CHAPTER V

DISCUSSION

*Enterococcus* has emerged as a prominent nosocomial pathogen since last decade worldwide (NNIS 1999., Murray 1990 &1998, MMWR 1993, Mundy et al 2000, Ike et al 1987). The results in our present study showed, *E. faecalis* (80 %) and *E. faecium* (11%) as the two predominant species in our clinical setup accounting for 91% of *enterococci* isolated. These findings were similar to several studies worldwide reflecting the general trend in prevalence of *enterococci* (Moellering Jr 1992, Murray 1990, Jones 1997, Davies et al 1992, Richards et al 2000, Low et al 2001). Prevalence of unusual enterococcal species (9%) as shown in our study was similar to that of other studies that showed the prevalence of non-*faecalis* and non-*faecium* *enterococci* as 2-10% (Gordon et al 1992, Murray 1998, Jones et al 1995). Some of the previous studies from India have reported *E. faecalis* and *E. faecium* as the only prevalent species (Nischal et al 1996, Gulati et al 1997, Devi et al 2002).

We have documented varied distribution by site of isolation for the 396 *enterococci* isolated through our study. Interestingly as shown in Table 9, 54 % of *enterococci* were isolated from urinary tract, while 39% and 7 % were from exudate and bloodstream specimens respectively. Several studies carried in different parts of the world shows that urinary tract remains the most common site of isolation for *enterococci* (Gordon et al 1992, Zervos et al 1986, Morrison Jr 1986, Bouza et al 2001- ESGNI-003 study, Davies et al 1992, Mathai et al 2001, Desai et al 2001, Miskeen et al 2001), as also the results of our study. The 80% prevalence of *E. faecalis* as the predominant species among urine specimens in our study is in concordance with the results of most of these studies.

The spectrum of enterococcal infections was diverse and occurred in a patient population among whom 52% were males, 48 % were females, while 75% were inpatients among them. The median age of the patients was 33 years (range,
Neonates to 90 years) that was comparable with the above mentioned studies, although variation in age occurred among studies on enterococcal UTI where the median age was >55-60 years mostly.

The highest prevalence of enterococci from urinary tract infections is of serious concern. Urinary tract infections (UTI) are the most common of all infections caused by enterococci particularly in hospitalized patients. Our results were concordant with many Indian studies over the years, while few studies contrasted our results. Desai et al in 2001 studied the prevalence and distribution of various species of enterococci in a tertiary care hospital in Mumbai, by processing various clinical specimens of catheterized patients with UTI. Enterococci were prevalent in 22% of the total specimens with Foley's catheters. In a one and a half year study from a tertiary care hospital in Mumbai, the incidence of Enterococcus species in urinary tract infections was found to be 7.3% with E. faecalis as the predominant species isolated (87%) followed by E. faecium (10.8%) and E. durans (2.14%) (Miskeen et al 2001). In 2003 Parvathi and Appalaraju in their one-year study showed a prevalence rate of 3.5% enterococci from various clinical samples in a tertiary care hospital in Coimbatore. The highest incidence was from isolates of urine (43%), pus (40%), wound swabs (11%), and the least incidence were noted in discharges from fistula (2%), blood cultures (2%) and peritoneal aspiration fluid (2%) while E. faecalis accounted to 88% of the isolates. The frequency of isolation of E.coli has declined over time from 35.6% in 1996, to 32.5% in 1998 and thereafter to 26.6% in 2001, on the other hand enterococci were isolated at an increasing frequency over the years from 11.8% in 1996, to 15.3% in 1998, and thereafter 22% in 2001 which authenticates it's emergence as a significant nosocomial uropathogen (Wazait et al 2003).

The increased prevalence of nosocomial enterococcal UTI in our study is probably the result of many predisposing factors like increasing use of catheterization and broad-spectrum antibiotics mostly among patients of older age group as shown by several studies (Bouza et al 2001, ESGNI-004 study, Moulin et al 1998, Desai et al 2001), but lesser prevalent (< 5%) among younger age group where catheter related infections accounted for upto 48% of all the nosocomial UTI.
Thus by and large enterococci continues to play a major role in the nosocomial UTI and the results of our study are in concordance with other studies.

The prevalence of enterococci from blood stream infections is 9% (36/396). 90% of blood stream infections were from cases of septicemia without endocarditis. The incidence of enterococcal bloodstream infections in our setup was highest in the pediatrics specialty, since majority (60%) of the blood culture samples which yielded enterococcus were from the pediatrics age group, and sepsis was most commonly reported from neonates with focal infections including meningitis, scalp abscess and pneumonia in this age group as shown by several studies (Yuce et al 2001, Lee et al 1999, Bilikova et al 2003, Bhat et al 1997, McNeeley et al 1996, Breton et al 2002, Chang Chien et al 2000). The results of a recent point prevalence survey of nosocomial infections in 29 Pediatric Prevention Network NICU conducted by CDC in U.S. has shown that enterococci ranks second only to coagulase-negative staphylococci as the leading cause of nosocomial infections of neonates in ICU (Sohn et al 2001). On the other hand, the emergence of vancomycin resistant enterococci (VRE) in neonatal infections as shown by other recent studies poses a therapeutic challenge and emphasizes the urgency for more effective prevention interventions (Yuce et al 2001, Malik et al 1999). Thus the significance of our findings is relevant in an era of increasing rates of antimicrobial resistance especially among the pediatric age group. 40% of enterococcal bloodstream infections in our study were distributed evenly among other medical and surgical wards in an elderly patient population, including cases of septicemia with endocarditis, which is in concordance with the results of other studies (Murray 1990, Megran 1992, Fernandez-Guerrero et al 2002, Kholeif et al 2002).

Although *enterococci* play a significant role in many nosocomial infections their role in polymicrobial infections still remains debatable, but several experimental studies have shown that *enterococci* contribute to the severity of the disease in case of polymicrobial infections like intra-abdominal and wound infections, burns or abscesses, surgical site infections, and bacteremia (Murray 1990, Chow et al 1993, Jett et al 1992). Sood et al in 1997 conducted a retrospective analysis of positive blood cultures obtained during the period of five years from 1991 to 1995 and depicted a change in the trend of causative organisms among the polymicrobial culture positive samples. Gram-negative bacteria were shown to predominate between 1991-94 with an average of 69% of cases, whereas in 1995 the gram-positive cocci isolation was nearly the same as gram-negative bacteria (45% vs. 55%) with *E. faecalis* isolates exhibiting multi-drug resistance, which had prognostic and therapeutic implications. Although the clinical significance of *enterococci* in intra-abdominal and pelvic infections remains debatable, several reports depict their role in peritonitis, intra-abdominal or pelvic abscess, surgical site infection, suppurative thrombophlebitis, acute salpingitis, and endometritis where *enterococcus* were isolated along with S. aureus, CoNS and gram negative bacilli which includes Pseudomonas, Klebsiella, E. coli and Acinetobacter commonly although at varying incidence (Johnson et al 1986, Harbarth et al 2004, Sitges-Serra et al 2002, Garcia-Rodriguez et al 1995, Dougherty 1984, Murthy et al 1998). Most of these studies depict that 5-70% of enterococcal infections as polymicrobial, which varies according to the type of infection. Only 44 % of enterococcal infections were polymicrobial in our study, while majority were non-bloodstream isolates that underscores the clinical significance of *enterococci* isolated. The polymicrobial cultures generally yielded one or more of the following pathogens: Escherchia coli, Klebsiella spp, Pseudomonas aeruginosa, Proteus species, Citrobacter spp, Enterobacter spp and Staphylococcus aureus among the bacteria and Candida species among the fungus.

The general risk factors for most of the enterococcal infections are a prolonged hospital stay by patients with severe underlying diseases and life threatening conditions which includes cancer, diabetes mellitus, chronic renal
failure, major trauma, surgical procedures and urinary and vascular catheterization as authenticated by several studies (Patterson et al 1995, Shlaes et al 1981, Malone et al 1986, Reid et al 2001, Noskin et al 1991, Graninger et al 1992, Zaas et al 2002). 68% of the patients with the bloodstream infections in our study had a peripheral or central catheter in place within 48 hours preceding the blood culture. Further, patients who had previously received antimicrobial agents like expanded or broad-spectrum cephalosporins that lack activity against enterococci, or a combination therapy, have three to five times greater risk of acquiring enterococcal bacteremia exhibiting resistance to antibiotics like gentamicin, or vancomycin (Fridkin et al 2001, Diekema et al 2002, Zervos et al 1987, Noskin et al 1991, Safdar et al 2002, McCarthy et al 1994, Jean et al 2001).

The necessity for rapid and precise identification of **enterococci** to species level has increased dramatically over the years for epidemiological studies. The most prominent and common among all species of **enterococci** are the **E. faecalis** and proper identification of this species is highly essential. The results of our study show that the prevalence of **enterococci** in our health care setup is highly significant and alarming. **E. faecalis** (80%) and **E. faecium** (11%) contributed to 91% of all enterococcal species isolated, while 9% of **enterococci** comprised three different unusual species, which included **E. gallinarum**, **E. hirae**, and **E. casseliflavus**. The distribution by site of isolation for the 396 **enterococci** predominantly included 213 (54%) from urinary tract, 153 (39%) from exudate specimens and 30 (7%) isolates from bloodstream infections while 75% of all the isolates were from inpatient specimens. The conventional biochemical phenotyping tests identified and specified majority of enterococcal species, which was confirmed by the commercially available API Rapid ID 32 Strep Kit. Thus, appropriate species identification of **enterococci** plays a vital role in epidemiological surveillance within hospitals, since the nosocomial transmission of **enterococcus** is steadily increasing worldwide. However after initial identification, studying the antimicrobial susceptibility pattern of **enterococci** is required to initiate appropriate antimicrobial therapy. With the emergence of multidrug resistance including aminoglycosides and
glycopeptides, the necessity for surveillance of this emerging nosocomial pathogen has become necessary to minimize patient morbidity.

