Chapter VI

Molecular Typing of High-level Aminoglycoside Resistant Enterococci

CHAPTER VI

18. MOLECULAR TYPING OF HIGH-LEVEL AMINOGLYCOSIDE RESISTANT ENTEROCOCCI

Enterococci rank among top three pathogens causing nosocomial infections worldwide, since last decade [13, 17]. Although initially thought to have evolved from patient's own flora, enterococci was later shown to be exogenously acquired from nosocomial settings by Zervos and his colleagues in 1986, using molecular epidemiological tools [18]. Since then, innumerable reports of nosocomial enterococcal infections were published and most studies used molecular epidemiological tools for typing enterococci to study the clonality of the isolates. In case of suspected outbreak conditions, the analysis of strain clonality helps in confirming the association between patients (hosts) and reservoirs for enterococci, and to determine the possible modes of transmission. In some instances, the phenotyping and antibiotyping results of enterococci may help presumptively in investigating whether the isolates studied have, or lack clonal relationship, although unwarranted. However, most of the inconclusive results obtained by other typing methods can be authenticated by the application of molecular epidemiological techniques, which gives a clear picture regarding the clonality of the isolates.

Although a variety of molecular epidemiological techniques have been applied for epidemiological typing of drug resistant enterococci "Pulsed field gel electrophoresis" (PFGE) is considered to be the gold standard for molecular epidemiological analysis of gram-positive cocci, since last decade [279]. Other techniques like plasmid DNA analysis-which was the first tool to be applied for epidemiological analysis of enterococci [18], PCR based typing and a more recent technique: AFLP have also been found to be effective in epidemiological typing of enterococci. Thus molecular typing of enterococci is inevitable to draw conclusive evidences regarding the epidemiology of drug resistant strains in any health-care setting.

OBJECTIVES

- To document the epidemiological pattern of enterococci by Molecular typing of the isolates.

MATERIALS AND METHODS

1. Molecular typing of HLAR enterococci

The molecular typing of HLAR enterococci was performed by two methods namely, chromosomal DNA restriction endonuclease digestion by PFGE and plasmid DNA restriction endonuclease digestion by agarose gel electrophoresis. Overall, we used the same set of randomly selected HLAR enterococci for both the molecular typing methods with occasional exceptions in few instances (where few strains were included/excluded in either typing method, albeit occasionally). The results of both the methods were compared and analyzed statistically to evaluate their significance.

A. Pulsed field gel electrophoresis-PFGE

The HLAR enterococci were subjected to pulsed field gel electrophoresis to compare the polymorphisms in the genomic DNAs among test strains as described previously with minor modifications [279]. Briefly, the procedure involves preparation of agarose-DNA plugs, processing and restriction-digestion of plugs as described below:

i. Preparation and processing of DNA plugs

a. Preparation of genomic DNA in agarose plugs

- Enterococci were grown overnight in 5 ml of Brain heart infusion broth-BHI (Hi Media, Mumbai, India) at 37°C and the cells were harvested by centrifugation at 6,000 rpm for 15 min at 4^0 C.
- The cells were suspended in an equal volume (5 ml) of PIV buffer (1 M NaCl, 10 mM Tris-HCl [pH 7.6]) and mixed well.

- A portion of this suspension (2.5 ml) was mixed with 2.5 ml of 1.6% Low melting temperature agarose (SEA-Plaque Agarose, FMC bioproducts, Rockland ME, USA) in sterile, distilled, deionized water at 40-50°C and stirred well to mix the suspension.
- This mixture was then pipetted (100 µl) into a plug mold (Bio-Rad Laboratories, Hercules, California) and allowed to solidify.
- The agarose plugs were removed carefully from the plug mold without causing damage to the plug and lysed by placing 1-4 plugs in 10 ml of fresh lysis solution (6 mM Tris-HCl [pH 7.6], 1 M NaCl. 100 mM EDTA [pH 7.5], 0.5% Brij 58, 0.2% deoxycholate, 0.5% sodium lauroyl sarcosine, 20 mg/ml of RNAse [DNAse free] and 1 mg/ml of Lysozyme and incubated overnight at 37°C with gentle shaking (N.B: *RNAse and Lysozyme were added freshly just prior to lysis step*).
- Following incubation the lysis solution was replaced with 10 ml of ESP solution (0.5 M EDTA [pH 9-9.5], 1% sodium lauroyl sarcosine and 100 mg/ml of proteinase K) and incubated overnight at 50°C with gentle shaking.
- After incubation the ESP solution was decanted completely and the plugs were washed thoroughly with dilute TE buffer (10 mM Tris-HCl [pH 7.5], 0.1 mM EDTA) thrice for 30 minutes with gentle shaking at 37°C.
- After washing, the plugs were transferred to a clean tube with fresh diluted TE buffer (5 ml) and stored at 40°C.

N.B. *Optimization*: *Various incubation conditions, and concentration of enzymes were tried for genomic DNA agarose plug preparation during standardization, and the above-mentioned conditions were followed throughout our study since they yielded the best results comparatively.*

***b. Restriction digestion of DNA plugs*:** The restriction digestion of enterococcal DNA embedded in agarose plugs were performed using *Sma*I (New England Biolabs, U.K) restriction enzyme following the recommendation of the manufacturer as follows:

- The restriction digestion was performed by placing a DNA plug in a sterile microcentrifuge tube with 200 µl of distilled water, 25 µl of restriction buffer (supplied along with the restriction enzyme) and 20 Units of *Sma*I restriction enzyme and incubated at 25°C in a water bath (Julabo, U.S.A) for 12 hours.
- After incubation, the restriction digested DNA plugs were washed with dilute TE buffer (1 ml) for 1 hour at 37°C and electrophoresed using a CHEF apparatus under optimal conditions.

ii. Contour-clamped homogenous electric field-CHEF electrophoresis

The restriction digested DNA plugs were subjected for CHEF electrophoresis as below mentioned.

a. Loading the sample agarose plugs

- Single plugs of agarose were placed on each tooth of the comb and the preparation was left at room temperature for some time (to facilitate adhesion).
- The gel was cast around the comb and the plugs remained in place when the comb was removed after solidification of the gel. A lambda ladder PFG marker (New England Biolabs, U.K) was loaded along with sample plugs in every run.

b. Casting the gel

- The casting stand (14 cm x 12.7 cm) supplied with the CHEF-DR II apparatus (Bio-Rad laboratories, U.S.A) was used to cast the agarose gel for CHEF electrophoresis as per manufacturers instructions.
- Briefly, 1.2% agarose gel was prepared (Low melting temperature agarose, SEA-Plaque Agarose, FMC bioproducts, Rockland ME, USA) in 0.5X TBE buffer and 80-100 ml of molten agarose was poured (for a 5 mm thick gel) and allowed to cool at room temperature for 1 hour.
- The comb was removed after solidification and the wells were overlaid with few milliliters of remaining molten agarose to fill the gap above the plug, and allowed to cool for 15 minutes before starting the electrophoresis.

