Characteristics of eye-origin *Streptococcus canis*: Correlation between antimicrobial resistance and epidemiological features

眼科領域由来犬レンサ球菌が保有する特性：抗菌薬耐性と疫学特性との連関

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I. General Introduction

1. Human-animal bond

It is posited that dogs understand human emotions (1). This capability has been instrumental in forging relationships between dogs and humans. Presently, dogs extend their roles beyond being guide and hearing assistance animals to encompass functions such as disaster rescue and even disease detection (2-5). Furthermore, the purview of canines is not confined to pragmatic tasks alone. Recent insights reveal that companion animals contribute significantly to the alleviation of depression and loneliness, the enhancement of social interactions and skills, and the reduction of anxiety and agitation, thereby yielding psychological benefits (6). After losing their jobs as rodent hunters, cats have often been perceived as providing fewer direct benefits to humans than dogs. Meanwhile, recent attention has been focused on the psychological connections and relationships cats foster with humans (7). Additionally, studies have begun to scientifically elucidate the health-enhancing effects of cats on humans, indicating that these effects may include stress reduction and increased alertness (8). Moreover, in response to the recent issue of an aging society in Japan, there has been growing interest in the economic aspects of elderly healthcare costs and their favorable relationship with pet ownership (9).

Thus, companion animals have become entities that provide significant psychological benefits to humans, not just practical advantages. This evolving recognition underscores the inextricable role of companion animals in human life (10).

2. Infectious diseases and the human-animal relationship

Historically, from the bubonic plague to COVID-19, infectious diseases have profoundly affected human lives. Many of these diseases, including notable ones, are zoonotic. Research shows that 61% (868 out of 1,415) of known human pathogens are zoonotic, and this figure rises to 75% (132 out of 175) for emerging infectious diseases (11).

The inevitably increasing closeness between humans and animals raises concerns about infectious diseases. While generally beneficial, this intimate relationship also increases the risk of zoonotic diseases, which are transmissible between animals and humans. Closer human-animal interactions, particularly with companion animals, allow these diseases to cross species barriers more easily. From such circumstances, a comprehensive approach to human and animal health, including their environment, would be incredibly beneficial for humanity. The growing interdependence of human and animal health emphasizes the necessity for an integrated approach that considers the well-being of both, leading to the concept of One Health.

3. The One Health approach

The One Health approach, postulated by the World Health Organization (WHO), is a globally recognized concept. It is a comprehensive approach to maintaining the health of humans, animals, and the environment in balance and optimal condition (12).

In this concept, "animals" encompasses a broad spectrum, including wildlife, industrial livestock such as cattle and swine, and companion animals like dogs and cats, maintaining a close relationship with humans.

Confronting zoonotic bacteria and antimicrobial resistance (AMR) in companion animals is also a critical challenge of this approach.

In companion animal medicine, where public health insurance does not cover treatments, the selection and use of antimicrobials largely depend on the individual veterinarian's judgment. As a result, drugs considered critically essential, such as fluoroquinolones and third-generation cephalosporins (13), may be used unrestrictedly as initial treatments despite their package inserts advising against such first-line use.

In spite of alerts from companion animal healthcare experts (13, 14), imprudent antimicrobial practices persist, often due to a lack of education on judicious use. The situation is aggravated by the free-market approach of veterinary care, which can lead to resistance to change by hospital administrators. Moreover, a greater awareness among leaders in the field, including veterinary specialists, is needed to curb the continuation of these issues.

Recently, there has been an increase in AMR rates of bacteria isolated from dogs and cats in Japan (15), probably due to the increased or inappropriate use of antimicrobials, partly in response to growing awareness of pet owners' rights and veterinarians' strong sense of mission to meet these demands. Furthermore, several papers have warned about the transmission of bacteria, including drug-resistant strains, between companion animals and humans (14-17). To address these situations, veterinary care professionals, including veterinarians and veterinary nurses for companion animals（VNCA), must possess accurate knowledge about bacterial infections and AMR, providing appropriate prevention and treatment.

4. *Streptococcus* species

Streptococcus spp. are gram-positive, non-spore-forming, spherical, or ovoid bacteria with a diameter of less than two µm. These nonmotile bacteria are typically in pairs or chains in liquid media. There are over 50 species

within this genus; most are facultatively anaerobic and possess a chemo-organotrophic nature with a fermentative metabolic pathway. They are further divided into six species groups: Anginosus, Bovis, Mitis, Mutans, Pyogenic, and Salivarius (18).

Their cell-wall polysaccharides form the foundational basis of the Lancefield antigenic grouping (19). These bacteria are also classified based on their hemolytic properties (18). Among them, β-hemolytic streptococci are important pathogens of companion animals and humans, although they are also members of normal flora (20, 21).

More research is needed regarding the infection status of these bacteria in companion animals because many aspects of their prevalence in the community are largely unknown.

Consequently, this study aims to ascertain the infection status of *Streptococcus* spp. in companion animals. Additionally, it seeks to elucidate the unique incidence of antimicrobial resistance in the ophthalmological domain, as revealed by the investigation. This research endeavors to fill the gaps in our understanding of the epidemiological landscape of streptococcal infections in companion animals, focusing on antimicrobial resistance patterns and their contributing factors.

II. Chapter 1

Prevalence and characteristics of β-hemolytic streptococci isolated from diseased dogs and cats

1. Introduction

Major species of β-hemolytic streptococci isolated from companion animals include *Streptococcus canis, Streptococcus agalactiae, Streptococcus dysgalactiae* subsp. *equisimilis*, *Streptococcus dysgalactiae* subsp. *dysgalactiae*, and *Streptococcus equi* subsp. *zooepidemicus* (22-24). They all belong to the pyogenic group and have been reported to cause various infections in both animals and humans (18, 20, 21).

Almost all the isolates of *S. canis* are categorized within the Lancefield group G, with a noted exception (25). This bacterium was first reported in 1986, and since then, there have been reports of its isolation from various infections (26). Significantly, catastrophic infections of S. canis in shelter cats have been documented (20), and canine cases of severe soft tissue infection (SSTI) have been reported (27, 28). Furthermore, *S. canis* has been implicated in human infectious diseases, including septicemia, cellulitis, and endocarditis (29-31).

S. agalactiae, a representative of Lancefield group B streptococci, is an essential cause of mastitis in dairy cattle, resulting in economic losses (32), and has also been isolated from companion animals, causing bacteremia, endocarditis, and neonatal sepsis in dogs (fading puppy syndrome) (20). In cats, it causes peritonitis and endometritis with bacteremia (20). In humans, it causes urinary tract infection and septicemia in pregnant women and septicemia and meningitis in newborns (18).

S. dysgalactiae is recognized to have subspecies *S. dysgalactiae* subsp. *equisimilis* and *S. dysgalactiae* subsp. *dysgalactiae*. *S. dysgalactiae* subsp. *equisimilis*, belonging to groups G, C, A, and L, is known to cause conditions

in dogs ranging from dermatitis to septicemia, and in humans, infections from minor cutaneous infection to life-threatening streptococcal toxic shock syndrome (STSS) (33). *S. dysgalactiae* subsp. *dysgalactiae* has been reported to cause neonatal death in dogs (20) and has also been associated with cases of septicemia in humans, albeit rarely.

