

*Original Scientific Article***ANTIMICROBIAL RESISTANCE OF *ENTEROCOCCUS FAECIUM* ISOLATED FROM THE URINARY SYSTEM OF DOGS**Sukru Kirkan<sup>1</sup>, Ugur Parin<sup>1</sup>, Gamze Balat<sup>2</sup><sup>1</sup>*Department of Microbiology, Faculty of Veterinary Medicine, Aydin Adnan Menderes University, Aydin, Turkey*<sup>2</sup>*Department of Microbiology, Health Sciences Institute, Aydin Adnan Menderes University, Aydin, Turkey*

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**ABSTRACT**

The purpose of the present study was to determine the antimicrobial susceptibility of vancomycin-resistant and *Enterococcus faecium* strains isolated from urine samples of dogs. A total of 22 *Enterococcus* sp. samples were isolated and identified from 100 urine samples collected by cystocentesis from dogs of both sexes. The identification with species specific primers for multiplex PCR revealed that all 22 isolates (100%) belonged to *E. faecium*. Vancomycin resistance was found in 10 (45%) samples of *E. faecium* strains with PCR study by *vanA* and *vanB* primers.

**Key words:** enterococci, *E. faecium*, vancomycin, *vanA*, *vanB*

**INTRODUCTION**

Enterococci, which can cause serious health problems in humans and animals, are opportunistic pathogenic microorganisms. Regarding pet animals, there are reports about infection caused by enterococci which were isolated from certain cases of urinary tract infections, periodontitis, osteomyelitis, gastroenteritis, peritonitis, and endocarditis (1, 2). Also, the presence of *E. faecalis* and *E. faecium* among dogs has been reported previously (3, 4, 5).

Among the enterococci, *Enterococcus faecalis* and *E. faecium* are the most common species isolated from clinical cases, and *E. durans*, *E. gallinarum*, *E. avium*, *E. casseliflavus*, *E. raffinosus*, *E. solitarius* and *E. hirae* are less common (3, 6).

Enterococci have gained resistance against some antibiotics. Antibiotic resistance in enterococci may be natural or acquired. Most enterococci naturally show resistance to antimicrobials such as  $\beta$ -lactams, clindamycin, aminoglycosides and fluoroquinolones. Enterococci are naturally susceptible to ampicillin and vancomycin, but they may develop resistance if exposed to antibiotics excessively. Similarly, enterococci can also develop resistance to macrolides, glycopeptides (vancomycin, teicoplanin), chloramphenicol, aminoglycosides and  $\beta$ -lactams (7).

Another type of acquired resistance that is very important in enterococci is glycopeptide resistance, which is expressed by different phenotypes that can vary from *vanA* to *vanG*. The phenotypic classification is based on whether the bacterium is resistant to vancomycin solely or vancomycin and teicoplanin as double resistance, whether or not the resistance is inducible or structurally transmissible to other bacteria. Among the glycopeptide resistance types mentioned, the best-defined resistance are *vanA*, *vanB*, *vanC* and *vanD* (8).

*Enterococcus* species can be transmitted through contamination of saliva, urine or faeces by direct contact from pets to humans. This transmission plays an important role in the distribution of resistant

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genes among bacterial species (9). The widespread use of antibiotics in pets has been reported to be an important reason for acquiring resistance to enterococcal species. There are different studies on the presence of enterococci in healthy dogs (nasal, rectal, oral) and antibiotic susceptibility (10). In humans, *E. faecalis* and *E. faecium* were identified and antibiotic susceptibilities of the isolates were determined by the disc diffusion method and vancomycin resistance was revealed (11).

The scope of this study was to determine the antimicrobial susceptibilities of vancomycin-resistant *E. faecium* strains isolated from urine specimens of dogs.