*Enterococci* have become predominant nosocomial pathogen due to their remarkable ability to acquire and disseminate antibiotic resistance by a variety of routes especially in hospital settings. The emergence of resistance to aminoglycosides and β-lactams/glycopeptides among nosocomial *enterococci* is of clinical significance, since resistance to a combination of the above mentioned antimicrobials poses a great therapeutic challenge (Murray 1990, 1998 & 2000). The trends in antimicrobial susceptibility vary within, as well, between countries and continents depending on various factors, which include the characteristics of the healthcare facility, infection control practices and antimicrobial use. Hence, continuous surveillance of this nosocomial pathogen helps in tracking the emergence of newer resistance in any healthcare setup, which enables to initiate and execute appropriate infection control measures at the right time.

We investigated the prevalence of resistance among *enterococci* to various antibiotics, by different phenotypic and genotypic methods. Disc diffusion testing results depicted that all 396 *enterococci* tested were highly susceptible to vancomycin, teicoplanin and linezolid, which is consistent with results of some Indian studies (Prakash et al 2005, Karmarkar et al 2004) but in contrary with many studies from U.S, U.K, Europe (NNIS, 1999, Dutka-Malen et al 1995, Murray 1998 & 2000, Leclercq et al 1988, Handwerger et al 1993, Thai et al 1998), where VRE is reported more frequently.

The present study showed 42% of all enterococcal isolates to be sensitive for ciprofloxacin, while another study from New Delhi showed that only 12% of *E. faecalis* were susceptible to ciprofloxacin (Mathur et al 2003). These results are consistent with the fact that ciprofloxacin are useful only in treating UTI, and is less effective in treating other serious enterococcal infections (Murray 1990, Gordon et al 1992). The administration of fluoroquinolones has an impact on gastrointestinal flora, with an associated increase in the carriage rate of *E. faecium*, apart from causing enterococcal superinfections (Dan et al 1997). Further, studies have shown an increase in the isolation rate of *enterococci* from urine samples in the hospital correlating with the heavy consumption of fluoroquinolones that may have a possible indirect impact on the selection and spread of vancomycin resistant *enterococci* in a hospital setup (Dan et al 1997). Hence appropriate use of fluoroquinolones by the clinicians based on the microbiological and antimicrobial susceptibility will help minimize the adverse effects of fluoroquinolones on hospital microbial ecology.

The MIC testing of β-lactam antibiotics by agar dilution method showed that 44% and 32% of all *enterococci* were resistant to penicillin and ampicillin respectively, which was concordant with the disc diffusion results. Our findings were similar to the results of many recent studies that have depicted a gradual increase in the resistance rates of penicillins over the years. But the resistance rates vary according to the country, with US isolates exhibiting comparatively higher resistance especially by *E. faecium* isolates. Boyce et al in 1992 in their study showed the incidence of ampicillin-resistant *enterococci* (ARE) increased sevenfold at a university-affiliated hospital in U.S between 1986 and 1988. Subsequently, many studies conducted in the early 90's showed between 5 to 30% of *enterococci* were ampicillin resistant and *E. faecium* isolates exhibited higher resistance than *E. faecalis* (Chirurgi et 1992, Louie et al 1992). Torell et al in 1999 and in 2001 showed the incidence of ARE among enterococcal isolates at a University Hospital in Sweden increased from 0.5% to 8.1% between 1991 and 1995. The SENTRY antimicrobial surveillance program conducted during 1997 to 1999 in U.S, Canada, Latin America, Europe and Asia-Pacific through reference laboratories showed that
76% to 99% of enterococci were susceptible to ampicillin, with highest resistance (24%) exhibited by U.S and Canadian isolates which was increasing gradually over three years of the study (Low et al 2001).

Enterococci are intrinsically resistant to β-lactams especially to semi-synthetic penicillinase-resistant penicillins and cephalosporins, due to low affinity of their penicillin-binding proteins to β-lactam antibiotics (Williamson et al 1985 & 1986, Fontana et al 1996). Among the penicillins, ampicillin is the most active in vitro. Studies have shown that E. faecium is more resistant than E. faecalis to penicillins with comparatively higher MICs (Murray 1990 & 1998). Our results were consistent with these facts, since 52% and 24% of E. faecium and E. faecalis respectively exhibited resistance to ampicillin. The prevalence of ampicillin resistant E. faecium (AREF) in several studies was in accordance to the results of our study. A surveillance of gram-positive bacteria isolated from patients with hospital-acquired infections or community acquired infections between 2000 to 2001 from China depicted the rate of AREF was 73.8% (31/42), that was significantly higher than ampicillin resistant E. faecalis (16.4%, 47/286) (Li et al 2003). Another study conducted in five Nordic hospitals showed that all E. faecalis were susceptible to ampicillin, although half the E. faecium (range 33 to 61%) were resistant to ampicillin (Simonsen et al 2003). Some studies have depicted nosocomial outbreaks with subsequent endemicity of AREF (Harthug et al 2000, Eliopoulos et al 1984).

Our results were concordant with many Indian studies showing a gradual increase in the resistance rates of penicillins over the years, while few studies contrasted our results. Only 10.2% of enterococci from urine cultures exhibited penicillin resistance in a study conducted in a tertiary care hospital in Vellore, in 1996 (Jesudason et al 1998), which subsequently increased to 32% as shown by another study from Nagpur (Agarwal et al 1999). A recent one-year study from a tertiary care center in New Delhi showed emergence of multi drug resistance in E. faecalis isolates depicting 66% resistance to ampicillin (Mathur et al 2003), which was almost double than the present study (31%). As none of the enterococci were β-lactamase producer, penicillin-binding protein appears to be the predominant mechanism of resistance to β-lactams by the isolates in this study that was
concordant with several studies worldwide (Chirurgi et al 1992, McCarthy et al 1994, Barisic et al 2000, Udo et al 2002). Marked difference was observed with other Indian studies (Parvathi et al 2000, Devi et al 2002), which have shown up to 50% β-lactamase associated resistance.

The emergence of vancomycin resistance among enterococci (VRE) brought this nosocomial pathogen into limelight. Since their first detection in 1986, there is a steady increase in vancomycin resistance among nosocomial isolates of enterococci worldwide. From 1989 through 1993, the proportion of VRE reported to CDC's National Nosocomial Infections Surveillance (NNIS) system increased from 0.3% to 7.9% (MMWR 1993) and subsequent studies showed up to 47% increase of VRE from 1994 to 1998 (McGeer et al 2000). While a surveillance data reported by the NNIS system for 1993-1997 compared with January-November 1998, showed a marked increase (55%) in VRE associated with nosocomial infections in ICU patients from U.S. hospitals (NNIS, 1999). But the prevalence of VRE varies among different countries and continents, which are governed by various factors including the use of glycopeptides in humans and animals (growth promoters). Several studies over the years show that U.S and Europe top the list in VRE, but it is yet to pose a serious threat in some countries including India, even though reports have already shown sporadic occurrence of VRE (Barisic et al 2000).

In our study, all 396 enterococci tested were susceptible to vancomycin, teicoplanin and linezolid by agar dilution method. Our results were consistent with some studies including those from India. The SENTRY antimicrobial surveillance program conducted during 1997 to 1999 in U.S, Canada, Latin America, Europe and Asia-Pacific through reference laboratories showed that 83% to 100% of enterococci were susceptible to vancomycin, with highest resistance (17%) exhibited by U.S isolates which increased gradually over three years of the study (Low et al 2001) and in the preceding year (1997) 14% of the U.S isolates exhibited vancomycin resistance in the same study (Pfaller et al 1999). These studies reflect the general trend of vancomycin resistance among enterococci in western countries. A study from Nagpur, India showed that 3.3% of 150 enterococci (129 E. faecalis and 21 E. faecium) exhibited low-level vancomycin resistance with MIC <16 µg/ml (Agarwal
et al 1999). Subsequently, Purva et al reported the first case of vancomycin-resistant *E. faecium* in 1999 from New Delhi isolated from the blood culture of a patient with non-Hodgkins lymphoma. Thereafter, studies showed 1% to 5% vancomycin resistance among *enterococci* (Mathur et al 2003, Parvathi et al 2000). Recently Taneja et al in 2004 showed that 5.5% of 144 urinary *enterococci* isolated from urinary specimens exhibited vancomycin resistance, which included five *E. faecium* and one each of *E. faecalis*, *E. casseliflavus* and *E. pseudoavium* with MIC ranging from 8 to 32 µg/ml. A study by Karmarkar et al in 2004 from Mumbai (Bombay) that showed 23% of 52 *enterococci* (ten *E. faecalis*, and forty-two *E. faecium*) isolated from clinical specimens, were resistant to vancomycin with an MIC > 4 µg/ml, but sensitive to teicoplanin depicting a van-B resistance.

In this context, the emergence of vancomycin resistance among *enterococci* in India is a cause for concern in the near future. Hence, in hospitals where VRE is yet to be detected, periodic culture surveys of stools or rectal swabs from patients at high risk of VRE infection or colonization are recommended (Low et al 2001). Furthermore, *enterococci* from all clinical specimens need to be tested by vancomycin agar screening method to detect them early to initiate appropriate infection control measures, as well as restructure the antibiotic policy, if needed.

