VI.

DISCUSSION
V. DISCUSSION

Enterococci are part of the normal flora of gastrointestinal tract, genital-tract and oral cavity of human beings. These are relatively less virulent organisms but can cause urinary tract infections, wound infections, intra abdominal infections, bacteraemia, septicaemia and endocarditis. Now a day’s these are being isolated more frequently from clinical specimens, particularly in hospitalized patients. Data from the CDC (1993) indicates that enterococci are the third most leading cause of nosocomial infection, joining E. coli, Pseudomonas and Staphylococcus aureus in the list of most prevalent species.

Recent studies showed increased interest in enterococci not only because of their ability to cause serious infections but also because of their increasing resistance to many antimicrobial agents. Serious enterococcal infections are refractory to antibiotic treatment due to increased resistance to antibiotics and the mortality rate is high. Limited number of antibiotics is available for treating enterococcal infections.

Urinary tract and wound infections are the most common enterococcal infections, both within and outside hospital settings. Species identification of Enterococci has gained importance in the last few decades. Enterococcus species have the ability to acquire new antibiotic resistance determinants including vancomycin resistance.

INcIDENCE

In the present study, of the total 9000 clinical specimens studied, over a period of three and half years, 3822 specimens yielded growth and of these 244 were enterococci (2.71%) [Table No. 2]. This incidence rate correlated well with the studies conducted by Mendiratta DK (1.16%) and Parvathi et al. (3.38%). It was lesser than that reported by Miskeen PA et al. (7.38%) and PJ Desai et al. (22.19%). These two workers studied patients those were hospitalized for prolonged period for various disorders which require catheterization. Sonal Saxena et al. compared hospitalized and non-hospitalized patients and reported that these organisms are known to have increasing role in nosocomial infections.

Enterococci tend to produce infection in patients who are elderly or debilitated. In the present study [Table No. 1], the highest incidence was seen in the age group of 51-70 years...
(32%) and neonates (1-10 days). Various associated risk factors are common in old age group. For example, obstructive uropathies with catheterization, abdominal surgeries for diverticulitis or
biliary tract infection are performed more commonly in elderly persons. The mean and median age of the patients with Enterococcal infection was 42 ± 24 SD. Barros et al. have reported high prevalence of enterococcal infections in the age group of 50-59 and mean age of the patients was around 60 years.[29,66] The enterococcal endocarditis occurs more commonly in older individuals, rarely in infants and occasionally in children.[1,15] The patients with enterococcal endocarditis are predominantly older males,[106] with an average age of 56 to 59 or more (65) years.[22] Enterococcal infection was found in 16 neonates [Table No. 1]. The infants with severe underlying disease, intravascular device, umbilical arterial or venous catheters, nasogastric endotracheal intubation and prematurity appear to be more susceptible.[1,3,108]

In general, enterococcal infections are distributed equally in both the sexes. In the present study also, there was no significant difference in the incidence of enterococcal infections among males (54%) and females (46%), but some studies have noted higher incidence of enterococcal UTI among females.[29,66] In the present study, the incidence of enterococcal urinary tract infection is significantly higher in males belonging to old age group (51 to 90 years). However, the incidence was higher (i.e. 16%) among sexually active females (age group 18 to 40 years) suffering from UTI [Chart No. 1]. During intercourse and after bowel movement (cleaning), there is possibility of entry of intestinal or vaginal enterococci (normal commensals) into urinary tract due to proximity of urethral, vaginal and anal openings [honeymoon cystitis]. The prevalence of nosocomial enterococcal UTI is increasing in a number of hospitals. In contrast, enterococci are the rare cause of infection such as uncomplicated cystitis in non-hospitalized women.[1,3] Enterococci cause less than 5% of UTIs in young, healthy women with cystitis those do not have structural abnormalities, instrumentation and / or recurrent infections.[11]

**Table A. Incidence (%) of Enterococci in Different Specimens at Different Places**

<table>
<thead>
<tr>
<th>Name, Place (Year)</th>
<th>Pus</th>
<th>Urine</th>
<th>Blood</th>
<th>Peritoneal Fluid</th>
<th>Other</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desai PJ, Mumbai (2001)[23]</td>
<td>27.8</td>
<td>28.57</td>
<td>--</td>
<td>7.10</td>
<td>12.9</td>
<td>22.19</td>
</tr>
<tr>
<td>Parvati S, Coimbatore (2003)[15]</td>
<td>5.03</td>
<td>4.48</td>
<td>0.35</td>
<td>11.11</td>
<td>0.5</td>
<td>3.38</td>
</tr>
<tr>
<td>Present study, Karad, Vijaypur (2012)</td>
<td>1.86</td>
<td>4.23</td>
<td>2.54</td>
<td>1.45</td>
<td>0.97</td>
<td>2.71</td>
</tr>
</tbody>
</table>

Among the 244 isolates obtained from various clinical samples, maximum isolates (incidence) were from urine (4.23%) followed by blood (2.54%), pus (1.86%) and peritoneal fluid (1.45%) [Table no. 2]. While one (each) isolate was from pleural fluid, CSF, bronchial lavage and bile. These incidence rates were correlated well with a study done by Parvathi et
*al.*[^15] who reported highest incidence in pus (5\%) and urine (4.48\%) while least incidence in
blood (0.35%) and peritoneal fluid. But PJ Desai et al.[23] have reported high prevalence in urine (8.92%) and ascitic fluid (7.10%). The infection due to enterococci were found to be extremely significant $[\chi^2 = 53.77, P<0.0001]$, when compared with different clinical specimens. Enterococci isolated from various clinical specimens do not reflect the true incidence of infection caused by this organism, but definitely suggest the increased frequency of their isolation from various clinical materials.[23]

In the present study, distributions of enterococcal isolates showed that majority of enterococci were obtained from urine (59%), pus (28%), blood (9%) and peritoneal fluid (2%). While very few isolates were obtained from pleural fluid, C.S.F., bronchial lavage and bile (all together 2%) [Table No. 2]. The present study correlates with Udo EE’s[154] study who has reported that majority of them were obtained from urine (36·6 %), stool (11·8 %), wound swabs (11·0 %), blood (10·3 %), high vaginal swabs (9·1 %), cervical swabs (3·1 %) and miscellaneous sources, including cervical swabs, catheter tips, endotracheal secretions and ear swabs (18·0 %).

**Urinary Tract Infection**

Urinary tract infection is the most common infection caused by enterococci. These organism tend to cause infection in patients in whom mucosal or epithelial barriers have been disrupted or the balance of normal flora altered by antibiotic treatment.[107] Urinary tract infections due to enterococci rises particularly among hospitalized patients, who have received antibiotic therapy (particularly cephalosporin),[23] or undergone instrumentation of urinary tract,[66, 107] urinary catheterization (8.92%),[23] having structural abnormalities, and / or recurrent urinary tract infections.[15] A twenty-fold increase in the incidence of enterococci causing nosocomial UTI is documented.[5]

In the present study, out of 3400 UTI patients screened, enterococci were isolated from 143 (4.23%) cases. Of these 19 (13%) isolates were from UTI in amenorrhea cases, 14 (10%) from catheterized patients and 13 (9%) were from hypertensive patients while 17 (12%) from other complications [Table No. 3]. In pregnant women due to physiological, hormonal and mechanical changes they are considered immunocompromised UTI host. The urinary stasis, vesicourethral reflux and enlarged belly increase the risk of UTI among pregnant women. Catheter-related UTI occurs because enterococci may enter in to the bladder during catheterisation. It also helps in colonization by providing surface for bacterial adhesion and causing mucosal irritation.[177]
The reported frequency of enterococcal UTI is variable in different studies. Miskeen et al.\cite{176} have isolated these organisms in 7.4% of the UTI patients and Jaylaxmi et al.\cite{178} has reported 2.12% enterococcal incidence in asymptomatic bacteriuria in pregnant women. Krishna KS et al.\cite{102} from Lucknow, has reported UTI as the most common form of bacterial infection affecting renal transplant recipients. Among the Gram positive cocci causing UTI in these patients, enterococci (58%) were the most common followed by staphylococci.\cite{178} The bladder, prostate and kidney are commonly infected by enterococci.\cite{3}

**Wound Infection**

Enterococci are frequently isolated from surgical wound infections, decubitus ulcers, diabetic foot infections and burns.\cite{22} They rarely cause cellulitis, deep tissue or diabetic wound infections.\cite{3}

In the present study, among the 3719 patients with wound infections, enterococci were responsible for 69 (1.86%) cases. Among these, post operative wound infection (29%) was the most common infection, followed by pyogenic skin infection (21.7%) and diabetic foot ulcers (17.4%). Infections of wounds on buttock (11.6%) and wounds caused by road traffic accident (8.7%) were also seen. [Table No. 4]. Smets YFC et al. reported surgical site wound infection in 30% of cases and claimed that enterococci were the major pathogen. Krishna KS et al. have reported, in wound infections among the renal transplant recipients staphylococci was most common followed by enterococci.\cite{102} Infections caused by enterococci often involve intra-abdominal and surgical sites.\cite{24}

Enterococci can survive for many days on fabrics. The contaminated hospital environment may play a significant role in the transmission of these microorganisms. Frequent washing, disinfection and changing of hospital garments play an important role in the prevention of transmission of enterococcal infections.\cite{122} Contaminated perineal skin and soil may be the source of these organism. Skin and soft tissue infections identified as the source of bacteraemia in 15 to 30% cases.\cite{22}

**Blood Stream Infection**

Enterococci may cause serious infections like blood stream infection.\cite{66} They are frequent cause of nosocomial bacteraemia in patients with intravascular catheters. The source of enterococcal bacteraemia without endocarditis is often the urinary tract infection, wound infection, haepatobiliary tract infection,\cite{22} vascular and / or catheters.\cite{1} In 1986-1990
according to the Centres for Disease Control and Prevention’s National Nosocomial Surveillance Survey, enterococci are reported to cause 8.3% of cases of nosocomial bacteraemia.\cite{22} These organisms account for 5 to 20% of bacterial endocarditis for both native and prosthetic valves.\cite{107} It is usually a disease of older men,\cite{106} occasionally occur in children and rarely in infants. In the present study, out of 867 blood samples, enterococci were isolated from 22 (2.54%) cases [Table No. 2], its more than reported by Roy I et al.\cite{103} (0.8%). Of these 22 isolates, 16 were from infection in neonates, 6 from septicaemia or bacteraemia in adults. [Table No. 8].

