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Drug resistance in *Enterococcus* species in a tertiary level hospital in Assam, India

Sir,

Non-specific use of broad-spectrum antibiotics is responsible for conversion of enterococci, the otherwise gut commensal to opportunistic nosocomial as well as community acquired pathogen. The emergence of high level resistance to aminoglycosides has made the therapeutic combination of penicillin and gentamicin ineffective[1]. Concomitant vancomycin resistance in enterococci not only leaves fewer options for infection management due to this organism but also is important due to potential risk of vancomycin resistant gene transfer from *Enterococcus* to *Staphylococcus aureus*[2]. This laboratory based cross-sectional study was conducted in a tertiary care hospital in Assam, India, from July 2012 to June 2013 to evaluate the frequency of clinical isolates of *Enterococcus* spp and their resistance pattern to different antibiotics. The ethical clearance for the study was obtained from institutional ethics committee.

Our study included isolates from heterogenous specimens such as urine, blood, pus, CSF, *etc*. The genus *Enterococcus* was identified by Gram stain, catalase test, hydrolysis of bile-esculin, PYRase test, heat tolerance at 60°C for 30 min in water bath and salt tolerance (6.5% NaCl)[1,3]. Speciation was done according to the conventional scheme of Facklam and Collins[3]. Isolates identified as *Enterococcus* were further subjected to identification by Vitek2 automated system (bioMerieux, France). Antimicrobial susceptibility to ampicillin (10 µg), penicillin (10U), vancomycin (30 µg), teicoplanin (30 µg), linezolid (30 µg) and ciprofloxacin (5 µg) was tested for all the isolates by modified Kirby-Bauer disk diffusion method[4] as per Clinical and Laboratory Standards Institute (CLSI) guidelines[5]. Nitrofurantoin (300 µg) was tested only for urinary isolates and erythromycin (30 µg) for isolates from specimens other than urine. High level aminoglycoside resistance to gentamicin (120 µg) and streptomycin (300 µg) was also determined[5]. Minimum inhibitory concentration (MIC) of vancomycin was determined by Etest® (bioMérieux, France). *Enterococcus faecalis* ATCC 29212 was used as control. Antimicrobial susceptibility was checked by Vitek2 automated system® for comparison. To detect the genotype of the vancomycin resistance gene, DNA was extracted from the culture using QIAamp DNA Purification Kit, (Qiagen; Hilden, Germany), and its quality was assessed on 1.2 per cent agarose gel, a single band of high molecular weight DNA was observed. Reference strain used was *E. gallinarum* ATCC 49573. Fragment of the *van* gene was amplified by PCR from the above isolated DNA using already published primers and PCR protocol for detection of *van C-1* gene[6]. The primer used was 5'-3' (+) GAAAGACAACAGGAAGACCGC and (--) ATCGCATCACAAGCACCAATC. PCR amplicon band of 800 bp was observed. DNA sequencing was done in Xcelris Laboratory, Ahmedabad, to confirm the genotype of *van C* gene. Forward and reverse DNA sequencing reactions of the PCR amplicon were carried out with VAN F and VAN R primers using BDT V3.1 cycle sequencing kit on ABI 3730xl Genetic analyzer (Waltham, Massachusetts, USA). Consensus sequence of 797 bp *van* gene was generated from forward and reverse sequence data using aligner software, USA. This *van* gene sequence was used to carry out BLAST with the NCBI genbank database (*http://www.ncbi.nlm.nih.gov*). Sequence showed 100 per cent similarity to *E. gallinarum* eS464 *vanC1* vancomycin resistance gene (Accession no. EU151772.1).

Conventional biochemical tests identified a total of 95 enterococcal isolates, which were subjected to

Vitek2 automated system identification that identified 93 as *Enterococcus*. The remaining two were identified as *Leuconostoc mesenteroides* spp *cremoris* and *Pediococcus pentosaceous*. Most of the isolates were from urine (88.17%, n=82) followed by five (5.38%) from pus, four (4.3%) from blood, one (1.08%) isolate each from CSF and duodenal aspirate. Majority of enterococcal isolates were from urine, similar to many studies done in India[2,7,8]. Speciation of the 93 enterococcus species by Vitek2 automated system was similar to that by conventional biochemical tests. Table I shows the species distribution of the *Enterococcus* species. *E. faecalis* was the commonest species (81.72%) isolated, followed by *E. faecium* (12.9%), which was similar to studies done elsewhere[9-11]. The other species isolated were *E. raffinosus* (3.23%, n=3), *E. avium* (1.08%, n=1) and *E. gallinarum* (1.08%, n=1) which have also been reported from India[10,12,13]. Table II shows the antibiotic susceptibility pattern of the enterococcal isolates. All 93 isolates were found to be resistant to penicillin. Many studies in India have reported resistance to penicillin in the range of 40-80 per cent[12-14]. A study by Jain *et al*[15] from north India has also reported penicillin resistance as 100 per cent. Ampicillin resistance was seen in 93.6 per cent isolates. This was a higher value as compared to many other studies[7,13,14]. Eighty two per cent urinary isolates were found to be sensitive to nitrofurantoin. A few studies have reported similar results[10,16]. Nitrofurantoin is an excellent drug against enterococcal urinary tract infection and has been used for past many years. It is both bacteriostatic and bactericidal. No cross-resistance was seen between nitrofurantoin and any other antibiotic[9]. All isolates were found to be sensitive to teicoplanin and linezolid. High level gentamicin resistance (HLGR) and high level streptomycin

