Original Research Article

Phenotypic speciation of clinical isolates of Enterococci with special reference to Vancomycin susceptibility by Broth Micro-dilution method

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Abstract

Background: *Enterococci*, once regarded as a commensal in the gastrointestinal tract, are now emerging as an important nosocomial pathogen. By intrinsic and acquired mechanisms of resistance, they pose a significant therapeutic challenge. **Objectives:** This study aims to characterize *Enterococci* up to species level based on their phenotypic characters and study their antibiogram with special mention to Vancomycin susceptibility. **Materials and Methods:** A cross-sectional descriptive study was conducted in the Department of Microbiology, Andhra Medical College. A total of 47 *Enterococci* isolates recovered from clinical specimens like Urine & Pus are included in this study. Specimen processing and speciation were done according to standard protocols. Kirby–Bauer disc diffusion technique was used to study antimicrobial susceptibility pattern with recommended drugs including high-level aminoglycoside resistance, whereas the minimum inhibitory concentration of vancomycin was determined by the Broth Microdilution method, with reference to the Clinical and Laboratory Standards Institute guidelines (CLSI). **Results:** Two different species of *Enterococci* were isolated, *E. faecalis* and *E. faecium* accounting 86% and 14% each. All the strains were sensitive to Vancomycin, Linezolid and Teicolplanin, while all the urine isolates are also sensitive to Nitrofurantoin. Disparities were not observed between the disc diffusion technique and Broth Microdilution method in determining vancomycin resistance. **Conclusion:** *E.faecalis* and *E. faecium* were the predominant species in causing Enterococcal infections. To maintain the low level of resistance, improvement of antibiotic policies and hospital infection control is essential.

Keywords: Broth Micro-dilution, MIC, Speciation, Enterococci, Glycopeptides

Introduction

The *Enterococci* are Gram-positive, spherical, oval, or coccobacillary, and their arrangement is in pairs and short chains. Most of the species are nonmotile and non-capsulated[1].

Though primarily, they are opportunistic pathogens, their constitutionally low virulence is well compensated for by their intrinsic resistance towards antibiotics and their ability to acquire resistance to several broad-spectrum antibiotics[2, 3]. Tracking the distribution of *Enterococci* and the information of their antibiogram is of utmost importance to prevent and control its spread[4, 5]. Especially in hospitalized patients, they mainly cause urinary tract infections. The most important predisposing factors are indwelling catheters and urinary tract instrumentation. They are frequently isolated from cases of wound infections, particularly intra-abdominal[1].

Identification and speciation of enterococcal isolates hold a substantial influence on therapeutic choice since antimicrobial susceptibility pattern varies between the species. Even though vancomycin-resistant enterococci (VRE) were first reported in 1986, from the UK and France, in recent years, they are disseminated all around the world[5, 6]. The incidence of vancomycin-resistant *E.faecium* in Asia is yet low; nevertheless, outbreaks have been reported. Limited views were expressed in various studies done on the prevalence of *Enterococci* and paucity of data is available on VRE[5, 7].

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The present study aims to characterize enterococci up to the species level and study their antibiogram with particular regard to vancomycin.

Materials and Methods

A cross-sectional descriptive study was conducted in the Department of Microbiology, Andhra Medical College, over a period of 7 months. A total of 47 enterococcal isolates were recovered from various clinical samples like urine and pus from routine culture.

All the specimens were inoculated on to Blood and MacConkey agar. (Image 1 & 2) Incubation of culture plates was done at 35℃ for 18-24 hours.

