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Implications of Virulence Factors, Biofilm Production and Antimicrobial Resistance Among Clinical and Commensal Isolates of Enterococcus species in Hospitalized Patients

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Abstract

Background: Increasing incidence of nosocomial pathogenicity and antibiotic resistance among commensal enterococci in recent years is posing therapeutic challenges. Identification of factors associated with pathogenicity and antibiotic resistance in fecal isolates of enterococci and its correlation with their clinical counterparts has become important. The present study was undertaken to compare virulence factors, biofilm production and antibiotic susceptibility among fecal and clinical isolates of enterococcus species at a tertiary care hospital.

Materials and Methods: This prospective study was conducted in July-August, 2018 on patients admitted in a tertiary care hospital. 25 isolates each from clinical and fecal specimens (commensals) were speciated and tested for virulence factors (gelatinase and hemolysin), biofilm production and antibiotic susceptibility as per standard guidelines.

Results: E. faecium was the most common species in both clinical (48%) and fecal (52%) isolates. 64% of fecal isolates were strong biofilm producers compared to clinical isolates (12%) ($t_{29.648} = 3.561$, $p = 0.001$), indicating that biofilm production contributes to colonization. High prevalence of VRE (vancomycin resistant enterococci) was noted in both fecal (40%) and clinical (36%) along with high HLAR (High Level Aminoglycoside Resistance) and MDR (Multi Drug Resistance) phenotype. Gelatinase production was seen only in clinical isolates (12%), hemolysin production was more in clinical (20%) than fecal (4%) isolates, indicating role of virulence factors in pathogenicity.

Conclusion: Gut colonization by enterococci (especially biofilm producers and VRE) is worrisome due to their nosocomial potential. High prevalence of virulence factors among clinical isolates may have a bearing on pathogenicity and severity of infection. High prevalence of VRE, MDRE, HLAR and linezolid resistance poses challenges in treatment of enterococcal infections.

Keywords: biofilm, enterococci, gelatinase, VRE, HLAR

Introduction

Enterococci are normal inhabitants of the bowel that have emerged as a leading cause of nosocomial infections. The emergence and spread of high-level aminoglycoside resistance (HLAR), vancomycin resistant enterococci (VRE) and multidrug-resistant (MDR) enterococci (MDRE) strains has increased in recent years posing therapeutic challenges [1, 2]. Vancomycin use seems to play a crucial role in increased fecal carriage and subsequently development of infection.

Among the virulence factors, Cytolysin (CylA), hemolysin and gelatinase (GelE) are implicated in enterococcal pathogenesis and play an important role in the severity of systemic enterococcal diseases. Extracellular surface protein (ESP) is an important

adhesin involved in colonization and biofilm formation [3]. Biofilm production has been shown to enhance the persistence of enterococci and increase tolerance to antimicrobial agents and host immune responses. [3, 4]

Given the importance of Enterococcus as a nosocomial pathogen and increasing prevalence of MDR enterococci, the identification of virulence factors associated with invasiveness and disease severity has become an important subject for research. It becomes important to understand their ecology, epidemiology and virulence for controlling infections caused by enterococci and also for stemming further development of antibiotic resistance [5, 6]. However, published studies on the prevalence, virulence factors, antibiotic susceptibility pattern and biofilm production of different species of enterococci, show conflicting data for hemolysis, association between biofilm production and source of isolate (pathogenic or colonizer), association between gelatinase and biofilm production, and association between biofilm production and MDR phenotype [3, 15, 16, 18, 20, 21].

With this background, the present study was undertaken to compare virulence factors, biofilm production and antibiotic susceptibility pattern among clinical isolates and fecal carriage of Enterococcus species at a tertiary care hospital.

Materials And Methods

This prospective study was carried out was carried out as part of ICMR STS-2018 (Reference ID:2018-00424) for two months (in July and August), 2018 at Department of Microbiology, Victoria Hospital, Bangalore Medical College & Research Institute, Bangalore, Karnataka, India. Institutional Ethical Clearance was obtained. Informed consent (in English and Kannada) was obtained from all the study participants.

Sample collection:

Total of 50 samples from admitted patients were included. Twenty-five Enterococcus spp. isolated from different patient specimens (urine, blood, pus, body fluids, endotracheal aspirate etc) in pure cultures and found to be the cause of true infection in all the cases and twenty-five commensal Enterococcus spp. isolated from fecal samples of hospitalized patients (known as colonizer isolates) were included in the study.

