A Study of Antimicrobial Resistance in Clinical Isolates of Enterococci

Dr. Saurabh Jain*, Dr. Yogyata Marothi**

*Assistant Professor Microbiology Department Chirayu Medical College and Hospital Bhopal (M.P.)

** Professor Microbiology Department R.D.Gardi Medical College Ujjain (M.P.)

Abstracts: Introduction: Recent years have witnessed a resurgence of interest in enterococci due to increasing resistance to antibiotics in term of both multiplicity of resistance and level of resistance to particular drugs. **Methodology:** This is a hospital based study, conducted in R.D. Gardi Medical College and C.R.G.Hospital Ujjain (M.P.). All clinical samples such as urine, blood, pus, sputum, ascitic fluid, stool etc. were collected from patients visiting OPD and admitted in CRGH in the study period of 1 and 1/2 year (January 2011-June 2012). Bacterial colonies suggestive of enterococci were further identified and antibiotic susceptibility testing done for each enterococcal isolates by DDT of Kirby Bauer on Muller Hinton Agar according to CLSI guideline.[3,5,6] MIC was also determine for ampicillin, gentamicin, streptomycin and vancomycin by Agar dilution.[6] **Result and Observation:** Only 2 enterococcal species isolated they were E.faecalis (86.62%) and E.faecium (15.18%). In pathogenic E.faecalis, 90.28% isolates showed resistance to penicillin, 65.28% to ampicillin, 62.5% to high level gentamicin (HLG) and 51.39% to high level streptomycin (HLS). One E.faecalis was resistant to vancomycin (VRE). All E.faecalis were sensitive to linezolid. In pathogenic E.faecium 84.61% isolates showed resistance to penicillin, 23.08 % to ampicillin, 53.85% to HLG and 69.23% to HLS. All E.faecium were sensitive to vancomycin and linezolid. In colonizing enterococci resistance is very low as compared with pathogenic. Multiple drug resistance (penicillinG, ampicillin, HLG, HLS) was more common (32.94%) in isolates of enterococci. **Discussion**: Study from Sevagram and Nagpur also isolated two species of Enterococci. Very high penicillin-G resistance was also observed in study from Nagpur.[15] In this study resistance to ampicillin in E.faecalis was 65.28% and in E.faecium 23.08%. A study done in Mumbai also find similar finding.[16] A study done by Rahangdale et al, which showed 49.59% high level resistance to gentamicin.[15] In this study HLSR in E.faecalis was 51.39% and in E.faecium 69.23%. Observation close to our study was reported from Nagpur.[15] Vancomycin resistance was not detected in E.faecium. Resistance to vancomycin is widely variable. Agrarwal et al, Titze-de-Almeida et al, Rahangdale et al, did not get any VRE in their study. [14,15,17] **Conclusion:** Multidrug resistances are the common problem. Although, at present, VRE is not a problem in our set up, its routine monitoring is essential, since it appears to be an emerging pathogen in India.[13] [Jain S NJIRM 2014; 5(6):81-87]

Key Words: Enterococcus, Antibiotic resistance, MIC

Author for correspondence: Dr. Saurabh jain, Assistant Professor, Dept. Of Microbiology, Chirayu Medical College and Hospital Bhopal, India **Mo:** +91-9893877252 **Email:** drsaurabhjainmd@gmail.com

Introduction: Recent years have witnessed a resurgence of interest in enterococci due to increasing resistance to antibiotics in term of both multiplicity of resistance & level of resistance to particular drugs. Enterococci particularly E.faecium, always have a high intrinsic level of resistance to antimicrobial agents.

As enterococci are present as commensal flora in GIT, enterococcal infection is thought to be endogenous, arising from patient own flora. Enterococci are the second most common cause of nosocomial urinary tract and the third most common causeof nosocomial bacteremia.[1,2] The mainstay in the treatment of serious enterococcal infection is the synergistic effect of penicillin/ampicillin (or vancomycin) & aminoglycoside.[3] However high level resistance i.e. MIC ≥2000 µg/ml to gentamicin & other aminoglycoside is seen with increased frequency.[3] In the recent years, enterococci resistant to vancomycin are also isolated.[4] Vancomycin resistant enterococci (VRE) commonly have penicillin/ampicillin & high level aminoglycoside resistance resulting in bacterial strain that may be untreatable with currently available antibacterial agents.

