Investigation of Faecal Carriage of High-Level Gentamicin Resistant Enterococci in Dogs and Cats

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ABSTRACT

The emergence and spread of high-level gentamicin resistant (HLGR) enterococci have been a concern due to the eliminatation of bactericidal effect between aminoglycosides and cell-wall-active antimicrobials. Therefore, this study to determine the fecal carriage of (HLGR) enterococci in dogs and cats, to investigate the antimicrobial resistance, resistance mechanisms implicated and virulence genes of the isolates. A total of 465 rectal swab samples from dogs (n=226) and cats (n=239) from three different cities (Istanbul, Ankara and Mersin) of Turkey were used for the analysis of HLGR enterococci. The antimicrobial susceptibilities of HLGR isolates were searched by disc diffusion method, resistance and virulence genes by polymerase chain reaction (PCR) and minimum inhibitory concentration (MIC) values for gentamicin were determined by macrodilution method. HLGR enterococci were detected in 25 (11.1%) dogs and in 28 (11.7%) cats. Based on PCR results, 12 were identified as *Enterococcus feacalis* and 41 as *Enterococcus faecium*. All of the isolates showed a MIC value of ≥2048 µg/ml for gentamicin, and except one isolate, the rest of the isolates showed multidrug resistance (MDR) phenotype. None of the isolates displayed vancomycin resistance phenotype. The bifunctional enzyme encoded by *aac(6)-Ie-aph(2)-Ia* were detected in HLGR isolates as well as other aminoglycoside resistance genes at varying rates. Virulens genes were only detected in 11 (20.8%) *E. faecalis* isolates with different combinations. However, none of the isolates carried the *hly* gene. The results showed that both dogs and cats are a potential reservoir for MDR HLGR enterococci which may play an important role in the spread of these nosocomial pathogens.

Keywords: Cat; Dog; Enterococci; Faecal carriage; High Level Gentamicin Resistance; Virulence genes.

INTRODUCTION

Enterococci are part of the microbiota of the gastrointestinal tract of animals and humans. In the last three decades, enterococci have emerged as one of the leading causes of nosocomial infections due to their increased ability to develop resistance to various classes of antimicrobials and their virulence factors (1). Underlying mechanisms causing antimicrobial resistance in enterococci may be intrinsic to species or may be acquired through mutation of target genes or horizontal transfer of genetic material encoding resistance genes (2).

High level aminoglycoside resistance (HLGR) in enterococci is mediated by bi-functional aminoglycoside-modifying enzyme (AME) with both 6′-acetyltransferase and 2″-phosphotransferase (AAC(6′)-Ie-APH(2″)-Ia) activities. This enzyme is encoded by *aac(6′)-Ie-aph(2″)-Ia* gene, which is part of a conjugative transposon called Tn5281, and frequent localization of this transposon on

the plasmid also facilitates rapid spread of resistance gene from cell to cell (3).

Numerous virulence determinants have been identified that play an important role in the pathogenesis of infections caused by enterococci (4). Of these, cytolysin (*Cyl*), aggregation substance (*asa*1), enterococcal surface protein (*esp*), hyaluronidase (*hyl*), and gelatinase (*gel*E) have been reported to render enterococci more prone to cause disease and exacerbate disease symptoms (5). Cytolysin is a bacteriocine-type exotoxin with hemolytic activity on erythrocytes, leukocytes and macrophages (6). Gelatinase is an extracellular zinc endopeptidase/protease that is capable of hydrolyzing gelatin, collagen, casein, hemoglobin, and other peptides, which has also been reported to contribute to adhesion and biofilm formation (7). Aggregation substance (AS) is a multifunctional adhesin consisting of closely related surface proteins that promotes bacterial adherence to renal tubular cells (8) and internalization by intestinal cells (9). In addition to mediating adherence and invasion of eukaryotic cells, AS allows donor and recipient strains to form tight aggregates, which permits strains to maintain close contact over a period of sufficient time for conjugative transfer (10). The *esp* gene participates in the formation of biofilm, which plays an important role in the transfer of genetic material between cells and increase resistance to antibiotics (4).

Pet animals have long been known to play an important role as a reservoir of antimicrobial resistant bacteria. Considering the potential for resistant bacteria to pass from pet animals to humans due to close physical contact, this issue is becoming an even more important public health issue (2, 11).

In Turkey, there are a few studies examining the reservoir status of dogs and cats for the presence of antimicrobial resistant enterococci. These studies aimed mainly either to identify specific antimicrobial-resistant enterococci species (12, 13, 14), or to determine the antimicrobial susceptibilities of isolated enterococci (15, 16). Estimating the true rate of resistant strains to an antimicrobial is closely related to the isolation procedures used. Morever, there are no studies to identify HLGR enterococci in dogs and cats to date. Therefore, it was aimed to investigate the feacal carriage of enterococci positive for HLGR in cats and dogs in different provinces (İstanbul, Ankara and Mersin), to search for mechanisms mediating HLG, tetracycline and erythromycin resistance as well as virulence genes of the isolates.

