International Journal of Research in Medical Sciences
Jayavarthinni M et al. Int J Res Med Sci. 2015 Oct;3(10):2623-2629
www.msjonline.org
pISSN 2320-6071 | eISSN 2320-6012

DOI: http://dx.doi.org/10.18203/2320-6012.ijrms20150802

Research Article

Phenotypic speciation of enterococci with special reference to prevalence, virulence and antimicrobial resistance

Jayavarthinni Manavalan[1]*, Kavitha Kannaiyan[2], Abarna Velayutham[3], Sindhanai Vadivel[4], Sethumadhavan Kuthalaramalingam[2]

[1]Department of Microbiology, Indira Gandhi Medical College and Research Institute, Pondicherry, India
[2]Department of Microbiology, Aarupadaiveedu Medical College and Hospital, Pondicherry, India
[3]Department of Microbiology, Sri Lakshmi Narayana Institute of Medical Sciences, Pondicherry, India
[4]Department of Microbiology, Dhanalakshmi Srinivasan Medical College and Hospital, Perambalur, Tamilnadu, India

Received: 12 August 2015
Accepted: 04 September 2015

***Correspondence:**
Dr. Jayavarthinni Manavalan,
E-mail: jayavarthinni@yahoo.co.in

ABSTRACT

Background: Now considered as one of the most important Nosocomial pathogen, enterococci have been found to possess virulence factors like biofilm formation and are increasingly exhibiting antimicrobial resistance in India. This study was undertaken to estimate the prevalence of enterococci from various clinical samples simultaneously correlating their virulence property and antimicrobial resistance, in addition to speciation.
Methods: A total of 126 enterococcal isolates from various clinical samples were included and processed according to standard protocols and speciation was based on Facklam and Collins conventional method. Virulence determinants like hemolysin, gelatinase and biofilm formation were assessed by phenotypic tests. Antibacterial susceptibility pattern was determined by Kirby Bauer disc diffusion method with recommended drugs including high level aminoglycoside resistance. Minimum inhibitory concentration (MIC) for vancomycin was done by E-test.
Results: Out of 1746 clinical samples, enterococci accounted for 7.22%. They consisted of *E. faecium* 52.38%, *E. faecalis* 32.54%, and *E. avium* 15.08% isolated from urine 8.26%, pus 8.44%, blood 0.56% and body fluids 1.28%. Study on virulence factors revealed that 19.84% strains produced gelatinase, 18.25% produced hemolysin and 73.81% produced biofilm. High level resistance to gentamycin and streptomycin were 4.76% and 5.56% respectively. Vancomycin resistance was 3.17%.
Conclusions: This study indicates the change in epidemiology of enterococcal infections from *E. faecalis* to *E. faecium* and low prevalence of vancomycin resistant enterococcus (VRE) in our region. To maintain the low level of resistance, improvement of antibiotic policies and hospital infection control is essential.

Keywords: Enterococci, Biofilm formation, Vancomycin resistance, E test

INTRODUCTION

Enterococci though considered as perpetual commensal organisms well suited for survival in intestinal and vaginal tracts and the oral cavity, possess certain properties that can be ascribed roles in pathogenesis. The natural ability of enterococci to readily acquire, accumulate, and share extra chromosomal elements encoding virulence traits or antibiotic resistance genes lends advantages to their survival under unusual environmental stresses and in part explains their increasing importance as nosocomial pathogens.[1]

Also enterococci have become a vexing problem in clinical medicine because of their ability to infect patients who are typically receiving antibiotic therapy for

unrelated underlying illness. Moreover, the acquisition of high level aminoglycoside resistance and vancomycin resistance has limited the therapeutic options available and infections have become extremely difficult to manage.[2] They also show intrinsic resistance to a number of commonly used antibiotics particularly the cephalosporins.[3] The ability of enterococci to cause disease is an intrinsic property of the organism or possibly subpopulations within enterococcal species. By altering endogenous bacterial flora, antibiotic therapy promotes increased colonization by antibiotic-resistant organisms. Therefore, antibiotic resistance and intrinsic virulence both contribute to disease, but in separate and complementary ways.[1]

A number of studies had determined the prevalence of enterococci in India.[4,5] However, only a very few studies focused on the prevalence of virulence factors of enterococci. This study was conducted to analyze the prevalence of *Enterococcus* species in various specimens and to correlate the virulence factors like gelatinase production, hemolysin production, and biofilm formation with antimicrobial resistance, in specific, vancomycin resistance and high level aminoglycoside resistance to guide infection control practices.

