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# Investigation of Vancomycin Resistance and Some Virulence Factors in *Enterococci* Strains Isolated from Dogs

Özgül Gülaydın[1,a,*], Ismail Hakkı Ekin[2,b], Ziya Ilhan[3,c], Muazzez Yeşilyurt[1,d]

[1]Siirt University, Faculty of Veterinary Medicine, Department of Microbiology, Siirt, Türkiye
[2]Van Yüzüncü Yıl University, Faculty of Veterinary Medicine, Department of Microbiology, Van, Türkiye
[3]Balıkesir University, Faculty of Veterinary Medicine, Department of Microbiology, Balıkesir, Türkiye

[a]ORCID: 0000-0001-8376-2008; [b]ORCID: 0000-0001-5029-8130;
[c]ORCID: 0000-0003-3638-9196; [d]ORCID: 0000-0003-4195-6335

*Corresponding Author
E-mail: ozgul.gulaydin@siirt.edu.tr

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**Abstract**

The aim of this study was to evaluate *in vitro* antimicrobial susceptibility of *Enteroccoccus faecalis* and *Enterococcus faecium* strains isolated from dog, as well as the phenotypic and genotypic characterization of vancomycin resistance and some virulence genes of the isolates. A total of 197 samples were analyzed, including 114 urine, 63 rectal swab and 20 vaginal swab samples. *Enteroccocus* spp. were isolated from 83 (42.1%) of the samples. By PCR test with species-specific primers, 42 (50.6%) isolates were identified as *E. faecalis* (95.2%) and *E. faecium* (4.8%). The highest resistance was found to erythromycin (16.66%) and tetracycline (11.9%) whereas vancomycin resistance was low (4.76%) by disc diffusion method. *GelE* (47.6%) and *asa1* (38.1%) genes associated with the virulence were detected in the isolates, while all isolates were negative for *cylA* gene. As a result, *E. faecalis* from canine samples was isolated at higher rate than that of *E. faecium*, and the resistance of the isolates to β-lactam antibiotics (ampicillin, penicillin, imipenem, and vancomycin) as well as multiple antibiotic resistance was low. The *gelE* gene was detected in more isolates than the *asa1* gene, and all isolates were negative for *cylA* gene.

**Keywords:** Antimicrobial susceptibility, dog, *E. faecalis*, *E. faecium*, vancomycin, virulence gene.

## INTRODUCTION

Enterococci are gram-positive, cocci shaped and catalase negative bacterial agents. Generally, these microorganisms are opportunistic pathogens and can be found in the natural flora of the intestinal systems of both humans and animals. Enterococci cause mastitis in cattle, urinary tract and ear infections in dogs, and endocarditis in sheep (Quinn et al. 2011). It is among the important members of nosocomial infections, especially in human medicine (Murray, 1990; Brinkwirth et al. 2021). Although there are many species in the *Enterococcus* genus, it has been reported that the strains isolated from dogs are mostly identified as *Enterococcus* (*E.*) *faecalis* and *E. faecium* (Jackson et al. 2009; Iseppi et al. 2015; Ben Said et al. 2017; Kubasova et al. 2017; Feßler et al. 2022).

Convantional bacteriological methods are used for the identification of *Enterococcus* spp. in terms of species level. But, the different biotypes of the field isolates reduce the reliability of these methods. Also, bacteriological conventional methods are expensive and time consuming. Molecular methods are more reliable for the identification of the bacteria. Variations in sequences of *sodA* genes are found to be greater between species and less within species (Jackson et al. 2004). Thus, isolates were identified by amplifying *sodA* gene by PCR, in this study.

Since enterococci have intrinsic resistance to many antimicrobial agents, effective antimicrobial therapy is critical in the treatment of infections caused by these agents. In general, aminoglycoside, β-lactam group antibiotics and vancomycin are recommended to be preferred for the treatment options (Chow, 2000; Li et al. 2015). Although vancomycin resistant enterococci have been isolated at low rates in dogs (Bondi et al. 2015; Torres et al. 2018; Van den Bunt et al. 2018), many researchers point out that dogs are carriers of multidrug resistant strains (Jackson et al. 2009; Iseppi et al. 2015; Ben Said et al. 2017; Kubašová et al. 2017; Stepien-Pysniak et al. 2021; Troscianczyk et al. 2021). Furthermore, Aarestrup et al. (2001) emphasized that vancomycin resistant *E. faecium* strains isolated from animals can be threaten public health.