Enterococcal endocarditis may present as an acute or subacute illness. Common risk factors are genitourinary and biliary portal complications. In Mandell’s study, 50% of the men had preceding genitourinary instrumentation or UTI and 30% of the women had preceding abortion or instrumentation.\cite{15} Chopadekar K et al.\cite{105} noted that 13% of E. faecalis were the cause of catheter related blood stream infection as well as biofilm formation. Krishna KS et al.\cite{102} from Lucknow, reported E. faecalis blood stream infection among the renal transplant recipient patients.\cite{88}

Enterococci are the second most common cause of infective endocarditis (6-10%) next to viridans streptococci.\cite{70} Life threatening infections like pericardial infection is thought to occur due to complex interplay between the magnitude of virulence of the strain and the host response. Virulence factors of E. faecalis like adhesins, cytolysins and others help the organism to multiply and spread widely. Pericarditis is usually secondary to the extension of an underlying condition such as infection from pleural cavity.\cite{38} In the present study, enterococci were isolated from pleural fluid and bronchial aspirates, one each [Table No. 2]. The associated risk factors for VRE bacteraemia include haemodialysis, organ transplantation, receipt of corticosteroids, chemotherapy, parenteral nutrition, surgery, severe illness, long-term antibiotic administration, indwelling urinary catheters, neutropenia, mucositis,\cite{101} heart diseases and / or drug addicts.

**Neonatal Sepsis**

Enterococci have been recognized as important pathogens in high risk nursery since 1997 but the incidence and number of reports of nursery outbreaks associated with E. faecalis and E. faecium have increased substantially since 1980s.\cite{179} Septicaemia continues to be a major cause of mortality and morbidity among the neonates around the world. Neonates are more susceptible to infection because of weak immunity. Blood stream infections were the most common infection in this age group.\cite{103} Strong evidence indicates that patients were infected by E.
faecalis and E. faecium. They were isolated simultaneously from blood and CSF among four patients. Out of these four patients one had three successive cultures positive for E. faecium over a period of 8 days. Septicaemia and meningitis due to enterococci was seen in neonates. Enterococcal infections occurred in infants of low-birth weight, premature infants with several severe underlying conditions as well as without underlying conditions.[108] Prolonged use of central venous catheter, necrotising enterocolitis, bowel resection and prior exposure to antibiotics are associated findings. Clinical manifestations of enterococci are nonspecific. Meningitis and endocarditis required prolonged (≥ 3 weeks, ≥ 6 weeks respectively) treatment with combination therapy for cure.[179]

In the present study, among 22 isolates from blood, 15 were from neonatal sepsis and one neonate who had other complication [Table No. 8]. The prevalence of E. faecium in the gastrointestinal flora of neonates was recorded from infants in the NICU.[108] Furthermore, in many of the cases source was endogenous, maternal (birth canal) or Nosocomial (hospital environment).

Other Infections

Chetana V et al. have reported 6.8% of enterococci from bacteriuria patients with gall bladder and biliary tract diseases.[109] Enterococcal central nervous system infections rarely develop in patients usually who have underlying complication.[3] In the present study, we could isolate enterococci from one patient each with biliary tract infection and meningitis.

POLYMICROBIAL

Enterococci are frequently isolated from mixed culture with Gram-negative bacilli and anaerobes from skin and soft tissue infection.[22] Among the 244 isolates of enterococci, only 162 (66%) were pure enterococci, the rest 82 (34%) were mixed with other bacteria and were isolated in association with E. coli, Klebsiella, Citrobacter, Proteus, Pseudomonas, Staphylococcus aureus and Candida species [Table No. 5]. Parvathi et al.[15] has reported 57% pure isolates and 43% were in combination with other organisms. Whereas PJ Desai et al.[23] reported 18% pure culture of enterococci and remaining 82% along with same organisms like our study. Intra-abdominal or pelvic wound infections and other wound cultures are usually polymicrobial[97] but the role of enterococci remains controversial. More often enterococci were found as one of the isolates in clinical specimens with polymicrobial etiology which are better established as pathogens and are primary target of subsequent therapy. Enterococci by virtue of being non-
invasive organisms, their presence with organisms like *Staphylococcus aureus* and other Gram negative organisms are suggestive of colonization rather than infective etiological agent. The source of this organism is commonly the faecal flora. Even though wound colonization was very high in burn patients but none had sepsis due to enterococci.[23] In the present study, enterococci were isolated from three burn cases. The mortality due to poly microbial bacteraemia is two times higher than mortality associated with bacteraemia due to enterococci alone. While mixed infection are frequently cured by antimicrobials not active against enterococci, but specific therapy directed against enterococci is warranted when these organisms are predominant species or isolated from blood cultures.[107]

**IDENTIFICATION**

The higher incidence of β-haemolytic strains of *E. faecalis* and *E. faecium* were noted in one study.[23] The enterococci were misidentified as coagulase negative staphylococci, streptococci or micrococcii (unpublished data). This advocates that the clinical specimens should be screened for the presence of enterococci by using catalase and bile esculin test. Prolonged incubation or modified bile esculin test (like Pfizer’s selective media which gives rapid results) should be used for presumptive identification. It indicates that there is very low index of suspicious observation for enterococcal colonies.

In the present study, the battery of tests identified 98.77% of enterococcal species isolated from human source. Vitek 2 were used to identify various enterococcal species and we were able to speciate about 241 (out of 244) isolates. Conventional tests proposed by Facklam and Collins[43,44,61,63,72,100,180] were thus successfully used to identify enterococci to the species level. We were unable to confirm identification of 23 enterococcal isolates by Facklam and Collins method. Doubtful and unidentified species were further identified by Vitek 2 automated method. Out of these 23 enterococcal isolates, three isolates remain unidentified.

The key tests are arginine hydrolysis and acid production from mannitol and sorbose. On the basis of these tests, enterococcal isolates are grouped into five groups.[42] In the present study [Table No. 6], most of the isolates belonged to Group II (94.67%), followed by group III (2.46%) and group I (1.64%). There was no single isolate belonging to group IV and V. Then additional tests were used to differentiate to species level. Strict adherence to the differentiation scheme of Facklam and Collins,[43,44,61,63,72,100,180] with key tests recommended for enterococcal identification, resulted in misidentification of 4 strains as *E. solitarius*. The automated Vitek 2 system identified these isolates as *E. faecalis* (not as *E. solitarius*). These isolates fermented the
ribose, reduced tellurite but failed to ferment the sugar lactose, suggesting that they are really lactose non fermenting *E. Faecalis*. Rouff KL *et al.*\textsuperscript{[100]} have also reported that 22 isolates were misidentified as *E. solitarius* by the same method but he confirmed as *E. faecalis* by Rapid Strip method (all 22) and Vitek system \textsuperscript{(20)}. Additional tests such as tellurite reduction and ribose fermentation test\textsuperscript{[73]} are useful and are essential for differentiation of lactose negative *E. faecalis* and *E. solitarius*. Lactose negative *E. faecalis* may be miss-categorized as *E. solitarius*.\textsuperscript{[23]}

We could not identify 3 (1.2\%) enterococcal isolates by the scheme of Facklam and Collins as well as by automated Vitek 2 method. Rouff KL *et al.*\textsuperscript{[100]} and Bhat KG\textsuperscript{[181]} were also unable to identify 0.3\% and 0.7\% isolates respectively up to species level. Therefore emphasis should be given on larger spectrum of tests in order to recognize species within genus. Accurate identification up to species level is very important as there is variation in resistance to antibiotics by particular enterococcal species.\textsuperscript{[23]}

![Chart No. 6. Showing Different Species of Enterococci with Percentage](image)

Enterococci are part of the normal flora of gastrointestinal and genitourinary tract colonizing the bowels of more than 90\% of the healthy human beings. Among the enterococcal species, *E. faecalis* and *E. faecium* are two major pathogens accounting for 60-90\% and 5-35\% of all enterococcal infection respectively.\textsuperscript{[5,13,99]} In the present study, *E. faecalis* (77\%) and *E. faecium* (17\%) were the major isolates among the enterococcal infections. Earlier, the ratio of *E. faecalis* to all other *Enterococcus* species was approximately 10:1. But in recent years, there has been a progressive decline (up to 3.7:1) in enterococcal bacteraemia and other infections.\textsuperscript{[115]} This is also true in the present study (3.4:1).
Table B. Place and Year wise Distribution of *Enterococcus* Species

