CHAPTER 1

INTRODUCTION

Enterococci, a Gram positive cocci, is a complex group of eubacteria that are diverse and possess a significant relationship with the humans. They occur as commensal in the alimentary tract of humans and animals, some of them cause serious infections in humans and animals while some strains are of economic importance, being used in the food industry (Gilmore et al 2002). In addition these bacteria are found in the soil, on plants, surface water, and other environments exposed to human or animal faeces (Khun et al 2003, Fisher & Philip 2009). Under normal conditions enterococci are reported to be stably co existing with the host. However with the advent of modern medicine, this balanced commensalism is being disrupted. Enterococci have been known for over a century on their potency to cause infections in humans (Sherman 1937, MaCallum & Hastings 1899). Initial reports on enterococci as pathogens have shown that enterococcal infections were limited in numbers and occurrence, and mostly caused by the single species, Enterococcus faecalis. E. faecalis accounts for 80% of enterococcal infections (Murray 1990). The first report on high level gentamicin resistant (HLGR) enterococci occurred in 1980 in hospital settings (Patterson & Zervos 1990). Subsequently, ampicillin resistant Enterococcus faecium started to emerge (Gallowy-Pena et al 2009), and in 1986 high-level vancomycin resistant enterococci (VRE) was discovered (Leclercq et al 1988, Uttley et al 1988). But in the last two decades its position as a major opportunistic pathogen contributing to an increased level of nosocomial infections is found to be escalating. Enterococci are reported to be the most
frequently isolated bacteria from hospital associated infections in the US and Europe (Hidron et al 2008). In the recent years there has been a rise in similar reports from other countries worldwide, France, Greece, Italy, China, Tehran, Japan and India (Casetta et al 1998, Daikos et al 2003, Zarrilli et al 2005, Qu et al 2006, Feizabadi et al 2007, Watanabe et al 2009, Upadhaya et al 2009).

The main enterococcal infections include urinary tract infections, infections in the intra-abdominal cavity, enterococcal bacteraemia and endocarditis (Fraser et al 2012). A variety of antimicrobial therapy is being used against enterococcal infections. Ampicillin is the drug of choice for monotherapy of susceptible enterococcal infections. Vancomycins are used under conditions of penicillin resistance and allergy, others like linezolid, daptomycin and tigecycline are used under conditions of VRE. Combination therapy of a cell wall active agent (ampicillin, vancomycin) and an enzyme modifying agent (gentamicin, streptomycin, etc.) is followed in the case of endocarditis and enterococcal meningitis (Fraser et al 2012).

Emergence of resistance to multiple antibiotics and its ability to survive at elevated levels of these drugs has significantly complicated the management of enterococcal infections. Enterococci have gained significance as an important nosocomial pathogen mainly due to their resistance to the commonly used antimicrobial agents such as aminoglycosides, cephalosporins, semisynthetic penicillins, etc. (Marothi et al 2005). The prime reason for this appears to be the ability of the organism to acquire resistance determinants from related strains and spontaneous mutations within the bacterium (Patterson & Zervos 1990). Acquiring resistance determinants from related strains has led to the genomic plasticity of the pathogen and hence contributed to their adaptation to the hospital environment (Zarrilli et al 2005).
High level gentamicin resistance (HLGR) has been a cause of concern in many hospital associated infections. There has been a tenfold increase in the prevalence of HLGR enterococci during 2003 – 2008, as reported by the Norwegian surveillance system for antimicrobial resistance (Rosevoll 2011). The HLGR gene could be easily acquired from within the nosocomial settings due to various factors. And the high rate of acquisition of HLGR by *E. faecalis* has been frequently reported from various countries (Lopes et al 2003, Simjee et al 1999).

The significance of *E. faecalis* as an infectious agent and challenges involving the multi drug resistance along with increasing levels of resistance to the regular antibiotic regimen is on the rise in India. Reports on the prevalence of this bacterium in clinical, nosocomial and environmental samples have been communicated from different parts of the country in the recent years (Mendiratta et al 2008, Upadhaya et al 2009, Sood et al 2009, Narayanaswamy et al 2011, Vinodhkumar et al 2011). In this context, analysing the nature and spread of antibiotic resistant *E. faecalis* among clinical isolates will enable efficient infection control measures. Genetic analysis of *E. faecalis* pertaining to HLGR genes and its transfer mechanism in a hospital set up has not been elucidated yet in India. This study aims at identification of the prevalent form of HLGR gene(s), the type of transposable element involved and also estimating the frequency of transfer of the resistance gene(s) and analysing the genetic diversity employing clinical isolates of *E. faecalis* collected over a time period of three years from a multispecialty hospital in Southern India.
1.1 REVIEW OF LITERATURE

1.1.1 Classification and Nomenclature of Enterococci

Early documentation system of microorganisms shows the genus enterococci classified under “Streptococci of faecal origin”. Early as in 1899, the genus was described as bacteria in pairs or short chains and isolated from patients with acute Endocarditis (MaCallum & Hastings 1899). They were also described as bacteria with very hard and tenacious life. This group later came to be called as Micrococcus zymogenes. In 1905, Gordon reported the occurrence of streptococci from air, contaminated with animal feces. Andrews and Horder coined the name Streptococcus faecalis in the year 1906. Further, there were descriptions of Streptococcus faecium (Orla-Jensen 1919) and Streptococcus durans (Sherman & Wing 1935) based on the organism’s fermentation patterns. They were later identified as different from streptococci and included as members of “enterococcal group”. In 1967, Nowlan and Deibel included Streptococcus avium to the enterococcal group. In 1970, it was proposed by Kalina that a separate genus be established for the enterococcal group based on the cellular features and phenotypic characteristics. However, the usage was continued until 1984 (Schleifer & Balz 1984) when a definite genotypic distinction was proven for both Streptococcus faecalis and Streptococcus faecium from the other members of the genus streptococcus. Since then the genus enterococcus came into valid acceptance. Enterococci are grouped under the phylum Firmicutes and the family of Enterococcaceae. As on date, there are 47 species in the Enterococcus genus registered in the Taxonomy browser gene bank database. (http://www.ncbi.nlm.nih.gov/taxonomy/?term=enterococcus).
1.1.2 Physiology and Distribution

Enterococci are Gram positive facultative anaerobic organisms that are catalase negative, with the ability to hydrolyse esculin in the presence of bile. They can grow under harsh conditions, such as wide temperature ranges of 10°C to 45°C, up to 6.5% NaCl, and pH 9.6 (Sood et al 2008). Enterococci are also capable of survival at 60°C up to a period of 30 minutes (Facklam et al 1989). *E. faecalis* has been observed to adapt to lethal levels of bile salts and detergents such as Sodium Dodecyl Sulfate (SDS). The above characteristics of enterococci make it a hardy bacterium enabling persistence and spread under nosocomial situations. Conditions of weeklong prevalence of enterococci as contaminants of hospitalized patients have been recorded by the health department of UK (Brown et al 2006).

1.1.2.1 Human Intestinal colonisation

Enterococci and group D streptococci form an essential part of the intestinal micro flora of humans and animals. The species distribution shows some characteristics with respect to site-specific colonisation and species distribution. In the human intestine *E. faecium* and *E. faecalis* are the most frequent species isolated. The isolation of *E. faecalis* and *E. faecium* has often been used to indicate fecal contamination of food (Klein 2003).