While *vanA* and *vanB* genes are responsible for acquired vancomycin resistance in *E. faecalis* and *E. faecium* isolates, it has been reported that *vanC* genes cause intrinsic vancomycin resistance in other species (Silva et al. 2011).

Many virulence-related factors such as gelatinase (*gelE*), aggregation factor (*asa1*) and cytolysis (*cyl*) play role in the pathogenesis of infections caused by enterococcus isolates (Vankerckhoven et al. 2004; Lopes et al. 2006). Gelatinase encoded by *gelE*, is an extracellular zinc-endopeptidase/protease and plays an important role in hydrolyzing gelatin, collagen, casein, hemoglobin, and other peptides (Kiruthiga et al. 2020). Aggregation subsistence is encoded by the *asa1* gene and facilitates the conjugative transfer of extra-chromosomal structures

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carrying the sex pheromone genes between isolates. In addition, it increases virulence by providing adhesion to the urogenital and gastrointestinal system cells (Comerlato et al. 2013). In *Enterococcus* spp., hemolyzing of bovine and horse erythrocytes are regulated by *cylA* and reported to be contributed to virulence. The cytolysin operon are consisted of two fragment as lysin (L) encoded by *cylL* genes and an activator (A) encoded by *cylA*. Also, the cytolysin has bactericidal activity against other Gram positive bacteria (Shankar et al. 2004).

In this study, investigating of *in vitro* antimicrobial susceptibility of *E. faecalis* and *E. faecium* strains isolated from urine, rectal and vaginal swab samples from dogs to various antimicrobial agents, phenotypic and genotypic characterization of vancomycin resistance and some virulence genes of the isolates were aimed.

## MATERIALS AND METHODS

### *Material*

In this study, a total of 197 samples collected from dogs housed in Van Metropolitan Municipality Animal Care and Rehabilitation Center were analyzed. The samples consisted of 114 (57.8%) urine, 63 (31.9%) rectal swabs, and 20 (1.1%) vaginal swabs.

This study has ethical approvement by Van Yuzuncu Yil University University Animal Researches Ethic Committee with the number of 2022/01-10.

### *Method*

#### *Isolation of Enterococcus* spp.

After the urine samples were centrifuged according to the method reported by Papini et al. (2006), they were inoculated on Columbia blood agar (Oxoid, CM 03331, England), containing 5-7% defibrinated sheep blood, and Slanetz Bartley Medium (Oxoid, CM0377, England). Rectal and vaginal swab samples were directly inoculated. The agar plates were incubated at 37°C under aerobic conditions for 24-48 hours. After examining the colonies according to macroscopic and microscopic morphology, the isolates were evaluated by Gram staining, catalase reaction, growth in 6.5% NaCl and hydrolyzing esculin (Quinn et al. 2011).

#### *Identification by PCR*

Identification of the isolates in terms of *E. faecalis* and *E. faecium* was performed by PCR. Genomic DNA used in PCR was obtained by boiling method described by Bang et al. (2017). Purity and amount of genomic DNA were determined spectrophotometrically (Nano-300, Allsheng). For the identification of *E. faecalis* and *E. faecium* isolates by PCR, species-specific primers reported by Jackson et al. (2004) were used (Table 1). To prepare of PCR mix, 1.5 µl of forward and reverse primer and 5 µl of DNA were added to 10 µl of mastermix (A.B.TTM 2X PCR Mastermix), and the total volume was made up to 25 µl with PCR water. The amplicons obtained from PCR were electrophoresed on agarose gel and analyzed in a UV transilluminator. *E. faecalis* ATCC 29212 and *E. faecium* ATCC 6057 strains were used as positive control in PCR, and PCR water was used as negative control.

