Chapter V

Cell-Cell Communication and Gene Transfer among Aminoglycoside Resistant Enterococci

CHAPTER V

17. CELL-CELL COMMUNICATION AND GENE TRANSFER AMONG AMINOGLYCOSIDE RESISTANT ENTEROCOCCI

The versatile genetic machinery of enterococci has enabled them to become a prominent nosocomial pathogen. The pheromone induced aggregation substance (AS) mediates efficient cell-cell (bacterium-bacterium) contact to facilitate plasmid exchange: mostly encoding antibiotic resistance and virulence traits, as part of a bacterial sex pheromone system in enterococci [17, 116]. However, the mere absence of this sex pheromone system among some clinical enterococcal isolates doesn't warrant that plasmid exchange would not occur, since alternate sex-pheromone independent plasmid transfer mechanisms have also been depicted by several studies [120]. Hence, the property of transferable multidrug resistance including aminoglycosides, glycopeptides and beta-lactams, is possibly one explanation for intra and inter hospital dissemination of enterococci [6, 17]. Further, the AS produced by the pheromone responding plasmids, have been shown to enhance the infectivity of enterococci by several means [136, 137]. Reports have shown that there lies disparity in the gene transfer mechanisms of enterococci in various geographical regions. Hence studies focusing on the gene transfer mechanisms in enterococci helps in understanding the dynamics of their genetic machinery, which in turn enables to formulate and implement strategies that minimizes the problems of enterococcal infections, as well the dissemination of virulence and antimicrobial resistant traits in any hospital setting.

OBJECTIVES

- To study the transferability and characterization of the genetic determinants in enterococci

MATERIALS AND METHODS

1. Pheromone response assay

Detection of clumping (aggregation) by donor cells (test isolates) in response to the pheromone was carried out as described previously [116, 127].

A. Pheromone recovery

- 5 ml N2GT broth (Oxoid Nutrient broth No.2 supplemented with 0.2% glucose and buffered to pH 7.0 with 0.1m Tris.HCl) was inoculated with 0.05 ml of an overnight culture of plasmid free *E. faecalis* recipient strain JH2-SS/FA2-2 RF.
- The cells were grown to mid log phase at 37°C with shaking.
- The cells were then pelleted by centrifugation at 12,000 rpm, and the culture filtrate (supernatant) was collected and boiled for 10 minutes and used as "pheromone" for the clumping assays.

B. Clumping assay

- 0.5 ml of the culture filtrate (pheromone) was mixed with 0.5 ml of fresh N2GT broth in 1:1 ratio, to which 20 µl of overnight cultured cells (test isolates) were added to test for pheromone response or their ability to clump.
- The mixture was incubated for 2-4 hours 1:10 with shaking and examined for clumps visually, as well microscopically.

C. DAPI staining: The mixture was also stained using 4'-6-Diamidino-2-phenylindole (DAPI) stain known to form fluorescent complexes with natural double-stranded DNA, and the clumping response was visualized under fluorescent as well phase contrast microscope briefly as follows:

- DAPI stain 5 µg/ml (DAPI stain - Sigma Aldrich, US, 1mg/ml stock made in double distilled water) was added to a final concentration to the mixture (1ml) and kept at room temperature for 10 minutes.
- 10 µl of this suspension was placed on a glass slide and covered with a coverslip and visualized under fluorescent microscope at excitation wavelength 350 nm, as well under phase contrast.

2. Conjugative in-vitro gene transfer assays

The in-vitro gene transfer assays by conjugation were carried out by broth matings [116], or filter matings [368] whichever appropriate as described below:

A. Broth matings

- Overnight cultures of donor (test isolates) and recipient strains (plasmid free recipient strain *E. faecalis* JH2SS/ FA2-2 RF) were grown in Todd-Hewitt broth.
- 0.5 ml of recipient cells and 0.05 ml of donor cells were mixed into 4.5 ml fresh N2GT broth in a 1:10 donor recipient ratio.
- This 1:10 mixture was incubated at 37°C with gentle agitation for 2-4 hours.
- After incubation the mixture was vortexed to obtain a uniform suspension and 10 fold serial dilutions were plated (0.1 ml) on Todd Hewitt broth solid medium supplemented with 5% sheep blood and appropriate selective antibiotics and colonies were counted after 48 hours of incubation at 37°C.
- The antibiotic concentrations used in the selective agar plates were as follows: streptomycin 250 µg and spectinomycin 250 µg (recipient markers), rifampicin 25 µg and fusidic acid 25 µg (recipient markers), gentamicin 500 µg (donor marker).
- Separate platings were done from the mixture to select donors (using donor marker alone) and recipients (using recipient marker alone) separately. This provides a basis for estimating plasmid transfer frequency as transconjugants per donor (or) recipient.
- Plasmid transfer frequency was calculated by appropriate differencing between transconjugants and donor/recipient count, i.e: transconjugants cfu/ml / donor (or) recipient cfu/ml.

