

Original Research Article

A study on virulence factors and antimicrobial resistance pattern among enterococci isolated from various clinical specimens from a tertiary care hospital

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ABSTRACT

Background: Enterococci, adult faeces commensal are important nosocomial pathogens. *E. faecalis* is the most common cause of infection, followed by *E. faecium*. In the past two decades, they have developed resistance to many commonly used antimicrobial agents. Understanding virulence factors and monitoring antimicrobial resistance among Enterococci is essential for controlling the spread of bacterial resistance and important for epidemiological surveillance within the hospital environment. The aim of the study is to evaluate antibiotic resistance and virulence factors exhibited by *Enterococcus sp.*

Methods: One hundred consecutive isolates of *Enterococci* isolated from different clinical samples of patients attending AVMC and H, a tertiary care center at Pondicherry in a period of 20 months were included in the study. *Enterococcus sp.* were identified as per standard conventional bacteriologic methods and detected for the production of virulence factors such as Hemolysin production, Gelatinase production. Antimicrobial susceptibility testing was carried out by disc diffusion method and MIC of vancomycin and teicoplanin was determined by E-test strips.

Results: Among 100 Enterococcal isolates included in the study, 81% were *E. faecalis* and 19% were *E. faecium* which were isolated from urine (44%), Pus (51%) and others specimen (5%, which includes blood 80% and drain tube 20%). In this study, overall 15% of *E. faecalis* and 1% of *E. faecium* showed hemolysin production and Gelatinase was produced by 6% of *E. faecalis* and 4% of *E. faecium*. Majority of *E. faecalis* and *E. faecium* strains isolated in our study, had increased sensitivity were to be exhibited for Linezolid, Vancomycin followed by high level gentamycin and high degree of resistance to penicillin, ciprofloxacin and cotrimoxazole. Analyzing the results of MIC of vancomycin and teicoplanin, 5 isolates were classified phenotypically as VanB phenotype that possess only moderate to high levels of vancomycin resistance and one isolate obtained from drain tube which showed MIC of vancomycin as 120µg/ml and teicoplanin 16µg/ml was grouped into VanA.

Conclusions: Though the prevalence of vancomycin resistant *Enterococci* (VRE) is very low in our study, yet regular monitoring of vancomycin resistance is very crucial for early detection, treatment, application of preventive and control measures and most importantly to check the spread of virulent multidrug resistant *Enterococcus species*.

Keywords: Antimicrobial susceptibility testing, *Enterococci*, Hemolysin production, Gelatinase production

INTRODUCTION

Enterococcus sp. are normal inhabitants of the intestinal tract of humans and animals with low intrinsic virulence

and second most common nosocomial pathogen associated with significant morbidity and mortality.¹⁻⁴ They are emerging nosocomial pathogens due to the increasing antibiotic pressure, high degree of resistance to

aminoglycosides, erythromycin, tetracycline and more recently vancomycin.¹ The characteristic of enterococci that makes them such formidable pathogens is their intrinsic resistance to a number of antimicrobial agents. *Enterococci* exhibit low levels of intrinsic resistance to penicillins, cephalosporins, carbapenems, carbacephems, aminoglycosides and lincosamides. They also have acquired genes to resist the action of glycopeptides such as vancomycin and teicoplanin.⁵

Multidrug resistant (MDR) enterococci exhibiting high level resistance to penicillin, glycopeptides, fluoroquinolones and aminoglycosides have emerged as an important cause of nosocomial infections and a formidable challenge to clinicians.¹ Major cause of intrinsic resistance is believed due to commonly used antibiotics and their ability to acquire resistance to all currently available antibiotics, either by mutation or by receipt of foreign genetic material through the transfer of plasmids and transposons.⁶

Patients with underlying malignancies, chronic renal disease on dialysis, transplant recipients and those with long term exposure to third generation cephalosporins and vancomycin are at an increased risk for development of enterococcal infections. Rapid spread of VRE due to patient-to-patient transmission in health care settings is of concern because infections due to VRE remaining difficult to treat, despite availability of new antibiotics tigecycline and linezolid.

Understanding the exact prevalence rate, virulence factors and monitoring antimicrobial resistance pattern among *Enterococci* isolated from various clinical specimens is essential for controlling the spread of bacterial resistance and important for epidemiological surveillance within the hospital environment.⁷ Despite the increasing reports of VRE in different countries, there is a distinct lack of data from rural population. With this background, the following study was undertaken to determine the prevalence of *Enterococcal species*, isolated from clinical samples along with their antimicrobial susceptibility patterns, virulence factors, and typing.

The objective of this study was to evaluate antibiotic resistance among *Enterococci spp* isolated from patients at AVMC and H and to study the virulence factors exhibited by them.

