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Vancomycin-resistant *Enterococcus* (VRE) isolates from dogs and cats in veterinary hospitals in Brazil

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Abstract

Background In veterinary medicine, particularly concerning dogs and cats, there is limited data regarding vancomycin-resistant *Enterococcus* (VRE). However, multidrug-resistant *Enterococcus* is frequently identified, raising concerns about the potential for spreading these resistant microorganisms to humans due to their zoonotic nature. This study aimed to identify VRE colonizing animals admitted to a veterinary hospital and to determine the presence of the major resistance genes responsible for vancomycin resistance.

Results *Enterococcus* was found to carry the *van*A gene in 52.54% of cases, the *van*B gene in 23.73%, the *van*C gene in 20.34%, and the *van*E gene in 3.39%. The antimicrobials with the lowest resistance were chloramphenicol (5.08%) and ampicillin (6.78%). In contrast, the highest resistance was observed with enrofloxacin (79.66%), rifampicin (67.80%), and ciprofloxacin (61.02%). Seven *Enterococcus* isolates showed resistance to vancomycin as well as high-level aminoglycoside resistance (HLAR).

Conclusion A total of 46 animals were found to be colonized by VRE, of which 16 were healthy. The *van*A gene was the most commonly isolated (52.54%), followed by *van*B (23.73%), *van*C (20.34%), and *van*E (3.39%). This represents the first report of the *van*E gene being identified in a dog in Brazil. Among the 59 *Enterococcus* isolates, 84.75% ($n=50$) were found to be multidrug resistant. The colonization of VRE and *Enterococcus* HLAR in dogs and cats poses a public health concern, as it increases the risk of environmental dissemination and has implications for One Health.

Keywords VRE, Small animals, Multidrug resistance, HLAR, Antimicrobial

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Background

Antimicrobial resistance is a major concern in One Health, as multidrug-resistant microorganisms can be found in animals, humans, and the environment. The World Health Organization estimates that by 2050, antimicrobial resistance will be responsible for 10 million deaths annually, with 1.27 million deaths already reported in 2019 [1, 2]. Among these microorganisms, *Enterococcus* is an emerging pathogen in veterinary medicine, causing healthcare-associated infections.

The microorganisms belonging to the genus *Enterococcus* are gram-positive, catalase-negative, facultative anaerobic cocci and are considered part of the normal intestinal microbiota of humans and animals [3, 4]. Of the 50 species described, *E. faecalis* and *E. faecium* are the most clinically relevant in both humans and animals [5]. *Enterococci* are commensal microorganisms with a worldwide spread [6], and for a long time, they were considered harmless, and only in recent decades were they considered an emerging bacterium of great importance in health, mainly with the increase in their incidence in hospital infections [7, 8].

The growing significance of *Enterococcus* in both veterinary and human medicine is due to its role in urinary tract and skin infections, endocarditis, and sepsis. It is now recognized as the third most prevalent pathogen in hospital infections worldwide, primarily because of its ability to acquire antimicrobial resistance—especially to vancomycin, one of the last-resort treatments for multidrug-resistant *Enterococcus* infections [9, 10]. The indiscriminate use of vancomycin in human medicine, along with the use of avoparcin (a glycopeptide-class antimicrobial) as a growth promoter in livestock, has significantly contributed to the emergence of vancomycin-resistant *Enterococcus* (VRE) [11, 12].

Beyond the emergence of vancomycin resistance, what has alarmed the scientific community is the rapid appearance of new vancomycin resistance genes in *Enterococcus* species. Notably, since its first isolation in France in 1988 [13], ten different variations of the VRE gene have been identified (*vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanF*, *vanG*, *vanL*, *vanM*, and *vanN*) [14], with *vanA* and *vanB* being the most prevalent.

In addition to glycopeptide resistance, including vancomycin, *Enterococcus* can exhibit intrinsic resistance to other antibiotic classes, such as polymyxins, lincosamides, cephalosporins, and low levels of aminoglycosides [15]. Resistance can also be acquired through plasmids, integrons, and/or transposons [14], facilitating the transmission of resistance genes among the environment, humans, and animals.

