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Research Article

Isolation of Vancomycin Resistant Enterococci from Animal Faeces, Detection of Antimicrobial Resistance Profiles and Vancomycin Resistant Genes [1] [2]

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Abstract

Infections caused by Vancomycin Resistant Enterococci (VRE) are important in human medicine in terms of treatment difficulties. Molecular studies in the last years revealed that VRE occurrence in animals might be important in epidemiology of infections in human. This study aims to detect VRE occurrence in various animals, examine antibiotic resistance profiles phenotypically, and determine the distribution of the vancomycin resistant genes, *vanA, vanB, vacC1, vanC2/C3*. For this purpose, rectal swabs were collected from farm and companion animals; and cloacal swab or litter were collected from chickens and they were processed for VRE isolation. Following the identification of the isolates, antimicrobial susceptibilities of the isolates were determined in accordance with the Clinical and Laboratory Standards Institute (CLSI) standards. Distribution of the vancomycin resistant genes; *vanA, vanB, vanC1* and *vanC2/C3* among enterococcus species and different animal species were determined by multiplex PCR. VRE were isolated from 17% of the feline samples, 20% of each of the other species, and 19% of all the samples. Those isolates were identified as *E. casseliflavus* (n=39), *E. gallinarum* (n=55) and *E. faecium* (n=3) as a result of multiplex-PCR. According to the antimicrobial susceptibility tests, most of the isolates were found to be resistant to penicillin G, ciprofloxacin and erythromycin. Eighteen (18.8%) of the isolates were found to be resistant against two antibiotic groups, while 69 (71 %) of them were resistant to three or more antibiotics.

Keywords: *Antimicrobial resistance, Enterococcus spp., animal faeces, vancomycin resistance*

Hayvan Dışkılarından Vankomisin Dirençli Enterokokların İzolasyonu, Antimikrobiyal Direnç Profillerinin ve Vankomisin Direnç Genlerinin Saptanması

Özet

Vankomisin dirençli enterokoklardan (VRE) kaynaklanan infeksiyonlar, tedavide karşılaşılan zorluklar nedeniyle insan hekimliğinde önemli bir yer tutmaktadır. Son yıllarda yapılan genetik düzeydeki çalışmalar, hayvanlardaki VRE varlığının da insanlardaki infeksiyonunun epidemiyolojisinde önemli olacağını vurgulamaktadır.Bu çalışmada farklı hayvan türlerinde VRE varlığı ve türlerinin dağılımının saptanması; antibiyotiklere duyarlılıklarının belirlenmesi; vankomisin direncinin kodlayan v*anA, vanB, vacC1, vanC2/C3* genlerinin dağılımının araştırılması amaçlanmıştır. Bu amaçla evcil hayvanlardan ve çiftlik hayvanlarından rektal svab ve tavuklardan kloakal svab/altlık örnekleri (n=500) toplandı ve VRE yönünden bakteriyolojik olarak incelendi. İzolatların identifikasyonun takiben, antimikrobiyal duyarlıkları Clinical and Laboratory Standards Institute (CLSI) standartlarına uygun yöntemlerle saptandı. Vankomisine direnç ile ilişkili *vanA, vanB, vanC1* ve *vanC2/C3* genlerinin *Enterococcus* türleri arasındaki dağılımı multipleks-PCR ile araştırıldı. Kedilerin %17'sinden, diğer gruplardaki hayvanların herbirinin %20'sinden, toplamda örneklerin %19'undan VRE izole edilmiştir. Yapılan multiplex PCR sonucunda izolatların 39'u *E. casseliflavus,* 55'i *E. gallinarum* ve 3'ü *E. faecium* olarak tanımlanmıştır. Antimikrobiyal duyarlılık testi sonuçlarına göre izolatların büyük çoğunluğu penicillin G, siprofloksasin ve eritromisine dirençli bulundu. İzolatların 18 (%18.6)'inin iki antibiyotik grubuna ve 69 (%71)'unun 3 ve daha fazla sayıda antibiyotik grubuna dirençli oldukları saptandı.

