Case Report

Enterococcus faecium septicemia in a dog: Clinical features and molecular screening of virulence genes of *Enterococcus*

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Abstract

A one-year-old Golden Retriever male dog manifested multi-systemic inflammation with a marked increment of plasma D-dimer. *Enterococcus* spp. was identified from hemoculture and it showed multidrug resistance (MDR), including amikacin, cephazolin, ceftriaxone, clindamycin, imipenem, penicillin G and sulfa-trimethoprim. Pure *Enterococcus* colonies were identified for species-specific DNA by PCR amplification and sequencing. It was 100% identical with the *Enterococcus faecium* strains in the National Center for Biotechnology Information (NCBI) database. Molecular screening of virulence genes of the isolated *E. faecium* strain exhibited four of nine genes including gelatinase (*gel*E), cell wall adhesion (*efaAfm*), second collagen adhesin (*scm*) and adhesin of collagen of *E. faecium* (*acm*). Although *Ehrlichia canis* and *Babesia canis vogeli* were also detected, the MDR *E. faecium* carrying virulence genes was important for aggravating the disease severity. The present study highlights clinical features and virulence genes associated with *E. faecium* septicemia in a dog.

Keywords: *Enterococcus faecium*, septicemia, virulence genes, canine

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Introduction

Enterococcus faecium is a Gram-positive facultative anaerobic bacterium presenting in gastrointestinal tract of dogs, cats, other animals, as well as humans. Currently, it has emerged as a crucial pathogen because of a relative increased rate of multi-drug resistance and production of virulence genes (Iseppi *et al*., 2015; Bang *et al*., 2017; Bello Gonzalez *et al*., 2017; Bertelloni *et al*., 2017; Kubasova *et al*., 2017; Pillay *et al*., 2018; Stępień-Pyśniak *et al*., 2021).

E. faecium isolates from fecal samples of healthy dogs represented multi-drug resistance, such as ampicillin, ciprofloxacin, erythromycin, rifampicin and tetracycline, at a high incidence (Kubasova *et al*., 2017). In other study in fecal and urine samples of healthy dogs and dogs with urinary tract infection, *E. faecium* resisted aminoglycosides, fluoroquinolones, oxacillin and quinupristin-dalfopristin (Stępień-Pyśniak *et al*., 2021). In addition, *E. faecium* virulence genes were detected in the isolates of fecal and urine samples of healthy dogs and dogs with urinary tract infection, respectively (Pillay *et al*., 2018; Stępień-Pyśniak *et al*., 2021).

Numerous putative virulence genes of *E. faecium* were extensively studied in human and animal sources, *for example*, enterococcus surface protein (*esp*), adhesin of collagen (*acm*), second collagen adhesin (*scm*), serine-glutamate-repeat-containing-protein A (*SgrA*), gene encoding glycoside hydrolase (*hyl*), aggregation substance (agg or *asa1*), gelatinase gene (*gelE*), cytolysin genes (*cyl*) and element IS (IS*16*) (Ferguson *et al*., 2016; Kubasova *et al*., 2017; Freitas *et al*., 2018; Kiruthiga *et al*., 2020; Revtovich *et al*., 2021; Stępień-Pyśniak *et al*., 2021). With the virulence potential of *E. faecium* using the number or score of the present virulence genes have been investigated (Strateva *et al*., 2016). Furthermore, studies have suggested that some virulence genes may impact the risk of *E. faecium* strains and they have investigated the different adaptive features of virulence genes between non-invasive and invasive strains and, later, have been classified as clinical (clade A1), commensal strains (clade B) and animal sources (clade A2) (Strateva *et al*., 2016; Freitas *et al*., 2018; Revtovich *et al*., 2021).

Regarding studies of pathogenic *E. faecium* strains on antimicrobial resistance accompanied with virulence genes in human and animal sources they have been extensively distributed, whereas to date, there is a lack of information at this point on dogs with pathogenic *E. faecium* causing septicemia. This case is the first report illustrating clinical features and virulence potential of *E. faecium* strain causing septicemia, representing a multi-drug resistance and virulence gene production, in a dog.

