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Original Research Article

VANCOMYCIN RESISTANCE AND VIRULENCE DETERMINANTS IN CLINICAL ISOLATES OF ENTEROCOCCUS SPECIES IN A TERTIARY CARE HOSPITAL, CENTRAL INDIA

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Abstract

Background: Enterococcus species are one of the diverse organisms present in the intestinal tract of human beings and are responsible for community and hospital-acquired infections. The present study determines the virulence factors and antibiotic sensitivity pattern of Enterococcus species against vancomycin. **Materials and Methods:** All the samples were collected from the admitted patients of Index Medical College Hospital and Research Centre, Indore, India, and the study was approved by the Institutional Ethical Committee (MU/Research/EC/Ph.D./2021/58). A total of 106 Enterococcal isolates were collected during December 2021 to November 2023 and were analyzed for species distribution, Detection of virulence factors like gelatinase and hemolysin production and biofilm formation were done with standard methods. Vancomycin-resistance detection in Enterococcus species was done using the E-test and the disc diffusion method. **Result:** All clinical specimens revealed 106 isolates of Enterococci; the most common species was Enterococcus faecalis (64.20%), followed by Enterococcus faecium (23.58%), Enterococcus durans (13.20%), and Enterococcus avium (8.49%). Of the 106 isolates of Enterococcus, 51.89% showed positive gelatinase production, 50.94% were for hemolysin production, and 44.34% showed the formation of biofilm. Vancomycin resistance among the tested isolates is observed in the disc diffusion method was 34.91%, while the E-test indicated 30.20%. Production of gelatinase and hemolysin, and formation of biofilm among the tested isolates of Enterococci are 45.4%, 38.8%, and 40.4%, respectively. Significant associations have been observed between virulence factors like gelatinase production and vancomycin resistance in the isolates of Enterococcus. **Conclusion:** Among the virulence factors examined on Enterococcus species isolated in this study, enzymes such as hemolysin and gelatinase were found in considerable quantities. Vancomycin resistance and virulence variables were significantly correlated. In order to stop the spread of vancomycin resistance, persistent pathogen surveillance is necessary in hospital settings.

INTRODUCTION

Enterococci, a gram-positive coccus, belonging to group D streptococci that occur singly or arranged in pairs or short chains. Considered as an emerging pathogenic organism, Enterococci frequently appear as commensal in the female vaginal area, gastrointestinal system, and oral cavity in humans as well as in animals.[1,2] The predominant Enterococcus species are E. faecalis, E. faecium, E.durans, and E. avium, making up 80–90% of hospital-acquired infections worldwide. E. faecium plays a role in the majority of human enterococcal illnesses.[3-4] Hospitalized patients are the main victims of Enterococcus infections. Urinary tract infections are the most widespread illnesses produced by Enterococcus species, and followed by bloodstream infections (BSI) like endocarditis, purulent

infections, intra-abdominal, and intra pelvic infections.[5,6]

The existence of Enterococci which are resistant to vancomycin, produce β-lactamase enzyme, illustrate high-level aminoglycoside resistance (HLAR), and are resistant to glycopeptides presents a global treatment problem in hospital settings.[7] Since Enterococci have developed various virulence factors, like hemolysins, cytolysins, gelatinases, enterococcal surface proteins (ESP), and aggregation compounds, they can infect individuals.[8,9] The finding provided compelling evidence for the uniqueness of extra virulence traits that could augment the virulence of Enterococcus linked to illnesses. In recent decades, possible Enterococcus virulence factors have been observed; these are mainly associated with E. faecalis.[10]

Furthermore, Enterococci are significant reservoirs of virulence factors for the emergence of antibiotic resistance between different Enterococci species, based on epidemiological knowledge.[11] Antimicrobial-resistant Enterococci, specifically VRE (Vancomycin Resistant Enterococci), are thus an ongoing clinical problem in healthcare settings. The goal of this study was to explore the virulence factors and patterns of antimicrobial susceptibility, especially with vancomycin in clinical isolates of Enterococcus species isolated from a tertiary care center.

