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Biofilm Formation and Virulence Genes in Clinical Isolates of *Enterococcus faecalis*

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Abstract

Enterococcus faecalis is a Gram-positive bacterium commonly found in the gastrointestinal tract that can cause serious infections. Many enterococci have broad resistance to antibiotics including penicillin, cephalosporins, aminoglycosides and glycopeptides. There are several adaptation mechanisms that bacteria can undergo to become more resistant, among them is the formation of biofilm. Several genes have been linked to the increase in the capacity of biofilm formation by bacteria such as *gelE*, *esp* and *asa1*. The aim of this research was to evaluate the biofilm formation of 12 *E. faecalis* isolates collected in hospitals and a standard strain, as well as to evaluate the hydrophobicity of its membrane and the presence of virulence genes. All the isolates formed biofilm and the characteristics of their membrane were variable. In addition, the presence of at least one virulence gene was found in all the 12 isolates, and none of the genes in the standard strain, indicating the acquisition of these genes in the hospital environment. With this, we can conclude that there is a close relationship between biofilm formation, acquisition of antibiotic resistance and the presence of virulence genes.

Keywords

Enterococci, Gelatinase, Esp, Aggregating Substance, Resistance

1. Introduction

Enterococcus faecalis is a Gram-positive bacterium, facultative anaerobic, that can be found as coccus alone or in pairs and is a commensal present in the human oral cavity and gastrointestinal tract. But this species is opportunistic, and can cause severe infections, being responsible for 12% of nosocomial infections

[1] [2].

It is the predominant species of *Enterococcus* genus, where around 85% of enterococci infections are caused by *E. faecalis*. Adding to that, the presence of intrinsic and acquired resistance gives the capacity to be resistant to many antimicrobial drugs used in the clinic, making the treatment of infections more complicated [3] [4] [5].

There are several adaptation mechanisms that microorganisms can use to survive in the environment, which can make them more resistant and virulent [6] [7]. One of these mechanisms is the formation of biofilm, which occurs when planktonic bacteria adhere to a substrate and begin to produce polymeric material. Biofilm works as a physical barrier against the immune system and protect bacteria against the action of antimicrobials [8]. In accordance with Mohamed and Huang [9], bacteria that are present in biofilms can survive concentration up to a thousand times higher of antibiotics, proving the importance of this structure in bacterial infections.

Studies show the relation between the presence of genes such as *gelE*, *esp* and *asa1* with the capacity of bacteria to form biofilm, increasing the resistance to antimicrobials and virulence [10] [11] [12] [13]. Besides the presence of these genes, bacterial cellular hydrophobicity is another virulence factor, related to the capacity of microorganisms to adhere to a substrate and form biofilm [14] [15]. For that reason, this research aims to evaluate biofilm formation, cellular hydrophobicity and the presence of virulence genes in clinical isolates of *Enterococcus faecalis.*

2. Methodology

2.1. Strains and Growth Condition

Twelve *E. faecalis* clinical isolates collected from a hospital in Recife (Pernambuco, Brazil) were used in this research and a standard strain (UFPEDA 09) from the Department of Antibiotics collection. The clinical isolates were collected from different sites of infection (**Table 1**). They grew in Brain-Heart Infusion (BHI) at 37˚C for 24 hours and were kept at −20˚C in BHI + 15% glycerol.

2.2. Confirmation of Cell Identity through MALDI-TOF

Bacterial species of isolates were confirmed through MALDI-TOF technique. Isolates were deactivated and the proteic fraction was obtained and applied on the MALDI plate. Cellular matrix was prepared with acid alpha-ciane-4-hydrocinnamic (10 mg/mL) in 50% acetonitrile and 0.3% trifluoroacetic acid and applied on the plate with sample at room temperature for crystallization.

The MS spectrum in positive linear mode (acceleration voltage: 20 kV and detection limit—m/z: 2000 - 20,000) was made through Flex Control Version 3.0 program in Autoflex III (Bruker Daltonics, Billerica, MA, USA) MALDI-TOF Mass Spectrometer. Spectrums were compared with MALDI Biotyper Version 3.1 system and was given a compatibility score ranging from 0.000 - 1.699 (unre-

liable identification), 1.700 - 1.999 (potential genus identification), 2.000 - 2.299 (safe genus identification and potential specie identification) and 2.300 - 3.00 (highly potential specie identification).