One of the biggest therapeutic challenges is treating serious enterococcal infections showing HLAR. A synergistic combination regimen is not possible even if the isolate is susceptible to either of the cell-wall active agents (β-lactams/glycopeptides) (Murray 1990, Moellering Jr 1990, Levison et al 2000). Hence, studies on the prevalence of high-level aminoglycoside resistance are of prime importance to choose an alternate therapeutic combination. The prevalence of high-level resistance to streptomycin was first reported in 1970, followed by several reports of streptomycin and kanamycin resistance (Moellering Jr et al 1971, Calderwood et al 1977, Courvalin et al 1978) and the first report of high-level plasmid-borne resistance to gentamicin came in 1979. It was reported in three strains of *Streptococcus faecalis* subspecies zymogenes that were also resistant to other aminoglycosides (Horodniceanu et al 1979). Since then several studies have been conducted worldwide on the prevalence of aminoglycoside resistance in *enterococci*
and the frequency of HLGR has increased from 4.5% to 65% over the years (Zervos et al 1987, Mederski-Samoraj et al 1983, Del Campo et al 2000).

The present study showed that 60% and 44% of all enterococci tested were resistant to high-level gentamicin and streptomycin respectively by agar screening method, which included 64% and 46% of E. faecalis, 60% and 36% of E. faecium and, 58% and 42% of the unusual enterococcal species exhibiting resistance to high-level gentamicin and streptomycin respectively. In 1995, the first report of high-level aminoglycoside resistance among enterococci from India was published (Cherian et al 1995). Later in 1997, Bhat et al, showed that among 41 strains of enterococci isolated from cases of neonatal bacteremia, 8.6% and 33.3% of E. faecalis and E. faecium strains respectively showed high level gentamicin resistance, while 6% and 50% of E. faecalis and E. faecium strains respectively showed high level streptomycin resistance. Another study from JIPMER reported endocarditis caused by high-level gentamicin resistant enterococci in 1998 (Khanal et al 1998), since then the rate of HLGR enterococci was steadily increasing, as evident from the results of the present study. Our study is consistent with another recent study by Randhawa et al in 2004, which showed a prevalence of 68% and 43% for HLGR and HLSR respectively, but their study did not differentiate the species prevalence of enterococci. Likewise, another study by Karmarkar et al in 2004 from Mumbai (Bombay) showed that 100% of E. faecalis and 86% of E. faecium (52 enterococci-ten E. faecalis and forty-two E. faecium) isolated from clinical specimens was resistant to high-level gentamicin.

The prevalence of HLGR was higher than HLSR in our study, which reflects the general trend as evident through many studies from European and Asian countries that includes Spain, Croatia, Taiwan, Greece, Japan, and India (Prakash et al 2005, Karmarkar et al 2004, Devi et al 2002, Barisic et al 2000, Del Campo et al 2000, Randhawa et al 2004, Schouten et al 1999, Tsakris et al 2001, Schmitz et al 1999, Teng et al 1998, Kobayashi et al 2001). But some studies contradict our findings, which show a higher prevalence of HLSR than HLGR among enterococci. A study from Canada showed that 4% and 29% of E. faecium strains exhibited HLGR, and HLSR respectively (Louie et al 1992) that was consistent with the
results of other studies from U.S. and Canada (Jones et al 1995). Subsequently the multi centered SENTRY antimicrobial surveillance program conducted in 1997 showed that 46% and 76% (Canada), 40% and 70% (U.S.), and 17% and 50% (Latin America) of *E. faecium* strains exhibited HLGR and HLSR respectively, while 46% and 52% (Canada), 27% and 32% (U.S.), and 43% and 33% (Latin America) of *E. faecalis* strains exhibited HLGR and HLSR respectively (Pfaller et al 1999). Further, HLGR rates may vary considerably between laboratories (1% to 28%) of different hospitals within the country as shown by a study from five Nordic hospitals (Simonsen GS et al 2003).

The excessive use of gentamicin in our setup (as standard prophylactic/therapeutic regimens) may be a reason for a higher prevalence (60%) of HLGR among *enterococci*, a fact that has been shown by some studies (Zervos et al 1986 & 1987, Huycke et al 1991). For example, mostly for all cases of pre-operative prophylaxis in our hospital, a dose of gentamicin along with metronidazole is given with/without ampicillin (or) cephalosporins depending on the type of surgery. Since the gentamicin prophylaxis is aimed to eradicate gram-negative (GN) organisms, the *enterococci* in the GI tract are tolerable to the dose regimen for GN bacteria. Hence they thrive and out compete other strains and create a niche for itself, and under debilitating conditions get disseminated through any possible foci leading to bacteremia or other associated infections (Zervos et al 1986 & 1987, Huycke et al 1991). Subsequently, the pre-operative prophylactic regimen is followed empirically for post-operative periods and remains unaltered, except on occasions of a culture report suggesting an alteration. In the pediatric specialty of our hospital, empirically all cases of neonatal sepsis are treated with ampicillin and amikacin or, gentamicin, and switched over to cefotaxime along with gentamicin if needed, or indicated otherwise. Cefotaxime and gentamicin are used to treat neonatal sepsis in neonatal ICU initially, and switched over to a combination of cefeperazone and sulbactum with or without vancomycin if needed, or indicated otherwise.

Although streptomycin is rarely used, 44% *enterococci* exhibited HLSR in the present study. One reason for the prevalence of HLSR may be the clinical isolates might carry the HLSR and HLGR traits on the same genetic element
The results of the present study depict concomitant resistance to different antimicrobials including streptomycin that is consistent with several studies. Agarwal et al in 1999 showed concomitant high-level resistance to penicillin and aminoglycosides in 16% of enterococci from Nagpur, while another study showed concomitant resistance to penicillin and aminoglycosides in 61% of E. faecalis isolates (Singh et al 2003). A recent study from New Delhi showed that HLGR and HLSR were present together in 43% of enterococci isolated from pediatric septicemic cases (Randhawa et al 2004). Thus the concomitant resistance to other antimicrobials along with HLAR narrows down the therapeutic choice, thereby posing difficulty in treating serious enterococcal infections exhibiting multidrug resistance.

The phenotypic analysis of antimicrobial resistance in enterococci may be sufficient for rapid initiation of appropriate antimicrobial therapy. However, for studying the geographical trends of molecular basis of antimicrobial resistance and to understand the epidemiology of antimicrobial resistant enterococci many a times this information alone is insufficient. Hence, further genotypic analysis (molecular characterization) of the genetic determinants encoding antimicrobial resistance is required to investigate the magnitude of this multifaceted problem.

More than 90% of clinical HLGR enterococci possess the bifunctional gentamicin resistance gene aac(6′)+aph(2′″) that encodes resistance to virtually all therapeutic aminoglycosides, including gentamicin, tobramycin, amikacin, kanamycin, and netilmicin, but not streptomycin (Horodniceanu et al 1979), while less than 10% possess other aminoglycoside-modifying enzymes (AME) like aph(2′″)-Ic, aph(2′″)-Id, and aph(2′″)-Ib (Chow 2000). HLSR is most commonly encoded by ant(6′)-I gene and can coexist with the gene(s) for HLR to other aminoglycosides (Chow 2000, Murray 1998). Occasionally HLSR in enterococci may be due to ant(3′″)-Ia gene (Chow 2000), or due to ribosomal resistance (Eliopoulos et al 1984). Hence genetic analysis of the HLAR among enterococci helps us to know the differences, if any, in the epidemiology of the HLAR genes between different countries and continents (Papapaskevas et al 2000, Kobayashi et al 2001, Simjee et al 2000).
The results of the present study show that bifunctional gentamicin resistance gene $aac(6')+aph(2")$ was present in 96% (71 of 74 isolates), 94% (24 of 26 isolates) and 22% (2 of 9 isolates) of HLGR $E. faecalis$, $E. faecium$ and unusual species of enterococci respectively. The $ant(6')$-I gene was present in 88% (53 of 60 isolates), 80% (12 of 15 isolates) and 33% (2 of 6 isolates) of HLSR $E. faecalis$, $E. faecium$ and unusual species of enterococci respectively. Both the genes were present concomitantly in 36 $E. faecalis$, eight $E. faecium$, and two unusual species of enterococci as depicted in table 13. Our results were consistent with several studies, although few studies showed some differences in the prevalence of HLAR genes. The results of a Greek study showed that $aac(6')+aph(2")$ gene was detected in 83% of $E. faecalis$, while the $ant(6)$-I gene was detected in all HLSR isolates of $E. faecalis$ and $E. faecium$ (Papaparaskevas et al 2000), which coincided with our results for the prevalence of both $aac(6')+aph(2")$ and $ant(6)$-I genes. The presence of other aminoglycoside resistance genes like $aph(2")$-Ic/ $aph(2")$-Id/ $aph(2")$-Ib/ $ant(3")$-Ia, apart from $aac(6')+aph(2")$ and $ant(6)$-I genes may be a contributing factor for the differences. Various studies from Germany (Kaufhold et al 1992), U.K (Simjee et al 2000), Greece (Pournaras et al 2000), Netherlands (van Den Braak et al 1999), and Kuwait (Udo et al 2002) showed that the bifunctional gentamicin resistance gene $aac(6')+aph(2")$ was present in all HLGR enterococcal isolates (100%) tested. Our results were similar to these studies with regard to the prevalence of $aac(6')+aph(2")$ gene, which is present in 96% and 94% of $E. faecalis$ and $E. faecium$ respectively.