Wada et al. conducted the first meta-analysis and systematic review to assess the prevalence of VRE in companion animals, reporting a prevalence of 18.2% in dogs and 12.3% in cats, with most studies conducted in Europe [16]. In veterinary medicine, particularly in cats and dogs, limited data exist on the dynamics of VRE infections. However, multidrug-resistant *Enterococcus* is frequently identified, contributing to the spread of multidrug-resistant microorganisms to humans due to its zoonotic potential [17, 18]. Therefore, this study aimed to identify vancomycin-resistant *Enterococcus* isolated from healthy and diseased animals admitted to a veterinary hospital and to determine the presence of major vancomycin resistance genes.

Results

Among the 81 dogs and 25 cats sampled, vancomycin-resistant *Enterococcus faecalis* (VREfs) was isolated from rectal swabs in 30.8% ($n=25$) of dogs and 52% ($n=13$) of cats. Vancomycin-resistant *Enterococcus faecium* (VREfm) was detected in 6.2% ($n=5$) of dogs. Additionally, 4.9% ($n=4$) of *Enterococcus gallinarum* (VREgg) isolates were found in dogs, and 4% ($n=1$) in cats. Two dogs were simultaneously colonized by both VREfs and VREfm.

Of the 46 animals (32 dogs and 14 cats) positive for VRE, 18 were positive only at admission to the Veterinary Clinic Hospital (HCV), 17 animals were positive only at discharged, and 12 were positive at both moments (at admission and discharge), totaling 59 samples. Of these same colonized animals, 16 (five dogs and 11 cats) were healthy and were hospitalized for the castration procedure (Table 1).

The *Enterococcus* strains exhibited the highest resistance to enrofloxacin (79.66%, $n=47$), rifampicin (67.80%, $n=40$), and ciprofloxacin (61.02%, $n=36$), followed by teicoplanin and erythromycin (55.93%, $n=33$ each). The lowest resistance rates were observed for chloramphenicol (5.08%, $n=3$), ampicillin (6.78%, $n=4$), gentamicin

Table 1 Identification of *Enterococcus* species isolated from healthy and sick dogs and cats hospitalized at HCV-CAV-UDESC

Animals	Nº animals	Nº (%) non-VRE	VREfs		VREfm		VREgg	
			Nº (%) sick positives	Nº (%) healthy positives	Nº (%) sick positives	Nº (%) healthy positives	Nº (%) sick positives	Nº (%) healthy positives
Dogs	81	49 (60.5)	21 (25.9)	4 (4.9)	5 (6.2)	0	3 (3.7)	1 (1.2)
Cats	25	11 (44)	3 (12)	10 (40)	0	0	0	1 (4)
Total	106	60 (56.6)	24 (22.6)	14 (13.2)	5 (4.7)	0	3 (2.8)	2 (1.9)