Fig. 1: Blood Agar Plate showing tiny non-hemolytic colonies

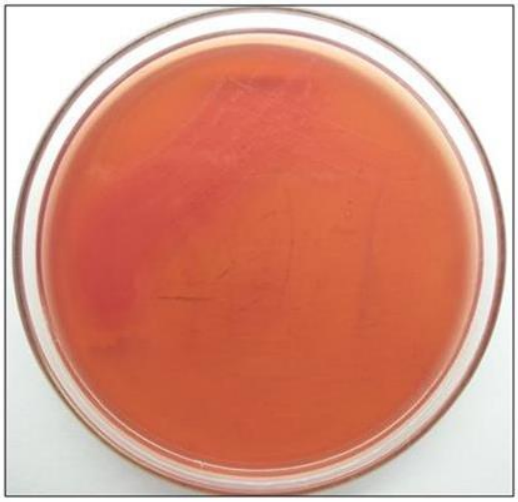

Fig. 2: MacConkey agar with tiny magenta-pink colonies

Enterococci were identified by colony morphology, Gram staining (Image 3), catalase test, bile-esculin test (Image 4), and salt tolerance test (6.5% NaCl).

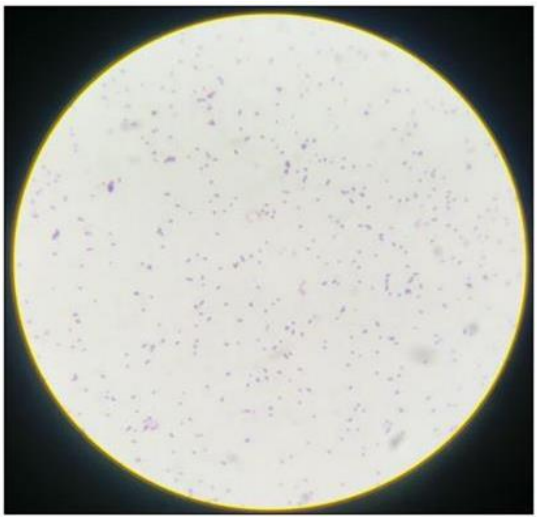

Fig. 3: Gram staining showing Gram positive Oval cocci arranged in angles and short chains [Internal scale: 100X Magnification]

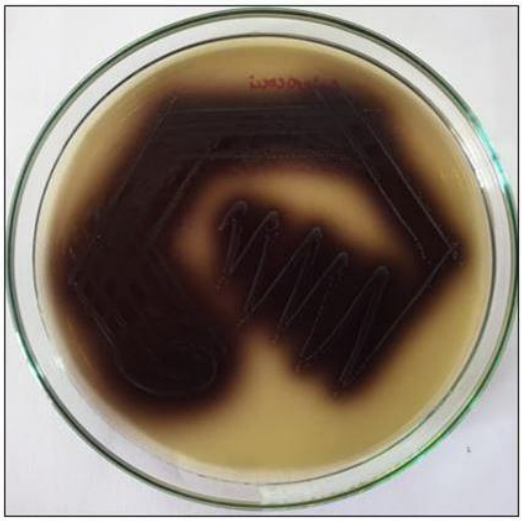

Fig. 4: Bile Esculin Agar showing black colour colonies

The strains isolated were identified and speciated in accordance with standard laboratory procedures as per the scheme of Facklam & Collins. (Image 5)[8, 9].