Identification:

Presumptive isolated colonies suggestive of enterococci from fecal samples were subjected to standard conventional biochemical tests, including Gram staining, catalase reaction, growth in the presence of 6.5% NaCl, bile esculin hydrolysis and sugar fermentation for speciation [7, 8]. Gram-positive and catalase negative cocci were presumptively identified as enterococci and confirmed by their growth on bile-esculin agar and in 6.5% NaCl broth, hydrolysis of esculin. Enterococci were speciated based on mannitol fermentation, raffinose fermentation, sorbitol fermentation, arabinose fermentation and growth on potassium tellurite agar. Isolates from clinical samples were processed by automated Vitek2 (BioMe´rieux, Marcy-l'Étoile, France) identification system.

Virulence factors:Gelatinase production: Isolates were inoculated into brain heart infusion broth containing 4% gelatin (40 g/L). After overnight incubation at 37ºC, the tubes were cooled at 4ºC for 30 min and gelatinase production was assessed by liquefying the gelatin [3]

Hemolysin production: The hemolytic activity was determined using sheep blood agar plates after 24 or 48 h of incubation at 37ºC. The appearance of a clear zone was considered indicative of β-hemolysis.

Biofilm production: Semi-quantitative microtiter plate assay was performed with minor modifications. Bacterial isolates were diluted (1: 100) in a trypticase soy broth supplemented with 1% glucose. From this culture, 200 µL was inoculated into 96-well polystyrene flat-bottom microtiter plates. After 24 h of incubation at 37ºC, the plates were gently washed thrice with sterile phosphate-buffered saline (PBS). The plates were inverted and dried at 25°C for 1 hour. For biofilm quantification, 200 µL of 2% aqueous safranin dye was added to each well and the plates allowed to stand for 40 min at room temperature. The excess safranin was washed off with sterile PBS, and the biofilm-bound-safranin was extracted with 200 ml of 95% ethanol. The absorbance of the extracted safranin was measured at 490 nm with an ELISA reader. Optical density (OD) ≥0.24 was considered indicative of strong biofilm

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formation, OD ≤0.12 was categorized as non-biofilm-forming. As a negative control, well-bound safranin was measured for wells exposed only to a medium containing TSB + 1% glucose, without any bacterial inoculation [3]

Antimicrobial susceptibility testing: For all the isolates was carried out by modified Kirby-Bauer disc diffusion method according to the CLSI guidelines [9] . The inoculum was prepared and adjusted to 0.5 McFarland's turbidity standard. The disk diffusion test was employed to determine the susceptibility of the isolates to vancomycin (30 µg), gentamicin (120 µg), linezolid (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), ampicillin (10 µg) and tetracycline (30 µg).

For the isolates that showed resistance phenotype to vancomycin by disk diffusion method, minimal inhibitory concentration (MIC) of vancomycin was determined by automated Vitek2 (BioMe´rieux, Marcy-l'Étoile, France) identification systems. The results were interpreted according to the CLSI guidelines[9] . Multidrug-resistance (MDR) was defined as resistance to three or more different classes of antibiotics. [10]

Data Analysis: Data was entered into an excel spreadsheet and analyzed. Data is presented in the form of figures, tables, graphs or pie charts.

Results

A total of 50 Enterococcus spp were included, of which 25 enterococci were isolated from various clinical specimens and 25 commensal enterococci from fecal samples of hospitalized patients. Locations of patients at the time of sample collection were as follows **(Table 1)**. Clinical specimens collected were as follows **(Table 2)**

The median age of patients from whom clinical specimens were isolated was 23 (interquartile range = 34.9) years. The median age of patients from whom fecal specimens were isolated was 20 (interquartile range = 33.5) years. The mean duration of hospitalization prior to isolation of Enterococcus species from fecal samples was 6.24 (range 2-14) days.

Among the 25 clinical isolates, 4 species were identified (E. faecium, E. faecalis, E. gallinarum and E. durans) E. faecium being the most common (48%). **(Figure 1).** Among the 25 fecal isolates, 3 species were identified, where E. faecium was the most common (52%), followed by E. faecalis and E. gallinarum **(Figure 1)**.

