A Study On High and Low level Drug Resistance Pattern among Clinical Isolates of Enterococci

Reena Rajan¹ and D Karthikeyan²

¹Department of Microbiology, Vinayaka Mission’s Kirupananda Variyar Medical College Salem, Tamil Nadu-636308, India.
²Department of Microbiology, Vinayaka Mission’s Medical College Karaikal, Tamil Nadu-609609, India.
*Corresponding author E-mail: reenarajan83@gmail.com

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The combined abilities of colonisation and both inherent and acquired resistance have made Enterococci a significant human pathogen. Aims & Objectives: This study was done to determine the Minimum Inhibitory Concentration of various antibiotics against Enterococci and to correlate the phenotypic and genotypic characteristics of Enterococci with low level and high level drug resistance. A total of 774 isolates of Enterococci obtained from various clinical samples were subjected to antimicrobial susceptibility testing by Kirby Bauer Disk Diffusion method. The Minimum Inhibitory Concentration of various antibiotics were determined by Vitek 2 automated system, agar dilution and E test.

Results: 15 out of 774 isolates showed the presence of vancomycin resistant genes by Multiplex PCR. 10 (90.91 %) isolates out of 11 E. faecalis with van A gene showed high level resistance to Penicillin (16-64 µg/ml). 8 (72.73 %) out of 11 isolates showed high level resistance to Gentamicin (512-1024 µg/ml). 6 (54.55 %), out of 11 isolates were resistant to â lactams. One isolate of E. faecalis from urine with van B gene showed high level resistance to Penicillin (32 µg/ml), Linezolid (≤ 8µg/ml), high level resistance to Gentamicin (1024 µg/ml), Fluoroquinolones (≤ 8µg/ml) and Macrolides (≤ 8µg/ml). Conclusion: Isolates of Enterococci resistant to glycopeptides, penicillin, Betalactams and aminoglycosides have important clinical implications in the treatment for infection.

Keywords: Minimum Inhibitory Concentration, Phenotype, Genotype, High Level drug resistance, Low level drug resistance.

The genus Enterococci have exhibited the potential to harbour and transfer drug resistant genes and have become an important pathogen in clinical settings. In Enterococci, lower affinity of Penicillin Binding Proteins (PBP’s) are responsible for decreased susceptibility to Penicillin’s. The Minimum Inhibitory concentration (MIC) of penicillin for Enterococci are higher than that for Streptococci and inhibition of PBP’s does not result in bactericidal activity in Enterococci ¹.

Enterococci display low to moderate level resistance to aminoglycosides due to slow uptake or permeability of these agents. All Enterococci have innate low level resistance to aminoglycosides with minimal inhibitory concentration ranging from 4 µg/ml to as high as 256 µg/ml. High level resistance to Gentamicin is associated with bifunctional enzyme possessing acetylase (6) and phosphotransferase (2) activities conferring resistance to all aminoglycosides.
except Streptomycin 2. Enterococci also exhibits acquired resistance via mutations in existing DNA or through acquisition of new DNA and high level resistance are usually due to the transferable plasmid mediated production of aminoglycoside inactivating enzymes 3.

Beta lactamase producing Enterococci exhibits inducible and constitutive low level resistance 4. In clinical isolates of E. faecium, a lactamase resistance is associated with mutations or overproduction of PBP5 with Ampicillin MIC of >256 mg/L in some strains. Isolates of E. faecium with MIC of Ampicillin d”64 mg/L may respond to high dose Ampicillin therapy 5.

Quinolone resistance occurs by mutation in the quinolone resistance determining regions of the genes that encodes gyrase and topoisomerase IV and have been observed in clinical isolates of Enterococci. These mutations prevent efficient binding of the antibiotics to the enzymes which enable DNA replication to continue despite the presence of antibiotics. The second mechanism contributes to quinolone resistance are Multidrug resistance efflux pumps (MDRs) on bacterial chromosomes. Inactivation of homolog of quinolone resistance (Qnr) identified in E. faecalis resulted in modest decrease in resistance to quinolones and over expression of gene results in increased resistance 6.

