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Investigation of vancomycin resistance genes in *Enterococcus* species isolated from bovine mastitis

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ABSTRACT

The aim of the present study was to isolate *Enterococcus* species from mastitis in cows, investigate the vancomycin resistance genes in the isolated species using PCR, and determine the antibiotic resistance of VRE strains to some antibiotics commonly used in Turkey. A total of 512 mammary quarter milk samples from 150 lactating cows were used. Following phenotypic typing by a commercial identification kit, multiplex PCR was applied to the strains using species specific primers. The *Enterococcus* isolation rate was found to be 13.9% (n=71). The most frequently isolated species was *E. faecalis* (n=40, 56.3%), followed by *E. faecium* (n=16, 22.5%), *E. solitarius* (n=6, 8.5%), *E. durans* (n=5, 7.1%) and *E. hirae* (n=4, 5.6%). Of 71 *Enterococcus* strains, 19 (26.7%) were determined to be VRE. While a total of five *vanA* (7%), 10 *vanB* (14%) and 12 *vanC2/C3* (16.9%) genes were detected in the strains, none of the strains harbored the *vanC1* gene. The vancomycin resistance gene was not found in any *E. solitarius* strain. While all of the VR 19 strains were phenotypically resistant to vancomycin and fusidic acid, high resistance rates were also determined in the strains to streptomycin (84.2%), erythromycin (73.7%), tetracycline (68.4%), penicillin G (68.4%), gentamicin (68.4%), lincomycin (57.9%), cephalothin (52.6%) and kanamycin (52.6%). Consequently, it was thought the VRE positive mastitic milk samples may comprise a potential risk for public health. To our knowledge, this is the first study showing the presence of VRE in milk with mastitis by PCR in Afyonkarahisar.

Key words: antimicrobial resistance; bovine mastitis; *Enterococcus* spp.; PCR; vancomycin resistance

Introduction

Enterococcus species, which are fecal flora agents in most mammals and birds, are known to be important pathogens causing nosocomial bacteremia and community-acquired infections (SCHABERG et al., 1991). Enterococci, especially *Enterococcus faecalis* and *Enterococcus faecium*, can also cause bovine mastitis, the most economically important disease in the dairy industry. In studies on the role of Enterococci in the etiology of mastitis, the incidence in cow mastitis has been reported to vary

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between 0.3% and 60% (JACKSON et al., 2010; NAM et al., 2010; ERBAS et al., 2016; WU et al., 2016; RÓŻAŃSKA et al., 2019; BUROVIĆ, 2020).

Vancomycin-resistant *Enterococcus* (VRE) strains (*E. faecalis* and *E. faecium*) were been first identified by UTTLEY et al. (1988) in England, and since then increasing VRE infections have been reported from many countries around the world (SCHABERG et al., 1991; CDC, 1993; ABELE-HORN et al., 2006; ADAMS et al., 2016; BUETTI et al., 2019). VRE species are among the most important nosocomial pathogens that have also been isolated from animal populations in recent years, and the ability of these species to transfer antibiotic resistance genes to other bacteria has been emphasized (KLARE et al., 2003; HEUER et al., 2006; MARSHALL and LEVY, 2011). In addition, there are studies showing that VRE strains carrying antibiotic resistance genes are transmitted to humans by animals and food of animal origin (STOBBERINGH et al., 1999; PHILLIPS et al., 2004; HEUER et al., 2006; HAMMERUM, 2012).

It has been seen that vancomycin resistance is generally determined at phenotypic level in *Enterococcus* strains isolated from animals with mastitis in Turkey, while studies using genotypic methods have focused on the *vanA* gene encoding resistance in strains. Therefore, this study aimed to investigate the etiology of bovine mastitis related to the *Enterococcus* species, and the presence of *vanA, vanB*, *vanC1* and *vanC2/C3* genes associated with vancomycin resistance in the strains.