B. Filter matings

- Overnight cultures of donor and recipient strains (plasmid free recipient strain *E. faecalis* JH2SS/ FA2-2 RF) were grown in Todd-Hewitt broth.
- 0.5 ml of recipient cells and 0.05 ml of donor cells were mixed into 4.5 ml fresh N2GT broth in a 1:10 donor recipient ratio.

- This 1:10 mixture was collected on a membrane filter (0.22 µm, Millipore, India), and the filter was placed on BHI agar plate supplemented with 5% sheep blood and incubated at 37°C overnight.
- After incubation the cells were suspended in 1.0 ml of N2GT broth, and 0.1 ml of this cell suspension was spread on Todd Hewitt broth solid medium supplemented with 5% sheep blood and appropriate selective antibiotics, and colonies were counted after 48 hours of incubation at 37°C.
- The antibiotic concentrations used in the selective agar plates were as follows: streptomycin 250 µg and spectinomycin 250 µg (recipient markers), rifampicin 25 µg and fusidic acid 25 µg (recipient markers), gentamicin 500 µg (donor marker).
- Separate platings were done from the mixture to select donors (using donor marker alone) and recipients (using recipient marker alone) separately. This provides a basis for estimating plasmid transfer frequency as transconjugants per donor (or) recipient.
- Plasmid transfer frequency was calculated by appropriate differencing between transconjugants and donor/recipient count, i.e: transconjugants cfu/ml / donor (or) recipient cfu/ml.

3. Molecular characterization of Transconjugants

The donors, recipients and transconjugants were molecular characterized to study the plasmid and genomic DNA profiles. The whole (undigested) plasmid DNA and the restriction digested plasmid DNA were extracted as described in **Chapter-III, Section-4**, and separated on 0.8 % agarose gel using 0.5 X TBE at 60 V stained, visualized and documented using a gel documentation system (Vilber-lourbet, France). The genomic/ chromosomal DNA of the donors, recipients and transconjugants were *Sma*I digested and separated by PFGE as described in **Chapter VI** and the gel patterns were subsequently analyzed and interpreted appropriately.

RESULTS

1. Pheromone response assay

The pheromone response of all 110 HLGR *E. faecalis* and 20 HLG sensitive *E. faecalis* were tested as per standard procedures [116]. 68 (62%) HLGR *E. faecalis* and four (20%) of twenty HLG sensitive *E. faecalis* exhibited clumping of cells that depicted a pheromone response by enterococci tested as shown in **Table 16**.

Table 16. Clumping response of *E. faecalis*

Isolates tested	No. tested	No. (%) of isolates positive
All HLGR enterococci	110	68 (62)
HLGm sensitive enterococci	20	4 (20)

2. Conjugative in-vitro gene transfer assays

The in-vitro gene transfer assays by conjugation were carried out by broth matings and filter matings. Although transconjugants were obtained in broth matings, the transfer frequency was comparatively lesser than filter mating assay. Hence, our subsequent experiments were switched to filter mating assay to check the plasmid transfer frequency from clinical donor isolates to a standard recipient *E. faecalis*-FA2-2 (rifampin and fusidic acid resistant). We subjected four groups of 30 randomly selected HLGR *E. faecalis* isolates for the in-vitro gene transfer assay by filter mating technique, the results of which are depicted in **Table 17**. The isolates of four groups included were as follows: I. Twenty aac(6')+aph(2")-HLGR, ant(6)-I-HLSR and pheromone response positive *E. faecalis*, II. Four aac(6')+aph(2") and pheromone response positive *E. faecalis*, III. Four aac(6')+aph(2") and ant(6)-I-HLSR positive but pheromone response negative *E. faecalis* and IV. Two aac(6')+aph(2") positive but pheromone response negative *E. faecalis*. The transconjugants were assayed for their antimicrobial resistance by phenotypic and genotypic assays. The transconjugants were assayed by PCR to check for the transfer of the virulence trait-"Esp".

