

## Identification and Antimicrobial Susceptibility of Enterococci Isolated from Dogs and Cats Subjected to Differing Antibiotic Pressures

Yasushi KATAOKA<sup>1</sup>\*, Chieko ITO<sup>1</sup>, Aya KAWASHIMA<sup>1</sup>, Miki ISHII<sup>1</sup>, Satoko YAMASHIRO<sup>1</sup>, Kazuki HARADA<sup>1,2</sup>, Hiroki OCHI<sup>1</sup> and Takuo SAWADA<sup>1</sup>

<sup>1</sup>)Department of Veterinary Microbiology, Nippon Veterinary and Life Science University, Tokyo 180–8602, Japan

<sup>2</sup>)Department of Veterinary Internal Medicine, Tottori University, Tottori 680–8550, Japan

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**ABSTRACT.** The purpose of the present study was to determine the prevalence of antibiotic-resistant enterococci in dogs and cats subjected to differing antibiotic pressures, and the prevalence of vancomycin resistance genes in isolates from these animals. Enterococci were isolated from fecal samples of 65 healthy dogs and 29 healthy cats brought to animal hospitals, from rectal swabs of 73 puppies and 15 kittens from five breeders and two pet shops, and from fecal samples of 20 dogs and 9 cats that were treated with antibiotics in Nippon Veterinary and Life Science University Animal Medical Center. The rates of resistance to ampicillin among isolates from the kitten–puppy group and healthy dog–cat group were 6.8 and 4.3%, respectively. In contrast, the rates of resistance to ampicillin in enterococci from the treatment group under antibiotic pressure were 37.5%. There was a significant difference between the antibiotic-treated group and the untreated group ( $P < 0.01$ ). Similarly, in the treatment group, the rate of resistance to enrofloxacin was extremely high (75.0%). In comparison, in the healthy group and kitten–puppy group, the rates of resistance to enrofloxacin were 23.4 and 12.1%, respectively. Among these groups, a significant difference was also observed in the apparent resistance rates ( $P < 0.01$ ). Vancomycin-resistant enterococci (VRE) harboring *vanA* or *vanB* were not detected in any groups. Therefore, contamination of VRE in dogs and cats is still considered to be minimal in Japan.

**KEY WORDS:** antibiotic pressure, canine, feline, susceptibility, vancomycin-resistant enterococci.

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Enterococci are normal microbial flora of the gastrointestinal tract of humans and animals, and the major species of enterococci are *Enterococcus faecalis*, *E. faecium* and *E. durans* [1, 2, 13–15]. Generally speaking, enterococci are weak pathogens that do not cause illness in healthy humans or animals. However, enterococci have emerged as an important cause of nosocomial infections and have been found to quickly acquire resistance to many antimicrobials [6, 21].

In recent years, the appearance of vancomycin-resistant enterococci (VRE) has caused serious problems both in humans and in veterinary medicine [11, 24]. Resistance to glycopeptides in enterococci is mediated by *vanA*, *vanB* and *vanC* cluster, and the *vanA* genotype is considered to be of major importance [3]. The *vanA* genotype is the predominant resistant genotype and is characterized by acquired inducible resistance to both vancomycin and teicoplanin. The *vanB* cluster confers inducible resistance to various levels of vancomycin, and isolates exhibit susceptibility to teicoplanin, because this antibiotic is not an inducer. The *vanC*-type glycopeptide resistance is characterized by chromosomally-encoded and constitutively expressed resistance to low levels of vancomycin, but susceptibility to teicoplanin. This resistance has been described as an intrinsic property of *E. gallinarum*, *E. casseliflavus* and *E. flavescens* [3].

Vancomycin-resistant *E. faecium* strains carrying the *vanA* gene have been isolated from chicken, pigs and cattle, as well as meat from these animals [7, 9, 17]. Some epidemiological studies suggest that animals carrying VRE in their gastrointestinal tract could be the source of VRE infections in humans [31]. These VRE of animal origin can colonize humans, being able then to transfer their resistance genes to other human intestinal bacteria [4, 28]. There are no reports about the propagation of VRE in companion animals to humans. However, there are reports that *Staphylococcus aureus* and *Campylobacter jejuni* were propagated from companion animals to humans [29, 32].