Antibiotic susceptibility pattern

Of 25 clinical isolates, 9 (36%) were Vancomycin resistant enterococci. Vancomycin resistance was more commonly seen in E. faecalis isolates (55%). Multiple drug resistance (resistance to 3 or more drugs) was observed in all VRE isolates and 7/9 (77%) were high level aminoglycoside resistant (resistance to high level gentamicin 120 µg by disk diffusion method, as per CLSI guidelines) [9] **(Figure 2)**. Mean Minimum Inhibitory Concentration (MIC) of clinical VRE isolates identified by Vitek was found to be 7.44 µg/ml **(Figure 3)**. Among 25 clinical isolates, 17 were high level aminoglycoside resistant, where it was most commonly seen in E. faecium (58.8%). Highest prevalence of HLAR among the 4 species followed the same pattern with highest being E. faecium with 83.3%. **(Table 4)**

Among 25 fecal isolates, 10 were Vancomycin resistant enterococci **(Table 3)**. Vancomycin resistance was most common in E. faecalis (55.5%). Multiple drug resistance was observed in 9 (90%) and HLAR in 4 (40%) of the VRE isolates. Linezolid resistance among the VRE isolates was quite high at 70 %, whereas the prevalence of linezolid resistance among all 25 fecal isolates was 40%. A Wilcoxon signed rank test (Z = -2.622, p = 0.009) and a Mann-Whitney U test (U = 212.5, p = 0.009) showed that there was a significant difference between Linezolid Resistance of the commensal isolates compared to the clinical isolates, which indicated that the former had greater Linezolid Resistance (The Mean Rank was 29.50 for the Commensal isolates compared to 21.50 for Clinical isolates). Among the 25 fecal isolates, 12 were HLAR, where it was most commonly seen in E. faecium (66.6%) and highest prevalence of HLAR also being seen in E. faecium with 61.5% being HLAR. **(Table 4)**

It was identified that there was significant evidence of an association between ICU admission and Multidrug Resistance, ($X^2(1) = 4.193$, p = 0.041). 34% of all MDR species were isolated from patients with history of ICU admission. 30% of VRE positive patients had recent ICU admission. A Wilcoxon signed rank test (Z = -2.609, p = 0.039) and a Mann-

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Whitney U test (U = 225, p = 0.039) showed that there was a significant difference between clinical and commensal isolates from patients with history of ICU Admission, with the former having a higher frequency. (The Mean Rank was 29 for the Clinical isolates compared to 22 for commensal isolates).

A Wilcoxon signed rank test (Z = -2.802, p = 0.005) and Mann-Whitney U test (U = 187.5, p = 0.005) identified that history of Penicillin use was more frequent among patients from whom clinical samples were isolated. [Mean Rank was 30.50 for the Clinical isolates compared to 20.50 for Commensal isolates]

Gelatinase production : (Table 5) Among clinical isolates, only 12 % (3) were gelatinase positive, where 22.2 % E. faecalis (2/9) and 8.3 % E. faecium (1/12) produced gelatinase. In contrast, among the fecal isolates, none of the enterococci produced gelatinase.

Hemolysin production : (Table 5) Incidence of α hemolysis was more in clinical isolates compared to fecal isolates. 20 (80 %) of clinical isolates were found to be non hemolytic, and 5 (20 %) were α-hemolytic, where all α-hemolytic isolates were E. faecium. In contrast, most fecal isolates were non hemolytic (24, 96 %), and only 1 isolate (4 %) was α-hemolytic, which again belonged to E. faecium.

Biofilm production

By analyzing the OD490 values, the isolates were classified into non-biofilm producing (OD value < 0.12), intermediate biofilm producing (OD value 0.12 - 0.24), and strong biofilm producing (OD value > 0.24) **(Figure 4)**. Among the 25 clinical isolates, 4 (16%) were non biofilm producing, 18 (72%) were intermediate biofilm producing and 3 (12%) were strong biofilm producers. On the other hand, among the 25 fecal isolates, most were strong biofilm producers (64%) and intermediate biofilm producers (24%). Only 3 (12%) were non biofilm producing. **(Table 5).** There was a significant difference in mean OD490 values between clinical and commensal isolates ($t_{29.648}$ = 3.561, p = 0.001). The average OD490 value for commensal isolates was 0.14696 greater than that of clinical isolates, indicating stronger biofilm production.

Discussion

Enterococci have emerged to be responsible for an increasing number of nosocomial and opportunistic infections such as urinary tract infections, surgical site infections, bacteremia, etc. Usually, the most common species isolated is E. faecalis, followed by E. faecium [2, 4, 5, 10, 11] However, the most common species isolated in our study in both clinical and fecal samples was E. faecium (48% and 52% respectively) followed by E. faecalis (36% and 36% respectively). Recent studies by Anjana Telkar [13] and Purohit G et al [14] have shown that E. faecium is observed to be the most common species isolated from samples such as blood, urine and stool. In clinical samples, E. gallinarum (8%) and E. durans (8%) were also isolated, whereas in fecal samples, only E. gallinarum (12%) was isolated apart from E. faecium and E. faecalis.

