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Molecular Identification of Vancomycin Resistance and Virulence Genes in Foodborne Enterococci

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ABSTRACT. The study was performed to determine the presence of vancomycin phenotyping genes and some virulence traits in enterococci species. For this purpose, a total of 42 enterococci including 6 vancomycin-resistant and 36 vancomycin-susceptible strains originated from meat/meat products and milk/dairy products were assessed for the *van*A, *van*B and *van*C genes and *agg, esp, gelE, ace* and *efa*A virulence genes by using polymerase chain reaction or multiplex polymerase chain reaction. The *van*A gene was found in 12% (n=5) of the strains and *van*C gene in 50% (n=21). From these, three *van*A- (*E. faecalis, E. durans, E. casseliflavus*) and two *van*C-positive (*E. durans*) strains had a minimum inhibitory concentration of > 256 µg/ml as previously determined with the E-test. The strains expressing vancomycin susceptibility originating from ready-to-eat food were found to carry *van*A (n=1) and *van*C (n=5) genes. On the other hand, the *van*B gene was not detected among strains. Moreover, no strain was found to harbor virulence traits studied. Our results indicated that resistant or susceptible enterococci from foods of animal origin can be a possible reservoir for resistance genes and may have a potential role for transfer of genetic elements among enterococci or to other bacteria. Furthermore, to develop epidemiological surveillance systems for foodborne antibiotic resistant pathogens as vancomycin-resistant enterococci and their genes responsible for resistance, primarily *van*A, *van*B, continues to be an essential issue all around the world. The present work provides data for foodborne enterococci isolates harboring *van*A gene from Turkey.

Keywords: enterococcus, food, vanA, vanC, vancomycin

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INTRODUCTION

ntibiotic resistance among the microorganisms And emergence of resistance is an ancient phenomenon. Glycopeptide (vancomycin) resistance gene vanA was detected from 30 000 years old permafrost sample in the Yukon (Canada) and its similarity to modern variants was clearly evidenced. As a result, antibiotic resistance is accepted as a natural phenomenon (D'Costa et al., 2011). In recent times, antibiotic resistant pathogens have become significant public health threat worldwide. The occurrence and spread of vancomycin resistant enterococci (VRE) is another concern because enterococci species are responsible for most of nosocomial infections (Oravcova et al., 2016). In particular, E. faecalis and E. faecium are the third and fourth prevalent hospital environment acquired pathogens all around the world. Enterococci demonstrate resistance to many different antibiotics, most particularly resistance to glycopeptides. Vancomycin is more important than other antibiotics since it is more frequently used to treat most of Gram-positive bacterial infections. Nine vancomycin resistance genotypes were detected in enterococci (vanA, vanB, vanC, vanD, vanE, vanG, vanL, vanM and vanN) (Werner, 2012). E. gallinarum and E. casseliflavus strains have vanC genotype associated with intrinsic vancomycin resistance (Gousia et al., 2015). Strikingly, vanA, vanB, vanG, vanN and vanM genotypes are genetically located on plasmid or chromosome and they can be transferred to other species and/or bacteria (Cattoir and Leclercq, 2013). VRE infections, especially caused by high-level resistant enterococci carrying vanA and/or vanB genotypes, can only be treated with a few numbers of effective medical agents. Therefore, they are accepted as one of the clinically important antimicrobial resistant pathogens. The vanA-type vancomycin resistance is very common among the enterococci and the encoding gene has been primarily identified in E. faecalis, E. faecium and secondly E. durans, E. hirae, E. gallinarum, E. casseliflavus, E. raffinosus, E. avium, E. mundtii, E. cecorum (Harada et al., 2012).

