CHAPTER 3

GENOTYPING OF CLINICAL ISOLATES OF Enterococcus faecalis FOR HLGR

This chapter describes the approaches made to screen the clinical isolates of Enterococcus faecalis and the results obtained to identify the prevalent form of the HLGR gene(s) and the transposable element(s) associated with the resistance gene.

High-level gentamicin resistance (HLGR) in enterococci is generally mediated by aminoglycoside-modifying enzymes (AMEs). The most common and clinically important AME is the bifunctional enzyme AAC(6')-Ie-APH(3'')-Ia with both adenyltransferase and phosphotransferase activities (Ferretti et al 1986). This enzyme is encoded by the fused gene aac(6')-Ie-aph(3'')-Ia. This gene in enterococci has also been reported to be carried by the conjugative transposon designated as Tn5281 (Hodel-Christian & Murray1991). The other genes that confer resistance to gentamicin includes, aph(3'')-Ia, -Ib and -Id. These genes though less frequently reported carries significance in determining the synergism of the antimicrobials used, that could be altered by the presence/absence of these genes in HLGR isolates (Kao et al 2000).

In this study 40 clinical isolates designated as HLGR (MIC>500mg/ml) were subjected to genotypic screening to detect the HLGR gene(s) and the transposable element conferring resistance to high-level gentamicin.
3.1 DETECTION OF HLGR GENE(S) IN THE CLINICAL ISOLATES OF E. FAECALIS

Gene specific PCR amplification of the genes responsible for conferring HLGR was performed for all the 40 isolates taken for this study. The fused gene \( \text{aac}(6')\text{-le-aph}(2'')\text{-Ia} \), and the aminoglycoside phosphotransferase genes, \( \text{aph}(2'')\text{-Ib}, -Ic \) and \( -Id \) were considered for screening the clinical isolates as the potential resistance determinants in \( E. \ faecalis \).

The amplification of a 1374bp product was observed for the fused gene \( \text{aac}(6')\text{-le-aph}(2'')\text{-Ia} \) in all the clinical isolates (Figure 3.1a). The amplicons corresponded to position 348-1722 of the coding sequences of Genbank ID: M13771. All the isolates designated phenotypically as HLGR showed amplification for the fused gene. The clinical isolate, S1 screened as sensitive for gentamicin did not have any amplification for the same PCR reaction. No amplification was seen in the plasmid free strain of FA2-2, and also for the control strain of ATCC 29212 that is sensitive to gentamicin (Figure 3.1 b). These results prove the presence of the \( \text{aac}(6')\text{-le-aph}(2'')\text{-Ia} \) gene as one of the genetic determinant for HLGR in the clinical isolates used. The prevalence of the bifunctional gene \( \text{aac}(6')\text{-le-aph}(2'')\text{-Ia} \) conferring HLGR in clinical isolates of \( E. \ faecalis \) has been frequently reported by various studies worldwide (Chow et al 1999, Simjee et al 2000, Qu et al 2006, Feizabadi et al 2008, Watanabe et al 2009).
Figure 3.1  (a) PCR amplification of the internal fragment (1374bp) of HLGR gene \textit{aac(6')} - \textit{Ie-aph(2'')} - \textit{Ia}.

Figure 3.1  (b) PCR amplification of the internal fragment (1374 bp) of HLGR gene \textit{aac(6')} - \textit{Ie-aph(2'')} - \textit{Ia}

PCR amplification for the other genes, \textit{aph(2')} - \textit{Ib}, -\textit{Ic} and -\textit{Id} were also performed for all the clinical isolates and the control strains involved in this study. Amplification was not observed for any of the above three aminoglycoside phosphotransferase genes, in all the forty clinical isolates and also the control strains However, the same DNA has shown amplification for
the bifunctional gene (Figure 3.2) and the 16S rRNA gene (Figure 3.3) indicating the absence of 3 aminoglycoside phosphotransferase genes in the HLGR clinical isolates used in this study. The above amplification experiments showed that all HLGR isolates taken for this study harboured only the \textit{aac(6’)-Ie-aph(2’’)-Ia} gene suggesting that this gene is the major HLGR deciding gene. Similarly, Chow (2000) declared as the bifunctional gene as the clinically most important AME gene due to the fact that this gene confers resistance to virtually all the clinically available aminoglycosides, including gentamicin, tobramycin, amikacin, kanamycin, and netilmicin.

![Figure 3.2](image1.png)  
**Figure 3.2**  
PCR amplification of \textit{aph(2’’)-Ib} gene.

