# Chapter III

## Molecular Characterization of High-level Aminoglycoside Resistant Enterococci

# CHAPTER III

## 15. MOLECULAR CHARACTERIZATION OF HIGH-LEVEL AMINOGLYCOSIDE RESISTANT ENTEROCOCCI

The high-level aminoglycoside resistance exhibited by nosocomial enterococci apart from posing therapeutic challenge exacerbates the issue further, since their machinery helps in dissemination or transfer of these determinants (drug resistance) via. plasmids and transposons to other closely related species and genus more rapidly in nosocomial settings [13, 121, 138]. The aminoglycoside resistant enterococci exhibit versatility in their genetic mechanism to encode resistance to a single antimicrobial (aminoglycoside) in more than one way [8]. Earlier studies have shown that there lies heterogeneity among the genetic determinants (plasmids) encoding HLAR in enterococci [18, 135, 292, 350, 354-356] however, several recent studies have shown that a predominant type of plasmid was present among many HLGR enterococci, which depicts their widespread dissemination in any given setting [18, 251, 254, 354, 357]. Although a number of plasmids have been associated with HLGR, the nature of the plasmids varies geographically due to several reasons [121, 122]. Hence after phenotypic and genotypic analysis of HLAR enterococci, molecular characterization of the genetic determinants encoding aminoglycoside resistance helps in revealing the differences, if any, in the geographical trends of molecular basis of aminoglycoside resistance in enterococci, to understand the epidemiology of HLAR enterococci.

### OBJECTIVE

- To detect and molecular characterize the genetic determinants encoding high-level aminoglycoside resistance in enterococci.

## MATERIALS AND METHODS

### 1. Molecular characterization of HLAR enterococci

#### *A. Plasmid DNA profiling of HLAR enterococci*

***i) Alkaline lysis method:*** The mini-preparations of plasmid DNA from HLAR enterococci were obtained by the standard alkaline lysis method [327] with minor modifications as described below.

***Cell harvesting***

- A single bacterial colony was inoculated into Todd Hewitt broth (THB 5ml) with gentamicin 500 µg/ml and incubated at 37°C with vigorous shaking overnight.
- 1.5 ml of THB culture was transferred to an eppendorf tube and centrifuged at 13,000 rpm for 30 seconds at 4°C (This step was repeated if necessary, by decanting the supernatant and adding 1.5 ml culture to the same eppendorf to increase the cell mass).
- Then the medium was removed by aspiration, and the bacterial pellet was left to dry.

***Cell lysis***

- The bacterial pellet was resuspended in a solution containing lysozyme 10 mg/ml in 10 mM Tris, 1 mM EDTA (pH 8.0), and 25% (w/v) sucrose and incubated in a water bath at 37°C for 1 hour.
- Then the mixture was centrifuged at 13,000 rpm for 30 seconds, and the supernatant was decanted without disturbing the cell pellet.
- The cell pellet was resuspended in 100 µl of ice-cold **Solution-I** containing RNAse 100 µg/ml by vigorous vortexing.
- Subsequently, 200 µl of freshly prepared **Solution-II** was added and mixed by inverting the tube rapidly for 5 times (without vortexing), and the tube was stored on ice.

- Then 150 µl of ice-cold **Solution-III** was added and vortexed gently for 10 seconds to disperse **Solution-III** through viscous bacterial lysate and the tube was stored on ice for 3-5 minutes.
- Then centrifuge at 13,000 rpm for 10 minutes at 4°C in a microfuge and transfer the supernatant to a fresh tube (If the supernatant was not clear re-centrifuge as mentioned above till the supernatant was clear).

***Recovery of Plasmid DNA***

- Equal volumes of Isopropanol was added to the supernatant and mixed, and incubated at room temperature for 15 minutes (for better recovery of plasmid DNA the tube was stored at - 20°C for 1 hour).
- The mixture was centrifuged at 13,000 rpm for 20 minutes at 4°C and the supernatant was carefully decanted leaving the (invisible) pellet undisturbed.
- 1.5 ml of 70 % ethanol was mixed to wash the (invisible) pellet thoroughly at 4°C and centrifuged at 13,000 rpm for 15 minutes at 4°C.
- The supernatant was removed by aspiration carefully and the pellet was allowed to air dry for 10 minutes.
- Finally, the pellet was redissolved in 40µl of Milli-Q water (by vortexing briefly) and stored at - 20°C till further analysis.

***Lysis/Extraction buffers and Solutions***

**Solution-I**

50 mM glucose

25 mM Tris-Cl (pH 8.0)

10 mM EDTA (pH 8.0)

*Solution-I was prepared in batches of 100 ml, autoclaved for 15 minutes at 15 psi on liquid cycle and stored at 4°C.*

**Solution-II**

0.2 N NaOH (freshly diluted form a 10 N stock)

1 % SDS

*Solution-II was prepared freshly before use.*

**Solution-III**

| | |
|---|---|
| 5 M Potassium acetate | 60.0 ml |
| Glacial acetic acid | 11.5 ml |
| Double distilled water | 28.5 ml |

*Solution-III was stored at 4°C and transferred to an ice bucket just before use.*

***ii) Restriction endonuclease digestion of plasmid DNA and Separation*:** The plasmid DNA isolated by alkaline lysis method was digested with restriction endonuclease *Eco*RI (Bangalore Genei, India) in accordance with the manufacturer's specifications as described below.

The following mixture was added to a microfuge tube

| | |
|---|---|
| 10X restriction enzyme buffer | 3 µl (to a final 1 X concentration) |
| Plasmid DNA | 10 µl |
| *Eco*RI restriction enzyme | 1 µl |
| Double distilled water | 16 µl |

(The final volume was made up to a 30 µl)

- The restriction enzyme was added finally and gently mixed by spinning for 1-3 seconds in microfuge. The mixture was incubated in a water bath set at 37°C for 5 hours.
- The whole (undigested) plasmids and restriction digested plasmid DNA were separated on 0.8 % agarose gel using 0.5 X TBE at 60 V stained with ethidium bromide, visualized under an UV-transilluminator and documented using a gel documentation system (Vilber-lourbet, France).

***B. Southern blotting of Plasmid DNA***

The experimental procedure was carried in Center for cellular and Molecular biology (CCMB), Hyderabad, India. The whole (undigested), and the restriction-digested plasmid DNA separated on agarose gels were transferred onto a nylon membrane by Vacuum blotting as described previously for Southern hybridization [327] with minor modifications.

