ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 18, Issue, 01, pp.36041-36045, January, 2026
DOI: https://doi.org/10.24941/ijcr.50037.01.2026

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION OF VANCOMYCIN BY AGAR DILUTION METHOD FOR DETECTION OF VANCOMYCIN RESISTANT ENTEROCOCCI ISOLATED FROM VARIOUS CLINICAL SPECIMENS

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ARTICLE INFO

Article History:
Received 20th October, 2025
Received in revised form
17th November, 2025
Accepted 28th December, 2025
Published online 30th January, 2026

Keywords:

Enterococci, Vancomycin Resistant Enterococci, MIC. Agar dilution method.

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ABSTRACT

Background: *Enterococci* plays an important role in nosocomial infections and also become resistant to a variety of antimicrobials through intrinsic and acquired mechanisms. Emergence of Vancomycin Resistant *Enterococci* (VRE) has possessed a greater threat in its treatment. Detection of MIC of Vancomycin is important to treat VRE. **Objective:** To determine the prevalence and the antibiotic susceptibility pattern of *Enterococci* isolated from various clinical specimens & to determine the MIC of VRE by agar dilution method in our institute. **Material And Methods:** The study was conducted at the Department of Microbiology at Shridevi Institute of Medical Sciences & Research Hospital over a period of one year from June 2022 to July2023. All the *enterococci* obtained from urine, pus, blood and body fluids were included in the study. The isolates were confirmed and speciation was done by arginine deamination and fermentation of arabinose, mannitol, raffinose, and sorbitol. Antimicrobial susceptibility was determined by Kirby Bauer disk diffusion .MIC of vancomycin was determined by agar dilution method for all the *enterococci* isolates which showed intermediate and resistance to Vancomycin by Kirby Bauer disc diffusion method. **Results:** Amongst the total 60 Enterococcal isolates, 37 isolates (61.66 %) were *Enterococcus faecalis*, 23 isolates (38.33%) were *Enterococcus faecium*. Out of the 60 isolates, 28 (46.66%) were from pus followed by 22 (36.66%) were from urine, 9 (15 %) from blood, 1(1.66%) from pleural fluid. Antibiotic susceptibility pattern of *Enterococci* showed high sensitivity Teicoplanin 59(98.33 %) followed by Linezolid 55(91.66%) and High-level Gentamicin 47(78.33%).Out of the 11 isolates intermediate or resistant to vancomycin by Kirby Bauer disc diffusion method, 10 were sensitive by agar dilution method, whereas the only one isolate was resistant with MIC of 32 µg/ml .Occurrence of VRE in our setting is 1.6 %. **Conclusion:** Disk diffusion testing for vancomycin in enterococci can be unreliable and requires confirmation by an MIC method. Early detection, treatment and preventive action will help to limit the serious consequences caused by vancomycin resistant enterococcal infection

Citation: Robin Samuel and Parimala, T.V. 2026. "Determination of Minimum Inhibitory Concentration of Vancomycin by Agar dilution method for detection of Vancomycin Resistant Enterococci isolated from various clinical specimens.". ***International Journal of Current Research, 18, (01), 36041-36045.***

INTRODUCTION

E*nterococci* are pairs or chains of facultatively anaerobic, gram-positive cocci that naturally inhabit the environment (soil, plants, etc.) and is an essential constituent of the normal human/animal gut flora[1]. Enterococcus species are an important agent causing complicated UTIs, bacterial sepsis, endocarditis, intra-abdominal and pelvic infections, post-operative wound infections and soft tissue infections, neonatal sepsis and rarely meningitis[2]. Although more than one dozen species of *enterococci* have been identified, *Enterococcus faecalis* was the most common species associated with nosocomial infections, followed by *Enterococcus faecium*, and both species are responsible for about 95% of infections caused by *enterococci*.Other Enterococcus species, *E. gallinarum, E. casseliflavus, E durans, E. avium, and E. hirae*, are isolated much less frequently and account for less than 5% of clinical isolates[3].