Aims: The aim of present study is to identify enterococci resistant to high level aminoglycosides, penicillin-G, ampicillin, vancomycin and linezolid.

Objectives: (1) Isolation & Species identification of enterococci. (2) To study antimicrobial resistance pattern of enterococci by Disc Diffusion method.

(3) To determine the level of resistance to penicillin, aminoglycoside and vancomycin.

Methodology: Current study done in Microbiology Department of R.D.Gardi Medical College, Ujjain (M.P.) in a period of one and half year from January 2011 to June 2012. Clinical specimens viz urine, blood, pus, CSF, stool, fluids and aspirate were collected aseptically as per the standard recommendation from patient admitted in various wards as well as patient attending O.P.D. of R.D.Gardi Medical college, Ujjain and transport to laboratory.[3] Every specimen received in the Microbiology laboratory was processed according to the recommended procedures for the isolation and identification of bacterial isolates.[3] Enterococci were selected by colony morphology from the primary isolation plates. Suspected colonies of the genus Enterococcus on blood agar were small (0.5-1mm) size, semitransparent, smooth, low convex discs.[3,4] It showed no hemolysis, sometimes showed α or β haemolysis. On gram staining enterococci appear as pairs of oval cocci, the cells in a pair arranged at an angle to each other. These colonies of enterococci are catalase negative.

Enterococci were identified on the basis of their ability to hydrolyse of L-pyrrolidonyl-b-naphthylamide (PYR), salt-resistant growth (6.5% NaCl), and growth resistant to 40% bile with esculin hydrolysis.[3] Antibiotic susceptibility testing done for each enterococcal isolates by DDT of Kirby Bauer on Muller Hinton Agar according to CLSI guideline.[5] Antimicrobial agents viz β-lactams (Penicillin, Ampicillin), Aminoglycosides (High level Gentamicin and High level Streptomycin), Glycopeptides (Vancomycin), Oxazolidinones (Linezolid) tested for enterococcal isolates from all sample and Nitrofurantoin, Ciprofloxacin, Levofloxacin, Norfloxacin, Tetracycline used only for urine.

Determination Of MIC Of Antomicrobials By Agar Dilution[6]: After autoclaving, in Mueller Hinton agar calculated volume of antibiotic solution were added and make a final concentration of 8/16/32/64 µgm/ml of ampicillin, 500/1000/2000 µgm/ml of gentamicin and 1000/2000/4000 µgm/ml streptomycin. For vancomycin MIC testing commercially avialable E test strip was used. Strip contained 2 to 256 µgm/ml vancomycin concentration in the form increasing gradient. Inoculum of each selected isolates was prepared as for disc diffusion test described earlier giving a final concentration of 10^5cfu/ml. A loopful of suspension of isolates under test was inoculated on a specified area as shown in photo on a series of MHA plates each containing different concentration of test antibiotic (ampicillin, gentamicin, and streptomycin). In addition an antibiotic free MHA plates was similarly inoculated to check the quality of the inoculum. Further, for quality control E.faecalis ATCC 29212 was also inoculated on both antibiotic incorporation and antibiotic free MHA plate on a specified area. The plates were incubated at 37ºC for 18 hours. For vancomycin, the dried surface of a Mueller-Hinton agar plate was inoculated with test strain and E-strip was applied on the agar surface with the help of forcep and slightly press the E-strip.

The plates were examined after 18 hours of inoculation. Those bacterial isolates that did not produce growth at the inoculum site were interpreted as sensitive to the concentration of antibiotic in that agar plate and those bacterial isolates which produce even a single colony at the inoculum site were interpreted as resistance to the concentration of the antibiotic in that agar plate. The MIC of the test antibiotic for the bacterial isolates under the test was defined as the lowest concentration of the test antibiotic that did not produce growth at inoculums site. In E-strip testing for vancomycin at the point where lowest zone of inhibition present around concentration of antibiotic was labelled as MIC of vancomycin for that isolates of enterococci.

