Virulence Gene’s Relationship With Biofilm Formation and Detection of \(aac\ (6')/aph\ (2")\) in Enterococcus faecalis Isolated From Patients With Urinary Tract Infection

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**Background:** Enterococcus faecalis is part of the human gastrointestinal tract normal flora, which is considered as a major cause of nosocomial infections. One of the important virulence factors of enterococci is their ability to biofilms formation. The presence of biofilm formation ability may increase their ability to colonize in patients and remain in infection sites. Biofilms are bacterial populations that are found as surface-attached aggregates enclosed in an extracellular matrix. Bacteria with biofilm forming ability are thought to be more resistant to antibiotics than the corresponding free-living bacteria, thus causing the problem of antimicrobial resistance. Furthermore, some studies report that over 65% of hospital-acquired infections occur due to biofilm producing ability of the infecting organisms (4). Several virulence factors such as enterococcal surface protein (ESP), cytolsin (Cyl), aggregation substances (Agg) and gelatinase (Gel) have been described in E. faecalis.

**Objectives:** We investigated the potential relationships between biofilm formation and prevalence of virulence genes (asa1, esp, cylA, and gelE/sprE), and antimicrobial resistance genes \([aac\ (6')/aph\ (2")]\) in Enterococcus faecalis isolated from patients with urinary tract infection.

**Patients and Methods:** In this survey 95 E. faecalis isolates from patients with urinary tract infections staying at Shahid Beheshti hospital in Kashan, Iran, between 2007 and 2008 were studied. We analyzed the prevalence of genes encoding virulence factors (asa1, esp, cylA and gelE/sprE), and antimicrobial resistance genes \([aac\ (6')/aph\ (2")]\) by PCR. In addition, the production of biofilm and extracellular enzymes, hemolysin (Hln) and Gelatinase were examined.

**Results:** The asa1, [aac\ (6')/aph\ (2")], esp, cylA, and gelE/sprE were detected in 94.7%, 68.4%, 61.1%, 50.5% and 21.1% of E. faecalis isolates, respectively. The hemolysin production and gelatinase activity were seen in 44.2% and 20% of isolates, respectively. 16.8% of E. faecalis isolates showed strong and 81.2% exhibited weak biofilm formation. The percentages of genes encoding virulence factors in E. faecalis which had the ability of strong biofilm formation were as follows: gelE/sprE 25%, esp22.4%, \([aac\ (6')/aph\ (2")]\) 18.5%, asa1 16.7% and cylA 14.6%. The presence of both aac \((6')/aph\ (2")\) and esp positive act as a risk factor for biofilm formation (P value < 0.001).

**Conclusions:** There was a significant relationship between biofilm formation and possession of esp and aac \((6')/aph\ (2")\) genes. There was no evidence between biofilm formation and presence of any other gene. Enterococcal infections associated with biofilm formation have been a serious problem in recent years.

**Implication for health policy/practice/research/medical education:** Enterococcal infections associated with biofilm formation have been a serious problem in recent years. We investigated the potential relationships between biofilm formation and prevalence of virulence genes (asa1, esp, cylA, and gelE/sprE), and antimicrobial resistance genes \([aac\ (6')/aph\ (2")]\) in E. faecalis isolated from patients with urinary tract infection.

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the virulence of human enterococcal infections (2, 7).
Cytolys in genes are found on a plasmid or are integrat-
ed into the bacterial chromosome (8). Another virulence
gene is the chromosomal gel E which encodes Gel. Gela-
tinase is an extracellular zinc endopeptidase that hydro-
lyzes collagen, gelatin, and small peptide chains (8). Ge-
latinase producing strains of E. faecalis have been shown
to contribute to the severity of endocarditis in an animal
model (8). Hemolysin is a cytolytic protein capable of ly-
sis of human, horse and rabbit erythrocytes. Hemolysin
producing strains are found to be associated with in-
creased severity of infections (6).

