Characterization of *Enterococcus faecalis* isolates originating from different sources for their virulence factors and genes, antibiotic resistance patterns, genotypes and biofilm production

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Summary

In this study, 72 *Enterococcus faecalis* isolates originating from humans (n=39), dogs (n=26) and cats (n=7) were investigated for some virulence factors, some virulence genes, antibiotic resistance phenotypes, randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) patterns and biofilm production. Of the isolates, 31 (43.1%) were positive for gelatinase, 11 (15.3%) for aggregation substance and cytolysine, 38 (52.8%) for *gelE* and 34 (47.2%) for *asa1* genes. All isolates were found to be negative for *hyl*, *esp* and *cylA* genes. All isolates were found to be resistant to nalidixic acid and kanamycin. On the other hand, all isolates were cited for susceptible to amoxicillin. Vancomycin resistance genes (*vanA*, *vanB*, *vanC1/C2* or *vanD*) have not been detected in any of the phenotypically vancomycin resistant isolates. Isolates from humans, dogs and cats were grouped into 8, 2 and 4 antibiotypes depending upon susceptibilities to 12 different antibiotics. In all human, dog and cat isolates, 9, 12 and 2 genotypes were determined by RAPD-PCR, respectively. Nine (34.6%) of the dog isolates were found to be positive for biofilm production. This study showed that multiple antibiotic resistance among human isolates is more frequent than in dog and cat isolates.

Key words: Antibiotyping, Biofilm, *Enterococcus faecalis*, Genotyping, Virulence

Introduction

Enterococci are a dominant bacterial group in the intestinal flora of humans and animals. They are increasingly associated with nosocomial infections (Sun *et al*., 2012).

Several virulence factors such as aggregation substance (AS), gelatinase, cytolysin, enterococcal surface protein (esp) and hyaluronidase have been described in enterococci (Gulhan *et al*., 2006; Hallgren *et al*., 2009; Gulhan *et al*., 2012). AS encoded by *asa1* which is carried on a plasmid that enables the conjugative transfer of sex pheromone gene-containing plasmids through the clumping of one *Enterococcus* to another (Olsen *et al*., 2012). Gelatinase encoded by the chromosomal *gelE* is an extracellular zinc endopeptidase that has been shown to exacerbate endocarditis (Tsikrikonis *et al*., 2012). The cytolysin operon consists of five genes, of which *cylL1*, *cylL2*, *cylM*, and *cylB* are relevant to the expression of component L, whereas *cylA* is necessary for the expression of component A (Vankerckhoven *et al*., 2004). Esp, encoded by the chromosomal *esp*, is associated with increased virulence, colonization and persistence in the urinary tract, and biofilm formation (Upadhyaya *et al*., 2011). Hyaluronidase encoded by chromosomal *hyl* has been cited to contribute to invasion of the nasopharynx and pneumococcal pneumonia (Lopez *et al*., 2013).

Most enterococci have resistance to various antibiotics such as cephalosporins, penicillins, amino-glycosides, glycopeptides and lincosamides (Boynukara *et al*., 2002). Studies have recently focused on enterococcal infections in veterinary medicine in parallel with coming out the animal factor in transmission of resistant enterococci to humans (Pourakbari *et al*., 2013).

Biofilm is a structured community of microorganisms encapsulated within a self-developed polymeric matrix and adherent to various biotic and abiotic surfaces irreversibly. Biofilm production has been reported in some enterococcal infections. The major clinical infections have been caused by *Enterococcus faecalis* capable of producing biofilms (Upadhyaya *et al*., 2011).

The aim of this study was to investigate the virulence factors and genes, antibiotic resistance patterns, genotypes, and biofilm production of *E. faecalis* isolates originated from humans, dogs and cats and determine the genetic diversity among them.