two decades, risen in importance and are now amongst the commonest organisms causing hospital-acquired infection (HAI). Serious enterococcal infections are often refractory to treatment and mortality is high[4]. *Enterococci* have become increasingly important not only because of their ability to cause serious infections but also because of their increasing resistance to many antimicrobial agents. The natural ability of enterococci to acquire, accumulate, and share extra chromosomal elements encoding virulence traits or antibiotic resistance genes, in part, explains their increasing importance as nosocomial pathogens[5]. Even though vancomycin resistant enterococci (VRE) were first reported in 1986, from the UK and France, in recent years, they have been found to be disseminated all around the world[6]. VRE infection is associated with large attributable burdens, including excess mortality, prolonged in-hospital stay, and increased treatment costs[7]. Pandemic spread of VRE and acquisition of resistance to newer antimicrobials warrant continued surveillance and early detection of VRE along with Minimum Inhibitory

Concentrations (MIC)[8]. Identification and specification of enterococcal isolates possess substantial impact on therapeutic choice since antimicrobial susceptibility pattern varies between the species[6].\

MATERIAL AND METHODS

This was a prospective cross-sectional study conducted in the Department of Microbiology, Shridevi Institute of Medical Sciences & Research Hospital, Tumkur, Karnataka, South India from June 2022 to July 2023. Ethical approval was obtained from the institutional ethical committee.

Inclusion criteria:

- *Enterococci* isolated from clinical samples such as Urine, Pus, Blood, pleural fluid, body fluids etc.
- *Enterococci* isolated from clinical specimens from Age group 1year to 80years

Exclusion criteria

- Isolates other than *Enterococci*.
- *Enterococci* isolates from the stool samples, vaginal swab, sputum

Sample collection and transportation: The details of each sample will be recorded carefully in each case like details of the patients' name, age, sex, registration number, ward, diagnosis and site from which sample was collected etc. All the clinical samples were collected from each study participant aseptically..All the collected samples were transported to the Central laboratory,Department of Microbiology,SIMS&RH.

Sample Processing: The blood culture bottles were incubated at 37 °C and were observed daily after 48 h for 5 consecutive days for presence of turbidity, hemolysis, gas formation or color changes which are evidence of microbial growth. If the culture bottle does not show any growth within 7 days, it was reported as negative. Whenever visible growth appears, the bottle was opened aseptically; a small amount of broth was taken with a sterile loop and subcultured on Blood Agar and MacConkey Agar. Urine samples were inoculated on Blood agar, MacConkey Agar with a 10 µl calibrated loop and also other clinical samples were directly inoculated on Blood Agar and MacConkey agar .All the inoculated plates were incubated at 37 °C for 24 h . Presumptive identification of *Enterococcus* was done on the basis of colony characteristics, Gram's staining & Catalase test. Confirmation was done by growth in 6.5% NaCl, Bile esculin hydrolysis, potassium tellurite reduction, arginine dihydrolase test and sugar fermentation test such as of glucose, mannitol , sucrose and arabinose fermentation. All the samples were processed according to a standard operating procedure[2].

Antimicrobial susceptibility testing: It was done by Kirby Bauer Disc Diffusion Method on 5% Muller-Hinton agar plates as per CLSI guidelines [9].The plates were overlaid with the inoculums turbidity equivalent to that of a 0.5 McFarland Standard. Antimicrobial discs applied on the surface of agar plates were incubated overnight at 30-35˚C in ambient air. The following antibiotics were tested- Pencillin (10 U), Ampicillin 10 µg), Vancomycin (30 µg), Teicoplanin (30 µg), Linezolid (30 µg), Ciprofloxacin (5 µg), Gentamicin (10 µg), High level Gentamic.in (120 µg), Tetracycline (30 µg), Erythromycin (15 µg). Antibiotic susceptibility with Fosfomycin (200 µg. Nitrofurantoin (300 µg) disc was done only for the urinary isolates. For Vancomycin disc isolates showing zone size ≥17 mm for considered as sensitive, 15-16mm as Intermediate sensitive and ≤14mm as Resistant by Kirby Bauer Disc diffusion method.