Table 17. Filter-Mating experiments of HLGR-PCR positive *E. faecalis*

Lab no. of donor	Drug resistance pattern of the donor	Clumping response	Transfer freq.[a]	TC phenotype	TC genotype[b]
5797	Pen, Amp, Gm, Sm	+	2.5×10^{-4}	Gm, Sm	Gm, Sm, Esp
9953	Pen, Amp, Gm, Sm	+	3.4×10^{-5}	Gm, Sm	Gm, Sm
1670	Van, Gm, Sm	+	NT	-	-
3846	Pen, Gm, Sm	+	4.5×10^{-6}	Gm, Sm	Gm, Sm
10792	Pen, Amp, Gm, Sm	+	1.8×10^{-6}	Gm, Sm	Gm, Sm
6275	Gm, Sm	+	7.0×10^{-5}	Gm	Gm
14535	Pen, Amp, Gm, Sm	+	3.2×10^{-6}	Gm, Sm	Gm, Sm
6276	Gm, Sm	+	2.0×10^{-6}	Gm, Sm	Gm, Sm
3280	Pen, Amp, Van, Gm, Sm	+	2.5×10^{-4}	Gm	Gm
14550	Pen, Amp, Gm, Sm	+	NT	-	-
11660	Pen, Amp, Gm, Sm	+	4.2×10^{-7}	Gm	Gm
8257	Pen, Amp, Gm, Sm	+	3.5×10^{-7}	Gm	Gm
6130	Pen, Amp, Gm, Sm	+	6.0×10^{-3}	Gm, Sm	Gm, Sm
1777	Pen, Amp, Van, Gm, Sm	+	4.2×10^{-5}	Gm, Sm	Gm, Sm, Esp
1002	Pen, Gm, Sm	+	4.9×10^{-5}	Gm, Sm	Gm, Sm
271	Gm, Sm	+	2.0×10^{-6}	Gm, Sm	Gm, Sm
8765	Pen, Amp, Gm, Sm	+	3.8×10^{-4}	Gm, Sm	Gm, Sm
5298	Pen, Gm, Sm	+	4.1×10^{-4}	Gm, Sm	Gm, Sm
11871	Gm, Sm	+	NT	-	-
4028	Gm, Sm	+	5.0×10^{-5}	Gm, Sm	Gm, Sm
5969	Pen, Van, Gm	+	NT	-	-
6,641	Pen, Amp, Van, Gm	+	3.4×10^{-3}	Gm	Gm
10,638	Gm	+	4.0×10^{-5}	Gm	Gm
4343	Gm	+	5.2×10^{-4}	Gm	Gm
6,660	Pen, Amp, Gm, Sm	-	4.2×10^{-3}	Gm, Sm	Gm, Sm
57	Pen, Amp, Gm, Sm	-	5.0×10^{-4}	Gm, Sm	Gm, Sm
6236	Gm, Sm	-	4.5×10^{-4}	Gm, Sm	Gm, Sm
4252	Gm, Sm	-	3.0×10^{-6}	Gm	Gm
5099	Pen, Amp, Gm	-	3.2×10^{-5}	Gm	Gm
11,122	Gm	-	4.0×10^{-5}	Gm	Gm

TC, Transconjugants; NT, No transconjugants obtained;

TC genotype[b], Positive for aac+aph (Gm-HLGR)/ ant-6 (Sm-HLSR)/ Esp gene by PCR;

Transfer freq[a], Transfer frequency- Transconjugants/donor cell

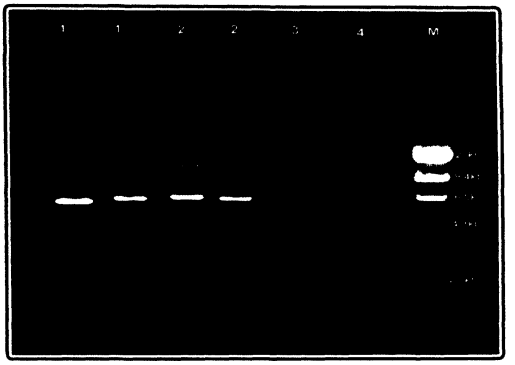
The frequency of transfer of the gentamicin resistance marker ranged from 10^{-3} to 10^{-7} transconjugants per donor cell, while four donor strains did not yield transconjugants in broth mating as well filter mating assays as depicted in **Table 17**. The transfer of "esp" gene-a putative virulence factor was demonstrated among transconjugants obtained from two of the thirty donor *E. faecalis* as confirmed by PCR. However in both instances, only few among the population of transconjugants showed the transfer of "esp" gene as evident by PCR, while the remaining transconjugants tested possessed the aminoglycoside resistance genes.

3. Molecular characterization of Transconjugants

The donors, recipients and five transconjugants were characterized to study the plasmid and genomic DNA profiles. The plasmid DNA was present in all the donor strains used in the in-vitro gene transfer assays, while the recipient strain and three of five transconjugants (which included two "esp" positive transconjugants) did not yield any plasmid DNA upon multiple extractions. The remaining two transconjugants showed a nearly identical plasmid DNA profile of donor strains after restriction digestion, supporting the transfer of plasmids from the donor to recipient as shown in **Figure 11**. The *Sma*I macrorestriction digestion of three transconjugants (including two "esp" positive transconjugants) and their corresponding donors and recipient were performed and separated by PFGE as shown in **Figure 11**. The macrorestriction PFGE pattern of the donors were heterologous, when compared with FA2-2 recipient and esp-negative, aminoglycoside resistant gene positive transconjugants, which had an identical pattern. On contrary "esp" and aminoglycoside resistance gene positive transconjugants showed a "closely related" pattern of the recipient with two band differences, leaving us to speculate that chromosome-to-chromosome transmission of "esp" gene might have occurred. On the other hand the FA2-2 recipient and the esp-negative, HLGR transconjugant showed an "identical" PFGE pattern, authenticating the involvement of plasmid DNA in transfer of gentamicin resistance.