Result and Observation: A total of 112 Enterococci were isolated from clinical specimens in study period. The highest yield was from urine (53.58%) followed by stool (24.11%), blood (9.82%), pus (9.82%), pleural fluid (0.89%), ascitic fluid (0.89%) and CSF (0.89%). Isolates obtained from urine (with significant bacteriuria), blood, pus, pleural fluid, ascitic fluid, CSF were considered as a pathogenic enterococccal isolates (75.89%) and from stool were considered as colonising enterococci (24.11%).[3] Only 2 enterococcal species isolated

they were E.faecalis (86.62%) and E.faecium (15.18%).

Table 1 Antimicrobial resistant profile for isolates of enterococci by DDT.

Antimirobial (For all sample)	Number of resistant isolates (n=112)											
	Pathogenic E.faecalis (n=72)			Colonising E.faecalis(n=23)			Pathogenic E.faecium (n=13)			Colonising E.faecium(n=4)		
	R	I	Total (%)	R	I	Total (%)	R	I	Total (%)	R	I	Total (%)
β-lactams												
Penicillin	65	0	65(90.28)	18	0	18(78.26)	11	0	11(84.61)	4	0	4(100)
Ampicillin	47	0	47(65.28)	5	0	5(21.74)	0	3	3(23.08)	0	0	0
Aminoglycosides												
Gentamicin	44	1	45(62.5)	1	0	1(4.35)	7	0	7(53.85)	2	0	2(50)
Streptomycin	37	2	37(51.39)	1	0	1(4.35)	9	0	9(69.23)	0	0	0
Glycopeptides												
Vancomycin	1	0	1(1.39)	0	0	0	0	0	0	0	0	0
Oxazolidinones												
Linezolid	0	0	0	0	0	0	0	0	0	0	0	0
Antimicrobial (only for urine) (n=60)												
Nitrofurantoin	20	0	20(40)				1	0	1(10)			
Ciprofloxacin	42	0	42(84)				10	0	10(100)			
Levofloxacin	44	0	44(88)				10	0	10(100)			
Norfloxacin	45	0	45(90)				10	0	10(100)			
Tetracycline	37	0	37(74)				9	0	9(90)			

• R=Resistance, I=Intermediate

Table 2: Pattern of multiple drug resistance (R-Pattern) in pathogenic strains of enterococci

R-Pattern	Number of isolates *E.faecalis*	*E.faecium*
One drug		
PG	9	1
HLS	1	1
HLG	0	1
Two drug		
PG+AMP	8	0
PG+HLG	1	2
PG+HLS	2	3
HLG+HLS	1	0
Three drug		
PG+AMP+HLG	11	1
PG+AMP+HLS	2	0
PG+HLG+HLS	4	2
Four drug		
PG+AMP+HLG+HLS	26	2
Five drug		
PG+AMP+HLG+HLS+VA	1	0

PG=penicillinG, AMP=ampicillin, HLG=high level gentamicin, HLS=high level streptomycin, VA=vancomycin.

E.faecalis was the most common in all type of sample including both pathogenic as well as colonising. Pathogenic as well as colonising isolates of enterococci were mostly isolated from female patient. Resistant patterrn for enterococcal isolates were shown in table 1. Table 2 showed that 28 (32.94%) isolates of enterococci were resistance to four drugs (penicillinG, ampicillin, HLG, HLS). 20 (23.53%) isolates were resistant to three drugs out of which 12 (14.11%) isolates were resistance to penicillinG, ampicillin, HLG. In E.faecalis all isolates (n=52) which were resistant to ampicillin by DDT, showed high level resistance (MIC ≥64µgm/ml) to ampicillin. In E.faecium all the three isolates, which showed decrease susceptibility by DDT were susceptible by agar dilution method with MIC of <8µgm/ml. Out of 95 isolates of E.faecalis HLG resistance by DDT was seen in 45 isolates (MIC >2000 µgm/ml) and one showed decreased susceptibility (MIC <500 µgm/ml). In E.faecium HLG resistance by DDT was seen 9 out of 17 isolates. Agar dilution method showed the same result and MIC for all isolates were >2000 µgm/ml. Out of 95 isolates of E.faecalis HLS resistance by DDT was seen in 36 isolates (MIC 4000->8000 µgm/ml) and 2 showed decreased susceptibility (MIC <2000 µgm/ml). In E.faecium HLS resistance by DDT was seen 9 out of 17 isolates. Agar dilution method show the same result and MIC for all isolates were >8000 µgm/ml. In E.faecalis High level resistant (HLR) to ampicillin, gentamicin and streptomycin seen in 27 out of 95 isolates (28.42%) (table 3).

