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Virulence Factors and Biofilm Formation in Vancomycin Resistant *Enterococcus faecalis* and *Enterococcus faecium* Isolates in Brazil

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Abstract

In this work, we evaluated biofilm formation of Vancomycin Resistant of *E. faecalis* and *E. faecium* (VRE) in different culture media and adhesion substrate, as well as cellular hydrophobicity and presence of virulence genes. For this, 35 isolates were collected from a public hospital in Recife, Pernambuco, Brazil and identified by the Matrix-Assisted Laser Desorption Ionization - Time-of-flight - Mass Spectrometry (MALDI-TOF-MS) technique. Biofilm formation was analyzed by the Crystal Violet (CV) method and fluorescence microscopy, cellular hydrophobicity by hydrocarbon interaction and the presence of *gel*E, *esp* and *asa*1 genes by Polymerase Chain Reaction (PCR). 12 isolates were identified as *E. faecalis* and 23 as *E. faecium*. Most were obtained in Coronary Units (40.0%) and Intensive Care Unit (31.4%). *E. faecium* isolates were more resistant to the antibiotics tested than *E. faecalis*; however, *E. faecalis* stood out as a biofilm producer. Regarding the presence and gene frequency, it was observed that *gel*E (54.3%) and *esp* (54.3%) were the most prevalent, followed by *asa*1 (22.9%). When comparing the gene frequency, it was observed that *gel*E and *esp* were predominant (48.6% for both species), while *asa*1 was more frequent in *E. faecalis* (20.0%). The data presented here are worrying, because they reveal the virulence potential of isolates VRE, which contributes to the dissemination and persistence of these pathogens in the hospital environment.

Keywords

Biofilm, Cellular Hydrophobicity, Gram-Positive, Hospital Environment,

Virulence Genes

1. Introduction

Enterococcus corresponds to Gram-positive microorganisms, whereupon 49 species are distributed in diverse environments, such as marine waters, plants, animal intestines, and others [1]. Among identified species, *E. faecalis* and *E. faecium* are those commonly found in the human intestinal tract. However, they are considered opportunistic pathogens being able to cause urinary tract infections, bacteremia, endocarditis, and neonatal meningitis [1] [2] [3] [4] [5].

Different factors contribute to the bacteria permanence in the hospital environment, mainly resistance [6] and virulence factors [7]. Vancomycin Resistance in *Enterococcus* spp. (VRE) was first reported in 1986 and has been associated with an increased mortality rate in patients suffering from bacteremia [8] [9] [10]. *E. faecium* and *E. faecalis* are the main species of this taxonomic group associated with resistance to vancomycin. There are reports of this type of resistance in other species of the genus (*E. gallinarum*, *E. casseliflavus*, *E. avium* and *E. raffinosus*); however, this resistance profile is less frequent [10] [11].

Virulence factors are related to the invasion of the pathogen in host tissue, persistence of infection, and biofilm formation potential. The biofilm architecture is influenced by several factors including hydrodynamic conditions, nutrient concentration, bacterial motility, and intercellular communication. Bacterial adhesion to the formation site is influenced by cell movement, electrostatic, and hydrophobic interactions, also adhesin expression [12] [13].

Over the years, a number of virulence genes have been described for *Enterococcus*, such as: aggregation substance, gelatinase, enterococcal surface protein, cytolysin, pheromones, and hyaluronidase [14] [16]. Gelatinase is a protein secreted by *E. faecalis* that potentially contributes to the virulence of this species, as well as the enterococcal adhesion encoded by the *esp* gene that contributes to colonization and persistence of *E. faecalis* during infection of ascending urinary tract [17]. The aggregation substance, on the other hand, corresponds to a pheromone-induced surface protein, which promotes the formation of conjugation aggregates during bacterial conjugation, contact between cell-cell, and between cell-host cell [18].

The aim of study was to evaluate and compare resistance profile and biofilm formation between *E. faecalis* and *E. faecium* clinical isolates in different culture media and adhesion substrate, and also to identify cell hydrophobicity and the presence of virulence genes.

2. Materials and Methods

2.1. Bacterial Samples

Clinical isolates were obtained according to the protocol established by the hos-

pitals and approved by the Research and Ethics Committee (CEP) of the Federal University of Pernambuco (UFPE), by protocol number: 2.581.568. Thirty-five isolates were collected from sectors of a public hospital in Recife, Pernambuco, Brazil, in 2018. An *E. faecalis* isolate obtained from the collection of the Department of Antibiotics of the UFPE (UFPEDA 09) was used as a control. All isolates were stored in Brain-Heart Infusion (BHI) broth and agar (2˚C - 8˚C), and in BHI liquid with 15% glycerol (−20˚C). The susceptibility profile of the isolates was identified using the VITEK 2 Compact automation equipment (BioMérieux®) and the interpretation of results was according to criteria recommended by the Clinical Laboratory Standards Institute [19].