<table>
<thead>
<tr>
<th>Name, Place (year)</th>
<th>Name of the species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. faecalis</td>
</tr>
<tr>
<td>Rouff KL, Boston (1990)[100]</td>
<td>87.8</td>
</tr>
<tr>
<td>Gordon S, Franklin (1992)[66]</td>
<td>90</td>
</tr>
<tr>
<td>Bhat KG, Mangluru (1997)[181]</td>
<td>87</td>
</tr>
<tr>
<td>Desai PJ, Mumbai (2001)[23]</td>
<td>59.5</td>
</tr>
<tr>
<td>Miskeen PA, Mumbai (2002)[176]</td>
<td>87</td>
</tr>
<tr>
<td>Udo EE, Kuwait (2003)[154]</td>
<td>85.3</td>
</tr>
<tr>
<td>Prakash VP, Pondicherry (2005)[112]</td>
<td>--</td>
</tr>
<tr>
<td>Mendiratta, Sevagram (2008)[30]</td>
<td>85.3</td>
</tr>
<tr>
<td>Rahangdale, Nagpur (2008)[153]</td>
<td>64.2</td>
</tr>
<tr>
<td>Shah L, Surat (2012)[182]</td>
<td>75</td>
</tr>
<tr>
<td>Ghosh R, Kolkata[183]</td>
<td>60</td>
</tr>
<tr>
<td>Present study, Karad &amp; Vijaypur</td>
<td>77.1</td>
</tr>
</tbody>
</table>

The non- *E. faecalis* & non- *E. faecium* species varies from 0 to 19%. In the present study we came across 6% non- *E. faecalis* & non-*E. faecium* species among the entercoccal isolates. We could recover *E. durans, E. avium, E. raffinosus, E. Gallinarum* and *E. hirae*. This is comparable with studies conducted by other authors.\[23,66,100,112,176,181\] *E. durans* (2%) was predominant among these species followed by *E. avium* and *E. raffinosus* (1% each) while we identified rare species *E. gallinarum* and *E. hirae* (1% together) [Table No. 6]. Miskeen *et al.*\[176\] has reported *E. durans* (2%) as the predominant species in her study. According to the study by Desai P J *et al.*,\[23\] 14.85% of isolates were identified as members of non-*faecalis* and non-*faecium* species and *E. avium, E. hirae, E. raffinosus, E. gallinarum* and *E. casseliflavus* [non-*faecalis* and non-*faecium*]. Some authors have also reported *E. casseliflavus*\[23,66,100,112\] and *E. mundtii*\[112\] but we did not come across these species. Vittal Prakash\[61\] and Ghosh R\[183\] have reported that prevalence of unusual species of enterococci is 19% in south India 24% in eastern India respectively, which is more than the present study. Vittal Prakash *et al.* and Desai P J *et al.*
have done studies on nosocomial infection and they recovered more isolates from burn wounds, surgical wounds, foley’s catheters and umbilical stump. Vittal Prakash used sodium dodecyl sulfate-polyacrylamide gel electrophoresis for species identification for all isolates.

*E. faecalis* constituted the predominant isolates. *E. faecium* was the commonest blood culture isolate while *E. faecalis* predominated among pus and urine samples. Other rare species *E. mundtii, E. dispar, E. durans, E. avium, E. raffinosus* and *E. gallinarum* were also reported. In the study conducted in New Delhi, *E. faecium* (66%) was the commonest isolate followed by *E. faecalis* (20%) in blood specimen. While in another study from Chandigarh, *E. faecalis* (55%) followed by *E. casselilavus* (24%) and *E. faecium* (12%) were reported from urinary isolates. In the present study, *E. faecalis* was the major species isolated followed by *E. faecium* from various clinical conditions such as UTI, wound infection, neonatal sepsis and septicaemia. *E. faecium* was more common species among peritonitis patients. While non-*E. faecalis* non-*E. faecium* were common in wound infection [Table No. 8].

*E. faecalis* and *faecium* are known to be significantly associated with clinical disease hence their isolation is of serious concern. Other species such as *E. avium, E. hirae, E. raffinosus, E. gallinarum* and *E. casselilavus*, though have been isolated from clinical specimens, their clinical significance is doubtful. *E. gallinarum* and *E. casselilavus* have been isolated from patients who were either chronically ill or immunosuppressed. The majority of cases of bacteraemia due to these organisms involved patients with underlying conditions such as renal failure, diabetes mellitus, hematologic malignancy, recipients of solid organ and bone marrow transplants, antithrombin III deficiency, astrocytoma and chronic osteomyelitis. Cases of vascular and IV line related infections have also been described. Bacteraemia due to *E. gallinarum* and *E. casselilavus/flavescens* are frequently polymicrobial. Now days, various new sophisticated identification tools (e.g. Vitek) are available to identify such unusual species.

Identification & classification of enterococci to species level is often useful for proper patient treatment, epidemiological study and infection control purpose. Isolates of *E. faecium* tend to be more resistant to penicillin and ampicillin than *E. faecalis* isolates and vast majority of vancomycin resistant enterococci (VRE) are strains of *E. faecium*. 
ANTIBIOTIC SUSCEPTIBILITY PATTERN

Enterococci are normal commensals and are of less intrinsic virulence, but they have adequate intrinsic and acquired resistance to many antibiotics. Enterococci have a striking capability to survive in an environment of heavy antibiotics. The enterococci intrinsically resistant to semi-synthetic penicillinase resistant penicillin, cephalosporin, low level of clindamycin, trimethoprim-sulphamethoxazole and fluoroquinolones and acquired resistance to chloramphenicol, erythromycin, tetracycline, fluoroquinolone and high level of penicillin, clindamycin, aminoglycosides and vancomycin. Antibiotic resistant enterococci are being reported with increasing frequency in United States and other parts of the world. Careful review of invitro susceptibility data is required to treat infections caused by multi-drug resistant E. faecium.

β-Lactam Antibiotics (Penicillins)

In the present study, 52% of enterococcal strains were resistant to penicillin and 51% were resistant to ampicillin [Table No. 9]. This resistance correlated well with the studies conducted by Bhat et al. and Parvathi et al. In the study conducted by Parvathi et al. 38% strains were resistant to ampicillin and 43% to penicillin. While, Miskeen PA et al. reported 23.13% strains were resistance to penicillin and ampicillin. Edo EU et al., have reported 12% of the isolates were resistant to penicillin. Jesudasan et al. have that 10.20% resistance to penicillin; these were less than present study.

In the present study, more than 88% of E. faecium were resistant to penicillin, imipenem, ampicillin, piperacillin, amoxyccillin and combination with penicillin inhibitors like clavulanate, sulbactum and tazobactum while only to 30-43% of E. faecalis showed resistance to same drugs [Table No. 9]. These differences were statistically extremely significant (P<0.0001). Bhat et al. reported 50% of E. faecalis were resistant to ampicillin and 50.8% to penicillin. While, 86.7% strains of E. faecium were resistant to ampicillin & 88.4% to penicillin. All E. faecalis isolates (including β-lactamase producers) were susceptible to imipenem, in contrast to E. faecium isolates. These findings resemble to those in the present study.

Species identification of isolates enables us to assess antimicrobial resistance characteristics. Penicillin resistance was also reported among non- E. faecalis & non- E. faecium species. In the present study, non- E. faecalis and non-E. faecium were significantly more resistant to the penicillins, ampicillin, ampicillin-sulbactum, piperacillin and piperacillin
tazobactum than *E. faecalis*. Among these amoxyclyv and piperacillin-tazobactum are the common drugs useful for treatment.
In the present study, there was no significant difference between penicillin and ampicillin resistance. Amoxicillin (69%), amoxycillin with clavulanic acid (69%), ampicillin with sulbactum (69%), piperacillin (66%), piperacillin with tazobactum (70%) and imipenem (70%) were significantly more active than penicillin (57%) and ampicillin (58%) alone against *E. faecalis* \( P<0.0001 \) and other *E. species*. [Table No. 9]. \( \beta \)-lactamase inhibitor combinations ampicillin-sulbactum, amoxyclav were more effective in 98.28% and 91.84% respectively.\[16\] The activity of ampicillin combined with sulbactum is slightly better when compared with that of ampicillin alone against the clinical isolates of *E. faecalis*,\[27\] but in the present study such difference was not noted against *E. faecium*. The reason might be that all isolates of *E. faecium* were \( \beta \)-lactamase non producer & highly (>88%) resistant to all \( \beta \)-lactam antibiotics. CLSI\[130\] designates penicillin and ampicillin as comparable agents that need not be duplicated in antibiotic sensitivity testing. We also recommend that any one among penicillin or ampicillin, second among amoxycillin, piperacillin or imipenem and third from amoxycillin with clavulanic acid, ampicillin with sulbactum or piperacillin with tazobactum can be included for testing.

