Is there any association between antibiotic resistance and virulence genes in Enterococcus isolates?

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Abstract

Objective: In this study, we aim to determine the frequency of antibiotic resistance and five virulence genes in Enterococcus species and the relationship between antibiotic resistance and virulence genes.

Material and Methods: A total of 86 Enterococcus strains isolated from inpatients between 2015 and 2016 were included. Identification and antibiotic susceptibilities of strains were determined using a BD Phoenix fully automated system. The presence of virulence-associated genes (esp, gel E, asa1, hyl, and cyl) were investigated by using PCR method.

Results: Of the 86 Enterococcus strains, 53 (61.6%) and 33 (38.4%) were Enterococcus faecium and Enterococcus faecalis, respectively. Vancomycin and high-level gentamicin resistance (HLGR) in E. faecalis strains were 0.6% and 60.6%, respectively. Furthermore, 52 of the 53 E. faecium strains were both vancomycin-resistant and HLGR. The frequency of esp, gel E, asa1, cyl, and hyl was 91.9%, 60.5%, 54.7%, 43%, and 26.7%, respectively. The asa 1, cyl, and gel E genes were detected at high frequencies in vancomycin-susceptible and non-HLGR strains, whereas hyl gene was detected at high frequencies in vancomycin-resistant and HLGR strains.

Conclusion: Virulence genes were more frequent in vancomycin-susceptible and non-HLGR Enterococcus strains than in the resistant strains. Although infections caused by multidrug-resistant strains are difficult to treat, it should be considered that susceptible strains have more virulence genes. This may reduce the in vivo efficacy of drugs and lead to treatment failures. Therefore, in addition to the in vitro susceptibilities of drugs, clinical efficacy should be monitored.

Key words: Antibiotic resistance, Enterococcus faecalis, Enterococcus faecium, virulence gene.

Introduction

Enterococci are Gram–positive, catalase-negative, no spore-forming facultative anaerobic bacteria. They can survive in a wide range of temperature (5°C–65°C) and pH (4.5–10) as well as in the presence of 6.5% NaCl (1,2). Enterococci are part of the human gut microbiota. After gastrointestinal colonization, they can cause blood-stream infections, endocarditis, and intra-abdominal and pelvic infections in critically-ill patients (2,3) In recently, enterococci are the second most common cause of nosocomial wound and urinary tract infections and the third most common cause of nosocomial bacteremia. (1,4,5) The Enterococcus faecalis and E. faecium are most prevalent species in human diseases (1-4).

Enterococci are intrinsically resistant to cephalosporins, sulfonamides, and low concentrations of aminoglycosides (6-8). In addition, acquired vancomycin and high-level aminoglycoside resistance limit the treatment options.

However, enterococci have various virulence factors such as enterococcus surface protein and aggregation substance that enable adhesion to host, colonization, and biofilm formation (1-3,9). Furthermore, hyaluronidase, cytolysin, and gelatinase enzymes secreted by enterococci contribute to invasion by causing damage to the host tissue (4,8,9).

Some of the genes encoding virulence factors are chromosomal origin, whereas others are subsequently acquired via plasmids (3,9). In this study, we aim to determine the frequency of antibiotic resistance and virulence genes in Enterococcus species and the relationship between antibiotic resistance and virulence factors.
Material and Methods

We included 86 Enterococcus strains isolated from rectal swabs and clinical samples of inpatients at Karabük Training and Research Hospital between January 2015 and December 2016. The strains were stored in tryptic soy broth containing 10% glycerol at −25°C until molecular tests were performed. Only one strain from each patient was included. The ethical approval was obtained from the Non-interventional Clinical Research Ethics Board of Karabük University (2016 13/3).