2.2. MALDI-TOF Mass Spectrometry

The technique MALDI-TOF-MS was used for taxonomic confirmation of the isolates. Bacterial colonies were suspended in 300 µL of Milli-Q water and added with 900 µL of absolute ethanol. The suspensions were centrifuged at 15,600 g for 2 min. The supernatant was removed, and the pellet was dried in SpeedVac for 20 min. At the samples were added 50 µL of formic acid (70%) and 50 µL of acetonitrile. The resulting mixture was homogenized on a vortex stirrer and centrifuged at 15,600 g for 2 min, and the supernatant transferred to a new microtube. The matrix prepared with alpha-cyano-4-hydroxycinnamic acid (10 mg/mL), 50% acetonitrile, and 0.3% trifluoroacetic acid was added to the MALDI plate containing the sample at room temperature (18˚C) for crystallization. MS spectra were acquired in a linear positive mode (acceleration voltage: 20 kV and detection range - m/z: 2000 - 20,000) using the Flex Control Version 3.0 Program in MALDI-TOF Autoflex III Mass Spectrometer (Bruker Daltonics, Billerica, MA, USA). The obtained mass spectra were compared to data obtained from the MALDI Biotyper Version 3.1 Database.

2.3. Virulence Gene Detection

Genomic DNA from each isolate was extracted using the commercial GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich/Merck, Darmstadt, Germany), quantified in NanoVue™ Spectrophotometer (General Eletric, Massachusetts, EUA), and stored at −20˚C. The virulence genes *gel*E, *esp* and *asa*1 were identified by PCR following the instructions of the SuperMix® (Thermo Fisher Scientific, Massachusetts, EUA) and the sequence of the primers are listed in **Table 1**. The PCR conditions were: Initial denaturation of 94˚C for 2 min, 35 cycles of: 94˚C for 30 s, 56˚C for 30 s and 72˚C for 1 min, added to a final extent of 72˚C for 5 min, followed by cooling the samples to 4˚C. The amplicons were analyzed by 1.2% agarose gel electrophoresis and 0.5X TBE buffer and visualized by SYBR® Green dye on the photodocumentator in UV light. The amplicons were purified by the Clean-Up PCR Purification Kit (Sigma-Aldrich/Merck, Darmstadt, Germany) and sequenced by the Automated DNA Sequencer (Applied Biosystems, Hitashi). The obtained sequences were deposited in GenBank

Table 1. Primers used to identify virulence genes from Vancomycin-resistant *Enterococcus* isolates.

Gene	Primers (5'→3')	Amplicon (pb)	Tm (˚C)
*gel*E	F: CGAAGTTGGAAAAGGAGGC R: GGTGAAGAAGTTACTCTGA	333	56
esp	F: AGATTTCATCTTTGATTCTTGG R: AATTGATTCTTTAGCATCTGG	188	
*asa*1	F: AAGAAAAAGAAGTAGACCAAC R: AAACGGCAAGACAAGTAAATA	261	

with the identification code MN508951, MN508952, MN508953, for the *gel*E, *esp* and *asa*1 genes, respectively.

2.4. Cell Surface Hydrophobicity Determination

Bacterial cell surface hydrophobicity was determined according to a method described above [20] with modifications. Bacterial strains were grown at 37˚C for 24 hours in BHI. Subsequently the growth was diluted 1:50 in 5 mL of fresh medium and was incubated further at 37˚C for 4 h. Log-phase bacteria were harvested by centrifugation, washed twice with PUM buffer (22.2 g of potassium phosphate trihydrate, 7.26 g of monobasic potassium phosphate, 1.8 g of urea, and 0.2 g of magnesium sulfate heptahydrate/liter [pH 7.1]), and absorbance was adjusted to 1.0 at 400 nm (OD_{400}). Then, 250 µL of n-hexadecane was added to 1 mL of bacterial cell suspension normalized. The mixtures were incubated at 30˚C for 10 min, subsequently vortexed vigorously for 2 min, and allowed to stand for 15 min at room temperature to ensure complete separation of the organic and aqueous phases. The absorbance of the aqueous layer was measured at 400 nm. The percent of cell surface hydrophobicity was calculated by formula: [1 − (final OD_{400}/initial OD_{400}) × 100].

2.5. Biofilm Formation

Biofilm of *Enterococcus* sp. was determined by CV method [21] with some modification, under different conditions: Hydrophilic (Glass Test Tube) and Hydrophobic (Polystyrene 96-well Microplates) substrates, and two culture media: BHI and Tryptic Soy Broth (TSB). 160 µL of the culture medium, 20 µL of distilled water and 20 µL of the adjusted bacterial inoculum 1.5×10^8 CFU/mL were added to the microtiter plates. In the test tubes, 800 µL of culture medium, 100 µL of distilled water and 100 µL of bacterial inoculum were mixed. For the sterility control of the two substrates, the bacterial inoculum was replaced by distilled water. After incubation for 24 hours at 37˚C, the two substrates were washed three times with saline (0.9%) to remove planktonic cells, and then incubated at 55˚C for biofilm fixation. Subsequently, 200 µL of crystal violet was added to the plates and 1 mL to the test tubes for 15 minutes. After this period, the plates

were washed with distilled water and eluted with 100% ethanol to obtain the optical density reading at a wavelength of 570 nm. From the readings (OD570), the mean of the absorbance values of each sample (ODs) in comparison with the absorbance of the sterility control (ODc) were determined. The samples were classified as strongly (4× ODc < ODs), moderately (2× ODc < ODs ≤ 4× ODc) and weakly (ODc < ODs ≤ 2× ODc) forming biofilms. Isolates that presented absorbance values equal to or less than the control were classified as non-biofilm producers.