Anahtar sözcükler: *Antimikrobiyal direnç, Enterococcus spp., hayvan dışkıları, vankomisin direnci*

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INTRODUCTION

Enterococci are a part of normal human and animal faecal flora. On the other hand, they can cause septicemia, endocarditis, meningitis, urinary and genital tract infections as opportunistic pathogens; and they have emerged as an increasingly important cause of nosocomial infection since 1980s. These bacteria have clinical importance because of their increasing acquired antimicrobial resistance along with intrinsic resistance [1-4]. In the last decade, studies on examination of nosocomial infectious agents such as methicillin resistant staphylococci, vancomycin resistant enterococci in different animals started to have clinical concern. The emergence of resistance to vancomycin has presented an increasingly important problem in treatment [1,2,5-10]. Various genes including *vanA, vanB, vanC, vanD*, *vanE, van G, vanN* and *vanM* encode vancomycin resistance among enterococci. There are two types of vancomycin resistance. The first is intrinsic resistance demonstrated as low-level resistance to vancomycin, which is observed among *E. gallinarum, E. casseliflavus* and *E. flavescens* species. These strains carry *vanC* gene and are susceptible to teicoplanin. The second one is acquired and inducible resistance, which is mostly observed in *E. faecium* and *E. faecalis*. These strains often carry transferable *vanA* or *vanB* genes. Strains with *vanA* genotype display inducible high-level resistance to both vancomycin and teicoplanin; while strains with *vanB* genotype have resistance only to vancomycin [2,4,8-11]. Although vancomycin resistant *E. gallinarum* strains commonly carry *vanC* gene, strains carrying both *vanA* and *vanC* genes have been reported [8,9]. Therefore, it is clinically and epidemiologically important to determine the gene encoding resistance and then to detect vancomycin resistance phenotypically [8,9]. There are various studies reporting the existence of VRE species in various animal species [2,4,6,11,12]. Recent molecular epidemiological studies suggest that VRE residing the gastrointestinal flora of animals can be a source of infection for human. Many researchers report that those VRE can be transmitted to human via contaminated raw or insufficiently treated food, or after a physical contact with a companion animal such as cats and dogs [7,10,12,13]. This study aims to detect VRE occurrence in various animals, to examine antimicrobial resistance profiles phenotypically, and to determine the distribution of vancomycin resistant genes, *vanA*, *vanB*, *vacC1*, *vanC2/C3*.

MATERIAL and METHODS

Fecal Samples

The animal species in this study were divided into three groups. The first group included farm animals (cows and sheep); the second group included companion animals (cats and dogs); the third group included poultry animals. One hundred rectal swabs were collected from each species in group one and two (totally 400 samples). In group three, rectal swabs or litter samples were collected from each flock. Samples from group-1 were collected from the farms were located mainly in Istanbul and Çatalca, Maşukiye, Tekirdağ. In those farms the most common antimicrobials were enrofloxacin, amoxicillin clavulanic acid, oxytetracycline, penicillin G and erythromycin. All of the canine and feline samples were collected from the animals in Istanbul. The most common antimicrobials were cephalosporins, aminoglycosides particularly gentamicin, azithromycin and enrofloxacin. Poultry samples were collected from Marmara region mainly, Istanbul, Balıkesir, Bandırma. Erythromycin, neomycin and tylosin were used in those flocks. Other information about the animals is shown in *Table 1*.

Culture

Swabs were inoculated into tubes containing Bile Esculin Azide Broth (BD BBL 212207) supplemented with 6 µl/ml vancomycin hydrochloride (Molekula) and incubated for 24 h at 37˚C. Five grams from the litter samples were homogenized in 45 ml saline water and 5 ml of it was transferred into Bile Esculin Broth supplemented with 6 µl/ml vancomycin hydrochloride (Molekula). Cultures

Table 1. *Animals included in the study*
Tablo1.*Çalışmaya dahil edilen hayvanlar*