In Japan, companion animals are being increasingly reared indoors [30], and as a result, pet owners have more opportunities for contact with companion animals. Potential transmission of pathogenic and/or antimicrobial resistant bacteria from companion animals to their keepers have been reported frequently [5, 23]. Therefore, monitoring of drug-resistant bacteria and pathogenic bacteria in companion animals has become important in public health and the veterinary medicine. However, only a few studies have reported VRE in companion animals in Japan and overseas [12, 16, 26].

In this study, we aimed to collect basic data derived from epidemiological survey of enterococci in dogs and cats as companion animals bred in Japan. In addition, we examined the relationship between antimicrobial susceptibility of enterococci and the selective pressure of antibiotics in these animals.

\*CORRESPONDENCE TO: KATAOKA, Y., Department of Veterinary Microbiology, Nippon Veterinary and Life Science University, Musashino, Tokyo 180–8602, Japan.  
e-mail: ykataoka@nvlu.ac.jp

## MATERIALS AND METHODS

**Sampling:** Fecal swabs of dogs and cats were collected and divided into the following three groups: group 1, healthy group; group 2, antibiotic exposure group; and group 3, group with no exposure to antibiotics. For the healthy group, fecal samples were collected from 65 dogs and 29 cats more than six months old that were brought into 22 animal hospitals (2 hospitals in Hokkaido, 3 in Fukushima, 1 in Ibaraki, 3 in Tokyo, 1 in Kanagawa, 3 in Yamanashi, 1 in Fukui, 5 in Aichi, 2 in Osaka and 1 in Okayama) for routine care, such as vaccination from 1999 to 2000. For the antibiotic exposure group, fecal samples were collected from 20 dogs and 9 cats more than 6 months old that were treated with antibiotics (penicilins; 14/29, cephalosporins; 4/29, aminoglycosides; 5/29, quinolones; 19/29) in Nippon Veterinary and Life Science University Animal Medical Center from 2006 to 2007. For the group with no exposure to antibiotics, fecal samples were collected from 73 puppies less than 3.5 months old and 15 kittens less than two months old from five breeders (43 puppies and 8 kittens; 2 breeders in Ibaraki, 3 in Saitama) and two pet shops (30 puppies and 7 kittens; 1 shops in Tokyo and 1 in Kanagawa). There was no history of antimicrobial exposure in the puppies and kittens, but it is possible that the dams were administered lincomycin for postpartum infection prophylaxis.

**Isolation and identification of enterococci:** All samples were plated onto EF agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) plates and Enterococcosel agar (Becton, Dickinson and Co., Tokyo, Japan) plates and incubated aerobically at 37°C for 48 hr. From each plate, one or more colonies with morphological characteristic of enterococci (i.e., dark brown halo) were initially tested by Gram staining, growth in 6.5% NaCl broth and bile esculin hydrolysis. All presumed enterococci were further identified as described by Facklam and Collins [10]. In group 1, because the number of hospitals varied with region, the following tests were used to one strain/head randomly selected in consideration of a regional difference. In the other two groups, multiple strains/head were also tested in the following experiments. Those strains were selected based on differences in the size of halo and morphology of colonies. Enterococcal isolates were stored at -80°C until used for testing.

