DISCUSSION
6.1. Distribution of enterococci among clinical specimens:

Enterococci are a common inhabitant of the intestinal and vaginal tract of humans and animals. As a result, they are released in large numbers in faeces, and may become the predominant contaminant microbiota in many food sources in developing countries (Giraffa, 2002).

Enterococci are the third most common cause of nosocomial bacteremia. In a recent study from Delhi, India (Jain et al., 2011) the prevalence of enterococcal bacteremia was observed to be 72% among hospitalized patient’s and 28% in outpatients.

Enterococci are second most common cause of nosocomial urinary tract infections not only in Western countries, but also in India, and are frequently recovered from urine samples.

In our study, enterococci were collected from clinical specimens such as blood, urine, pus, CSF, vaginal, vulval and semen swabs, except from stool. Majority of the specimens in our study included urine 55.26% (126/228) and blood 32.3% (73/228).

6.2. Prevalence of Enterococcus species:

More than seventeen species had been identified in enterococci. So far reports from India and other parts of the world have shown E. faecalis as the most common species followed by E. faecium. Though the prevalence of E. faecium is considered lesser compared to E. faecalis, E. faecium carries
greater intrinsic resistance to most of the antibiotic classes and thus MDR naturally.

Species identification of enterococci is usually determined phenotypically using characteristics such as motility, pigment production, and carbohydrate utilization (Facklam et al., 1989). However, this becomes difficult for identifying unusual strains with few characteristic reactions such as motile *E. gallinarum* or *E. casseliflavus* species that are encountered in samples.

From 1995 a PCR based method for the identification of enterococci was made available by Dutka-Malen et al., 1995 which targets the species specific ligase gene for the identification of *E. faecalis*, *E. faecium*, *E. casseliflavus*, and *E. gallinarum*. However, this technique is available to identify only four species mentioned above, leaving the other enterococcal species unidentifiable except by biochemical methods.

Due to importance of speciation in enterococci, we have subjected all the isolates for biochemical testing and PCR with primers specific for species such as *E. faecium* (ddl*\_E\_faecium*), *E. faecalis* (ddl*\_E\_faecalis*), *E. gallinarum* (vanC1), *E. casseliflavus*, *E. flavescens* (vanC2/C3). The other species such as *E. avium*, *E. dispar*, *E. durans* were identified based on their biochemical characteristics.

The results of species identification by phenotypic and molecular methods were discordant due to the variation shown by *E. faecium* to the sugar sorbitol and arabinose. A total of 117/228 (51.31%) *E. faecium* and 99/228 (43.42%) *E. faecalis* were identified in our isolates.
In our study, *E. faecium* (82%), *E. faecalis* (10.95%), *E. avium* (2.8%), *E. gallinarum* (1.36%) and *E. casseliflavus* (1.36%) were obtained from blood sample, thus *E. faecium* was the predominantly observed species among blood isolates. *E. faecalis* (56.34%) was the predominant species observed among enterococci obtained from urine followed by *E. faecium* (39%).

In our study *E. faecium* was the predominant species isolated than *E. faecalis* by PCR method (Angeletti et al., 2001).

However, species identified by molecular method showed good correlation with the documented resistance profile of the species especially to high level aminoglycosides. Though biochemical methods are gold standard as well as routinely used for the identification of enterococci up to species level, being a nosocomial agent, exact identification of *E. faecium* by PCR could help not only in treatment but also in managing outbreak situations.

Majority of the studies from India had reported more number of *E. faecalis* by phenotypic identification method of Facklam and Collins (1989).

Biswa et al., in 2013 from Bihar has compared clinical and fecal isolates of enterococci and found almost same number of *E. faecalis* (45%) and *E. faecium* (43%) in clinical sample and in faecal samples along with other species viz. *E. gallinarum, E. faecium, E. raffinosus* and *E. dispar*.

A study by Narayanaswamy et al., in 2011 had reported 316 (88%) *E. faecalis* followed by 44 *E. faecium* from 360 clinical isolates collected from a tertiary care hospital in Chennai. All their study isolates were shown to be
sensitive to vancomycin, teicoplanin and linezolid by phenotypic method. They have reported 56% HLGR and 42% HLSR by agar screening method.

In a recent study by Fernandes and Dhanashree, in 2013 from Mangalore, South India 56% of *E. faecalis* and 34% of *E. faecium* were recovered from 180 clinical enterococcal isolates. 123/180 (82%) of the isolates were hemolysin producers and showed 8.6% of VRE and 50% HLARE by agar screening method. The 13 VRE included 4 *E. faecalis*, 6 *E. faecium*, 2 *E. dispar* and one *E. avium* isolate. However, VRE isolates were susceptible to high level gentamicin, streptomycin and teicoplanin.

There are also studies from India with *E. faecium* as the predominant isolate and occurrence of other species.

Baragundi *et al.*, from Bagalkot, Karnataka in 2010 had reported (57/120) *E. faecium* (47.5%) and (53/120) *E. faecalis* (44.16%) and other species. They showed the presence of *E. mundti* (5), *E. durans* (2), *E. dispar* (1) and *E. gallinarum* (1) by biochemical characterization. Similarly, Kapoor *et al.*, 2005 from New Delhi (66%) and Kamarkar *et al.*, 2004 from Mumbai had reported *E. faecium* (81%) as the predominant isolate and other species by conventional biochemical testing methods.