Materials and methods

Sampling and phenotypic isolation of Enterococcus spp. from milk samples. A total of 512 mammary quarter milk samples were collected from 150 lactating cows on different family farms in the central town and villages of Afyonkarahisar province, Turkey. No antibiotics had been applied to the animals in the previous three months. Following the physical examination of each mammary quarter, CMT was applied for each mammary quarter and test scores were evaluated as +1, +2, +3 and negative, according to the method described by SCHALM et al. (1971). After CMT scoring, animals with a positive CMT reaction in at least one mammary quarter were evaluated as infected for mastitis, and milk samples were collected from these animals under aseptic conditions. For this purpose, the teat ends were cleaned with 70% alcohol, dried, the first streams of foremilk discharged, and then 10 mL of milk from each mammary quarter was aseptically collected into sterile vials. Samples were immediately transported to the laboratory in a cool box on ice. For isolation of *Enterococcus* spp. from milk samples, the stages of pre-enrichment and inoculation onto selective solid medium were performed. Ten µL of each milk sample were taken and transferred into an Enterococcosel broth for pre-enrichment. The samples were incubated under aerobic conditions for 24 h at 35°C. A 10 µL aliquot was taken from the pre-enrichment broth and inoculated onto Enterococcosel agar. The plates were aerobically incubated at 35°C for 24 h. Following the incubation, the samples that formed at least five black pigmented colonies on the agar were evaluated, and these colonies were examined macroscopically and microscopically. From the suspected colonies, Gram staining, catalase activity and growth ability in nutrient broth containing 6.5% NaCl were tested (QUINN et al., 1999; HOLT et al., 2000). Specific phenotypic identification of the isolates was achieved using a Crystal™ Identification Systems Gram-Positive ID kit (Becton, Dickinson and Company, NJ, USA). The strains were stored at -20°C in trypticase soy broth containing 15% glycerol until DNA extraction.

Genotypic identification and determination of vancomycin resistance genes (vanA, vanB, vanC1, vanC2/C3). DNAs were extracted from all test isolates using a genomic DNA purification kit (Thermo Scientific, Lithuania), as described by the manufacturer. For identification of *E. faecalis*, *E. faecium*, *E. durans*, *E. solitarius*, *E. hirae* and *E. avium,* multiplex PCR was applied using species-specific primers (JACKSON et al., 2004). Multiplex PCR was also used in the detection of *vanA* (DUTKA-MALEN et al., 1995)*, vanB* (ELSAYED et al., 2001)*, vanC1* (DUTKA-MALEN et al., 1995) and *vanC2/C3* (SATAKE et al., 1997) genes. All oligonucleotide primers used in the study are listed in Table 1.

The multiplex PCR was performed in a total volume of 25 µl containing 10x PCR buffer, 3 mM $MgCl_2$, 200 µM dNTP mix, 1 mM each primers, 2 U Taq DNA polymerase, 5 µl template DNA and deionized water. The amplification conditions for *E. faecalis*, *E. faecium, E. durans*, *E. solitarius*, *E. hirae*, *E. avium* and vancomycin resistance genes are shown in Table 2. All PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized using ethidium bromide (5µl/ml) under U.V. light.

Table 1. Multiplex PCR primers used in this study

Target gene		Oligonucleotide sequence (5'−3')	Product size (bp)	Reference
E. faecalis	F R	ACTTATGTGACTAACTTAACC TAATGGTGAATCTTGGTTTGG	360	Jackson et al., 2004
E. faecium	F R	GAAAAAACAATAGAAGAATTAT TGCTTTTTTGAATTCTTCTTTA	215	Jackson et al., 2004
E. durans	F R	CCTACTGATATTAAGACAGCG TAATCCTAAGATAGGTGTTTG	295	Jackson et al., 2004
E. solitarius	F R	AAACACCATAACACTTATGTGACG AATGGAGAATCTTGGTTTGGCGTC	371	Jackson et al., 2004
E. hirae	F R	CTTTCTGATATGGATGCTGTC TAAATTCTTCCTTAAATGTTG	187	Jackson et al., 2004
E. avium	F R	GCTGCGATTGAAAAATATCCG AAGCCAATGATCGGTGTTTTT	368	Jackson et al., 2004
vanA	F R	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	732	Dutka-Malen et al., 1995
vanB	F R	AAGCTATGCAAGAAGCCATG CCGACAATCAAATCATCCTG	536	Elsayed et al., 2001
vanC1	F R	GGTATCAAGGAAACCTC CTTCCGCCATCATAGCT	822	Dutka-Malen et al., 1995
vanC2/C3	F R	CGGGGAAGATGGCAGTAT CGCAGGGACGGTGATTTT	484	Satake et al., 1997

