Ankara Üniv Vet Fak Derg, **61**, 133-140, 2014

The relationship between virulence factors and vancomycin resistance among Enterococci collected from food and human samples in Southern Turkey

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Summary: The aims of this research were to study the prevalence of potential virulence factors, vancomycin resistance and also to evaluate a possible correlation that can exist between vancomycin resistance and potential virulence factors between 51 *Enterococcus* spp. isolated from food and 50 *Enterococcus faecium* strains from human in southern Turkey. Identification of the isolates was determined by Vitek-II system. Antimicrobial susceptibility tests were performed by Vitek-II system and disc diffusion method. The presence of *vanA* and *vanB* as well as enterococcal virulence genes of cytolysin (*cylA*), the aggregation substance (*asa1*), gelatinase (*gelE*), enterococal surface protein (*esp*), hyaluronidase (*hyl*) were investigated by Polymerase Chain Reaction (PCR) method. Haemolysin production was also studied phenotypic method. Apart from one isolate, none of the food originated enterococci were resistant to vancomycin, and none carried *vanA* and *vanB* resistance genes. All clinical isolates were resistant to vancomycin and 84% of them carried *vanA*; 2%, *vanB;* and 14%, neither *vanA* nor *vanB* genes. Except for the *cylA* gene, all other virulence genes and vancomycin resistance were higher in human strains, and a positive correlation was observed between multivirulence genes and hemolytic activity. For all strains, a positive correlation existed between the *esp* gene positivity and vancomycin resistance, while for only *E. faecium, esp, hyl* gene positivity and vancomycin resistance a positive correlation could be seen. Furthermore, "silent *cylA*" genes were found in two food and one intestinal strains. Based on our findings, we can suggest that virulence increases in parallel to vancomycin resistance, and food may be a potential source for dissemination of *gelE*, *asa1* and *hyl* virulence genes. Finally, *esp* and *hyl* genes presence should carefully be monitored in food originated enterococci.

Key words: Enterococci, relationship, vancomycin resistance, virulence factors.

Türkiye'nin güneyinde gıda ve insan örneklerinden toplanan Enterokok izolatlarının vankomisin direnci ve virülans faktörleri arasındaki ilişki

Özet: Bu çalışmanın amaçları, Türkiye'nin güneyinde gıda kaynaklı 51 adet *Enterococcus* spp. ile insan kaynaklı 50 adet *Enterococcus faecium* izolatlarının potansiyel virülans faktörlerinin, vankomisin direnç özelliklerinin prevalansının belirlenmesi ve vankomisin direnci ile virülans faktörler arasında muhtemel ilişkinin değerlendirilmesidir. İzolatlarının identifikasyonu Vitek-II sistemiyle, antimikrobiyel direnç testleri Vitek-II sistemi ve disk difüzyon metodlarıyla çalışıldı. *vanA* ve *vanB* ile sitolizin (*cylA*), agregasyon faktörü (*asa1*), jelatinaz (*gelE*), entrokok yüzey proteini (*esp*), hiyolüronidaz (*hyl*), genlerinden oluşan enterokokal virülans genler Polimeraz Zincir Reaksiyonu (PCR) metoduyla, hemolizin üretimi fenotipik yöntemle çalışıldı. Bir izolat dışında, gıda izolatlarının hiçbiri vankomisine dirençli değildi ve hiçbiri *vanA* ve *vanB* geni taşımamaktaydı. Klinik izolatların tümü vankomisine dirençliydi ve bu izolatların %84'ü *vanA*, %2'si *vanB* genlerini taşımakta; %14'ü *vanA* ve *vanB* genlerini taşımamaktaydı. *cylA* geni dışındaki test edilen diğer virülans genler ve vankomisin direnci, klinik izolatlarda daha yüksekti ve çoklu virülans genler ile vankomisin direnci arasında pozitif bir korelasyon gözlendi. Tüm izolatlar arasında *esp* gen pozitifliği ile vankomisin direnci, *E. faecium* türlerinde ise *esp, hyl* gen pozitiflikleri ile vankomisin direnci arasında pozitif bir korelasyon olduğu görüldü. Ayrıca, iki gıda ve bir intestinal enterokok suşunda ''sessiz *cylA* geni'' bulundu. Bulgularımıza göre, virülans faktörler vankomisin direncine paralel olarak artabilir ve gıda kaynaklı enterokoklar *gelE*, *asa1, hyl* virülans genlerinin yayılmasında potansiyel kaynak olabilir. Sonuç olarak, gıda kaynaklı enterokoklarda *esp* ve *hyl* gen varlığı dikkatle izlenmelidir.