A pilot study conducted by Sekar *et al.*, in 2008, had analyzed 31 (71%) enterococci obtained from 40 stool samples from a tertiary care hospital in Chennai. 22/31 were *E. faecium* and 9/31 were *E. faecalis*. 
In the present study, we have also reported species other than *E. faecium* and *E. faecalis* such as *E. avium*, *E. hirae*, *E. durans*, *E. dispar*, *E. casseliflavus* and *E. gallinarum*.

Prakash *et al.*, 2005 from Puducherry, South India were the first to report unusual species such as *E. avium*, *E. hirae*, *E. durans*, *E. mundtii*, *E. raffinosus*, *E. casseliflavus* and *E. gallinarum* amongst 242 clinical enterococcal isolates. They performed whole cell protein profiling for the identification of species.

A recent study by Nepal HP, *et al.*, in 2013 had reported 66.3% *E. faecalis*, 22.7% *E. faecium*, each of *E. avium* (0.9%), *E. dispar* (0.9%), *E. mundtii* (0.9%), *E. gallinarum* (0.9%), *E. hirae* (0.9%) along with *E. cecorum* (1.82%) and *E. saccharolyticus* (4.54%) among 110 enterococcal isolates from different clinical specimens collected from a tertiary care hospital in Eastern Nepal. They characterized enterococci by standard biochemical testing. Majority of the isolates were obtained from urine (48%) which is similar to our study, followed by blood, pus, vaginal swabs etc. But, they have not discussed about the antibiotic profile of species other than *E. faecalis* and *E. faecium*.

In recent times occurrence and clinical significance of other species of enterococci are gaining importance around the globe. In India, there are sporadic reports on the isolation and management of infections due to unconventional species of enterococci. This emphasizes the importance of the role of clinical microbiology laboratories in speciation of *Enterococcus isolates* in order to provide the clinician with the correct choice of antibiotics.
A study by Telkar et al., in 2012 from Karnataka had reported one *E. durans* from blood culture which was high level gentamicin and streptomycin resistant. Majority of the isolates observed in their study was *E. faecium* 35/50 (70%), followed by *E. faecalis* 14/50 (28%) and *E. durans* 1/50 (2%). They had also documented 6/50 (12%) of VRE and 20/50 (40%) of HLAR by disc diffusion not by agar screening method.

6.3. Antibiotic resistance pattern of enterococci:

Emergence of antibiotic resistance in *Enterococcus* species especially multi-drug resistance (MDR) to almost all available antibiotics has led to limitations in treating severe enterococcal infections. Due to their capability to acquire resistance genes and also presence of unique enzyme encoded mechanisms, they confer resistance to antibiotics such as aminoglycosides and glycopeptides.

Gordon et al., (1992) from Atlanta worked on antimicrobial susceptibility of unusual species of enterococci observed that *E. faecalis* accounted for about (90%) isolates, followed by *E. faecium* (8%) and other species such as *E. gallinarum, E. avium, E. casseliflavus, E. raffinosus* and *E. hirae*. They have reported that all the other species of enterococci were also high level resistant to aminoglycosides.

We have observed majority of our isolates were resistant to ciprofloxacin (68.85%), pristinomycin (66.2%) and erythromycin (56.14%) by disc diffusion method. 53.3% and 49.4% of resistance were observed to gentamicin (120mg) and amikacin (30mg) (CLSI, 2011- *S. aureus* breakpoint
for amikacin) respectively. 25.87% ampicillin resistance and 5.26% of resistance to nitrofurantoin was observed in our study isolates.

SENTRY Antimicrobial surveillance programme conducted in 1997-1999 among many countries observed that nitrofurantoin was active against majority of the isolates tested and it could be suggested for enterococcal urinary tract infections (Low et al., 2001).

In our study, all the isolates were susceptible to teicoplanin and linezolid.

Jada et al., 2012, from Chennai have reported 100 E. faecalis with a range between 71% - 83% resistance to most of the drugs tested viz. gentamicin, penicillin, erythromycin and ciprofloxacin. However, all their study isolates were susceptible to vancomycin.

Quinupristin/dalfopristin could be used in the treatment of VRE in case if it is E. faecium but this drug is less effective against vancomycin resistant E. faecalis. Linezolid is preferred over quinupristin/dalfopristin due to the side effects (Grim et al., 2009).

Daptomycin a lipopeptide antibiotic, currently considered as a reliable therapeutic alternative to treat VRE isolates (Arias et al., 2011). It is still a sparingly used drug for enterococcal infections in many centres moreover; linezolid seems to be an effective drug among all the high level and low level resistant isolates.

Also, there is only MIC breakpoints available for daptomycin and the unavailability of daptomycin discs and also they are not frequently prescribed
for enterococcal isolates, hence we have not included daptomycin for the susceptibility testing.

Few studies have been carried out on the use and susceptibility profile of daptomycin to enterococcal infections.

In India, 14/450 (3.11%) of the isolates were reported to have high daptomycin resistance in enterococci (Vidyalakshmi et al., 2012) by MIC method.

6.4. High level aminoglycoside resistance in Enterococcal isolates:

The high-level resistance to the aminoglycoside can eliminate the synergistic bactericidal effect obtained by antibiotic combination (Leelaporn et al., 2008).

