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Species identification of β-hemolytic streptococci from diseased companion animals and their antimicrobial resistance patterns in Japan (2021)

Goro Kurita[1)], Yuzo Tsuyuki[1,2)], Sachiko Shibata[3)], Megumi Itoh[2)], Mieko Goto[1)], Haruno Yoshida[1)] and Takashi Takahashi[1,*)]

[1)] Laboratory of Infectious Diseases, Graduate School of Infection Control Sciences & Ōmura Satoshi Memorial Institute, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan
[2)] Division of Clinical Laboratory, Sanritsu Zelkova Veterinary Laboratory, 3-5-5 Ogibashi, Koto-ku, Tokyo 135-0011, Japan
[3)] Division of Clinical Laboratory, Sanritsu Laboratory, 1353-25 Kamikouya, Yachiyo, Chiba 276-0022, Japan

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Abstract

We aimed to identify species and evaluate antimicrobial resistance (AMR) of ß-hemolytic streptococci recovered from Japanese companion animals in 2021. Strains were recovered from clinical specimens of 109 companion animals that exhibited symptoms/signs between April and May, 2021. We identified strains by 16S rRNA sequencing and evaluated their AMR phenotypes using the broth microdilution method. Macrolide/lincosamide/tetracycline resistance genes, including *erm*(A), *erm*(B), and *mef*(A), in addition to *tet*(M), *tet*(O), *tet*(K), *tet*(L), and *tet*(S), were amplified by polymerase chain reaction. The 16S rRNA sequencing identified ß-hemolytic streptococcal strains as *Streptococcus canis* (*n* = 102, 93.6%), *S. dysgalactiae* subsp. *equisimilis* (*n* = 4), *S. agalactiae* (*n* = 2), and *S. dysgalactiae* subsp. *dysgalactiae* (*n* = 1). Overall AMR rates were 34.9% for minocycline, 22.0% for erythromycin, 22.9% for azithromycin, 21.1% for clindamycin, and 10.1% for levofloxacin. We found macrolide/lincosamide/tetracycline resistance gene detection of *tet*(M) (9.2%), *tet*(O) (26.6 %), *tet*(L) (1.8%), *erm*(B) (22.0%), and *mef*(A) (1.8%). Our findings support the unique features of β-hemolytic streptococci from companion animals (dominant *S. canis* isolation and dominant resistance to tetracycline), along with dominant tetracycline resistance genes, in agreement with those of ß-hemolytic streptococci in 2017.

Key Words: species identification, antimicrobial resistance, β-hemolytic streptococci, companion animals, Japan

Introduction

To identify streptococcal strains, cell wall carbohydrate antigen grouping by Lancefield antisera is used, along with the properties of β-/α-/γ-hemolysis on sheep blood agar plates in veterinary clinical situations. For example, outside veterinary laboratories (with a microorganism testing room) provide identification results of β-hemolytic groups G/C/B/A streptococci to veterinarians. In a previous study[6)], we reported species-level identification of

* Corresponding author:
Prof. Takashi Takahashi
Laboratory of Infectious Diseases, Graduate School of Infection Control Sciences & Ōmura Satoshi Memorial Institute, Kitasato University
5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan
Tel: +81-3-5791-6428, Fax: +81-3-5791-6441, E-mail: taka2si@lisci.kitasato-u.ac.jp
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β-hemolytic streptococcal strains from diseased companion animals (i.e., dogs and cats), based on 16S rRNA sequencing data. Group G strains included *Streptococcus canis* and *S. dysgalactiae* subsp. *equisimilis*, while group C consisted of *S. dysgalactiae* subsp. *equisimilis*, *S. dysgalactiae* subsp. *dysgalactiae*, and *S. equi* subsp. *zooepidemicus*. Groups B and A strains included only one species for each: *S. agalactiae* and *S. dysgalactiae* subsp. *equisimilis*, respectively. Thus, groups G/C strains possess multiple streptococcal species and their antimicrobial resistance (AMR) data should be evaluated for each species (not for each group).

S. canis, which was first reported in 1986[3], forms large, smooth, gray-white-colored colonies with strong β-hemolysis on blood agar. It also shows a Christie-Atkins-Munch-Peterson (CAMP) positive reaction as a biochemical property. This bacterium is an emerging pathogen that causes self-limiting dermatitis. However, *S. canis* is associated with multiple documented pyogenic syndromes in cats by an institutional boarding facility[14]. In some animals, this bacterial infection may lead to severe diseases in companion animals, including arthritis, streptococcal toxic shock syndrome, necrotizing fasciitis, septicemia, and pneumonia[4,11]. *S. canis*-related invasive infections may be underdiagnosed because species-level identification has not been conducted for the same.

