarland's turbidity and the inoculum was spot inoculated on the surface of Brain heart infusion agar with increasing concentrations of gentamicin, streptomycin, kanamycin and amikacin antibiotics (Himedia Labs, Mumbai, India). The plates were incubated at 37 °C for 24hrs and inspected for more than one colony forming units in the spotted area. High level streptomycin resistant isolates were confirmed by further incubation upto 48 hrs. *E. faecalis* ATCC 29212 was used as a negative control strain. The isolates were confirmed as high-level aminoglycoside-resistant enterococci (HLARE) by considering growth >512 µg/ml for gentamicin, >2048 µg/ml for streptomycin, >2048 µg/ml for kanamycin and >512 µg/ml for amikacin. Vancomycin resistance was confirmed by Hi-Comb MIC E-strips (HiMedia Labs, Mumbai, India) on MHA plates lawn cultured with suspected strains of enterococci matching 0.5 McFarland standards.

2.3. Plasmid Replicon Typing in Enterococci

Plasmid replicon typing was carried out to characterize all the high level aminoglycoside resistant isolates of enterococci as described previously by Jensen *et al.*, 2010 without modifications. Six multiplex-PCRs with the specific primers for 18 *rep*-families (Table 1) and three simplex PCR for *rep*10b (prototype pSK6)- 200bp (56°C), *rep*12 (prototype pBMB67)- 470bp (52°C), *rep*19 (prototype pUB101)- 543bp (52°C) were used for plasmid classification. All the PCR amplicons were resolved by electrophoresis along with 100bp molecular weight DNA marker (Real Biotech Corporation, Taiwan) in a 1.2% agarose-Tris-borate-EDTA gel stained with ethidium bromide (0.5µg/ml) and visualized under Gel Documentation system (BioRad, USA) and the gel images were documented. Each PCR product irrespective of the presence or absence of resolved gene product were cross checked by simplex PCR for the presence of appropriate amplicon sizes. Positive PCR products were sent for sequencing at Xcelris labs, Ahmedabad, India. The sequenced results were further analysed using GenBank tool for 90-100% sequence similarity in the database.

Table 1: List of Multiplex PCR, primers, annealing conditions and their respective amplicon sizes for plasmid replicon typing.

Primers used and their respective amplicon size (bp)	Multiplex PCR 1: 56°C	Multiplex PCR 2: 52°C	Multiplex PCR 3: 52°C	Multiplex PCR 4: 56°C	Multiplex PCR 5: 52°C	Multiplex PCR 6: 52°C
	*rep*1 (pIP501)- 624bp	*rep*4 (pMBB1)- 430bp	*rep*3 (pAW63)- 403bp	*rep*2 (pRE25/pEF1)- 630bp	*rep*15 (pUSA03)- 327bp	*rep*5 (pSAS pN315)- 637bp
	*rep*9 (pCF10)- 201bp and	*rep*7 (pUSA02)- 227bp	pMG1 (unique) (pHTβ)- 199bp.	*rep*6 (pS86)- 551bp	*rep*16 (pSAS)- 592bp	*rep*11 (pEF1071)- 500bp
	*rep*10 (pIM13)- 382bp	*rep*14 (pR1)- 164bp		*rep*8 (pAM373)- 394bp	*rep*13 (pC194)- 402bp	
		*rep*17 (pRUM)- 604bp		*rep*18 (pEF418)- 462bp		

3. RESULTS AND DISCUSSION

3.1. Species distribution of enterococci:

Out of 228 isolates collected, *E. faecium* was the predominant species 117/228 (51.31%), followed by *E. faecalis*99/228 (43.42%), *E. avium*4/228 (1.75%), *E. durans*1/228 (0.43%), *E. hirae*2/228 (0.87%), *E. gallinarum*2/228 (0.87%), *E. casseliflavus* 1/228 (0.43%) and *E. dispar* 2/228 (0.87%). Studies from India have shown *E. faecalis* as the common species by biochemical characterization. In our study, this may be due to the biochemical variations observed among different species of enterococci which required further confirmation with molecular methods for accurate documentation of species (Padmasini *et al.*, 2014). The prevalence of *E. faecium* is lower in India compared to *E. faecalis*. In our study, *E. faecium* carries greater intrinsic resistance to most of the antibiotic classes tested and thus MDR naturally.