**Antimicrobial susceptibility tests:** Minimum inhibitory concentrations (MICs) were obtained with a microdilution test according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Fifteenth Informational Supplement. Villanova, PA: CLSI; 2005 Publication No. M100-S15). The following eight antibiotics were tested: ampicillin (ABPC), streptomycin (DSM), gentamicin (GM), kanamycin (KM), erythromycin (EM), chloramphenicol (CP), enrofloxacin (ERFX) and vancomycin (VCM). For each antibiotic, two-fold dilutions were performed to obtain final concentrations from 512 to 0.125 µg/ml. MIC breakpoints were set according to CLSI guidelines. Isolates were considered resistant when MICs were equal to or greater than the following val-

ues (mg/l): ABPC, 16; DSM, 128; GM, 32; KM, 128; EM, 8; CP, 32; ERFX, 4 and VCM, 32. *E. faecalis* ATCC 29212 was used as a control microorganism in each set of tests.

**Detection of vancomycin resistance genes using multiplex PCR:** Detection of *vanA*, *vanB*, *vanC1* and *vanC2* by PCR was attempted for all enterococcal strains. For amplification of *vanA*, *vanB*, *vanC1* and *vanC2/C3*, the PCR described by Clark *et al.*, Dutka-Malen *et al.* and Satake *et al.* was modified as follows [6, 8, 27]. After incubation in trypticase soy broth (Becton, Dickinson and Co.) overnight at 37°C, a 25 µl of culture volume was mixed with 25 µl of InstaGene Matrix kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Cell suspensions were heated for 10 min at 100°C and centrifuged. A volume (2.5 µl) of the supernatant was then used for PCR amplification. The four primer sets shown in Table 1 were added to the reaction mixtures as follows: 5 pmol of the *vanA* primers; 2.5 pmol each of the *vanB*, *vanC1* and *vanC2/C3* primers. A multiplex PCR assay was performed in a total volume of 25 µl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP and dTTP) and 0.625 U of *Taq* DNA polymerase (Takara Bio Inc., Otsu, Japan). A TaKaRa PCR Thermal Cycler Dice® mini thermocycler was used and programmed as follows: initial denaturation at 94°C for 5 min, 30 cycles of amplification (denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 1 min) and a final extension at 72°C for 10 min. PCR products were analyzed by the agarose gel electrophoresis. *Enterococcus faecium* ATCC 51559 (*vanA*-containing reference strain), *E. faecalis* ATCC 51299 (*vanB*), *E. gallinarum* ATCC 49573 (*vanC1*) and *E. casseliflavus* ATCC 25788 (*vanC2/3*) were used as VRE controls.

**Statistical analysis:** The statistical software Stat View ver5.0 was used for all analyses. Categorical variables were analyzed by univariate analysis using the Chi-square method or Fisher's exact test. Differences were considered significant when the *P* value was less than 0.05.

## RESULTS

**Identification of enterococci:** Table 2 shows the results of identification of *Enterococcus* species in each group. In group 1 dogs, the most prevalent enterococcal species was *E. faecalis* (52.3%); *E. faecium* and *E. durans* were isolated from 27.7% (18 of 65) and 13.8% (9 of 65) of subjects, respectively. Similarly, the microbe most frequently isolated from healthy cats was *E. faecalis* (48.3%), followed by *E. faecium* (37.9%) and *E. durans* (10.3%). In group 2 dogs, the most prevalent species was *E. faecium*, which accounted for 60.4% of subjects. In group 2 cats, *E. faecium* was also most prevalent (61.1%). These adult dogs and cats were mainly held by *E. faecalis* and *E. faecium*. In contrast, in puppies that had not been exposed to antibiotics, *E. faecalis* was the most prevalent (66.4%) enterococcal species. In kittens, *E. hirae* was the most prevalent species, present in 40.0% of subjects.

