Correlation between biofilm formation and gelE, esp, and agg genes in Enterococcus spp. clinical isolates

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Enterococci are a leading cause of nosocomial infections. Enterococcus faecalis is the species most frequently isolated, and it is commonly recovered from surgical wounds, intra-abdominal infections, the bloodstream, and especially urinary tract infections (UTIs).1,2

Virulence factors contribute to enterococcal fitness and its persistence as a pathogen in the nosocomial environment. These factors are mainly related to adherence, including biofilm formation, and include gelatinase (encoded by gelE), enterococcal surface protein (encoded by esp), and aggregation substance (encoded by agg).3,4

In addition to their involvement with biofilm formation, GelE hydrolyzes collagen, casein, and hemoglobin,5-7 and Agg mediates the formation of aggregates during conjugation,8 promoting the transference of mobile genetic elements and contributing to pathogenesis.9,10

Biofilm is a structured and complex community of microorganisms adhering to a biotic or abiotic surface.11 Biofilms are a vital strategy for all kinds of microorganisms, allowing them to survive adverse conditions. Enterococci have an extraordinary ability to form biofilms, and this characteristic is strongly related to specific conditions, such as urinary tract infections.11 Most isolates (87.5%) were from urine (87.5%), and 1% were biofilm formers (75%), and the gelE and agg genes were frequently observed (100% and 75%, respectively). Among MDR Enterococcus faecium, the most frequent site of recovery was blood (35.7%), and with the exception of esp (75%), neither biofilm (13%) nor virulence factors were common features.

The distribution of virulence factors is shown in Table 3. Overall, 73.3% of isolates harbored gelE, 70% harbored esp, and 58.3% harbored agg. Only 42.6% of the isolates positive for gelE produced gelatinase. Although not statistically significant (P = 0.09), esp was more frequent in isolates of noninvasive sites for both species. Among biofilm formers, 85.3% (174/204) were from noninvasive site (urine), and 58.3% (15/36) were from invasive sites (Table 1). The association between noninvasive site isolation and biofilm formation was statistically significant (P < 0.0001). In contrast, the other virulence genes and gelatinase production were randomly distributed in invasive and noninvasive sites (no statistical significance). Among Enterococcus faecalis, gelE and gelatinase were only found in isolates recovered from invasive sites, and agg was not found in any strain.

The ability to form biofilm was observed in 81.25% (n = 195) of all isolates; 75.4% (n = 147) were classified as moderately or strongly adherent and 24.6% (n = 48) as weakly adherent. Only 2 Enterococcus faecium (10.5%) showed the ability to form biofilm, and they were weakly adherent.

The association between each virulence gene and biofilm formation is shown in Table 4. All associations (gelE/biofilm+, esp/biofilm+, and agg/biofilm+) were statistically significant (P = 0.009, P < 0.001, and P = 0.001, respectively). Six isolates (2.5%) encoded all tested virulence genes and did not form biofilm, while 4 isolates (1.67%) were biofilm producers with no detected virulence genes.

Among the biofilm formers, the highest resistance rates were 22.05% and 17.44% to gentamicin (high levels) and ciprofloxacin,
respectively. For other antimicrobials (linezolid, ampicillin, vancomycin, and nitrofurantoin), few biofilm formers were resistant (6.66%, 2.56%, 1.03%, and 0.51%, respectively). There were no statistically significant differences between adherent/non-adherent isolates and antimicrobial resistance ($P = 0.29$).

This study evaluated biofilm formation and its relationship to virulence genes and antimicrobial resistance in *Enterococcus* spp. Similar to previous studies, enterococci were commonly recovered from urinary tract sites, where *E. faecalis* was prevalent. Higher antimicrobial resistance was observed in *E. faecium* compared with *E. faecalis*.15-24

Most of the virulence genes examined in our study were related to biofilm formation, specifically in the adhesion step. Johansson and Rasmussen25 observed that normal flora isolates (noninvasive) produce more biofilm than isolates from infective endocarditis (invasives), which our study confirmed. For invasive isolates, other virulence traits may be more relevant than adherence. For example, the degradation of collagen and other proteins may be extremely relevant for invasion and dissemination, which may explain the association we found between invasive isolates, other virulence traits may be more relevant than adherence.

We observed that *E. faecalis* produced biofilm more often than *E. faecium*, as previously reported.13,26 Although not statistically significant ($P = 0.29$), biofilm producers showed lower resistance rates compared with non-adherent isolates, most likely due to bacterial fitness. It is important to note that cells were exclusively tested in their planktonic form. The MICs of cells in biofilm were not determined.