S. equi subsp. *zooepidemicus*, while primarily known as the causative agent of upper respiratory disease in horses (34), has also been isolated from dogs (23) and is implicated in septicemia and endocarditis in humans (35).

However, in Japanese companion animal practice, skin and urinary tract diseases are relatively prevalent (36), and the high AMR rates of *Staphylococcus* spp. and *Escherichia coli* (15), which are predominantly isolated from these sources, may lead to a focus primarily only on these bacteria. Consequently, the significance of β-hemolytic streptococcal infections might be underestimated.

2. Purpose

The author conducted this investigation to comprehend the isolation status and antimicrobial resistance of β-hemolytic streptococci in companion animals, which closely interact with humans and serve as a zoonotic reservoir for cross-species infections. By comparing our findings with the prior research (23), our objective was to discern trends in antimicrobial resistance and to furnish veterinary professionals with critical information pertinent to therapeutic interventions and the implications for human health. To actualize this objective, the author conducted the following study.

1. Survey of the isolation status of β-hemolytic streptococci in diseased dogs and cats
2. Identification of isolates by 16s rRNA and species-specific genes

3. Evaluation of the phenotypic and genotypic profiles of the isolates' antimicrobial susceptibility

3. Materials and methods

3-1. Collection of the isolates

3-1-1. Collection of the clinical specimens

The study used β-hemolytic streptococci isolated from diseased dogs and cats at the Sanritsu Zelkova Veterinary Laboratory from April 1 to May 31, 2021. These samples were collected by companion animal veterinary practitioners and sent to the laboratory for bacteriological analysis with order sheets detailing the animal's residence, species, age, gender, and isolation site. Among the collected specimens, isolates that demonstrated β-hemolysis on blood agar plates and morphologically identified as streptococci were determined grouping by the Lancefield classification kit (Seroiden Strepto Kit Eiken®; Eiken Chemical Co., Tokyo, Japan) in the laboratory (Figure 1).

3-1-2. Preservation of the isolates

The provided isolates were suspended in a Brain Heart Infusion(BHI) liquid medium supplemented with concentrated glycerin. They were then preserved at temperatures ranging between -80°C and -70°C in our laboratory.

3-1-3. Collection of the comparative data

We also used the dataset from Fukushima and colleagues' 2019 paper on β-hemolytic streptococci (23) to compare and evaluate each element of the data.

3-2. Species identification

3-2-1. DNA extraction

After storage, each preserved isolate was cultured on sheep blood agar plates (Kohjin Bio Co., Ltd. Saitama, Japan) under 5% CO_2 at 35°C overnight. All isolates were suspended in 100 μL of TE (Tris-EDTA) buffer (Table. 1) to achieve turbidity visually equivalent to a McFarland standard of 0.5. Subsequently, lysis of the bacteria was performed at 97°C for 10 minutes using a thermal cycler (LifeECO TC-96/G/H(b)C, Hangzhou Bioer Technology Co. Ltd.) (37). After lysis, the samples were centrifuged at 10,000 rpm for 2 minutes by a benchtop centrifuge to harvest supernatant for DNA solution for the polymerase chain reaction (PCR) template.

3-2-2. Analysis of 16S rRNA sequence

Amplification of the DNA encoding 16S rRNA was conducted using the template DNA solutions under the conditions specified in Tables 2 and 3 (23, 24). The resulting PCR amplification products were subjected to gel electrophoresis, followed by ethidium bromide staining, and their presence was confirmed by a U.V. transilluminator（Vilber-Lourmat ECX-F15.M). The amplified products were purified using the QIAquick PCR Purification Kit (QIAGEN K.K.). The concentration and purity of the purified DNA were measured using a NanoDrop™ spectrophotometer（Thermo Scientific). Subsequently, sequencing reactions and purification were conducted using BigDye Terminator v3.1 and BigDye Xterminator, followed by electrophoresis utilizing the 3130x1 Genetic Analyzer. The sequence waveforms obtained were verified using the Finch TV (freeware by Geospiza: https://digitalworldbiology.com/finchtv; last accessed December 31, 2023). Homology analysis of the acquired sequences was performed on the National

Library of Medicine's online platform (https://blast.ncbi.nlm.nih.gov/Blast.cgi; last accessed December 31, 2023).

Based on the 16S rRNA sequencing results, we conclusively identified the β-hemolytic streptococcal isolates at the species/subspecies level. These isolates were distinctly recognized, having a similarity of ≥ 98.7% to the 16S rRNA sequence of their respective type strains (23, 24).

3-2-3. Confirmation of species identification

Species-specific genes inherent to each β-hemolytic streptococcal species were amplified to further identification precision. For *S. canis*, the *cfg* gene (encoding CAMP-factor) (38); for *S. agalactiae*, the *dlt*S gene (encoding histidine kinase membrane sensor protein) (39); and for *S. dysgalactiae*, the *emm* gene (encoding M-protein) was detected (40). The primers used and the reaction conditions are shown in Tables 4 and 5**.**

3-3. Evaluation of antimicrobial susceptibility

3-3-1. Antimicrobial susceptibility testing (AST)

Minimum inhibitory concentrations (MICs, μg/ mL) of 14 antimicrobials (penicillin G, ampicillin, cefepime, cefotaxime, ceftriaxone, cefozopran, 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) (Figure 1) (23), based on the Clinical and Laboratory Standards Institute (CLSI) guidelines for β-hemolytic streptococci (41). When determining minocycline resistance, we used the tetracycline breakpoint in accordance with CLSI guidelines. The quality of AST was controlled using two strains of American Type Culture Collection (ATCC) (*Enterococcus faecalis* ATCC 29212 and *S. pneumoniae* ATCC 4961). The

MIC_{50} and MIC_{90} were calculated for minocycline, erythromycin azithromycin, clindamycin, and levofloxacin against *S. canis*.

3-3-2. Detection of AMR genes

The study investigated AMR genes for macrolides and tetracyclines in all enrolled isolates along with AST. Specific primers were employed to amplify the macrolide resistance genes *erm*(A) (encoding inducible-type methylase), *erm*(B) (encoding constitutive-type methylase), and *mef*(A) (encoding transmembrane domains of ABC transporter), and the tetracycline resistance genes *tet*(M) (encoding ribosomal protection protein), *tet*(O) (encoding ribosomal protection protein), *tet*(K) (encoding membrane-associated efflux pump), *tet*(L) (encoding membrane-associated efflux pump), and *tet*(S) (encoding ribosomal protection protein) by PCR (42, 43). Table 6 outlines the primers used for the amplification, and Table 7 describes the reaction mixture composition and condition for these targets.

3-4. Statistical analysis

To compare the acquired data with Fukushima and colleagues' data in 2017 (23), we applied Fisher's exact probability test (two-sided) to determine significant variations in categorical variables. The analysis used the Statcel 4 application（OMS Publisher, Tokyo, Japan). A P-value of less than 0.05 was considered statistically significant.

3-5. Ethical statement

The study design was approved by the Ethics Committee of the Sanritsu Zelkova Veterinary Laboratory, ensuring the confidentiality of the enrolled animals. Approval number: SZ20210825.