Together with *E. faecium* and *E. faecalis*, the incidence of other enterococci species isolated from patients display an alarming increase. This is mainly correlated with their increased putative virulence traits and multiple antibiotic resistances (Biswas *et al.*, 2016). The presence of virulence factors in enterococci gives them different roles both as commensal and as pathogen bacteria for human health (Farahani, 2016). Enterococcal virulence factors are divided into two groups; promot-

ing colonization traits such as aggregation substance (*aga*), collagen binding protein (*ace*), endocarditis specific antigen (*efaA*), surface protein (*esp*) and affecting tissues such as cytolysin (*cyl*), gelatinase (*gelE*), hyaluronidase (*hyl*). In addition to these, sex pheromone genes (*cpd*, *cob*, *ccf*, *cad*) work together with other virulence genes help to trigger infection reactions (Chajecka-Wierzchowska *et al.*, 2017). Animal originated food isolates of enterococci may harbor many of above mentioned virulence genes thus, these foods may play important role as potential source for human infections (Yilmaz *et al.*, 2016).

The goals of this study were to investigate the vancomycin resistance profile, to determine the presence of virulence genes in vancomycin-resistant/susceptible enterococci from food of animal origin, and to raise public awareness about the possible health risks.

MATERIALS AND METHODS

Strains

Bacterial strains were from the Food Hygiene and Technology Department collection, Veterinary Faculty. A total of 42 strains consisting of 36 *E. faecium*, 4 *E. avium* and 2 *E. gallinarum* were selected among enterococcci collected from foods of animal origin between September and December 2011 from different cities (Istanbul, Bursa, Yalova, Balikesir) in Marmara Region. API identification and vancomycin/ teicoplanin MIC's results (Cetinkaya *et al.*, 2013) of the selected strains are summarized in Table 1. Stock cultures were kept frozen (-20°C) in Brain Heart Infusion broth (Oxoid CM1135, England) containing 20% (v/v) glycerol. The cultures were activated in Brain Heart Infusion broth at 37°C.

PCR confirmation of strains and Determination of vancomycin resistance and virulence genes

Total DNA from bacterial strains was extracted by using Chelex 100 (Sigma Aldrich, USA). The PCR was processed in a ThermoCycler (Runik, SCM 96G). Each 25 μ l reaction mixture consisted of 1 μ l template DNA, 1.25 U of Hot Start Taq DNA polymerase (Bioron, Germany), 10 mM of Tris-HCl pH 8.9, 22 mM of KCl, 1.8 mM of MgCl₂ (Fermentas, USA), 200 μ M of dNTPs (Biolabs, UK) and 0.5 mM of each primers (Sentegen, Turkey). The PCR method was used to confirm previously API identified strains at the genetic level by using species-specific primers (*E. faecium, E. faecalis, E. durans, E. gallinarum, E. casseliflavus, E. avium*) and to investigate the presence of *vanA*, *vanB* and *vanC* resistance genes, and the virulence trait genes *agg*, *esp*, *gelE*, *ace* in all strains as well as $efaA_{fs}$, $efaA_{fm}$ in *E. faecalis* and *E. faecium*. The primers, their sequences, products sizes and amplification procedures for PCR conditions are presented in Table 2. The PCR products were electrophoresed (Thermo Scientific EC300XL, USA) on 3% agarose gel (Biomax, Dubuque, USA) and visualized (BioRad Gel DocXR+, USA) by ethidium bromide staining.

 Table 1. Description of the strains used in this study and the results of screening for vancomycin resistance genes.

