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Virulence Factors of *Enterococcus faecium* and *Enterococcus faecalis* Strains Isolated from Humans and Pets

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Abstract: The virulence factors of 146 *Enterococcus faecium* and 32 *Enterococcus faecalis* strains isolated from faecal samples of humans, dogs, and cats were investigated. In total, 178 strains were examined by gelatinase (GelE), aggregation substance (AS), cytolysin, and slide haemagglutination tests. The results of detected virulence factors of *E. faecium* and *E. faecalis* strains were: GelE: 17.1% vs. 37.5%; AS: 13% vs. 12.5%; cytolysin: 7.5% vs. 12.5%; haemagglutination activities with rabbit erythrocytes: 9.6% vs. 9.4%; haemagglutination activities with human erythrocytes: 17.1% vs. 21.9%, respectively.

Key Words: Enterococci, gelatinase, aggregation substance, cytolysin, haemagglutination

İnsan ve Pet Hayvanlarından İzole Edilen *Enterococcus faecium* ve *Enterococcus faecalis* Suşlarının Virülens Faktörleri

Özet: Bu çalışmada, insan, köpek ve kedilere ait dışkı örneklerinden izole edilen 146 *Enterococcus faecium* ve 32 *Enterococcus faecalis* suşunun virülens faktörleri araştırıldı. Toplam 178 suş, jelatinaz, agregasyon substansı, sitolizin ve lam hemaglutinasyon testleri yönünden incelendi. *E. faecium* ve *E. faecalis* suşlarında incelenen virülens faktörleri sırasıyla jelatinaz % 17,1, % 37,5; agregasyon substansı % 13, % 12,5; sitolizin % 7,5, % 12,5; tavşan eritrositleri ile hemaglutinasyon % 9,6, % 9,4 ve insan eritrositleri ile hemaglutinasyon ise % 17,1, % 21,9 olarak belirlendi.

Anahtar Sözcükler: Enterokok, jelatinaz, agregasyon substansı, sitolizin, hemaglutinasyon

Introduction

Enterococci are commensal organisms of the gastrointestinal and urogenital systems of humans, several other mammals, and birds (1). They are common in environments contaminated by human and animal faecal materials (e.g. urban sewage, recipient water, and soil receiving fertilisers of animal origin), as well as in food products derived from animals (2-4). Some enterococcal strains are used as probiotic agents and are thought to have beneficial effects on a number of gastrointestinal and systemic diseases (5); however, on some occasions, enterococci may cause serious diseases when the commensal relationship with its host is disrupted (6). *E. faecium* and *E. faecalis* associated colonisation and infection in animals cause the vast majority of clinical enterococcal infections in humans,

including important nosocomial infections, especially in immunocompromised patients (7).

A number of enterococcal virulence factors have been described. Among them, gelatinase (GelE), aggregation substance (AS), and cytolysin have been studied most intensively (6,8).

GelE is an extracellular zinc metalloendopeptidase secreted by *E. faecalis* that shares homologies with GelE of *Bacillus* species and *Pseudomonas aeruginosa* elastase (9). GelE can hydrolyse gelatine, casein, haemoglobin, and other bioactive peptides, which provides a clue to its potential role as a virulence factor in enterococci (10).

AS of *E. faecalis* is involved in the conjugative transfer of plasmids, which can be observed as a clumping reaction. It has been demonstrated to mediate adhesion

to cultured renal cells, suggesting that it might be important in the pathogenesis of infection (11). In 2 rabbit endocarditis models, both the combination of cytolysin and AS (12), and the combination of AS and enterococcal binding substance (13) were associated with increased virulence. In contrast, in a rat model, the presence of AS was not correlated with the establishment of endocarditis (14). Results of epidemiological studies in clinical enterococcal isolates are also contradictory. Agglutination of erythrocytes by bacteria is a convenient measure of adherence (15). In addition to AS, haemagglutination properties may contribute to the attachment to host cells.

Enterococci secrete molecules that are putative virulence factors. For example, cytolysin is a bacterial toxin that is encoded by an operon consisting of 8 genes localised on a pheromone-responsive plasmid or chromosome (16-18). Cytolysin shows haemolytic (against human, horse, and rabbit erythrocytes) and bactericidal activity against other Gram-positive bacteria (9).

