Original Article

DOI: 10.7860/JCDR/2021/48616.15077

Microbiology Section

Phenotypic Characterisation, Virulence Determination and Antimicrobial Resistance Pattern of *Enterococcus* Species Isolated from Clinical Specimen in a Tertiary Care Hospital in Kolkata

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ABSTRACT

Introduction: Enterococci are usually normal human commensal of gastrointestinal tract predominantly. They are considered as an important nosocomial pathogen now a day due to its intrinsic as well as increasing acquired antibiotic resistance resulting in a great threat to modern Medicine.

Aim: To determine prevalence of Enterococci isolated from clinical specimens with special reference to its virulence and antibiogram conventionally.

Materials and Methods: A cross-sectional observational study was conducted over a period of two years (January 2019 to December 2020) with 326 Enterococci, isolated from various clinical specimens received by Department of Microbiology. Enterococci isolated from stool samples were excluded. They were identified and speciated conventionally as per standard laboratory protocol. Gelatinase, haemolysin and biofilm formation was determined for each isolate. Their antibiogram was also determined by disc diffusion methods over blood agar followed by Minimum Inhibitory Concentration (MIC) testing (as per Clinical and Laboratory Standards Institute (CLSI) guideline). All statistical analysis was done by Chi-square test using Software Statistical Package for the Social Sciences (SPSS) version 22.0.

Results: Among the total 4516 samples collected, growth of Enterococci was noted in 7.22% cases. Out of them, *Enterococcus faecalis (E. faecalis)* (84.05%) out numbered *Enterococcus faecium (E. faecium)*. Urine was the most predominant (55.22%) sample. A 73.93% isolates produced biofilm whereas 18.40% produced haemolysin and 19.94% produced gelatinase. Most of the isolates were susceptible to vancomycin (94.79%) and linezolid (98.77%). High level gentamicin resistance was seen in 54.6% cases. Ciprofloxacin was the most resistant antibiotic. Vancomycin Resistance *Enterococcus* (VRE) was detected in 5.21% cases only, out of which Van A type was detected phenotypically in most cases.

Conclusion: The high rate of resistance to high level gentamicin could fail treatment of gentamicin in combination with penicillin group of antibiotics. In clinical samples, the emergence of VRE strains makes treatment options more challenging.

Keywords: Antibiogram, Enterococci, Prevalence, Virulent

INTRODUCTION

Enterococci are gram positive, facultatively anaerobic ovoid cocci that may occur in pair or short chains [1]. It was previously classified as Group D *Streptococcus*, but later in 1984, a separate genus classification was introduced [2]. Although, it was considered as commensal in intestinal canal, vaginal tracts and the oral cavity, but it possesses certain features that may have roles in pathogenesis [3]. The increasing incidence of Enterococci as nosocomial pathogen is due to its natural ability to obtain and share extra chromosomal elements encoding virulence traits or antibiotic resistant genes [4]. There are so many Enterococcal pathogenic factors including secreted virulence factors and adhesion factors have been detected in the last few years [5]. The predominant factors are adhesion of collagen from *E. faecalis (ace)*, aggregation substance *(asa)*, extracellular surface protein *(esp)*, and endocarditis and biofilm associated pilli (ebp) [6,7]. This aggregation substance increases bacterial adherence to renal tubular cells [8]. Enterococcal colonisation and biofilm formation were promoted by esp, leading to resistance to stresses and adhesion to cells as seen in endocarditis and Urinary Tract Infection (UTI) [9]. The gene cluster responsible for formation of pili by Enterococci is *ebp*. Adheison of collagen from *E. faecalis (ace)* is a collagen binding protein, belonging to the Microbial Surface Components Recognising Adhesive Matrix Molecules (MSCRAMM) family, helping in the pathogenesis of endocarditis [10].

Secreted virulence factors are hyaloronidase (hyl), cytolysin (cyl) and gelatinase (gelE) [11,12]. Gelatinase, an extracellular zinc-containing metalloproteinase, helps in degrading host tissue and provides nutrients [12]. Cytolysin is a beta haemolytic enzyme in human. Hyaluronidase (Hyl), a degradative enzyme, causes damage to the tissues made of hyaluronic acid thus promoting spread of Enterococci and their toxins through host tissue [8].