*c. **CHEF electrophoresis***

- The CHEF-DR II electrophoresis chamber (Bio-Rad laboratories, U.S.A) was filled with 2 liters of pre chilled 0.5X TBE running buffer 30 minutes before electrophoresis.
- A variable speed pump connected to the gel tank was turned on, to equilibrate the circulating buffer to desired temperature. The temperature of the circulating TBE buffer was maintained at 14°C with the pump tube coiled in a temperature controlled water bath (Julabo, U.S.A).
- The pump was turned off, and the gel was placed in the chamber so that the bottom rested against the two gel stops inside the chamber. It was checked to ensure that about 2 mm buffer covered the gel and then the pump was turned on.
- The buffer flow rate was maintained by adjusting the variable speed pump knob, at 1 liter per minute without disturbing the gel.
- The lid was replaced onto the electrophoresis chamber, and the Pulsewave 760 Switcher and Model 200/2.0 power supply were turned on.
- The switching times were set using the Pulsewave 760 switcher with the pulse time ramped from 1 second to 35 seconds and a start ratio of 1.0 over 27 hours at 200 V, and the run was carried by maintaining the circulating buffer temperature at 14°C.
- After electrophoresis, the gel was removed carefully and stained with ethidium bromide (0.5 mg/ml) for 20 minutes and destained in distilled water for 1 hour.
- Then the gel was transferred onto an UV transilluminator visualized and documented for further analysis.

B. Plasmid restriction endonuclease analysis-REA typing

i) Plasmid REA typing: The plasmid DNA of the HLAR enterococci along with control strains were isolated by alkaline lysis method, digested with restriction endonuclease *Eco*RI, separated by agarose gel electrophoresis and documented as described previously in Chapter III.

C. Visual and Computational analysis of the gels

The PFGE and Plasmid gels were visually interpreted based on the differences in banding pattern as per the consensus guidelines of Tenover et al. [294]. The computerized interpretation of the banding patterns was performed, by analyzing the captured gel images using Bionumerics software, Version 2.5 (Applied Maths, Kortrijk, Belgium). The dice coefficient of similarity was calculated and a dendrogram constructed for phylogenetic (cluster) analysis using the unweighted pair group method with arithmetic averages (UPGMA).

D. Statistical analysis of typing methods

i. Comparison of PFGE and Plasmid REA typing methods

The concordance between PFGE and Plasmid REA typing was determined based on the similarity of clusters/groups obtained by visual interpretations matching the consensus guidelines of Tenover et al. [294], from the isolates for which both PFGE and Plasmid RE profiles were available.

ii. Simpson's index of diversity and Confidence interval

The Simpson's index of diversity was used to test the discriminatory power (D) of the typing methods. The calculation of single numerical index of discrimination (D), was based on the probability that two unrelated strains sampled from the test population will be placed into different typing groups as described by Hunter and Gaston [371]. This index was derived from elementary probability theory and is given by the following equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j - 1)$$

where N is the total number of strains in the sample population, s is the total number of types described, and n_j is the number of strains belonging to the jth type. This equation was derived as follows. The probability that a single strain sampled at random will belong to the jth group is n_j/N. The probability that two strains sampled consecutively will belong to that group is $n_j(n_j - 1)/ N(N - 1)$. These probabilities can be summed for all

the described types to give the probability that any two consecutively sampled strains will be the same type. This summation can be subtracted from 1 to give the equation above. No correcting factor for small populations has been made, as typing schemes are not validated with small samples. A Simpson's index close to zero indicates that there is little diversity as shown by the typing method (index= 0 indicates no diversity at all) whereas a Simpson's index approaching 1 indicates a high diversity as shown by the typing technique (index= 1 indicates maximum diversity where no two isolates are similar).

An approximate 95% confidence interval was calculated as proposed by Grundmann et al. [372]. Briefly, the Simpson's index of diversity D, for the assessment of the discriminatory power of typing techniques is an unbiased estimate of the true diversity λ of a population based on a sample of n individuals. Inferences on the diversity of the population involve a sampling process. Simply by chance, different samples will give different results, the difference being due to sample variation and by drawing repeated samples the precision of the mean estimate for D will improve. If repeated samples of a fixed size n are drawn from the sample population, the values of D will be distributed about λ with the variance σ^2:

$$\sigma^2 = \frac{4}{n}[\sum \pi_j^3 - (\sum \pi_j^2)^2],$$

where π_j is the frequency n_j/n, n_j is the number of strains belonging to the jth type, and n is the total number of strains in the sample population. An estimate of the standard deviation of λ is given by the square root of σ^2, and we propose the following as approximate 95% confidence interval (CI):

$$CI = [D - 2\sqrt{\sigma^2}, D + 2\sqrt{\sigma^2}]$$

We applied these equations for calculations using a simple in-house program written in Microsoft Excel to determine the diversity index (D) and confidence intervals to compare the discriminatory power of PFGE typing with that of Plasmid REA typing for:

- Visually interpreted PFGE gels Vs. Plasmid REA gels and,
- Computationally interpreted PFGE gels Vs. Plasmid REA gels.

RESULTS

1. Molecular typing of HLAR enterococci

The molecular typing of HLAR enterococci was performed by two methods namely, PFGE and plasmid REA by agarose gel electrophoresis. The same set of randomly selected HLAR enterococci for both the molecular typing methods were used with occasional exceptions in few instances (where few strains were included/excluded in either typing method, albeit occasionally). The comparative results of both the typing methods are depicted in **Table 18 a** and **b**, and elaborated in the following sections.

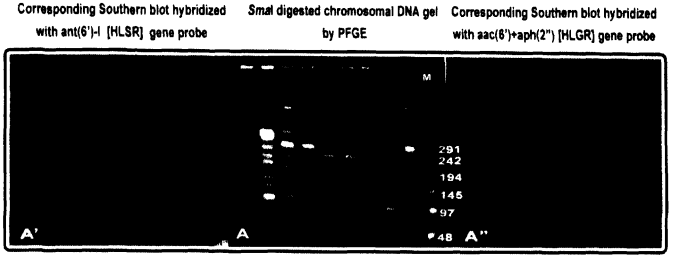
A. PFGE typing

The chromosomal DNA restriction endonuclease digestion of HLAR enterococci was performed using an infrequently cutting enzyme *Sma*I and the digested DNA plugs were separated by pulsed field gel electrophoresis. The 76 isolates of enterococci subjected to pulsed field gel electrophoresis included, 66 randomly selected HLAR clinical isolates apart from ten standard strains and transconjugants. The 66 clinical isolates included fifty four *E. faecalis*, six *E. faecium*, four *E. gallinarum*, one *E. avium* and one *E. durans* exhibiting resistance to both or either of the aminoglycosides (gentamicin/ streptomycin) separately. The *Sma*I digestion yielded approximately 12-20 DNA fragments of various sizes ranging from 50-450 kb in size by PFGE as shown in **Figure 13**. The PFGE patterns of the isolates were analyzed visually and computationally as per standard consensus guidelines [294].

