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Araştırma Makalesi ***Research Article***

Examination of Vancomycin Resistant Enterococci (VRE) Isolated from Canine and Feline Rectal Swabs

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Abstract

The aim of this study was to determine the occurrence of vancomycin resistant enterococci (VRE) in dogs and cats, examine the antimicrobial resistance profiles phenotypically, and the distribution of the vancomycin resistance associated genes by PCR. For this purpose, rectal swabs from animals were collected and processed for VRE isolation. After the identification of the isolates, antimicrobial susceptibility of the isolates were determined according to the Clinical and Laboratory Standards Institute (CLSI) standards. Finally, distribution of the vancomycin resistance associated genes; *vanA, vanB*, *vanC1* and *vanC2/C3* were determined by PCR. Totally 157 (86 canine, 71 feline) rectal swab samples were examined. VRE were isolated from 12 canine and 8 feline samples. The VRE isolation rate in companion animals was 12.7%. Antimicrobial susceptibility results varied among the isolates; however, enrofloxacin resistance was common in both species. Multidrug resistance was also detected. As a conclusion, screening of distribution of VRE among pet animals would be useful to detect any emerging antimicrobial resistance problem related to public health.

Özet

Kedi ve Köpek Rektal Sıvaplarından İzole Edilen Vankomisin Dirençli Enterokokların (VRE) Araştırılması

Bu çalışmanın amacı kedi ve köpeklerde vankomisin dirençli enterokokların (VRE) varlığının araştırılması, fenotipik olarak antimikrobiyal direnç profillerinin incelenmesi ve vankomisin direnci ile ilişkili genlerin dağılımının PCR ile saptanmasıdır. Bu amaçla kedi ve köpeklerden rektal sıvap örnekleri toplandı ve VRE izolasyonu yönünden incelendi. İzolatların identifikasyonunu takiben, Clinical and Laboratory Standards Institute (CLSI) standartlarına göre antimikrobiyal duyarlılıkları araştırıldı. Son olarak, vankomisin dirençle ilişkili genleri; *vanA, vanB*, *vanC1* ve *vanC2/C3* gen bölgeleri PCR ile saptandı. Toplam 157 (86 köpek, 71 kedi) sıvap örneği incelendi %12,7' sinden VRE izole edildi. 12 köpek ve 8 kedi örneğinden VRE izole edildi. Antibiyotik duyarlılık sonuçları izolatlar arasında farklılıklar göstermekle birlikte tüm izolatlar arasında enrofloksasin direnci en sık karşılaşılan dirençti. Aynı zamanda çoklu antibiyotik direnci de saptandı. Sonuç olarak, pet hayvanlarında VRE dağılımının sürekli taranması, antibiyotik direnci ile ilişkili olası halk sağlığı problemlerinin önlenmesi açısından önemi vurgulandı.

Introduction

Antimicrobial resistance amongst companion animals is a complex research subject that is of increasing importance because of both animal health and public health issues. Due to the close contact of people with companion animal particularly household pets, there is concerns about the animal and human health risks associated with multidrug resistant (MDR) infections (Bağcıgil et al., 2012; Weese, 2008). Enterococci are Gram positive opportunistic pathogens that are a part of normal human and animal faecal flora. They can cause various 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 (Ghosh et al., 2012; Ke et al., 1999; Weese, 2008). Genes including *vanA, vanB, vanC1, vanC2/vanC3, vanD*, *vanE, van G, vanL, vanN* and *vanM* encode vancomycin resistance among enterococci. There are two type of resistance to vancomycin: intrinsic and acquired resistances. The intrinsic resistance is observed among *E. gallinarum, E. casseliflavus* and *E flavescens* species. These species carry *vanC* gene, have

low-level resistance to vancomycin and susceptible to teicoplanin. The acquired and inducible resistance is mostly observed in *E. faecium* and *E. faecalis* strains carrying transferable *vanA* or *vanB* genes. Although vancomycin resistant *E. gallinarum* strains commonly carry *vanC* gene, strains carrying both *vanA* and *vanC* genes have been reported. Therefore, the determination of genes encoding the resistance is both clinically and epidemiologically important (Coombs et al., 1999; Corso et al., 2005; Lopez et al., 2013). The aim of this study was to examine occurrence of VRE in companion animals, to determine their antimicrobial resistance profiles phenotypically, and the distribution of vancomycin resistant genes.