MATERIALS AND METHODS

Ethical Statement

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

Sample collection: Between March 2018 and April 2018, rectal swab samples were collected from 226 dogs and 239 cats from three different provinces (İstanbul, Ankara and Mersin) of Turkey (Figure 1). During the collection of the samples, information related with location, age and gender of the animals was also recorded.

Isolation and identification: For the presence of HLGR enterococci, based on Clinical and Laboratory Standards Institute criteria (CLSI) (17), the rectal swab samples were inoculated into Enterococcosel broth (BD, UK) supplemented with 500 µg/ml gentamicin, and incubated for 48 h at 37°C. When growth occured in the Enterococcosel broth, a loopfull of culture was inoculated on VRE agar (Oxoid, UK) plates supplemented with 500 µg/ml gentamicin, incubated for 48 h at 37°C (9). A presumptive colony showing typical morphology was selected and passaged onto blood agar supplemented with 5% defibrinated sheep blood. The selected colony was first screened by Gram staining, catalase test, and growth in brain-heart infusion broth (BHIB) containing 6.5% NaCl (18). Identification of enterococci at the species level was carried out by multiplex PCR (19). PCR primers used for *Enterococcus faecium* and *Enterococcus faecalis* identification in this study are given in Table 1.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibilities of the isolates were tested for eight antimicrobials using disc diffusion method following CLSI guidelines (17). The antimicrobials used were: ampicillin (AM, 10 µg), ciprofloxacin (CIP, 5 µg), erythromycin (E, 15 µg), rifampin (RA, 5 µg), quinopristin-dalfopristin (SYN, 15 µg), tetracycline (TE, 30 µg), chloramphenicol (C, 30 µg) and vancomycin (VA, 30 µg). A gentamicin disc (120 µg) was also included in antimicrobial susceptibility testing for confirmation of HLGR. ATCC *E. faecalis* 29212 was used as quality control strain. MIC values of the isolates were determined by macrodilution method (17). The isolates, which were resistant to three or more antimicrobials from

Figure 1: Map of Turkey depicting provinces from which samples were taken

different classes, were evaluated as multiple drug resistant (MDR) (20).

DNA isolation

Total genomic DNA was extracted from the overnight cultures of the isolates using a commercial DNA isolation kit (GeneJET Genomic DNA Purification Kit, Lithuania) according to the manufacturer's recommendations. Concentration and purity of DNA was measured by NanoDrop (ThermoFischer Scientific, USA).

Detection of resistance genes

Aminoglycoside, macrolide and tetracycline resistance genes were searched as previously reported by Vakulenko *et al.* (20) and Malhotra-Kumar *et al*. (21). Primers used for the detection of resistance genes in the study are presented in Table 2.

Detection of Virulence Genes

Presence of virulence genes (*asa*1, *gel*E, *cyl*A, *esp*, and *hyl*) have been investigated by Vankerckhoven *et al*. (23). Primers used for the detection of virulence genes in the study are given in Table 2.

Statistical analysis

The statistical analyses were carried out using SPSS 16 (SPSS Inc., Chicago, IL, USA). The frequencies of the variables were presented as numbers and percentages. The chi-square (χ^2) test, Fisher's exact test and Fisher-Freeman-Halton test

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

Primer Name	Sequence (5'-3')	Target Taxon	Target Gene	Product Size (bp)	Reference
E1	TCAACCGGG GAGGGT	*Enterococcus* spp.	16S rRNA	733	Layton
E2	ATTACTAGCGATTCC GG				
FL1	ACTTATGTGACTAAC TTAACC	*E. faecalis*	sodA	360	
FL2	TAATGGTGAATCTTG GTTTGG				
FM1B	ACAATAGAAGAATTATTA TCTG	*E. faecium*	sodA	214	
FM2B	CGGCTGCTTTTTTGA ATTCTTCT				

Table 2. Primers used for the detection of virulence and resistance genes in the study