The most common form of acquired resistance to macrolides is production of an enzyme (erm B gene) that methylates a specific adenine in the 23 S rRNA of the 50S ribosomal subunit there by reducing the binding affinity of this drug for the ribosome, which also reduces the binding of Lincosamide and Streptogramin to the ribosome. An efflux pump encoded by transferrable mefA gene is known to pump macrolides out of the cell and confers low level resistance in Enterococci. 7

Linezolid has been reported to cure VRE endocarditis and other serious intravascular infections, bacteremia, UTI and skin and soft tissue infections 8. Tigecycline displays broad spectrum of activity and potency against Staphylococcus, Streptococci and Enterococci with MIC of 1.2µg/mL. Tigecycline may play a role in combination therapy with bactericidal agents such as Vancomycin, Gentamicin, Rifampicin or Daptomycin. Combinations of Daptomycin (8 mg/kg) plus Ampicillin plus Gentamicin and Daptomycin plus Gentamicin plus Rifampicin have been reported to be successful in cases of Vancomycin resistant endocarditis 9.

One of the major concerns that physicians face during treatment of Enterococcal infection is the probability of developing resistance during therapy, which may lead to therapeutic failures and contributes to patient mortality. Monitoring the resistance pattern of clinical isolates of Enterococci is a useful tool to obtain information on the prevalence of multidrug resistant isolates and thereby limiting the spread of bacterial resistance.

**Aim & Objectives**

1. To determine the Minimum Inhibitory Concentration of various antibiotics against Enterococci by Vitek 2 automated system.
2. To detect High Level Gentamicin Resistance among Enterococci by various phenotypic methods.
3. To correlate phenotypic and genotypic characteristics of Enterococci with low level and high level drug resistance.

**MATERIALS AND METHODS**

A total of 774 isolates of Enterococci obtained from various clinical samples were subjected to antimicrobial susceptibility testing on Muller Hinton Agar by Kirby-Bauer disk diffusion method and the plates were incubated for 16-18 hrs at 37°C. The diameter of the zone of inhibition was measured and zone size interpreted according to CLSI standards 10.

The antibiotic disks used were Penicillin (10 U), Amoxicillin Clavulanic acid (20/10ig), Erythromycin (15ig), High Level Gentamicin (120 ig), Linezolid (30ig), Teicoplanin (30 ig), Ciprofloxacin (5ig), Levofloxacin (5 ig) and Nitrofurantoin (300ig). Zones of inhibition were measured and recorded and the organism was interpreted as sensitive or resistant as per the recommendations from CLSI guidelines using Enterococcus faecalis ATCC 29212 as control.

Screening for isolates resistant to Vancomycin and High Level Gentamicin was done on Brain Heart Infusion agar containing 6µg/ml of vancomycin and 500 μg/ml of Gentamicin respectively. Inoculum of 0.01 mL of 0.5 McFarland’s was spot inoculated. Presence of more than 1 colony was interpreted as a resistant strain 11. Multiplex PCR was performed to detect the presence of vancomycin resistance
genes., vanA and vanB using the following primers (Sigma Aldrich, USA).  
Gene: VanA (732 bp)  
Primer: F (+) - GGGAAAACGACAATTGC (position: 176–192)  
R (-) - GTACAATGCGGCCGTTA (position: 907–891)  
Gene: VanB (647 bp)  
Primer: F (+) - ACGGAATGGGAAGCCGA (position: 169–185)  
R (-) – TGCACCCGATTTCGTTC (position: 815–799)

Minimum Inhibitory Concentration for Gentamicin was determined by agar dilution method and Epsilometer test. Agar dilution was performed by on Brain heart infusion agar by using 500 ig , 1000 ig and 2000 ig of Gentamicin. To detect Gentamicin resistance by E test, MIC strips coated with Gentamicin in a concentration gradient of 0.064-1024 ig/ml was used . MIC was read when the ellipse intersects MIC scale on the strip.  
Turbidometrically controlled bacterial pure growths were suspended into sterile physiological saline and this suspension was used to fill Vitek 2 Compact system and antimicrobial susceptibility testing cards. For biochemical identification of isolates in Vitek system, the following parameters were used: Growth in 6.5 % NaCl, â-glucuronidase, Trehalose, Arginine dihydrolase, D-sorbitol, Urease, Raffinose, D-galactose, D-mannitol, Sucrose, â-galactosidase, Salicin, L-pyrorbidinyl arylamidase, D-xylose, D-maltose, Methyl- â-D-glycopyranoside, D-ribose, â-glucosidase, â-mannosidase, Phosphatase etc.  