Case description

A one-year-old Golden Retriever male dog, weighing 29.3 kg, was presented at the Veterinary Teaching Hospital, Khon Kaen University with anorexia, weakness, lateral recumbency and red eyes for three days. The dog received complete vaccinations. Physical examination revealed fever (104^{o}F), 7% dehydration, subconjunctival hemorrhage, with no obvious congestion of bulbar vessels, tongue necrosis, scrotal edema and hemorrhage and limb edema. Normal heart sound and increased lung sound were noted. On the second day of hospitalization, regurgitation, cough and a progression of severe chemosis with serosanguineous ocular discharge, and edema of both upper and lower eyelids were noticed.

Diagnostic workup

Abnormalities of blood tests were mild anemia (PCV 36%, reference range 37-55%), mild leukocytopenia (5.4x10^{3} cell/µl, reference range 6-15x10^{3} cell/µl), mild thrombocytopenia (107,000 cells/µl, reference range 150,000-500,000 cells/µl), hypoalbuminemia (serum albumin 2.1 g%, reference range 2.3-3.8 g%) and mild hypokalemia (3.48 mmol/l, reference range 4.1-5.3 mmol/l). Two out of four systemic inflammatory response syndrome (SIRS) criteria, consisting of fever and leukocytopenia (total white blood cell < 6x10^{3} cell/µl), were noted (Montealegre and Lyons, 2021). A marked increase of plasma D-dimer was found (5,462 ng/ml, reference range 80-390 ng/ml). Quantitative canine pancreatic lipase immunoreactivity (Spec cPL®, IDEXX Laboratories, Westbrook, ME, USA) was within the reference range (93 ng/ml, reference range 0-200 ng/ml). Serum creatinine and alanine aminotransferase (ALT) were within the reference ranges. Venous blood gas analysis showed mild metabolic acidosis. Urinalysis revealed proteinuria with inflammatory sediment, consistent with cystitis, hematuria, hemoglobinuria and bilirubinuria. Radiographic findings showed bronchial patterns of lung infiltration.

Enterococcus spp. was identified from hemoculture. The antimicrobial susceptibility pattern of *Enterococcus* spp. was examined using the disc diffusion method; it was susceptible to doxycycline and amoxy-clavulanic acid. This isolated *Enterococcus* strain displayed multi-drug resistance (MDR) including amikacin, cefazolin, ceftriaxone, clindamycin, imipenem, penicillin G and sulfa-trimethoprim.

Pure colonies of the bacterium were confirmed for *Enterococcus* spp. with further identification of the species-specific DNA by PCR amplification and sequencing. DNA isolation was performed by heat lysis. Ent1 and Ent2 (Ke *et al*., 1999), EM1A and EM1B primers (Cheng *et al*., 1997) were used for *Enterococcus* spp. and *E. faecium* amplification respectively. PCRs were carried out in a total volume of 25 µl, including 12.5 µl of the 2X master mixture (Dreamtaq™ Green PCR master mixture (2X), Thermo Scientific Inc., Waltham, MA, USA) containing Dream Tag DNA polymerase, 2X Dream Tag buffer, 0.4 mM each of dATP, dCTP, dGTP and dTTP, and 4 mM of $MgCl_2$, then adding 0.5 µl of each primer (5 pmol each), and 2.5 µl of DNA template. DNase-free water was added to bring the reaction volume to 25 µl. PCRs were conducted using a Thermal cycler (T100 Thermal cycle, Bio-Rad, Hercules, CA, USA) under the following condition: initial denaturation at 95^{o}C for 3 mins, followed by 40 cycles of denaturation at 95^{o}C for 30 secs, annealing at 55^{o}C for 30 secs, extension of 72^{o}C for 1 min and the final extension step at 72^{o}C for 5 mins. The negative controls of each reaction were also made.

PCR products generated were 112 and 658 DNA bp products for *Enterococcus* spp. and *E. faecium* respectively in 1.5% agarose gel electrophoresis (Fig 1). The sequences were analyzed by Illumina Miseq using Barcode Taq sequencing (Illumina, Celemics, Seoul, South Korea). All gene sequences were compared with the known sequence in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST). It was 100% identical to *E. faecium* strains (*e.g.*, PR05720-3, 18-465).