## MATERIAL AND METHODS

One hundred urine samples (10 ml) were collected from 72 sick dogs (suspected of having a urinary tract infection) and 28 healthy dogs (with no clinical sign) by means of cystocentesis, which were brought to the to the Adnan Menderes University, Veterinary Faculty Research and Practice Hospital for examination. The samples were immediately taken to the Routine Diagnosis Laboratory of the Department of Microbiology of the Faculty of Veterinary Medicine of the University of Menderes in an insulated box containing ice cubes. Adnan Menderes University Animal Experiments Local Ethics Committee (ADU-HADYK) report dated 14.08.2015 and numbered 64583101/2015/103 did not show any penalty in conducting the research.

### Isolation and identification of Enterococci

The undiluted urine samples were cultured on 5% sheep blood agar and incubated at 37°C for 24 hours under aerobic atmosphere. At the end of this period, Gram staining method and catalase test were applied to the colonies. Catalase-negative colonies were regarded as *Streptococcus* sp. and inoculated into a bile esculin agar (Enterocococel agar) for identification of enterococci. The petri dishes were incubated at 37°C for 24 hours under aerobic atmosphere. After that, black colonies were selected and inoculated into a brain heart infusion agar. *Enterococcus* sp. isolates were tested for oxidase test, PYR test, 6.5% NaCl salt tolerance test and identified by genus level. The identified colonies were inoculated to broth medium for identification and stored at -20 °C for PCR tests.

### DNA isolation

DNA isolations of strains were conducted via Genomic DNA purification kit (Fermentas®) appropriate to procedure. The extracted DNA has been kept in cryo tubes in deep freeze at -20°C.

### Primers

The primers used for the detection of *E. faecium*-*E. faecalis* and the presence of the vancomycine resistance genes are shown in Table 1.

### Positive control

*E. faecalis* ATCC 29212 and *E. faecium* ATCC 19434 strains were used as positive control.

**Table 1.** Oligonucleotide primer pairs, amplicon size and target genes

Primer	Target gene	Primer sequences (5'-3')	Amplicon size (bp)
<i>Enterococcus</i> sp.	<i>tuf</i>	TACTGACAAACCATTTCATGATG	112
		AACTTCGTCACCAACGCGAAC	
<i>E. faecium</i>	<i>ddl</i> <sub><i>E. faecium</i></sub>	F: TAGAGACATTGAATATGCC	550
		R: TCGAATGTGCTACAATC	
<i>E. faecalis</i>	<i>ddl</i> <sub><i>E. faecalis</i></sub>	F: ATCAAGTACAGTTAGTCT	941
		R: ACGATTCAAAGCTAACTG	
Vancomycin resistance	<i>vanA</i>	F: GGGAAAACGACAATTGC	732
		R: GTACAATGCGGCCGTTA	
Vancomycin resistance	<i>vanB</i>	F: ACCTACCCTGTCTTTGTGAA	300
		R: AATGTCTGCTGGAACGATA	

### PCR

Identification of *Enterococcus* sp. was performed by a PCR assay to detect the *tuf* gene, with primer pairs previously described (12). Identification of *E. faecalis* and *E. faecium* was carried out by a PCR assay to detect *ddl<sub>E. faecalis</sub>* and *ddl<sub>E. faecium</sub>*, respectively, with primer pairs previously described (13). Vancomycin resistance genes (*vanA* and *vanC*) were detected by a multiplex PCR assay as described elsewhere (14).

### Detection of the amplification product

The 10 µl amplified products were detected by staining with 0.5 µg/ml ethidium bromide after electrophoresis at 80 Volt for 40 min in 2% agarose gel. The expected base pair size of *Enterococcus* sp., *E. faecalis* and *E. faecium* were 112 bp, 941 bp and 550 bp respectively. For detection of the broad vancomycin resistance genes, 732 bp for *vanA*, 300 bp for *vanB* amplicon sizes were examined.

### Antibiotic susceptibility

The E-Test method was used to determine the antibiotic susceptibility of the isolated *E. faecium*. Tetracycline, tigecycline, clindamycin, ceftriaxone and ampicillin E-test strips supplied by Oxoid® were used for the antibiogram. The MIC values obtained for the antibiotics were evaluated in accordance with the recommendation of CLSI (15).