3.2. Antibiotic resistance exhibited among enterococci:

The results obtained for MIC of aminoglycosides out of 228 isolates analyzed. 106 (46.49%) isolates were high level gentamicin resistant (HLGR) with ≥512 µg/ml; 96 (42.10%) were high level streptomycin resistant (HLSR) with ≥2048 µg/ml; 60 (26.31%) were high level amikacin resistant (HLAR) with ≥512 µg/ml and 116 (50.87%) were high level kanamycin resistant (HLKR) with ≥2048 µg/ml were observed. The species distribution and high level aminoglycoside resistance were compared in table 2.

Table 2: represents high level aminoglycoside resistance distributed in *Enterococcus* species

Clinical Source (n=228)	HLGR (>500µg/ml) (n=106)	HLSR (>2000µg/ml) (n=96)	HLAkR (>2000µg/ml) (n=60)	HLKR (>500µg/ml) (n=116)
E. faecium (117)	64 (60.3%)	58 (60%)	42 (70%)	67 (58%)
E. faecalis (99)	40 (37.7%)	35 (36.4%)	15 (25%)	47 (40.5%)
E. avium (4)	-	1 (1.4%)	1 (1.6%)	-
E. durans (1)	1 (0.94%)	1 (1.4%)	1 (1.6%)	1 (0.86%)
E. hirae (2)	-	1 (1.4%)	1 (1.6%)	1 (0.86%)
E. gallinarum (2)	-	-	-	-
E. casseliflavus (1)	1 (0.94%)	-	-	-
E. dispar (2)	-	-	-	-

3.3. Plasmid replicon typing in Enterococci:

We analyzed all the enterococcal isolates for the distribution of twenty different replicon types (Table 3) by PBRT method targeting 20 *rep* familiesof which 16 *rep* types were distributed in our isolates.

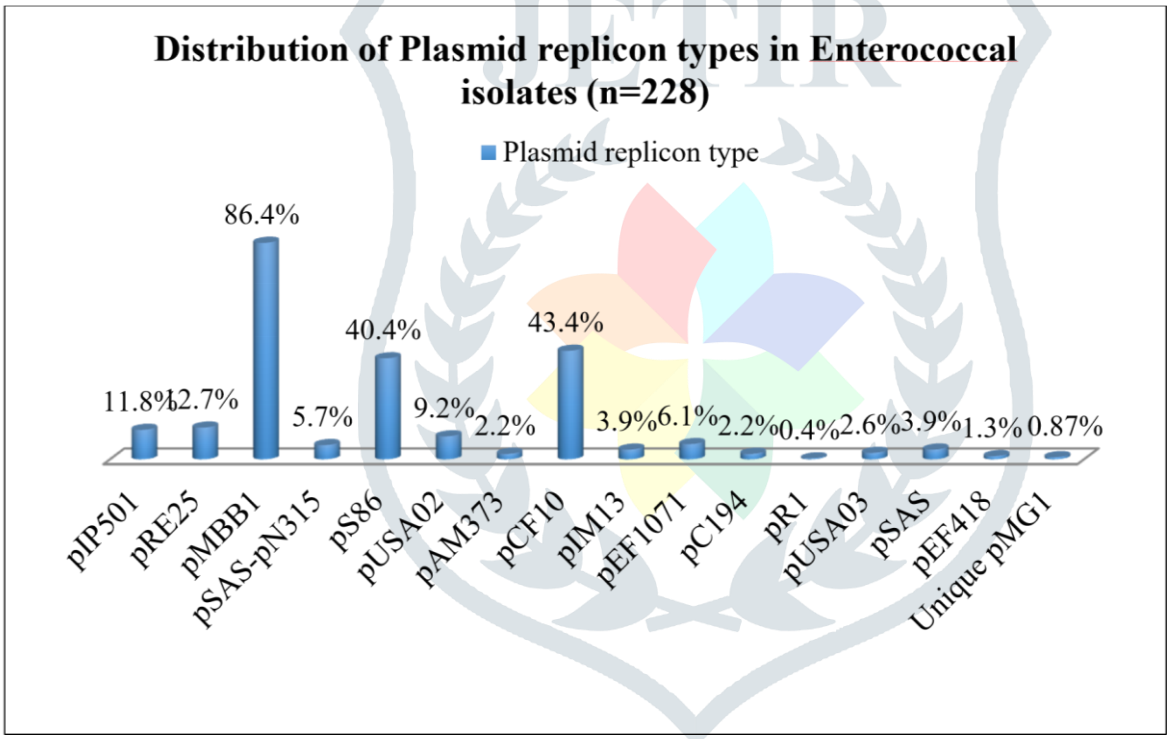
Table 3: represents the different types of plasmid replicon types and their function in gram positive bacteria