Gene	Sequence (5'-3')	Product size (bp)	Reference
*asa*1	GCACGCTATTACGAACTATGA	375	
	TAAGAAAGAACATCACCACGA		
gelE	TATGACAATGCTTTTTGGGAT	213	
	AGATGCACCCGAAATAATATA		
cylA	ACTCGGGGATTGATAGGC	688	
	GCTGCTAAAGCTGCGCTT		
esp	AGATTTCATCTTTGATTCTTGG	510	
	AATTGATTCTTTAGCATCTGG		
hyl	ACAGAAGAGCTGCAGGAAATG	276	
	GACTGACGTCCAAGTTTCCAA		
erm(A)	CCCGAAAAATACGCAAAATTTCAT	590	
	CCCTGTTTACCCATTTATAAACG		
erm(B)	TGGTATTCCAAATGCGTAATG	745	
	CTGTGGTATGGCGGGTAAGT		
mef(A/E)	CAATATGGGCAGGGCAAG	317	
	AAGCTGTTCCAATGCTACGG		
tet(M)	GTGGACAAAGGTACAACGAG	406	
	CGGTAAAGTTCGTCACACAC		
tet(O)	AACTTAGGCATTCTGGCTCAC	515	
	TCCCACTGTTCCATATCGTCA		
tet(K)	GATCAATTGTAGCTTTAGGTGAAGG	155	
	TTTTGTTGATTTACCAGGTACCATT		
tet(L)	TGGTGGAATGATAGCCCATT	229	
	CAGGAATGACAGCACGCTAA		
aac(6')-Ie-aph(2")-Ia	CAGGAATTTATCGAAAATGGTAGAAAAG	369	
	CACAATCGACTAAAGAGTACCAATC		
aph(2")-Ib	CTTGGACGCTGAGATATATGAGCAC	867	
	GTTTGTAGCAATTCAGAAACACCCTT		
aph(2")-Ic	CCACAATGATAATGACTCAGTTCCC	444	
	CCACAGCTTCCGATAGCAAGAG		
aph(2")-Id	GTGGTTTTTACAGGAATGCCATC	641	
	CCCTCTTCATACCAATCCATATAACC		
aph(3')-IIIa	GGCTAAAATGAGAATATCACCGG	523	
	CTTTAAAAAATCATACAGCTCGCG		
ant(4')-Ia	CAAACTGCTAAATCGGTAGAAGCC	294	
	GGAAAGTTGACCAGACATTACGAACT		

Table 3: Distribution of HLGR isolates according to age groups, genders and cities among dogs

Variables	Ankara (n=96)				İstanbul (n=66)				Mersin (n=64)				Total (n=226)			
	Ex.[a]	Neg.[b]	Pos.[c]	p value	Ex.	Neg.	Pos.	p value	Ex.	Neg.	Pos.	p value	Ex.	Neg.	Pos.	p value
Sex																
Female	38	35	2	0.532[d]	21	15	6	0.532[d]	25	24	1	1.00[d]	84	74	9	0.972[d]
Male	58	53	5		45	39	9		39	38	1		142	130	15	
Age																
1<	16	15	–	0.034[f]	12	9	4	0.884[f]	18	–	–	0.105[f]	46	24	4	0.073[1]
1-3	44	42	1		21	16	5		16	15	1		81	73	7	
3-6	25	23	2		22	26	6		10	–	–		57	49	8	
6-9	5	5	2		5	5	–		11	–	–		21	10	2	
9-12	4	4	1		1	1	–		6	6	–		11	11	1	
>12	2	1	1		5	5	–		3	2	1		10	8	2	

[a]Examined, [b]Negative, [c]Positive, [d]Pearson Chi-Square, [e]Fisher Exact Test, [f]Fisher-Freeman-Halton test

Table 4: Distribution of HLGR isolates according to age groups, genders and cities among cats

Variables	Ankara (n: 78)				İstanbul (n: 80)				Mersin (n:81)				Total (n: 239)			
	Ex.[a]	Neg.[b]	Pos.[c]	p value	Ex.	Neg	Pos	p value	Ex.	Neg	Pos	p value	Ex.	Neg	Pos	p value
Sex																
Female	33	32	2	0.571[e]	39	12	17	0.776[d]	43	43	–	NA	115	114	19	0.045[d]
Male	45	44	1		41	32	9		38	38	–		124	47	10	
Age																
1<0	16	16	1	0.067[f]	14	8	6	0,196[f]	46	46	–	NA	76	54	6	0.269[f]
1-3	43	43	–		40	27	13		13	13	–		96	40	13	
3-6	16	15	1		21	17	4		17	17	–		54	34	5	
6-9	1	1	–		3	1	2		3	3	–		7	4	2	
9-12	3	2	1		1	1	0		1	1	–		5	4	1	
>12	–	–	–		1	–	1		2	2	–		3	3	1	

[a]Examined, [b]Negative, [c]Positive, [d]Pearson Chi Square, [e]Fischer Exact Test, [f]Fisher-Freeman-Halton test

were used to compare categorical variables where appropriate. A p value of 0.05 was considered as statistically significant.

