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Vancomycin Resistance of *Enterococcus faecalis* and *Enterococcus faecium* Isolated from cattle milk*

Tekin KECECI[1], Kadir Semih GUMUSSOY[1], Harun HIZLISOY[2]

[1] Erciyes University, Faculty of Veterinary Medicine, Department of Microbiology, Kayseri- TURKEY
[2] Erciyes University, Faculty of Veterinary Medicine, Department of Veterinary Public Health, Kayseri-TURKEY

Summary: In this study the presence of *Enterococcus* spp. in raw cattle milk and the detection of the resistance to vancomycin of the isolates by using phenotypic and molecular methods were investigated. Totally, 150 milk samples were collected from healthy animals or animals with mastitis scored with California Mastitis Test. Eighty four *Enterococcus* spp. were isolated and 57 (68%), 8 (9%) and 19 (23%) of the isolates were identified by Polymerase Chain Reaction as *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus* spp., respectively. None of the isolates were resistant to vancomycin with E test. However, 11 (19%) *E. faecalis* and 7 (88%) *E. faecium* isolates were positive for *Van*B. *Van*C2, *Van*C3 and *Van*B, *Van*C2, *Van*C3 genes were found together in 1 and 2 *E. faecium* isolates, respectively. In this study, *Enterococcus* spp. were significantly found in cattle milk. Because of the detection of vancomycin resistance by molecular test, this method was found to be more effective in the detection of antibiotic resistance.

Key words: Cattle, *Enterococcus* spp., mastitis, milk, PCR, vancomycin

İnek Sütlerinden İzole Edilen *Enterococcus faecalis* ve *Enterococcus faecium*'un Vankomisin Direnci

Özet: Bu çalışmada çiğ inek sütlerinden *Enterococcus* spp'lerin izolasyonu ve identifikasyonu ile elde edilen izolatlarda vankomisin direncinin fenotipik ve moleküler yöntemlerle saptanması amaçlandı. Toplam 150 adet süt örneği sağlıklı ve mastitisli hayvanlardan California Mastitis Test ile skorlanarak toplandı. Seksen dört *Enterecoccus* spp. izolatı elde edildi ve bunların 57 (%68), 8 (%9) ve 19 (%23)'ü sırasıyla *Enterococcus faecalis*, *Enterococcus faecium* and *Enterococcus* spp. olarak polimeraz zincir reaksiyonu ile identifiye edildi. Etest ile izolatların hiçbiri vankomisine direnç göstermedi. Bununla birlikte, *E. faecalis* izolatlarının 11 (%19)'inde ve *E. faecium* izolatlarının 7(%88)'sinde *Van*B geni pozitif olarak tespit edildi. Ayrıca, 1 izolatta *Van*C2, *Van*C3 genleri ve 2 *E. faecium* izolatında *Van*B, *Van*C2, *Van*C3 genleri birlikte bulundu. Bu çalışmada inek sütlerinde önemli derecede *Enterococcus* spp. varlığı saptanmıştır. Vankomisin direncinin moleküler testlerle tespitinden dolayı bu test, antibiyotik direncin tespitinde daha etkili olduğu sonucuna varıldı.

Anahtar kelimeler: *Enterococcus* spp., mastitis, PZR, sığır, süt, vankomisin

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Introduction

Enterococci are ubiquitous bacteria found in the normal intestinal flora of humans and animals, and are common in environments contaminated by human and animal fecal materials (16). They are also readily recovered from foods such as milk and meat products and various environmental sources (29). These agents are found in the digestive tract of animals and are natural bacterial flora, especially in cases where milking hygiene is inadequate. *Enterococci* entering the mammary gland and colonize through the ducts of the udder and generate clinical signs related to infection in the mammary gland (17,21). Vancomycin resistant *enterococci* (VRE) are currently emerging as a global threat to public health. The first clinical isolates of VRE were reported in Europe in 1988 (27). To date, various types of VRE were characterized phenotypically and genotypically (*Van*A, B, C1, C2, C3, D, E, G, L, M and N). *Van*A-type glycopeptide resistance is characterized by acquired inducible resistance to both vancomycin and teicoplanin. *Van*B type glycopeptide resistance is characterized by acquired inducible resistance to various concentrations of vancomycin but typically not to teicoplanin (30). *Van*A and *van*B clusters have been primarily found in *E. faecalis* and *E. faecium*. The *van*C genotype corresponds to the intrinsic glycopeptides resistance seen in *Enterococcus gallinarum*, *Enterococcus casseliflavus* and *Enterococcus flavescens* (28).