Several surveys of clinical isolates between 1970 and 1977 found no high-level gentamicin resistance (Mollering et al., 1970, Basker et al., 1977, Caldrwood et al., 1977). Later, it was reported from Western countries.

In a study by Zervos et al., 1986 at University of Michigan Hospital have shown that, incremental increase of gentamicin resistance was from 0.4% in 1981 to 13% in 1985. Three-quarters of patients with serious underlying illness were infected or colonized with GRE.

In the present study, we have analyzed both high level and low level resistance among our study isolates by MIC. For gentamicin and amikacin the dilution used was from 0.5-512µg/ml while for streptomycin and kanamycin 0.5-2048µg/ml was used.
Gentamicin being the widely used aminoglycoside for combinational therapy along with penicillin group of drugs, showed 46.49% (106/228) of HLGR phenotype with MIC >512µg/ml. 50.87% (116/228) of strains were HLKR with >2000µg/ml which were the highest amongst aminoglycosides tested. Whereas, 42.10% (96/228) of strains were HLSR with >2048µg/ml and 26.31% (60/228) of strains were HLAkR with >512µg/ml.

High level aminoglycoside resistance among *Enterococcus* species is commonly reported worldwide, Indian reports had detected about 40-60% high level resistance to aminoglycosides.

44.4% high level gentamicin resistance and 16.6% for penicillin resistance in enterococci has been reported from Mumbai (Shindae *et al*., 2012).

A study from South India has reported a very high percentage of (65.6%) of HLARE from diabetic foot infections (Vinodkumar *et al*., 2011).

In another study from Maharashtra, HLAR was found to be 46%, in which high HLGR were observed in *E. faecalis* and high HLSR in *E. faecium* isolates (Mendiratta *et al*., 2008).

A previous study by Sekar *et al*., 2008, Chennai India has reported a low fecal carriage of enterococci with 2% HLGR and 4% HLSR in enterococci. But their study showed *E. faecalis* as the predominant species than *E. faecium* when screened with 32 enterococcal isolates.

High level resistance to aminoglycosides were shown in species other than *E. faecium* and *E. faecalis*.
In our study, 1 *E. hirae* was HLKR, HLSR and HLAKR, 1 *E. casseliflavus* was HLGR, 2 *E. avium* were HLSR, HLAKR and 1 *E. durans* was high level resistant to all the aminoglycosides tested (Padmasini et al., 2014).

The pattern of HLAR in our study shows that around 41/228 (18%) of isolates were resistant to all the aminoglycosides and 78/228 (34.21%) of isolates were low level or not HLARE.

The high level resistant phenotypes detected in our study were widely distributed among the clinical sources. Increased number of HLR isolates obtained from urine (>50% - 62% among HLR strains) were high level resistant phenotypes followed by isolates obtained from blood (>28%-43%), pus (>5%-7%), semen (2%) and vaginal isolates (1%) respectively.

In a study by Dhallal et al., 2008, 425 UTI cases were screened for enterococci and found *E. faecalis* (70%) as the predominant isolate than *E. faecium* (30%). They used both biochemical method and genus-species specific target (rrs) PCR which showed similar results on comparison.

High level gentamicin resistance is primarily due to the presence of bifunctional aminoglycoside resistance mediating gene $\text{aac(6')-Ie-aph(2'')-Ia}$ which also confers high level resistance to clinically useful aminoglycosides such as amikacin, tobramycin, kanamycin, netilmicin, dibekacin except streptomycin (Chow., 2000). $\text{aph(2'')-Ib}$ was first detected in *E. faecium* and *E. coli* and found to confer high level resistance to gentamicin, tobramycin, amikacin, kanamycin, netilmicin and dibekacin but not to amikacin.
*aph(2’’)-Ic* confers HLR to gentamicin, tobramycin and kanamycin while the strains carrying them can be treated with amikacin, netilmicin and streptomycin in combination with cell wall inhibitors. Earlier this gene was first shown to be present in *E. gallinarum* in 1997 obtained from farm animals and in *E. faecalis* and *E. faecium* isolates obtained from humans (Donabedian *et al.*, 2003).

*aph(2’’)-Id* gene was first reported in *E. casseliflavus* in 1998 and the isolate showed high level resistance to gentamicin but not to amikacin. This gene has similar mechanism to that of *aph(2’’)-Ib* and had been reported in VRE- *E. faecium* isolates from clinical settings (Vakulenko & Mobashery., 2003).

In the present study, the overall percentage of *aac(6’)-Ie-aph(2’’)-Ia* gene positivity was (79/228) 34.64%. Out of 106 strains of HLGR identified by MIC method, 69 strains (65%) carried *aac(6’)-Ie-aph(2’’)-Ia* gene.

In a previous study (Zarrilli *et al.*, 2005), all the high level gentamicin resistant *E. faecalis* and *E. faecium* isolates were found to carry *aac(6’)-Ie-aph(2’’)-Ia* gene.

Interestingly, the rare aminoglycoside phosphotransferase resistance gene *aph(2’’)-Ic* was found in 1.31% (3/228) isolates of enterococci in our study.