#### *Determination of Antimicrobial Susceptibility*

The susceptibility of *E. faecalis* and *E. faecium* isolates to ampicillin (10 µg, Oxoid), penicillin (10 IU, Oxoid), erythromycin (15 µg, Oxoid), chloramphenicol (30 µg, Oxoid), tetracycline (30 µg, Oxoid), imipenem (10 µg, Oxoid), ciprofloxacin (5 µg, Oxoid) and vancomycin (5 µg, Oxoid) was determined by disk diffusion test. The criteria of Clinical Laboratory Standards Institute (CLSI, 2018) for ampicillin, penicillin, erythromycin, chloramphenicol and tetracycline, and the criteria of European Commitee on Antimicrobial Susceptibility Testing (EUCAST, 2019) for imipenem, ciprofloxacin and vancomycin were taken into account in the evaluation of the test. Accordingly, the isolates were evaluated as susceptible (S), intermediate (I) and resistant (R).

#### *Determination of Vancomycin Resistance Genes*

The presence of *vanA* and *vanB* genes were investigated by PCR in isolates that were resistant to vancomycin (Table 1). PCR mix was prepared as same as the protocol described in the identification of isolates by PCR.

#### *Determination of Virulence Genes*

The presence of some virulence-related genes such as *asa1, gelE* and *cylA* in enterococci isolates was determined by PCR. Gene specific primers used in PCR were given in Table 1. PCR mix was prepared as same as the protocol described in the identification of isolates by PCR.

**Table 1.** Primer sequences used in PCR.

| Genes | Oligonucleotides (5'-3') | bp | PCR Cycles (denauration / anneling / elongation) | References |
|---|---|---|---|---|
| *Sod A* *E. faecalis* | **F:** ACTTATGTGACTAACTTAACC **R:** TAATGGTGAATCTTGGTTTGG | 360 | 94°C 1 min / 54°C 1 min / 72°C 1 min 35 cycles | Jackson et al. 2004 |
| *Sod A* *E. faecium* | **F:** GAAAAAACAATAGAAGAATTAT **R:** TGCTTTTTTGAATTCTTCTTTA | 215 | 94°C 1 min / 50°C 1 min / 72°C 1 min 35 cycles | Jackson et al. 2004 |
| **Vankomisin direnç genleri** | | | | |
| *vanA* | **F:** ATGAATAGAATAAAAGTTGC **R:** TCACCCCTTTAACGCTAATA | 1032 | 94°C 1 min / 50°C 1 min / 72°C 1 min 35 cycles | Saha et al. 2008 |
| *vanB* | **F:** GTGACAAACCGGAGGCGAGGA **R:** CCGCCATCCTCCTGCAAAAAA | 433 | 94°C 1 mn / 63°C 1 min / 72°C 1 min 35 cycles | Handwerger et al. 1992 |
| **Virülens ile ilişkili genler** | | | | |
| *asa1* | **F:** GCACGCTATTACGAACTATGA **R:** TAAGAAAGAACATCACCACGA | 375 | 94°C 1 min / 50°C 1 min / 72°C 1 min 35 cycles | Vankerckhoven et al. 2004 |
| *gelE* | **F:** ACCCCGTATCATTGGTTT **R:** ACGCATTGCTTTTCCATC | 419 | 94°C 1 min / 52°C 1 min / 72°C 1 min 35 cycles | Lopes et al. 2006 |
| *cylA* | **F:** ACTCGGGGATTGATAGGC **R:** GCTGCTAAAGCTGCGCTT | 688 | 94°C 1 min / 57°C 1 min / 72°C 1 min 35 cycles | Vankerckhoven et al. 2004 |

## RESULTS

### *Isolation*

Within the scope of the study, 83 (42.1%) of 197 samples were found to be suspected as *Enterococcus* spp. by culture method. The suspected isolates were obtained from 42 (66.66%) of 63 rectal swab samples, 35 (30.70%) of 114 urine samples and 6 (30%) of 20 vaginal swab samples.

### *Identification by PCR*

By PCR using species specific primers, 42 (50.6%) of 83 *Enterococcus* spp. suspected isolates were identified at the species level but, it was observed that 41 (49.4%) isolates did not amplify with these primers. Of the isolates, 40 (95.2%) were identified as *E. faecalis* and 2 (4.8%) as *E. faecium*. Of *E. faecalis* strains, 22 (55%) were detected in urine, 17 (42.5%) in rectal swab and 1 (2.5%) in vaginal swab samples. *E. faecium* isolates were identified from 2 rectal swab samples (Table 2, Figure 1).