Molecular screening of nine genes encoding virulence factors of *Enterococcus* spp. in the present study included production (*cylM*), transportation (*cylB*) and activation (*cylA*) of cytolysin, gelatinase (*gelE*), extracellular surface protein (*esp*), cell wall adhesion (*efaAfm*), second collagen adhesin of *E. faecium* (*scm*), cell wall anchored protein (*sgrA*) and adhesin of collagen of *E. faecium* (*acm*). Primers for *cylM*, *cylB*, *cylA*, *gelE*, *esp* and *efaAfm* were described by Eaton and Gasson (2001), while *scm*, *sgrA* and *acm* were quoted from Soheili *et al.* (2014). PCRs of each gene were done twice. Sequencing was performed and analyzed. All protocols were described as mentioned above. The oligonucleotides, product length and annealing temperature (Tm) of each gene are shown in Table 1.

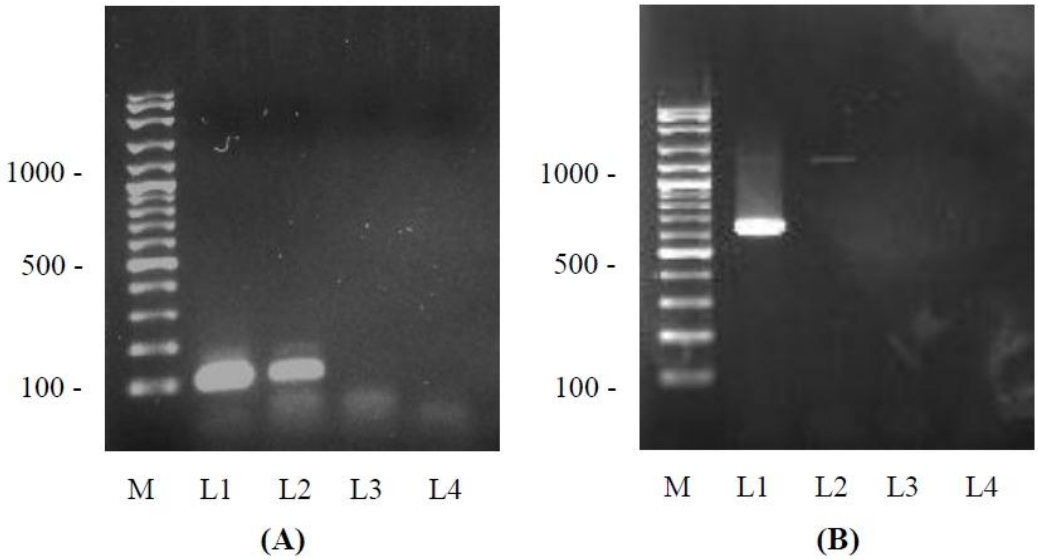

Figure 1 **(A)** Agarose gel electrophoresis of PCR amplification of *Enterococcus* spp. Lane M, 100-bp DNA ladder; L1, pure colonies of *Enterococcus* spp. isolated from blood; L2, eye swab sample; L3, urine sample; L4, negative control. PCR generated 112 DNA bp product for *Enterococcus* spp. Blood and eye swab samples were positive for *Enterococcus* spp. **(B)** Agarose gel electrophoresis of PCR amplification of *E. faecium*. Lane M, 100-bp DNA ladder; L1, pure colonies of *Enterococcus* spp. isolated from blood; L2, eye swab sample; L3, urine sample; L4, negative control. PCR generated 658 DNA bp product for *E. faecium*. The blood sample was positive for *E. faecium*.

Table 1 The oligonucleotides, product length and annealing temperature (Tm) of each gene