Table 2. Multiplex PCR amplification conditions used in this study

Step	Cycle		Temperature		Time	
	E. faecalis, *E. faecium*, *E. durans*, *E. solitarius*, *E. hirae*, *E. avium*	*vanA*, *vanB*, *vanC1*, *vanC2/C3*	*E. faecalis*, *E. faecium*, *E. durans*, *E. solitarius*, *E. hirae*, *E. avium*	*vanA*, *vanB*, *vanC1*, *vanC2/C3*	*E. faecalis*, *E. faecium*, *E. durans*, *E. solitarius*, *E. hirae*, *E. avium*	*vanA*, *vanB*, *vanC1*, *vanC2/C3*
Initial denaturation	1	1	95 °C	94 °C	4 min	2 min
Denaturation	35	30	95 °C	94 °C	30 sec	60 sec
Annealing	35	30	55 °C	54 °C	60 sec	60 sec
Extension	35	30	72 °C	72 °C	60 sec	60 sec
Final extension	1	1	72 °C	72 °C	7 min	10 min

Antibiotic susceptibility test. The antibiotic resistances of the strains determined to be genotypic resistant to vancomycin were tested on Mueller Hinton agar using the Kirby-Bauer disc diffusion method. The tested antibiotics were amoxicillin/clavulanic acid (30µg), tetracycline (30µg), penicillin G (10U), erythromycin (15µg), cephalothin (30µg), gentamicin (10µg), vancomycin (30µg), ampicillin (10µg), chloramphenicol (30µg), streptomycin (10µg), ciprofloxacin (10µg), kanamycin (30µg), lincomycin (10µg), teicoplanin (30µg) and fusidic acid (10µg). The evaluation of the disc zone diameters was made according to Clinical and Laboratory Standards Institute (CLSI, 2013; 2017) standards.

Results

Phenotypic isolation findings. In this study, 71 (13.9%) *Enterococcus* suspected isolates were obtained from a total of 512 mammary quarter milk samples. According to the phenotypic isolation results using a commercial identification kit, of the 71 isolates 39, 16, six, five, four and one were determined to be *E. faecalis, E. faecium*, *E. solitarius, E. durans*, *E. hirae* and *E. avium,* respectively.

Genotypic identification findings. According to the multiplex PCR results using species specific primers for verification of the phenotypically identified species, of the 71 isolates, 40 were classified as *E. faecalis*, 16 as *E. faecium*, six as *E. solitarius*, five as *E. durans* and four as *E. hirae*. One strain determined as *E. avium* with the commercial identification kit was identified to be *E. faecalis* by PCR. The gel electrophoresis images of *E. faecalis* (360 bp), *E. faecium* (215 bp), *E. solitarius* (371 bp), *E. durans* (295 bp) and *E. hirae* (187 bp) are shown in Fig. 1.

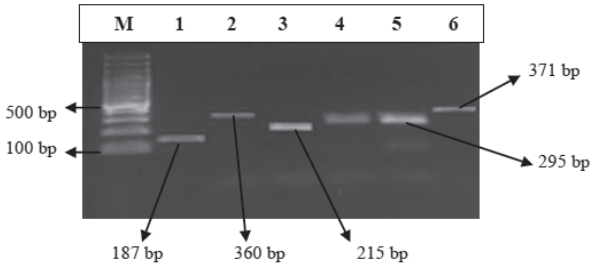

Fig. 1. *Enterococcus* species specific mPCR. M: 100 bp DNA ladder; lane 1: *E. hirae* (187 bp); lane 2: *E. faecalis* (360 bp); lane 3: *E. faecium* (215 bp); lane 4,5: *E. durans* (295 bp); lane 6: *E. solitarius* (371 bp)

