DIFFERENCES IN GENOTYPE OF E. FAECALIS AND E. FAECIUM CLINICAL ISOLATES REGARDING CYTOLYSIN AND GELATINASE PRODUCTION IN BULGARIAN PATIENTS

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ABSTRACT
Purpose - compare the phenotype and genotype correlation of cytolysin and gelatinase production in clinical isolates Enterococcus spp. Materials and methods - 100 Enterococcus strains collected over a period of one year from inpatients of two Bulgarian university hospitals, were tested for phenotype production of cytolysin and gelatinase. Multiplex PCR was performed to screen the presence of gelE and cylA virulence genes. Results – 17% of the enterococcal isolates demonstrated only cytolysin production phenotypically. Gelatinase activity was found in 21% of the isolates. Only E. faecalis showed combined phenotypic production of cytolysin plus gelatinase (21%). Forty-five percent of the tested enterococci were identified negative for both hemolysin and gelatinase activity. GelE was the most prevalent virulent gene (48% of the isolates). CylA gene was present alone only in four non-invasive E. faecalis isolates. Twenty-six percent of the isolates possessed both cylA and gelE genes and 21% did not harbor any of the virulence factors genotypically. Conclusion - our results prove that it is appropriate to perform both phenotypic and genotypic analysis of the enterococci virulence profile in parallel in order to better characterize the strains, which in turn may serve to develop more effective methods to limit the spread of infections caused by these microorganisms.

Key words: Enterococcus spp., virulence factors, virulence genes

INTRODUCTION
Enterococcus are Gram-positive, catalase-negative, non-spore-forming lactic acid bacteria, that are common commensal organisms in the gastrointestinal tract of humans and animals. Moreover, they may cause community-acquired infections and have been reported to be one of the leading causes of nosocomial infections with an increasing mortality rate (1). Although many Enterococcus species can cause human infections (2), most frequently isolated species in up to 90% of enterococcal infections is Enterococcus faecalis (3), followed by Enterococcus faecium, which has shown an increase in prevalence in recent years (4). These microorganisms have been associated with urinary tract infections, bacteremia and sepsis, endocarditis, surgical wound infections and burn wound infections, cholecystitis, peritonitis, neonatal meningitis (2). The therapeutic options of infections caused by Enterococcus species are limited because of their intrinsically or acquired via transposons or plasmids resistance to many antibiotics, including to the glycopeptide Vancomycin (3). In recent years, there has been a rapid occurrence of multidrug resistant enterococci (5), which pose real therapeutic difficulties.

The numerous virulent determinants that Enterococcus possesses and are able to share among other species (2) helps this microorganism to survive in a very crude environment and are considered significantly important in exacerbating diseases caused by them. Therefore, the greater understanding of the virulence traits of circulating Enterococcus strains has become more and more important
and may help to understand better the pathogenesis of these microorganisms.

In this study, we compared the phenotype and genotype correlation of cytolysin and gelatinase production in clinical isolates *Enterococcus* spp.

**MATERIALS AND METHODS**

**Collection and identification of study isolates**

A total of 100 *Enterococcus* strains were collected over a period of one year from inpatients of University Hospital “Prof. Dr. Stoyan Kirkovich” AD, Stara Zagora, and University Hospital “St. George” EAD, Plovdiv, Bulgaria. All of the strains were from clinical relevant sites and from various specimens. Most of the enterococcal isolates were recovered from urine (n=45). Other specimens included wound secretions (n=31), blood (n=10), vaginal swabs (n=6), body fluids (n=5) and respiratory samples (n=3).

All the samples were inoculated onto blood agar plates (HiMedia Laboratories, Mumbai) and incubated 24 hours at 37°C. *Enterococcus* suspected colonies were identified by standard microbiological methods including Gram staining, catalase test, esculin hydrolysis (6). Species identification was performed according to the Facklam & Collins scheme (7) by a standard conventional biochemical test based on the carbohydrate fermentation of 1% sugar mannitol solution and arabinose, pyruvate hydrolysis (PYR test), arginine decarboxylation, growth of media containing potassium tellurite and via Vitek-MS automated systems (bioMérieux, France).

**Phenotypic detection of virulence factors**

All *Enterococcus* isolates were tested for the presence of virulence determinants cytolysin and gelatinase.