There was a high prevalence of VRE in both clinical isolates (36%) and fecal isolates (40%). The prevalence rate is higher than other studies; study by Purohit G et al, 2017 [14] had VRE prevalence of 22.8%; study by Saffari F et al, 2017 [3] had VRE prevalence rate of 4% in pathogenic isolates and no VRE was found in fecal isolates; study by A Tripathi et al, 2016 [15] showed a prevalence rate of 7.9%. E. faecalis was found to have the highest prevalence of Vancomycin Resistance in both clinical isolates (55.5 %) and fecal isolates (55.5 %), which is significantly higher than other studies. [3, 14, 15] Resistance to linezolid was also significantly higher among the VRE isolates at 22 % (2/9) in clinical VRE isolates, and 70 % (7/10) in fecal VRE isolates. Overall linezolid resistance was significantly higher in fecal isolates (40 %) compared to clinical isolates (12 %) [Wilcoxon signed rank test (Z = -2.622, p = 0.009) and a Mann-Whitney U test (U = 212.5, p = 0.009)]. Studies conducted in Europe, the United States and Taiwan demonstrated similar findings [16] of linezolid non-susceptibility. Gut colonization with VRE can be attributed to longer hospital stays, prior treatment with systemic antibiotics, recent ICU admission and or surgery.

Highest rates of antibiotic resistance among clinical isolates was seen towards erythromycin (23/25, 92 %) and ciprofloxacin (23/25, 92 %) followed by high level gentamicin (17/25, 68 %) and tetracycline (16/25, 64 %), whereas highest rates of antibiotic resistance among fecal isolates was seen towards erythromycin (19/25, 76 %) and ampicillin (19/25, 76

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%) followed by tetracycline (17/25, 68 %) and ciprofloxacin (17/25, 68 %). MDR phenotype was seen in 92 % (23/25) of clinical isolates compared to 84 % (21/25) of fecal isolates, and found to be associated with a history of ICU admission ($X^2(1) = 4.193$, $p = 0.041$).

In our study, only 6% of the enterococci were gelatinase positive, where all 3 gelatinase producing enterococci were from clinical isolates (12%) and none among the fecal isolates produced gelatinase. Most commensal isolates were strong biofilm producers (64 %) in contrast to clinical isolates where only 12 % were strong biofilm producers, evidenced by a higher mean biofilm OD490 value of among commensal isolates ($t_{29.648} = 3.561$, $p = 0.001$). These results are corroborated by other similar studies where gelatinase production appeared to be associated with the quantity of biofilm produced by human fecal E. faecalis isolates, where 90% of the fecal isolates were able to produce biofilm, but had no gelatinase activity[18] and where gelatinase activity was detected in only 34% of pathogenic isolates and none of the fecal isolates produced this enzyme[3]. These results indicated that gelatinase had a role in the pathogenesis of enterococci, as suggested in another study [19]. Also, study by Safari et al [3] observed that most fecal isolates (90%) were moderate or strong biofilm formers. Johansson and Rasmussen [20] had shown that isolates from normal flora produced more biofilm than isolates from samples with infective endocarditis, which is consistent with our results. Because biofilm formation is regulated by a complex network of transcriptional factors further investigations are required to find out the relationship between virulence factors or genes and biofilm production, as well as its relationship with antibiotic resistance.

Most of isolates were non haemolytic (88 %), 12 % were mildly α-hemolytic (all α-hemolytic isolates were E. faecium) and no isolate was ß-haemolytic. 80 % of clinical isolates were non hemolytic, and 20 % were mildly α-hemolytic, in contrast, most fecal isolates were non hemolytic (96 %), and only 1 isolate (4 %) was α-hemolytic. The incidence of hemolysin in our study was much lower than that reported in other studies [21]. However, High prevalence of non-hemolytic isolates and absence of ß-haemolysis was also seen in other studies; study by Anjana Telkar et al, 2012 [13] observed 78 % of isolates to be non-hemolytic, and 22% were α-hemolytic. Similarly, in a study by Saffari F et al [3] , incidence of hemolysin was much lower, although hemolysis had a tendency to be present more often in clinical isolates than in commensal isolates, which corroborates with our results.

Limitations of the study: Our study was limited by the small sample size.