Anahtar sözcükler: Enterokok, ilişki, vankomisin direnci, virülans faktörler.

Introduction

Enterococci, widespread in nature, are part of the communal flora of gastrointestinal and genitourinary tracts of humans as well as digestive tract of animals. enterococci can also present in foods, especially in those of animal origin such as meat, fermented sausages and cheeses. Several studies have indicated that strains of enterococci may have beneficial effects on the

production and ripening of cheeses as they may contribute to cheese flavor and taste with their metabolic and technological traits. However, enterococci may cause spoilage problems in certain cheeses or processed meat products (12, 20). Although fermented foods with enterococci contribution have a long history of safe use, the presence of enterococci in foods is still an important issue for the food industry and public health.

In some reports, enterococci are considered as emerging pathogens of humans and are often noted as important agents of hospital acquired infections with an increasing mortality rate of up to 61%. The reason behind this rise is their ability to develop resistance against a wide variety of antibiotics, especially, glycopeptides (vancomycin and teicoplanin) (9). In general, vancomycin resistance is found in enterococci and encoded by the *vanA* gene cluster that carried on the mobile genetic element Tn1546. This resistance can be transferred by conjugative plasmids (9, 12, 20). Although vancomycin has been used in the treatment of enterococci for many years, recently, vancomycin resistant enterococci (VRE) strains have emerged worldwide as important nosocomial agents. This has posed a serious problem in the treatment of enterococcal infections and other Gram positive bacteria such as *Staphylococcus aureus* as enterococci have the ability to transfer resistant determinants to other vancomycin-susceptible species horizontally (20).

Several studies have shown that enterococci may harbour putative virulence traits and virulence genes such as production of aggregation substances (*agg*), gelatinase (*gelE*), hemolysin, adhesine (*ace*), the adhesinlike *E. faecalis* and *E. faecium* antigen A (*efaAfs and efaAfm*, respectively) and enterococcal surface protein (*esp*) (12, 15, 21). Eventually, enterococci are not considered as "generally recognized as safe" (GRAS) microorganisms in the food industry due to reasons such as; spoilage problems, biogenic amine production and acquired resistance problems in combination with the virulence factors mentioned above.

In this study, we investigated the prevalence of phenotypic and genotypic (*vanA* and *vanB* genes) vancomycin resistance and five virulence genes of enterococal surface protein (*esp),* gelatinase *(gelE),* aggregation substances *(asa1),* cytolysin production *(cylA),* hyaluronidase (*hyl)* genes and hemolysis in human blood among enterococci derived from food products and human clinical samples to evaluate a possible correlation between vancomycin resistance and potential virulence factors.

Materials and Methods

Materials: A total of 101 enterococci isolates were studied for the presence of virulence traits, vancomycin susceptibility and hemolytic activity. In this study, 51 *Enterococcus* spp., were isolated and identified from 80 food samples (*n*=28 cheese, *n*=26 fruit and vegetables *n*=21 sucuk and *n*=5 chicken meat) purchasing in Çukurova region from March to September 2010. Distribution of identified enterococci of food originated isolates; 24 from cheese samples, 10 from sucuk (a traditional Turkish meat product), 4 from raw chicken meat and 13 from fruit & vegetables foods. Clinical strains were collected over a period of 3 years (2010-2012) from clinical laboratories of the Balcalı Hospital Central Laboratory (Southern Turkey). A total of 50 clinical VRE strains, one isolate from each patient, were collected from infected hospitalized patients (n=25) and intestinally colonized hospitalized patients (n=25).

Isolation and identification of food and clinical enterococci: Isolation of food isolates was evaluated, as described, by Abriouel et al.(1). Further species level identification of isolates with a typical enterococci morphology on kanamycin esculin azide agar (KEA) (Merck KGaA, Germany) media were examined by biochemical tests such as catalase production, Gram staining, gas production from glucose and growing in 6.5% NaCl. Clinical isolates were routinely grown on Columbia agar (Becton-Dickinson, Sparks, MD) supplemented with 5% defibrinated sheep blood. For identification, cultures were examined with VITEK-2 automated identification system in the University central laboratory.

All identified strains (food & clinical) were stored in Brain Hearth Broth (BHI) including 10% of human blood and 10% of glycerol at -20ºC until PCR analysis.

Screening of enterococci for glycopeptide resistance and virulence genes: Genomic DNA was extracted mechanically by the "Mickle Sytem" (The Mickle Lab. Engeneering Co. Ltda, Gomshall, Surrey, UK) according to manufacturer's instructions after overnight cultures in 5% defibrinated sheep-blood agar of enterococci. A spectrophotometer (UV-VIS Spectrophotometer CHEBIOS) was used for quantitation of DNA samples (100ng/µL DNA for each sample). Extracted DNAs of enterococci were stored at -20ºC until to use as a template for PCR amplifications.