MATERIALS AND METHODS

A total of 106 enterococcal isolates were obtained from various clinical samples in the Department of Microbiology, Index Medical College, Hospital & Research Centre (IMCHRC), Indore, Madhya Pradesh, from October 2021 to September 2023. Ethical clearance of the study protocol was taken from the institutional Ethics Committee (MU/Research/EC/Ph.D./2021/58), before the commencement of the study. All other samples were inoculated on blood agar and MacConkey agar. After incubating for 24 to 48 hours at 37°C, culture plates were examined. Identification and speciation of Enterococci are done by studying the colony morphology, gram staining, bile esculin testing, salt tolerance testing, and carbohydrate fermentation testing.[12]

Antibiotic susceptibility testing: Antibiotic susceptibility testing for penicillin (10 units), chloramphenicol (30µg), erythromycin (15 µg), tetracycline (30 µg), doxycycline (30 µg), ciprofloxacin (5 µg), high-level gentamycin (120 µg), vancomycin (30 µg) and linezolid (15 µg) was performed employing the Kirby-Bauer disk diffusion method on Mueller-Hinton agar and Mueller-Hinton Agar with 5% Sheep or human blood (MHSA) for Streptococci species using the Clinical and Laboratory Standards Institute (CLSI-2022) recommendations, and the data were interpreted.[13]

Detection of Vancomycin resistance in Enterococci

To determine the MIC (E-test) value, the antimicrobial gradient examine combines the principles of diffusion or dilution techniques. The basis of it is the ability to produce an antibacterial agent concentration gradient in the agar medium. This method is available commercially with the product of E-test.13 In the process, an agar surface that has been previously inoculated with the microorganism under test is covered with a strip that has been impregnated with an antimicrobial agent gradient of increasing concentration from one end to the other. The MIC of vancomycin, can be measured with this technique. The growth inhibition ellipse and the strip intersect to determine the MIC value.13 Because it is easy to use, it is frequently employed to satisfy the needs of physicians. According to the CLSI-2022, and Himedia manual, Enterococci are classified as resistant to vancomycin if their MIC is more than 32 µg/ml, as intermediately resistant if it is between 8 and 16 µg/ml, and as susceptible if it is less than 4 µg/ml.[14,15]

Detection of virulence factors

Different virulence factors like Biofilm production, Hemolysin (cytolysin) production, and, Gelatinase production were detected in all Enterococcal isolates.

Microtiter plate method for detection of Biofilm production.

Enterococcal colonies were grown overnight on blood agar, and then were added with 2% sucrose in trypticase soya broth (TSB) from Hi-media laboratories in Mumbai, India. The whole night was allocated in the incubator at 37°C. The overnight growth was diluted to 1:100 in the TSB using sucrose. 200µl of this diluted inoculum was added at a time to sterile microtitre plate made of polystyrene that had a flat bottom. The first well, which was free of bacterial inoculum, was used as a control. The plates were then incubated for 48 hours at 37°C in an aerobic environment. After that, the nonadherent planktonic cells were removed from the wells by giving them a thorough PBS (phosphate buffer saline; pH 7.2) wash. The adhering biofilms were then fixed with 2% sodium acetate for 20 minutes. The plates were dried after being stained with 0.1% safranin for 20 minutes. An ELISA microtitre plate reader readout at 490 nm and five washing steps. If the absorbance was more than 0.2 OD, it was considered to be high or highly positive; if it was between 0.20 and 0.10, it was considered to be moderate; and if it was less than 0.10, it was considered to be weak or absent.[14]

Hemolysin (cytolysin) production

The enterococcal inoculation was put onto Todd-Hewitt agar supplemented with 10% human-defibrinated blood to verify it. Hemolysin synthesis is marked by a distinct zone of β hemolysis surrounding the bacterial colonies on the culture plate.[11]

Gelatinase production

30 g of gelatine per liter of Todd-Hewitt agar (Hi Media Laboratories, Mumbai, India) was used. The identification of the gelatinase production was

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detected by spot inoculation of the Enterococcus colonies on the gelatine agar plates, the plates were incubated at 37°C for eighteen hours. Once the plates were cooled to 4°C for five hours, the presence of a cloudy halo around the colony showed the degree of hydrolysis.[15]

Statistical Analysis

The statistical analysis was done using descriptive statistical methods such as graphical representation, frequency, and percentage distribution. The statistical software SPSS version 11.0 was used to tabulate and analyze the data to compare the virulence factors of Enterococcus spp. and the antibiotic resistance of various clinical isolates. Analysis of the categorical variables, the chi-square test was employed, and a p-value < 0.05 was considered significant.