Resistance of *Enterococci* to various antibiotics is increasing and vancomycin-resistant *enterococci* are being increasingly observed.

In Europe, in particular, the use of avoparcin which is a glycopeptide, used as a growth factor in some animal feeds, has led to vancomycin resistant *Enterococcus* strains spreading to humans through the food chain from animals (16,18,20). However, after the prohibition of avoparcin in various European countries, the isolation of the *Van*A genotype has been reduced (13).

In the present study, our aims were to isolate and identify *Enterococcus* spp. from the milks of healthy cattles and cattles with subclinical mastitis and to detect vancomycin resistance in isolates with phenotypic and molecular methods.

Material and Methods

The Collection of Milk Samples

In the study, a total of 150 milk samples were collected from small and large dairy farms between March 2011 and April 2011 in Nevsehir province. Fifty of the samples were taken from healthy cattles and 100 were taken from cattles with subclinical mastitis according to CMT scoring. The samples were brought to the laboratory in cold chain in sterile 50 mL tubes and bacteriological inoculations were carried out on the same day.

California Mastitis Testing (CMT)

For the test, 2 mL of milk was taken from each teat of the cattle into a CMT container. CMT reagent was dropped onto this and the results were scored. Dove gray colored milk samples were considered as normal. According to the manufacturer's recommendation, the samples given a score of one are weak positive (+), those with a score of two are certain positive (++) and those with a score of three are strong positive (+++); samples are scored in terms of gel formation and change of color to blue-purple (11).

The Isolation of Enterococcus *spp.*

For the isolation of *Enterococcus* spp. 5 mL milk samples were inoculated onto Chromocult *Enterococci* Broth (Merck1.10294) and kept for incubation at 37° C for 48 hours. The changing

of the tubes' color to blue-green was scored as one (+) to three positive (+++). After rating of the samples, from the medium assigned as positive, 0.1 mL was taken and inoculated onto m-Enterococcus Selective Agar and Bile-Aesculin-Azide Agar (Coccosel agar, bioMerieux). Inoculated petri dishes were then incubated at 37° C for 48 hours. In this study, three suspected colonies from each positive samples were subcultured on blood agar and tested phenotypically and genotypically (23).

The Identification of Enterococcus spp. with mPCR at Genus and Species Level and Detection of VanA, VanB, VanC1 and VanC2 Genes

For the genotypical identification of *Enterococcus* spp. isolates and the determination of *van*A, *van*B, *van*C1 and *van*C2 genes were carried out according to the methods described by Dutka-Malen et al. (7). The positions and sequences of the oligodeoxynucleotides were shown in table 1.

Total genomic DNA was extracted from the isolates, using a commercial DNA extraction kit (Axygen Bioscience, Union City, CA) as per the manufacturers' directions.

PCR was performed on a DNA thermal cycler (Techne TC-512, UK) in a final volume of 25 ul containing 2.5 µl of DNA template, 10X PCR Buffer (670 mM Tris-HCl (pH 8.3), 100 mM 2-mercaptoethanol and 167 mM $(NH4)_2SO_4$), 0.8 mM dNTPs; 1.5 mM $MgCl_{2,}$ 50 pmol of each primer and 0.5 U of Taq DNA polymerase.

The samples were subjected to an initial denaturation step (94°C for 2 min), followed by 30 amplification cycles. Each amplification cycle consisted of 1 min at 94°C (denaturation), 1 min at 54°C (primer annealing), 1 min at 72°C (primer extension) and the final extension (72°C for 10 min) cycle. The amplified products were resolved in 1.5% (wt/vol) Tris-acetate-EDTA (TAE) agarose gel, and the band patterns were examined in the gel documentation system (Vilber-Lourmat, France) (7).

Antibacterial Susceptibility Testing For Vancomycin

The antibiotic susceptibilities of *Enterococcus* spp. to vancomycin were evaluated by E test. In the study, the vancomycin E test strip (Liofilchem, Italy) was used. The isolates were grown on blood agar (Merck, Germany) at 37° C for 24 h. Then, the suspension of the isolates was adjusted to McFarland 0.5 by using physiological saline. The suspensions were spread onto Mueller Hinton Agar (Merck, Germany). E test strips were placed onto the agar and incubated at 37° C for 24 h aerobically.

An elliptic inhibition zone formed around the strip; the intersection point with the scale on the strip was considered as the MIC value. When evaluating the results, the Clinical and Laboratory Standards Institute (5) was taken into account and the isolates were evaluated as susceptible, intermediate and resistant.

