Original Article

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Determination of Enterococcal Virulence Factors Expression and Impact of Biofilm Formation on Antimicrobial Susceptibility Pattern

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Abstract

Introduction: Enterococcus spp. has become recognized as a significant cause of hospital-acquired infections. Two main virulence factors namely gelatinase and hemolysin of Enterococci have been proved to cause severe infections. In addition, biofilm formation is causing infection by enhancing the persistence of Enterococci in medical indwelling devices. Therefore, the study aimed to evaluate the presence of hemolysin, gelatinase, and biofilm formation in Enterococcus spp. and the impact of biofilm on antimicrobial susceptibility patterns.

Materials & Methods: Total 104 Enterococcal isolates obtained from different clinical samples were included in the study for expression of virulence factors. All isolates were evaluated for biofilm formation by the tissue culture plate method. Hemolysin production was checked by using 5% sheep blood agar and gelatinase production by peptone yeast extract agar containing 3% gelatin. Antimicrobial susceptibility testing was done by the Vitek2 compact automated system.

Results: Out of 104 isolates, 1(1%) were strong biofilm producers while 4(3.85% %) and 54(52%) were moderate and weak biofilm producers respectively. Hemolysin production was observed in 19(18%) isolates and gelatinase production was universal.

Conclusion: Biofilm-producing strains showed higher resistance to beta-lactam drugs and high-level aminoglycosides. Hence, amongst three virulence factors, studying biofilm formation can be an important tool to develop a hospital's antibiotic policy and other virulence factors can be helpful to understand the pathogenesis of infection caused by Enterococcus spp. as well as for antimicrobial usage strategies.

***Keywords**: Antimicrobial susceptibility, Biofilm, Enterococci, Gelatinase, Hemolysin, Virulence factors*

Introduction

Enterococci are gram-positive cocci commonly considered normal inhabitants of the human gastrointestinal tract. The property of intrinsic resistance to many antibiotics and their ability to acquire resistance by mutations or by the acquisition of plasmids make them unique. *Enterococcus* spp. has been identified as a significant cause of hospital-acquired infections worldwide. [1] This is attributed to the acquisition of multidrug resistance (MDR) and the expression of virulence factors by *Enterococcus* spp.

The most important virulence factors of *Enterococcus* spp. are hemolysin, gelatinase, aggregation substances, lipoteichoic acid, capsular polysaccharides, and cell wall carbohydrate. Hemolysin is a cytolytic protein lysing erythrocytes and was found to be associated with increased severity of infections. [2] Gelatinase is a protease hydrolyzing collagen, casein, hemoglobin, and other peptides. [3] Gelatinase-producing strains of *Enterococcus faecalis* have been proved to enhance the virulence of endocarditis. Alongside, it has been noted that the frequency of the gene coding for Enterococcal surface protein (ESP) has been higher among clinical isolates than among commensal isolates. [4] Esp gene promotes primary attachment and biofilm formation of *E.faecalis* on the surfaces. Hence, biofilm formation is an important factor causing infection by enhancing the persistence of *Enterococci* on medical indwelling device infections. [5] Increased prevalence of *Enterococci* as a nosocomial pathogen and emergence of MDR strains emphasize on evaluation of virulence factors associated with invasiveness and disease severity.

Table 1: Panel of biochemical tests used for identification of Enterococcus species

Enterococcus spp.	Arginine Deamination	Pyruvate utilization	Mannitol fermentation	Arabinose fermentation	Raffinose fermentation	Sucrose fermentation	Pigment on tryptic soy agar	Motility
Enterococcus faecalis	+	+	+	-	-	+	-	-
Enterococcus faecium	+	-	+	+	V	+	-	-
Enterococcus avium	-	-	+	+	-	+	-	-
Enterococcus hirae	+	-	-	-	+	+	-	-
Enterococcus raffinosus	-	+	+	+	+	+	-	-
Enterococcus dispar	+	+	+	-	+	+	-	-
Enterococcus durans	+	-	-	-	-	-	-	-
Enterococcus mundtii	+	-	+	+	+	+	+	-
Enterococus casseliflavus	+	V	+	+	+	+	+	+
Enterococcus gallinarum	+	-	+	+	+	+	-	+

Hence, the present study aims at the study of three major virulence factors of *Enterococcus* spp. and the impact of biofilm formation on antimicrobial susceptibility testing.

