Comparison of Virulence Gene Profiles of *Enterococcus faecium* and *Enterococcus faecalis* Chicken Neck Skin and Faeces Isolates

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Summary

The objective of this study was to find out the distribution of major virulence determinants *asa1*, *gelE*, *cylA*, *esp*, and *hyl* by multiplex PCR in 132 *Enterococcus faecium* and 67 *Enterococcus faecalis* isolates originated from chicken neck skin samples at slaughterhouse and faeces samples from intensive broiler enterprises and rural poultry establishments. In the study, 31.2% (62/199) of the enterococcal strains harbored at least one virulence determinant. The *gelE* gene was the predominant (30.2%) virulence trait among the enterococci investigated followed by *asa1* (15.6%). Both *gelE* and *asa1* genes were significantly higher in *E. faecalis* than *E. faecium*. The *hyl*, *esp* and *cylA* genes were detected with percentages of 1.5%, 1.5% and 0.8% in *E. faecium* isolates. None of the *E. faecalis* strains harbored *cylA*, *esp* and *hyl* genes. The results indicate that a clear difference was observed in the kind of virulence factor present in strains between faecal samples and skin samples. Also, *E. faecium* strains isolated from both chicken skin samples and faeces presented lower pathogenicity potential than did *E. faecalis*.

Keywords: *Chicken, Enterococcus faecalis, E. faecium, multiplex PCR, Virulence genes*

**Enterococcus faecium** ve **Enterococcus faecalis** Tavuk Boyun Derisi ve Dışkı İzolatlarının Virülens Gen Profillerinin Karşılaştırılması

Özet

Bu çalışmada, mezbahada tavuk boyun derisinden, entansif broyler çiçikleri ve köylerde aile işletmelerindeki tavukların dışkı örneklerinden izole edilen 132 *Enterococcus faecium* ve 67 *Enterococcus faecalis*’in başlıca virülens genleri olan *asa1*, *gelE*, *cylA*, *esp* ve *hyl* genlerinin multiplex PCR ile tespiti amaçlanmıştır. Enterokok izolatlarının %31.2’sinin en az bir virülens genine sahip olduğu belirlenmiştir. *gelE* geninin dominant (%30.2) virülens faktörü olduğu ve bunu %15.6 ile *asa1’in takip ettiği saptanmıştır. Hem *gelE* hem de *asa1 genlerinin* *E. faecalis’te* *E. faecium’a oranla önemli ölçüde yüksek olduğu tespit edilmiştir. *E. faecium* izolatlarının %1.5, %1.5 ve %0.8’inde sarsılsyla *hyl*, *esp* ve *cylA* genleri belirlenmiştir. *E. faecalis* izolatlarının hiçbirinde *cylA*, *esp* ve *hyl* genleri belirlenmemiştir. Çalışma bulguları, tavuk dışkı örneklerinden izole edilen entansif kokular ile boyun derisi örneklerinden izole edilenler arasında virülens faktörleri açısından önemli bir fark olduğunu ifade etmektedir. Ayrıca, hem tavuk boyun derisinden hem de dışkıdan elde edilen *E. faecium* izolatlarının *E. faecalis’é oranalı daha düşük patojenite potansiyeline sahip olduklarını ortaya konmuştur.

Anahtar sözcükler: Tavuk, *Enterococcus faecalis, E. faecium, multiplex PCR, Virulence genler*

**INTRODUCTION**

Genetical similarities between animal and human originated enterococci have been reported and role of natural transmission of enterococci from food animals and contaminated foods to human tract can not be ruled out. Enterococci can cause food intoxication through production of biogenic amins and worrisome opportunistic infections because of the virulence traits. Some strains are resistant to many antibiotics, but antibiotic resistance alone cannot explain the virulence of enterococci. The differentiation of apparently safe and non-safe enterococcal strains is not simple, especially because of effective horizontal gene transfer mechanisms. *Enterococcus faecalis* and *E. faecium* are the most relevant species of *Enterococcus* genus with...
regard to clinical aspects. Also, virulence of Enterococcus spp. may be linked to these species 4.

A number of genes encoding for virulence factors including asa1, esp, hyl, gelE, and cyl in E. faecalis and E. faecium have been described and their effects have been shown in human and animal studies 24. Aggregation substance (AS), a surface protein adhesin encoded by the gene asa1 has a contribution to virulence together with cytolsin 6, facilitates the aggregation of the donor and recipient bacteria for efficient transfer of transmissible conjugative plasmids 25. Another enterococcal adhesin is the “enterococcal surface protein” (ESP), encoded by the gene esp 1, that plays a role in biofilm formation and adherence to abiotic surfaces 1. Hyaluronidase, which is expressed by the hyl gene, acts on hyaluronic acid and increases bacterial invasion 8. The gelE gene encodes for an extracellular Zn-metalloendopeptidase that is capable of hydrolysing gelatin, collagen, casein, hemoglobin and other biological peptides 9. The cytolysin (Cyl) is a cellular toxin and capable of lysing a range of prokaryotic and eukaryotic cells 10.