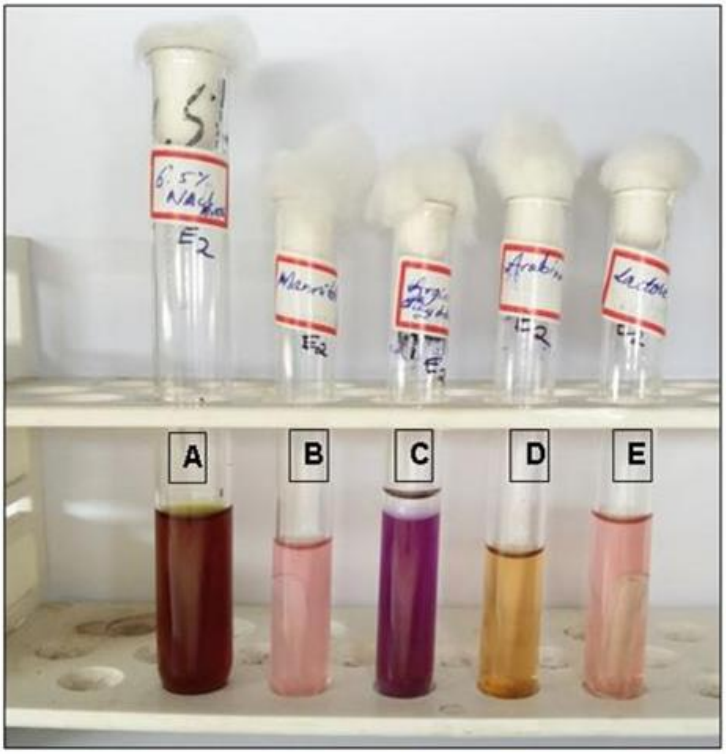

Fig. 5: Reactions of E.faecalis: A – 6.5% NaCl Broth – Positive, B – Mannitol – Fermented, C – Arginine dihydrolase – Positive, D – Arabinose – Not Fermented, E – Lactose – Fermented

Antimicrobial susceptibility testing was done using Disc diffusion technique with reference to Clinical and Laboratory Standards Institute (CLSI) guidelines against ampicillin (10 µg), vancomycin (30 µg), linezolid (30 µg), teicoplanin (30 µg), ciprofloxacin (5 µg), and high-level gentamicin (120 µg) and also, nitrofurantoin (300 µg) for urinary isolates. (Image 6)[10].

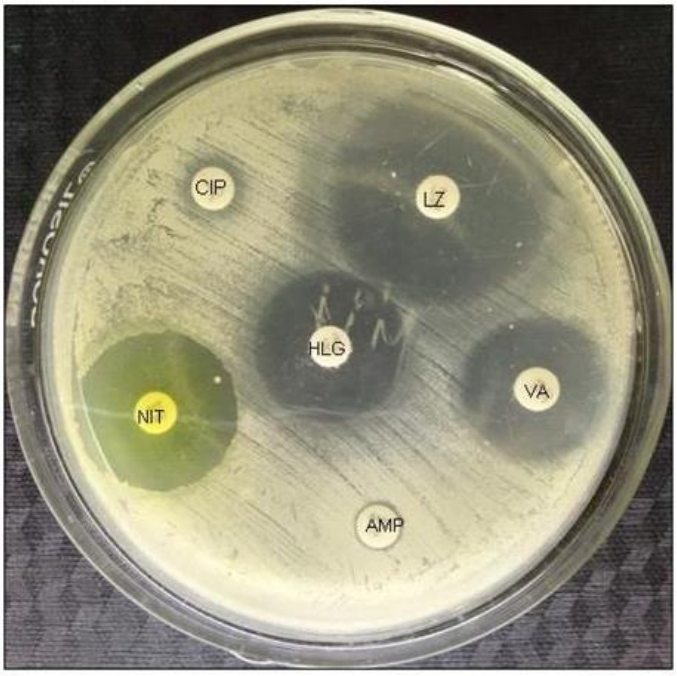

Fig. 6: Antimicrobial Susceptibility testing on MHA. (CIP – Ciprofloxacin, HLG – High-level Gentamycin, LZ – Linezolid, VA – Vancomycin, AMP – Ampicillin)

Broth Micro-dilution method was used to determine the minimum inhibitory concentration (MIC) of Vancomycin with a concentration range of 0.125 µg/ml to 256 µg/ml. For this procedure, cation adjusted Muller-Hinton broth media and vancomycin powder with a potency of 950 µg/mg acquired from HiMedia Laboratories Pvt. Limited, were used. 96 well micro test plates with u-bottom wells were obtained from Tarsons Products Pvt. Ltd. All the procedures were performed as recommended by the standard microbiological guidelines, and results were reported with reference to CLSI guidelines[10]. The lowest concentration of the drug, inhibiting visible growth after incubation, was regarded as MIC (Image 7). *E. faecalis* ATCC 29212 has been used as a standard strain for control.