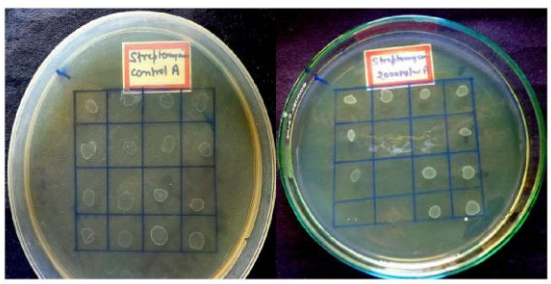
Table 3 Concomitant HLR to ampicillin and aminoglycosides in pathogenic/ colonising isolates of enterococci

Species of enterococci	Number of isolates (n=112) HLR to ampicillin	HLR to gentamicin	HLR to streptomycin	HLR to ampicillin, gentamicin and streptomycin
E.faecalis (95)	52(54.74%)	45(47.37%)	36(37.89%)	27(28.42%)
E.faecium (17)	0	9(52.94%)	9(52.94%)	0

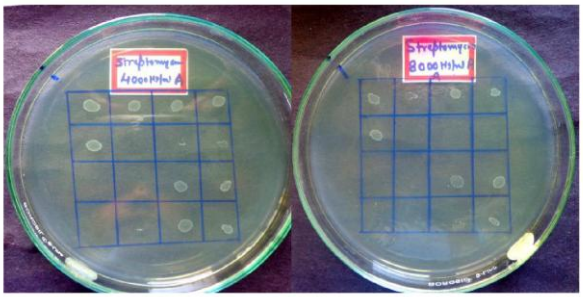
*HLR= high level resistance

In E.faecium HLR to ampicillin was not seen but HLR to streptomycin more common than E.faecalis as shown in table 3, one E.faecalis showed no zone in Disc Diffusion Test was labelled as VRE. On MIC, this isolate showed HLR for vancomycin (>256 µgm/ml). If any isolate of enterococci has MIC ≥64 µgm/ml for vancomycin, it was labelled as HLR vancomycin (CLSI, 2010).

