

The Prevalence of *Enterococcus* spp., Resistance Profiles, the Presence of the *VanA* and *VanB* Resistance Genes in Chicken Meats

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ABSTRACT

The aim of the present study was to investigate the prevalence of *Enterococcus* spp., resistance profiles, and the presence of the *VanA* and *VanB* resistance genes in the chicken meat samples that were collected from the Van market, Turkey. A total of 100 chicken meat samples were used. Among the samples, 27 (27%) were *Enterococcus* spp. positive. A total of 67 isolates were obtained from 27 chicken meat samples, which were positive for *Enterococcus* spp. Among the 67 isolates, 53 (79.10%) were identified to be *E. faecalis* and 14 (20.90%) were identified to be *E. faecium*. The analysis of antibiotic resistance revealed that 48 isolates (71.74%) exhibited resistance to multiple antibiotics and 19 isolates (28.36%) were resistant to at least one antibiotic. At least 50% of the *E. faecalis* and *E. faecium* strains were intermediately sensitive to ampicillin, penicillin, chloramphenicol, vancomycin, and gentamicin. Moreover, the presence of the *VanA* and *VanB* genes in 13 strains that were phenotypically and intermediately resistant to vancomycin was examined by PCR test. The PCR analysis revealed that no isolate had the *VanA* and *VanB* genes. As a result, the detection of *Enterococcus* spp. in chicken meat is an indication of not paying attention to hygienic conditions. At the same time, the existence of multiple antibiotic resistance in isolates obtained from these foods also suggests that phenotypically determined resistances may threaten public health.

Keywords: Chicken meat, *Enterococcus* spp. *VanA*, *VanB*

Tavuk Etlerinde *Enterococcus* spp. Prevelansı, Direnç Profilleri, *VanA* ve *VanB* Direnç Genlerinin Varlığı

ÖZ

Türkiye’de Van ili piyasasından toplanan tavuk eti örneklerinde *Enterococcus* spp. prevelansı ve antibiyotik dirençliliği ve *VanA* ve *VanB* direnç genlerinin belirlenmesi amaçlandı. Çalışmada 100 adet tavuk eti örneği kullanıldı. Bunların 27’si (%27) *Enterococcus* spp. pozitif bulundu. *Enterococcus* spp. için pozitif olan 27 tavuk eti örneğinden toplam 67 izolat elde edildi. Bunlardan 53’ü (%79.10) *E. faecalis*, 14’ü (%20.90) ise *E. faecium* olarak tespit edildi. Antibiyotik dirençlilikleri incelenen analizler sonucunda *Enterococcus* spp. izolatlarının 48’sinin (%71.64) iki veya daha fazla antibiyotiğe dirençli olduğu, 19’sinin (%28.36) ise en az bir antibiyotiğe dirençli olduğu tespit edilmiştir. *E. faecalis* ve *E. faecium* suşlarının en az %50’si ampisilin, penisin, kloramfenol, vankomisin ve gentamisine duyarlı ve orta düzeyde olduğu tespit edildi. Ayrıca fenotipik olarak vankomisine dirençli ve orta düzeyde olan 13 izolatta *VanA* ve *VanB* geni varlığı PCR testi ile araştırıldı. PCR testi ile analizi yapılan izolatların hiçbirinde *VanA* ve *VanB* geni tespit edilemedi. Sonuç olarak, tavuk etlerinde *Enterococcus* spp. tespit edilmesi hijyenik koşullara dikkat edilmediğinin göstergesidir. Aynı zamanda bu gıdalardan elde edilen izolatlarda çoklu antibiyotik dirençliliğinin var olması ayrıca fenotipik olarak belirlenen dirençliliklerin halk sağlığını tehdit edebileceğini düşündürmektedir.