Multiplex PCR was performed to screen specific virulence genes (*esp, hyl, asa1, cylA* and *gelE*) and vancomycin resistance genes (*vanA*, *vanB*) as described previously (25, 5, 14). The specific primers and PCR conditions were presented in Table 1. Amplicons were analyzed by electrophoresis on 2% agarose gels [PegGOLD Universal Agarose, 91052 Ertangen Deutschland, 2%(w/v)] containing 0.5% ethidium bromide in TBE buffer (40mM tris, 20mM boric acid, 1 mM EDTA, pH 8.3) for 30/60 minutes at 120/150 V in the presence of 50-100bp DNA ladder (Fermentas SMo.323-Lithuania). The gel was photographed on an UV transilluminator (Kodak Gellogic-1500 imaging system).

Table 1. List of primers and amplification conditions used in the present study.
Tablo 1. Çalışmada kullanılan primerler ve amplifikasyon koşulları.

Gene	Primers sequence (5'-3')	Product size (bp)	Amplification conditions	Reference
gelE	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA	213	Initial cycle of 94ºC for 5 min.; 45 cycles of: 94ºC for 60 s, 48ºC for 80s,72ºC for 90 s;1 cycle: of 72ºC for 15 min.	(25)
hyl	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTCCCAA	276		
asa1	GCACGCTATTACCAACTATGA TAAGAAAGAACATCACCACGA	375		
esp	AGATTTCATCTTTGATTCTTGG AATTGATTCTTAGCATCTGG	510		
cylA	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	688		
vanA	TCTGCAATAGAGATAGCCGC GGAGTAGCTATCCCAGCATT	375	Initial cycle of 94ºC for 5 min; 30 cycles of 94ºC for 30 s, 48ºC for 30 s, 72ºC for 30 s; 1 cycle of 72ºC for 7 min.	(5)
vanB	TCTGCAATAGAGATAGCC GC GGAGTAGCTATCCCAGCA TT	527	Initial cycle of 94ºC for 5 min; 30 cycles of 94ºC for 40 s, 58ºC for 60 s, 72ºC for 30 s; 1 cycle of 72ºC for 30 min.	(14)

Assay of hemolytic activity: Production of hemolysis was determined by streaking the *Enterococcus* spp. cultures on Mueller Hinton Agar plates supplemented with 5% human blood. Plates were aerobically incubated at 37ºC for 18-24 hours and were examined for hemolysis. Presence of zone of clearing around the colonies was interpreted as beta-hemolysis (19).

Vancomycin susceptibility test: Vancomycin susceptibility of food originated enterococci was examined by disk diffusion method using the standard antibiotic discs with a concentration of 30 µg (Oxoid). Susceptibility or resistance was determined according to the recommendation of Clinical and Laboratory Standards Institute (CLSI) guidelines (7).

Vancomycin resistance patterns of clinical strains and confirmation for suspected results of food isolates were evaluated by using gram positive antibiotic susceptibility cards (Biomerieux Vitek-2-AST-P534-SA-France) in VITEK-2 automated identification system (Biomerieux, Durham, North Carolina, USA). The results were recorded after 18-24 h of incubation at 37ºC and evaluated by the manufacturer instructions using the breakpoints for enterococci proposed by the Clinical and Laboratory Standards Institute (7). *Enterococcus faecalis* 1047387 (vancomycin sensitive) and *Enterococcus faecium* 1045803 (vancomycin resistant) were used as reference strains in phenotypic antibiotic resistance tests.

Minimal inhibitory concentration (MIC) testing: For all (resistant or suspected) isolates, MICs of vancomycin was determined by VITEK-2 AST compact panel (Biomerieux Vitek-2-AST-P534-SA-France) and confirmed by E-test (Biodisk, Solana, Sweden) according to the CLSI's (7) guidelines.

Statistical analysis: Statistical analysis of data was performed with S-PLUS 18 statistical program (S-PLUS 18.00 for Windows, Professional Edition). Number and percentage of categorical measures were summarized. Chi-square test was used to compare categorical measures between groups (Fisher's exact test where appropriate). A *P* value of <0.05 was regarded as statistically significant.

Results

Among the food samples tested,a total of 51 isolates were identified as the following species; *E. faecium* (20 isolates), *E. faecalis* (12 isolates), *E. casseliflavus* (7 isolates), *E. gallinarum* (4 isolates), *E. durans* (7 isolates) and *E. raffinosus* (1 isolates). A total of 50 clinical enterococci (infection and intestinal isolates) were identified as *E. faecium*.