Some studies have indicated a variety of distribution profiles of AME genes among enterococci. A recent study from a university hospital in Japan showed that $aac (6')+aph (2")$ gene was present in 42.5% of $E. faecalis$ and 4.3% of $E. faecium$, while almost half of $E. faecalis$ and $E. faecium$ isolates were shown to possess $ant (6)$-Ia and $aph (3')$-IIIa genes. The profile of AME gene(s) detected most frequently in individual strains of $E. faecalis$ was $aac (6')aph(2") + ant(6)$-Ia + $aph(3')$-IIIa, and isolates with this profile showed high level resistance to both gentamicin and streptomycin. In contrast, AME gene profiles of $aac (6')$-Ii+ $ant (6)$-Ia+ $aph (3')$-IIIa, followed by $aac (6')$-Ii alone, were predominant in $E. faecium$. 
Only one AME gene profile of \( \text{ant(6)-Ia}+\text{aph(3')-IIIa} \) was found in \( E. \text{avium} \) (Kobayashi et al 2001). Another study from Spain depicted heterogeneity in the distribution of AME genes like the previous study among HLAR strains, while more than one AME gene was detected in 71% of the strains (Del Campo et al 2000). Another study from U.S showed that the \( \text{aac(6')}+\text{aph(2'')} \), \( \text{aph(2'')}\text{-Ic} \), and \( \text{aph(2'')}\text{-Id} \) genes were present in enterococcal isolates from animals, food, and humans. \( \text{aac(6')}+\text{aph(2'')} \) gene was the most common gene among the HLGR isolates evaluated in their study and was detected in various enterococcal species, including the \( E. \text{faecalis}, E. \text{faecium}, E. \text{gallinarum}, \) and \( E. \text{casseliflavus} \). These isolates were collected from human stool, chicken, and pork purchased in grocery stores and chickens, dairy cattle, swine, and turkeys on farms. These observations provide evidence of a large reservoir for these resistance genes in human, food and food-producing animals, indicating widespread dissemination of these resistance determinants (Donabedian et al 2003).

Multiplex PCR for detecting the AME genes from bacterial colonies directly was standardized and the Colony PCR was followed. It gives rapid results, within six hours after identification of \( \text{enterococci} \). Although our study shows a very high prevalence of \( \text{aac(6')}+\text{aph(2'')} \) and \( \text{ant(6')-I} \) genes among the HLAR \( \text{enterococci} \), the presence of other AME genes among these isolates cannot be ruled out, since we did not test the isolates for presence of those genes which have been shown to be present along with the \( \text{aac(6')}+\text{aph(2'')} \) and \( \text{ant(6')-I} \) genes among HLAR \( \text{enterococci} \) (Del Campo et al 2000, Kobayashi et al 2001).

The results of our study showed that all the isolates tested were susceptible to vancomycin, teicoplanin and linezolid. While 58% and 69% isolates were susceptible to penicillin and ampicillin respectively, only 44% and 58% of the isolates were susceptible to the aminoglycosides gentamicin (high-level) and streptomycin (high-level) respectively. Susceptibility against ciprofloxacin was exhibited by 42% of all \( \text{enterococci} \), while the 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 58% of \( E. \text{faecalis}, E. \text{faecium} \) and unusual enterococcal species.
respectively. However, there were differences in the results between disc diffusion testing and agar screening/agar dilution method. None of the penicillin and ampicillin resistant enterococcal isolates tested positive for beta-lactamase production.

Genotypic detection of aminoglycoside resistance genes by multiplex PCR, showed that the bifunctional gentamicin resistance gene $aac(6')+aph(2'\text{")}$ was present in 96% and 94% of HLGR $E. \text{faecalis}$ and HLGR $E. \text{faecium}$ isolates respectively, while $\text{ant(6')-I}$ gene (streptomycin resistant) was detected in 88% and 80% of HLSR $E. \text{faecalis}$ and HLSR $E. \text{faecium}$ respectively. The $aac(6')+aph(2'\text{")}$ and the $\text{ant(6')-I}$ gene were present together in 36 of $E. \text{faecalis}$ and eight of $E. \text{faecium}$ isolates respectively. Among unusual species of enterococci tested for aminoglycoside resistant genotypes, only two isolates (two $E. \text{gallinarum}$) possessed $aac(6')+aph(2'\text{")}$ gene, while another two isolates (two of $E. \text{gallinarum}$) possessed the $\text{ant(6')-I}$ gene, which also possessed $aac(6')+aph(2'\text{")}$ gene in them. Overall, absence of these genes in some suggests alternate aminoglycoside resistance mechanisms among the enterococcal species exhibiting HLAR.

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

Enterococci, a normal human commensal though catapulted as a prominent nosocomial pathogen owing to their versatility of antimicrobial resistance, it was their property of pathogenicity addressed a century back-in 1899 that first underscored their emergence as a human pathogen (Murray 1990, Jett et al 1994). Since then several studies started exploring their versatility in causing human infections. To facilitate any clinical infection, enterococci must first be able to colonize, primarily by adhering to the host mucosal surfaces with the help of adhesin systems like aggregation substance-AS, enterococcal surface protein-esp,
adhesin and surface carbohydrates, while much of recent attention is on esp. After successful adherence/colonization the organism must then evade the host clearance causing an array of infections like bacteremia, UTI, endocarditis, soft tissue infections and other miscellaneous infections and ultimately produce pathologic changes in the host, either through direct toxic activity by secretory products like cytolysin, gelatinase/ protease, bacteriocin / hemolysin, or indirectly by inducing an inflammatory response with the help of lipoteichoic acids, complement and neutrophils (Jett et al 1994, Mundy et al 2000). While most of the colonizers or the commensals do not possess all the "virulence factors" needed for successful evasion and subsequent infection process, those that possess them emerge to become a successful nosocomial pathogen facilitated by other predisposing factors. Hence, knowledge about the prevalence of various virulence factors would help us to assess the clinical significance of enterococci. Thus, the purpose of the present study was to perform a molecular epidemiological survey by investigating the presence of known and novel potential virulence factors in enterococci isolated from different sources by phenotypic and genotypic methods and also to study the possible correlations between potential virulence factors possessed by strains and their source of isolation. In India, no report has been published so far concerning the occurrence of known virulence determinants from different clinical sources.

The prevalence rate of various virulence factors by phenotypic assays, in the present study may not reflect the true incidence, as it would not be able to reveal unexpressed virulence factors due to the presence of silent/mutated genes as shown by some studies (Eaton et al 2001, Creti et al 2004). Hence genotypic analysis was a better choice for characterization of the virulence determinants in enterococci from infection derived isolates that may aid in determining the outcome of the disease.

The virulence determinants were present in different proportions between the E.faecalis & E.faecium isolates in our study. E.faecalis isolates carried multiple virulence genes, whereas the E.faecium isolates were devoid of these virulence genes except esp and hyl. A multiplex PCR developed for the simultaneous detection of enterococcal genes has not been described before. The presence of the genes that encode gelatinase (gelE), hyaluronidase (hyl) aggregation substance (asa1),
extracellular surface protein (esp) and cytolysin activator (cyl A) were investigated by multiplex PCR. The multiplex protocol for these five genes provides a reliable and rapid alternative to phenotypic testing and uniplex PCRs. (Vankerckhoven 2004).

One of the aims of this work was to identify the role of the aggregation substance (AS) in the process of adhesion of enterococci to renal and intestinal epithelial cells in vitro (Chow et al 1993, Kreft et al 1992, Sußmuth et al., 2000). AS protein may be involved in virulence and is expressed on the surface of E. faecalis. The AS gene asa1, is present in pheromone-responsive plasmids (Sußmuth et al 2000, Wells et al 2000, Clewell 1993). AS facilitates aggregation of donor and recipient bacteria and helps in the transfer of conjugative plasmids (Chow et al 1993).

The asa1 is present in 92% of E. faecalis and by phenotypic assay only 59% of the asa1 positive adhered to the Vero-Cell lines. Among the E. faecium, the asa1 gene was totally absent but 61% of them adhered to Vero-cell lines though less effectively than E. faecalis isolates. This was in agreement with a study by Coque et al in 1995, which observed approximately one-half of the genetic lineages of the species from infections of hospitalized patients possessed genes for aggregation substance and the protease gelatinase. This suggests that these traits entered the species earlier than did other toxins like hemolysin/cytolsin, bacteriocin and gelatinase. Aggregation substance is an integral component of the pheromone-responsive plasmid exchange system ((Sußmuth et al 2000, Wells et al 2000, Clewell 1993). Therefore, nosocomial strains of E. faecalis may be those best equipped to participate in genetic exchange and may be selected by the presence of antibiotic resistance determinants on such plasmids.

To our knowledge this is the first study from India that investigates the adherence of enterococci to Vero cell lines. 54% of the Enterococcal isolates had the ability to adhere Vero cell lines, of which 58% and 61% were E. faecalis and E. faecium respectively. E. faecalis was highly adherent than E. faecium (more than 10 bacteria per cell). Previous studies of the incidence of asa1 in enterococcal isolates
are in agreement with the results of our study: some studies indicated a higher prevalence of \textit{asa1} in clinical isolates (Coque et al 1995, Waar et al 2002), whereas in others, \textit{asa1} did not occur more frequently in invasive than in commensal strains (Huycke & Gilmore 1995, Archimbaud et al 2002). Among the \textit{E.faecalis} 57\%, 40\% and 3\% were from urine, exudates and bloodstream infections respectively and among \textit{E.faecium} 44\%, 30\% and 26\% were from urine, bloodstream and exudates respectively.