Identification and antimicrobial susceptibility testing: Clinical samples sent to the microbiology laboratory were inoculated on Columbia agar supplemented with 5% sheep blood, eosin-methylene blue (EMB) agar, and chocolate agar, whereas rectal swabs were cultured only on Enterococcus agar. [all, Becton Dickinson (BD) and Company Franklin Lakes, NJ, USA]. Blood samples were collected in BD BACTEC Plus vials and incubated in Bactec FX 40 (BD, MD, USA) fully automated blood culture system for seven days. The vials that were positive for bacterial growth were cultured on blood agar, EMB agar, and chocolate agar. After incubation for 24–48 h, Gram staining was performed on calsate-negative, pyrrolidinyl aminopeptidase-positive colonies. Colonies that were found to be Gram-positive cocci on microscopic examination were further analyzed for identification and antibiotic susceptibility using a BD Phoenix™ (Becton Dickinson and Company BD, Sparks, MD, USA) fully automated system. Vancomycin resistance was also confirmed using gradient minimum inhibitory concentration (MIC) method with E-test strips (bioMérieux, Marcy-l’Etoile, France). Antibiotic susceptibility test results were evaluated according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (10). Enterococcus faecalis ATCC 29212 was used as the quality control strain.

PCR detection of virulence genes: The stock cultures of Enterococcus strains were inoculated on 5% sheep blood agar. The pure bacterial culture was obtained after 24 hours of incubation. PCR analysis of each virulence gene was separately performed. The genomic DNA was isolated from pure bacterial cultures using a GF Medical gel, using an EV231 (Consort, Belgium) device.

The amplified products were separated by electrophoresis for 1 h at 100 V in a 1.5 % agarose gel, on 1.5% agarose gel, using an EV231 (Consort, Belgium) device.

Ethidium bromide-stained products were imaged using Syngene™ IG3 (Syngene, Cambridge, UK) gel DOC instrument under UV light. A 100-bp DNA ladder (New England Biolab, Ipswich, MA, USA) was used to compare the band size of each gene. Enterococcus faecalis MMH 594, and Enterococcus faecium C68 were used as positive control strains [E. faecalis MMH 594 (esp, cyl, asal1 and gel E (+), E. faecium C68 hyl (+)].

Statistical analysis: The data were analyzed using the Minitab 17 (Minitab, Inc., PA, USA) statistical software program. The Anderson Darling test was performed to determine whether the data were normally distributed. Descriptive statistics were shown as numbers and percentages. The correlation between virulence genes and antibiotic resistance of strains was calculated using Chi-square test. A P-value ≤ .05 was considered statistically significant.

Results

The 86 Enterococcus species were obtained from rectal swab (n=41), blood (n=22), urine (n=19) and wound (n=4) samples. The 53 (61.6%) strains were E. faecium and, 33 (38.4%) strains were E. faecalis. Moreover, 54 (62.8%) and 72 (83.7%) strains were resistant to vancomycin and high levels of gentamicin, respectively.

The frequency of vancomycin resistance and HLGR in E. faecalis were 0.6% (2/33) and 60.6% (20/33), respectively; 52 (98.1%) of 53 E. faecium were vancomycin-resistant and HLGR. Of the 86 strains, 91.9% (n=79) carried esp gene. This was followed by 60.5% gel E (n=52), 54.7% asal1 (n=47), 43% cyl (n=37), and 26.7% hyl (n=23).

The distribution of virulence genes in Enterococcus species has been shown in Table 2. The esp gene was detected in >90% strains in both species (P = .88).

However, asal1, cyl, and gel E virulence genes were significantly more frequent in E. faecalis strains than in E. faecium strains (P < .001). Furthermore, 90.9% of E. faecalis strains carried asal1, whereas this rate was 32% for E. faecium strains (P < .001). Similarly, gel E was more frequent in E. faecalis strains, and the rates were 84.8% and 45.3% in E. faecalis and E. faecium, respectively. In addition, cyl gene was detected in 78.8% of E. faecalis strains and 20.8% of E. faecium strains (P < .001). In contrast, hyl gene was significantly more frequent in E. faecium strains than in E. faecalis strains (39.6% vs. 6%, P < .001).

The distribution of virulence genes in HLGR and non-HLGR strains has been shown in Table 4. The hyl gene was significantly more frequent in HLGR strains, whereas asal1, cyl, and gel E genes were significantly more frequent in non-HLGR strains.
Table 1. Primer sequences used for the amplification of the target genes.