Animal Species	Samples	Age		Antibiotic Usage		
		<1 year	≥1 year	Used	Not Used	Not Known
Sheep	100 rectal swabs	28	72	1[a]	8[a]	0
Cattle	100 rectal swabs	8	92	32	52	16
Cat	100 rectal swabs	27	73	37	32	31
Dog	100 rectal swabs	24	76	14	47	39
Poultry	3 layer flocks, 45 cloacal swabs	-[c]	-[c]	3[a]	0	0
	53 broiler flocks, 53 litter samples	-[c]	-[c]	26	27	0
	2 individual samples[b]	-[c]	-[c]	0	2	0

[a] The numbers indicate the farm/flock number; [b] Intestinal content from a pigeon and a layer chicken after necropsy; [c] Layers: between 3 to 11 months-old; broilers: between 10 to 45 days-old; individual samples: 4 months-old pigeon and 30 days-old broiler chicken

with colour change were subcultured onto Bile Esculin Agar (BD BBL 299068) and incubated for 24 h at 37˚C. Presumptive *Enteroccocus* spp. with black colour was subcultured onto Nutrient agar (BD Difco 269100) plates supplemented with 7% sheep blood to achieve pure cultures. Catalase negative, aesculin hydrolysis positive and growth of 6.5% in NaCl positive colonies were evaluated as presumptive *Enterococcus* species. Further identification was performed through API 20 STREP along with pigment production; and by methyl-α D-glucopyranoside acidification test and motility test. Due to the inadequacy of API 20 STREP test in differentiation of some strains, the final identification was completed after the multiplex PCR results [9,11,12,14,15].

Detection of Vancomycin Resistance Genes

After phenotypical confirmation of vancomycin resistance of the isolates by macro-dilution method [17], vancomycin resistant enterococci were further examined by multiplex PCR according to Kariyama et al.[15] for the detection of genes encoding vancomycin resistance. Primers suggested by Elsayed et al.[16] were used in order to detect *vanB* gene. Fifty µl from VRE cultures from Tryptic Soya Broth after 24 h of incubation at 37˚C were mixed with the equal volume of 7.5% Chelex 100 (BioRad). The mixture was heated for 10 minutes at 100˚C and centrifuged; and a 2.5 µl volume of the supernatant was then used for PCR amplification. Primer sets shown in *Table 2* were included into the reaction mixtures as follows: 5 pmol of *vanA* primers, 2.5 pmol of each, *vanC1*, *vanC2/C3* and *rrs* primers, 7.5 pmol of *E. faecalis* specific primers, 1.25 pmol of *vanB, E. faecium* specific primers. The multiplex PCR was performed in a total volume of 25 µl containing 10 mM Tris HCL (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM per deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 0.625 U *Taq* DNA polymerase (TaKaRa Taq, 250 U). DNA amplification was carried out according to the following protocol: initial denaturation at 94˚C for 5 min, 30 cycles of amplification (denaturation at 94˚C for 1 min, annealing at 54˚C for 1 min, extension at 72˚C for 2 min) and final extension at 72˚C for 10 min in a (MaxyGene Gradient Therm-1000) system. PCR products were analyzed on a 1.5% Agarose B Low EEO (Bio Basic Inc.) with 0.5 x Tris-borate-EDTA buffer.

Control strains which were kindly provided by Dr. Luca Guardabassi (Life University, Copenhagen), including *E. faecium* BM4147 (VanA), *E. faecalis* V583 (Van*B*), *E. gallinarum* BM4174 (VanC1), *E. casseliflavus* DSMZ 20680 (VanC2/C3), *E. faecium* CCUG542 (vancomycin susceptible) were used in PCR assays.

Antimicrobial Susceptibility Test

The isolates were examined by disc diffusion method according to the standards of Clinical and Laboratory Standards Institute (CLSI) for detection of penicillin (10 mg), ampicillin (10 mg), erythromycin (15 mg), tetracycline (30 mg), ciprofloxacin (5 mg), doxycycline (30 mg) and rifampicin (5 mg) susceptibilities. In addition, Minimal Inhibition Concentration (MIC) values for teicoplanin were determined by broth macro dilution method. To detect high level of aminoglycoside resistance (HLAR), the growths in gentamicin (600 mg/ml) and streptomycin (1.000 mg/ml) were evaluated. In order to detect β-lactamase producing isolates, beta-lactamase (Nitrocefin) disks (Bio Chemika, Fluka) were used. *E. faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923 strains were used as control strains in antimicrobial susceptibility tests [17].