## RESULTS

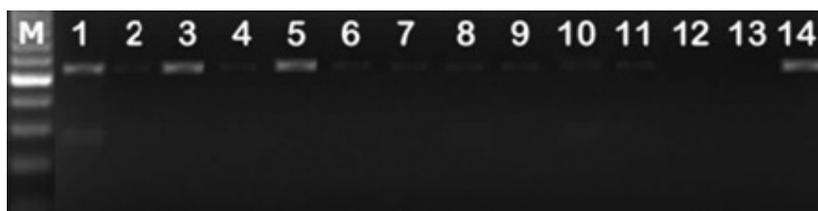
Twenty-two *Enterococcus* sp. isolates were identified by PCR as *E. faecium* (Table 2; Fig. 2). The electrophoresis image of the isolates is shown in Fig. 1.

**Table 2.** Identification rates of *Enterococcus* sp.

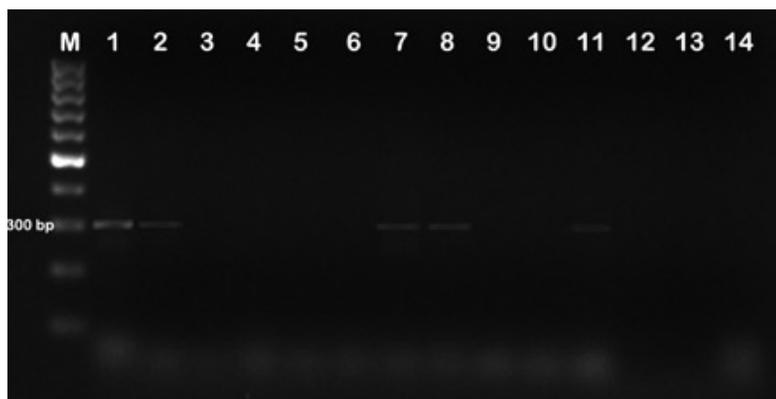
Isolate (n)	Identification number	Identification rate
<i>E. faecalis</i>	-	-
<i>E. faecium</i>	22	100



**Figure 1.** *Enterococcus* sp. electrophoresis gel image M:100 bp DNA ladder, 1-11: *Enterococcus* sp. positive samples, 12: *E. faecalis* ATCC 29212 positive control, 13: *E. faecium* ATCC 19434 positive control, 14: Negative control



**Figure 2.** *E. faecium* electrophoresis gel image M:100 bp DNA ladder, 1-11: *E. faecium* positive samples, 12: *E. faecalis* ATCC 29212 positive control, 13: Negative control, 14: *E. faecium* ATCC 19434 positive control



**Figure 3.** Vancomycin resistance electrophoresis gel image M:100 bp DNA ladder, 1-2-7-8-11: *vanB* positive samples, 3-4-5-6-9-10-12: *vanB* negative samples 12: *E. faecalis* ATCC 29212 positive control, 13: *E. faecium* ATCC 19434 positive control, 14: Negative control

**Table 3.** MIC of *E. faecium* isolates

Antimicrobial agent	MIC Range ( $\mu\text{g/ml}$ )	MIC 50 ( $\mu\text{g/ml}$ )	MIC 90 ( $\mu\text{g/ml}$ )	Resistance (%)
<b>Ampicillin</b>	4-0.12	0.5	1	100
Tetracycline	128-2	16	128	100
<b>Tigecycline</b>	16-0.12	2	4	100
<b>Clindamycin</b>	32-0.06	16	32	100
<b>Ceftriaxone</b>	128-2	128	128	100

Ten (45%) *E. faecium* isolates had vancomycin resistance to the *vanB* gene (Fig. 3). The *Enterococcus* sp. were isolated from clinically sick animals. The other bacterial isolates identified from this study were *Bacillus* sp. (n=17), *Streptococcus* sp. (n=18), *Staphylococcus* sp. (n=13) and *Klebsiella* sp. (n=12).