Each colonised body site represents an ecosystem defined by unique physiochemical and microbial constituents. Much interest is focused on the bacterial community of the large bowel of the humans (Tannock & Cook 2002). Culture techniques were augmented in order to document the bowel colonising micro flora, and the viable but non culturable group of bacteria were analysed using molecular methods (Suau et al 1999). These results revealed that enterococci make up 1% of the intestinal micro flora. Enterococci are found in colons of nearly all humans in numbers as high as
10^8 colony forming units (CFU) (Nobel 1978). However the medical significance of enterococci is much greater as they are rated as the most common cause of nosocomial infections in the humans.

### 1.1.2.2 Other sites of human infections

Microbial communities of the skin, respiratory tract, oral cavity and vagina have been investigated particularly from the perspective of their disease producing capability. Enterococci are commonly reported from various sources of infection as part of polymicrobial flora. Enterococcal occurrences are frequently reported from urine samples, though they may or may not be associated with urinary tract infections (Taneja et al 2004, Mohanty et al 2005). Wound infections and wound pus also show a good number of enterococcal colonisation. The association is often related with prolonged stay in the hospital coupled with other serious illness conditions of the patient (Granado 1998). Giacometti (2000) had related enterococci to be the main source of surgical wound infections leading to complicated situations in hospital settings. Blood stream infections also show a good number of enterococcal colonisation. Separate studies conducted by Suppli et al (2011) in Denmark and by Vithiya et al in India (2011) revealed a large number of enterococci associated with blood stream infections. About 10-15% of endocarditis is attributed to *Enterococcus* species (Landman & Quale 1997).

In humans, internal colonisation of enterococci seems to happen preferentially due to implants or indwelling devices used for treatment of various diseases. Kurup et al (2001) had observed the entry of enterococci to the central nervous system (CNS) through a peritoneal shunt. Intra vascular catheter related blood stream infections were reported by Sandoel et al (2002). The Infectious Disease Society of America (ISDA) had suggested enterococci as one of the major sources of intravascular catheter related infections.
(Mermel et al 2009). These kinds of infection pose a serious threat as they present a complex situation to the medical practitioners. In all these conditions, *E. faecalis* is the most common cause of infection, followed by *E. faecium*, and has been associated with either high level antimicrobial resistance or possession of multi drug resistance mechanisms.

1.1.3 **Non human reservoirs**

Enterococci are normally found in a variety of non human reservoirs. Enterococci are a natural part of the intestinal flora of a majority of mammals and birds with some sporadic existence in reptiles and insects. In addition they are found in soil, plants and in water (Aarestrup et al 2003). Their primary source of entry to water is through faecal contaminants. *E. faecalis* and *E. faecium* dominate the list of isolated species of Enterococci from water. *E. faecalis* and *E. faecium* are again the most common enterococci typically associated with animals and birds. Singh (2009) reported enterococci of equine origin, of which 97% carried resistance to more than five antimicrobials used commonly in livestock industry. The use of antimicrobial agents on livestock has created an increasing awareness for the analysis of resistant bacteria from non human survivors. Enterococci of non human origin also play a critical role in acquisition and dissemination of antibiotic resistance determinants.

1.1.4 **Enterococcal Diseases**

Enterococci, as an opportunistic pathogen is of importance in causing several infections (with or without association of other microorganisms present in the clinical situations) which under prolonged colonisation leads to a disease condition in debilitated individuals. Currently, enterococci rank third or even second in frequency of bacteria isolated from hospitalised patients (Deshpande et al 2007).
Nosocomial enterococcal disease occurs by a two-step process. There is an initial, usually asymptomatic colonisation of patients, mainly of the gastrointestinal tract or occasionally the skin, by strains endemic in a hospital. These strains can come from other patients, from the hospital personnel harbouring these strains in their own gastrointestinal tract, or from environmental sources in the hospital. Such nosocomial strains often possess one or more virulence traits and/or antibiotic resistance. As the host immune suppression increases, the requirements for particular traits of the infecting strain for disease manifestation decreases. Subsequently, the exogenous enterococcal population expands often facilitated by elimination of competitors through antibiotics. Once colonised with enterococci, patients may carry them for months or even years. For a number of patients, however, the second step follows, i.e. tissue invasion from the enterococcal reservoir and eventually disease (Kayser 2003). Table 1.1 lists the various disease conditions created by enterococcal infections along with their clinical synopsis.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Clinical Synopsis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary tract infections</td>
<td>Dysuria and pyuria, frequently nosocomial and derived from patients using urinary catheters</td>
<td>Chen and Zervos 2009</td>
</tr>
<tr>
<td>Bacteraemia</td>
<td>Caused by organism entering blood stream from sources such as UTI or intra abdominal abscess, can increase likelihood of endocarditis</td>
<td>Chen and Zervos 2009, Ubeda et al 2010</td>
</tr>
<tr>
<td>Endocarditis</td>
<td>Infection of heart endothelium or valves, can lead to significant morbidity and mortality, occurs from sources of infection involving genitourinary and gastrointestinal tracts, wounds, bacteraemia</td>
<td>Megran 1992</td>
</tr>
</tbody>
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Table 1.1 (Continued)

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<thead>
<tr>
<th>Disease</th>
<th>Clinical Synopsis</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Meningitis</td>
<td>Rare, primarily diagnosed in neonates</td>
<td>Durand et al 1992, Kayser 2003</td>
</tr>
<tr>
<td>Wound infections</td>
<td>Largely attributed to surgery, decubitus ulcers, and burns</td>
<td>Giacometti 2000</td>
</tr>
<tr>
<td>Peritonitis</td>
<td>Intra abdominal or pelvic infections, abdominal swelling, polymicrobial, patients typically acutely ill</td>
<td>Iaria et al 2005</td>
</tr>
</tbody>
</table>

1.1.5 Epidemiology

Although enterococci do not reach the top-ten list of nosocomial outbreak pathogens (Gastmeier et al 2005, Gastmeier et al 2006), European Centre for Disease Prevention and Control (ECDC) has placed them on the list of pathogens posing major threat to healthcare systems. The distribution of enterococci is prominent worldwide and it is the most commonly isolated species from both clinical and environmental samples (Kuhn et al 2003). The species is highly known for its opportunistic pathogenesis. A study from Denmark (Mutnik et al 2003) has shown 57% occurrence of enterococci in hospitalised patients and 40% in healthy individuals. Hospitalised patients show a general incidence of the infection not only due to the enterococcal virulence factors, but also because the hospitals could be a hub incubating the causative agent (Fisher & Phillips 2009). Enterococcal infections have been reported with high frequency from countries like the United States of America, the United Kingdom, Japan, Greece, Ireland, Italy, France, Iran, etc (Sood et al 2009). European countries have shown more than 30% prevalence of enterococci in their hospitals (Werner et al 2008).
In India, enterococcal infections have been reported in the northern parts of the country. Bhat et al (1997) had reported the presence of enterococci that were resistant to high levels of gentamicin from hospital settings in North India. In 1998, Khanal et al isolated HLGR *E. faecalis* from endocarditis in South India. In the same hospital setting, Prakash et al (2005) reported isolation of five other species of enterococci apart from *E. faecalis* and *E. faecium* from hospitalised patients. Upadhyaya et al (2009) had observed 85.5% occurrence of *E. faecalis* in a tertiary care hospital in Bangalore, which showed high level resistance to gentamicin. Mendiratta et al (2009) reported an increase in the occurrence of High Level Aminoglycoside Resistance (HLAR) enterococci from central India. The above reports indicate the significance of enterococci as an emerging infectious agent of high clinical significance in India.