2.6. Fluorescence Microscopy Biofilm Analysis

To confirm biofilm formation on the different substrates, the assays were repeated using the 6-well polystyrene plate (hydrophobic substrate) and glass coverslips (hydrophilic substrate). It was chosen a microorganism that produces biofilm strongly in hydrophobic and hydrophilic substrates (*E. faecalis* 19185). In the hydrophobic substrate, it was added 4 mL of TSB, 0.5 mL of distilled water, and 0.5 mL of bacterial inoculum (1.5×10^6 CFU/mL). In the hydrophilic substrate the coverslips were placed in Petri dishes and 8 mL of TSB, 1 mL of distilled water, and 1 mL of bacterial inoculum were added. For sterility control, the bacterial inoculum was replaced by distilled water. Substrates were washed three times with 0.9% saline to remove planktonic cells. Followed by an addition of SYBR® Green (Dilution of 20 µL for each 1 mL of milliQ water) and Calcofluor White (1:1 with 10% KOH) dyes to analyze the biofilm cells and the polysaccharide structure, respectively. The images were obtained by epifluorescence microscopy (LEICA) on filter 2 (BP 515 - 560) for SYBR® Green and filter 1 (BP 480/401) for Calcofluor White.

2.7. Statistical Analysis

All tests were performed in triplicate, mean and standard deviation were calculated. Graphs and significance analysis ($p < 0.05$) were determined using GraphPad Prism version 5.0 software.

3. Results

The taxonomic identity of all isolates was confirmed by the MALDI-TOF-MS technique. Of the 35 isolates analyzed, 12 (34.3%) corresponded to *E. faecalis* and 23 (65.7%) to *E. faecium* (Table 2). It is possible observed in Table 2 that identified isolates were obtained from different origins and sectors of a public hospital in Recife, Pernambuco, Brazil. Most isolates were collected from the Coronary Units (COU = 40.0%) and the Intensive Care Unit (ICU = 31.4%). Others were obtained from the medical clinic (14.3%), cardiology (11.4%) and emergency (2.9%). Regarding the colonization site, it was observed that most bacteria (71.4%) were isolated from rectal swab. However, other sites of infection were also reported, but with a lower percentage. 14.3% were collected from blood, 11.4% from urine, and 2.9% from catheter. *E. faecalis* was most obtained

Table 2. General characteristics of *E. faecalis* and *E. faecium* isolates.

Identification	Species Identification (MALDI-TOF)	Origin	Source	Cellular hydrophobicity	Biofilm formation				Virulence genes		
					Hydrophobic substrate		Hydrophilic Substrate				
					TSB	BHI	TSB	BHI	*gelE*	*esp*	*asa*1
UFPEDA 09	*E. faecalis*	UFPEDA	Collection	M HFB	+++	+++	+	−	−	−	−
08850	*E. faecalis*	Blood	Medical clinic	M HFB	+	+++	+	+	−	−	+
11233	*E. faecalis*	Rectal swab	COU	HFL	+++	++	+++	+	+	−	−
11705	*E. faecalis*	Blood	Medical clinic	M HFB	+++	++	+	++	−	−	−
13241	*E. faecalis*	Catheter	Cardiology	M HFB	+++	++	++	++	−	−	+
17870	*E. faecalis*	Blood	ICU	HFB	+++	++	++	++	−	−	−
18576	*E. faecalis*	Rectal swab	Medical clinic	HFL	+++	+++	++	+++	−	+	+
00640	*E. faecalis*	Rectal swab	COU	M HFB	+++	+++	++	++	−	−	−
01014	*E. faecalis*	Rectal swab	ICU	M HFB	+++	+++	++	++	−	−	+
04757	*E. faecalis*	Rectal swab	ICU	M HFB	++	+	+	+	−	−	−
06430	*E. faecalis*	Blood	ICU	M HFB	+++	++	++	−	−	−	+
06941	*E. faecalis*	Rectal swab	ICU	M HFB	++	++	−	+	−	−	+
19185	*E. faecalis*	Rectal swab	COU	M HFB	+++	+++	++	+	+	+	+
11170	*E. faecium*	Rectal swab	ICU	HFL	+	+	−	+	+	+	−
10964	*E. faecium*	Rectal swab	COU	HFL	++	−	−	−	+	+	−
11574	*E. faecium*	Rectal swab	COU	HFL	+++	+	−	++	+	+	−
12455	*E. faecium*	Rectal swab	COU	HFL	++	++	+	+	+	−	−
14872	*E. faecium*	Urine	ICU	M HFB	+++	−	−	+	−	−	−
02089	*E. faecium*	Rectal swab	COU	HFL	+	+	−	+	−	−	−
03376	*E. faecium*	Rectal swab	Cardiology	HFL	+++	+++	−	++	−	+	−
12805	*E. faecium*	Rectal swab	ICU	HFL	++	+	++	−	+	+	−
15353	*E. faecium*	Rectal swab	COU	HFL	+	+	+	++	+	+	−
16184	*E. faecium*	Rectal swab	COU	HFL	+++	++	+	+	+	+	−
16206	*E. faecium*	Rectal swab	COU	HFL	−	−	−	++	+	+	−
16598	*E. faecium*	Rectal swab	COU	HFL	+	−	−	+	+	+	−
17281	*E. faecium*	Urine	Medical clinic	HFL	+	−	−	+	+	+	−
18008	*E. faecium*	Rectal swab	COU	HFL	+	+++	−	+	+	+	−
18300	*E. faecium*	Blood	ICU	HFL	+	−	+	+	+	+	−
00821	*E. faecium*	Rectal swab	Cardiology	HFL	+	+	+	+	+	−	−
00931	*E. faecium*	Rectal swab	COU	HFL	+	+	+	+	+	+	+
01236	*E. faecium*	Rectal swab	ICU	HFL	+	+	+	+	−	+	−