*E. faecalis* can be inhibited by concentrations of penicillin achievable in plasma (MIC of 1 to 8 \( \mu \)g/ml) but this is usually not the case in *E. faecium*.\[32\] This is true in our study also, *E. faecium* was significantly more resistant than *E. faecalis* \( P<0.0001 \) as well as other species of enterococci \( P<0.01 \) to penicillin by agar dilution method. Four out of 42 *E. faecium* isolates (9.5%) verses 110 of 188 *E. faecalis* isolates (58.5%) were inhibited by penicillin at MIC ranges \( \leq 16 \mu \)g/ml and were susceptible to it. While it drastically increased (>MIC 32 \( \mu \)g/ml) in case of 38 of 42 *E. faecium* isolates (90.5%) versus 78 of 188 *E. faecalis* isolates (41.5%) were inhibited by penicillin at MIC ranges at 64, 128 \( \mu \)g/ml or above 128 \( \mu \)g/ml (or not) and were resistant to it. While 43% strains of non-faecalis non-faecium species enterococci were inhibited at MIC ranges of \( \leq 16 \mu \)g/ml and were susceptible and 57% were inhibited at an MIC range of \( \geq 32 \mu \)g/ml and were resistant [Table No.12]. In other studies over all more than 50% strains tested were inhibited at an MIC range \( \geq 32 \mu \)g/ml and were resistant. Exactly same observation was reported by other workers.\[166,176\]

For ampicillin MICs of \( >100 \mu \)g/ml are now common, and this concentration is close to the upper limit of concentrations achievable in the serum. Grayson *et al.* have noted that penicillin MICs for *E. faecium* is often 1 to 2 dilutions higher than those of ampicillin.\[124\] Penicillin resistance has increased in the recent years. As it is the mainstay of therapy for enterococcal infections, development of high level resistance to penicillin would have important
clinical implications. Penicillin and ampicillin resistance are usually intrinsic, primarily due
to low affinity of the penicillin binding proteins (PBPs) and it results in loss of synergistic effect between β-lactams and aminoglycosides leading to treatment failures. E. faecalis, expressing β-lactamase enzyme and High Level Resistance to Penicillin (HLPR) and ampicillin (MIC ≥ 256 µg/ml) have been reported from various locations.

Table C. Antibiotic Resistance Pattern

<table>
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<tr>
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<tr>
<td>Penicillin</td>
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<td>43</td>
<td>--</td>
<td>44</td>
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<td>38</td>
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<td>--</td>
<td>--</td>
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<tr>
<td>Amoxyclav</td>
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<td>8</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>40.57</td>
</tr>
<tr>
<td>Erythromycin</td>
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<td>--</td>
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<td>--</td>
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<td>82.4</td>
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<tr>
<td>Tetracycline</td>
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<td>56</td>
<td>33</td>
<td>40</td>
<td>62</td>
<td>84.8</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>--</td>
<td>0.78</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>6.56</td>
</tr>
<tr>
<td>Vancomycin</td>
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<td>0.00</td>
<td>0.05</td>
<td>2.6</td>
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<td>1</td>
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<tr>
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<td>--</td>
<td>2.6</td>
<td>0.00</td>
<td>1.23</td>
</tr>
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<td>Gentamicin*</td>
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<td>37</td>
<td>--</td>
<td>13.9</td>
<td>53</td>
<td>54.5</td>
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<tr>
<td>Streptomycin*</td>
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<td>--</td>
<td>20</td>
<td>40</td>
<td>38.5</td>
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<tr>
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<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.00</td>
<td>1.23</td>
</tr>
</tbody>
</table>

*High Level Aminoglycoside Resistance

β-Lactamase Production

Enterococci have acquired β-lactamase (gene) from staphylococci.[16,176] It is usually plasmid mediated and cannot be detected by routine disc diffusion or dilution method.[30,100] In the present study, two strains showed production of β-lactamase enzyme by iodometric and nitrocefin method [table No. 13] and showed MIC for penicillin ≥128 µg/ml. These two strains were resistant to all the penicillin group of drugs as well as HLAR. They were susceptible to ampicillin-sulbactum, amoxycillin-clavulanic acid and piperacillin-tazobactum. These observations are similar to that reported by Gordon S et al.[66] While Jesudasan MV et al.[16] have not reported β-lactamase production by enterococci by both iodometric and nitrocefin
method. Parvathi S et al. have reported 32% and 34% enterococcal isolates as positive by the
acidometric and clover leaf technique respectively (biological method) which is much higher than other studies (including present study). Both these methods are easy to perform but acidometric is less practical, since pH and buffer controls must be included and large amount of penicillin G is required. The clover leaf method is comparatively cost effective.[155] Unfortunately, we have not used any these method. However, when the organism is β-lactamase positive, it should be considered resistant to all beta-lactam drugs.[124]

The incidence of β-lactamase production [Table No. 13] is less (1.5%) among penicillin resistant enterococci in the present study. The screening of enterococcal isolates for β-lactamase production should be practiced more routinely in clinical laboratories by using nitrocefin based test, particularly those isolates with high level aminoglycoside resistance that are recovered from normally sterile body sites. β-Lactamase producing enterococci have been reported from at least nine cities in the United States, including an outbreak of β-lactamase producing Enterococcus faecalis in infants and toddler surgical ward in Boston, Mass.[47,66]

The CLSI recommends that all isolates of enterococci from blood and cerebrospinal fluid must be tested for β-lactamase production by using nitrocefin method and by using inoculum of $10^7$ CFU/ml. Recently, Handwerger et al. described a β-lactamase-producing (by bioassy), vancomycin-resistant *E. faecalis* isolate that did not give a positive result by nitrocefin test. Although the nitrocefin test may not be 100% sensitive, it still remains the most effective method for screening for β-lactamase-producing enterococci in the clinical laboratory and the only method recommended by the CLSI.[124]

Most of the Vitek users mention, enterococci were resistant to penicillin but moderately susceptible to ampicillin, since the β-lactamase is effective in hydrolyzing both the drugs. While some β-lactamase negative enterococci show differential resistance to penicillin and ampicillin, for which the reference MIC testing indicated penicillin resistance [MIC= 32 µg/ml] but moderate susceptibility to ampicillin [MIC = 4 µg/ml], the clinical significance of this is not clear.[90] Similar differential susceptibility patterns have been noted by other workers.[15,181]

**Other Antibiotics**

In the present study, enterococci were more resistant to ciprofloxacin (85%), gatifloxacin (66%), azithromycin (84%), erythromycin (82%), and tetracycline (75%) than doxycycline (48%) and rifampicin (34%) [Table No. 10]. Our resistance patterns resembled to the resistance patterns of study conducted by Mendritta DK *et al.*[30] but our isolates showed more resistance to these antibiotics than other workers.[176,181] Parvathi *et al.*[15] reported 33% of the strains resistant...
to ciprofloxacin, 41% strains to tetracycline. In a study by Udo EE et al.\textsuperscript{154} among the 415 isolates, 63, 60 and 40% were resistant to erythromycin, tetracycline and chloramphenicol, respectively.

\textit{E. faecium} (>90%) was more resistant to azithromycin, erythromycin, ciprofloxacin and gatifloxacin than \textit{E. faecalis} (<84%), but there is no statistical significant difference. \textit{E. faecium} (33 and 71%) was significantly more susceptible than \textit{E. faecalis} (23 and 46%) to tetracycline and doxycycline (P<0.005) respectively in the present study [Chart No. 3]. Bhat KG et al.\textsuperscript{181} have reported that 19.9% of \textit{E. faecalis} were resistant to ciprofloxacin, 29.8% to tetracycline. In the same study 66.7% of \textit{E. faecium} were resistant to ciprofloxacin, 68.9% to tetracycline. Gordon \textit{et al.}\textsuperscript{66} also have noted that \textit{E. faecium} was found to be more resistant to commonly used antibiotics as compared to \textit{E. faecalis}.

**Quinolone**

Ciprofloxacin, considered the drug of choice for empirical treatment of UTI, was effective (earlier) in 55.78% of strains tested.\textsuperscript{176} In the present study, majority (88.5%) of the isolates were resistant to ciprofloxacin with MICs of $\geq 4$ $\mu$g/ml [Table No. 14]. There was no significant difference between \textit{E. faecalis} and \textit{E. faecium} for susceptibility to ciprofloxacin in the present study. Ciprofloxacin was less effective than penicillin alone or in combination with gentamicin.\textsuperscript{66} In the present study too, penicillin (48%) was more effective than ciprofloxacin (15%). This observation resembles to the observation made by Miskeen PA \textit{et al.}\textsuperscript{176} and Udo EE \textit{et al.}\textsuperscript{154} Therefore, ciprofloxacin may not be the best alternative drug for the treatment of serious enterococcal infections.

Ciprofloxacin and other quinolones like ofloxacin, norfloxacin and enoxacin introduced in the same time have only modest activity against enterococci. Bactericidal effect of quinolones is inoculum dependent and may be seen only at higher concentrations which are unachievable systemically in clinical use. Effectively, their use is limited to the treatment of urinary tract infections. Among these new fluoroquinolones clinafloxacin is the most active agent against enterococci.\textsuperscript{27}

**Tetracycline**

In the present study, \textit{E. faecium} was significantly more susceptible than \textit{E. faecalis} to tetracycline and doxycycline (P<0.005) by disc diffusion method. The \textit{E. faecalis} (78%) strains were more resistant to tetracycline (MIC $\geq 16$ $\mu$g/ml) than \textit{E. faecium} (74%) by agar dilution method [Table No. 11]. Tetracycline was active against some vancomycin resistance
enterococcal isolates. The higher rate of tetracycline resistance, especially among clinically important Gram-positive cocci, has limited its effectiveness. Doxycycline and minocycline have been used in the treatment of VRE infections, often with other agents successfully, however it is difficult to assess their overall effectiveness. Other investigational agents with in vitro activity against VRE include glycyclyclines. Tetracycline derivatives known as glycyclyclines have excellent activity against enterococci including multi-drug resistant strains.\[^{27}\] Doxycycline has the inhibitory activity of quinupristin-dalfopristin (when given in combination) against a number of \textit{E. faecium} isolates.\[^{28}\]

**Erythromycin**

Erythromycin (82\%) and azithromycin (84\%) were the most non-effective drug against enterococci, in the present study. This resistance pattern is higher than the study by Udo EE \textit{et al.}\[^{154}\] who have reported 63\% enterococcal isolates resistant to erythromycin.