1.1.6 Clinical Significance

1.1.6.1 Hospital Acquired Infections

Hospital acquired infection (HAI) is a condition where the infection was neither present nor incubating at the time of hospital admission and the infection had occurred during the initial 48 hours after hospitalization of the individual. HAI lead to various direct and indirect consequences for the affected patients. It could lead to conditional deterioration of the patient, prolonged hospital stay and escalated cost of the treatment process due to the extension of the stay period. Also there arises ample risk of various secondary complications due to the acquired infection. Increased rates of morbidity and mortality are also evident in such situations (Bradley & Fraise 1996).

Acquired infections resulting in multiple resistances to antimicrobials escalate the significance of enterococci in hospital environments. The propensity of the organism to survive over various hardy situations enables it to be a persistent inhabitant of hospital environments thus increasing the risk of immune compromised individuals. Eradication of
enterococci through simple disinfection techniques does not result in successful control of hospital-acquired infections.

1.1.6.2 Antimicrobials Used for Control of Enterococci

The discovery of antibiotics is considered one of the most significant health related achievements of modern times and antibiotic therapy is one of the corner stone’s in modern medicine. Microbial control is achieved through varied mechanism of action by the specifically classified group of antibiotics. In the last decade several antimicrobials with effect on enterococci have been developed, that exhibit about 70% clinical and microbiological success (Wang & Hsueh 2009). In order to improve their efficacy and reduce the development of resistance in clinical conditions, it is preferable to employ individual class of the antimicrobial drug as part of a combination regimen (Arias et al 2010). The Figure 1.1 shows the classification of antibiotics based on chemical structure and function.

Figure 1.1 Classification of antibiotics based on chemical structure and function
(Adapted from http://www.pharmamicroresources.com)
1.1.6.2.1 Aminoglycosides

Aminoglycoside antibiotics were one of the early-discovered antibiotics and have been in use for over 60 years. Aminoglycosides have a broad antimicrobial spectrum covering a wide variety of aerobic Gram negatives and few Gram positives (Vakulenko & Mobashery 2003). They display concentration-dependent bactericidal activity and are effective even when the bacterial inoculum is large (Ebert & Craig 1990).


Gentamicin is the aminoglycoside most often used, because of its low cost and reliable activity against Gram negative aerobes (Rougier et al 2004). The major limitations of aminoglycosides is a relatively low therapeutic index with both nephrotoxicity and ototoxicity, and that they are not absorbed orally due to their cationic nature and thus must be given by either an intravenous or intramuscular route (Jana & Deb 2006, Rougier et al 2004).
1.1.6.2.2 Glycopeptides

Glycopeptides include a group of drugs that are composed of glycosylated cyclic or polycyclic non-ribosomal proteins. This class of drugs inhibits the synthesis of cell walls in susceptible microbes by inhibiting peptidoglycan synthesis. They bind to the amino acids within the cell wall preventing the addition of new units to the peptidoglycan. Significant glycopeptide antibiotics include vancomycin, teicoplanin, telavancin, bleomycin, ramoplanin, and decaplanin.

These antibiotics are effective against most Gram-positive cocci and are bactericidal only against enterococci. Glycopeptide antibiotics are generally administered in patients who are critically ill, have demonstrated hypersensitivity to the β-lactams and for patients infected with β-lactam resistant species (Van Bambeke 2004). Vancomycin and teicoplanin are two glycopeptide antibiotics that are routinely used to treat Gram-positive bacterial infections. In 1986, the first Vancomycin resistant E. faecium was reported in France followed by a case in England (Leclercq et al 1988, Uttley et al 1988). Since then, the glycopeptides’ resistance has spread steadily in enterococci. Occurrence of vancomycin resistant enterococci is in the rise in hospitals across the world (Mutters et al 2013). Resistance to glycopeptide antibiotics is inducible and often mediated by genes on plasmids (Leclerq et al 1992). Six different gene clusters have been reported in enterococci that confer resistance to vancomycin. The reason for emergence and dissemination of vancomycin resistance in humans has been attributed to the widespread use of vancomycin in clinical practice and in animal husbandry (Rubinstein & Keynan 2013).
1.1.6.2.3 β-lactams

β-lactams are a broad class of antibiotics that contain a β-lactam ring in their structure. This class includes penicillin derivatives, cephalosporins, monobactams and carbapenems. Most β-lactams act by inhibiting the cell wall biosynthesis. This group of antibiotics is widely used and hence is of high commercial importance (Elander 2003). Bacteria often tend to develop resistance to β-lactam antibiotics by synthesising a β-lactamase enzyme that cleaves and thereby inactivates the β-lactam ring. The β-lactamase gene expression is seen to be induced by exposure to β-lactams (Kak & Chow 2002). β-lactamase inhibitors are generally employed to overcome this resistance. The genes encoding β-lactamase enzymes may be inherently present or may be acquired through plasmid transfers between bacteria.

Of all the β-lactams, penicillin has the highest activity against enterococcal colonisation, followed by carbapenems. Cephalosporins provide very limited activity against enterococci. Ampicillin is the most active penicillin in therapeutic use. Enterococci often possess an intrinsic resistance to β-lactam antibiotics and hence are often used in combination with aminoglycosides (Ravizolla et al 1997).

1.1.6.2.4 Other Antibiotics

Linezolid inhibits protein synthesis and is active against all clinically important Gram positive bacteria, although it only displays a bacteriostatic effect (Leach et al 2011, Livermore 2003). Daptomycin interferes with the cytoplasmic membrane causing depolarization and cessation of protein, DNA and RNA-synthesis (Enoch et al 2007, Silverman et al 2003). It has concentration-dependent bactericidal activity against enterococci (Akins & Rybak 2001). Daptomycin is active against
glycopeptides resistant enterococci and a synergistic combination with gentamicin has shown to have bactericidal activity (Leclercq et al 1991). Quinupristin-dalfopristin (Q/D) is a streptogramin antibiotic that is only active against *E. faecium*. It inhibits protein synthesis and is considered bacteriostatic against enterococci (Blondeau & Sanche 2002). Tigecycline is a broad-spectrum antibiotic that inhibits the protein synthesis. It is reported to be more effective against enterococci than other Gram positive bacteria, in cases of infections, in skin and soft tissues and intra-abdominal infections (Tasina et al 2011, Yahav et al 2011).

Chloramphenicol is a bacteriostatic, broad spectrum antimicrobial effectively used against Gram positive and gram negative bacteria. It functions by inhibiting bacterial protein synthesis. The high prevalence of multidrug resistant enterococci in many hospital settings had led to the use of chloramphenicol as an alternative therapeutic agent. Enterococci resistant to chloramphenicol have emerged due to the ability of the bacteria to produce chloramphenicol acetyl transferase (CAT) enzyme (Jones et al 2001). Hence this antibiotic is not a preferred choice for first line treatment of enterococcal infections due to its various adverse side effects (Wiest 2012). The Figure 1.2 represents the cellular targets of various antibiotics used against enterococci.

Increased usage of antibiotics in human medicine and animal husbandry over the past 70 years has led into a precarious situation. It has resulted in creating a constant selection pressure that has been the cause of an increasing number of microorganisms gaining resistance to these medicines. The use of antimicrobials in infection control is significantly correlated to the emergence of resistant bacteria (Davies & Davies 2010, Levy 2002). Multiple drug resistant bacteria are seen in the hospital settings worldwide (Hidron et al 2008), leading to a scanty scope in treatment options. Hence, the
development of antimicrobial resistance by bacteria constitutes a major threat to human health (http://www.who.int/drugresistance/en/).