Anahtar Sözcükler: *Enterococcus* spp., Tavuk eti, *VanA*, *VanB*

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INTRODUCTION

Enterococcus spp. are naturally found in the environment and the normal intestinal microbiota of both animals and humans. Due to fecal contamination, these species can reach media such as soils, fertilizers of animal origin, and sewer systems and, thus, contaminate waters and vegetables. The species can invade the intestinal tracts of domestic and wild animals. Thus, enterococcus can be found in any type of food from vegetables to raw meat and cheese due to cross-contamination between food processing stages (Giraffa 2002, van Schaik and Willems 2010, Boehm and Sassoubre 2014).

Enterococci were previously known as Lancefield D group streptococci or fecal streptococci. *Enterococcus* spp. are Gram-positive, oxidase and catalase negative, facultative anaerobic and homofermentative bacteria that produce lactic as the final product of glucose fermentation. Most enterococci are grow at a NaCl concentration of 6.5%, pH value of 9.6, and temperatures between 10 °C and 45 °C. At least 50 different *Enterococcus* species have been identified (Foulquié-Moreno et al. 2006, Semedo-Lemsaddek et al. 2010 Lawlwy et al. 2012, Bonacina et al. 2017). The most common *Enterococcus* spp. in human intestines are *Enterococcus faecalis* and, to a lesser level, *Enterococcus faecium* whereas the most common species in foods and animals are *E. cecorum*, *E. faecalis*, *E. hirae*, and *E. faecium* (Prieto et al. 2016).

Enterococci are accepted as the agents of a series of clinical infections that are not food-borne such as bacteriemia and endocarditis. Moreover, they are also of importance in food microbiology. Enterococci are important spoilage microorganisms, especially in meat and dairy products, are known to have virulence factors, their antibiotic resistance is known to be species-specific, and most *Enterococcus* species are not fully pathogenic (Lawlwy et al. 2012, Halkman 2019).

Certain desirable metabolic properties of enterococci attach importance to their use in the production of various foods. The role of enterococci in food-borne diseases is debatable, but they can be regarded as hospital pathogens and threats to public health due to their antimicrobial resistance stemming from their effective ability to transfer genetic material and the emergence of virulence factors. Acquired infections, virulence determinants, and antimicrobial-resistant strains not only occur in hospitals, humans, and veterinary clinics but also soils, waters, insects, plants such as vegetables, and raw and fermented food products due to environmental contamination (Semedo-Lemsaddek et al. 2010, Lawlwy et al. 2012). Moreover, enterococci can decarboxylate amino acids and are known to produce biogenic amines such phenylamine and as tyramine (Nieto-Arribas et al. 2011, Vidal-Carou et al. 2011).

E. faecalis and *E. faecium* are the most commonly isolated species from meat and meat products while *E. gallinarum*, *E. durans*, *E. mundtii*, *E. casseliflavus*, and

E. hirae, are more rarely isolated (Semedo-Lemsaddek et al. 2010). The presence of such isolates in chicken meats poses a threat to food safety and public health (Aslam et al. 2012).

The vancomycin-resistant enterococcus strains pose serious problems. The identified and characterized vancomycin-resistant phenotypes are *VanA*, *VanB*, *VanD*, *VanE*, and *VanG* (Švec and Devriese 2015). *VanA* and *VanB* are the most common resistance types in clinical Enterococci (Çöleri and Çökmüş 2008). The vancomycin-resistant strains of *E. faecalis* and, to a lesser degree, *E. faecium* are thought to be related to human pathogenesis (Semedo-Lemsaddek et al. 2010).

The aim of this study is to isolate *Enterococcus* spp. from chicken meat samples taken from the Van market in Turkey, and to identify *E. faecalis* and *E. faecium* strains in isolates. In addition, the determination of *VanA* and *VanB* genes, which are known to be the most common causes of resistance in isolates that are phenotypically resistant to vancomycin.

MATERIAL AND METHODS

Bacterial Strains

E. faecalis (ATCC® 51299), *E. faecium* (ATCC® 6057), *E. faecium* (vanA+) and *E. faecalis* (vanB+) that were procured from the Food Hygiene and Technology Department of the Veterinary Faculty of Van Yüzüncü Yıl University were used as the reference strains.