Vancomycin resistance genes (vanA, vanB, vanC1, vanC2/C3) findings. Vancomycin resistance genes were investigated with multiplex PCR in 71 *Enterococcus* strains that were definitively identified by PCR, and 19 (26.7%) of the strains were determined to be VRE. Of 19 VRE strains, five, nine, three and two were *E. faecalis*, *E. faecium*, *E. durans* and *E. hirae*, respectively. The vancomycin resistance gene was not found in any of the *E. solitarius* strains. While a total of five *vanA* (7.0%), 10 *vanB* (14.0%) and 12 vanC2/C3 (16.9%) genes were detected in the strains, none of the strains harbored the *vanC1* gene. It was determined that the *vanC2/C3* gene was the most common resistance gene in the tested strains. The distribution of *vanA, vanB, vanC1 and vanC2/C3* genes detected by mPCR and PCR products are shown in Table 3 and Fig. 2, respectively.

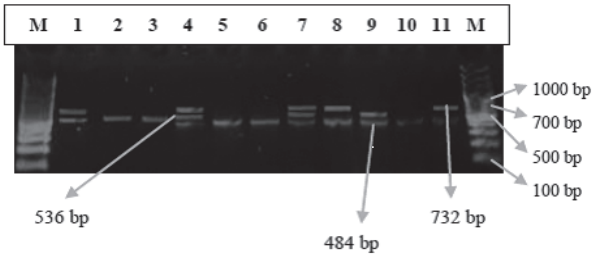

Fig. 2. Detection of *vanA* (732 bp), *vanB* (536 bp) and *vanC2/C3* (484 bp) genes by mPCR. M: 100 bp DNA ladder; lane 1: *vanA* and *vanB* positive *E. faecium*; lane 2: *vanB* positive *E. faecalis*; lane 3: *vanB* positive *E. faecium*; lane 4: *vanA*, *vanB* and *vanC2/C3* positive *E. faecalis*; lane 5: *vanC2/C3* positive *E. durans*; lane 6: *vanC2/C3* positive *E. hirae*; lane 7: *vanA*, *vanB* and *vanC2/C3* positive *E. faecium*; lane 8: *vanA* and *vanC2/C3* positive *E. faecalis*; lane 9: *vanB* and *vanC2/C3* positive *E. faecium*; lane 10: *vanC2/C3* positive *E. faecalis*; lane 11: *vanA* and *vanC2/C3* positive *E. faecium*

Table 3**.** Distribution of vancomycin resistance genes

Gene	*E. faecalis* (n=40)	*E. faecium* (n=16)	*E. solitarius* (n=6)	*E. durans* (n=5)	*E. hirae* (n=4)	Total (n=71)
Alone *vanA*	-	-	-	-	-	0
Alone *vanB*	2 (5.0%)	4 (25.0%)	-	-	-	6 (8.4%)
Alone *vanC2/C3*	1 (2.5%)	1 (6.2%)	-	3 (60.0%)	2 (50.0%)	7 (9.9%)
vanA+vanB	-	1 (6.2%)	-	-	-	1 (1.4%)
vanA+vanC2/C3	1 (2.5%)	1 (6.2%)	-	-	-	2 (2.8%)
vanB+vanC2/C3	-	1 (6.2%)	-	-	-	1 (1.4%)
vanA+vanB+vanC2/C3	1 (2.5%)	1 (6.2%)	-	-	-	2 (2.8%)
Total	5 (12.5%)	9 (56.2%)	0	3 (60.0%)	2 (50.0%)	19 (26.7%)