As previously reported,27,28 esp was the only virulence gene present in *E. faecium*. Esp is an important feature of clonal-complex 17 (CC17), which is present in a special lineage of *E. faecium* and is strongly associated with hospital outbreaks around the world.24,29 This gene may be important for biofilm formation;7 Di Rosa26 suggests that synergy between esp and biofilm formation helps establish a successful infection. However, it does not seem to be essential for biofilm formation,30 which is a multifactorial process.

Other virulence factors include the collagen binding protein (encoded by ace), which performs an important role in forming the microbial extracellular matrix;30,31 the cytolysin activator (bacterial toxin with hemolytic activity against eukaryotic cells,32 encoded by cylA); the biofilm enhancer in *Enterococcus* (encoded by bee), which is involved in pili formation and enhances biofilm formation in *Enterococcus faecalis*33 and the endocarditis and biofilm-associated a pili (encoded by ebp).34 These factors participate in invasion and colonization and may contribute to biofilm formation.35

The small number of *E. faecium* isolates is a limitation of our study. However, this study reinforces the well-known characteristics of *E. faecalis* and *E. faecium* as species with high virulence and resistance, respectively. It also confirms that biofilm formation is a multifactorial process requiring different genes and their products. To elucidate biofilm formation, these studies are essential for determining the role of each molecule in the virulence process and the local epidemiological characteristics of enterococci.

Enterococci were recovered from patient infections or surveillance sites at Complexo Hospitalar Santa Casa de Porto Alegre (Porto Alegre, Brazil) as part of an epidemiological surveillance study (September 2012 to March 2013). Genus identification was performed through the observation of phenotypic characteristics according to Teixeira et al.16 Species identification was determined by duplex PCR assay22 as follows: 5 pmol of the *E. faecalis-*specific primers (5′-ATCAAGTACAGTTGATCTTT ATTAG-3′ and 5′-ACGTTCAAACGTAATCAGCA TCGAT-3′), 1.25 pmol of the *E. faecium-*specific primers (5′-TTGAGGCAGATTCAAGTAGCA ACTCCGATT TCGATGACCTCC-3′), 10 mM TRIS-HCl (pH 8.3), 50 mM KC1, 1.5 mM

### Table 1. Different sites from isolation of *Enterococcus faecalis* and *Enterococcus faecium*

| Sites            | *E. faecalis n (%) | *E. faecium n (%) | Total n (%) |
|------------------|--------------------|-------------------|-------------|
| Urine            | 197 (82.08)        | 6 (2.5)           | 203 (84.58) |
| Blood            | 10 (4.17)          | 7 (2.91)          | 17 (7.08)   |
| Abdominal secretion | 7 (2.91)          | 1 (0.42)          | 8 (3.33)    |
| Catheter         | 1 (0.42)           | 1 (0.42)          | 2 (0.84)    |
| Pelvic collection | 2 (0.83)           | 0 (0)             | 2 (0.84)    |
| Others*          | 4 (1.67)           | 4 (1.67)          | 8 (3.33)    |
| Total            | 221 (92.1)         | 19 (7.9)          | 240 (100)   |

*Sites with one isolate each (bile, bone, secretions, cervical fluid, pleural fluid, rectal fluid, sputum).

### Table 2. Susceptibility profile of enterococcal isolates, according to the species

| Antibiotics       | S n (%) | I n (%) | R n (%) | Total n (%) |
|-------------------|---------|---------|---------|-------------|
| Ampicillin        | 218 (98.6) | 3 (1.4) | -       | 221 (92.1) |
| Gentamicin        | 176 (79.6) | -       | 45 (20.4) | 19 (7.9)  |
| Ciprofloxacin     | 118 (53.4) | 67 (30.3) | 36 (16.3) | 203 (84.58) |
| Nitrofurantoin    | 196 (99.5) | 1 (0.5) | -       | 203 (84.58) |
| Vancomycin        | 213 (96.4) | 8 (3.6) | -       | 221 (92.1) |
| Linezolid         | 204 (92.3) | 4 (1.8) | 13 (5.9) | 221 (92.1) |
| Quinupristin/dalfopristin | NT | NT | NT | 221 (92.1) |