From a starting collection of 50 clinical isolates (corresponding to 25 patients with acute VRE infection and 25 patients without infection but with VRE colonization). A Total of 50 isolates were identified as *E. faecium.* Most of isolates (9; 36%), (2; 8%), (2; 8%), (2; 8%) and (10; 40%) in nosocomial environment were from intensive care internal medicine, pediatric hematology, urology, brain surgery, and other clinics respectively, while most of the intestinal isolates (15; 60%), (4; 16%), (4; 16%) and (2; 16%) were from pediatric hematology and oncology, brain surgery, intensive care and burn unit clinic respectively.

In the present study, 39 (76.4%) of food enterococci were found to be sensitive to vancomycin, and 11 (21.5%) of these were found intermediate sensitive to vancomycin. Only one strain identified as *E. casseliflavus* and collected from a lettuce sample was found to be resistant to vancomycin. Its MIC value was found to be 64 µg/mL. *E. faecium* strains from all clinical samples were found to be resistant to vancomycin and teicoplanin, and their MIC values were above 128 µg/mL.

Table 2. Occurrance of virulence determinants among enterococci isolates.
Tablo 2. Enterokok izolatları arasında virülans determinantların varlığı.

	Virülence Determinants					
N[a]	*asa1*	*esp*	*hyl*	*gelE*	*cylA*	*β-hemolysis.*
E.faecalis	5(41.7)	1(8.3)	7(58.3)	10(83.3)	1(8.3)	2(16.7)
E.faecium	22(31.4)	11(15.7)	22(31,4)	22(31.4)	6(8.6)	8(11.4)
E.gallinarum	1(25)	0	0	1(25)	0	0
E.casselliflavus	3(42.9)	0	1(14.3)	0	0	0
E.durans	5(71.4)	1(14.3)	1(14.3)	2(28.6)	1(14.3)	3(42.9)

a: Number of tested isolates; values in parenthesis indicate percentage

Table 3. The distribution of multi virulence genes of enterococci from different sources.
Tablo 3. Farklı kaynaklı enterokoklarda çoklu virülans genlerin dağılımı.

		Number of virulence genes					Total	*P* value
		0	1	2	3	4		
Food	N[a]	23	10	13	3	2	51	
	%	45.10	19.60	25.50	5.90	3.90	100.00	*P*<0.001
Human Clinical	N[a]	3	4	4	5	9	25	
	%	12.00	16.00	16.00	20.00	36.00	100.00	*P*<0.001
Human intestinal	N[a]	18	7	0	0	0	25	
	%	72.00	28.00	0.00	0.00	0.00	100.00	*P*<0.001
Total	N[a]	44	21	17	8	11	101	
	%	43.60	20.80	16.80	7.90	10.90	100.00	*P*<0.001

[a]: Number of isolates

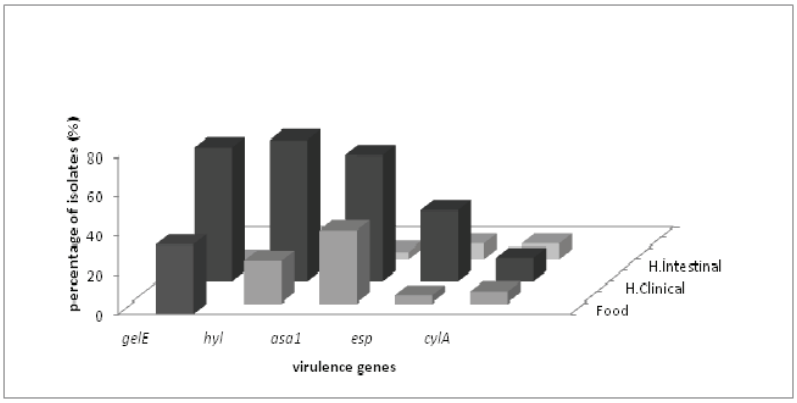

Figure 1. The distribution of five virulence genes of enterococci from food, human clinical and intestinal isolates.
Şekil 1. Gıda, klinik enfeksiyon ve kolonizasyon kaynaklı enterokoklarda *esp, hyl, gelE*, *asa1* ve *cylA* virülans genlerinin dağılımı.