Material and methods

All Enterococcal isolates were tested by the tissue culture plate method for biofilm production. [6] All strains were subcultured on sheep blood agar for recovery of pure growth. Then strains were inoculated into trypticase soy broth with 0.5% glucose and incubated at 37ºC for 24 hours. The next day, the culture was diluted with TSB-0.5% glucose up to 1:40. The uniform suspension was obtained by proper mixing and 200µl of the diluted solution was added to flat bottomed polystyrene microtiter well and the plate was incubated at 37ºC for 48hr. After two days, wells were washed three times by adding 300 µl of distilled water and allowed to air dry in an inverted position at room temperature for 1 hr. After complete drying, wells were stained with 200 µl of 0.1% safranin and allowed to stand for 20min at room temperature. The absorbance of biofilm formation on the bottom surface of each well was determined at 490nm in an ELISA reader. The test was carried out in quadruple and an average of four optical density (OD) values was taken. Culture medium without organism was taken as blank. Controls were processed in duplicate and the mean OD value of positive control (ODc) was used as standard. [Figure-1] Values were interpreted as weak, moderate, and strong biofilm producers. [Table-2]

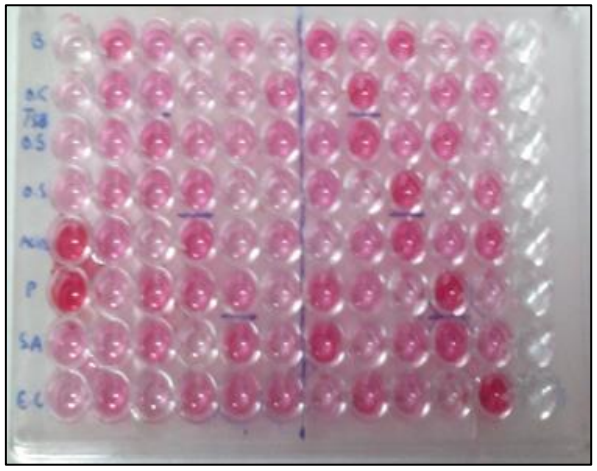


Figure 1: Microtiter plates having biofilm formation after staining with safranin

Table 2: Classification of bacterial adherence by Tissue Culture Plate method

Mean OD value	Adherence
ODc ≤ OD ≤ 2xODc	Weak
2xODc ≤ OD ≤ 4xODc	Moderate
OD > 4xODc	Strong

Hemolysin production was detected by inoculating Enterococcal isolates on 5% sheep blood agar (HiMedia

Labs). Plates were incubated overnight at 37ºC in a candle jar to create a capnophillic environment and screening of plates was done at 24hr and 48hr. A clear zone of complete hemolysis was considered to be a positive indicator of hemolysin production. [Figure-2] Gelatinase production was detected by inoculating the isolate in peptone yeast extract agar containing 30g/l of gelatin. [7] Plates were incubated overnight at 37ºC and then allowed to cool for 2hr at 22-25ºC. The appearance of a turbid halo around the line of stab was considered to be a positive indication of gelatinase production. [Figure-3]

Figure 2: Hemolysin production on blood agar: Complete hemolysis was considered positive.

Figure 3: Gelatinase test: Zone of haze around the stabline was considered positive.