**Antimicrobial susceptibility tests:** Results of drug susceptibility tests are shown in Table 3. No significant differences

Table 1. Multiplex PCR primers for detection of VRE

Primer specificity	Size of PCR product (bp)	Primer pair sequences	Reference
<i>vanA</i>	1,030	5'-CATGAATAGAATAAAAAGTTGCAATA-3' 5'-CCCCTTTAACGCTAATACGATCAA-3'	[6]
<i>vanB</i>	433	5'-GTGACAAACCGGAGGCGAGGA-3' 5'-CCGCCATCCTCTGCAAAAAA-3'	[6]
<i>vanC1</i>	822	5'-GGTATCAAGGAAACCTC-3' 5'-CTTCGCCATCATAGCT-3'	[8]
<i>vanC2/C3</i>	484	5'-CGGGGAAGATGGCAGTAT-3' 5'-CGCAGGGACGGTGATTTT-3'	[28]

Table 2. Identification of enterococci isolated from three groups

Group		Number of animals	Number of bacteria	<i>E. faecalis</i>	<i>E. faecium</i>	<i>E. durans</i>	<i>E. avium</i>	<i>E. gallinarum</i>	<i>E. hirae</i>	<i>E. casseliflavus</i>
1	Dogs	65	65	34 (52.3%)	18 (27.7%)	9 (13.8%)	1 (1.5%)	2 (3.1%)		1 (1.5%)
	Cats	29	29	14 (48.3%)	11 (37.9%)	3 (10.3%)		1 (3.4%)		
2	Dogs	20	48	16 (33.3%)	29 (60.4%)	2 (4.2%)		1 (2.1%)		
	Cats	9	18	6 (33.3%)	11 (61.1%)	1 (5.6%)				
3	Puppies	73	107	71 (66.4%)	17 (15.9%)	3 (2.8%)			16 (15.0%)	
	Kittens	15	25	8 (32.0%)	5 (20.0%)			2 (8.0%)	10 (40.0%)	

Group 1: Healthy group; This group, there is almost no exposure to antimicrobial agents. Group 2: Antibiotic exposure group; This group has a history of treatment of antibiotics. Group 3: Group with no exposure to antibiotics; This group is puppies, and kittens have no exposure to antimicrobial agents.

Table 3. Results of susceptibility tests

Antibiotics	Group	MIC range (mg/l)	Resistant (%)
ABPC (16) <sup>a</sup>	1	0.5–32	4/94 <sup>b</sup> (4.3%)
	2	2–512	24/64 (37.5%)
	3	<0.125–256	9/132 (6.8%)
DSM (128)	1	16–128	33/94 (35.1%)
	2	16–512	22/64 (34.4%)
	3	8–512	41/132 (31.1%)
GM (32)	1	0.5–512	42/94 (44.7%)
	2	0.5–512	31/64 (48.4%)
	3	4–512	56/132 (42.4%)
KM (128)	1	32–128	40/94 (42.6%)
	2	16–512	28/64 (43.8%)
	3	16–512	55/132 (41.7%)
EM (8)	1	0.125–128	31/94 (33.0%)
	2	0.25–512	29/64 (45.3%)
	3	0.125–512	58/132 (43.9%)
CP (32)	1	2–128	12/94 (12.8%)
	2	4–64	23/64 (35.9%)
	3	2–128	38/132 (28.8%)
ERFX (4)	1	1–128	22/94 (23.4%)
	2	1–512	48/64 (75.0%)
	3	0.25–64	16/132 (12.1%)
VCM (32)	1	0.5–8	0/94
	2	0.5–4	0/64
	3	0.5–8	0/132

a) ( ):break point (mg/l). b) resistant strains/all of isolates. Group 1: Healthy group. Group 2: Antibiotic exposure group. Group 3: Group with no exposure to antibiotics.

were observed among the three groups in the rates of resistance of enterococci to DSM, GM, KM, EM or CP. However, rates of resistance to ABPC in group 1 and group 3 were 4.3 and 6.8%, respectively. In contrast, the rate of resistance to ABPC in the enterococci from group 2 was as high as 37.5%. There was a significant difference between the two groups that were not under antibiotic pressure and the group that had been exposed to antibiotics ( $P<0.01$ ). Similarly, in group 2, the rate of resistance to ERFX was 75.0%. In contrast, in the other two groups, the rates of resistance to ERFX were 23.4 and 12.1%, respectively. Among these groups, a significant difference was also observed in the apparent resistance rates ( $P<0.01$ ). Differences of resistant rates between any groups were not related to differences of enterococcal species.

