Summary
The results of our study conducted between July 2001 to June 2003 in Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), a 900-bedded tertiary care hospital at Pondicherry, South India are summarized as follows:

**Enterococci in Clinical infections**

The clinical prevalence of enterococci in our health care setup was highly significant and alarming. Among the 242 enterococci isolated, *E. faecalis* (71%) and *E. faecium* (10%) contributed to 81%, while remaining 19% of enterococci comprised seven different unusual species, which included *E. gallinarum, E. avium, E. raffinosus, E. hirae, E. mundtii, E. casseliflavus* and *E. durans*. The distribution by site of isolation for the 242 enterococci predominantly included 111 isolates (46%) from bloodstream, 72 (30%) from urinary tract, and 59 (24%) from exudate specimens, while 75% of the all the isolates were from inpatient specimens. The infections were polymicrobial only in 46 (19%) of the 242 cases from which enterococci were isolated. The conventional biochemical phenotyping tests identified and speciated majority of enterococcal species, while the molecular phenotyping using WCP fingerprinting by SDS-PAGE validated the authenticity of the unusual species, and the exact taxonomic status of the atypical phenotypic variant strains and strains that showed weak saccharolytic reactions biochemically.

**Antimicrobial resistance in enterococci**

The antimicrobial susceptibility testing by disc-diffusion method showed that all the isolates tested were susceptible to teicoplanin and linezolid, and 92% were susceptible to vancomycin. While 58% and 69% isolates were susceptible to penicillin and ampicillin respectively, only 42% and 54% of the isolates were susceptible to the aminoglycosides gentamicin (high-level) and streptomycin (high-level) respectively. Minimal susceptibility against ciprofloxacin was exhibited by 38% of all enterococci, while the
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Urinary isolates tested for nitrofurantoin and ciprofloxacin showed 78% and 32% susceptibility respectively. Of clinical significance was the high-level gentamicin resistance exhibited by 58%, 60% and 43% of *E. faecalis*, *E. faecium* and unusual enterococcal species respectively. However, there were differences in the results between disc diffusion testing and agar screening/agar dilution method while testing enterococci for vancomycin resistance. None of the penicillin and ampicillin resistant enterococcal isolates tested for beta-lactamase production using a nitrocefin disc yielded a positive result.

The genotypic detection of aminoglycoside resistance genes by Multiplex PCR, showed that the bifunctional gentamicin resistance gene aac(6')+aph(2") was present in 96% of HLGR *E. faecalis* isolates, while ant(6')-1 gene (streptomycin resistant) was detected in 94% of HLSR *E. faecalis* (including many HLGR isolates). The aac(6')+aph(2") and the ant(6')-1 gene were present in 87% and 89% of *E. faecium* isolates respectively. Among unusual species of enterococci tested for aminoglycoside resistant genotypes, only eight isolates (two *E. gallinarum* and six *E. avium*) possessed aac(6')+aph(2") gene, while four isolates (two *E. gallinarum* and two *E. avium*) possessed the ant(6')-1 gene, which also possessed aac(6')+aph(2") gene in them. The absence of these genes in some suggests alternate aminoglycoside resistance mechanisms among the enterococcal species exhibiting HLAR.

**Molecular Characterization of High-level aminoglycoside resistant enterococci**

The plasmid DNA was present 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 ≥70 kb to 2 kb. The restriction-digested plasmids were classified into seven groups (groups A-G) comprising 27 isolates, while 26 isolates that exhibited unique *EcoRI* restriction plasmid profile could not be clubbed with any groups and hence classified as "unique" restriction profiles.
The genetic elements conferring HLAR were located (localized) by DNA-DNA Hybridization studies using \textit{aac(6')-aph(2'')} probe and \textit{ant(6')-1} gene probes. The restriction-digested plasmid DNA from HLAR \textit{E. faecalis} isolates was southern transferred and hybridized with the respective DNA probes. The plasmids classified into different groups with respect to the \textit{EcoRI} 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 \textit{EcoRI} 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 \textit{Smal} 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/streptomycin resistance determinants on their chromosome among the isolates from our hospital setup.

\textit{Virulence factors in enterococci}

The presence of various virulence factors was depicted among enterococci isolated in our study. Bacteriocin production was found in 42\% of the \textit{E. faecalis} isolates and was confined mostly to this single species with the exception of one \textit{E. faecium} isolate. The \textit{E. faecalis} bacteriocin showed a narrow spectrum of activity, active only against two of the species-specific indicators and not against non-species specific indicator strains tested. The \textit{E. faecium} bacteriocin showed a broad spectrum of activity, against all genus-specific indicator strains used in the study, but not against non-genus specific indicator strain tested. Hemolysin production was depicted among 14\% \textit{E. faecalis} isolates and 100\% \textit{E. durans} isolates, while none other species depicted production of hemolysin. Gelatinase production was detected among 58\% of \textit{E. faecalis} isolates, while none other species produced gelatinase. Most of the bacteriocin and gelatinase producing \textit{E. faecalis} concomitantly exhibited HLAR.
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The "Esp" gene was detected among 50% of all isolates of enterococci, which included 65% *E. faecalis* and 40% *E. faecium* isolates, while none other species depicted the presence of "esp" gene. The bifunctional gentamicin resistance gene-aac(6')+aph(2'" and the streptomycin resistant gene-ant(6')-l were present together in 61% and 80% "esp" positive isolates of *E. faecalis* and *E. faecium* respectively. Biofilm formation was found among 26% of *E. faecalis* isolates, while none other species produced biofilm. Thus various virulence traits depicted had permeated *E. faecalis* extensively in our clinical setup. In *E. faecalis* the highest prevalence of virulence factors were exhibited by the urinary isolates (46%) followed by bloodstream isolates (38%), while in *E. faecium* bloodstream isolates exhibited the highest prevalence (50%) of virulence factor, followed by urinary isolates.

**Cell-Cell communication & Gene transfer among aminoglycoside resistant Enterococci**

The enterococci isolated from our setup were highly capable of transferring genetic determinants as evident by conjugation assays. Pheromone responsiveness (for cell-cell communication) was depicted 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 a 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. 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.
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**Molecular typing of High-level aminoglycoside resistant enterococci**

The PFGE typing of the chromosomal DNA of HLAR enterococci yielded approximately 12-20 DNA fragments of various sizes ranging from 50-450 KB in size. The PFGE patterns were analyzed visually and computationally as per standard guidelines. The visual analysis classified 33 of 62 clinical isolates along with 2 transconjugants into nine groups (A-I) based on the PFGE pattern, while the remaining 29 isolates were classified as "unique" based on their restriction profiles, since their profiles could not be clubbed with any groups. The dendrogram constructed by computational analysis of PFGE gels depicted several clusters of isolates, which were partly concordant with the clusters formed by visual interpretation. However differences surfaced between results of clustering with either interpretation methods.

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