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Research Article

Isolation of Ampicillin and Vancomycin Resistant *Enterococcus faecium* from Dogs and Cats [1][2]

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Abstract

In this study, it was aimed to determine the occurence of ampicillin and vancomycin resistant enterococci (ARE and VRE) species in dogs and cats, antimicrobial susceptibility and virulence genes *(asa*1, *esp, gel*E, *hyl, cyl*A) of the isolates. Minimal inhibitor concentration (MIC) values of ampicillin and vancomycin were determined by macro dilution method and E-test, respectively. For this purpose, 531 rectal swabs collected from dogs (n=276) and cats (n=255) from three different cities (İstanbul, Ankara and Mersin) were examined. ARE was detected in 60 (21.7%) of dogs and in 47 (18.4%) of cats. VRE was detected in one dog and two cats. All ARE and VRE isolates were identified as *Enterococcus faecium* by polymerase chain reaction (PCR), and showed multi-drug resistance (MDR) phenotype. A small number of ARE*fm* isolates (4.7%) carried virulence gene. To the authors' knowledge, the study is first reporting *van*A gene harboring VRE*fm* in dogs in Turkey. The results indicated that both dogs and cats were frequent carriers of ARE*fm*. Due to close contact with humans, dogs and cats may play an important role in the spread of these nosocomial pathogens in the community. Therefore, further molecular studies are needed to elucidate the possible role of animal originated ARE*fm* and VRE*fm* strains in human nosocomial infections.

Keywords: *Ampicillin resistance, Cat, Dog, Enterococcus faecium, Vancomycin resistance*

Köpek ve Kedilerden Ampisilin ve Vankomisin Dirençli *Enterococcus faecium* İzolasyonu

Öz

Bu çalışmada, köpek ve kedilerde ampisilin ve vankomisine dirençli enterokokların (ARE ve VRE) izolasyonu, izolatların antimikrobiyal duyarlılıklarının ve virülans genlerinin *(asa*1, *esp, gel*E, *hyl, cyl*A) belirlenmesi amaçlandı. Ampisilin ve vankomisin dirençli izolatların minimal inhibitör konsantrasyonları (MİK) sırasıyla makrodilüsyon metodu ve E-test ile belirlendi. Bu amaçla üç farklı ildeki (İstanbul, Ankara ve Mersin) köpeklerden (n=276) ve kedilerden (n=255) toplanan 531 rektal svab örneği çalışmaya dahil edildi. Köpeklerin 60'ında (%21.7) ve kedilerin 47'sinde (%18.4) ARE tespit edildi. VRE bir köpek ve iki kedide saptandı. Tüm ARE ve VRE izolatları, polimeraz zincir reaksiyonu (PZR) ile *Enterococcus faecium* olarak identifiye edildi ve bu izolatlar çoklu ilaç direnç (MDR) fenotipi gösterdi. Az sayıdaki izolatta (%4.7) virülans geni saptandı. Yazarların bilgisine göre, bu çalışma ile ilk olarak Türkiye'de köpeklerden *van*A geni taşıyan VRE*fm* izolasyonu bildirilmektedir. Sonuçlar, hem köpeklerin hem de kedilerin ARE*fm* ile yüksek oranda kolonize olduklarını göstermektedir. Köpekler ve kediler yakın fiziksel temaslarından dolayı, insanlara bu nozokomiyal patojenlerin yayılmasında önemli bir rol oynayabilir. Bu nedenle, hayvanlardan izole edilen ARE*fm* ve VRE*fm* suşlarının insan nozokomiyal enfeksiyonlarındaki olası rolünü aydınlatmak için ileri moleküler çalışmalara ihtiyaç vardır.