Standard Strain

In the study, for the phenotypic and molecular analysis, *Enterococcus faecalis* ATCC 29212 and *Enterococcus faecium* ATCC 6057 were used as reference strains.

Results

Results of Isolation and Identification

Eighty four (56%) of the 150 milk samples scored by CMT were determined as positive in terms of *Enterococcus* spp. with phenotypic methods. *Enterococcus* spp. was isolated from 24 (16%), 60 (40%) of healthy and mastitic cattle milk samples, respectively. In mPCR analysis, 84 of the isolates yielded 57 (68%), 8 (9%) and 19 (23%) *E. faecalis, E. faecium* and other *Enterococcus* species, respectively.

Table 1. Primers used in this study for the detection of resistance genes by PCR-based method.

Gene	Nucleotide sequence (5' to 3')	Amplicon size (bp)	Literature
*van*A	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	732	7
*van*B	ATGGGAAGCCGATAGTC GATTTCGTTCCTCGACC	635	7
*van*C1	GGTATCAAGGAAACCTC CTTCCGCCATCATAGCT	822	7
*van*C2, C3	CTCCTACGATTCTCTTG CGAGCAAGACCTTTAAG	439	7
ddl E. faecalis	ATCAAGTACAGTTAGTCTT ACGATTCAAAGCTAACTG	941	3,7
ddl E. faecium	GCAAGGCTTCTTAGAGA CATCGTGTAAGCTAACTTC	550	7
rrs(16S rRNA)	GGATTAGATACCCTGGTAGTCC TCGTTGCGGGACTTAACCCAAC	320	3

Table 2. The distribution of *Enterococcus* spp. isolated from milk samples.

Milk samples	Positive samples	mPCR results	Distribution of isolates		
			E. faecalis	*E. faecium*	*Other Enterococcus species*
Healthy (n=50)	24 (16%)	24 (16%)	14 (17%)	2 (2%)	8 (10%)
Mastitic (n=100)	60 (40%)	60 (40%)	43 (51%)	6(7%)	11 (13%)
Total	84 (56%)	84 (56%)	57 (68%)	8 (9%)	19 (23%)

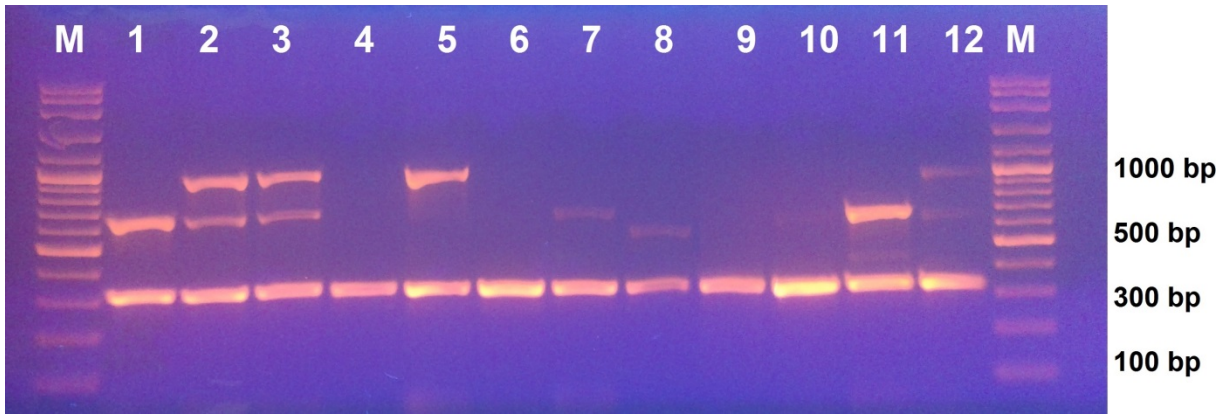

Figure 1. mPCR products of *Enterococcus* spp. and their Van genes.

M: DNA ladder (100 bp plus Thermo); 1-12: *Enterococcus* spp. (320 bp); 2, 3, 5, 12: *E. faecalis* (941 bp); 8-11: *E. faecium* (550 bp); 1-3, 7, 10-12: *Van*B (635 bp); 11: *Van*C2, *Van*C3 (439 bp).