Although chicken meat consumption is estimated around a million tone 11, there is a lack of information on virulence genes of enterococci from poultry in Turkey. Therefore, the objective of this study was to investigate and compare the distribution of major virulence determinants cylA, hyl, asa1, esp, and gelE in E. faecalis and E. faecium strains isolated from neck skin samples and faeces of chicken.

**MATERIAL and METHODS**

**Bacterial Strains**

A total of 199 Enterococcus including 132 E. faecium and 67 E. faecalis strains were investigated. Faecal strains consisted of 36 E. faecium and 41 E. faecalis from intensive broiler enterprises, and 56 E. faecium and 10 E. faecalis from rural poultry establishments in Kirikkale district 12. Additionally, previously PCR verified 40 E. faecalis and 16 E. faecium strains that were isolated from chicken neck skin samples at slaughter in Ankara 13 were included.

Reference strains that were used in multiplex PCR assays were E. faecalis MMH594 (gelE+, asa1+, esp+ and cylA+), E. faecalis ATCC 29212 (gelE+ and asa1+), E. faecium C68 (hyl+ and esp+), E. faecium C38 (esp+) and E. faecalis 217 (gelE+, asa1+, esp+ and cylA+). Enterococcus faecalis MMH594, E. faecalis 217, E. faecium C68, and E. faecium C38 were kindly provided from Vanessa Vankercckhoven from University of Antwerp, Vaccine and Infectious Disease Institute Medical Microbiology, Antwerp, Belgium.

**Species Verification of Faecal E. faecium and E. faecalis Strains By Multiplex PCR Assay**

Faecal E. faecium and E. faecalis strains previously isolated from intensive broiler enterprises and rural poultry establishments were verified by multiplex PCR. The extraction of DNA from the isolates was done with Chelex-100 (Bio-Rad, Hercules, CA, USA) resin based technique 13. Resulting supernatant was used as template DNA for amplification procedures in the multiplex PCR assays. For the verification of E. faecium and E. faecalis, primer pairs (Alpha DNA, Montreal, Canada) and multiplex PCR protocol of Kariyama et al.14 was used (Table 1).

**Detection of Virulence Genes By Multiplex PCR**

The extraction of DNA was done as mentioned

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**Table 1.** Primer sequences used in this study for verification of Enterococcus species and virulence determinants

<table>
<thead>
<tr>
<th>Target</th>
<th>Oligonucleotide Sequence (5′-3′)</th>
<th>Product Size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddl E. faecalis</td>
<td>ddlE1- ATCAAGTACAGTTAGTCTTTATTAG</td>
<td>941 bp</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>ddlE2- ACCGATTCAAGGTAATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ddl E. faecium</td>
<td>ddlF1- TTAGACCCGACAGTGCG</td>
<td>658 bp</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>ddlF2- TATGACCCGACAGTGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>asa1</td>
<td>ASA11- GGAGGATTCCAGAACTATGGA</td>
<td>375 bp</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>ASA12- TAAGAAAGAATCACCACCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gelE</td>
<td>GEL1- TATGACCCGTCATCCGGGAT</td>
<td>213 bp</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>GEL2- AGATGACCCGAAATATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cylA</td>
<td>CYT1- GTCTGGAGAGGTAATTGAGGC</td>
<td>688 bp</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>CYT1b- GCTGAGACATCCGCGCCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>esp</td>
<td>ESP 14F- AGATTTTACCTATCTTCCTTGG</td>
<td>510 bp</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>ESP 12R- AATGGATCTTATGCACTCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hyl</td>
<td>HYL n1- ACAGAAGAGCTGAGGAAATGA</td>
<td>276 bp</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>HYL n2- GCTGACGTCCAAGTTCCAA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
above. Virulence genes specific primers (Alpha DNA) (Table 1) were used in the multiplex PCR according to Vankerckhoven et al. However, different from Vankerckhoven et al.'s protocol, 2.5 U Taq polymerase (Bioron GmbH, Ludwigshafen, Germany) and 1 x PCR Buffer [10 mmol l⁻¹ Tris-HCl (pH 8.3), 50 mmol l⁻¹ KCl, 0.01% Tween-20] (Bioron) were used and initial denaturation time was decreased from 15 to 5 min. In every multiplex PCR analysis positive controls were used in order to eliminate false negative results.

**Electrophoresis of the Multiplex PCR Products**

A 20 µl aliquot of each PCR products stained with 6x loading dye (Promega, Madison, USA) were analyzed by agarose gel (1.5% Agarose-Basica LE, Prona, Spain) electrophoresis (CSL MSMix-Duo, Corston, UK), stained with 0.1 µg ml⁻¹ ethidium bromide (BioChemica GmbH, Darmstadt, Germany), at 85 V for 1.5 h and visualized by a gel documentation and analysis system (Sygene Ingenius, Cambridge, UK).

**Statistical Analysis**

Comparison between *E. faecalis* and *E. faecium* isolates from the incidence of virulence genes including *asa1*, *gelE*, *cylA*, *esp* and *hyl* were analyzed with Fisher Exact statistical analysis.