Antibiotic susceptibility test findings. According to the Kirby-Bauer disc diffusion test results, all of the 19 strains that were determined to be VRE by PCR were resistant to vancomycin and fucidic acid. High resistance rates to streptomycin (84.2%), erythromycin (73.7%), tetracycline (68.4%), penicillin G (68.4%) gentamicin (68.4%), lincomycin (57.9%), cephalothin (52.6%) and kanamycin (52.6%) were also found in the VRE strains (Table 4). In addition, multidrug resistance (defined as resistance to at least three antibiotic classes) against the tested antibiotics was observed in the VRE strains. Resistance to at least seven antibiotics was determined in the *E. faecalis* strains and at least four antibiotics in *E. faecium*, *E. durans* and *E. hirae* strains. The most common multiple resistance profiles were to vankomycin, fusidic acid, tetracycline, erythromycin, streptomycin, penicillin G and gentamicin.

Table 4. Antibiotic resistance of VRE strains

Antibiotic	VR *E. faecalis* (n=5)		VR *E. faecium* (n=9)		VR *E. durans* (n=3)		VR *E. hirae* (n=2)		Total (n=19)	
	R n (%)	I n (%)	R n (%)	I n (%)	R n (%)	I n (%)	R n (%)	I n (%)	R n (%)	I n (%)
Amoxicillin/clavulanic acid (30µg)	1 (20.0)	2 (40.0)	2 (22.2)	2 (22.2)	0	0	0	0	3 (15.8)	4 (21.0)
Tetracycline (30µg)	4 (80.0)	1 (20.0)	6 (66.7)	3 (33.3)	2 (66.7)	0	1 (50.0)	0	13 (68.4)	4 (21.0)
Penicillin G (10U)	4 (80.0)	*-	7 (77.8)	*-	1 (33.3)	*-	1 (50.0)	*-	13 (68.4)	*-
Erythromycin (15µg)	4 (80.0)	1 (20.0)	7 (77.8)	1 (11.1)	2 (66.7)	1 (33.3)	1 (50.0)	1 (50.0)	14 (73.7)	4 (21.0)
Cephalothin (30µg)	3 (60.0)	1 (20.0)	6 (66.7)	1 (11.1)	0	0	1 (50.0)	0	10 (52.6)	2 (10.5)
[a]Gentamicin (10µg)	4 (80.0)	1 (20.0)	6 (66.7)	2 (22.2)	2 (66.7)	1 (33.3)	1 (50.0)	1 (50.0)	13 (68.4)	5 (26.3)

Table 4. Antibiotic resistance of VRE strains (continued)

Antibiotic	VR *E. faecalis* (n=5)		VR *E. faecium* (n=9)		VR *E. durans* (n=3)		VR *E. hirae* (n=2)		Total (n=19)	
	R n (%)	I n (%)	R n (%)	I n (%)	R n (%)	I n (%)	R n (%)	I n (%)	R n (%)	I n (%)
Vancomycin (30µg)	5 (100)	0	9 (100)	0	3 (100)	0	2 (100)	0	19 (100)	0
Ampicillin (10µg)	1 (20.0)	*-	0	*-	0	*-	1 (50.0)	*-	2 (10.5)	*-
Chloramphenicol (30µg)	2 (40.0)	0	4 (44.4)	1 (11.1)	0	0	0	0	6 (31.6)	1 (5.2)
Streptomycin (10µg)	5 (100)	*-	7 (77.8)	*-	2 (66.7)	*-	2 (100)	*-	16 (84.2)	*-
Ciprofloxacin (10µg)	1 (20.0)	0	2 (22.2)	0	0	0	0	0	3 (15.8)	0
Kanamycin (30µg)	3 (60.0)	2 (40.0)	5 (55.5)	2 (22.2)	1 (33.3)	1 (33.3)	1 (50.0)	0	10 (52.6)	5 (26.3)
Lincomycin (10µg)	4 (80.0)	0	5 (55.5)	1 (11.1)	1 (33.3)	0	1 (50.0)	0	11 (57.9)	2 (10.5)
Teicoplanin (30µg)	2 (40.0)	0	3 (33.3)	0	0	0	0	0	5 (26.3)	0
Fusidic acid (10µg)	5 (100)	0	9 (100)	0	3 (100)	0	2 (100)	0	19 (100)	0

[a]Low-level gentamicin resistance
*-: No standard defined for zone diameter; R: Resistant; I: Intermediate

Discussion

The present study investigated the presence of VRE species in bovine mastitic milk samples by PCR, and the antibiotic resistance of VRE strains to antibiotics commonly used in Turkey.

