

Investigation of Virulence Genes of *Enterococcus faecalis* Strains Isolated from Mastitic Bovine Milk

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ABSTRACT

In this study it was aimed to investigate the potential virulence genes (gelatinase [*geE*]), adhesion-associated protein [*EfaAfs*], enterococcal surface protein [*esp*], cytolysins [*cyIA*, *cyIM*, *cyIB*], sex pheromones [*cpd*, *cob*, *ccf*], aggregation substance [*aggA*], enhanced expression of pheromone [*eep*]) of *Enterococcus faecalis* strains isolated from mastitic bovine milk samples with polymerase chain reaction (PCR). A total of 56 *E. faecalis* isolates, which were isolated from 600 bovine mastitic milk samples, were used as material. After the isolation of enterococci in selective media, identification based on genus and species were also performed using PCR. Later, the *E. faecalis* isolates were tested for the presence of eleven virulence factors. The *efaAfs* gene was the predominant (94.6%) virulence gene among the enterococci investigated followed by *cpd* (91.0%), *geE* (87.5%), *esp* (51.7%), *ccf* (42.8%), *cob* (10.7%), *eep* (8.9%), *aggA* (7.1%), *cyIA* and *cyIM* (1.8%). *cyIB* gene were not detected in any of isolates. 1.8% and 3.6% of the strains harboured eight and seven virulence determinants, while there was no isolate having no virulence genes. Studies on the prevalence of enterococci in dairy cattle have been reported however there is still a lack of information regarding virulence genes of enterococci isolated from mastitic bovine milk. To the best knowledge of the authors this is the first report describing virulence genes of *E. faecalis* isolated from bovine mastitic milk in Turkey. It was concluded that *E. faecalis* strains isolated from mastitic bovine milk were found to be highly pathogenic with potential risk factors for consumer health. Further epidemiological studies are necessary to investigate the status of virulence factors of other enterococci isolated from mastitic bovine milk in the veterinary field.

Keywords: *Enterococcus faecalis*; Virulence Genes; Bovine Mastitis

INTRODUCTION

Enterococcus faecalis is a Gram-positive bacterium that inhabits the oral cavity and gastrointestinal flora of humans and animals. Although enterococci are ubiquitous in nature as normal commensals they are also of medical importance. Enterococci are a leading cause of nosocomial infections in humans (1) and have been associated with bovine mastitis in dairy cattle (2). Little information is available on enterococcal pathogens isolated from mastitic milk samples (2), and if any, most studies focus on isolation and identification of major species (3).

Though enterococci as opportunistic pathogens do not have strong virulence factors comparing with more virulent bacteria, several factors conferring enhanced virulence have been identified in *E. faecalis* (3). A number of genes encoding for virulence factors in *E. faecalis* strains including adhesion-associated protein (*efaAfs*), sex pheromones (*cpd*, *cob*, *ccf*), gelatinase (*geE*), enterococcal surface protein (*esp*), enhanced expression of pheromone (*eep*), aggregation substance (*aggA*), cytolysins (*cyIA*, *cyIB*, *cyIM*) have been described (4, 5). The *geE* gene encodes for an extracellular Zn-metalloendopeptidase that is capable of hydrolysing

gelatin, collagen, casein, hemoglobin and other biological peptides (1). The other virulence genes are represented by *efaAfs*, cell wall adhesins expressed in serum in *E. faecalis* (6). The enterococcal surface protein *esp* gene has a large surface protein with high molecular weight of unknown function whose presence is increased in frequency among infection arising from *E. faecalis* isolates (5, 7). Sex pheromones (*cpd*, *cob*, *ccf*) are chemotactic for human leukocytes and facilitate conjugation (8). *eep* (enhanced expression of pheromone) gene does not contain the pheromone sequence but is necessary for pheromone expression and is a membrane protein of *E. faecalis* (4). Aggregation substances encoded by *aggA* are responsible for increased bacterial adhesion to eukaryotic cells (9). The cytolysins (*cytA*, *cytM*, *cytB*) lyse a broad range of eukaryotic and prokaryotic cells and enhances the *E. faecalis* virulence (10).

Studies on the prevalence of enterococci in dairy cattle have been reported worldwide (11-13) as well as in Turkey (14, 15). However, little information is available on enterococcal virulence genes, and if any, most studies focus on the three major origins; medical, environmental or food isolates, in the world (3, 5) as well as in Turkey (16).

