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Phenotypic Detection and Genotypic Characterization of Vancomycin-Resistant Enterococci in a Tertiary Care Hospital

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ABSTRACT
The prevalence of vancomycin-resistant enterococci (VRE) in hospitals has escalated into a severe threat to patients due to the problem of multiple drug resistance. These strains possess various resistance genes, leading to high-level inducible or low-level intrinsic resistance to vancomycin. Infections caused by VRE strains are associated with elevated mortality rates. The study aimed to characterize phenotypically and genotypically VRE strains isolated from diverse clinical samples. 150 enterococcus strains were isolated from different clinical samples. Phenotypic and genotypic methods were used to identify and specify strains. Broth microdilution methods determined the MIC of vancomycin and genotypic characterization was performed using PCR. out of 150 Enterococcus species isolated, were subjected to vancomycin resistance using conventional microbiological methods. The incidence rate of male VRE was 7/11 (63.6%) and female VRE was 4/11 (34.4%). Most VREs were from male patients of different age groups. Of these, 7 were E. faecalis and 4 were E. faecium. When these 11 strains of VRE were tested for the presence of the Van A gene, 9(81.8%) were positive and 2(18.2%) were negative. The present study showed the prevalence of van A genes. These findings have profound and practical implications for healthcare professionals and researchers, equipping them with actionable knowledge to effectively address the growing threat of VRE infections.

INTRODUCTION

Enterococci, a part of the normal human focal flora, have become a global concern. In hospitalized patients, soft-tissue wounds, ulcers and the gastrointestinal tract are the main sites of colonization. On the other hand, the oral cavity, genitourinary tract and skin are sites less often colonized by enterococci. The emergence of vancomycin-resistant enterococci (VRE) as hospital-associated pathogens, first identified during the mid-1980s in Europe, has since become a rapidly spreading global threat.

The prevalence of vancomycin-resistant enterococci (VRE) inhospitalshas escalated into a severe threat to patients due totheproblemofmultiple drug resistance.Three distinct glycopeptide resistance phenotypes (Van A, Van B, Van C) exist and they can be differentiated based on the level and inducibility of resistance to vancomycin and teicoplanin. The VanA type has acquired inducible resistance to both vancomycin and teicoplanin, while the VanB type has acquired inducible resistance to vancomycin but not to teicoplanin. These distinctions are crucial in understanding and managing the resistance patterns in enterococcal infections[3].

The antibiotic resistance patterns of Enterococcus isolates have been thoroughly investigated; however, there is a significant gap in our understanding regarding the phenotypic and genotypic characterization of vancomycin resistance among enterococcal isolates. This study, crucial in filling this gap, provides valuable insights into the prevalence of vancomycin-resistant enterococci and the study of vanA genes, which are essential to understanding and managing the resistance patterns in enterococcal infections. The findings of this study could potentially inform future strategies for managing antimicrobial resistance in hospital settings and contribute to the broader field of antimicrobial resistance research.

MATERIAL AND METHODS

The present study, conducted in the Department of Microbiology, Index Medical hospital and Research Centre after approval from the ethical committee, was a meticulously comprehensive investigation. A Total of 150 enterococcus species isolated from different clinical samples were subjected to vancomycin-resistant enterococcus (VRE) screening using conventional microbiological methods. The clinical Specimens included blood, urine, sputum, pus, wound swabs, throat swabs, nasal swabs and endotracheal tip etc.

Phenotypic Identification: The enterococci isolates were identified and speciated based on colony morphology, Gram stain and various biochemical reactions, such as the catalase test, the bile-esculin test, growth in 6.5% NaCl and pigment production.

Antibiotic susceptibility testing was conducted using the Modified Kirby Bauer disc diffusion method, following the guidelines outlined by the Clinical and Laboratory Standards Institute (CLSI 20124). Antibiotic discs (Himedia) with specified concentrations were employed for testing, including penicillin (10U/disc), ampicillin (10µg), high-level gentamicin (120µg), ciprofloxacin (5µg), vancomycin (30µg) and linezolid (30µg).