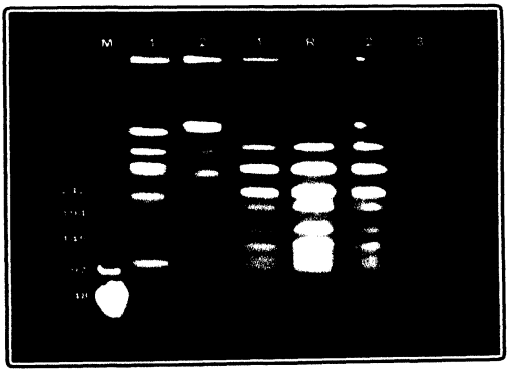
Figure 11. Molecular Characterization of *E. faecalis* donors, recipients and transconjugants

A. Plasmid DNA restriction [*Eco*RI] profiles of donors, recipients and transconjugants



1- 6275 [donor]; 2- 6130 [donor]; 1' and 2'- Corresponding Transconjugant
3- Recipient *E. faecalis* FA2-2; 4- Recipient *E. faecalis* JH2 SS
M- Hind III digested Lambda DNA marker.

B. Chromosomal DNA restriction [*Sma*I] profiles of donors, recipients and transconjugants by PFGE



1 and 2- donors; 1' and 2'- esp-negative, HLAR genes positive transconjugants
3' - esp- positive, HLAR genes positive transconjugant
R- Recipient *E. faecalis* FA2-2; M- Lambda ladder PFG marker.

DISCUSSION

Enterococci exhibit resistance to an array of antimicrobials including the most recent molecules, a property that has catapulted them as one of the leading clinical challenges [193]. The sex-pheromone based gene transfer system has made them "unique" among all nosocomial pathogens. Since their discovery, sex-pheromone induced/mediated gene transfer especially those of antimicrobial resistance, between *E. faecalis* isolates have been demonstrated [6, 127]. Small peptide sex pheromones that are specific for different types of plasmids are secreted by plasmid free strains of *E. faecalis* (recipient cell) into the culture medium. A potential donor cell containing a pheromone-responsive plasmid (generally encoding antibiotic resistance) comes into contact with its corresponding pheromone, following which the transcription of a gene on the plasmid is turned on, resulting in the synthesis of a sticky substance called aggregation substance (AS) on its surface. Subsequently, formation of some sort of mating channels between the cells leads to transfer of genetic determinants encoding antibiotic resistance/virulence determinants between donor and recipient strains [6, 17, 127]. In addition to mediating bacterial binding to other enterococci, AS also plays a role in binding of *E. faecalis* to eukaryotic cells, including pig renal tubular cells and human intestinal epithelial cells [199].

Mostly, *E. faecalis* strains from hospitalized patients possessing pheromone responsive plasmids encoding antibiotic resistance have shown a clumping response, leading to transfer of genetic determinants (encoding antibiotic resistance) between the donor and recipient strains [129-132]. In our study 68 (62%) of the HLGR *E. faecalis*, and 4 (20%) of 20 HLG sensitive *E. faecalis* exhibited clumping depicting production of aggregation substance (AS) in response to a culture filtrate known to contain numerous pheromones, which suggests their potential to transfer plasmid DNA between clinical isolates, although the degree of clumping varied between test strains.

Our results matched a study from Japan, where 85% of the hemolytic strains exhibited a clumping response and about 90% of these hemolytic strains were resistant to one or more drugs, whereas this was true for only about 54% of non-hemolytic strains

[235]. While several studies from Greece, Japan, U.S, have shown that 95% to 100% of pheromone responsive plasmids encoded gentamicin resistance among clinical enterococci [133-135, 161]. These studies concluded that these pheromone responsive plasmids might play a role in the spread of gentamicin resistance especially in a hospital set up.

