

Drug resistance & virulence determinants in clinical isolates of *Enterococcus* species

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Background & objectives: Enterococci are the leading cause of nosocomial infections, and are thus a persisting clinical problem globally. We undertook this study to determine the virulence factors and the antibiotic resistance in *Enterococcus* clinical isolates.

Methods: One hundred and fifty *Enterococcus* isolates obtained from various clinical specimens were speciated biochemically and subjected to antibiotic susceptibility testing using Kirby-Bauer disk diffusion method. Resistance to vancomycin was determined by using agar screen method. Haemolysin and gelatinase productions were detected using 5 per cent sheep blood agar and 12 per cent gelatin agar, respectively.

Results: Among the 150 *Enterococcus* isolates, 84 (56%) were *E. faecalis*. 51(34%) *E. faecium*, and 15 (10%) were other *Enterococcus* spp. Haemolysin production was seen among 123 (82%) isolates while 61 (40.6%) isolates produced gelatinase. Nearly 50 per cent of the isolates showed high level aminoglycoside resistance (HLAR). A total of 13 (8.6%) isolates showed vancomycin resistance, of which 11(7.3%) had an MIC >8 µg/ml.

Interpretation & conclusions: Presence of VRE was found to be low among the isolates studied. However, occurrence of VRE along with HLAR calls for regular detection of vancomycin resistance promptly and accurately to recognize VRE colonization and infection. Early detection of VRE and HLAR along with their virulence trait will help in preventing the establishment and spread of multidrug resistant *Enterococcus* species.

Key words Gelatinase - haemolysin - HLAR - vancomycin resistant enterococci

Enterococci, recognized as opportunistic pathogens, are natural inhabitants of the oral cavity, gut and the female genital tract in both humans and animals^{1,2}. These are an important global cause of nosocomial infections³. Of the two most common *Enterococcus* species, *E. faecalis* has been found to be responsible

for 80-90 per cent⁴ and *E. faecium* for the remaining human enterococcal infections⁵. The most frequent infections caused by these organisms include urinary tract infections followed by intra-abdominal or intra-pelvic abscesses. Blood stream infections are the third most common infections caused by these organisms⁶.

Enterococci with high level aminoglycoside resistance (HLAR), β -lactamase production and glycopeptides resistance including vancomycin resistant enterococci (VRE) have emerged, thus posing a therapeutic challenge to physicians^{7,8}. Epidemiological data also suggest that enterococci are important reservoirs for transmission of antibiotic resistance genes among different species of bacteria⁹. Thus, the occurrence of antimicrobial resistant enterococci, especially VRE is a persisting clinical problem in health care facilities in all geographical areas. This study was undertaken to know about the antimicrobial susceptibility patterns and virulence factors in clinical isolates of enterococci.

Material & Methods

A total of 150 enterococcal isolates obtained from clinical samples (urine, pus, sputum, blood, vaginal swabs) sent to the Department of Microbiology, Kasturba Medical College, Mangalore, India, for routine culture and sensitivity, during July-September 2011, were included in the study by following convenient sampling method. The power of the study was 80 per cent. Ethical clearance of the study protocol was taken from the institutional Ethics Committee prior to the commencement of the study.

All the culture media, antibiotics discs and standard strains of bacteria used in study was procured from Himedia Laboratories Pvt. Ltd., Mumbai, India.

Urine samples were cultured by semiquantitative method on Cysteine Lactose Electrolyte Deficient (CLED) medium and blood agar. Blood samples were processed by BacT/ALERT 3D (BioMerieux Inc. USA) automated system. All other samples were inoculated on blood agar and MacConkey's agar. All culture plates were incubated at 37°C for 24-48 h and examined for growth. Identification of genus *Enterococcus* was done using colony morphology, Gram staining, bile esculin test and salt tolerance test. Speciation was done according to Manero and Blanch¹⁰ using biochemical tests like tellurite resistance and fermentation of mannitol and sorbitol.

Haemolysin and gelatinase production were studied using the method adopted by Upadhyaya *et al*¹¹. Briefly, a single colony of biochemically identified *Enterococcus* spp. was inoculated on 5 per cent sheep blood agar and 12 per cent gelatinase agar. The presence of a clear zone of β -haemolysis around the colonies indicated the production of haemolysin while a clear halo around the colonies on gelatinase agar indicated gelatinase activity. *Serratia mercenses* ATCC 13880

(Himedia Laboratories Pvt. Ltd., Mumbai, India) was used as positive control for gelatinase activity.