Screening of vancomycin-resistant enterococci was done with vancomycin 30 µg disc was done. The vancomycin's minimum inhibitory concentrations (MICs) were determined using the E test (Himedia) and the agar dilution method. As part of quality control measures, E. faecalis ATCC 29212 and E. faecalis ATCC 51299 were included as reference strains.

Molecularmethods for VRE Detection: Polymerase chain reaction (PCR) assays, known for their precision, were performed to detect vancomycin resistance genes in enterococci, especially E. faecium and E. faecalis. PCR is a widely used molecular biology technique that amplifies a specific segment of DNA, allowing for the detection of specific genes. A PCR Kit procured from Helini Biomolecules, Chennai, was used for this purpose. DNA was extracted from the enterococcal isolates using the Helini Pure Fast Bacterial Genomic DNA Mini Spin Prep Kit and subjected to real-time PCR for gene detection, ensuring the accuracy of our results.

Forward Primer: 5'-TGCGCGGAATGGGAAAACGACA-3'

Reverse Primer:5'-CAGCCCGAAACAGCCTGCTCAA-3'

The PCR Product size is 473 bp, representing the Van Age ne, a key determinant of vancomycin resistance in enterococci. An optimal negative control was employed using 1 µL of molecular-grade water.

RESULTS AND DISCUSSIONS

Out of 150 Enterococcus species isolates, the majority were collected from male patients, with 95 and 55 isolates from females. The most isolates of Enterococcus species were from the 51-60 age group patients 37(24.6%), followed by 41-50 age group patients 28(18.6%),61-70 age group 25916.6%), 31-40 age group 21(14%), 21-30 age group 16(10.6%), 71-80 age group 15(10%) and 10-20 age group 8(5.3%). (Table1).

Out of 150 enterococcus isolates, ,68(45.3%) were E. faecalis, 52(34.6%) were E. faecium and 30(20%) were other enterococcus species by phenotypic methods. The most common clinical samples from which enterococcus was isolated were pus 37(24.6%), ear swab 23(15.3%), urine 32(21.3%), Fluids & other 21(14%), Blood 19(12.6%) & sputum 18(12%). Where 11(7.3%) were VRE and the highest number of VRE

Table 1: Age and sex-wise distribution of different isolates.

Age group (In year)	Enterococcus species		
	male	Female	Total
10-20	5	3	8(5.3%)
21-30	11	5	16(10.6%)
31-40	13	8	21(14%)
41-50	17	11	28(18.6%)
51-60	24	13	37(24.6%)
61-70	16	9	25(16.6%)
71-80	9	6	15(10%)
Total	**95**	**55**	**150**

Table 2: Prevalence of vre isolates from different clinical samples

clinical samples	Number of Enterococcus (n=150)	VRE(n=11)	VRE %
Pus	37(24.6%)	3	27.2%
Urine	32(21.3%)	3	27.2%
Blood	19(12.6%)	1	9.1%
Ear Swab	23(15.3%)	1	9.1%
Fluids & other	21(14%)	2	18.1%
Sputum	18(12%)	1	9.1
Total	**150**	**11**	**7.3%**

Table 3: Distribution of enterococcus isolated in different clinical samples.

Clinical samples	Enterococcus species		
	E. faecalis	E. faecium	Other enterococcus species
Pus	17	13	7
Urine	15	11	6
Blood	9	7	3
Ear Swab	11	8	4
Fluids & other	11	9	1
sputum	5	4	9
Total (n=150)	68(45.3%)	52(34.6%)	30(20%)

Table 4: Minimum Inhibitory Concentration (MIC) values of Vancomycin against the VRE strains.