Most of the studies including ours have used phenotypic assays for studying the pheromone responsiveness of the clinical isolates. While some of the recent studies have used genotypic assays to detect the presence of Aggregation Substance (AS/agg) gene- a product of pheromone response by plasmids, which also contribute substantially to the increased pathogenicity of enterococci. Coque et al. [129] in a study among 192 *E. faecalis* showed that AS was found among 52% of isolates from endocarditis, 72% of isolates from other infections, 56% of hospital fecal isolates and among 30% of fecal isolates from healthy volunteers, while none of the 86 non- *E. faecalis* isolates were positive for AS. Another study from Italy revealed that AS gene was found among 33% of *E. faecalis* isolates, but none among the *E. faecium* isolates [203]. These studies also emphasize that the phenotypic assays may not reveal the exact prevalence of pheromone response among enterococci, since many a times "silent AS genes" may not enable enterococci to exhibit a pheromone response that depicts a lesser prevalence of AS among enterococci otherwise. Hence it is unwarranted that all the pheromone response negative strains may not be true negative, although the percentage of such strains if prevalent would be very less.

The clumping response exhibited by 20% of the HLG sensitive *E. faecalis* in our study suggests, that apart gentamicin resistance plasmids, other plasmids encoding hemolysin/bacteriocin, UV resistance and resistance to other antibiotics like tetracycline, kanamycin, erythromycin, vancomycin (but not gentamicin resistance) can also be pheromone responsive as shown by several studies [6, 26, 235]. Our finding is concordant with the results of these studies since the four pheromone responsive *E. faecalis* isolates (20%) in our study were sensitive to other antibiotics tested including HLG, but were hemolysin/bacteriocin positive. Thus our results depict that apart

facilitating the transfer of antimicrobial resistance determinants, pheromone responsive strains may facilitate transfer of virulence determinants between cells, which is of prime concern in a hospital setting.

Although aggregation substance (AS) is a pheromone-inducible surface protein that mediates binding of donor cells to plasmid free recipients, which is essential for high-efficiency conjugation of sex pheromone plasmids, it has also been shown to act as a virulence factor during host infection and AS remains to be the most well studied adhesin of enterococci [199]. Several studies depict the significance of AS, as an important virulence factor in the pathogenesis of enterococcal infections like UTI and endocarditis. Our results shows that all the AS positive *E. faecalis* isolates possess one or more of the virulence factors: esp/gelatinase/bacteriocin studied, depicting these infection derived isolates may represent a unique genetic lineage capable of being disseminated within the hospital setup.

The other major function exhibited by AS is adherence of the *E. faecalis* isolates to host tissues, since in vitro studies have shown that AS mediates adhesion to various eukaryotic cell surfaces, such as cultured pig renal tubular cells and promotes internalization by cultured human intestinal cells, suggesting that AS-expressing cells may likely form larger aggregates in vivo than cells not expressing this trait [195-198]. Furthermore, AS have been shown to exhibit resistance to killing by polymorphonuclear leukocytes and macrophages, thereby promoting intracellular survival of *E. faecalis* inside neutrophils [199]. While Isenmann et al. [201, 202] have shown that interaction of fibronectin and aggregation substance promotes adherence of *E. faecalis* to human colon. Thus studies focusing on the prevalence of AS, either by indirect phenotypic clumping assays, or through genotypic assays to detect the presence of Aggregation Substance/agg gene would be a good indicator to know the appalling characteristics of the clinical enterococcal isolates.

The transferability of various genetic determinants especially by conjugation has enabled nosocomial strains of enterococci to outcompete indigenous commensal

enterococci, thereby increasing the presence of nosocomial strains in the gastrointestinal tract, as well dissemination of these strains in a nosocomial setup. Our preliminary results depicted that the donor isolates could transfer plasmids in both broth and filter matings [132], however in each case plasmids were transferred at a higher frequency to the *E. faecalis* recipient by filter mating than by broth matings, which has been shown previously by some studies [6, 7, 251]. Hence, our subsequent experiments were switched to filter mating assays unless otherwise specified (as broth matings). The four groups of 30 randomly selected HLAR *E. faecalis* isolates subjected for the gene transfer assays were having both pheromone response positive and pheromone response negative strains, the combinations of which are depicted in **Table 17**. The transconjugants were assayed for their antimicrobial resistance as well for the transfer of the virulence trait "Esp" by phenotypic and genotypic assays.