No.	Source	API Identification ^a	PCR Identification	Vancomycin MIC's ^a (ug/ml)	Teicoplanin MIC's ^a (ug/	Genes
110.					ml)	Genes
1	Meatball	E. gallinarum	E. casseliflavus	> 256	> 256	vanA
2	Meatball	E. avium	E. durans	> 256	> 256	vanA
3	Meatball	E. faecium	E. faecium	<4	≤ 8	vanC
4	Meatball	E. faecium	E. faecium	<4	≤ 8	vanC
5	Meatball	E. avium	E. durans	> 256	> 256	vanC
6	Meatball	E. avium	E. durans	> 256	> 256	vanC
7	Meatball	E. feacium	E. faecalis	> 256	> 256	not detected
8	Minced meat	E. faecium	E. faecium	4	≤ 8	vanA
9	Minced meat	E. faecium	E. faecium	<4	≤ 8	vanC
10	Minced meat	E. faecium	E. faecium	<4	<u>≤ 8</u>	vanC
11	Minced meat	E. faecium	E. faecium	<4	<u> </u>	vanC
12	Minced meat	E. faecium	E. faecium	<4	<u>≤</u> 8	not detected
13	Minced meat	E. faecium	E. faecium	<4	<u> </u>	not detected
14	Minced meat	E. faecium	E. faecium	<4	<u>< 8</u>	not detected
15	Beef	E. avium	E. faecalis	> 256	> 256	vanA
16	Beef	E. faecium	E. faecium	<4	< 8	vanC
17	Beef	E. faecium	E. faecium	<4	< 8	vanC
18	Beef	E. faecium	E. faecium	<4	< 8	not detected
19	Lamb	E. faecium	E. faecium	<4	< 8	vanC
20	Lamb	E. faecium	E. faecium	<4	< 8	not detected
21	Salami	E. faecium	E. faecium	<4	< 8	not detected
22	Raw cow's milk	E. faecium	E. faecium	<4	< 8	vanC
23	Raw cow's milk	E. faecium	E. faecium	<4	< 8	vanC
24	Raw cow's milk	E. faecium	E. faecium	<4	< 8	vanC
25	Raw cow's milk	E. faecium	E. faecium	<4	< 8	vanC
26	Raw cow's milk	E. faecium	E. faecium	<4	< 8	vanC
27	Raw cow's milk	E. faecium	E. faecium	<4	< 8	not detected
28	Raw goat's milk	E faecium	E faecium	<4	< 8	vanC
29	Raw goat's milk	E faecium	E faecium	<4	< 8	not detected
30	Village cheese	E faecium	E faecium	<4	< 8	vanA
31	Urfa cheese	E gallinarum	E gallinarum	<4	< 8	vanC
32	Kashar cheese	<u> </u>	E faecium	<4	< 8	vanC
33	Cottage cheese	E faecium	E faecium	<4	< 8	vanC
34	Mihalic cheese	E faecium	E faecalis	<4	< 8	not detected
35	Anten cheese	E faecium	E faecium	<4	< 8	not detected
36	Cottage cheese	E. faecium	E faecium	<4	< 8	not detected
37	Kashar cheese	E faecium	E faecium	<4	< 8	not detected
38	Cottage cheese	E. faecium	E faecium	<4	< 8	not detected
39	White cheese	E faecium	E faecium	<4	< 8	not detected
40	Butter	E. faecium	E. faecium	<4	< 8	not detected
41	Butter	E. Juccium	E. juccium	×۳ <1	< 8	vanC
42	Butter cream	E. Juccium	E. juccium	<u></u>	<u> </u>	vanC
74	Butter creatin	јисстит	<u>.</u> . јисстит	т^	<u> </u>	vunC

^apublished in elsewhere (Cetinkaya et al., 2013)