S, sensitive; I, intermediate; R, resistant; NT, not tested.
Table 3. Presence of virulence factors in isolates of E. faecalis and E. faecium recovered from different anatomical sites

| Species       | Site          | n (%)    | gelE   | gelatinase | esp   | agg   | Biofilm |
|---------------|---------------|----------|--------|------------|-------|-------|---------|
| E. faecalis   | Invasive      | 24 (10.9)| 20 (83.3)| 8 (33.3)  | 14 (58.3)| 16 (66.7)| 20 (83.3)|
|               | Noninvasive   | 197 (89.1)| 154 (78.2)| 66 (33.5) | 141 (71.6)| 124 (62.9)| 173 (87.8)|
|               | Total         | 221 (100)| 174 (78.7)| 74 (33.5) | 155 (70.1)| 140 (63.4)| 193 (87.3)|
| E. faecium    | Invasive      | 13 (68.4)| 2 (15.4) | 1 (7.7)   | 8 (61.5) | -     | 1 (7.7)  |
|               | Noninvasive   | 6 (31.6) | -       | -         | 5 (83.3) | -     | 1 (16.7) |
|               | Total         | 19 (100)| 2 (10.5) | 1 (5.3)   | 13 (68.4)| -     | 2 (10.5) |

*Association between biofilm formation and invasive/noninvasive sites (P < 0.0001).

Table 4. Association between the presence of virulence factors and biofilm formation by chi-square test

| Virulence factors | Biofilm n (%) | P value |
|-------------------|---------------|---------|
|                   | Positive      | Negative|
| gelE<sup>+</sup>  | 150 (62.5)    | 26 (10.8)|< 0.009  |
| gelE<sup>-</sup>  | 45 (18.8)     | 19 (7.9) |         |
| esp<sup>+</sup>   | 147 (61.25)   | 21 (8.75)|< 0.001  |
| esp<sup>-</sup>   | 48 (20)       | 24 (10)  |         |
| agg<sup>+</sup>   | 124 (51.7)    | 16 (6.6) | 0.001   |
| agg<sup>-</sup>   | 71 (29.6)     | 29 (12.1)|         |

MgCl<sub>2</sub>, 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), and 0.625 U of Taq DNA polymerase. Amplifications were performed in a Life Pro thermocycler (Bioer) using 54 °C as the annealing temperature. The presence of virulence genes, esp (5'-TTGGCTAATGC TATGGCCCA GA-3') and gelE (5'-ACCCCGGTATC TTGGATTCCG CA-3'), gelE (5'-ACCCCGGTATC TTGGATTCCG CA-3') and gelE (5'-ACCCCGGTATC TTGGATTCCG CA-3') and agg (5'-AAGAAAAAGA AGTAGGCAAA C-3' and 5'-AACGGCAAG ACAAGTAAAT A-3') were investigated by PCR as previously described<sup>38,39</sup> with the following adaptations. The annealing temperature was 54 °C for esp and 56 °C for gelE and agg. Positive control strains Enterococcus faecalis ATCC 29212 and Enterococcus faecium SS1274 were used. Gelatinase production was phenotypically determined according to Marra et al.,<sup>40</sup> using BHI (Brain Heart Infusion) with 4% gelatin.

Susceptibility tests were performed according to CLSI (2013),<sup>41</sup> Ampicillin (10 μg), ciprofloxacin (5 μg), gentamicin (120 μg), linezolid (30 μg), and quinupristin–dalfopristin (15 μg) were tested by disk diffusion; strains from urinary sites were also tested for nitrofurantoin (300 μg). The Minimum Inhibitory Concentration (MIC) was determined for vancomycin by the broth microdilution method (BMD).<sup>42</sup> MDR isolates were defined as presenting resistance to three or more different antimicrobial classes.<sup>42</sup>

Biofilm formation was tested on polystyrene microtiter plates, and the optical density results were interpreted as described previously.<sup>43</sup> First, 180 μL of trypticase soya broth (Becton Dickinson) supplemented with 1.5% glucose was added to each well of a sterile 96-well polystyrene microtiter plate, and then 20 μL of bacterial suspension was added. The plates were incubated for 24 h at 35 ± 2 °C under static conditions. After incubation and broth removal, wells were washed 3 times with sterile saline, and the adherent bacteria were fixed with methanol for 30 min. Adherent bacteria were stained with 0.5% crystal violet for 15 min, and biofilm was eluted with ethanol for 30 min. The absorbance was measured at 492 nm using an Expert Plus microtiter plate reader (ASYS Hitech). Staphylococcus epidermidis ATCC 35984 was the positive control strain.

All analyses were performed with SPSS version 19.0 (USA). A chi-square test (or Fisher exact test, when appropriate) was performed, and P < 0.05 was considered statistically significant.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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