S. dysgalactiae subsp. *equisimilis* strains also form large, smooth, gray-white-colored colonies with strong β-hemolysis, which has microbiological properties similar to those of *S. canis*. This bacterium is zoonotic and is transmitted among some animals (i.e., healthy dogs, cats, and horses) and humans[1,17]. On the other hand, *S. dysgalactiae* subsp. *dysgalactiae* shows a variety of α-/β-/γ-hemolysis, which is mainly recovered from pigs and cows[21]. Both subspecies indicate β-D-glucuronidase positive reaction as a biochemical property.

S. agalactiae strains exhibit β-hemolytic activity, along with a CAMP-positive reaction. This bacterium can grow in milk as well as in rectal and vaginal specimens[18] and causes intramammary infections (mastitis) among cows. A recent study demonstrated that *S. agalactiae* clones were shared by cows and herdspersons[18]. This microorganism is a part of the host commensal flora in the urogenital and lower gastrointestinal tracts in 10%–40% of healthy human adults[5]. Thus, human and veterinary clinicians should be aware of the potential risk of infectious diseases caused by this bacterium.

We documented significant changes in AMR rates among *S. canis* strains recovered in 2017 and 2015[6]. Tetracycline and macrolide/lincosamide resistance rates seem to have increased rapidly among *S. canis* strains in Japan. The prevalence of tetracycline resistance was 41.0% vs. 22.1% ($P = 0.01$), the prevalence of macrolide resistance 18.3% vs. 5.9% ($P = 0.016$), and the prevalence of lincosamide resistance 17.1% vs. 5.9% ($P = 0.039$). According to a report from the National Veterinary Assay Laboratory[13], quinolone and tetracycline antimicrobials constituted 7.0% and 1.9% of the overall antibiotics (converted weight in kilograms to bulk powder) used for dogs and cats in 2016. Therefore, we need to monitor the changes in AMR rates among β-hemolytic streptococci, in particular *S. canis*, in the future.

Few studies have assessed all β-hemolytic streptococci from diseased dogs/cats in Japan in terms of species-level identification and their AMR phenotypes or macrolide/lincosamide/tetracycline resistance genotypes. The purpose of this study was to determine the species-level identification and AMR patterns of β-hemolytic streptococcal strains recovered from companion animals in 2021.

Materials and Methods

Collecting β-hemolytic streptococcal strains and host information

Clinical specimens (submitted by veterinarians), along with culture request sheets (including host information), were immediately

Table 1. Oligonucleotide primers for targeted genes and their polymerase chain reaction (PCR) amplicon sizes

Targeted gene (specific species)	Primer	Direction	Sequence (5'→3')	Expected amplicon size (bp)	Reference
16S rRNA (universal)	27F[1)]	Forward	AGAGTTTGATCMTGGCTCA	1,497	(6,22)
	1485R[1)]	Reverse	G TACGGTTACCTTGTTACGAC		
cfg (S. canis)	camp-canis-I	Forward	CAATTAACTAATAAGGTAGAACAG	238	(8)
	camp-canis-II	Reverse	CTCTCTCAAAACGGGTG		
dltS (*S. agalactiae*)	dlts-F	Forward	CTGTAAGTCTTTATCTTTCTCG	199	(16)
	dlts-R	Reverse	TCCATTCGCTTAGTCTCC		
emm (S. dysgalactiae)	emm1[1)]	Forward	TATTSGCTTAGAAAATTAA	Variable and 180 bp used for genotyping	(20)[2)]
	emm2	Reverse	GCAAGTTCTTCAGCTTGTTT		

[1)] The same primers are used for both PCR amplification and sequencing.