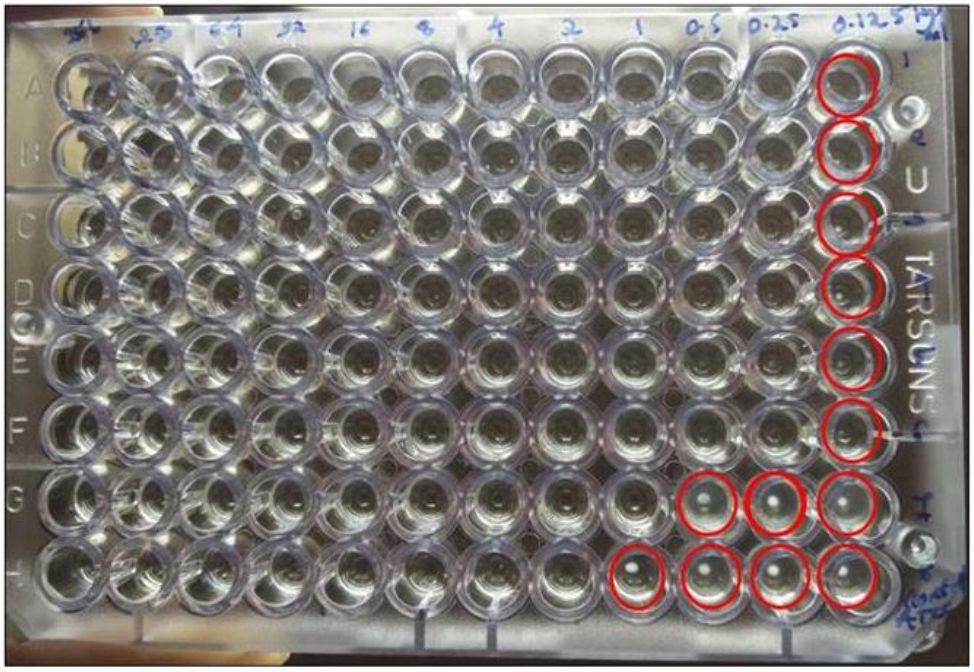

Fig.7: Broth Microdilution for Vancomycin Susceptibility Testing

Collected data were entered in Microsoft Excel 2019, and statistical analysis was performed using IBM SPPS 20.

Results

A total of 47 strains of enterococci recovered from different clinical specimens were analyzed in this study. It was observed that enterococci species were predominantly isolated from 41-60 years of age group (38%) followed by 0-20 (20%) and 21-40 (26%), while the lowest number of enterococci are isolated from age group 61 years and above (16%) (Table1).

Table 1: Age-wise distribution of the isolates

S.No	Age(in years)	No. of isolates	Percentage(%)
1	0-20	9	20
2	21-40	12	26
3	41-60	18	38
4	> 61	8	16
	Total	47	100

The isolates have female preponderance (73%). The highest frequency of isolates was recovered from urine (65%), followed by pus (29%). Among these isolates, 60% were obtained from hospitalized patients, 10% from the emergency department and 30% were from the outpatient department.

Out of the total enterococci strains isolated, 40 (86%) of the strains came out be *E.faecalis*, and 7 (14%) were *E.faecium* on species-level identification (P=0.0001).

All the strains had shown sensitivity to Vancomycin, Linezolid and Teicolplanin, while all the urine isolates are also sensitive to Nitrofurantoin.

Among the susceptibilities of other antibiotics, Ampicillin (38.2%) was found to be least susceptible, followed by High-Level Gentamycin (57.4%) & Ciprofloxacin (61.7%).

MIC range for vancomycin (µg/mL) in all the isolates of Enterococci are less than 4 µg/mL, indicating susceptibility to Vancomycin and no errors were detected between Disk diffusion and Broth Microdilution method (Table 2).

Table 2: MIC range for vancomycin (µg/mL) in different clinical isolates

	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256
E.faecalis (n=40)	19	2	4	11	5	0	0	0	0	0	0	0
E.faecium (n=7)	6	0	1	0	0	0	0	0	0	0	0	0

Discussion

The enterococci are commonly found in the gastrointestinal and genital tract of humans. Enterococci are now emerging as important agents of nosocomial infection. Majority of the Enterococci isolated in this study are from urine samples which correlated with studies like Ashish Karna et al. and Jayavarthini M et al. (Table 3) (P = 0.003)[5,14].