Several studies have determined the prevalence of Enterococci in India (Pondicherry 7.22% [13], Lucknow 1.46% [14], Kolkata 10% [15], Mumbai 5.5% [16], Kolkata 4.8% [17]) However, only a very few studies focused on the virulence factors of Enterococci Suchi SE et al., and Jayavarthinni M et al., [5,13].

This study was conducted to analyse the prevalence of *Enterococcus* species in various specimens, to detect various virulence factors like gelatinase, haemolysin and biofilm formation and to study antimicrobial resistance, in specific, VRE and high level aminoglycoside resistance to guide infection control practices.

MATERIALS AND METHODS

The present cross-sectional and observational study was conducted for a duration of two years, from January 2019 to December 2020 in

the Department of Microbiology, NRS Medical College and Hospital, Kolkata. West Bengal, India.

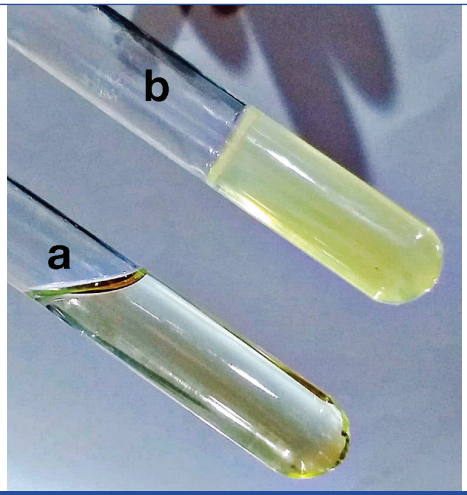
Inclusion criteria: All the heterogeneous clinical samples (urine, pus, blood, body fluids) from patients of indoor and Outpatient Department, received by the Department during this period were processed by standard laboratory protocol and isolated *Enterococcus* were included in this study [18].

Exclusion criteria: Enterococci isolated from stool samples were excluded.

Study Procedure

A total of 326 Enterococci isolates were included in this study. The genus *Enterococcus* was confirmed by gram stain, i.e., gram positive cocci in pairs and short chains, colony characters, pH, temperature, catalase test and biochemical tests like bile esculin hydrolysis, salt tolerance test using 6.5% NaCl, Arginine Decarboxylation, sugar fermentation using D (-) Arabinose, D-Mannitol, L (-) Sorbose, D- Sorbitol and D (+) Raffinose were carried out on colonies grown [18,19]. Strains were further identified to species level by using Vitek®2 compact system (BIOMERIEUX). All isolates were stocked in glycerol broth at (-) 80˚C for further testing of virulence factors determination and antimicrobial susceptibility testing. All epidemiological parameters were analysed including prevalence rate, age and sex criteria.

Test for virulence factors: Production of gelatinase was assessed by the ability to liquefy gelatine [20]. For detection, nutrient gelatin gel containing 12% gelatine was used. Organism inoculated by stab culture within it. After overnight incubation at 37°C, liquefaction was tested by tilting the tube [Table/Fig-1].