Sample Collection

A total of 100 chicken meat samples comprising breasts and drumsticks were used as the study material. The samples were bought from the sales places under aseptic conditions, brought to the laboratory at +4 °C, and analyzed immediately.

Isolation of *Enterococcus* spp.

A total of 10 g sample was obtained from the chicken meat samples that were collected under aseptic conditions and brought to the laboratory in a cold chain. Then, was homogenized with 90 ml sterile peptone water for 2 minutes. A total of 0.1 ml homogenate was inoculated into the Slanetz&Bartley Medium (LABM LAB166, UK) using the spread plate method. The petri dishes were firstly incubated at 37 °C for 4 h and then at 44 °C for 24-48 h under aerobic conditions. Then, the colonies that were larger than 1-2 mm and with colors ranging from pink-red to brown were identified as *Enterococcus* spp. Five typical colonies that grew on the SB medium were individually inoculated onto the %0.6 Yeast Extract (YE) (LABM, MC001, UK) containing Tryptone Soya Agar (TSA) (LABM, LAB011, UK) and biochemical assays were performed (Anonymous 2015, Švec and Devriese 2015). The isolates that were identified to be *Enterococcus* spp. were confirmed using PCR.

The Conformation of *Enterococcus* spp. and Isolation of *E. faecalis* and *E. faecium*

A commercial kit (GeneAll, Exgene™ Cell SV, South Korea) was used for DNA extraction from the *Enterococcus* spp. colonies that were isolated from the chicken meat samples. The specific primer pair, namely Ent1 and Ent2, that was developed by Ke et al. (1999) for the *tuf* gene region was used for the confirmation of the *Enterococcus* spp. isolates. The gene regions that were developed by Jackson et al. (2011) were used for the identification of *E. faecalis* and *E. faecium* using PCR

(Table 1). For the preparation of the PCR mixture, 10 µl mastermix (A.B.T™ 2X PCR MasterMix, Turkey), 1.5 µl of each primer, and 5 µl genomic DNA were added and the total volume was brought to 25 µl using PCR water. Table 2 shows the PCR protocol that was followed during the analysis. The amplicons were run on 1.5% agarose gel (jelde (Vivantis, USA+Bioshop, TAE Buffer 50X Liquid concentrate, Canada) and the positive control bands were examined using an imaging device.

Table 1. Primers used in PCR analysis

Microorganism	Oligonükleotid Sequence	bp	Referans
<i>Enterococcus</i> spp.	Ent1-TACTGACAAACCATTTCATGATG Ent2-AACTTCGTCACCAACGCGAAC	112	Ke et al. (1999)
<i>E. faecalis</i>	FL1-ACITTATGTGACTAACTTAACC FL2- TAATGGTGAATCTTGGTTTGG	360	Jackson et al. (2011)
<i>E. faecium</i>	FM1-GAAAAAACAATAGAAGAATTAT FM2-TGCTTTTGAATCTTCTTTA	215	
Gene			
<i>vanA</i>	A1-ATGAATAGAATAAAAAGTTGC A2-TCACCCCTTTAACGCTAATA	1032	Saha et al. (2008)
<i>vanB</i>	B1-GTGACAAACCGGAGGCGAGGA B2-CCGCCATCCTCCTGCAAAAAA	433	Handwerger et al. (1992)

Antibiotic Resistance

Antibiotic Resistance tests were tested by the standard disk diffusion method of Kirby-Bauer (Bauer et al., 1966) on Mueller Hinton Agar (Oxoid CM0337, UK. Ampicillin (AMP, 10 µg, Liofilchem®, Italy), penicillin (P, 10 U, Liofilchem®, Italy), erythromycin (E, 15 µg, Liofilchem®, Italy), chloramphenicol (C, 30 µg, Liofilchem®, Italy), tetracycline (TE, 30 µg, Liofilchem®, Italy), vancomycin (VA, 30 µg, Liofilchem®, Italy), and gentamicin (CN, 120 µg, Liofilchem®, Italy) were used to determine the antibiotic resistance of the *Enterococcus* isolates. The results were evaluated according to the disk diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI 2018).