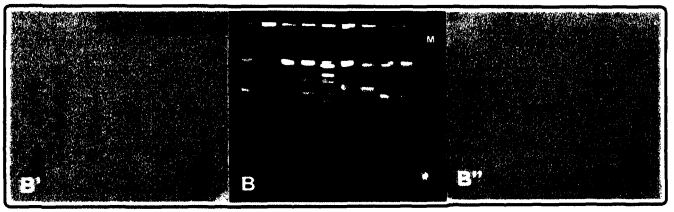
i. Visual interpretation of PFGE gels

The PFGE gels were visually analyzed and interpreted based on the consensus guidelines of Tenover et al. [294], although with more stringency with no band differences between isolates as one PFGE type. Four out of the sixty-six clinical isolates (which included one isolate each of *E. faecalis*, *E. gallinarum*, *E. avium* and *E. durans*) were excluded from visual analysis and interpretation, since these isolates were either refractory for restriction digestion, or did not yield a satisfactory separation upon repeated testing.

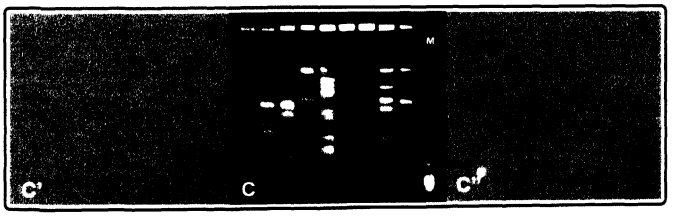
Figure 13. Representative gel images of *SmaI* macrorestriction digested chromosomal DNA of HLAR Enterococci by pulsed field gel electrophoresis [PFGE].



A. Lab number of enterococci from lanes 1- 9 in respective order:
An-1, 15,332, 9953, 1002, 5342, 9478, 14,550, 8670, 14,535.



B. Lab number of enterococci from lanes 1- 9 in respective order:
6130, 6265, 8765, 11,660, 11,871, 11,861, 11,869, 8257, 891,



C. Lab number of enterococci from lanes 1- 9 in respective order:
4343, 3844, 5969, 6275, 10,638, 7132, 881, 271, 6276.

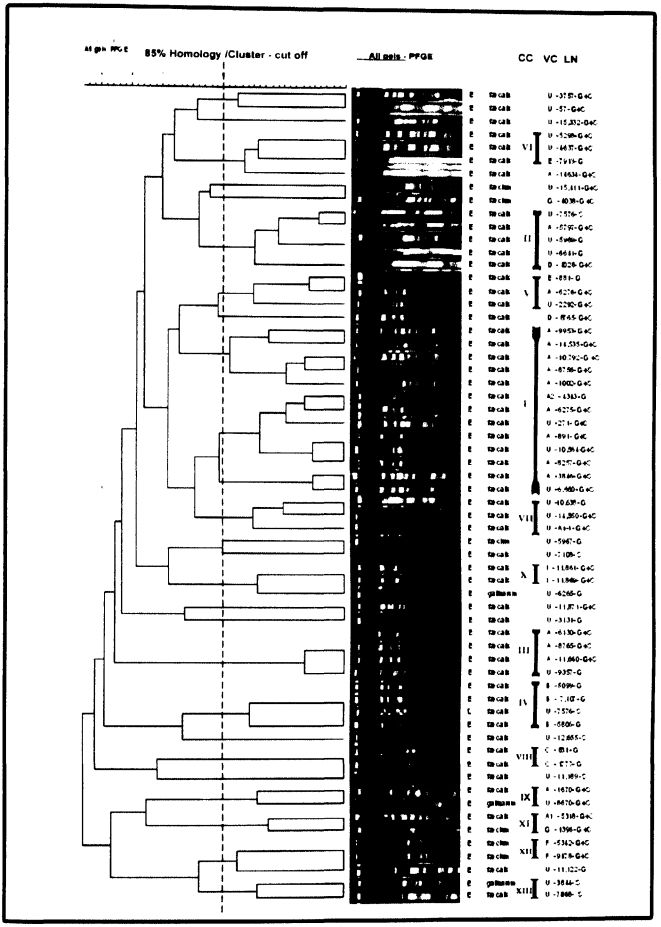
M- Lambda ladder PFG marker

The enterococci were classified into groups with respect to the restriction endonuclease (*Sma*I) digestion profiles of their genomic DNA as depicted in **Table 18 a** and **b**. The groups were designated alphabetically (in caps) for more than one isolate exhibiting the same/ indistinguishable *Sma*I restriction profile. The 33 of 62 clinical isolates along with two transconjugants studied were classified into nine groups (A-I), while the remaining 29 isolates exhibited unique *Sma*I restriction profiles that couldn't be clubbed with any groups and hence classified as "unique" restriction profiles. The 35 isolates were classified as follows; 16 isolates in group-A, two subtypes of group-A which showed a nearly identical PFGE pattern as group A isolates, three isolates in group-B and two isolates each in group-C, D, E, F, G, H and I. The group F and G included two isolates each of *E. faecium*, and group H isolates included two transconjugants, while the isolates in other groups were *E. faecalis*.

ii. Computational analysis of PFGE gels

The visually interpreted PFGE gels were subjected further for computational analysis using the Bionumerics Software 2.5, Applied Maths, Belgium. The PFGE gels were scanned and the cluster analysis was performed, by measuring the Dice coefficient similarity of the bands. The dendrogram was constructed using Unweighted Pair group method using Arithmetic averages (UPGMA) method with different position (band) tolerance and optimization settings (0.5-3.0%). Four out of the sixty-six clinical isolates (which included one isolate each of *E. faecalis*, *E. gallinarum*, *E. avium* and *E. durans*) were excluded from computational analysis and interpretation, since these isolates were either refractory for restriction digestion, or didn't yield a satisfactory separation upon repeated testing. The enterococci were classified into groups/clusters based on a homology of >85% with respect to the *Sma*I digestion profiles of their genomic DNA as shown by the dendrogram in **Figure 14** and as depicted in **Table 18 a** and **b**. The groups/clusters were designated in Roman numerals for more than one isolate exhibiting >85% homology in their *Sma*I restriction profile at different position (band) tolerance settings. A 2.0% position tolerance and 1.0% optimization setting of the gel, which was considered an optimal setting (standardized after trying different combinations of band settings), yielded a total of thirteen clusters (I-XIII).

Figure 14. Computational Cluster analysis[a] of PFGE gels of HLAR Enterococci using Dice coefficient and UPGMA method (Bionumerics, Applied Maths, Belgium).



[a] The settings used for computational cluster analysis were 2.0% (band) Tolerance, 1.0% Optimization and a Homology/Cluster cut off of 85%.

CC, Computational Clusters; VC, Visual Clusters; LN, Lab Number of test strains

N.B: The phenotypic and genotypic details of the test strains are depicted in Table 18.

The 47 of 62 clinical isolates analyzed were classified into 13 clusters (I-XIII), while the remaining 15 isolates exhibiting < 85% homology in their *Sma*I restriction profile were classified as "unique" profiles. The 47 (clustered) isolates were classified as follows; 13 isolates in Cluster I, five isolates in Cluster II, four isolates each in Clusters III and IV, three isolates each in Clusters V, VI and VII, and two isolates each in Clusters VIII-XIII. There were one each *E. faecalis* and *E. gallinarum* in Cluster IX and XIII, while Cluster XI had one each of *E. faecalis* and *E. faecium*. Cluster XII had two isolates of *E. faecium*, while the isolates in other clusters were *E. faecalis*, and the "unique" isolates had all three species among them. The clusters were subsequently compared with the visual interpretation results based on the consensus guidelines of Tenover et al. [294].