![Figure 3.3](image2.png)  
**Figure 3.3**  
PCR amplification for \textit{aph(2’’)-Id} and 16S rRNA gene.
Similarly, absence of the three aminoglycoside phosphotransferase genes in the clinical isolates was also reported earlier by Qu et al (2006) in HLGR *E. faecalis* isolated in a Chinese hospital. *aph(2·")-Ia*, *aph(2·")-Ib* and *aph(2·")-Id* genes are found to be comparatively rare in enterococci (Chow et al 1999, Tai et al 1998, Kao et al 2000). However, the presence of these genes in association to HLGR has been frequently reported in other species of enterococci like, *E. faecium*, *E. casseliflavus* and *E. gallinarum* (Qu et al 2006). According to Donabedian et al (2003), the *aph(2·")-Id* and *aph(2·")-Ib* genes, which are rarely found in human isolates, are more prevalent in several diary and meat producing animals, and are transmitted to humans through the food supply. Besides, gentamicin is widely used in swine, chickens and turkeys. The above reports imply these genes to be present in niches other than clinical environments. Hence, it could be presumed that these novel aminoglycoside-modifying genes in enterococci can be easily acquired from the environment. Chow et al 2001 had reported on *aph(2·")-Ib* conferring resistance to HLGR in a clinical isolate of *Escherichia coli*. This provides evidence for the transfer of these genes to gram-negative bacteria also. Thus the common occurrence on these HLGR genes in places that favour easy contact to humans and possibility of horizontal transfer to unrelated bacteria emphasised the need to detect for the presence of the *aph(2·")* genes in our isolates. These genes have been so far reported rarely in *E. faecalis* by relatively small surveys done worldwide (Kao et al 2000).

The detection of resistance gene(s) is employed to predict bactericidal synergism in enterococci (Chow et al 1997, Tai et al 1998, Kao et al 2000). The *aph(2·")*- genes encoding aminoglycoside phosphotransferase modifies gentamicin, tobramycin, kanamycin, netilmicin, and dibekacin. Enterococci that possess only *aph(2·")-Id* would be susceptible to ampicillin-amikacin synergism. Hence, the routine screening protocols for HLGR, *aac(6·')-Ie-aph(2·")-Ia* gene could be falsely deemed to carry resistance to
amikacin (Kao et al 2000). There is also a potential possibility of these genes to spread to other species of enterococci if they are carried by the clinical isolates. Hence, it is thought of as imperative to screen the clinical isolates for the presence of the \( a_{ph}(2^\prime)ib \), \( Ic \) and \( Id \) genes even though the fused gene \( a_{ac}(6^\prime)-Ie-ap_{h}(2^\prime)-Ia \) could be the major factor for HLGR in \( E. faecalis \).

As it was observed that the HLGR clinical isolates taken for study harboured only the fused gene, \( a_{ac}(6^\prime)-Ie-ap_{h}(2^\prime)-Ia \), further analysis on determining the conservation of the gene in the clinical isolates was carried out. It has been well documented that these conserved motifs in the aminoglycoside-modifying enzymes play an important role in resistance determination (Rice et al 1995). PCR RFLP of the gene from the clinical isolates indicated that the gene is conserved amongst all the isolates taken for study. Figure 3.4 shows the uniform PCR RFLP profile using restriction enzyme, \( ScaI \) for the isolates used. The same was confirmed with other restriction enzymes, \( TaqI, MnlI, HincII \) etc (results not shown). Sequencing of the PCR amplified product from four different clones generated with the \( a_{ac}(6^\prime)-Ie-ap_{h}(2^\prime)-Ia \) gene fragment of four isolates, \( U \) 3, \( U \) 201, \( M \) 66 and \( B \) 1053 was carried to verify the conserved nature. The sequence of the 1374bp PCR amplicon of the HLGR gene from our clinical isolates produced 100% similarity with the earlier reported coding sequence of the gene \( a_{ac}(6^\prime)-Ie-ap_{h}(2^\prime)-Ia \) (Swenson et al 1995), Genbank ID: M13771. Reports of any variation within the HLGR gene have not been reported till date. Sequencing analysis of the HLGR gene from our clinical isolates also confirms the same.
3.2 IDENTIFICATION OF INSERTION SEQUENCE (S) IN HLGR CLINICAL ISOLATES

3.2.1 Detection of IS256

Reports on the presence of insertion sequences are common in the enterococcal genome. Presence of three IS elements, viz., IS256, IS257 and IS1272 have been reported in enterococci and are known to be present in multiple copies which encode transposases that are involved in the mobility of antimicrobial resistance determinants (Gilmore et al 2002). Hence all 40
clinical isolates taken for this study were screened for the presence of IS elements IS256 and IS257. Screening for IS1272 was not attempted as no direct association of the IS element with HLGR have been reported in clinical isolates of *E. faecalis*.