Cats and dogs have played an important role in the human community, which allows them to have a good relationship with humans and contribute to their welfare; however, this relationship also poses serious risks of transmission of infectious agents to man. Pathogenic zoonotic bacteria and intestinal flora from pets can infect or reach the human population by direct contact. These bacteria can colonise in humans and transfer their resistance genes to other bacteria belonging to the endogenous flora of humans (19).

This study was performed to demonstrate the GelE, AS, cytolysin, and haemagglutination properties of *E. faecium* and *E. faecalis* isolated from humans, dogs, and cats.

Materials and Methods

Materials

Enterococcal strains: In this study, 146 *E. faecium* and 32 *E. faecalis* strains were investigated. All the strains were isolated from healthy humans, dogs, and cats in the Van district of Turkey in 2003 and 2004, during our previous study (20) (Table 1).

Table 1. Distribution of *E. faecium* and *E. faecalis* strains by source.

Source	<i>E. faecium</i>	<i>E. faecalis</i>
Humans	55	8
Dogs	64	15
Cats	27	9
Total	146	32

Reference strains: The reference strains of enterococci were kindly provided by Dr. D.B. Clewell (Department of Biologic and Materials Sciences, School of Dentistry, University of Michigan, Ann Arbor, Michigan, USA) (Table 2).

Methods

Detection of gelatine hydrolysis (GelE)

GelE activity of enterococci was tested in gelatine medium, as described by Su et al. (10). GelE-positive colonies on gelatine medium were surrounded by a turbid halo after 2 days of incubation at 37 °C. To measure the hydrolysed gelatine in the agar plates, 0.5-1.0 ml of Frazier solution (mercuric chloride, 15.0 g; hydrochloric acid (37%), 20 ml; distilled water, 100 ml) was poured on the surface of the medium to precipitate the unhydrolysed gelatine. *E. faecalis* OG1RF was used as a positive control.

Detection of aggregation substance (AS)

Measurement of the AS of the enterococci was performed by clumping assay, as described by Chow et al. (12). The plasmid-containing reference *E. faecalis* OG1X (pAM9058) strain was incubated in Todd Hewitt Broth

Table 2. Standard *E. faecalis* derivatives and contents of isogenic strains, plasmid/transposon, and virulence factors.

Strain/Isogen	Plasmid	Transposon	Virulence Factors
OG1X	None	None	None
OG1RF	None	None	GelE
OG1X (pAM9058)	pAD1	Tn917	AS
OG1X (pAM944)	pAD1	Tn917	Cytolysin
OG1X (pAM714)	pAD1	Tn917	Cytolysin +AS
OG1SSP	PCF10	Tn915	GelE +AS

(THB) at 37 °C for 18 h. After centrifugation at 10,000 xg for 10 min at 4 °C, the plasmid-containing supernatant obtained was sterilised by autoclaving for 15 min. The supernatant was diluted 1:5 in sterile THB and used in clumping assays in microtitre plates. The enterococcal strains were inoculated in 200 ml of plasmid-containing suspension and incubated at 37 °C for 24 h. Then, each well was examined for cell clumping at 2, 4, 8, and 24 h. The plasmidless reference *E. faecalis* OG1X strain was used as a negative control, whereas 2 variants of *E. faecalis* OG1X containing either plasmid pAD1 or pCF10 were used as positive controls.

Detection of cytolysin production

Brain heart infusion agar (Difco, USA) supplemented with 5% horse blood was used for the detection of cytolysin activity. Plates were incubated at 37 °C for 24 h and cytolytic activity was observed as β -haemolysis surrounding bacterial colonies. *E. faecalis* OG1X (pAM944) was used as a positive control (12).

Slide haemagglutination test

The isolates were cultivated in 10 ml of THB for 18 h at 37 °C. The cultures were washed 3 times in 0.002 M PBS (pH 6.8) and suspended in 200 ml of the same buffer. The suspension was pre-treated with trypsin (5

mg per 200 ml of bacterial suspension) for 1 h at 37 °C, then washed and resuspended in the initial volume of PBS. Haemagglutination tests were carried out with 20 ml of 2% erythrocytes (rabbit, sheep, bovine, horse, and human; Sigma, USA) and 20 ml of trypsinised culture on slides. The suspensions were mixed and the slides were rotated gently, and within 30 s the haemagglutination was recorded as strong agglutination (++), agglutination (+), or no agglutination (15).