B. Plasmid REA typing

The plasmid DNA restriction endonuclease (RE) digestion of HLAR enterococci was performed using *Eco*RI a restriction enzyme that digests the gentamicin resistance plasmids but not the transposons flanking these plasmids if any, thereby enabling us to type the plasmids to assess their clonal relatedness. The 66 isolates of enterococci subjected to plasmid restriction endonuclease analysis included, 57 randomly selected HLAR clinical isolates apart from 9 standard strains and transconjugants. The 57 clinical isolates included forty five *E. faecalis*, six *E. faecium*, four *E. gallinarum*, one *E. avium* and one *E. durans* exhibiting resistance to both or either of the aminoglycosides (gentamicin/ streptomycin) separately. The *Eco*RI digestion yielded approximately 5-15 DNA fragments of various sizes ranging from 1.5-65 kb in size by agarose gel electrophoresis (**Figure 8**- Chapter IV). The plasmid RE patterns of the isolates were analyzed visually and computationally as per standard guidelines.

i. Visual interpretation of plasmid RE gels

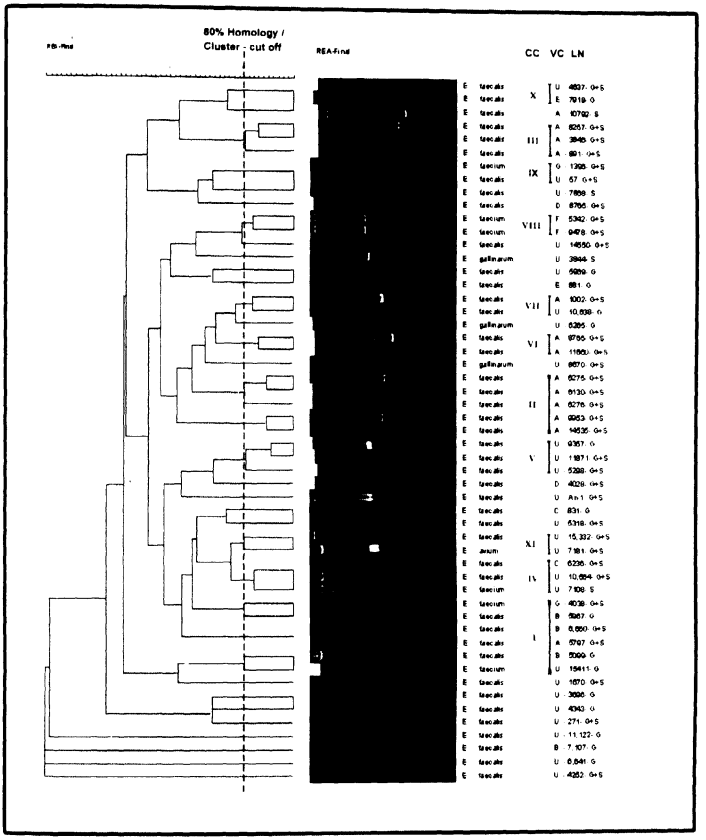
The plasmid RE gels were visually analyzed and interpreted based on the molecular weight standards run along with each gel as described previously [120, 286]. Four out of the 57 clinical isolates (which included two *E. faecalis* and one isolate each of *E. gallinarum* and *E. durans*) were excluded from visual analysis and interpretation, since these isolates didn't yield plasmids upon repeated extractions or were either refractory for

restriction digestion. The enterococci were classified into groups with respect to the restriction endonuclease (*Eco*RI) digestion profiles of their plasmid DNA as depicted in **Table 18 a** and **b**. The groups were designated alphabetically (in caps) for more than one isolate exhibiting the same/ indistinguishable *Eco*RI restriction profile. 27 of 53 clinical isolates studied were classified into seven groups (A-G), while the remaining 26 isolates exhibited unique *Eco*RI restriction plasmid profile that couldn't be clubbed with any groups and hence classified as "unique" restriction profiles. The 27 isolates were classified as follows; 13 isolates in group-A, four isolates in group-B and two isolates each in group-C, D, E, F and G. The group F and G included two isolates each of *E. faecium*, while the isolates in other groups were *E. faecalis*. The "unique" isolates had four different enterococcal species among them as depicted in **Table 18 a** and **b**.

ii. Computational analysis of plasmid RE gels

The visually interpreted plasmid RE gels were subjected further for computational analysis using the Bionumerics Software 2.5, Applied Maths, Belgium. The plasmid RE gels were scanned and the cluster analysis was performed, by measuring the Dice coefficient similarity of the bands. The dendrogram was constructed using Unweighted Pair group method using Arithmetic averages (UPGMA) method with different position (band) tolerance and optimization settings (0.5-3.0%). Four out of the 57 clinical isolates (which included two *E. faecalis* and one isolate each of *E. gallinarum* and *E. durans*) were excluded from computational analysis and interpretation, since these isolates were either refractory for restriction digestion, or didn't yield a satisfactory separation upon repeated testing. The enterococci were classified into groups/clusters based on a homology of >80% with respect to the *ECo*RI digestion profiles of their genomic DNA as shown by the dendrogram in **Figure 15** and as depicted in **Table 18 a** and **b**. The groups/clusters were designated in Roman numerals for more than one isolate exhibiting >80% homology in their *ECo*RI restriction profile at different position (band) tolerance settings. A 2.0% position tolerance and 1.0% optimization setting of the gel, which was considered an optimal setting, yielded a total of eleven clusters (I-XI).

Figure 15. Computational Cluster analysis[a] of Plasmid RE gels of HLAR Enterococci using Dice coefficient and UPGMA method (Bionumerics, Applied Maths, Belgium).



[a] The settings used for computational cluster analysis were 2.0% (band) Tolerance, 1.0% Optimization and a Homology/Cluster cut off of 80%.

CC, Computational Clusters; VC, Visual Clusters; LN, Lab Number of test strains

N.B: The phenotypic and genotypic details of the test strains are depicted in Table 18.