This was described as novel gentamicin resistance gene by Kobayashi *et al.*, 2001 but this gene was not detected among the *E. faecium, E. faecalis* and *E. avium* isolates on analysis.
The strains carrying \textit{aph}(2'')-Ic gene is assumed to show susceptibility to the combination of ampicillin along with amikacin, netilmicin or dibeakacin but it is resistant to ampicillin-gentamicin synergism even though the MIC for gentamicin is less than $\geq$500 $\mu$g/ml - 256 $\mu$g/ml (Chow, 2000).

At the same time, these three isolates carrying \textit{aph}(2'')-Ic gene in our study also carried the bi-functional enzyme coding \textit{aac}(6')-\textit{Ie-aph}(2'')-Ia and \textit{aph}(3')-\textit{IIIa} genes.

The isolates carrying the other aminoglycoside phosphotransferase resistance genes such as \textit{aph}(2'')-Ib and \textit{aph}(2'')-Id had been reported to encode high level resistance to gentamicin (>500 $\mu$g/ml) but these genes were not detected among our study isolates.

19/93 (20.4\%) enterococcal isolates carried \textit{aph}(2'')-Ib and 26/93 (27.9\%) isolates carried \textit{aph}(2'')-Id in a study conducted by Vakulenko \textit{et al.}, 2003 from Michigan, USA.

Another most important aminoglycoside phosphotransferase gene tested in our study is \textit{aph}(3')-\textit{IIIa} which confers resistance to kanamycin and streptomycin was detected among 36.4\% (83/228) isolates of enterococci.

Aminoglycoside nucleotidyltransferase genes such as \textit{ant}(6')-Ia and \textit{ant}(9')-Ia confers high level resistance to streptomycin and spectinomycin antibiotics. \textit{ant}(6')-Ia was detected in 57/228 (25\%) which is responsible for HLSR. Out of 228 isolates screened, only 2 isolates (0.87\%) were positive for \textit{ant}(9')-Ia gene even though all other resistance encoding genes were tested. Their MIC for gentamicin was 16$\mu$g/ml and for streptomycin 1024$\mu$g/ml.
ant(9')-Ia gene was also reported by Kobayashi et al., 2001 from each of E. faecalis and E. faecium. This gene was previously reported from staphylococci and gram negatives by LeBlanc et al., 1990.

ant(4')-Ia gene which is responsible for resistance to tobramycin, amikacin and kanamycin synergism but susceptible to synergistic effect of other aminoglycosides. ant(4')-Ia was reported in E. faecium and E. faecalis isolates by Ounissi et al., 1990 and Kobayashi et al., 2001.

In our study, ant(4')-Ia gene was not present in any of the isolates tested.

Aminoglycoside acetyl transferase aac(6')-Ii gene was detected in 46/228 (20.17%) isolates. This gene was shown to confer susceptibility to ampicillin-gentamicin, amikacin and streptomycin synergism but resistant to synergism with kanamycin, netilmicyn and tobramycin.

Among the aac(6')-Ii gene positives, 70% (32/46) isolates were from blood and the remaining were from urine (13/46) and CSF (1/46) samples. This gene was observed in 42/46 (91.3%) E. faecium isolates and 4/46 (8.7%) E. faecalis isolates. This gene was present in combination with other aminoglycoside resistance genes, in some cases; they appeared as the only resistant gene (with absence of other genes analyzed) in E. faecium isolates in our study with HLR phenotype.

Studies have reported presence of aac(6')-Ii gene in E. faecium isolates conferring intrinsic resistance in these isolates and has not been found in other enterococcal species (Costa et al., 1993, Kobayashi et al., 2001, Vakulenko et
They had reported $aac(6')-Ii$ in 53.7% $E. faecium$ isolates. But in our study, we observed the presence of this gene in 8.7% $E. faecalis$. Further understanding of the mechanism and function of this gene would throw light on this gene activity.

$E. faecium$ with $aac(6')-Ii$ and $ant(6')-Ia$ may also restrict the choice of therapy, because AAC(6')-I also reduce the synergy between beta-lactams and aminoglycosides with free 6'-amino groups (Kobayashi et al., 2001).

3/106 (0.02%) isolates that were high level gentamicin resistant and 9/96 (9.3%) isolates that were high level streptomycin resistant did not carry any of the genes tested. The majority of these strains were $E. faecium$ and the reason for the high level resistance and absence of genes analyzed in this study could not be explained.

A similar expression of HLAR phenotypes with absence of HLR genes was also observed by Kobayashi et al., 2001. The isolates with HLAR phenotype did not carry the genes tested among $E. faecalis$ and $E. faecium$.

High-level gentamicin resistance in enterococci may not in all instances preclude the therapeutic use of other aminoglycosides (Chow et al., 1997).

Our study has also proved upon analyzing four aminoglycosides such as gentamicin, streptomycin, kanamycin and amikacin. High level aminoglycoside resistance was observed in 10/228 (4.38%) kanamycin, 4/228 (1.75%) amikacin and in one isolate with kanamycin and amikacin that were low level resistant to gentamicin and streptomycin.
6.5. Glycopeptide resistance in Enterococcal isolates:

26 years ago Uttley et al., in 1989 was the first to report vancomycin resistant enterococci (8 VRE isolates) from patients with end stage renal disease. The vanA gene is plasmid-borne and confers high-level resistance to vancomycin. VanB differs phenotypically in that it retains susceptibility to teicoplanin. Most of the VRE cases from Europe, the United States, and Korea were due to vanA, whereas the epidemic in Singapore and Australia has predominantly found to be vanB.

