Antibiogram Pattern and Virulence Trait Characterization of *Enterococcus* Species Clinical Isolates in Eastern India: A Recent Analysis

Srujana Mohanty[1] Bijayini Behera[1]

[1] Department of Microbiology, All India Institute of Medical Sciences, Bhubaneswar, Odisha, India

Address for correspondence Srujana Mohanty, MD, Department of Microbiology, All India Institute of Medical Sciences, Bhubaneswar, 751019, Odisha, India (e-mail: srujana_micro@yahoo.co.in).

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Abstract

Objective We aimed to evaluate the current antimicrobial susceptibility pattern and characterize putative virulence traits among *Enterococcus species* isolates from various clinical specimens in view of their increased isolation rates in both community-related and serious nosocomial infections, as well as resistance to many antibiotics.

Methods Study (April 2017–March 2018) included consecutive, nonrepeated, discrete, and clinically significant isolates of enterococci. Susceptibility testing included detection of high-level aminoglycoside-resistant (HLAR) and glycopeptide-resistant enterococci (GRE). All screen-positive GRE isolates were investigated by polymerase chain reaction for species confirmation and presence of *vanA*/*vanB* genes. Virulence genes *ace*, *asa1*, *cyt*, *efa*, *esp*, *gelE*, and *hyl* were investigated by molecular methods. Hemolysin and biofilm production were studied using phenotypic methods.

Results Of 111 isolates, 89 (80.1%), 16 (14.4%), and 6 (5.4%) were from urine, pus, and blood, respectively, consisting predominantly of *E. faecalis* (67, 60.4%) and *E. faecium* (32, 28.8%). *E. hirae* (5, 4.5%) was the predominant non-*E. faecalis* non-*E. faecium* isolate. Other species were *E. durans* (4, 3.6%), *E. avium* (2, 1.8%), and *E. mundtii* (1, 0.9%). Seven (6.3%) out of the 111 isolates were GRE, all *vanA* genotype. HLAR was observed in 70 (63.1%) isolates, significantly higher in *E. faecium* than *E. faecalis* (81.2 vs. 58.2%; $p < 0.05$). All were susceptible to daptomycin. Hemolysin activity and biofilm production were observed in 38 (34.2%) and 36 (32.4%) isolates. Most frequent virulence genes were *efa* (77, 69.4%), *ace* (71, 63.9%), *asa1* (67, 60.3%), and *gelE* (66, 59.4%). There was a predominant association of *esp* and *hyl* genes with *E. faecium* and that of the other genes with *E. faecalis*.

Conclusion The study will contribute to the existing limited data on virulence trait characterization of clinical *E.* spp. isolates in India. At the same time, it will help to serve as a guide in the choice of empirical therapy in enterococcal infections leading to favorable clinical outcomes by decreasing the clinical failure, microbiological persistence, and associated mortality, and will lead to future studies on controlling the spread of virulent and multiresistant isolates.

Keywords

- *Enterococcus faecalis*
- *Enterococcus faecium*
- Glycopeptide-resistant enterococci
- nosocomial infection
- vancomycin-resistant enterococci

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Introduction

Enterococci, normally considered commensal members of healthy intestinal microbiota of humans and animals, have gained widespread importance due to their increased isolation rates in both community-related and nosocomial infections with substantial morbidity and mortality.[1,2] Worldwide, enterococci are considered the second most common etiologic agent of urinary tract infections and third of nosocomial bacteremia.[1,3] Other significant infections caused by enterococci include peritonitis, cholecystitis, meningitis, wound, and soft tissue infections, catheter-related infections, endocarditis, neonatal sepsis, intra-abdominal and pelvic infections, and endodontic and medical device–associated infections.[2–6] Of more than 50 species known, *Enterococcus faecalis* and *E. faecium* together account for the majority of approximately 90% of clinical isolates (*E. faecalis* 80–85% and *E. faecium* 10–15%).[1–3] Other less commonly isolated species include *E. gallinarum*, *E. casseliflavus*, *E. avium, E. durans, E. raffinosus, E. mundtii*, and *E hirae*, accounting for approximately 5 to 10% infections.[1,3,7,8]