<table>
<thead>
<tr>
<th>Virulence factor</th>
<th>Target gene</th>
<th>Primer sequences (5’-3’)</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggregation substance</td>
<td>asa1</td>
<td>GCACGCTATTACGAAACTATGA</td>
<td>375</td>
<td>[11]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TAAGAAAGAACATCACCCAGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelatinase</td>
<td>gel E</td>
<td>CGA AGT TGG AAA AGG AGG C</td>
<td>372</td>
<td>[13]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGT GAA GAA GTT ACT CTG A</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AATTGATCTTTACAGTCTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>hyl</td>
<td>CCCGAGCAGACATGAAATGGCG</td>
<td>605</td>
<td>[12]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGCATTGGGGATTGATAGGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytolysin</td>
<td>cyl</td>
<td>ACTCGGGATTGATAGGC</td>
<td>688</td>
<td>[11]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GCTGCTAAAGCCTGCGTT</td>
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<td></td>
</tr>
</tbody>
</table>

Table 2: Distribution of virulence genes among *Enterococcus* species (n %)

<table>
<thead>
<tr>
<th>Virulence gene</th>
<th><em>E. faecalis</em> (n=33)</th>
<th><em>E. faecium</em> (n=53)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>asa1</td>
<td>30 (90.9)</td>
<td>17 (32.1)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>cyl</td>
<td>26 (78.8)</td>
<td>11 (20.8)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>esp</td>
<td>30 (90.9)</td>
<td>49 (92.5)</td>
<td>.88</td>
</tr>
<tr>
<td>gel E</td>
<td>28 (84.8)</td>
<td>24 (45.3)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>hyl</td>
<td>2(6.0)</td>
<td>21 (39.6)</td>
<td>&lt; .001</td>
</tr>
</tbody>
</table>

Table 3: Distribution of virulence genes among *VRE and **VSE enterococcal strains (n %)

<table>
<thead>
<tr>
<th>Virulence gene</th>
<th>VRE (n=54)</th>
<th>VSE (n=32)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>asa1</td>
<td>17 (32.1)</td>
<td>30 (90.9)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>cyl</td>
<td>11 (20.8)</td>
<td>26 (78.8)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>esp</td>
<td>50 (94.3)</td>
<td>29 (87.8)</td>
<td>.74</td>
</tr>
<tr>
<td>gel E</td>
<td>24 (45.3)</td>
<td>28 (84.8)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>hyl</td>
<td>21 (39.6)</td>
<td>2 (6.0)</td>
<td>&lt; .001</td>
</tr>
</tbody>
</table>


Table 4: Distribution of virulence genes among *HLGR and non HLGR enterococcal strains (n %)

<table>
<thead>
<tr>
<th>Virulence gene</th>
<th>HLGR (n=72)</th>
<th>non HLGR (n=14)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>asa1</td>
<td>34 (47.2)</td>
<td>13 (91.6)</td>
<td>.002</td>
</tr>
<tr>
<td>cyl</td>
<td>24 (33.3)</td>
<td>13 (91.6)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>esp</td>
<td>66 (91.7)</td>
<td>13 (91.6)</td>
<td>.88</td>
</tr>
<tr>
<td>gel E</td>
<td>40 (55.5)</td>
<td>12 (85.7)</td>
<td>.02</td>
</tr>
<tr>
<td>hyl</td>
<td>22 (30.6)</td>
<td>1 (7.1)</td>
<td>.04</td>
</tr>
</tbody>
</table>

*HLGR: High level gentamicin resistant

Figure 1. a) Agarose gel electrophoresis images of *gel E* (372 bp) in *Enterococcus* strains. Note: Nc: Negative control (ultrapure H2O). Pc: Positive control (*E. faecalis* MMH 594) M:100 bp DNA Ladder (New England Biolabs, USA) b) Agarose gel electrophoresis images of *hyl* (605 bp) in *Enterococcus* strains. Note: Nc: Negative control (ultrapure H2O). Pc: Positive control (*E. faecium* C68) M:100 bp DNA Ladder (Product code :N3231S, New England Biolabs, USA)
Discussion

Although enterococci are members of the normal human intestinal flora, they cause severe infections such as sepsis, endocarditis, and intra-abdominal abscess following gastrointestinal colonization in patients hospitalized for a long time, exposed to invasive intervention, and those using broad-spectrum antibiotics (2,3,6,7). Enterococcus faecalis is the most frequently isolated species in clinical specimens, and a high level of antibiotic resistance is common in E. faecium strains (2,7,14).