RESULTS

Identification of bacterial isolates

A total of 53 HLGR enterococci were isolated from 465 faecal samples obtained from dogs and cats [25 (11.1%) from dogs and 28 (11.7%) from cats]. Distribution of HLGR enterococci isolated from dogs and cats was determined and presented according to location, age and gender in Table 3 and 4. Isolation rates of HLGR enterococci in dogs (p=0.001) and cats (p<0.001) were found statistically significant according to provinces. PCR based identification of the isolates showed that 12 (22.6%) isolates were *E. feacalis* and 41 (77.4%) isolates were *E. faecium* (Figure 2).

Antimicrobial susceptibility testing

The rates of resistance for antimicrobials tested are presented in Table 4. None of the isolates was positive for vancomycin. As seen Table 5, nearly all isolates showed MDR phenotype, except one isolate. Gentamicin MIC values of the isolates was determined as ≥2048 µg/ml.

Table 5: Antimicrobial resistance phenotypes observed among the HLGR enterococci

Resistance phenotype	No of isolates	*Enterococcus* spp.			
		E. faecium		*E. faecalis*	
		Dog	Cat	Dog	Cat
AM, TE, CIP, RA, E, SYN	15	5	9	–	1
AM, TE, CIP, RA, E	10	7	3	–	–
AM, TE, RA, E, SYN	1	1	–	–	–
AM, TE, CIP, RA	1	1	–	–	–
TE, CIP, RA, E, SYN	5	–	–	2	3
AM, TE, RA, E	2	1	1	–	–
TE, C, E, SYN	1		–	–	1
AM, TE, CIP, RA, E	4		4	–	–
AM, CIP, RA, E, SYN	2	1	1	–	–
AM, TE, RA, C, E, SYN	1	–	–	–	1
TE, CIP, E, SYN	2	–	–	–	2
TE, RA, C, E, SYN	2	–	–	–	2
TE, RA, E	1	1	–	–	–
AM, CIP, E, SYN	1	–	1	–	–
AM, CIP, E	2	2	–	–	–
AM, TE, E, SYN	1	1	–	–	–
AM, CIP, RA, E	1	1	–	–	–
AM	1	1	–	–	–

Distribution of resistance genes

All HLGR isolates carried *aac(6)-Ie-aph(2)-Ia*, together with *aac(6')-Ie-aph(2")-Ia*, *aph(3")-IIIa*, *aph(2")-Ic*, *aph(2")-Id* genes in various rates (Figure 3). In addition, tetracycline and erythromycin resistant isolates harbored *tet*M, *tet*L and *erm*B genes alone or in combination (Table 6) (Figure 4).

Detection of virulence genes

Among the 53 HLGR isolates, 11 were positive for *asa*1 (20.8%), 5 (9.4%) for *gel*E, 5 for (9.4%) *esp* and 5 (9.4%) for *cyl*A (Figure 5). Virulence gene profile analysis revealed that 7 (13.2%) isolates contained two genes and four (7.5%)

Table 7: Distribution of the virulence genes among HLGR *E. faecalis* isolates

Virulence genes	Animal Species	
	Dog	Cat
*gel*E, *asa*1	1	4
*cyl*A, *esp*, *asa*1	–	4
*asa*1, *esp*	1	–
*asa*1, *cyl*A	–	1
Total	**2**	**9**

Table 6: Distribution of resistance genes detected among the isolates

Resistance genotype	No of the isolates	Species	Other resistance genes detected
aac(6')-Ie-aph(2")-Ia, aph(3")-IIIa, aph(2")-Ic, aph(2")-Id	1 (Dog)	*E. faecium*	*tet*M, *tet*L, *erm*B
aac(6')-Ie-aph(2")-Ia, aph(3")-IIIa, aph(2")-Id	1 (Cat)	*E. faecium*	*tet*M, *tet*L, *erm*B
aac(6')-Ie-aph(2")-Ia, aph(3")-IIIa, aph(2")-Ic	2 (Dog)	*E. faecium*	*tet*M, *tet*L, *erm*B
aac(6')-Ie-aph(2")-Ia, aph(3")-IIIa, aph(2")-Ic	1 (Dog)	*E. faecium*	*tet*L, *erm*B
aac(6')-Ie-aph(2")-Ia, aph(3")-IIIa	1 (Cat)	*E. faecium*	*tet*M, *tet*L, *erm*B
aac(6')-Ie-aph(2")-Ia, aph(3")-IIIa	5 (Dog), 3 (Cat)	*E. faecium*	*tet*M, *erm*B
aac(6')-Ie-aph(2")-Ia, aph(3")-IIIa	1 (Dog), 3 (Cat)	*E. faecalis*	*tetM, ermB*
aac(6')-Ie-aph(2")-Ia, aph(3")-IIIa	1 (Dog), 1 (Cat)	*E. faecalis*	*tet*M, *tet*L, *erm*B
aac(6')-Ie-aph(2")-Ia, aph(3")-IIIa	2 (Cat)	*E. faecalis*	*tet*M, *tet*L, *erm*B
aac(6')-Ie-aph(2")-Ia, aph(3")-IIIa	1 (Cat)	*E. faecalis*	*tet*M, *erm*B
aac(6')-Ie-aph(2")-Ia, aph(2")-Ic	2 (Dog), 3 (Cat)	*E. faecium*	*tet*M, *tet*L, *erm*B
aac(6')-Ie-aph(2")-Ia	4 (Dog), 6 (Cat)	*E. faecium*	*tet*M, *tet*L, *erm*B
aac(6')-Ie-aph(2")-Ia	2 (Cat)	*E. faecium*	*tet*L, *erm*B
aac(6')-Ie-aph(2")-Ia	3 (Dog), 2 (Cat)	*E. faecium*	*tet*M, *tet*L
aac(6')-Ie-aph(2")-Ia	2 (Cat)	*E. faecalis*	*tet*M, *erm*B
aac(6')-Ie-aph(2")-Ia	4 (Dog)	*E. faecium*	*tet*M, *tet*L, *erm*B
aac(6')-Ie-aph(2")-Ia	1 (Dog)	*E. faecium*	*erm*B