Results

The distribution of virulence factor results for *E. faecium* and *E. faecalis* strains, by origin, are given in Table 3. Of the 146 *E. faecium* isolates, 25 (17.1%) were positive for GelE, 19 (13%) were positive for AS, and 11 (7.5%) were positive for cytolysin. According to the haemagglutination test, while 14 (9.6%) were positive with rabbit erythrocytes, 25 (17.1%) were positive with human erythrocytes. On the other hand, of the 32 *E. faecalis* strains, 12 (37.5%) were GelE, 4 (12.5%) were AS, and 4 (12.5%) were cytolysin positive. The haemagglutination test showed that 3 (9.4%) were positive with rabbit erythrocytes, whereas 7 (21.9%) were positive with human erythrocytes.

Table 3. The distribution of virulence factors of *E. faecium* and *E. faecalis* strains.

Tests	<i>Enterococcus faecium</i>				<i>Enterococcus faecalis</i>				
	Human n = 55 (%)	Dog n = 64 (%)	Cat n = 27 (%)	Total n = 146 (%)	Human n = 8 (%)	Dog n = 15 (%)	Cat n = 9 (%)	Total n = 32 (%)	
GelE	7 (12.7)	17 (26.6)	1 (3.7)	25 (17.1)	1 (12.5)	9 (60)	2 (22.2)	12 (37.5)	
A S	15 (27.2)	1 (1.6)	3 (11.1)	19 (13)	3 (37.5)	1 (6.7)	0 (0)	4 (12.5)	
Cytolysin	10 (18.2)	0 (0)	1 (3.7)	11 (7.5)	4 (50)	0 (0)	0 (0)	4 (12.5)	
Haemagglutination	Rabbit	2 (3.6)	10 (15.6)	2 (7.4)	14 (9.6)	1 (12.5)	2 (13.3)	0 (0)	3 (9.4)
	Sheep	0 (0)	1 (1.6)	0 (0)	1 (0.7)	0 (0)	1 (6.7)	0 (0)	1 (3.1)
	Bovine	2 (3.6)	2 (3.2)	2 (7.4)	6 (4.1)	1 (12.5)	1 (6.7)	0 (0)	2 (6.2)
	Horse	0 (0)	2 (3.2)	0 (0)	2 (1.4)	0 (0)	1 (6.7)	0 (0)	1 (3.1)
	Human	5 (9)	16 (25)	4 (14.8)	25 (17.1)	3 (37.5)	4 (26.7)	0 (0)	7 (21.9)

Discussion

Several virulence factors, such as GelE, enterococcal surface protein (Esp), AS, cytolysin, lipase, and haemagglutinin, are possibly associated with the colonisation and pathogenesis of enterococci (10-12,18).

GelE is a protease produced by *E. faecalis* that is capable of hydrolysing gelatine, collagen, casein, haemoglobin, and other peptides (11). It might play an important role in the severity of systemic disease, as shown in several independent studies (12,21). GelE was also shown to be enriched in clinical isolates in some studies (55%-100%) versus 27%-66% in stool isolates from healthy volunteers (2,6,8,22), but a contradicting observation has also been reported (23). In a previous study (9), 54% of *E. faecalis* from endocarditis isolates, 68% from other blood culture isolates, and 27% from community-acquired faecal isolates were GelE-positive. Kanemitsu et al. (24) reported that GelE was detected in 42 (45%) of 93 *E. faecalis* strains from humans, but was not detected in *E. faecium* isolates. Archimbaud et al. (3) also found that 17 (58.6%) of 29 *E. faecalis* strains isolated from clinical isolates were GelE-positive. In the present study, GelE was detected in 12.7% of *E. faecium* strains from humans, in 26.6% from dogs, and in 3.7% from cats, whereas 12.5% of *E. faecalis* from humans, 60% from dogs, and 22.2% from cats were GelE-positive.