Screening by Agar dilution method: The isolates which are vancomycin resistant enterococci and vancomycin intermediate resistant by disc diffusion method were screened by agar dilution method. Procedures are undertaken as recommended by standard microbiological guidelines, and results was reported with reference to CLSI guidelines[10]. For agar dilution method, Muller hinton broth were incorporated with vancomycin was prepared in different concentrations i.e. 0.5ug, 1ug, 2ug,, 4ug, 8ug, 16ug and 32ug per ml. Lowest concentration of antibiotic, inhibiting visible growth after recommended incubation, is regarded as MIC. Isolates with MIC ≤4µg/ ml were considered as a sensitive, with MIC of 8 to 16µg/ ml as intermediate and MIC of ≥32µg/ml as considered as Resistant .Muller Hinton agar media and vancomycin powder of potency 950 µg/mg obtained from HiMedia Laboratories, India, were used in this study.

Quality control: All culture media were prepared following the manufacturer's instruction. Batch of prepared media was checked for sterility by incubating samples of the plate at 37 °C for 24 h. *E. faecalis* ATCC 29212 and *S.aureus* ATCC 25923 is used as an ATCC control.

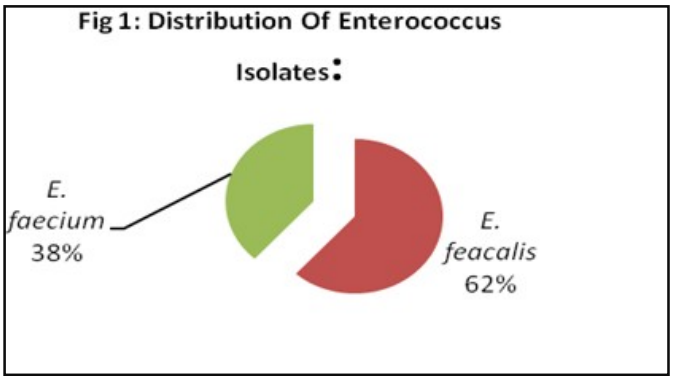
Statistical analysis: Data was entered and analysed in Microsoft Excel and percentages of Enterococcal species and their antibiotic susceptibility was calculated.

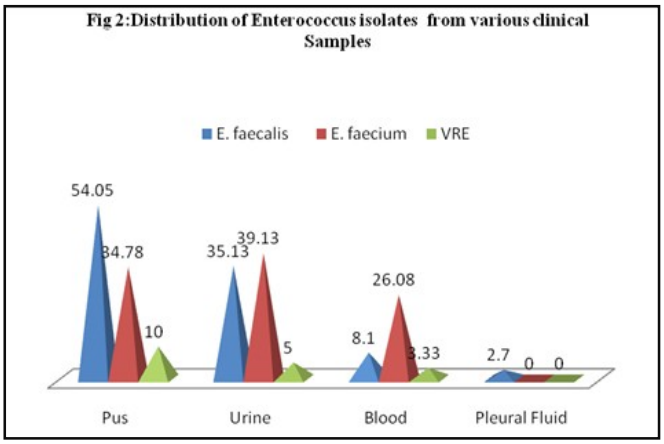
RESULTS

A total of 2600 culture samples were received from the microbiology laboratory during the study period. Out of these, 60 samples showed *Enterococcus* species growth from various clinical specimens.Of these, 37(61.66 %) were *E. faecalis* and 23 (38.33 %) were *E. faec*ium (Table 1).

