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RESEARCH ARTICLE

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

Aylin KASIMOĞLU DOĞRU * Yılmaz Emre GENÇAY * Naim Deniz AYAZ *

* Kırıkkale Üniversitesi Veteriner Fakültesi, Besin Hijyeni ve Teknolojisi Anabilim Dalı, TR-71450 Kırıkkale - TÜRKİYE

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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 çiftlikleri 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 multipleks 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 sırasıyla *hyl*, *esp* ve *cylA* genleri belirlenmiştir. *E. faecalis* izolatlarının hiçbirinde *cylA*, *esp* ve *hyl* genleri belirlenememiştir. Çalışma bulguları, tavuk dışkı örneklerinden izole edilen enterokoklar ile boyun derisi örneklerinden izole edilenler arasında virülens faktörleri açısından önemli bir fark olduğunu ifade etmektedir. Ayrıca, hem tavuk boyun derisinden hem de dışkısından elde edilen *E. faecium* izolatlarının *E. faecalis*'e oranla daha düşük patojenite potansiyeline sahip oldukları ortaya konulmuştur.

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

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 [1]. Enterococci can cause food intoxication through production of biogenic amins and worrisome opportunistic infections because of the virulence traits [2].

Some strains are resistant to many antibiotics, but antibiotic resistance alone cannot explain the virulence of enterococci [1]. The differentiation of apparently safe and non-safe enterococcal strains is not simple, especially because of effective horizantal gene transfer mechanisms [3,7]. *Enterococcus faecalis* and *E. faecium* are the most relevant species of *Enterococcus* genus with

İletişim (Correspondence)
☎ +90 318 3573301/3161
✉ akasimoglu@superonline.com

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 [2,5]. Aggregation substance (AS), a surface protein adhesin encoded by the gene *asa1* has a contribution to virulence together with cytolysin [6], facilitates the aggregation of the donor and recipient bacteria for efficient transfer of transmissible conjugative plasmids [4,7]. Another enterococcal adhesin is the "enterococcal surface protein" (ESP), encoded by *esp* gene 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. faecium* and 16 *E. faecalis* 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 Vankerckhoven 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 establisments 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

Table 1. *Primer sequences used in this study for verification of Enterococcus species and virulence determinants*
Tablo 1. *Enterokok türlerinin doğrulanmasında ve virülens genlerinin tespitinde kullanılan primer dizileri*

Target	Oligonucleotide Sequence (5'-3')	Product Size	Reference
ddl E. faecalis	ddlE1- ATCAAGTACAGTTAGTCTTTATTAG ddlE2- ACGATTCAAAGCTAACTGAATCAGT	941 bp	14
ddl E. faecium	ddlF1- TTGAGGCAGACCAGATTGACG ddlF2- TATGACAGCGACTCCGATTCC	658 bp	15
asa1	ASA11- GCACGCTATTACGAACTATGA ASA12- TAAGAAAGAACATCACCACGA	375 bp	8
gelE	GEL 11- TATGACAATGCTTTTTGGGAT GEL 12- AGATGCACCCGAAATAATATA	213 bp	8
cylA	CYT I- ACTCGGGGATTGATAGGC CYT IIb- GCTGCTAAAGCTGCGCTT	688 bp	16
esp	ESP 14F- AGATTTCATCTTTGATTCTTGG ESP 12R- AATTGATTCTTTAGCATCTGG	510 bp	17
hyl	HYL n1- ACAGAAGAGCTGCAGGAAATG HYL n2- GACTGACGTCCAAGTTTCCAA	276 bp	8

above. Virulence genes specific primers (Alpha DNA) *(Table 1)* were used in the multiplex PCR according to the Vankerckhoven et al.[8]. However, different from Vankerckhoven et al.[8]'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 MSMixi-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 [18].

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).