![Diagram of antibiotic targets in Enterococci]

**Figure 1.2 Antibiotic targets in Enterococci**

1.1.7 **Antimicrobial Resistance**

1.1.7.1 **Intrinsic Resistance**

Intrinsic resistance is a characteristic feature of a species wherein all members of the same species would be a carrier for the same set of
resistance mechanisms. Resistance of enterococci to most β-lactam antibiotics is governed by a penicillin-binding protein (PBP) that has a low affinity for beta-lactam agents (Fontana et al 1992, Fontana et al 1996). The resistance is of a lower level for antimicrobials like ampicillin, ureidopenicillins, penicillin and imipenem. Minimum inhibitory concentration (MIC) of antibiotics of E. faecium is generally higher than that of E. faecalis (Murray 1990). Members of enterococci display low level intrinsic resistance to lincosamides and aminoglycosides (Murray 1990). Resistance to lincosamides and dalfopristin is attributed by the efflux pump in E. faecalis (Singh et al 2002). Also, many of the wild type enterococci possess endogenous efflux pumps that excrete chloramphenicol making them low level resistant (Lynch et al 1997). Most enterococci are susceptible to co-trimoxazole in vitro, but it is not the same in vivo, because enterococci are able to incorporate exogenous folic acid which enables them to bypass the inhibition of folate synthesis caused by co-trimoxazole (Murray 1990). Thus like most of the bacteria, enterococci posses a low level inherent mechanism to surpass the control measures of a few of the antibiotics in clinical usage.

1.1.7.2 Acquired Resistance

Acquired resistance is the ability of a bacterium to display resistance to antimicrobials by means of gene(s) acquired from their existing environment. This acquired resistance complements the already available intrinsic resistance mechanism of the bacterium. Hence acquisition of any resistance gene(s) by organisms that are regular inhabitants of infectious environments makes it difficult for them to be controlled. This could create a limitation to the choice of agents employed for their infection control. Enterococci belong to the normal gut flora of all humans. Therefore it was previously thought that infections due to these organisms were endogenously acquired from the patient’s own flora. However, analysis of enterococcal
infections in recent years has shown, however, that most infecting strains appear to be exogenously acquired from other strains that share their environment. The human gut flora exhibits a diversity of antimicrobial resistance genes (Sommer et al 2009). Thus, enterococci experience a favourable situation to acquire resistance genes from the diverse gut community.

Enterococci have evolved with enhanced resistances to many antimicrobial agents by acquiring antibiotic resistance gene(s) on plasmids/transposons from other organisms or by spontaneous mutations. The significant case of increased resistance in enterococci, specifically in *E. faecalis* is well reported in the case of aminoglycoside resistance (Kak & Chow 2002). Major risk factors for acquiring nosocomial enterococcal infections are a serious underlying disease and a prolonged hospital stay. This would increase the complications in a hospital setting if there is also a history of a preceding antibiotic therapy. Table 1.2 provides a summary of the different antibiotics used against *E. faecalis*, their resistance, and mechanism of action, phenotypes and type of resistance involved.

### 1.1.8 Aminoglycoside Resistance in Enterococci

All enterococci and other facultative anaerobes have intrinsic low-level resistance to aminoglycosides because of impaired uptake (Moellering 1991). MIC concentrations of aminoglycosides range from 4 μg/ml to 256 μg/ml, and for gentamicin it ranges from 6 to 48μg/ml. These strains generally exhibit susceptibility to synergism in case of ampicillin-gentamicin or vancomycin-gentamicin whenever they do not display high level resistance to ampicillin or vancomycin (Chow 2000). The major mechanism of high level aminoglycoside resistance in clinical isolates of both Gram negative and Gram positive bacteria is enzymatic modification of the aminoglycosides. There are three families of enzymes that perform this function. The bacterial
cytoplasm displays a co-factor dependent drug modification that is carried out by the following enzymes *viz.*, aminoglycoside phosphotransferases (APHs), which are the primary cause for high-level resistance, aminoglycoside acetyltransferases (AACs) and aminoglycoside nucleotidyltransferases (ANTs) (Gallowy-Pena et al 2009).
Table 1.2  Antibiotics used against *Enterococcus faecalis*, mechanism of action, phenotypes and type of resistance involved.

<table>
<thead>
<tr>
<th>Antibiotic resistance</th>
<th>Mechanism of resistance</th>
<th>Associated enzyme</th>
<th>Phenotype</th>
<th>Intrinsic, sporadic or associated Mobile Genetic Elements</th>
<th>References</th>
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<tr>
<td></td>
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<tr>
<td>Ribosome mutation</td>
<td></td>
<td>-</td>
<td>High-level aminoglycoside resistance with MIC 128,000 μg/ml</td>
<td>Sporadic</td>
<td>Arias et al 2007.</td>
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<tr>
<td>AME (Aminoglycoside Modifying Enzyme)</td>
<td></td>
<td>High-level gentamicin resistance</td>
<td>Tn5281</td>
<td>Rice 1998</td>
<td></td>
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<tr>
<td>Antibiotic resistance</td>
<td>Mechanism of resistance</td>
<td>Associated enzyme</td>
<td>Phenotype</td>
<td>Intrinsic, sporadic or associated Mobile Genetic Elements</td>
<td>References</td>
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<tr>
<td>Destruction of β-lactam ring</td>
<td>-</td>
<td>-</td>
<td>β-lactam resistance</td>
<td>Tn552 and others</td>
<td>Sava et al 2010</td>
</tr>
<tr>
<td>Linosamides</td>
<td>ABC-efflux pump</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Altered ribosome</td>
<td>ermA</td>
<td>MLSA phenotype</td>
<td>Tn554</td>
<td>Snydman et al 2005.</td>
</tr>
<tr>
<td>Altered ribosome</td>
<td>ermB</td>
<td>MLSR phenotype</td>
<td>Tn917, Tn1545</td>
<td>Krogstad and Pargwette 1980, Geraci and Martin 1954</td>
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<tr>
<td>Antibiotic resistance</td>
<td>Mechanism of resistance</td>
<td>Associated enzyme</td>
<td>Phenotype</td>
<td>Intrinsic, sporadic or associated Mobile Genetic Elements</td>
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<tr>
<td>Linezolid</td>
<td>rRNA point mutations</td>
<td>g2576T, g2505A, l4 (F101L)</td>
<td>Linezolid resistance</td>
<td>Sporadic</td>
<td>Bozdogan and Leclercq 1999, Hersberger et al 2004</td>
</tr>
<tr>
<td>Methylated rRNA</td>
<td></td>
<td>cfr</td>
<td>Linezolid, lincosamides, streptogramin A resistance</td>
<td>pEF-01</td>
<td>Pogue et al 2007</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>Altered membrane bound protein</td>
<td>Cardiolipin synthetase</td>
<td>Contributes to Daptomycin resistance through an unknown mechanism</td>
<td>Sporadic</td>
<td>Canton et al 2010, Crank et al 2010</td>
</tr>
<tr>
<td></td>
<td>Altered membrane bound protein</td>
<td>gdpD</td>
<td>Daptomycin resistance, effect is amplified in combination liaF mutation</td>
<td>Sporadic</td>
<td>Crank et al 2010</td>
</tr>
<tr>
<td></td>
<td>Altered membrane bound protein</td>
<td>liaF</td>
<td>Daptomycin resistance when combined with gdpD mutation</td>
<td>Sporadic</td>
<td>Crank et al 2010</td>
</tr>
</tbody>
</table>