Mathur et al., from New Delhi, was among the first to report VRE from India in 2003.

A study by Werner et al., in 2012 has observed 80 VRE carriers from 600 neonatal ICU patients. Almost 71 E. faecium isolates were identified to be vancomycin resistant by E-test and they were subjected to PCR identification for their van genotype which showed 67 isolates carried vanB type and 4 isolates neither carried vanA or vanB genes. All these isolates were positive for virulence factors such as esp and hylEfm virulence factors. Thus their specimens had 56 VRE outbreak isolates upon analyzing by molecular methods with IS16 element.

Teicoplanin hetero resistance in vanA VRE strains was described by Khan et al., in 2008 and several genomic rearrangements and deletions within the vanA gene cluster elements and mutational changes within the two-component regulator genes vanS and vanR were identified in glycopeptides heteroresistant strains. However, these changes were not experimentally proven
and were not functionally linked to the described heteroresistance phenotype (Alam et al., 2001, Qu et al., 2009).

In our study, VRE was observed in 3/228 (1.3%) isolates. All the three were *E. faecium* and found to carry vanA genotype. They all were high level vancomycin resistant by MIC ranging from 128 to $\geq 256\mu g/ml$ but their teicoplanin resistance was very low (MIC $4\mu g/ml$ and two strains even lesser, hence susceptible) (Padmasini et al., 2014). These isolates were obtained from blood (2) and vulval swab (1). These VRE isolates were also high level resistant to aminoglycosides irrespective of the specimen sources they were obtained from.

A recent study by Ira et al., from Puducherry, in 2013 had screened 367 enterococcal isolates from various clinical sources from both inpatients and outpatients. They had reported 32/367 isolates (8.7%) as vancomycin resistant (128$\mu g/ml$) by the MIC method compared to 34 isolates (9.26%) by the disc diffusion method. 29/32 VRE carried vanA gene whereas 2/32 VRE, carried vanB gene.

*vanC* genes are commonly present in motile species of enterococci such as *E. gallinarum* and *E. casseliflavus*. The presence of this gene indicates the intrinsic / intermediate-level resistance to vancomycin with an MIC range of 8-16$\mu g/ml$ (CLSI guidelines, 2013).

The isolates with *vanC1* gene are *E. gallinarum* and *vanC2/C3* gene is *E. casseliflavus* (Bell et al., 1998).
In our study, MIC for vancomycin was 2µg/ml for *E. casseliflavus* and 4µg/ml for *E. gallinarum*.

There are a few unconventional findings of phenotypic and genotypic analysis of vancomycin resistance in enterococci e.g., Park *et al.*, from Seoul in South Korea had reported an outbreak at a tertiary care hospital where six VanB phenotype and *vanA* genotype in *E. faecium* isolates having heterogenous expression of teicoplanin resistance were isolated. This heterogeneity may be due to the presence of mutations, either in the *vanA* gene cluster or in the *vanS* regulatory element. Such isolates have not yet been reported from India.

In India linezolid resistance is very scarcely reported. The first report on linezolid resistant enterococci with an MIC >256µg/ml by E-test method and MIC 1024µg/ml by agar dilution method has been documented by Smit Kumar *et al.*, in 2013 from Kolkata.

**6.6. Macrolide resistance in Enterococcal isolates:**

Macrolide and lincosamide antibiotics are chemically distinct inhibitors of bacterial protein synthesis. Modification of the ribosomal target of this antibiotic confers cross-resistance to all these antibiotics exhibit MLS\_B resistant phenotype.

The occurrence of an antagonism between erythromycin and clindamycin, by the double-disc diffusion test, indicates inducible production of methylase.
In our study, 41.22% of the isolates showed constitutive $\text{MLS}_B$ resistance phenotype, 1.75% of the isolates showed inducible $\text{MLS}_B$ resistance phenotype. We did not observe any M-phenotype of resistance among our isolates.

A report from Netherland by Schmitz et al., 2000 displayed greater resistance in $E. \text{faecium}$ towards erythromycin which showed 100% constitutive $\text{MLS}_B$ phenotype.

43.25% of our study isolates were sensitive to both erythromycin and clindamycin by D-test method. 96/178 (53.9%) of isolates showed resistance with MIC range of $>256 \mu g/ml$ towards erythromycin. The MIC results for erythromycin had shown that 17 isolates which showed intermediate resistance to erythromycin by disc diffusion had turned out to be resistant by MIC method.

Reports on macrolide resistance in India are comparatively lesser than that of the other parts of the world. In a study conducted by Jain et al., 2011, from Delhi on the antibiotic resistance profile of enterococci showed 76% resistance to erythromycin.

Erythromycin resistance determinants include Erm methylases, efflux pumps and inactivating enzymes. Detection of these erythromycin resistant determinants by PCR is used to distinguish the different mechanisms of erythromycin resistance in clinical isolates and also used in surveillance studies of erythromycin resistant determinants (Sutcliffe et al., 1996).
Among the three macrolide resistant genes analyzed, \textit{ermB} was the only gene found in 32.45\% of the isolates and the other genes such as \textit{ermA} & \textit{ermC} were absent.