**RESULTS**

A total of 199 Enterococcus including 132 *E. faecium* and 67 *E. faecalis* originated from intensive broiler enterprises, rural poultry establishments and chicken neck skin samples at slaughter level were analyzed for the presence of virulence genes *cylA*, *hyl*, *asa1*, *esp* and *gelE* (Fig. 1). Virulence gene distributions of *E. faecium* and *E. faecalis* strains from intensive broiler enterprises, rural poultry establishments and chicken neck skin samples are shown in Table 2.

In the present study, the percentage of enterococci harboring at least one virulence determinant was 31.2% (62/199) and was significantly (P<0.0001) high in *E. faecalis* (33/67, 49.3%) than *E. faecium* (29/132, 22.0%). Statistically, the *E. faecium* strains of intensive broilers (18/36, 50.0%) were significantly more virulent than the *E. faecium* strains of either rural establishments (9/56, 16.1%) (P<0.0001) or slaughter level (2/40, 5.0%) (P<0.0001). No significant difference was observed between the virulent strain percentages of *E. faecium* strains isolated from rural establishments and neck skin samples. *E. faecalis* strains of intensive broiler origin (15/41, 36.6%) were significantly (P<0.0001) less virulent than *E. faecalis* strains originated from neck skin samples (16/16, 100.0%) while, were significantly (P=0.0079) more virulent than strains of rural establishments (2/10, 20.0%). A significant (P<0.0001) difference was observed between the virulence gene distributions of *E. faecalis* (16/16, 100.0%) and *E. faecium* (2/40, 5.0%) strains from neck skin samples. Additionally, no significant difference was found in virulence gene prevalences between the strains of *E. faecalis* and *E. faecium* isolated from rural establishments (P=0.4624) and intensive broilers (P=0.0946).
The *gelE* gene was the predominant (60/199, 30.2%) virulence trait among the enterococci investigated followed by *asa1* (31/199, 15.6%). Both *gelE* (*P*<0.0001) and *asa1* (*P*<0.0498) genes were significantly higher in *E. faecalis* (31/67, 46.3% for *gelE*; 14/67, 20.9% for *asa1*) than *E. faecium* (29/132, 22.0% for *gelE*; 17/132, 12.9% for *asa1*).

While none of the *E. faecalis* strains harbored *cylA*, *esp* and *hyl* genes, *E. faecium* strains harbored the *hyl*, *esp* and *cylA* genes as 1.5% (2/132), 1.5% (2/132) and 0.8% (1/132), respectively.

**DISCUSSION**

Since chicken meat and products are highly consumed and influx of virulence genes from enterococci of chicken origin to human intestinal tract is a possible route, this study has an impact on understanding the distribution of major virulence genes among *E. faecium* and *E. faecalis* of chicken origin.

According to the results of present study, *E. faecium* strains isolated from both chicken neck skin samples and faeces have lower potential pathogenicity than *E. faecalis*. However, virulence genes in *E. faecium* isolates presented more variable genotypes than did *E. faecalis* strains, as none of the *hyl*, *esp* or *cylA* genes were detected in *E. faecalis* isolates. On the other hand, the gene *gelE* and *asa1* were present in both analyzed species. A clear difference was observed in the kind of virulence factor present in strains between faecal samples and neck skin samples. Franz et al. previously reported that the presence of virulence factors is a strain specific character. In the present study, *gelE* gene was determined as the predominant (30.2%) virulence trait among all of the enterococcal strains, and especially in *E. faecalis*. Similarly, the high distribution of the *gelE* gene in *E. faecalis* reported by Franz et al. and Poeta et al. for the faecal poultry samples. Also, results of the present study show that the prevalence of *gelE* and *asa1* genes were higher in *E. faecalis* than *E. faecium*. Similarly some researchers stated that *gelE* appear to be relatively frequent among *E. faecalis* strains coming from various sources.

In the present study, the *hyl*, *esp* and *cylA* genes were detected with percentages of 1.5% (2/132), 1.5% (2/132) and 0.8% (1/132) respectively. Moreover, all strains of *E. faecium* harboring *hyl* were also harboring *gelE*. Also, none of the *E. faecalis* strains harbor the *hyl*, *esp* and *cylA* genes. Poeta et al. reported 30% *cylA* positivity for *E. faecalis* and *E. faecium* strains of poultry origin. The results of the present study for the *esp* negativity in *E. faecalis* is in compliance with previous reports. According to the literature review, we could not find any previous report about *esp* gene in *E. faecium* strains of poultry faeces origin.

Consequently, the results indicate that, a clear difference was observed in the kind of virulence factor present in strains between faecal samples and neck skin samples. Also, *E. faecium* strains isolated from both chicken neck skin samples and faeces have lower pathogenicity potential than *E. faecalis*. Therefore, *E. faecium* strains of poultry origin may play no or only a minor role in this increasing virulence trend.
Acknowledgements

The authors would like to thank Vanessa Vankerckhoven (University of Antwerp, Vaccine and Infectious Disease Institute Medical Microbiology, Antwerp, Belgium) for providing the reference strains E. faecalis MMH594, E. faecalis 217, E. faecium C68 and E. faecium C38 and Mehmet Gülşen (Diagen Co. Inc.) for supplying primers.

REFERENCES