2.3. Biofilm Formation

Biofilm formation of *E. faecalis* isolates was evaluated in 96-well plates in BHI, with Crystal Violet method as Stepanovic *et al.* [16] described. Using the absorbance (Abs), we determined the medium of absorbance value of each sample (Abss) compared with the absorbance of control (Absc). Biofilm formation was classified as strong (4× Absc < Abss), moderate (2× Absc < Abss ≤ 4× Absc) and weak (Absc < Abss ≤ 2× Absc). Isolates with equal or smaller absorbance than control were classified as non-biofilm producers.

2.4. Determination of Bacterial Hydrophobicity

Hydrophobicity of *E. faecalis* isolates was evaluated through microbial adhesion to solvent, proposed by Tendolkar [17], that is based on microbial affinity to a non-polar solvent (ρ-xylene). Bacterial cells grew 24 hours in BHI broth at 37˚C. After growth, bacteria were diluted in BHI at 1:50 and incubated again for four hours and spun at 5000 rpm for 5 minutes and resuspended in PUM (pH 7.1) with final OD 1.0 (~108 UFC·mL^{-1}, OD400). 1 mL of bacteria suspension was mixed with 250 µL of ρ-xylene and incubated for 10 minutes at 30˚C. After incubation, samples were mixed well in the vortex for 120 seconds and incubated again for 30 minutes at room temperature for phase separation. 200 µL of aqueous phase was measured in OD400. All samples were done in triplicate. Percentage of microbial adhesion to hydrophobic solvent (AMSH) is expressed following the formula: AMSH = [1 − (OD2/OD1) × 100].

2.5. Detection of Virulence Genes

Genomic DNA of isolates was extracted using GenElute Bacterial Genomic DNA (Sigma®) kit, quantified and stored at −20˚C. Virulence genes gelE (F: CGAAGT TGGAAAAGGAGGC; R: GGTGAAGAAGTTACTCTGA), esp (F: AGATTT CATCTTTGATTCTTGG; R: AATTGATTCTTTAGCATCTGG) and asa1 (F: AAGAAAAAGAAGTAGACCAAC; R: AAACGGCAAGACAAGTAAATA) were identified using PCR following SuperMix (Invitrogen®) kit instructions. PCR conditions were: initial denaturation at 94˚C for 2 min and 35 cycles of: 94˚C for 30 s, 56˚C for 30 s and 72˚C for 1 min, with final extension of 72˚C for 5 min. Amplicons were analyzed by electrophoresis in 1.2% agarose and 0.5× TBE and visualized in SYBR® Green. Samples were classified as positive when compared with a positive control (strain 19185 in GenBank as MN508951, MN508952, MN508953).

3. Results

Results are resumed in **Table 1**, where isolates are identified and with the source

Table 1. Results from analysis of tested isolates.

ID	MALDI (score)	Origin	Local of collection	Resistance profile	Classification	Hydrophobicity	Biofilm formation (BHI)	Virulence genes		
								gelE	*esp*	*asa1*
09	2.278	UFPEDA	Collection	Susceptible to all antimicrobials		M HFB	+++	−	−	−
135.4	2.463	Urine	Medical clinic	CIP-ERI-EST-GEN-MOX-NOR	MDR	HFB	+++	+	+	+
729.4	2.458	Urine	Medical clinic	ERI-EST		M HFB	+++	+	+	−
958.3	2.510	Urine	Ambulatory	ERI		HFL	+++	−	−	−
961.3	2.411	Blood	Medical clinic	AMP-CIP-ERI-GEN-MOX-NOR-TEI-VAN	MDR	HFL	+++	−	−	+
34.4	2.525	Surgical wound	Orthopedics	CIP-ERI-EST-MOX-NOR	MDR	HFB	+++	−	−	+
563.4	2.468	Urine	Vascular bed	ERI		M HFB	+++	−	+	+
253.4	2.481	Urine	ICU	ERI-EST		HFL	+++	+	+	−
111.7	2.262	Urine	Medical clinic	CIP-ERI-EST-GEN-MOX-NOR	MDR	HFL	+++	−	+	+
797.6	2.290	Peritoneal fluid	Vascular bed	CIP-ERI-EST-MOX		HFB	+++	+	+	+
946.6	2.371	Urine	Ambulatory	GEN		HFB	++	−	+	+
879.6	2.446	Urine	ICU	ERI-EST		M HFB	+++	−	+	−
794.6	2.501	Urine	Medical clinic	ASB-ATM-CAZ-CFO-CFZ-CRO-CRX-TIC-TOB	MDR	HFB	+++	−	−	+