In the present study, 53 (61.6%) and 33 (38.4%) of the 86 Enterococcus strains were identified as E. faecium and E. faecalis, respectively. The majority of clinical isolates of E. faecalis and E. faecium strains were vancomycin-resistant that were isolated from the rectal swabs (n = 41). Vancomycin resistance and HLGR were 62.7% and 83.7% in E. faecium strains and 0.6% and 60.6% in E. faecalis strains, respectively. Consistent with our results, several studies from Turkey have also reported higher rates of vancomycin resistance and HLGR in E. faecium strains (15-17).

The esp was the most frequently virulence gene encoding the enterococcal surface protein. In 1999, the enterococcal surface protein was first identified in gentamicin resistant E. faecalis species (18). The esp gene secreted from the pathogenicity islands of both E. faecalis and E. faecium (1,3,18). It is associated with adhesion, invasion, and evasion (9,19). In addition, it contributes to biofilm formation. Biofilm formation is closely-associated with the development of endocarditis and urinary tract infection (1,9,19).

In the present study, esp was detected in 90.9% and 92.5% of E. faecalis and E. faecium strains, respectively (P = 0.8). Studies conducted in Turkey have reported esp frequencies of 25.6%–87% (15,17,20,22). The high frequency of esp, might be due to regional differences and the collection date of the strains. In enterococci isolates, the acquisition of virulence genes increases in time parallel to the increase in antibiotic resistance. The prevalence of esp gene reported in other countries were 46.6% in Iran, 76% in Brazil, 81.5% in Australia, and 100% in Trinidad (23-26). Vankercikoven et al. reported that the frequency of esp gene was 65% in 271 E. faecium isolates collected from European countries (11). Udo et al. reported a lower rate (31.5%) in 466 E. faecalis isolates collected from eight hospitals in Kuwait (19). Oancea et al. reported that esp gene is transferred among the enterococcal species via the conjugative plasmid in vitro (27). We detected esp gene in >90% strains in both species, supporting this finding.

In the present study, gel E gene was the second most common virulence gene (60.5%). The gel E is of chromosomal origin and encodes gelatinase, also known as a metalloprotease that hydrolyze small peptides such as collagen, elastin, and casein. Thus, it provides nutrients to the bacteria and contributes to invasion (1-3). In this study, the frequency of gel E gene was 84.8% in E. faecalis and 45.3% in E. faecium strains (P = 0.000). Similarly, Al Taib et al. detected gel E in 76.5% and 66.7% of E. faecalis and E. faecium strains, respectively, isolated in Malaysia (13). The frequency of gel E gene is between 0% and 22.8% in studies conducted in Turkey (15-17,21). However, a higher frequency (0%–78%) is reported in studies around the world (13,19,24,25). The gel E gene among representative strains is shown in Figure 1.

In the current study, asa1 gene, encoding the aggregation substance, was the third most common virulence gene with a frequency of 54.7%. Aggregation substance is a glycoprotein expressed on the surface of E. faecalis. It provides contact between cells during adherence to eukaryotic cells and conjugation. It increases the adhesiveness of the enterococcal surface and enables escape from the immune system by preventing the fusion of lysosomal vesicle and phagosome (3,11,19).

The asa1 gene was found to be significantly more frequent in E. faecalis strains than in E. faecium strains (90.9% vs. 32.1%, respectively, P = 0.000). Similarly, Baylan et al. (20) and Coşkun (16) from Turkey also detected asa1 gene in E. faecalis strains at significantly higher rates. In a study conducted in Iran, Hasani et al. reported the prevalence of asa1 as 92.4% in E. faecalis and 7.6% in E. faecium (28). In the present study, asa1 was significantly more frequent in vancomycin-susceptible strains than vancomycin-resistant strains (90.9% vs. 32%, respectively, P = 0.000). This can be attributed to the fact that vancomycin-susceptible strains are mostly E. faecalis and vancomycin-resistant strains are mostly E. faecium. Mete et al. did not detect asa1 in vancomycin-resistant strains (15). The asa1 was significantly more frequent in non-HLGR strains than HLGR strains (91.6% vs. 47.2%, respectively, P = 0.002).

