

## PREVALENCE, IDENTIFICATION AND DISTRIBUTION OF VARIOUS ENTEROCOCCAL SPECIES ISOLATED IN KATIHAR DISTRICT, BIHAR WITH SPECIAL REFERENCE TO VRE.

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**ABSTRACT: BACKGROUND:** Identification of enterococci to species level in order to determine the species prevalent in this geographic region and also to determine the species-specific antimicrobial susceptibility pattern. **OBJECTIVE:** To detect and determine glycopeptide resistance by screening for vancomycin resistant enterococci (VRE) in both colonized and infected patients. **METHODS:** A total of 123 isolates of enterococcus species were recovered from various clinical and faecal samples of hospitalized patients, from September 2010 to June 2011. Various species of enterococci were identified by standard methods. Vancomycin susceptibility in enterococci was detected by disc diffusion method (DDM), vancomycin screen agar method (VSAM) and agar dilution method to determine minimum inhibitory concentration (MIC). **RESULTS:** *E. faecalis* was the predominant isolate from the clinical and faecal samples. Multidrug resistance was more in *E. faecium* than *E. faecalis*. MIC method could detect 7 VRE and 27 strains with reduced susceptibility to vancomycin. Disk diffusion test and vancomycin screen agar failed to detect 50 % and 29.4% of resistant strains respectively. **CONCLUSION:** Vancomycin resistance was also detected in less virulent strains of enterococcus like *E. gallinarum* and *E. dispar*. In laboratories where performance of MIC studies is not feasible, VSAM method should be preferred over the DDM for detection of vancomycin resistance in enterococci.

**KEY WORDS:** Enterococcus species, vancomycin resistant enterococci (VRE), multi drug resistant enterococci (MDRE)

**INTRODUCTION:** Although considered benign and opportunistic pathogens, Enterococci have long been known to cause endocarditis in hospitalized patients. Their resistance to several antimicrobial agents, whether intrinsic (low level resistance to penicillin, cephalosporins and aminoglycosides) or acquired ((high level aminoglycoside resistance) is of great concern. <sup>(1)</sup> This increasing prevalence has been paralleled by the occurrence of vancomycin-resistant strains, which were first reported in 1988. <sup>(2)</sup> Recently VRE and MDRE have emerged as a leading cause

of nosocomial infections in patients who are either debilitated or as superadded infection especially in surgical and tertiary care units, particularly after organ transplants. These strains are emerging as causes of meningitis and other infections of the central nervous system in neonates and as osteomyelitis, lung infection, urinary tract and pelvic infections in adults.<sup>(1)</sup>

Antibiotics that achieve high gastrointestinal concentration but are inactive against enterococci favour colonization of the gastro intestinal tract with VRE. Colonization with VRE can lead to serious diseases like urinary tract infections, bacteremia and VRE sepsis which can be fatal.<sup>(3)</sup>

To the best of our knowledge no study on enterococcus has been carried out in Bihar and Eastern parts of India regarding its characterization to species level and the burden of VRE in this region. The main objectives of the present study were to identify the species of enterococcus isolated from both clinical and faecal samples, which indicated colonization, determine their antimicrobial susceptibility pattern and look for the presence of VRE in the strains isolated.

### **MATERIAL AND METHODS:**

**STUDY POPULATION:** A total of 123 isolates of enterococcus species were recovered from various clinical and stool samples of hospitalized patients, from September 2010 to June 2011. 60 strains of enterococcus species were isolated from various samples like urine, catheter tip, pus, drainage fluid, tracheal aspirate and blood. An additional 63 strains of enterococcus were also isolated from fecal samples of hospitalized patients who were otherwise not suffering from any other infection, to look for colonization with enterococcus species. The criteria for VRE infection or colonization were: hospitalization for 5 days or more, use of antimicrobials (cephalosporins, aminoglycosides and fluoroquinolones).