METHODS

All the heterogeneous clinical samples received by the Department of Microbiology in Aarupadai Veedu Medical College and Hospital, Puducherry, for a period of one year, from March 2010 to February 2011 in which enterococci was isolated were included in the study. A total of 126 *Enterococcus* species was isolated from various clinical samples. The genus *Enterococcus* was confirmed by Gram stain, i.e. gram positive cocci in pairs and short chains, esculin hydrolysis, hydrolysis of L-pyrrolidonyl-β-napthylamide (PYR) test and salt tolerance.[2] Enterococcal strains were further identified to species level by using conventional physiological tests devised by Facklam and Collins.[6]

Test for virulence factors:

Production of gelatinase was assessed by the ability of the enterococci to liquefy gelatin.[7] Hemolysin production was measured by the macroscopic appearance of complete zone of hemolysis (beta hemolysis) in blood agar plate supplemented with 5% sheep blood.[8] Biofilm production was assessed by Congo red agar method by viewing the black colour colonies and by tube method[9] using Brain Heart Infusion (BHI) broth and crystal violet (0.1% stain).

Antibiotic susceptibility testing:

Antibiotic susceptibility testing of the clinical isolates was performed using Kirby-Bauer disk diffusion method. Mueller-Hinton agar supplemented with 5% sheep blood was used.[10] The antibiotic discs were purchased from Hi Media, Mumbai. The antibiotic discs and their potency were as follows: penicillin (10 units), gentamycin-high content (120 µg), streptomycin-high content (300 µg), ciprofloxacin (5 µg), vancomycin (30 µg), nitrofurantoin-for urinary isolates only (300 µg), ceftriaxone (30 µg) and linezolid (30 µg).

E-test was done to determine the minimum inhibitory concentration of vancomycin for all the clinical isolates of enterococci. The E-test is comprised of two strips: Strip A: 240-0.01 µg and Strip B: 4-0.001 µg (Hi Comb, MIC test, HIMEDIA laboratories).The results were interpreted as per NCCLS guidelines.[11]

RESULTS

The study of enterococci in varied infections revealed that out of 1746 clinical samples, enterococci accounted for 7.22%, isolated from urine 8.26%, pus 8.44%, blood 0.56% and body fluids 1.28% (Table 1).

Table 1: Prevalence of enterococci in various clinical materials.

Clinical material (No.)	No. of enterococci isolates (%)
Urine (n=872)	72 (8.26)
Pus (n=616)	52 (8.44)
Blood (n=180)	1 (0.56)
Ascitic fluid (n=78)	1 (1.28)
Total (n=1746)	126 (7.22)

They consisted of *E.faecium* 52.38%, *E.faecalis* 32.54%, and *E.avium* 15.08% (Figure 1).

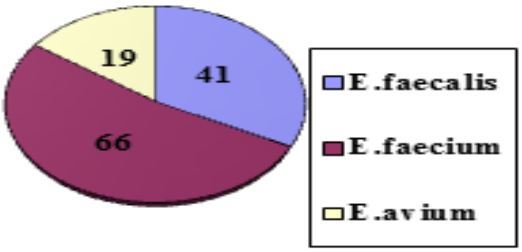

Figure 1: Distribution of *Enterococcus* species.

They occurred more commonly in the age group of more than 50 years with female preponderance (Figure 2).

Study on virulence factors revealed that 19.84% strains produced gelatinase, 18.25% produced hemolysin and 73.81% produced biofilm (Table 2).

Tube method was more sensitive for the detection of biofilm and 73.81 % were positive by tube method (Figure 3). By congored agar method, 40.47 % were positive (Figure 4).