Table 1.2 (Continued)
1.1.8.1 Mechanism of Aminoglycoside Resistance

1.1.8.1.1 Modification of the Bacterial rRNA

High level resistance (MIC >2000mg/ml) to virtually all the clinically available aminoglycoside antibiotics, except streptomycin and to some extent, arbekacin is given by the bifunctional enzyme AAC (6’)-APH (2”) in enterococci, streptococci and staphylococci (Leclercq et al 1992, Chow 2000). The primary target of aminoglycosides in the case of prokaryotes is the 30S small ribosomal subunit. They bind specifically to the 16S ribosomal RNA of the small ribosomal subunit (Abajy et al 2007, Abraham and Rood 1987, Al-Nassir et al 2008, Alekshun and Levy 2007). The 16S rRNA is involved in protein translation and is responsible for the proof reading mechanism that control the correct codon-anticodon match. The interactions between the codon of the mRNA and anticodon of the tRNA is stabilised by the “flip-out” mechanism of the bases A1492 and A1493 of rRNA (Amyes 2007). The ability of aminoglycosides to also displace these adenines that interact with codon-anticodon pairing is crucial to increase the protein translation infidelity (Amyes 2007). This is the first phase of reaction that ultimately leads to cell death. Bacteria may protect themselves by adopting various means against the action of aminoglycosides. This could include the expression of efflux pumps and target modification of important rRNA bases by methylation (Archer & Niemeyer 1994).

1.1.8.1.2 Enzymatic Modification of Aminoglycosides

However, the most clinically important mode of resistance is through the enzymatic modification of the antibiotics themselves (Arias et al 2010, Arias & Murray 2009). A substantial reduction in the affinity of the antibiotic to the 16S rRNA has been reported as a result of aminoglycoside modification through acetylation and/or phosphorylation (Arthur et al 1993).
The structure for all three classes of aminoglycoside resistance enzymes has been elucidated (Bell et al 1998). These structural studies have established similarities of aminoglycoside acetyltransferases with the members of the large Gcn-5 histone N-acetyltransferase (GNAT) related superfamily (Baquero et al 2003, Bell et al 1998), and aminoglycoside phosphotransferases to the Ser/Thr/Tyr protein kinases (Bae et al 2006). There also exists functional similarity of the aminoglycoside modifying enzymes to other families of enzymes, where both aminoglycoside acetyltransferases and phosphotransferases exhibit protein/peptide modification capabilities (Barie 1998, Bellanger et al 2009). Further similarities between the family members of resistance causing enzymes have been validated through enzyme kinetic and chemical mechanism studies (Arias & Murray 2009, Bellanger et al 2009, Bertram et al 1991, Biavasco et al 2007, Björholt & Haglind 2004, Bonora et al 2004, Bonora et al 2006, Bonten et al 2001). These studies have been integral in the designing of drug modifications to counter aminoglycoside resistance and to identify inhibitors of enzymes responsible for resistance to aminoglycosides (Brandis et al 1985, Caillaud et al 1987, Bruand et al 1991, Braun et al 2000, Burrus et al 2002, Bonora et al 2004, Brochet et al 2008, ). This would aid in overcoming the resistance mechanisms and help in retaining efficacy to the proven antibiotics in use.

The most important, yet most difficult, aminoglycoside resistance protein to overcome is arguably the bifunctional enzyme AAC(6′)-APH(2′″), which possesses both acetyl CoA-dependent acetylation and ATP-dependent phosphorylation activities (Carmeli et al 2002). The protein is the most common determinant of high level resistance (MIC of over 2000 μg/ml) in Gram positive pathogens such as Enterococcus and Staphylococcus. It is also showing a growing importance in the development of resistance profiles of Gram negative organisms (Carrico et al 2005). Initial sequence alignments of
the gene followed by the confirmation with gene truncation experiments suggested that there are two distinct active sites conferring the gene function in AAC(6′)-APH(2″). It was confirmed that the N-terminal carried the acetyltransferase activity and the phosphotransferase activity resides in the C-terminal region of the protein (Chopin et al 2001, Chopin et al 2005). There is considerable evidence that the bifunctional enzyme is a product of a fusion between the aac and aph gene (Chopin et al 2001, Christensen et al 2001). Sequence analyses have shown that both the acetyltransferase and phosphotransferase domains have closely related monofunctional enzymes (Christensen et al 2001, 2003).

Boehr et al (2004) analysing the domain-domain interaction of the AAC and APH domains have stated that both these domains make important interactions that are required for proper structure and thermo stability, however, there is no evidence that there are functional interactions between the two domains. Sequences of the aac(6′)-Ie-aph(2″)-Ia have been cloned and further characterised from various sources of E. faecalis (Chopin et al 2001) and Staphylococcus aureus (Chopin et al 2005) revealing that both catalytic domains carry distinctive properties. The APH domain, with much broader region specificity, can catalyze the phosphorylation of hydroxyls on four different aminoglycoside ring systems (Cosgrove et al 2003). The AAC domain on the other hand has much stringent region specificity, catalyzing the O-acetyltransfer (Courvalin 2006). This is the only member of the AAC(6′) subclass possessing this functionality. The protein is also capable of doubly modifying (6′-acetyl, 2″-phosphoryl) aminoglycosides (Courvalin 2006). Thus, both the AAC and APH domains have broad substrate profiles for their class, and together, they detoxify nearly all known clinically relevant aminoglycosides.
1.1.8.2 Genetics of Aminoglycoside Resistance in Enterococci

1.1.8.2.1 \textit{aac(6')-Ie-aph(2'')-Ia}

Aminoglycoside resistance is primarily delivered by the aminoglycoside modifying enzymes. The major resistance contributing gene is the \textit{aac(6')-Ie-aph(2'')-Ia}, encoding the bifunctional aminoglycoside modifying enzyme. This enzyme is encoded by the fused gene \textit{aac(6')-Ie-aph(2'')-Ia} that renders resistance to all the commonly used aminoglycosides like gentamicin, amikacin, tobramycin, kanamycin and netilmicin, excluding streptomycin and eliminates synergism between all the aminoglycoside and cell wall acting agents. This gene is found to be located in plasmids and/or chromosomes and usually reported as a part of a transposable element (Qu et al, 2006, Watanabe et al 2009). This gene is found to be the prime reason for HLGR particularly in \textit{E. faecalis} (Watanabe et al 2009). Over 90% of clinical isolates of enterococci possess \textit{aac(6')-Ie-aph(2'')-Ia} and about 10% carry \textit{aph(2'')-Ib}, \textit{Ic} and \textit{Id} genes in Japan. The clinical isolates are reported to be possessing gentamicin resistance with a MIC of more than 2000 μg/ml.

1.1.8.2.2 \textit{aph(2'')-Ib}

Gene \textit{aph(2'')-Ib} mediates high level resistance to gentamicin, tobramycin, kanamycin, netilmicin, dibekacin and generally reported in vancomycin resistant \textit{E. faecium} (Kao et al 2000). This gene shows a good variation in the activity level of the phosphotransferase enzyme to all the above stated aminoglycoside substrates as well as to amikacin, isopamicin and arbekacin. The predicted amino acid sequence of \textit{aph(2)-Ib} gene shows 32% identity and 49% similarity with \textit{aph(2)-Id}, 33% identity and 51% in the \textit{aph (2'')} domain of the \textit{aac(6')-Ie-aph(2'')-Ia} gene and 25% identity and 42% similarity with \textit{aph(2'')-Ic} (Kao et al 2000). Reports have accounted the occurrence of this gene in close proximity to the \textit{aac(6'')-Im} gene in
Enterococci. The same situation has also been detected in a plasmid from *E. coli* of clinical origin. This provides a direct clue to the mechanism of horizontal gene transfer of these potential genes between gram positive and gram negative bacteria.