To conclude, gut colonisation by enterococci (especially biofilm producers and VRE) is worrisome because of their nosocomial potential and therapeutic challenges. Possible correlation between the high prevalence of MDR phenotype and biofilm production (possibly necessary for colonization) in these colonisers needs to be further explored. High prevalence of VRE, MDRE, HLAR and linezolid resistance is a cause for concern as we are heading towards post antibiotic era. Further, virulence factors among clinical isolates of enterococci complicate the scenario due to increased pathogenicity and severity of enterococcal infections. Antibiotic stewardship, coupled with appropriate infection control practices to reduce and limit fecal enterococcal carriage may help in decreasing severity of enterococcal infections. Due to small sample size and multifactorial nature of this study, further research is required.

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Tables And Figures :

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Table 1

Distribution of Enterococci and VRE Isolates Among Different Clinical Departments

Location	Clinical Isolates		Commensal Isolates		Total
	VSE	VRE	VSE	VRE	
Emergency ICU	2	0	0	0	2 (4%)
Medicine Ward	1	0	3	1	5 (10%)
Nephrourology ICU	1	0	1	0	2 (4%)
NICU	2	3	1	1	7 (14%)
Paediatric Ward	2	1	1	6	10 (20%)
PICU	1	0	1	0	2 (4%)
OBG ICU	1	2	1	0	4 (8%)
OBG Ward	2	0	0	0	2 (4%)
Ophthalmology Ward	1	0	0	0	1 (2%)
Orthopedic Ward	0	1	4	0	5 (10%)
Plastic Surgery Ward	1	0	0	0	1 (2%)
Surgery Ward	2	1	3	2	8 (16%)
Not Traceable	0	1	0	0	1 (2%)
Total	16 (64%)	9 (36%)	15 (60%)	10 (40%)	50

Table 2

Distribution of Enterococcus Species in Various Clinical Specimens

Nature of specimen	Frequency (Percent of total)				Total
	E. faecium	*E. faecalis*	*E. gallinarum*	*E. durans*	
Urine	5 (20%)	2 (8%)	0	1 (4%)	8 (32%)
Pus	2 (8%)	3 (12%)	1 (4%)	0	6 (24%)
Blood	2 (8%)	3 (12%)	0	1 (4%)	6 (24%)
Vaginal swab	1 (4%)	1 (4%)	0	0	2 (8%)
Conjunctival swab	1 (4%)	0	0	0	1 (4%)
CSF	1 (4%)	0	0	0	1 (4%)
Peritoneal fluid	0	0	1 (4%)	0	1 (4%)
Total	12 (48 %)	9 (36 %)	2 (8 %)	2 (8 %)	25 (100%)

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Table 3

Frequency of Vancomycin Resistance among species

Species	VRE		VSE		Total
	Count	% within row	Count	% within row	
			Clinical isolates		
E. *faecalis*	5	55.5%	4	44.4%	9
E. *faecium*	2	16.60%	10	83.3%	12
E. *gallinarum*	1	50%	1	50%	2
E. *durans*	1	50%	1	50%	2
Total	9	36%	16	64%	25
			Commensal isolates		
E. *faecalis*	5	55.5%	4	44.4%	9
E. *faecium*	4	30.7%	9	69.3%	13
E. *gallinarum*	1	33.3%	2	66.6%	3
Total	10	40%	15	60%	25

Table 4

Frequency of High-Level Aminoglycoside Resistance (HLAR)

Species	HLAR		NON-HLAR		Total
	Count	% within row	Count	% within row	
			Clinical isolates		
E. *faecalis*	5	55.5%	4	44.4%	9
E. *faecium*	10	83.3%	2	16.6%	12
E. *gallinarum*	1	50%	1	50%	2
E. *durans*	1	50%	1	50%	2
Total	17	68%	8	32%	25
			Commensal isolates		
E. *faecalis*	4	44.4%	5	55.5%	9
E. *faecium*	8	61.5%	5	38.5%	13
E. *gallinarum*	0	0.0%	3	100.0%	3
Total	12	48%	13	52%	25