RESULTS

A total number of 106 Enterococci were obtained from various clinical samples. Most of the Enterococcus species isolated were Enterococcus faecalis (64.20%) followed by Enterococcus faecium (23.58%), Enterococcus durans (13.20%), and Enterococcus avium (8.49%). (Figure 1) The isolation rate of the organism was more in female gender (52.83%) compared with males (47.17%). The highest ratio of infection was noted among the age group 46-60 yrs. [Table 1]

Species distribution of Enterococci out of 106 of various clinical samples tested E. faecalis, was the highest isolated species (64.15%), In blood and pus, its distribution was (83.33%) and (63.63%) respectively. Body fluids yielded equal number of E. faecalis and E.faecium (50%). and from the swabs one each of E.faecalis and E.avium were isolated. (50%) [Table 2].

Detection of vancomycin-resistance in clinical isolates of Enterococcus species was done using the Disc Diffusion and E-test method. In the disc diffusion method, the vancomycin resistance detected was 34.91% (n=37), and in E-test showed 30.20% (n=32) (Figure 2&3). Virulence factors were detected in various clinical isolates of Enterococcus species by using phenotypic methods were hemolysis production, Gelatinase production, and Biofilm production. Out of 106 Enterococcus isolates, 51.89% showed positive gelatinase production, followed by positive hemolysin production in 50.94% and 44.34% showed biofilm production respectively (Figure 4&5). When the virulence factors of the organism detected were compared with the specimens tested, in urine samples, 74.55%, 66.67%, and 72.34% showed gelatinase production, hemolysin production, and biofilm production respectively. Isolates from blood and pus showed 10.9%, 11.1% and 10.6% respectively. The association of organisms isolated from samples and virulence factors like gelatinase production and biofilm production showed statistically significant value whereas the hemolysin production was insignificant among the tested strains. [Table 3]. (p-Value <0.05 is significant)

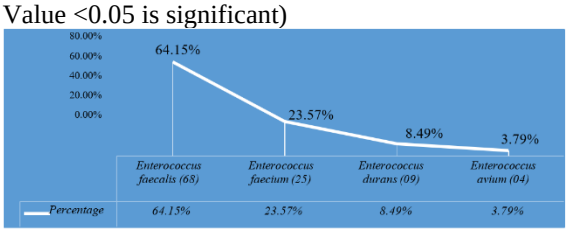

Figure 1: Distribution of Enterococcus species in all clinical isolates

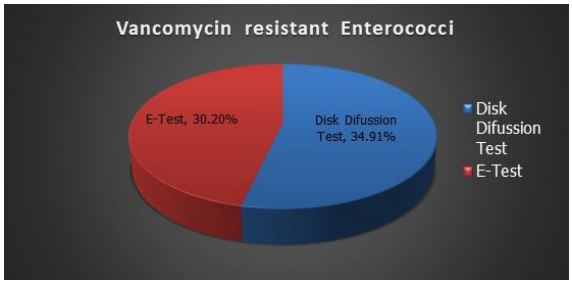

Figure 2: Percentage of vancomycin resistance observed in enterococci (VRE) with disc diffusion and E-test.