Therapy of infections caused by enterococci is problematic because of their intrinsic reduced susceptibility to several frequently used antimicrobial agents such as aminoglycosides (except for high-level resistance), clindamycin, cephalosporins, and trimethoprim/sulfamethoxazole.[9–11] Moreover, acquired resistance through lateral gene transfer to other agents, (β-lactams, macrolides, glycopeptides, and oxazolidinones) with subsequent emergence of multidrug-resistant (MDR), high-level aminoglycoside-resistant (HLAR) and glycopeptide-resistant enterococci (GRE), including vancomycin-resistant enterococci (VRE), make it more challenging.[9–11] VRE infections are associated with higher mortality, longer hospital stay, and higher costs compared with vancomycin-susceptible isolates and are recognized as a leading cause of outbreaks of hospital-acquired infections and intensive care unit (ICU) hospitalized patients.[12–15] Of nine types of vancomycin-related operons/genetic elements (*vanA*, *vanB*, *van C1/C2/C3*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, and *vanN*), associated with glycopeptide-resistance in enterococci, *van A*, and *van B* are by far the most prevalent types and *E. faecium* is the predominant species of GRE.[8–11]

It is important to perform accurate molecular identification of *van* types along with accurate species identification since at times, enterococci exhibit different phenotypic profile of glycopeptide-resistance which may pose infection control problems.[16–18] For example, *vanA* genotype VRE strains exhibiting vanB phenotype pattern have been reported from South Korea, Japan, China, as well as India.[16–18] Sometimes, unexpected outbreaks with an unanticipated *van* type may occur representing a change in local epidemiology and necessitating major changes in infection control policies and responses.[19] A recent study, in fact, has highlighted the importance of adjusting for *E. species* when assessing the burden of vancomycin resistance.[20] Additionally, though linezolid and daptomycin have been the drug of choice for management of infections caused by VRE,[8,10,11,21] both linezolid- and daptomycin-resistant enterococci have emerged recently with simultaneous resistance to both vancomycin and linezolid, as well as to vancomycin and daptomycin.[8,22–25]

Study of another aspect of enterococcal infections, that is, the pathogenic mechanisms or virulence factors (VFs) is gaining importance as the process of invasion is usually facilitated by damage to host tissues and presence of VFs such as adhesins, colonization factors, and cell aggregates, such as biofilms.[2,5,26,27] The various VFs encoded by their respective genetic elements consist of both extracellular proteases, as well as cell surface–associated proteins of which gelatinase (*gelE*), hyaluronidase (*hyl*), cytolysin (*cylA*), enterococcal surface protein (*esp*), accessory colonization factor (*ace*), aggregation factor (*asa1*), and endocarditis antigen (*efaA*) have been studied most intensively.[26–29] Phenotypic characteristics, such as hemolysis and biofilm formation, have also been recognized as critical for in vivo bacterial growth.[26–29] Some studies show a relation between the presence of virulence genes and multiple antibiotic resistance, whereas others speculate that virulence genes did not affect the pattern of antimicrobial resistance.[30–32] Hence, we undertook this study to determine the current pattern of species distribution, antimicrobial susceptibility, and virulence determinants among clinical isolates of enterococci.

Methods

The study, approved by Institutional Ethical Committee, was conducted over a period of 1 year from April 2017 to March 2018 in a tertiary-care research, referral, and teaching hospital in Eastern India.

Isolate Identification and Susceptibility Testing

Consecutive, nonrepeated, discrete, and clinically significant isolates of *E. species* identified by standard microbiological techniques were included in the study.[1,3]

Identification was based on the typical magenta-colored colonies on the MacConkey agar, gram-positive reaction, catalase-negativity, growth on and blackening of bile-esculin agar, growth in the presence of 6.5% sodium chloride, heat tolerance test, motility testing, pigment production, and various biochemical tests including arginine dihydrolase reaction and carbohydrate fermentation reactions in purple broth. Susceptibility testing to antimicrobial agents was performed as per the latest Clinical and Laboratory Standards Institute guidelines using discs of standard concentration.[33] Susceptibility to ampicillin, vancomycin, teicoplanin, linezolid, daptomycin, and fosfomycin was confirmed by gradient minimum inhibitory concentration (MIC) method with EzyMIC strips (HiMedia, Mumbai, India; ►**Fig. 1A**). HLAR included detection of both high-level gentamicin resistance (HLGR) and high-level streptomycin resistance (HLSR) using discs of gentamicin (120 µg) and streptomycin (300 µg) and confirmed by EzyMIC (gentamicin MIC $\geq$ 500 µg/mL and streptomycin MIC $\geq$ 2,000 µg/mL).[33] Strains with intermediate resistance were included in the percentage of resistant isolates. Multidrug-resistance was defined as nonsusceptibility to at least one