4. Results

4-1. β-hemolytic streptococcal isolates and patient background

Between April 1 and May 31, 2021, 2,112 clinical specimens were submitted to the Sanritsu Zelkova Veterinary Laboratory, of which 109 isolates (5.2%) were identified as β-hemolytic streptococci (Tables 8-10, Figure 2).

Of all 2,112 specimens, 648 were from cats, and 1,464 were from dogs (Figure 3).

The breakdown by Lancefield classification included group G (*n* = 103), group C (*n* = 3), group B (*n* = 2), and group A (*n* = 1) (Figure 4).

Figure 5 shows the geographical distribution of the patients from whom these isolates were isolated. The regions included Tokyo (*n* = 42), Chiba (*n* = 23), Saitama (*n* = 11), Aichi (*n* = 10), Kanagawa (*n* = 8), Ibaraki (*n* = 3), and Miyagi/Tochigi/Gifu (*n* = 2 each).

Some isolates were from sites considered to be sterile, such as uterine contents (*n* = 7) and ascites (*n* = 1). Specimens from dogs (*n* = 97) and cats (*n* = 12) included open pus (*n* = 36), ear/nose origin (*n* = 28), urogenital tracts (*n* = 24), eyes (*n* = 9), anal gland fluid (*n* = 2), and others (*n* = 2) (Figures 6, 7). The patient profile was as follows: average age, 10.9 years; age range, 1-17 years; 50 males and 59 females (Tables 8-10). Figures 8-10 indicate the age distribution of the cases. Dogs and cats exhibit a similar composition of ages, with the highest frequency observed in the senior age range of 13-15 years.

4-2. Species/subspecies identification

4-2-1. 16S rRNA analysis

Based on the 16s rRNA sequencing data for identifying β-hemolytic streptococci species/subspecies, *S. canis* (*n* = 102, 93.6%) belonging to

group G was the most predominant. It was followed by *S. dysgalactiae* subsp. *equisimilis* (*n* = 4, 3.7%) from groups C and G, *S. agalactiae* (*n* = 2, 1.8%) from group B, and *S. dysgalactiae* subsp. *dysgalactiae* (*n* = 1, 0.9%) from group A (Table 11). No significant difference was observed in the isolation rates when compared with the data from the 2017 study.

4-2-3. Confirmation of identification by species-specific genes

All isolates confirmed as *S. canis* through 16S rRNA sequencing possessed the *cfg* gene.

For the four isolates of *S. dysgalactiae* subsp. *equisimilis*, the *emm* genotypes identified were *stG840.0, stC9431.0, stC37.0,* and *stL1929.1*. One isolate of *S. dysgalactiae* subsp. *dysgalactiae* had the *emm* genotype of *stC46.2* (GenBank accession no. LC649931) (Tables 12-14).

4-3. Antimicrobial susceptibility

4-3-1. AMR phenotypes

Table 15 shows the data on the prevalence of AMR patterns within β-hemolytic streptococcal isolates analyzed in 2021 and 2017. In 2021, resistance to any of the antimicrobials was observed in 39 isolates. The overall AMR profiles revealed a 34.9% (*n* = 38) resistance rate 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. One isolate was non-susceptible to chloramphenicol with an MIC of 8 μg/mL.

A comparative analysis indicates no substantial variation in resistance rates for these antimicrobials between 2021 and 2017. No isolates were resistant to β-lactams including carbapenems, or vancomycin.

The MIC_{50}/ MIC_{90} values for minocycline, erythromycin, azithromycin, clindamycin, and levofloxacin against *S. canis* isolates (*n* = 102) were as follows: 1/>4 µg/mL, ≦0.12/>2 µg/mL, ≦0.25/>4 µg/mL, ≦0.12/>1 µg/mL, and 0.5/>8 µg/mL, respectively (Table 16).

Of the 38 isolates resistant to minocycline, 35 were *S. canis,* two were *S. agalactiae*, and one was *S. dysgalactiae* subsp. *equisimilis*. These resistant isolates were obtained from either sterile sites (uterine content, *n* = 3) or non-sterile sites (open pus, ear/nose origin, eye origin, urogenital tract origin, tooth origin, and skin origin, *n* = 36). They were collected from nine out of a total of 15 prefectures.

4-3-2. AMR genotypes

Table 17 shows the data on the prevalence of AMR gene patterns within β-hemolytic streptococcal isolates of the 2021 study.

Our analysis identified AMR genes for macrolide, lincosamide, and tetracycline, specifically *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%) in the isolates. No evidence of amplification was seen for *tet*(K), *tet*(S), or *erm*(A) in any of the isolates. The detection frequency of these AMR genes remained consistent between 2017 and 2021 with no significant difference. Of the samples, 37 (36.3%) *S. canis* isolates carried the AMR genes. Of the total isolates possessing AMR genes, 41 were sourced either from sterile sites (*n* = 3) or non-sterile sites (*n* = 38) and were found across nine out of 15 surveyed prefectures.

5. Discussion

We isolated 109 strains of β-hemolytic streptococci, identifying four different species and subspecies. There is a trend of higher isolation

numbers in the urban areas of the Kanto and Chubu districts, which may be associated with the number of companion animal hospitals in those areas (44).

Although there are more households with cats than those with dogs (45), requests for bacterial culture for dogs were more than double those for cats. This trend could be attributed to cat owners' fundamental behavior or awareness, or it may be a characteristic inherent to the species. Moreover, the isolation rate of β-hemolytic streptococci in dogs was significantly higher compared to cats. Further detailed investigation is required to understand these trends.

According to the survey results by the Japan Pet Food Association (45), the proportion of dogs and cats aged 13-15 years in the total canine and feline populations in 2021 was 14.9% and 9.7%, respectively. The peak age for bacterial isolation was 13-15 years for both dogs (n = 32, 34.4%) and cats (n = 4, 36.4%), significantly higher than overall population structures. From these findings, it can be inferred that infections by β-hemolytic streptococci are more prevalent in older animals, and considering the opportunistic nature of these bacteria, the involvement of age-related factors such as immunosenescence is suggested (46).

In this survey, further detailed analysis necessitates additional information on breeds or neutering status. Modifying the methodology in future surveys may be necessary to confirm this critical factor.

Among 109 β-hemolytic isolates, *S. canis* belonging to Lancefield group G was the most prevalent with 102 isolates, emphasizing the importance of this bacteria in companion animal medicine. The remaining three species, in descending order of prevalence, were *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 the 2017 study by

Fukushima et al., *S. equi* subsp. *zooepidemicus* (group C) was also identified (23) in addition to the species identified in our study. Ultimately, they confirmed five species: *S. canis, S. agalactiae, S. dysgalactiae* subsp. *equisimilis, S. dysgalactiae* subsp. *dysgalactiae, and S. equi* subsp. *zooepidemicus*.

The similarity in isolation patterns observed between 2021 and 2017 holds clinical implications, with *S. canis* being the most frequently identified species. Fluoroquinolones, represented by enrofloxacin, which is a veterinary product, are contraindicated in treating *S. canis* infections due to their association with severe adverse conditions like STSS and necrotizing fasciitis (NF) (20). To mitigate the risk of administering contraindicated pharmacological agents, veterinary practitioners should endeavor to identify the bacterial species present precisely, given that a considerable proportion of streptococcal infections are attributable to *S. canis*. This identification can be relatively easily achieved through microscopic observation of bacterial morphology (48), employing not only Gram staining but also Giemsa staining or other rapid staining techniques.