RESULTS

Isolation and Identification of Vancomycin Resistant Enterococci

Of 500 fecal samples 97 (19%) VRE were isolated. Isolation rate of VRE were 17% for cats, 20% for each of

Table 2. *Multiplex PCR primers*
Tablo 2.*Multipleks PCR primerleri*

Target Gene	Size of PCR Product	Primer Pair Sequence	Reference
vanA	1.030 bp	5′-CATGAATAGAATAAAAGTTGCAATA -3′ 5′-CCCCTTTAACGCTAATACGATCAA -3′	[14]
vanB	536 bp	5′-AAGCTATGCAAGAAGCCATG -3′ 5′-CCGACAATCAAATCATCCTC -3′	[10]
vanC1	822 bp	5′-GGTATCAAGGAAACCTC -3′ 5′-CTTCCGCCATCATAGCT -3′	[14]
vanC2/C3	484 bp	5′-CGGGGAAGATGGCAGTAT -3′ 5′-CGCAGGGACGGTGATTTT -3′	[14]
E. faecalis	941 bp	5′-ATCAAGTACAGTTAGTCTTTATTAG -3′ 5′-ACGATTCAAAGCTAACTGAATCAGT -3′	[14]
E. *faecium*	658 bp	5′-TTGAGGCAGACCAGATTGACG -3′ 5′-TATGACAGCGACTCCGATTCC -3′	[14]
rrs (16SrRNA)	320 bp	5′-GGATTAGATACCCTGGTAGTCC -3′ 5′-TCGTTGCGGGACTTAACCCAAC -3′	[14]

the other groups. Biochemical tests and API results were sufficient only for the identification of the isolates in the genus level, but the differentiation of *E. faecalis, E. gallinarum* or *E. casseliflavus* was performed after multiplex PCR assay. Final identification results are shown in *Table 3*.

Group 1 - Farm Animals: VRE were isolated from 20% of sheep samples examined. The samples were collected from six different farms; in only one of these farms, antimicrobial treatment was applied to the animals, and six VRE were isolated from the sheep from that farm. The VRE isolation rate for the cow was 20%. Twenty percent (n=4) of those isolates were from the farms in which antibiotic treatment had been applied and 60% were from the antibiotic unused farms. Among these 20 isolates, 20% of them were isolated from cattle that had an antimicrobial treatment within one year, while 60% of them from non-antimicrobial used animals. For the remaining four VRE positive animals, the farmers gave no information about antimicrobial usage.

Group 2 - Companion animals: VRE were isolated from 20% of the dogs. The isolation rate was 29% in dogs with antimicrobial therapy history within one-year period, and was 19% in dogs with non-antimicrobial therapy background. VRE were isolated from 4 of 24 dogs that were younger than 1-year age; 16 of 76 one-year age and older dogs. Of dogs from which VRE were isolated, 75% were owned dogs; remaining 25% were from kennels. VRE were isolated from 17% of the sampled cats. The isolation rate was 24% in the cats with antimicrobial therapy history within one-year period, and was 19% in cats without any therapy. VRE were isolated from 10 of 27 cats that were younger than 1-year age; 7 of 73 one-year age and older ones. It was remarkable that VRE were isolated from five of the six cats that were younger than one-year and with antimicrobial therapy history.

Group 3 - Poultry animals: VRE were isolated from 12 of 46 layers, 7 of 53 litter samples and one pigeon. It was informed that in 26 of the 53 broiler flocks antimicrobial agents were being used and VRE were isolated from 6 of those flocks. In 29 flocks no antimicrobial therapy were applied and VRE were isolated only from one of those flocks. Antimicrobial agents were used in all of the layers.