Anahtar sözcükler: *Ampisilin direnci, Enterococcus faecium, Kedi, Köpek, Vankomisin direnci*

INTRODUCTION

Enterococci, for many years, have been considered as commensal inhabitants of the gastrointestinal tract of animals and humans. However, during last three decades, *Enterococcus* spp., especially *E. faecium*, has emerged one of the important nosocomial pathogens worldwide due to the acquired high resistance profiles such as amino-glycosides, ampicillin and vancomycin, making therapy options very limited [1,2]. Of these resistance mechanisms,

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high level ampicillin resistance (>256 µg/mL) is important problem, especially when associated with high level aminoglycoside and glycopeptide resistance. The most common mechanism for high level ampicillin resistance is multiple mutations in the active site of the penicillin binding protein (PBP5) [3-5].

The first isolation of vancomycin resistant *E. faecium* (VRE*fm)* from humans was first reported in Turkey by Başustaoğlu et al.[6] and the first VRE*fm* outbreak was announced in a tertiary hospital in Ankara by Çolak et al.[7]. Subsequently, hospital-acquired infections and outbreaks caused by VRE*fm* have been reported [8,9]. According to the 2015 and 2016 national hospital infections surveillance network reports, VRE isolation rates were reported as 14.03% and 13.33%, respectively [10,11]. In contrast to human studies, there is a paucity of studies on the isolation and molecular characterization of VRE*fm* from dogs and cats. VRE*fm* was first reported in a 3-year-old male cat with urinary system problem by Bağcıgil et al.[12]. Similarly, there is only one study of isolation and molecular characterization of ampicillin resistant *E. faecium* (ARE*fm)* from cats and dogs, in which occurence of ARE*fm* was found in 20.9% of dogs and in 25.4% of the cats [13].

Enterococci have the ability to produce a number of virulence factors**,** playing important role in their pathogenesis such as aggregation substance *(asa*1), gelatinase *(gel*E), cytolysin *(cyl*A), enterococcal surface protein *(esp)*, hyaluronidase *(hyl)* [14]. Of these factors, *esp* was reported to be more frequently related with infections and nosocomial infections caused by ARE*fm* and VRE*fm*. The reason for this has been shown as increased ability of adherence to epithelial surfaces and biofilm formation of *esp* carrying isolates.

Dogs and cats are close contact with humans, and may transmit resistant bacteria to their owners. The data on the occurence of ARE*fm* ve VRE*fm* in dogs and cats have remained largely unknown in Turkey. Therefore, current study was conducted to investigate the occurence of ARE*fm* and VRE*fm* in pet animals to elucidate possible public health implications.

MATERIAL and METHODS

Ethical Statement

The study was approved by the Animal Ethical Committee of Hatay Mustafa Kemal University 2018/3-7

Sample Collection

Rectal swab samples were collected from dogs (n=276) and cats (n=255) from three different provinces (İstanbul, Ankara and Mersin) between March 2018 and April 2018. The rectal swabs were taken from both healthy and sick pet animals.