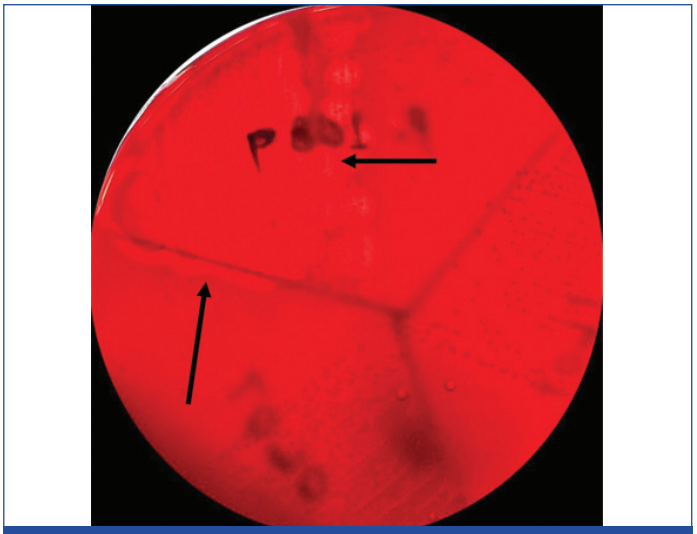


[Table/Fig-1]: Showing gelatin hydrolysis test of *Enterococcus*; a) Gelatin hydrolysis Negative, b) Gelatin hydrolysis Positive.

Haemolysin production was measured by the macroscopic appearance of complete zone of haemolysis (beta haemolysis) in blood agar plate supplemented with 5% sheep blood [Table/Fig-2] [21].

Biofilm production was assessed by christensen tube method using trypticase soy broth with 2% sucrose [22]. A loopful of microorganisms was inoculated within it from overnight culture and incubated for 24 hours at 37°C. The tubes were decanted thereafter and washed thrice with Phosphate Buffer Saline (PBS) (pH 7.2). Then they were dried and stained with 0.1% crystal violet for 30 minutes. Excess stain was washed with deionised water. The tubes were dried then and observed for biofilm production. If a visible film of stain lines the sides and bottom of each tube, biofilm was considered to be positive [Table/Fig-3] [23].

Antibiotic susceptibility testing: Kirby-Bauer disk diffusion method was used for antimicrobial susceptibility testing. Mueller-Hinton agar supplemented with 5% sheep blood was used [24]. The antibiotic discs were purchased from Hi-Media. The antibiotic discs and their potency were as follows: ampicillin (10 µg), gentamicin high content (120 µg), streptomycin high content (300 µg), ciprofloxacin (5 µg),



[Table/Fig-2]: Showing haemolysis (shown by black arrow) produced by *Enterococcus*.

[Table/Fig-3]: Showing biofilm formation of *Enterococcus* (in the tube shown by black arrow).

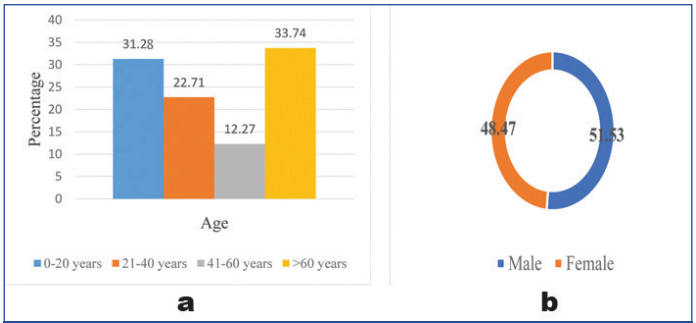
vancomycin (30 µg), nitrofurantoin for urinary isolates only (300 µg), ceftriaxone (30 µg), teicoplanin (30 µg), and linezolid (30 µg). E-test was done to determine the MIC of vancomycin for all the clinical isolates of Enterococci. Different genotypes of Van gene were analysed by looking into resistance patterns of vancomycin and teicoplanin. The results were interpreted as per CLSI guidelines [25]. *E. faecalis* ATCC 29212 and *E. faecalis* ATCC 51299 were included as a quality control strain. All culture media, reagents and chemicals were obtained from Hi-Media Private Limited, Mumbai, Maharashtra India.

STATISTICAL ANALYSIS

Microsoft Excel and Microsoft word (version 10) were used to generate the tables and figures. All statistical analysis was done using Chi-square test. The software used for the statistical analysis was SPSS version 22.0.

RESULTS

Out of 4516 heterogeneous clinical specimens, 326 Enterococci were isolated and identified, having prevalence rate of 7.22%. Among 326 *Enterococcus* species, 274 (84.05%) species were *E. faecalis* and 52 (15.95%) species are *E. faecium*. Highest prevalence of *Enterococcus* was seen in males 168 (51.53%) followed by females 158 (48.47%), with M:F=1.06:1. The maximum percentage of isolation was seen among the age group >60 years (33.74%) [Table/Fig-4a,b]. The [Table/Fig-5] shows sample distribution of cases, maximum *Enterococcus* was isolated from urine specimen.