**ISOLATION & IDENTIFICATION:** Enterococci were identified using standard methods based on Gram staining, catalase reaction, hydrolysis of bile esculin, growth in 6.5% NaCl, growth at 10°C and 40°C, growth at pH 9.6, heat test, hydrolysis of leucine-beta-naphthalamide (LAP) and L-pyrrolidonyl-β-naphthalamide (PYR). Further identification to species level were based on carbohydrate fermentation using 1% solution of following sugars: glucose, lactose, mannitol, sucrose, arabinose, sorbose, sorbitol, raffinose, ribose, trehalose, xylose, melibiose, glycerol; by pigment production, motility test, pyruvate utilization in 1% pyruvate broth, acidification of methyl-alpha-D-glucopyranoside, Voges-Proskauer test, arginine decarboxylation, hippurate hydrolysis, reduction of potassium tellurite and tetrazolium chloride.<sup>(4, 5, 6)</sup>

**ANTIMICROBIAL SUSCEPTIBILITY TESTING:** Antibiotic susceptibility test was done by Kirby Bauer disc diffusion method on Muller Hinton agar. Inoculum was prepared and adjusted to 0.5 Mc Farland's turbidity standard. Antibiotic disc were obtained from the Hi Media Laboratories (Mumbai) viz ampicillin (10 µgm), ampicillin/sulbactam (10/10 µgm), penicillin (10 units), piperacillin (100µgm), tetracycline (30 µgm), ciprofloxacin (5 µgm), erythromycin (15 µgm), vancomycin (30µgm), teicoplanin (30 µgm), linezolid (30 µgm) & imipenem (30 µgm). The test was quality controlled using *E. faecalis* ATCC 51299 and *E. faecalis* ATCC 29212.<sup>(7)</sup>

### **Detection of vancomycin resistance in enterococci by vancomycin screen agar method:**

## ORIGINAL ARTICLE

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For the agar screen method brain heart infusion agar (Hi Media, Mumbai) was supplemented with 6 µg/ml of vancomycin. The test organisms were grown in peptone water and the turbidity was matched with 0.5 Mc Farland's standard. The bacterial strains were spot inoculated on the agar medium using 10 µl of bacterial culture. The plates were incubated at 37°C for 24 hours. Presence of more than one colony or a haze of growth after 24-hour incubation was read as resistance.<sup>(7,8)</sup>

**Determination of MIC by agar dilution method:** Agar dilution was used to determine MIC of vancomycin to enterococci. Brain-heart infusion agar (Hi Media, Mumbai) was supplemented with different concentrations of vancomycin. The test organism was grown in broth and the turbidity matched with Mc Farland's 0.5 standard. The bacterial strains were spot inoculated on the surface of agar medium using 10 µl of bacterial culture. The plates were incubated at 37°C for 24 hours. The minimum concentration of vancomycin, which inhibited bacterial growth, was considered MIC. Enterococci which had MIC ≥ 32 µg/ml were considered resistant; MIC of 8-16 µg/ml as intermediately resistant; and MIC of ≤ 4 µg/ml as susceptible to vancomycin.<sup>(8)</sup>

**RESULTS:** Out of the 60 enterococcal strains isolated from clinical samples, 27(46%) were identified as *E. faecalis*, 26 (43%) as *E. faecium*, 3 (5%) as *E. solitarius* and 2 (3.3%) each as *E. raffinosus* and *E. gallinarum*. The majority of the isolates from the fecal samples were *E. faecalis* 28 (44.4%) followed by *E. gallinarum* 22 (34.9%), *E. faecium* 11 (17.7%) and 1(1.5%) each of *E. raffinosus* and *E. dispar*.

Enterococcal strains, both clinical isolates and isolates from faeces of colonized patients showed resistance to various antibiotics. Antibiotics to which maximum resistance was seen were piperacillin, ciprofloxacin, penicillin, ampicillin and imipenem. Clinical isolates showed least resistance to vancomycin, teicoplanin and linezolid whereas faecal isolates showed least resistance to linezolid, teicoplanin and vancomycin. However vancomycin resistance was seen in 14.2% of faecal isolates as compared to 1.6% of clinical isolates. **(Table 1)** Amongst the faecal isolates, 27.2% of *E. faecium* were found to be VRE and 10.7% of *E. faecalis*. Multidrug resistance amongst clinical isolates was more in *E. faecium*; 73% of these strains showing complete resistance to imipenem. **(Table 1)** Out of the total of 123 clinical and faecal isolates, 17 and 24 isolates were resistant to vancomycin by disc diffusion method(DDM) & vancomycin agar screen method(VASM). **(Table 2)** The highest resistance was observed among *E. faecalis* followed by *E. faecium* and *E. gallinarum* by agar screen method.