Materials and Methods

Samples

One hundred and fifty seven rectal swab samples (86 canine, 71 feline) were collected within 6-month period. The age of the animals were varying between less than 1 year and up to 15 years. Almost half of the animals (48%) in both species were clinically healthy; the purposes of the visit were mostly vaccination, anti-parasitic treatment, or some of them were residents of the clinics. Other reasons were viral, bacterial or parasitic infections. Twenty one of 71 cats and 22 of 86 dogs had antimicrobial therapy history because of various health problems.

Isolation of vancomycin resistant enterococci

Swabs were inoculated into tubes containing Bile Esculin Azide Broth (BD BBL 212207) supplemented with 6 µg/ml vancomycin hydrochloride (Molekula) and incubated for 24 hours at 37°C. Presumptive *Enterococcus* spp. with black colour sub-cultured onto Nutrient agar (BD Difco 269100) plates supplemented with 7% sheep blood to achieve pure cultures. Catalase negative, esculin hydrolysis positive and growth in 6.5% NaCl positive colonies evaluated as presumptive *Enterococcus* species and then confirmed by PCR using *Enterococcus* specific primers (Table 1) as described previously (Ke et al., 1999). DNA extracts were prepared by boiling method (Kariyama et al., 2000). Briefly; 50 µl from VRE cultures from Tryptone Soya Broth (Oxoid CM0129) after 24 hours of incubation at 37°C were mixed with the equal volume of 6% Chelex 100 (BioRad). The mixture was heated for 10 minutes at 100°C and centrifuged; and a 2 µL volume of the supernatant was then used for PCR amplification. The PCR assay 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), 0,5 pmol of each primers, 1 U *Taq* DNA polymerase (FIREPol® DNA Polymerase, Solis BioDyne). DNA amplification was carried out with the following protocol: initial denaturation at 95˚C for 3 min, 35 cycles of amplification (denaturation at 95˚C for 30 sec, annealing at 55˚C for 30 sec, extension at 72 ˚C for 1 min) and final extension at 72 ˚C for 10 min in a (MaxyGene Gradient Therm-1000) system. PCR products were analysed on a 1.5% Agarose B Low EEO (Bio Basic Inc.) with 0.5 x Tris-borate-EDTA buffer to see PCR products with 112 bp lenght (Ke et al., 1999).

Detection of vancomycin resistance related genes

Before examining the vancomycin resistance related genes by PCR, vancomycin resistance of *Enterococcus* species (n=20) were confirmed by macro-dilution method according to CLSI protocols (CLSI 2006). All the confirmed vancomycin resistant enterococci were examined by multiplex PCR according to Kariyama et al. (2000) and Elsayed et al. (2001). DNA extracts were prepared by boiling method (Kariyama et al., 2000), as described above. The multiplex PCR assay was performed in a total volume of 25 µL containing 2 µL template DNA, 1xPCR buffer without $MgCl_2$ (Solis BioDyne), 0.625 U *Taq* DNA polymerase (FIREPol® DNA Polymerase, Solis BioDyne), 1.5 mM $MgCl_2$, 0.2 mM per deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 5 pmol of *vanA*, 1.25 pmol of *vanB,* 2.5 pmol of *vanC1,* 2.5 pmol of *vanC2/C3,* 2.5 pmol of *rrs* primers, 7.5 pmol of *E. faecalis*, 1.25 pmol of *E. faecium* specific primers. Primer sets shown in Table 1. DNA amplification was carried out with the following protocol: 94˚C for 5 min, 30 cycles of amplification (94˚C for 1 min 54˚C for 1 min, 72 ˚C for 2 min) and final extension at 72 ˚C for 10 min in thermocycler (MaxyGene Gradient Therm-1000). PCR products were analysed on a 1.5% Agarose B Low EEO (Bio Basic Inc.) with 0.5 x Tris-borate-EDTA buffer. *E. faecium* BM4147 (*VanA*), *E. faecalis* V583 (*VanB*), *E. gallinarum* BM4174 (*VanC1*), *E. casseliflavus* DSMZ 20680 (*VanC2/C3*), *E. faecium* CCUG542 (vancomycin susceptible) were used as reference strains in PCR assays.