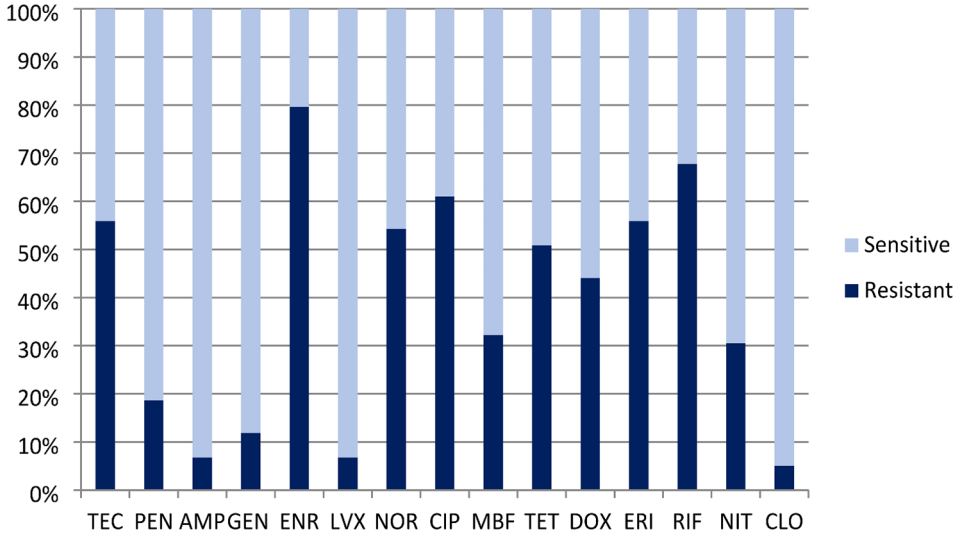

Fig. 1 Profile of sensitivity to different antimicrobials of VRE colonizing hospitalized dogs and cats. TEC = Teicoplanin; PEN = Penicillin; AMP = Ampicillin; GEN = Gentamicin; ENR = Enrofloxacin; LVX = Levofloxacin; NOR = Norfloxacin; CIP = Ciprofloxacin; MBF = Marbofloxacin; TET = Tetracyclin; DOX = Doxycycline; ERI = Erythromycin; RIF = Rifampicin; NIT = Nitrofurantoin; CLO = Chloramphenicol

Table 2 Detection of the main genes related to Vancomycin resistance in different *Enterococcus* species colonizing hospitalized dogs and cats

Enterococcus identification	vanA (%)	vanB (%)	vanC (%)	vanE (%)	Total
E. faecalis	27 (45.76)	13 (22.03)	6 (10.17)	2 (3.39)	48 (81.36)
E. faecium	4 (6.78)	1 (1.7)	0	0	5 (8.47)
E. gallinarum group	0	0	6 (10.17)	0	6 (10.17)
Total	31 (52.54)	14 (23.73)	12 (20.34)	2 (3.39)	59 (100)

120 mcg (11.86%, $n = 7$), and penicillin G (18.64%, $n = 11$). The resistance percentages of all tested antimicrobials are shown in Fig. 1.

Among the 59 *Enterococcus* isolates (48 *E. faecalis*, 5 *E. faecium*, and 6 *E. gallinarum*), 84.75% ($n = 50$) were multidrug-resistant (MDR), meaning they exhibited resistance to at least one antimicrobial from three or more different classes. All *E. faecium* and *E. gallinarum* isolates were classified as MDR. Additionally, seven *Enterococcus* isolates (5 *E. faecalis*, 1 *E. faecium*, and 1 *E. gallinarum*) exhibited high-level aminoglycoside resistance (HLAR) in addition to vancomycin resistance.

Regarding the detection of vancomycin resistance genes, 52.54% ($n = 31$) of the samples were positive for the *van*A gene, 23.73% ($n = 14$) for the *van*B gene, 20.34% ($n = 12$) for the *van*C gene, and 3.39% ($n = 2$) for the *van*E gene, as shown in Table 2. The *van*D and *van*G genes were not detected.

According to the identification of the species of *Enterococcus* and the presence of the gene that confers resistance to vancomycin and the results of MIC, it was possible to distribute the isolates in seven groups (Table 3): 1) *E. gallinarum* group with the presence of the *van*C gene, which was identified in six isolates from five different animals (one animal was positive in both, admission and discharged); 2) *E. faecium* with the *van*B gene identified in only one animal; 3) *E. faecium* with the presence of the *van*A gene identified in four different animals and with distinct antimicrobial susceptibility profiles; 4) *E. faecalis* with the *van*E gene, present in two samples and both belonging to the same animal, but with different resistance profiles; 5) *E. faecalis* with the presence of the *van*C gene present in four different animals, and in one of them it was isolated the same microorganism at the hospital admission, three days after admission and at the time of patient's discharged; 6*) E. faecalis* with the *van*B gene, this profile was present in 12 different animals, and in one of them the same microorganism was isolated in both collections with the same antimicrobial susceptibility profile, and; 7) *E. faecalis* with the *vanA* gene.