### 1.1.8.2.3 *aph (2")-Ic*

The *aph(2")-Ic* gene encodes aminoglycoside phosphotransferase and mediates clinical resistance to gentamicin, tobramycin, kanamycin dibekacin and not amikacin and netilmicin (Chow et al 2001). The gene was originally detected in *E. gallinarium* but since then also found in *E. faecalis*. This gene confers specific resistance to ampicillin-gentamicin synergism. MIC for gentamicin is restricted to approximately 250 µg/ml. Laboratories that routinely screen for HLGR enterococci are likely to ignore this gene. A site directed mutation caused in the carboxy half of the protein had given a fourfold rise in the MIC value for gentamicin, tobramycin, amikacin and kanamycin (Kak & Chow 2002). This finding is of significance in the clinical environment as these mutations could warrant a major difference in the therapeutic regimens of routine antibiotic concentrations followed.

### 1.1.8.2.4 *aph(2")-Id*

The *aph(2")-Id* gene encodes aminoglycoside phosphotransferase that provides high level resistance to gentamicin, tobramycin, kanamycin, netilmicin and dibekacin (Tsai et al 1998). This gene was initially reported in *E. casseliflavour*, subsequently reported in *E. faecium* also.
1.1.8.2.5 Other genes

Other genes like \textit{aph(3')-IIIa} encoding aminoglycoside phosphotransferase confers high level resistance to kanamycin and also mitigates the ampicillin amikacin synergism (Boehr et al 2001). \textit{aac(6')-Ic} encodes aminoglycoside acetyltransferase and eliminates synergism between cell wall active antibiotics with tobramycin, kanamycin, netilmicin, sisomicin etc. This gene is located in the chromosome and so far been found only in \textit{E. faecium} (Gilmore et al 2002). \textit{ant(4')-Ia} – the aminoglycoside nucleotidyl transferase, a less prevalent gene in enterococci gives resistance to tobramycin, amikacin, kanamycin and dibekacin and also alleviates the ampicillin, amikacin synergism. \textit{ant(6')-Ia} genes confer high level resistance to streptomycin in \textit{E. faecalis} isolates to an alarmingly high level of 128000ug/ml (Eliopoulos 1984). The presence of multiple genes capable of imparting resistance to aminoglycosides and their ability to exist in different enterococcal species is a concern towards infection control.

1.1.9 Mobility of Resistance Factors in \textit{Enterococcus faecalis}

\textit{Enterococcus faecalis} is the most common enterococcal species isolated from patients with hospital-acquired infection. Plasmids and transposons harboured by \textit{E. faecalis} facilitate the spread of resistance genes and virulence factors. Horizontal gene transfer amongst bacteria could be a major player leading to prevalence and spread of the notorious genes in the nosocomial environments. With the increased knowledge on genomics and molecular phylogeny it has become evident that horizontal gene transfer has taken a critical lead in directing the evolution of bacterial species (Doolittle 2000). They show well established mechanisms that are responsible for the evolution of pathogenic potential, metabolic diversity, and perhaps even the operon structure of the genome. The factors that are predominant for the mobilisation of bacterial genes include bacteriophages, plasmids, and
transposons/insertion sequences (Weaver et al 2002). There could be a
difference in the level of contribution of these individual factors to gene
transfer, however all species carry representatives of each class. A good
variety of plasmids and transposons have been identified and characterised in
enterococci.

1.1.9.1 Pheromone Responsive Plasmids

The pheromone responsive plasmids are a family of approximately
20 conjugative plasmids whose transfer mechanisms are controlled by peptide
sex pheromones secreted by the plasmid free cells. The size of these plasmids
range between 37 to 91kb, with low copy number of only 2 to 4 per cell,
exhibiting a narrow host range, restricted only to the enterococci (Gilmore
2002). The repA genes encode the replication initiator proteins for these
plasmid replicons. The 5’ region of the gene is highly conserved while the 3’
region is not well conserved, across the group of plasmids but is highly
conserved within the plasmids. The conserved regions carry out significant
functions unique to these plasmids (Gering et al 1996). The central region
exhibits limited sequence conservation but carries multiple repeat units’
characteristic for each plasmid, which has also been shown to be involved in
the origin of conjugative transfer. This kind of arrangement is found to be
unique in case of conjugative plasmids (Weaver et al 2002).

Conjugative plasmids in *E. faecalis* confer a mating response to the
sex pheromones secreted by potential recipient cells. This mating signal
induces the synthesis of a surface aggregation substance that facilitates the
formation of mating aggregates and plasmid transfer. 60% of the drug
resistant strains harbour pheromone responsive plasmids and exhibit a
clumping response with a culture filtrate of a plasmid free recipient strain of
*E. faecalis* (Ma et al 1998). The physical aggregations of the cells favour
exchange of genetic material between the strains. The well characterised
pheromone responsive conjugative plasmids include pCF10, pAD1, pTEF1, pTEF2, pJH2 and pAM373 (Clewell 2007, Dunny 2007, Jensen et al 2010). The pheromone responsive conjugative plasmids have a significant role in the horizontal spread of genes between strains of *E. faecalis* (Writh 1994). Pournaras et al (2000) had reported that the pheromone responsive plasmids could be the major reason for the conjugal transfer of HLAR in *E. faecalis*.

1.1.9.2 Transposable Elements

*Enterococcus faecalis* strain V583 (Paulsen et al 2003), contains mobile and exogenously acquired DNA for over 25% of the genome. They include conjugative and composite transposons, a pathogenicity island, integrated plasmid genes and high number of Insertion Sequence (IS) elements. A number of transposable elements, both conjugative and composite, have been identified and characterized in enterococci. Many of these elements are the potential carriers of the HLGR gene and result as important sources of horizontal exchange of the resistance (Sood et al 2008).

1.1.9.2.1 Insertion Sequences

Insertion sequences (IS), or insertion-sequence elements, are segments of bacterial DNA that can move from one position on a chromosome to a different position on the same chromosome or to a plasmid within the cell. The distribution of three typical IS elements which have been described as components of enterococcal and staphylococcal genome are IS256, IS257, and IS1272. These elements are reported to occur in multiple, independent copies in the genomes of staphylococci and enterococci (Kozitskaya et al 2004).

IS256 is a characteristic element in the genome of multi-drug resistant nosocomial *Staphylococcus epidermidis* isolates that might be
involved in the flexibility and adaptation of the genome in clinical isolates (Kozitskaya et al 2004). IS256 are present in many strains of enterococci that carry antibiotic resistance, and is highly correlated with the presence of HLGR. But, they have also been found as a part of the chromosome in strains that do not harbor HLGR (Rice & Marshall 1992). The presence of IS256 in multiple copies speculate that new IS256 based composite transposons can emerge and could lead to mobility of considerably larger segments of DNA that may include other resistance genes (Rice et al 1995).

IS257 is the other insertion element frequently reported in enterococci and staphylococci. Transposon Tn4003, carrying the trimethoprim resistance is reported to carry repeat sequences of IS257 (Leelapor 2008). IS257 is also subsequently noted in *E. faecalis* in situations of Transposon Tn4001 as a carrier of HLGR in clinical isolates. However, occurrence of IS257 in association with HLGR has not been shown to follow a designated pattern as that of IS256 (Kozitskaya et al 2004, Watanabe et al 2009). Occurrence of IS257 has also been reported in clinical isolates sensitive to gentamicin (Kozitskaya et al 2004).