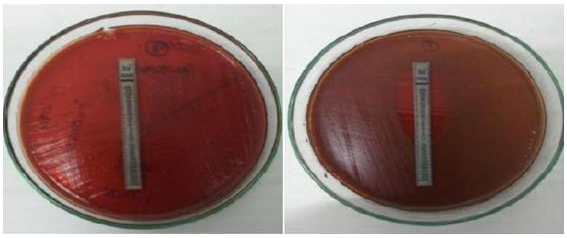

Figure 3: E-test done for Vancomycin showing Resistance and Sensitive results. Enterococcus isolates show resistance to vancomycin by E-test (MIC method). Enterococcus isolates shows sensitive to vancomycin by E-test (MIC method)

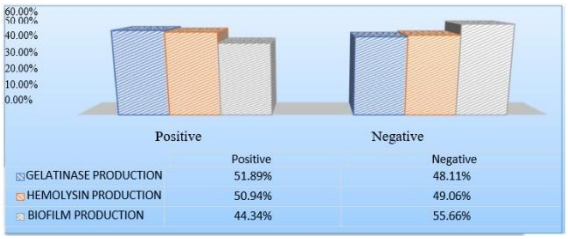

Figure 4: Virulence factors of Enterococcus species in all clinical samples.

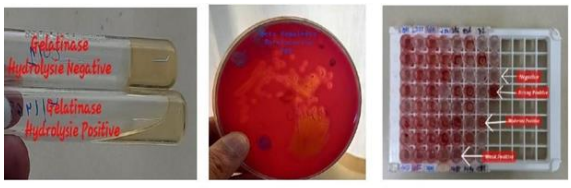

Figure 5: Virulence factors of Enterococcus species, (A) Gelatinase production Negative and Positive (B) hemolysin indicated by clear halo around colonies on human blood agar (C) Picture shows biofilm production using by tissue culture plate method.

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When the association between virulence factors and vancomycin resistance was compared, showed positive 45.4%, 38.8% & 40.4% in gelatinase production, hemolysin production, and biofilm production and vancomycin sensitivity showed positive 54.5%, 61.1% & 59.5% respectively. The association of only Gelatinase production shows significant value and hemolysin production & biofilm production were not significant [Table 4]. (p-value <0.05 is significant).

Table 1: Distribution between age group and gender of Enterococcus species.

Gender/ Age group	0-15	16-30	31-45	46-60	Above 60
Female 56 (52.83%)	4(7.14%)	6(10.71%)	17(30.36%)	13(21.43%)	17(30.36%)
Male 50 (47.14%)	1(2.0%)	4(8.0%)	11(22.0%)	20(40.0%)	14(28.0%)

Table 2: Species distribution of Enterococci among various samples studied.

S. No.	Number and percentage of samples tested	Enterococcus species n=106				Total (106)
		E.faecalis	E.faecium	E.durans	E.avium	
1	Urine	48 (62.33%)	21 (27.27%)	06 (7.79%)	02 (2.59%)	77 (72.64%)
2	Blood	10 (83.33%)	00 (00%)	02 (16.67%)	00 (00%)	12 (11.32%)
3	Pus	07 (63.63%)	02 (18.19%)	01 (09.09%)	01 (09.09%)	11 (10.38%)
4	Body Fluids	02 (50%)	02 (50%)	00 (0%)	00 (0%)	04 (3.77%)
5	Swabs	01 (50%)	00 (0%)	00 (0%)	01 (50%)	02 (1.89%)
6	Total (106)	68 (64.15%)	25 (23.57%)	09 (08.49%)	04 (03.79%)	106 (100%)

Table 3: Association between virulence factors and Enterococcus species isolated from various samples. (p-Value: - <0.05 is significant)

Samples	Gelatinase Production n=55 (51.89%)	Hemolysin production n=54 (50.94%)	Biofilm production n= 47 (44.34%)
Urine	41 (74.5%)	36 (66.6%)	34 (72.3%)
Blood	6 (10.9%)	8 (14.8%)	5 (10.6%)
Pus	6 (10.9%)	6 (11.1%)	4 (8.5%)
Fluid	1 (1.8%)	2 (3.7%)	2 (4.2%)
Swab	1 (1.8%)	2 (3.7%)	2 (4.2%)
Chi-Square	13.71	9.26	13.05
p-Value	0.045	0.27	0.50
Significance	Significant	Insignificant	Significant