Isolation and Identification

For the presence of ARE and VRE, the rectal swab samples were inoculated into two different Enterococcosel broth (BD, UK), one with 32 µg/mL ampicillin to detect ARE isolates, and the other with 6 µg/mL vancomycin to detect VRE isolates. Both were incubated for 48 h at 37°C. In the case of growth in the Enterococcosel broth for ARE detection, a loopfull of culture was inoculated on VRE agar (Oxoid, UK) plates supplemented with 32 µg/mL ampicillin. In the case of growth in the Enterococcosel broth for VRE detection, a loopfull of culture was inoculated on VRE agar plates supplemented with 6 µg/mL vancomycin. Both plates were incubated for 48 h at 37°C. Subsequently, one putative colony from each plate was randomly selected and identified by a species-specific multiplex polymerase chain reaction (mPCR) method [15]. mPCR assays confirming the presence of the genus *Enterococcus* and identifying *E. faecalis* and *E. faecium* were performed in a total volume of 25 µL, consisting of 10× PCR buffer [750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], 1.5 mM $MgCl_2$, 200 µM each dNTP, 20 pmol of E1-2 and FMB1-2 primer, 32 pmol of FL1-2 primer, 10 µL template DNA and 2 U Taq DNA polymerase. PCR amplification was carried out in following thermal cycling conditions: initial denaturation at 94°C for 3 min and 30 cycles of amplification consisting of denaturation at 94°C 1 min, annealing at 55°C 1 min, and extension at 72°C 1 min, with a final extension step at 72°C for 7 min. The presence and size of the amplified products were analyzed by electrophoresis in 1x TBE buffer on 1.5% agarose gels. The species-specific primers for mPCR of *E. faecium* and *E. faecalis* are given in *Table 1*.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of the isolates were tested for eight antimicrobials using disc diffusion method following Clinical and Laboratory Standards Institute (CLSI, 2012) criteria [16]. The antimicrobials used were: ampicillin (AM, 10 µg), ciprofloxacin (CIP, 5 µg), erythromycin (E, 15 µg), gentamicin (CN, 120 µg), rifampin (RA, 5 µg), tetracycline (TE, 30 µg), chloramphenicol (C, 30 µg) and vancomycin (VA, 30 µg). MIC values of ARE and VRE isolates were determined by macrodilution method and E-test (Bioanalyse, Turkey), respectively. The isolates, which were resistant to three or more antimicrobials from different classes, were evaluated as multiple resistance (MDR).

Determination of Vancomycin Resistance Genes

The isolates found to be phenotypically as vancomycin resistant, resistance genes mediating vancomycin resistance were investigated by multiplex PCR as previously described by Depardieu et al.[17]. Briefly, PCR reaction was carried out in a total volume of 50 µL, consisting of 10× PCR buffer [750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], 2 mM $MgCl_2$, 200 µM each dNTP, 40 pmol of

Table 1. *PCR primers used for E. faecium and E. faecalis identification in this study*

Primer Name	Sequence (5'-3')	Target Taxon	Target Gene	Amplicon Lenght (bp)
E1	TCA ACC GGG GAG GGT	*Enterococcus* spp.	16S rRNA	733
E2	ATT ACT AGC GAT TCC GG			
FL1	ACT TAT GTG ACT AAC TTA ACC	*E. faecalis*	sodA	360
FL2	TAA TGG TGA ATC TTG GTT TGG			
FM1B	ACA ATA GAA GAA TTA TTA TCT G	*E. faecium*	sodA	214
FM2B	CGG CTG CTT TTT TGA ATT CTT CT			

Table 2. *Primers used for detection of the vancomycine resistance genes*

Primer Name	Sequence (5'-3')	Gene	Amplicon Lenght (bp)
EA1	GGGAAAACGACAATTGC	*van*A	732
EA2	GTACAATGCGGCCGTTA		
EB3	ACGGAATGGGAAGCCGA	*van*B	647
EB4	TGCACCCGATTTCGTTC		
EC5	ATGGATTGGTAYTKGTAT	*van*C1/C2	815/827
EC8	TAGCGGGAGTGMCYMGTAA		
ED1	TGTGGGATGCGATATTCAA	*van*D	500
ED2	TGCAGCCAAGTATCCGGTAA		
EE1	TGTGGTATCGGAGCTGCAG	*van*E	430
EE2	ATAGTTTAGCTGGTAAC		
EG1	CGGCATCCGCTGTTTTTGA	*van*G	941
EG2	GAACGATAGACCAATGCCTT		

each primer, 10 µL template DNA and 2 U Taq DNA polymerase. PCR amplification was carried out in following thermal cycling conditions: initial denaturation at 94°C for 3 min and 30 cycles of amplification consisting of denaturation at 94°C 1 min, annealing at 54°C 1 min, and extension at 72°C 1 min, with a final extension step at 72°C for 7 min. The presence and size of the amplified products were analyzed by electrophoresis in 1xTBE buffer on 1.5% agarose gels. Primers used for the detection of the vancomycine resistance genes are shown in *Table 2*.