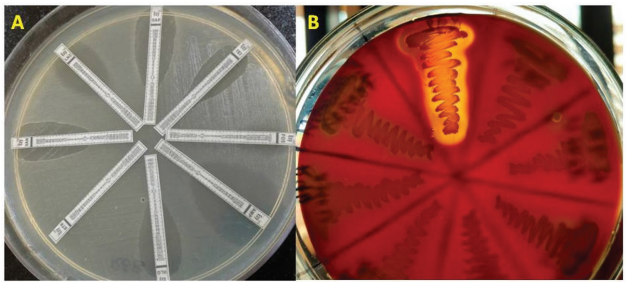

Fig. 1 *Enterococcus species* showing (**A**) susceptibility to various antimicrobial agents and (**B**) hemolysin activity.

agent in three or more antimicrobial categories.[34] Standard strains of *E. faecalis* ATCC 29212 (vancomycin susceptible), *E. faecium* ATCC 35667 (vancomycin susceptible), *E. faecalis* ATCC 51299 (vancomycin-resistant and HLAR), and *E. casseliflavus* ATCC 700327 were used as controls.

Phenotypic Detection of Virulence Traits

Hemolysin Activity

A brain–heart infusion agar plate supplemented with 5% human blood was inoculated with pure isolates and incubated at 37°C for 24 hour. A clear zone of β-hemolysis around the bacterial colonies indicated the production of hemolysin (►**Fig. 1B**).[29,32]

Biofilm-Forming Assay

Isolates were tested for biofilm-production by semiquantitative microtiter-plate adherence assay as per Stepanović et al and interpreted as follows: less than 0.12, nonbiofilm producer; 0.12–0.24, moderate biofilm producer; and greater than 0.24, strong biofilm producer.[35] *Staphylococcus epidermidis* strains ATCC 35984 (strong biofilm producer) and ATCC 12228 (nonbiofilm producer) were used as controls.

Molecular Investigations

All isolates were investigated by polymerase chain reaction (PCR) for species confirmation using species-specific primers and for presence of virulence-encoding genes using a panel of oligonucleotide primer pairs (Sigma-Aldrich Ltd, St. Louis, Missouri, United States) with their expected amplicon sizes as listed in ►**Table 1**.[28,29,36] To detect the presence of genes encoding the virulence factors, one triplex PCR (*asa1*/*gelE*/*esp*), one duplex PCR (*hyl*/*cylA*), and two single PCRs (*ace* and *efaA*) were performed. All phenotypic screen-positive GRE isolates were investigated for presence of *vanA* and *vanB* genes using primer pairs shown in ►**Table 1**.[36] Control

Table 1 Oligonucleotide primers used to amplify genes for species confirmation, *van* gene characterization, and virulence factor detection in enterococci

Target gene	Virulence factor/ resistance determinant	Oligonucleotide sequence (5′–3′)	Amplicon size (bp)	Annealing temperature (°C)	Reference
gelE	Gelatinase	TAT-GAC-AAT-GCT-TTT-TGG-GAT AGA-TGG-ACC-CGA-AAT-AAT-ATA	213	56	[28]
hyl	Hyaluronidase	ACA-GAA-GAG-CTG-CAG-GAA-ATG GAC-TGA-CGT-CCA-AGT-TTC-CAA	276	56	[28]
cylA	Cytolysin	ACT-CGG-GGA-TTG-ATA-GGC GCT-GCT-AAA-GCT-GCG-CTT	688	56	[28]
esp	Enterococcal surface protein	AGA-TTT-CT-CTT-TGA-TTC-TTG-G AAT-TGA-TTC-TTT-AGC-ATC-TGG	510	56	[28]
asa1	Aggregation substance	GCA-CGC-TAT-TAC- GAA -CTA-TGA TAA-GAA-AGA-ACA-TCA-CCA-CGA	375	56	[28]
ace	Collagen binding protein	GGA-ATG-ACC-GAG-AAC-GAT-GGC GCT-TGA-TGT-TGG-CCT-GCT-TCC-G	616	62	[29]
efaA	endocarditis antigen A	GCC-AAT-TGG-GAC-AGA-CCC-TC CGC-CTT-CTG-TTC-CTT-CTT-TGG-C	688	60	[29]
vanA	*vanA* gene	CT-GAA-TAG-AAT-AAA-AGT-TGC-AAT-A CCC-CTT-TAA-CGC-TAA-TAC-GAT-CAA	1,030	55	[36]
vanB	*vanB* gene	GTG-ACA-AAC-CGG-AGG-CGA-GGA CCG-CCA-TCC-TCC-TGC-AAA-AAA	433	60	[36]
E. faecalis	Species identification	ATC-AAG-TAC-AGT-TAG-TCT-TTA-TTA-G ACG-ATT-CAA-AGC-TAA-CTG-AAT-CAG-T	941	55	[36]
E. faecium	Species identification	TTG-AGG-CAG-ACC-AGA-TTG-ACG TAT-GAC-AGC-GAC-TCG-GAT-TCC	658	58	[36]