1. **Cytolysin production**

Cytolysin production was tested by a spot inoculation of a pure culture of the enterococci onto Todd-Hewitt agar, supplemented with 5% human blood and incubated 48 hours at 37°C. A presence of clear zone of β-hemolysis surrounding the colonies was considered as a positive result (8).

2. **Gelatinase production**

Gelatinase production was determined by a spot inoculation of the *Enterococcus* strains onto Todd-Hewitt agar containing 30 g gelatin per 1 liter. Plates were incubated overnight at 37°C. The appearance of a clear zone/turbid halo around the colonies after the plates were cooled for 2 hours at 4°C was considered to be a positive indication of gelatinase production (8).

**Genotypic detection of virulence genes**

For the Polymerase Chain Reaction (PCR) experiments, genomic DNA was extracted using an appropriate DNA extraction kit (GenneMATRIX Gram Plus & Yeast Genomic DNA Purification Kit, Poland), according to the manufacturer’s instructions and was stored at -80°C until use. The concentration and purity of the DNA samples were measured spectrophotometrically at 260 nm/280 nm using a GeneQuant 1300 spectrophotometer (GE Healthcare Life Sciences, Switzerland).

Multiplex PCR was performed to screen the presence of *gelE* and *cylA* (activator of cytolysin) virulence genes. The specific primers were used according to a previous study (9). The primers were supplied by Biomed Future, Sofia, Bulgaria. The sequences of primers are shown in Table 1.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer</th>
<th>Oligonucleotide sequence (5’-3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>gelE</em></td>
<td>GEL 11</td>
<td>TATGACAATGCTTTTTTGGGAT</td>
<td>213</td>
</tr>
<tr>
<td></td>
<td>GEL 12</td>
<td>AGATGCACCCCGAAATAATA</td>
<td></td>
</tr>
<tr>
<td><em>cylA</em></td>
<td>CYT I</td>
<td>ACTCGGGGATTGATAGGC</td>
<td>688</td>
</tr>
<tr>
<td></td>
<td>CYT IIb</td>
<td>GCTGCTAAAGCTGCGCTT</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.** PCR primers used to detect virulence genes (*gelE* and *cylA*) of *Enterococcus* spp. were selected by Vankerckhoven et al. (2004).

PCR was applied in a total volume of 20 μl PCR mixture containing 2 μl PCR buffer, 0.8 μl MgCl2, 2 μl dNTP, 0.1μl of Gel 11 and Gel 12 primers, 0.3 μl of Cyt I and Cyt IIb primers, 0.5 μl Taq DNA polymerase, 2 μl of DNA and...
adding sterile DNA/RNAse free water up to 20 µl.

The PCR was performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The PCR reagents were provided by Thermo Fisher Scientific, USA. Amplification for PCR products were done as follows: initial denaturation at 94°C for 7 minutes followed by 35 cycles consisting of denaturation (94°C for 1.0 minute), annealing (56.4°C for 1.0 minute), and extension (72°C for 1.0 minute), and final extension step at (72°C for 7 minutes). The resulting PCR amplicons were analyzed by electrophoresis on 2% agarose gel containing ethidium bromide in TBE buffer for 40 minutes at 120 V in the presence of 100 bp DNA ladder.

RESULTS
Totally, among 100 clinical isolates of Enterococcus spp. obtained in our study, 85% (n=85) were identified as E. faecalis whereas 15% (n=15) harbored E. faecium. Most of the patient specimens (45%) were recovered from urine (E. faecalis, n=38 and E. faecium, n=7), followed by wound secretions (31%) (E. faecalis, n=28 and E. faecium, n=3). Other specimens included blood (n=10), vaginal swabs (n=6), body fluids (n=5) and respiratory samples (n=3).

The one hundred enterococci were tested for their haemolytic and gelatinase activity by phenotype methods. The presence of genes that encode the both virulence factors cytolysin (cylA) and gelatinase (gelE) was evaluated by multiplex PCR (Figure 1a, b). The virulence determinant content of each isolate under consideration was found both phenotypically and genotypically in varying proportions as reported in Tables 2 and 3.