To the best of our knowledge, this is the first report about virulence genes in *E. faecalis* of isolated from bovine mastitic milk in Turkey. In this study, it was aimed to investigate the potential virulence genes (*efaAfs*, *cpd*, *cob*, *ccf*, *gelE*, *esp*, *aggA*, *eep*, *cytA*, *cytM*, *cytB*,) of *E. faecalis* strains isolated from mastitic bovine milk samples with polymerase chain reaction (PCR).

MATERIALS AND METHODS

Bacterial strains

A total of 242 dairy cattle at 38 dairy farms were investigated and 600 milk samples were obtained with mastitis (clinical or subclinical). The milk samples were from family farms in Aydin region. Aydin is a county of 8007 km² situated along the Aegean Sea on the western part of Turkey.

Diagnosis of mastitis

Clinical mastitis was diagnosed by changes in the udder and milk by veterinary practitioners. Changes in the udder included pain, swelling, warmth and abnormal appearance (blood tinged milk, watery secretions, clots, pus) of milk. Cows that did not have clinical mastitis were subjected to further investigation for subclinical mastitis by using

California Mastitis Test. The procedures and interpretations have been described previously (17). For collection of milk samples, teat ends were cleaned using 70% alcohol moistened swabs and allowed to dry. After discarding the first few streams, 2-5 ml of the milk samples were collected into sterile 5 ml glass flasks.

Identification of enterococci

Ten µl of milk was transferred in 5 ml Chromocult Enterococci Broth (Merck, Germany) and incubated for 48 h 37°C for selective enrichment of the enterococci. Positive cultures (a strong blue-green colour of the broth indicated the presence of enterococci) were transferred to Enterococcosel Agar (Becton Dickinson, Germany) for selective isolation. Plates were incubated overnight at 37°C. One presumptive (small, translucent with brownish-black to black zones) colony was passed to blood agar. Bacteria were identified by standard methods using morphological and biochemical characteristics (17). All isolates were stored at -80°C in Brain Heart Infusion Broth (Oxoid, UK) with 20% glycerol until use for molecular confirmation.

DNA extraction

For genomic DNA from individual pure culture isolates were extracted with InstaGene™ DNA extraction kits (Bio-Rad, Brazil) according to the manufacturer's instructions. DNA sample concentrations were determined by spectrophotometry at the wave length of 260 nm and 280 nm.

Detection of Enterococci, *E. faecalis* and virulence genes

The enterococcus suspect colonies were confirmed genetically using genus (18) and species specific PCR (19). Uniplex PCR was used for detection of *EfaAfs*, *cpd*, *cob*, *ccf*, *gelE*, *cytA*, *cytM*, *cytB*, *aggA* (5), *esp* (7), *eep* (20) genes. All primers (Macrogen, Holland) used in this work are listed in Table 1. All the PCR reactions were carried out in a final volume of 20 µl containing 1X PCR buffer, 2 mM MgCl₂, 200 mM each of the four dNTPs (Fermentas, USA), 0.5 mM of each primer and 1.25 units of Taq DNA polymerase (Fermentas, USA). PCR conditions are given in Table 2. Reference strains *E. faecalis* ATCC 29212 (*gelE*+, *EfaAfs*+, *cpd*+, *cob*+, *cytA*+, *cytM*+, *cytB*+, *ccf*+, *eep*+) were used as positive controls (21) and *Escherichia coli* ATCC 25922 was used as negative control strain. The positive control strains for genes of *esp*