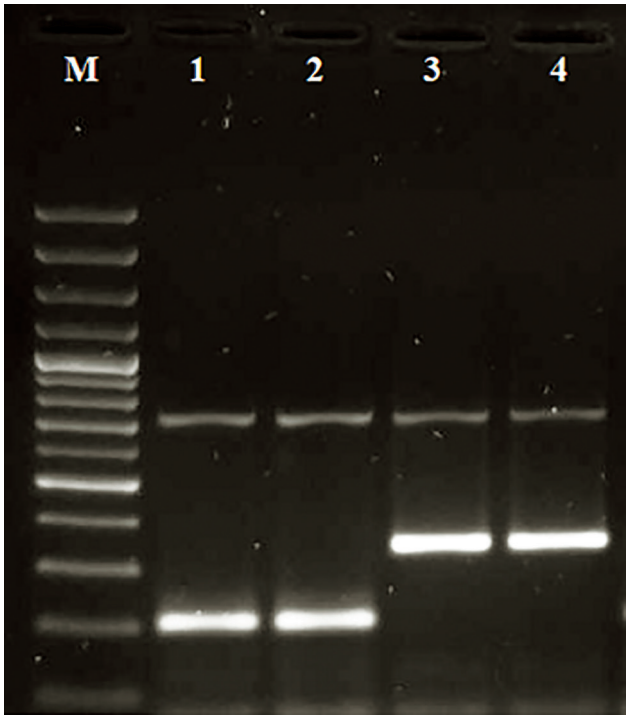

Figure 2. Genus and species-specific mPCR with primer pair E1/E2, FL1/FL2 and FM1B/FM2B. Lane M: 100 bp plus molecular marker, Lane 1-2: *Enterococcus* spp. (733 bp) and *E. faecium* (214 bp), Lane 3-4: *Enterococcus* spp. (733 bp) and *E. faecalis* (360 bp)

isolates harbored three genes (Table 7). None of the isolates harbored *hly* gene.

DISCUSSION

The increasing rate of antimicrobial resistant bacteria in pet animals is a growing public health concern. Since close contact between pets and their owners facilitates the transmission of resistant bacteria. Enterococci can acquire resistance to different classes of antimicrobials and play a role as hub for the dissemination of resistance genes (11).

Aminoglycosides together with cell wall active agents such as penicillin, ampicillin or vancomycin have been used for the treatment of infections caused by enterococci due to their synergetic activity. The presence of HLGR make therapy options of enterococcal infections very limited (22). In the current study, HLGR enterococci were isolated from 12.2% (53/435) of the faecal samples. In Turkey, aminoglycosides are not widely used parenterally in cats and dogs due to their kidney cytotoxicity, but are used in topical applications. Therefore, presence of HLGR enterocci could be attributed