**Rifampicin**

\textit{E. faecium} (71\%) was significantly more resistant than \textit{E. faecalis} (26\%) to rifampicin (P<0.0001) in the present study. Rifampicin alone has very limited usefulness in the treatment of enterococcal infections because of its poor bactericidal activity, presence and emergence of subpopulations of resistant bacteria, both in-vitro and in-vivo. Since rifampicin remains active against many strains of multi-resistant enterococci, it is often tested in combination with other agents.\[^{27}\]

**Nitrofurantoin, Fosfomycin, Vancomycin and Linezolid**

The present study revealed that all species of enterococci were more susceptible (more than 93\%) to nitrofurantoin, fosfomycin, vancomycin and linezolid [Table No. 10]. Among the 244 enterococcal isolates 99.58\% were sensitive to teicoplanin, 99.18\% to vancomycin, 98.77\% to linezolid, 96.72\% to fosfomycin and 93.44\% to nitrofurantoin by Kirby Bauer disc diffusion method. Compared to all other antibiotics tested, these four antibiotics were more effective, and the difference is statistically significant [P<0.005]. \textit{E. faecalis} was less resistant to these antibiotics than \textit{E. faecium}. Latika \textit{et al.}\[^{23}\] and Baldir G \textit{et al.}\[^{184}\] also reported less than 2\% enterococcal strains were resistant to linezolid.
Nitrofurantoin and fosfomycin have activity against some isolates including VRE especially from cystitis and other UTI. Miskeen PA et al. also showed that nitrofurantoin resulted in complete inhibition of growth in 99.22% of the isolates tested. Same observation was found in the present study. Even though, fosfomycin has activity against enterococci, but rapid emergence of resistance, limits its usefulness as a single agent.

Glycopeptide

The most recent and disturbing resistance reported among enterococci is vancomycin resistance. It has been increasingly reported from all parts of the world. There are various phenotypes of glycopeptide resistance in enterococci: Van A phenotype- inducible, high level resistance to both vancomycin (MIC \( \geq 64 \) to \( >1,000 \) \( \mu g/ml \)) and teicoplanin (MIC \( \geq 16 \) to 512 \( \mu g/ml \)), Van B- inducible, variable resistance to only vancomycin (MIC \( \geq 4 \) to 1,024\( \mu g/ml \)), Van C- intrinsic, constitutive low level resistant to only vancomycin (2 to 32 \( \mu g/ml \)), Van D- inducible, moderate resistance to vancomycin (MIC 64 to 128 \( \mu g/ml \)) and teicoplanin (MIC 4 to 32 \( \mu g/ml \)), Van E- \textit{E. faecalis} low level resistance to vancomycin (MIC 16 \( \mu g/ml \)) and Van G- \textit{E. faecium} low level resistance to vancomycin (MIC 16 \( \mu g/ml \)). Except Van A and Van D (some time) phenotype, all phenotypes are sensitive to teicoplanin (MIC \( \leq 0.5 \) \( \mu g/ml \)). Van A Phenotype is more widely distributed and thus the predominant type of resistance reported. Additionally, vancomycin resistance occur more commonly in \textit{E. faecium}, which is inherently more resistant to multiple drugs, making therapy extremely problematic. In the present study, both the VRE (1\%) isolates showed high level resistance to vancomycin as well as teicoplanin and belonged to Van A phenotype, of these one belonged to \textit{E. faecium} and another to \textit{E. durans}. Even though, seven (3\%) isolates of enterococci belonged to Van B phenotype, they were sensitive to both vancomycin and teicoplanin [Table No. 15].

In the present study, all the isolates (100\%) of \textit{E. faecalis} were sensitive to vancomycin and teicoplanin while up to 87.62\% isolates of \textit{E. faecium} were sensitive to vancomycin and teicoplanin. This exactly correlates with the study conducted by Bhat KG et al. The majority of VRE are encountered in \textit{E. faecium}. Resistance to vancomycin has also been reported among \textit{E. gallinarum} & \textit{E. faecalis}. Emergence of vancomycin resistance wave seen reported in few more studies. Miskeen PA et al. (100\%), Gordon et al. and Udo EE et al. (99.6\%) reported that many isolates were susceptible to both vancomycin and teicoplanin.
and all the organisms were inhibited at concentration ranging from 0.5 µg/ml to 4 µg/ml.
In the present study, both the VRE isolates were also resistant to penicillins alone, combination of penicillin with penicillin inhibitors and gentamicin as well as vancomycin and aminoglycoside. These VRE isolates were also resistant to azithromycin, erythromycin and ciprofloxacin but susceptible to linezolid, nitrofurantoin and fosfomycin. Vancomycin resistant *E. faecium* [VREF] was susceptible to tetracycline & doxycycline and intermediately susceptible to gatifloxacin but vancomycin resistant *E. durans* remained resistant to all these three drugs. Udo EE *et al.*\textsuperscript{[154]} also have reported that VRE isolates expressed low MIC value but they were resistant to ampicillin, erythromycin, ciprofloxacin and high-level streptomycin & gentamicin.

**Methods for Detection:**

There are several methods available to study the vancomycin resistance like disk diffusion, agar screen, E- Test, WalkAway, MicroScan, Vitek and PCR.\textsuperscript{[28,33,123,124]} All these methods are able to detect the high level vancomycin resistance (*vanA*). The prevalence of *vanB* strains may be underestimated in many hospitals; previous studies noted that the MicroScan (rapid) and Vitek (GSP-TA) systems were unable to detect the low level vancomycin resistance (*vanB*).\textsuperscript{[33,123]} In addition, there is also problem in detecting intrinsic vancomycin resistance in *E. gallinarum* (*vanC1/C2*) with these automated methods. Vitek results are highly consistent than the WalkAway and MicroScan panels and readers.\textsuperscript{[124]} The new Vitek 2 and 45-well Vitek GPS-101 shows improved sensitivity compared to the Vitek GPS-TA, without significant loss of specificity.\textsuperscript{[123]} In the present study, two isolates were resistant to vancomycin and teicoplanin by Vitek 2 automated method.

The agar screen method (6 µg/ml) appears to be the most reliable and easy method for routine screening in the detection of *vanA*, *vanB*.\textsuperscript{[123]} But it continues to show problems in the detection of *vanC1/C2* VRE. The agar screen method a simple and sensitive test for detecting vancomycin resistance and are currently being evaluated and recommended by the CLSI working group.\textsuperscript{[28,124]} Resistance at moderate and higher concentrations of vancomycin is easily detected by standard susceptibility testing procedures. Modifications have improved the detection of low-level vancomycin resistance (Van B, van C and other). The E-test method and PCR\textsuperscript{[28]} are an accurate, alternative for the confirmation of vancomycin resistance. In the present study, we used 1 µg/ml (for only *vanC1/C2*), 2 & 4 µg/ml concentrations for screening VRE and 4 & 8 µg/ml concentrations in agar dilution for screening TRE. Then we confirmed by agar dilution, Hi-combi and Vitek 2 methods. This helped to avoid the missing of VRE isolates. Hiramatsu *et al.*\textsuperscript{[129]} suggested using BHI agar with 4 µg/ml vancomycin. However, this method
is probably an inappropriate means of screening for VRE owing to high number of false positives as well as it fails to detect some strains of VanC.

In the present study, 100% *E. faecalis* strains were susceptible to vancomycin as well as teicoplanin. One isolate from *E. faecium* was intermediately susceptible to teicoplanin by disc diffusion method but the same isolate was resistant to teicoplanin by agar dilution, Hi-Combi and Vitek 2 techniques. Another single strain from *E. durans* was resistant to vancomycin and teicoplanin by all above mentioned methods. We missed one teicoplanin resistant isolate by disc diffusion method, which was detected by other (dilution) methods. Agar dilution, Hi-Combi and Vitek 2 techniques showed comparable result with each other.

By using the CLSI breakpoints, 1.9% strains were categorized as susceptible by disk diffusion and resistant by MIC (very major error); 9.6% strains were categorized as susceptible by disk diffusion and intermediate by MIC, and 1.9% strains were categorized as intermediate by disk diffusion and resistant by MIC (minor errors). In the present study, 24 of 244 (9.84%) enterococcal isolates were categorised intermediately susceptible to vancomycin by disc diffusion but susceptible by dilution (MIC ≤ 4) method (minor error). Seven of 244 (2.87%) enterococcal isolates were categorized intermediately susceptible to teicoplanin by disc diffusion but susceptible by dilution (MIC ≤ 8) method (minor error). Single (0.41%) strain was categorized as intermediately susceptible to teicoplanin by disk diffusion but was categorized resistant by MIC (minor error) [Table No. 16].