Materials and Methods

Bacterial isolates

A total of 72 *E. faecalis* isolates, including 39 humans, 26 dogs and seven cats' origins, were used in the study. All isolates were phenotypically identified to the species level using conventional methods and were confirmed by polymerase chain reaction (PCR)

(Furlaneto *et al*., 2014).

Detection of gelatinase, AS and cytolysin production

A single-colony inoculum was streaked on Todd-Hewitt agar plates containing 3% gelatin and incubated aerobically at 37°C for 48 h. A positive result was recorded when a clear halo was seen around each colony (Gulhan *et al*., 2012). Measurement of the AS of the enterococci was performed by clumping assay, as described previously (Gulhan *et al*., 2006). Brain heart infusion agar supplemented with 5% horse blood was used for the detection of cytolysin activity as defined elsewhere (Gulhan *et al*., 2012).

PCR detection of virulence genes

PCR amplification was subjected to detect the presence of genes involved in the expression of *cylA*, *gelE*, *esp*, *asa1* and *hyl* using the primers described by Vankerckhoven *et al*. (2004).

Antibiotic susceptibility test

All isolates were tested against 12 different antibiotics using disc diffusion method. A susceptibility test result of each antibiotic was evaluated according to CLSI interpretive standards (CLSI, 2011).

Detection of *van* genes

The genes responsible for resistance to vancomycin (*vanA*, *vanB*, *vanC1/2* and *vanD*) were investigated by PCR, as described previously (Sharifi *et al*., 2013).

Antibiotyping of isolates

This procedure was performed by means of the Pearson product moment correlation coefficient and the unweighted pair group method using arithmetic averages (UPGMA) cluster analysis. The antibiotic susceptible/ resistance patterns were analyzed to obtain dendrogram with cut-off value of 70%.

Randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) amplification

RAPD-PCR analysis was done using the primer M13 (5′-GAG GGT GGC GGT TCT-3′) as described previously (Versalovic and Lupski, 2002). Grouping of the RAPD-PCR patterns was performed by means of the UPGMA cluster analysis. The strains grouping co-efficients of similarity of 70% for RAPD typing were applied.

Biofilm formation

Congo red agar was used to detect biofilm production. Black colonies on Congo red agar were evaluated as biofilm positive, colorless colonies were evaluated as negative (Ciftci *et al*., 2009).

Results

Virulence factors and genes

The distribution of the virulence factors and genes of *E. faecalis* by their origins are given in Table 1.

Antibiotic susceptibility and phenotype

Antibiotic resistance/susceptibility patterns of 72 *E. faecalis* isolates are presented in Table 2. None of the 14 vancomycin resistant isolates, vancomycin resistance genes (*vanA*, *vanB*, *vanC1/2* or *vanD*) has been detected. Multiple antibiotic resistance phenotypes of isolates are presented in Table 3.

Antibiotyping

Antibiotyping of isolates performed by UPGMA and the human, dog and cat isolates were grouped into 8, 2 and 4 antibiotypes, respectively (Figs. 1, 2 and 3). Human isolates were divided into eight groups (A-H) based on 70% similarity. Groups were represented as AHA1-A3 (n=3); AHB (n=1); AHC1-C2 (n=2); AHD1-D3 (n=3); AHE1-E2 (n=2), AHF1-F3 (n=3); AHG1-G4 (n=5) and AHH1-17 (n=20). Dog isolates were divided into two main groups (A and B) to 70% similarity rate. Groups were represented as ADA1-A7 (n=8) and ADB1-B15 (n=18). Cat isolates were grouped into four main groups (A-D) based on 70% similarity. Groups were represented as ACA (n=1); ACB1-B2 (n=2); ACC (n=1) and ACD1-D3 (n=3).