[2)] Basic local alignment search tool of obtained *emm* sequence was performed at the following URL: https://www2.cdc.gov/vaccines/biotech/strepblast.asp.

sent to the Sanritsu Zelkova Veterinary Laboratory to determine the causative bacteria. These specimens were taken from diseased companion animals, which visited clinics/hospitals during the study period from April 1st through May 31st, 2021, with significant symptoms/signs observed by their pet owners/veterinarians[6)]. The specimens were derived from either sterile or non-sterile sites. Each specimen was inoculated on sheep blood agar plates and incubated in 5% CO_2 at 35°C for 24 h. Gray-white colonies with β-hemolysis were subjected to latex agglutination testing with specific antisera for Lancefield carbohydrate antigen grouping (Seroiden Strepto Kit, Eiken Chemical Co., Ltd., Tokyo, Japan). All β-hemolytic streptococcal strains (one strain/one animal) were stored between –70°C and –80°C until further genotypic/phenotypic analyses were performed. Host information (species, sex, age, clinical specimen, date collected, and Japanese prefecture in which veterinarians worked) was obtained from the request sheets. Streptococcal strains (with host information) were sent to the Laboratory of Infectious Diseases, Graduate School of Infection Control Sciences and Ōmura Satoshi Memorial Institute.

Determining streptococcal species identification

Genomic DNA was extracted from the β-hemolytic streptococcal strains by suspending in Tris-EDTA buffer and boiling at 97°C for 10 min, as reported previously[10)]. We finally identified β-hemolytic streptococcal strains at the species/subspecies level on the basis of 16S rRNA sequencing results[6,22)] (Table 1). The strains were unambiguously identified, based on only one choice with ≥98.7% similarity to the 16S rRNA sequence of the corresponding type strain.

Confirming accurate species-level identification

To confirm the accuracy of the species-level identification by 16S rRNA sequencing, we performed the polymerase chain reaction (PCR)-based amplification of an *S. canis*-specific *cfg* gene (encoding CAMP-factor)[8)], *S. agalactiae*-specific *dltS* gene (encoding histidine kinase membrane sensor protein)[16)], and *S. dysgalactiae*-specific *emm* gene (encoding M protein)[20)]. Table 1 shows oligonucleotide primers for these genes and their PCR amplicon sizes. *emm* genotyping was conducted using the Basic Local Alignment Search Tool at the Strep Laboratory of Centers for Disease Control and Prevention (https://www2.cdc.gov/vaccines/biotech/strepblast.asp).

Determining AMR phenotypes and macrolide/lincosamide/tetracycline resistance genotypes

Minimum inhibitory concentrations (MICs, μg/mL) of 14 antimicrobials (penicillin G, ampicillin, cefepime, cefotaxime, ceftriaxone, cefozopran,