Target	Primer	Sequence (5′- 3′)	Product length (bp)	Tm (°C)	Reference
Enterococcus spp.	Ent1	TACTGACAAACCATTCATGATG	112	55	(Ke *et al.*, 1999)
	Ent2	AACTTCGTCACCAACGCGAAC			
E. faecium	EM1A	TTGAGGCAGACCAGATTGACG	658	55	(Cheng *et al.*, 1997)
	EM1B	TATGACAGCGACTCCGATTCC			
cylM	TE13	CTGATGGAAAGAAGATAGTAT	742	40	(Eaton and Gasson, 2001)
	TE14	TGAGTTGGTCTGATTACATTT			
cylB	TE15	ATTCCTACCTATGTTCTGTTA	843	40	(Eaton and Gasson, 2001)
	TE16	AATAAACTCTTCTTTTCCAAC			
cylA	TE17	TGGATGATAGTGATAGGAAGT	517	40	(Eaton and Gasson, 2001)
	TE18	TCTACAGTAAATCTTTCGTCA			
gelE	TE9	ACCCCGTATCATTGGTTT	419	40	(Eaton and Gasson, 2001)
	TE10	ACGCATTGCTTTTCCATC			
esp	TE34	TTGCTAATGCTAGTCCACGACC	933	40	(Eaton and Gasson, 2001)
	TE36	GCGTCAACACTTGCATTGCCGAA			
efaAfm	TE37	AACAGATCCGCATGAATA	735	40	(Eaton and Gasson, 2001)
	TE38	CATTTCATCATCTGATAGTA			
scm (*fms10*)	scm_F	GTTTACTAGTCCTAGTTGC	1,015	40	(Soheili *et al.*, 2014)
	scm_R	TCTGTACTGTCGCTTGTGTC			
sgrA	sgrA_F	CTGATCGGATTGTTTATGA	150	40	(Soheili *et al.*, 2014)
	sgrA_R	AATAAACTTCCCCAATAACTT			
acm (*fms8*)	fms8_F	AGACGAGCAGATGAACAGCC	765	40	(Soheili *et al.*, 2014)
	fms8_R	CCCGTCAATCGTCGTACTGT			

Enterococcus DNA identification from eye swab and urine samples were additionally performed by PCR as previously described. Sequencing analysis of *Enterococcus* DNA detected from ocular swab was 98.86% similar to *Enterococcus villorum* strain ATCC 700913 using BLAST at NCBI database. A urine sample was negative both by bacterial culture and *Enterococcus* DNA PCR amplification. This data manifested that there was no relation between the bloodstream and the eyes, as well as the urinary tract system.

In addition, whole blood was further diagnosed for *Ehrlichia canis*, *Babesia canis* vogeli, *Anaplasma platys*, and *Leptospira* spp. by DNA amplification. The primers of these DNA pathogens have been previously described (Wen *et al.*, 1997; Martin *et al.*, 2006; Victoria *et al.*, 2008; Harrus *et al.*, 2011). *E. canis* and *B. canis vogeli* DNA were detected, while *A. platys* was negative. Blood and urine samples were negative for *Leptospira* spp. (data not shown).

Treatment plan

Intravenous fluid therapy, tolfenamic acid and omeprazole were given for the initial treatment. On the second day of hospitalization, 200 ml of platelet-rich plasma (PRP) was administered intravascularly. Serosanguineous ocular discharge and eyelid edema improved after 24-48 hours of PRP treatment. In the first week of treatment, doxycycline was given. The topical antibiotic drug was given every 4-6 hours in both eyes. Two days after PRP administration, blood transfusion was administered because of progressive anemia (PCV 24%), hypoproteinemia (total protein 4.7 g%, reference range 5.4-7.7 g%), and hypoalbuminemia (serum albumin 1.9 g%). Regurgitation and cough disappeared on the fourth day of hospitalization. After doxycycline treatment for six days, because of a positive *Enterococcus* hemoculture when the blood collection according to the aseptic technique was performed, the antimicrobial drug was changed to amoxy-clavulanic acid. All clinical signs improved, and the dog was discharged from hospital on the ninth day. Two weeks later, hemoculture was re-evaluated and it was negative for *Enterococcus* spp. Subsequently, *E. canis* treatment with doxycycline for 28 days was given. Diffuse redness of the conjunctiva was gradually eliminated and cleared up by the third week.

Discussion

In dogs, endocarditis, wound and urinary tract infections caused by enterococcus have been reported (Arias *et al.*, 2017; Wood *et al.*, 2020; Stępień-Pyśniak *et al.*, 2021; Reagan *et al.*, 2022), while the literature concerning dogs with enterococcal septicemia has rarely been distributed.

The causes and risk factors of enterococcal infections in dogs are unclear. According to human literature, endogenous translocation of enterococci from the gut to other organs may be involved with infections including bacteremia and significant risk factors include immunosuppressive drugs and in situ device use (Kajihara *et al.*, 2015).

The actual origin of the *E. faecium* septicemia, in this case, is unknown. The dog had not been hospitalized, had no previous history of anti-microbial drug use and no evidence of other organ infection related to *E. faecium*. It was noteworthy that the dog appeared to have been in contact with pigeons' feces, before the presentation with severe illness. Since bacterial isolation and genomic identification from the environment and animals' guts, in this case, had not been done, the origin of infection could not be identified precisely. The exact route of natural infection-causing enterococcus septicemia has not been clearly described.