**Table 2.** Bacteriological culture and PCR results of urine, rectal and vaginal swab samples.

| Sample (n) | Culture (suspected as *Enterococcus* spp.) | | PCR | |
|---|---|---|---|---|
| | + (%) | - (%) | *E. faecalis* (%) | *E. faecium* (%) |
| Urine (114) | 35 (30.7) | 79 (69.3) | 22 (52.3) | - |
| Rectal swab (63) | 42 (66.6) | 21 (33.4) | 17 (42.5) | 2 (4.7) |
| Vaginal swab (20) | 6 (30) | 14 (70) | 1 (2.5) | - |
| **Total** | **83 (42.1)** | **114 (57.9)** | **40 (95.2)** | **2 (4.8)** |

+: The number of samples obtained from *Enterococcus* spp. suspected isolate by bacteriological methods.
-: The number of samples from which *Enterococcus* spp. suspected isolates were not obtained by bacteriological methods.

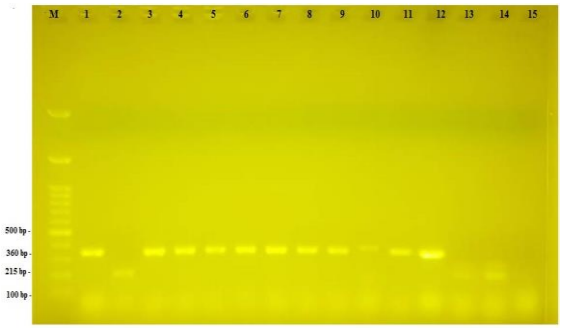


**Figure 1.** Image of amplicons obtained from *E. faecalis* and *E. faecium* isolates on agarose gel. (M: 100 bp DNA Ladder, 1: *E. faecalis* ATCC 29212, 2: *E. faecium* ATCC 6057, 3-12: *E. faecalis* isolates (360 bp), 13-14: *E. faecium* isolates (215 bp), 15: Negative Control).

### *Determination of Antimicrobial Susceptibility*

Of the 42 *Enterococcus* spp. isolates examined by disc diffusion in the study, 1 (2.38%), 7 (16.66%), 1 (2.38%), 5 (11.90%), 1 (2.38%), 1 (2.38%) and 2 (4.76%) were found to be resistant to penicillin, erythromycin, chloramphenicol, tetracycline, imipenem, ciprofloxacin and vancomycin, respectively (Figure 2). While it was determined that the resistance profile against the antimicrobial agents was not high in isolates, it was determined that 1 (2.38%) isolate had multi-drug resistance against penicillin, erythromycin, tetracycline and vancomycin.

### *Determination of Vancomycin Resistance Genes*

While *vanA* gene was detected in 1 (50%) of the 2 isolates determined to be resistant to vancomycin in the study, *vanB* gene was not detected in both isolates.

### *Determination of Virulence Genes*

The *asa1* gene was found positive in 16 (38.09%) and *gelE* gene was found in 20 (47.61%) of 42 enterococci that were identified by PCR at the species level. The *cylA* gene could be detected in none of the isolates.

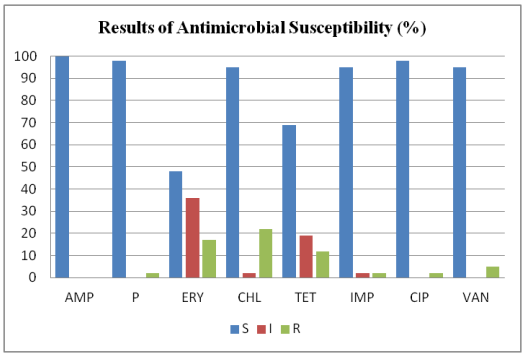


**Figure 2.** Distribution of antimicrobial susceptibility results of *Enterococcus* spp. isolates. **AMP:** Ampicillin, **P:** Penicillin, **ERY:** Erythromycin, **CHL:** Chloramphenicol, **TET:** Tetracycline, **IMP:** Imipenem, **CIP:** Ciprofloxacin, **VAN:** Vancomycin **S:** Susceptible, **I:** Intermediate, **R:** Resistance

## DISCUSSION AND CONCLUSION

Human beings come in close contact with cats and dogs. The fact that some business sector have switched to a home office work program because of the COVID-19 pandemi and this situation will probably continue in the future, will increase the direct contact of cats and dogs with people as with other pets.