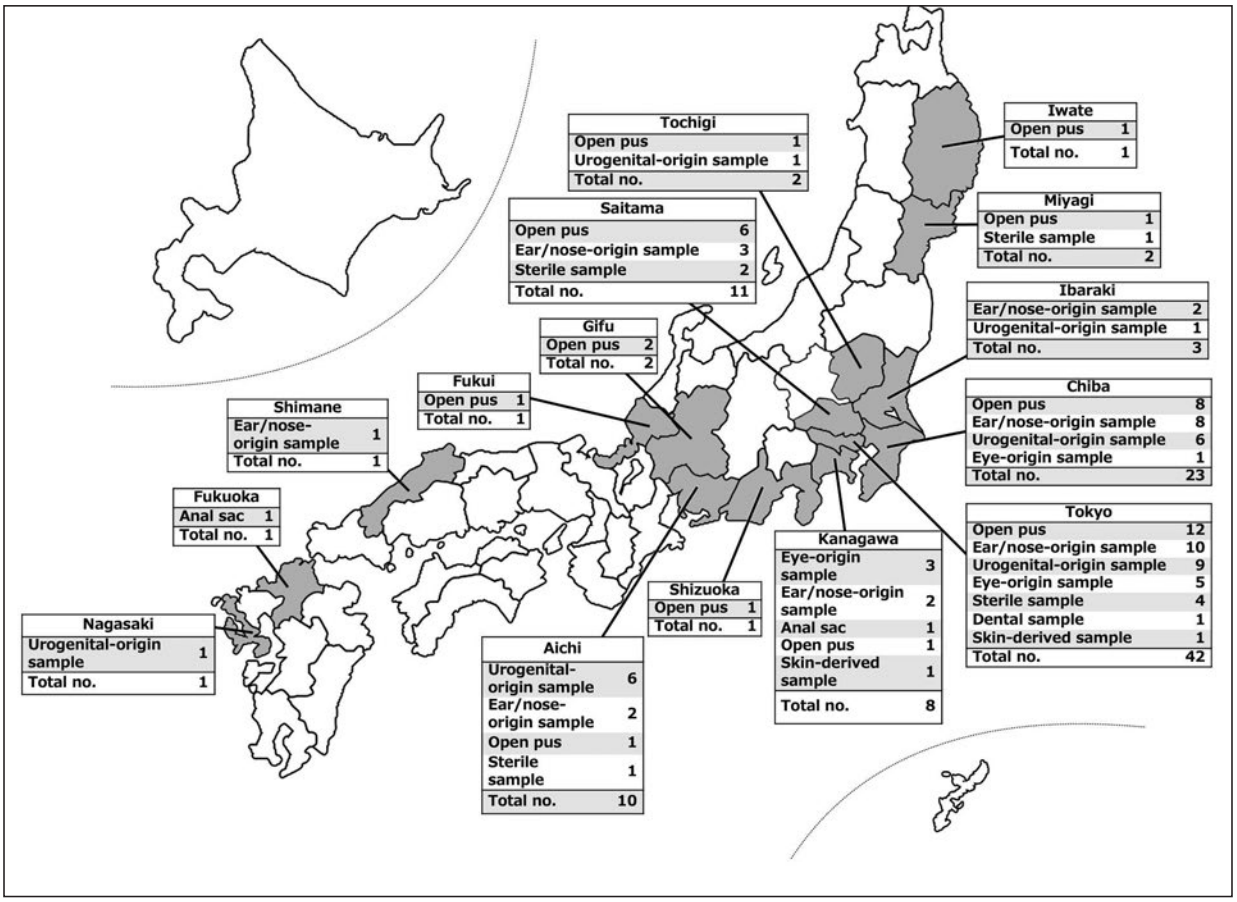

Fig. 1.
Japanese geographical distribution of clinical specimens collected between April and May in 2021, to recover β-hemolytic streptococcal strains from diseased dogs and cats. The main prefectures were Tokyo (n = 42), Chiba (n = 23), Saitama (n = 11), Aichi (n = 10), and Kanagawa (n = 8). The specimens were from sterile sites (n = 8), whereas those were from non-sterile sites (n = 101).

meropenem, minocycline, erythromycin, azithromycin, clindamycin, levofloxacin, vancomycin, and chloramphenicol) were examined using broth microdilution method (MICroFAST Panel Type 7J for *Streptococcus* spp., Beckman Coulter Inc., Tokyo, Japan) as previously described[6], according to the Clinical and Laboratory Standards Institute (CLSI) guidelines for β-hemolytic streptococci[2]. When determining minocycline resistance, we used the tetracycline breakpoint in accordance with CLSI guidelines. The quality of antimicrobial susceptibility testing was controlled using two American Type Culture Collection (ATCC) strains (*Enterococcus faecalis* ATCC 29212 and *S. pneumoniae* ATCC 4961). The AMR rate and MIC values were measured for each β-hemolytic streptococcal strain against the antimicrobial agents. We also calculated the lowest antimicrobial concentrations at which 50% (MIC_{50}) and 90% (MIC_{90}) of each species were inhibited.

The presence of macrolide/lincosamide resistance genes [*erm*(A), *erm*(B), and *mef*(A)], in addition to tetracycline resistance genes [*tet*(M), *tet*(O), *tet*(K), *tet*(L), and *tet*(S)] among all β-hemolytic streptococcal strains was confirmed by PCR[7,12]. The correct sequences of these resistance genes from several PCR-positive strains were confirmed via direct sequencing analysis of purified amplicons. The significance of differences between AMR phenotypes and macrolide/lincosamide/tetracycline resistance genotypes in 2021 and 2017 were assessed.

Table 2. Species/subspecies ID of dog/cat-origin β-hemolytic streptococci based on 16S rRNA sequencing data according to cell wall carbohydrate antigenicity

Lancefield group	ID data of the streptococci isolated in 2021	ID data of the streptococci isolated in 2017
G	*S. canis* (93.6%), *S. dysgalactiae* subsp. *equisimilis* (0.9%)[1)]	*S. canis* (89.3%), *S. dysgalactiae* subsp. *equisimilis* (1.5%)
C	*S. dysgalactiae* subsp. *equisimilis* (2.8%)[1)]	*S. dysgalactiae* subsp. *equisimilis* (1.5%), *S. dysgalactiae* subsp. *dysgalactiae* (0.8%), *S. equi* subsp. *zooepidemicus* (0.8%)
B	*S. agalactiae* (1.8%)	*S. agalactiae* (5.3%)
A	*S. dysgalactiae* subsp. *dysgalactiae* (0.9%)[2)]	*S. dysgalactiae* subsp. *equisimilis* (0.8%)
Total no. (%)	109 (100%)	131 (100%)

ID, identification. The parentheses indicate the percentages of species/subspecies ID among the total streptococci. We cited ID data of the streptococci isolated in 2017 from our previous study **(6)**.