Continued

18984	*E. faecium*	Urine	Emergency	HFL	+	+	−	+	+	+	−
15088	*E. faecium*	Urine	Medical clinic	HFL	++	+	−	−	+	+	−
11496	*E. faecium*	Rectal swab	ICU	HFL	−	−	−	+	+	+	−
13679	*E. faecium*	Rectal swab	COU	HFL	+	+++	+	++	−	−	−
10800	*E. faecium*	Rectal swab	Cardiology	HFL	++	+++	+	++	−	−	−

UFPEDA—Department of Antibiotics of Federal University of Pernambuco, ICU—Intensive care unit, COU—Coronary unit, HFL—hydrophilic, M HFB—Moderately hydrophobic, MFB—Hydrophobic. In biofilm formation: Weak (+), Moderate (++) e Strong (+++) biofilm producers and no biofilm producers (−). In virulence genes: Presence (+) e Absence (−).

from blood cultures (11.4%) and ICUs (14.3%), while isolates of *E. faecium* were more frequent from rectal swab (51.4%) and COUs (31.4%).

Resistance profile showed a higher resistant to ampicillin and penicillin G, 62.9% were resistant to ampicillin, but only one *E. faecalis* isolate was resistant to this antibiotic (19185), 77.1% were resistant to penicillin G, mainly in *E. faecium* isolates (62.8%). All isolates were resistant to vancomycin, 34.3% were resistant to daptomycin, and 14.3% were resistant to linezolid (Table 3).

Ability to form biofilm is showed in Table 2 and Figure 1. It was observed that *E. faecium* had a lower potential when compared to *E. faecalis*. All *E. faecalis* isolates were able to produce biofilm. However, only seven of 23 *E. faecium* isolates produced biofilm under the conditions tested. From the investigated substrates (plate and tube), it was observed that isolates of both species showed better performance for biofilm production on the hydrophobic surface (Plate = 48.6%) when compared to the hydrophilic surface (Tube = 5.7%). Regarding the culture media, it was found that in the TSB the isolates had higher formation potential, regardless of the species analyzed (Figure 1).

Table 4 shows the general characteristics of the isolates in relation to total samples, source, origin, biofilm formation, cellular hydrophobicity, and virulence genes (distribution and frequency). From this table, it is possible to compare the resistance profile and virulence potential of each species.

For the following variables, cell hydrophobicity and presence of virulence genes, *E. faecalis* and *E. faecium* also presented distinct profiles. Most isolates of *E. faecalis* were moderately hydrophobic (M HFB = 25.7%), while all of *E. faecium* were hydrophilic (HFL = 62.9%), excepting isolate 14872 which also presented as M HFB.

In the present study, we also investigated the presence and frequency of virulence genes: *gel*E, *esp* and *asa*1. The gelatinase gene, *gel*E (54.3%), and the *Enterococcus* surface protein gene, *esp* (54.3%), were most prevalent. Followed by the aggregation substance gene, *asa*1 (22.9%). *E. faecalis* showed higher positivity for the *asa*1 gene (20%), followed by *gel*E (5.7%) and *esp* (5.7%) genes. In *E. faecium* isolates, the frequency of *gel*E and *esp* was higher (48.6% for each gene), followed by *asa*1 that was detected in only one isolate (00931), see Table 2.