Miskeen PA *et al.* also analyzed the result of in vitro inhibition experiment with glycopeptide antibiotics and reported the fact that a great degree of discrepancy appeared in the disc diffusion technique. The disc diffusion experiments indicated the presence of false resistance among enterococcal strains [50 out of 147 cases]. All these 50 strains were however susceptible to glycopeptides when tested by E-test and agar dilution methods, and showed MIC values <4 µg/ml. Several recent reports do not recommend the disc diffusion method for detecting susceptibility for enterococci to at least glycopeptide antibiotics which is not satisfactory method for reporting (at least VRE). Similarly, 24 to 48 hours incubation and the use of strong transmitted light to read the plates have improved the accuracy of the disk diffusion method.
Clinical significance of VRE

VRE isolates should be tested for antibiotic susceptibility to all potentially active and commercially available drugs. **Vancomycin in combination** with an aminoglycoside has demonstrated synergistic activity against enterococci both in vitro and in vivo, and it is recommended as the drug of choice in patients with serious, penicillin allergy or in the treatment of ampicillin- and penicillin-resistant bacteria. However, enterococci are becoming progressively more resistant to traditional antibiotic therapy. In addition to high-level aminoglycoside resistance and rapid spread of vancomycin resistance have resulted in limited therapeutic alternatives. Treatment of VRE infections, particularly *E. faecium*, is tremendously challenging, because these organisms are resistant to many antibiotics. Penicillin or ampicillin with or without a synergizing aminoglycoside would be a rational option in the nonallergic patient infected with vancomycin resistant *E. faecalis*.\(^{27}\)

Clinically, vancomycin resistance is associated with more **frequent episodes** of recurrent bacteraemia and other infections, persistent isolation from primary sites of infections, increased frequency of endovascular infection and mortality.\(^{32}\) Since beginning, the prevalence of VRE has increased from 0.3% to 47% in United States\(^{152}\) and European countries.\(^{27}\) The use of avoparcin (glycopeptide) as growth promoter in animal feed seems to be the major contributor to vancomycin resistance in European countries. In contrast, US has not permitted avoparcin in animal feed but the use of vancomycin in treating human infections has increased more than 100 fold in last 40 years.\(^{27,117}\) While in India neither avoparcin was / is used as feed supplement in animal nor vancomycin is used much in clinical practice (data not available). Therefore, studies from India report a meagre percentage of vancomycin resistance,\(^{5,15,23,176,181}\) as also seen in our hospital. But monitoring is needed since it’s an emerging (VRE) pathogen in India,\(^{30}\) not today but in future it may create significant problem in clinical practice.

The **difficulty in treating** VRE when they are recovered from infected sites and the fact that enterococci are not highly virulent, the question sometimes arises: Do we really need to treat the patient with antibiotics active against VRE? Certainly yes, infective endocarditis, urinary tract infections, and any infection of a sterile space with VRE should be treated aggressively. Patients with VRE infective endocarditis may be benefited from early valve removal. VRE bacteraemia related to intra venous catheters may resolve spontaneously after removal of the catheter. However, treatment with the best available drug for any VRE bacteraemia is probably warranted and highly recommended for patients with prosthetic or abnormal heart valves in an
attempt to prevent endocarditis. Linezolid and quinupristin-dalfopristin are most active (the latter against *E. faecium* only), but tetracycline may also be considered for therapy.[28]

**Risk factors**

There are various risk factors associated with acquisition of vancomycin resistant enterococci include preceding vancomycin or cephalosporin therapy, exposure to a VRE carrier (nurse) and proximity to a patient with a known infection. Enterococci have an ability to **transfer** the *vanA* and *vanB* gene to self- transferable (with in genus to other species)[33] as well as other Gram positive organisms like staphylococci,[186] streptococci and listeria.[117] The transfer of vancomycin resistance may occur at the time of plasmid transfer or without acquisition of plasmid DNA suggests that transfer of vancomycin resistance may occurs via conjugative transposons. The genetic transfer may occur among organism in hospital environment. The rapid spread of VRE organisms occurring among hospitalized patients suggest that enterococci with vancomycin resistance may emerge rapidly as nosocomial pathogens.[33]

**Vancomycin Dependent Enterococci (VDE)**

VDE arises following prolonged therapy with vancomycin. The strains have previously been isolated from stool, urine, blood and peritoneal fluid.[50,162] Lisa Dever[160] reported that initial VREF, stool isolate from patient had the same biochemical reactions and antibiotic susceptibility patterns as VDEF. Nosocomial infections and outbreaks have been reported from bone marrow transplant units and such strains can be typed by using pulsed field gel electrophoresis.[50] DNA sequencing has detected DDL gene mutation or deletion (*D-Ala*;*D-Ala ligase* or 18 pb deletion of *vanSB gene*) among the VDE strain.[50,161] The growth of these fastidious organism can be supported by vancomycin, ristocetin and *D-Ala; D-Ala*, not by teicoplanin. Spontaneous reverent away from vancomycin dependence can occur[161] at frequency of 1 in 10⁶. Awareness of the existence of these strains is important, especially in the context of long term vancomycin therapy and suspected presence of “nutritionally deficient” organism.[162]

**Prevention and control**

The rapid increase in the incidence of infections with VRE in the western hemisphere is of great concern. The Hospital Infection Control Practices Advisory Committee (HICPAC) recently published recommendations for preventing the spread of vancomycin resistance.[27,28] There is an important and sought role of microbiology laboratories, as they through accurate and timely detection of resistance, are the first line of defence. Occurrence of VRE is increasing in
the United States, Europe\textsuperscript{131} and now it is also occurring in India. It is essential to optimize the laboratories capacity to identify vancomycin resistance. There were dramatic increase in vancomycin resistance in enterococci, CDC,s HICPAC had published recommendations (February 1995) to control the nosocomial transmission of VRE.\textsuperscript{28,32}

**These recommendations mainly focused on**

i. Prudent use of vancomycin,

The use of vancomycin is appropriate or acceptable conditions are as follows

a. For treatment of serious infections due to metronidazole and β-lactam resistant Gram positive microorganisms or patients with penicillin allergy.

b. Prophylaxis of major surgical procedures, e.g. cardiac or total hip replacement at institutions with a high rate of infections due to MRSA.

The use of vancomycin should be discouraged in the following situation:

a. Prophylaxis- in Routine surgical procedure, low birth weight infants, during dialysis.

b. Empirical antimicrobial therapy including antibiotic associated colitis or renal failure.

c. Selective decontamination of the digestive tract.

d. Eradication of MRSA colonization.

e. Application of topical solution or irrigation.

ii. Education of hospital staff,

Continuing educational programs for hospital staff (including attending and consulting physicians, medical residents, students, pharmacy personnel, nurses, laboratory personnel, and other direct patient caregivers) should include information about the epidemiology of VRE and the potential impact of this pathogen on the cost and outcome of patient care. Since detection of VRE require a very powerful approach and high performance standards for hospital personnel, special awareness and educational sessions should be implicated.

iii. Effective use of the microbiology laboratory

Clinical isolates should be screened for vancomycin resistance. Agar screening method (6 \( \mu g/ml \) of vancomycin in brain-heart infusion agar) is a simple, sensitive test for vancomycin resistance and recommended by CLSI.

iv. Implementation of infection control measures.

It includes the use of gloves and gowns and isolation or cohorting of patients, as appropriate to specific conditions.
Aim of this recommendation is to minimize nosocomial transmission of VRE; hospitals must use a multidisciplinary approach that requires participation by a variety of departments and personnel.

**Newer Drugs**

**Daptomycin**

With the limited therapeutic options, VRE and VRSA infections can be treated with totally newer antibiotic modification of vancomycin or teicoplanin. Daptomycin, an acidic lipopeptide, gave promising result in vitro.[27] It could be an efficient and appropriate alternative drug especially in the treatment of skin and soft tissue[28] as well as blood stream infections.[187]

**Quinupristin-Dalfopristin**

Quinupristin-dalfopristin (pristinomycin) is a streptogramin antibiotic that has been studied in the treatment of infections due to vancomycin-resistant E. faecium. It has bacteriostatic activity against E. faecium but is inactive against E. faecalis.[28] It was the first antibiotic approved for the treatment of patients with serious or life-threatening infections associated with VREF bacteraemia.[27] Unfortunately, in the present study, only 21% enterococcal isolates were sensitive to dalfopristin-quinupristin, and E. faecalis (24%) was more sensitive than E. faecium (12%) to the same [Table No.10].