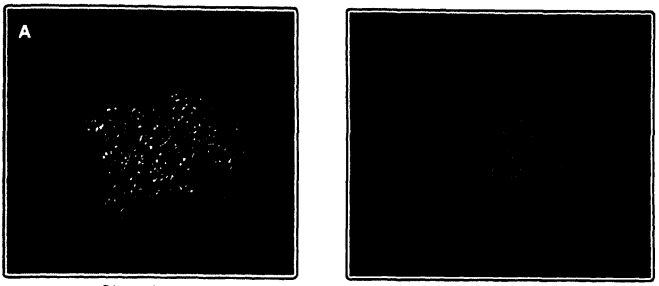
The group III and IV HLAR *E. faecalis* isolates in our conjugation studies were pheromone response negative as evident by clumping assay results. Hence we suggest the prevalence of a different plasmid system among these isolates, although the possibility of non-responsiveness to the corresponding pheromone due to a "silent gene" cannot be ruled out as shown by some studies [25, 210, 241]. The mating experiments between plasmid free recipient *E. faecalis* (FA2-2 RF) and, group III and IV donor cells did not exhibit visible mating aggregates unlike the group I and II donors, although transfer of plasmids occurred between donor and recipient cells. However, small mating aggregates were observed when the broth mating mixture was stained using 4'-6-Diamidino-2-

phenylindole (DAPI) and visualized under fluorescent as well phase contrast microscope, but aggregate of donor cells (self-clumping) were not even observable by microscopy in the DAPI stained clumping assay after exposure to a culture filtrate of a standard plasmid free recipient strains *E. faecalis* JH2 SS/ FA2-2 RF known to contain multiple pheromones as shown in **Figure 12**. Further, these strains failed to transfer plasmids when *E. faecium* BM 4105 RF/BM 4105 SS were used as recipient strains, confirming that they do not possess broad-host-range plasmids like pAMβ1 or pIP501 [125]. Thus, our results predict that these isolates may possess non-pheromone responsive plasmids or a pheromone-independent plasmid system as described recently [120, 139], although we have not done hybridization studies with DNA probes specific for these plasmids to confirm the existence of these plasmid groups among enterococci in our hospital setup.

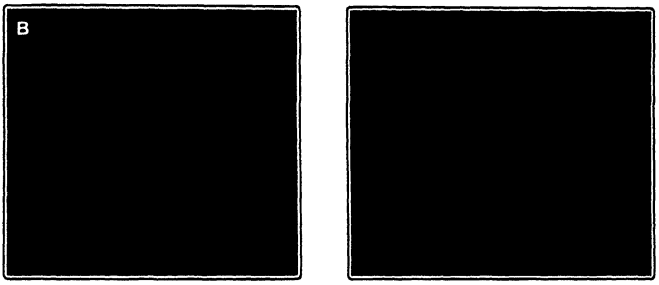
Few studies including ours, have depicted the nature and mode of the plasmid transfer [6, 7, 120, 132, 139], while most other studies carried among nosocomial enterococci worldwide have investigated only the ability of the enterococci to transfer antimicrobial resistant plasmids between strains without probing the pheromone responsiveness of the HLGR plasmids [251, 350, 356-358]. Generally in a clinical setup the demonstration of the transferability of genetic determinants alone would be sufficient enough to underscore the significance and their potential for dissemination of antimicrobial resistance. However, investigating the nature and mode of gene transfer would further contribute in understanding the basics, which enable us to redefine current strategies to prevent and control the spread of multidrug resistance among enterococci.

High-level plasmid mediated gentamicin resistance in *E. faecalis* was first reported in 1979 in France [254]. In 1983 a study from U.S showed that nine clinical isolates of *E. faecalis* encoded high-level resistance to gentamicin as well other aminoglycosides, and depicted that the genetic determinants for HLGR were carried on conjugative plasmids that transferred at a high frequency [258]. Later in 1990, the HLGR genes were shown to be located on transposons, which increased the transferability of the HLAR isolates [369]. Thereafter, several studies showed the transferability of gentamicin resistance and the role of transposons mediating this process.

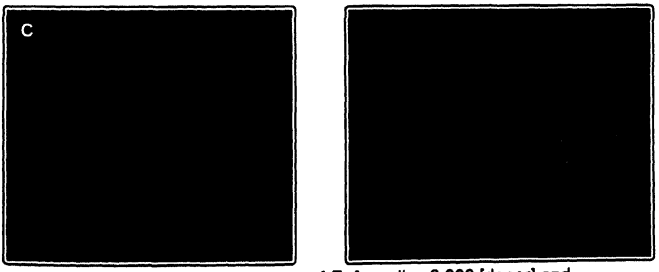
Figure 12. Representative Phase contrast and Fluorescent Microscopy images of Clumping and Mating Assays of *Enterococcus* test strains



Clumping response of *E. faecalis* - 5797 Group-I test strain against *E. faecalis* JH2 SS pheromones



No self-clumping by *E. faecalis* - 6,660 Group-II test strain against *E. faecalis* JH2 SS pheromones



Small mating aggregates of *E. faecalis* - 6,660 [donor] and *E. faecalis* FA2 RF [recipient] in a broth mating mixture.