Only two isolates, one from *E. faecalis* (19185) and one from *E. faecium*

Table 3. Resistance profile of *E. faecalis* and *E. faecium* isolates.

Identification	AMP		PEN G		VAN		DAP		LIN	
	RIS	MIC	RIS	MIC	RIS	MIC	RIS	MIC	RIS	MIC
UFPEDA 09	S	-	S	-	S	-	S	-	S	-
08850	S	4	R	>8	R	256	*	4	S	2
11233	S	2	S	4	R	96	S	1	S	≤0.5
11705	S	4	R	>8	R	256	S	2	S	2
13241	S	4	R	>8	R	>32	*	4	S	1
17870	S	2	S	4	R	>32	S	2	R	>4
18576	S	1	S	4	R	>32	S	1	S	1
00640	S	1	S	4	R	>32	S	2	S	1
01014	S	2	S	8	R	>32	*	>4	S	2
04757	S	1	S	4	R	>32	S	>4	S	2
06430	S	2	R	>8	R	96	S	2	S	2
06941	S	2	S	8	R	>32	*	4	S	2
19185	R	>16	R	>8	R	>32	S	2	S	2
11170	R	>16	R	>8	R	96	S	2	R	>4
10964	R	>16	R	>8	R	>32	S	2	S	1
11574	R	>16	R	>8	R	>32	S	2	S	1
12455	R	>16	R	>8	R	>32	S	2	S	1
14872	R	>16	R	:8	R	>32	S	1	S	≤0.5
02089	R	>16	R	>8	R	>32	*	>4	S	2
03376	R	>16	R	>8	R	>32	*	>4	R	>4
12805	R	>16	R	>8	R	>32	*	4	R	>4
15353	R	>16	R	>8	R	>32	S	2	S	1
16184	R	>16	R	>8	R	>32	S	2	S	1
16206	R	>16	R	>8	R	>32	S	2	S	2
16598	R	>16	R	>8	R	256	S	2	S	2
17281	R	>16	R	>8	R	>32	S	2	S	2
18008	R	>16	R	>8	R	>32	*	>4	S	1
18300	R	>16	R	>8	R	>32	S	2	S	1
00821	R	>16	R	>8	R	>32	*	>4	R	>4
00931	R	>16	R	>8	R	>32	*	>4	S	1
01236	R	>16	R	>8	R	>32	S	2	S	2
18984	R	>16	R	>8	R	96	*	>4	S	≤0.5
15088	R	>16	R	>8	R	>32	S	2	S	2

Continued

11496	R	>16	R	>8	R	>32	*	4	S	2
13679	S	2	R	>8	R	>16	S	1	S	2
10800	S	2	S	2	R	>32	S	0.5	S	1

UFPEDA—Department of Antibiotics of Federal University of Pernambuco. Antibiotics: AMP—ampicillin, PEN G—penicillin G, VAN—Vancomycin, DAP—daptomycin, LIN—linezolid. RIS—Resistance International System, MIC—Minimum inhibitory concentration (μg/mL). R—resistant, S—susceptible. *Note: When clinical breakpoints for daptomycin were originally set there was insufficient evidence to set a susceptible-resistant breakpoint. In our study we considered daptomycin resistant with MIC ≥ 4[19].