Table 18 a. Molecular typing results by PFGE and Plasmid REA

S. No.	Lab no.	Species	VA-Plas. group	VA-Pfge group	CA-Plas. group	CA-Pfge group	HLAR	Esp.	Gel.	Bac.	Biofilm	Date	Section	Age/Sex	Hos. No.	Ward/OPD	Diagnosis
1	8756	*E. faecalis*	No Plas.	A	No Plas.	I	G+S	+	+	-	-	25/09/02	Exd-Pus	65/M	D 331667	S-II	Hydatidosis
2	5797	*E. faecalis*	A	A	I	II	G+S	+	+	+	+	19/08/02	Blood	25/M	D 169100	CTVS	RHD
3	1670	*E. faecalis*	Unique	A	Unique	IX	G+S	+	+	-	-	19/02/02	Ex-Asc.flu.	40/F	D 247333	M3/43	Peritonitis
4	9953	*E. faecalis*	A	A	II	I	G+S	+	+	+	+	12/9/01	Urine	21/M	C 486594	Uro	NA
5	1002	*E. faecalis*	A	A	VII	I	G+S	+	+	+	+	11/2/02	Blood	55/M	D 13753	Inf.Dis.-210	Seps./Degrow.Injuries
6	14535	*E. faecalis*	A	A	II	I	G+S	+	+	+	+	3/12/02	Urine	20/M	D 561007	Uro/OPD	UTI
7	6275	*E. faecalis*	A	A	II	I	G+S	+	+	+	+	9/9/02	Blood	25/M	D 169100	CTVS	Post AVR fever
8	6276	*E. faecalis*	A	A	II	V	G+S	+	+	+	+	9/9/02	Blood	25/M	D 169100	CTVS	Post AVR fever
9	6130	*E. faecalis*	A	A	II	III	G+S	+	+	+	+	3/9/02	Blood	25/M	D 169100	CTVS	Post AVR fever
10	8765	*E. faecalis*	A	A	VI	III	G+S	+	+	+	-	20/0702	Urine	7/ M	C 982290	NA	UTI
11	11660	*E. faecalis*	A	A	VI	III	G+S	+	+	+	+	25/09/02	Urine	65/M	B 8509	M-III	Sepsis
12	8257	*E. faecalis*	A	A	III	I	G+S	+	+	+	-	12/9/02	Ex-Wn.Sw	30/F	D 765567	S-4	Duodenal perforation
13	891	*E. faecalis*	A	A	III	I	G+S	+	+	+	-	12/1/02	Ex-Pus	54/M	D 507887	S-31	Post op.wnd. infl'n
14	3846	*E. faecalis*	A	A	III	I	G+S	+	+	+	-	7/6/02	Blood	3/M	D 545765	Pd-II/21	? Meningitis
15	10792	*E. faecalis*	A	A	Unique	I	G+S	+	+	+	+	15/12/01	Ex-Pus	36/F	D 760267	S3 Casu.	Peritonitis (Bac++)
16	14634	*E. faecalis*	ND	A	ND	Unique	G+S	+	+	-	-	7/12/02	Urine	50/F	D 801885	M-II/46	UTI
17	5318	*E. faecalis*	Unique	A1	Unique	XI	G+S	+	+	+	-	31/07/02	Blood	50/M	D 548148	S1-SICU	Ulcer.Colitis+sepsis
18	4343	*E. faecalis*	Unique	A2	Unique	I	G	+	-	-	-	16/04/02	Urine	23/M	D 270181	NA	?UTI
19	7,107	*E. faecalis*	B	B	Unique	IV	G	+	+	-	+	9/11/01	Blood	1day/M	D 755975	NICU	Neonatal sepsis
20	5099	*E. faecalis*	B	B	I	IV	G	-	-	-	-	31/08/01	Blood	32/F	D 747123	Paed-I	VSD
21	5806	*E. faecalis*	ND	B	ND	IV	G	+	+		+	22/09/01	Blood	6/F	D 192261	Paed-II	Inf.endocarditis
22	831	*E. faecalis*	C	C	Unique	VIII	G	+	+	+	-	4/2/02	Blood	NA	B 642563	Med	NA
23	1777	*E. faecalis*	ND	C	ND	VIII	G	+	+	+	+	11/3/02	Blood	NB/ F	D 540193	Pd-I -NICU	Sepsis
24	4028	*E. faecalis*	D	D	Unique	II	G+S	+	+	+	-	11/5/02	Exudate	13/F	D 777157	Med-1/43	Poisoning
25	8765	*E. faecalis*	D	D	Unique	Unique	G+S	+	+	+	+	7/12/02	Blood	65/M	D 555534	M-III/42	Sepsis
26	881	*E. faecalis*	E	E	Unique	V	G	+	-	+	-	29/01/02	Exudate	12/F	D 526358	Pd-21	Peritonitis
27	7918	*E. faecalis*	E	E	X	VI	G	+	+	+	-	2/9/02	Ex-Wn.Sw	60/M	D 549725	S-4	Amputated diab.foot
28	5342	*E. faecium*	F	F	VIII	XII	G+S	+	-	-	-	5/9/01	Blood	2/F	D 748862	Paed-II	? Enteric fever
29	9478	*E. faecium*	F	F	VIII	XII	G+S	+	-	-	-	31/08/01	Urine	32/F	D 747567	M-II	?Pneumonia
30	4038	*E. faecium*	G	G	I	Unique	G+S	+	-	-	-	11/5/02	Exudate	3/F	D 773400	OG-2/16	Bacterial vaginosis
31	1396	*E. faecium*	G	G	IX	XI	G+S	+	-	-	-	7/2/02	Ex-Asc.flu.	24/F	D 764749	Med-1/46	Malabsorption
32	13514-TC	*E. faecalis*	No Plas.	H	No Plas.	NA	G+S	-	-	-	-	7/12/01	Urine	53/M	D 220541	NA	UTI
33	10564-TC	*E. faecalis*	No Plas.	H	No Plas.	NA	G+S	-	-	-	-	25/09/01	Urine	39/M	D 192942	Urology	B/L renal stone+ UTI
34	11861	*E. faecalis*	ND	I	ND	X	G+S	+	+	+	-	30/09/02	Urine	23/F	D 794441	OG-2	Post op. LSCS
35	11869	*E. faecalis*	ND	I	ND	X	G+S	+	+	+	-	30/09/02	Urine	36/M	D 341963	Uro	Neovas.Dystrop.Syn.

VA, Visual analysis; CA, Computational analysis; Plas, plasmid; Gel, gelatinase; Bac, bacteriocin
NB, No bands; ND, Not done; TC, Tranconjugant; G, HLGR; S, HLSR

D. Simpson's index of diversity and Confidence interval

The Simpson's index of diversity as shown in **Table 19**, depicts that both typing methods: Plasmid REA and PFGE were having high and equal discriminatory power. The visually interpreted results showed a marginally lesser diversity index (D) of 0.93 and 0.91 for Plasmid REA and PFGE respectively when compared with the computationally interpreted results, which showed a diversity index (D) of 0.97 and 0.93 for Plasmid REA and PFGE respectively. Since the 95% confidence intervals (CI) were overlapping as depicted in the bar graph **|Graph 1|,** the differences were not statistically significant.

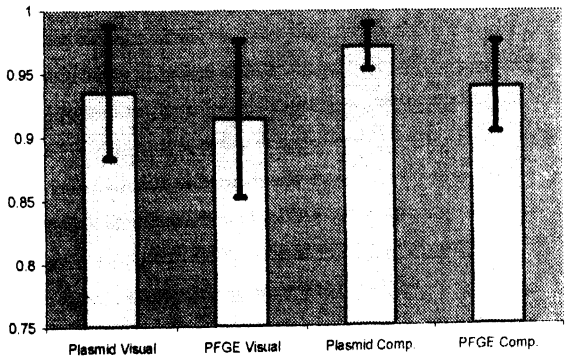
Table 19. Statistical analysis-Simpson's index of diversity and Confidence interval

Analysis	DI	95% CI	2 SE
Plasmid Visual	0.935	0.88-0.99	0.052
PFGE Visual	0.914	0.85-0.98	0.062
Plasmid Comp[a]	0.971	0.95-0.99	0.018
PFGE Comp.	0.939	0.90-0.97	0.036

DI, diversity index; CI, confidence intervals

SE, Standard error; [a]Comp. computational.