***i) Vacuum transfer*:** The plasmid DNA separated on agarose gel was vacuum transferred onto nylon membranes by an alkaline transfer method using an in-house Vacuum blotting apparatus (CCMB, Hyderabad, India) and Hybond-N+, which is a positively charged nylon membrane (Amersham biosciences) where nucleic acid samples may be fixed by simple alkali treatment or alkali blotting, rather than UV exposure or baking. The brief step-wise procedure followed was as below mentioned:

- The vacuum-blotting unit (apparatus) was kept in proper orientation to carry out the blotting procedure.
- First, the pump inlet on the front panel was connected to the liquid trap, which in turn was connected to the base of the vacuum blotter.
- Then a 3MM Whatman filter paper was placed on the vacuum plate after wetting it with distilled water, onto which the Nylon membrane (Hybond N+ membrane) cut according to the dimensions of the agarose gel, was placed after wetting it with distilled water.
- A plastic mask (made from a polyethylene sheet) with a window in the center, cut according to the dimensions of the agarose gel was placed on the membrane in such a way that it overlaps each side of the membrane by approximately 5 mm.
- Then the frame was placed on top of the unit and the clamps were tightened.
- The agarose gel was placed onto to the membrane by gradually sliding the gel without entrapping air bubbles.
- It was made sure that the gel and mask overlapped by at least 2 mm, while small cracks or leakages in the agarose gel were sealed with low melting point agarose.
- ***Depurination***- The gel was covered fully with about 20 ml (depending on gel size) of **Solution-I** (with a pipette) and immediately the vacuum pump was switched on and adjusted to exert 20 pounds pressure.
- The depurination step for plasmid DNA was carried until the bromophenol blue on the agarose gel turned yellow (about 20 minutes) while the depurination for chromosomal DNA from PFGE gels was carried for a longer duration (about 40 minutes). Excess **Solution-I** over the gel was removed after depurination by wiping the gel surface with a gloved finger or by using pipette.

- ***Nucleic acid transfer-*** Immediately **Solution-II** was poured onto the gel to cover it fully and transferred for about 1- 1.5 hours depending on the size of the DNA.
- It was made sure that the gel remains immersed all the time with **Solution-I** or **Solution-II** whichever applicable during the depurination or transfer process.
- Once the transfer was completed, excess solution was removed from the gel and the pump was turned off.
- The gel was removed carefully and stained with Ethidium bromide (0.5 μg/ml) for 20 minutes and examined under UV-transilluminator for checking the efficiency of nucleic acid transfer.
- Finally, the nylon membrane was removed (a lower right corner cut was made, to mark the orientation of the transfer of nucleic acid from agarose gel) and washed in **Solution-III** for 5-10 minutes with agitation, air-dried and stored at 4°C for subsequent hybridization experiments.

**Solutions and Buffers**

| | |
|---|---|
| **Solution-I** | 0.22 M HCl (Depurination solution) |
| **Solution-II** | 0.44 N NaOH (Alkaline transfer solution) |
| **Solution-III** | 2 X SSC |

*C. **Preparation of DNA probes for HLAR genes***

The DNA probes for high-level gentamicin resistance [aac(6')+aph(2")] and high-level streptomycin resistance [ant(6)-I] genes were prepared as per standard procedures using kits wherever applicable. Briefly, the aac(6')+aph(2") and ant(6)-I genes were amplified from a standard strain by PCR and separated on a 2 % agarose gel. The gene specific fragments were gel purified using QIAGEN gel DNA extraction kit as per manufacturer's instructions (QIAGEN, Germany) and stored at-20°C till the radiolabelling of the probes were done.

***i) Gel DNA extraction procedure:*** The gel DNA extraction protocol using a microcentrifuge was followed as per manufacturers instructions, with all centrifugation steps carried out at 13,000 rpm on a tabletop microcentrifuge (Biofuge, France).

- Briefly, the HLGR and HLSR gene specific DNA fragments were excised from the agarose gel with a clean, sharp scalpel by placing the gel on a UV transilluminator using the reflector lights in the system.
- Then the gel slice was weighed in a colorless tube, and three volumes of Buffer QG (containing guanidine thiocyanate) was added to one volume of gel and incubated at 50°C for 10 minutes with intermittent vortexing during incubation.
- After dissolving the gel slice completely, the color of the mixture was checked to be yellow. If so, one gel volume of Isopropanol was added to the sample and mixed.
- Then the sample was applied to a QIAquick column to bind DNA, which was placed in the 2 ml collection tube provided, centrifuged for 1 minute and the flow-through was discarded.
- Then 0.75 ml of Buffer PE was added to the column for washing, centrifuged twice for a minute and the flow-through discarded.
- Finally, the QIAquick column was placed into a clean, sterile 1.5 ml microcentrifuge tube and the DNA was eluted by adding 50 µl of Milli-Q water to the center of the column, and let the column stand for a minute before centrifuging for a minute. The eluted DNA collected in the microcentrifuge tube was stored at - 20°C till the radiolabelling of the probes were done.
- The eluted DNA was confirmed by running the sample on a 2 % agarose gel with a molecular weight marker used previously.

***ii) Radiolabeling of the DNA probe by Random priming method***: The radiolabeled DNA probes were generated by using a random primer kit and a radiolabeled dNTP- [α-32P] dATP (BARC, Mumbai, India) as per manufacturer's instructions.

- Briefly, 2 µl of template DNA was added (gel purified and eluted DNA corresponding gentamicin and streptomycin genes, and lambda DNA [New England Biolabs, UK]) in 20 µl of sterile water using a sterile 1.5 ml microfuge tube.

- The DNA was denatured at 94°C for 2 minutes by placing the tube in a boiling water bath, and the tube was removed and snap-freezed immediately by placing the tube on ice.
- Then add appropriate volumes of reagents were added in the following order:

| | |
|---|---|
| Random primer buffer solution | 5 µl |
| Random primer solution | 5 µl |
| dNTP mix (4 µl each) | 12 µl |
| Radiolabeled dNTP-[α-32P]dATP | 4 µl |
| Klenow enzyme | 2 µl |

  The final volume of the reaction mix was made up to 50 µl.
- The components were mixed gently by inverting the tube several times, and quick-spun for 2 seconds in a microfuge at maximum speed.
- Then the tube was incubated with the reaction mixture at 37°C for 1 hour in a water bath.