Antimicrobial susceptibility test

The isolates were examined through disc diffusion method according to the standards of Clinical and Laboratory Standards Institute (CLSI) for detection of penicillin (10 µg), Ampicillin (10 µg), erythromycin (15 µg), tetracycline (30 µg), enrofloxacin (5 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), nitrofurantoin (300 µg) and rifampicin (5 µg) susceptibilities. In addition to this, Minimal Inhibition Concentration (MIC) values for teicoplanin (Sanofi-Aventis) were determined by broth macro dilution method.

Table 1. Primers used in the study.

Tablo 1. Çalışmada kullanılan primerler.

Primer specificity	Size of PCR product	Primer pair sequence	Reference
Enterococcus spp.	112 bp	5'-TACTGACAAACCATTCATGATG-3' 5'-AACTTCGTCACCAACGCGAAC-3'	Ke et al., 1999
vanA	1,030 bp	5'-CATGAATAGAATAAAAGTTGCAATA -3' 5'-CCCCTTTAACGCTAATACGATCAA -3'	Kariyama et al., 2000
vanB	536 bp	5'-AAGCTATGCAAGAAGCCATG -3' 5'-CCGACAATCAAATCATCCTC -3'	Elsayed et al., 2001
vanC1	822 bp	5'-GGTATCAAGGAAACCTC -3' 5'-CTTCCGCCATCATAGCT -3'	Kariyama et al., 2000
vanC2/C3	484 bp	5'-CGGGGAAGATGGCAGTAT -3' 5'-CGCAGGGACGGTGATTTT -3'	Kariyama et al., 2000
E. faecalis	941 bp	5'-ATCAAGTACAGTTAGTCTTTATTAG -3' 5'-ACGATTCAAAGCTAACTGAATCAGT -3'	Kariyama et al., 2000
E. faecium	658 bp	5'-TTGAGGCAGACCAGATTGACG -3' 5'-TATGACAGCGACTCCGATTCC -3'	Kariyama et al., 2000
rrs (16SrRna)	320 bp	5'-GGATTAGATACCCTGGTAGTCC -3' 5'-TCGTTGCGGGACTTAACCCAAC -3'	Kariyama et al., 2000

To detect high level of aminoglycoside resistance (HLAR), the growth in gentamicin (600 µg/mL) and streptomycin (1000 µg/mL) was evaluated. *E. faecalis* ATCC 29212, Staphylococcus *aureus* ATCC 25923 strains were used as controls in antimicrobial susceptibility tests (NCCLS, 2004; CLSI, 2006).

Results

Of 157 faecal samples, 20 (12.7%) VRE were isolated. Isolation rate of VRE were 11.3% (n=7) for cats and 14% (n=12) for dogs. VanA *E. faecium* was isolated from a 3-year-old male cat with urinary system problem and using ciprofloxacin at the time of sampling. Isolated species were shown in Table 2.

MIC values of 7 feline isolates were 8-16 µg/ml and 0.5-1 µg/mL for vancomycin and teicoplanin, respectively. MIC value of VanA *E. faecium* isolate was 128 µg/mL for vancomycin and >64 µg/mL for teicoplanin. The MIC values of all canine isolates were 8-16 µg/mL and 0.25-0.5 µg/mL for vancomycin and teicoplanin, respectively.

All canine isolates were resistant to enrofloxacin (100%), erythromycin (83.3%) and rifampicin (75%) resistance followed this. Ampicillin had the lowest resistance rate. Six isolates had high level of aminoglycoside resistance, and all canine isolates were multidrug resistant (resistant to three or more antimicrobial agents). Similarly enrofloxacin resistance (87.5%) was dominant among feline isolates; followed by erythromycin (75%), ciprofloxacin (75%) and rifampicin (75%) resistances. None of the feline isolates were resistant to chloramphenicol, three feline isolates showed high level of aminoglycoside resistance and 75% of the isolates were multidrug resistant. VanA *E. faecium* isolate showed resistance to all tested antimicrobials, except chloramphenicol. Antimicrobial resistance rates of the all vancomycin resistant enterococci were shown in Table 3.

Table 2. Distribution of vancomycin resistant isolates.

Tablo 2. Vankomisin dirençli izolatların dağılımı.