Antimicrobial susceptibility testing of all isolates was done by using an automated vitek2 compact system (BioMerieux, Marcy l'Etoile, France). The antibiotics tested were Penicillin, Ampicillin, High-level gentamycin, High-level streptomycin, Ciprofloxacin, Levofloxacin, Erythromycin, Quinopristin/Dalfopristin, Linezolid, Vancomycin, Tetracyclin, Tigecycline, and Nitrofurantoin. *E.coli* ATCC 25922 and *S.aureus* ATCC 25923 were used as quality control strains. All ATCC strains were procured from Microbiologics, USA. The antibiotic susceptibility results were divided into resistant, intermediate, or sensitive as per the Clinical & Laboratory Standards Institute (CLSI) guidelines. [8]

Result

A total of 104 Enterococcal strains were isolated from different clinical samples. Out of 104, 62 (59%) were from urine followed by 22(21%) from blood cultures and 10(10%) from pus swabs. [Figure-4]

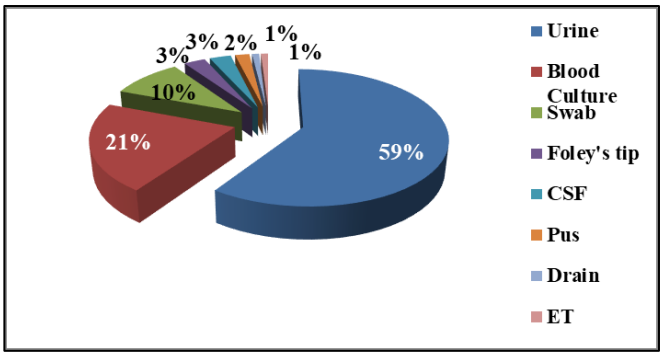
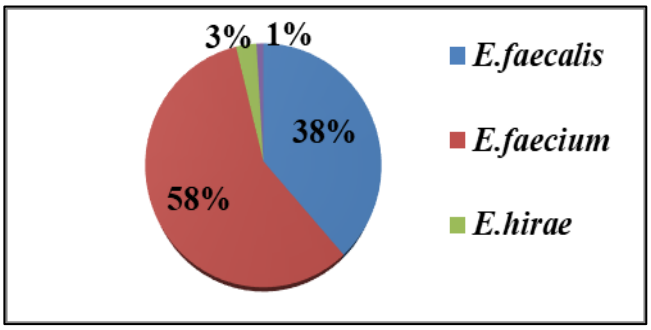


Figure 4: Distribution of Enterococcus spp. recovered from different clinical specimens

All strains were identified up to species level. Amongst them, 60(58%) were *E.faecium* followed by 40(38%) *E.faecalis*, 3(3%) *E.hirae*, and 1(1%) *E.raffinosus*. [Figure-5]



Out of total 10 commensal Enterococcal strains isolated, 5 were *E. faecium* and 5 were commensal isolates. It was noted that amongst clinical isolates, 19 (18%) were

hemolysin producers, 59 (57%) were biofilm producers and 104 (100%) isolates were gelatinase producers. [Table-3]

Table 3: Comparison of hemolysin, gelatinase, and biofilm production in clinical and commensal isolates

Virulence factors	Clinical isolates (n= 104)	Commensal (n=10)
Hemolysin	19	0
Gelatinase	104	10
Biofilm		
Strong	1	0
Moderate	4	0
Weak	54	3

Out of a total of 59 biofilm-producing *Enterococcus* spp., 1(1.7%) showed strong adherence, while 4(6.7%) and 54(91%) were moderate and weak biofilm producers respectively. [Figure-6].

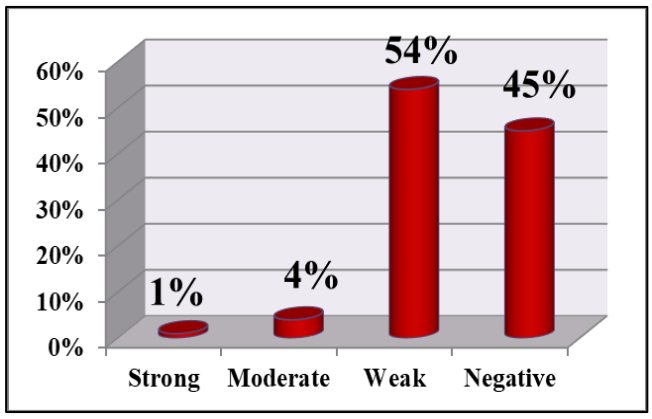


Figure 5: Percentage of biofilm production by Enterococcus spp. from clinical specimens