Table 2 Distribution and species identities of enterococci isolated from clinical specimens

Specimen	Number (%) of isolates						Total no. of isolates
	Enterococcus. faecalis	*E. faecium*	*E. hirae*	*E. durans*	*E. avium*	*E. mundtii*	
Urine	58 (65.1)	24 (26.9)	4 (4.5)	3 (3.4)	–	–	89
Pus	6 (37.5)	5 (31.2)	1 6.2)	1 (6.2)	2 (12.5)	1 (6.2)	16
Blood	3 (50)	3 (50)	–	–	–	–	6
Total	67 (60.4%)	32 (28.8%)	5 (4.5%)	4 (3.6%)	2 (1.8%)	1 (0.9%)	111

strains used were *E. faecium* ATCC 35667, *E. faecalis* ATCC 29212 (positive control for *asa1* and *gelE*), and *E. faecalis* ATCC 51299 (*vanB* genotype, positive control for *cylA*, *efaA*, *ace*).

Results

Patient Demographics

A total 111 *E. species* were isolated during the study period, 89 (80.1%) from urine, 16 (14.4%) from pus, and 6 (5.4%) from blood. ►**Table 2** displays the species identities along with specific sources of the isolates, consisting of *E. faecalis* (67, 60.4%), *E. faecium* (32, 28.8%), *E. hirae* (5, 4.5%), *E. durans* (4, 3.6%), *E. avium* (2, 1.8%), and *E. mundtii* (1, 0.9%). Ten (9.0%) were from outpatient department, 84 (75.6%) from admitted patients, and 17 (15.3%) from ICUs. Forty one (36.9%) were from male patients, whereas 70 (63.1%) were from females. The lowest and highest age at which an *E. species* was isolated was *E. faecalis* from blood sample of a 7-day-old female child and *E. durans* from urine sample of a 77-year-old female elderly patient, respectively.

Antimicrobial Resistance Profile and Distribution of Glycopeptide-Resistance Genes

Resistance profile and MIC characteristics of the isolates to various antimicrobial agents are shown in ►**Tables 3** and **4**, respectively. Overall, 107 isolates were resistant to one or more agents; erythromycin (103, 92.8%), ciprofloxacin (98, 88.2%), levofloxacin (95, 85.6%), and doxycycline (72, 64.8%). Compared with *E. faecalis*, the *E. faecium* isolates were

Table 3 Comparative resistance profile of *Enterococcus species* to various antimicrobial agents

Antimicrobial/or resistant phenotype (disc strength in µg)	*n* (%) of resistant isolates among			
	E. faecalis ($n=67$)	*E. faecium* ($n=32$)	Other enterococci ($n=12$)	Total ($n=111$)
Ampicillin (10)	5 (7.5)	31 (96.9)[e]	1 (8.3)	37 (33.3)
Vancomycin (30)	1 (1.5)	6 (18.7)[e]	0	7 (6.3)
Teicoplanin (30)	1 (1.5)	6 (18.7)[e]	0	7 (6.3)
HLAR[a] (120 and 300)	39 (58.2)	26 (81.2)[e]	5 (41.6)	70 (63.1)
Ciprofloxacin (5)	61 (91.0)	31 (96.9)	6 (50.0)	98 (88.2)
Levofloxacin (5)	58 (86.6)	31 (96.9)	6 (50.0)	95 (85.6)
Doxycycline (30)	52 (77.6)[e]	16 (50.0)	4 (33.3)	72 (64.8)
Chloramphenicol (30)	26 (38.8)	7 (21.9)	1 (8.3)	34 (30.6)
Erythromycin (15)	64 (95.5)	31 (96.9)	8 (66.6)	103 (92.8)
Rifampicin (5)	26 (38.8)	30 (93.7)[e]	4 (33.3)	60 (54.1)
Linezolid (30)	1 (1.5)	4 (12.5)[e]	0	5 (4.5)
Nitrofurantoin[b] (300)	3/58 (5.2)	14/24 (58.3)[e]	1/7 (14.3)	18/89 (20.2)
Fosfomycin[c] (200)	3/58 (5.2)	–	–	3/58 (5.2)
Daptomycin[d]	0	0	0	0
Multidrug resistance	35 (52.2)	30 (93.7)[e]	5 (41.6)	70 (63.1)