RAPD-PCR

Among human, dog and cat isolates nine, 12 and 2 different profiles were determined by RAPD-PCR, respectively (Figs. 4 and 5). Analysis of RAPD-PCR patterns of human isolates revealed the presence of nine RAPD types (A-I) based on 70% similarities. Isolates were represented in four clusters: C (n=3), D (n=2), F

Table 1: Distribution of virulence factors and genes among *Enterococcus faecalis* isolates

Virulence factors/genes	Origin			Total n=72 (%)
	Human n=39 (%)	Dog n=26 (%)	Cat n=7 (%)	
Gelatinase	9 (23.1)	19 (73.1)	3 (42.9)	31 (43.1)
Aggregation substance	9 (23.1)	2 (7.7)	0	11 (15.3)
Cytolysin	11 (28.2)	0	0	11 (15.3)
gelE	27 (69.2)	10 (38.5)	1 (14.3)	38 (52.8)
asa1	21 (53.8)	13 (50)	0	34 (47.2)
hyl	0	0	0	0
esp	0	0	0	0
cylA	0	0	0	0

Table 2: Antibiotic resistance/susceptibility patterns of *Enterococcus faecalis* isolates

Antibiotics	Human (n=39)		Dog (n=26)		Cat (n=7)		Total (n=72)	
	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)
AMP	1 (2.6)	38 (97.4)	0 (0)	26 (100)	0 (0)	7 (100)	1 (1.4)	71 (98.6)
P	1 (2.6)	38 (97.4)	0 (0)	26 (100)	0 (0)	7 (100)	1 (1.4)	71 (98.6)
VAN	13 (33.3)	26 (66.7)	1 (3.8)	25 (96.2)	0 (0)	7 (100)	14 (19.4)	58 (80.6)
B	24 (61.5)	15 (38.5)	8 (30.8)	18 (69.2)	1 (14.3)	6 (85.7)	33 (45.8)	39 (54.2)
OTET	33 (84.6)	6 (15.4)	15 (57.7)	11 (42.3)	2 (28.6)	5 (71.4)	50 (69.4)	22 (30.6)
KAN	39 (100)	0 (0)	26 (100)	0 (0)	7 (100)	0 (0)	72 (100)	0 (0)
ERY	22 (56.4)	17 (43.6)	0 (0)	26 (100)	0 (0)	7 (100)	22 (30.6)	50 (69.4)
AMX	0 (0)	39 (100)	0 (0)	26 (100)	0 (0)	7 (100)	0 (0)	72 (100)
NOR	9 (23.1)	30 (76.9)	4 (15.4)	22 (84.6)	2 (28.6)	5 (71.4)	15 (20.8)	57 (79.2)
NAL	39 (100)	0 (0)	26 (100)	0 (0)	7 (100)	0 (0)	72 (100)	0 (0)
CEP	10 (25.6)	29 (74.4)	4 (15.4)	22 (84.6)	1 (14.3)	6 (85.7)	15 (20.8)	57 (79.2)
CIP	18 (46.2)	21 (53.8)	0 (0)	26 (100)	3 (42.9)	4 (57.1)	21 (29.2)	51 (70.8)

AMP: Ampicillin (30 µg), P: Penicillin G (10 µg), VAN: Vancomycin (30 µg), B: Bacitracin (10 µg), OTET: Oxytetracyclin (30 µg), KAN: Kanamycin (5 µg), ERY: Erythromycin (15 µg), AMX: Amoxicillin (25 µg), NOR: Norfloxacin (30 µg), NAL: Nalidixic acid (30 µg), CEP: Cephalothin (30 µg), and CIP: Ciprofloxacin (5 µg)

Table 3: Antimicrobial resistance phenotypes detected in *Enterococcus faecalis* isolates by their origins