Antibiotic susceptibility testing for ampicillin, chloramphenicol, erythromycin, co-trimoxazole and teicoplanin was done by Kirby-Bauer disk diffusion method¹² on Mueller- Hinton agar and the results were interpreted as per Clinical and Laboratory Standards Institute (CLSI) guidelines¹³. *E. faecalis* ATCC 29212 (Himedia Laboratories Pvt. Ltd., Mumbai, India) was used as a quality control strain. HLAR was determined by disk diffusion method using high level gentamicin (120 μ g) and streptomycin (300 μ g) disks (Himedia Laboratories Pvt. Ltd., Mumbai, India). A diameter of the zone of inhibition <6 mm indicated resistance, 7-9 mm indicated that the test was inconclusive and \geq 10 mm indicated susceptibility¹⁴.

Vancomycin agar screen test was performed using Brain Heart infusion (BHI) agar with 6 μ g/ml vancomycin to look for resistance to vancomycin. One or more colony or a film of growth indicated resistance to vancomycin. Vancomycin sensitive strain *E. faecalis* ATCC 29212 was used as negative control and vancomycin resistant strain *E. faecalis* ATCC 51299 was used as positive control¹⁴.

Determination of minimum inhibitory concentration (MIC) of vancomycin for enterococcal isolates which grew on vancomycin agar screen was done by agar dilution method¹³. Brain-heart infusion agar (Hi Media, Mumbai) was supplemented with different concentrations of vancomycin. Ten microliter of bacterial culture was spot inoculated after adjusting the turbidity with McFarland 0.5 standard. The plates were incubated at 37°C for 24 h and examined for growth. The minimum concentration of vancomycin which inhibited bacterial growth was considered MIC. Enterococci which had MIC >32 μ g/ml were considered resistant; 8-16 μ g/ml as intermediately resistant and MIC of 4 μ g/ml as susceptible to vancomycin as per CLSI guidelines¹³.

Results

E. faecalis was the commonest (56%) species isolated followed by *E. faecium* (34%) (Table I). Haemolysin was produced by 82 per cent and gelatinase by 40.6 per cent of the isolates. Haemolytic activity was seen in all the species studied whereas gelatinase production was not demonstrated in *E. durans* and *E. avium*. The production of haemolysin was seen in more number of isolates in each species; 43.9 per cent of *E. faecalis* and 29.5 per cent of *E. faecium* produced both the virulence factors tested.

Table I. Distribution of *Enterococcus* spp. in various clinical specimens

Sample (n=150)	<i>E.faecalis</i>	<i>E.faecium</i>	<i>E.durans</i>	<i>E.dispar</i>	<i>E.avium</i>	<i>E.ceconum</i>	Total (%)
Urine (n=59)	37	20	1	1	0	0	39.3
High vaginal swab (n=53)	31	14	2	4	1	1	35.3
Wound swab (n=13)	3	8	1	0	1	0	8.6
Pus (n=10)	6	3	1	0	0	0	6.6
Sputum (n=6)	3	2	1	0	0	0	4
Blood (n=3)	2	1	0	0	0	0	2
Catheter tip (n=3)	1	2	0	0	0	0	2
Bile (n=3)	1	1	0	0	1	0	2
Total (%)	56	34	4	3.3	2	0.6	100

Resistance of *E. faecium* isolates was higher than *E. faecalis* to all antibiotics except chloramphenicol. HLSR was higher in *E. faecium* (58.8%) than *E. faecalis* (48.8%) whereas HLGR was almost similar in both (Table II). HLGR was detected in 51.3 per cent and HLSR in 49.3 per cent of the total isolates. Fifty one isolates (34%) showed both HLGR and HLSR which included 35.7 per cent (n=30) of the *E. faecalis* and 35.3 per cent (n=18) of the *E. faecium* isolates. Thirteen isolates were found to be vancomycin resistant, of which 11 had an MIC > 8 µg/ml and <16 µg/ml which can be considered as vancomycin intermediately resistant. The VRE included four *E. faecalis*, six *E. faecium*, two *E. dispar* isolates and one *E. avium* isolate. Among the VRE, three *E. faecium* isolates (two from urine and one from blood) and one isolate each of *E. faecalis* and *E. avium* were found to be resistant to teicoplanin.

Discussion

The isolation rate of *E. faecalis* in our study was more than that of *E. faecium*. Similar results have been

reported from central and south India^{15,16}. However, studies carried out in north India have shown *E. faecium* to be responsible for a larger number of enterococcal infections than *E. faecalis*¹⁷. In our study 82 per cent of the isolates were tested positive for haemolysin and 40.6 per cent for gelatinase. Haemolysin producing strains were also found to be more than those producing gelatinase by Klibi *et al*¹⁸.