[Table/Fig-4]: Showing age wise distribution (a) and Sex wise distribution (b) of cases (n=326).

Samples	Number of cases	%
Urine	180	55.22
Pus	64	19.63
Blood	48	14.72
Fluid	34	10.43
Total	326	100

[Table/Fig-5]: Showing sample wise distribution of cases (n=326).

Out of total 326 isolates, 19.94% were gelatinase producer, whereas, 18.40% isolates produced haemolysin and 73.93% formed biofilm. *E. faecalis* was found to be significantly more virulent [Table/Fig-6].

Virulence factors	*E. faecalis* (%)	*E. faecium* (%)	Total (%)	p-value (Chi-square test)
Gelatinase	36 (11.04)	29 (8.90)	65 (19.94)	0.0001*
Haemolysin	52 (15.95)	8 (2.45)	60 (18.40)	0.6760
Biofilm formation	216 (66.26)	25 (7.67)	241 (73.93)	0.0001*

[Table/Fig-6]: Showing virulence factors produced by various *Enterococcus* species.

The VRE cases were detected in 5.21% isolates out of which Van A type (MIC values in the range of 64-256 µg/mL) was detected phenotypically in most cases (64.71%) followed by Van B (35.29%) (MIC values in the range of 64-128 µg/mL). Antimicrobial resistance patterns showed resistivity to ampicillin, ciprofloxacin and gentamicin [Table/Fig-7].

Antibiotic discs	*E.faecalis* (N=274) (%)	*E. faecium* (N=52) (%)	Total (%)
Ampicillin (10 µg)	177 (64.6)	28 (53.85)	205 (62.88)
Ciprofloxacin (5 µg)	210 (76.64)	32 (61.54)	242 (74.23)
Nitrofurantoin (300 µg)	16 (5.84)	4 (7.69)	20 (6.13)
Gentamicin (120 µg)	160 (58.39)	18 (34.62)	178 (54.60)
Streptomycin (300 µg)	84 (30.66)	22 (42.31)	106 (32.52)
Teicoplanin (30 µg)	65 (23.72)	16 (30.77)	81 (24.85)
Linezolid (30 µg)	1 (0.36)	3 (5.77)	4 (1.23)
Vancomycin (30 µg)	9 (3.28)	8 (15.38)	17 (5.21)

[Table/Fig-7]: Showing antimicrobial resistance patterns among the clinical isolates of *Enterococcus* (n=326).

DISCUSSION

The changing clinical patterns of the *Enterococcus* infections and their antimicrobial susceptibility patterns have become an important topic of discussion, as it is emerging as nosocomial pathogen nowadays [26]. In present study, prevalence rate of Enterococci isolated from various clinical specimens was 7.22%, which was consistent with the study of Jayavarthinni M et al., [13]. The overall prevalence of Enterococcal infection varies across continents, countries and within hospitals. In India, the occurrence varies from 1-36% [15]. Das S, in Kolkata showed prevalence rate 10% [15]. Agarwal J et al., in Lucknow showed prevalence rate of *Enterococcus* to be 1.46% [14], whereas Shinde RS et al., in Mumbai showed 5.5% [16]. Anbumani N et al., from Southern India showed it only 2% [27], whereas Desai PJ et al., stated a higher prevalence of 22.19% [26].

In present study, *E. faecalis* was the predominant species. This finding was similar with findings of Fernandes SC and Dhanashree B and also with Bose S et al., [28,29]. *E. faecalis* was found to be the predominant isolate in Das S, Sharma S et al., Mule P et al., and Bose M et al., [15,30-32]. But there are few studies which showed *E. faecium* as predominant species by Karmarkar MG et al., and Jain S et al., [8,33], Jayavarthinni M et al., and Jaiswal S et al., [13,34].