Number of antibiotics	Antimicrobial resistance phenotype	Number of isolates with phenotype		
		Human	Dog	Cat
8	VAN-B-OTET-KAN-ERY-NOR-NAL-CIP	1	-	-
	VAN-B-OTET-KAN-ERY-NAL-CEP-CIP	2	-	-
	B-OTET-KAN-ERY-NOR-NAL-CEP-CIP	1	-	-
7	VAN-B-OTET-KAN-ERY-NOR-NAL	1	-	-
	VAN-B-OTET-KAN-ERY-NAL-CIP	1	-	-
	VAN-B-OTET-KAN-NOR-NAL-CEP	-	1	-
	VAN-B-OTET-KAN-NOR-NAL-CIP	1	-	-
	VAN-B-OTET-KAN-NAL-CEP-CIP	1	-	-
	VAN-OTET-KAN-ERY-NOR-NAL-CIP	1	-	-
	B-OTET-KAN-ERY-NOR-NAL-CIP	1	-	-
6	VAN-B-OTET-KAN-ERY-NAL	2	-	-
	B-OTET-KAN-ERY-NOR-NAL	1	-	-
	B-OTET-KAN-ERY-NAL-CIP	3	-	-
	B-OTET-KAN-NOR-NAL-CEP	-	1	-
	OTET-KAN-ERY-NAL-CEP-CIP	1	-	-
5	VAN-B-OTET-KAN-NAL	1	-	-
	VAN-OTET-KAN-ERY-NAL	1	-	-
	P-B-OTET-KAN-NAL	1	-	-
	B-OTET-KAN-ERY-NAL	1	-	-
	B-OTET-KAN-NOR-NAL	-	2	-
	B-OTET-KAN-NAL-CEP	-	1	-
	B-OTET-KAN-NAL-CIP	2	-	-
	OTET-KAN-ERY-NOR-NAL	1	-	-
	OTET-KAN-NOR-NAL-CIP	-	-	1
	OTET-KAN-NAL-CEP-CIP	1	-	-
4	AMP-OTET-KAN-NAL	1	-	-
	B-OTET-KAN-NAL	1	3	-
	B-KAN-NAL-CIP	1	-	1
	OTET-KAN-ERY-NAL	2	-	-
	OTET-KAN-NAL-CEP	2	-	-
	OTET-KAN-NAL-CIP	-	-	1
	KAN-ERY-NAL-CEP	1	-	
	KAN-NOR-NAL-CEP	-	-	1
3	B-KAN-NAL	1	-	-
	OTET-KAN-NAL	1	7	-
	KAN-ERY-NAL	1	-	-
	KAN-NAL-CEP	-	1	-
2	KAN-NAL	2	10	3

AMP: Ampicillin (30 µg), P: Penicillin G (10 µg), VAN: Vancomycin (30 µg), B: Bacitracin (10 µg), OTET: Oxytetracyclin (30 µg), KAN: Kanamycin (5 µg), ERY: Erythromycin (15 µg), AMX: Amoxicillin (25 µg), NOR: Norfloxacin (30 µg), NAL: Nalidixic acid (30 µg), CEP: Cephalothin (30 µg), and CIP: Ciprofloxacin (5 µg)

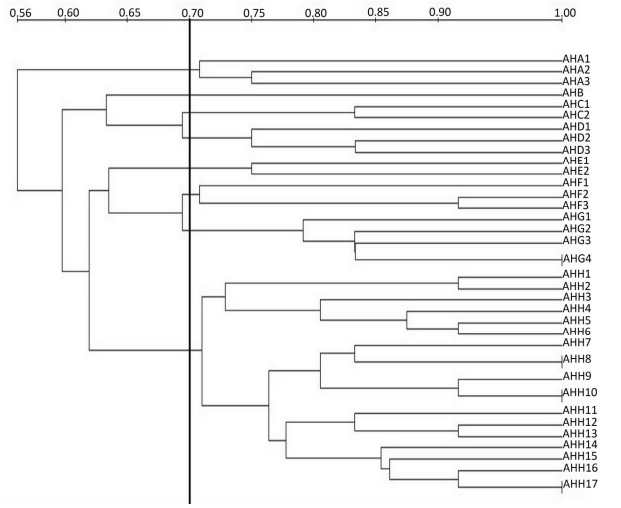

Fig. 1: Antibiotype patterns of *Enterococcus faecalis* isolated from human and dendrogram obtained by UPGMA