Detection of Virulence Genes

Presence of virulence genes *(asa*1, *gel*E, *cyl*A, *esp*, and *hyl)* were investigated by mPCR [14]. PCR reactions were performed in a total volume of 50 µL containing 10× PCR buffer [750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], 2 mM $MgCl_2$, 200 µM each dNTP, 20 pmol of *asa1*, *gelE* and *hyl* primers, 40 pmol of *cylA* and *esp*, 10 µL template DNA and 2 U Taq DNA polymerase. After initial denaturation at 95°C for 5 min, 30 cycles of amplification were performed with denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min, followed by one cycle of a final extension at 72°C for 10 min. The amplified products were detected by electrophoresis 1× TBE buffer on 1.5% agarose gels.

Pulsed Field Gel Electrophoresis (PFGE) Analysis

Clonal relationship of the VRE isolates were determined by PFGE, which was performed in Public Health Institution of Turkey (Ankara) as described previously by Morrison et al.[18]. Briefly, bacterial cells (approximately 2×10^9 cells/mL) were mixed with an equal volume of low-melting-point agarose. The plugs were lysed with lysozyme and proteinase K, and then chromosomal DNA was digested with 40 U *Sma*I (Fermentas). Fragmented DNA samples were electrophoresed in 1% pulsed-field certified agarose (Bio-Rad) using a CHEF-DR II system(Bio-Rad) with 5-30 s pulse times, for 20 h at 14°C at 6 V cm^{-2}. The gel was stained with ethidium bromide (5 mg mL^{-1}), visualized under UV light, and photographed using a gel logic 2200 imaging system (Resolution: 1708×1280 pixel; Kodak). The DNA band profiles were analysed with GelCompar software (version 3.0; Applied Maths). Band tolerances of 1.5% and 1% normalization were used for comparison of DNA profiles.

Statistical Analysis

Differences in frequencies of isolation rates according to cities, different age groups and genders were evaluated using Pearson's chi-square test. SPSS 14.01 was used for statistical analysis. Any P value equal to/or less than <0.05 was considered as statistically significant.

RESULTS

Ampicillin resistant enterococci was detected in 60 (21.7%) of dogs and in 47 (18.4%) of cats *(Table 3, 4)*. All ampicillin resistant isolates were identified as *E. faecium* by PCR *(Fig. 1)*. Isolation rates between cities were found statistically significant (P<0.001). But, no statistically significant differences was observed among age groups and genders. VRE was isolated from two cats and one dog in Mersin. No VRE was isolated from other cities. All VRE isolates were identified as vancomycin resistant *E. faecium* (VRE*fm)* and positive for *van*A gene by PCR *(Fig. 2)*.

All ARE*fm* and VRE*fm* isolates were MDR phenotype *(Table 5)*. Ampicillin MIC values was between 64 and ≥256 µg/mL. Sixty isolates showed ≥256 µg/mL, 31 isolates 256 µg/mL, 13 isolates 128 µg/mL and three showed 64 µg/mL. All VRE*fm* isolates showed a MIC value of ≥256 µg/mL for vancomycin.

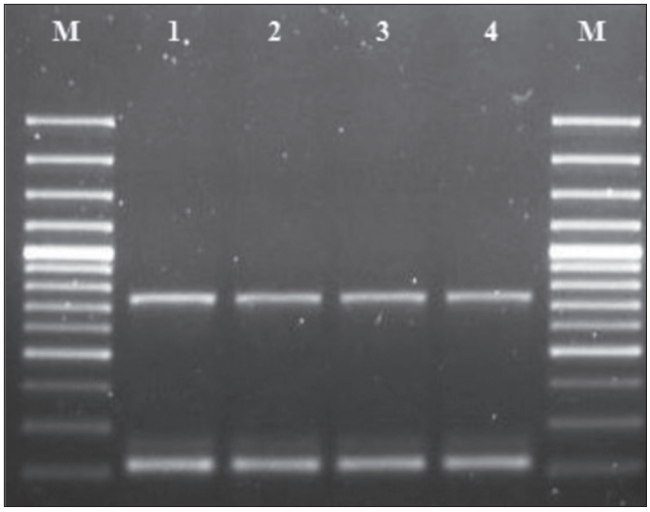

Fig 1. Agarose gel electrophoresis of *E. faecium* isolates. Lane M: 100 bp plus molecular marker, Lane 1-4: *E. faecium* (214 bp) plus *Enterococcus* spp. (733 bp)