The adhesion experiments clearly confirmed that \textit{E.faecalis} is more virulent than \textit{E.faecium}. In particular, a large number of \textit{E. faecalis} isolates were adhesive on Vero cells. Bacterial adherence was associated significantly with that of other virulence factors studied, the \textit{gel E} and \textit{esp}, in agreement with the observations of other authors (Archimbaud et al 2002, Chow et al 1993, Coque et al 1995, Heaton et al 1996, Jett et al 1994).

Our results depicted that 69\% of \textit{E. faecalis} and 66\% of \textit{E. faecium} isolates possessed the \textit{esp} gene, while none other species tested showed the presence of \textit{esp} gene, which is concordant with other studies from U.S., U.K, Italy, Netherlands, and Germany (Shankar et al 1999-Toledo-Arana et al 2001, Hammerum et al 2002, Coque et al 2002), although some recent studies have shown the presence of \textit{esp} additionally among other species like \textit{E. raffinosus}, and \textit{E. durans} (Semedo et al 2003, Harrington et al 2000). The cell wall-associated protein-\textit{esp} was initially shown to be associated only among infection-derived \textit{E. faecalis} isolates and not in clinical \textit{E. faecium} isolates, or in three other less pathogenic enterococcal species tested (Shankar et al 1999). But subsequently another study showed that a subpopulation of epidemic vancomycin-resistant \textit{E. faecium} isolates contained a variant of the \textit{esp} gene that was absent among all non-epidemic and animal isolates (Willems et al 2001). Following this study \textit{esp} variants were reported in clinical isolates of \textit{E. faecium} from different parts of the world (Woodförd et al 2001, Baldassarri et al 2001). It is quite interesting but not surprising considering the fact, that emergence of genetic lineages with enhanced/newer virulence traits among other enterococcal species is not impossible, since vertical inheritance (virulence)
can slowly penetrate into other lineages of the species by further DNA transfer as shown for other bacteria (Mundy et al 2000).

The prevalence of *esp* varied between different studies depending on several factors. Our results matched that of a study from Italy, which revealed that *esp* gene were found in 72% and 60% of *E. faecalis* and *E. faecium* isolates respectively (Dupre et al 2003). Another prospective study of 398 patients with *E.faecalis* bacteremia screened for various virulence markers showed that 32% of *E. faecalis* isolates carried *esp* gene, however there was no significant association between 14-day mortality and the virulence markers studied, singly or in combination (Vergis et al 2002). In another study, 29 *E. faecalis* isolates from patients with endocarditis or bacteremia or from stools of healthy volunteers were investigated for their ability to adhere to Int-407 and Girardi heart cell lines and for the presence of known enterococcal virulence factors. The incidence of *esp* was shown to be 72.4%, which remained the highest than other virulence factors studied. But the authors concluded that bacterial adherence was not significantly associated with any of these virulence factors (Archimbaud et al 2002). However, these studies (Dupre et al 2003, Archimbaud et al 2002) investigated the prevalence of *esp* using a lesser sample size than our study, thus making it difficult to make a conclusive statement regarding the significance of *esp*. The higher prevalence (69%) of *esp* among *E.faecalis* from our setup depicts that this virulence trait may have permeated more deeply into the species by horizontal transfer and would have acquired it comparatively earlier, thereby enhancing the ability of the organism to cause disease beyond that intrinsic to the species background as hypothesized previously (Mundy et al 2000). The diversity of *esp* positive *E. faecalis* isolates from different sources viz., urine (61%), exudates (37%) and blood (2%) in our study authenticates the enhanced ability of this organism to cause disease.

Another study demonstrated that *esp* occurs more frequently among ampicillin-resistant and vancomycin-susceptible *E. faecium* clones from hospitalized patients, an observation, which indicates that antibiotic-resistant variants may frequently arise under antibiotic selective pressure among *esp*-positive clones reaching ecological abundance in the nosocomial habitat (Coque et al 2002). Our
results showed a very strong association between gentamicin resistance and esp (P = 0.0001, χ² test value-34.34; OR = 7.98; 95% CI=3.7-17.41) among E. faecalis isolates reflecting this fact. Statistically (OR—7.98) our study showed that the isolates with esp gene could exhibit eight times greater resistance to gentamicin, than those without esp gene.

The aac(6')+aph(2") and ant(6')-I genes were present together among 61% and 80% of esp positive isolates of E. faecalis and E. faecium isolates respectively, while many of these isolates exhibited ampicillin resistance. The aac(6')+aph(2") gene alone was present among 20% each of esp positive E. faecalis and E. faecium isolates respectively, while the ant(6')-I gene alone was present in only among 2% of E. faecalis isolates. 18% of esp positive E. faecalis isolates were sensitive to other antibiotics tested.

Several studies have investigated the relationship between the presence of esp and vancomycin resistance in enterococci (mostly in E. faecium), since vancomycin resistance is the most significant problem encountered by developed countries, although many developing countries are yet to experience the consequences of VRE. Leavis et al in 2003 showed that the presence of variant esp gene in vancomycin resistant E. faecium (VREF) and VSEF was strongly associated with a specific epidemiologic source because the presence of esp was higher in clinical infections and epidemic associated isolates, than in surveillance isolates. Some studies have shown that esp gene plays a major role in dissemination of vancomycin-resistant E. faecium since predominant epidemic strains harbored the esp gene, while most of the non-epidemic strains were esp gene negative (Leavis et al 2003,Routsi et al 2003). Furthermore, the genetic machinery that enables dissemination of antimicrobial resistance determinants between enterococci is always of serious concern, since the same machinery is capable of transferring virulence determinants (Oancea et al 2004).

Although several virulence determinants have been depicted over the years in enterococci, recently depicted property of biofilm formation by enterococci has gained momentum due to their clinical significance in nosocomial settings
(Toledo-Arana et al 2001). Even though inappropriate antibiotic use has contributed to the emergence of *enterococci* in hospitals, studies have shown that acquisition of new virulence traits may have played an important evolutionary role (Jett et al 1994). The capacity of *enterococci* to cause infections is enhanced by the property of biofilm formation especially on indwelling medical devices in hospitalized patients, since the proportion of enterococcal bacteremia associated with central venous catheters/urinary catheters has increased dramatically over the years with significant morbidity and mortality (Murray 1990, Patterson et al 1995, Jett et al 1994). Once a catheter has become colonized with microorganisms, invasion of the bloodstream can occur. However, for an effective colonization (of catheters), a microorganism must have the capacity to form a biofilm on the device material, and studies have shown the biofilm-forming characteristics of bloodstream enterococcal isolates in vitro and the type of infection caused in vivo (Sandoe et al 2003).

Our results depicted biofilm formation by 68% of *E. faecalis*, and 54% of *E. faecium* while none other species tested depicted biofilm formation on abiotic surface in a phenotypic assay using polystyrene microtiter plates based on the approach of Toledo-Arana et al 2001. A strong biofilm formation was depicted by 60% and 28% & 12% of *E. faecalis* formed moderate and weak biofilms respectively. Our results were concordant with the spectrum of biofilm formation as shown by Toledo et al in 2004 and with another study from U.K. that showed 42% of *E. faecium* isolates could produce biofilm, apart from *E. faecalis* (Sandoe et al 2003), and none of the non-faecalis non-faecium *enterococci* produced biofilm in their study (Toledo-Arana et al 2001). The prevalence of biofilm formation (68%) by *E. faecalis* in our study was very much within the range, when compared with other studies showing 100% (Sandoe et al 2003), 93% (Mohamed et al 2004), and 57% (Toledo-Arana et al 2001) of their isolates producing biofilm. Further, 71% of biofilm forming *E. faecalis* were urinary isolates, while 21% were from exudates and 8% from bloodstream in our study with majority of the patients catheterized, which authenticates the significance of biofilm formation by these isolates as shown by other studies (Sandoe et al 2003).
The genetic determinants controlling biofilm formation in *enterococci* are yet to be unraveled completely, but several studies have shown the involvement of previously characterized virulence determinants in biofilm formation (Toledo-Arana et al. 2001, Kristich et al. 2004, Mohamed et al. 2004, Waar et al. 2002). The analysis of the relationship between biofilm formation and the presence of other virulence factors in *E. faecalis* depicted interesting results through our study. Many studies have depicted that *esp* determinant is highly associated with the ability to form a biofilm at abiotic surfaces (Toledo-Arana et al. 2001), and *esp* induced biofilms had increased antimicrobial resistance (Waar et al. 2002). But the results of a recent study contradict the earlier hypothesis where the authors demonstrated that in vitro biofilm formation occurs not only in the absence of *esp*, but also in the absence of the entire pathogenicity island that harbors the *esp* coding sequence. They concluded that *E. faecalis* forms complex biofilms by a process that is sensitive to environmental conditions and does not require the *esp* surface protein (Kristich et al. 2004). Our results showed that 29% of the *esp* positive isolates, as compared to 20% of the *esp* negative isolates (P=0.324, $\chi^2$ test value=0.97) showed biofilm formation, while 22% of the *esp* positive and gelatinase negative isolates, as compared to 6% of the *esp* and gelatinase negative isolates (P=0.086, Fisher's exact test) showed biofilm formation, which indicates that presence or absence of the *esp* gene does not greatly influence the biofilm formation by *E. faecalis* statistically as shown by other recent studies. Another study showed that endocarditis isolates of *E. faecalis* produced biofilm significantly more often than non-endocarditis isolates. Furthermore, their results showed that *esp* was not required, but its presence was associated with higher amounts of biofilm (Mohamed et al. 2004). The absence of *esp* among 51% of biofilm formers in the same study motivated the investigators to look for other genes that might influence biofilm formation. Their results showed that disruptions in other genes like *epa* (enterococcal polysaccharide antigen), *atn* (autolysin), *gelE* (gelatinase), and *fsr* (quorum sensing locus) resulted in fewer attached bacteria and lesser biofilm formation as determined using phase-contrast microscopy, thereby emphasizing the significant role of other determinants (apart *esp*) in biofilm formation in *enterococci* (Mohamed et al. 2004).
However a recent study that depicted an esp-independent biofilm formation, subsequently demonstrated that gelE (gelatinase) enhances biofilm formation by *E. faecalis* (Kristich et al 2004), which was further authenticated by other studies (Mohamed et al 2004, Pillai et al 2004). In our study 34% of the gelatinase positive isolates, as compared to 14% of the gelatinase negative isolates (P=0.004, $\chi^2$ test) showed biofilm formation, while 42% of the esp negative and gelatinase positive isolates, as compared to 6% of the esp and gelatinase negative isolates (P=0.001, Fisher's exact test) showed biofilm formation, which indicates that presence of gelatinase could enhance biofilm formation even in the absence of esp gene. On the other hand, 32% of the esp and gelatinase positive *E. faecalis* isolates depicted biofilm formation, while only 6% of the esp and gelatinase negative *E. faecalis* isolates depicted biofilm formation (P=0.005), which was highly significant indicating that the presence of both the factors could influence the production of biofilm. However, it is difficult to predict the exact role of these virulence factors in biofilm formation unless the genetic machinery of the biofilm formation is unraveled completely. The prevalence of gelatinase production, in our study genotypically is 100% and by phenotypic assays is 60%, depicting the presence of the determinants encoding gelatinase production (Eaton et al 2001, Creti et al 2004). Based on our results we conclude that the gelatinase production enhances biofilm formation by clinical *E. faecalis* isolates.