***iii) Purification of radiolabeled DNA probe by Spun-Column chromatography***: The Spun column chromatography was used to separate the labeled DNA that passes through the gel-filtration matrix, from lower molecular weight substances (viz, radioactive precursors) that are retained on the column as per standard procedures [327].

- Briefly, a 1-ml disposable syringe was plugged with a small amount of sterile glass wool (Supelco), which was accomplished by using the barrel of the syringe to tamp the glass wool in place.
- Then the syringe was filled with Sephadex G-50 (Amersham biosciences, U.S.A) equilibrated in 1X TEN buffer (pH 8.0) and the buffer flown through by tapping the side of the syringe barrel. The resin was added till the syringe was completely full.
- The syringe was inserted into a 15-ml disposable plastic tube and centrifuged at 1600g for 4 minutes at room temperature in a swinging-bucket rotor in a bench-top centrifuge.

- The resins were packed down and became partially dehydrated during centrifugation, and the steps were repeated till the volume of packed column was 0.9 ml.
- The DNA sample was applied to the center of the column in a total volume of 0.1 ml (50 µl of Milli-Q water was added to the 50 µl of the random primed mix to make up the total volume to 0.1 ml), and the spun column was placed in a fresh disposable tube containing a decapped microfuge tube. The centrifugation was carried out as in previous step and the effluent DNA was collected into the decapped microfuge tube.
- The syringe was removed which contained unincorporated radiolabeled dNTPs and other small components, and disposed off safely in a radioactive waste.
- The decapped tube was removed carefully using forceps, recapped and labeled appropriately and stored at -20°C until needed.
- The rough estimate of the proportion of radioactivity that has been incorporated into the template DNA was obtained by holding the tube with eluted DNA to a hand-held radioactivity minimonitor.

**Buffer**

10X TEN buffer

0.1 M Tris-Cl (pH 8.0)

0.01 M EDTA (pH 8.0)

1 M NaCl

***D. DNA-DNA hybridization studies***

The nylon membranes transferred with the digested and undigested plasmid DNA, as well the restriction digested chromosomal DNA (separated by PFGE) from the enterococcal test isolates and standard strains were hybridized with the DNA probes (HLGR gene probe, HLSR gene probe and lambda DNA marker probe) prepared previously, as per standard procedures [327] with minor modifications.

***i) Prehybridization***

- The nylon membranes were rolled into the shape of cylinder and placed inside the roller bottle together with the plastic mesh provided by the manufacturer (Hybaid, Thermo Hybaid, U.S.A).
- Approximately 0.1 ml of Pre-hybridization solution (containing equal volumes of SDS and $Na_2PO4$, i.e: 7 % SDS and 0.5 M $Na_2PO4$) was added for each square centimeter of the membrane (20 ml in total) and the bottles were closed tightly.
- Then the hybridization tubes were placed inside the pre-warmed hybridization oven (Hybaid, Hybaid, U.S.A) at 65°C for 15-20 minutes with agitation.

***ii) Hybridization***

- The Prehybridization solution was decanted from the hybridization bottle and replaced with same volume of fresh solution containing the radiolabeled DNA probe.
- Then the bottles were closed tightly and replaced in hybridization oven quickly at 65°C, and the hybridization was carried out for 18 hours with agitation.
- Before adding the probe to the hybridization solution, it was denatured at 100°C for 5 minutes by placing the tube in a boiling water bath (since the probe has double stranded DNA) and snap-frozen by placing it on ice.

***iii) Post Hybridization***

- After hybridization the membranes were removed from the hybridization bottles, and excess hybridization solution were briefly drained from the membrane by holding the corner of the membrane with forceps to the lip of the bottle/container.
- The hybridization solution with the radiolabeled probe was decanted into a dark bottle, sealed and stored at -20°C for reuse.
- The membrane was rinsed thrice for 2 minutes with **Wash Solution-I** (2X SSC and 0.5 % SDS) using fresh solution for every rinse, and decanted into a radioactive disposal container.

- Then 25 ml of **Wash Solution-I** (1 ml/cm2 membrane) was added fresh to the membranes in the roller bottles, and kept in the hybridization oven at 65°C for 20 minutes with agitation.
- The **Wash Solution-I** was decanted and replaced with 25 ml of **Wash Solution-II** (0.1X SSC and 0.5 % SDS) and kept in the hybridization oven at 65°C for another 20 minutes with agitation.
- Finally, the membranes were removed from the bottles and the liquid drained off from them by placing it on a pad of towels.
- Then the damp membrane was placed on a sheet of Saran Wrap to cover it, and exposed appropriately to obtain an autoradiographic image.
- The radioactivity of the membrane after hybridization was measured using a hand-held radioactivity minimonitor, before Autoradiography.

**Solutions**

**Prehybridization Solution** (20 ml)

14 % SDS - 10 ml (7 %)

1 M $Na_2PO4$ - 10 ml (0.5 M)

**Wash Solution-I** (100 ml)

20X SSC - 10 ml (2X)

10 % SDS - 5 ml (0.5X)

Make up the volume to 100 ml with double distilled water

**Wash Solution-II** (200 ml)

20X SSC - 1 ml (0.1X)

10 % SDS - 10 ml (0.5X)

Make up the volume to 200 ml with double distilled water.

***E. Autoradiography/Phosphor imaging***

The hybridized membranes were exposed and images were developed using a phosphor imager (FUJIFILM, Kanagawa, Japan). The phosphor imaging has an edge over the conventional autoradiography since it is a robust and rapid method consuming very

minimal time for exposing and developing the radiographic images, without any hazard for the user.

- Briefly, the hybridized membranes covered with Saran wrap were exposed onto a Phosphor Imaging Plate- IP (FUJI FILM, Kanagawa, Japan) by placing the IP in a Cassette - 20 X 25 (BAS Cassette- 2025, FUJIFILM, Kanagawa, Japan) for 1-2 hours. After the exposure the IP was removed from the Cassette and scanned by a laser releasing photons that were collected to form an image by the Phosphor Imaging device (Phosphor Imager, FUJIFILM, Kanagawa, Japan).
- The images were captured electronically, viewed and stored in the computer attached for further analysis.