RESULTS  
Out of 774 samples studied , 726 (93.80 %) isolates were Enterococcus faecalis, followed by Enterococcus avium, 33 (4.26 %), Enterococcus faecium, 12 (1.55 %) and Enterococcus durans, 3 (0.39 %)  
Out of 30 Vancomycin resistant isolates obtained by agar screen method from 774 samples, resistant phenotype was detected in 18 isolates by Vitek 2 automated system. Out of 18 isolates showed the presence of vancomycin resistant genes by multiplex PCR.  
10 (90.91 %) isolates out of 11 E. faecalis with van A gene showed high level resistance to Penicillin (16-64 µg/ml), 8 (72.73 %) out of 11

| Sample | Isolates (n=15) | Genotypes | Resistance profile (MIC) |
|--------|----------------|-----------|-------------------------|
|        | Penicillin     | HLG       | β lactams               |
| Urine E. faecalis | vanA | 8-64 (R) | 512-1024 | Positive |
| (11 isolates) |       |           |                       |
| Pus E. faecalis | vanA & vanB | 32 (R) | 1024 (R) | - |
| Urine E. faecalis | vanB | 32 (R) | 1024 (R) | - |
| (1 isolate) |       |           |                       |
| Urine E. faecium | vanA | 2 (S) | 1024 (R) | - |
| Urine E. durans | vanA | 16 (R) | 1024 (R) | - |

| Isolate   | Genotype | Low Level Resistance ≤8µg | High Level Resistance (16-64 µg) |
|-----------|----------|---------------------------|---------------------------------|
| E. faecalis (11) | vanA | 1 (9.09) | 10 (90.9) |
| E. faecium (1) | vanA | 0 (0.00) | 0 (0.00) |
isolates showed high level resistance to Gentamicin (512-1024 µg/mL), 6 (54.55 %) out of 11 isolates were resistant to a lactams due to the modification of Penicillin binding proteins [Table 1]. One (9.09 %) out of 11 isolates were resistant to Linezolid, 10 (90.91 %) isolates showed resistance (e” 8µg/ml) and one isolate showed intermediate resistance (2 µg/ml) to Ciprofloxacin.

Nine (81.82 %) isolates showed resistance (e” 8µg/ml) and 2 (18.18 %) showed intermediate resistance (4 µg/ml) to Levofoxacin, 9 (81.82 %) isolates showed resistance (e”8µg/ml) and 2 (18.18 %) showed intermediate resistance (1-4 µg/ml) to Erythromycin. 6/11 (54.55 %) showed resistance and 2 (18.18 %) were with intermediate resistance to Nitrofurantoin.

One isolate of E. faecalis from urine with van B gene showed high level resistance to Penicillin (32 µg/ml), Linezolid (e” 8µg/ml), high level resistance to Gentamicin (1024 µg/ml), Fluoroquinolones (e” 8µg/ml) and Macrolides (e” 8µg/ml). One E. faecium isolate with van A gene showed susceptibility to Penicillin with high level resistance to Gentamicin (1024 µg/ml). One E. faecalis isolate from pus with both van A and van B gene showed high level resistance to Penicillin (32 µg/ml), Linezolid (e” 8 µg/ml), high level resistance to Gentamicin (1024 µg/ml), Fluoroquinolone(e” 8µg/ml) and intermediate resistance to macrolides(4 µg/ml).

No significant association was found between Vancomycin resistant genotypes of Enterococci and their high and low levels of resistance to Penicillin, Gentamicin and a lactams (p value >0.05) [Table:2, 3&4].

**DISCUSSION**

Enterococci possess remarkable ability to acquire antibiotic resistance determinants. The ability of these organisms to colonise the gastrointestinal tract of hospitalised patients for prolonged period is a crucial factor that influences the development of drug resistance. With the increased use of Vancomycin and wide spectrum antibiotics in the health care setting, these organism have been identified as common etiology of nosocomial infection.

**Enterococci exhibit decreased susceptibility to Penicillins and Ampicillins as a result of expression of low level Penicillin Binding Proteins (PBPs).** Isolates of Enterococci susceptible to Penicillin are predictably susceptible to Ampicillin, Amoxicillin, Ampicillin-Sulbactam, Amoxycillin-Clavulanate, Piperacillin and Piperacillin-Tazobactam for non a lactamase producing Enterococci. Isolates of Enterococci susceptible to Ampicillin cannot be assumed to be susceptible to Penicillin.