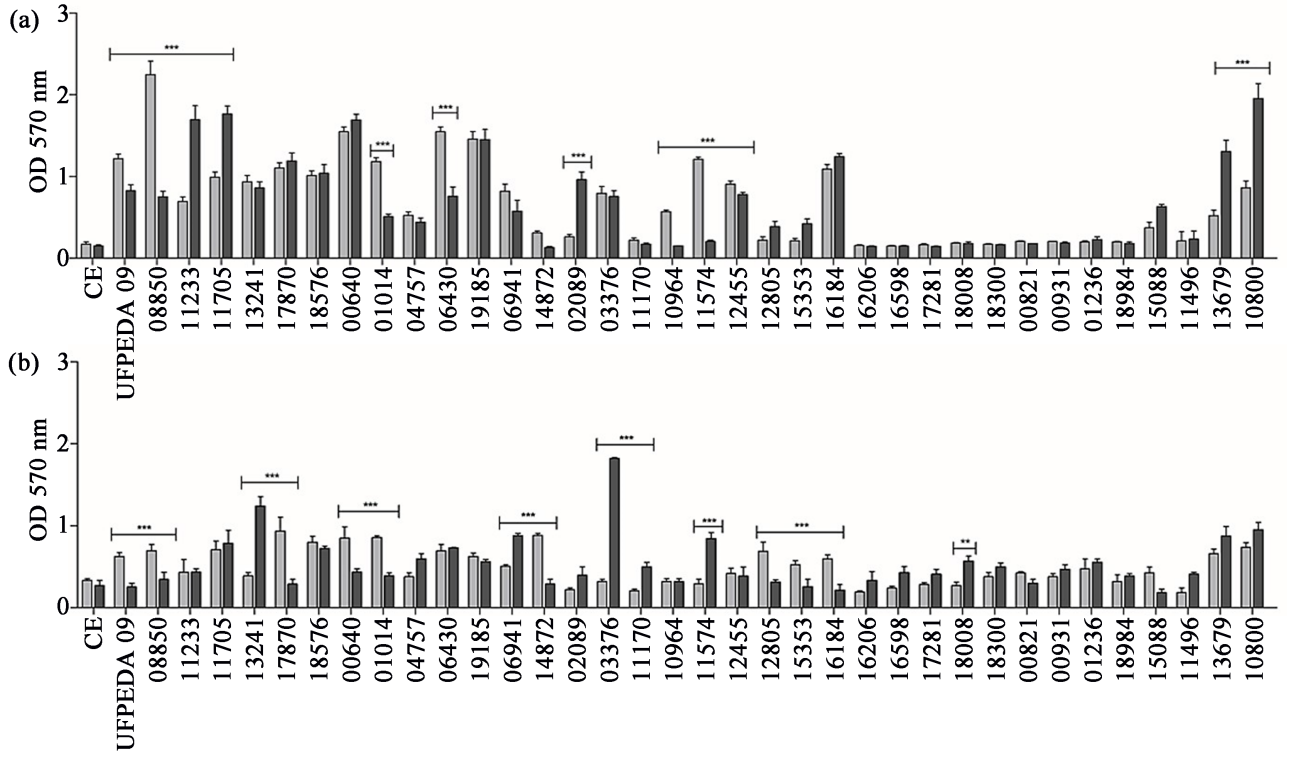

Figure 1. Comparison of biofilm formation between the culture media used. Gray bars represent TSB culture medium and black bars represent BHI culture medium. (a) Hydrophobic surface. (b) Hydrophilic surface. ***$p < 0.005$.

(00931) were positive for three genes tested. The other isolates presented different genetic profiles. The profile (*esp* + *asa*1) was identified in only one isolate (18576) belonging to the species *E. faecalis*. While the profile (*gel*E + *esp*) was detected in 14 isolates of *E. faecium*. Isolates containing only one gene were also found, 6 in *E. faecalis* and 4 in *E. faecium* (see Table 2).

Interestingly, five isolates, two from *E. faecalis* (17870, 00640), three from *E. faecium* (02089, 13679, 10800), and control 09 did not show any of the genes investigated. These same samples presented themselves as moderate to strong biofilm producers in hydrophobic subtracts. On the other hand, eight isolates were positive for at least two of the three genes investigated and were classified as weak or non-producer of biofilm (see Table 2).

Table 4. Frequency of main characteristics of *E. faecalis* and *E. faecium* isolated from a public hospital in Recife, PE, Brazil.

Characteristics	*E. faecalis*	*E. faecium*	Total
Total n. (%)	12 (34.3)	23 (65.7)	35 (100.0)
Source n. (%)			
ICU	5 (14.3)	6 (17.1)	11 (31.4)
COU	3 (8.6)	11 (31.4)	14 (40.0)
Medical Clinic	3 (8.6)	2 (5.7)	5 (14.3)
Emergency	0 (0.0)	1 (2.9)	1 (2.9)
Cardiology	1 (2.9)	3 (8.6)	4 (11.4)
Origin n. (%)			
Rectal swab	7 (20.0)	18 (51.4)	25 (71.4)
Blood	4 (11.4)	1 (2.9)	5 (14.3)
Urine	0 (0.0)	4 (11.4)	4 (11.4)
Catheter	1 (2.9)	0 (0.0)	1 (2.9)
Resistance profile			
AMP	1 (2.9)	21 (60.0)	22 (62.9)
PEN G	5 (14.3)	22 (62.8)	27 (77.1)
VAN	12 (34.3)	23 (65.7)	35 (100.0)
DAP	4 (11.4)	8 (22.9)	12 (34.3)
LIN	1(2.9)	4 (11.4)	5 (14.3)
Biofilm formation n. (%)			
Strong biofilm on hydrophobic substrate	10 (28.6)	7 (20.0)	17 (48.6)
Strong biofilm on hydrophilic substrate	2 (5.7)	0 (0.0)	2 (5.7)
Strong TSB biofilm	9 (25.7)	4 (11.4)	13 (37.1)
Strong BHI biofilm	5 (14.3)	4 (11.4)	9 (25.7)
Cellular hydrophobicity n. (%)			
HFL	2 (5.7)	22 (62.9)	24 (68.6)
M HFB	9 (25.7)	1 (2.9)	10 (28.6)
HFB	1 (2.9)	0 (0.0)	1 (2.9)
Virulence genes n. (%)			
*gel*E	2 (5.7)	17 (48.6)	19 (54.3)
esp	2 (5.7)	17 (48.6)	19 (54.3)
*asa*1	7 (20.0)	1 (2.9)	8 (22.9)

ICU—Intensive care unit, COU—Coronary unit, AMP—ampicillin, PEN G—penicillin G, VAN—vancomycin, DAP—daptomycin, LIN—linezolid, HFL—hydrophilic, M HFB—Moderately hydrophobic, MFB—Hydrophobic. TSB—Tripyc Soy Broth media, BHI—Brain-Heart infusion media.