***F. Stripping/Deprobing membranes***

The nylon membranes after phosphor imaging were reused for the subsequent experiment with a different probe, after successful removal/stripping off the probes from the membranes as per the manufacturer's instructions (Hybond-N+, Amersham biosciences).

- Briefly, the nylon membrane was placed in a glass tray onto which 500 ml of 0.1% hot boiling SDS solution was poured and kept for 1 hour with shaking.
- The procedure was repeated if the membrane exhibited significant radioactivity as detected by a hand held minimonitor.
- After stripping, the membrane was exposed to a phosphor imager as described previously to quantify the radioactivity before reusing the membrane with another probe.

## RESULTS

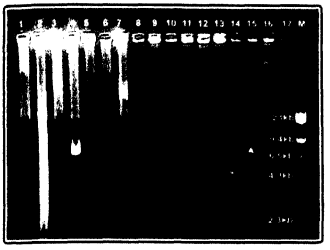
### 1. Molecular characterization of HLAR enterococci

#### A. Plasmid DNA profiling of HLAR enterococci

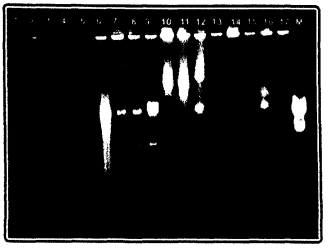
The genotyped enterococcal isolates were subjected for molecular characterization to study the genetic basis of their aminoglycoside resistance. Briefly, the plasmid DNA was isolated from 66 isolates of enterococci, which included 57 randomly selected HLAR clinical isolates and nine standard strains and transconjugants. The 57 HLAR (as confirmed by PCR) isolates included forty-five *E. faecalis*, six *E. faecium*, four *E. gallinarum*, one *E. avium* and one *E. durans*. The whole plasmids, as well the restriction digested plasmids were separated, electrophoresed and interpreted accordingly.

The whole plasmid and the *Eco*RI digested plasmid profiles of the enterococcal isolates are depicted in **Figures 7** and **8**. Most of the HLAR enterococci yielded between one to five plasmids. Majority isolates possessed at least two plasmids and the molecular weight of the plasmids ranged approximately $\geq$ 70 kb to 2 kb. The restriction-digested plasmids were classified into groups with respect to the *Eco*RI profiles of their plasmids as depicted in **Table 13.a and b**. The groups were designated alphabetically (in caps) for more than one isolate exhibiting the same *Eco*RI restriction plasmid profile. Four out of the fifty-seven test isolates (two *E. faecalis* and one isolate each of *E. gallinarum* and *E. durans*) did not yield plasmids upon repeated extractions or were either refractory for restriction digestion, hence excluded from further analysis and interpretation. 27 of 53 isolates were classified into seven groups (groups A-G) as follows: thirteen isolates in group-A, four isolates in group-B and two isolates each in group-C, D, E, F and G. Group F and G included two isolates each of *E. faecium*, while the isolates in other groups were *E. faecalis*. 26 isolates exhibited unique *Eco*RI restriction plasmid profile that could not be clubbed with any groups and hence classified as "unique" restriction profiles. While only three among the remaining nine isolates tested, which include three transconjugants and the standard/control strains yielded plasmids. However, the three transconjugants included in this panel failed to yield plasmid DNA upon multiple extractions.

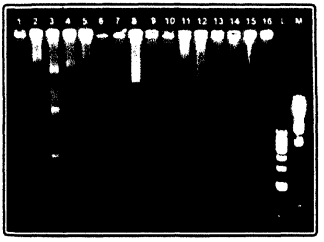
# Figure 7. Whole Plasmid DNA analysis of HLAR Enterococci.



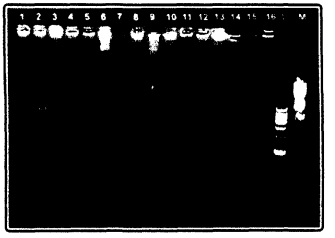
A. Lab number of Enterococci from lanes 1- 17 in respective order: 9357, 6,477, 7108, 7181, 8756, 6236, 4252, 5967, 10,564, 6,660, 7,107, 831, 5099, 5318, 5797, 4038, 11,122.



B. Lab number of Enterococci from lanes 1- 17 in respective order: 6,641, 7868, 3696, 1670, 1396, An-1, 15,332, 9953, 1002, 5342, 9478, 14550, 8670, 14535, 4343, 3844, 5969.



C. Lab number of Enterococci from lanes 1- 16 in respective order: 6275, 10,638, 7132, 881, 271, 6276, 6130, 6265, 8765, 11660, 11871, 8257, 891, 3846, 5298, 15411.



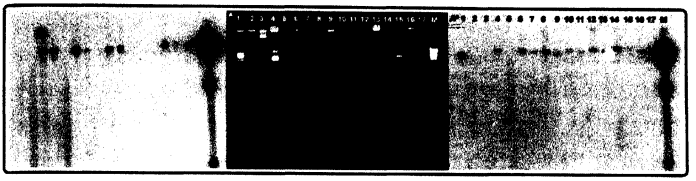
D. Lab number of Enterococci from lanes 1- 16 in respective order: 4637, 10792, 13514-TC, 9118- TC, 10564- TC, 4028, 3757, 8765, 7918,57, CDC-SS-1273- *E. faecalis*, EF 1002- Esp-Positive control, *E. faecalis*- pAM, JH2-SS, FA2-2 RF, *E. faecalis*- MTCC-439.

TC- Transconjugant; M- Hind III digested Lambda DNA marker; L- 1KB ladder.

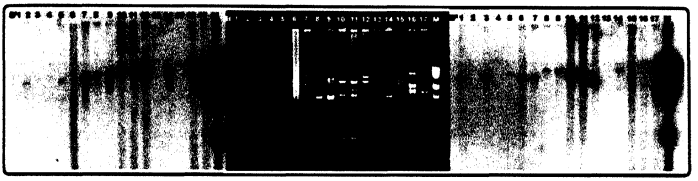
N.B: The details of antimicrobial resistance of the enterococcal test strains are depicted in Table 13.