The aggregation substance, expressed by asat, which is
located on a plasmid, is a pheromone-inducible protein
that enables the conjugative transfer of sex pheromone
gene-containing plasmids through the clumping of one
Enterococcus to another. As a virulence factor, the ag-
gragation substance enhances bacterial adherence to re-
nal tubular cells and endocardial cells (8). The enzymes
aac (6') and aph (2") inactivate the most frequently used
aminoglycosides in clinical practice. These modifying
enzymes include aminoglycoside acetyltransferase (aac)
and aminoglycoside phosphoryltransferase (aph).

2. Objectives

In this study we investigated the relationships between
biofilm formation and harboring virulence genes (asa1,
estp, cyIA, gelE, sprE) and the resistance-modifying enzyme
genes, aac (6') and aph (2")

3. Patients and Methods

The E. faecalis isolates used in this study were isolated
from hospitalized patients with complicated UTI stay-
ing at Shahid Beheshti hospital in Kashan, Iran, between
2007 and 2008. A total of 600 patients, which confirmed
pyuria (WBC ≥ 5/ hpf) with ≥10⁴ CFU/ml in their uri-
nary culture were selected for this study. Urine samples
were cultured on blood agar and Mac Conkey agar and
incubated at 37°C for 24 hours. Positive cultures were
confirmed by gram staining and standard biochemical
tests such as: catalase, PYR and growing on media con-
taining 6.5% salt. Biofilm formation was examined as de-
scribed previously (2). The culture was diluted to 1:100 in
medium and 200µl of this cell suspension was used to in-
oculate sterile flat-bottomed 96-well polystyrene microti-
tre plates (BD Biosciences). After 24 h at 37°C, wells were
gently washed three times with 300µl of distilled water,
dried in an inverted position, and stained with 300µl of
3% crystal violet solution in water for 45min. After stain-
ing, plates were washed 3 times with distilled water.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5’ to 3’)</th>
<th>Cycle</th>
<th>Initial Denaturation</th>
<th>Cycling</th>
<th>Final Extension</th>
<th>Length (bp)</th>
</tr>
</thead>
</table>
| E. faecalis | F:5’ATCAAGTACAGTGGTATTAGCTTTATTAG-3’
R:5’ACGATTCAAGCTAAGCTGATC-3’ | 30 | 5 min, 94°C | 1 min, 9°C; 1 min, 72°C | 10 min, 72°C | 941 |
| asat       | F:5’GATTCTCTGATGTTGTTGAAAC-3’
R:5’GGTGGTCAAATGATTAATG-3’ | 35 | 2 min, 95°C | 1 min, 95°C; 1 min, 46°C; 1 min, 72°C | 10 min, 72°C | 380 |
| esp        | F:5’TGATGCTAATGTCAGTCAGGAAC-3’
R:5’GCGTCAACACTTGCATTGCCGA-3’ | 35 | 2 min, 95°C | 1 min, 95°C; 1 min, 46°C; 1 min, 72°C | 10 min, 72°C | 955 |
| cyIA       | F:5’GGGGATTGAGTAGTCGCTCATCC-3’
R:5’GCACCGACGGTAATACAGCTCTAGTCTCC-3’ | 35 | 2 min, 95°C | 1 min, 95°C; 1 min, 46°C; 1 min, 72°C | 10 min, 72°C | 432 |
| gelE/sprE  | F:5’ATGAGGGGATATGCGTGCTTCC-3’
R:5’CTGCTGCGACAGCGGATA-3’ | 35 | 2 min, 94°C | 30 sec, 94°C; 30 sec, 48°C; 3 min, 72°C | 6 min, 72°C | 2428 |
| Acc(6’)/aph(2’) | F:5’ACCAAGCCGAAATACACAGATC-3’
R:5’CAGTATACATAACCACACCAC-3’ | 5 min, 94°C | 1 min, 94°C; 1 min, 55°C; 1 min, 72°C | 10 min, 72°C | 220 |
Bacterial DNA was extracted by a boiling method. Primers reported by Kariyama et al., were used for amplification of the genes of *E. faecalis* (9). For detection of *esp*, *gelE/sprE*, *cylA*, *asa1*, *acc(6')/aph(2')* genes in *E. faecalis* strain, we used the primers reported by Seno et al. (2). The primers used in this study for detecting *E. faecalis*, *esp*, *gelE/sprE*, *cylA*, *asa1*, *acc(6')/aph(2')* genes are summarized in Table 1.