All the HLGR isolates taken for the study gave positive results for the presence of the insertion sequences IS256. IS256 primers also showed amplification for the control strains FA2-2 and ATCC 29212. A single product to the size of (approximately) 1000bp was seen in all the HLGR resistant isolates, which is absent in the sensitive isolate S1 (Figure 3.5).

Multiple copies of IS256 dispersed throughout the genome are a common occurrence in enterococci and staphylococci (Kozitskaya et al 2004). The occurrence of IS256 in the genome has been correlated with a higher frequency in the clinical specimens rather than in the commensal of *Staphylococcus epidermidis* (Kozitskaya et al 2004). Our results detected the presence of IS256 in all the clinical samples and also in the control strains used and absent in the sensitive isolate S1. The non-infectious nature of the sensitive isolate S1 could have accounted for the absence of the IS element. Relevance of IS256 with the HLGR gene has been vividly described by Simjee et al (2000) during the process of locating truncations in IS256 associated with HLGR clinical isolates in UK. Our study also further confirms the occurrence of this insertion sequence with the resistance gene (Figure 3.5).
Figure 3.5  PCR amplification of IS256.

3.2.2 Detection of IS257

Detection of IS257 in the clinical isolates is significant as the occurrence of IS257 with the HLGR gene has been shown in a number of transposable elements. Rice & Carias (1998) identified IS257 to be located in the transposon Tn5385 carrying the HLGR gene. IS257 has been highly associated in the modified forms of the transposons Tn5281 and alike structures also carrying the HLGR gene *aac(6')-Ie-aph(2''')-Ia* (Hodel-Christian & Murray 1992). In our analysis, IS257 elements are detected in all the isolates (including the sensitive isolate) and showed multiple products amplified by PCR for all the samples (Figure 3.6). Isoforms of this insertion element have been reported earlier in *S. epidermidis* and *S. aureus* (Kozitskaya 2004), which could implicate for the amplification of varying size of products by PCR and our clinical isolates also depict a similar result. IS257 had also been reported in a few strains susceptible to aminoglycosides (Kozitskaya 2004) and is similarly found in our sensitive isolate too.
Figure 3.6  PCR amplification for IS257-(Amplification repeated for lane 4 and found positive with 4 products)

3.2.3 Identification of Transposon Tn5281

3.2.3.1 Intact form of Tn5281

HLGR resistance was reported to be carried by the transposon Tn4001 in *Streptococcus pasteurians* (Patterson & Zervos 1990) and in *Staphylococcus aureus* (Chow et al 2007). The same was reported to be present in transposon Tn5281 in *E. faecalis* (Watanabe et al 2009). PCR based screening of Tn5281 was done in all the clinical isolates and the control strains. Presence of the transposon Tn5281in the intact form was checked by PCR amplification using a single primer from IS256 (Figure. 3.7). Presence of IS256 in inverted orientation in the transposon Tn5281 aids in amplifying the transposon with a single primer as well, resulting in a 3.5kb product (Figure 2.1).
Figure 3.7  PCR amplification for Tn5281 using single primer P10.

PCR amplification resulted in a 3.5kb product along with other multiple products for all the isolates, excluding the sensitive clinical isolate. Strains, FA2-2 and ATCC 29212 also displayed multiple products for this PCR amplification experiment. Nonspecific PCR products could be due to multiple copies of IS256 elements (Hodel-Christian & Murray 1992) distributed throughout the genome of *E. faecalis*. In silico PCR analysis with V583 genome revealed products of various sizes ranging from 900bp to 7.5Kb. Similar results were reported by Simjee et al (2002) and Feizabadi et al (2008) in clinical isolates of *E. faecium* and *E. faecalis* respectively. Hallgreen et al (2003) also presented similar results studying the isolates from a Swedish intensive care unit. They have stated that products of 2.5kb and smaller obtained using the single primer of IS256 could indicate the presence of other IS256 flanked structures in the isolates.