The frequency of transfer of the gentamicin resistance marker ranged from 10^{-3} to 10^{-7} transconjugants per donor cell, which is concordant with several studies. A recent study from Greece [134] had depicted a similar plasmid transfer frequency of 10^{-3} to 10^{-8}. But, all the HLGR isolates in their study have been shown to exhibit clumping response, proving that all HLGR plasmids were pheromone responsive, in concordance with several other studies [133, 135, 161]. However, the present study showed that all the HLGR plasmids were not pheromone responsive as described in the previous section. Some studies have shown a very high plasmid transfer frequency between 10^{-0} to 10^{-3} among the pheromone responsive plasmids in broth matings [133, 135], while other studies that did not differentiate the pheromone responsiveness of plasmids have shown a plasmid transfer frequency (gentamicin resistance) between 10^{-2} to 10^{-8} by filter mating assays [251, 350, 356-358], which matches the transfer frequency of our study.

Apart from the type of plasmid, the transfer frequency depends on several other factors, including the association of the plasmids with various transposable elements like Tn4001/Tn5281, which are found commonly in enterococci [369]. Studies have shown that variant forms of these transposons can be generated by molecular rearrangements during the process of transposition from the chromosome to a plasmid, or during in vivo conjugative transfer [356]. Interestingly, the frequencies of transfer of gentamicin resistance for the isolates containing truncated variants of Tn4001 (generally located on plasmids) are significantly higher than those isolates containing non-truncated variants (mostly carried on the chromosome) as shown by some studies from France and Greece [355, 356].

In our study few pheromone responsive HLGR *E. faecalis* isolates were not able to transfer the HLGR/HLSR marker both by filter and broth matings. The conjugative transfer of gentamicin resistance may not be possible if the aac(6')+aph(2") gene is carried on non-truncated transposons regardless of their location on a plasmid or on the chromosome [356], which can be quoted as a possible reason for non-transfer of the HLGR markers by some of our isolates.

Although majority of the donor isolates could transfer the HLSR marker along with HLGR marker, few donors failed as depicted by the profiles (phenotypic/genotypic) of transconjugants (**Table 17**). The possible explanation for this phenomenon may be that HLSR marker might be encoded by a different non-conjugative/non-pheromone responsive plasmid, or else HLSR marker might be present on the chromosome of the clinical isolates that couldn't be mobilized. This fact was authenticated by our hybridization studies, since different fragments of the *EcoR*I digested plasmids hybridized to the HLSR and HLGR gene probes respectively for the donor *E. faecalis* isolates that failed to transfer HLSR marker. The failure to transfer HLSR marker by some *E. faecalis* isolates is in agreement with previous reports [287, 357], which had quoted similar reasons.

Thus as depicted above, even though sex pheromones appear to play a significant role in the evolution and transfer of certain plasmids in enterococci, it should be kept in mind that the importance of aggregation substance in enterococcal matings relates to its ability to initiate contact in liquid suspensions, and it is not an essential component in matings that occur on solid surfaces [370]. In natural environments, interspecies contact on surfaces may be a common occurrence (e.g., within biofilms), and potential signals that may be present in such environments still influence the induction of multiple components of a given mating system leading to transfer of genetic determinants between cells. Further, the existence of an alternate conjugative plasmid transfer system that is "pheromone-independent", but capable of transferring the antibiotic resistant plasmid efficiently during broth matings [120, 125, 139] is also of concern. Thus, the versatility of the genetic machinery of enterococci promotes dissemination of resistance and favors stability of resistance genes even in the absence of exposure to antibiotics.

Most interestingly, the transfer of "esp" gene-a putative virulence factor was demonstrated among transconjugants obtained from two of the 30 donor *E. faecalis* as confirmed by PCR. However in both instances, only a subpopulation of transconjugants acquired the "esp" gene as evident by PCR, i.e. only 2 among 18 transconjugants tested acquired the "esp" determinant from *E. faecalis* as depicted by PCR, while all the

transconjugants tested possessed the aminoglycoside resistance genes, indicating that the transferred antibiotic resistance determinants were not directly linked with the "esp" gene. This is not surprising, since antibiotic resistance determinants have not been identified in the Pathogenicity island (PAI) that encodes the "esp" gene in enterococci [214]. This fact was authenticated by our study, since the plasmid DNA isolated from the donor strains could not show the presence of the "esp" gene by PCR, which otherwise were positive for the HLGR determinants by PCR and DNA hybridizations.