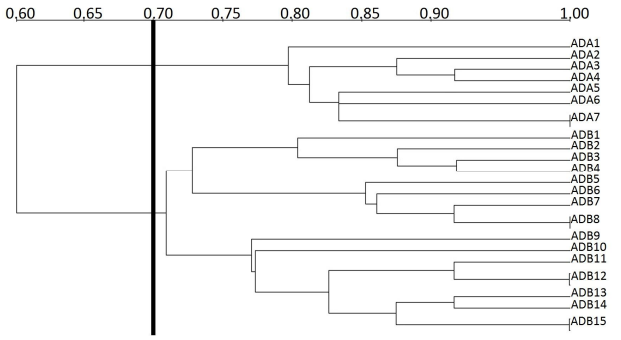

Fig. 2: Antibiotype patterns of *Enterococcus faecalis* isolated from dogs and dendrogram obtained by UPGMA

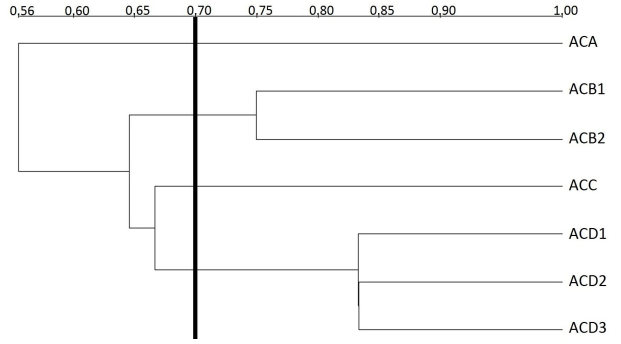

Fig. 3: Antibiotype patterns of *Enterococcus faecalis* isolated from cats and dendrogram obtained by UPGMA

(n=10), I (n=19) and 5 unique types (each consisted of one isolate). Dog isolates were grouped into 12 unique types (A-L) based on 70% similarities: type A (n=1), B (n=1), C (n=1), D (n=2), E (n=2), F (n=2), G (n=7), H (n=3), I (n=1), J (n=3), K (n=2), and L (n=1). Cat isolates were classified into two groups (A and B) with 70% similarities. Isolates were presented in two major types: type A (n=2), and type B (n=5).

Biofilm production

Nine (34.6%) of 26 dog isolates were found to be positive for biofilm production. Whereas it was not detected biofilm production in any human and cat isolates.

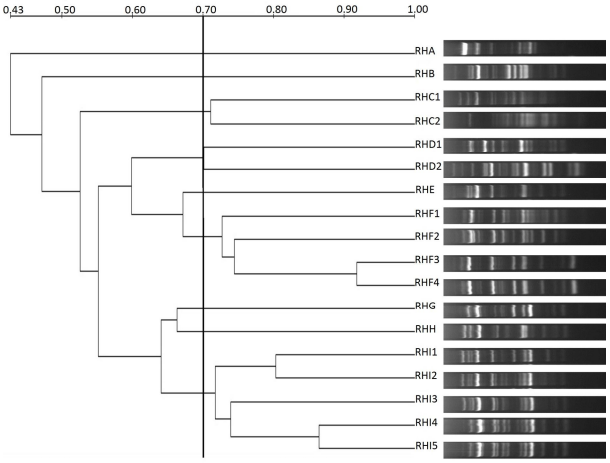

Fig. 4: RAPD patterns of *Enterococcus faecalis* isolated from human and dendrogram obtained by UPGMA

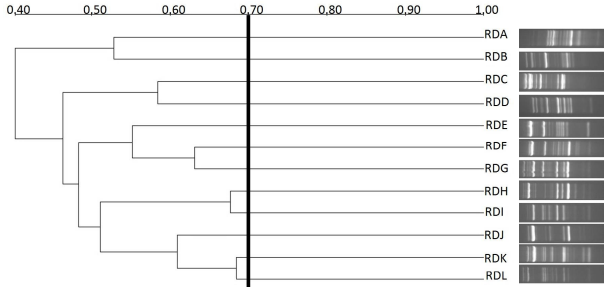

Fig. 5: RAPD patterns of *Enterococcus faecalis* isolated from dog and dendrogram obtained by UPGMA

Discussion

Enterococcus faecalis is an opportunistic pathogen in both humans and animals. The natural ability of enterococci to acquire, accumulate, and share extra-chromosomal elements encoding virulence traits (Lopez *et al*., 2013).