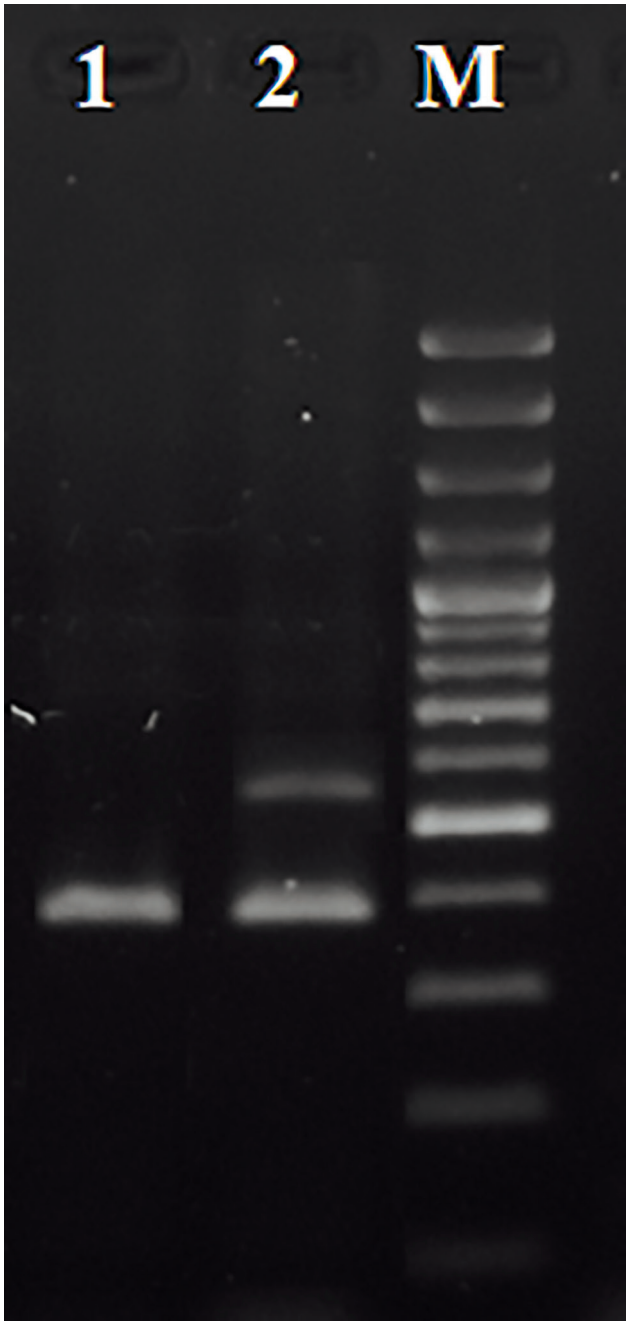

Figure 3. Agarose gel electrophoresis of mPCR for aminoglycoside resistance genes. Lane M: 100 bp molecular marker, Lane 1: *aac(6')-Ie-aph(2")-Ia* (369 bp), Lane 2: *aac(6')-Ie-aph(2")-Ia* (369 bp) and *aph(3')-IIIa* (523 bp) *tet*L (229 bp)

to environmental contamination or their close contact with their owners. This result is in agreement with the findings of Ben Said *et al.* (25), who reported that 14.9% of the isolates were HLGR. However, in another study, Bertelloni *et al.* (26) isolated 222 enterococci from dog faecal samples and de-

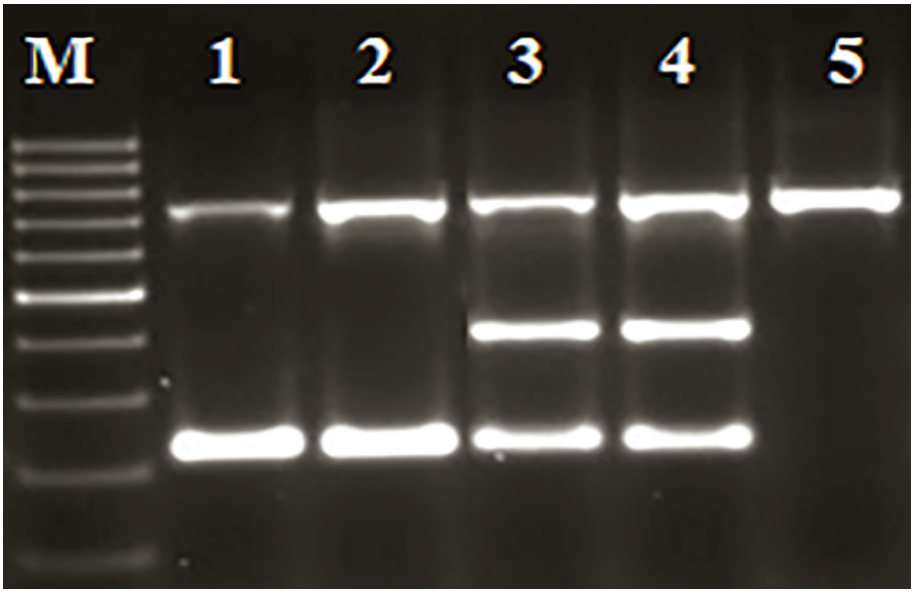

Figure 4. Agarose gel electrophoresis of mPCR for tetracycline and macrolide resistance genes. Lane M: 100 bp molecular marker, Lane 1-2: *tet*L (229 bp) and *erm*B (745 bp), Lane 3-4: *erm*B (745 bp), tetM (406 bp) and *tet*L (229 bp), Lane 5: *erm*B (745 bp)