Discussion

Currently, several studies worldwide have highlighted the occurrence of VREs isolated from animal and human feces. However, most animal studies are based on the phenotypic resistance of VRE but do not relate the different genes responsible for this resistance [19]. In Brazil, most studies are related to the isolation of VRE in production animals, food and humans, but there are no consistent data in dogs and cats that investigate colonization in these species during hospitalization. Interestingly, VRE*fs* was the most isolated species in the feces of dogs and cats (35.85%) in the present study. Our results are noteworthy because studies by Ossiprandi et al. [20]

Table 3 Characterization of Vancomycin-resistant *Enterococcus* (VRE) colonizing hospitalized dogs and cats

Sample	Animal	Species Enterococcus	Antimicrobial resistant	Van gene	Vancomycin MIC (µg/mL)
1	Dog	*E. faecalis*	GLI / FQ / TET / RIF / NIT	A	≥ 256
2	Cat	*E. faecalis*	GLI / FQ / TET / ML / NIT	A	≥ 256
3	Cat	*E. faecalis*	GLI / FQ / NIT	A	128
4	Dog	*E. faecalis*	GLI / FQ / ML / RIF	A	≥ 256
5	Dog	*E. faecalis*	GLI / FQ / RIF / NIT	A	≥ 256
6	Dog	*E. faecalis*	GLI / HLAR / FQ / TET / ML / RIF	A	≥ 256
7	Dog	*E. faecalis*	GLI / HLAR / FQ / TET / ML / RIF	A	≥ 256
8	Dog	*E. faecalis*	GLI / FQ / TET / ML	A	≥ 256
9	Cat	*E. faecalis*	GLI / TET / ML / NIT	A	≥ 256
10	Cat	*E. faecalis*	CLI / PEN / AMP / RIF	A	≥ 256
11	Dog	*E. faecalis*	GLI / FQ / ML / RIF / NIT	A	≥ 256
12	Cat	*E. faecalis*	GLI / PEN / HLAR / FQ / TET / ML / RIF	A	≥ 256
13	Dog	*E. faecalis*	GLI / AMP / FQ / TET / ML / RIF	A	≥ 256
14	Dog	*E. faecalis*	GLI / FQ / RIF / NIT	A	≥ 256
15	Dog	*E. faecalis*	GLI / TET	A	128
16	Cat	*E. faecalis*	GLI / TET	A	128
17	Cat	*E. faecalis*	GLI / FQ	A	128
18	Dog	*E. faecalis*	GLI / FQ / ML / RIF / NIT	A	≥ 256
19	Cat	*E. faecalis*	GLI / PEN / FQ / TET / RIF / NIT	A	≥ 256
20	Dog	*E. faecalis*	GLI / FQ / RIF / NIT	A	≥ 256
21	Dog	*E. faecalis*	GLI / FQ / RIF / NIT	A	≥ 256
22	Cat	*E. faecalis*	GLI / FQ	A	128
23	Cat	*E. faecalis*	GLI / FQ	A	128
24	Cat	*E. faecalis*	GLI	A	128
25	Cat	*E. faecalis*	GLI	A	128
26	Dog	*E. faecalis*	GLI / NIT	A	128
27	Dog	*E. faecalis*	GLI / NIT	A	128
28	Cat	*E. faecalis*	GLI / FQ / ML / RIF	B	128
29	Dog	*E. faecalis*	GLI / FQ / TET / ML	B	≥ 256
30	Dog	*E. faecalis*	GLI / FQ / ML / RIF	B	128
31	Dog	*E. faecalis*	GLI / FQ / ML / RIF	B	128
32	Dog	*E. faecalis*	GLI / PEN / FQ / TET	B	≥ 256
33	Dog	*E. faecalis*	GLI / FQ / ML / RIF	B	128
34	Dog	*E. faecalis*	GLI / PEN / HLAR / FQ / TET / ML / RIF	B	≥ 256
35	Dog	*E. faecalis*	GLI / HLAR / FQ / TET / ML / RIF	B	≥ 256
36	Cat	*E. faecalis*	GLI / PEN / FQ / TET / RIF	B	≥ 256
37	Dog	*E. faecalis*	GLI / PEN / HLAR / FQ / TET / ML/ RIF	B	≥ 256
38	Cat	*E. faecalis*	GLI / FQ / ML / RIF	B	128
39	Cat	*E. faecalis*	GLI / FQ / ML / RIF	B	128
40	Dog	*E. faecalis*	GLI / FQ / ML / RIF	B	128
41	Dog	*E. faecalis*	GLI / FQ / TET / ML / RIF	C	32
42	Dog	*E. faecalis*	GLI / FQ / TET / ML / RIF / CLO	C	32
43	Dog	*E. faecalis*	GLI / FQ / TET / ML / RIF	C	32
44	Dog	*E. faecalis*	GLI / FQ / ML / RIF	C	32
45	Dog	*E. faecalis*	GLI / FQ / TET	C	32
46	Dog	*E. faecalis*	GLI / FQ / TET / ML / RIF	C	32
47	Dog	*E. faecalis*	GLI / FQ / NIT	E	32
48	Dog	*E. faecalis*	GLI / FQ / TET	E	32
49	Dog	*E. faecium*	GLI / HLAR / FQ / TET / ML / RIF	A	≥ 256
50	Dog	*E. faecium*	GLI / PEN / TET / ML / NIT / CLO	A	≥ 256
51	Dog	*E. faecium*	GLI / PEN / AMP / FQ / TET / ML / RIF / NIT	A	≥ 256
52	Dog	*E. faecium*	GLI / PEN / AMP / FQ / TET / ML / RIF	A	≥ 256
53	Dog	*E. faecium*	GLI / PEN / FQ / TET / ML / RIF / CLO	B	≥ 256