MIC test of various clinical strains of enterococcus to vancomycin showed 8 strains to have reduced susceptibility to vancomycin i.e. MIC ranging from 8 to 16 µg/ml. Only one strain of *E. faecium* had MIC ≥ 32 µg/ml and was considered as VRE. **(Table 3)** Faecal isolates on the other hand were found to be more resistant to vancomycin with 19 strains showing reduced susceptibility and six showing resistance i.e. MIC ≥ 32 µg/ml. **(Table 4)**

Amongst the 9 clinical VRE and or VIE, 4 (44.4%) strains were isolated from urinary tract infections (UTI), 3 (33.3%) from wound infection and 2 (22.2%) from blood stream infection (BSI). On the other hand 25 faecal isolates were found to be VRE/VIE. The predominant clinical presentations in the VRE positive cases were appendicitis, hernia, burn, granuloma, swelling of body and hepatosplenomegaly.

**DISCUSSION:** Combination of colonizing abilities and drug resistance both inherent and acquired, has made Enterococci attain greater significance as human pathogens. In the present

study, *E. faecalis* was the predominant species isolated from both clinical & fecal specimens. Other studies done on Enterococci also support the same finding. This is probably due to predominance of *E. faecalis* in the endogenous flora of human body. <sup>(9,10)</sup>

The major sources of VRE are from the clinical environment. <sup>(11, 12)</sup> In our study, VRE strains were isolated from faecal samples of colonized patients. The VRE species isolated from these samples were *E. faecium* followed by *E. faecalis*, *E. gallinarum* and *E. dispar*. Patients infected or colonized with VRE did not show any clear correlation between the use of antimicrobials or gastrointestinal tract surgery.

The incidence of infection with strains of enterococcus with glycopeptides resistance has increased dramatically. In the present study DDM and VASM test failed to detect 50% and 29.4% strains respectively. The DDM failed to recognize as resistant those strains that have reduced susceptibility to vancomycin. The MIC test not only detected 7 VRE but also another 27 enterococcal strains with reduced susceptibility to vancomycin. The MIC test may therefore be considered as the gold standard. This observation is consistent with that made in a previous report. <sup>(8)</sup>The treatment of vancomycin resistant enterococci is a major problem. Vancomycin resistance eliminates the synergistic activity usually achieved by aminoglycoside combination, thus leaving beta-lactams as the only choice to combine with aminoglycoside. Moreover many of the vancomycin resistant enterococci are multidrug resistant. The antibiotic of choice for such multidrug resistant enterococci is currently not known. <sup>(8)</sup>

Out of the 63 stool isolates, 9 strains were resistant to 9 antibiotics out of the 10 antibiotics used. The outcome of infections with such multidrug resistant Enterococcus strains might be fatal. In most of the earlier reports, such high MDR rates were rarely observed in enterococci. High resistance of the clinical strains of *E. faecalis* to penicillin could be due to low affinity of penicillin binding proteins or production of beta lactamases. <sup>(9)</sup>

We conclude that enterococcal strains with high rate of resistance to multiple drugs are not only prevalent in the clinical environment but also in the gastrointestinal tract of the colonized patients. However patients with VRE infections and those showing resistance to multiple drugs could not be followed up, so the actual outcome of the infections with these strains could not be found out.

The prevalence of VRE was quite high amongst the colonized patients. This situation makes it mandatory for the clinical microbiologists to try to identify the most useful active antibiotic for treatment. The time has come for proper control measures to be taken to prevent the spread of such infections. MIC for vancomycin should be performed, in laboratories equipped to perform these tests to keep record of increasing resistance of enterococci to vancomycin.