Antimicrobial Susceptibility Test Results

Group 1 - Farm animals: The MIC values of ovine isolates were 8-16 µg/ml and 0.5-1 µg/ml for vancomycin and teicoplanin, respectively. All the ovine isolates were susceptible to tetracycline, and none of them showed high level of aminoglycoside resistance, however, 30% of the isolates were multidrug resistant (resistant to three or more antimicrobial agents).

MIC value was 512 µg/ml for vancomycin and 64 µg/ml for teicoplanin for one bovine isolate *(Table 3)* and the values of the remaining bovine isolates were 8-32 µg/ml and 0.5-16 µg/ml for vancomycin and teicoplanin, respectively. None of the bovine isolates had high level of aminoglycoside resistance, and 60% of them were multidrug resistant.

Group 2 - Companion animals: For canine isolates; 18 isolates had 8-16 µg/ml and 0.5-1 µg/ml MIC values, while two high level vancomycin resistant isolates *(Table 3)* had 512 µg/ml and 128 µg/ml MIC values for vancomycin and teicoplanin, respectively. Among the isolates, 75% were multidrug resistant, and more than 50% of those isolates were resistant to penicillin, rifampicin, ciprofloxacin and erythromycin. High level of gentamicin resistance were observed in one isolate and both streptomycin and gentamicin resistance in two isolates. Two *E. gallinarum* isolates with high level of vancomycin resistance were also resistant to all other antimicrobial tested by disc diffusion; and in addition, one of them had HLAR.

MIC values of feline isolates were 16-32 µg/ml and 0.25-1.0 µg/ml for vancomycin and teicoplanin respectively. Ninety four percent of the isolates were multidrug resistant. High level of aminoglycosides (both gentamicin and streptomycin) was observed in five isolates, only streptomycin resistance was observed in three isolates.

Group-3: Poultry animals: The MIC values of 17 avian isolates were between 8-16 µg/ml and 0.5-2 µg/ml for vancomycin and teicoplanin, respectively. The remaining three isolates showed high level of vancomycin and teicoplanin (256-512 µg/ml and 32-128 µg/ml) *(Table 3, Table 5)*. All avian isolates were multidrug resistant. Among 20 isolates, in 10 (50%) only streptomycin, in five (25%)

Table 3. *Distribution of vancomycin resistant enterococci species*
Tablo 3. *Vankomisin dirençli enterokok türlerinin dağılımı*

Source	*E. faecium & vanA*	*E. casseliflavus*	*E. gallinarum*	Total
Dog	0	6	14[a]	20
Cat	0	3	14	17
Cow	0	19[a)]	1	20
Sheep	0	11	9	20
Poultry	3	0	17	20

[a] High level of vancomycin resistance (MIC value = 512 µg/ml) was detected in two canine and one bovine isolates, but vanA or vanB genes were not detected

only gentamicin and in five (25%) both streptomycin and gentamicin resistance were detected.

Isolates with High Level of Vancomycin Resistance

We detected high level of vancomycin resistance in two canine *E. gallinarum* isolates and one bovine *E. casseliflavus* isolate, however, we did not detect *vanA* or *vanB* genes by PCR. The two dogs were from a kennel and from the same cage. Since they were street dogs, there were no information about their previous health status, and antimicrobial therapy history.

The other isolate, *E. casseliflavus* with *vanC* gene was from a 3 to 6 years old cattle raised in a semi intensive system. Ten animals from the same farm were examined and VRE were isolated from 5 (50%) of them, however, high level of vancomycin resistance were observed from only one animal. The farmers informed us that oxytetracycline and penicillin products had been used in this farm in the sampling year, but they had not been applied to this animal.

There were three *E. faecium* isolates carrying *vanA* gene, two of them were isolated from the litters of two different flocks including 90-day-old layers in a breeding unit. The other isolate was from a litter of a 10 to 15 day-old broiler flock in which enrofloxacin application was being performed at the sampling time.