Table 3 (continued)

Sample	Animal	Species Enterococcus	Antimicrobial resistant	Van gene	Vancomycin MIC (µg/mL)
54	Dog	*E. gallinarum* group	GLI / FQ / RIF / NIT	C	32
55	Dog	*E. gallinarum* group	GLI / FQ / RIF / NIT	C	32
56	Cat	*E. gallinarum* group	GLI / FQ / TET / RIF	C	32
57	Dog	*E. gallinarum* group	GLI / TET / RIF / CLO	C	32
58	Dog	*E. gallinarum* group	GLI / FQ / ML / RIF	C	32
59	Dog	*E. gallinarum* group	GLI / HLAR / FQ / TET / ML / RIF	C	32

and Bertelloni et al. [21] did not isolate VRE in dog feces from Italy and Hungary, respectively. The only difference between these studies and ours is that they directly sampled feces, while our study collected rectal swabs for analysis. This discrepancy may be linked to the use of antimicrobials in dogs and cats across different countries. The number of animals colonized by this microorganism found in the present study is alarming since they serve as potential disseminators of this important hospital-infection pathogen in humans.

Zoonotic transmission of multidrug-resistant microorganisms between dogs, cats, and humans is already established, though it is often challenging to determine the direction of transmission [16]. However, some studies have shown that VRE strains isolated from dogs are identical to those found in human hospital infections, carrying the same transposon responsible for harboring the van gene [22, 23]. Some authors have demonstrated the capacity for horizontal transfer of antimicrobial resistance genes from *Enterococcus* to other bacterial species, enabling the dissemination of these genes to humans, which could have a significant clinical impact on medicine [24, 25]. Possible impacts are supported by studies showing similarities between clinical *Enterococcus* isolates from humans and pets [26].

Although some studies have demonstrated similarities between *Enterococcus* isolates from pets and humans, we have not found any studies identifying the transmission route of *Enterococcus*-related infections between these animals and humans. This contrasts with products of animal origin, which have already been shown to transmit vancomycin-resistant *Enterococcus* (VRE) to humans due to the extensive use of glycopeptide-class antimicrobials in food-producing animals in the 1990s.