This isolation status is also vital in human medicine. It has been pointed out that *S. canis* is involved in various human infections (16). However, in clinical practice, identification of streptococci often only goes as far as the Lancefield classification, and further identification is not commonly done. There is a suggestion that *S. canis* might be more prevalent than currently thought in samples from humans that are simply identified as group G streptococcus (49). Considering this, veterinary practitioners must take ample precautions regarding infection control for companion animal owners and animal hospital staff who handle animals.

The *emm* gene codes the M protein on the bacterial surface, significantly influencing the virulence of *S. dysgalactiae* subsp. *equisimilis* and host

immune response interactions (50). Typing of this gene is essential for monitoring the pathogen's etiology and understanding its evolutionary dynamics. The *emm* type of *S. dysgalactiae* subsp. *equisimilis* is inherently linked to its lineage, serving as a vital marker for tracing infection routes (16, 51). In this study, we have identified the *emm* genotypes for four *S. dysgalactiae* subsp. *equisimilis* isolates (s*tG840.0, stC9431.0, stC37.0,* and *stL1929.1*) (Tables 12-14). To derive broader epidemiological insights, ongoing surveillance and analysis of a more comprehensive array of isolates are required. Thus, the current data set, while informative, represents a narrow step; a more expansive and long-term genotype profiling is imperative for comprehensive epidemiological mapping and for informing public health interventions.

An investigation into the status in resistance rates of the most prevalent *S. canis* isolates, based on data from 2017 and 2015 (23, 24), revealed consistently high resistance rates to tetracycline antimicrobials. This resistance pattern may be associated with clinical veterinarians' preference for prescribing tetracycline antimicrobials. In particular, doxycycline or minocycline are sometimes selected for treatment against methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) (52, 53), raising concerns about the potential inappropriate use of these drugs in various infections. Consequently, there is an imperative for clinical veterinarians to practice judicious use of all antimicrobial agents, not solely those within this class of antimicrobials. A slight increase has been observed in MIC_{50} with minocycline and MIC_{90} with levofloxacin compared to the 2017 survey, necessitating continued observation in the future.

These findings also represent a critical aspect of AMR in this bacterium as a zoonotic infection affecting humans, warranting ongoing investigation (54).

The construction of complete genome sequences of the streptococcus isolates is necessary to verify the accuracy of the 2021 PCR-based resistance genotyping data. Optimal results would be achieved by integrating short-read Illumina sequencing (using the MiSeq platform) with long-read Nanopore sequencing (using the MinION device), followed by hybrid assembly (utilizing Unicycler) to obtain the complete circular genome sequences, including any plasmids.

Yoshida et al. reported constructing draft genome sequences for seven isolates of *S. canis* isolated from dogs and cats (55). The genotypes of resistance profiles were confirmed by incorporating their contig sequences into the application of the Center for Genomic Epidemiology's ResFinder version 3.2 (https://cge.cbs.dtu.dk/services/ResFinder/). None of the four isolates harbored macrolide, lincosamide, or tetracycline resistance genes; however, *tet*(S), *erm*(B), and *erm*(B)+*tet*(O) were identified in the remaining three isolates, corroborating the PCR-based resistance genotype data. Furthermore, they identified a variant sequence of *erm*(B) in an *S. agalactiae* isolate resistant to clindamycin yet sensitive to erythromycin, inferred from whole genome sequences rather than direct sequencing of PCR-purified amplicons. These findings underscore the necessity of whole genome analysis in the current study as well.

Two significant limitations marked the study. Initially, the breadth of host-related data was restricted, including only primary identifiers such as species, sex (without neutering status), age, type of clinical specimen, date of bacterial isolation, and the geographical area of the veterinary practice. Forthcoming research should endeavor to compile exhaustive data to confirm the impact of antimicrobial use on resistance patterns, particularly concerning the former antimicrobial regimens prescribed by companion animal practitioners.

Secondly, the cohort of isolates from presumed sterile sources was quite limited, with only seven from uterine contents and one from peritoneal fluid. Murata and colleagues observed in Japan a critical case of SSTI progressing to septic shock in a miniature dachshund induced by *S. canis* isolated from the bloodstream and necrotic tissue (27). Prospective investigations should include a broader spectrum of sterile samples from severe pathological conditions to elucidate serious streptococcal infections, such as STSS and NF.

In addition, as the only data available for comparison with this study are from 2015 (24) and 2017 (23), continued surveillance will be necessary to ascertain the proportion of isolated bacterial species and their resistance trends more accurately.

6. Conclusion

In conclusion, *S. canis* was the most dominant species among β-hemolytic streptococci isolated from diseased dogs and cats in Japan from April 1 to May 31, 2021. They carried tetracycline-resistance genes with the dominant phenotype of tetracycline resistance. Companion animal veterinarians in Japan must consider these unique features when treating animals displaying clinical symptoms or signs of streptococcal infections. Continued monitoring of these bacteria is essential to understand the characteristics of infections caused by these species and to prevent the further emergence of AMR.

III. Chapter 2

Characteristics of eye-origin *Streptococcus canis*

1. Introduction

Among β-hemolytic streptococci, *S. canis* is the most frequently isolated species from dogs and cats (23, 24). *S. canis* is distributed as commensal bacteria in the skin, oral cavity, and reproductive organs of companion animals (20). However, it can also act as an opportunistic pathogen, infecting tissues such as the skin, cornea, and urogenital tract, as well as sterile sites like blood and ascites, thereby causing diverse lesions (29). One particularly severe condition it causes is NF, predominantly occurring in the limbs of dogs (20, 27). This disease is characterized by sudden onset, causing intense local inflammation followed by systemic effects like bacteremia, and without aggressive treatment, it can be highly lethal.

While such fulminant diseases receive considerable attention, *S. canis* also occupies an important position in ophthalmic disorders. Among ophthalmologic clinical cases, Goss et al. reported that *S. canis* is the predominant species in canine ulcerative keratitis, with an isolation rate of 29.3% (56). Similarly, in a study on canine ulcerative keratitis by Hewitt et al., *S. canis* was identified as the second most commonly isolated bacteria, following *Staphylococcus pseudintermedius* (57). When consolidating various reports, the isolation rate of *S. canis* in ophthalmic areas is found to be 7% to over 30%（58-60). Cloet et al. have reported that diagnostic use of PCR has revealed a higher prevalence of *S. canis* in the eyes than previously recognized, becoming a concern for veterinary ophthalmologists (61).

In 2020, Enache and colleagues reported identifying a specific clonal complex of the MLST in the isolates of *S. canis* from ulcerative keratitis in four Pug dogs (62). They concluded that clonal complex (CC) 13 was associated

with ulcerative keratitis in the dog, and there might be some etiological importance.

In this chapter, to concretely apply the findings from Chapter 1 to companion animal medicine, the author focused specifically on isolates from ophthalmic diseases, investigating their phenotypes, genotypes, and AMR profiles.