METHODS

This prospective study was carried on to 100 consecutive isolates of *Enterococci* isolated from different clinical samples (exudates, urine, blood and body fluids) of patients attending Aarupadai Veedu Medical College and Hospital, a tertiary care center at Pondicherry. This study was done from May 2015 to December 2016 in the microbiology department of AVMC and H. The study protocol was approved by the institutional ethics sub-committee after which the study was initiated. Informed

verbal consent was taken from the patients before including into the study.

Identification of *Enterococcus sp.*

Collection, processing, isolation and speciation of *Enterococcus* species from different clinical specimens were carried out as per standard conventional bacteriologic methods.⁸ All gram-positive cocci that are catalase negative are confirmed as *Enterococcus* genus with growth on and blackening of bile-esculin agar, growth in the presence of 6.5% sodium chloride (salt tolerance test) and heat tolerance test. Further *Enterococcus* species were identified by potassium tellurite reduction, arginine dihydrolase test and sugar fermentation test including glucose, lactose, mannitol and arabinose.⁸

Detection of virulence factors

Hemolysin production will be detected by inoculating *Enterococci* on to freshly prepared beef heart infusion agar supplemented with 5% human blood. Plates will be incubated overnight at 37°C in a carbon dioxide chamber and evaluated at 24 and 48 hours. A clear zone of β -hemolysis around the streak on blood agar will be considered to be a positive indication of hemolysin production. Gelatinase production will be detected by inoculating the organism on to freshly prepared peptone-yeast extract agar containing 30 g/L of gelatin. Plates will be incubated overnight at 37°C and then cooled to ambient temperature for 2 hours. The appearance of a turbid halo or zone around the colonies will be considered to be a positive indication of gelatinase production.

Antimicrobial susceptibility testing for *Enterococcus sp.*

Antimicrobial susceptibility testing was performed on Mueller Hinton agar as per CLSI guidelines. The following antibiotics were tested- Vancomycin (30 μ g), Ciprofloxacin (30 μ g), Gentamicin (10 μ g), Linezolid (30 μ g), Doxycycline (30 μ g), Nitrofurantoin (30 μ g), Cefuroxime (30 μ g), Cefoperazone (30 μ g), Norfloxacin (10 μ g), Piperacillin (10 μ g), Amikacin (30 μ g), Clindamycin (2 μ g), Amoxicillin (30 μ g) and Teicoplanin (30 μ g) as per CLSI guidelines.⁹

Minimum inhibitory concentration for vancomycin, teicoplanin resistant *Enterococcus sp.*

MIC of vancomycin and teicoplanin was determined by E-test strips. MIC values were interpreted in accordance with CLSI guidelines as follows: for vancomycin: (susceptible \leq 4 μ g/mL; intermediate, 8-16 μ g/mL; and resistant, \geq 32 μ g/mL). For teicoplanin: (susceptible \leq 8 μ g/mL; intermediate -16 μ g/mL and resistant \geq 32 μ g/mL). *E.faecalis* ATCC 29212 will be used for quality control. Based on E-test, the isolates of VRE will be defined phenotypically as Van A, Van B, Van C and Van

D on the basis of their vancomycin MIC and susceptibility to teicoplanin.⁴

RESULTS

Enterococcal isolates

A Total of 100 Enterococcal isolates were isolated in a period of 20 months. These isolates were from different clinical samples like pus, urine, blood, drain tube. Two species of *Enterococci* were speciated, i.e., *E. faecalis* and *E. faecium*. There were 81 isolates (81%) of *E. faecalis* and 19 isolates (19%) of *E. faecium*. There were 81 isolates (81%) of *E. faecalis* and 19 isolates (19%) of *E. faecium* which were isolated from urine (44%), Pus (51%) and others specimen (5%, which includes blood 80% and drain tube 20%). Specimen wise distribution of *Enterococcus sp* is shown in Table 1.

The mean age group of the study participants was 41-50 years; n=22 (22%); 55 of them (55.0%) were females and rest were males. The age wise distribution of isolates was shown in Table 2.

Virulence factors

Exhibited by urine isolates

Among *E. faecium* isolates from urine isolates, 25% (n=2/8) were gelatinase positive whereas among *E.*

faecalis, 2.7% (n=1/36) were gelatinase positive and 14.2% (n=5/36) were hemolysis positive.

Table 1: Specimen wise distribution of enterococcus sp.

Specimen	Species	
	No. of <i>E. faecalis</i> (%)	No. of <i>E. faecium</i> (%)
Urine (44)	36 (81.8)	8 (18.2)
Pus (51)	41 (80.4)	10 (19.6)
Others (5)	4 (80.0)	1 (20.0)
Total	81	19

Table 2: Age wise distribution of Enterococcal isolates.