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Table 5

Hemolytic Activity, Gelatinase activity and Biofilm Production

Species	Non hemolytic	α-hemolytic	Gelatinase activity	Non biofilm forming	Intermediate biofilm	Strong biofilm
			Clinical isolates : Counts (% within row)			
E. faecalis (n=9)	9 (100%)	0 (0%)	2 (22.2%)	1 (11.1%)	6 (66.6%)	2 (22.2%)
E. faecium (n=12)	7 (58%)	5 (42%)	1 (8.3%)	1 (8.3%)	11 (91.6%)	0 (0%)
E. gallinarum (n=2)	2 (100%)	0 (0%)	0 (0%)	0 (0%)	1 (50%)	1 (50%)
E. durans (n=2)	2 (100%)	0 (0%)	0 (0%)	2 (100%)	0 (0%)	0 (0%)
Total (n=25)	20 (80%)	5 (20%)	3 (12%)	4 (16%)	18 (72%)	3 (12%)
			Commensal isolates : Counts (% within row)			
E. faecalis (n=9)	9 (100%)	0 (0%)	0 (0%)	1 (10%)	3 (30%)	5 (50%)
E. faecium (n=13)	12 (92%)	1 (8%)	0 (0%)	1 (7.7%)	3 (23%)	9 (69.2%)
E. gallinarum (n=3)	3 (100%)	0 (0%)	0 (0%)	1 (33.3%)	0 (0%)	2 (66.6%)
Total (n=25)	24 (96%)	1 (4%)	0 (0%)	3 (12%)	6 (24%)	16 (64%)

Figure 1

Distribution of Species in Clinical and Commensal Isolates

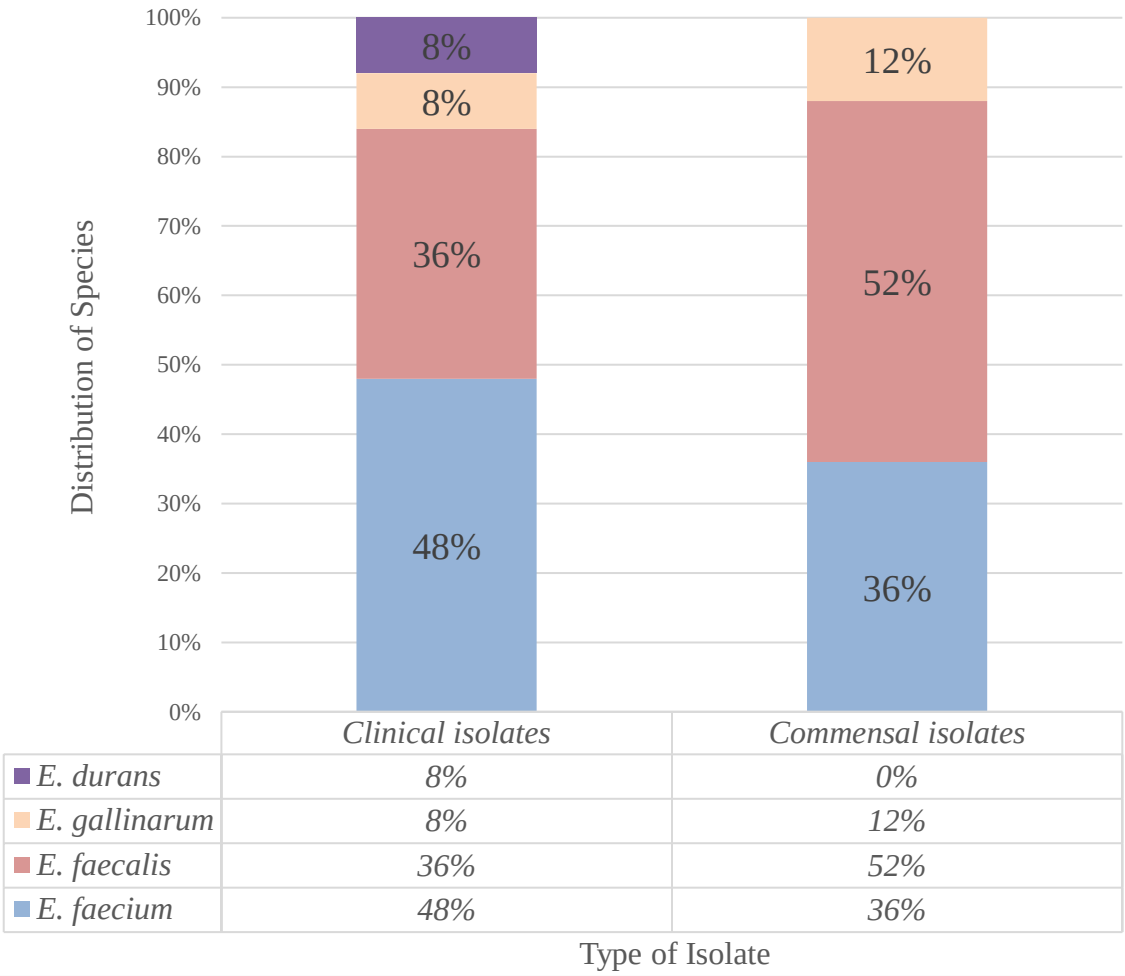

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Figure 2

Antibiotic Resistance Patterns Among Vancomycin Resistant (VRE) and Vancomycin Sensitive Enterococci (VSE) in Clinical and Commensal Isolates