Antibiotic Susceptibility Testing Results

The MIC results of *E. faecalis* and *E. faecium* isolates are demonstrated in table 3. Although all of the isolates were found as intermediate and susceptible to vancomycin, none of the isolates were detected as resistant by using E test. The MIC values of *E. faecalis* isolates for vancomycin were between 0.016µg/mL and 24 µg/mL and those of *E. faecium* isolates were between 1 µg/mL and 24 µg/mL. While 18 (32%) of *E. faecalis*, two (25%) of *E. faecium* isolates were intermediate to vancomycin, 39 (68%) of *E. faecalis*, six (75%) of *E. faecium* isolates were susceptible.

Molecular Evaluation of Vancomycin Resistance

Although vancomycin resistance was not detected in any of the *enterococci* isolates by phenotypic and molecular tests, 11 (19%) of 57 *E. faecalis* isolates were found positive for presence of the *Van*B gene. In addition, while, the *Van*B gene was found in 7 (88%) of eight *E. faecium* isolates, *Van*C2, *Van*C3 genes found in one (12%) *E. faecium* isolate, the *Van*B, *Van*C2, *Van*C3 genes were found together in two *E. faecium* isolates (Figure 1), (Table 4).

Discussion

Enterococcal infections are currently thought to be caused by endogenous bacteria in human flora. However, *enterococci* have recently begun to be called nosocomial infection pathogens. Commonly used antibiotics such as vancomycin, cephalosporins, and aminoglycosides have been reported to be associated with an increase in nosocomial enterococcal infections (25). *Enterococci* have a broad host range and located in the digestive tract of animals as the natural bacterial flora. Due to mainly failure in the regular cleaning of barns, the teats are easily infected and caused mastitis. *Enterococci*, by entering the mammary gland and colonize through the teats and generate clinical signs related to infection in the mammary gland (17,21). *Enterococci* found in milk and dairy products cause diseases in humans, which could result as serious public health problem (24).

The prevalence of *enterococci* in milk has been demonstrated by several authors (19,22). These studies generally focused on the contamination of raw milk and mastitis (14). Araya et al. (1), isolated *Enterococcus* spp. in 38% of milk in

Table 3. MIC distributions of *Enterococcus* spp. for vancomycin.

Species	No of isolates	MIC ranges µg/ml																						
		S																I				R	
		0.016	0.023	0.032	0.047	0.064	0.094	0.125	0.19	0.25	0.38	0.50	0.75	1	1.5	2	3	4	8	12	16	24	32	256
Efs	57 (68%)	6	2	4	2	2	2	1	1	1	1	2	2	1	1	3	5	3	4	5	6	3		
Efm	8 (9%)													1		1	2	2	1			1		

Efs: *E. faecalis;* Efm: *E. faecium* S: Susceptible; I: Intermedier; R: Resistant

Table 4. The distribution of resistance genes in the isolates.

Resistance genes	*E. faecalis*	*E. faecium*
*Van*A	-	-
*Van*B	11	7
*Van*C2, *Van*C3	-	1
*Van*B, *Van*C2, *Van*C3	-	2

the analysis of 105 raw milk samples available for consumption. The distribution of isolates at species level were as follows: 71% *E. faecalis*, 19% *E. faecium*, 4% *E. durans*, 4% *E. gallinarum* and 2% *E. avium*.

In a study about mastitis etiology in cattle's milk conducted by Pitkala et al. (22), the authors detected 4237 bacteria from 12661 samples and 1.2% of these samples were *Enterococcus* spp. In Italy, Cenci Goga et al. (3) performed a study about *Enterococcus* spp. isolation in which 7 (53%) of 13 samples and 27 (77%) of 35 samples were found as *E. faecalis* and *E. faecium*, respectively.

In our study, 84 (56%) of the 150 milk samples analyzed were found to be positive for *Enterococcus* spp. with phenotypic methods. Eighty four *Enterococcus* spp. isolates were obtained from 84 positive milk samples. All isolates were determined as *Enterococcus* spp. at genus level by PCR and 57 (68%) and eight (9%) of the isolates were identified as *E. faecalis* and *E. faecium,* respectively. The differences between the results of the present study and those of other studies (1,3,22) were probably due to the different numbers of samples collected. In addition, environmental problems, such as the presence of a sewage system in the area where milk samples were collected, are thought to have an effect on the results obtained.

In the study reported by Devrise et al. (6), authors found that 61 *E. faecalis*, three *E. faecium*, one *E. durans* and one *E. hirae* isolates were identified from 248 milk samples taken from cattles with subclinical mastitis in Belgium. In total, this accounts for 26% of identified bacteria. In contrast, the rate of *E. faecalis* was reported relatively high in our study (51%) in the samples of cattle with mastitis. This difference might be due to the prevalence of *E. faecalis* in various countries, farm management, climatic factors and the high sensitivity of the detection methods.