Enterococci are opportunistic pathogens found in the normal flora of the gastrointestinal tract of humans and animals, and are isolated from different infections of various animal species, especially cats and dogs (Aarestrup et al. 2000; Wong et al. 2015; Torres et al. 2018; Van den Bunt et al. 2018). On the other hand, these factors also cause community-acquired nosocomial infections and create an important health problem with increasing antibiotic resistance (Banerjee and Anupurba, 2016; Gawryszewska et al. 2016). Acquired antibiotic

resistance in enterococcal isolates and the ability to transfer this phenomen to other microorganisms constitute an important problem in the treatment of infections caused by agents (Arias and Murray, 2012).

In this study, *in vitro* antimicrobial susceptibility to various antimicrobial agents and characterization of vancomycin resistance by phenotypic and genotypic methods and analysis of some virulence genes in *E. faecalis* and *E. faecium* strains isolated from urine, rectal and vaginal swab taken from dogs were performed.

There are various studies on the isolation of *Enterococcus* spp. from rectal swab and/or urine samples of dogs (Damborg et al. 2008; Türkyılmaz et al. 2010; Bang et al. 2017; Kubasova et al. 2017; Ben Said et al. 2017; Pillay et al. 2018; Troscianczyk et al. 2021). In these studies it has been reported that the majority of the strains were identified as *E. faecalis* (Damborg et al. 2008; Bang et al. 2017; Pillay et al. 2018; Troscianczyk et al. 2021; Stepien-Pysniak et al. 2021). In this study, *E. faecalis* was isolated from 20.30% of 197 samples taken from dogs by bacteriological culture and PCR analysis, and this rate was found to be significantly higher than the isolation rate of *E. faecium* (1.01%) ($p<0.05$). In the study, it was found that the isolation rate of *E. faecalis* was consistent with the data reported by Türkyılmaz et al. (2010), Said et al. (2017), and Pillay et al. (2018) but, lower than revealed by Damborg et al. (2008), Bang et al. (2017), and Troscianczyk et al. (2021). Although the isolation rate of *E. faecium* was reported to be high in various studies (Türkyılmaz et al. 2010; Kubasova et al. 2017) it is noteworthy that the number of positive *E. faecium* samples in the present study (1.01%) is considerably lower than reported by other studies. This may be related to the age of the animals, their nutritional habits and the methods applied.

Unconscious use of antibiotics is an important factor that increases the development of resistance in bacterial agents (Sweileh, 2021). In this context, the importance of antibiotic resistance developing in bacterial agents in the natural flora of pet animals is frequently emphasized (Jackson et al. 2009). Generally, the use of antimicrobials that inhibit cell wall synthesis, such as ampicillin and vancomycin, is recommended for the treatment of infections caused by enterococcal strains (Vu and Carvalho, 2011).

It has been reported that β-lactam (such as ampicillin, penicillin, and imipenem) resistance is not high in *Enterococcus* spp. strains isolated from dogs (Türkyılmaz et al. 2010; Gülhan et al. 2015; Bang et al. 2017; Ben Said et al. 2017; Troscianczyk et al. 2021). However, some researchers reported that more than 30% of the strains were resistant to ampicillin or penicillin (Damborg et al. 2008; Kubasova et al. 2017; Stepien-Pysniak et al. 2021). In the present study, all *E. faecalis* and *E. faecium* strains were found to be susceptible to ampicillin, while only 1 (2.38%) of the isolates were resistant to penicillin and imipenem.