Table 1: Oligonucleotide primers used to in the study

	Primer	Sequence (5'-3')	Amplicon size (bp)	Reference
1	Ent1 Ent2	TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	112	(18)
2	DDF DDR	CACCTGAAGAAACAGGC ATGGCTACTTCAATTTCCAG	475	(19)
3	<i>gel</i> E ^F <i>gel</i> E ^R	ACCCCGTATCATTGGTTT ACGCATTGCTTTTCCATC	419	(5)
4	<i>cy</i> L ^{AF} <i>cy</i> L ^{AR}	TGGATGATAGTGATAGGAAGT TCTACAGTAAATCTTTTCGTCA	517	(5)
5	<i>cc</i> F ^F <i>cc</i> F ^R	GGGAATTGAGTAGTGAAGAAG AGCCGCTAAAATCGGTAAAAT	543	(5)
6	<i>efa</i> A ^{Fs} ^F <i>efa</i> A ^{Fs} ^R	GACAGACCCTCACGAATA AGTTCATCATGCTGCTGTAGTA	705	(5)
7	<i>cy</i> L ^{MF} <i>cy</i> L ^{MR}	CTGATGGAAAGAAGATAGTAT TGAGTTGGTCTGATTACATTT	742	(5)
8	<i>cp</i> d ^F <i>cp</i> d ^R	TGGTGGGTATTTTTCAATTC TACGGCTCTGGCTTACTA	782	(5)
9	<i>cy</i> B ^F <i>cy</i> B ^R	ATTCCTACCTATGTTCTGTTA AATAAACTCTTCTTTTCCAAC	843	(5)
10	<i>esp</i> F ^F <i>esp</i> F ^R	TTGCTAATGCTAGTCCACGACC GCGTCAACACTTGCATTGCCGAA	933	(7)
11	<i>eep</i> F ^F <i>eep</i> F ^R	GAGCGGGTATTTTAGTTCGT TACTCCAGCATTGGATGCT	937	(20)
12	<i>cob</i> F ^F <i>cob</i> F ^R	AACATTCAGCAAACAAAGC TTGTCATAAAGAGTGGTCAT	1405	(5)
13	<i>agg</i> AF ^F <i>agg</i> AR ^R	AAGAAAAAGTAGACCAAC AACGGCAAGACAAGTAAATA	1553	(5)

Table 2: PCR conditions for the detection of virulence genes, *Enterococcus* sp. and *E. faecalis* strains.

Action	PCR ¹			PCR ²		
	Temperature (°C)	Duration (min)	Cycle	Temperature (°C)	Duration (min)	Cycle
Initial denaturation	95	5	1	95	5	1
Denaturation	95	0.5		95	0.5	
Annealing	53	0.5	30	57	0.5	30
Extension	72	1		72	1	
Elongation	72	15	1	72	15	1
Cooling	4	infinite		4	infinite	

¹ *Enterococcus* sp., *E. faecalis*, *gelE*, *cy*L^A, *cc*F, *Efa*A^{Fs}, *cy*L^M, *cp*d, *cy*L^B, *eep*, *cob*

² *esp*, *aggA*

and *aggA* could not be obtained and for this study *esp* and *aggA* positive PCR amplicon was sequenced by a private company (Macrogen, Holland). The amplification products were analysed by electrophoresis on 1.5% agarose gel at 100 V for 30 min in Tris-acetate-EDTA buffer and revealed in ethidium bromide (20 µg/ml).

RESULTS

Isolation and identification

A total of 600 milk samples were taken from 38 farms and tested for presence of enterococci. Among samples tested a total of 96 (16.0%) *Enterococcus* spp. were detected biochemically.

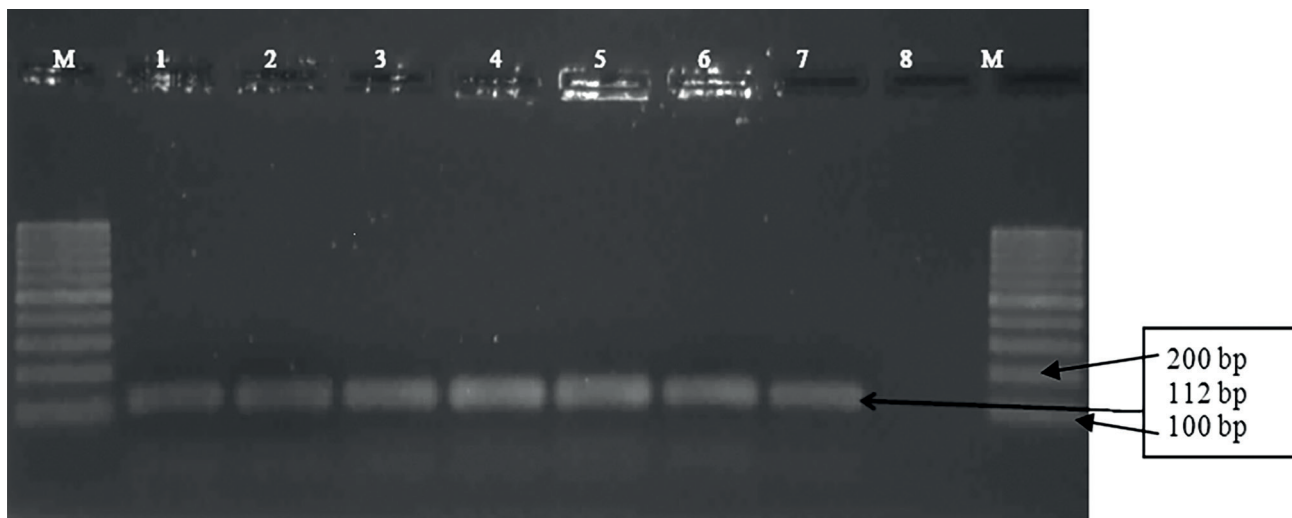


Figure 1: PCR detection of *Enterococcus* spp. isolated strains **1-6**: *Enterococcus* sp. field isolates **7**: Positive control (*E. faecalis* ATCC 29212) **8**: Negative control (*E. coli* ATCC 25922) **M**: 100 bp DNA ladder.