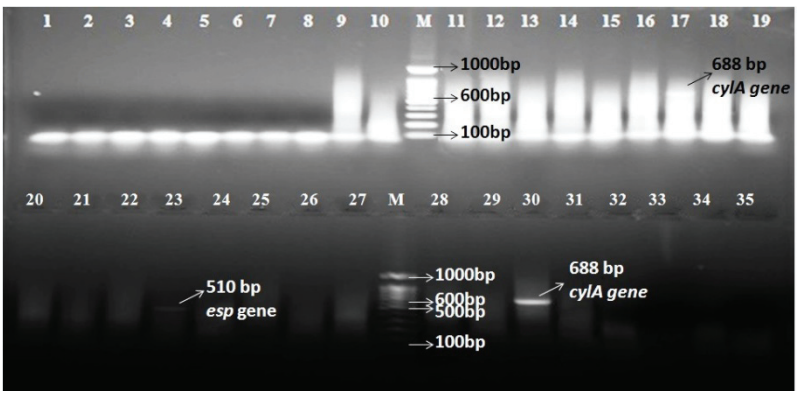

Figure 2. Ethidium bromide-stained agarose gel electrophoresis of PCR-amplified products of enterococci. Lanes **11** and **28:** 100 bp DNA markers; **17:** *cylA* positive *E. durans* (parmesan cheese) isolate; **30:** *cylA* positive *E. faecalis* (Urfa Cheese) isolate; **23:** *esp* positive *E. faecalis* isolate (home made sucuk). The other strains are negative.
Şekil 2. Enterokok izolatlarının PCR ürünlerinin etidyum bromür ile boyanmış agaroz jel elektroforezi ile görüntüsü. Sıra **11** ve **28:** 100 bp DNA cetveli; **17:** *cylA* pozif *E. durans* (kaşar peyniri) izolatı; **30:** *cylA* pozitif *E. faecalis* (Urfa Peyniri) izolatı; **23:** *esp* positive *E. faecalis* (home made sucuk) izolatı. Diğer isolatlar negatiftir.

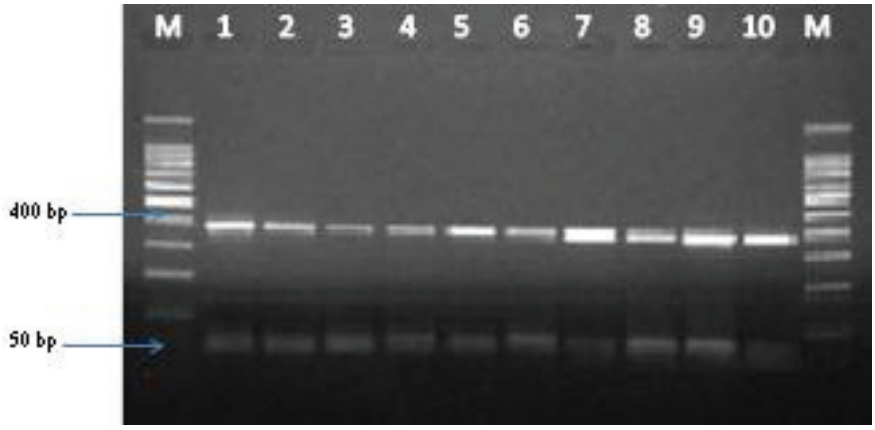

Figure 3. Agarose gel electrophoresis showing positive amplification of 375 base fragments specific for *vanA* of VR*E. Faecium* strains from clinical specimens. Lane M: Size marker (50-bp DNA ladder); lanes 1-10: VR*E. Faecium* strains
Şekil 3. Agaroz jel elektroforezi ile klinik kaynaklı VR*E. faecium* türlerinin 375 bp'de *vanA* gen fragmentleri görülmektedir. M: Moleküler belirteç (50-bp DNA ladder); **1-10:** VR*E. faecium* türleri.

Table 4.The correlation of vancomycin resistance and virulence genes of *E. faecium (n=70)* isolates in present study.
Tablo 4. Çalışmada izole edilen *E. faecium* izolatlarında (n=70) vankomisin direnci ile virülans genler arasındaki korelasyon.

		E. faecium				
	T[a]	*esp*	*asa1*	*hyl*	*gelE*	*cylA*
S+I[b]	20	0	5	2	5	1
R[c]	50	11	17	20	17	5
T[d]	70	11	22	22	22	6
P[e]		0.03[f]	0,57	0.02[f]	0.574	0.666

[a]. Total number of isolates
[b]. Isolates sensitive or intermediate sensitive to vancomycin
[c]. Isolates resistant to vancomycin
[d]. Total number of *E. faecium* isolates
[e]. *p* values between number of isolates and virulence genes obtained with fisher`s exact test.
[f]. Statistically significant correlation between vancomycin resistance and virulence gene

Table 5. The correlation of vancomycin resistance and virulence genes of all enterococci isolates.
Tablo 5. Tüm enterokoklarda vankomisin direnci ile virülans genler arasındaki korelasyon.