After the initial adherence to the host tissues with the help of adhesins like *asa1* and esp, *Enterococcus* invades and cause systemic infections and modulates the host inflammatory responses. The potentially toxic secreted products like hemolysin/cytolysin, bacteriocin, hyaluronidase and gelatinase causes direct tissue damage, which contributes to the severity of enterococcal infections (Jett et al in 1992 & 2004, Johnson 1994). Studies have depicted that hemolysin/cytolysin synthesis is linked to the same genetic determinant as bacteriocin production in *Enterococcus* (Brock et al 1963, Brock & Peacher 1963, Dunny et al 1975, Ike et al 1992), and its production has been depicted as a pathogenic marker by many studies (Libertin et al 1992, Vittal Prakash et al 2003, Ike et al 1987).
Enterococcal cytolysin is a structurally novel bacterial toxin expressed by some strains of *E. faecalis* and is distantly related to the class of bacteriocins known as lantibiotics. The cytolysin can be encoded by large pheromone-responsive plasmids, or on the chromosome within pathogenicity islands. A complex process that involves the products of eight genes, designated cylR1, cylR2, cylLL, cylLS, cylM, cylB, cylA, and cylI produces it. The cytolysin operon within the *E. faecalis* pathogenicity island is associated with other virulence determinants, including aggregation substance and surface protein *esp*. The simultaneous deletion of chromosomal regions encoding surface protein *esp* and secreted cytolysin is very similar to the coordinated deletion of chromosomal regions coding for fimbriae and hemolysin in pathogenicity islands I and II of pathogenic E. coli (Hacker et al 1990). It appears that such deletions represent a general mechanism of bacterial virulence modulation.

Our study showed that 17% of all *enterococci* produced hemolysin/cytolysin, although the property was confined to *E. faecalis* and *E. faecium* isolates. Our results reflect the general pattern of hemolysin/cytolysin production by *E. faecalis* and *E. faecium* only among medical isolates (Libertin et al 1992, Ike et al 1987). Studies among food isolates have shown that non-faecalis and non-faecium *enterococci* too can produce hemolysin/cytolysin (Semedo et al 2003, Franz et al 1999). Our results showed that 19% of *E. faecalis* and 16% of *E. faecium* produced hemolysin/cytolysin respectively.

The diversity of hemolysin producing *E. faecalis* isolates from different sources viz., urine (87%), exudate (8%) and blood (5%), in our study authenticates the enhanced ability of this organism to cause disease, since a number of independent studies using different model systems have consistently found a role for the *E. faecalis* bacteriocin/hemolysin in the toxicity of enterococcal infections (Jett et al 1992, Chow et al 1993). Galvez et al in 1985 found that among 90 *enterococci* strains of human origin, 36 strains produced hemolysin. Later Libertin et al in 1992 screened the clinical isolates and concluded that hemolysin/bacteriocin produced by *enterococci* could be considered as a marker of pathogenicity.
Although the hemolysin and bacteriocin properties are encoded by the same genetic determinant among most of the *E. faecalis* isolates, both properties may not be expressed concomitantly (Dunny et al 1975). Diagnostically, this toxin (hemolysin) causes a beta-hemolytic reaction on human and horse blood agar, but does not hemolyze sheep blood agar, which is frequently used in clinical microbiology laboratories (Jett et al 1994, Facklam et al 1999). Thus assays using human/horse blood are required for screening beta-hemolysin production by *enterococci*.

Some studies on *enterococci* isolated from endocarditis and bacteremic patients showed that hemolysin/cytolysin occurs at a frequency of 45-60% (Huycke et al 1991 & 1995, Ike et al 1987), while our results were highly concordant with other studies that showed much lesser prevalence of hemolysin/ cytolysin (7%). A recent study from U.S. showed that 11% of 219 *E. faecalis* isolates from patients with enterococcal bacteremia were positive for hemolysin (Vergis et al 2002), while Elsner et al in 2000 reported the presence of cytolysin among 16% of *E. faecalis* blood culture isolates. Another study showed that cytolysin was present only in 17% of *enterococci* isolated from patients with endocarditis or bacteremia or from stools of healthy volunteers (Archimbaud et al 2002). Coque et al in 1995 showed that cytolysin was more common in non-endocarditis clinical isolates (37%) and in hospital fecal isolates (31%) than among endocarditis (16%) and community fecal isolates (20%). The lesser incidence of hemolysin production (19%) by *E. faecalis* isolates in the present study is of significance and further genotypic analysis of these determinants would help us to know the discordance in the concomitant expression of the properties. However from the clinical microbiological perspective, qualitative analysis of these virulence determinants would be sufficient enough to predict the prognosis of serious enterococcal infections.

The *cylA* gene was present in 22% of all isolates of *E. faecalis* and totally absent in *E. faecium* isolates. It was distributed among the urinary tract isolates (23%) more often than among bloodstream isolates (8%). It was never detected in isolates from endocarditis. Its absence or low prevalence in endocarditis strains has already been reported by other authors (Huycke & Gilmore 1995, Archimbaud et al
2002). cylA was always associated with aggregation substance genes asa1, whereas the reverse was not observed. Hemolytic activity was detected in 19% of strains, with a tendency to be present more often in urinary isolates (87%) than bloodstream (5%) isolates. The lack of cytolysin phenotypic/genotypic congruence may suggest the occurrence of missing genes in the cyl operon among cylA-positive/haemolysin-negative strains.

The gelatinase production in enterococci was depicted a century back, when this property was used to classify Streptococcus faecalis into subspecies, although the procedure was stopped since the advent of molecular taxonomical methods (Murray 1990). But over the years, the virulence of enterococcal gelatinase has been proven in animal models (Dupont et al 1998, Engelbert et al 2004), which was authenticated by several studies depicting gelatinase production by enterococci from human infections. Our study depicted gelatinase production among 60% of E. faecalis isolates, while none other species tested produced gelatinase in the phenotypic assay. The aac(6′)+aph(2′) and ant(6′)-I genes were present together in 52% gelatinase positive (Gel+) E. faecalis. The aac(6′)+aph(2′) gene was present separately in 16% Gel+ E. faecalis isolates, while 8% of Gel+ E. faecalis isolates were streptomycin resistant, and 24% Gel+ isolates were sensitive to other antibiotics tested.

The results of our study were concordant with a study from U.S. that depicted gelatinase production by 54% of E. faecalis isolates from endocarditis, 58% of isolates from other infections, 62% of hospital fecal isolates, and in 27% of fecal isolates from healthy volunteers, but was absent in all the 86 non-E. faecalis isolates studied (Coque et al 1995). The gelE gene was in all the E. faecalis isolates tested. In another study there was no significant association between 14-day mortality and any of the virulence markers studied among patients with bacteremia due to E. faecalis, even though 64% of isolates produced gelatinase (Vergis et al 2002). Several studies have shown that 45-60% of the E. faecalis isolates from human infections produced gelatinase, whereas none of the non- E. faecalis isolates did. But most of these studies did not address whether gelatinase affects the severity of disease, as it does in animal models (Elsner et al 2000, Archimbaud et al 2002, Roberts et al 2004,
Kanemitsu et al 2004, Creti et al 2004). However, some studies have shown the role of the extracellular gelatinases (proteases) in human infections is of clinical significance since they produce pathologic changes in the host, by degrading lipids, deoxyribonucleic acid and hyaluronic acid (a component of the connective tissues), or by disrupting the equilibrium by inducing an inflammatory response with the help of lipoteichoic acids, complement and neutrophils (Jett et al 1994, Mundy et al 2000, Semedo et al 2003). Our results showed that majority of the HLGR enterococci possessed more than one virulence factor.