Antimicrobial resistance rates of the all vancomycin resistant enterococci and of the isolates with high level of vancomycin resistance were shown in *Table 4* and *Table 5*, respectively.

DISCUSSION

There are various studies on occurrence of VRE in different animal species or their products. The different isolation rates or diversity of species can be resulted from different breeding facilities, management procedures and environmental factors [2,4,10-12,18-20]. The presence of VRE in companion animals is becoming a high clinical concern due to the high transmission risk of VRE via close contact with their owners. Herrero et al.[12] have examined randomly selected 87 dogs for the presence of VRE for 5 years, and have isolated VRE from 15 samples and have reported that *vanA* originated glycopeptides resistance was common among the canine *E. faecium* isolates. Boynukara et al.[6] have detected vancomycin resistance in 91.3% of *Enterococcus* species isolated from human, dog and cat faeces. Lopez et al.[10] have sampled 126 canine faecal samples and have not detected VRE with acquired resistance. In the present study, no *Enterococcus* species carrying *vanA* and/or *vanB* genes were isolated; however, *E. gallinarum* and *E. casseliflavus* with *vanC1* and *vanC2/3* genes were detected. Besides, in two dogs, *Enterococcus* species representing VanA phenotypic resistance (high-level resistance to vancomycin and teicoplanin) were detected.

De Leener et al.[13] have reported that combination of different resistance against two and more antimicrobial agents detected more frequently from cats and dogs from kennels than the ones from private owners. There are some reports documenting the presence of VRE in dogs living on farms where VRE were present among the other farm animals. However, Herrero et al.[12] have reported that ten of the eleven VRE harbouring dogs did not have any contact with farm animals. Abbott et al.[1] have described a high-level gentamicin resistant and vancomycin resistant *E. faecium* in a dog. The authors have suggested that the origin of the agent was from an external source, possibly from the oral cavity or faeces of an attacking dog, a veterinary health care profession, the owner or the environment. In the present study, 75% of the VRE positive dogs were owned dogs and they did not have any direct contact with any other animals. The VRE isolation rate

Table 4. *Antimicrobial resistance rates of vancomycin resistant enterococci based on animal species*
Tablo4. *Vankomisin dirençli enterokokların hayvan türlerine gore antimikrobiyal dirençlilik oranları*

Source	Number (%)	P10	AM 10	E15	T30	CIP5	DO30	RA5	GM-HLAR	S-HLAR	B-LACTAMASE
CAT (n=17)	n	17	9	13	8	12	7	6	5	8	-
	%	100	53	76	47	71	41	35	29	47	-
DOG (n=20)	n	18	6	12	9	10	7	11	2	3	-
	%	90	30	60	45	50	35	55	10	15	-
COW (n=20)	n	16	2	12	3	13	1	11	-	-	-
	%	80	10	60	15	65	5	55	-	-	-
SHEEP (n=20)	n	16	2	4	-	14	3	12	-	-	-
	%	80	10	20	-	70	15	60	-	-	-
POULTRY (n=20)	n	20	8	20	19	12	15	14	10	15	-
	%	100	40	100	95	60	75	70	50	75	-
TOTAL (n=97)	n	87	27	61	39	61	33	54	17	26	-
	%	90	28	63	40	63	34	56	18	27	-

P10 *= penicillin (10 mg),* ***AM 10*** *= ampicillin (10 mg),* ***E15*** *= erythromycin (15 mg),* ***T30*** *= tetracycline (30 mg),* ***CIP5*** *= ciprofloxacin (5 mg),* ***DO*** *= 30 doxycycline (30 mg),* ***RA5*** *= rifampicin (5 mg),* ***GM-HLAR*** *= gentamicin-high level of aminoglycoside resistance,* ***S-HLAR*** *= streptomycin- high level of aminoglycoside resistance*

Table 5. *Antimicrobial susceptibilities of the five Enterococcus species with high level of vancomycin resistance*
Tablo 5. *Yüksek vankomisin direncine sahip beş Enterokok türünün antimikrobiyal duyarlılık profili*