Although this was not the objective of our study, the colonization of dogs and cats by VRE is a concern and highlights the need for further research on the potential transmission of these genes to microorganisms in the human microbiota. The identification of the first VRE strain carrying the *vanE* gene in a dog in Brazil underscores the need for epidemiological studies in dogs and cats to determine the transmission routes of these multidrug-resistant microorganisms. Additionally, it emphasizes the importance of epidemiological surveillance in veterinary hospitals, which is currently insufficient in Brazil.

According to a survey conducted by Wada et al. [16], which reviewed several studies worldwide, the prevalence of VRE in cats was 12.3%, while in dogs it was 18.2%. Alarmingly, our results revealed a prevalence of 41.97% in dogs and 56% in cats, all of which had no clinical signs of VRE infection but were colonized in the gastrointestinal tract. This highlights the importance of surveillance cultures for detecting multidrug-resistant microorganisms.

Another very important finding was that 34.78% ($n = 16$) of the animals that presented VRE colonization were healthy animals that performed castration procedures. However, the CAV-UDESC HCV is a school hospital, and these animals are hospitalized for more time for students to practice procedures, which facilitates the dissemination of the microorganism and the colonization of other animals. This fact is strengthened in Brazil because there is no official legislation requiring the establishment of Infection Control Commissions in Veterinary Hospitals, making it more difficult to detect multidrug-resistant microorganisms and implement control measures. In our study, 36.96% ($n = 17$) of the animals were only able to identify VRE samples at hospital discharge, suggesting that the hospital is an important source in the epidemiology of transmission. According to Verwilghen and Singh [27], the longer an animal is hospitalized, the greater the risk of colonization by multiresistant microorganisms present in the environment or of spreading these bacteria to other susceptible animals. This is because the animals are mostly hospitalized in crates.

The *E. gallinarum* and *E. casseliflavus/flavenscens* species belong to the *E. gallinarum* group and are intrinsically resistant to vancomycin, with the vanC gene responsible for a low level of antimicrobial resistance but maintaining sensitivity to teicoplanin [28]. However, the vanC gene is not exclusive to this group of Enterococcus and has also been identified in *E. faecalis* by Schwaiger et al. [29] in Germany. Similarly, our study observed the presence of the vanC gene in all samples from the *E. gallinarum* group ($n = 6$) and in 10.17% ($n = 6$) of *E. faecalis* samples.

Despite having been described in other works, the *van*E genes in *E. faecalis* [30], *van*B gene in *E. faecium* [31] and

*van*C gene in *E. faecalis* [32] are not common in humans and animals, much less in dogs and cats, which demonstrates the importance of our findings for animals that have frequent proximity to humans. The animals that harbor these genes serve as reservoirs for compromising One Health.

According to Adhikari [33], meningitis, endocarditis and other important infections caused by *Enterococcus* in immunosuppressed patients are treated with vancomycin associated with aminoglycosides, including gentamicin, but when the microorganism has high levels of resistance to aminoglycoside (HLAR), treatment becomes ineffective. Bertelloni et al. [21] found 6.7% of *Enterococcus* HLAR, a value below that found in the present study, 11.86%, which not only showed resistance to aminoglycosides but also vancomycin.

In Brazil, enrofloxacin is used indiscriminately in the clinical routine of dogs and cats. Interestingly, in the present study, this antimicrobial was the one that showed the greatest resistance in VRE strains. Another extremely worrying factor is that 84.75% of *Enterococcus* were characterized as multiresistant, making antimicrobial therapy even more difficult. On the other hand, *Enterococcus*, like *Streptococcus*, are not microorganisms that produce beta-lactamase, which justifies the low resistance to penicillin and ampicillin found in the present study.

Conclusion

The presence of multidrug-resistant Enterococci and resistance genes for vancomycin, particularly genes that have not yet been identified in dogs and cats in Brazil, raises concerns about the effectiveness of control measures within veterinary hospital environments. Professionals in this field are in constant contact with animals and may serve as reservoirs, potentially disseminating these genes into the community.