2. Purpose

We conducted the following study to elucidate the clinical significance of *S. canis* in veterinary ophthalmology, provide information for the proper treatment and further offer strategies to reduce antimicrobial resistance.

1. Survey of the isolation status of *S. canis* from the canine ocular lesions
2. Hemolytic activity (HA) measurement and virulence-associated gene (VAG) profiling of the eye-origin isolates to evaluate the pathogenesis of the isolates.
3. Analysis of clonal complexes in *scm* and Multi Locus Sequence Typing (MLST) of the eye-origin isolates to investigate the relationship among them.
4. Examination of the antimicrobial susceptibility profile of the eye-origin isolates

3. Materials and methods

3-1. Collection of the isolates

The bacterial isolates in Chapter 1 were included. Among these isolates, we selected nine eye-origin isolates (8.8%) and used 20 isolates from ears (19.6%) as controls. Additionally, 13 eye-origin isolates from Fukushima and colleagues' 2017 study (23) and the National Collection of Type Cultures (NCTC) 12191(T) were used as controls. In addition to these isolates, 17 randomly selected non-eye and non-ear isolates from the 2021 survey were

enrolled for controls in the MLST and AMR phenotype analysis (Figures 11, 12).

3-2. HA measurement

Isolates were inoculated from the sheep blood agar and cultivated in Todd-Hewitt broth and yeast extract overnight. After centrifuging the culture solution, the supernatant was mixed with 5% sheep red blood cells. Following incubation at 37°C, the mixture was centrifuged, and the supernatant was transferred to 96-well plates. The absorbance was measured at 545 nm, and values ≥0.5 were considered high-level HA, and values <0.5 were considered low-level HA (63, 64). An outline of the procedure is shown in Figure 13.

3-3. VAG profiling

The PCR-primer sets to confirm the isolates' VAG profiles were constructed. The primer sequences were designed from data on Identical Protein Groups annotated by the National Center for Biotechnology Information, which includes whole genome sequences (WGSs) of *S. canis* (65). These sets included primers for *inl* (encoding internalin), *sagA* (encoding streptolysin S), *slo* (encoding streptolysin O), *scp* (encoding C5a peptidase), *lbp* (encoding laminin-binding protein), *fbp* (encoding fibronectin-binding protein), *gbp* (encoding glucan-binding protein), *ap1* (encoding pilus ancillary protein 1), *fp1* (encoding fimbrial protein), and *brp* (encoding biofilm regulatory protein) (Table 18). PCR reactions were conducted under conditions described in Tables 19 and 20. As positive controls, NCTC 12191(T) and FU6, TA4, FU53, and FU97 from the 2017 study with whole genome sequences were included in the study. For some VAG-positive isolates, direct sequencing further confirmed the amplified VAG sequences.

3-4. SCM allele typing

For analysis of the *scm* gene, the study used specific primers for PCR amplification, resulting in amplicons with sizes ranging from 1,700 to 2,100 bp (Tables 21-23) (66). After purification, sequencing was performed directly. From the resulting nucleotide sequences, an unrooted phylogenetic tree was constructed based on the deduced amino acid sequences using the Neighbor-Joining method (67). The associated taxa were then clustered in the bootstrap test (1,000 replicates), following which the tree was scaled by the Poisson correction method and branch lengths, reflecting the same units as distances. Finally, we conducted allele typing based on variable or conserved amino acid sequences in the phylogenetic tree. All analyses were performed using MEGA X software (version 10.0.5) (68).

3-5. Multi-Locus Sequence Typing (MLST)

MLST analysis was conducted on all enrolled isolates. For the six genetic loci other than *xpt*, primers were prepared according to the description in the study by Pinho et al. (69). For the *xpt* locus, primer sequences were determined from the report by Fukushima et al. (2020), adopting the M13 universal sequencing primer to enable sequencing across all *S. canis* isolates (Table 24) (68). The rationale for this choice is that previous primers had binding sites too close to the *xpt* allele-determining sequences, which sometimes precluded the complete reconstruction of the gene after sequencing. PCR reactions were conducted under conditions described in Tables 25 and 26.

The results were uploaded to the pubMLST website (70), and each isolate's Sequence Type (ST) was determined. In addition to identical allele types, single locus variants differing in only one housekeeping gene were classified as clonal complexes (CCs). Whenever there were novel allele-

determining sequences or allele combinations, we registered them with isolate/host information in the *S. canis* PubMLST isolates database.

3-6. AST and detection of AMR genes

The study used data for 14 antimicrobial agents (penicillin G, ampicillin, cefepime, cefotaxime, ceftriaxone, cefozopran, meropenem, minocycline, erythromycin, azithromycin, clindamycin, levofloxacin, vancomycin, and chloramphenicol) in Chapter 1. In addition, the AMR rates were calculated for each class of antimicrobial agents.

In addition, the presence of AMR genes against macrolides, lincosamides, and tetracyclines [*erm*(A), *erm*(B), *and mef*(A); *tet*(M), *tet*(O), *tet*(K), *tet*(L), and *tet*(S)] through PCR analysis were assessed by data obtained in Chapter 1 as well.

3-7. Statistical analysis

To compare the 2021-eye with the 2021-ear, the 2017-eye, and the 2021 non-eye-non-ear group, we applied Fisher's exact probability test (two-sided) to determine significant variations in categorical variables. The analysis used the Statcel 4 application（OMS Publisher, Tokyo, Japan). A *p*-value of less than 0.05 was considered statistically significant.

3-8. Ethical statement

The study design was approved by the Ethics Committee of the Sanritsu Zelkova Veterinary Laboratory, ensuring the confidentiality of the affected animals. Approval number: SZ20211126-2.

4. Results

As mentioned in Chapter 1, we obtained 2,112 clinical specimens from dogs (*n* = 1,464) and cats (*n* = 648) from April 1 to June 30, 2021. Among these, β-hemolytic streptococci were 109 isolates with an isolation rate of 5.2%. Out of these isolates, the 2021-eye-origin *S. canis* (the 2021-eye) were recovered from the cornea and eye discharge of nine dogs, with a mean age of 8.6 years, while the 2021-ear-origin isolates (the 2021-ear) were from the ear of 20 dogs, with a mean age of 11.0 years. In addition, we collected the 2017-eye-origin isolates (the 2017-eye) of Fukushima and colleagues' study (23). They were isolated from the cornea, conjunctiva, and eye discharge of 13 dogs, with a mean age of 8.6 years, as a control. The profiles of the isolates used are presented in Table 27, and the collection locales are shown in Figure 14.

4-1. HA measurement

One isolate of the 2017-eye had high-level HA, while the rest, including the NCTC 12191(T) strain, had low-level HA.

HA values of all enrolled isolates and NCTC 12191(T) strain are shown in Tables 28-30.

4-2. VAG profiling

The detection of the VAGs resulted in a nearly uniform distribution across all groups, with no association of the 2021-eye with the detection rate of each VAG. Sequencing of the VAGs from selected isolates confirmed that the amplified genes were identical to the target genes.

The detection rates of each VAGs for the 2021-eye, the 2021-ear, and the 2017-eye are presented in Table 31-33. Table 34 represents the prevalence of VAGs in each group.