Enterococcaceae is an uncommon family of the microbiome presented on the ocular surface of normal dogs (Rogers *et al.*, 2020). Even though *E. villorum* might cause ocular infection, we could not yet definitively conclude that ocular lesions in this dog were caused solely by *E. villorum* rather than by polymicrobial ocular infections or secondary response to sepsis. However, the factual results only support that there was no relation of pathogens between the eyes and bloodstream as mentioned above.

Regarding the virulence genes, cytolysin or hemolysin, a bacterial toxin, has β-hemolytic properties and bacteriocin activity (Yordanova and Stanilova, 2020). The *gel*E gene encodes the gelatinase enzyme, which is responsible for the hydrolysis of hemoglobin, fibrinogen, collagen and other proteins. The *esp* gene is associated with biofilm formation and colonization (Kubasova *et al.*, 2017). The role of *efaAfm* may be related to cell wall adherence. The *scm* gene binds to collagen type V and fibrinogen. A surface adhesion *sgrA* binds to nidogen 1 and 2, which are ubiquitous basement membrane components, related to biofilm formation. The *acm* binds to collagen types I and IV (Soheili *et al.*, 2014)

The virulence potential of *E. faecium* indicated by the number or score of present virulence genes has been determined (Strateva *et al.*, 2016). *E. faecium* isolated from blood, in this case, exhibited four out of nine virulence genes: *gel*E, *efaAfm*, *scm,* and *acm* genes (Fig 2).

Studies have shown the diverse incidence of virulence genes of *E. faecium* strains with the differences in species, types of isolates and geography (Strateva *et al.*, 2016; Kubasova *et al.*, 2017; Kiruthiga *et al.*, 2020; Stępień-Pyśniak *et al.*, 2021). The study in 92-healthy dogs showed the incidence of the *efaAfm* gene detected from fecal isolates was 88 (95.65%). The presence of only the *efaAfm* gene may not be a risk indicator for pathogenic strains. This study also revealed the low incidences of *esp*, *gelE* genes of *E. faecium* strains at 2 (2.17%) and 3 (3.26%), respectively (Kubasova *et al.*, 2017). A recent study showed no statistical differences in virulence genes (such as *cylA*, *gelE*, *esp*, *efaAfm,* and *sgrA*) of *E. faecium* between the isolates from fecal samples of fifteen healthy dogs and urine samples of three dogs with urinary tract infection, suggesting microflora in the gut is possibly considered as the main source of enterococcal urinary tract infection (Stępień-Pyśniak *et al.*, 2021). However, the number of *E. faecium* isolates from dogs in that study was a small group. To identify commensal and pathogenic *E. faecium* strains in dogs, more studies are required to investigate the diverse virulence gene distribution in a large group.

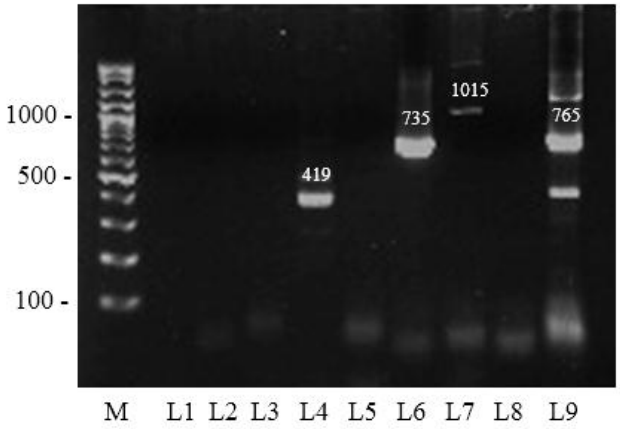

Figure 2 Agarose gel electrophoresis of PCR amplification of virulence genes of *E. faecium* from the blood sample. Lane M, 100-bp DNA ladder; L1, *cylM*; L2, *cylB*; L3, *cylA*; L4, *gelE*; L5, *esp*; L6, *efaAfm*; L7, *scm*; L8, *sgrA*; L9, *acm*. Virulence genes of *gelE*, *efaAfm*, *scm* and *acm* generated 419, 735, 1015, and 765 DNA bp products respectively. Negative control of each gene was done. No band was presented in the negative control (data not shown).