IS1272 is the third insertion element often reported in transposable elements carrying the HLGR gene and also resistance to erythromycin. IS1272 is reported to be a part of the 65 kb transposon Tn5385 that carries multiple antibiotic resistance genes (Rice 1998), which also harbors the HLGR gene *aac(6′)-Ie-aph(2′′)*. There has been no reports with direct reference to IS1272 being a possible cause of HLGR dissemination.

1.1.9.2.2 Tn4001

Transposon Tn4001 was originally identified in Australian isolates of *Staphylococcus aureus*. The same was observed on a 70 kb mobile element located in a gentamicin resistant and Beta-lactamase producing plasmid pBEM10.
of \textit{E. faecalis} HH22 (Simjee 2000). Lyon et al (2004) reported the presence of Tn4001 in the plasmid pSK1 of \textit{S. aureus} that existed in two forms pSK1a and pSK1b. pSK1b has a tandem duplication of IS256 which was well correlated with an increased resistance to gentamicin.

Transposon Tn4001 is composed of a central region containing the \textit{aac(6')-Ie-aph(2'')-Ia} gene, flanked on each side by inverted copies of insertion sequence IS256 (Daikos et al 2003). This conjugative transposon is either located in the chromosomal DNA or on a plasmid. The complete (4768 bp) sequence of the transposon has been determined (Dyke et al 1992). Early reports of transposons identified in \textit{E. faecalis} found Tn4001 to be the main carrier of HLGR resistance. Later this was found confined in Staphylococci and the \textit{E. faecalis} Tn4001 was designated as a similar but not identical element and was designated as Tn5281.

1.1.9.2.2.1 Modified forms of Tn4001

Several variants of transposon Tn4001 have been identified in enterococci from diverse geographic locations, the Tn4001-IS257 hybrid and the Tn4001-truncated elements (Hodel-Christian et al 1991). In the North American isolates of \textit{Staphylococcus aureus}, region encoding \textit{aac(6')-Ie-aph(2'')-Ia} is bound on each side by 425 bp of the IS256 elements with both sides missing the external 900 bp of IS256. In place of the external portion of IS256, these isolates have three copies of a commonly occurring staphylococcal insertion sequence, IS257 (Byrne et al 1990). Three different types of Tn4001-like elements in which the IS256 elements were largely truncated and replaced by IS257 elements were identified on large conjugative and non-conjugative plasmids of 33-43 kb in the staphylococcal strains from chickens. They are, a 38kb of pGTK1 from \textit{Staphylococcus warneri}, a 43kb of pGTK2 from \textit{Staphylococcus sciuri} and a 33kb of pGTK3 from \textit{Staphylococcus warneri} (Lange et al 2003).
The frequencies of transfer of gentamicin resistance for the isolates containing truncated variants of Tn4001 differed significantly in clinical isolates of E. faecalis. Twenty four of the thirty isolates containing truncated variants transferred the gentamicin resistant gene where as only 3 of 34 isolates transferred the non truncated form (Daikos et al 2003). The non-truncated elements are reported to be more stable, where as the truncated variants are mobile and transfer the resistance gene more efficiently (Daikos et al 2003).

1.1.9.2.3 Tn5281

In Tn5281, the aac(6′)-Ie-aph(2″)-Ia bifunctional aminoglycoside resistance determinant is flanked by inverted copies of staphylococcal insertion element IS256. It was found to be similar, if not identical, to the transposon Tn4001 found in Staphylococcus aureus, consisting of the aac(6′)-Ie-aph(2″)-Ia gene flanked on both sides by the insertion element IS256 in inverse orientations. The genetic determinant encoding gentamicin resistance on pBEM10 of E. faecalis HH22 is carried on a transposon Tn5281, that is highly related to the staphylococcal gentamicin resistant transposon Tn4001 found in Australian isolates of Staphylococcus aureus and Tn4031 found in United States isolates of Staphylococcus epidermidis (Hodel-Christian et al 1992). The transposon Tn5281 from pBEM10 is reported to have a tandem duplication of IS256 at one terminus of Tn4001 (Hodel-Christian et al 1992).

The three transposons Tn5281, Tn4001 and Tn4031 have shown similar restriction profiles for the enzymes, HindIII, Clal and HaeIII. Restriction endonuclease digestion patterns of Tn5281 generated with HincII, Scal, and Alul were also consistent with Tn4001 and Tn4031 (Hodel-Christian et al 1992). Hence, Tn5281 is the transposon that is similar but not identical to the staphylococcal transposon Tn4001. All recent reports of
transposon carrying the HLGR gene \textit{aac(6')-le-aph(2’)-Ia} in \textit{E. faecalis} is designated as Tn5281 (Feizabadi et al 2006, Watanabe et al 2009)

1.1.9.2.3.1 Modified Forms of Tn5281

Transposon Tn5281 has been reported to exist in modified forms in \textit{E. faecalis} with respect to the number and type of IS elements associated. Three types of Tn5281-truncated structures have been reported in clinical isolates. One of them lacks the left IS256 element (Leelapron et al 2008), the other lacks the right IS256 element and the third, lacking IS256 at both ends (Feizabadi et al 2008). Tn5281-truncated structures are found to spread more efficiently among enterococci (Watanabe et al 2009).

1.1.9.2.4 Transposon Tn5384

\textit{Enterococcus faecalis} has been found to harbor a good number of transposable elements. Rice et al (1995) identified a 26 kb mobile element from \textit{E. faecalis} strain CH116. This was designated Tn5384, which confers resistance to erythromycin and to high levels of gentamicin. Tn5384 is a composite element containing three copies of insertion element IS256. Among three copies, two of IS256 flanked the \textit{aac(6')-le-aph(2’)-Ia} bifunctional aminoglycoside modifying enzyme gene in the inverted orientation, forming a structure similar to staphylococcal gentamicin resistance transposon Tn4001. One of the IS256 elements involved in the Tn4001-like structure forms the left end of Tn5384 and the right end carries a directly repeated insertion of IS256, approximately 23kb downstream of the leftmost insertion. Rice et al (1995) have validated that IS256 can form mobile elements when the tandem sequences are in a directly repeat orientation, as earlier reported in many gram negative bacteria.
1.1.9.2.5 Transposon Tn5385

Transposon Tn5385 of size 65 kb have been reported in *E. faecalis*. Regions of Tn5385 are linked by a series of insertion sequence elements (IS256, IS257 and IS1216) of staphylococcal and enterococcal origin. Tn5385 is a highly complex element that contains several other composite and conjugative transposable elements earlier reported in both enterococci and staphylococci. Tn5385 is found to carry the conjugative transposon Tn5381, composite transposon Tn5384 and elements indistinguishable from staphylococcal transposons Tn4001 and Tn552 (Rice 1998). Though reported as a transposable element relevance of Tn5385 as a possible disseminator of antibiotic resistance genes in both enterococci and staphylococci have not been reported.

1.1.10 Molecular typing of *Enterococcus faecalis*

Bacterial isolates and strains have been classified based on genotyping using several techniques. The identified genetic profile of any bacteria by a specific genotyping method has to be as unique as a fingerprint (Li et al 2009). Genotyping could be done based on various approaches which could include DNA banding based methods / PCR banding methods (Yildirim et al 2011).