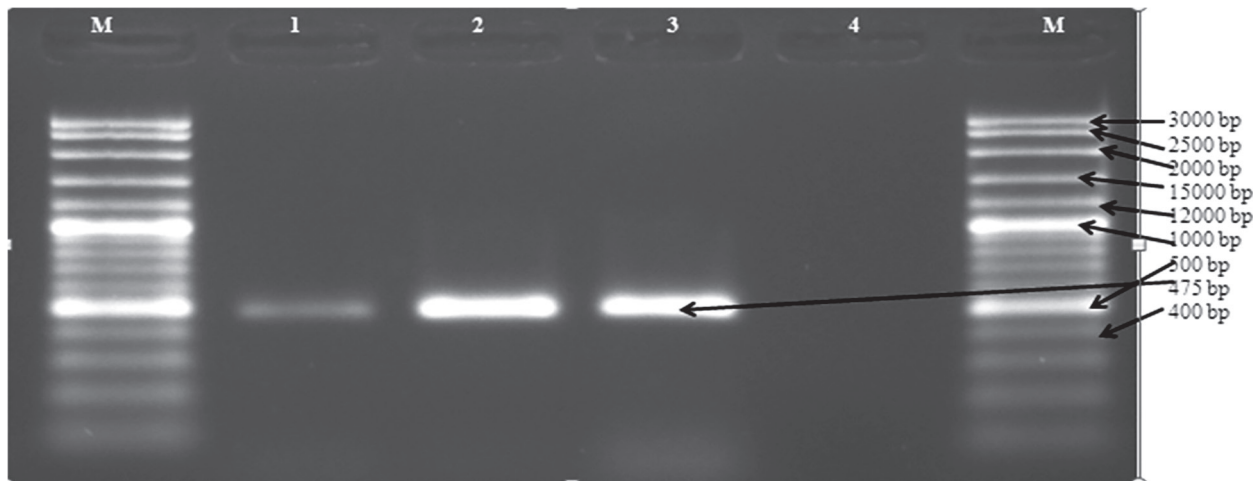


Figure 2: PCR detection of isolated *E. faecalis* strains **1-2**: *E. faecalis* field isolates **3**: Positive control (*E. faecalis* ATCC 29212) **4**: Negative control (*E. coli* ATCC 25922) **M**: 100 bp DNA ladder.

PCR

Of these 96 isolates, all of them were *Enterococcus* spp. PCR positive (Figure 1) and of these 56 samples (58.3%) were *E. faecalis* isolates (Figure 2).

The *E. faecalis* isolates were tested for the presence of eleven virulence genes. The *cylB* genes were not detected in any of the isolates. The frequency of the other ten virulence genes ranged in prevalence from 1.8% cytolysins (*cylA* and *cylM*) to 94.6% (*efaAfs*). The *efaAfs* gene was the most widespread virulence determinant. The second most frequently occurring virulence gene, *cpd*, was found in 91.0% the enterococci investigated followed by 87.5% (*gelE*),

51.7% (*esp*), 42.8% (*ccf*), 10.7% (*cob*), 8,9% (*eep*), 7.1% (*aggA*). Furthermore, multiple virulence genes co-existed in the *E. faecalis* isolates. One (1.8%) and two (3.6%) of the strains harboured eight and seven virulence determinants. All the isolates were carrying at least one virulence gene (Figure 3). The distribution of virulence genes is presented in Figure 4.