Table 1. Distribution Of Enterococcus Isolates:

S/N	Name of the species	Number of isolates	PERCENTAGE
1.	*E. feacalis*	37	61.66
2.	*E. faecium*	23	38.33





Majority of enterococcus isolates were obtained from pus 28(46.66%) followed by urine 22(36.66%) and blood 9(15%).Similarly most of of *E.faecalis* isolates were from Pus 20 (54.05%) followed by Urine 13(35.13%). Whereas maximum *E. faecium* isolates were isolated from Urine 9(39.13 %) followed by Pus 8 (34.78%). Also most of

Table 2. Distribution of Enterococcus isolates from various clinical Samples

Specimen Type	*E. faecalis* (N=37)	Percentage	*E. faecium* (N=23)	Percentage	Total	Percentage	VRE (N=11)	Percentage
Pus	20	54.05	8	34.78	28	46.66	6	10
Urine	13	35.13	9	39.13	22	36.66	3	5
Blood	3	8.10	6	26.08	09	15.00	2	3.33
Pleural Fluid	1	2.70	0	0	01	1.66	0	0

Table 3. Antibiotic Sensitive Pattern of Enterococcus isolates

S/N	ANTIBIOTICS	*E.faecalis* (N=37)		*E.faecium* (N=23)	
		Sensitive	Resistant	Sensitive	Resistant
1	Ampicillin (10 µg)	15(40.54%)	22(59.45%)	10(43.47%)	13(56.52%)
2	Penicillin (10unit)	20(54.05%)	17(45.94%)	12(52.17%)	11(47.82%)
3	High Level Gentamycin (120µg)	29(78.37%)	8(21.62%)	18(78.26%)	05(21.73%)
4	Ciprofloxacin (5µg)	27 (72.97%)	10(27.02%)	16(69.56%)	07(30.43%)
6	Teicoplanin (30 µg)	37(100%)	0(0%)	22(95.65%)	01(4.34%)
7	Linezolid (30 µg)	35(95.59%)	2(5.40%)	20(86.95%)	03(13.04%)
8	Tetracycline(30 µg)	24(64.86%)	13(35.13%)	15(65.21%)	08(34.78%)
	Urine isolates	N=13		N=9	
9	Nitrofurantoin(300µg)	12(92.30%)	1(7.69%)	6(71.42%)	3(33.33%)
10	Norfloxacin(10µg)	10(76.92%)	3(23.07%)	4(44.44%)	5(55.55%)
11	Fosfomycin(200 µg)	12(92.30%)	1(7.69%)	-	-

Table 4. Screening of Enterococcal Isolates which were intermediate or resistance to vancomycin with Kriby Bauer disc diffusion method by Agar dilution method

No of Isolates	Kirby Bauer disc diffusion Method		Agar dilution method	
	Zone of inhibition (mm)	**Interpretation**	**MIC(µg /ml)**	**Interpretation**
1	15	I	4µg	S
2	15	I	2µg	S
3	16	I	1µg	S
4	15	I	0.5µg	S
5	16	I	2 µg	S
6	15	I	**0.5µg**	S
7	15	I	**1 µg**	S
8	16	I	**2 µg**	S
9	10	R	1 µg	S
10	9	R	4 µg	S
11	7	R	32 µg	R

6(26.08%) of the isolates from blood were *E. faecium* (Table-2). Antibiotic Susceptibility pattern of *E. faecalis* showed high sensitivity Teicoplanin 32(86.48 %) followed by Linezolid 35(95.59%) and High-Level Gentamicin (HLG) 33(89.18%). Majority 12 (92.30%) of them were sensitive Nitrofurantoin and Fosfomycin for urine samples. The maximum number of isolates was resistant to Penicilllin 20(54.05%) and Ampicillin 22(59.45%). Similarly Most of *E. faecium* isolates were sensitive to Teicoplanin 22 (95.65%) followed by Linezolid 21(91.30%) and by HLG 20(86.95%) . The majority of urine isolates were sensitive to Nitrofurantoin 6 (71.42%) Most of them were resistant to Pencillin 11(47.82%) and Ampicillin 13(56.52%) (Table 3). Out of the 3 isolates resistant to Vancomycin by Kirby Bauer disc diffusion method, two were sensitive by agar dilution method, whereas the other one resistant with MIC of 32 µg/ml . All 8 isolates which were intermediately sensitive by Kirby Bauer disc diffusion were found to be sensitive by agar dilution method with MIC values less than 4 µg/ml. The present study showed that the isolate which was resistant to Vancomycin both by disc diffusion and MIC by agar dilution was *E.faecium* (Table-4)