Table 3: Comparative analysis sample wise distribution of Enterococci Species isolated

Author	Year	Highest %
Present study	**2019**	**Urine (65%)**
Ashish karna et al	2019	Urine (61.53%)
Jayavarthinni M et al	2015	Urine (57.14%)
Biswas et al	2015	Urine (36.70%)
Nautiyal S et al	2013	Urine (46.70%)

The prevalence of *E.faecalis* is more than *E.faecium* in the present study which correlates with Parameswarappa et al. and Ambumani N et al. (Table 4) (P = 0.0001)[2, 13].

Table 4: Comparison of Prevalence of Enterococci Species isolated

Author	Year	*E.faecalis*	*E.faecium*	Others
Present study	**2019**	**86%**	**14%**	**Nil**
Aashish karna et al	2019	38%	37%	75%
Jayavarthinni M et al	2015	32.5%	52.38%	15.08%
Parameswarappa et al	2013	63.4%	36.6%	Nil
Nautiyal S et al	2013	75.60%	11.10%	13.3%
Ambumani N et al	2011	88%	12%	Nil

Enterococci are generally seen in elderly age group, which is also seen in this study and correlated with Biswas et al. and Parameswarappa et al. (Table 5)[12,13].

Table 5: Comparison of Age wise distribution of Enterococci Species isolated

Author	Year	Age group with Highest isolation rates[Years (%)]	Age group with Lowest isolation rates[Years (%)]
Present study	**2019**	**41-60 (38%)**	**>61 (8%)**
Aashish karna et al	2019	0-10 (20.9%)	51-60 (8.7%)
Biswas et al	2015	41-50 (45.8%)	31-40 (12.5%)
Parameswarappa et al	2013	41-60 (63.4%)	>61 (29.3%

In this study, all isolates are sensitive to Vancomycin, Linezolid, Teicoplanin & Nitrofurantoin and were relatively resistant to High-level Gentamycin, Ampicillin & Ciprofloxacin, which correlates with Ambumani et al., which was not statistically significant (P = 0.424). (Table 6)[2].

Table 6: Comparative analysis of High-Level Gentamycin resistance among Enterococci Species isolated

Author	Year	HLG Resistance
Present study	**2019**	**46%**
Aashish karna et al	2019	18.7%
Nautiyal S et al	2013	77.7%
Ambumani N et al	2011	56%
Mohanty S et al	2011	73.3%

For all the antibiotics there was no significant difference between resistance patterns of *E.faecalis* & *E.faecium*. MIC Testing Results of Enterococci by Broth Microdilution towards Vancomycin showed 100% susceptibility which correlated with Jayavarthinni M et al. & Ambumani N et al. (Table 7) indicating Vancomycin resistance is low in our region[2, 14].

Table 7: MIC Testing Results of Enterococci by Broth Microdilution to Vancomycin (%Susceptibility)

Author	Year	Species tested	
		E.faecalis	E.faecium
Present study	2019	100% (<2µg/ml)	100% (<2µg/ml
Jayavarthinni M et al	2015	100% (<2µg/ml)	97% (<2µg/ml)
Ambumani N et al	2011	100% (<4µg/ml)	100% (<4µg/ml)

Conclusion

Combination therapy with cell wall active agent and (Penicillin, Ampicillin & Vancomycin) & Aminoglycoside (Gentamycin & Streptomycin) is recommended for treatment of severe enterococcal infections.

But High-level resistance to Aminoglycosides could nullify this combination. Therefore to distinguish these High-level aminoglycoside resistant strains, which might have been merely intrinsic resistant strains, routine laboratory testing of for High-level Gentamycin is garnering importance.

The problem of VRE may not be very high in India as also seen in our institution at present, but monitoring of VRE is the need of the hour since it appears to be an emerging pathogen in India.

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