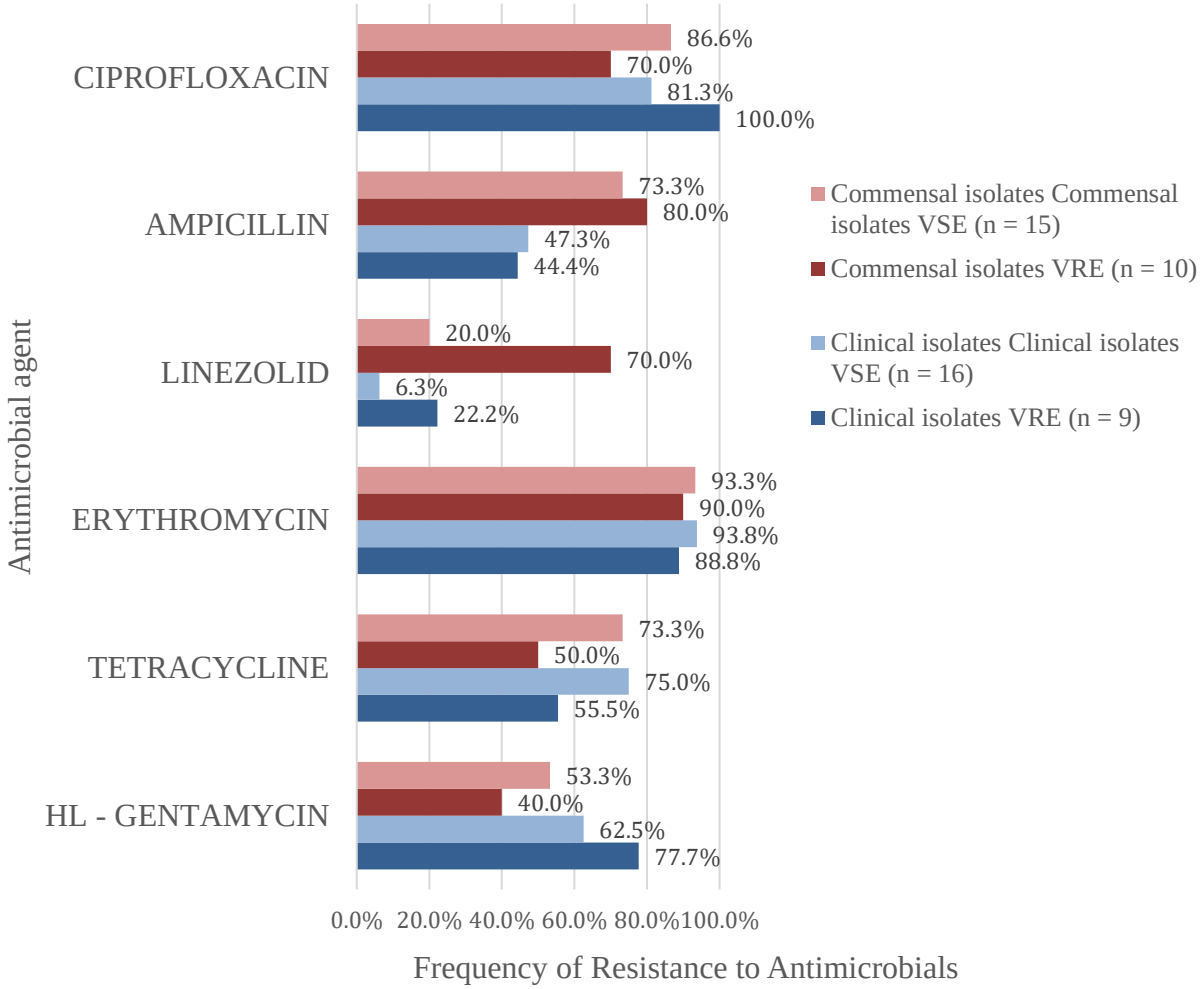

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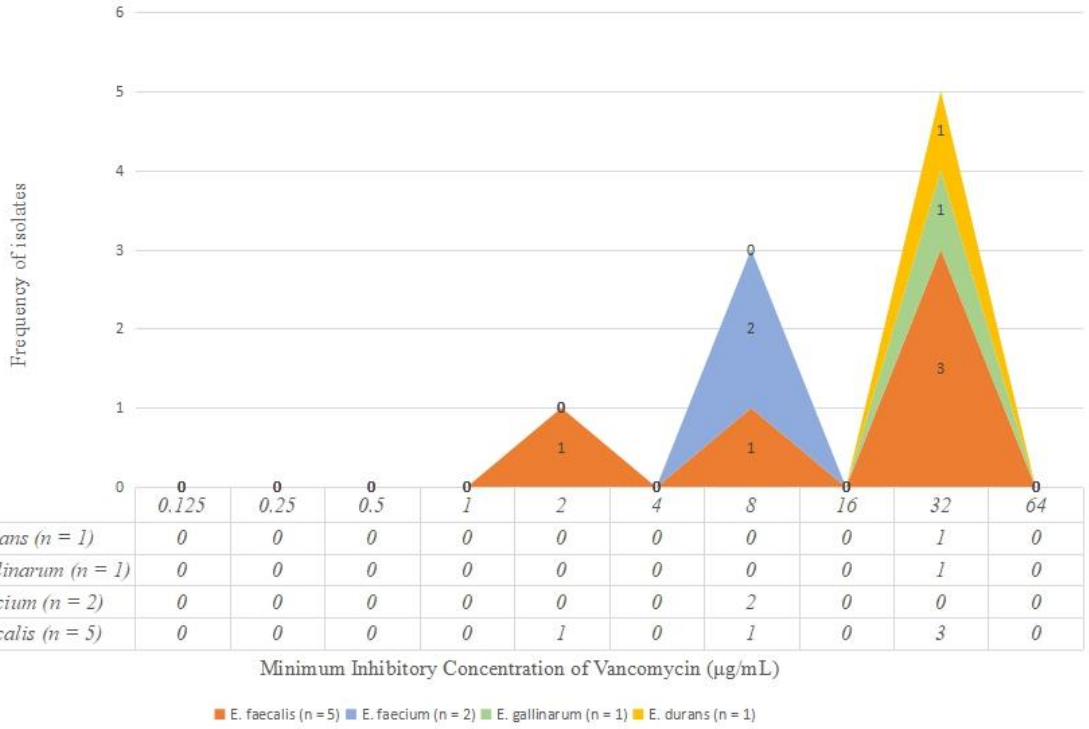
Figure 3

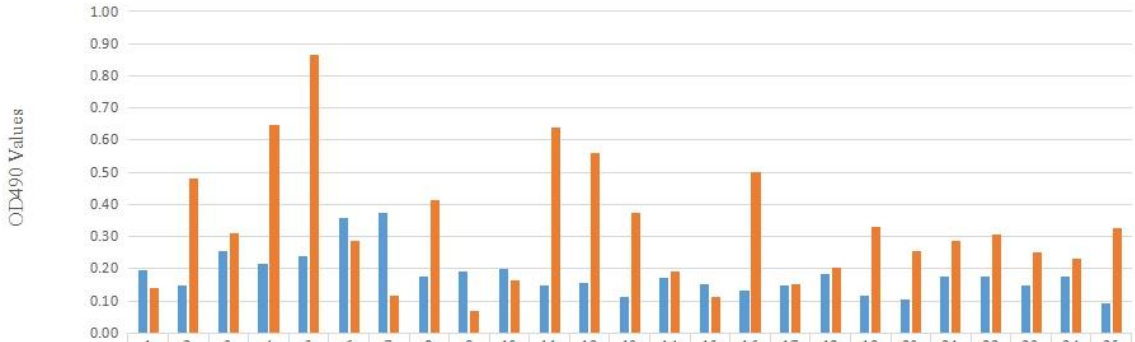
Minimum inhibitory concentrations (MICs) of Vancomycin in VRE (from clinical isolates)



	0.125	0.25	0.5	1	2	4	8	16	32	64
E. durans (n = 1)	0	0	0	0	0	0	0	0	1	0
E. gallinarum (n = 1)	0	0	0	0	0	0	0	0	1	0
E. faecium (n = 2)	0	0	0	0	0	0	2	0	0	0
E. faecalis (n = 5)	0	0	0	0	1	0	1	0	3	0

Figure 4

OD490 Values Among Clinical and Commensal Isolates



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Clinical isolates	0.20	0.15	0.26	0.22	0.24	0.36	0.37	0.17	0.19	0.20	0.15	0.16	0.11	0.17	0.15	0.13	0.15	0.18	0.12	0.11	0.18	0.17	0.15	0.18	0.09
Commensal isolates	0.14	0.48	0.31	0.65	0.87	0.29	0.12	0.42	0.07	0.17	0.64	0.56	0.37	0.19	0.11	0.50	0.15	0.20	0.33	0.26	0.29	0.31	0.25	0.23	0.33

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