**Detection of vancomycin resistance genes:** Results of the detection of vancomycin resistance genes by multiplex PCR are shown in Table 2. *VanA* and *vanB*, which indicate high-level resistance to vancomycin, were not detected in any of the groups. In group 1, three strains of *E. gallinarum* carried *vanC1* and one strain of *E. casseliflavus* possessed the *vanC2/3*. In group 3, two strains of *E. gallinarum* was also detected *vanC1*. A similar result was obtained in group 2: one strain of *E. gallinarum* carried *vanC1*. There was no significant difference in the presence of *van* genes among the three groups.

## DISCUSSION

In this study, we clarified the differences observed in the antibiotic-resistance rates of enterococci in dogs and cats under various antibiotic selective pressures. Among three groups tested, significant differences were observed in the

rates of resistance to ABPC and ERFX. Specifically, in dogs and cats under antibiotic selective pressure, the rates of resistance to these drugs were 37.5 and 75.0%, respectively. In contrast, in groups under little or no antibiotic selective pressure, rates of resistance to these antibiotics were lower than that in a group under antibiotic selective pressure. These results suggest that the history of ABPC (penicilins; 14/29) and ERFX (quinolones; 19/29) use had a significant impact on resistance rates. In our past survey, cephalexin, ERFX, amoxicillin and ABPC were used in more than 75 percent of animal hospitals in Japan [18], implying that high rates of resistance are most likely due to the frequent use of ABPC and ERFX as antimicrobials in veterinary medicine.

In group 3, which had not been exposed to antibiotics, resistance to certain antimicrobial agents was confirmed, and we speculate that resistance to enterococci may have been propagated from parental animals. In support of this notion, *Staphylococcus aureus* on parental skin readily establishes in the infantile gut [22]. In this way, normal flora is easily transmitted from parental animals to kittens or puppies, perhaps due to poor competition from other gut bacteria. In our study, we note that the resistance rates of enterococci in the kitten-puppy group were similar to that of the healthy group.

Rodrigues *et al.* have reported that pets are important reservoirs of drug-resistant enterococci in Portugal [26], detecting resistance to ABPC in 21.2%, to EM in 100% and to ERFX in 76.9% of strains, respectively. Furthermore, Jackson *et al.* reported that ciprofloxacin-, CP- and GM-resistant enterococci were isolated from 90, 85 and 79% of dogs and cats in the United State, respectively [16]. These results are similar to the resistance rates of the antibiotic pressure group in our study. This may be probably derived from the same antibiotics used to treat dogs and cats in other countries and Japan.

In this study, VRE harboring *vanA* or *vanB* in any of the groups was not detected. In contrast, Herrero *et al.* reported that 15 VRE strains, all resistant to vancomycin and harboring *vanA*, were obtained from 12.6% of dogs at the Animal Hospital of the School of Veterinary Medicine in Madrid, Spain [12]. Based on these results, contamination of VRE in dogs and cats is considered to be minimal in Japan. As shown in the report of Kuhn *et al.* [20], Spain has a particularly high rate of VRE contamination among European countries: contamination has been estimated at 36% in humans, and at 30% in pigs. In contrast, only 5% of humans are contaminated in Japan [25], whereas contamination in food-producing animals has not been detected [19]. Collectively, these results indicate that VRE was not isolated from fecal samples of dogs and cats in Japan without horizontal transfer of *van* genes from human and livestock and without environmental pollution of *van* genes. However, in Spain, highly resistant VRE colonization has been observed in dogs and cats. If antimicrobial agents used in foreign countries are approved and gain use in Japan, there is a possibility the carrier rate of VRE in companion animals may increase in the future. Therefore, it will be necessary to monitor the presence of drug-resistant bacteria including VREs in companion animals.

In conclusion, given that overuse of antibiotics in clinical small animal medicine can cause the spread of resistance genes and resistant enterococci, we suggested that antibiotics should be used more appropriately by each veterinarian.

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