To confirm the potential for biofilm formation, 19185 *E. faecalis* isolate was selected and analyzed on two substrates (plate and tube) by fluorescence microscopy. The results confirmed that 19185 is a strong biofilm producer, regardless of the condition tested, be it a hydrophobic substrate (**Figure 2(c)**) or a hydrophilic substrate (**Figure 2(f)**). **Figure 2(a)** and **Figure 2(d)** show the microorganisms. Also, in **Figure 2(b)** and **Figure 2(e)** it is possible to notice the production of extracellular matrix secreted by bacterial cells. The **Figure 2(c)** and **Figure 2(f)** is the overlay of previous images.

4. Discussion

Since 2017, a reduction in hospital infection rates in general has been observed. However, there is an increase in the number of infections caused by multi-resistant microorganisms. This phenomenon has been occurring not only in Brazil, but worldwide. In adult and neonatal ICU, *Enterococcus* spp. vancomycin resistance are among the microorganisms that cause the most health care-related infection (HAI) and increase in-patient mortality [22] [23]. According to the World Health Organization (WHO), *E. faecium* VRE isolates are more frequent in hospital outbreaks than *E. faecalis*, since they have a larger gene arsenal related to resistance and virulence factors [10] [24]. As well as *E. faecium* is more resistant to AMP than *E. faecalis* according by EUCAST and CLSI protocols. It was not different in our study [19] [25].

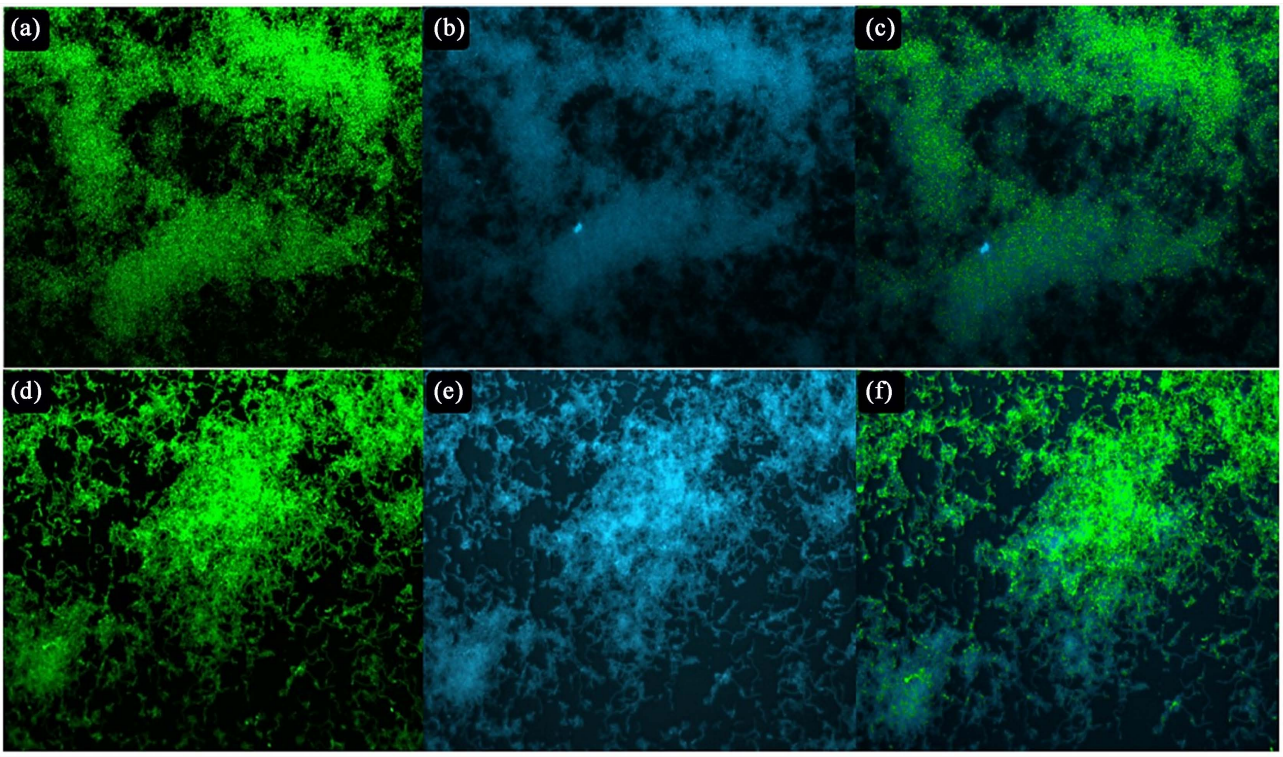

Figure 2. Analysis of biofilm formation through epifluorescence. *E. faecalis* strain 19185. Upper line hydrophobic substrate and lower line hydrophilic substrate. (a) and (d) Images were dyed with Calcofluor White (Filter 1). (b) and (e) Images were dyed with SYBR Green. (c) Overlay images (a) and (b). (f) Overlay images (d) and (e).