Total cellular DNA was prepared as follows: *E. faecalis* culture grown overnight on Blood agar or TSB was transferred to a 250µl Eppendorf vial, then centrifuged at 10000 rpm for 10 min. The supernatant was discarded and 250µl of distilled water was added to the pellet, and resulting solution was heated for 15 min at 100 °C, and centrifuged at 10000 rpm for 10 min. The supernatant was transferred to a new microtube, and stored at -20 °C. Primers were added to the reaction mixture; the 25µl reaction volume contained 0.2mM of each dNTP (dATP, dCTP, dGTP, dTTP), 10 mM Tris-HCL (PH 8.3), 50 mM KCl, 1.5 mM Mgcl 2 and1U Top DNA polymerase, PCR conditions are shown in Table 1. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel. After electrophoresis, gel was stained with ethidium bromide and photographed under a UV trans-illuminator (INGENIUS, SYNGENE). A 100-bp DNA (Bio NEER, Korea) ladder was used as a molecular size marker. The data were compared using the chi-square test or Fisher's exact test with SPSS version 16 for Windows (SPSS, Chicago, IL). A P value of < 0.05 was considered significant.

### 4. Results

From the total of 114 isolated enterococcus strains 95 (83.3%) were recognized as *E. faecalis* and 19 (16.7%) of them were *E. faecium*. The mean age for the studied population was 39.20 years (± 20.06), which ranged between 1 and 91 years. *E. faecalis* isolates were recovered from 95 patients including 48 men (50.5%) and 47 women (49.5%). By using PCR, *asa1*, *acc(6')/aph(2')*, *esp*, *cylA*, *gelE/sprE* genes were found in 90 (94.7%), 65 (68.4%), 58 (61.1%), 48 (50.5%) and 20 (21.1%) of the isolates, respectively. The findings indicate that 58.3% of isolates (28 out of 48) possessing the *cylA* gene, produced hemolysin (P value < 0.005). Biofilm formation assay showed that 16.8% (16 out of 95) of *E. faecalis* isolates contribute to a strong (OD570 ≥ 0.2), while 83.2% (79 out of 95) exhibit weak (OD570 < 0.2) biofilm formation. The relationship between biofilm-forming capacities and virulence genes are summarized in Table 2.