## Figure 8. Plasmid-Restriction endonuclease analysis and Southern hybridization of HLAR enterococci

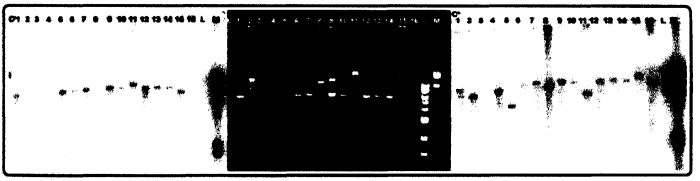
Corresponding Southern blot hybridized with ant(6')-I [HLSR] gene probe. | Plasmid-DNA Restriction endonuclease analysis | Corresponding Southern blot hybridized with aac(6')+aph(2") [HLGR] gene probe.



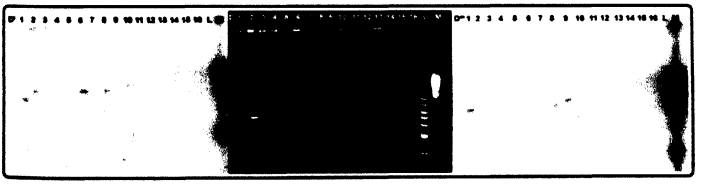
A. Lab number of Enterococci from lanes 1- 17 in respective order: 9357, 6,477, 7108, 7181, 8756, 6236, 4252, 5967, 10,564, 6,660, 7,107, 831, 5099, 5318, 5797, 4038, 11,122.



B. Lab number of Enterococci from lanes 1- 17 in respective order: 6,641, 7868, 3696, 1670, 1396, An-1, 15,332, 9953, 1002, 5342, 9478, 14550, 8670, 14535, 4343, 3844, 5969.



C. Lab number of Enterococci from lanes 1- 16 in respective order: 6275, 10,638, 7132, 881, 271, 6276, 6130, 6265, 8765, 11660, 11871, 8257, 891, 3846, 5298, 15411.



D. Lab number of Enterococci from lanes 1- 16 in respective order: 4637, 10792, 13514-TC, 9118- TC, 10564- TC, 4028, 3757, 8765, 7918,57, CDC-SS-1273- *E. faecalis*, EF 1002- Esp-Positive control, *E. faecalis*- pAM, JH2-SS, FA2-2 RF, *E. faecalis* MTCC-439.

M- Hind III digested Lambda DNA marker; L- 1 KB Ladder

**B. Results of DNA-DNA Hybridization studies**

***i. Cross-hybridization***

The bifunctional gentamicin and streptomycin resistance gene probes were used in cross-hybridization studies with the PCR amplified AME gene products from the HLAR enterococci used for plasmid DNA profiling. The DNA probes showed extensive homology with the corresponding amplified AME gene product from all the HLAR enterococcal isolates subjected for DNA Hybridization studies.

***ii. Plasmid DNA Hybridization of HLAR enterococci***

The summary of the different restriction digested plasmid fragments hybridizing with the aac(6')+aph(2") and ant(6')-I gene probes are depicted in **Table 13.a** and **b**. Briefly, the plasmids classified into different groups with respect to the *Eco*RI profiles exhibited a unique DNA hybridization pattern for the respective gene probe with occasional variations within the group. The sizes of hybridizing fragments depicted in **Table 13.a** and **b** are approximate measurements derived from the molecular weight standards run with every gel.

The hybridization patterns of the restriction digested plasmid DNA from HLAR test isolates with gentamicin and streptomycin DNA probes are shown in **Figure 8**. Thirteen *E. faecalis* categorized as Group-A based on their similarity in the *Eco*RI restriction plasmid profiles showed approximately a 11-kb *Eco*RI fragment hybridizing with gentamicin as well streptomycin gene probe, comprising the largest number of isolates showing a similar hybridization pattern in a single group. The four *E. faecalis* Group-B isolates showed a 15-kb *Eco*RI fragment hybridizing with gentamicin gene probe, while the streptomycin gene probe hybridized to approximately 15-kb *Eco*RI fragment in one isolate (the other three isolates being sensitive for streptomycin). The two *E. faecalis* Group-C isolates showed a 20-kb *Eco*RI fragment hybridizing with gentamicin gene probe, while the streptomycin gene probe hybridized to approximately

12-kb *Eco*RI fragment in one isolate (the other isolate being sensitive for streptomycin). The two *E. faecalis* Group-D isolates showed a 7.5-kb *Eco*RI fragment hybridizing with gentamicin as well streptomycin gene probe. The two HLGR *E. faecalis* Group-E isolates showed an 8-kb *Eco*RI fragment hybridizing with gentamicin gene probe. The two *E. faecium* Group-F isolates showed a 13-kb *Eco*RI fragment hybridizing with gentamicin gene probe, while the streptomycin gene probe hybridized with two *Eco*RI fragments approximately of 20-kb and 70-kb in size in both the isolates. The two *E. faecium* Group-G isolates showed a 13-kb *Eco*RI fragment hybridizing with gentamicin gene probe, while the streptomycin gene probe hybridized to approximately a 9-kb *Eco*RI fragment in both the isolates.

Only four of twenty-six isolates (15%) possessing "unique" *Eco*RI restriction plasmid profiles showed *Eco*RI fragments of same size encoding resistance for both the aminoglycoside genes by hybridization. However, every isolate had a hybridizing fragment of different size ranging from 6 to 12-kb as depicted in **Table 13.a** and **b**. For the remaining 22 isolates, gentamicin and streptomycin gene probes hybridized to *Eco*RI fragments of the different sizes ranging from 5 to 70-kb for the same isolate. There were no fragments showing hybridization with the gene probes for isolates that did not yield plasmids.

***iii. Chromosomal DNA Hybridization studies of HLAR enterococci***

The *Sma*I restriction digested chromosomal DNA of the HLAR enterococci separated by PFGE did not show any fragments hybridizing with either of the DNA probes tested as shown in **Figure 13** (Chapter VI). This confirms that none of the HLAR enterococci from our hospital carried the gentamicin/streptomycin resistance determinants on their chromosome.