[1)] Four strains of *S. dysgalactiae* subsp. *equisimilis* possessed *stG840*.0, *stC9431*.0, *stC37*.0, and *stL1929*.1.

[2)] One strain of *S. dysgalactiae* subsp. *dysgalactiae* possessed *stC46*.2 (GenBank accession no. LC649931).

Ethical statement

The Ethics Committee at the Sanritsu Zelkova Veterinary Laboratory reviewed and approved the study design (approval number SZ20210825) to ensure the privacy of affected dogs and cats.

Statistical analysis

We used Fisher's exact probability test (two-sided) to assess significant differences in categorical variables between data obtained in 2021 and 2017. Statcel4 application (OMS Publisher, Tokyo, Japan) was used for this analysis. Statistical significance was set at $P<0.05$.

Results

β-hemolytic streptococcal strains and host information

Of the 2,112 clinical specimens obtained from the dogs (n = 1,464) and cats (n = 648), 109 β-hemolytic streptococcal strains (isolation rate 5.2%), including groups G (n = 103), C (n = 3), B (n = 2), and A (n = 1), were collected from April 1st, 2021 through May 31st, 2021. Fig. 1 shows the geographical distribution of clinical specimens collected during the study period. The prefectures sampled were Tokyo (n = 42), Chiba (n = 23), Saitama (n = 11), Aichi (n = 10), Kanagawa (n = 8), Ibaraki (n = 3), and Miyagi/Tochigi/Gifu (n = 2 for each). Strains were obtained from sterile specimens of uterine content (n = 7) and ascites (n = 1). There were also those obtained from open pus (n = 36), ear/nose-origin (n = 28), urogenital tracts-derived (n = 24), eye-origin (n = 9), anal glandular fluid (n = 2), and other specimens (n = 2) of dogs (n = 98) and cats (n = 11). The companion animal demographics were as follows: mean age, 10.9 years; age range, 1–17 years; sex, 50 males and 59 females.

Identification by 16S rRNA sequencing

Table 2 indicates the species/subspecies identification of β-hemolytic streptococci based on 16S rRNA sequencing data according to the Lancefield group. A total of 109 streptococcal strains consisted of *S. canis* (n = 102, 93.6%) belonging to group G, *S. dysgalactiae* subsp. *equisimilis* (n = 4, 3.7%) belonging to groups C and G, *S. agalactiae* (n = 2, 1.8%) belonging to group B, and *S. dysgalactiae* subsp. *dysgalactiae* (n = 1, 0.9%) belonging to group A. There was no significant difference in the prevalence between different species identified in 2021 and 2017.

Evaluation of identification validity

All *S. canis* strains possessed the specific *cfg*, and two *S. agalactiae* strains contained the specific *dltS*. Four *S. dysgalactiae* subsp. *equisimilis* strains included *emm* genotypes of *stG840*.0, *stC9431*.0, *stC37*.0, and *stL1929*.1. One

Table 3. Antimicrobial resistance phenotypes and macrolide/lincosamide/tetracycline resistance genotypes among β-hemolytic streptococcal strains in 2021/2017

Resistance phenotype and genotype	Percentage of β-hemolytic streptococcal strains in 2021 (n = 109)	Percentage of β-hemolytic streptococcal strains in 2017 (n = 131)
Resistance to minocycline	34.9 (n = 38)	39.7 (n = 52)
Resistance to erythromycin	22.0 (n = 24)	19.8 (n = 26)
Resistance to azithromycin	22.9 (n = 25)	19.8 (n = 26)
Resistance to clindamycin	21.1 (n = 23)	17.6 (n = 23)
Resistance to levofloxacin	10.1 (n = 11)	4.6 (n = 6)
tet(M)	9.2 (n = 10)	16.0 (n = 21)
tet(O)	26.6 (n = 29)	29.8 (n = 39)
tet(L)	1.8 (n = 2)	2.3 (n = 3)
tet(S)	0	2.3 (n = 3)
erm(B)	22.0 (n = 24)	18.3 (n = 24)
mef(A)	1.8 (n = 2)	3.8 (n = 5)

Antimicrobial resistance was determined based on the Clinical and Laboratory Standards Institute (CLSI) guidelines (M100-S25).