Abbreviation: HLAR, high-level aminoglycoside resistance.
[a]HLAR includes both high-level gentamicin resistance and/or high-level streptomycin resistance.
[b]Tested only in urinary isolates.
[c]Tested only in urinary isolates of *E. faecalis.*
[d]Tested by Etest only.
[e]$p < 0.05$ (significant) for difference in resistance between *E. faecalis* and *E. faecium* by Chi-square test.

Table 4 Minimum inhibitory concentration characteristics of *Enterococcus species* to various antimicrobials

Antibiotic	No. of isolates with MIC (µg/mL)															MIC range (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	No. (%) resistant strains
	0.032	0.125	0.19	0.25	0.38	0.5	0.75	1	1.5	2	4	6	16	24	32	48	64	128	> 256				
Ampicillin ($n = 111$)	–	–	–	2	–	8	19	25	13	6	–	–	–	–	–	–	2	3	32	0.032–> 256	1.5	> 256	37 (33.3)
Vancomycin ($n = 111$)	–	–	–	3	1	14	32	33	16	5	–	–	–	2	–	–	–	–	5	0.25–> 256	1	2	7 (6.3)
Teicoplanin ($n = 111$)	–	–	–	6	8	60	13	13	1	3	–	–	3	–	–	–	–	–	4	0.25–> 256	0.5	1	7 (6.3)
Linezolid ($n = 111$)	–	–	–	–	–	3	7	21	28	47	3	–	1	–	–	–	–	–	1	0.5–> 256	1.5	2	5 (4.5)
Daptomycin ($n = 111$)	–	11	16	17	22	15	19	10	1	–	–	–	–	–	–	–	–	–	–	0.125–1.5	0.38	0.75	0
Fosfomycin ($n = 58$)[a]	–	–	–	–	–	–	–	–	–	–	6	17	16	5	4	3	4	1	2	6–> 256	16	64	3 (5.2)

Abbreviation: MIC; Minimum inhibitory concentration.
[a]Tested only in urinary isolates of *E. faecalis*.

significantly more resistant to most of the tested antimicrobials except doxycycline to which resistance was significantly higher in *E. faecalis* (►**Table 3**). Resistance to ciprofloxacin, levofloxacin, and erythromycin was similar in both the species. Isolated HLGR and HLSR was observed in 33 (29.7%) and 11 (9.9%) isolates, respectively, with both in 26 (23.4%) isolates. Thus, HLGR occurred in 59 (53.1%), while HLSR was displayed by 37 (33.3%) isolates. In toto, a total of 70 (63.1%) isolates displayed HLAR (both HLGR and HLSR) comprising of 39 *E. faecalis*, 26 *E. faecium*, 3 *E. durans*, 1 *E. avium*, and 1 *E. mundtii*. As regard to MICs, in case of ampicillin, maximum isolates (32, 28.8%) demonstrated high MICs of greater than 256 µg/mL followed by 1 µg/mL (25, 22.5%). In case of vancomycin, maximum isolates (33, 29.7%) displayed MIC of 1 µg/mL, while for teicoplanin, majority (60, 54.1%) had MIC 0.5 µg/mL (►**Table 4**). Multidrug-resistance was observed in 63.1% isolates, significantly higher in *E. faecium* than *E. faecalis* (93.7 vs. 52.2%, $p < 0.05$).

Seven isolates (6.3%) were glycopeptide-resistant (six *E. faecium* and one *E. faecalis*), three from blood (two *E. faecium* and one *E. faecalis*), and four from urine (all *E. faecium*; ►**Table 5**). *E. faecium* was thus accounted for 85.7% (six of seven) of GRE, all from inpatients, including three from ICUs. Furthermore, all GRE exhibited vanA phenotype and harbored the *vanA* gene cluster demonstrating complete agreement between phenotypic susceptibility test results and resistance genotypes (►**Table 5**). All GRE displayed HLGR along with resistance to ampicillin, ciprofloxacin, levofloxacin, and rifampicin. One, two, three, and five GRE isolates retained susceptibility to erythromycin, high-level streptomycin, chloramphenicol, and doxycycline, respectively (►**Table 5**). All *Enterococcus* isolates were susceptible to daptomycin, while 106 (95.5%) were susceptible to linezolid (►**Tables 3** and **4**). Two (1.8%) *E. faecium* isolates, one each from blood and urine exhibited simultaneous resistance to glycopeptides and linezolid.