### Table 2. Relationship Between Strong Biofilm-Forming and Virulence Factors in 95 *E. faecalis* Isolates

<table>
<thead>
<tr>
<th>Virulence Factors</th>
<th>Number of Isolates</th>
<th>Strong Biofilm Formation No.16</th>
<th>Weak Biofilm Formation No. 79</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acc (6')/aph (2')</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>65</td>
<td>12</td>
<td>53</td>
<td>0.381</td>
</tr>
<tr>
<td>Negative</td>
<td>30</td>
<td>4</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td><strong>asa 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>90</td>
<td>15</td>
<td>75</td>
<td>0.611</td>
</tr>
<tr>
<td>Negative</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><strong>esp</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>58</td>
<td>13</td>
<td>45</td>
<td>0.059</td>
</tr>
<tr>
<td>Negative</td>
<td>37</td>
<td>3</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td><strong>cylA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>48</td>
<td>7</td>
<td>41</td>
<td>0.375</td>
</tr>
<tr>
<td>Negative</td>
<td>47</td>
<td>9</td>
<td>38</td>
<td></td>
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<tr>
<td><strong>gelE/sprE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>20</td>
<td>5</td>
<td>15</td>
<td>0.218</td>
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<tr>
<td>Negative</td>
<td>75</td>
<td>11</td>
<td>64</td>
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<tr>
<td><strong>Hemolysing</strong></td>
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<td></td>
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<td></td>
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<tr>
<td>Hemolysing producing</td>
<td>42</td>
<td>4</td>
<td>38</td>
<td>0.076</td>
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<tr>
<td>Hemolysing non producing</td>
<td>53</td>
<td>12</td>
<td>41</td>
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<tr>
<td><strong>Gelatinase</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Gelatinase producing</td>
<td>19</td>
<td>3</td>
<td>16</td>
<td>0.599</td>
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<tr>
<td>Gelatinase non producing</td>
<td>76</td>
<td>16</td>
<td>63</td>
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<tr>
<td><strong>Catheter</strong></td>
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<tr>
<td>Catheter-related</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>0.611</td>
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<tr>
<td>Catheter-unrelated</td>
<td>90</td>
<td>15</td>
<td>75</td>
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</table>
There were no statistically significant differences between asa1, acc (6')/aph (2''), esp, cylA, and gelE/sprE genes with application of catheter on biofilm formation. The occurrence of E. faecalis isolates possessing acc (6')/aph (2''), which inactivate all of the most frequently used aminoglycosides in clinical practice, were high in our study. The Percentage of positive virulence genes (asa1, esp, cylA and gelE/sprE) and acc (6')/aph (2'') isolates in 95 of the E. faecalis isolates are presented in Figure 1. Comparison of strong and weak biofilm-formation ability of the 95 isolates of E. faecalis based on the presence of virulence genes (asa1, esp, cylA and gelE/sprE) and acc (6')/aph (2'') are shown in Figure 2.

5. Discussion

Our data indicated that E. faecalis possessing acc (6')/aph (2''), esp and asa1 were predominant. The study results confirm the presence of a statistically significant relationship between esp and acc (6')/aph (2'') genes (P value < 0.001), but the relationship between the presence of esp and acc (6')/aph (2'') other virulence genes was not significant. There was a significant relationship between the presence of esp and acc (6')/aph (2'') genes and biofilm formation. The presence of both acc (6')/aph (2'') and esp positive act as a risk factor for biofilm formation (P value < 0.001).

The odds ratio (OR) was 51.6 (95% confidence interval [CI], 10.4 to 255.11), which suggests a significant influence of acc (6')/aph (2'') and esp positive on biofilm formation. With regard to biofilm formation, there have been different reports on the role of Esp and Gel. A strong association between the presence of Esp and the ability of enterococcal strain to form biofilms in vitro has been described (10). These findings are in agreement with the results of Seno et al. in their article the biofilm forming abilities were significantly higher in esp-positive isolates.

Kristich et al. demonstrated that in vitro biofilm formation occurs, not only in the absence of esp, but also in the lack of the entire pathogenicity island that harbors the esp coding sequence (12). Mohamed et al. reported that esp was not required to form biofilm (13). They showed that all 74 esp-positive isolates produced biofilm, and 77 of 89 esp-negative isolates also produced biofilm (13). The other studies confirmed that this protein is a predisposing or an important factor for biofilm formation, but is not completely necessary (10, 14). The capacity of biofilm formation is not restricted to the esp positive isolates. Hammerum et al., reported gentamicin-resistant E. faecalis in esp-positive isolates from human, which were encoded by acc (6')-Ie-aph (2'')-Ia from patients in a Danish hospitals (15).

In this study, there were no statistically significant differences between biofilm-forming capacities and the presence of catheter. Biofilm formation by E. faecalis occurs in response to any bacterial factors that is related to the extracellular matrix of the host. This study showed a statistically significant relationship between esp and acc (6')/aph (2'') genes. Our study indicates that E. faecalis isolates that have both esp and acc (6')/aph (2'') genes form strong biofilms in the urinary tract. This study suggests that further investigation is necessary to better understand the relationship of virulence genes of this pathogen.
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References

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