MDR—Multidrug resistant; CIP—Ciprofloxacin; ERI—Erythromycin; EST—Streptomycin; GEN—Gentamicin; MOX—Moxifloxacin; NOR—Norfloxacin; AMP—Ampicillin; TEI—Teicoplanin; VAN—Vancomycin; ASB—Ampicillin + Sulbactam; ATM—Aztreonam; CAZ—Ceftazidime; CFO—Cefoxitin; CFZ—Cefazolin; CRO—Ceftriaxone; CRX—Cefuroxime; TIC—Ticarcillin; TOB—Tobramycin; HFB—Hydrophobic; M HFB—Moderate hydrophobic; HFL—Hydrophilic.

described. MALDI scores affirm the cellular identity of *Enterococcus faecalis*, with scores ranging from 2.262 to 2.525.

Five of the isolates were collected in a medical clinic, two in ambulatory, vascular bed or ICU and one in orthopedics. Furthermore, nine of 12 samples were collected from urine. Resistance profile was also analyzed, showing that all strains collected in hospitals already have resistance to at least one antimicrobial. Five of 12 strains were classified as multidrug-resistant (MDR).

Regarding biofilm formation, all strains form biofilm, being 12 classified as strong and one moderate. And as for hydrophobicity, five were classified as hydrophobic, four as moderately hydrophobic and four as hydrophilic.

As for the virulence genes associated with biofilm, four of the isolates have the *gelE* gene and eight have the *esp* or the *asa1* gene, indicating the presence of

these genes in isolates that form biofilm. One of the isolates has all three genes and is classified as hydrophobic (797.6) and only one isolate doesn't have any of the genes and is classified as hydrophilic (958.6).

4. Discussion

The highest prevalence of isolates found in urine samples could be explained by the fact that the species has a preference for the urinary system. Urinary tract infections are one of the most common infections in humans, and previous studies showed that *E. faecalis* can invade and colonize this particular system, causing infections [18] [19]. Five of the MDR samples were strong biofilm formers and three of them were collected from urine. About the virulence genes, *gelE* was present in one of the isolates, *esp* in two and *asa1* in all MDR isolates.

Previous studies have already reported the presence of *E. faecalis* resistance to many antimicrobials used in clinics. Reporting also the incidence of MDR strains, which makes it even more difficult to treat infections caused by this pathogen [20] [21] [22]. Most of enterococci classified as MDR are from hospital lineages, showing the increasing presence of resistant organisms in this environment. Adding to this, *Enterococcus* spp. can transmit resistance genes to many other bacterial genera, including Gram-positives and Gram-negatives, making more concerning the presence of this organism in hospitals [23] [24].

This work shows that the tested *E. faecalis* isolates have high capacity to form biofilms, corroborating with Hashem *et al.* [25] and Zheng *et al.* [12]. We also tried to find a relation for the high capacity to form biofilms, either through hydrophobicity assessment or the presence of virulence genes. As it was shown, both hydrophobic and hydrophilic isolates can be strong biofilm formers. Therefore, it is not possible to create a relation between the characteristics of membranes with formation of biofilm in different surfaces. Tendolkar *et al.* [17] explained that many factors can act together to influence structure and biofilm formation, such as adherent surface, molecular interactions of bacteria and protein synthesis.

The *gelE* gene produces gelatinase, one enzyme responsible for collagen, casein and hemoglobin hydrolysis. *esp* encodes enterococcal surface protein, and your presence is related to increase in colonization and persistence of bacteria in the urinary tract. And *asa1* gene encodes aggregation substance, responsible to make bacterial conjugation easier. In general, the presence of these genes is related to a higher virulence, and studies connect their presence with also a higher capacity to form biofilm [11] [17] [26].

With the results shown in this study, we can observe the absence of genes related to virulence in the standard isolate but the presence in 11/12 of *Enterococcus faecalis* clinical isolates, indicating the high prevalence in hospital isolates. Regarding the relation between the presence of genes and the formation of biofilms, with the presence of any of three virulence genes tested, the isolates had strong formation of biofilm. However, it is not possible to identify a relation

between the genotypes and phenotypes of isolates. Biofilm formation is a multifactorial process, where many mechanisms work together influencing its structure and formation [17].

In conclusion, it is possible to affirm that *E. faecalis* isolates can strongly form biofilm, and we can confirm the presence of virulence genes on these isolates and the incidence of *E. faecalis* classified as MDR in hospital, making infection more aggressive and difficult to treat. More studies need to be done searching for a deeper understanding in biofilm formation mechanism and consequently making it possible to find solutions to inhibit the formation of this important bacterial structure.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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