Study from Korea, by Lim \textit{et al.}, 2002 has also documented the prevalence of \textit{ermB} gene among enterococci. 55.4\% of the isolates with \textit{ermB} gene positivity and \textit{ermA} gene in 5.2\% and \textit{mefA} 3.5\% of isolates.

Study by Reyes \textit{et al.}, 2007 from Colombia showed, isolates with high level of resistance to erythromycin, 62\% of \textit{E. faecalis} and 82\% of \textit{E. faecium} with MIC of \textit{\geq 128}\mu g/ml. All the resistant enterococcal isolates contain \textit{ermB} gene. None of the isolates were found to harbor the \textit{mefA} gene.

Emaneini \textit{et al.}, 2009 had reported 82\% and 45\% of enterococcal isolates were resistant to erythromycin with MIC range of \textit{\geq 128}\mu g/ml and \textit{512}\mu g/ml. \textit{ermB} gene (52\%) was the most prevalent gene observed in erythromycin resistant enterococcal isolates followed by \textit{ermA} gene (2\%).

**6.7. Prevalence of virulence factors in Enterococci:**

Enterococci though an opportunistic bacteria possess various virulence factors viz. enterococcal surface protein (Esp) and aggregation substance (Agg) which could enhance the colonization process in the host and binding to the host epithelium, respectively (Johnson, 1994). Others such as cytolysin, enterolysin A, gelatinase, hyaluronidase, Zinc metallo endopeptidase, enhanced expression of pheromone (Eep), and adhesion-associated protein EfaA (\textit{E. faecalis} endocarditis antigen A) have been reported to be among the most

Several studies with in vivo and in vitro host models to determine the nature of virulence factors had been carried out by several workers globally.

In the present study, we had analyzed the virulence factors phenotypically and genotypically.

Upon phenotypic screening of virulence factors, majority of the isolates were hemolytic on blood agar (31.57%). cylA gene which encodes for cytolysin/hemolysin activation was present in 25.8% of our study isolates.

26.75% isolates were positive for gelatin hydrolysis and 48.7% of the enterococcal isolates carried gelE gene that encodes for gelatinase.

But, a study by Eaton and Gasson in 2001 had described the presence of silent gelE genes that is, the presence of gelE gene in 8 E. faecalis strains and 2 E. faecium strains but lack phenotypic activity of gelatinase. They discussed that this may be due to inactive gene product or due to down regulation of gene expression.

Earlier studies had reported that this silent genes gets activated by conditions found in gastro intestinal tract, or by the intestinal flora and their synergism and also may be due to the presence and persistence of large number of enterococcal colonization (Finlay et al., 1997).

Johansson et al., from Sweden (2012) conducted a study on virulence factors in isolates of E. faecalis from infective endocarditis and from the normal flora stated that the production of virulence factors were less in
commensal enterococci than the clinical isolates. But their study results have shown that biofilm production for commensal isolates were higher than clinical isolates. This indicated the biofilm formation helps the enterococci to colonize in the gastrointestinal tract.

In our study, 7% of the isolates were strong biofilm formers, 29% were moderate biofilm formers. asa1 gene that is responsible for aggregation substance was present in almost 121/228 (53%) and they were analyzed for correlation with biofilm formation and hemagglutination. There was no correlation between the presence of asa1 and biofilm formation.

Further, enterococcal surface protein Esp was analyzed which were previously considered to be responsible for biofilm formation by various research workers. We had found that 94 isolates (41.2%) were positive for esp gene. Previous studies have shown that hospital isolates carried esp more than that of community isolates (Eaton et al., 2001).

5.26% of enterococcal isolates were DNase positive, 38.2% showed hemagglutination with AB’ blood group and 52.84% showed hemagglutination to O’ group of blood.

Apart from phenotypic detection of virulence factors, certain virulence factors were detected only by molecular analysis such as hyl gene for hyaluronidase, ace gene for collagen binding protein and efaA gene for endocarditis antigen.

Amongst all virulence factors analyzed in our study, 173 strains (75.8%) carried endocarditis antigen coding gene efaA. Earlier studies had reported that
The efaA gene was present in all clinical *E. faecalis* isolates analyzed (Eaton et al., 2001; Creti et al., 2004).

The second most common gene was *ace*, which is present in 63.2% of isolates. The most infrequent gene in our isolates was *hyl* gene that was observed in (12/228) 5.2% isolates of *E. faecium* especially in one of the vancomycin resistant *E. faecium* isolate (Ef090). Interestingly, all these isolates were obtained from blood, none from other specimen sources. Of the 12 positive isolates, *hyl* gene was the only virulent gene present in 11 isolates and one isolate carried *hyl* along with *esp*.

Our findings show that there was an even distribution of virulence factors among low level and high level resistant aminoglycoside resistant enterococcal isolates.

**6.8. Plasmid replicon typing in enterococci:**

Enterococci are prone to acquire antibiotic resistance either by mutation (especially point mutations) or by horizontal transfer of mobile genetic elements (especially resistant plasmids or transposons) (Jensen et al., 2010). The pheromone responsive plasmids were first described by Dunny et al., in 1978. Plasmid incompatibility types were first classified by Novick et al., in 1987.