Vancomycin is used for methicillin-resistant *Staphylococcus* spp. infections as well as for *Enterococcus* spp. infections. On the other hand, it is stated that this antibiotic is rarely used due to the lack of sufficient information about its pharmacodynamics and pharmacokinetics in pet animals (Wijesekara et al. 2017). In this study, 4.76% of *Enterococcus* spp. strains isolated from dogs were resistant to vancomycin. It was seen that the data obtained were compatible with other studies reported a limited number of resistant strains (Damborg et al. 2008; Türkyılmaz et al. 2010; Gülhan et al. 2015; Ben Said et al. 2017; Stepien-Pysniak et al. 2021; Troscianczyk et al. 2021). While Stepien-Pysniak et al. (2021) reported that they did not detect *vanA* gene by PCR in vancomycin resistant isolates, in the present study, *vanA* gene was found to be positive in 1 of the 2 vancomycin resistant isolates. Another resistance mechanism such as *vanD*, *vanE*, *vanG*, and *vanL* (Silva et. 2011) may play role in the other vancomycin resistant isolate.

In this study investigating the antimicrobial susceptibility of *Enterococcus* spp. strains isolated from urine, rectal and vaginal swab samples of dogs, erythromycin resistance was determined to be the highest in the isolates (16.66%). Tetracycline resistance rate was determined as 11.90%. In the previous studies consistent with the presented study, erythromycin and tetracycline resistance was found to be high. However, it is noteworthy that the resistance rate found in other studies was higher (>40%) than in this study (Damborg et al. 2008; Türkyılmaz et al. 2010; Bang et al. 2017; Kubasova et al. 2017; Ben Said et al. 2017; Stepien-Pysniak et al. 2021; Troscianczyk et al. 2021). This may be related to whether the animals have antibiotic therapy before, or to the fact that the methods applied are not the same, or to the genetic differences of the strains.

As reported in studies (Damborg et al. 2008; Bang et al. 2017; Kubasova et al. 2017; Ben Said et al. 2017; Stepien-Pysniak et al. 2021; Troscianczyk et al. 2021) conducted on the detection of ciprofloxacin resistance in *Enteroccocus* spp. strains isolated from rectal swab samples of dogs, ciprofloxacin resistance was found to be low in this study. Considering the studies on this subject (Damborg et al. 2008; Türkyılmaz et al. 2010; Kubasova et al. 2017; Said et al. 2017), it was determined that a similar data was valid for chloramphenicol resistance.

It has seen that different data have been reported in studies conducted on the determination of virulence factors that play a role in the pathogenesis of enterococcal infections. It had been reported that the *gelE* gene responsible for gelatinase production, was found in 23-52% of the isolates and the aggregation factor (*asa1*) was found in more than 50% (Gülhan et al. 2015; Kubasova et al. 2017; Said et al. 2017; Pillay et al. 2018; Stepien-Pysniak et al. 2021; Troscianczyk et al. 2021). Similarly, in the present study, *gelE* gene was detected in 47.61% of the isolates, while the *asa1* gene was detected at a lower rate (38.09%). These result showed that dogs could be reservoir of potentially pathogenic *Enterococcus* spp. which harboured virulence factors.

The cytolysin gene (*cylA*) had been reported to be detected as low (5%) by some researchers (Kubasova et al. 2017) but high (55%) by some ones (Troscianczyk et al. 2021). Haemolysin that encoded by *cylA* was associated with the endocarditis cases (Cox et al. 2005). Also, Olsen et al. (2012) reported that *cylA* gene was found to be higher rate in strains isolated from disease cases than commensal strains. In this study, *cylA* gene was not found in the examined isolates. Isolating strains from clinically healthy dogs might cause this situation in this study.

As a result, in this study, it was determined that *E. feacalis* was isolated at a higher rate than *E. faecium* from canine samples and the susceptibility of the isolates to β-lactam group antibiotics (such as ampicillin, penicillin, imipenem and vancomycin) was high and the rate of multiple antibiotic resistance was low. However, it was determined that the resistance developed against tetracycline and erythromycin in isolates should be paid

attention. In addition to being found in the natural flora, it was thought that antimicrobial resistance in strains isolated from disease cases should be investigated in future studies.

**Conflict of Interest**
The authors declare that they have no competing interests.

**Authorship contributions**
Concept: Ö.G., İ.H.E., Design: Ö.G., İ.H.E., Data Collection or Processing: Ö.G., M.Y., Analysis or Interpretation: Ö.G., İ.H.E., Z.İ., Literature Search: Ö.G., M.Y., Writing: Ö.G., Z.İ.

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