Hyaluronidase has been proposed as a potential virulence factor in several gram-positive bacteria, including S. aureus, S. pneumoniae, and S. pyogenes (Hynes et al 2000). Recent data from pneumococcal models of infection suggest that hyaluronidase may contribute to invasion of the nasopharynx that precedes central nervous system infection and contribute to pneumococcal pneumonia [Polissi et al 1998, Berry et al 2000].

Both $hyl_{Efm}$ and $esp_{Efm}$ were found overwhelmingly in E. faecium strains isolated from cultures from humans. E. faecium has traditionally been considered a bacterial species of limited virulence due to its involvement in a relatively low percentage of enterococcal infections (10%) and the difficulty of establishing animal models of infection (Polissi et al 1998, Berry et al 2000). The increase in the percentage of enterococcal infections caused by E. faecium over the past decade in the United States (30%–40% in several surveys) suggests that these bacteria may have become more virulent. Although the increased resistance to antimicrobial agents in E. faecium over the same period of time should not be overlooked as a predisposing factor in the modern hospital, it is probably not the only explanation. After all, data from Europe in the 1990s suggested that fecal colonization by vancomycin-resistant E. faecium was quite frequent in some areas of Europe (van der Auwera et al 1996, Klare et al 1999) but was almost nonexistent in community dwellers from the United States. Yet the rates of infection and colonization by vancomycin -resistant enterococci in the hospital were far greater in the United States than in Europe over the same period.
The association between the presence of \( \text{esp}_{Ef} \) and \( \text{hyl}_{Ef} \) and the expression of antibiotic resistances seen in our study are consistent with a scenario in which \( E. \text{faecium} \) isolates from the United States that are prevalent in the nosocomial setting had acquired resistance (e.g., to ampicillin) and virulence before the introduction of vancomycin resistance operons. Published reports document the rise of ampicillin resistance in \( E. \text{faecium} \) before the recognition of vancomycin resistance in this species (Chirurgi et al 1992). It is worth noting that TX0016, the strain analyzed for the partial \( E. \text{faecium} \) database, is an ampicillin-resistant, vancomycin-sensitive isolate from a case of endocarditis (Murray, unpublished data). The introduction of the van A and van B operons into \( E. \text{faecium} \) strains rich in antimicrobial resistance and (presumably) virulence determinants, was then prompted by widespread use of orally administered vancomycin in the United States during the 1980s. This co-existence of virulence determinants and multi resistance in the United States yielded a group of strains particularly suited to causing clinical infection in the modern hospital. The situation in Europe is different because the population of vancomycin-resistant \( E. \text{faecium} \) in Europe was created through use of the growth-promoting antibiotic avoparcin in animals during the 1970s through 1990s. These \( E. \text{faecium} \) strains, which as animal colonizers were devoid of virulence determinants, were sufficient to colonize the human population in Europe. The presence of substantial numbers of these less virulent vancomycin-resistant strains may have precluded the rapid emergence of the more pathogenic varieties by competing for one of the selective niches (growth in glycopeptide-rich environment) enjoyed exclusively by the more pathogenic strains in the United States.

At present, we cannot say with certainty whether and to what extent \( E. \text{faecium} \) actually makes hyaluronidase and under what conditions this protein may be synthesized or exported. Northern hybridization experiments indicate that the hyaluronidase ORF is transcribed under nonselective growth conditions in vitro (data not shown). Therefore, we have compelling reason to believe that the protein is synthesized at least under some environmental conditions. Experiments to characterize the extent of hyaluronidase production by \( \text{hyl}_{Ef} \)-positive strains are ongoing.
Comparison of different S. aureus genome sequences indicates that substantial variation exists between strains, with much of the variability involving antimicrobial resistance determinants and pathogenicity islands (Fitzgerald et al 2001). Our data suggest that E. faecium should be added to the growing list of human pathogens with a variety of genomic inventories, some of which have evolved within and are particularly suited to surviving and causing infections in the modern hospital environment.

In our hands, results obtained by phenotypic tests always revealed a lower percentage of strains that produced haemolysin, gelatinase or aggregation substance, compared to genotypic characterization. This may be due to the presence of silent genes that are expressed only under in vivo conditions, to the presence of undetected gene mutations or to the fact that detection by PCR of a single gene inside an operon, may overlook the absence of other genes that are necessary for phenotypic expression. Techniques such as RT-PCR may provide useful information on the level of expression of the target DNA.

In the present study, the results revealed that the clinical strains carried at least one and concomitantly up to as many as four virulence markers. The majority of the strains harbored between two and three virulence determinants. None of the strains examined showed all of the virulence genes investigated in the study. In general the urinary as well as exudates strains carried predominantly three virulence genes concomitantly. On the contrary, two virulence genes were found to co-exist preferentially in strains isolated from blood and exudates. Generally the genotype gelE+, asa1+, esp+ was predominantly found in 153 (48%) of the 316 clinical strains studied, where it was more prevalent in strains isolated from urinary strains and exudates than those isolated from blood. Therefore in some manner, it was observed that the gene gel E is frequently associated with asa1 or esp and to a lesser extent with the virulence marker cylA. hyl gene was totally absent in E. faecalis isolates. In conclusion, our data indicate that E. faecalis strains isolated from different sources possess distinctive patterns of potential virulence factors, with a larger number of genes that encode potential virulence factors among isolates from UTIs.
Among the *E. faecium* strains gelE, *asa1*, cylA genes were totally absent. The only genes that were present were *hyl* and *esp*. In general the urinary as well as exudates strains carried predominantly both the virulence genes concomitantly. On the contrary, only the *hyl* virulence gene was found to exist preferentially in strains isolated from blood isolates. Generally the genotype *hyl+ & esp+* was predominantly found in 14 (32%) of the 44 clinical strains studied, where it was prevalent in strains isolated from urinary strains and exudates and *esp* was not present in any of those isolated from blood. Therefore, it was observed that the *hyl & esp* were the only genes associated with *E. faecium*. This is in concordance with studies from Brazil and Italy (Creti et al 2004).

*Enterococci* rank among top three pathogens causing nosocomial infections worldwide, since last decade (Murray 1990 & 1998). Although initially thought to have evolved from patient's own flora, *enterococci* was later shown to be exogenously acquired from nosocomial settings by Zervos and his colleagues in 1986, using molecular epidemiological tools. Since then, innumerable reports of nosocomial enterococcal infections were published and most studies used molecular epidemiological tools for typing *enterococci* to study the clonality of the isolates. In case of suspected outbreak conditions, the analysis of strain clonality helps in confirming the association between patients (hosts) and reservoirs for *enterococci*, and to determine the possible modes of transmission. In some instances, the phenotyping and antibiotyping results of *enterococci* may help presumptively in investigating whether the isolates studied have, or lack clonal relationship. However, most of the inconclusive results obtained by other typing methods can be authenticated by the application of molecular epidemiological techniques, which gives a clear picture regarding the clonality of the isolates.

A variety of molecular epidemiological techniques have been applied for epidemiological typing of drug resistant *enterococci*. "Pulsed field gel electrophoresis" (PFGE) is considered to be the gold standard for molecular epidemiological analysis of gram-positive cocci, since last decade (Murray et al 1990). Other techniques like plasmid DNA analysis—which was the first tool to be applied for epidemiological analysis of *enterococci* (Zervos et al 1986), PCR based
typing and a more recent technique: AFLP have also been found to be effective in epidemiological typing of enterococci. Thus molecular typing of enterococci is inevitable to draw conclusive evidences regarding the epidemiology of drug resistant strains in any health-care setting.

Microbial identification although undoubtedly plays a major role in determining the clinical outcome of any disease, it is the typing of the microbe identified which provides insights into epidemiological aspects of these infections, especially those of nosocomial origin in relation to endemicity/outbreak due to strains that exhibit clinically important antimicrobial resistance. Enterococcus remains to be a "perfect fit" in this category, leading the race among all nosocomial pathogens (Murray 1990). The recent genotyping methods are considerably more powerful than most of the phenotype-based typing systems providing a finer level of epidemiological discrimination, differentiating both closely and distantly related independent isolates that otherwise may appear as identical, since the approach focuses on genetic determinants rather than phenotypic characteristics alone. The results of the microbial typing along with other supporting clinico-epidemiological details helps in initiating and executing appropriate infection control measures for multidrug resistant nosocomial pathogens like enterococci in any health care setting at the appropriate time contributing to a substantial decrease in morbidity and mortality.