Sample Number	Species and Resistant Gene	MIC-Van (ug/ml)	MIC-Tei (ug/ml)	P10	AM 10	E15	T30	CIP5	DO30	RA5	GM-HLAR	S-HLAR	B-LACTAMASE
132[a]	*E. faecium, VanA*	256	64	R	R	R	O	S	S	S	R	S	N
135[a]	*E. faecium, VanA*	256	32	R	S	R	S	S	S	S	R	S	N
61[a]	*E. faecium, VanA*	512	128	R	S	R	R	S	R	S	S	R	N
147[b]	*E. gallinarum VanC1*	512	256	R	R	O	R	O	O	R	S	S	N
148[b]	*E. gallinarum VanC1*	512	128	R	R	R	R	R	O	R	R	R	N
82[c]	*E. casseliflavus VanC2*	512	64	R	S	O	O	S	S	R	S	S	N

MIC-Van *= Minimal İnhibitory Concentration for vancomycin,* ***MIC-Tei*** *= Minimal İnhibitory Concentration for teicoplanin,* ***P10*** *= penicillin (10 mg),* ***AM 10*** *= ampicillin (10 mg),* ***E15*** *= erythromycin (15 mg),* ***T30*** *= tetracycline (30 mg),* ***CIP5*** *= ciprofloxacin (5 mg),* ***DO*** *= 30 doxycycline (30 mg),* ***RA5*** *= rifampicin (5 mg),* ***GM-HLAR*** *= gentamicin-high level of aminoglycoside resistance,* ***S-HLAR=*** *streptomycin- high level of aminoglycoside resistance; [a] avian isolate; [b] canine isolate; [c] bovine isolate*

among the dogs treated with an antimicrobial therapy within a year was 29%, while it was 19% among the dogs without any therapy. Besides, it was remarkable that the two isolates with high-level vancomycin and teicoplanin resistance were both from two dogs sharing the same box in a kennel. There was no information, for example, about the antimicrobial therapy background of those two dogs. However, as they are sharing the same box, horizontal transmission of the agent is highly possible.

Seo et al.[4] have detected *vanA* gene in six of VRE isolates showing high-level vancomycin resistance (MIC: >256 µg/ml) from poultry farms, and four of those isolates were resistant to other antimicrobials in addition to vancomycin and teicoplanin. Ünal et al.[21] were isolated high-level vancomycin resistant *E. faecium* from one of 400 swab samples collected from commercial broiler farms. In a study in Brazil, Xavier et al.[18] have not isolated any *Enterococcus* species carrying *vanA* or *vanB* genes, but they have detected *vanC1* in 13% and *vanC2/C3* in 5.5% of the isolates. Kaya et al.[22] have reported that none of the 80 *Enterococcus* species from chicken intestinal content showed resistance to vancomycin and teicoplanin; however, they have detected that 17.5% of them were resistant to high-level aminoglycosides. In the present study, *vanA* carrying *E faecium* (n=3) and *vanC1* carrying *E. gallinarum* (n=19) were isolated from poultry samples. Two of the *E. faecium* isolates were from layer flocks having neomycin sulphate administration, the other one was from a broiler flock with enrofloxacin administration at the time of sampling. It was observed that VRE isolation rate was higher in the flocks with intense antimicrobial usage.

Kempf et al.[23] have reported that all *vanA* carrying avian originated *Enterococcus* spp. isolates were resistant to tetracycline, 66 % of them were resistant to erythromycin, but none of them was resistant to ampicillin or gentamicin. Herrero et al.[12] have reported that all vancomycin resistant *E. faecium* strains from dogs were highly resistant to vancomycin and harboured the *vanA* gene; moreover, 11 of those strains were resistant to tetracycline, and 10 were resistant to erythromycin. Kaya et al.[22] have detected resistance to erythromycin in 45% of the chicken VRE strains. In the present study, 55% of ovine, 60% of bovine, 75% of the canine, 94% of the feline and all of the avian isolates were resistant to three or more antimicrobial agents. It was remarkable that all the chicken isolates were resistant to penicillin and erythromycin and 95% of them were resistant to tetracycline. This resistance profiles especially in avian isolates, are good examples of adverse effect of antimicrobial usage for preventive purposes in poultry flocks.