PCR based typing methods targeting specific replicons of plasmids in gram negative bacteria have been developed by Gotz et al., in 1996, which was further refined by Carattoli et al., in 2005. This method was used in molecular epidemiology studies of R-plasmids carrying broad spectrum β-
lactamases in Enterobacteriaceae family (Carattoli et al., 2009; Carattoli et al., 2006; Hopkins et al., 2006).

Transposon insertions in repA gene prevented the replication in E. faecalis, suggesting that the RepA protein functioned as the replication initiator protein for the replicon. This suggestion was later confirmed by Francia et al., 2004.

Loss of plasmid stability and an increase in plasmid copy number was observed by transposon insertions in repB and repC genes which facilitate replication segregational stability and functions to control the replication frequency of the plasmid (Weaver et al., 1993). However, RepA type replicons are widely distributed among low G+C gram positive bacteria but are said to be restricted to their native host range.

In our study, we have analyzed enterococcal isolates for twenty different replicon types by Plasmid based replicon typing method (PBRT) targeting the 20 different types of rep families of which 16 rep types were positive.

It was shown that, the presence of multiple replication initiation proteins (rep) in many strains of enterococci with multiple drug resistance and the sources of the isolates had a significant correlation with the distribution of plasmid replicon types.

Our finding proves circulation of numerous types of plasmids that are often present in clinical enterococci. To the best of our knowledge there are no documented reports on rep families in enterococci from India. This study may
initiate epidemiological documentation of findings on the prevalence, analysis of plasmid types among enterococci.

Jensen and his co-workers USA had previously detected ten rep-families from 28 *E. faecalis* and 51 *E. faecium* strains with the same method.

The most prevalent replicon type observed among our enterococcal isolates was pMBB1 197/228 (86.4%) rep4 gene type.

In a study by Wyckoff *et al.*, 1996 from Oregon had observed the presence of pMBB1 as a stable cryptic plasmid which was 2.85 kb in 95% of isolates and was stably maintained even after 100 generations without selection.

Secondly, pCF10 (rep9) replicon was observed in 43% (99/228) of our enterococcal isolates. pCF10 is a 58 kb conjugative plasmid encoding for tetracycline resistance. Genes involved in pheromone inducible conjugation is found to be located in these plasmids.

pAD1, pAM373 and pCF10 are grouped as plasmids which are pheromone responsive. Pheromone responsive plasmid free cells induce these plasmids for the intracellular aggregation and high frequency DNA transfer (Hegstad *et al.*, 2010). The pheromone inducible conjugative hemolysin plasmid pAD1 was previously observed to share homology to pCF10.

In our study, 43% pCF10 and 3% pAM373 were detected in enterococcal isolates. These types were present in enterococcal species other than *E. faecium* and *E. faecalis*. 
pIP501 (*repI*) is a broad-host-range conjugative plasmid. It was originally isolated from a clinical strain of *Streptococcus agalactiae* by Horodniceanu *et al.*, in 1975. The regions of the plasmid that encode antibiotic resistance, replication and mobilization function have been sequenced previously by Brantl *et al.*, in 1990.

A 2.2 kb sequence in this plasmid is capable of supporting plasmid replication contained the RepR protein determinant and a putative replication origin.

In our study, pIP501 was observed in 11% (27/228) of the isolates, out of which 22 were *E. faecium*, 1 *E. gallinarum* and 4 *E. faecalis*. Among 27/228 pIP501 positive isolates, 8 (30%) were HLAR and 1 (3.7%) VRE.

Study by Rosvoll *et al.*, 2010 had observed *vanA* positive isolates to posse’s pIP501.

Previous reports on a limited number of *vanA*-containing plasmids in enterococci suggests that the pRE25 and pIP501 replicons are commonly present in VRE (Sorum *et al.*, 2006; Garcia-Migura *et al.*, 2007; Sletvold *et al.*, 2007, 2008).

In our study, pRE25 was observed in 12.7% (29/228) isolates, out of which 2 were *vanA* positive.

A study by Qu *et al.*, in 2012 had also observed pRE25, pEF418, pRUM and pB82 plasmids are the most prevalent among VRE isolates thus associated with transmission in nosocomial settings. They detected the
presence of VRE from clinical samples (47 VRE from 13 hospitals in China) but not from samples collected in healthy individuals.

13/228 (5.7%) isolates carried pSAS-PN 315 (rep5) and majority of the positive isolates were \textit{E. faecium} (11/13) with one VRE and 2 \textit{E. faecalis}. 8/13 of rep5 type was detected along with a group of rep types (Table 5.8) which had conferred antibiotic resistance in enterococci. This plasmid was previously reported in two of the MRSA isolates (Jensen \textit{et al.}, 2010).

92/228 (40\%) isolates carried pS86 (rep6) which is a rolling circle replication plasmid. Martinez-Bueno \textit{et al.}, in 2000 reported these in \textit{E. faecalis} as small cryptic theta replication plasmids with complete nucleotide sequence of 5149 bp.

pUSA02 was detected in other enterococcal isolates such as \textit{E. durans} and \textit{E. hirae} isolates by Lopez \textit{et al.}, 2012. Plasmids in rep7 family had been observed to contain antimicrobial resistance genes such as tetracycline, chloramphenicol and macrolide.