Collection with rectal swab is indicated by the *Agência Nacional de Vigilância Sanitária* (ANVISA) as a practice of epidemiological surveillance after the patient was hospitalized, which is a concern, since most isolates of *E. faecium* of this study came from this collection, and all isolates were VRE. *Enterococci* are already in the normal microbiota, and in this case VRE isolates may be the main transmission mechanisms within the hospital sectors [26]. This is a worrying factor for immunocompromised patient. According to a study [27], hospitalized patients have a high incidence of enterococcal infections, not only due to the virulence of isolates, but also due to the circulation of health professionals and the hospital area. This represents a risk environment, which can justify the spread of species in different sectors of a hospital or from different hospitals as previously demonstrated [28].

Among the strategies to combat antimicrobial resistance (ADR) are socio-educational interventions, monitoring access to these drugs and rational prescriptions [29]. According to Pan American Health Organization (PAHO), ADR surveillance work should be continuous and multidisciplinary [30]. Thus, the collective effort of the hospital unit investigated in our study and also of local health agencies could result in a more effective control of ADR.

Regarding of biofilm formation, the composition of the medium is probably the most important factor that influences the ability of bacteria to produce biofilm under *in vitro* conditions. Thus, TSB is the most widely used medium for the cultivation of Gram-positive biofilms [21]. The relationship between biofilm formation and cellular hydrophobicity is still difficult to confirm. The determination of bacterial surface hydrophobicity alone is not a sufficient factor to characterize biofilm formation. Other factors are needed to stimulate or develop this ability in microorganisms. Considering that, in enterococcal cells this trait seems to be no different [20].

The biofilm formation is a multifactorial process related to both gene expression and interference of environmental factors, which can be aggravated, as these genes as well as resistance genes can be propagated between species through mobile DNA elements [31]. The presence of virulence genes may be correlated with biofilm formation, which has been reported over the years [22] [27] [32]. The prevalence of *gel*E, *esp* and *agg* (other aggregation substance) genes is high when it comes to clinical isolates [32].

Furthermore, biofilm formation is more related to the presence of *gel*E gene, regardless of whether or not isolates present *esp* and *agg* genes in *E. faecalis* species. However, studies demonstrated biofilm formation in mutated strains for the *esp* gene (Esp-negative) and confirmed that neither *esp* nor *gel*E appear to be necessary to develop this factor *in vitro*. In addition, also confirm that the presence of these genes seems to be more related to the successful establishment of an infection [22] [33]. These data are interesting because they indicate that the presence or absence of the genes investigated in this study does not seem to be the primary or only characteristic for biofilm formation in these species. It is

noteworthy that, the ability to form biofilm contributes to the pathogenicity of infections, since it enables the mediation of adhesion, colonization, and invasion of host tissue. Thereby, modulating immunity, producing enzymes, and toxins that help the installation and increase of severity of infection [13] [14].

It was observed that in **Figure 2** it is possible to evidence the formation of biofilm by fluorescence. For this, we used SYBR Green (capable of intercalating in the hydrogen bridges of double strand of bacterial DNA) and Calcofluor White dyes (which binds the β1-6 polysaccharide bonds that are formed in the exopolysaccharide matrix or EPS) [34] [35] [36]. The fluorescence technique is widely used, these dyes together in our work were capable to show the biofilm and prove that *Enterococcus* isolates can be able to form biofilm in different surfaces.

In general, the formation of biofilms depends on several factors such as the types of microorganisms, surfaces and environmental conditions such as pH and temperature [13]. The biofilm matrix is mainly composed of EPS, but also contains water, lipids, nucleic acids and extracellular proteins, forming a porous architecture with channels that allow the passage of nutrients [12]. In addition, electrostatic and hydrophobic attractive forces, van der Waals interactions, hydrogen bonds and covalent bonds, as well as flagella, fimbrial adhesin and polymers are considered for biofilm formation [12] [37]. Biofilms are a suitable microenvironment for microbial survival, being a protective barrier, including against antimicrobials [38]. Therefore, studies on the subject are crucial to understand the functioning of this mechanism in different microorganisms and help in the development of effective therapies.

5. Conclusion

Our findings show the importance of characterizing virulence factors and biofilm formation related to clinical isolates of *Enterococcus* that present resistance mainly to vancomycin, daptomycin, and linezolid. Also, it demonstrates genetic and biochemical alternatives, which facilitate the adaptation and survival of *Enterococcus* isolates. In addition, the presence and high frequency of *gel*E, *esp* and *asa*1 genes are an indicative that monitoring studies in the hospital setting should occur frequently. These factors increase the spread and severity of infection, making treatment difficult and increasing rates of infection morbidity and mortality of hospitalized patient.

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Conflicts of Interest

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

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