VRE isolates n= 11	Vancomycin MIC values (µg/ml)						
	Intermediate (8-16 µg/ml)		Resistant (≥32µg/ml)				
	8 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml	256 µg/ml	512 µg/ml	Total
E. faecalis			3	2	1	1		7
E. faecium			2		2			4
Total			5	2	3	1		11

Table 5: Antibiotic sensitive pattern of E. faecalis and E. faecium

Antibiotics	E. faecalis (n=68)		E. faecium (n=52)	
	Sensitive	Resistant	Sensitive	Resistant
Ampicillin	67(98.5%)	1(1.5%)	17(32.7%)	35(67.3%)
Penicillin	15(22.1%)	53(77.9%)	9(17.3%)	43(82.6%)
Vancomycin	**61 (89.7%)**	**7(10.3%)**	**38(92.3%)**	**4(7.7%)**
Linezolid	100 (100%)	0%	100%	0%
Ciprofloxacin	19(27.9%)	49(72.1%)	14(26.9%)	38(73.1%)
Gentamycin (HLG)	**56(82.3%)**	**9(17.64%)**	**42(82.7%)**	**9(17.3%)**
Antibiotics	**Antibiotics sensitive and resistant pattern of E. faecalis & E. faecium (only for urine isolates) N=9**			
	E. faecalis (n=15)	E. faecium (n=11)		
Tetracycline (U)	12(80%)	3(20%)	8(72.7%)	3(27.3%)
Doxycycline (U)	12(80%)	3(20%)	9(81.8%)	2(18.2%)
Minocycline (U)	12(80%)	3(20%)	9(81.8%)	2(18.2%)
Fosfomycin (U)	14(93.3%)	1(6.7%)	10(91.9%)	1(9.1%)
Nitrofurantoin (U)	13(86.7%)	2(13.3%)	10(91.9%)	1(9.1%)

Table 6: Minimum Inhibitory Concentration (MIC) values of Vancomycin against the VRE strains.

VRE isolates n= 11	Vancomycin MIC values (µg/ml)						
	Intermediate (8-16 µg/ml)		Resistant (≥32µg/ml)				
	8 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml	256 µg/ml	512 µg/ml	Total
E. faecalis			3	2	1	1		7
E. faecium			2		2			4
Total			5	2	3	1		11

Table 7: Van a genotype distribution in vre isolates

Enterococcal isolates	Total Isolates tested	Van A genotype	
		Present	Absent
E. faecalis	7/11(63.6%)	6	1
E. faecium	4/11(36.4%)	3	1
Total (n=11)	11	9(81.8%)	2(18.2%)

were isolated from pus and urine culture 3(27.2%) and 3(27.2%), respectively, followed by fluids and other 2(18.1%), blood 1(9.1%), ear swab 1(9.1%) and 1(9.1%) were from sputum samples.- (Table 2).

Out of 150 Enterococcus sp. Isolated from different clinical samples, 68(45.3%) were E. faecalis, 52(34.6%) were E. faecium, and 30(20%) were other enterococcus species. - (Table 3).

A total of 11(7.3%) isolates were found to be vancomycin-resistant using the broth microdilution technique as per CLSI guidelines. 7/68(10.2%) were vancomycin-resistant enterococcus faecalis, 4/52(7.7%) were vancomycin-resistant enterococcus faecium, and no VRE was isolated from other enterococcus species. All 11 isolates found vancomycin-resistant by the broth microdilution method showed MIC between ≥32µg/ml to ≥256µg/ml.- (Table 4)

The sensitivity pattern of E. faecalis and E. faecium shows that 100% were sensitive to linezolid. Where E. faecalis shows that 67(98.5%) were sensitive to ampicillin, followed by 56(82.3%) were to gentamycin (HLG), 61 (89.7%) were to vancomycin, 19(27.9%) were to ciprofloxacin, 15(22.1%) were to penicillin. In the case of E. faecium38(92.3%) were sensitive to vancomycin followed by 42(82.7%) were to gentamycin (HLG), 14(26.9%) were to ciprofloxacin, 9(17.3%) were to penicillin. - (Table 5)

Out of 11 vancomycin-resistant enterococci (VRE), 7 were E. faecalis &4 were E. faecium. All the VRE E. faecalis and E. faecium isolates expressed high-level vancomycin resistance (MIC =32µg/ml). The minimum inhibitory concentration to vancomycin was as follows: 5 had a MIC (≥32µg/ml), 2 had a MIC (≥64µg/ml), 3 had a MIC (≥128µg/ml) and 1 had a MIC (≥256µg/ml)-(Table 6).