Though multiple products for the single primer had been described previously confirmation of the 3.5kb amplicon to posses the HLGR gene was
done by PCR RFLP using restriction enzymes *HindIII* and *HincII* on the amplified products (Figure 3.8). Restriction with *HindIII* resulted in two products of sizes 2.5kb and 500bp (two fragments of 500bp) in all the clinical isolates. *HincII* digestion yielded fragments of sizes approximately 2.2kb and 1.2kb for all the clinical isolates. Simjee et al (1999) had described similar results with isolates of *E. faecium*. The restricted fragment sizes were determined by *in-silico* analysis using the sequence of Tn4001 from *Staphylococcus aureus* plasmid sequence pSK1 (GenBank: GU565967.1).

![Image of gel electrophoresis](image)

**Figure 3.8**  PCR RFLP Profile of Tn5281 amplified using single primer-P10.

Other fragments with lower intensity could be from the multiple products obtained using the single primer of IS256. The above experiments with our clinical isolates confirm the transposon Tn5281 to be carrying the HLGR gene. The restriction profile obtained could be indicative that Tn5281 from our isolates resembles the transposable element reported in the *E.*
faecalis strain HH22 on plasmid pBEM10 as discussed by Simjee (2000) analysing clinical isolates of E. faecalis from the UK.

Identification of the transposon Tn5281 was also confirmed by another PCR based approach, in which amplification was achieved using primer pairs (Table 2.2, Figure 2.1) involving IS256 and the HLGR gene aac(6’)-le-aph(2’’)-Ia. Tn5281 is reported to carry the HLGR gene aac(6’)-le-aph(2’’)-Ia flanked by inverted repeats of IS256 (Watanabe et al 2009). This Tn5281 amplification was uniform for all the HLGR isolates and V583. Primer sets P3 and P8 (Table 2.2, Figure 2.1) resulted in a product of size 2.5 kb and primer pair P3 and P9 (Table 2.2, Figure 2.1) produced a fragment of 1.8 kb size (Figure 3.9 and 3.10 respectively) in all the HLGR clinical isolates. The sizes of the amplicons were determined by Primer-3 tool utilising the sequence information of Tn4001. There was no amplification with the sensitive isolate, FA2-2 and ATCC 29212. The above results indicate that Tn5281 is the HLGR carrier in clinical isolates of E. faecalis taken for this study.

![PCR amplification of Tn5281 (P3 and P8).](image)

**Figure 3.9**  PCR amplification of Tn5281 (P3 and P8).
Figure 3.10  PCR amplification of Tn5281 (P3 and P9)

The uniform products obtained for all the clinical isolates used suggest that Tn5281 exists in a non truncated form in our clinical isolates. Non truncated elements have been reported to be of chromosomal origin than plasmids of *E. faecalis* (Casetta et al 1998, Daikos et al 2003, Woodford et al 2003).

3.2.3.2  Confirmation of Tn5281 by Southern hybridisation

The PCR amplified products using both the single primer of IS256 and the primer pairs involving IS256 and HLGR gene from the clinical isolates were confirmed to carry the HLGR gene also by Southern hybridisation of the same with a probe prepared from the HLGR gene fragment. The 1374bp HLGR gene fragment was restricted with *ScaI* and *HincII* enzymes to release a 616bp product that carried the coding region of the HLGR gene devoid of the primers as ends. The probe labelled and hybridised with the PCR amplicons of both the primer pairs involving P3-P8 and P3-P9 gave positive results confirming the presence of the HLGR gene in the amplified products (Figure 3.11).
Figure 3.11 Southern hybridisation of PCR amplified products to verify HLGR gene. Lane 8: plasmid DNA of HLGR clinical isolate (not seen in the hybridised profile)

The PCR amplicons of the single primer P10 were also subjected to confirmation by Southern Hybridisation using the above mentioned probe. The results (Figure 3.12) clearly demarcate the amplified products of the HLGR isolates and the control strains FA2-2 and ATCC 29212 that are sensitive to gentamicin. There were no signals detected from the multiple products amplified from FA2-2 and ATCC 29212 for the same primer (Figure 3.10). The 3.5kb product amplified using the single primer showed hybridisation with the probe from V583 and the other clinical isolates taken for study (Figure 3.13).
Figure 3.12 Southern hybridisation to confirm presence of HLGR gene in Tn5281 (amplified using primer P10).

Figure 3.13 Southern hybridisation showing HLGR gene in 3.5kb product of Tn5281. (Lane 3,4 and 11 –was repeated and verified for the same primer).
The strength of the hybridisation signal in the HLGR clinical isolates however varies in our experiment as compared to the earlier report by Feizabadi et al (2008). Their results had highlighted the 3.5kb by southern hybridisation. The reason could be due to the variation in the probe region used for the hybridisation analyses. Feizabadi & Co workers (2008) used the probe from the region corresponding to the transposase function of the HLGR gene (sequence position 1687–2055 of the bifunctional gene sequence) which is also present in the IS256 sequence. But this study involved the internal region of the gene (sequence position 337 – 993 of the gene within the region 304 - 1743 corresponding to the coding region) as the probe. However, the other smaller signals picked up by the probe have to be verified further in order to confirm other IS256 flanking sequences that carry any similarity with the coding region of the HLGR gene.