Although various species of enterococci were isolated at regular intervals throughout our study period as depicted in previous chapters, we could find clustering of particular species during various time periods from specific units/wards. E. faecalis was the commonest and predominant of all species and was isolated consistently throughout our study period, but we were unable to cluster them merely by their antibiotype. Hence the molecular typing technique of chromosomal DNA analysis by PFGE was performed to determine the clonality of these isolates. The profiles of PFGE gels were analyzed both, visually as per Tenover et al 1995, guidelines and computationally using the Diversity database software in our study.
Overall, the results of molecular typing of this study depicted that the strains belonging to various groups/clusters were isolated from individual patients in different wards. The wards along with their corresponding ICUs were located on different floors of the same building of the hospital block in SRMC. The medicine wards (with their ICU) were located on the third floor, the surgical wards on the second floor, pediatrics wards and the gynecology wards on the first floor. The same nurses work on a single ward. Although unusual enterococcal species were prevalent in our hospital, it was the emergence of HLAR among \textit{E. faecalis} that was of serious concern and needed special focus. Hence molecular typing of randomly selected HLAR \textit{enterococci} was performed by PFGE for epidemiological investigations, and to track the dissemination and evolution of multi-drug resistant strains more efficiently in our hospital setting. The chromosomal DNA analysis by PFGE is considered a gold-standard technique for molecular epidemiological studies since last decade. Hence, we followed this method for molecular epidemiological studies of drug resistant \textit{enterococci} (Zervos et al 1987, Vittal Prakash et al 2004, Ma et al 1998, Mayer 1988, Wanger et al 1990, Tomita et al 2002 & 2003).

The visual interpretations of PFGE gels based on the similarity of clusters/groups obtained by matching the consensus guidelines of Tenover et al. showed concordance results, which had some clinico-epidemiological significance when analyzed. The PFGE typing results showed that approximately 50% (30) of the (60) HLAR enterococcal isolates were homogeneous and formed eight clusters (I - VIII), while 50%( 30) of the isolates were non-clusterable depicting heterogeneity/diversity among the HLAR isolates. The PFGE genotypic cluster-A isolates were found to be "endemic" in our hospital during our study period, since they were present in different medical and surgical wards situated in I, II and III floors for more than a year from October 2003 to August 2004 as shown in Table 19. Incidentally, three consecutive \textit{E. faecalis} isolates from a patient with fever after aortic valve replacement were included for molecular typing, and all three isolates were proven to be genotypically similar and grouped under cluster-A. Interestingly, two \textit{E. faecalis} isolates showed a "closely related" PFGE pattern of cluster-A isolates with 2-3 band differences and classified as A1 and A2 according to
the definitions of Tenover et al in 1995. These Al and A2 E. faecalis isolates would have originated from cluster-A isolates after a possible genetic event, i.e., a point mutation or an insertion/deletion of DNA, since these two strains were isolated from same wards during the same time period when cluster-A strains were prevalent (Tenover et al 1995). Thus our results depict that enterococcus has high possibilities for genomic rearrangements, which would be evident only through molecular typing techniques like PFGE. This evidence would be highly helpful to trace the outbreak related isolates, like those of Al and A2 as shown in the present study.

Three E. faecalis PFGE Cluster-B strains were isolated from pediatrics ward and the New block ICU (NBICU) within a span of three months during 2003, but not isolated there after. This suggests that routine sanitation measures (without the knowledge of the prevalence of E. faecalis isolates) practiced by the particular ward was sufficient enough to prevent (or eradicate) the dissemination of antimicrobial resistant nosocomial pathogens. The E. faecalis strains from clusters C, D and E were isolated from the same or different wards during 2004, suggesting the circulation/dissemination of the HLAR E. faecalis in our hospital. The HLAR E. faecium isolates from clusters A, F, G & H, were isolated within a short span during 2003 and 2004 respectively, which were also possessing esp gene. The prevalence of the strains possessing esp gene all through our study period indicates widespread dissemination of these HLAR enterococci in our hospital setup (intrahospital strain dissemination) as shown by several studies (Huycke et al 1991, Leavis et al 2003, Seetulsingh et al 1996, Donabedian et al 1992).

The predominance of strains from cluster A, along with strains from other clusters (B, C, D, E) in particular ward(s) during specific time periods suggest that these isolates were derived from a common source and spread from patient to patient, however we do not know the method of transient carriage in the nosocomial transmission of the commonly prevalent aminoglycoside resistant enterococci, since the reservoir and mode of transmission of the HLAR enterococci were not determined in our study. However, several researchers have studied the epidemiology of nosocomial environmental reservoirs of multidrug resistant
*Enterococci*. *Enterococci* have been shown to be capable of prolonged survival on hands, gloves, thermometers, blood pressure handcuffs, IV fluid pumps, bedrails and linen and various hospital environmental surfaces (Bonten et al 1996, Noskin et al 1995, Livornese Jr et al 1992, Brooks et al 1998). Further, nosocomial *enterococci* were shown to be resistant to heat (upto 80°C for 1 min), and could withstand routine disinfection procedures (150ppm chlorine) followed for infected linen, which underscores the significance of *enterococci* to survive and disseminate in the hospital environment (Freeman et al 1994). Most of the studies have shown concordance between hospital environmental strains and the patient isolates often resistant to vancomycin or high-level gentamicin, which were confirmed using molecular epidemiological tools like PFGE and/or Plasmid typing (Takahashi et al 1999, Dicuonzo et al 2001, Kuriyama et al 2003, Martinez et al 2003, Mayer et al 2003, Reisner et al 2000, Smith et al 1998). Thus as suggested in these studies, any of the above mentioned source(s) could have been the reason for “intrahospital dissemination” of *enterococci* in our hospital.

The 50% (30) non-clusterable isolates with "unique" PFGE patterns were isolated from various wards (including those wards where the predominant clusters of *enterococci* were found) throughout our one-year study period. Although few of these "unique" strains were "possibly related" with the isolates from different clusters based on the consensus guidelines (Tenover et al 1995), the genomic heterogeneity exhibited by majority of the non-clusterable isolates depicts the diversity of HLAR *enterococci* as shown by several studies from U.S, Netherlands, Norway, Greece, U.K, (Bopp et al 1999, van den Braak et al 2000, Eliopoulos et al 1984, Papaparaskevas et al 2000, Hall et al 1992). The diversity (50%) of the isolates as shown by PFGE typing in our study suggests that, apart from patient-to-patient spread that was equally (50%) a major cause for dissemination of clusterable (homogenous) HLAR isolates, other possible sources can be due to colonizer isolates or fecal contamination, or they could be community acquired isolates. Several epidemiological studies conducted in human subjects from community have yielded *enterococci* resistant to various antimicrobials like ampicillin, gentamicin and vancomycin (D’Agata et al 2001, Eliopoulos et al 1984). Thus screening the inpatient population for fecal carriage of antimicrobial resistant *enterococci*, and
conducting point-prevalence studies would be highly significant and necessary in the wake of HLAR and emergence of vancomycin resistance among *enterococci*. This would help in initiating appropriate infection control measures and restructuring the hospital antibiotic policy, if needed.

Studies have shown that epidemic drug resistant *enterococci* may possess specific genetic characteristics (encoding virulence determinants) resulting in a distinct lineage, which could facilitate enhanced colonization/infection of the host (Mundy et al 2000). The permeation of these virulence genetic characteristics into different species differs according to the setup, patient demographics and other extrinsic factors. The *esp* gene and associated virulence factors have permeated deeply into the *E. faecalis*, since 69% of these isolates exhibited the presence of this putative virulence factor. Our PFGE typing results, which depicts the clonality of the HLAR isolates, reveals that majority (60%) of the clusterable isolates (I-VIII) possessed *esp* gene (including two *E. faecium* clusters), while they were absent among the 40% of the other isolates. The clustering of these *esp* positive isolates proves that strains of this chromosomal lineage are related, and may have been derived from a common ancestral strain as described by Lund et al, 2003. However, the presence of the *esp* gene among 40% of unique/non-clusterable *E. faecalis* isolates also depicts that the pathogenic potential due to the presence of *esp* gene need not be confined to a single genetic lineage as shown by a recent study (Leavis et al 2003), since closely related lineages/genotypes can be equally virulent resulting in epidemicity.

Our findings suggest a strong association between the presence of *esp* gene, and high-level gentamicin resistance, although both have been shown to reside on entirely different genetic determinants (chromosomal and extra-chromosomal [plasmid] respectively). The prevalence of this combination is of high clinical significance in any health-care setup, since several studies have shown that specific genetic lineages (as shown by PFGE) exhibited the presence of *esp* with vancomycin and/or ampicillin resistance among clinical *E. faecalis* and *E. faecium* isolates (Willems et al 2001, Coque et al 2002, Leavis et al 2003, Harrington et al 2000). These studies suggests that prior treatment/exposure to the antibiotics like vancomycin and/or ampicillin would have selected these clones facilitating to reach
ecological abundance in the nosocomial habitat due to the presence of \textit{esp} gene, although studies are yet to analyze the significance of the combination of HLGR and \textit{esp} gene. Thus, our findings suggest that the higher prevalence of gentamicin resistance in our hospital would have contributed to selection of those clones/lineages with \textit{esp} gene resulting in "intrahospital dissemination". Thus appropriate measures to contain the antimicrobial resistance in any health care setup would by all means decrease the probability of selection and dissemination the \textit{esp} positive clones, which poses a tough challenge ahead.

PFGE was performed in a single stretch using the isolates collected during this study period. Molecular typing results of this study, depict that concomitant performance of molecular typing (by PFGE typing) during any suspected outbreak/increase in the prevalence of nosocomial pathogens would be highly helpful in tracking and preventing the dissemination of multi-drug resistant strains/ clones, more efficiently at a given point of time in a hospital setup. Some studies have shown that multicenter PFGE studies with a harmonized protocol and centralized server for interpretation can address epidemiological questions effectively (de Lencastre et al 1996). Thus a cooperative venture for molecular typing of \textit{enterococci} would provide a rapid tracking system to assist hospitals, clinics and health care facilities in controlling the spread of multidrug-resistant \textit{enterococci} locally, nationally as well globally.