4-3. SCM allele typing

The allele types of the *scm* gene for the 2021-eye, the 2021-ear, and the 2017-eye, along with their respective accession numbers registered in the DNA Data Bank of Japan (DDBJ), are presented in Table 35.

As shown in Table 36, allele 2 (n = 6, 66.7%) was the most predominant of the *scm* sequence in the 2021-eye. On the other hand, the 2021-ear and the 2017-eye exhibited a significant distribution of allele 1 (n = 8, 40.0%)/ allele 2 (n = 6, 30.0%) and allele 1 (n = 4, 30.8%)/allele 2 (n = 3, 23.1%)/ allele 4 (n = 3, 23.1%), respectively. There was no statistically significant association with allele 2 prevalence between the 2021-eye compared to the 2021-ear (p = 0.106) and the 2017-eye (p = 0.079).

Figure 15 depicts the phylogenetic tree of the deduced amino acid sequences of the M-like protein of *S. canis* in the 2021-eye, the 2021-ear, and the 2017-eye, constructed using the neighbor-joining method with MEGA10 software. Though no statistical significance was observed, each group exhibits a certain distribution tendency within the tree. Notably, the 2021-eye tended to have a higher occurrence of allele 2.

4-4. MLST

Table 37 displays the allele profiles, STs, and CCs in three groups.

CC46, consisting of ST46 (n = 6) and ST2 (n = 1), of MLST was the most prevalent (n = 7, 77.8%) among the 2021-eye. The 2021-ear exhibited a predominant distribution of CC9 (n = 6, 30.0%) as well as CC46 (n = 6, 30.0%) consisting of ST2 (n = 3), ST46 (n = 2), and ST69 (n = 1). The 2017-eye also showed the most prevalent distribution of CC9 (n = 3, 23.1%) as well as CC46 (n = 3, 23.1%) consisting of ST46 (n = 2) and ST2 (n = 1). There was a statistically significant association of the 2021-eye with CC46 compared to the 2021-ear (p = 0.041) and the 2017-eye (p = 0.027).

Randomly selected non-eye and non-ear isolates from the 2021 survey (Table 38) showed a predominant prevalence of CC9 (*n* = 9, 52.9%), and CC46 was observed in only one isolate (5.9%). There was a significant difference in the prevalence of CC46 between the 2021-eye and this group (*p* = 0.0004).

The summarized data on the CC of MLST and the relationship between the 2021-ear and the 2017-eye are presented in Table 39.

Figure 16 represents the population structure of CCs by goe-BURST diagram (71). The diagram illustrates the relationship between each isolate and its corresponding CCs, with the size of the circle being proportional to the number of isolates. Red represents the 2021-eye, yellow corresponds to the 2017-eye, and blue indicates the 2021-ear. The circles encased by the red dashed line denote CC46, those in yellow are CC56, and the blue ones are CC9. The 2021-eye samples are concentrated within CC46.

4-5. AST and detection of AMR genes

Tables 40-43 show the profiles of AMR phenotypes in four groups, including the 2021 non-eye and non-ear.

The 2021-eye showed higher AMR phenotype levels than the 2021-ear and the 2017-eye (Tables 40-42). The most frequent resistance was to minocycline (*n* = 7, 77.8%), followed by clindamycin (*n* = 6, 66.7%), erythromycin/azithromycin (*n* = 5, 55.6%), and levofloxacin (*n* = 4, 44.4%) (Table 44).

The corresponding AMR genotypes were also prevalent (Table 45). A total of seven isolates (77.8%) of the 2021-eye carried at least one of the AMR genes, including *tet*(O)-erm(B) (*n* = 5), *tet*(M)-*mef*(A) (*n* = 1), and *tet*(O) (*n* = 1). Statistical analysis revealed a significant correlation between the

prevalence of AMR phenotypes/genotypes in the 2021-eye and those in the 2021-ear (p = 0.014), and the 2017-eye (p = 0.027) (Tables 45, 46).

Phenotypically, non-eye and non-ear isolates from the 2021 survey showed the same trend as the 2021-ear and the 2017-eye, with a significant difference from the 2021-eye (p = 0.038) (Table 43). On the other hand, the prevalence of the AMR genotype within this population (Table 47) did not show a statistically significant difference when compared to that of the 2021-eye group (p = 0.097).

Though some isolates had slightly high MIC within the sensitive range, all the enrolled isolates remained sensitive to β-lactam antimicrobials. The reference strain NCTC 12191(T) exhibited no AMR phenotypes and genotypes in this study.

5. Discussion

In both HA and VAG analysis, no significant differences were observed across the three groups (the 2021-eye, the 2021-ear, and the 2017-eye), suggesting that these pathogenic factors might not be involved in the variation of infection sites in this study. Regarding VAGs, it may be necessary to broaden the range of gene types covered in future investigations to gain a more comprehensive understanding.

The *scm* gene codes SCM protein on the bacterial surface of *S. canis.* The SCM protein is also a virulence factor of *S. canis*, possessed by all isolates of this species (69), although a report suggests that it may not influence bacterial pathogenicity (72). This protein is also utilized for the classification of *S. canis* based on its sequence. Current classification based on the *scm* sequence predominantly falls into two types (29). One approach by Pinho and colleagues divides the overall into two subgroups, Group 1 and 2, assigning seven types to Group 1 and five to Group 2. Previously, the *scm* in Group 2

was not entirely readable due to the proximity of primer binding sites to the gene, which hindered detection. Pinho and colleagues resolved this issue by modifying the primers, subsequently reporting the ubiquitous presence of *scm* across all isolates (69). Fukushima et al. expanded upon this classification, dividing the total into 15 types, with types 1-9 constituting Group 1 and types 10-15 for Group 2. Recent studies revealed that Group 1 binds to immunoglobulin while Group 2 to fibrinogen (73, 74). Additionally, Timoney JF et al. also classified *scm* from *S. canis* isolated from cats into four types, discussing its correlation with pathogenicity (75).

We employed the classification by Fukushima et al. in our current study (68). While our study observed certain trends between *scm* type and ocular diseases, no strong correlation was found. However, combining this with MLST could allow for more refined classification and contribute to clarifying the pathogenesis.

MLST is crucial for bacterial classification and is highly valuable for numerous clinical applications, including epidemiological data analysis, tracking investigations during infectious disease outbreaks, and understanding the spread of AMR isolates (62, 76-78).

In this study, CC46 was predominant in the 2021-eye, and all isolates of ST46 were of the *scm* allele 2 (Tables 35, 37). This finding suggests a profound association between ophthalmic diseases and the clonal spread of these isolates.

Fukushima et al. reported on cases of quinolone-nonsusceptible *S. canis* with CC46, including eight isolates (six of ST46 and one each of ST2 and ST69) (79). The sampling sites among these eight cases were predominantly open pus and ear discharge (three cases each), with urine accounting for two cases and no isolates from the eye. Among these, ST46 isolates were nonsusceptible to all or some of the following: ciprofloxacin, levofloxacin, and

norfloxacin. While ST2 and ST69 isolates were phenotypically susceptible to fluoroquinolones, mutations were observed in the quinolone resistance-determining regions (79). These findings suggest an association of CC46 with AMR, and the prevalence in eyes in the current study might be attributable to certain selective pressures.