The above results obtained from the clinical isolates and the control strains clearly indicate the presence of Tn5281 in an intact form as reported to carry the \textit{aac(6')-le-aph(2'')-Ia} gene flanked by IS256 in inverted orientation. There has been no evidence (PCR RFLP results of all isolates) to the presence of truncated forms of Tn5281 with a loss of IS256 in one of the sides of HLGR gene as was identified by Watanabe et al (2009) in a few of Japanese isolates. Similar kind of truncations involving absence of IS256 as a part of Tn5281 were also reported by Simjee et al (1999) in \textit{E. faecium}, from UK and Daikos et al (2003) in \textit{E. faecalis} from Greece.

\subsection{3.2.3.3 Modified forms of Tn5281}

Truncated or modified structures of Tn5281, involving the presence of IS256 and IS257 along with the HLGR gene \textit{aac(6')-le-aph(2'')-Ia}, in varying positions and numbers were reported by Leelaporn et al (2008) in clinical isolates of Enterococci. IS256 elements in association with HLGR gene, replaced by IS257 elements were identified on large conjugative and
non conjugative plasmids of 33–43 kb in the staphylococcal strains from chickens (Khan et al 2005). The clinical isolates taken for this study yielded products of varying sizes involving primer pairs from IS257/IS256 and HLGR gene (Table 2.1,Figure 2.2), but Southern hybridisation with the HLGR gene probe on these amplified products were negative (Figure 3.14). Presence of the insertion sequences in multiple positions in the genome could have resulted in multiple products in PCR amplification. However the occurrence of the HLGR gene carried by hybrid structures of Tn5281 was not identified in our isolates when probed with the HLGR gene fragment (Figure 3.14).

Tn5281-truncated structures that lack IS256 at either side, or at both sides, partial deletions and re arrangements within IS256 have also been reported (Leelaporn et al 2008, Watanabe et al 2009, Simjee et al 1999, Daikos et al 2003) from many countries. The results of this study differ significantly with respect to truncations or modified forms of Tn5281. All the clinical isolates screened in this study showed uniformity in carrying the intact form of Tn5281. However, significance of the intact or truncated structures of Tn5281 is as yet unknown (Watanabe 2009).

The rationale for the present investigation is to provide information on the molecular basis of high-level gentamicin resistance in *E. faecalis* isolated in a multi specialty hospital in order to understand the spread of HLGR. All the HLGR clinical isolates taken for analysis have responded uniformly to the PCR based detection and confirmation for the HLGR gene(s), Insertion elements IS256, IS257 and the transposons Tn5281. This infers 100% occurrence of the resistance determinants in the isolates selected randomly from the hospital. Exploring the presence of other transposable elements was not warranted as considerable evidence has been generated for the intact form of Tn5281 and also absence of association of IS257 with the HLGR gene.
Figure 3.14  Southern Hybridisation of PCR amplified products to identify modified forms of Tn5281.

3.3 SUMMARY OF THE GENOTYPIC ANALYSIS

All the HLGR clinical isolates carry the fused gene *aac(6’)-Ie-aph(2’’)-Ia* coding for the bifunctional aminoglycoside modifying enzyme. The other genes, *aph(2’)-Ib*, -Ic and -Id which also confers resistance to gentamicin, were not detected. It could be concluded that *aac(6’)-Ie-aph(2’’)-Ia* is the only genetic determinant conferring high level gentamicin resistance in clinical isolates of *E. faecalis*. The gene was highly conserved and functional in all the HLGR isolates confirmed phenotypically by the disc diffusion test. Presence of Insertion sequences IS256 and IS257 was observed. However, IS256 was the only insertion element identified to be involved with HLGR. Detection of IS256 and the HLGR gene *aac(6’)-Ie-aph(2’’)-Ia* warranted further analysis to detect and confirm transposon Tn5281 to harbour the HLGR gene. The transposon Tn5281 was found to be present in an intact form carrying the *aac(6’)-Ie-aph(2’’)-Ia* gene flanked by IS256. Association of IS257 with the HLGR gene was not established. Modified forms or hybrid structures of transposable element Tn5281 was also absent.