DISCUSSION

In this study, we have evaluated *E. faecalis* strains isolated from bovine milk and their presence of virulence genes was investigated by PCR. Enterococci can cause many economi-

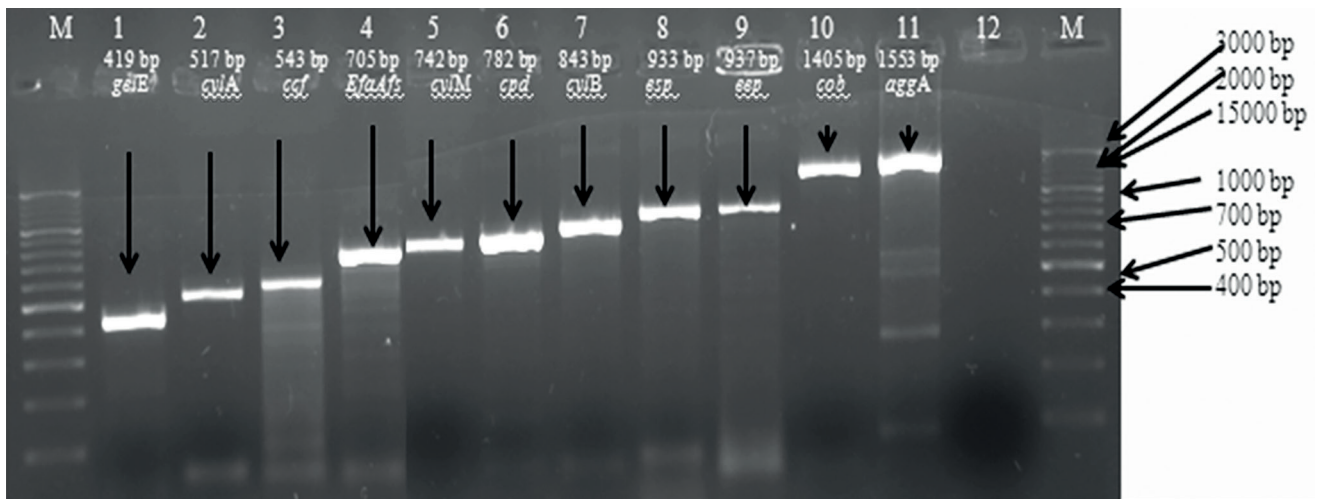


Figure 3: PCR detection of virulens genes. 1. *gelE* 2. *cylA* 3. *ccf* 4. *EfaAfs* 5. *cyIM* 6. *cpd* 7. *cyIB* (Positive control strain *E. faecalis* ATCC 29212) 8. *esp* 9. *eep* 10. *cob* 11. *aggA* gene positive isolates 12. Negative control (*E. coli* ATCC 25922) M: 100 bp DNA ladder.

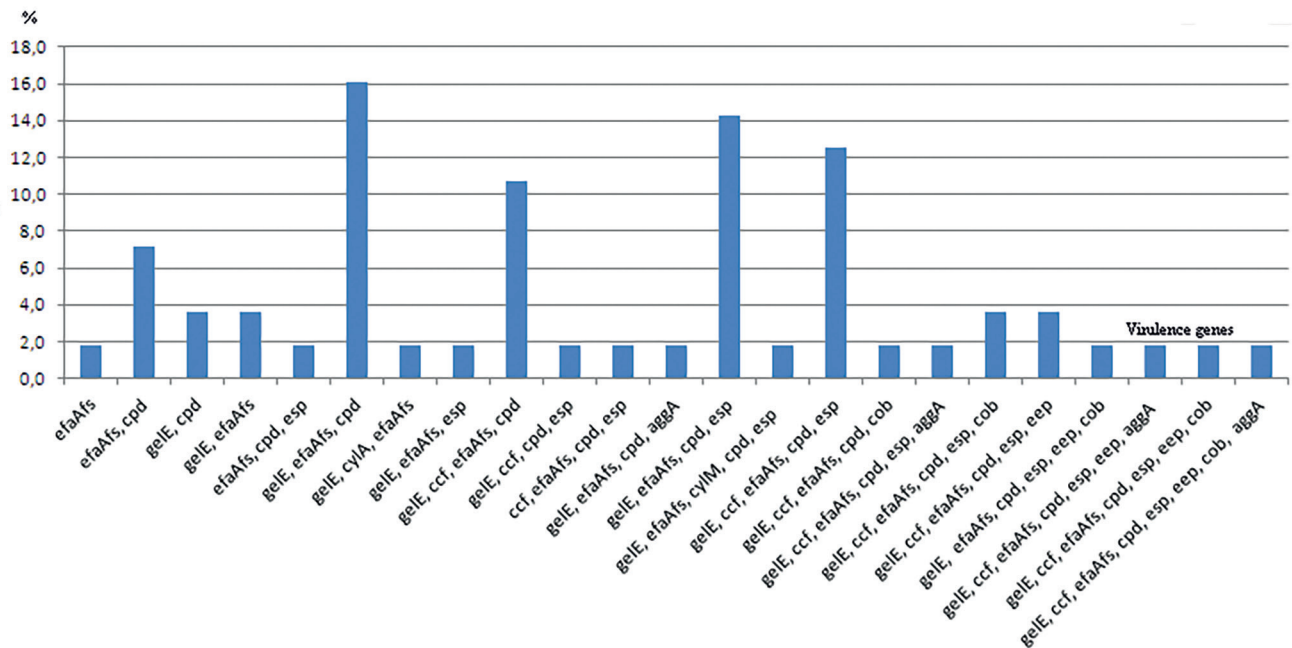


Figure 4: The distribution of virulence genes in *E. faecalis* strains isolated of mastitic bovine milk.