Enterococcus species, which were previously ignored in the etiology of mastitis, have taken their place among the bacterial agents in mastitis etiology in recent years. Various studies related to the role of *Enterococcus* species in mastitis etiology have shown that the prevalence of these species ranges between 0.3% and 60% in cow mastitis (NAM et al., 2010; KATEETE et al., 2013; ERBAS et al., 2016; KECECİ et al., 2016; WU et al., 2016; RÓŻAŃSKA et al., 2019; BUROVIĆ, 2020). Although the distribution of *Enterococcus* species shows diversity in these reports, *E. faecalis* and *E. faecium* have been pointed out as the most common species isolated from mastitis. In our study, the *Enterococcus* isolation rate was found to be 13.9% (n=71) in 512 mammary quarter milk samples obtained from 150 lactating cows. Following phenotypic identification using a commercial identification kit, mPCR was used for the definitive identification of the isolates. According to the mPCR results, the most frequently isolated species was *E. faecalis* (n=40; 56.3%), followed by *E. faecium* (n=16; 22.5%), *E. solitarius* (n=6; 8.5%), *E. durans* (n=5; 7.1%) and *E. hirae* (n=4; 5.6%). When compared with other investigations, similarity was found in terms of the high isolation

rate of *E. faecalis*, while differences were seen in the diversity and isolation rates of other species. The sample size, isolation and identification methods used, geographical variations and differences in origin of the strains may be the reason for the discrepancy in the distribution of species. In addition, in the present study, one strain identified as *E. avium* in phenotypic identification performed using a commercial identification kit was found to be *E. faecalis* by mPCR. This result showed that species specific identification should not be limited to the use of phenotypic methods, but should be supported and confirmed by genotypic techniques.

In recent years, it has been emphasized that foods of animal origin can act as reservoirs for VRE infections in humans, and strains carrying antibiotic resistance genes can play an active role in the spread of resistance to vancomycin. Therefore, an increase has been noticed in studies on vancomycin resistance in *Enterococcus* strains isolated from milk samples with mastitis. On the other hand, it is seen that vancomycin resistance is generally determined at the phenotypic level, and there are a limited number of studies using genotypic methods (KHAN et al., 2005; JUNG et al., 2007; ERBAS et al., 2016; KECECİ et al., 2016). JUNG et al. (2007) from Korea reported that they isolated seven (0.4%) VRE strains from 1981 mammary quarter milk samples, and typed all of these strains as *E. gallinarum* carrying *vanC1* gene. Similarly, KHAN et al. (2005) reported that 22 (88%) of 25 *E. gallinarum* strains isolated from mastitic milk samples carried only the *vanC1* gene, and no resistance gene was found in the five *E. faecalis* strains isolated. In a study conducted in Turkey, it was reported that the *vanA* gene was detected in only one (1.8%) of 56 *E. faecalis* strains isolated from bovine mastitis, but no vancomycin resistance was found in any of 20 *E. faecium*, 11 *E. hirae* and seven *E. durans* strains (ERBAS et al., 2016). In another study from Turkey, KECECİ et al. (2016) stated that *vanB* gene positivity was determined in 11 (19%) of 57 *E. faecalis* strains, and seven (88%) of eight *E. faecium* strains. In the same study the authors emphasized that *vanC2/C3* genes were found in one *E. faecium* strain, and *vanB, vanC2/C3* genes were found together in two *E. faecium* strains. In our study, we tested for vancomycin resistance genes in 71 *Enterococcus* strains by mPCR, and 19 (26.7%) of the strains were determined to be VRE. Of 19 VRE strains, nine, five, three and two were *E. faecium*, *E. faecalis*, *E. durans* and *E. hirae*, respectively. No vancomycin resistance gene was found in any of the *E. solitarius* strains. While a total of five *vanA* (7%), 10 *vanB* (14%) and 12 *vanC2/C3* (16.9%) genes were detected in the strains, none of the strains had the *vanC1* gene. It was determined that *vanA* and *vanB* gene positivity was only found in *E. faecalis* and *E. faecium* strains, while the *vanC2/C3* genes were positive in all the VRE strains (Table 3). The VRE isolation rate of 26.7% obtained in this study was considerably higher than the rates reported in similar studies. In addition, the results of our study differed from the findings of other investigators in terms of the diversity and distribution rates of vancomycin resistance genes in the strains. It was thought that the high VRE ratio and these differences obtained from our study might be related to the difference and origin of the isolated strains, the differences in the primer and amplification conditions used, and the geographical variations. In this study, the detection of *vanA* and *vanB* gene positivity in *E. faecium* and *E. faecalis* strains was consistent with the view that *vanA* and *vanB* resistance genes are more common in clinical origin *E. faecium* and *E. faecalis* strains isolated from human infections (GHOLIZADEH and COURVALIN, 2000; JELJASZEWICZ et al., 2000; SHEPARD and GILMORE, 2002; KLEIN, 2003; COURVALIN, 2005; LEVINE, 2006). Since *vanA* and *vanB* resistance genes can be transferred by transposons or plasmids, it was thought that *vanA* and *vanB* positive *E. faecalis* and *E. faecium* strains isolated in the study could mediate the spread of resistance genes and thus pose a potential risk to public health.