Table 3. *Distribution of AREfm isolates according to age groups, genders and cities among dogs*

Variables	Mersin					Ankara					İstanbul					Total				
	Ex.[a] (n)	Neg.[b] (n)	Pos.[c] n (%)	X^2 Value	P Value[d]	Ex. (n)	Neg. (n)	Pos. n (%)	X^2 Value	P Value	Ex. (n)	Neg. (n)	Pos. n (%)	X^2 Value	P Value	Ex. (n)	Neg. (n)	Pos. n (%)	X^2 Value	P Value
Sex																				
Female	41	38	3 (7.3)	0.240	0.624	47	37	10 (21.3)	1.447	0.229	21	4	17 (81)	11.803	0.01	109	79	30 (27.5)	3.542	0.06
Male	59	53	6 (10.2)			63	55	8 (12.7)			45	29	16 (35.6)			167	137	30 (18)		
Age																				
<1	27	25	2 (7.4)	3.392	0.183	24	19	5 (20.8)	0.909	0.635	11	5	6 (54.5)	0.121	0.941	62	49	13 (21)	0.872	0.647
1-3	20	16	5 (25)			60	52	8 (13.3)			33	18	16 (54.5)			113	84	29 (25.7)		
>3	34	32	2 (5.9)			26	21	5 (19.2)			22	11	11 (50)			82	36	16 (19.5)		

[a] *Examined,* [b] *Negative,* [c] *Positive,* [d] *Probability level*

Table 4. *Distribution of AREfm isolates according to age groups, genders and cities among cats*

Variables	Mersin					Ankara					İstanbul					Total				
	Ex.[a] (n)	Neg.[b] (n)	Pos.[c] n (%)	X^2 Value	P Value[d]	Ex. (n)	Neg. (n)	Pos. n (%)	X^2 Value	P Value	Ex. (n)	Neg. (n)	Pos. n (%)	X^2 Value	P Value	Ex. (n)	Neg. (n)	Pos. n (%)	X^2 Value	P Value
Sex																				
Female	42	41	1 (2.4)	2.156	0.142	44	41	3 (6.8)	0.060	0.806	39	15	24 (61.5)	9.785	0.002	125	97	28 (22.4)	2.569	0.109
Male	40	36	4 (10)			49	45	4 (8.2)			41	30	11 (26.8)			130	111	19 (14.6)		
Age																				
<1	47	45	2 (4.3)	4.894	0.087	22	18	4 (18.2)	5.108	0.078	14	6	8 (57.1)	1.834	0.400	83	69	14 (26.4)	0.622	0.733
1-3	18	15	3 (16.7)			58	55	3 (5.1)			52	32	20 (38.5)			128	102	26 (20.3)		
>3	17	17	0 (0)			13	13	0 (0)			14	7	7 (50)			44	37	7 (15.9)		