**Oxazolidinone**

The oxazolidinone (group of antibiotics) are newer synthetic antibiotics with good anti-enterococcal activity and are different from any other class. They were approved by the US and Europe food and drug administration in April 2000 for the treatment of infection caused by VREF including bacteraemia. Other members of this class are in different stages of preclinical development at many companies. These antibiotics are bacteriostatic against staphylococci and enterococci, and they act by inhibiting bacterial protein synthesis. They bind to the 23S rRNA of the 50S subunit on the bacterial ribosome.[27,151,152]

**Linezolid**

Linezolid and eperzolid are two oxazolidinone which showed excellent activity against multi drug resistance enterococci. Linezolid is a more versatile drug. Its antibacterial activity is not less than quinupristin-dalfopristin. It is active against both E. faecium as well as E. faecalis.
It can be given orally, which does not only increases the patient comfort but also decrease the costs and risks of intravenous therapy. In the present study, 99% enterococci were sensitive to
linezolid [Table No. 10]. Same susceptibility pattern was reported in study conducted by Rahangdale et al.\textsuperscript{[153]} who reported 100% sensitivity to linezolid. Even though it is highly active drug, it should not be used indiscriminately. It has also some potentially important drug-drug interaction, therefore careful review of the patients in consultation with a pharmacist, is recommended before prescribing.\textsuperscript{[15]}

Linezolid exhibits limited in-vitro bactericidal activity against enterococci. Resistance has been seen among \textit{S. aureus} and enterococci. The enterococci have been demonstrated to spread linezolid resistance in hospital environment. This favours the emergence of linezolid-resistance.\textsuperscript{[127,28,151]} The emergence of linezolid resistance (LR) occurred infrequently among VREF isolates recovered during compassionate-use program (1.8% isolates) in Chicago. The most important risk factors identified were receipt of multiple antibiotics and a protracted course of linezolid therapy. Duration of therapy is also the most important risk factor for bone marrow suppression due to linezolid use.\textsuperscript{[152]} Vancomycin and teicoplanin are reserved drugs. Fosfomycin and nitrofurantoin can be recommended only for urinary tract infection while linezolid can be used for other infections.\textsuperscript{[27,28]}

\section*{Old Drugs}

Novobiocin and bacitracin are two “old” drugs that have been used against enterococci. Both have been used in attempts to eliminate stool carriage of VRE with equivocal success and the former was combined with doxycycline to successfully treat VRE bacteraemia in a handful of patients.\textsuperscript{[28]}

\section*{Antibiotic Combinations (Synergism)}

The enterococcal infection in non-immunocompromised host can be treated with a single antibiotic. Penicillin or ampicillin remains the drug of choice. Glycopeptide and linezolid are the alternative agents. However, primary failure or relapse occurs in case of mono-therapy, as it produces bacteriostatic rather than bactericidal effect. Currently, there is no ideal therapy which yields bactericidal activity for serious infection.

There are a number of new approaches of combination therapy to enterococcal infections, including \(\beta\)-lactam-\(\beta\)-lactam, \(\beta\)-lactam-glycopeptide and \(\beta\)-lactam-fluoroquinolone combinations explored in experimental animal models. Each approach has limitations.\textsuperscript{[28]} Combinations involving double \(\beta\)-lactams have been examined, and the combination of ampicillin and
imipenem appeared to have a positive interaction in an experimental model of endocarditis in
rabbits. The combination of a glycopeptide and a β-lactam is an interesting one, whose use is derived from the observation that some strains of *E. faecium*, although, resistant to ampicillin and vancomycin, are still inhibited by the combination of these two drugs. For such strains, the MIC of ampicillin decreases in presence of vancomycin. In the treatment of experimental penicillin and glycopeptide-resistant *E. faecium* endocarditis combination of ceftriaxone-vancomycin and gentamicin was reported to be appreciably more efficient than either penicillin-vancomycin-gentamicin or penicillin-teicoplanin-gentamicin.\[27\]

The synergy of ciprofloxacin plus gentamicin or rifampicin or both was seen in vitro and in vivo against strains of VREF even though organisms were resistant to each antibiotic. Triple therapy (ciprofloxacin-rifampicin- gentamicin) was most effective for sterilizing vegetations. The combination of ampicillin with clinafloxacin also had bactericidal activity against similar strains when the drugs were present in serum at concentrations that were easily attainable.\[27\]

**High Level Aminoglycoside Resistance [HLAR]**

Enterococci exhibit intrinsically low level resistance to all aminoglycosides which is due to low uptake of these agents. Penicillin or other agents (like cycloserine, bacitracin, and vancomycin) inhibit bacterial cell wall synthesis (L forms) and thus overcome the permeability barrier and increase uptake (facilitation) of aminoglycosides (streptomycin) and kill the bacteria.\[32\] The increased uptake of streptomycin in the presence of penicillin occurred only during actively dividing cells. The high level streptomycin resistance ("nonsynergistic") in enterococcal strain is interesting. The failure of synergism in this strain was not due to failure of increased streptomycin uptake, but rather inability of the streptomycin to act once it entered the cell. This explains the failure of synergism of penicillin and streptomycin against enterococci which exhibit very high level resistance to streptomycin.\[165\]

Aminoglycoside modifying enzymes (AME) are responsible for HLAR coded on plasmid and are transferable. a) Streptomycin resistance is mediated by ribosomal mutation or enzymatic (6′ adenylytransferase) inactivation of only streptomycin, can be used to screen for streptomycin-penicillin synergy. b) The dual bifunctional enzyme (i.e., 2′-phosphotransferase- 6′-acetyltransferase) that mediates high-level gentamicin resistance also mediates tobramycin, netilmicin, amikacin and kanamycin resistance (except streptomycin). A gentamicin screen alone currently is sufficient to reliably predict synergy between penicillin and gentamicin, tobramycin, netilmicin, amikacin and kanamycin. c) In addition, this enzyme also mediates
resistance to kanamycin and amikacin so that gentamicin resistant *E. faecalis* isolates are also resistant to
these two aminoglycosides. Some *E. faecalis* isolates are susceptible to gentamicin (*i.e.*, lack 2\(^{-}\)-phosphotransferase-6'-acetyltransferase), but are resistant to kanamycin and amikacin. *These isolates produce 3'-phosphotransferase*, an enzyme that inactivates **kanamycin and amikacin but not gentamicin**. Therefore, although all gentamicin resistant isolates are also resistant to kanamycin and amikacin, not all gentamicin susceptible isolates are susceptible to kanamycin and amikacin. The gentamicin susceptible strains whether susceptible to kanamycin and amikacin can be determined only by screening for high level kanamycin resistance. There is no single enzyme that can inactivate all available aminoglycoside.\(^{[32,125,132]}\) Only streptomycin, gentamicin and occasionally, kanamycin should be considered for synergy screening.

**Activity of aminoglycoside-modifying enzymes in *E. faecalis* showing HLAR phenotype**

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Activity on aminoglycoside</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gentamicin</td>
</tr>
<tr>
<td>Streptomycin adenyltransferase</td>
<td>Absent</td>
</tr>
<tr>
<td>3' Phosphotransferase</td>
<td>Absent</td>
</tr>
<tr>
<td>2' Phosphotransferase &amp; 6' acetyltransferase (acetylase)</td>
<td>Present</td>
</tr>
</tbody>
</table>

HLAR enterococci were first reported from France in 1979 and since then it has been reported from all the countries.\(^{[29,30]}\) HLAR enterococci often have plasmid which also carries determinants encoding resistance to other antibiotics, besides limiting the option of using a combination of cell wall active antibiotics and aminoglycosides. The drug combinations depend on the synergistic bactericidal activity between the two antibiotic groups. It is often used empirically in serious infections. There are some evidences, patients treated with synergistic combinations of antibiotics have lower mortality rate than those receiving individual antibiotic therapy.\(^{[134]}\) A high occurrence of HLAR necessitates routine testing of the enterococcal isolates for it (HLAR). Arslan U showed that some of the *E. faecium* strains with *aac-aph* gene (for AME) were reported as susceptible by Vitek 2 and Phonix. So concentration used by automated, agar dilution or E-test methods to detect gentamicin resistant enterococci should be re-evaluated.\(^{[188]}\)

In the present study, a total of 54.5% isolates showed high level resistance to gentamicin and 38.5% to streptomycin by high content disc diffusion [Table No. 17]. This correlated well with study conducted by Mendiratta DK *et al.* (46% HLGR).\(^{[30]}\) Rouff KL *et al.*\(^{[100]}\) and Udo EE
et al.,\textsuperscript{154} reported 14\% HLGR. Combination therapy with cell wall active agents (penicillin,
ampicillin or vancomycin) and aminoglycoside (gentamicin or streptomycin) will improve the outcome of enterococcal infection & is recommended for the treatment of serious enterococcal infections such as meningitis or endocarditis especially caused by VRE.

In the present study, the high level gentamicin resistance (55%) was higher than high level streptomycin resistance (39%) [Table No. 17]. Similar observation reported in the study conducted by Rahangdale et al.[154] who reported high level resistant isolates to gentamicin (50%) which was higher in comparison to streptomycin resistance (48%). This ratio was reverse in study conducted by Miskeen PA et al.[176] who reported 42% resistance to streptomycin and 37% to gentamicin. Gordon et al.[66] also has reported that 45% isolates with high level gentamicin resistance were not highly resistant to streptomycin; therefore, cell wall inhibitors in combination with streptomycin may be useful in the treatment of serious infection. Streptomycin susceptibility has been reported in 25 to 30% of other high gentamicin resistant enterococcal isolates. In the present study, among high level gentamicin resistant enterococcal isolates 23% by disc diffusion and 18% by agar dilution method were susceptible to high level streptomycin. Certain strains with HLGR may not have HLSR.[66] [Table No. 19].

Since amikacin is sometimes considered for combination therapy, it is important to note that amikacin can be used to determine E. faecalis susceptibility to amikacin-penicillin combination. Kanamycin is known to predict amikacin-penicillin synergy more accurately than amikacin.[125] Among VRE, 30% strains can produce multiple enzyme type and are highly resistant to all known aminoglycosides.[32]

High level gentamicin and streptomycin resistance among E. faecium (79% and 60%) were significantly [P<0.001] more than among E. faecalis (50% and 31% respectively) [Table No. 17 & 18]. Mendiratta DK et al.[30] and Rouff KL et al.[100] also reported same observation. The increase prevalence of HLGR in metropolitan hospitals may be due to longer therapy, increase stay of chronic cases and wider usage of broad spectrum antibiotics.