Graph 1. Representation of Simpson's index of diversity (D) for Plasmid REA and PFGE along with 95% confidence intervals (CI) according to Grundmann et al [372]



DISCUSSION

Microbial identification undoubtedly plays a major role in determining the clinical outcome of any disease.

The results of the microbial typing along with other supporting Clinico-epidemiological details helps in initiating and executing appropriate infection control measures for multidrug resistant nosocomial pathogens like enterococci in any health care setting at the appropriate time contributing to a substantial decrease in morbidity and mortality.

Although various species of enterococci were isolated at regular intervals throughout our study period as depicted in previous chapters, we could find clustering of particular species during various time periods from specific units/wards. *E. faecalis* was the commonest and predominant of all species and was isolated consistently throughout our study period, but we were unable to cluster them merely by their antibiotype. Hence molecular typing techniques like plasmid DNA analysis and chromosomal DNA analysis by PFGE were performed to determine the clonality of these isolates. The profiles of plasmid REA and PFGE gels were analyzed both, visually as per consensus guidelines [294] and using the Bionumerics software in our study. However for practical purposes, the visual interpretation of plasmid REA and PFGE gel profiles (based on the consensus guidelines of Tenover et al.) was uncomplicated as well concordant for most isolates, when compared to the computational analysis of the gel images.

Several reasons could be quoted for the higher significance of the visual interpretation of the gels especially those of PFGE. A major reason is the non-availability of costly software (Bionumerics) for analysis of gels in most of the health-care settings. The visual interpretation can normalize the gels authentically rather then arithmetically (as done by software based on the programmed settings), thereby helping to distinguish very minor differences that go undetected due to the (band) tolerance settings chosen by computer software. This was shown in our study during the standardization of the optimal (band) tolerance settings using our PFGE and Plasmid REA gel images. The optimal settings of 2.0% (band) tolerance and 1.0% optimization, and a homology cut off of 80% and 85% (for Plasmid REA and PFGE dendrograms respectively) followed in our study, was able to give clusters which helped us to discriminate/distinguish the HLAR enterococci isolated in our study. But there were instances when a decrease/increase in the settings (between 0.5-3.0%) yielded concordance in clustering for some isolates, but resulted in a diminished/discordant clustering (as compared with the clustering by visual interpretation) for the remaining isolates. Thus an optimal setting of 2.0% band tolerance and 1.0% optimization and a homology cut off of 80% or 85% was followed, which could take care of justifiable, if not stringent clustering of the isolates by computational analysis matching the consensus guidelines of categorizing isolates as indistinguishable, closely related, possibly related, or different, based on the differences in number of bands [294]. The stringent visual clustering we followed based on the consensus guidelines could not cluster 29 isolates, hence classified them as "unique", while the computational analysis yielded only 15 non-clusterable "unique" isolates which had a homology level of >80% for all the isolates in the cluster, and non-clusterable "unique" isolates were exhibiting 60-85% of homology. When we decreased the homology cut off to 75% and increased the band tolerance settings up to 3.0%, the number of clusters formed was reduced thereby grouping more number of isolates with one to six band differences into one or two clusters. The densitometric curve based Pearson product moment correlation when used for constructing dendrograms using the optimized tolerance settings yielded highly discordant clustering, since the curves obtained from different PFGE gels were not homologous due to differences in signal strength, and background noise (signals), which

were visible as non-specific peaks even though they exhibited same banding patterns visually.

The major reason for the discordance of the typing results by either method using computational analysis was due to minor inevitable differences in the separation patterns of the gel (due to minor variations/differences in running conditions of the PFGE) which could be normalized only collectively (for all gels grouped together for analysis) and not individually according to the gel pattern, on the other hand visual normalization was done as per individual gel patterns which gave authentic clustering of the HLAR enterococci. Thus for further comparisons and discussions we used the visual interpretation results of molecular typing as recommended and followed by several studies [110, 279-282, 286, 291, 293, 294], however some studies have used computational analysis alone, or in conjunction with visual interpretations for studying the clonality or epidemiology of enterococci [282, 283, 292, 293, 364]. Even though some studies have depicted some standard protocols for PFGE typing of enterococci [279, 294] there is a lack of a harmonized protocol that could be followed worldwide, which would help in minimizing the errors in the performance and interpretation of PFGE experiments.

Overall, our molecular typing study depicted (**Table 18 a** and **b**) that the strains belonging to various groups/clusters were isolated from individual patients in different wards. The wards along with their corresponding ICUs were located on different floors of the same building of the hospital block in JIPMER. The medicine wards (with their ICU) were located on the third floor, the surgical wards on the second floor, pediatrics wards on the first floor and the gynecology wards in the ground floor. The same nurses work on a single ward. Interestingly, ten of the fifteen isolates of *E. gallinarum* were from pediatrics unit, while seven of the ten isolates exhibiting a similar antibiotype were from the same ward within a span of two months. The remaining three of the ten *E. gallinarum* were isolated from the same ward in the preceding three months, one of which showed an antibiotype similar to the cluster of seven isolates. The same was the case of three *E. casseliflavus* isolated from the same pediatrics unit within a span of two months in the preceding year. Most of these (eight of ten *E. gallinarum*, and all three *E. casseliflavus*)

isolates were from cases of septicemia, and the clinical and microbiological testing results of these isolates depict the nosocomial spread of these species. Although unusual enterococcal species were prevalent in our hospital, it was the emergence of HLAR among *E. faecalis* that was of serious concern and needed special focus. Hence molecular typing of randomly selected HLAR enterococci were performed by PFGE and Plasmid REA for epidemiological investigations, and to track the dissemination and evolution of multi-drug resistant strains more efficiently in our hospital setting.

The plasmid REA typing was performed in conjunction with the PFGE typing of chromosomal DNA for many reasons. Firstly, the molecular characterization of HLAR enterococci showed that gentamicin resistance was encoded by plasmids and these plasmids were capable of conjugation, hence there lie every possibility of plasmid transfer/dissemination leading to plasmid epidemic/outbreak in any hospital setup. Secondly, some studies have depicted that plasmids can influence the outcome of PFGE typing, since plasmids if possessing the same restriction site of the enzyme used to digest the genomic DNA for PFGE, then it can appear as a same or additional band in the chromosomal DNA pattern leading to erroneous interpretation of PFGE results [280, 293]. The DNA hybridization studies performed with the PFGE gels using Gm and Sm DNA probes showed that none of the bands separated encoded Gm/Sm resistance, while plasmid DNA from all the isolates encoded Gm/Sm resistance as evident by DNA hybridization. Thus it was authenticated that the bands resolved by PFGE were exclusively of chromosomal origin and none were of plasmid origin, thereby ruling out that plasmids did not influence the results of PFGE typing in our study (**Figure 13**). Moreover, plasmid fingerprinting is most useful for epidemiological studies that are limited both temporally and geographically.