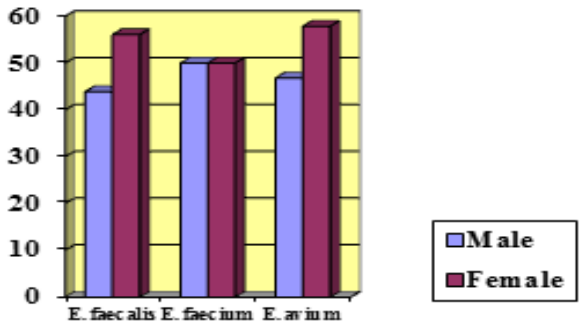

Figure 2: Age and Sex distribution of enterococcus species.

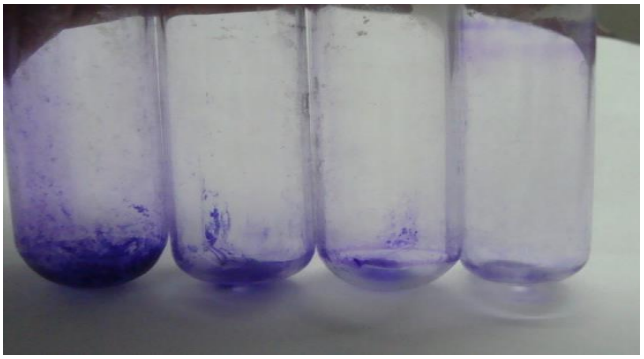

Figure 3: Tube test for biofilm formation (strongly positive, moderately positive, weakly positive, negative).

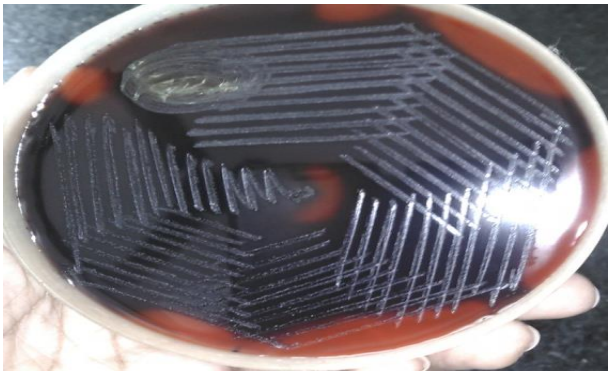

Figure 4: Congo red agar showing black coloured colonies indicative of biofilm formation.

Trends in antimicrobial susceptibility to a variety of agents were also determined. All the isolates were sensitive to linezolid (100% sensitivity) and all were resistant to ceftriaxone (100% resistance), 107 (84.92%) isolates were resistant to penicillin, 84 (66.67%) isolates were resistant to ciprofloxacin, 29 (40.27%) isolates were resistant to nitrofurantoin (for urinary isolates). High level resistance to gentamycin and streptomycin were exhibited by 6 (4.76%) and 7 (5.56%) isolates respectively. Isolates resistant to vancomycin was 4 (3.17%) (Table 3) * Vancomycin resistance detected by Disc diffusion and E- Test.

Table 2: Production of the three virulence factors in isolates from different clinical conditions.

Clinical samples	Gelatinase (%)	Hemolysin (%)	Biofilm (%)
Urine (n=72)	16(22.22%)	16(22.22%)	52(72.22%)
Pus (n=52)	8(15.38%)	7(13.46%)	39(75.0%)
Blood (n=1)	1(100%)	0	1(100%)
Ascitic fluid (n=1)	0	0	1(100%)
Total (n=126)	25(19.84%)	23(18.25%)	93 (73.80%)

Minimum inhibitory concentration for Vancomycin was studied for all the isolates. All of them were within the range of 2 microgram, except 4(6.06%) isolates of *E. faecium* in which MIC was in between 30 to 60 microgram (Figure 5).

DISCUSSION

Despite the fact that enterococci have been considered to have relatively low virulence, in the past few years these organisms, among all nosocomial pathogens have emerged as a significant concern.

Screening of various clinical specimens like pus, urine, blood and body fluids in our institution for a period of one year revealed that enterococci were prevalent in 7.8% of the total specimens, with wound and urine to be the major site of isolation (Table 1). The prevalence rate in our study was in accordance to the study conducted in Mumbai by Rupali S Shinde *et al* where enterococci caused approximately 5.5% of all infections.[12]

But a study by Anbumani *et al* from Southern India[5] reported that enterococci were prevalent in only 2% of the total specimens, with urine and wound to be the major site of isolation similar to our study. In contrast a study by Desai *et al*[4] stated a higher prevalence of 22.19% in

Northern India. He also affirmed that enterococci isolated from various clinical specimens do not reflect the true incidence of infection caused by this organism.