	T[a]	*esp*	*asa1*	*hyl*	*gelE*	*cylA*
S+I[b]	50	2	19	11	18	3
R[c]	51	11	17	20	17	5
T[d]	101	13	36	31	35	8
P[e]		0.02[f]	0.68	0.08	0.836	0.715

[a]. Total number of isolates
[b]. Isolates sensitive or intermediate sensitive to vancomycin
[c]. Isolates resistant to vancomycin
[d]. Total *Enterococcus* spp. isolates
[e]. p values between number of isolates and virulence genes obtained with fisher`s exact test
[f]. Statistically significant correlation between vancomycin resistance and virulence gene

The prevalence of genes encoding vancomycin resistance in enterococci; none of food isolates carried *vanA* and *vanB* resistance genes and 84% of clinical enterococci carried *vanA*, 2% of them *vanB* and 14% them neither carried *vanA* nor *vanB* genes.

Hemolytic activities of food enterococci were found 32 (62.7%) non-hemolytic, 13 (25.4%) α-hemolytic, 6(11.7%), β –hemolytic, respectively. A total of 16(64%) human intestinal isolates were found α-hemolytic and 9(36%) were non-hemolytic; but 50% of clinical *E. faecium* isolates were found to be α-hemolytic and 14% were β-hemolytic.

In present study, *E. faecium* carried more virulence genes than *E. faecalis* and other *Enterococcus* spp. Occurrence of virulence determinants among enterococcal isolates were presented in Table 2. In addition, the presence of strains harbouring multiple virulence genes is shown in Table 3. A summary of the prevalence of the virulence genes among the *Enterococcus* spp. and their origin is shown in Figure1. The gel image of some virulence genes of different reservoirs were also shown in Figure 2. The gel image of *vanA* genes of clinical isolates were presented in Figure 3.

A summary of correlations between virulence factors and vancomycin resistance is presented in Tables 4 and 5.

Discussion and Conclusion

In previous studies, antimicrobial resistance and virulence were characterized in enterococci isolates from food, but some researchers have observed a correlation between the presence of antimicrobial resistance and of virulence determinants (10, 11, 13, 15, 17). Few studies have been conducted on resistance, virulence determinants and/or correlation of virulence and resistance in enterococci of food and clinical origins in Turkey (4, 18). In this study, vancomycin resistance and five virulence profiles of *Enterococcus* spp. isolated from food and clinical samples were characterized, and correlation between resistance to vancomycin was investigated.

Literature suggests that *E. faecalis* and *E. faecium* have significant differences in terms of incidence of virulence factors as follows: (i) Franz et al., (11) reported that 10.4% of *E. faecium* strains were positive for one or more virulence determinants compared to 78.7% of *E. faecalis* strains, (ii) *E. faecalis* strains tested by Eaton and Gasson (10) possessed multiple determinants (between 6 and 11) and (iii) Vankerckhoven et al., (26) have concluded that *E. faecium* strains were generally free of virulence factors. By contrast, in our study, *E. faecium* strains had significantly more virulence determinants than *E. faecalis* strains and possessed multiple determinants (between 3 and 4 virulence genes), and were found with lower incidence (between 2, 3, and only one strain from Urfa cheese included 4 virulence genes) of multiple virulence determinants ($p<0.05$).

After multiplex-PCR amplification of selected virulence genes among 51 food and 50 human originated strains, possessing suspected virulence genes (*esp, hyl, gelE, asa1* and *cylA*) were more frequent among human clinical infection isolates than food and human intestinal isolates (Figure 1). There was a significant difference in occurrence of genes except for the *cylA* gene between human clinical, food and human intestinal isolates ($p<0.001$). Eaton and Gasson, (10) have reported that enterococci isolated from food sources have fewer virulence genes than those of clinical sources in accordance with our results.

Aggregation Substance (AS) is a pheromone-inducible surface protein of *E. faecalis* promoting the mating aggregate formation during bacterial conjugation (24). The *asa1* gene was very common within *E. durans* (71.4%) and 42.9% of *E. casseliflavus* isolates from fruit and vegetables. In addition, 31.4% of *E. faecium* strains were collected from human samples. These results differ from those of previous studies reporting the absence or low rates of *asa1* genes among *E. faecium* and *E. faecalis* strains (1, 17, and 26).