Rep Type	Plasmid	Function
1	pIP501	Dissemination of resistance. belong to Inc 18 plasmids
2	pRE25	Plasmids in enterococci transferring antibiotic resistance. Conjugative plasmids
3	pAW63	4 rep like plasmids present in Bacillus cereus-transmits virulence factors
4	pMBB1	Small cryptic plasmids
5	pSAS pN315	Present in clinically important *S. aureus*
6	pS86	Cryptic plasmids with tetracycline resistance
7	pUSA02	Antimicrobial resistance to tetracycline, macrolide and chloramphenicol
8	pAM373	2 sex pheromone plasmids found in *E. faecalis*
9	pCF10	Well characterized sex pheromone based conjugation
10	pIM13	Contained erm gene encoding macrolide, lincosamide and streptogramin B resistance
11	pEF1071	Contains genes for toxin production
12	pBMB67	3 plasmids all found in *B. thurungiensis*
13	pC194	4 plasmidsin Staphylococci
14	pR1	3 plasmids all found in E. faecium- small mobilizable plasmids
15	pUSA03	First isolated from MRSA known to cause community outbreaks
16	pSAS	Identified in a community acquired *S. aureus*
17	pRUM	It is conjugative multi-resistant plasmid, tetracycline resistant
18	pEF418	Contained ORFs from 2 enterococcal plasmids
19	pUB101	Resistant to fusidic acid

Unique	pMG1	Virulent plasmid

27/228 (11.8%) isolates carried *rep*1 (pIP501) plasmid, 29/228 (12.7%) *rep*2 (pRE25), 197/228 (86.4%) *rep*4 (pMBB1), 13/228 (5.7%) *rep*5 (pSAS-PN 315), 92/228 (40%) *rep*6 (pS86), 21/228 (9.21%) *rep*7 (pUSA02), 5/228 (2%) *rep*8 (pAM373), 99/228 (43%) *rep*9 (pCF10), 9/228 (3.94%) *rep*10 (pIM13), 14/228 (6.1%) *rep*11 (pEF1071), 6/228 (2.6%) *rep*13 (pC194), 1/228 (0.4%) *rep*14 (pRI), 6/228 (2.6%) *rep*15 (pUSA03), 9/228 (3.9%) *rep*16 (pSAS), 3/228 (1.3%) *rep*18 (pEF418) and 2/228 (0.87%) isolates carried unique plasmid pMG1. None of the isolates had *rep*3 (pAW63), *rep*10b (pSK6), *rep*12 (pBMB67), *rep*17 (pRUM), and *rep*19 (pUB101) as shown in Fig 1. Upon analysis, the rep types detected in our study replication initiation proteins (rep) had a significant correlation with the distribution of plasmid replicon types. Pattern of replicon prototypes was derived by the number of replicons detected in each strain. We obtained seven patterns of rep combinations (Table 4) of which, the first major pattern included with combination of 7 replicon prototypes were present in 4/228 (1.75%) isolatesof *E. faecium*. All these isolates were observed to be HLR to all the aminoglycosides (>500µg/ml for gentamicin and amikacin; >2000µg/ml for streptomycin and kanamycin) and macrolides (>256µg/ml for erythromycin) tested.