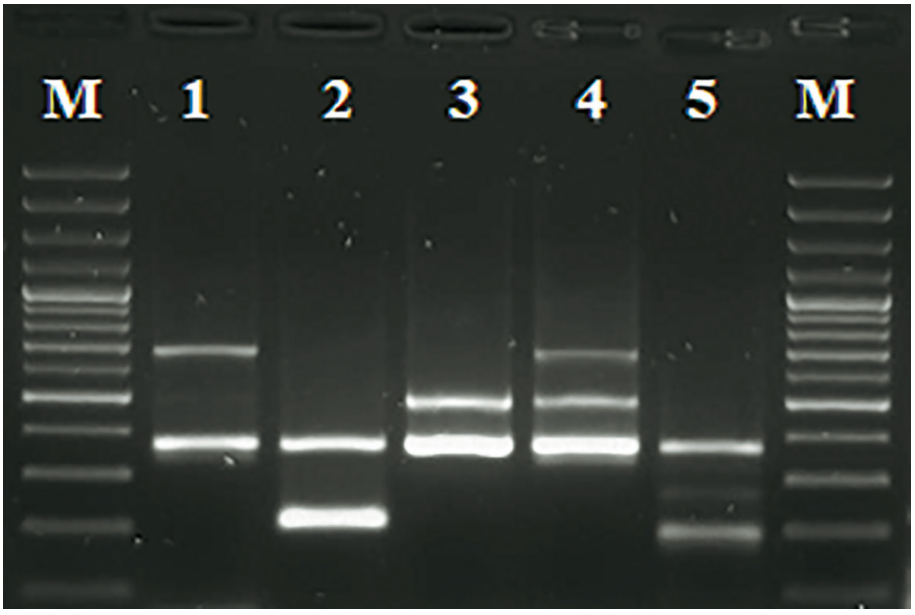

Figure 5. Agarose gel electrophoresis of mPCR for virulence genes. Lane M: 100 bp plus molecular marker, Lane 1: *cyl*A (688 bp) and *asa*1 (375 bp), Lane 2: *asa*1 (375 bp) and *gel*E (213 bp), Lane 3: *asa*1 (375 bp) and *esp* (510 bp), Lane 4: *asa*1 (375 bp), *esp* (510 bp) and *cyl*A, Lane 5: *asa*1 (375 bp), *hyl*A (276 bp) and *gel*E (213 bp)

tected that 6.7% (15) of the isolates were HLGR enterococci, consisting of eight isolates of *E. faecium*, six of *E. faecalis* and one isolate of *Enterococcus durans*. Marques *et al.* (24) detected in 15.2% of the *Enterococcus* spp. as HLGR. Main resistance mechanism of HLGR in enterococci is related with synthesis of bifunctional enzyme *aac(6)-Ie-aph(2)-Ia*, which is linked to *Tn*5281 carried on a plasmid (20). In this study, all isolates carried this gene alone or in various combinations of other genes encoding aminoglycoside resistance. De Leener *et al.* (27) detected *aac(6)-Ie-aph(2)-Ia* gene in 14 of 16 HLGR

enterococci from cats and dogs. Similar results indicating bifunctional aminoglycoside resistance genes in gentamicin resistant enterococci were also reported by Marques *et al.* (24), Ben Said *et al.* (25) and Jackson *et al.* (28).

Almost all of the HLGR isolates (except one isolate) showed MDR phenotype in this study. Similar observations have been previously reported by Bertelloni *et al.* (26) and Jackson *et al.* (28). This could be explained by co-localization of antimicrobial resistance genes on the mobile genetic elements (MGEs) such as plasmids, transposoons and integrons (11). In the current study, acquired resistance mechanisms against macrolide and tetracycline (commonly used antibiotics) were frequently observed among the isolates. The *erm*B gene, responsible for methylation of 23S rRNA, was detected in 46 (90.2%) of the 51 erythromycin resistant isolates. In previous studies, it has been reported that the *erm*B gene is the most common gene causing macrolide-lincosamide-streptogramin (MLS_B) resistance in enterococci (28).

Following erythromycin resistance, tetracycline resistance (88.7%) was the second common resistance observed in the study. In tetracycline resistant isolates, *tet*M and *tet*L genes were detected alone or in combination. It has been reported that these two resistance genes are synthesized through plasmids (30). Of these genes, while overexpression of *tet*M (a ribosomal protection protein) mediates tetracycline resistance, expression of *tet*L, encoding the major facilitator superfamily (MFS) efflux pump, leads to expel tetracyclines complexed with Mg^{2+} in exchange for H^+ (20). Widespread distribution of these genes was also reported by Ben Said *et al.* (25), who detected *tet*M in five isolates, *tet*M-*tet*L in 16 isolates and *tet*L in four isolates. In another study, dominance of these two genes was also reported by Jackon *et al.* (28), who detected *tet*M and *tet*L genes in 60.2% and 38.4% of the isolates, respectively.