Although chromosomal DNA analysis by PFGE is considered a gold-standard technique for molecular epidemiological studies since last decade, the plasmid DNA fingerprinting was the first molecular method used as a bacterial typing tool. Zervos et al. [18] authenticated nosocomial transmission and exogenous acquisition of *S. faecalis* for the first time using plasmid DNA content as an epidemiological marker. Since then,

several studies applied plasmid DNA fingerprinting for molecular epidemiology of nosocomial enterococci [47, 284, 285]. The evaluation of whole plasmid DNA although shown to be helpful in plasmid epidemics [286] have some limitations, since the undigested profiles often show multiple forms of plasmids, with circular and linear forms of plasmids migrating at different rates than the covalently closed circular form, which leads to erroneous interpretation of plasmid DNA fingerprinting results. Hence, restriction digestion of plasmid DNA is recommended and being followed for molecular epidemiological studies of drug resistant enterococci [47, 125, 132, 135, 284, 285, 373].

The visual interpretations of the Plasmid REA and PFGE gels based on the similarity of clusters/groups obtained by matching the consensus guidelines of Tenover et al. showed concordance between both typing methods, barring few exceptions, which had some clinico-epidemiological significance when analyzed. Most of the clusters obtained were concordant for both the typing methods in our study, since isolates grouped in Clusters A-F by either typing method exhibited highly homologous patterns resulting in identical clusters, which depicts a possible "intrahospital strain dissemination" as shown by other studies [280, 286], while the isolates exhibiting "unique" REA profile were also possessing "unique" PFGE profile. Two HLGR *E. faecalis* isolates that exhibited an identical plasmid REA pattern and grouped as cluster B showed "unique" heterologous PFGE patterns of their chromosomal DNA that were different from other isolates in PFGE group B cluster. The discordant plasmid REA typing results were of epidemiological significance, since intrahospital and interhospital (HLGR) plasmid dissemination has been depicted by few studies [286, 354]. The versatile genetic machinery of enterococci facilitates the transmission/dissemination of the conjugative plasmids between closely related strains [17, 138]. Hence, the same plasmid type (group B) present among two *E. faecalis* isolates with different chromosomal restriction endonuclease pattern (unique) as evident by PFGE, depicts that the intrahospital dissemination of (HLGR) plasmids from group B isolates to closely related strains (which would have been otherwise sensitive to aminoglycosides) could have occurred as shown by studies from U.S. and Japan [135, 286]. Our results were further authenticated because both the discordant isolates were pheromone responsive, and were able to transfer the

HLGR determinants in vitro at a high frequency. Thus our Plasmid REA typing results depict that antimicrobial resistant determinants encoded by plasmids can be disseminated separately (intrahospital plasmid dissemination) leading to plasmid outbreak/epidemic, apart from the commonly occurring intrahospital strain dissemination.

The Molecular typing results were instrumental in deriving the much needed clinical and epidemiological significance of the HLAR isolates. The PFGE typing showed that approximately 50% (33) of the (62) HLAR enterococcal isolates were homogeneous and formed clusters/groups (A-I), while the remaining 50% of the isolates were non-clusterable depicting heterogeneity/diversity among the HLAR isolates. The PFGE genotypic Cluster-A isolates were found to be "endemic" in our hospital during our study period, since they were present in different medical and surgical wards situated in I, II and III floors for more than a year from September 2001 to December 2002 as shown in **Table-18 a** and **b**. Incidentally, three consecutive *E. faecalis* isolates which were genotypically similar and grouped under Cluster-A were from a single patient with fever after Aortic valve replacement. Thus, our results authenticate that in majority of the Infective endocarditis cases when consecutive blood culture samples yield the same organism with a similar antibiotype it could be considered as empiric evidence of the etiogen as shown by several studies [162-164]. Interestingly, two *E. faecalis* isolates showed a "closely related" PFGE pattern of cluster-A isolates with 2-3 band differences and classified as A1 and A2, since we followed a stringent definition for strain clustering [294]. As depicted in the consensus guidelines, these A1 and A2 *E. faecalis* isolates would have originated from cluster-A isolates after a possible genetic event, i.e., a point mutation or an insertion/deletion of DNA, since these two strains were isolated from same wards during the same time period when cluster-A strains were prevalent [294]. Our results depict that enterococcus has high possibilities for genomic rearrangements, which would be evident through molecular typing techniques like PFGE. This evidence would be highly helpful to trace the outbreak related isolates, like those of A1 and A2 as shown in the present study. Furthermore, the endemicity of Cluster-A isolates documented by PFGE typing would be remarkably useful for complementing the clinical and epidemiological analysis of HLAR enterococcal isolates or any nosocomial outbreak.

Three *E. faecalis* PFGE Cluster-B strains were isolated from pediatrics ward and the neonatal ICU (NICU) within a span of three months during 2001, but not isolated there after. This suggests that routine sanitation measures (without the knowledge of the prevalence of *E. faecalis* isolates) practiced by the particular ward was sufficient enough to prevent (or eradicate) the dissemination of antimicrobial resistant nosocomial pathogens. The *E. faecalis* strains from clusters C, D, E and I were isolated from the same or different wards during 2002, suggesting the circulation/dissemination of the HLAR *E. faecalis* in our hospital. The HLAR *E. faecium* isolates from clusters F and G were isolated within a short span during 2001 and 2002 respectively, which were also possessing "esp" gene. The prevalence of the strains with same pulsotype all through our study period indicates widespread dissemination of these HLAR enterococci in our hospital setup (intrahospital strain dissemination) as shown by several studies [161, 220, 280, 286].

The predominance of strains from cluster A, along with strains from other clusters (B, C, D, E) in particular ward(s) during specific time periods suggest that these isolates were derived from a common source and spread from patient to patient, however we do not know the method of transient carriage in the nosocomial transmission of the commonly prevalent aminoglycoside resistant enterococci, since the reservoir and mode of transmission of the HLAR enterococci were not determined in our study. However, several researchers have studied the epidemiology of nosocomial environmental reservoirs of multidrug resistant enterococci. Enterococci have been shown to be capable of prolonged survival on hands, gloves, thermometers, blood pressure handcuffs, IV fluid pumps, bedrails and linen and various hospital environmental surfaces [65, 70, 73, 74]. Further, nosocomial enterococci were shown to be resistant to heat (upto 80^0C for 1 min), and could withstand routine disinfection procedures (150ppm chlorine) followed for infected linen, which underscores the significance of enterococci to survive and disseminate in the hospital environment [71]. Most of the studies have shown concordance between hospital environmental strains and the patient isolates often resistant to vancomycin or high-level gentamicin, which were confirmed using molecular epidemiological tools like PFGE and/or Plasmid typing [54, 75-80]. Thus as suggested in

these studies any of the above mentioned source(s) could have been the reason for "intrahospital dissemination" of enterococci in our hospital.