The most important characteristics of human origin clinical VRE isolates, apart from their pathogenicity and clinical significance, are considered to be the intrinsic or acquired type multiple antibiotic resistances in strains, the spread of antibiotic resistance, and the limitations of treatment options (CLEVEL, 1990; NOBLE

et al., 1992; MUNDY et al., 2000). As in many infections, the most important problem in the treatment of mastitis is the increasing problem of resistance to antibiotics, in addition to the presence of antibiotic residues in milk (BENIĆ et al., 2018; LAMARI et al., 2021; MIMOUNE et al., 2021). However, there is limited research on the antibiotic resistance profiles of strains isolated from bovine mastitis genotypically determined to be VRE (JUNG et al., 2007; ERBAS et al., 2016; ATTIA et al., 2017). JUNG et al. (2007) stated that seven *vanC1* gene positive *E. gallinarum* strains were resistant to vancomycin (14%), chloramphenicol (14%), ciprofloxacin (29%), erythromycin (14%) and tetracycline (42%). In a study conducted in Egypt, it was reported that resistance to at least two different antibiotics was detected in *vanA* and *vanB* gene positive VRE strains isolated from mastitic milk samples, and it was emphasized that the multiple antibiotic resistance profile in the strains was more common against ampicillin, ciprofloxacin, gentamicin and erythromycin (ATTIA et al., 2017). ERBAS et al. (2016) from Turkey stated that one *vanA* gene positive *E. faecalis* strain isolated from bovine mastitis was also resistant to tetracycline and erythromycin. In our study, the antibiotic resistance of 19 VRE strains was investigated by the Kirby-Bauer disc diffusion test. While all the strains were resistant to vancomycin and fusidic acid, high resistance rates in VRE strains were also found to streptomycin (84.2%), erythromycin (73.7%), tetracycline (68.4%), penicillin G (68.4%) gentamicin (68.4%), lincomycin (57.9%), cephalothin (52.6%) and kanamycin (52.6%) which are commonly used in veterinary medicine in Turkey. When evaluated on the basis of strains, resistance was detected to at least seven antibiotics tested against VR *E. faecalis* strains and to at least four antibiotics against VR *E. faecium*, *E. durans* and *E. hirae* strains. The most common multiple phenotypic resistance profile in the strains was against vancomycin, fusidic acid, tetracycline, erythromycin, streptomycin, penicillin G and gentamicin. It was thought that the high and multiple antibiotic resistance rates obtained may be due to the use of nonspecific and intensive antibiotics in the treatment of mastitis, without agent isolation or any antibiotic sensitivity test in the veterinary field. In Turkey, the widespread use of antibiotics, such as tetracycline, erythromycin, streptomycin, penicillin and gentamicin, in animals with mastitis seems to support our findings.