When determining resistance to minocycline, we used the tetracycline breakpoint in accordance with CLSI guidelines.

We cited resistance data of streptococci isolated in 2017 from our previous study [6].

S. dysgalactiae subsp. *dysgalactiae* strain had an *emm* genotype of *stC46*.2 (GenBank accession no. LC649931).

AMR phenotypes and macrolide/lincosamide/tetracycline resistance genotypes

The prevalence of antimicrobial resistance and resistance genes among β-hemolytic streptococcal strains in 2021 are shown in Table 3. Overall AMR rates were 34.9% (n = 38) for minocycline, 22.0% (n = 24) for erythromycin, 22.9% (n = 25) for azithromycin, 21.1% (n = 23) for clindamycin, and 10.1% (n = 11) for levofloxacin. There were no significant differences between the rates of resistance to the five antimicrobials in 2021 and those in 2017. None of the strains were resistant to β-lactams (including meropenem) and vancomycin: one strain was non-susceptible to chloramphenicol (MIC 8 μg/mL). We found MIC_{50}/MIC_{90} of minocycline (1/>4 μg/mL), erythromycin (<0.12/>2 μg/mL), azithromycin (<0.25/>4 μg/mL), clindamycin (<0.12/>1 μg/mL), and levofloxacin (0.5/>8 μg/mL) against *S. canis* strains (n = 102). Thirty-eight minocycline-resistant strains were either *S. canis* (n = 35), *S. agalactiae* (n = 2), or *S. dysgalactiae* subsp. *equisimilis* (n = 1). These minocycline-resistant strains also exhibited macrolide/lincosamide and/or quinolone resistance, except for one strain that showed only macrolide/lincosamide resistance. Thirty-nine resistant strains were recovered from sterile (uterine content, n = 3) and non-sterile (open pus, ear/nose-origin, eye-origin, urogenital tracts-origin, tooth-origin, and skin-origin, n = 36) sites and were confirmed in nine of 15 prefectures.

We found the macrolide/lincosamide/tetracycline resistance genes *tet*(M) (n = 10, 9.2%), *tet*(O) (n = 29, 26.6 %), *tet*(L) (n = 2, 1.8%), *erm*(B) (n = 24, 22.0%), and *mef*(A) (n = 2, 1.8%). There was no amplification of either *tet*(K), *tet*(S), or *erm*(A) among the strains. We observed no significant differences between the rates of detection of resistance genes in 2021 and of those in 2017. *S. canis* included 37 (36.3%) strains with these resistance genes. Forty-one strains with resistance genes were recovered from sterile (n = 3) and non-sterile (n = 38) sites and were confirmed in nine of 15 prefectures.

Discussion

Of the 109 β-hemolytic streptococci, we identified four different species/subspecies (*S.*

Table 4. Changes in resistance rates among *S. canis* strains from 2021, 2017, and 2015

Antimicrobial	Resistance rate (%) in 2021 (n = 102)	Resistance rate (%) in 2017 (n = 117)	Resistance rate (%) in 2015 (n = 68)
Tetracycline	34.3 (n = 35)	41.0 (n = 48)	22.1 (n = 15)
Macrolide	22.5 (n = 23)	18.8 (n = 22)	5.9 (n = 4)
Lincosamide	15.6 (n = 16)	17.1 (n = 20)	5.9 (n = 4)
Quinolone	10.8 (n = 11)	3.4 (n = 4)	4.4 (n = 3)

Antimicrobial resistance was determined based on the Clinical and Laboratory Standards Institute (CLSI) guidelines (M100-S25/M100-S22).

When determining resistance to minocycline, we used the tetracycline breakpoint in accordance with CLSI guidelines.