Since the time of discovery of this putative virulence factor-"esp" studies have tried unsuccessfully to demonstrate transfer of this gene [214]. Recently Oancea et al. [223] from Germany demonstrated the conjugative transfer of "esp" among *E. faecalis* and *E. faecium* isolates. During the same time period we demonstrated the transfer of "esp" between *E. faecalis* isolates, however there were some differences between both the studies. Our study could depict the conjugative transfer of "esp" gene between (two) clinical donor *E. faecalis* (HLAR) and a standard *E. faecalis* isolate (FA2-2 RF: rifampicin and fusidic acid resistant) with a transfer frequency of 4.2×10^{-5} and 2.5×10^{-4} for both the isolates respectively, while attempts with *E. faecium* BM 4105 RF as a recipient strain failed to yield any transconjugant. In contrast, Oancea et al. [223] showed that one *E. faecalis*, and none of the *E. faecium* when mated with recipients of the other species (*E. faecium*) generated esp-positive subpopulation of transconjugants, i.e.: only one among fourteen transconjugants acquired the esp determinant from *E. faecalis* as depicted by PCR.

Molecular characterization of the plasmid DNA from 30 donors in our study revealed that they did not encode "esp" as evident by PCR (but encoded HLGR/HLSR), while the recipient strain and three (which included 2 "esp" positive transconjugants) of the five transconjugants tested did not yield any plasmid DNA upon multiple extractions. The remaining two HLGR transconjugants showed a nearly identical plasmid DNA profile of their corresponding donor strains after restriction digestion, supporting the transfer of plasmids (HLAR) from the donor to recipient as confirmed by hybridization with HLGR/HLSR probe. Thus these results led us to speculate a chromosome-to-

chromosome transfer of the "esp" gene from the donor to recipient *E. faecalis* strains. Hence, molecular characterization of the chromosomal DNA of three donors, recipient and corresponding transconjugants were performed by PFGE after *Sma*I macrorestriction digestion. The macrorestriction PFGE pattern of all three donors were heterologous, when compared with *E. faecalis* FA2-2 recipient and esp-negative, aminoglycoside resistant gene positive transconjugants, which had an identical pattern. These results authenticate the involvement of plasmid DNA in transfer of gentamicin resistance, as well non-transfer of the "esp" gene to the recipient. On contrary "esp" and aminoglycoside resistance gene positive transconjugants showed a "closely related" pattern of the recipient with two band differences (from recipient), leaving us to speculate that chromosome-to-chromosome transmission of "esp" gene might have occurred.

Although the chromosome-to-chromosome transmission of "esp" gene as shown in the present study is concordant with another study by Oancea et al. [223], the plasmid localization of the "esp" gene shown by them was divergent from our results. They had explained that the "esp" gene was mobilized from the donor chromosome, integrated into a conjugative plasmid, which was then transferred into the recipient, giving an esp-positive transconjugant. This could be of high clinical significance, since in our study majority of the donors possessed conjugative plasmids encoding gentamicin resistance, as well "esp" positive, thus having every chance of mobilizing other determinants including that of "esp", although none tested in our study depicted the same. However, the possibility of the "esp" gene transfer among other isolates is unwarranted since they were not tested. But, hybridization with an "esp" gene probe, which we did not perform on our isolates, would have further authenticated our results, although the molecular characterization of the plasmid and chromosomal DNA prove to be qualitative evidences for our study. To our knowledge, this is the second experimental evidence to prove that chromosomally encoded virulence traits like "esp" is capable of being transferable by conjugation, which may give rise to newer genetic lineages.

SUMMARY

The results of our study depict that the enterococci isolated from our setup were highly capable of transferring genetic determinants as evident by conjugation assays. The phenotypic assay depicted pheromone responsiveness by 62% of the all HLGR *E. faecalis* isolates and 20% of HLG sensitive *E. faecalis* isolates tested as evident by a clumping response. Four groups of 30 randomly selected HLGR *E. faecalis* isolates subjected to in-vitro gene transfer assay showed that the gentamicin resistance marker transferred at frequencies ranging between 10^{-3} to 10^{-7} transconjugants per donor cell for 26 donors. The transfer of "esp" gene-a putative virulence factor was demonstrated among transconjugants obtained from 2 of the 30 donor *E. faecalis* as confirmed by PCR. However in both instances, only few among the population of transconjugants showed the transfer of "esp" gene as evident by PCR, while all the transconjugants tested possessed the aminoglycoside resistance genes. The PFGE patterns of the donor strains were heterologous, when compared with FA2-2 recipient and esp-negative, aminoglycoside resistant gene positive transconjugants, which had an identical pattern, authenticating the involvement of only the plasmid DNA in transfer of gentamicin resistance. On contrary "esp" and aminoglycoside resistance gene positive transconjugants showed a "closely related" pattern of the recipient with two band differences, leaving us to speculate that chromosome-to-chromosome transmission of "esp" gene might have occurred.

The gene-transfer mechanisms in enterococci facilitate the dissemination of the genetic determinants encoding antimicrobial resistance and virulence in any hospital setup. However, only molecular typing of the isolates authenticates the relationship between the isolates disseminated in a hospital setup. This would help us to know the clonality of the isolates and to prove the transmission of the clones, if any, between patients in our hospital setup, during the study period.