Hemolysin and biofilm production vary as per the type of infections too. Distribution of isolates based on clinical infections showed that urinary tract infections had 39(60%) biofilm producers and 10(15%) hemolysin producers followed by septicemia which had 13(59%) biofilm producers and 5(23%) hemolysin producers. [Table-4] The comparison of virulence factors amongst clinically significant *Enterococcus* spp. and commensal *Enterococci* showed variations too. It was observed that clinical isolates showed higher expression of virulence factors, while commensal *Enterococcus* spp. showed weak biofilm formation. [Table-5,6] Comparison of antimicrobial susceptibility pattern between biofilm producers and non-producers showed that antimicrobial resistance was higher in biofilm-producing *Enterococci.* [Figure-7]

Table 4: Production of hemolysin and biofilm in isolates from the different clinical specimen

Syndromes	Hemolysin	Biofilm
UTI (65)	10	1 (S)*, 3 (M)*, 35 (W)*
Septicemia (22)	5	13 (W)*
Wound infections (12)	4	1 (M)*, 5 (W)*
Endotracheal tube infection (1)	0	0
Meningitis (3)	0	0
Drain (1)	0	1 (W)*
* S-Strong, M-Moderate, W-Weak		

Table 5: Production of virulence factors hemolysin and biofilm in different Enterococcus spp. isolated from clinical specimens

Enterococcus spp.	Hemolysin	Biofilm*
E.faecalis (40)	18	1(S), 2(M),24(W)
E.faecium (60)	0	29(W),1(M)
E.hirae (3)	1	1(W)
E.raffinosus (1)	0	1(M)
* S-Strong, M-Moderate, W-Weak		

Table 6: Production of virulence factors hemolysin and biofilm by commensal Enterococcus spp.

Enterococci spp.	Hemolysin	Biofilm*
E.faecalis (5)	0	2 (W)
E.faecium (5)	0	1 (W)
* W-Weak		

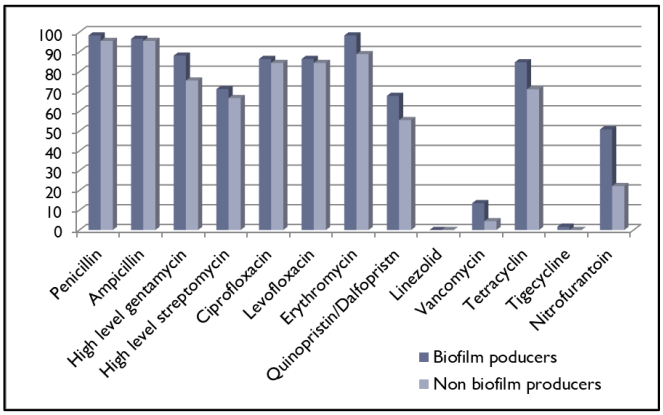


Figure 6: Comparison of resistance pattern of biofilm-producing enterococcal isolates and non-producing isolates

Discussion

Enterococcus spp. are the normal inhabitant of the gastrointestinal tract, but its significance increased due to the occurrence of nosocomial infections. In the present study, three virulence factors of *Enterococcus* spp. namely hemolysin, gelatinase, and biofilm formation were studied.