**Table 13 a**. Plasmid REA and DNA hybridization results

| S. No. | Lab no. | Species | HLAR | Plasmid group | Gm. Frag, Kb | Sm. Frag, Kb |
|---|---|---|---|---|---|---|
| 1 | 8756 | *E. faecalis* | G+S | No Plasmid | NA | NA |
| 2 | 5797 | *E. faecalis* | G+S | A | 11 | 11 |
| 3 | 1670 | *E. faecalis* | G+S | Unique | NH | NH |
| 4 | 9953 | *E. faecalis* | G+S | A | 11 | 11 |
| 5 | 1002 | *E. faecalis* | G+S | A | 11 | 11 |
| 6 | 14535 | *E. faecalis* | G+S | A | 11 | 11 |
| 7 | 6275 | *E. faecalis* | G+S | A | 11 | 11 |
| 8 | 6276 | *E. faecalis* | G+S | A | 11 | 11 |
| 9 | 6130 | *E. faecalis* | G+S | A | 11 | 11 |
| 10 | 8765 | *E. faecalis* | G+S | A | 11 | 11 |
| 11 | 11660 | *E. faecalis* | G+S | A | 11 | 11 |
| 12 | 8257 | *E. faecalis* | G+S | A | 11 | 11 |
| 13 | 891 | *E. faecalis* | G+S | A | 11 | 11 |
| 14 | 3846 | *E. faecalis* | G+S | A | 11 | 11 |
| 15 | 10792 | *E. faecalis* | S | A | 11 | 11 |
| 16 | 5318 | *E. faecalis* | G+S | Unique | 24 | 15 |
| 17 | 4343 | *E. faecalis* | G | Unique | 10 | NA |
| 18 | 7,107 | *E. faecalis* | G | B | 15 | NA |
| 19 | 5099 | *E. faecalis* | G | B | 15 | NA |
| 20 | 831 | *E. faecalis* | G | C | 20 | NA |
| 21 | 4028 | *E. faecalis* | G+S | D | 7.5 | 7.5 |
| 22 | 8765 | *E. faecalis* | G+S | D | 7.5 | 7.5 |
| 23 | 881 | *E. faecalis* | G | E | 8 | NA |
| 24 | 7918 | *E. faecalis* | G | E | 8 | NA |
| 25 | 5342 | *E. faecium* | G+S | F | 13 | 20, 70 |
| 26 | 9478 | *E. faecium* | G+S | F | 13 | 20, 70 |
| 27 | 4038 | *E. faecium* | G+S | G | 13 | 9 |
| 28 | 1396 | *E. faecium* | G+S | G | 13 | 9 |
| 29 | 13514-TC | *E. faecalis* | G+S | No Plasmid | NA | NA |
| 30 | 105640-TC | *E. faecalis* | G+S | No Plasmid | NA | NA |
| 31 | 9357 | *E. faecalis* | G | Unique | 11 | NA |
| 32 | 7108 | *E. faecium* | G+S | Unique | NH | 20, 70 |
| 33 | 5967 | *E. faecalis* | G | B | 15 | NA |
| 34 | 10,564 | *E. faecalis* | G+S | Unique | 12 | 12 |

**Table 13 b** Plasmid REA and DNA hybridization results

| S. No. | Lab no. | Species | HLAR | Plasmid group | Gm. Frag, Kb | Sm. Frag, Kb |
|---|---|---|---|---|---|---|
| 35 | 6,660 | *E. faecalis* | G+S | B | 15 | 15 |
| 36 | 11,122 | *E. faecalis* | G | Unique | 11 | NA |
| 37 | 6,641 | *E. faecalis* | G+S | Unique | 9.5 | NH |
| 38 | 7868 | *E. faecalis* | G+S | Unique | NH | 8 |
| 29 | An-1 | *E. faecalis* | G+S | Unique | 10 | NH |
| 40 | 15,332 | *E. faecalis* | G+S | Unique | 10.5 | 10.5 |
| 41 | 14550 | *E. faecalis* | G+S | Unique | 8 | 16 |
| 42 | 8670 | *E. gallinarum* | G+S | Unique | NH | NH |
| 43 | 3844 | *E. gallinarum* | G+S | Unique | NH | 23 |
| 44 | 5969 | *E. faecalis* | G | Unique | NH | NA |
| 45 | 10,638 | *E. faecalis* | G | Unique | 6 | NA |
| 46 | 271 | *E. faecalis* | G+S | Unique | 4.5 | 9 |
| 47 | 6265 | *E. gallinarum* | G | Unique | 9 | NA |
| 48 | 11871 | *E. faecalis* | G+S | Unique | 6.5 | 15 |
| 49 | 5298 | *E. faecalis* | G+S | Unique | 18 | 9 |
| 50 | 15411 | *E. faecium* | G+S | Unique | 11 | 11 |
| 51 | 4637 | *E. faecalis* | G+S | Unique | 6 | 6 |
| 52 | 9118-TC | *E. avium* | G+S | No Plasmid | NA | NA |
| 53 | 3757 | *E. faecalis* | G+S | No Plasmid | NA | NA |
| 54 | 57 | *E. faecalis* | G+S | Unique | 5 | 8 |
| 55 | 6236 | *E. faecalis* | G+S | C | 20 | 12 |
| 56 | 4252 | *E. faecalis* | G+S | Unique | 14 | 10 |
| 57 | 6,477 | *E. durans* | S | No Plasmid | NA | NA |
| 58 | 7181 | *E. avium* | G+S | Unique | 20 | 12 |
| 59 | 7132 | *E. gallinarum* | G+S | No Plasmid | NA | NA |
| 60 | 3696 | *E. faecalis* | G | Unique | 11 | NA |
| 61 | CDC-1273 | *E. faecalis* | - | Control | NH | NH |
| 62 | EF-1002 | *E. faecalis* | - | Control | NH | NH |
| 63 | pAM | *E. faecalis* | - | Control | NH | NH |
| 64 | JH2-SS | *E. faecalis* | - | Control | NH | NH |
| 65 | FA2-2 RF | *E. faecalis* | - | Control | NH | NH |
| 66 | MTCC-439 | *E. faecalis* | - | Control | NH | NH |

G, High-level Gentamicin resistance; S, High-level Streptomycin resistance

NH, No hybridization; NA, Not applicable; TC, transconjugant

## DISCUSSION

Enterococci exhibiting aminoglycoside resistance pose one of the biggest therapeutic challenges in treating serious infections. A synergistic combination regimen is impossible even if the isolate is susceptible to either of the cell-wall active agent (β-lactams/glycopeptides) [40, 131, 341]. Present study showed that 60% and 43% of all enterococci tested were resistant to high-level gentamicin and streptomycin respectively. The high-level plasmid-borne resistance to gentamicin was first reported in 1979 in three strains of *S. faecalis* [254] following which studies reported that HLGR conferring plasmids in *E. faecalis* isolated from diverse geographic locations were heterogeneous as determined by molecular genetic studies [18, 354]. Thus genotypic analysis and molecular characterization of HLAR determinants is highly essential to know the differences, if any, in the genetic basis of the HLAR in enterococci between different countries and continents [292, 349, 350].