The 50% (29) non-clusterable isolates with "unique" PFGE patterns were isolated from various wards (including those wards where the predominant clusters of enterococci were found) throughout our two-year study period. Although few of these "unique" strains were "possibly related" with the isolates from different clusters based on the consensus guidelines [294], the genomic heterogeneity exhibited by majority of the non-clusterable isolates depicts the diversity of HLAR enterococci as shown by several studies from U.S, Netherlands, Norway, Greece, U.K, [52, 282, 283, 292, 363]. The diversity (50%) of the isolates as shown by PFGE typing in our study suggests that, apart from patient-to-patient spread that was equally (50%) a major cause for dissemination of clusterable (homogenous) HLAR isolates, other possible sources can be due to colonizer isolates or fecal contamination, or they could be community acquired isolates. Several epidemiological studies conducted in human subjects from community have yielded enterococci resistant to various antimicrobials like ampicillin, gentamicin and vancomycin [81-83]. Thus screening the inpatient population for fecal carriage of antimicrobial resistant enterococci, and conducting point-prevalence studies would be highly significant and necessary in the wake of HLAR and emergence of vancomycin resistance among enterococci. This would help in initiating appropriate infection control measures and restructuring the hospital antibiotic policy, if needed.

The discriminatory power of a typing method is its ability to distinguish between unrelated strains. As discussed above, it is determined by the number of types defined by the test method, and the relative frequencies of these types. These two facets of discrimination are not generally presented as a single numerical value and therefore cannot be used for a straightforward comparison of different methods. Hence, we followed a method previously described by Hunter and Gaston [371] to give a single numerical index of discrimination of the typing method. An approximate 95% confidence interval was calculated as proposed by Grundmann et al. [372]. Our statistical results depicted (**Table 19**) that both Plasmid and PFGE typing methods, as well visual and

computational analysis of these results had an equally high discriminatory index. Hence, the choice of method depends on the user's need and the resources they are provided with. Our statistical typing results were highly concordant with another recent study, which used AFLP and PFGE for typing ampicillin resistant *E. faecium* [283].

Studies have shown that epidemic drug resistant enterococci may possess specific genetic characteristics (encoding virulence determinants) resulting in a distinct lineage, which could facilitate enhanced colonization/infection of the host [193]. The permeation of these virulence genetic characteristics into different species differs according to the setup, patient demographics and other extrinsic factors. The "esp" gene and associated virulence factors (**Table 14-** Chapter IV) have permeated deeply into the *E. faecalis*, since 65% of these isolates exhibited the presence of this putative virulence factor. Our PFGE typing results, which depicts the clonality of the HLAR isolates, reveals that majority (except one) of the clusterable isolates (A-I) possessed "esp" gene (including two *E. faecium* clusters), while they were absent among 41% of the unique/non-clusterable isolates. The clustering of these "esp" positive isolates proves that strains of this chromosomal lineage are related, and may have been derived from a common ancestral strain as shown previously [216]. However, the presence of the "esp" gene among 60% of unique/non-clusterable *E. faecalis* isolates also depicts that the pathogenic potential due to the presence of "esp" gene need not be confined to a single genetic lineage as shown by a recent study [220], since closely related lineages/genotypes can be equally virulent resulting in epidemicity.

Our findings suggest a strong association between the presence of "esp" gene, and high-level gentamicin resistance, although both have been shown to reside on entirely different genetic determinants (chromosomal and extra-chromosomal [plasmid] respectively). The prevalence of this combination is of high clinical significance in any health-care setup, since several studies have shown that specific genetic lineages (as shown by PFGE) exhibited the presence of "esp" with vancomycin and/or ampicillin resistance among clinical *E. faecalis* and *E. faecium* isolates [207, 219, 220, 364]. These studies suggests that prior treatment/exposure to the antibiotics like vancomycin and/or

ampicillin would have selected these clones facilitating to reach ecological abundance in the nosocomial habitat due to the presence of "esp" gene, although studies are yet to analyze the significance of the combination of HLGR and "esp" gene. Thus, our findings suggest that the higher prevalence of gentamicin resistance in our hospital would have contributed to selection of those clones/lineages with "esp" gene resulting in "intrahospital dissemination". Since, majority of these clones possess conjugative gentamicin resistance plasmids and the "esp" gene was transferable in vitro, the possibilities for "interhospital strain dissemination" remains very high. Thus appropriate measures to contain the antimicrobial resistance in any health care setup would by all means decrease the probability of selection and dissemination the "esp" positive clones, which poses a stiff challenge ahead.

Although we performed PFGE in a single stretch using the isolates collected during our study period, our molecular typing results depict that concomitant performance of molecular typing (by PFGE and/or Plasmid REA typing) during any suspected outbreak/increase in the prevalence of nosocomial pathogens would be highly helpful in tracking and preventing the dissemination of multi-drug resistant strains/ clones, or plasmid determinants (encoding resistance) more efficiently at a given point of time in a hospital setup. Some studies have shown that multicenter PFGE studies with a harmonized protocol and centralized server for interpretation can address epidemiological questions effectively [296]. Thus a cooperative venture for molecular typing of enterococci would provide a rapid tracking system to assist hospitals, clinics and chronic care facilities in controlling the spread of multidrug-resistant enterococci locally, nationally as well globally.

SUMMARY

Our molecular typing results depict that PFGE in conjunction with Plasmid REA typing would be highly helpful in tracking the intrahospital strain dissemination, as well dissemination of genetic determinants. The PFGE typing of the chromosomal DNA of HLAR enterococci yielded approximately 12-20 DNA fragments of various sizes ranging

from 50-450 kb in size. The PFGE patterns of the isolates were analyzed visually and computationally as per standard guidelines. The visual analysis classified 33 of 62 clinical isolates along with 2 transconjugants into 9 groups (A-I) based on the PFGE pattern, while the remaining 29 isolates were classified as "unique" based on their restriction profiles, since their profiles could not be clubbed with any groups. The computational analysis of PFGE gels depicted several clusters of isolates through the dendrogram constructed by the dice-coefficient and UPGMA method. They were partly concordant with the clusters formed by visual interpretation, although differences surfaced between results of clustering with either interpretation methods.

The plasmid REA typing of HLAR enterococci after *Eco*RI digestion yielded approximately 5-15 DNA fragments of various sizes ranging from 1.5-65 kb in size. The plasmid RE patterns of the isolates were analyzed visually and computationally as per standard guidelines. The visual analysis classified 27 of 53 clinical isolates studied into 7 groups (A-G) based on the plasmid REA pattern, while the remaining 26 isolates were classified as "unique" based on their plasmid restriction profiles, since their profiles could not be clubbed with any groups. The computational analysis of plasmid RE gels depicted several clusters of isolates through the dendrogram constructed by the dice-coefficient and UPGMA method. They were partly concordant with the clusters formed by visual interpretation, although differences surfaced between results of clustering with either interpretation methods. The Simpson's index of diversity depicted that both typing methods, Plasmid REA and PFGE used in our study were having a high and equal discriminatory power, while the interpretation of the molecular typing results, visually as well computationally showed approximately the same diversity index and the differences were not statistically significant. Thus the choice of interpreting molecular typing results depends on the users need and objective, although we prefer visual analysis of the gels for lesser sample size. Finally, the concordance between PFGE typing and Plasmid REA depict that a combination of both the typing methods would help in better discrimination of HLAR enterococci, when we speculate an involvement of both chromosomal and extra-chromosomal elements in antimicrobial resistance.