The most frequent infections caused by enterococci are urinary tract infections. The second most frequent enterococcal infections generally have been intra-abdominal and pelvic abscesses or post-surgery wound infections.[13] However, the role played by enterococci in these cases has not been fully defined, since infections of surgical wounds and of the urinary tract often resolve without specific therapy.

In this study the prevalence rate of enterococci in pus sample was 8.44% and in urine was 8.22% (Table 1). A study by Orett *et al* also showed a similar prevalence rate of 11 % in urine.[14]

Longer hospital stay and immunocompromised conditions are known risk factors for nosocomial infections like enterococcal infections.[13] Among the hospitalized patients the post-surgical patients have longer hospital stay and have more chance of cross infection. So it may be the cause for higher incidence of enterococcal isolation from surgical ward samples.

Table 3: Antibiotic resistance pattern in *Enterococcus* species.

Antibiotics	*E. faecalis* (n=41)		*E. faecium* (n=66)		*E. avium* (n=19)		Total No. of isolates, n=126(%)
	No	(%)	No	(%)	No	(%)	
Penicillin	31	75.61	63	95.45	13	68.42	107(84.92)
Gentamycin (120 ug)	0	0	6	9.09	0	0	6 (4.76)
Streptomycin (300 ug)	0	0	7	10.60	0	0	7 (5.56)
Ciprofloxacin	28	68.30	42	63.64	14	73.68	84 (66.67)
Vancomycin*	0	0	4	6.06	0	0	4 (3.17)
Nitrofurantoin (n=72)	7 (n=25)	28.0	19 (n=41)	46.34	3 (n=6)	50	29 (40.27)
Ceftriaxone	41	100	66	100	19	100	126 (100)
Linezolid	0	0	0	0	0	0	0 (0)

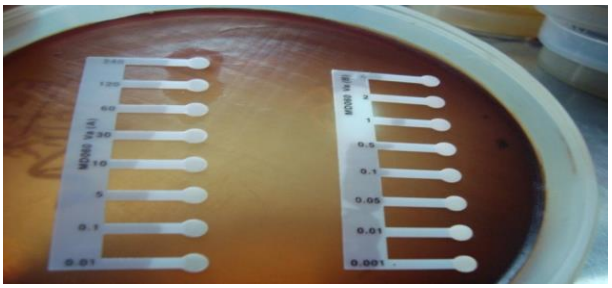

Figure 5: E Test for determination of minimum inhibitory concentration (MIC) for vancomycin.

Epidemiologic studies appear to conflict with respect to the association between enterococcal species and disease. Historically, the ratio of infections due to *E. faecalis* to those due to all other *Enterococcus* species was approximately 10:1. In recent years, there has been a progressive decline in this ratio of enterococcal infections. In our present study also, *E. faecium* (52.3%) was more common than *E. faecalis* (32.5%). Yet another study in Mumbai also stated that *E. faecium* was more prevalent than *E. faecalis*.[15] This microbiologic shift is likely to be explained in part by the emergence of VRE, in particular, the predominance of the species *E. faecium* among this subset of enterococcal isolates.

In general, enterococcal infections are distributed equally between the sexes. Although urinary tract infections are more common in healthy women than in healthy men, enterococci are an uncommon cause of uncomplicated cystitis in our settings. In accordance in our present study the incidence in females (53.17%) were found to be slightly increased as compared to the males (46.83%). This is partly in accordance with a study conducted by Katharine Bar *et al*[16], who stated that 50% of their cases were females.

Virulence among enterococci appears to have evolved in a mode and tempo that are no different from the emergence of pathogenic lineages of other species. Enterococci virulence factors include the phenotypic markers gelatinase, hemolysin, and biofilm formation.

Hemolysin is a cytolytic protein capable of lysing human, horse, and rabbit erythrocytes. Gelatinase is a protease produced by *E. faecalis* that is capable of hydrolyzing gelatin, collagen, casein, hemoglobin, and other peptides.