Figure 1 a), b). Gel electrophoresis of multiplex PCR product of amplification of cylA and gelE Enterococcus spp. virulence genes at 688 and 213 bp, respectively.
Lane M – 100 bp DNA ladder, lanes 1-12 – virulence genes in Enterococcus spp., lane 13 - negative control (no DNA added).
Table 2. Phenotype detection of virulence factors in Enterococcus spp. isolates from clinical samples.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cytolysin production N (%)</th>
<th>Gelatinase production N (%)</th>
<th>Both cytolysin and gelatinase production N (%)</th>
<th>Both cytolysin and gelatinase negative N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. faecalis N=85</td>
<td>15 (18%)</td>
<td>20 (23%)</td>
<td>18 (21%)</td>
<td>32 (38%)</td>
</tr>
<tr>
<td>E. faecium N=15</td>
<td>2 (13%)</td>
<td>1 (7%)</td>
<td>0 (0%)</td>
<td>12 (80%)</td>
</tr>
<tr>
<td>Total N=100</td>
<td>17 (17%)</td>
<td>21 (21%)</td>
<td>18 (18%)</td>
<td>44 (44%)</td>
</tr>
</tbody>
</table>

Table 3. Genotype detection of virulence determinants in clinical isolates of enterococci.

<table>
<thead>
<tr>
<th>Species</th>
<th>CylA+/GelE- N (%)</th>
<th>CylA-/GelE+ N (%)</th>
<th>Both CylA/GelE positive N (%)</th>
<th>Both CylA/GelE negative N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. faecalis N=85</td>
<td>4 (5%)</td>
<td>38 (45%)</td>
<td>25 (29%)</td>
<td>18 (21%)</td>
</tr>
<tr>
<td>E. faecium N=15</td>
<td>0 (0%)</td>
<td>10 (67%)</td>
<td>1 (7%)</td>
<td>4 (26%)</td>
</tr>
<tr>
<td>Total N=100</td>
<td>4 (4%)</td>
<td>48 (48%)</td>
<td>26 (26%)</td>
<td>22 (22%)</td>
</tr>
</tbody>
</table>

“+” – presence of virulence gene tested, “−” – absence of virulence gene tested

Our results determined that 17% (n=17) of 100 Enterococcus spp. isolates evaluated, 18% E. faecalis (n=15) and 13% E. faecium (n=2) respectively, demonstrate only cytolysin production phenotypically (Table 2). Gelatinase activity was only found in 21% (n=21) of the analyzed isolates with a tendency to be present more often in E. faecalis (24%, n=20) than E. faecium (7%, n=1). Cytolysin and gelatinase activity prevalence was higher in urinary tract and wound secretions enterococcal isolates than from other sources. Eighteen (21%) E. faecalis showed combined production of cytolysin plus gelatinase and more than a half of them (56%, n=10) were urine isolates. There were not any E. faecium showed both virulence factors. Overall, 44% (n=44) of the Enterococcus isolates (37% E. faecalis, n=32 and 80% E. faecium, n=12) were identified negative for both hemolysin and gelatinase activity.

Among the virulence genes that we tested, gelE was the most prevalent one. It was found alone in 45% of E. faecalis (n=38) and 67% of E. faecium (n=10) respectively as shown in Table 3. GelE was recovered in higher number isolates from urinary tract (22 strains), followed by isolates from wound secretions (11 strains) but was detected only in three from 10 (30%) invasive isolates. CylA gene was present alone only in four E. faecalis isolates (three wound secretion isolates and one isolate from urine). It was never detected alone in blood samples. Twenty-six percent of Enterococcus isolates (25 E. faecalis and only 1 E. faecium) possessed both cylA and gelE genes. However, 21% of the isolates (18 E. faecalis and 4 E. faecium) did not harbor any of the virulence factors genotypically.

Phenotype and genotype correlation in Enterococcus spp. tested isolates is reported in Table 4. The highest percentage of correspondence (17%) between the phenotype and the genotype (cylA −, gelE +) in the studied isolates was observed among the phenotypically gelatinase producing enterococci. In 3 of the strains (3%) demonstrated only cytolysin production phenotypically, there was a match with the genotype (cylA +, gelE −). In enterococci that demonstrated simultaneous phenotypically production of cytolysin and gelatinase and in those that did not phenotypically showed these two virulence factors, we observed almost the same percentage of genotype correspondence - 15% (15 strains; cylA +, gelE +) and 14% (14 strains; cylA −, gelE −), respectively. The differences between phenotype and genotype are also detailed in Table 4.
Table 4. Phenotype and genotype correlation in Enterococcus spp. clinical isolates regarding pathogenicity.