High level (≥256 μg/ml) of ampicillin resistance among HLGR and vancomycine resistant enterococci (VRE) strongly limit therapy options in enterococcal infections (31). Ampicillin resistance was found to be caused by multiple mutations in active sites of the *pbp*5 gene (23). The results of the current study showed high ampicillin resistance (77.4%) among HLGR enterococci. van den Bunt *et al.* (32) reported the prevalence of ARE_{fm} in cats and dogs 13% (11 of 85) and 30% (24 of 79), respectively. In contrast, a low resistance rate (12.2%) was reported by Bertelloni *et al.* (16). In addition, it has been reported that ampicillin resistance in *E. faecium* was more common rather than in other than *Enterococcus* spp. (12, 29). Similarly, ampicillin resistance is most common in *E. faecium* isolates in this study.

Fluoroquinolones are one of the last resort of antimicrobials used for the treatment of infections in dogs. Resistance to fluoroquinolones in *E. faecium* and *E. faecalis* strains is due to mutations in the quinolone-determining regions of *gyr*A and *par*C genes (31). In the present study, high resistance rate to ciprofloxacin were observed among HLGR (67.9%) isolates. Bertelloni *et al.* (26) reported occurence of resistance to ciprofloxacin and enrofloxacin as 34.7% and 49.5%, respectively.

Virulence genes expressed by enterococci are one of the main concerns contributing to the severity of infections. In this study, interestingly, virulence genes were only detected in HLGR *E. faecalis* isolates. Similarly, low rate of virulence factors in *E. faecium* isolates was also reported by Çelik *et al.* (14). Of the examined virulence genes, 5 (41.7%) *E. faecalis* isolates harbored the *gel*E gene encoding gelatinase, responsible for the hydrolysis of gelatine, elastin, collagen, haemoglobin, and play an important role in the development of endocarditis (34). Gelatinase synthesis in *E. faecalis* is considered to facilitate migration and spread via damaging the host tissue (35). Boyar *et al.* (15) reported that 44.9% of the isolates were positive for *gel*E gene, consisting mostly *E. faecalis* (89.7%). Another study carried out by Gülhan *et al.* (39), gelatinase activity was detected in 26.6% and 60% of *E. faecium* and *E. faecalis* isolates, respectively.

The enterococcal surface protein (ESP), chromosomally encoded, is responsible for an increased adhesion, biofilm formation, colonisation and immune evasion (35). The frequency of *esp* gene shows wide variation in various studies. The *esp* gene was detected in 41.7% of *E. faecalis* isolates in this study. Boyar *et al.* (15) found 33.6% of the isolates as positive for *esp* gene. A low rate of *esp* gene was reported by Oliveira *et al.* (37), who reported a prevalence rate of 10% among enterococci from dogs with periodontal disease. On the other hand, Iseppi *et al.* (28) and Gülhan *et al.* (39) did not find this gene among the isolates.

Aggregation substance (AS), encoded by *asa*1, enhances virulence by facilitating the conjugative transfer of sex pheromone gene-containing plasmids (34). In this study, nearly all HLGR *E. faecalis* isolates (91.7%) were positive for *asa*1

gene. In contrast, previous studies showed absence or low prevalence of *asa*1 gene among enterococci of pet animals. Iseppi *et al*. (38) found no positivity for this gene and attributed this by reduced transfer (resistance and virulence) capability of the isolates by mating. A very low ability of AS synthesis was reported among *E. faecium* (1.6%) and *E. faecalis* (6.7%) isolates by Gülhan *et al*. (39).

The cytolysin, encoded by *cyl*, is a two-peptide lytic toxin produced by some strains of *E. faecalis*, and has cytotoxic activity on human, horse and rabbit erythrocytes. The strains that have the ability to produce cytolysin are reported to increase the severity of the infections (35). In this study, cytolysin activity was only detected in 5 *E. faecalis* isolates. However, some reports have indicated the absence or low occurence of *cyl* gene both in *E. feacalis* and *E. faecium* isolates (36, 39).

In summary, the results of this study showed that a significant proportion of HLGR enterococci colonising a healthy dog and cat population in Turkey were resistant to several classes of antimicrobials. Moreover, virulence genes playing an important role in the pathogenesis and were frequently observed in clinical isolates. Thus, pet animals could act as a reservoir for antimicrobial resistance and virulence genes and cause the spread of these agents to distribute these genes to the environment, humans and susceptible animal populations. For the prevention of emergence and dissemination of MDR and virulent enterococci, prudent use antimicrobials should be promoted and continiuos antimicrobial monitoring programs should be applied in animals.

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