The results of whole plasmid and *Eco*RI digested plasmid profiles of our study depict heterogeneity among plasmids in HLAR enterococci, similar to several studies carried out in different parts of the world including U.S, France, U.K, Japan, and Greece [18, 135, 292, 350, 354-356]. Most of these studies have also shown that a predominant type of plasmid was present among many HLGR enterococci depicting their widespread dissemination, which is concordant with our study. The whole plasmid profiles depicted that the molecular weight of the plasmids ranged approximately between 70 kb to 2 kb among the HLAR isolates in our study, similar to other studies showing the presence of same range of plasmids, while most of the smaller molecular weight plasmids were cryptic [18, 251, 254, 354, 357]. A number of plasmids have been associated with HLGR, but the nature of the plasmids harboring the resistance genes does not appear uniform. The role of the bifunctional gentamicin resistance gene aac(6')+aph(2") encoded by plasmids were depicted since late 90s by many studies. The cause for such diversity in plasmids conferring the same phenotype within a species is unclear. A possible explanation may be that prolonged prevalence of HLGR in clinical isolates of enterococci could have allowed time for the aac(6')+aph(2") gene to become associated with

transposons and this would account for some degree of diversity observed in *E. faecalis* as evidenced [121, 122].

Several studies have depicted plasmid heterogeneity among HLGR *E. faecalis*, while some studies have shown homogeneity among HLGR plasmids in *E. faecium* authenticating the widespread dissemination of a single gentamicin resistance plasmid and its derivatives through localization of the genetic determinants by hybridization [357]. Our study shows that two homogenous groups of plasmids were prsent among four HLAR *E. faecium* isolates, while other two *E. faecium* isolates possessed a unique plasmid digestion profiles, which is concordant with the plasmid profiles of other studies [357]. But the chances of a single *E. faecium* isolate with HLGR plasmid getting circulated within the same hospital cannot be ruled out, since our study dealt only with isolates from a single hospital unlike other studies which investigated isolates from different hospitals [287, 357]. Other possible reason for this plasmid homogeneity may be because HLGR among *E. faecium* is a relatively new and infrequent event since early 90s [287, 357]. It is postulated that given sufficient time the homogeneity displayed by *E. faecium* plasmids may be replaced as in *E. faecalis* by a heterogeneous set of plasmids, thereby limiting the therapeutic choices available [121, 358]. The data from the present study authenticates this fact with comparatively lesser HLGR among *E. faecium*, but with the rise of HLAR this may emerge as an important clinical problem in the near future.

Although HLAR was proposed to be encoded by plasmids initially [254], subsequent studies provided genetic evidence by DNA-DNA hybridization experiments to confirm that HLAR determinants are usually encoded on plasmid DNA in *E. faecalis*, *E. faecium*, *E. avium*, *E. hirae*, *E. raffinosus*, *E. gallinarum* and *E. casseliflavus* [121, 122, 251, 287, 350, 355-357, 359]. Hence in the present study, DNA-DNA hybridization experiments were carried to map the genetic locations of the HLAR determinants among the clinical isolates using bifunctional gentamicin (Gm) resistance gene probe, and streptomycin (Sm) resistant gene probe.

The whole plasmid DNA was hybridized with Gm and Sm probes in our study, but the pattern of hybridization was not clear enough to be conclusive about the role of specific plasmids encoding HLAR. Thus, for conclusive evidences the restriction-digested plasmids were southern transferred and hybridized with respective DNA probe, which showed different plasmid fragments hybridizing with the Gm and Sm gene probes as depicted in **Table 13.a** and **b**. The plasmids classified into different groups (A-G) with respect to the *Eco*RI profiles exhibited a unique DNA hybridization pattern for the respective gene probe with occasional variations within the group, while isolates having "unique" *Eco*RI restriction plasmid profiles showed heterogeneous hybridization patterns of different sizes ranging from 5 to 70-kb for the respective gene probe.

Apart from 13 *E. faecalis* categorized as Group-A that showed approximately an 11-kb *Eco*RI fragment hybridizing with gentamicin as well streptomycin gene probe, four isolates categorized as "Unique" based on the *Eco*RI restriction profiles too showed an 11-kb *Eco*RI fragment hybridizing with the Gm probe. But, none except one "unique" isolate hybridized with the Sm probe while other isolates were sensitive to streptomycin. Along with the two *E. faecalis* Group-C isolates, one *E. avium* isolate with "unique" restriction profile too exhibited the same hybridization pattern showing a 20-kb and 12-kb *Eco*RI fragment hybridizing with Gm and Sm probes respectively. While another "unique" profiled *E. faecalis* isolate showed an 8-kb *Eco*RI fragment hybridizing with Gm probe like the two *E. faecalis* Group-E isolates, however a 16 kb fragment of the "unique" isolate hybridized with the Sm probe, while the Group-E isolates being sensitive to streptomycin did not hybridize. The transferability of the HLAR determinants between *E. faecalis* isolates [6, 116, 120, 127] may be one reason for the hybridization of identical/near identical (molecular weight wise) fragments even among different plasmid types present in clinical enterococcal isolates as shown in the present study.