Gene	Oligonucleotid sequences (5'-3')	Product size (bp)	Amplification procedure	
fcm	GAAAAAACAATAGAAGAATTAT	215		
Jem	TGCTTTTTTGAATTCTTCTTTA	215	_	
fls		360	An initial cycle of: 95°C for 4 min.	
			- followed by 30 cycles of: 95°C for	
dur		295	$20 = 55^{\circ}C$ for 1 min 72°C for 1 min and	
	TTACTTGCTGATTTTGATTCG	173		
gal	TGAATTCTTCTTTGAAATCAG			
	TCCTGAATTAGGTGAAAAAAC	288		
cas	GCTAGTTTACCGTCTTTAACG			
avi	GCTGCGATTGAAAAATATCCG	368		
	AAGCCAATGATCGGTGTTTTT	500		
		a 1030	An initial cycle of: 94°C for 5 min,	
vanA			followed by 30 cycles of: 94°C for	
			1 min, 54°C for 1 min, 72°C for 1 min, and	
	CCCCTTTAACGCTAATACGATCAA		final cycle 72°C for 10 min	
			(Evers et al., 1993)	
		433	An initial cycle of: 94°C for 5 min,	
			followed by 30 cycles of: 94°C for	
vanB	GTGACAAACCGGAGGCGAGGA		$1 \min 54^{\circ}C$ for $1 \min 72^{\circ}C$ for $1 \min$ and	
	CCGCCATCCTCCTGCAAAAAA		final axis $72^{\circ}C$ for 10 min	
			(Handwarger et al. 1002)	
		822	(Haldweiger <i>et ut.</i> , 1992) An initial cycle of: 04° C for 5 min	
C	GGTATCAAGGAAACCTC		followed by 30 cycles of: 94°C for	
vanC	CTTCCGCCATCATAGCT		1 min, 54°C for 1 min, 72°C for 1 min, and	
			final cycle 72°C for 10 min	
			(Dutka-Malen <i>et al.</i> , 1995)	
	TTACCAAGATGGTTCTGTAGGCAC	432	$30 \text{ cycles of: } 94^{\circ}\text{C} \text{ for } 30 \text{ s},$	
esp	CCAAGTATACTTAGCATCTTTTGG		58°C for 30 s, 72°C for 30 s	
			(Shankar <i>et al.</i> , 1999)	
	AAAGTAGAATTAGATCCACAC	822 432 320 402	33 cycles of: 94°C for 1 min,	
асе	TCTATCACATTCGGTTGCG		56°C for 1 min, 72°C for 1 min	
			(Mannu <i>et al.</i> , 2003)	
	AGTTCATGTCTATTTTCTTCAC		$30 \text{ cycles of: } 94^{\circ}\text{C} \text{ for } 30 \text{ s},$	
gelE	CTTCATTATTTACACGTTTG	402	56°C for 30 s, 72°C for 30 s	
			(Mannu <i>et al.</i> , 2003)	
	AAGAAAAAGAAGTAGACCAAC	1553	$30 \text{ cycles of: } 94^{\circ}\text{C} \text{ for } 30 \text{ s},$	
agg	AAACGGCAAGACAAGTAAATA		58° C for 30 s, /2°C for 30 s	
			(Eaton and Gasson 2001) An initial cycle of: 94° C for 2 min	
		705	52°C for 2 min 72°C for 2 min, followed by	
$efaA_{fs}$	GACAGACCCTCACGAATA		$27 \text{ cycles of: } 94^{\circ}\text{C} \text{ for}$	
	AGTTCATCATGCTGTAGTA		15 s. 52°C for 15 s. 72°C for 15 s	
			(Eaton and Gasson 2001)	
		735	An initial cycle of: 94°C for 2 min,	
$efaA_{fm}$			52°C for 2 min, 72°C for 2 min, followed by	
	ΑΛΟΛΟΛΙΟΟΟΛΙΟΛΛΙΑ ΓΑΤΤΤΟΑΤΟΑΤΟΤΘΑΤΔΩΤΛ		27 cycles of: 94°C for	
			15 s, 52°C for 15 s, 72°C for 15 s	
			(Eaton and Gasson 2001)	

 Table 2. Oligonucleotide primer sequences and amplification conditions.

Gene	Numbers of <i>van</i> A, <i>van</i> B and <i>van</i> C positive strains (%)	Strains	Vancomycin/teicoplanin MIC's (μg/ml)
		E. casseliflavus (n=1)	> 256 / > 256
•	5 (11.9%)	<i>E. durans</i> (n=1)	> 256 / > 256
vanA		<i>E. faecalis</i> (n=1)	> 256 / > 256
		<i>E. faecium</i> (n=2)	\leq 4 / \leq 8
vanB	0		
		<i>E. durans</i> (n=2)	> 256 / > 256
vanC	21 (50%)	<i>E. faecium</i> (n=18)	< 4 / ≤ 8
		<i>E. gallinarum</i> (n=1)	< 4 / ≤ 8
	16 (38.1%)	<i>E. faecalis</i> (n=1)	> 256 / > 256
Not detected		<i>E. faecalis</i> (n=1)	< 4 / ≤ 8
		<i>E. faecium</i> (n=14)	< 4 / ≤ 8

Table 3. Distribution of vancomycin resistance genes in enterococci (n=42) and their MIC's.