Fig 1. Shows the distribution of plasmid replicon typing in enterococcal isolates by PBRT method



The second pattern with 6 replicon prototypes was observed in 5/228 (2.19%) isolates of *E. durans* (1), *E. faecium* (2) and *E. faecalis* (2). The third pattern with 5 replicon prototypes was detected in 10/228 (4.4%) isolates of which majority of them were *E. faecium.* The 2 *E. faecalis* isolates had similarity in distribution of plasmid prototypes except for the presence of amikacin resistance in a strain (Ef23). The fourth pattern with 4 replicon prototypes was observed in 17/228 (7.5%) isolates of which 6 *E. faecalis*, 9 *E. faecium*, 1 *E. avium* and 1 *E. hirae*. The fifth pattern with 3 replicon prototypes was observed in 40/228 (17.5%) isolates of enterococci 17 *E. faecium,* 22 *E. faecalis* and 1 *E. avium* isolates.

Secondly, *rep*9 (pCF10) replicon was observed in 43% (99/228) our enterococcal isolates and *rep*8 (pAM373) in 3% of the isolates. pAD1, pAM373 and pCF10 were grouped as plasmids which are pheromone responsive. In our study *rep*8 and *rep*9 replicon types were present in *Enterococcus* species other than *E. faecium* and *E. faecalis*. *rep*1 (pIP501) has been identified as a multi resistance broad-host-range Inc18 plasmid. In our study, *rep*1 was observed in 11% (27/228) of the isolates, out of which 22 were *E. faecium*, 1 *E. gallinarum* and 4 *E. faecalis*. Among 27/228 *rep*1 positive isolates, 8 (30%) were HLAR and 1 (3.7%)

VRE. Study by Rosvoll *et al.*, 2010 had observed *vanA* positive isolates to possess*rep*1. Previous reports on a limited number of *vanA*-containing plasmids in enterococci suggests that the *rep*1 and *rep*2 replicons are commonly present in VRE (Sorum *et al.*, 2006; Garcia-Migura *et al.*, 2007; Sletvold *et al.*, 2007, 2008). In our study, *rep*2 was observed in 12.7% (29/228) isolates, of which 2 were *vanA* Positive. 13/228 (5.7%) isolates carried *rep*5 (pSAS-PN 315) and 11/13 of the positive isolates were *E. faecium* with one VRE and 2 *E. faecalis*. 8/13 of *rep*5 type isolates has shown high level resistance to all of the aminoglycosides and vancomycin tested in our study might probably due to presence of this rep type along with other rep prototypes. This plasmid was previously reported in two of the MRSA isolates (Jensen *et al.*, 2010). 92/228 (40%) isolates carried *rep*6 (pS86) which is a rolling circle replication plasmid. Martinez-Bueno *et al.*, in 2000 reported *rep*6 in *E. faecalis* as small cryptic theta replication plasmids with complete nucleotide sequence of 5149 bp.

Table 4: represents the major patterns (5, 6 and 7) of plasmid replicons along with their resistance pattern observed in enterococcal isolates.