Many experimental studies highlighted the correlation of virulence factors with morbidity and mortality. Dupont H *et al* [9] studied three virulence factors namely cytolysin, gelatinase, and aggregation substance in animal models and noted increased inflammatory response. Stevens SX *et al* [10] studied hemolysin-encoding plasmid in isogenic *E. faecalis* strains and concluded a significant association with aggressive infections. Schlievert *PM et al* [11] studied the importance of enterococcal aggregation substance (AS) and enterococcal binding substance (EBS) of *Enterococcus faecalis* in animals. In the present study, it was observed that clinical isolates were forming biofilms while it was absent in commensals. Hence, it is likely that biofilm formation plays an important role in the pathogenicity of *Enterococci* by helping in the colonization of indwelling medical devices that further lead to nosocomial infections. Few studies like Fluit *et al* [12] and Fridkin *et al* [13] reported 7 % and 10 % of bloodstream infections respectively caused by *Enterococci*. These studies indicate the impact of biofilm formation in indwelling catheters.

Comparing the virulence of different *Enterococcus* species, *E.faecalis* produce significantly more biofilms than *E.faecium* and are more likely to cause catheter-related bloodstream infections. Sandoe *et al* [14] reported that the ability of enterococcal isolates to form biofilm in vitro is a marker of virulence trait that enhances the ability of isolated to cause infections. In the present study, *E.faecalis* showed more hemolysin and biofilm production as compared to *E.faecium.*

Considering urinary tract infections, many conflicting data are available highlighting the correlation of enterococcal surface protein (esp) gene producing biofilms and symptomatic urinary tract infections. Gard S *et al* [15] did not find any correlation between the strain forming biofilm and urinary tract infections. In the present study, *Enterococcus* spp. were isolated from different clinical specimens, and it was observed that expression of virulence factors was higher in urinary tract isolates followed by blood cultures.

In the present study, both clinical and commensal isolates were tested for hemolysin and gelatinase phenotypically and quantitative biofilm production by a microtitre method. It was observed that hemolysin and biofilm production were more in clinical isolates while gelatinase formation was similar. Tsikrikonis G *et al* [16] observed that isolates from surveillance and clinical samples produced biofilm significantly more often than animal isolates, along with hemolysin production. Whilst, similar proportions of animal and human *E. faecalis* produced gelatinase. Hence, it was concluded that gelatinase does not play a significant role in the pathogenicity of enterococcal infections.

The property of intrinsic resistance makes *Enterococcus* spp. unique in antimicrobial susceptibility pattern. Gilmore *et al* [17] had reported multidrug-resistant *Enterococcus* spp. highlighting the major problem worldwide. In the present study, a comparison of the antimicrobial susceptibility pattern of biofilm producers and non-biofilm producing strains showed that antimicrobial resistance was higher amongst *Enterococcus* spp. forming biofilms. Fallah *et al* [18] reported that resistance to some antibiotics including penicillin G, ampicillin, vancomycin, nitrofurantoin and chloramphenicol, and ciprofloxacin was significantly higher among biofilm producers. In the present study aminoglycosides, erythromycin, vancomycin, quinupristin/dalfopristin, tetracycline, nitrofurantoin showed higher resistance among biofilm producing *Enterococcus* spp. Ampicillin and linezolid may be used as an effective treatment for infections caused by biofilm producers *Enterococci*. Hence, the present study reinforces the role of biofilm formation in resistance to antimicrobial agents.

The identification of virulence factors associated with *Enterococci* invasiveness and disease severity will be an important subject of future investigations. Study of other virulence factors may further provide therapeutic alternatives and a better way to treat the ubiquitous microorganisms like *Enterococcus* spp.

The present study had a limitation that clinical correlation of virulence factors produced by *Enterococcus* spp. was not done. Further studies are required to understand the association of virulence factors and the clinical outcome of the patients. Secondly, only three virulence factors were studied, it was likely that other virulence factors too might play a significant role in the pathogenesis of Enterococcal infections. Third, more commensal isolates are required to understand the significant differences.

Conclusion

Hemolysin and biofilm production is one of the important virulence factors commonly found in E.faecalis and E.faecium. Strains producing biofilms are more resistant to antibiotics, hence treatment differs. A better understanding of the role of the virulence factors of Enterococcus spp. in infections may help in the development of new treatment strategies to prevent infection by this species.

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