Nautiyal S et al., showed that male was more affected than female [35]. This finding was similar with present study showing male preponderance (51.53%). Tripathi A et al., also showed male preponderance in their study [36].

In present study, the maximum percentage of isolation was seen among the age group >60 years (33.74%). Jayavarthinni M et al., showed that more commonly affected age group of more than 50 years [13]. The maximum percentage of isolation was seen among the age group 40-60 years, in the study of Sharma S et al., [30]. Though there are some studies showing that young age group was more commonly affected, such as Nautiyal S et al., [35].

In present study, isolates were highest from urine (55.22%), followed by pus and blood. This finding was consistent with Jayavarthinni M et al., Sharma S et al., Bose M et al., and Jaiswal S et al., [13,30,32,34].

In this study, 19.94% were gelatinase producer, whereas, 18.40% isolates produced haemolysin and 73.93% formed biofilm. *E. faecalis* was found to be significantly more virulent. Jayavarthinni M et al., also showed that study on virulence factors revealed that 19.84% strains produced gelatinase [13], 18.25% produced haemolysin and 73.81% produced biofilm. Banerjee T and Anupurba S also revealed in their study that 9.03% strains produced gelatinase, 31.61% produced haemolysin and 26.12% produced biofilm and *E. faecalis* was the most virulent strain among all *Enterococcus* species [37]. Higher percentage of haemolysin and gelatinase production was noted in some other studies also [27,38,39]. Fernandez SC and Dhanashree B, showed haemolysin production in 82% cases and gelatinase production in 40.6% of the isolates [28]. Whereas, Tellis R and Muralidharan S showed 44% haemolysin production and 32% gelatinase production in their study [38]. Higher rates of biofilm formation were noted in Upadhyaya GPM et al., (86.6%) [39].

In the present study, majority of the *Enterococcus* isolates were resistant to ciprofloxacin (74.23%) and ampicillin (62.88%). Only 6.13% isolates were resistant to nitrofurantoin (for urinary isolates). A 54.60% isolates were resistant to high level gentamicin and 32.52% to streptomycin (elevated level). Similar finding was also noted in Parameswarappa J et al., Jayavarthinni M et al., Sharma S et al., and Mendiratta DK et al., [1,13,30,40].

The most recent and important resistance in Enterococci is VRE which has been increasingly reported from all parts of the world [17]. In present study 5.21% the isolates are VRE which showed significant similarity to results reported from other studies ranging between 1.7-20% in tertiary care hospitals in other parts of India [17,30,32,35]. In the present study authors have phenotypically isolated 64.71% strain of Van A, and 35.29% strains of Van B. Similar finding was also noted in the study of Nautiyal S et al., [35].

In this study, authors found that all clinical isolates of VRE were susceptible to linezolid. Linezolid nonsusceptible Enterococci (1.23%) may be an emerging clinical problem in other countries. Similar finding was noted in Tripathi A et al., [36]. Overall, *E faecium* was found to be more resistant than *E. faecalis*, in present study, which was also similar with the study of Mule P et al., and Jaiswal S et al., [31,34].

Limitation(s)

One of the major limitations of present study was not able to use molecular methods for identification, virulence factor determination and antimicrobial susceptibility testing. As there was very low

number of *E. faecium* isolates found in present study, data could not be generalised.

CONCLUSION(S)

Various studies have shown an increase in the rate of infection and antibiotic resistance in *Enterococcus* species. High resistivity to commonly used antibiotics and emergence of VRE strains has further aggravate the situation. Thus, we suggest more rational use of antibiotics and infection control in our health care settings.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]

- Plagiarism X-checker: Jan 24, 2021
- Manual Googling: Apr 19, 2021
- iThenticate Software: May 25, 2021 (23%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:

- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? No
- Was informed consent obtained from the subjects involved in the study? No
- For any images presented appropriate consent has been obtained from the subjects. No

Date of Submission: **Jan 20, 2021**
Date of Peer Review: **Mar 01, 2021**
Date of Acceptance: **Apr 30, 2021**
Date of Publishing: **Jul 01, 2021**