We cited resistance data of streptococci recovered from our previous studies [6,22].

canis belonging to group G, *S. dysgalactiae* subsp. *equisimilis* belonging to groups C and G, *S. agalactiae* belonging to group B, and *S. dysgalactiae* subsp. *dysgalactiae* belonging to group A). In contrast, the following five species and subspecies were identified in our previous study[6]: *S. canis* (group G), *S. agalactiae* (group B), *S. dysgalactiae* subsp. *equisimilis* (groups C, G, and A), *S. dysgalactiae* subsp. *dysgalactiae* (group C), and *S. equi* subsp. *zooepidemicus* (group C). In agreement with these observations, the prevalent streptococcal strains recovered from dogs in the USA were *S. canis*, *S. dysgalactiae* subsp. *equisimilis*, and *S. equi* subsp. *zooepidemicus*[11]. We need to collect and analyze more β-hemolytic streptococci recovered from dog/cat-derived clinical specimens to confirm the variations of the isolation and AMR rates.

Four *emm* genotypes (*stG840*.0, *stC9431*.0, *stC37*.0, and *stL1929*.1) were detected in the *S. dysgalactiae* subsp. *equisimilis* strains in the 2021-investigation. Additionally, three *emm* types (*stC9431*.0, *stG245*.0, and *stG485*.0) were observed in 2017[6], and two *emm* types (*stC9431*.0, *stG6792*.3, and *stC1929*.1) were found in 2015[22]. These *emm* genotypes seem to contribute to the understanding of the transmission profiles of *S. dysgalactiae* subsp. *equisimilis* between dogs/cats and humans, although these studies had small numbers of strains. We should further increase the *emm* genotype data in the animal-derived *S. dysgalactiae* subsp. *equisimilis*.

Table 4 shows the changes in resistance rates among *S. canis* strains from 2021, 2017[6], and 2015[22] because of the dominant *S. canis* isolation among β-hemolytic streptococci from 2021. Three investigations revealed the dominant resistance to tetracycline. Therefore, Japanese veterinarians should be informed of the necessity of careful administration of tetracycline in practice.

We constructed draft genome sequences of seven *S. canis* strains from dogs and cats that were collected during the study period of April–May 2017[23]. The resistance genotypes were determined by inserting our contig sequences into the Web-based application ResFinder version 3.2 (https://cge.cbs.dtu.dk/services/ResFinder/), which is managed by the Center for Genomic Epidemiology[24]. Although there were no macrolide/lincosamide/tetracycline resistance genes in the four strains, we found *tet*(S), *erm*(B), and *erm*(B)+*tet*(O) in the three strains, showing the same resistance genotyping data by PCR. In addition, we found the *erm*(B) variant sequence of *S. agalactiae* clindamycin-resistant erythromycin-susceptible strain based on whole genome sequences, not direct sequencing data with purified amplicons by PCR[19]. This variant sequence contained the insertion of an IS1216E element at nucleotide position 642, which resulted in the deletion of a segment spanning nucleotides 642–738 (97 bp). To confirm the validity of resistance genotyping data by PCR in 2021, we needed to construct whole genome sequences of the 2021-streptococcal strains. It would be better if we could obtain the complete circular genome sequences containing plasmid(s) by conducting both Illumina sequencing (using MiSeq) for short-reads and Nanopore sequencing (using MinION) for long-reads, followed by the hybrid assembly

(using Unicycler)[9].

Our study has two limitations. First, this investigation had limited host information (species, sex, age, clinical specimen, bacterial isolation date, and prefecture in which veterinarians worked). In future investigations, detailed information, in particular antimicrobial dosing used for the animals, needs to be collected from veterinarians to estimate the antimicrobial selective pressure. Second, the number of strains from sterile sites of uterine content (n = 7) and ascites (n = 1) was small. We experienced severe soft tissue infection with septic shock caused by *S. canis* isolated from the blood/necrotic tissue of a miniature dachshund[15]. Further investigation including sterile specimens from severe diseases should be conducted to understand the situations of severe infections (streptococcal toxic shock syndrome, severe soft tissue infection, etc.).

In conclusion, our findings support dominant *S. canis* isolation and dominant resistance to tetracycline, along with dominant tetracycline resistance genes among β-hemolytic streptococcal strains from diseased dogs and cats in Japan (April–May, 2021). These unique features could be useful to Japanese veterinarians when examining and treating animals with clinical symptoms/signs suggestive of streptococcal infections. In the future, these strains should be monitored throughout the country sequentially and additional β-hemolytic streptococcal strains need to be characterized.

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Conflict of interest

None to declare.

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