Tsikrikonis *et al*. (2012) have reported that 28.6% of human isolates and 26.9% of animal isolates were positive for gelatinase. Similar results have also been reported by other researchers (Han *et al*., 2011; Olsen *et al*., 2012; Sun *et al*., 2012). In this study, gelatinase and *gelE* were detected in 23.1% and 69.2%; in 73.1% and 38.5%; in 42.9% and 14.3% of human, dog and cat isolates, respectively. The *gelE* gene was detected in 52.8% of all isolates and was thus the most common of the factors that we tested as cited by some authors (Dupre *et al*., 2003; Ghosh *et al*., 2012).

AS encoded by *asa1* has found additional roles of this protein in enterococcal virulence (Sun *et al*., 2012). A high incidence of this gene in *E. faecalis* has been reported in previous studies (Hallgren *et al*., 2009; Sharifi *et al*., 2013). By contrast, this gene was not found in *E. faecalis* isolates (Kafil *et al*., 2013). In our study, AS and *asa1* were detected in 23.1% and 53.8% of human isolates; 7.7% and 50% of dog isolates,

respectively. In our cat isolates absence of AS and *asa1* gene suggest low virulence and reduced capability of strains to virulence traits.

Cytolysin-producing *E. faecalis* have been shown to be virulent in animal and human infections and associated with increased severity of infection (Hallgren *et al*., 2009). Tsikrikonis *et al*. (2012) showed that 28.6% of human isolates were hemolytic compared to 6.4% of animal isolates. The incidence of cytolysin in our study was lower than that previously reported by some researchers (Ghosh *et al*., 2012; Sun *et al*., 2012).

The *esp* was the least frequently detected virulence gene in dog and cat isolates, these observations are in accordance with previous reports (Hallgren *et al*., 2009; Lopez *et al*., 2013). Still, the low prevalence in human isolates is in contrast to other studies (Dupre *et al*., 2003; Vankerckhoven *et al*., 2004). The *esp* gene was not detected in this study, which was in compliance with findings of other studies (Harada *et al*., 2005; Olsen *et al*., 2012; Lopez *et al*., 2013). However, this was in contrast to the findings of some researchers (Upadhyaya *et al*., 2011; Tsikrikonis *et al*., 2012; Sharifi *et al*., 2013).

An open reading frame (hyl_{Efm}) with homologies to previously described hyaluronidase genes has been identified in *E. faecium* isolates. Then this factor and genes have been investigated by some authors in *E. faecalis* isolates (Vankerckhoven *et al*., 2004; Lopez *et al*., 2013). In the present study, all isolates were found to be negative for *hyl* gene.

Results obtained by phenotypic tests revealed a lower percentage of strains that produced haemolysin, gelatinase or AS, compared to genotypic characterization. This may be due to the presence of silent and undetected genes or to the fact that detection by PCR of a single gene inside an operon. The conflicting results from our study and of other investigations concerning occurrence of virulence factors among isolates might be due to differences in the reservoir of the various countries or the ecological origin of strains, the sensitivity of detection methods, number and kinds of examined samples in these studies.