<table>
<thead>
<tr>
<th>Correlation between phenotype and genotype</th>
<th>Cytolysin production, CylA+/GelE- N (%)</th>
<th>Gelatinase production, CylA-/GelE+ N (%)</th>
<th>Both cytolysin and gelatinase positive, both CylA and GelE positive N (%)</th>
<th>Both cytolysin and gelatinase positive, both CylA and GelE negative N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHENOTYPE AND GENOTYPE CORRESPONDENCE</td>
<td>3 (3%)</td>
<td>17 (17%)</td>
<td>15 (%)</td>
<td>14 (%)</td>
</tr>
<tr>
<td>PHENOTYPE AND GENOTYPE DIFFERENCE</td>
<td>14 (14%)</td>
<td>5 (5%)</td>
<td>3 (3%)</td>
<td>30 (30%)</td>
</tr>
<tr>
<td></td>
<td>3 strains: CylA + GelE +</td>
<td>2 strains: CylA + GelE +</td>
<td>2 strains: CylA - GelE -</td>
<td>6 strains: CylA + GelE +</td>
</tr>
<tr>
<td></td>
<td>3 strains: CylA - GelE -</td>
<td>3 strains: CylA - GelE -</td>
<td>1 strain: CylA - GelE +</td>
<td>23 strains: CylA - GelE +</td>
</tr>
<tr>
<td></td>
<td>8 strains: CylA - GelE +</td>
<td></td>
<td>1 strain: CylA - GelE +</td>
<td></td>
</tr>
</tbody>
</table>

“+” – presence of virulence gene tested, “-” – absence of virulence gene tested

DISCUSSION

Enterococci are one of the most important bacterial species, major opportunistic infectious agents that cause a wide range of nosocomial infections, especially in intensive care units worldwide (10). E. faecalis and E. faecium are known to be the major causative agents of enterococcal infections (11). The virulence factors of enterococci are the main focus in many studies over the past few years. The virulence in this genus is usually considered a multifactorial process that involves several genes and their products and is associated with different factors such as cytolysin and gelatinase production, presence of hyaluronidase, Enterococcus surface protein (Esp), aggregation substance (AS), ability to form biofilm, etc. (12). Their incidence among the strains varied widely from study to another, probably because of their different clinical and geographical origin.

This study evaluated a hundred clinical isolates, of which the prevalence of E. faecalis among the clinical isolates was higher than E. faecium (85% and 15% respectively). Similar distribution of species was observed in other studies in North and Latin America, some European and Asian countries (13, 14), while other authors reported the opposite trend in India (15).

The cytolysin was the first and the best-studied virulence factor in enterococci. It is a protein toxin capable of lysing human, horse, rabbit, but not sheep erythrocytes (16) and it also has bacteriocin activity against other Gram-positive bacteria (17). The cytolysin is encoded usually on pheromone-responsive plasmids (18) but also can be located in the bacterial chromosome (19). It is encoded by cytolysin operon and several genes are essential for its functional production. The cylA gene is responsible for the expression of whole operon (20). E. faecalis strains producing cytolysin have been shown to be virulent in animal and nematode models and human infections (21, 22) and have been associated with increased severity of infection (22), and subsequent increased mortality risk.

The prevalence of cytolysin and gelatinase virulence determinants is having been shown to be higher in E. faecalis than E. faecium isolates even though in some studies in Europe E. faecium was reported generally free of virulence factors (23, 24). In our study, phenotype production incidence of cytolysin among Enterococcus spp. isolates was 35 % (33% E. faecalis and 2% E. faecium, respectively). Iran authors (25) reported a similar rate of hemolysin positive isolates, but the rate of the two species was different from the one reported by us (39% E. faecium and 27% E. faecalis, respectively). Seventeen percent of all the tested enterococci possessed the cytolysin production only. We observed a higher cytolysin activity prevalence in the urinary tract and wound secretions isolates than from other sources. Invasive cytolysin
positive enterococcal isolates were not observed in contrast to the French and American studies (26, 27), which reported a high percentage of invasive isolates that produce cytolysin (40% and 50%, respectively).