Identification of the *VanA* and *VanB* genes

The presence of the *VanA* and *VanB* resistance genes in the phenotypically and intermediately strains that were identified by applying the disc diffusion method to the confirmed *Enterococcus* spp. was investigated. The specific primer pair that was developed by Handwerger et al. (1992) and Saha et al. (2008) was used for this purpose (Table 2). For the preparation of the PCR mixture, 10 µl mastermix (A.B.T™ 2X PCR MasterMix, Turkey), 1.5 µl of each primer, and 5 µl genomic DNA were added and the total volume was brought to 25 µl using PCR water. Table 3 shows the PCR protocol that was followed during the analysis.

Table 2. PCR protocol applied in analyzes

Microorganism	Initial Denaturation (°C/min)	Amplification (Denaturation/Annealing/Extension)	Final Extension (°C/min)	Cycle
<i>Enterococcus</i> spp.	95/10	95 °C 45 sec/59 °C 45 sec/72 °C 45 sec	72/5	35
<i>E. faecalis</i>	95/10	95 °C 45 sec/55 °C 60 sec/72 °C 60 sec	72/7	35
<i>E. faecium</i>				
Gene				
<i>VanA</i>	94/10	94 °C 45 sec/50 °C 45 sec/72 °C 60 sec	72/10	30
<i>VanB</i>	94/10	94 °C 45 sec/62 °C 45 sec/72 °C 60 sec	72/10	30

RESULTS

A total of 27 samples (27%) were determined to be *Enterococcus* spp. positive and a total of 67 *Enterococcus* spp. isolates were obtained from the 27 samples. Among the 67 isolates, 53 (79.10%) were identified to

be *E. faecalis* and 14 (20.90%) were identified to be *E. faecium* (Figure 1). Table 3 shows the antibiotic resistance percentages of the 67 *Enterococcus* spp. isolates.

Table 3. Antibiotic resistance percentages of *Enterococcus* spp. isolates (%)

n	AMP (%)			P (%)			E (%)			C (%)			TE (%)			VA (%)			CN (%)		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
<i>E. faecalis</i> (53)	52 (98.11)	-	1 (1.89)	50 (94.33)	-	3 (5.67)	15 (28.30)	16 (30.19)	22 (41.51)	48 (90.57)	3 (5.66)	2 (3.77)	10 (18.87)	5 (9.43)	38 (71.70)	44 (83.01)	2 (3.77)	7 (13.22)	51 (96.23)	-	2 (3.77)
<i>E. faecium</i> (14)	13 (92.86)	-	1 (7.14)	12 (85.71)	-	2 (14.29)	8 (57.14)	1 (7.14)	5 (35.72)	13 (92.86)	-	1 (7.14)	2 (14.29)	2 (14.29)	10 (71.42)	10 (71.43)	1 (7.14)	3 (21.43)	14 (100)	-	-
Total (67)	65 (97.01)	-	2 (2.99)	62 (92.54)	-	5 (7.46)	23 (34.33)	17 (25.37)	27 (40.30)	61 (91.04)	3 (4.48)	3 (4.48)	12 (17.91)	7 (10.45)	48 (71.64)	54 (80.60)	3 (4.48)	10 (14.92)	65 (97.01)	-	2 (2.99)

n: number of positive isolates, AMP: Ampicillin, P: Penicillin, E: Erythromycin, C: Chloramphenicol, TE: Tetracycline, VA: Vancomycin, CN: Gentamicin