Age in Years	No. of isolates
<1 year	2
1-10 years	5
11-20 years	7
21-30 years	16
31-40 years	15
41-50 years	22
51-60 years	13
61-70 years	13
> 70 years	7

Table 3: Virulence characteristics of *Enterococcus sp.*

Virulence Factors	Urine (44)		Pus (51)		Others (5)	
	<i>E. faecalis</i> (N=36, %)	<i>E. faecium</i> (N=8, %)	<i>E. faecalis</i> (N=41, %)	<i>E. faecium</i> (N=10, %)	<i>E. faecalis</i> (N=4, %)	<i>E. faecium</i> (N=1, %)
Hemolysis	5 (14.2%)	0 (0%)	10 (24.3%)	1(10%)	0 (0%)	0 (0%)
Gelatinase	1 (2.7%)	2 (25%)	5 (12.1%)	1(10%)	0 (0%)	1 (100%)
Potassium tellurite	36 (100%)	8 (100%)	41 (100%)	10 (100%)	4 (100%)	1 (100%)
Arginine dihydrolase	36 (100%)	8 (100%)	41 (100%)	10 (100%)	4 (100%)	1 (100%)
Glucose fermentation	36 (100%)	8 (100%)	41 (100%)	10 (100%)	4 (100%)	1 (100%)
Lactose fermentation	36 (100%)	8 (100%)	41 (100%)	10 (100%)	4 (100%)	1 (100%)
Mannitol fermentation	36 (100%)	8 (100%)	41 (100%)	10 (100%)	4 (100%)	1 (100%)
Arabinose fermentation	0 (0%)	8 (100%)	0 (0%)	10 (100%)	0 (0%)	1 (100%)

Exhibited by pus isolates

In overall of 51 pus isolates, *E. faecalis* isolates were 41 (80.4%) which showed 24.3% (n=10/41) positivity for hemolysis and 12.1% (n=5/41) positivity for gelatinase. Totally, 10 (19.6%) *E. faecium* were isolated from pus; one (10%) was tested positive for each hemolysis and gelatinase production.

Exhibited by others isolates

Out of 5 isolates belonging to others category includes 4 blood sepsis isolates and one isolate was from drain tube

which includes 4 *E. faecalis* (80%) and one *E. faecium* (20%). None of the *E. faecalis* isolates were positive for hemolysis and gelatinase production where as one isolate (100%) of *E. faecium* from blood tested positive for gelatinase production. Virulence characteristics of *Enterococcus sp* is shown in Table 3.

Antimicrobial susceptibility pattern of Enterococcal isolates

Among 81 *E. faecalis* strains isolated in our study, higher degree of sensitivity were exhibited for Linezolid

(96.2%), Vancomycin (96.2%) followed by high level gentamycin (120mcg; 83.9%), whereas high degree of resistance were exhibited to Penicillin (76.5%) followed by Ciprofloxacin (51.8%) and Cotrimoxazole (50.6%). Out of 19 *E. faecium* isolates included in the study, higher degree of sensitivity were exhibited for

Linezolid (94.7%), Vancomycin (84.2%) followed by high level Gentamycin (120mcg; 68.4%), whereas high degree of resistance were exhibited to Penicillin (84.2%) followed by Ciprofloxacin (68.4%) and Cotrimoxazole (52.6%). Antimicrobial susceptibility pattern of *Enterococcus sp* is shown in Table 4.

Table 4: Antimicrobial susceptibility pattern of *Enterococcus sp*.

Antibiotics	<i>E. faecalis</i> (81)		<i>E. faecium</i> (19)	
	Resistant (%)	Sensitive (%)	Resistant (%)	Sensitive (%)
Penicillin	62 (76.5)	19 (23.4)	16 (84.2)	3 (15.7)
Ciprofloxacin	42 (51.8)	39 (48.1)	13 (68.4)	6 (31.5)
Hi level Gentamycin	13 (16.0)	68 (83.9)	6 (31.5)	13 (68.4)
Amikacin	28 (34.5)	53 (65.4)	9 (47.4)	10 (52.6)
Cotrimoxazole	41 (50.6)	40 (49.4)	10 (52.6)	9 (47.4)
Linezolid	3 (3.7)	78 (96.2)	1 (5.26)	18 (94.7)
Vancomycin	3 (3.7)	78 (96.2)	3 (15.7)	16 (84.2)

Table 5: Antimicrobial susceptibility and vancomycin typing of resistance *Enterococcus sp*.