Hemolysin and gelatinase producing strains of enterococcus might play a role in virulence and associated with increased severity of infection.[17]

The incidence of hemolysin and gelatinase production in our study was 18.25% and 19.84% respectively. This was similar to the study conducted by Masoud Alebouyeh *et al*[8] who demonstrated the hemolysin and gelatinase production as 16.4% and 12% respectively, in clinical isolates. However Coque *et al*[17] reported a significantly higher frequency of gelatinase positive phenotype in clinical isolates that is 54% of endocarditis isolates and 58% of nonendocarditis clinical isolates. His study also showed that as many as 16% of the infection-derived *Enterococcus* isolates were hemolytic. All of the isolates we studied were clinically virulent, because all were associated with bacteremic illness. However the significance of these factors in the pathogenesis of enterococcal infection needs to be elucidated in further studies.

Biofilm production is an important factor which helps the organism to adhere onto surfaces, which facilitates later in invasion and causing infection. The ability to form biofilm on medical devices is a potential virulence trait that may allow enterococci to cause infections in the expanding population of patients managed with such devices. In the present study 73.61% of the enterococcal isolates formed biofilm. According to the National Institutes of Health, biofilms are medically important, accounting for >60% of microbial infections in the body.[18]

In our study by means of tube method, 93 (73.81%) isolates were positive by biofilm formation; while only 51 (40.47%) isolates were positive by congored agar method and 33 (26.19%) were negative by both methods. Also in the present study, by congored agar method very different results were obtained, 59.52% of the strains displayed red (pink to orange) colonies while 40.47% of the isolates showed black colonies, but no dry crystalline morphology was observed.

Although, there are many studies related to methods which are used to determine the biofilm production in bacteria, very few data exist on comparison of the biofilm screening methods for enterococcus. However the use of CRA test for determination of *S. aureus* biofilm formation yields inconsistent results. Mathur *et al*[9] tested 152 clinical isolates of staphylococci by three in vitro screening procedures (tissue culture plate, TM and CRA) for their ability to form biofilm. CRA method showed very little correlation with either of the two methods and the parameters of sensitivity (6.8%), specificity (90.2%) and accuracy (40.9%) were found as very low. Moreover in our study also, the diversity in colony colours by CRA (from red to pink or white) was variable and sometimes it was difficult to differentiate. Therefore, based on our results we are unable to recommend CRA to screen for biofilm formation.

The prevalence of three virulence factors namely biofilm, gelatinase and hemolysin was compared and contrasted (Table 2). Nosocomial strains of organisms develop various mechanisms in colonizing to cause infection. Biofilm production is an important factor which helps the organism to adhere onto surfaces, which facilitates later in invasion and causing infection. This study shows that more than 70% of the isolates produced biofilm formation.

A comparative study among the different clinical isolates with respect to production of the three virulence factors shows that biofilm production is very high (more than 70%) among isolates grown from urinary tract infection and wound infection. Thus we can say that the biofilm production in nosocomial strains of organisms is an important pathogenic factor in causing infection in the hospital environment. Other virulence factors also have a role in the occurrence of infection and in the clinical outcome.

Enterococci are intrinsically resistant to many antimicrobial agents and this intrinsic resistance to commonly used antimicrobial agents may have allowed them a cumulative advantage for further acquisition of genes encoding high-level resistance to aminoglycosides, penicillins, tetracycline, chloramphenicol, and now vancomycin.

Penicillin resistance has increased in the recent years. In the present study (Table 3), 107 (84.92%) enterococcal strains showed penicillin resistance. This was similar to a study in India where they showed 89.43% isolates resistant to pencillin.[3] However in a cross sectional study Simonsen *et al*[19] stated *E. faecalis* isolates were uniformly susceptible to ampicillin, and also only 40% of *E. faecium* isolates were resistant to ampicillin. Because penicillin is the main stay of therapy for infections due to enterococci, the organism's development of high level resistance to this drug would have important clinical implications.

Combination therapy with cell wall active agent (penicillin, ampicillin and vancomycin) and aminoglycoside (gentamycin or streptomycin) is recommended for the treatment of serious enterococcal infections. But high level resistance to aminoglycoside could nullify this combination. Therefore to distinguish this high level aminoglycoside resistance strains from simple intrinsic resistant strains is of utmost importance.