In contrast, Al Hasan et al. found asa1 at similar rates in HLGR (54.1%) and non-HLGR strains (52.8%) in 220 enterococcal isolates in Iran (28). The frequency of asa1 in enterococcal strains of human origin is between 11.2% and 45% in Turkey (15,17,20). In other studies, asa1 frequency was reported to be 38% in Brazil, 54.3% in Iran, 79% in Sweden, and 100% in China (4,24,29,30). In a study conducted in Australia, Worth et al. did not detect asa1 gene in vancomycin-resistant E. faecium strains (25).

Cytolysin is a toxin whose expression is under the control of eight genes defined as cyl (15,19). Cytolysin is hemolysin in the human, rabbit, and horse erythrocytes. However, it is not active against to sheep erythrocytes (1,3,15). Moreover, it is a bacteriocin that shows bactericidal effect against many Gram-positive bacteria. Cyl has a lytic effect in retinal and intestinal cells as well as macrophages and neutrophils (1,3). The cyl genes are transmitted via plasmids (3). However, cyl genes can also be found in the pathogenicity islands integrated into the bacterial chromosome (3,15). In the present study, the frequency of cyl gene was 78.8% in E. faecalis strains and only 20.8% in E. faecium strains (P = 0.000). Additionally, the frequency of cyl gene was extremely high in vancomycin-susceptible and non-HLGR strains. The frequency of cyl gene was reported to be 6.9%–33.2% in Turkey (15-17), 13% in Sweden (29), and 30.4% in Iran (4).
The *hyl* gene encoding the hyaluronidase enzyme is found especially in *E. faecium* species and causes tissue damage by degrading the hyaluronic acid (3). Furthermore, disaccharides formed as a result of hyaluronic acid degradation may be a source of food in bacteria. (3,12,19) In the present study, we detected *hyl* gene at a frequency of 26.7%. The *hyl* gene among representative strains has been shown in Figure 1. This frequency was 39.6% in *E. faecium* strains and 6% in *E. faecalis* strains (P = 0.000). Coşkun (16) and Çopur et al. (17) reported the frequency of *hyl* gene as 8.7% and 12.9% in Turkey. On the other hand, Gözalan et al. did not detect *hyl* gene in any of the 55 vancomycin-resistant *E. faecium* isolates (21). In other studies, *hyl* gene frequency was reported to be 2.4% in Australia (25), 27.5% in China (30), and 35.4% in Iran (12). Bilström et al. reported a frequency of 4% in 267 *E. faecium* isolates in Sweden (8).

There are some limitations of the study. The study was conducted at a single center and had a small sample size. Furthermore, vancomycin-resistance genes were not investigated using molecular methods.

**Conclusion**

We detected *esp* gene in >90% *Enterococcus* species. The *asa1*, *cyl*, and *gel E* genes were significantly more frequent in *E. faecalis* strains, whereas *hyl* gene was significantly more frequent in *E. faecium* strains. Vancomycin resistance and HLGR were lower in *E. faecalis* strains, but virulence genes (except *hyl*) were more common. All virulence genes (except *hyl*) studied in vancomycin-susceptible and non-HLGR strains were more frequent than those in vancomycin-resistant and HLGR strains. The limited of treatment options for multidrug-resistant enterococci infections is a great concern. Vancomycin-susceptible and non-HLGR strains have multiple virulence factors, which may reduce the *in vivo* efficacy of antibiotics, leading to treatment failures. Therefore, clinical efficacy as well as *in vitro* efficacy of antibiotics used in the treatment of enterococcal infections should be monitored.

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**Conflict of Interest:** The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Author’s Contributions:** NA, ET; Research concept and design, Research the literature, ET; Genetic analyses NA; preparation of the article, Revision of the article.

**References**