Distribution of Virulence Traits

As regard to the virulence traits tested, hemolysin activity was displayed by none of *E. faecium*; but significantly by 52.2% *E. faecalis* isolates (►**Table 6**). The ability to form a biofilm was detected in 36 (32.4%) of which 19 (17.1%) were strong and 17 (15.3%) were moderate biofilm producers. A significant difference in biofilm-formation capacity was observed between *E. faecalis* and *E. faecium* isolates ($p < 0.05$), significantly more in *E. faecalis* (►**Table 6**). Molecular testing showed 96 isolates (86.5%) harboring at least one virulence gene; 42 (37.8%) carried five genes, 19 (17.1%) had four genes, 7 (6.3%) carried three virulence genes, 15 (13.5%) had two genes, and 13 (11.7%) isolates possessed a single gene. Up to 74.6% (50/67) *E. faecalis* had 3 or higher virulence-encoding genes, whereas the same was observed in only 34.4% (11/32) *E. faecium* isolates. No virulence-encoding gene was detected in 15 isolates. Frequency of *efa*, *ace*, *asa1*, *gelE*, and *cylA* was significantly more in *E. faecalis* while that of *esp* gene was more in *E. faecium* (►**Table 6**). A comparative analysis showed vancomycin-sensitive enterococci (VSE) isolates to be significantly associated with hemolysin

Table 5 Specimen types, patient details, and microbiological characteristics of glycopeptide-resistant enterococci ($n = 7$)

Strain no.	Species	Specimen	Age (y) and sex	Location	Vancomycin MIC (μg/mL)	Teicoplanin MIC (μg/mL)	Resistance phenotype	Type of *van* gene	Susceptibility to other antibiotics
R22	*Enterococcus faecium*	Urine	33, M	Ward	> 256	> 256	vanA	*vanA*	LZ, DM
R36	*E. faecium*	Blood	11, F	Ward	> 256	> 256	vanA	*vanA*	DC, LZ, DM
R59	*E. faecium*	Urine	51, M	ICU	> 256	> 256	vanA	*vanA*	DC, LZ, DM
R82	*E. faecium*	Blood	65, F	ICU	24	16	vanA	*vanA*	HLS, DC, CP, DM
R106	*E. faecium*	Urine	49, F	ICU	> 256	16	vanA	*vanA*	HLS, EM, DC, CP, LZ, DM
R107	*E. faecium*	Urine	71, M	Ward	> 256	> 256	vanA	*vanA*	DM
R108	*E. faecalis*	Blood	64, M	Ward	24	16	vanA	*vanA*	DC, CP, LZ, DM

Abbreviations: CP, chloramphenicol; DC, doxycycline; DM, daptomycin; EM, erythromycin; HLS, high-level streptomycin; ICU, intensive care unit; LZ, linezolid; MIC; minimum inhibitory concentration.

production, biofilm-formation, and *asa1*, *gelE*, and *cylA* genes while VRE isolates were significantly associated with only *esp* gene (►**Table 7**).

Discussion

The present study provides an estimate of the recent pattern of species distribution, antimicrobial susceptibility, and virulence trait profiles of clinical enterococcal isolates in an Indian tertiary care hospital. Frequency of isolation was predominantly from urine specimens followed by wound exudates or blood, as has been observed in other studies from India and abroad.[18,27,37,38] *E. faecalis* as the overall predominant isolate is congruent with previously published literature.[37–43] The proportion of *E. faecium* (28.8%), however, appears moderately high in our institute. This might be due to the increased use of antibiotics expected in a tertiary care institute such as ours which selects out the more resistant species. Recent studies from India and outside have reported rising rates of *E. faecium* as high as 44.5% (49/110) to 48.3% (80/178).[39,43] At other places, however, *E. faecium* still constitutes only approximately 4 to 10% of the enterococcal isolates.[41,42] *E. hirae* was found as the predominant non-*E. faecalis* and non-*E. faecium* isolate in the current study comprising of 4.5% of the total isolates. *E. hirae* as one of the non-*E. faecalis* and non-*E. faecium* isolates from clinical specimens has been described only on few instances before, ranging from 1.6 to 3.03%.[37,39–41] So the relative distribution of *Enterococcus* may vary from place to place and also between the institutions. As in previous studies, majority isolates were from admitted patients and ICUs.[18,44] In Iran, the frequency of VREs isolated from ICUs, nephrology, and internal wards were 33.3, 20.8, and 16.7%, respectively.[44] In an Indian setting, 291 (79.3%) of 367 isolates were obtained from inpatients with rest from outpatients.[18]