DNA banding based methods classifies isolates according to the size of fragments generated through digestion of the genomic DNA with the use of restriction endonucleases and separated through electrophoresis employing constant electrical field. But DNA fragments larger than 20kb show the same mobility with conventional electrophoresis and tend to migrate in a size independent manner. Hence, separation of larger sized DNA fragments was achieved by the application of alternating electric fields at different angles through Pulsed Field Gel Electrophoresis (PFGE). PFGE
employs the use of restriction enzymes of uncommon recognition sequences that results in DNA fragments of larger sizes and the banding pattern generated after the electrophoresis reflects the DNA polymorphisms at the recognition sites.

Typing methods fall into two broad categories viz., i) phenotypic and ii) genotypic methods. Phenotypic methods are those that characterize the products of gene expression in order to differentiate strains. Genotypic methods are those based on the analysis of genetic structure of an organism and include polymorphisms in DNA restriction. Molecular subtyping has become an essential component of epidemiologic investigations of infectious diseases. This widespread use of molecular typing has resulted in a plethora of techniques and protocols for subtyping. The process of subtyping is important epidemiologically for, recognizing outbreaks of infection, detecting the cross transmission of nosocomial pathogens, determining the source of the infection and identifying particularly virulent strains of organisms and monitoring vaccination programs.

1.1.10.1 PFGE Based Subtyping

PFGE is a powerful tool for characterizing various strains at the DNA level, obtaining relevant information on genome size and constructing the physical and genetic map of the chromosome of bacteria that are poorly understood at the genetic level as well as in separating chromosomes in microorganisms, and in the long range mapping of mammalian genes. PFGE has been repeatedly shown to be more discriminating and can be applied as a universal generic method for subtyping of bacteria. Only the choice of the restriction enzyme and conditions for electrophoresis need to be optimized for each species. DNA restriction patterns generated by PFGE are stable and reproducible at the intra and inter laboratory levels.
Among the many known enterococcal molecular typing methods, PFGE has proven to be a highly reproducible and accurate typing method, which can distinguish clonal populations, and hence is considered a “gold standard” for subspecies discrimination of *E. faecalis* clinical isolates. However, the results obtained by PFGE are not readily transportable, making it difficult to compare results among different laboratories and thus limiting studies involving inter-laboratory comparisons (Werner 2013).

### 1.1.10.2 Subtyping by PCR

The polymerase chain reaction (PCR) amplification pattern attempts to employ regions of the genome that could exemplify the variations within by amplifying the same. Repetitive DNA sequences are dispersed in multiple copies throughout the bacterial genomes. The consensus sequences of these elements could be utilised for amplifying the differences within the genomes. The banding patterns obtained from these amplifications are used for the fingerprinting of bacteria (Park et al 2004). Typing methods targeting tandem repeats or mobile elements such as insertion sequences have been reported and have proved to be a valuable tool for molecular typing and strain characterisation of some bacteria (Yildirim et al 2011). The methodology involved carries effectiveness in its approach and is easily performed.

Genetic mutation and rearrangement caused by Insertion Sequences (IS) is one of the important mechanisms for generating genetic diversity in prokaryotes. Molecular methods analysing bacterial strains using these sequences can detect the existence, copy number and locations of insertion sequences but also reflect the overall genomic organisation of those strain. Insertion sequences have been described in enterococci (Rice et al 1995) and are highly correlated with the mobility of anti microbial resistance determinants (Gilmore et al 2002). They are reported to be potentially active for genetic rearrangements and could be associated with strain variations.
IS256 and IS1216 of enterococci have been greatly responsible towards movement of resistance determinants. IS256 was first described as inverted repeats flanking the \( aac(6\prime)\text{-}aph(2\prime) \) bifunctional aminoglycoside modifying enzyme gene in \textit{S. aureus} (Kozitskaya et al 2004) and also in \textit{E. faecalis} since then.

1.2 \textbf{SUMMARY OF LITERATURE REVIEW}

In summary, the reviewed literature regarding the scope of the study provided the following insights.

- Enterococci, a Gram positive, facultative anaerobic bacteria, an usual commensal found in various locations capable of survival under hardy situations.

- Enterococci are a major group of opportunistic pathogens capable of infecting humans and live stocks.

- Enterococcal infections, in situations of prolonged colonisation may lead to fatal disease conditions.

- Of all the enterococcal species \textit{E. faecalis} accounts as a major causative agent and is often associated with hospital acquired infections.

- Several antimicrobials are used for the control of Enterococcus faecalis.

- \textit{E. faecalis} possess due intrinsic resistance mechanisms that permit them to be potential agents as persistent infectious agents.

- They harbour and have the potential to acquire genetic factors favouring their resistance to antimicrobials to levels much
higher than those normally used in routine infection control under clinical situations.

- Aminoglycoside resistance is of due importance that requires attention in case of *E. faecalis* associated drug resistance. Gentamicin is the most commonly used aminoglycoside.

- High level gentamicin resistance in *E. faecalis* is primarily attributed to the bifunctional fused gene *aac(6′)-Ie-aph(2″)-Ia*.

- The genes responsible for high level gentamicin resistance in *E. faecalis* are harbored in chromosomes/ plasmids and are highly influenced by transposable elements.

- The plasmids in *E. faecalis* could directly or indirectly facilitate the transfer of the resistant trait(s).

- Transfers from chromosomal origin are also feasible through pheromone responsive plasmids, conjugative plasmids, composite and conjugative transposons.

- Conjugation is a favourable mechanism of genetic exchange that enables transfer of resistance genes and is highly influenced by various insertion elements and transposons.

- The exchange of resistance determinants among related isolates could lead to genome plasticity eventually leading towards genotype evolution favouring better adaptability of the organism in clinical conditions.

- Subtyping of strains/isolates from different sources of infection could help in analyzing distinctive genotypes responsible for outbreak situations and also establishes a genetic lineage among strains/isolates studied.
• PFGE is a much preferred approach for subtyping *E. faecalis* at molecular level, however simpler techniques like PCR based approaches are thought of as much simpler techniques performed with ease to achieve the same.

• Knowledge of distinctive subtypes obtained pave way for understanding or predicting the potential spread of infection through spread of a high multidrug resistant strain/ isolate in a nosocomial situation and hence could be valuable inputs for stringent control measures.

1.3 **OBJECTIVE OF THE STUDY**

The objective of this study was to understand the molecular basis of the high level gentamicin resistance, in clinical isolates of *E. faecalis* by analysing the genetic determinants for HLGR, its ability of dissemination influenced by the location of the gene and to analyse the relatedness of the HLGR isolates.

**The Specific objectives of the thesis include,**

• The identification of the HLGR gene(s), (*aac(6’)-Ie-aph(2’)*, *aph(2’)-Ib*, *aph(2’)-Ic* and *aph(2’)-Id*).

• Determination of the type of transposable element(s) associated with the HLGR.

• Ascertain the transfer ability of the resistance gene.

• Investigation on the location of the gene(s).

• Genetic relatedness/diversity of the isolates.
The thesis is organized into five chapters which cover the following topics of the work.

**Chapter 1** provides the background of the work, evidence in the literature for the significance of HLGR in *E. faecalis*.

**Chapter 2** describes the materials and methods used in this study.

**Chapter 3** describes the results of the strategies and methods used to identify the gene and the transposon involved with the isolates and discuss its relevance in determining the virulence of *E. faecalis*.

**Chapter 4** states the approaches made for determining the location and transfer of the HLGR gene in the isolates, and discusses the results.

**Chapter 5** describes the establishment of the genetic relatedness of the isolates using PFGE, PCR and post PCR based approaches and discusses the potential of this analysis.

**Chapter 6** summarises the findings with relevance to the HLGR gene being the source of gentamicin resistance and its potential to be disseminated within the nosocomial environment and the clonal relatedness of the isolates taken for study.