The detection of cylA by PCR was not strictly correlated with its phenotypic expression. In our work, 35% Enterococcus spp. (n=35) showed hemolytic activity on blood agar plates, although cylA-positive were 30 of them (E. faecalis, n=29 and E. faecium, n=1, respectively). These results correlate with the data reported in India and the USA (26, 28). The lack of cytolysin phenotypic/genotypic compatibility may suggest missing genes in the cyl operon among cylA-positive/haemolysin-negative strains or the presence of the silent cylA gene (29). We detected cylA gene alone only in four E. faecalis isolates (three wound isolates and one isolate from urine) but it was never detected alone in invasive blood isolates. Our results showed a notably lower frequency of cylA detection (4%) compared to results reported in South Brazil (54.4%, n=31) (30), and in Italy (26.4%) (31).

Another well-known virulent factor in Enterococcus spp. is gelatinase. It is an extracellular zinc metallo-endopeptidase capable to disintegrate gelatin, collagen, casein, hemoglobin and other bioactive peptides (32). Gelatinase is encoded by the chromosomal gelE gene (33) and is regulated by the quorum-sensing fsr locus in a cell-density-dependent manner (34). The gelatinase detection was higher present in clinical isolates than in fecal isolates from healthy volunteers (35), which indicate its virulence potential.

Gelatinase production is mediated by gelE gene and phenotypically expressed in vitro by liquefaction of a culture medium containing the substrate (36). Similar to the results obtained for the cytolysin activity, the gelatinase activity was present more often in E. faecalis (45%, n=38) than E. faecium (7%, n=1). A higher rate of gelE-positive E. faecalis (67%, n=64) than ours result and lack of gelE occurrence in E. faecium was reported in Iran (25). We observed a lower number enterococcal isolates (n=3) from blood with phenotype gelatinase activity compared to non-invasive isolates (22 isolates from urine and 11 from wound secretions), which is in accordance with other authors who also reported its low prevalence or absence in invasive strains (26).

Because of their ability to remain silent (37) the expression of enterococcus gelE genes similar to reported before results for cylA genes, vary in-vivo and in-vitro. The presence of the gelE gene and the lack of gelatinase activity in Enterococcus spp. have been also associated with manipulation in the laboratory and low level expression or down regulation of the gelE gene (38). Our results obtained by phenotypic tests always revealed a lower number of isolates that produced gelatinase in phenotypic test, even though gelE was detected by PCR - 39 (53%) of total 74 gelE-positive strains (63 E. faecalis and 11 E. faecium).

These results correspond to data obtained in Iran where 49.3% of the E. faecalis isolates exhibit gelE did not show gelatinase activity (25). In another study, gelE genes were not detected among enterococci at all (9). It suggests that geographic features might be the reason for the variation among different rates of the virulence factors. Some authors reported that other genes might be associated with gelE expression control and indicated that mutated genes affect gelE gene expression and possibly regulate gelatinase production (39). Seventy-five percent of the tested invasive isolates possessed the gelE gene, but as it reported previous, only 25% of them expressed the gene. When comparing the results of phenotypic and genotyping analysis it was observed that 72 isolates carried the gelE gene and 39 were gelatinase producers, but it is important to highlight that only 18% and 26% of them were positive for phenotypic and genotypic characteristics, respectively.

CONCLUSION
Due to the increasing importance of Enterococcus spp. as causative agents of severe infections, including nosocomial infections, the identification of their virulence factors related to the invasiveness and severity of the disease is a subject of great importance and merits particular attention. Due to the existence of silent virulence genes in these microorganisms (cylA, gelE), in order to better characterize the strains and in order to prevent nosocomial infections, Enterococcus spp. must be constantly monitored and phenotypic and genotypic analysis of their virulence pattern need to be performed in parallel. This may serve as a basis for the development of new and more effective strategies to prevent the
spread of enterococcal infections, such as inhibiting the action of the virulence factors and may provide an appropriate treatment and eradication methods.

REFERENCES