A high resistance rate to various antimicrobials (erythromycin, ciprofloxacin, levofloxacin, and doxycycline) was observed in the current study which is a cause of concern and precludes their use in routine treatment of enterococcal infections in this region. On the other hand, moderate-to-low resistance was observed to nitrofurantoin (20.2%), fosfomycin (5.2%), and linezolid (4.5%) and none to daptomycin. These latter antimicrobials may therefore be indicated for treatment of enterococcal infections, especially nitrofurantoin and fosfomycin may be recommended for empirical treatment of urinary tract infection due to *E. species* in our region. Similar high resistance to various antimicrobials has been observed in Iran, Egypt, Turkey, and in another hospital in Eastern India.[38,42,43,45]

Multidrug-resistance, as well as HLAR was observed in 63.1% isolates (HLGR in 53.1% and HLSR in 33.3%) in our study. HLGR and HLSR were detected in 50 and 34% isolates in the study from Iran with MDR observed in 36%.[42] In Egypt, all *E. faecium* and 74.6% of *E. faecalis* were MDR with HLGR detected in 79.6% and HLSR seen in 36.9% isolates.[38] Frequency of HLAR in India ranges from 47.41 to 72.47%.[32,39] Since enterococcal resistance to gentamicin and streptomycin occur by different mechanisms of enzymatic inactivation,

Table 6 Distribution of virulence traits/genes among *Enterococcus species*

Virulence trait/gene	No. (%) of isolates			
	Enterococcus faecalis ($n = 67$)	*E. faecium* ($n = 32$)	Other enterococci ($n = 12$)	Total ($n = 111$)
Hemolysin	35 (52.2)[a]	0	3 (25)	38 (34.2)
Biofilm	30 (44.8)[a]	4 (12.5)	2 (16.7)	36 (32.4)
efaA	55 (82.1)[a]	14 (43.8)	8 (66.7)	77 (69.4)
ace	50 (74.6)[a]	14 (43.8)	7 (58.3)	71 (63.9)
asa1	48 (71.6)[a]	11 ((34.4)	8 (66.7)	67 (60.3)
gelE	48 (71.6)[a]	10 (31.2)	8 (66.7)	66 (59.4)
cylA	31 (46.2)[a]	4 (12.5)	4 (33.3)	39 (35.1)
esp	7 (10.4)	12 (37.5)[a]	4 (33.3)	23 (20.7)
hyl	3 (4.8)	4 (12.5)	0	7 (6.3)

[a]$p < 0.05$ (significant) for difference in frequency of virulence traits between *E. faecalis* and *E. faecium* by Chi-square test.

Table 7 Comparative distribution of virulence traits/genes between VRE and VSE isolates

Virulence trait/gene	No. (%) of isolates	
	VRE ($n = 7$)	VSE ($n = 104$)
Hemolysin ($n = 38$)	0	38 (36.5) [a]
Biofilm ($n = 36$)	1 (14.3)	35 (33.6) [a]
efaA ($n = 77$)	5 (71.4)	72 (69.2)
ace ($n = 71$)	3 (42.8)	68 (65.4)
asa1 ($n = 67$)	1 (14.3)	66 (63.5) [a]
gelE ($n = 66$)	1 (14.3)	65 (62.5) [a]
cylA ($n = 39$)	0	39 (37.5)[a]
esp ($n = 23$)	5 (71.4)[a]	18 (17.3)
hyl ($n = 7$)	1 (14.3)	6 (5.8)

Abbreviations: VRE, vancomycin-resistant enterococci; VSE, vancomycin-sensitive enterococci.
[a]$p < 0.05$ (significant) for difference in resistance between VRE and VSE by Chi-square test.

it is important to test susceptibilities to both agents. Prevalence of GRE (6.5%) is comparable to previous Indian studies which have detected a VRE rate of 7.09 to 8.7%.[18,32] However, recent studies from Western and North-East India identified higher rates of vancomycin resistance (14.6 and 24%, respectively) with *E. faecium* accounting for the majority of GRE infections.[46,47] VRE frequency in other studies outside India ranges from 4.5 to 21%.[42,43] A point of note is that, similar to our finding, only *vanA* gene was detected among GRE in various studies from India and outside.[27,42,46,48,49] Interestingly, a recent study from Egypt has described the presence of only *vanB* and *vanC*$_1$ gene clusters in VRE isolates.[38]