It is noteworthy to find that the four (2 each) Group-F and Group-G *E. faecium* isolates showed a 13-kb fragment hybridizing with Gm probe, but the Sm probe hybridized with 20-kb and 70-kb fragments of the Group-F isolates, and with a 9-kb fragment of the Group-G isolates. Another interesting result to be pondered was the

hybridization patterns of three *E. faecium* isolates (two Group-F, and one "unique" profiled isolate) that showed two fragments-20-kb and 70-kb hybridizing with the Sm probe. At the first instance we thought that the residual undigested plasmid DNA resulted in hybridization of Sm probe with two fragments (since other isolates depicted only a single fragment hybridizing with Sm probe). Hence we repeated the experiment for these three *E. faecium* isolates by redigesting the plasmids with *Eco*RI and hybridized with Sm probe which yielded the same result and confirmed that ant(6')-I Sm probe hybridized with two plasmid fragments. A study by Ounissi et al. [360] had previously depicted this type of discrepancy with the ant(6')-I probe which hybridized with streptomycin susceptible enterococci and staphylococci. They suggested that this could result from the presence in the strain a silent/remnant ant-6 gene, or from hybridization to another portion of the genome. The gene for ANT(6) was nearly always (99.8% in staphylococci and 99.6% in enterococci) associated with that encoding an APH(3'). This observation, combined with the fact that the streptomycin-susceptible strains that hybridized with the ant(6')-I probe were all kanamycin resistant, suggested a physical link between the two resistance genes, resulting in cross-hybridization. Further as a general estimate, probes will hybridize to fragments that have >80% complementarity and hybridization is therefore less affected by minor nucleotide changes and can detect related groups of alleles [327, Neil Woodford, Personal communication, 2005]. Taking all these facts into consideration we tested the susceptibility of three *E. faecium* isolates to kanamycin and found them resistant. Thus we concluded that these three *E. faecium* isolates from our study might possess the aph(3')-IIIa gene conferring high-level kanamycin resistance and were plasmid encoded [361], which hybridized with the ant(6')-I Sm probe, although PCR detection of aph(3')-IIIa gene was not done to validate further. Only four (including one *E. faecium* isolate compared with Group-A isolates) among 26 isolates possessing "unique" *Eco*RI restriction plasmid profiles showed hybridizing fragments of 6 kb, 10.5 kb, 11 kb, and 12 kb with Gm and Sm probes for each of four isolates. However for the remaining 22 isolates, Gm and Sm gene probes hybridized to *Eco*RI fragments of the different sizes ranging from 5 to 70-kb.

As authenticated by preliminary cross-hybridization studies with PCR products of HLAR enterococci, there were no false-positive/false-negative results in the DNA-DNA hybridization experiments with plasmid DNA from majority of clinical enterococcal isolates, with the exception of ant(6')-I Sm probe with plasmid DNA of three *E. faecium* isolates that showed two fragments (20 and 70kb) hybridizing with the Sm probe. There were no fragments showing hybridization with the gene probes for negative controls, or isolates that did not yield plasmids emphasizing the sensitivity of our hybridization experiments.

The HLGR determinants apart being encoded by plasmids could also be carried on the chromosome in *E. faecalis* via different transposons [123, 261, 355, 358, 362]. However in our study, *Sma*I restriction digested chromosomal DNA of the HLAR enterococci separated by PFGE did not show any fragments hybridizing with either of the DNA probes tested, thus confirming that HLAR determinants are encoded only by plasmid DNA and not by the chromosomes among the isolates from our hospital.

Thus the *Eco*RI restriction plasmid profiles and the hybridization patterns of the aminoglycoside resistant enterococci especially those of *E. faecalis* from our hospital set up in South India shows heterogeneity among plasmids, while some plasmids showed homogeneity among the isolates studied which may be due to dissemination of the plasmid determinants, or the plasmid possessing strain within our hospital. Although the aac(6')+aph(2") gene conferring the HLGR phenotype appears to be conserved, there may be substantial differences in the flanking regions immediately adjacent to the fused gene, which can be another cause for the diversity in plasmids conferring the HLAR as shown in the prsent study. The prolonged prevalence of HLGR in clinical isolates of enterococci that has enabled the aac(6')+aph(2") gene to become associated with transposons accounting to some degree of plasmid diversity among *E. faecalis*, which is concordant with other studies that have authenticated this fact [121, 122]. Although we did not use any Insertion Sequence (IS) probes (IS 256/257) to confirm the involvement of transposons among the HLAR plasmids in our study, the diversity of plasmids and the plasmid hybridization patterns with Gm and Sm probes provide an indirect evidence to

authenticate the involvement of Transposons. The restriction enzyme *EcoR*I used in our study are not known to digest within the HLGR gene or within the Transposon carrying the gene. Thus the plasmid (hybridization) fragment size differences indicate that the DNA sequences flanking the gene were different. Hence different plasmid types may be involved in the dissemination of the strain, or, the HLAR determinants among enterococci in our clinical setup in South India [363, Neil Woodford, Personal communication, 2005].

## SUMMARY

The results of our study depict the presence of plasmid DNA among most of the HLAR *E. faecalis* isolates (53 of 60 isolates tested), which yielded between one to five plasmids, while majority isolates possessed at least two plasmids. The whole plasmid profiles depicted that the molecular weight of the plasmids ranged approximately between 70 kb to 2 kb. The restriction-digested plasmids were classified into seven groups (groups A-G) comprising 27 isolates based on their homogeneity in digestion pattern, while 26 isolates that exhibited heterogeneous *Eco*RI restriction plasmid profiles could not be clubbed with any groups and hence classified as "unique" restriction profiles.

To determine the location of the genetic elements conferring HLAR, the restriction digested and the whole plasmid DNA from HLAR *E. faecalis* isolates were Southern transferred and subjected for DNA-DNA Hybridization using aac(6')+aph(2") and ant(6')-I gene probes. The plasmids classified into different groups with respect to the *Eco*RI profiles exhibited an identical DNA hybridization pattern for the respective gene probe with occasional variations within the group. Gentamicin and streptomycin gene probes hybridized to *Eco*RI fragments of the different sizes ranging from 5 to 70-kb for the same isolate. The sizes of hybridizing fragments were approximate measurements derived from the molecular weight standards run with every gel after hybridizing with the lambda DNA probe. The *Sma*I digested chromosomal DNA of the HLAR enterococci separated by PFGE did not show any fragments hybridizing with either of the DNA probes tested confirming that none of the HLAR enterococci carried the gentamicin, or,

streptomycin resistance determinants on their chromosome. Thus our study depicts that HLAR determinants are encoded only by plasmid DNA among the isolates from our hospital setup.

The antimicrobial resistance although undoubtedly catapulted enterococci to become a prominent nosocomial pathogen since last decade, there are several other factors in enterococci that enhances the prospects of their pathogenicity even in the presence or absence of antimicrobial resistance. Hence, it is imperative to assess the role of such (virulence) factors and their contribution to the pathogenicity of enterococci.