The enterococcal gene *esp*, encoding the high-molecular-weight surface protein, has been detected widely in enterococci isolated from patients with infections such as bacteremia or endocarditis isolates, yet is rare in stool isolates from healthy individuals (2, 21). The contribution of the surface protein *esp* in terms of colonization and persistence of *E. faecalis* in urinary tract infections has been shown in an animal model (22). The highest *esp* gene rate was detected in 15.7% (11/70) of *E. faecium* strains. Our clinical *E. faecium* isolates, harbouring high rates of the *esp* gene, were urinary tract infection agents, and the intestinal *E. faecium* isolates were found to have *esp* genes with very low rates (1.9%). These results support the findings in the aforementioned studies (2, 21).

Gelatinase activity, involved in the hydrolysis of gelatine, collagen and some other proteins, was most frequent in *E. faecalis* (83.3%) and *E. faecium* (31.4%) strains in our study. *gelE* gene positive strains occurred with statistically significant differences among food and clinical isolates ($p<0.005$). In our study, *gelE* presence of *E. faecalis* was found to be 100% among vegetable food isolates. These results are higher than those reported by Abriouel et al. (1), reporting that *gelE* presence in *E. faecalis* stains from vegetable food isolates was 76.5%; in addition, *E. faecium* in which *gelE was* present was found to be higher in our clinical isolates (18/50; 72%). These findings differ from those of Abriouel et al. (1) who reported no relation of *E. faecium* isolates for the *gelE* gene in their research.

Hyaluronidase, encoded by the *hyl* gene, acts on hyaluronic acid (hyaluronate, hyaluronan); is mainly a degradative enzyme associated with tissue damage (12). The *hyl* gene was identified within 58.3% of *E. faecalis* and 31.4% of *E. faecium* strains. This gene was identified in each of seven strains of *E. casseliflavus* and *E. durans* isolated from food. These values are higher than those mentioned in a study conducted by Vankerckhoven et al. (26) who found *hyl* gene presence in only one fecal isolates but none of probiotic and nonfecal isolates.

In the present study, *gelE* and *hyl* gene positivity was found significantly higher in *E. faecalis* isolates ($P<0.001$); and *hyl*, *asa1* and *gelE* gene positivity was observed to be equal in *E. faecium* isolates. With the rates of 35.6 %, 34.6 %, respectively, *asa1* and *gelE* were the most frequent virulence genes. According to a recent study in Turkey, Baylan et al. (4) reported that *hyl* gene positivity was found higher in *E. faecium* isolates ($P<0.01$). There were no enterococci strains including five virulence genes. Similarly, Baylan et al. (4) found no more than 4 virulence genes. Our values are higher than those of Baylan et al. (4) as we found that the *hyl* and *asa1* genes had the same ratio, and the *esp* gene was found in half of the urinary infection strains (7/12). Furthermore, in Baylan et al.`s study (4) *asa1* and *esp* were the most frequent virulence factors with rates of 26.7% and 25.6% respectively, while there were 32 (35.6%) strains without any of the investigated virulence factors. In our study, only two urine isolates (16%) had no virulence genes.

cylM, *cylB* and *cylA* genes are responsible for modification, secretion and activation of cytolysin. In our study, the *cylA* gene was identified in only 14.3% (1/7) of *E. durans*, 8.6% (6/70) of *E. faecium* and 8.3% (1/12) of *E. faecalis* strains with no significant differences among the three groups. With a range of 9-44% among *E. faecalis* isolates, our results are lower than those obtained in previous studies (10, 17). *E. casseliflavus*, *E. gallinarum*, and *E. raffinosus* from fruit and vegetables isolates were also found free from the *cylA* gene in the present study. These results are similar to previous reports of Abriouel et al. (1) regarding the absence of the *cylA* gene among vegetable food isolates. High rate of *asa1* and *cylA* genes among our *E. durans* isolates is a

worrying output as there are some reports suggesting that the presence of virulence traits in species other than *E. faecalis* and *E. faecium* is important for proper evaluation of virulence evolution within the *Enterococcus* genus (16, 23). No significant difference was observed between *cylA* gene positivity and β-hemolytic activity in our study.

Expression of gelatinase and cytolysin aggregation substances of enterococci may vary between in-vivo and in-vitro as it is well known that these genes have the ability to remain silent; thus, it is so important to take this into consideration during laboratory detection (8, 10, 23). In our study, two of the non-hemolytic *E. faecium* and one non-hemolytic *E. faecalis* strains had the "silent *cylA* gene", a finding parallel to a previous study conducted by Eaton and Gasson (10), in which the researchers found two *E. faecalis* strains to have no hemolytic activity, yet carried *cyl* genes. Similar results were also observed by Barbosa et al. (3). This is possibly the first study reporting on "silent *cylA* genes'' among Turkish food and clinical isolates. The occurrence of β-hemolysis was identified only within 42.9% of *E. durans*, 16.7% of *E. faecalis* and 11.4% of *E. faecium* strains with no significant difference among food and human isolates. These results are similar to those emerged in previous studies showing a high incidence of β-hemolysis among *E. faecalis* strains (10, 11).