RESULTS

PCR identification of tested strains evidenced differences from previous API results. According to PCR, three *E. avium* were identified as *E. durans*; two *E. faecium* as *E. faecalis*, one *E. avium* as *E.faecalis*, and one *E. gallinarum* as *E. casseliflavus* (Table 1).

Among the tested strains *van*A and *van*C genes were found in five (12%) and 21 strains (50%) respectively, meanwhile *van*B gene was not detected. Strains carrying *van*A genes were from cheeses (*E. faecium*), meatballs (*E. durans* and *E. casseliflavus*), minced meat (*E. faecium*) and beef (*E. faecalis*). *Van*C gene was determined in strains derived from five different ready-to-eat dairy products (three cheeses, butter and butter cream). The prevalence of *van*C gene was more common among *E. faecium* (n=18) compared to *E. durans* (n=2) and *E. gallinarum* (n=1). Interestingly, an important percentage (38%) of *E. faecium* (14 strains) and *E. faecalis* (2 strains) did not give any band for these genes.

Data related to the distribution of vancomycin resistance genes in enterococci and their respective minimum inhibitory concentrations (MIC) values for vancomycin/teicoplanin antibiotics is shown in Table 3. Three of five *van*A-positive strains exhibited high MICs to vancomycin/teicoplanin. Among the *van*C gene positive strains, only two *E. durans* had MIC

values higher than 256 μ g/ml for vancomycin/teico-planin.

The strains were also screened for the presence some virulence factors such as *agg*, *esp*, *gel*E, *ace* and *efa*A, nonetheless the searched virulence genes were not detected in any tested strain.

DISCUSSION

Different types of acquired vancomycin resistance are known in enterococci, meanwhile the vanA followed by vanB are the most prevalent resistance genotype (Werner, 2012). In this study we examined acquired resistance genes including vanA, vanB, and vanC responsible for intrinsic resistance in vancomycin-resistant/susceptible enterococci isolates from animal originated food. The results indicated that strains belonged to *E. faecalis*, *E. faecium*, *E. durans* and *E. casseliflavus* species carried vanA gene with a prevalence of 11.9% (5 strains). Among these strains an *E. faecium* isolated from ready-to-eat food (village cheese) showed susceptibility to vancomycin (MIC, $\leq 4 \mu g/ml$).

Several studies from different countries reported the presence of *vanA* gene in foodborne enterococci. Lopez *et al.* (2009) reported the prevalence of *vanA* gene as 22.6% (two *E. faecium*, three *E. durans* and two *E. hirae*) in 31 VRE isolates. Likewise, Gousia et al. (2015) stated that 22 E. faecium (15.6%) among 141 enterococci carried vanA gene. Relatively lower prevalence (2.4% of enterococci from meat and poultry) was reported by Yilmaz et al. (2016). Another work revealed the presence of vanA gene in three vancomycin-susceptible E. faecalis isolates and one E. hirae isolate (Perin et al. 2014). Osman et al. (2016) and Harada et al. (2012) detected vanA gene in one E. faecalis strain from fish and one E. cecorum strain from poultry samples, respectively. Contrary results were reported by Kasımoğlu-Doğru et al. (2010) and Chajecka-Wierzchowska et al. (2016). Contrary results were given by Kasımoğlu-Doğru et al. (2010) and Chajecka-Wierzchowska et al. (2016) suggesting that the strains from food and livestock samples did not harbor vanA gene. In our study, tested strains had negative results for vanB gene. These results are similar to those obtained by Kasımoğlu-Doğru et al. (2010), Perin et al. (2014), Yılmaz et al. (2016) and Chajecka-Wierzchowska et al. (2016). Nevertheless, Gousia et al. (2015) and Lopez et al. (2009) reported the presence of vanB gene in E. faecium (1.4%) and E. faecium (6.4%) respectively. Another study conducted by Perin et al. (2014) indicated the presence of both vanA and vanB genes at seven E. faecalis strains.