Species	*E. faecium* *vanA*	*E. casseliflavus* *vanC2*	*E. gallinarum* *vanC1*
Dogs (n=86)	0	4 (33.3%)	8 (66.7%)
Cats (n=71)	1 (12.5%)	2 (25%)	5 (62.5%)

Discussion

In the last decade, examination of nosocomial infectious agents such as methicillin resistant staphylococci, vancomycin resistant enterococci in different animal species started to have clinical concern (Bağcıgil et al., 2012; Boynukara et al., 2002; Coombs et al., 1999; Corso et al., 2005; Ghosh et al., 2012). Enterococci are intrinsically resistant to a variety of antimicrobial agents, such as cephalosporins, some penicillins, clindamycin and trimethoprim, which makes

Table 3. Antimicrobial resistance of the isolates.
Tablo 3. İzolatların antimikrobiyal direnç dağılımları.

	F	E	EX	CF	C	RD	P	AM	TE	GM-HLAR	S-HLAR
Dogs (n=12)	4	10	12	8	4	9	4	2	8	1*	6
%	33.3	83.3	100	66.7	33.3	75	33.3	16.7	66.7	8.3	50
Cats (n=8)	3	6	7	6	0	6	3	3	5	1*	3
%	37.5	75	87.5	75	-	75	37.5	37.5	62.5	12.5	37.5

F= nitrofurantoin (300 μg); E= erythromycin (15 μg); EX= enrofloxacin (5 μg); CF= ciprofloxacin (5 μg); C= chloramphenicol (30 μg); RD= rifampicin (5 μg); P= penicillin (10 μg); AM= ampicillin (10 μg); TE= tetracycline (30 μg); GM-HLAR: Gentamycin– High Level of Aminoglycoside Resistance; S-HLAR: Streptomycin– High Level of Aminoglycoside Resistance; *: indicated isolates showed both streptomycin and gentamycin resistance.

the implications of acquired resistance even greater. There are studies reporting that dogs and cats might act as reservoirs of antimicrobial resistance genes that can be transferred from pets to human (Ghosh et al., 2012; Jackson et al., 2009; Lopez et al., 2013; Weese, 2008). Jackson et al. (2009), predominantly isolated enterococci from three sites of cats and dogs: rectal, hindquarters and belly. However they also isolated enterococci from teeth, nasal areas and suggested that different areas of animals can be contaminated with enterococci and this case increase the importance of risks of transmission from animals to human.

There are various studies on the detection of VRE in different animal species or their products. Due to the different breeding facilities, management procedures and environmental factors in those studies, VRE isolation rates or diversity of isolated species were varying (Boynukara et al., 2002; Ghosh et al., 2012; Herrero et al., 2004; Lopez et al., 2013; Jackson et al., 2009). Jackson et al. (2009), examined various samples from 155 dogs and 145 cats, isolated enterococci from 80% and 60% of them, respectively; but they reported that none of the isolates were resistant to vancomycin, daptomycin, or linezolid. Lopez et al. (2013), reported that *vanA* containing enterococci had been detected in 22 and 13% of tested animals in the previous studies in Spain and in their study which has been conducted after 10-12 years of avoparcin ban, they didn't detect any vancomycin resistant enterococci with *vanA* or *vanB.* The authors isolated intrinsic-resistant *E. gallinarum* and *E. casseliflavus* isolates in 12% of tested samples. Boynukara et al. (2002) detected vancomycin resistance in 91.3% of *Enterococcu*s species isolated from human, dog and cat feces. Herrero et al. (2004) isolated 15 VRE from 87 dogs examined within 5 year-period and indicated that *van*A originated glycopeptides resistance was common. Ghosh et al. (2012) detected vancomycin resistant *E. faecalis* with *vanB* from a healthy resident cat in a small animal hospital. In a previous study, which has been performed in our laboratory (Bağcıgil et al., 2015), no *Enterococcus* species carrying *vanA* and/or *vanB* genes were isolated; however, *Enterococcus* species with *vanC1* or *vanC2/3* genes were detected from dogs (20%) and cats (17%). Besides, in two dogs, *Enterococcus* species representing VanA phenotypic resistance (high-level resistance to vancomycin and teicoplanin) were detected (2015). In the present study VRE were isolated from 14% of dogs and 11.3 % of cats and *vanA* harbouring *E. faecium* was recovered from a cat.