P	CIP	HLG	AMI	CO	LIN	VAN	NIT	TET	VAN MIC	TEI MIC	Type	Organism
R	R	S	S	R	S	R	NA	NA	10µg/ML	0.5µg/ml	VAN B	<i>E. faecalis</i>
R	R	R	R	S	S	R	NA	NA	10µg/ml	0.5µg/ml	VAN B	<i>E. faecium</i>
R	S	S	S	R	S	R	NA	NA	30µg/ml	0.5µg/ml	VAN B	<i>E. faecalis</i>
R	R	S	S	R	R	R	NA	NA	30µg/ml	0.5µg/ml	VAN B	<i>E. faecalis</i>
R	R	S	R	S	S	R	NA	NA	60µg/ml	0.5µg/ml	VAN B	<i>E. faecium</i>
R	S	S	R	R	R	R	NA	NA	120µg/ml	16µg/ml	VAN A	<i>E. faecium</i>

MIC for vancomycin, teicoplanin resistant *Enterococcus sp* by E-test method

MIC of vancomycin and teicoplanin was determined by using E-test strips. Based on E-test, the isolates of VRE were defined phenotypically as Van A, Van B, Van C and Van D. Among 100 Enterococcal isolates included in our study, three isolates each of *E. faecalis* and *E. faecium* showed resistance to vancomycin hence tested for MIC of vancomycin and teicoplanin. Out of the 6 isolates tested, 2 isolates showed MIC of vancomycin as 10µg/ml and teicoplanin 0.5µg/ml, 2 isolates showed MIC of vancomycin as 30µg/ml and teicoplanin 0.5µg/ml and 1 isolate showed MIC of vancomycin as 60µg/ml and teicoplanin 0.5µg/ml where grouped into VanB. One isolate from drain tube showed MIC of vancomycin as 120µg/ml and teicoplanin 16µg/ml was grouped into VanA. Antimicrobial susceptibility and Vancomycin typing of resistance *Enterococcus sp* is shown in Table 5.

DISCUSSION

Enterococci have evolved as second most common nosocomial pathogen associated with significant morbidity and mortality.¹⁰ Our prospective study was carried out on 100 Enterococcal isolates, during a 20

months period from May 2015 to December 2016 at Aarupadai Veedu medical college and hospital. The predominant species isolated in this study were *E. faecalis* constituting 81% of isolates followed by 19% of *E. faecium*. The distribution of ratio of *E. faecalis* to *E. faecium* is similar to the study conducted by other authors.^{4,11-14}

According to study conducted by Marothi et al, most frequent infections caused by enterococci are urinary tract infections followed by infections of intra-abdominal and pelvic abscesses or post-surgery wound infections whereas in our study most of the Enterococcal isolates were isolated from urine (44%), Pus (51%) and others specimen (5%, which includes blood 80% and drain tube 20%).¹⁵ These results were similar to the results conducted by Sanal et al, were the maximum number of isolates was obtained from urine.¹⁶

The mean age group of the study participants was 41-50 years; n=22 (22%); 55 of them (55.0%) were females and rest were males. This is similar to a study conducted by Jayavarthini et al, the incidence in females (53.17%) were found to be slightly increased as compared to the males (46.83%).¹⁷ This is partly in accordance with a

study conducted by Katharine Bar et al, who stated that 50% of their cases were females.¹⁸

In this study, overall 15% of *E. faecalis* and 1% of *E. faecium* showed hemolysin production. Higher percentage of hemolysin production was seen among pus isolates (5%). Gelatinase was produced by 6% of *E. faecalis* and 4% of *E. faecium*. Haemolysin producing strains were found to be more than those producing gelatinase by Klibi et al.¹⁹ Among *E. faecalis* isolates 4% of isolates showed positivity for both hemolysin and gelatinase production. The ability to produce haemolysin and gelatinase helps these organisms to acquire adequate nutrition in the host tissues as well as further to the spread of infection in the host body, thus increasing the severity of infection.^{10,16,17}

Majority of *E. faecalis* and *E. faecium* strains isolated in our study, had increased sensitivity for Linezolid, Vancomycin followed by hi level gentamycin and high degree of resistance to Penicillin, Ciprofloxacin and Cotrimoxazole. Our results correlated with the other studies conducted by Salem-Bekhit et al, Jayavarthinni et al, Prakash VP et al, Gupta V et al.^{4,17,20,21}

Analyzing the results of MIC of vancomycin and teicoplanin, 5 isolates were classified phenotypically as Van B phenotype that possess only moderate to high levels of vancomycin resistance and one isolate obtained from drain tube which showed MIC of vancomycin as 120µG/ml and teicoplanin 16µg/ml was grouped into VanA. In enterococci, two principal phenotypes of acquired vancomycin resistance have been described, VanA and VanB. The VanA determinant is carried on transposon Tn1546 or close relatives that are transferable in conjugation experiments.⁴

CONCLUSION

Though the prevalence of Vancomycin resistant *Enterococci* (VRE) is very low in our study, yet regular monitoring of vancomycin resistance is very crucial for early detection, treatment, application of preventive and control measures and most importantly to check the spread of virulent multidrug resistant *Enterococcus species*.

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