Table 4: Association between virulence factors and Vancomycin resistance in Enterococcus species. (p-Value: - <0.05 is significant)

Virulence Factors		Vancomycin (30µg)		p-Value
		Sensitive	Resistance	
GELATINASE	Positive (55)	30 (54.5%)	25 (45.4%)	0.018
	Negative (51)	39 (76.4%)	12 (23.5%)	0.047
HEMOLYSIN	Positive (54)	33 (61.1%)	21 (38.8%)	0.38
	Negative (52)	36 (69.2%)	16 (30.7%)	0.39
BIOFILM FORMATION	Positive (47)	28 (59.5%)	19 (40.4%)	0.30
	Negative (59)	41 (69.4%)	18 (30.5%)	0.29

DISCUSSION

In the present study, clinical isolates of Enterococci were studied for different virulence factors along with vancomycin resistance in Enterococci, which is implicated in the treatment of complicated and drug-resistant Enterococcal infections. Most of the Enterococcus species were Enterococcus faecalis (64.20%) followed by Enterococcus faecium (23.58%), Enterococcus durans (13.20%), and Enterococcus avium (8.49%), similar results were found in the studies of Sanal C. Fernandes et al. and Hashem YA et al.[16,17]

In this study, most of the Enterococcus isolates were Enterococcus faecalis (64.20%) were from urine samples followed by Enterococcus faecium (27.27%), Enterococcus durans (7.79%) and Enterococcus avium (2.59%), and among 2 swab samples tested Enterococcus faecalis and Enterococcus avium were isolated in equal number. These results are in comparison with study of Tsering Yangzom et al.[18]

Enterococcus isolates in our study, 51.89% showed positive for gelatinase production, 50.94% demonstrated positive hemolysin production, and 44.34% demonstrated biofilm production, relevant study observed that organisms that produced hemolysin were more prevalent in another investigation than those that produced gelatinase by Klibi et al.[19] These organisms' ability to create hemolysin and gelatinase enhances their uptake of enough nutrients from the host tissues and promotes the infection's spread throughout the host body, hence increasing the infection.[20]

Additionally, our study revealed a correlation between virulence factors and vancomycin resistance, with positive results for gelatinase production, hemolysin production, and, biofilm production of 45.4%, 38.8%, and 40.4%, respectively. Detection of vancomycin-resistant drug in Enterococcus species disc diffusion method and E-

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test by MIC method. In the disc diffusion method, the vancomycin resistance enterococci (VRE) were found 34.91% and in E-test by MIC showed 30.20%. According to another study in AIIMS, New Delhi by G. Mathur et al,[21] 29.05% & 22.22% isolates were found to be resistant to vancomycin by disk diffusion and agar screen methods. Contrasting findings were seen in another study, where the majority of E. faecalis 94.3%, and E. faecium 15.16% showed vancomycin-resistant by E-test as reported by Khafil et al.[22] Another study from Bangalore, by Sreeja et al,[23] reported that 27% of the enterococci which showed resistance to vancomycin by E-test (MIC method). Another study conducted in south India by Praharaj Ira et al,[24] found that the percentage of Enterococcus isolates was 35.01%, the percentage of total isolates that produced hemolysin was approximately 19.2%, and the percentage of Enterococcus isolates that produced biofilm was 23.55% of the entire number of vancomycin-resistant enterococci (VRE). Enterococci, especially VRE in all isolates, have the ability to hydrolyze collagen and certain bioactive peptides, suggesting that they play a function in initiating and maintaining the inflammatory process.[25]

Vancomycin resistance enterococci (VRE) vary in different geographic regions which are ruled by manifold factors including the use of which act as growth promoters, and glycopeptides in accordingly acquired community infections in health care settings.

CONCLUSION

The present study describes the virulence factors of Enterococci and analyzes their correlation with vancomycin-resistance in Enterococci, however, there have been varying findings from previous research. In our study, we discovered that the majority of the clinical isolates of Enterococci tested positive for both gelatinase and biofilm production. Vancomycin resistance in Enterococci species has a significant association with the formation of virulence factors, as shown in through the gelatinase production.

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