In their investigation of *S. canis* isolated from pyoderma, Imanishi et al. also studied oral isolates as a control group (76). Out of 26 isolates from the canine oral cavity, 11 (42.3%) belonged to CC46, though they primarily counted most of them as CC2, focusing on ST2, comprising seven ST46, three ST2, and one ST69. Given the rapid change in prevalence observed in the current study, CC46 isolates may not be consistently present in the oral cavity. However, considering the proximity of the mouth to the eyes, it is plausible that these isolates could translocate to ocular regions via mediums such as saliva.

In this study, no statistical association was observed between predominant CC46/allele2 and either HA values or VAGs in the 2021-eye. The findings suggest that these isolates do not possess strong pathogenicity; instead, their relevance seems to lie primarily with AMR.

We demonstrated a stronger association of the the 2021-eye with minocycline resistance compared to the 2021-ear and the 2017-eye. Moreover, four isolates were resistant to levofloxacin, while none showed quinolone-nonsusceptibility among the 2017-eye (Table 44), suggesting the rapid spread of quinolone-nonsusceptible strains. Similarly, for AMR genes, a statistically significant prevalence in the 2021-eye group was confirmed when compared with the 2021-ear and the 2017-eye (Table 45). Though no significant difference in the prevalence rate was observed between the 2021-eye and 2021 non-eye non-ear isolates, a subsequent comparison between the 2021-eye and all other 2021 isolates revealed a statistically significant

difference in the prevalence of AMR genes (p = 0.010). Therefore, no significant difference between these two groups is believed to be attributable to a sampling discrepancy by pure chance.

According to a paper on Japanese veterinary practitioners (80), tetracyclines, including those for human use, are the fourth most commonly used class of antimicrobials. As mentioned in Chapter 1, this tendency may be due to corresponding to an antibiogram for methicillin-resistant staphylococci (52). Moreover, fluoroquinolones are the third most frequently used antimicrobials, although they are restricted to use only when the first-choice drugs are ineffective (80).

It is crucial to inform companion animal practitioners about the rational use of antimicrobials, with a particular emphasis on systemic and local applications, especially in the field of ophthalmology.

The study's limitations include insufficient host information and the therapeutic course.

In the study by Enache et al., clinical signs of cases were meticulously examined by ophthalmologists, and an investigation into the patient's medical history, prior use of antimicrobial agents, and treatment progression were analyzed (62). Similarly, Leis ML et al., who reported an association between the failure of corneal ulcer treatments and ST43 isolates of *S. canis*, investigated a detailed history of underlying conditions and the use of antimicrobial treatments before and after therapy (78). In our current study, the accessible information was limited, and it was impossible to investigate some critical elements, such as the breed and detailed therapeutic course, including the history of antimicrobial application.

In the future, more detailed information is necessary to clarify the association of the features of the eye-origin isolates with their clinical implication. Moreover, the most critical aspect is the continuation of broader

surveillance efforts. Particularly in instances where the proliferation of specific AMR isolates is identified, it is imperative to promptly communicate comprehensive data, including recommendations for antimicrobials, to authorities and veterinary associations. To facilitate this, appropriate programs will be necessary to enable sustained and effective reporting and intervention.

Nevertheless, this study is the first to report the occurrence of the ST46 isolates carrying multiclass antimicrobial resistance phenotypes with genotypes of *tet*(O)-*erm*(B) among the eye-origin *S. canis* isolates.

6. Conclusion

The study first documented an instance of ocular isolates predominantly containing ST46 with multiclass AMR phenotypes and *tet*(O)-*erm*(B) or *tet(*O) genotypes. In addition, fluoroquinolone resistance was highly prevalent, which will be a grave concern in veterinary and human medicine.

As challenges of this study, there is a need for further information gathering and analysis to clarify epidemiological characteristics such as host information and treatment history. In any case, companion animal practitioners need better to understand the microbiological and epidemiological characteristics of *S. canis*, and the author believes the study's results will benefit future veterinary and human clinical settings.

IV. General Discussion

This study provides essential insights for the application of companion animal healthcare in the following respects:

1. The importance of microscopic observation of bacteria
2. The selection of antimicrobials for empiric use
3. The significance of AST and antibiograms
4. The rational use of topical agents
5. Perspectives on zoonotic infections affecting both humans and animals

1. The importance of microscopic observation of bacteria

As mentioned in Figure 1 of Chapter 1, morphological observation plays the most significant role in bacterial identification.

According to a survey conducted by the Japan Small Animal Veterinary Association in 2017 (81), only 10.2% (15/147) of veterinarians consistently observe microscopically samples from affected tissues or discharges when encountering bacterial infections. Differentiating, at least between cocci and bacilli, is one of the most crucial processes, as well as identifying the focus of infection. However, it is thought that in most cases, empiric use of antimicrobials is based solely on the presumed focus of infection, even if the specimen is available.

When enough bacteria are collected for microscopic visualization, observing their morphology becomes crucial for following decision-making. It helps differentiate staphylococci from other bacteria in dermatology or *S. canis* from other species in ophthalmology. Such differentiation is vital to initiating the appropriate treatment (48). Especially in diseases requiring immediate aggressive intervention, such as NF, this can be a matter of life or death (27).

Given its importance as a critical indicator for suspected infections and antimicrobial selection, morphological observation should be incorporated more routinely into companion animal practice.

2. The selection of antimicrobials for empiric use

In a series of investigations, including precedent studies, it has been found that β-hemolytic streptococci account for more than 5% of the total number of cultures sent to Sanritsu Zelkova Veterinary Laboratory, with a majority identified as *S. canis* (23). In the context of companion animal healthcare in Japan, infections caused by *Staphylococcus* spp. in dermatology and *Escherichia coli* in urology have been focused on due to concerns over drug resistance (15). In contrast, streptococci, particularly *S. canis*, have not been extensively studied.

S. canis is typically susceptible to β-lactam antimicrobials, while fluoroquinolones are generally contraindicated (20). The rationale for this contraindication is multifaceted. Firstly, a significant number of cases of severe *S. canis* infections, such as STSS and SSTI, have been associated with the use of enrofloxacin, a fluoroquinolone (82). It is hypothesized that the use of fluoroquinolones in strains possessing prophages may induce an SOS response, leading to phage induction and the expression of pokeweed mitogen-like superantigens coded by the phage, contributing to the severity of the disease (28, 83). Furthermore, a report indicates a statistically significant increase in treatment failures for ophthalmic diseases involving *S. canis*, particularly post-surgical conjunctival grafts, when treated with second-generation fluoroquinolones such as ofloxacin and ciprofloxacin (84). This evidence further elucidates the importance of careful antimicrobial selection and the potential risks associated with fluoroquinolones in treating *S. canis* infections.