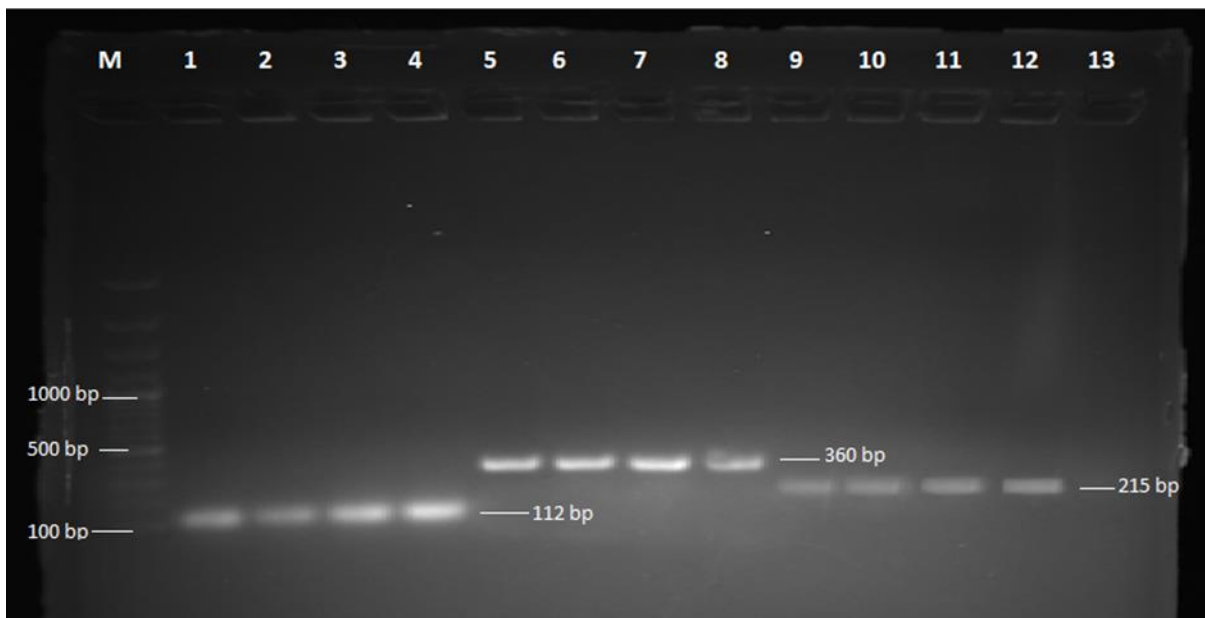


Figure 1: The agarose gel image of the amplicons that were identified in the *Enterococcus* spp. (112 bp), *E. faecalis* (360 bp) and *E. faecium* (215 bp) isolates using PCR (M: 100 bp DNA marker; 1: *E. faecalis* ATCC® 51299; 2-4: *Enterococcus* spp. isolats; 5: *E. faecalis* ATCC® 51299; 6-8: *E. faecalis* isolats; 9: *E. faecium* ATCC® 6057; 10-12: *E. faecium* isolates; 13: Negative control)

The analysis revealed that 48 isolates (71.74%) of the *Enterococcus* spp. isolates were resistant to two or more antibiotics while 19 isolates (28.36%) of the

isolates exhibited resistance to at least one antibiotic (Table 4).

Table 4. Number of *E. faecalis* and *E. faecium* isolates resistant to multiple antibiotics

Species (n)	Number of isolates (%)	Multiple antibiotic resistance
<i>E. faecalis</i> (53)	1 (1.89)	C, TE
	3 (5.66)	E, C, TE
	24 (45.28)	E, TE
	2 (3.77)	TE, VA
	1 (1.89)	E, C, TE, VA
	1 (1.89)	A, P, E, TE, VA
	2 (3.77)	P, E, TE, VA
	3 (5.66)	E, TE, VA
	2 (3.77)	E, CN
<i>E. faecium</i> (14)	4 (28.57)	E, TE
	1 (7.14)	A, P, TE, VA
	1 (7.14)	TE, VA
	1 (7.14)	E, C, TE
	1 (7.14)	P, TE, VA
	1 (7.14)	E, TE, VA

AMP: Ampicillin, P: Penicillin, E: Erythromycin, C: Chloramphenicol, TE: Tetracycline, VA: Vancomycin, CN: Gentamicin

The presence of *VanA* and *VanB* genes in 13 phenotypically vancomycin resistant isolates was investigated by PCR method. According to PCR analysis, *VanA* and *VanB* genes were not detected in any of the isolates.