MIC OF STREPTOMYCIN FOR 46 ISOLATES OF ENTEROCOCCI

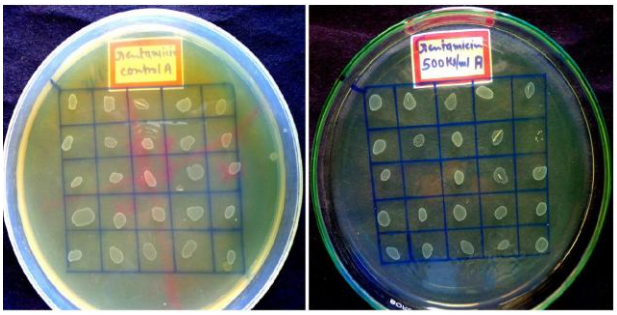


CONTROL 2000µgm/ml

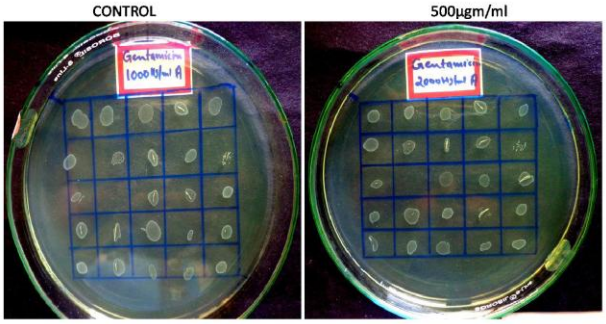


4000 µgm/ml 8000 µgm/ml

MIC OF GENTAMICIN FOR 52 ISOLATES OF ENTEROCOCCI

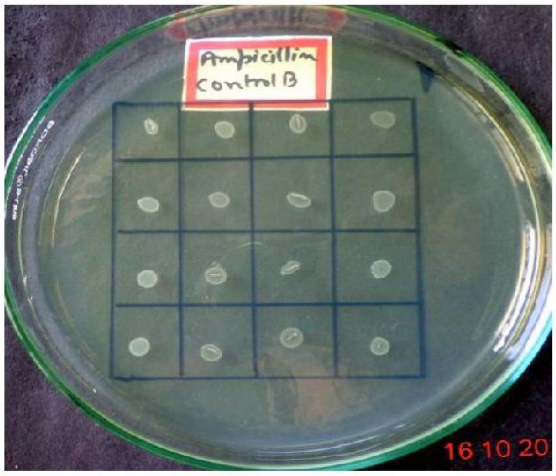


CONTROL 500µgm/ml

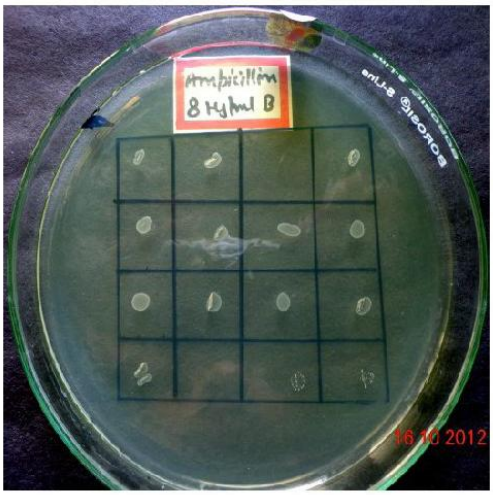


1000 µgm/ml 2000 µgm/ml

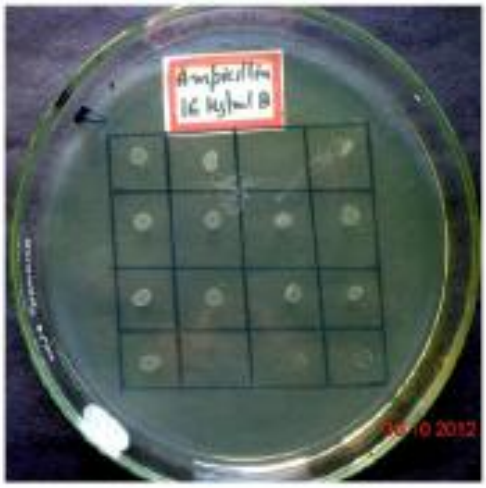
MIC OF AMPICILLIN FOR 50 ISOLATES OF ENTEROCOCCI