Vancomycin is, in some cases, the only antibiotic still effective in the treatment of nosocomial enterococcal infections (Sharifi *et al*., 2013). In the field of antibiotic resistance, one of the most challenging recent issues is the worldwide emergence of vancomycin-resistant enterococci (VRE) (Lopez *et al*., 2013). Besides several existing reports of VRE in farm animals (Han *et al*., 2011), there are a limited number of studies dealing with the colonization of VRE in companion animals (Lopez *et al*., 2013), even though VRE have been recorded in the intestinal tract of dogs and cats (Boynukara *et al*., 2002). No resistance to vancomycin was found in several studies on enterococci from dogs and cats (Ossiprandi *et al*., 2008; Ghosh *et al*., 2012). In our study only 13 human and one dog isolates were found to be resistant to vancomycin phenotypically. However, all isolates were negative for *van* genes as recently reported (Furlaneto *et al*., 2014). In present study all isolates were found to be resistant to kanamycin and nalidixic acid. Bacitracin and oxytetracyclin resistances were observed most frequently as compared to the other antibiotics. Similar results have been reported by other researchers (Boynukara *et al*., 2002; Ossiprandi *et al*., 2008; Ghosh *et al*., 2012). The present study showed that among human isolates resistance to multiple antibiotics was observed at a greater frequency than dog and cat isolates as previously reported (Gulhan *et al*., 2006). Fortunately, our isolates remain highly susceptible to ampicillin, penicillin, amoxicillin, norfloxacin and cephalothin.

Antibiotyping of *Enterococcus* isolates by several methods were performed based on their different antibiotic resistance profiles (Jackson *et al*., 2009; Ghosh *et al*., 2012). Antibiotic-resistant *E. faecalis* isolates have been grouped and a scattered distribution has been noted, indicating that resistance was not related to a particular clone (Furlaneto *et al*., 2014). In the present study human, dog and cat isolates were grouped into in 8, 2 and 4 groups, respectively.

Genotyping of *Enterococcus* species can be made by RAPD-PCR has been reported in previous studies (Getachew *et al*., 2012; Ghosh *et al*., 2012; Pourakbari *et al*., 2013). In a study carried out on human in Iran (Pourakbari *et al*., 2013) cited the similarity pattern built for *E. faecalis* isolates by RAPD-PCR, has demonstrated the presence of four distinct clusters (A, B, C, D). Getachew *et al*. (2012) have reported that VRE species showed diverse RAPD profiles with some clustering of strains based on the individual's background. In this study RAPD-PCR profiles in human isolates showed 9 types, of which 4 were predominant. This suggests that isolates were polyclonally disseminated in our setting. On the basis of RAPD-PCR, 12 main groups could be distinguished in dog isolates and 2 in cat isolates. These findings imply that enterococci are genetically and phenotypically diverse which are consistent with findings of other authors who have reported considerable genetic variability in *Enterococcus* species (Harada *et al*., 2005; Getachew *et al*., 2009).

The prevalence of biofilm production reported previously for commensal isolates has been variable (Ciftci *et al*., 2009; Upadhyaya *et al*., 2011; Tsikrikonis *et al*., 2012). In this study, biofilm production was detected in only nine (34.6%) of 26 dog isolates. These results indicated that there may be more than one factor determining the production of biofilms in enterococci.

In conclusion, results obtained by phenotypic tests revealed a lower percentage of strains that produced haemolysin, gelatinase or AS, compared to genotypic characterization. In some cases, our strains also possessed silence virulence genes and it is now known that environmental signals may play a role in gene expression. Nevertheless, none of the detected biological characters should be considered definitive marker of pathogenicity; they could contribute to the virulence potential of enterococci, but this may be dependent on additional virulence factors present. The results from this study indicated that healthy dogs and cats are a source of antimicrobial resistant enterococci and may act as a reservoir. The results also demonstrated that the RAPD-

PCR patterns among *Enterococcus* strains from humans, dogs and cats were heterogeneous and considerably diverse. The demonstration of diversity of RAPD patterns at the species level will be essential for understanding the molecular ecology of enterococci in the intestine of animals and humans.

Conflict of interest

No conflict of interest to declare.

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