In the present study, 82.17 % isolates were resistant to Penicillin by disk diffusion method. About 19.90 % (154/774) isolates were with MIC range of 16-64 µg. In a similar study from Delhi, S Jain et al., 2011 have reported 100 % isolates resistant to Penicillin by disk diffusion method. In the present study, out of E. faecium isolates, 33.33 % showed MIC of e”

| Table 3. Relation between van genotypes and high and low level Gentamicin resistance |
|-----------------------------------------------|
| Isolate   | Genotype | Low Level Resistance (64-512 µg) | High Level Resistance (512-1024µg) |
|------------|----------|---------------------------------|-----------------------------------|
| E. faecalis (11) | vanA | 0 (0.00) | 8 (72.73) |
| E. faecium (1) | vanA | 0 (0.00) | 1 (100) |

| Table 4. Relation between van genotypes and high and low level resistance to a lactams |
|-----------------------------------------------|
| Isolate   | Genotype | Low Level Resistance (Penicillin Binding Protein modification) | High Level Resistance (a lactamase) |
|------------|----------|-------------------------------------------------|-----------------------------------|
| E. faecalis (11) | vanA | 6 (54.55) | 0 (0.00) |
| E. faecium (1) | vanA | 0 (0.00) | 0 (0.00) |
16 µg and 19.42 % of *E. faecalis* were resistant to Penicillin with MIC e” 16 µg. In a study from Chandigarh by V Gupta et al., 2007, 14 % isolates were with MIC of > 16 µg/ml for Penicillin 16. In a study reported by Triveda , 2016 from South India, Penicillin resistance was found to be higher among *E. faecalis* (64.51 %) isolates compared to *E. faecalis* (48.45 %) 17. In the present study no significant difference in resistance to Penicillin was found between *E. faecalis* and *E. faecium* isolates (p value >0.05).

High level resistance to â lactam antibiotics are seen in *E. faecium* due to the over production of low affinity Penicillin Binding Proteins (PBP’s). A variety of point mutation in PBP has been described in *E. faecalis* and *E. faecium* 18. In our study, by vitek 2 automated system, resistance to â lactams due to modification of Penicillin Binding Proteins was detected in 40.70 % isolates. A similar study by S Sivasankari et al., 2013 have reported 72.70 % *E. faecium* isolates resistant to Ampicillin compared to *E. faecalis* (24.30 %) 19. In a North Indian study, C S Aher et al., 2014 have reported 80.70 % *Enterococci* isolates resistant to Ampicillin 20.

The prevalence of *Enterococci* with high level Gentamicin resistance are increasing and varies by geographical region and are generally resistant to all aminoglycosides with the occasional exception of Streptomycin. Aminoglycoside Modifying Enzymes (AME’s) are the most important mechanism mediating high level resistance. These enzymes confer high level resistance to more than one aminoglycoside. A single strain of *Enterococci* may acquire several AME’s. Both *E. faecalis* and *E. faecium* may acquire â- adenyl transferase which confer HLR to Streptomycin 13. By disk diffusion method, 53.49 % showed high level Gentamicin resistance. In a study from North India, D K Mendratta et al., 2008 have reported, 46 % isolates resistant to both high Level Gentamicin(HLG) and High Level Streptomycin(HLS) by both disk diffusion and agar dilution method and combined resistance to both HLG and HLS was higher in *E. faecium* compared to *E. faecalis* 21. In the present study no significant difference in combined resistance to HLG and HLS was observed between *E. faecalis* and *E. faecium* (p value >0.05) where as HLGR was higher among *E. faecium* isolates. In our study 15.89 % isolates showed high level resistance to Gentamicin (2000 µg/ml) by agar dilution method and 33.66 % isolates showed resistance to both Penicillin and High Level Gentamicin.

By E test 18.22 % isolates showed MIC of 512 µg/ml and 16.67 % were with MIC e” 1024 µg/ml for Gentamicin. In the present study the results of Minimum Inhibitory Concentration for HLG were in concordance with disk diffusion method. A similar finding was reported in a study by V P Prakash, 2005 22. In a South Indian study P Jyothi et al., 2014 have reported 40 % isolates from urine showed high level resistance to Gentamicin and Streptomycin by both disk diffusion and Enz MIC strip method 23. In a South Indian study by E Padmasini et al., 2014 among clinical isolates of *Enterococci , 42.7 % were HLG (MIC e” 512 µg/ml) by E test 24. In a study report by S Mittal et al., 2016, high level gentamicin resistance was more common among *Enterococci* isolated from urine sample (41.50 %) compared to other clinical samples and HLGR was found to be more common in VRE isolates compared to VSE 25 where as our study showed no significant association between VRE and HLGR (p value >0.05).