[a] *Examined,* [b] *Negative,* [c] *Positive,* [d] *Probability level*

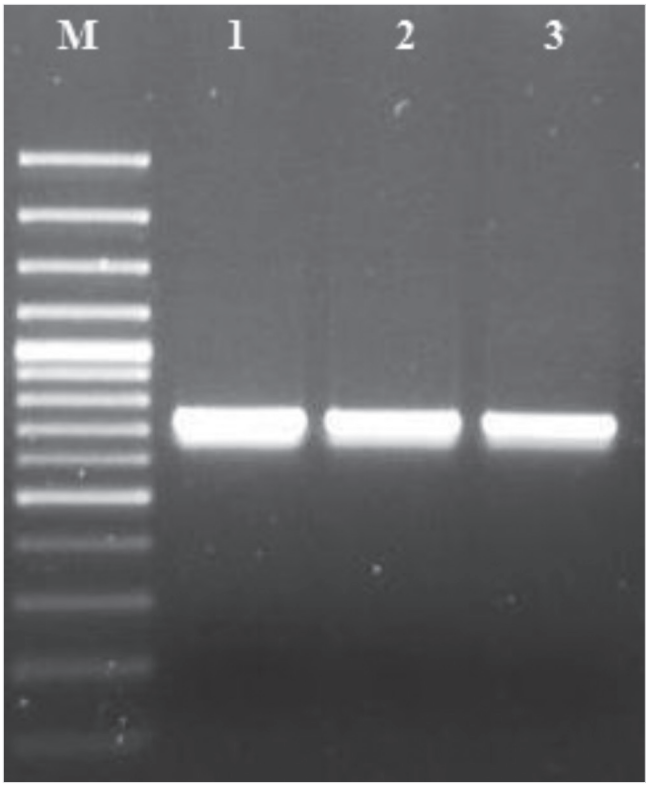

Fig 2. PCR amplification of vanA gene of VREfm isolates. Lane: 100 bp plus molecular marker, Lane 1-3: vanA gene (732 bp) positive isolates

Table 5. *Antimicrobial resistance phenotypes of AREfm isolates*

Resistance Phenotype	Species	
	Dog (n=60)	Cat (n=47)
AM, RA, CN, CIP, TE, E	25	18
AM, CN, CIP, TE, E	7	4
AM, RA, CIP, TE, E	5	6
AM, RA, CN, CIP, E	1	-
AM, RA, CN, TE, E	3	4
AM, RA, CN, TE	-	1
AM, CN, CIP, TE	-	1
AM, RA, CIP, TE	2	-
AM, CN, TE, E	1	-
AM, CIP, TE, E	2	2
AM, RA, TE, E	3	2
AM, CN, CIP, E	1	-
AM, CN, TE, E	-	2
AM, CIP, TE	2	-
AM, TE, E	7	6
AM, RA, TE	1	1

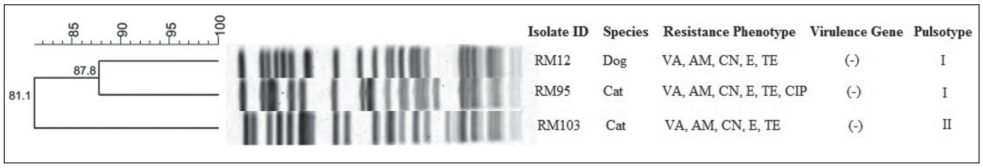

Fig 3. Dendogram showing the results of SmaI PGFE VREfm isolates. Pulsotypes are indicated as Roman numerals. Based on a similarity coefficient ≥85%, the isolates indicated two pulsotypes

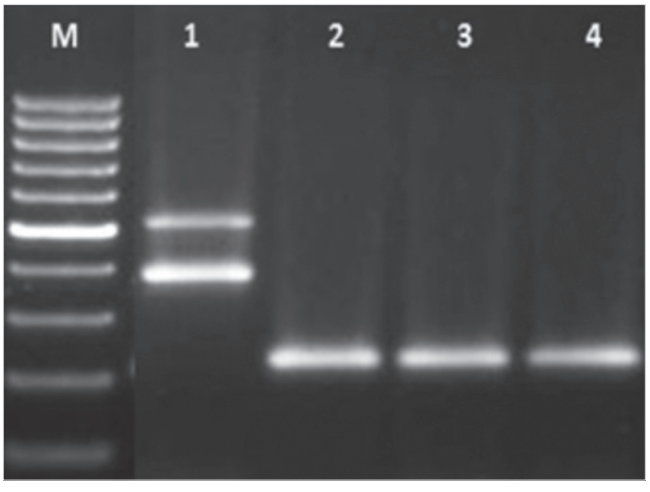

Fig 4. Agarose gel electrophoresis of virulence genes detected in ARE*fm* isolates. Lane M: 100 bp molecular marker, Lane 1: *esp* (510 bp) + *asa*1 (375 bp), Lane 2-4: *hly* (276 bp)

A shown in *Fig. 3*, PFGE typing of three VRE*fm* isolates showed two distinct PGFE pulsotypes based on a similarity coefficient of ≥85.