DISCUSSION

Enterococci have emerged from relatively innocuous organisms to medically important multidrug-resistant nosocomial pathogens that are considered a serious public health threat. It is due to their inherent resistance to antibiotics, ability to adhere to indwelling medical devices, and ability to survive in adverse environmental conditions[11] .The widespread use of vancomycin and extended-spectrum cephalosporins in hospitals leads to the worldwide emergence of VRE[12]. Hence in this study we analysed the changing pattern of enterococcal infections and their resistance to antibiotics with emphasis on VRE. In the present study, Out of the 2600 samples 60 samples positive for *Enterococcus* species.So, the prevalence for *Enterococcus* species in the whole sample was 2.30%. This prevalence rate was consistent with the findings of other authors who found the prevalence rate in Egypt (3.3%), in Bangladesh (3.2%), in India (2.3%) and in Asian pacific (3.6%) [13]. Where as the study conducted in Kolkata in 2011 by Chakraborthy et al [14] found a 7.3% prevalence rate, and one other study conducted by Phukan et al[15]. found 7.4% prevalence rate .The lower prevalence in the present study might be due to the variation in the study participants and the methods employed for detection of *enterococci. E. faecalis* was the most commonly isolated Enterococcus species, followed by *E. faecium*; this finding corresponds with the results of a worldwide surveillance report on Gram-positive pathogens and supports the idea that *E. faecalis* is the leading cause of Enterococcal infections< 90% [16]. In our study, rate of isolation of *E. faecalis* was 37 (61.66%) and *E. faecium* was 23 (38.33%).Similarly Dilshad Arif et.al.[17] reported *E. faecalis* (60.3%) and *E. faecium as* (39.6%).Whereas S. Sreeja et al.[18] reported *E. faecalis* (76%) and *E. faecium as* (24%). However,the recent trends show an increase in the isolation rate of *E faecium* which is alarming as its intrinsic resistance to many antimicrobial agents may lead to a treatment failure. In the present study 28(46.66 %) enterococcus species were isolated from pus sample followed by urine (36.66 %) and blood (15%) which is similar to study by Sreeja.S et.al[18] the maximum number of isolates were obtained from pus (43%), followed by urine (31%). Study done by Sengupta M. et al.[19] in 2023 found the most frequent sample for isolation of bacteria was urine at 60.11% followed by blood at 20.48%, pus at 18.33%, and body fluids at 1.08%. According to study conducted by Marothi et al[20] most frequent infections caused by *enterococci* are urinary tract infections followed by infections of intra-abdominal and pelvic abscesses or post-surgery wound infections. One of the most probable cause for the high isolation rate of *enterococc*i from urine was it reside as commensals in GIT.Urinary catheterization may also have contributed to higher isolation of *enterococci* from urine specimens.