Therefore, the colonization of VRE and *Enterococcus* HLAR in dogs and cats is a concern for public health, increasing the risk of environmental dissemination and impacting the One Health concept. For the authors, this report represents the first documentation of the *van*E gene in a dog in Brazil.

Methods

Samples

The Brazilian Veterinary Medical Teaching Hospital of the University of the State of Santa Catarina (CAV-UDESC) treats approximately 5000 animals per year. Rectal swabs of 81 dogs and 25 cats were collected at least two times: at admission for HCV and after discharge of patients from the veterinary hospital from September to December 2017. Therefore, all dogs and cats that were hospitalized, regardless of the reason, for at least two days and that did not die during this period were considered for sampling. For collection, swabs were previously humidified with sterile 0.85% saline solution for collection and deposited in tubes containing the selective and differential medium, Enterococcosel broth, supplemented with 6 µg of vancomycin and carried to the Center for Diagnostic Animal Microbiology (CEDIMA) of the CAV-UDESC for incubation at 37 °C for 48 h.

Phenotypic detection of VRE

The tubes containing *Enterococcosel* broth supplemented with vancomycin that showed blackish coloration after 48 h incubation were plated on 5% sheep blood agar with vancomycin discs in the center of the plate. Samples that presented growth in blood agar and formed halos less than or equal to 17 mm were submitted to the Gram test to confirm gram-positive cocci in addition to the catalase test to identify microorganisms belonging to the genus *Enterococcus*.

Antimicrobial susceptibility test of Enterococcus and detection of a high level of aminoglycoside resistance (HLAR)

Strains identified as *Enterococcus* were tested for antimicrobial susceptibility profile using the disk diffusion technique using the following disks: teicoplanin (30 µg), penicillin G (10 UI), ampicillin (10 µg), enrofloxacin (5 µg), levofloxacin (5 µg), marbofloxacin (5 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), tetracycline (30 µg), doxycycline (30 µg), rifampicin (5 µg), nitrofurantoin (300 µg) and chloranphenicol (30 µg). For the identification of high-level aminoglycoside resistance *Enterococcus* (HLAR), a gentamicin disk (120 µg) was used. The zones of growth inhibition around each antibiotic disk were measured and interpreted using the criteria established by CLSI for humans and animals [34, 35]. The *Escherichia coli* ATCC 25,922 reference strain was used as a quality control to determine susceptibility to antimicrobial agents. In addition to the disk diffusion test for vancomycin, the minimum inhibitory concentration (MIC) technique for this antimicrobial was also performed, according to CLSI [34].

Bacterial DNA extraction

DNA extraction from all phenotypically suggestive samples of vancomycin-resistant *Enterococcus* isolated from the animals was performed through the protocol described by Parussolo [36] to identify the *Enterococcus* species and to detect the gene that confers resistance to vancomycin. Bacterial isolates were inoculated in BHI broth at 37ºC for 24 h and stored at -20ºC. A volume of 200 µL of each isolated was transferred to a sterile microtube, mixed with 500 µL of chloroform: isoamyl alcohol (24:1), and incubated in water bath at 56ºC for 30 min. After that, microtube was centrifuged at 12,000 rpm

Table 4 Primers for detecting Vancomycin resistance genes and identifying the main *Enterococcus* species