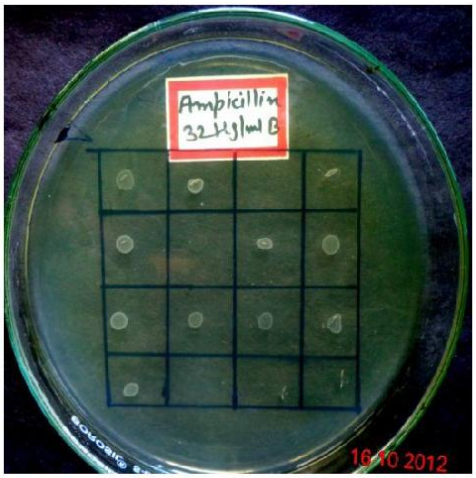
CONTROL



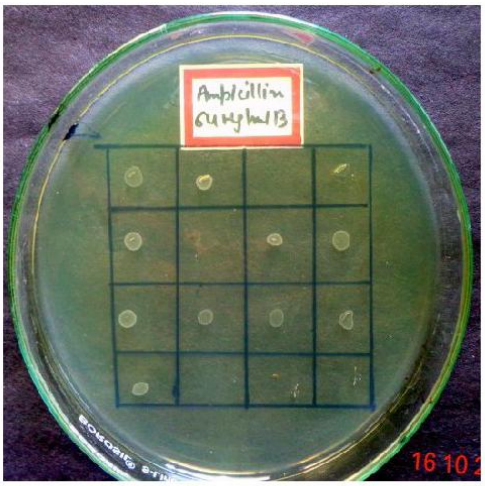
8 µgm/ml



16 µgm/ml



32 µgm/ml



64 µgm/ml

Discussion: Recent years have witnessed 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.[7,8,9] In the present study, enterococci were mostly isolated from urine (53.58%), pus (9.82%) and blood (9.82%). Recently, Bose et al from Maharashtra published the similar finding. According to them enterococci were isolated most commonly from urine (62.13%), blood (27.02%), pus (7.9%).[10] Similar finding were also reported from Manipal (Sikkim) and by Agarwal et al from Lucknow.[11,12] Two enterococcal species were identified: *E.faecalis* (86.62%) and *E.faecium* (15.18%). Other enterococcal species were not isolated in present study. A study done by Bose et al reported similar finding.[10] Study from Sevagram and Nagpur also isolated two species of Enterococci namely *E.faecalis* (most common) and *E.faecium*.[13,14] Recently from Manipal (Sikkim) a study done by Adhikari et al isolated additionally three more species of Enterococci, namely *E.casseliflavus, E.durans*, *E.dispar*. In their study *E.faecium* was 3rd most common.[11] Similarly Agarwal et al also isolated *E.avium*, *E.dispar*, *E.cecorum*, *E.hirae* from various clinical samples.[12] *E. gallinarum* (2.44%) and *E. raffinosus* (0.81%) were isolated from Nagpur.[15] *E.faecium* was the most common (80.77%) species of enterococci in the study of Karmarkar et al.[16]

In our study resistance to penicillinG was very high 90.28% in *E.faecalis* and 84.61% in *E.faecium*. Similar finding was observed in study from Nagpur where 89.43% isolates of enterococci were resistant to penicillinG.[15] Titze-de-Almeida et al from Brazil found *E.faecium* which was less resistant (11.1%) than *E.faecalis* (27.6%).[17] Karmarkar et al found *E.faecium* (71.43%) to be more resistant to penicillinG as compared to *E.faecalis* (40%).[14] Similarly finding are also reported from Nagpur.[14] In this study resistance to ampicillin in *E.faecalis* was 65.28% and in *E.faecium* 23.08%. A study done in Mumbai also find similar finding.[16] Rahangdale et al found 43.9% enterococci were resistant to ampicillin.[15] In a study from Lucknow, *E.faecium* (61.11%) was found to be more resistant to ampicillin than *E.faecalis* (16.67%).[12] Similar result were also published by Agrarwal et al. Exact reason for this difference with our study is not known but this may be due to geographic variation.[14] In this study all isolates (n=52) of *E.faecalis* which were resistant to ampicillin by DDT, showed high level resistance (MIC ≥64µgm/ml) to ampicillin. Williamson JC et al also found high level ampicillin resistance in all isolates of enterococci. Similarly *E.faecium* strains were susceptible to ampicillin in Brazil study as against studies of Lucknow and Nagpur.[17]

In our study HLGR in *E.faecalis* was 62.5% and in *E.faecium* 53.85%. A study done by Rahangdale et al, which showed 49.59% high level resistance to gentamicin.[15] But in study from Lucknow lower prevalence of HLGR was noted in *E.faecalis* (10.53 %) as well as in *E.faecium* (6.45%).[12] Similarly Salem Bekhit et al also found low prevalence of HLGR in *E.faecalis* (22.3 %) as well as in *E.faecium* (18.5%) as compared to our study.[18] In contrast, high prevalence of HLGR was noted in *E.faecalis* (100%) as well as in *E.faecium* (85.71%) in Mumbai.[16]

In this study HLSR in *E.faecalis* was 51.39% and in *E.faecium* 69.23%. Observation close to our study was reported from Nagpur.[15] But a study from Lucknow showed low prevalence of HLSR in *E.faecalis* (21.05%) as well as in *E.faecium* (22.22%).[12] Recently Salem Bekhit et al and study from Sevagram also found low prevalence as compared to our study.[13,18]