No. of Rep families Present	Strain and Pattern of replicons present		Resistant to
	Strain No.	Pattern	
7	051- Efm*	pIP501, pMBB1, pRE25, pUSA03, pC194, pSAS-PN315, pEF1071	HLGR, SR, KR, AkR, Ery
	061- Efm	pIP501, pMBB1, pRI, pMG1 unique, pS86, pEF418, pSAS-PN315	HLGR, SR, KR, AkR, Ery
	062- Efm	pIP501, pCF10, pMBB1, pUSA02, pRE25, pS86, pSAS-PN315	HLGR, SR, KR, AkR, Ery
	075- Efm	pIP501, pMBB1, pRE25, pUSA03, pC194, pSAS-PN315, pEF1071	HLGR, SR, KR, AkR, Ery
6	Ed1- *E. durans*	pCF10, pRE25, pS86, pAM373, pSAS, pEF1071	HLGR, SR, KR, AkR, Ery
	023- Efm	pCF10, pMBB1, pRE25, pUSA03, pC194, pEF1071	HLGR, SR, KR, AkR, Ery
	063- Ef*	pIP501, pCF10, pMBB1, pUSA02, pRE25, pSAS-PN315	HLGR, SR, KR, AkR, Ery
	070- Efm	pMBB1, pUSA03, pRE25, pMG1 unique, pC194, pEF1071	HLGR, SR, KR, AkR, Ery
	157- Ef	pIP501, pMBB1, pRE25, pS86, pSAS, pC194	HLGR, SR, KR, AkR,
5	Ef11- Ef	pIP501, pMBB1, pCF10, pIM13, pS86	HLGR, SR, KR
	Ef23- Ef	pIP501, pMBB1, pCF10, pIM13, pS86	HLGR, SR, KR, AkR
	Efm52- Efm	pIP501, pMBB1, pS86, pSAS-PN315, pEF1071	Not HLAR
	Ed3- Efm	pRE25, pS86, pAM373, pSAS, pEF1071	HLGR, SR, AkR,
	008- Efm	pMBB1, pS86, pUSA03, pC194, pEF1071	HLGR, KR, AkR,
	085- Efm	pIP501, pCF10, pMBB1, pUSA02, pSAS-PN315	HLGR, SR
	090- Efm	pIP501, pMBB1, pRE25, pSAS-PN315, pEF1071	HLGR, SR, KR, AkR,
	091- Efm	pIP501, pCF10, pMBB1, pUSA02, pRE25	HLGR, SR, KR
	109- Efm	pIP501, pMBB1, pUSA02, pRE25, pEF1071	HLKR

	154- Efm	pMBB1, pMG1 Unique, pRE25, pS86, pSAS	HLGR, SR, KR, AkR

*****Efm- *E. faecium,* Ef- *E. faecalis;* HLARE –high level aminoglycoside resistance to gentamicin, amikacin, streptomycin and kanamycin; HLGR- high level gentamicin resistance; SR- high level streptomycin resistance; KR- high level kanamycin resistance; AkR- high level amikacin resistance; Not HLAR- the strains were not high level aminoglycoside resistant.

Table 5. To detect the prevalence of plasmid replicon types and their distribution in resistant and virulent enterococcal isolates.

Plasmids	**Rep Type**	**No. of HLARE* carrying plasmids (n=41)**
pMBB1	4	36
pRE25	2	21
pCF10	9	21
pS86	6	19
pEF1071	11	8
pIP501	1	8
pUSA02	7	7
pSAS-PN315	5	5
pC194	13	4
pUSA03	15	3
pIM13	10	3
pEF418	18	3
pSAS	16	3
pMG1	Unique	2
pAM373	8	1
pR1	14	1

32/228 isolates in this study had a combination of *rep*6 and *rep*4 and 24/228 isolates with *rep*9, *rep*4, *rep*6 replicon types circulating which requires further insight. *rep*7 was detected in other species of enterococci such as *E. durans* and *E. hirae* isolates by Lopez *et al.*, 2011. Plasmids with *rep*7 replicon type had been observed to carry genes conferring resistant to antibiotics such as tetracycline, chloramphenicol and macrolides. In our study, 21/228 (9%) isolates with *rep*7 were resistant to tetracycline and erythromycin antibiotics. Out of 21 isolates, 16 of them were *E. faecium*, 1 *E. dispar* and 4 *E. faecalis* isolates. Majority of the isolates with *rep*7 were antibiotic resistant especially HLARE (Table 5). *rep*10 (pIM13 prototype) was first isolated from *Bacillus subtilis* and is a naturally occurring plasmid. They are stably maintained at high copy numbers and are found to constitutively express resistance to macrolide lincosamide-streptogramin B antibiotics especially *ermC* gene (Projan*et al.,* 1987). In our study, 3% (9/228) of the isolates carried *rep*10 (pIM13) plasmid. All these 9 isolates were obtained from urine and amongst which 6 were *E. faecalis* and 1 *E. hirae.* Though these isolates did not carry *ermC* gene, all were constitutively resistant to erythromycin and clindamycin and only one isolate carried *ermB* gene responsible for macrolide resistance. 14/228 (6.1%) isolates carried *rep*11 (pEF1071) in our study. This plasmid was earlier reported in enterococci which carried genes for toxin production (Balla and Dicks, 2005). Out of these 14 positive isolates, 13 were *E. faecium* and one *E. durans*.