All of the 22 *E. faecium* isolates were found to be 100% resistant to tigecycline, ampicillin, tetracycline, clindamycin and ceftriaxone. The antimicrobial resistance results of *E. faecium* isolates are shown in Table 3.

## DISCUSSION

Antimicrobial resistance is a noteworthy concern in animal health throughout the world. Indeed, the enterococci are known to be ubiquitous microorganisms found in various habitats of animals. They recently emerged as a significant agent of multiple drug resistance infections (16, 17). In dogs, enterococci are known to be both bacterial flora elements and infections (18).

The present study investigated the urogenital carriage of enterococci among pet dogs, with reference to antimicrobial resistance. The evaluation of urine culture of dogs with UTIs revealed that *E. faecium* was the only identified enterococcal species from dogs in this study. Similarly, *E. faecium* was reported to be most common species isolated from dogs in previous studies carried out in Turkey (19, 20). However in another study, *E. faecalis* has been identified to be predominantly isolated from urinary tract infections of dogs as a causative agent (21). The results of the current study showed greater isolation rates of *E. faecium* in the urine samples of dogs (22%) compared with *E. faecalis* (0%). Such results agree with the findings of Rodrigues et al. (3) but contradict the results of Jackson et al. who found that *E. faecalis* was the predominant species among the examined dogs (22).

Furthermore, in a study conducted with molecular typing methods, a high degree of diversity was observed between similar and related strains isolated from human and animal specimen. It has been reported that the transposon Tn1546, which is found in human enterococcal isolates, is also shown

in the urine-infected enterococcus species of dogs, and can be a proof of gene mutation between human and animal-bearing species resistant to vancomycin (19, 23).

A large proportion of enterococcus strains are naturally resistant to antimicrobial agents used in the treatment of Gram-positive bacterial infections (24). Numerous antibiotics such as penicillins, cephalosporins, quinolones and low levels of aminoglycosides have been shown to exhibit natural resistance, as well as that enterococci can produce antibiotic resistance through new mechanisms and transfer through these resistance plasmids (25). In our study, we found that all strains were resistant to tigecycline, ampicillin, tetracycline, clindamycin and ceftriaxone, and this should be considered when suggesting antimicrobial therapy options for urinary tract infections in dogs.

Although vancomycin has been reported to be the most effective antibiotic against enterococci, the increase in the number of vancomycin-resistant strains has been reported as significant. There are multiple vancomycin resistant phenotypes, including *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, and *vanG*. The most clinically important strains are *vanA* and *vanB* resistant strains. Strains with the *vanA* gene show the highest resistance to vancomycin and teicoplanin, while strains with the *vanB* gene show only resistance to vancomycin (24).

In this study, *vanB* resistance was detected in 10 (45%) *E. faecium* isolates. As a result of the E-test, *E. faecium* isolates were 100% resistant against tigecycline, ampicillin, tetracycline, clindamycin and ceftriaxone. The impact of the results indicates an emergency for pet owners, since the antimicrobial resistant *E. faecium* strains may infect people living with dogs.

## CONCLUSION

Multiple drug resistant *E. faecium* was isolated and identified from dogs with UTIs in this study. The identification of *E. faecium* from dogs with UTIs supports the claim of enterococci being a true uropathogen rather than solely an opportunistic organism. Besides, the gross resistance to multiple antimicrobials strongly indicated that treatment of UTIs should not be initialised before the results of urine culture and antibacterial susceptibility are reported, especially because random applications can result in overgrowth of non-susceptible bacteria. However, when random use of antibiotics is inevitable, reasonable use is necessary to

reduce or exclude the increase of antimicrobial-resistant organisms and to maintain the efficacy of antimicrobials in veterinary medicine. Hence, antimicrobial susceptibility monitoring programmes are essential tools for developing appropriate therapy protocols for urinary tract infections of dogs.

## CONFLICT OF INTEREST STATEMENT

The authors declared that they have no potential conflict of interest with respect to the authorship and/or publication of this article.

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