In this study high level resistance to both gentamicin and streptomycin were 33.68% for *E.faecalis* and 23.53% for *E.faecium.* Mendiratta et

al from Sevagram, Maharashtra reported combined resistance to both drug were 7.8% in *E.faecalis* and 59.1% in *E.faecium.*[13]

Only one isolate of *E.faecalis* (1.39%) was found resistant to vancomycin by DDT and resistance was high level (MIC >256µgm/dl). Vancomycin resistance was not detected in *E.faecium*. Resistance to vancomycin is widely variable. Agrarwal et al, Titze-de-Almeida et al, Rahangdale et al, did not get any VRE in their study.[14,15,17] But in a study from Mumbai 10% vancomycin resistance in *E.faecalis* and 28.57% in *E.faecium* was noted.[16] Similarly, Salem Bekhit et al found 1.8% vancomycin resistance in *E.faecalis* and 18.5% in *E.faecium*.[18] Agarwal et al from Lucknow found 9.52% vancomycin resistance in *E.faecalis* but did not find any vancomycin resistance in *E.faecium*.[12] Although, at present, VRE is not a problem in our set up, its routine monitoring is essential, since it appears to be an emerging pathogen in India.[13] Some of VRE strains remain susceptible to tetracycline, erythromycin, chloramphenicol, fluoroquinolones, or rifampicin and used as monotherapy or usually combining 2 or 3 antibiotics.[19]

Tetracycline is effective drug in the enterococcal isolates of urine only. In this study resistance to tetracycline in *E.faecalis* was 74% and in *E.faecium* 90%. Salem Bekhit et al also found 62.7% tetracycline resistance in *E.faecalis* and 85.2% in *E.faecium* [18]. Similar result are also published from Brazil.[17] In a study from Lucknow, *E.faecalis* was more resistant to tetracycline showing 76.19% resistance as compared to *E.faecium* (72.73%).[14]

In our study resistance to ciprofloxacin in *E.faecalis* was 84% and in *E.faecium* 100%. Agarwal et al from Nagpur also found just similar resistance profile to ciprofloxacin in *E.faecalis* (64.3%) and in *E.faecium* (95.2%).[14] But some authors found decreased resistance to both species of enterococci as compare to this study. Like in a study from Saudi Arabia, 49.4% resistance in *E.faecalis* and 51.9% in *E.faecium* was found.[18] Similarly, some authors also found that *E.faecalis* was more resistance to ciprofloxacin then *E.faecium*. Agarwal et al found 64.29% resistance in *E.faecalis* and 51.51% resistance in *E.faecium*.[12] Similar finding showed in a study from Brazil and by Karmarkar et al.[3,17]

Our study showed that only 40% *E.faecalis* and 10% of *E.faecium* were resistant to nitrofurantoin. Similar result published from Nagpur by Rahangdale et al.[15] According to them enterococci showed only 22.76 % resistant to nitrofurantoin. Nitrofuratoin is a reserve drug for treatment of enterococccal urinary tract infection as shown in our observation. In a study of Canada, it was found that all VRE were susceptible to nitrofurantoin.[20]

Conclusions: Enterococcal infection continously rising and most common factor is antibiotic resistant to various antibiotic. PenicillinG is not effective antibiotic in our setup. Resistance to ampicillin and amminoglycosides are also at high level. MDR in enterococci is very high in our study place. High level resistance in aminoglycoside as well as in ampicillin is very high that may lead to failure in synergism. Because of intrinsic resistance to much antibiotic and high level resistance to effective antibiotic very few antibiotics are left for treatment of enterococccal infection. Although prevalence of VRE is low in our study place at present but this may rise. So continuous monitoring is required and recommended. Although, at present, VRE is not a problem in our set up, its routine monitoring is essential, since it appears to be an emerging pathogen in India.[4] Nitrofuratoin is a reserve drug for treatment of enterococccal urinary tract infection as shown in our observation.

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Conflict of interest: None

Funding: None