Many species of enterococci, as stated for *Cory*nebacterium spp., Arcanobacterium haemolyticum and Lactococcus spp. were reported to harbor vanA ligase gene while vanB has been primarily determined in *E. faecium* and *E. faecalis*. The difference observed in the dissemination of vanA and vanB resistance genes may be attributed to the fact that vanA gene is mostly located on transposon, a mobile genetic element, in comparison to vanB gene cluster (Cetinkaya *et al.* 2000). VanA gene cluster responds to both vancomycin and teicoplanin resistance but vanB gene cluster is responsible for resistant to vancomycin but not for teicoplanin (Lefort *et al.*, 2004). In our study, strains resistant to vancomycin were also resistant to teicoplanin and they were not carrying vanB gene.

The presence of *van*C gene in enterococi has been characterized as the intrinsic resistance (Gousia *et al.*, 2015). As seen in Table 1, *van*C gene was found in 10 (eight *E. faecium*, two *E. durans*) meat/meat products and 11 (ten *E. faecium*, one *E. gallinarum*) milk/dairy originated strains. Among the strains carrying *van*C gene, only two *E durans* isolated from meatball samples had vancomycin-resistance with a MIC value of $> 256 \mu g/ml$ while the others were vancomycin-sus-

ceptible (MICs, < 4 µg/ml). Previously, the presence of *van*C1 and *van*C2/3 genes in vancomycin-susceptible *E. faecalis* isolated from broilers in Brazil (De Moura *et al.*, 2013) and *van*C gene in *E. gallinarum* in Canada (Diarra *et al.*, 2010) was published. Chajecka-Wierzchowska *et al.* (2016) also reported the presence of *van*C2/3 genes in *E. casseliflavus* isolates from ready-to-eat meat products but not *van*C1 gene. Moreover, a study in Egypt demonstrated *van*C gene carrying *E. gallinarum* and *E. faecalis* strains from fish samples (Osman *et al.*, 2016). Conversely, lack of *van*C gene in animal originated food was recently reported by Yilmaz *et al.* (2016) in Turkey and by Gousia *et al.* (2015) in Greece.

Enterococci strains carrying virulence factor genes cause more severe infections than the strains lacking these pathogenicity traits. Virulence genes have been frequently observed in E. faecalis strains (Chajecka-Wierzchowska et al., 2017). In our study, virulence traits (agg, esp, gelE, ace and efaA) were not found in any of the tested strains. This can be explained by the limited number of E. faecalis tested in the study. In contrast to our results, virulence genes gelE, esp, ace, asa1, efaA and hyl in E. faecalis, E.faecium, E. durans and E. hirae isolates from milk and dairy products were observed by Perin et al. (2014), Hammad et al. (2015) and Gaglio et al. (2016). A study performed by Klibi et al. (2013) in Tunisia indicated the location of hyl, esp and gelE genes in meat isolates of enterococci species (E. faecalis, E. faecium, E. gallinarum). Another report revealed the presence of *efaA*, *agg*, esp, gelE, cyl, cop, cpd, ccf genes in E. faecalis and E. faecium isolated from ready-to eat fermented foods in Turkey (Toğay *et al.*, 2010).

CONCLUSIONS

The presence and prevalence of *van*A and *van*C genes and the absence of *van*B and virulence trait genes in vancomycin-resistant/susceptible enterococci strains were proved. Our findings may be evaluated from two different points: firstly, detection of *van*A gene in VRE strains and particularly in one ready-to-eat food isolate is a matter of interest. These strains can be a part of transmission the high level vancomycin resistance to other strains and/or bacteria. Secondly, the role of food isolates on the spread of pathogenicity genes continues to raise public health concerns. Thus, the lack of any investigated virulence genes in the strains constitutes positive part of the study. Furthermore, monitoring the presence of virulence and *van* genes in different food isolates is essential

to evidence their spreading speed and possible public health risks all over the world.

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CONFLICT OF INTEREST

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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