A small number of isolates (4.7%) carried virulence genes among ARE*fm* isolates. Among the isolates, *esp*, *asa*1 and *hly* genes were only virulence genes detected, but *gel*E and *cyl*A were not detected in any of the isolates tested *(Fig. 4)*. None of VRE*fm* isolates were positive for virulence genes tested.

DISCUSSION

The present study revealed a high intestinal carriage rate of ARE*fm* in dogs (21.7%) and cats (18.4%) in Turkey. In a previous study, Çelik et al.[13] reported comparable colonization rate in dogs and cats (20.9% and 25.4%, respectively) in İstanbul. In a countrywide population-based study in Netherland, de Regt et al.[19] reported that prevalence of intestinal carriage of ARE*fm* was 25.6% in dogs and 5.1% in cats. In a cross-sectional study carried out in the United Kingdom and Denmark, the prevalence rates of ARE*fm* in dogs were reported as 23% and 76%, respectively [20].

The *van*A carrying VRE*fm* was isolated from 0.13% of dogs and 0.8% of cats in this study. In Japan, Kataoka et al.[18] did

not detect VRE in dogs and cats subjected to different antibiotic regimens. In contrast, Devriese et al.[22] investigated the presence of faecal carriage of VRE in 87 dogs treated at the Animal Hospital of the School of Veterinary Medicine in Madrid, Spain, detected 11 (12.6%) *van*A carrying VRE*fm*. Since no information on the living conditions, contact with different animal species and eating habits, previous treatment records was available in this study, it was not possible to determine the origin of VRE transmission to cats and dogs. Guardabassi et al.[23] suggested that VRE isolates was generally resistant to different classes of antimicrobials such as macrolides, aminoglycosides, tetracyclines, the use of such antimicrobials in pet animals might lead the co-selection of VRE. Although carriage rate of MDR VRE was found to be very low in this study, it should be ruled out that this microorganism might emerge as a nosocomial pathogen in veterinary medicine, might play role as a source of VRE for humans, and might be able to promote the horizontal dissemination of resistance genes among strains of animals and humans [24].

A small number of ARE*fm* isolates were positive for virulence genes, and any VRE*fm* isolates carried virulence genes in this study. Similar observation was reported by Çelik et al.[13], who detected only *efa*A (13.8%) and *gel*E (11.1%) as virulence genes among ARE*fm* isolates. Leavis et al.[25] reported that the *esp* gene carrying ARE*fm* isolates are generally epidemic and cause severe nosocomial infections in hospitals. In this study, *esp* gene was detected only in one isolate from a cat together with *asa*1. Similarly, Damborg et al.[20] also didn't detect *esp* gene in any ARE*fm* isolate. Leavis et al.[25] explained this with two different views: (i) the dog ARE*fm* isolates might be evolved by acquiring virulence genes such as *esp* and *hyl* and adapted to hospital settings, (ii) human ARE*fm* strains might be ancestors of dog strains and lost their virulence factors outside hospital settings.

In conclusion, to the authors' best knowledge, this is first report of VRE*fm* carrying *van*A in dogs in Turkey. MDR bacteria including ARE*fm* and VRE*fm* in pet animals should be monitored by national surveillance programs. To elucidate the possible role of these bacteria in human nosocomial infections, the isolates from both pet animals and human nosocomial infections should be compared using advanced molecular techniques.

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