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## ORIGINAL ARTICLE

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**Table 1: Resistance pattern of different enterococcus species**

Antibiotics	Clinical Isolates (n=60)					Fecal Isolates (n=63)				
	No. of resistant strains					No. of resistant strains				
	<i>E.faecalis</i>	<i>E.faecium</i>	<i>E.solitarius</i>	<i>E.raffinosis</i>	<i>E.gallinarum</i>	<i>E.faecalis</i>	<i>E.faecium</i>	<i>E.gallinarum</i>	<i>E.dispar</i>	<i>E.raffinosis</i>
<b>Penicillin</b>	22 (81.4)	12 (46.1)	0	0	0	20 (71.4)	3 (27.2)	0	0	0
<b>Ampicillin</b>	7 (25.9)	12 (46.1)	0	0	0	13 (46.4)	4 (54.5)	9 (40.9)	1 (100)	0
<b>Ampicillin/sulbactam</b>	10 (37)	10 (38.4)	0	0	0	16 (57.1)	7 (63.6)	11 (50)	1 (100)	0
<b>Tetracycline</b>	10 (37)	10 (38.4)	1 (33.3)	1 (50)	1 (50)	20 (71.4)	6 (54.5)	7 (31.8)	0	0
<b>Piperacillin</b>	25 (92.5)	20 (76.9)	1 (33.3)	1(50)	1(50)	28 (100)	10 (90.9)	19 (86.3)	1 (100)	1 (100)
<b>Ciprofloxacin</b>	24 (88.8)	12 (46.1)	2 (66.6)	1 (50)	0	27 (96.4)	9 (81.8)	15 (68.1)	0	1 (100)
<b>Linezolid</b>	2 (7.4)	3 (11.5)	0	0	0	1 (3.5)	2 (18.1)	1(4.54)	0	0
<b>Imipenem</b>	10 (37)	19 (73)	0	0	0	20 (71.4)	7 (63.6)	19 (86.3)	0	0
<b>Vancomycin</b>	0	1 (3.84)	0	0	0	3 (10.7)	3 (27.2)	2 (9.1)	1 (100)	0
<b>Teicoplanin</b>	1 (3.7)	0	0	0	0	3 (10.7)	2 (18.1)	0	0	0

Figures in parenthesis indicate percentages

**Table 2: Distribution of resistant strains of enterococcus species detected by both DDM & VSAM:**

Methods used to detect vancomycin resistance	Clinical Isolates (n=60) No. of resistant strains					Faecal Isolates (n=63) No. of resistant strains				
	<i>E.faecalin</i> (27)	<i>E.faecium</i> n (26)	<i>E.solitarius</i> n (3)	<i>E.raffinosis</i> n (2)	<i>E.gallinarum</i> n (2)	<i>E.faecalis</i> n (28)	<i>E.faecium</i> n (11)	<i>E.gallinarm</i> n (22)	<i>E.dispar</i> n (1)	<i>E.raffinosis</i> n (1)
Disc-diffusion method (strains showing intermediate-resistance & completee resistant)	2	3	0	0	0	5	4	2	0	1
<b>TOTAL</b>	5					12				
Vancomycin gar screen method	3	4	0	0	0	7	5	4	0	1
<b>TOTAL</b>	7					17				

**Table-3: MIC range for vancomycin (µgm/ml) in different clinical isolates**

Clinical isolates	MIC Values (µgm /mm)						
	≤ 0.5	1	2	4	8	16	≥32
<b>E. faecalis (N=27)</b>	0	0	16	9	3	0	0
<b>E. faecium (N=26)</b>	0	2	16	5	4	0	1
<b>E. raffinosus (N= 2)</b>	0	0	2	0	0	0	0
<b>E. solitarius (N= 3)</b>	0	0	3	0	0	0	0
<b>E. gallinarum (N= 2)</b>	0	0	1	0	1	0	0

**Table-4: MIC range for vancomycin (µgm/ml) in different faecal isolates**

Stool Isolates	MIC Values (µgm /mm)						
	≤ 0.5	1	2	4	8	16	≥ 32
<b>E. faecalis (N=28)</b>	0	1	9	9	2	6	1
<b>E. faecium (N=11)</b>	0	1	4	3	2	4	2
<b>E. gallinarum (N=22)</b>	0	7	7	0	0	4	2
<b>E. raffinosus (N=1)</b>	0	0	1	0	0	0	0
<b>E. dispar (N=1)</b>	0	0	0	0	1	0	1