Gentamicin can be used in combination with β-lactams or glycopeptides for treatment of enterococcal infections in humans. But, this synergism is lost in case of high-level of gentamicin resistance (de Leener et al., 2005; Sayıner, 2008). High-level of aminoglycoside resistance can develop by two mechanisms. The first mechanism is the alteration of aminoglycoside binding region on the ribosomes. This is non-transferable resistance only causes high level of streptomycin resistance (S-HLAR). The second resistance mechanism is observed as transferable gentamicin resistance (GM-HLAR) and enzymes such as phosphotransferase, adenyltransferase, acetyltransferase are involved. Such strains will be resistant to all other aminoglycosides except streptomycin (Sayıner, 2008). In the current study, S-HLAR was detected in 3 feline and 6 canine isolates. Beside this, GM-HLAR was detected in one of those feline and one canine isolates. enterococci from the intestinal tract of cats and dogs may act as a reservoir of resistance genes for animal and human pathogens (de Leener et al., 2005; Sayıner, 2008). In a case of transmission of such isolates to human, it would be unavoidable to have some problems in the treatment of these cases. Therefore, the HLAR detected in this study should not be ignored.

Enterococci are intrinsically resistant to a variety of antimicrobial agents, such as cephalosporins, some penicillins, clindamycin and trimethoprim. Dogs and cats might act as reservoirs of antimicrobial resistance genes that can be transferred from pets to people (Ghosh et

al., 2012; Jackson et al., 2009; Lopez et al., 2013; Türkyılmaz et al., 2010; Weese, 2008). Lopez et al. (2013), isolated intrinsic-resistant *E.gallinarum* and *E. casseliflavus* isolates in 12% of tested samples, and emphasized that some of those isolates were showing a multi-resistant phenotype. Ghosh et al. (2012) performed a study to determine whether resident cats in small animal veterinary hospital carry multidrug resistant enterococci and they detected multidrug resistance in 48.9% of all enterococcal isolates. Türkyılmaz et al. (2010), reported that out of 91 *Enterococcus spp.* isolated from companion animals 41 of them were multidrug resistant. In the present study, 75% of the feline and all of the canine isolates were resistant to three or more antimicrobial agents. There is always close contact between companion animals and the owners, and therefore the presence of intrinsic vancomycin and multidrug resistant *Enterococcus* species should never be ignored. Herrero et al. (2004) reported that all canine vancomycin resistant *E. faecium* strains were highly resistant to vancomycin and carried the *vanA* gene; furthermore, 10 of those isolates were resistant to erythromycin and 11 were resistant to tetracycline. Türkyılmaz et al. (2010), detected clindamycin (100%), tetracycline (70.3%) and erythromycin (69.2%) resistances in enterococcal species isolated from cats and dogs. There are some studies providing evidences that resistance in enterococci to gentamicin, flouroquinolones, and quinupristin/dalfopristin in different animal species is correlated with the consumptions of those antimicrobials in animals (Hershberger et al., 2005). In the present study, resistance rates for erythromycin, enrofloxacin, ciprofloxacin, rifampicin and tetracycline were over 65% in all enterococcal species from companion animals. While enterococci are not commonly associated with infections in small animals, due to their ability to horizontally transfer resistance traits to other bacteria, enterococcal resistance reported in this study is important especially that to enrofloxacin and tetracycline since those agents are very commonly used in small animal veterinary medicine to treat various bacterial infection. Therefore it is important to pay attention to a well-considered use of this antibiotic in companion animals.

Ghosh et al. (2012) revealed that some of the feline isolates from the clinically healthy resident cats of small animal hospital were genotypically identical or closely related to isolates from surfaces of different environments of the same hospital. They also indicated that some studies showed that enterococci can survive on inanimate dry hospital surfaces for up to 4 months. In the present study, the isolation rate of *E. faecium* with *vanA* gene was low, on the other hand, the rate of multi-resistant *E. casseliflavus* and *E. gallinarum* isolates were high. Commensal bacteria such as enterococci, staphylococci etc. have natural gene transfer mechanism and can harbour multiple resistance. It is important to reveal those strains from animals, particularly companion animals that have close contact with their owners. Therefore proper use of disinfectants is very important for maintaining hygiene standards in hospitals, particularly for reducing nosocomial infections.

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