Biofilm produced by bacteria is an important factor to adhere to the biotic and abiotic surface and in protecting bacteria from host defense. The *asa1* or *agg*, *cylA* and *esp* genes of *E. faecium* isolates from dogs have been found frequently in strong biofilm formation strains (Stępień-Pyśniak *et al.*, 2021). No detection of *esp* and *gelE* genes of *E. faecium* isolates from fecal samples of 16-healthy dogs (El-razik *et al*., 2020).

The *acm* and *scm* genes, also known as *E. faecium* surface proteins (*fms*), *fms*8 and *fms10* respectively (Sillanpää *et al.*, 2009) are members of the microbial surface component recognizing adhesive matrix molecules (MSCRAMMs), a family of microbial adhesive matrix molecules (Freitas *et al.*, 2018). To establish host colonization, adherence and invasion, microorganisms express cell surface adhesins to adhere to the extracellular matrix proteins (ECMs), a complex mixture of glycoproteins and proteoglycans, including collagens, fibronectin and laminin which are the structural support of epithelial and endothelial cells of animal tissues (Nallapareddy *et al.*, 2003).

There is no information about the detection of the *acm* and *scm* genes in clinical isolates of dogs. This case was the first report to detect these genes from the blood sample of a dog with *E. faecium* septicemia. The adherent ability to the ECMs regulated by the *acm* and *scm* genes, suggesting that *E. faecium* can invade epithelial and endothelial cells. Subsequently, the *gelE* gene is involved in colonization, hydrolysis of collagen and other proteins (hemoglobin and fibrinogen), degrading host immunity and inducing inflammation.

Studies have reported the existence of silent genes and the presence of the target gene without the phenotypic detection. Similarly, detection of the phenotypic activity has been observed in more isolates than the presence of one target gene suggesting the possible role of other genes (Iseppi *et al.*, 2015; Kubasova *et al.*, 2017). Additional study on the virulence genes and their biological characteristics is necessary for the investigation of pathogenicity of dogs with *E. faecium* septicemia in a large population.

E. canis and *B. canis vogeli* infections have been shown to present with diverse clinical manifestations. Acute *E. canis* infection persists for 2-4 weeks and commonly presents with fever, weight loss, lymphadenopathy, splenomegaly and vasculitis, while subclinical and chronic infections can be asymptomatic (Rodríguez-Alarcón *et al.*, 2020). Clinical signs of *B. canis vogeli* infection have been reported to be identical to *E. canis* infection (Salem and Farag, 2014). Although *E. canis* and *B. canis vogeli* were also detected in this dog, the disease severity was potentially aggravated by *E. faecium*, leading to marked plasma D-dimer increment.

Coagulopathy accompanied by an aggravated host inflammatory response, is a leading cause of pathological conditions that induce disseminated intravascular coagulopathy (DIC) (Papageorgiou *et al.*, 2018), which is diagnosed by presenting at least four of the following criteria: low platelet count, prolonged prothrombin time or activated partial thromboplastin time, low fibrinogen concentration or increased fibrin degradation products concentration and the presentation of schistocytes. Increment in plasma D-dimer concentration has been proposed to be a potentially accurate diagnosis criterion of DIC (Machida *et al.*, 2010) and has been seen to increase significantly in dogs with sepsis, suggesting hemostatic disorder (De Laforcade *et al.*, 2003). Moreover, the increment of plasma D-dimer (> 2,000 ng/ml) has correlated significantly with disease severity in humans (Yao *et al.*, 2020; Zhou *et al.*, 2020). In this case, the disease severity associated with *E. faecium* septicemia was of concern because of the presentation of multi-systemic inflammation, hemorrhage, thrombocytopenia and markedly increased plasma D-dimer, which raised suspicions of having DIC.

In conclusion, the present study illustrates the severity of *E. faecium* septicemia that is related to its MDR phenotype accompanied by the detection of *gelE*, *efaAfm*, *scm,* and *acm* virulence genes. This data points toward the significance of enterococcal infection in the clinical field that could be associated with disease aggravation, stressing the importance of treatment of virulent and drug-resistant strains of *Enterococcus* infection in dogs.

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