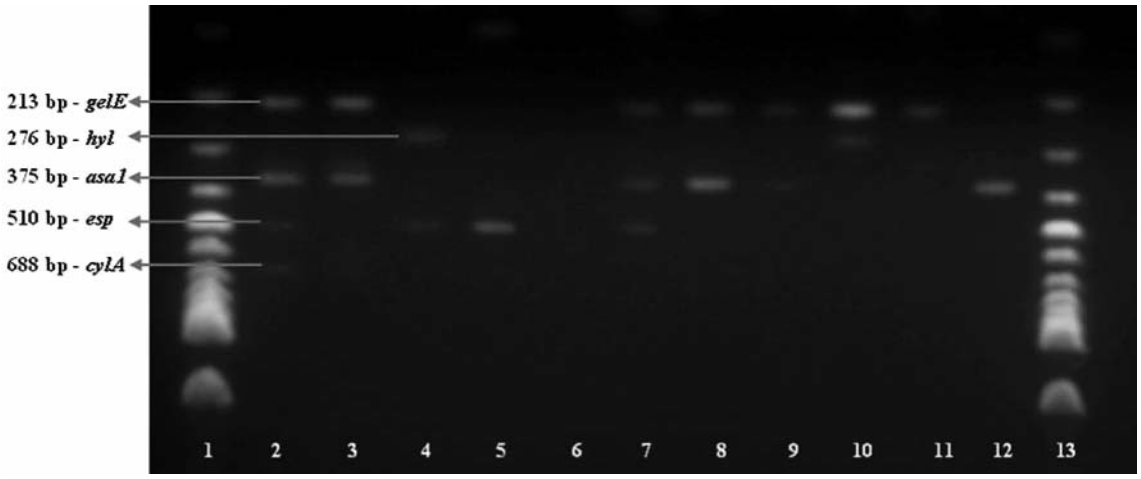

Fig 1. Major virulence genes of *E. faecium* and *E. faecalis*

(Lanes **1** and **13:** 100 bp DNA marker; **2:** *E. faecalis* MMH 594; **3:** *E. faecalis* ATCC 29212; **4:** *E. faecium* C68; **5:** *E. faecium* C38; **6:** negative control; **7:** *gelE, asa1* and *esp* positive E. faecium isolate; **8:** *gelE* and *asa1* positive *E. faecalis* isolate, **9:** *gelE* and *asa1* positive *E. faecium* isolate; **10:** *gelE* and *hyl* positive *E. faecium* isolate; **11:** *gelE* positive *E. faecalis* isolate; **12:** asa1 positive *E. faecalis* isolate)

Şekil 1. *E. faecium* ve *E. faecalis*'in başlıca virülens genleri

(Sıra **1** ve **13:** 100 bp DNA cetveli; **2:** *E. faecalis* MMH 594; **3:** *E. faecalis* ATCC 29212; **4:** *E. faecium* C68; **5:** *E. faecium* C38; **6:** negatif kontrol; **7:** *gelE, asa1* ve *esp* pozitif *E. faecium* izolatı; **8:** *gelE* ve *asa1* pozitif *E. faecalis* izolatı, **9:** *gelE* ve *asa1* pozitif *E. faecium* izolatı; **10:** *gelE* ve *hyl* pozitif *E. faecium* izolatı; **11:** *gelE* pozitif *E. faecalis* izolatı; **12:** *asa1* pozitif *E. faecalis* izolatı)

Table 2. *Sources and virulence gene distributions of E. faecium and E. faecalis*
Tablo 2. *E. faecium ve E. faecalis'lerin orijinleri ve virülens gen dağılımları*

Enterococcus faecium			***Enterococcus faecalis***		
Origin of Strains *	**n of Strains**	**Virulence gene Profile (n) (%)**	**Origin of Strains ***	**n of Strains**	**Virulence gene Profile (n) (%)**
I	36	*gelE* (4) (11.1) *gelE* + *asa1* (11) (30.6) *gelE* + *hyl* (2) (5.6) *gelE* + *asa1* + *esp* + *cylA* (1) (2.8)	I	41	*gelE* (9) (22.0) *gelE* + *asa1* (6) (14.6)
R	56	*gelE* (4) (7.1) *gelE* + *asa1* (4) (7.1) *gelE* + *asa1* + *esp* (1) (1.8)	R	10	*gelE* (1) (10.0) *gelE* + *asa1* (1) (10.0)
CNS	40	*gelE* (2) (5.0)	CNS	16	*gelE* (9) (56.3) *asa1* (2) (12.5) *gelE* + *asa1* (5) (31.3)

**** I:*** *intensive broiler enterprise;* ***R:*** *rural poultry establishment;* ***CNS:*** *chicken neck skin samples*

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.[19] 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.[19] and Poeta et al.[20,21] 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 [3, 19].

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) in *E. faecium* strains, 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.[21] 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 [20,22,23]. 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.

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