It was also observed that, nearly 44 per cent of the *E. faecalis* isolates possessed both haemolysin and gelatinase enzymes while production of both the factors was substantially lesser in the case of *E. faecium* isolates. This may possibly be one of the reasons why the species of *E. faecalis* is responsible for a greater number of infections than *E. faecium*. The ability to produce haemolysin and gelatinase helps these organisms to acquire adequate nutrition in the host tissues as well as further the spread of infection in the host body, thus increasing the severity of infection¹¹. Gelatinase production alone was considerably higher (50%) among the *E. faecalis* isolates than in *E. faecium*

Table II. Antibiotic resistance (%) among the enterococcal isolates

Antibiotic	<i>E.faecalis</i> (n=84)	<i>E.faecium</i> (n=51)	<i>E.durans</i> (n=6)	<i>E.dispar</i> (n=5)	<i>E.avium</i> (n=3)	<i>E.ceconum</i> (n=1)
Ampicillin	36.9	52.9	33.3	0	66.7	100
Erythromycin	81.0	90.1	0	100	33.3	100
Chloramphenicol	45.2	37.2	0	0	0	0
Co-trimoxazole	64.2	70.5	0	20.0	33.3	100
HLSR	48.8	58.8	16.7	40.0	0	0
HLGR	53.5	53.0	16.7	60.0	0	100
Teicoplanin	1.2	5.8	0	0	33.3	0
Vancomycin	4.7	11.7	0	40.0	33.3	0

HLSR, high level streptomycin resistance; HLGR, high level gentamicin resistance

isolates (34%). Majority of the wound infections in the present study was found to be caused by *E. faecium* isolates.

Antibiotic resistance among enterococci is a global problem. In our study, the highest resistance was seen against erythromycin, which is in agreement with other studies carried out in India^{19,20}. Forty two per cent of the enterococcal isolates were resistant to ampicillin. This was in concurrence with the findings of Karmarkar *et al*²¹. However, other studies^{17,20} have reported higher rates of ampicillin resistance. Overall, *E. faecium* isolates were more resistant than *E. faecalis* in our study, as has been reported earlier^{15,16}.

The occurrence of HLGR among the enterococcal isolates in our study was seen to be 53 per cent with no significant difference seen between *E. faecalis* and *E. faecium* isolates. Mendiratta *et al*¹⁵ have reported greater resistance to HLG among *E. faecium* as compared to *E. faecalis* isolates. Studies conducted in New Delhi and Mumbai have reported HLGR prevalence to be as high as 70 and 100 per cent, respectively^{21,22}.

High level streptomycin resistance was found in 49.3 per cent of the isolates in our study, with *E. faecium* (59%) showing greater resistance as compared to *E. faecalis* (49%). HLSR in our study was observed to be higher than that reported from other Indian studies^{17,19,22}, thus reflecting greater usage of streptomycin in this region.

In India, the prevalence of VRE has been reported to be between 0- 30 per cent^{17,19,20-23}. In our study, 13 isolates were found to be resistant to vancomycin with *E. faecium* (11.7%) showing higher resistance than *E. faecalis* (4.7%). Karmarkar *et al*²¹ had also reported greater resistance among *E. faecium* isolates though Agarwal *et al*¹⁹ found vancomycin resistance to be greater among *E. faecalis* isolates.

Resistance to teicoplanin among the VREs was seen to be much lesser in our study than reported by Karmarkar *et al*²¹. Of the thirteen VRE isolates, five showed resistance to teicoplanin. Seven VREs were found to be susceptible to either high level streptomycin or gentamicin. Hence, these infections could be treated with a combination of a high level aminoglycoside and a β lactam antibiotic. However, the other six isolates showed both HLGR and HLSR, thus making the treatment in such cases extremely difficult.

Vancomycin resistant enterococci can be expected to be a major problem in the coming years and hence it

is essential that adequate measures be taken in all health care settings to contain the dissemination of the resistant strains. Routine testing of all enterococcal isolates for vancomycin resistance at least by vancomycin agar screen test, judicious use of vancomycin, rapid isolation of patients suspected to have VRE infections and effective surveillance mechanisms will go a long way in limiting the spread of VRE. Further studies are necessary to characterize the virulence factors and the drug resistance genes of enterococcal isolates by molecular methods to know their role in the pathogenesis of nosocomial infections.

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