In our study, 21/228 (9\%) isolates carried pUSA02 plasmid and all these isolates were resistant to tetracycline and erythromycin antibiotics. Out of 21 isolates, 14 were from blood, 6 from urine and one from vulval swab samples and 16 were \textit{E. faecium}, 1 \textit{E. dispar} and 4 \textit{E. faecalis} isolates. Thus majority of the isolates were observed to be antibiotic resistant especially HLARE.

Rep10 plasmids contain 8 plasmids observed in wide range of species. pIM13 was first isolated from \textit{Bacillus subtilis} and is a naturally occurring plasmid. They are stably maintained at high copy numbers and are found to
constitutively express resistance to macrolide lincosamide-streptogramin-B antibiotics especially *ermC* gene (Projan *et al.*, 1987).

In our study, 3% (9/228) of the isolates carried pIM13 (*rep10*) plasmid. All these 9 isolates were obtained from urine culture and amongst which 6 were *E. faecalis* and 1 *E. hirae*. Though these isolates did not carry *ermC* gene, all were constitutively resistant to erythromycin and clindamycin and only one isolate carried *ermB* gene responsible for macrolide resistance.

14/228 (6.1%) isolates carried pEF1071 (*rep11*) in our study. This plasmid was previously reported in enterococci which carried genes for toxin production (Balla and Dicks, 2005). Interestingly out of these 14 positive isolates, 13 were *E. faecium* and one *E. durans* and majority of the isolates did not carry any of the virulence genes tested.

pC194 is a *rep13* type small plasmid that encodes for chloramphenicol resistance in staphylococci (Ballester *et al.*, 1989). In our study, this replicon type was detected in 6/228 (2.6%) *E. faecium* isolates. On observation, all these 6 isolates were high level resistant to gentamicin, streptomycin, kanamycin and amikacin. 5/6 isolates carried *aph(3’)- IIIa* gene along with other aminoglycoside resistance encoding genes analyzed.

pRI (*rep14*) was detected in only one isolate (0.4%) out of all the enterococcal isolates tested. This isolate was *E. faecium* with high level resistance to gentamicin, streptomycin, kanamycin and amikacin and the only virulent gene observed was *hyl* gene which encodes for hyaluronidase activity.
This isolate was also found to carry \textit{aac(6')- aph(2'')-Ia} bifunctional aminoglycoside resistance encoding gene, \textit{ermB} gene and \textit{ant(6')-Ia} genes respectively.

pUSA03 plasmid were first detected in \textit{S. aureus} with a function of dihydropteroate synthase which was resistant to clindamycin, tetracycline and mupirocin (McDougal \textit{et al.}, 2010). pUSA03 plasmid carried \textit{ileS2} gene which was found to play a major role in high level mupirocin resistance in community-acquired outbreak strain of \textit{S. aureus} (Perez-Roth \textit{et al.}, 2010).

In our study, 6/228 (2.6%) isolates carried pUSA03 (\textit{rep15}) in 5 \textit{E. faecium} and one \textit{E. avium} isolate. All the \textit{E. faecium} isolates were high level aminoglycoside resistant (HLGR, HLKR, HLAkR) while \textit{E. avium} was not a resistant isolate but was obtained from blood culture. 1 \textit{E. faecium} was from blood and the remaining 4 isolates were from urine specimen.

Rep16 family was observed in three plasmids from \textit{Staphylococcus}. Of which pSAS prototype was identified in a community acquired \textit{S. aureus} isolate. In our study, 9/228 (3.9%) isolates carried pSAS (\textit{rep16}).

3/228 (1.3%) isolates carried pEF418 (\textit{rep18}) was present in 2 \textit{E. faecium} and one \textit{E. faecalis} but all were resistant to high level aminoglycoside resistant phenotypes (HLGR, HLSR, HLAkR, HLKR).

pMG1-like plasmids are widely disseminated in vancomycin-resistant \textit{E. faecium} clinical isolates obtained from a hospital in the United States. It was first described by Ike \textit{et al.}, 1998 as pheromone-independent
gentamicin resistance conjugative plasmid pMG1 (65.1 kb) from a clinical isolate of *E. faecium* in Japan.

In a study by Song *et al.*, in 2013 from Norway, 120 amplified replicons were detected among 93 *E. faecalis* isolates by PCR based typing method. They detected 7 rep- families such as rep1, rep2, rep6, rep7, rep8, rep9 and rep17. Whereas, rep3, rep4, rep5, rep10, rep11, rep13, rep14, rep15, rep15, rep16, rep18, rep19 and unique replicon pMG1 were absent. Rep9 (pCF10) was the most predominant rep- family in their study.

In our study, 2/228 (0.8%) isolates carried unique plasmid pMG1. Interestingly, both isolates were high level resistant to all aminoglycosides tested and also carried gentamicin resistance encoding genes: *E. faecium* (1) obtained from urine carried bifunctional aminoglycoside resistance gene *aac(6')-aph(2'')-Ia, aph(3')-IIla, aac(6')-Ii* genes and *ermB* gene and *E. faecalis* (1) obtained from semen swab carried *aac(6')-aph(2'')-Ia, aph(2'')-Ic, aph(3')-IIIa, ermB* gene and *aac(6')-Ii* genes.

Apart from these sixteen identified plasmid replicons, four replicon types were not observed in our isolates which include pAW63 (*rep3*), pBMB67 (*rep12*), pRUM (*rep17*), and pUB101 (*rep19*).