Quinolone resistance in *Enterococci* is due to mutation in the quinolone resistance determining regions of the genes that encodes gyrase and topoisomerase IV enzymes. In the present study, 417 (57.44 %) *E. faecalis* isolates were resistant to Ciprofloxacin (MIC e” 8 µg/ml) and 414 (57.02 %) isolates to Levofloxacin (MIC e” 8 µg/ml). About 57.23 % isolates of *E. faecalis* were resistant to both Ciprofloxacin and Levofloxacin. 72.73 % *E. faecium* isolates were resistant to the fluoroquinolones tested. Among the fluoroquinolone resistant isolates, no significant difference was observed between *E. faecalis* and *E. faecium* isolates (p value >0.05). In a study from Tamilnadu S Sivasankari et al., 2013, 73.10 % *E. faecalis* and 81.80 % *E. faecium* isolates were resistant to Ciprofloxacin by disk diffusion method 19. In a North Indian study, N Gangurde et al., 2016 have reported 80.5 % *E. faecalis* and 86.2 % *E. faecium* resistant to Ciprofloxacin 26.

The most common form of acquired resistance to macrolides is production of enzymes that methylase a specific adenine in the 23S rRNA of 50 S ribosomal subunit which reduces binding affinity of macrolides to ribosomes. 77.27
% isolates of *E. faecalis* and 72.73 % *E. faecium* isolate were resistant to Erythromycin (MIC e" 8µg/ml) in this study. In the present study no statistically significant difference in Erythromycin resistance observed between *E. faecalis* and *E. faecium* (p value >0.05). Various Indian studies have reported Erythromycin resistance higher among *E. faecium* compared to *E. faecalis* 19,26.

Nitrofurantoin is active against *E. faecium* and *E. faecalis* and is effective in the treatment of VRE infection associated with urinary tract. A study conducted by G Zhanel et al., 2001 have reported that Nitrofurantoin is active against urinary isolates of *E. faecalis* and *E. faecium* 27. In the present study from urinary isolates, 14.93 % *E. faecalis* were resistant to Nitrofurantoin with MIC of 128 µg/ml. The MIC for 1.81% isolates of *E. faecalis* and 20.00 % *E. faecium* were 256 µg/ml and 128 µg/ml respectively. About 2.07 % isolates showed resistance to Nitrofurantoin in a study from Maharashtra by S Bose et al., 2012 28

A similar study by D Atray et al., 2016 have reported 80 % urinary isolates susceptible to Nitrofurantoin 29.

Linezolid resistance may be due to the presence of transferable plasmid borne cfr gene encoding methyl transferase or due to selective pressure imposed by antibiotic treatment. Linezolid is used in the treatment of infections caused by resistant gram positive bacteria particularly Vancomycin - resistant *Enterococcus faecalis*. Linezolid inhibits protein synthesis but at a different site from other agents that target the ribosome (Chloramphenicol, Macrolides, Lincosamides, Streptogramin, Aminoglycosides, Tetracycline). As a result, existing mechanisms of resistance to these agents do not confer cross-resistance to Linezolid 30. Our study reports 1.16 % isolates resistant to Linezolid. In a North Indian study by G Reena et al., 2013, 95 % isolates were susceptible to Linezolid 31. In a South Indian study, I Praharaj et al., 2013 have reported isolates with 100 % susceptibility to Linezolid 32. In a similar study by K Archana Rao et al., 2014, 12 % isolates showed resistance to Linezolid 33.

Tigecycline shows in vitro bacteriostatic activity against Vancomycin resistant *Enterococci*. Tigecycline was found to be the most effective drug against *Enterococci* in our study with 100 % susceptibility. Similar finding were reported from Indian studies 31,32.

As a result of mutation of chromosomal genes, Daptomycin resistance following therapy has been observed in clinical isolates of *Enterococci*. Daptomycin synergy has been described in vitro with Ampicillin, Cephalosporins, Imipenem, Rifampin and Gentamicin 34. In our study, 96.12 % isolates showed susceptibility to Daptomycin with MIC d" 4 µg/ml.

The overall antimicrobial pattern of the isolates showed significantly higher (p value < 0.05) percentage resistance to Penicillins, High level aminoglycosides, Macrolides, Fluoroquinolones and Linezolid among the clinical isolates of *Enterococci*.

**CONCLUSION**

The present study reports significantly higher percentage resistance to Penicillins, High level aminoglycosides, Macrolides, Fluoroquinolones and Linezolid among the clinical isolates of Enterococci. The most effective drug against Vancomycin Resistant *Enterococci* was found to be Tigecycline. No significant association was found between Vancomycin resistant genotypes of *Enterococci* and high and low levels of resistance to Penicillin, Gentamicin and â lactams.

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