AS is a virulence factor that seems to mediate the specific binding of enterococci to intestinal epithelium, renal epithelial cells, human neutrophils, and macrophages (1,11). In some studies, AS seems to be more common in clinical isolates than in stool isolates (9,25), but in other studies no difference was found (2,3). Coque et al. (9) reported that AS is the most prevalent single factor in clinical *E. faecalis* isolates (52%). Faecal strains were much less common in healthy volunteers (20%) than in patients (30%) in Spain. Similar results were found in 33% of clinical *E. faecalis* isolates and 4% of isolates from municipal waste water (25). In another study (2), all 48 *E. faecium* strains from food were AS-negative, whereas 24 (51.1%) of 47 *E. faecalis* strains were positive. Archimbaud et al. (3) reported that 14 (48.3%) of 29 *E. faecalis* strains isolated from clinical isolates were AS-positive. In the present study, AS was detected in 27.2% of *E. faecium* strains isolated from humans, in 1.6% from dogs, and in 11.1% from cats. AS was also detected in 37.5% of *E. faecalis* strains from

humans and in 6.7% from dogs, but was not detected in strains from cats.

Cytolysin is a cytolytic protein capable of lysing human, horse, and rabbit erythrocytes (16-18). Cytolysin is thought to have an important role in human infections, which is produced in 11%-70% of strains (3,8,22), compared to 0%-25% in stool isolates (9,22). It has been described as a virulence factor of *E. faecalis* in animal models and human infections (9,16-18). Ike et al. (17) reported that 60% of clinical *E. faecalis* isolates were cytolysin-positive in Japan. These findings suggest that this factor may play an important role in human infections. Evidence in support of this conjecture was provided by the results of a study (26) in which 40% of *E. faecalis* blood culture isolates were found to be haemolytic. In contrast, another study found that 16% of *E. faecalis* isolated from blood cultures were haemolytic (8). Similar results were found in the study by Coque et al. (9). Archimbaud et al. (3) reported that 5 (17.2%) of 29 *E. faecalis* strains isolated from clinical isolates were positive for cytolysin. In another study (2), 8.3% of *E. faecium* and 21.3% of *E. faecalis* strains were positive for β -haemolysin production. Eaton and Gasson (22) observed that 44% of *E. faecalis* strains from food showed cytolysin activity, whereas none of the *E. faecium* strains did. In the present study, cytolysin activity was detected in 18.2% of *E. faecium* isolates from humans and 3.7% from cats, but it was not detected in dog isolates. On the other hand, this factor was detected in only 50% of *E. faecalis* strains isolated from humans.

GelE was found to be the most common factor in both *E. faecium* and *E. faecalis* strains. It was concluded that GelE, AS, and cytolysin could not be considered important virulence factors by themselves. The rate of GelE, AS, and cytolysin factors in human isolates were lower compared to the results of previous studies. These differences might be based on hosts. The present study used strains that were isolated from healthy humans and pets. No further information was obtained from the literature concerning virulence factors of enterococci isolated from dogs and cats. Previous reports have shown a relative heterogeneity in the distribution of virulence factors of enterococci; therefore, the distribution of virulence factors of enterococci can vary according to geographical region.

Bacterial adherence to host cells appears to be a multifactorial phenomenon involving specific and non-

specific interactions. The ability of bacteria to attach to and agglutinate erythrocytes may be used as an in vitro model for studying host bacterium interaction and the mechanism of attachment (27,28). In addition to AS, haemagglutination may contribute to the attachment to host cells. Elsner et al. (8) reported that 86 (97%) of 89 *E. faecalis* strains isolated from human blood were haemagglutination-positive, while all of 24 *E. faecium* isolates were negative with rabbit erythrocytes. In a similar study (29), 81 (94.2%) of 86 *E. faecalis* strains were positive with agglutinated rabbit erythrocytes. Kurl et al. (28) observed that 42 group D streptococci did not agglutinate human erythrocytes. In the present study, 178 enterococcal strains were tested for haemagglutination activity by rabbit, sheep, bovine, horse, and human erythrocytes. While 14 (9.6%) *E. faecium* and 3 (9.4%) *E. faecalis* isolates agglutinated

rabbit erythrocytes, 25 (17.1%) *E. faecium* and 7 (21.9%) *E. faecalis* isolates agglutinated human erythrocytes. We also found that the examined strains agglutinated human erythrocytes more than rabbit erythrocytes. The rates of haemagglutination activity of strains examined in this study were lower than those in previous reports. This difference might be based on the presence of different adhesion molecules of local strains.

With this study some virulence factors were determined in *E. faecium* and *E. faecalis* strains from humans, dogs, and cats. The results of this study also indicate that enterococci from pets could be a potential risk factor for transmission to humans. Further studies of biochemical and molecular characterisations should be conducted to determine other pathogenic properties of enterococci.

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