The number of infections caused by *E. faecalis* among enterococcal infections is more than ten times compared to other species. However, in recent years, due to the emergence of vancomycin-resistant *enterococci* (VRE), this ratio has gradually decreased and *E. faecium* strains have begun to increase. Initially, avoparcin, a glycopeptide derivative, which was previously used as a growth promotor in animal feed in Europe, was considered to have led to an increase in vancomycin resistance (2).

In a study using the disc diffusion test which was conducted by Kuyucuoglu (14), while the vancomycin resistance of *E. faecalis* isolates

was 4.3%, all of the *E. faecium* isolates were found to be susceptible. However, in the study of Trivedi et al. (26), vancomycin resistance was not been determined in *Enterococcus* species isolated by using disc diffusion test. In the study reported by Kateete et al. (12), in which 16 (28%) of the *enterococci* strains were isolated from animals with clinical mastitis, three (28%) were found to be resistant to vancomycin. In the study of Li et al. (15), the vancomycin susceptibilities of isolates of *enterococci* were examined with E test and the MIC values of the four isolates with the *Van*B gene were determined to be between 8 and 256 µg/mL and six isolates with the *Van*C1 gene were found to be between 4 and 8 µg/mL, which is similar to the results of our study. In a study performed by Janoskova and Kmet (9), the antibiotic susceptibilities of *enterococci* were determined by the agar dilution method. At the end of the test, the MIC values of *enterococci* were lower than that of ours and detected to be between 0.5 and 4µg/mL. In the present study, the MIC values of *E. faecalis* and *E. faecium* isolates were examined by using E test. Although, all of the isolates were found to be moderately susceptible and susceptible at the end of the test, there was no resistance to vancomycin in any of the isolates. The MIC values of *E. faecalis* and *E. faecium* isolates were detected as 0.016 µg/mL- 24 µg/ml and 1 µg/ml-24 µg/mL, respectively. It was detected that 18 (32%) and 39 (68%) of the *E. faecalis* isolates were moderately susceptible and susceptible to vancomycin; two (25%) and six (75%) of the *E. faecium* isolates were moderately susceptible and susceptible to vancomycin. This might be caused by low uptake of the vancomycin and derivatives for the treatment of mastitis.

In the study conducted by Choi et al. (4), the *Van*C gene was found in 19 of 24 vancomycin-resistant *enterococci* isolated from milk. Unlike our study, in the study of Jung et al. (10), which they determined the presence of vancomycin resistance genes in 243 vancomycin-resistant *enterococci*, and the presence of the *Van*A gene was demonstrated. However, being similar to our study, the occurrence of the *Van*C2 gene was shown and additionally none of the resistance genes in the resistant isolates were reported. In a study by Franciosi et al. (8), the *Van*A and *Van*B genes were not found in *enterococci* isolated from raw cow's milk and cheese.

Although vancomycin resistance was detected from none of the *enterococci* isolates in our study by using phenotypic testing, the *Van*B gene was found in 11 (19%) of the *E. faecalis* and seven (88%) of the *E. faecium* isolates with molecular testing. In addition, the *Van*C2*, Van*C3 genes were found in one *E. faecium* isolate and the *Van*B*, Van*C2 and *Van*C3 genes were found together in two *E. faecium* isolates. In the light of this information, we concluded that the molecular testing was more effective in the determination of the antibiotic resistance.

In conclusion, our findings were similar to the results of other researchers and *Enterococcus* spp. presence in cattle milk was detected. In particular, it is of the utmost importance that the pharmacological properties and the spectrum of antibiotics used in the treatment of mastitis should be improved in order to prevent the proliferation of vancomycin-resistant *enterococci*. The most important weapons in the fight against the disease are to use preventive medicine, herd management and farm hygiene. The teat health and the hygiene should not be ignored.

In our country, testing for *Enterococcus* species isolated from the milk of cows with mastitis is not presently considered to be necessary. Samples are usually evaluated for *Staphylococcus* spp. and *Streptococcus*

spp. infections. However, in the evaluation of recently conducted studies, particularly in the spectrum of antibiotics used in mastitis infections, enterococci isolates have also been found.

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Corresponding author
Assist. Prof. Dr. Harun HIZLISOY
Erciyes University, Faculty of Veterinary Medicine
Department of Veterinary Public Health
Melikgazi 38039, Kayseri-TURKEY
GSM: +90 505 918 49 44
E-posta: harunhzlsoy@hotmail.com