pC194 is a *rep*13 type small plasmid that encodes for chloramphenicol resistance in staphylococci (Ballester*et al.,* 1989). This replicon type was detected in 6/228 (2.6%) *E. faecium* isolates in our study. On observation, all these 6 isolates were high level resistant to gentamicin, streptomycin, kanamycin and amikacin. 5/6 isolates carried*aph (3')-IIIa*gene along with other aminoglycoside resistance encoding genes analyzed. *rep*14 (pRI) was detected in only one isolate (0.4%) out of all the enterococcal isolates tested. This isolate was *E. faecium* with high level resistance to gentamicin, streptomycin, kanamycin and amikacin which carried *aac(6')-aph(2'')-Ia* bifunctional aminoglycoside resistance gene, *ermB* and *ant(6')-Ia* genes. pUSA03 plasmid were first detected in *S. aureus* with a function of dihydropteroate synthase which was resistant to clindamycin, tetracycline and mupirocin (McDougal *et al.,* 2010). pUSA03 plasmid carried *ileS2* gene which was found to play a major role in high level mupirocin resistance in community-acquired outbreak strain of *S. aureus* (Perez-Roth *et al.,* 2010). This plasmid can be of a broad host range, however its function in enterococci should be further analysed. In our study, 6/228 (2.6%) isolates carried *rep*15 (pUSA03) in 5 *E. faecium* and one *E. avium* isolate. All the *E. faecium* isolates were high level aminoglycoside resistant (HLGR, HLKR, HLAkR). Rep16 family have been reported in three plasmids from *Staphylococcus* community acquired isolates, In our study, 9/228 (3.9%) isolates carried *rep*16 (pSAS) which is an indicative of community spread of resistance genes. 3/228 (1.3%) isolates carried *rep*18 (pEF418) with 2 *E. faecium* and one *E. faecalis*all the three were high level aminoglycoside resistant phenotypes (HLGR, HLSR, HLAkR, HLKR). pMG1-like plasmids are detected in vancomycin-resistant *E. faecium* clinical isolates obtained from a hospital in the United States. It was first described by Ike *et al.,* 1998 as the pheromone-independent gentamicin resistance conjugative plasmid pMG1 from a clinical isolate of *E. faecium* in Japan. In our study, 2/228 (0.8%) isolates carried unique plasmid prototype pMG1 and both isolates were high level resistant to all aminoglycosides tested and also carried gentamicin resistance encoding genes: *E. faecium*(1/2) carried bifunctional aminoglycoside resistance gene*aac(6')-aph(2'')-Ia*, *aph(3')-IIIa, aac(6')-Ii* genes and*ermB* gene and *E. faecalis* (1/2) carried *aac(6')-aph(2'')-Ia*, *aph(2'')-Ic,aph(3')-IIIa*, *ermB* gene and *aac(6')-Ii* genes.

4. CONCLUSION

Classifying plasmids based on the diversity of replicon associated genes is called rep typing or replicon typing. Rep typing is considered to be a marker for incompatibility typing although the relationship between rep types and incompatibility types is not always well defined. We emphasize the circulation of various conjugative plasmids that are scarcely reported in clinical isolates of enterococci. Vancomycin resistant isolates carried pRE25 plasmids (rep2) in common which transfers resistance against kanamycin, neomycin, streptomycin, clarithromycin, roxithromycin, tylosin, chloramphenicol, azithromycin, erythromycin and clindamycin. To the best of our knowledge there are no documented reports on distribution of *rep* families in resistant enterococcal isolates from Indian settings. This study has thrown light into the role of plasmids in understanding the changing epidemiology and spread of antibiotic resistance within species among clinical isolates of enterococci.

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