Conclusions

In conclusion, the genotypic identification of *Enterococcus* and VRE strains isolated from bovine mastitic milk samples was achieved for the first time in Afyonkarahisar province of Turkey. Our study showed that Enterococci are emerging as one of the main species causing dairy cow mastitis in Afyonkarahisar province, and in routine diagnosis of mastitis etiology milk samples should be examined not only for specific agents but also for *Enterococcus* species. Considering that the greatest hazard in terms of VRE species is the transmission of vancomycin resistance genes and the acceptance of animal origin foods as reservoirs in the spread of resistance, it should not be ignored that dairy products that have not been sufficiently heat treated may play a role in the transmission of VRE species to humans. In addition, when considering the safety of Enterococci in food or probiotic use, strict monitoring mechanisms are necessary to guarantee consistent safety for consumers. In our study, multiple antibiotic resistance was also determined in VRE strains. Following the definitive identification of mastitis pathogens, an antibiotic sensitivity test should be conducted and the common use of nonspecific antibiotics for the treatment of mastitis should be restricted because of the increasing problem of resistance to antimicrobials all over the world.

Conflict of interest

The authors declare that they have no conflict of interest.

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SAŽETAK

Cilj ovog istraživanja bio je izolirati vrste bakterija iz roda *Enterococcus* u krava s mastitisom, zatim PCR-om analizirati gene rezistencije na vankomicin (VRE) u izoliranim vrstama te odrediti rezistenciju sojeva enterokoka na antibiotike koji se najčešće upotrebljavaju u Turskoj. Ukupno je upotrijebljeno 512 uzoraka mlijeka uzetih iz četvrti vimena 150 krava u laktaciji. Nakon fenotipske tipizacije komercijalnim identifikacijskim kitom primijenjen je multipleks PCR upotrebom početnica specifičnih za vrstu. Vrste iz roda *Enterococcus* pronađene su u 13,9 % uzoraka (n = 71). Najzastupljenija je vrsta bila *E. faecalis* (n = 40; 56,3 %), zatim *E. faecium* (n = 16; 22,5 %), *E. solitarius* (n = 6; 8,5 %), *E. durans* (n = 5; 7,1 %) i *E. hirae* (n = 4; 5,6 %). Od 71 soja iz roda *Enterococcus* 19 sojeva (26,7 %) bilo je rezistentno na vankomicin. U sojevima je otkriveno ukupno pet gena *vanA* (7 %), 10 gena *vanB* (14 %) i 12 gena *vanC2/C3* (16,9 %), no ni jedan soj nije nosio gen *vanC1*. Gen rezistencije na vankomicin nije pronađen ni u soju *E. solitarius*. Iako je svih 19 VR sojeva bilo rezistentno na vankomicin i fusidatnu kiselinu, visoka stopa rezistencije utvrđena je i na streptomicin (84,2 %), eritromicin (73,7 %), tetraciklin (68,4 %), penicilin G (68,4 %), gentamicin (68,4 %), linkomicin (57,9 %), cefalotin (52,6 %) i kanamicin (52,6 %). Zaključeno je da su uzorci mlijeka, koji potječu od krava s mastitisom i pozitivni su na VRE, potencijalno rizični za javno zdravlje. Prema našim saznanjima ovo je prvo istraživanje koje pokazuje prisutnost rezistencije na vankomicin kod enterokoka u uzorcima mlijeka krava s mastitisom izoliranima PCR-om u pokrajini odnosno okrugu Afyonkarahisar.

Ključne riječi: antimikrobna rezistencija; goveđi mastitis; *Enterococcus* spp.; PCR; rezistencija na vankomicin