DISCUSSION

While it is controversial that foodborne enterococci are bacterial pathogens, they can serve as potential virulence and antimicrobial resistance gene reservoirs for host-adapted strains. Studies have revealed that *Enterococcus* spp. contaminated retail meat products to a great degree and the differences in the antimicrobial resistance phenotypes were attributed to the antimicrobials that were used in animal food production environments (Hayes et al. 2003).

The consumption of chicken meat contaminated with enterococci is dangerous for public health, and especially the presence of strains with resistance genes can play a role in the transfer of these genes to consumers (Aslam et al. 2012).

Among the 100 chicken meat samples, 27% were determined to be *Enterococcus* spp. positive, which is close to the value of 28.6% that was reported by Pesavento et al. (2014) and the value of 30% that was reported by Onaran et al. (2019). Yüksel et al. (2013), Kilonzo-Nthenge et al. (2015), Donado-Godoy et al. (2015), Şentürk (2017), Kim et al. (2018), and Sanlibaba et al. (2018) reported higher prevalence values of 43%, 82.2%, 94%, 91.66%, 77.7%, and 79.50%, respectively, while Bayram et al. (2011) and Gousia et al. (2015) reported lower prevalence values of 12.5% and 21.7%, respectively. Enterococci are sensitive to sanitation and can be eliminated when effective cleaning procedures are applied. The constant negligence of cleaning practices will allow the growth of enterococci by causing the formation of a mineral residue that protects organisms from disinfectants (Adams and Moss 2008). The differences in the reported values are attributable to the lack of adherence to hygienic conditions during the production and storage of chicken meats.

E. faecalis and *E. faecium* are known to be the cause of the majority of the human enterococcus infections of hospital and food origin (Cetinkaya et al. 2000; Lawlwy et al. 2012; Lebreton et al. 2013).

In the study, the most prevalent strain was *E. faecalis* with a rate of 79.10% while the rest of the isolates (20.90%) was identified to be *E. faecium*. In agreement with this study, many studies have reported *E. faecium* and *E. faecalis* to be the most identified species (Hayes et al. 2003, Bayram et al. 2011, Kim et al. 2018, Sanlibaba et al. 2018, Molechan et al. 2019, Manson et al. 2019).

Robredo et al. (2000) reported that the dominant species was *E. durans*, followed by *E. faecalis* and *E. faecium*. This difference in our findings is attributable to the differences in the analysis methods as well as to regional differences.

At least 50% of the *E. faecalis* and *E. faecium* strains showed intermediate resistance to ampicillin, penicillin, chloramphenicol, vancomycin, and gentamicin. The antibiotic sensitivity of the *E. faecalis* and *E. faecium* isolates differ depending on geographical conditions. Various studies have been conducted in Turkey (Kasimoglu-Dogru et al. 2010, Onaran et al. 2019; Gökmen and Ektik, 2022), Canada (Aslam et al. 2012), Amerika-Tennessee (Kilonzo-Nthenge et al. 2015), Northwest Greece (Gousia et al. 2015), Colombia (Donado-Godoy et al. 2015), and South Korea (Kim et al. 2018; Kim et al. 2019). In the present study, 40.3% of the isolates were resistant to erythromycin and 71.64% of the isolates exhibited resistance to tetracycline. Molechan et al. (2019) determined that 76% of the chicken meat samples were resistant to erythromycin while all samples were resistant to tetracycline. Kim et al. (2018) reported that most isolates were resistant to erythromycin and tetracycline. In another study, 58% of the *E. faecalis* isolates were determined to be resistant to erythromycin while 71.4% were resistant to tetracycline; 48.6% of the *E. faecium* samples were resistant to erythromycin while 40.5% were resistant to tetracycline (Donado-Godoy et al. 2015). In another study on poultry meat, the majority of the *E. faecalis* and *E. faecium* isolates were determined to be resistant to erythromycin and tetracycline (Gousia et al. 2015). The results of this study agree with those reported in previous studies.