Gentamicin is an antimicrobial agent used in combination with b-lactams or glycopeptide antibiotics for treatment of enterococcal infections in humans. However, this synergistic bactericidal effect is lost in case of high-level of gentamicin resistance. Transmission of gentamicin resistant enterococci from food-producing animals to human through food chain was discussed. Besides, it was also mentioned that enterococci from the intestinal microbiota of cats and dogs might act as a reservoir of resistance genes for animal and human pathogens. Therefore it is important to pay attention to this type of resistance and a well-considered use of this antibiotic in companion animals is needed [3,13]. High-level aminoglycoside resistance occurs in two mechanisms. The first one is the resistance resulted by alteration of aminoglycoside binding region on the ribosomes. This kind of resistance only causes high level of streptomycin resistance (S-HLAR) and is not transferable. In the second resistance mechanism, which is observed as transferable gentamicin resistance (GM-HLAR), adenyltransferase, phosphotransferase, acetyltransferase enzymes are involved. The strains with GM-HLAR are resistant to all other aminoglycosides except streptomycin [3]. In the current study, GM-HLAR was detected in five feline, two canine and 10 avian isolates. The contamination risk at the poultry slaughter houses or close contact of the companion animals with their owners increases the importance of the detection of this transferable resistance in this study. Furthermore, resistance to both streptomycin and gentamicin was detected in five canine, two feline and five avian isolates. In any case of transmission of such isolates to human, it

would be unavoidable to have some problems in the treatment of these cases.

E. casseliflavus and *E. gallinarum* represent significant percentage of the faecal enterococci population of various animal species [4,10,11]. Khan et al.[24] have isolated *E. gallinarum* from milk samples of animals with mastitis and litters from 28 different flocks. In addition to the intermediate level of vancomycin resistance, those isolates were resistant to 6 to 8 antimicrobial agents among 13 different antimicrobials. The researchers have commented that the situation occurs because of previous usage of those antimicrobial agents or transmission of some resistance markers from another bacterial species. Although isolates with low level of vancomycin resistance (MIC: 4-8 µg/ml) were evaluated as unimportant isolates some cases such as endocarditis, bacteremia caused by *E. gallinarum* and *E. casseliflavus* strains with *vanC* intrinsic resistance particularly in immunosuppressed people has been reported recently [4,9]. Moreover, Corso et al.[9] have revealed that the clones of two *E. gallinarum* isolates with *vanA* gene had successfully transferred their resistance gene to one previously vancomycin susceptible *E. faecium* strain. Haenni et al.[25] have reported the first isolation of *E. casseliflavus* S8702 strain with vanB/*vanA-vanC* complex resistance from three different calves. Lopez et al.[10] have recovered *E. gallinarum* and *E. casseliflavus* in 12% of dog and healthy human faecal samples. Çetinkaya et al.[7] have detected MIC values higher than 256 µg/ml for vancomycin and teicoplanin in *E. gallinarum*, *E. avium* isolates. There is no particular protocol suggested by CDC for the patients that are infected or colonized by *E. gallinarum*. However, most researchers emphasize that in spite of the lack of any instructions for those patients, the ability of *E. gallinarum* strains to catch the genes encoding the high level of vancomycin resistance and to transfer them to important clinical strains such as *E. faecium* should not be omitted [4,9,10,24].

In conclusion, in the present study, the isolation rate of *E. faecium* carrying *vanA* gene was low, however, we detected both *E. casseliflavus* and *E. gallinarum* isolates with multidrug resistance in both examined animal groups. When the close contacts between companion animals and the owners, or among farm animals and the farmers, or the cross contamination at the slaughterhouses from intestinal content through to carcasses are considered, the presence of those intrinsic vancomycin resistance and multidrug resistant *Enterococcus* species should never be ignored. Therefore, the importance of general hygiene and management rules as well as routine screening tests is increasing in different breeding facilities.

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