Streptomycin and gentamycin resistance in the present study was 5.56% and 4.76% respectively (Table 3). All the resistant strains belonged to *E. faecium* alone. In India (Nagpur), a study by Agarwal *et al*[20] reported a prevalence of high level gentamicin resistance in enterococci to be 12% whereas high level streptomycin resistance was reported to be 24.7%. However in two other studies conducted in Delhi, 36% of *E. faecium* and

50% of *E. faecalis* isolates exhibited HLAR[21], and in another study 73.3% of HLAR isolates were detected.[22]

The emergence of vancomycin resistant enterococci (VRE) is a cause for concern because of the limited therapeutic options for treating serious infections and because of their potential to transfer vancomycin-resistance genes to other organisms, such as methicillin-resistant Staphylococcus aureus. Also, VRE has spread throughout the world to become a major cause of nosocomial infections. Moreover infections due to VRE may be associated with greater morbidity, mortality, lengths of stay and hospital costs than those due to vancomycin-susceptible enterococci (VSE) and independent of co-morbid conditions that may have led to infection.[23]

In our study, determination of vancomycin resistance was performed in all the 126 isolates of enterococci by disc diffusion assay and MIC by E-test. The E-test is a new in vitro susceptibility testing method used for quantitative determination of susceptibility to antimicrobial agents. E-test technology is based on the diffusion of a defined continuous antimicrobial gradient from a thin plastic strip, producing an organism inhibition ellipse whose intercepts with the graded test carrier indicate MICs.[24]

All the isolates of *E. faecalis*, 41(100%) and *E. avium*, 19(100%), minimum inhibitory concentration (MIC) for vancomycin were less than 2 microgram. Whereas, among the 66 isolates of *E. faecium*, 62 (93.94%), MIC was less than 2 microgram, while the remaining 4 (6.06%) MIC was in between 30 to 60 microgram (Figure 6). Only 4 isolates of *E. faecium* were resistant to vancomycin both by disk diffusion and E-test, which is only 3.17 % were resistant to vancomycin of MIC value in between 30 to 60 microgram. It indicates that vancomycin resistance is low in our region which was in accordance to a study conducted by Ragandale *et al*.[3]

Other antibiotics like ciprofloxacin and nitrofurantoin showed high resistance (Table 3). Resistance was 66.67% for ciprofloxacin and 40.27% for Nitrofurantoin. This was in accordance with the study conducted by Vittal P Prakash *et al*[25] who found ciprofloxacin resistance to be 63%. All the isolates were resistant to Ceftriaxone (Table 3). This is due to factor that all the enterococci exhibit intrinsic resistance to cephalosporins.[13] In the present study 100% sensitivity to linezolid was observed (Table 3). This was in accordance with the study conducted by Gupta V *et al*.[26] Linezolid may be of utmost utility for multidrug resistant strains.

CONCLUSION

This study confirms the worldwide trend in increasing occurrence of enterococci and the emerging pattern of antimicrobial resistance among such isolates. Our study demonstrated that *E. faecium* is now emerging as the predominant enterococcal isolate from human infections and thus the changing epidemiology of enterococcal infections. Biofilm was the most common virulence factor seen in our isolates. Given the growing importance of Enterococcus species as nosocomial pathogens the identification of virulence factors associated with enterococcal invasiveness and disease severity will be an important subject of future investigations. Development of other mechanisms like blocking of Enterococcal biofilm production or inhibiting the action of other virulence factors may provide an alternate method of therapy in the face of antimicrobial resistance infections. Finally 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.

ACKNOWLEDGEMENTS

Authors sincerely thank all the faculty and staff of Department of Microbiology, Aarupadai Veedu Medical College and Hospital, Puducherry, for their constant support. Authors also acknowledge the immense help received from the scholars who articles are cited and included in references of this manuscript. The authors are also grateful to authors/editors/ publishers of all those articles, journals and books from where the literature for this article has been reviewed and discussed.

Funding: No funding sources
Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

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Cite this article as: Jayavarthinni M, Kannaiyan K, Abarna V, Sindhanai V, Sethumadhavan K. Phenotypic speciation of enterococci with special reference to prevalence, virulence and antimicrobial resistance. Int J Res Med Sci 2015;3:2623-9.