Clinical failures have been reported for fluoroquinolone, erythromycin, tetracycline, and chloramphenicol for the treatment of *Enterococcus* spp. infections, which has been attributed to the widespread resistance of enterococci to erythromycin, clindamycin, and tetracyclines (Korten 2002; Moellering 2005). Moreover, the difference in findings of the studies on antibiotic resistance is attributable to the unconscious and illegal use of antibiotics in addition to geographical differences.

Enterococcus infections have been treated using glycopeptide antibiotics, especially using vancomycin, as they are approved for use in human treatment. However, there has been a drastic upsurge in vancomycin resistance with the widespread clinical use of vancomycin in hospitals (Kirst et al. 1998).

The concern about the increasing number of vancomycin-resistant enterococcus strains have been increasing (Lawlwy et al. 2012). The *VanA* phenotype is mostly found in *E. faecalis* and *E. faecium* and shows highly inducible resistance to vancomycin and teicoplanin while *VanB* shows intermediately inducible resistance to vancomycin (Švec and Devriese 2015). Furthermore, enterococci are known to gain antibiotic resistance through genetic mobile elements such as plasmids, integrons, and transposons, mutations, chromosomal, and exchange. The species can develop acquired resistance to many antibiotics through their various resistance properties (Hegstad et al. 2010, Hollenbeck and Rice 2012).

As the best-identified resistance genes in enterococci, the presence of the *VanA* and *VanB* genes was examined in the present study. However, the *VanA* and *VanB* genes were not detected in the phenotypically and intermediately vancomycin-resistant strains. Kasimoglu-Doğru et al. (2010) also did not detect the *VanA* and *VanB* genes. In another study, 93.5% of the *E. faecium* isolates that were obtained from different foods and phenotypically resistant to vancomycin had the *VanA* gene while 29% had the *VanB2,3* gene; the researchers did not detect *VanA*, *VanB*, or *VanB2,3* in the *E. faecalis* isolates (Gousia et al. 2015). Onaran et al. (2019) identified the *VanA* gene in 16.7% of the *Enterococcus* spp. isolates and the *VanB* gene in 8.3% of the isolates. In addition to the *VanA* and *VanB* genes, which are the best-identified vancomycin resistance genes in enterococci, *VanC*, *VanD*, *VanE*, *VanG*, *VanL*, *VanM*, and *VanN* resistances have also been observed (Arthur and Courvalin 1993, Ahmed and Baptiste 2018). Gökmen and Ektik (2022) found 31.5% *VanA*, 8.2% *VanB* and 23.3% *VanC2/C3* resistance genes in *Enterococcus* spp isolates. This explains the phenotypical resistance to vancomycin that was found in this study. Furthermore, the differences in the findings of the studies are attributable to differences in genetic mobile elements, chromosomal exchange, and mutations.

The presence of *Enterococcus* spp. in the chicken meats indicates the lack of adherence to hygienic conditions. The multiple antibiotic resistance of the isolates will complicate treatments and add to antibiotic resistance. No vancomycin resistance genes were detected in the study. The resistance in different phenotypes and the detection of vancomycin-resistant genes in enterococci that were isolated from foods in different studies pose a threat to public health. To prevent these undesired outcomes, inspections should become firmer, the use of antibiotics should be controlled, and strict policies should be employed.

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