There is a drastically increasing resistance to commonly used antimicrobial agents by *enterococci* i.e.an increase in the penicillin resistance to 95% and an increase in the ampicillin resistance to 95%[14]. Our study showed that 58.33% isolates were resistant to Ampicillin, 46.66 % to penicillin, 35% to tetracycline and 28.33% to ciprofloxacin . This is similar to the study by Dilshad Arif et.al[17]. who reported 58.2% isolates were resistant to Ampicillin. Whereas study by Kaarthiga S et al[21]. observed that 90% isolates were resistant to Penicillin and 81% to Ciprofloxacin . In the current study, High Level Gentamicin resistance (HLGR) among *Enterococcus* was observed in 21.66% of enterococcal isolates by DDT that is comparable with result Karna A et al[6]who reported 18.7% of HLGR in their samples and Kanithashree et al[22] accounted to 22.4 % and 30.8 % for *E. faecalis* and *E. faecium*. However, the incidence of HLGR *enterococci* was found to be higher in a study by *Kaarthiga S et al.* 69% [21].Higher resistance to gentamicin leads to the failure of synergistic combination therapy with gentamicin and beta-lactam antibiotics/glycopeptides for serious infections. Majority of *E. faecalis and E. faecium* strains isolated in our study, had increased sensitivity for Teicoplanin (98.33%) and Linezolid (91.66%) which is similar with the study of Darji SM et al[23] as they found 100 per cent susceptibility to Teicoplanin and Linezolid. Resistance against nitrofurantoin was noted in 6.66% of enterococcal isolates. This finding is lower than a report from Nepal(17.9%)[6]. This lower prevalence of nitrofurantoin resistance in our study indicates that it can be used as a therapeutic option in urinary tract infections caused by enterococcal species. Also in our study we observed that the isolates of *E.faecium* 3(33.33%) were more resistant to different antibiotics as compared to *E. faecalis* which is similar to Darji SM et al[23]. Out of the 11 isolates which were intermediate and resistant to vancomycin by disc diffusion method, one was resistant by agar dilution method with MIC of 32 µg/ml whereas the others were sensitive.The only isolate which was resistant to vancomycin both by disc diffusion and MIC by agar dilution was *E. faecium*. Similar observation has been made by Kanthishree B. et al [22] and suggested to supplement Kirby Bauer disc diffusion method with determination of MIC by dilution methods for detection of vancomycin resistance in the microbiology laboratory . Study by Valentina Y[24] also observed that among all VRE detected by disc diffusion method only 90% were confirmed by the agar dilution method. Patients with bacteremia with VRE were about 2.5 times more likely to die than those with VSE (Vancomycin Sensitive Enterococci) bacteremia, indicating that the development of Vancomycin resistance is a poor prognostic sign in critically ill patients[25]. Prevalence of VRE in US and European countries was reported as 5.0 % to 50 % and 13.0 % respectively. However, in India, the overall prevalence of *VRE* was found to be ranging from 1.0 to 45.6 % between1999 and 2021[1]. . The zonal council regions of India showed the prevalence of vancomycin resistant Enterococcus (VRE), and it was highest in north-east (24.7%), north (16.3%), western (10.1%), central (9.2%), and eastern (9.0%) India. The lowest prevalence was in south India (2.6%)[26]. Prevalence of VRE in the present study is 1.6% in contrast to 13.72% in Ohri S [27]et al. Studies from India are limited, and a scaling up is essential to identify patterns of resistance as well as to quantify the nationwide mortality burden owing to AMR.

CONCLUSION

The present study concludes that the result of the disc diffusion method can be inaccurate and lead to unwarranted utilization of vancomycin as a part of the treatment regimens. Therefore, the agar dilution method is recommended for laboratory screening and monitoring of VRE in resource poor settings. Although prevalence of VRE is low in our study at present, regular monitoring is essential for detection of vancomycin resistant enterococci which will help to limit the serious consequences.

Conflict of interest: None declared

Funding: No funding sources

Abbreviations

- **ATCC -** American Type Culture Collection
- **CLSI-** Clinical and Laboratory Standards Institute
- **DDT-**Disk Diffusion Test
- **HLG-** High Level Gentamicin
- **HLGR -**High Level Gentamicin resistance
- **MIC-** Minimum Inhibitory Concentration
- **VRE-** Vancomycin Resistant Enterococci

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