Table I. Species distribution of enterococcal isolates

Enterococcal species	Number of isolates (%)		
	Total	Inpatient	Outpatient
Enterococcus faecalis	76 (81.72)	61 (65.59)	15 (16.13)
E. faecium	12 (12.9)	6 (6.45)	6 (6.45)
E. raffinosus	3 (3.23)	2 (2.15)	1 (1.08)
E. avium	1 (1.08)	1 (1.08)	0 (0.00)
E. gallinarum	1 (1.08)	1 (1.08)	0 (0.00)
Total	93 (100)	71 (76.34)	22 (23.66)

Table II. Antibiotic susceptibility pattern of enterococcal isolates

Antibiotic	Sensitive (%)			Intermediate sensitive (%)			Resistant (%)			Total
	Total	Inpatient	Outpatient	Total	Inpatient	Outpatient	Total	Inpatient	Outpatient	
Ciprofloxacin (5 µg)	25 (26.9)	17 (18.3)	8 (8.6)	3 (3.2)	2 (2.2)	1 (1.1)	68 (69.9)	52 (55.9)	13 (18.0)	93
Penicillin (10U)	0 –	0 –	0 –	–	–	–	93 (100)	71 (76.3)	22 (23.7)	93
Ampicillin (10 µg)	6 (6.4)	1 (1.1)	5 (5.4)	0 –	0 –	0 –	87 (93.6)	70 (75.3)	17 (18.3)	93
Vancomycin (30 µg)	92 (99.0)	70 (75.3)	22 (23.7)	0 –	0 –	0 –	1 (1.1)	1 (1.1)	0 –	93
Teicoplanin (30 µg)	93 (100)	71 (76.3)	22 (23.7)	0 –	0 –	0 –	0 –	0 –	0 –	93
Linezolid (30 µg)	93 (100)	71 (76.3)	22 (23.7)	0 –	0 –	0 –	0 –	0 –	0 –	93
Erythromycin (30 µg)*	5 (45.5)	5 (45.5)	0 –	2 (18.2)	2 (18.2)	0 –	4 (36.4)	4 (36.4)	0 –	11
Nitrofurantoin (300 µg)**	67 (81.7)	46 (56.1)	21 (25.6)	2 (2.4)	2 (4)	0 –	13 (15.9)	13 (15.9)	0 –	82

*Tested for isolates from specimen other than urine , ** Tested for isolates from urine

resistance (HLSR) were found to be 53.76 and 33.33 per cent, respectively, similar to that reported earlier[2,10].

In our study, 71 (76.34%) enterococcal isolates were from hospitalized patients, while only 22 (23.66%) were from outpatients. Of the 32 urinary isolates from inpatients with more than three days of hospital stay, 84.38 per cent (n=27) were catheterized on the day of admission, with no sign and symptom of urinary tract infection. Urinary tract infection by *Enterococcus* in catheterized patients was found to be significantly associated with more than 72 h of hospitalization ($P<0.01$). Therefore, the enterococcal urinary tract infection in catheterized patients may be of nosocomial nature.

Though *E. faecalis* and *E. faecium* are more commonly isolated, we isolated *E. gallinarum* from the urine sample of a hospitalized patient, which is seldom isolated from clinical specimens. It was found to be resistant to vancomycin with MIC of 4 µg/ml and susceptible to teicoplanin and therefore, phenotype of glycopeptide resistance was of VanC (intrinsic resistance). The VanC phenotype, as found in *E. gallinarum*, *E. casseliflavus*, and *E. flavescens*, is characterized by intrinsic low-level resistance to vancomycin. The nucleotide sequences of the *vanC-1* gene in *E. gallinarum*, the *vanC-2* gene in *E. casseliavus*, and the *vanC-3* gene in *E. flavescens* have been reported, although there is some disagreement as to whether *E. flavescens* is actually an enterococcal species[5].

Though *E. gallinarum* is intrinsically resistant to vancomycin by virtue of *Van C1* gene, determination of genotype is of utmost importance, as acquisition of other *Van* gene may confer high level vancomycin resistance to this species and subsequent transfer to other species as well. Presence of *VanA* gene along with *vanC1* gene in *E. gallinarum* isolates has been reported[17].

In conclusion, vancomycin resistance was not found to be a major resistance in enterococcal isolates in this area. All isolates were resistant to penicillin and high level aminoglycoside resistance in enterococcal isolates made this combination ineffective as treatment option for this infection. Use of vancomycin and linezolid can increase the selective pressure of these antibiotics in near future to form resistance. As an alternative, nitrofurantoin can be a better treatment option if found sensitive for the category of urinary infections in catheterized hospitalized patients.

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Conflicts of Interest: None.

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