Daptomycin seems to be an alternative therapeutic option for GRE with over 99.8% isolates worldwide being susceptible from 2009 to 2013.[21,23] None of 47 VRE obtained from rectal, blood, and urine samples from Turkey were resistant to daptomycin.[49] Recently, however, the proportions of daptomycin-resistant *E. faecalis* and *E. faecium* were 3.23 and 10.53%, respectively, in a national collaborative study performed in Spain.[23] Linezolid, fosfomycin, and chloramphenicol are some of the other few agents that retain in vitro activity against many strains of multiple-drug resistant *E. species*.[50,51] Praharaj et al found 37.5% of VRE isolates to be susceptible to chloramphenicol; same has been observed in the current study.[18] With regard to the linezolid, though it is highly active against gram-positive cocci (GPC) and has good tissue penetration, the rapid emergence of linezolid-resistant GPC is alarming and requires ongoing surveillance. Recent literature review shows linezolid resistance varying from 0.2 to 9.7% among enterococci.[22,38]

Analysis of the virulence traits in the current study showed that majority of the virulence-encoding genes (*efa*, *ace*, *asa1*, *gelE*, and *cylA*) were significantly more prevalent in *E. faecalis* compared with *E. faecium* ($p < 0.05$), with only *esp* and *hyl* genes more prevalent in *E. faecium.* These findings are in accordance with previous reports which state the predominant association of *esp* and *hyl* genes with *E. faecium* and that of the other genes with *E. faecalis*.[28,30,44,52,53] The *esp* gene was also significantly more prevalent ($p = 0.05$) among VRE than among the VSE in Malaysia, with six of seven

(85.7%) VRE versus 95 of 215 (44.2%) VSE isolates carrying the gene.[53] In fact, "*esp*" is considered as a marker for an epidemic clone of *E. faecium* that has spread across the countries.[54] Overall, our results are similar to a study in Turkey, wherein *efa* gene was the most frequently detected virulence gene (92.7%), followed by *ace* (83.6%) in 110 isolates and all except *hyl* were significantly higher in *E. faecalis* isolates ($p < 0.05$).[43] The least prevalent virulence-encoding gene in the current study was *hyl* which was detected in only seven (6.3%) isolates and may have little role in pathogenicity in comparison to other genes.

As regard to the phenotypic virulence traits, 31.61 and 26.12% of 310 enterocccal isolates in a study from North India demonstrated hemolysis and biofilm production, respectively, slightly lower than in the current study.[32] In Egypt, the ability to form a biofilm was detected in almost all clinical isolates examined (97/103, 94.2%) with vancomycin- and linezolid-resistant enterococci more likely to exhibit strong/moderate biofilm formation than vancomycin- and linezolid-sensitive ones.[38] This difference in behavior could be due to local strain-to-strain variation between different geographical regions or different rates of adaptability of the isolates to the local environments. Overall, we found an inverse relationship between antimicrobial resistance and virulence traits; the frequency of majority of virulence traits being lower in isolates displaying higher resistance to antibiotics.

Conclusion

In view of increasing resistance to glycopeptides in enterococci and emerging resistance to currently available alternative therapeutic options for GRE, such as linezolid and fosfomycin, the susceptibility status of various antibiotics among clinical *E. species* isolates needs to be investigated periodically. To prevent infection and transmission of virulent and resistant enterococcal isolates in the hospital setting, appropriate surveillance and strict infection control measures need to be followed. The present study will contribute to the existing limited data on virulence trait characterization of clinical *E. species* isolates in India. At the same time, it will help to serve as a guide in the choice of empirical therapy in enterococal infections leading to favorable clinical outcomes by decreasing the clinical failure, microbiological persistence, and associated mortality and will lead to future studies on controlling the spread of virulent and multiresistant isolates.

Authors' Contributions

S.M. provided substantial contribution to the conception and design of the study, contributed to the acquisition, analysis and interpretation of data for the work, drafted the manuscript, and gave final approval of the version to be published.

B.B. helped in the literature search, contributed in the analysis and interpretation of data, and critically revised the work for important intellectual content.

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Conflicts of Interest

There are no conflicts of interest. The funding source had no role in the design, data acquisition, analysis and interpretation of the study, as well as writing of the manuscript.

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