Gene	Primer	Oligonucleotides (5′ – 3′)	Base pairs (bp)
*van*A	Forward	GGGAAAACGACAATTGC	732 bp
	Reverse	GTACAATGCGGCCGTTA	
*van*B	Forward	ACGGAATGGGAAGCCGA	647 bp
	Reverse	TGCACCCGATTTCGTTC	
*van*C/2	Forward	ATGGATTGGTAYTKGTAT	815/827 bp
	Reverse	TAGCGGGAGTGMCYMGTAA	
*van*D	Forward	TGTGGGATGCGATATTCAA	500 bp
	Reverse	TGCAGCCAAGTATCCGGTAA	
*van*E	Forward	TGTGGTATCGGAGCTGCAG	430 bp
	Reverse	ATAGTTTAGCTGGTAAC	
*van*G	Forward	CGGCATCCGCTGTTTTTGA	941 bp
	Reverse	GAACGATAGACCAATGCCTT	
ddlFaecalis	Forward	CACCTGAAGAAACAGGC	475 bp
	Reverse	ATGGCTACTTCAATTTCACG	
ddlFaecium	Forward	GAGTAAATCACTGAACGA	1091 bp
	Reverse	CGCTGATGGTATCGATTCAT	

for 10 min, and supernatant transferred to a new sterile microtube. We then added 600 µL of 70º alcohol, and further centrifuged the tube at 13,500 rpm for 20 min. After this procedure, supernatant was carefully discarded by inversion. Once DNA sample was completely dried, we added 200µL of Milli-Q water. DNA concentration measurements were performed on a NanoDrop (Thermo Fisher, Waltham, USA). To perform PCR tests, the DNA concentration was adjusted to 15–100 ng.

Multiplex PCR of Enterococcus faecium and Enterococcus faecalis and detection of genes of resistance to Vancomycin

Two multiplex PCR techniques, one for identification of the two main species of *Enterococcus* (*E. faecium* and *E. faecalis*) and another for the detection of vancomycin genes (*van*A, *van*B, *van*C, *van*D, *van*E and *van*G), were performed. Specific primers described in a previous study were used [37], as shown in Table 4. Samples identified as *Enterococcus* but negative for PCR identification and the presence of the *van*C gene were identified as belonging to the *Enterococcus gallinarun* group.

To identify the *Enterococcus* species, amplification reactions were conducted in a 25 µL final volume containing PCR buffer (Tris-HCl – 20 mM, KCl – 50 mM), MgCl2 (1.5 mM), dNTP (200 mM of each), Taq DNA polymerase (1 U), primers (4 pmol of each), and bacterial DNA (2 µL). For identification of vancomycin resistance genes, the following protocol was followed: 50 µL final volume containing PCR buffer (Tris-HCl – 20 mM, KCl – 50 mM), MgCl2 (1.5 mM), dNTP (200 mM of each), Taq DNA polymerase (1 U), primers (4 pmol of each), and bacterial DNA (3 µL).

For both reactions, the PCR program was carried out as follows: initial denaturation at 94 °C for 10 min; 40 cycles of denaturation at 94 °C for 40 s, annealing of the primer at 56 °C for 40 s, and 1 min of extension at 72 °C. The last cycle extension was at 72 °C for 7 min.

Amplified fragments were subjected to electrophoresis in a 2% agarose gel (100 V, 300 mA) for 1 h, stained with GelRedTM and visualized in a transilluminator. *Enterococcus faecium* (ATCC 51299) and *Enterococcus faecalis* (ATCC 700221) reference strains were used to assure quality control for detection (Fundação Oswaldo Cruz – Fiocruz). The *Escherichia coli* ATCC 25,922 reference strain was used as a negative control.

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Author contributions
RAPS, FZS, UMC, DGGS and SMF contributed to the study conception and design. Data collection was performed by RAPS, LP, FDM and MFS, and analysis was performed by RAPS, LP, DGGS, SMF. The first draft of the manuscript was written by RAPS. All authors read and approved the final manuscript. FZS and SMF were Master's supervisors to RAPS.

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Data availability
All data generated or analysed during this study are included in this published article in tables and figure.

Declarations

Ethical approval
This study was approved and conducted under the supervision by the ethical committee of Universidade do Estado de Santa Catarina (2042290617), Brazil and performed accordingly. This study was performed in accordance with the recommendations and it was carried out in compliance with the ARRIVE guidelines. Samples were collected only from animals for laboratory analyses, avoiding unnecessary pain and suffering of the animals and all owners were informed and consented to the collections.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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