Genotypic Confirmation of VRE by the Van A Gene:

When these seven strains of VRE were expressed for the presence of the Van A gene, 4(57%) were positive, and 3(43%) were negative (Table 7).

In our study, 11(7.3%) isolates were found to be vancomycin-resistant by the disc diffusion method. The MICs of vancomycin for all isolates were determined by broth microdilution technique. All 11 isolates showed resistance to vancomycin (MIC (≥32µg/ml-(≥256 µg/ml)and only 9 possessed the vanA gene.

Among the 11 VRE isolates, six isolates of E. faecalis and three isolates of E. faecium were found to have the van A genotype, as determined by the Rt-PCR assay for the van A gene and two were not detected-van A gene.

A study from North India reported only 1% vancomycin resistance in Enterococcus[4]. Another study from Chandigarh showed only 5.5% VRE isolates from urine specimens. In this study, VRE isolates exhibited a low-degree vancomycin resistance ranging from 8-32 µg/ml[5]. Yet another study from North India indicated only 2% VRE prevalence[6].

However, Mathur *et al.* reported 1% isolates of E. faecalis resistant to vancomycin by disc diffusion and agar screening method[7]. In another study by Taneja *et al*., eight (5.55%) VRE were detected by the E-test and agar dilution method[8].

Similarly, Fernandes and Dhanashree, in a study of 150 enterococcal isolates, reported a prevalence of vancomycin resistance of 8.6%[9]. Shafiyabi *et al*. also reported a prevalence of 5% of vancomycin resistance among enterococci isolates in a tertiary care centre in South India[10]. Vancomycin resistance is low in India compared to Western countries, although its rising rate is concerning[11].

Our study aims to evaluate the frequency of vancomycin resistance and their gene in the clinical isolates of Enterococci obtained from patients in this region. Approximately 150 confirmed cases of Enterococcal isolates were obtained from various clinical samples, encompassing urine, blood and pus.

In our study, out of 150 enterococcus isolates, the maximum isolates were from pus 37(24.6%), ear swab 23(15.3%), urine 32(21.3%), Fluids & other 21(14%), Blood 19(12.6%) & sputum 18(12%). Where 11(7.3%) were VRE, and the highest number of VRE were isolated from pus and urine culture 3(27.2%) and 3(27.2%), respectively, followed by fluids and other 2(18.1%), blood 1(9.1%), ear swab 1(9.1%) and 1(9.1%) were from sputum samples.

A study conducted by Akhter[12] observed a VRE isolation rate of 4.65% in E. faecalis. Their investigation [13] reported a VRE isolation rate of 1.4%, all of which were E. faecium. This rate is lower than the rate observed in our study.

The Van A resistance type was determined by using Vancomycin MIC and genotypic detection. A study[14] has demonstrated a 100% concordance between these two approaches for detecting van A VRE, like our work. Another study[15] reported a concordance rate of 95% in their investigation, which is comparatively lower than the concordance rate seen in our research.

Vancomycin resistance in our isolates is a stark reminder of the importance of strictly enforcing antibiotic policies and robust infection control measures. It underscores the need for vigilant measures to prevent the emergence and spread of multi-drug-resistant bacteria, highlighting the significance of judicious antibiotic use and stringent infection control practices in healthcare settings.

CONCLUSION

Enterococci have emerged as a pathogen associated with severe nosocomial infections recently. Urinary tract infections are the most frequent infections

caused by enterococci, followed by wound infections and bloodstream infections. E. faecalis and E. faecium cause most clinical infections. For ß-lactam-and aminogly cosides-resistant, Gram-positive bacteria, vancomycin is generally used. Treating severe infections caused by vancomycin-resistant enterococci has emerged as one of the leading clinical challenges for physicians because of limited therapeutic options. In this study, 11 isolates were vancomycin-resistant. These isolates are highly resistant to vancomycin using the broth microdilution method.

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