HLGR has also been linked to β-lactamase production, resistant to ciprofloxacin and chloramphenicol.[30] Our (two) isolates were β-lactamase producers which showed concomitant resistance to ciprofloxacin. The enterococci are intrinsically resistant to penicillin due to β-lactamase production. Penicillin increases uptake of aminoglycoside and becomes susceptible to combination of these two drugs.[165] The enterococci produce β-lactamase enzyme which neutralise the penicillin and hence aminoglycoside may not enter inside, even though they have capacity to (after entry) kill bacteria and become resistant to penicillin - aminoglycoside synergy.
Microbiologists need to assess the abilities of their routine susceptibility testing methods to detect resistance pattern in enterococci, since the CLSI had not yet completed their studies on methods for determining HLAR. In the present study, correlation among disc diffusion and agar dilution (recommended) method for detecting high level gentamicin and streptomycin resistance was statistically extremely significant (P<0.0001) [Table No. 20 & 21]. Our findings were consistent with those of an earlier study. Sahm DE et al. have reported good correlation of disk diffusion with broth dilution method in their studies, and concluded that high-content aminoglycoside disks can be used to accurately differentiate *E. faecalis* isolates that are resistant or sensitive to the synergy of various aminoglycoside-penicillin combinations. Some time there may not be correlation between zone of inhibition (i.e. zone diameter, 6 mm) of high-content aminoglycoside disks and high-level (i.e. MIC 2,000 µg/ml) aminoglycoside resistance.

The high-content disks prepared in huge quantity, stored at either 4 or -20°C for long period without major loss of activity (data not shown), and retrieved for use as needed. This method, therefore, offers a suitable technique by which many laboratories would be able to screen clinically significant *E. faecalis* isolates for synergy testing. The greater resistance of *E. faecium* to β-lactam and aminoglycoside antibiotics, the disk diffusion results presented here should be applied only to *E. faecalis* isolates and not to *E. faecium* strains. Further studies are required to determine the reliability of the disk test for detecting synergy resistance among important clinical isolates of *E. faecium*. We have adopted this procedure in our clinical laboratories, however, we recommend that the quality of the disks must be examined periodically by testing known synergy-susceptible (*Enterococcus faecalis* ATCC 29212) and resistant (*E. faecalis* ATCC 51299) strains.

**Antibiotic Synergisms**

There were some incidences of false resistance and false susceptibility occurring in HLAR, when we used various media and inoculum sizes. High incidence of false susceptibility has been reported by Basker and Calderwood et al. They reported some *E. faecalis* strains failed to grow in the presence of gentamicin (500 µg/ml) and streptomycin (2,000 µg/ml), but when tested by time-kill curve (TKC) method, these strains were refractory to gentamicin or streptomycin -penicillin synergy and vice-versa. Why these strains are inhibited by gentamicin or streptomycin but are not synergistically killed by the combination of penicillin and serum-achievable concentrations of gentamicin or streptomycin are not known. Our observation resembles to Basker and Calderwood *et al*. They studied amikacin - penicillin by broth dilution
and TKC methods.
High level gentamicin and streptomycin resistance were more in penicillin resistance than penicillin susceptible isolates by disc diffusion and agar dilution but the striking observation was that HLAR was also found in penicillin susceptible strains up to 20% to 35% by both high content disc diffusion and agar dilution method [Table No. 22]. According to present study there is no correlation between MIC ranges of penicillin and HLAR [Table No. 23, Chart No. 4]. So, these methods can be used only for screening of HLAR. These methods are not recommended to confirm the exact synergy between penicillin and aminoglycosides.

In the present study, there was wide variation noted in high level resistance to gentamicin (11 to 55%) as well as in streptomycin (12 to 47%) by different workers [Table C] and methods. There was no correlation found between HLAR by disc diffusion, agar dilution and broth dilution methods with antibiotic synergy by time kill curve and combined disc diffusion methods [Table No. 26 & 27]. Correlation between high level aminoglycoside resistance and antibiotic synergy is not statistically significant [P>0.1].

Due to lack of data concerning the treatment of multi-drug resistant enterococcal infections, it is probably prudent to test potential drug combinations in vitro using different techniques and apply the results to modify therapy. Two most common methods used for determining synergy are checkerboard and time kill curve test. These are, however, too cumbersome, time consuming and labour intensive for routine use in laboratories.[32]

Ryan RW et al.[134] reports data of 32 strains of enterococci which were evaluated using synergy screen (SS), checkerboard and TKCs with gentamicin and penicillin. Among the six aminoglycosides tested by them, only two (kanamycin and streptomycin) showed high-level resistance in 28% (9/32) and 33% (11/32 respectively) of the isolates, by synergy screen method. There was approximately a 100 x reduction [synergy] in colony count with the penicillin-gentamicin combination compared to either drug alone. When all TKCs were evaluated (32 isolates), a 10 x reduction [low level synergy] in colony count of the combination versus the individual antibiotics resulted in 100% low level synergy, and a 100 x reduction resulted only in 60% (19/32) synergy. He had not recommended checkerboard method which is less reliable.

In the present study, among the 124 penicillin resistant enterococcal isolates, only 7 (5.65%) strains were synergically susceptible to penicillin and gentamicin as well as penicillin and streptomycin in combination by combined disc diffusion method as well as time kill curve method. While, 2 strains were synergically low level susceptible to these combinations [Table No. 24]. Correlation between combined disc diffusion and time kill curve method for antibiotic
Synergy testing is statistically extremely significant (P<0.0001). Penicillin and aminoglycosides combined disc and time kill curve technique can be compared with routine achievable concentration by human serum. Antibiotic synergy has very good correlation between MIC ranges of penicillin. MIC ranges of penicillin and antibiotic synergism of penicillin with aminoglycoside is negatively correlated with each other [Table No. 25 & Chart No. 5]. This correlation is statistically very significant (P=0.007).

The correlation of SS and TKC was acceptable for gentamicin-penicillin (89%).[^134] SS is a yes / no test. If the isolate fails to grow in the presence of 2,000 µg/ml of gentamicin, bacterial ribosomes are presumably susceptible to the bactericidal effects of gentamicin. If cell wall integrity is destroyed by a cell wall-active agent, the lesser concentrations of aminoglycoside will effectively bind to 30S portions of the affected ribosomes. The interpretive agreement between this screening test and TKC is not surprising since both assays measure cell killing and not only inactivation. There is no statistically significant correlation between high level aminoglycoside resistance by disc diffusion, agar dilution and broth dilution methods with antibiotic synergy by time kill curve and combined disc diffusion methods [Table No 26 & 27].

The present study does not recommend the synergy screening as well as checkerboard[^133] method for testing of penicillin and aminoglycoside combination.[^134] Since the results of these tests do not consistently correlate well with bactericidal activity in test time-killing curves[^27] A reduction of MIC in both antibiotics of at least two dilution intervals (fourfold) showed it’s synergism by checkerboard dilution method[^134] but this fore fold reduced dilution may not be achievable in human serum.

The various combinations of antibiotics can be studied by double disc, modified E-test, checkerboard and time kill curve techniques. Combined disc technique could be applied to studies of penicillin G, ampicillin, carbenicillin, kefelin, ceftriaxone or vancomycin in combination with streptomycin, kanamycin, gentamicin or ciprofloxacin. These combinations may be studied on the same plate, many more drug combinations could be tested. Preliminary studies also indicate that the use of this method is not limited to enterococci, but may be applied to other organisms.

Penicillin and aminoglycosides combined disc technique, therefore, offers an easy, convenient, requires less time and is cost effective as high content disc concentration (HLAR) for routine screening test for enterococci in laboratories. This method for determining synergism is simple enough to be performed by any laboratory which is performed by Kirby-Bauer method.
Discussion

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for antibiotic susceptibility determinations. The area of synergistic effect can be readily and easily visualized preferably under transmitted light. We are recommending combined disc method for routine laboratory testing and time kill curve method for its confirmation but yet these methods need to be standardized.

Although methods for synergy analysis are not standardized from laboratory to laboratory, care was taken to insure "In-house" standardization of media, inoculum, time and temperature of incubation and reading of results. The data suggested that even if methods were standardized, difficulties still might exist, such as interpretation of results, setting of synergy criteria, and methodological and biological variation.

The acquired multi-drug resistance (glycopeptide) in enterococci with potential spread of enterococcal resistance determinants to other species will remain a concern. Continued development of new drugs by the pharmaceutical industry, aided by genomics screening, and rational drug design, offers the prospect of effective bactericidal monotherapy for enterococci, including VRE, HLAR and MDR enterococci. Wiser use of antimicrobial drugs, possibly guided by novel techniques for rapid and accurate microbiological diagnosis, and the nascent trend towards the development of narrower-spectrum antimicrobials may diminish some of the selective pressures. Novel therapies, such as vaccine-based immuno-therapies, phage therapy, and gene therapies[28] to reverse drug resistance, may offer long-term solutions to the problem of multi-drug resistance enterococci.

We have focused on the emergence of enterococcal antimicrobial resistance by various techniques, which have been less often in E. faecalis and are detected often in E. faecium. In addition, clinical investigations are needed to clarify the current strategies to prevent and control the spread of multi-drug resistance, inclusive of cost-effectiveness analyses that can substantiate such recommendations. Empirical therapy for enterococcal infections should be guided by local patterns of drug resistance.

However, emergence of in vitro resistance to many antimicrobial agents among enterococci has also been observed in several prospective studies; thus, we encourage clinicians to request susceptibility testing for all isolates recovered from patients, while they are receiving antimicrobial therapy for serious Gram positive cocci infections especially enterococci.