cally important animal diseases including bovine mastitis (2) and little information is available on enterococcal pathogens isolated from milk samples. In cases of mastitis where a causative agent has been identified, 0–21.2% of those was reportedly caused by enterococci (11, 13–15). *Enterococcus* spp. were isolated in 16% in the study. When we analysed the enterococci by species, *E. faecalis* was found to be the most prominent species. These results were similar to other

studies in which *E. faecalis* was reported as the most common isolated species (12, 16).

In this study, presence of virulence genes that encode *EfaAfs*, *cpd*, *cob*, *ccf*, *gelE*, *esp*, *aggA*, *eep*, *cylA*, *cyIM*, *cyIB*, was investigated by PCR. The adhesion-associated protein *EfaAfs* was present in 94.6% of all our isolates. This gene has been associated with endocarditis and is suspected to be involved in the surface adhesion mechanism in enterococci, and in

immune system evasion (6, 22). For *EfaAfs*, this was in agreement with a preceding study in which the gene was always found in medical *E. faecalis* isolates (5, 20, 22), whereas the majority (89%) of *E. faecalis* strains were found in food (5).

Sex pheromone genes (*cpd*, *ccf*, *cob*) and sex pheromone-related genes (*agg*, *eep*) are considered virulence determinants (23). While these genes are responsible for the conjugative transfer of sex pheromones plasmids, they can also be involved in the pathogenicity process as well. Among the sex pheromone genes in the study, *cpd* was the gene which had the highest frequency among the *E. faecalis* in our isolates, followed by *ccf* and *cob*. Previous studies reported frequent detection of these three sex pheromone genes in *E. faecalis* strains of various origins (5, 16, 22, 23). Both sex pheromone-related genes *eep* (coding for a protein enhancing the expression of pheromones) and *aggA* (coding for the aggregation substance) were detected in 8.9% and 7.1% in our isolates respectively. To the best of our knowledge, a few study examined the frequency of *eep* among *E. faecalis*, revealing that *eep* was present in more than half of clinical and environmental isolates (20, 21). The information related to the contribution of this virulence determinant is limited, but it may play a role in cow mastitis (24). Previous studies do not agree on the frequency of *aggA* among enterococci. Some studies have shown *aggA* in a high proportion among *E. faecalis* isolates from food and clinical origin (5, 23). In some of sex pheromone genes' positive strains, the *aggA* gene were also present (5).

The *gelE* gene, coding for an extracellular gelatinase, was also found to occur among mastitic bovine milk *E. faecalis* isolates in the study. This gene has been found previously in water, but also in food isolates (5, 20, 21, 25). The *esp* genes have been associated with urinary tract infections (15, 20). The *esp* gene has also been shown to occur with varying frequency in enterococci from other sources such as clinical, food and the environmental samples (5, 7, 22).

In the present study, frequency of cytolysin genes (*cytA*, *cytM*, and *cytB*) was low (1.8%, 1.8%, 0.0%, respectively). In a previous study (21), only 7 of 1558 isolates (0.4%) carried *cytABM* in environmental isolates. The frequency of the *cytA* gene among enterococci is highly variable and does not correlate with clinical or food isolates (25).

In conclusion, *E. faecalis* was the major enterococci species isolated from mastitic bovine milk samples in western of Turkey. Our results demonstrate that genes coding for

adhesion-associated protein, sex pheromones, gelatinase, enterococcal surface protein, enhanced expression of pheromone and aggregation substance can also occur in a high proportion of mastitic bovine milk isolates. The presence of these virulence determinants in *E. faecalis* strains may be significant in the evolution of pathogenic strains.

The findings of this study suggest that milk origin strains of *E. faecalis* may be potential risk factors for consumer health in terms of virulence genes. More detailed studies should be performed to investigate the status of virulence factors of other enterococci isolated from mastitic bovine milk in the veterinary field.

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