In some studies, the presence of *vanA*, including one or more virulence genes in the same genome, have favored particular clusters of *E. faecium* in clinical origin (6). Similar results were observed in our clinical infection isolates as well (Table 2 and 3). The vancomycin resistance patterns of the two groups of strains were very different with a significantly higher rate of resistance among the enterococci of human origin ($p<0.001$). While only one *Enterococcus casseliflavus* from lettuce was resistant to vancomycin, yet carrying neither *vanA* nor *vanB* genes, and with no occurrence of vancomycin resistant strains among cheese, raw chicken meat, sucuk, fruit or vegetable isolates confirms previous studies with low rates of vancomycin resistance among food enterococci (1, 15, 17) suggesting that food enterococci cannot be considered the main potential sources for dissemination of vancomycin resistance. On the other hand, Finding of 7 (14%) clinical *E. faecium* strains with non *vanA* and non *vanB* genotype, but resistant to vancomycin show that some polymorphism might occur in detection of resistance genes or other resistant genes (*vanC, vanD,...*etc.) might also be important in the vanA phenotype of VRE isolates in present study.

The relationship between antibiotic resistance and virulence genes of enterococci may vary from country to country. In a study by Vankerchoven et al. (26) covering a large geographical area, Italy and England, *esp* gene positivity was found to be 68% among vancomycin sensitive (VS) *E. faecium* isolates and 91% among vancomycin resistant (VR) *E. faecium* isolates ($p<0.05$) in Italy, and in England, *hyl* gene positivity was found to be 29% in VS *E. faecium* isolates and 71% in VR *E. faecium* isolates ($p<0.05$). In this study, we found *esp* gene positivity to be 15.7% in VR *E. faecium* isolates and *hyl* gene positivity, 28.6% among VR *E. faecium* isolates, and 2.9% among vancomycin sensitive and intermediate *E. faecium* isolates (Table 4; $p=0.03$, $p=0.02$ respectively). Similarly, among all enterococci isolates, *esp* gene positive isolates were found to be more resistant to vancomycin than sensitive and intermediate isolates (Table 5; $p= 0.02$). A significant correlation was also observed within vancomycin resistance and virulence genes of *esp* and *hyl* in clinical *E. faecium* strains. These results correlate with those obtained by Vankerchoven et al. (26), yet differ from those of Baylan et al (4), who found a correlation with resistance to different antibiotics and their *asa1*, *esp* virulence genes of enterococci, and no correlation between vancomycin resistance and virulence factors of clinical enterococci.

It is interesting to note that the co-existence of virulence genes such as *esp+hyl* and *gelE+esp+asa1* was found to be a contrubiting factor to vancomycin resistance in our study. There was also a correlation between the number of virulence genes and vancomycin resistance ($p<0.05$).

This study showed that foodborne enterococci can harbor potential virulence genes but not *vanA* and *vanB* resistance genes, and may rarely be resistant to vancomycin. While with variable frequencies, virulence determinants, mainly associated with species of *E. faecium*, *E. faecalis* and *E. durans,* were more common among cheese, sucuk and raw chicken meat isolates than those among human isolates. *E. faecalis*, *E. faecium* and *E. durans* strains from food may be potential sources for dissemination of virulence genes (especially *asa1*, *gelE* and *hyl*). A positive correlation was found between the number of virulence genes and vancomycin resistance as well as beta hemolytic activity. Positive correlation of vancomycin resistance and *esp* gene positivity observed in all enterococci and *esp*, *hyl* gene positivity in *E. faecium* (especially clinical) isolates yield significant data in this region of Turkey. This is possibly the first study to investigate the relationship between vancomycin resistance and virulence factors among *Enterococcus* spp. isolates originated from various food and clinical samples in Turkey. Further studies of more enterococci isolates of food and human origin with more virulence genes and antibiotics covering wider geographical areas are needed to better aid the food industry and contribute to clinical microbiology.

Acknowledgments

This study was supported by Çukurova University Research Fund (BAP Project number: *ZF2010D12*). Some part of this study was presented as a poster with "l12" code in European Biotechnology Congress 2011 (Istanbul, Turkey). Some part of this study was presented as a poster with "abstract cod.: 8" in International

Conference on Environmental, Industrial and Applied Microbiology- BioMicroWorld2013, 257p/734p, Madrid (Spain), 2-4 October.

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Geliş tarihi: 28.06.2013 / Kabul tarihi: 20.01.2014

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