Considering these factors, in infections of β-hemolytic streptococci, β-lactam antimicrobials such as penicillins and cephalosporins should be the first line of treatment. This selection is particularly pertinent in fields like ophthalmology, making it a crucial guideline for selecting empiric antimicrobial therapy. However, there is an issue to consider here in the ophthalmology setting. In Japan, there are only two approved ophthalmic antimicrobial products (85) for companion animal use. One of them is an ointment, which is less convenient to apply at home. The remaining formulation is an eye drop, which is more accessible for pet owners to administer than ointment; however, this formulation is a fluoroquinolone. According to directives from the Ministry of Agriculture, Forestry, and Fisheries (86), companion animal practitioners are advised to "preferentially use antimicrobials approved for use in companion animals. The use of antimicrobials not approved for companion animals or the use of unapproved drugs should be avoided as much as possible. The use of antimicrobials other than those approved for companion animals should be limited to cases where, based on the results of drug susceptibility testing, there are no existing veterinary drugs approved for companion animals" (translated by the author). Following this, only veterinary medications should be used until the results of the drug susceptibility tests are available. Acknowledging these circumstances, it is plausible that there will be an increased propensity for fluoroquinolones.

In any case, considering that approximately one out of 20 cases of all infections in companion animals would contraindicate the use of fluoroquinolones, a more judicious approach to antimicrobial selection is warranted. This emphasizes the importance of tailored antimicrobial therapy based on understanding the microbial landscape and resistance patterns in treating infections of β-hemolytic streptococci.

3. The significance of AST and antibiograms

In Chapter 2, we demonstrated that the resistance to fluoroquinolones in the field of ophthalmology has rapidly increased from 2017 to 2021. The swift changes in bacterial populations suggest that both identification of bacteria and AST should be conducted for all infections. However, in the JSAVA survey, 71 out of 158 respondents indicated that they do not routinely perform drug susceptibility testing (81).

AMR trends typically change in proportion to spacio-temporal coordinates. Bearing this in mind, it is necessary to create a hospital-specific antibiogram to make informed choices about antimicrobial use until susceptibility test results are available. Establishing an antibiogram facilitates the appropriate use of antimicrobials and allows for understanding the regional epidemiological characteristics at the time by providing crucial information.

Our former research indicated that the appropriate use of antimicrobials based on susceptibility testing, explicitly establishing a hierarchy of antimicrobial usage, could reduce the resistance rates of the *Staphylococcus intermedius* group and *E. coli* (87). Although we have presented the hierarchy of antimicrobial use, considering our findings, it appears necessary to further demote fluoroquinolones, currently a second-choice treatment for infections suspected to involve β-hemolytic streptococci, to a third-choice option. This adjustment will enable more appropriate drug selection in ophthalmic diseases using the hierarchy and will also serve as a reference in choosing treatment drugs using the hospital-specific antibiogram.

4. The rational use of topical agents

Topical medications, including ophthalmic drops, are considered to have less impact on other body parts than systemic administration; however, the high resistance rates in ophthalmology suggest that the same or greater level

of caution is required as with systemic administration. According to the JSAVA survey, the selection of fluoroquinolones among topical drugs is second only to gentamicin formulations (81). This predominance is concerning. If such medications are indiscriminately used in diseases appropriate for topical treatment, such as in ophthalmology, otolaryngology, and dermatology, it could potentially disadvantage animals and their owners. Veterinary practitioners must be fully aware of these implications and exercise judicious selection and use of these agents.

5. Perspectives on zoonotic infections affecting both humans and animals

β-hemolytic streptococci are a significant zoonotic agent in humans and animals. It should be assumed that animals infected with these bacteria are continually shedding them. Prompt and effective treatment is essential not only for reducing the chance of transmission to humans, including pet owners, but also for mitigating the emergence of AMR. Such efforts are essential for reducing the risks to human life and health posed by AMR, inadvertently arising from veterinary practices. Additionally, communicating to pet owners that bacteria are present in various secretions, not only in socially perceived contaminants such as pus or urine but also in tears and ocular discharges, is fundamental for infection control. This awareness highlights the importance of comprehensive hygiene and preventive strategies in managing pet health.

In such contexts, the role of VNCA, who are closest in terms of rapport with the animal owners, has become increasingly important. A survey report on their awareness demonstrates a high level of interest in antimicrobial stewardship (88). In Japan, similar surveys should be conducted in nursing

colleges and other relevant institutions to devise effective strategies based on the results, necessitating the concerted efforts of educators.

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VII. Tables

Table 1. The composition of TE buffer

Reagent	Volume	Final concentration
1 M Tris-HCl Buffer	5 mL	10 mM
0.5 M EDTA	1 mL	1 mM
D.W.	494 mL	
	500 mL	

Table 2. Primer set for species identification

Targeted gene	Primer	Direction	Sequence5'–3'	Expected amplicon size (bp)	Ref.
16S rRNA (universal)	27F	Forward	AGAGTTTGATCMTGGCTCAG	1,497	(23, 24)
	1485R	Reverse	TACGGTTACCTTGTTACGAC		

M = A, C

Table 3. Reaction mixture composition and cycling conditions for 16s rRNA gene amplification

Reagents	Volume
10x Ex-Taq Buffer	5 μL
10mM dNTP mix	4 μL
primer F (5 μM)	4 μL
primer R (5 μM)	4 μL
Template	2 μL
Ex-Taq HS DNA polymerase (TaKaRa)	0.25 μL
MiliQ	30.75 μL
Total	50 μL

94°C	1 min	
94°C	30 sec	35 cycles
62°C	90 sec	
72°C	90 sec	
72°C	10 min	

Table 4. Primer sets for species-specific genes

Targeted gene (specific species)	**Primer**	**Direction**	**Sequence5'–3'**	**Expected amplicon size(bp)**	**Ref.**
cfg **(*S. canis*)**	camp-canis-I	Forward	CAATTAACTAATAAGGTAGAACAG	238	(38)
	camp-canis-II	Reverse	CTCTCTCAAAACGGGTG		
dltS **(*S.galactiae*)**	dlts-F	Forward	CTGTAAGTCTTTATCTTTCTCG	199	(39)
	dlts-R	Reverse	ATCCATTCGCTTAGTCATCC		
emm **(*S. dysgalactiae*)**	emm1	Forward	TATTSGCTTAGAAAATTAA	Variable and 180 bp used for genotyping	(40)
	emm2	Reverse	GCAAGTTCTTCAGCTTGTTT		

S = C or G

Table 5. Reaction mixture composition and cycling conditions for species-specific gene amplification

Reagents	**Volume**
10x Ex-Taq Buffer	5 μL
10mM dNTP mix	4 μL
primer F (5 μM)	4 μL
primer R (5 μM)	4 μL
Template	2 μL
Ex-Taq HS DNA polymerase (TaKaRa)	0.25 μL
MiliQ	30.75 μL
Total	50 μL

Cycling condition for *cfg* amplification

94℃	2 min	
94℃	1 min	30 cycles
58℃	1.5 min	
72℃	1.5 min	
72℃	5 min	

Cycling condition for *dltS* amplification

94℃	3 min	
94℃	30 sec	30 cycles
52℃	30 sec	
72℃	40 sec	
72℃	50 min	

Cycling condition for *emm* amplification

94℃	5 min	
94℃	30 sec	30 cycles
52℃	60 sec	
72℃	120 sec	
72℃	10 min	

Table 6. Primer sets for AMR genes

Primers	Direction	Sequence (5'→3')	Length (mer)	Amplicon size (bp)	Ref
***erm*A-F**	forward	CCCGAAAAATACGCAAAATTTCAT	24	590	(42, 43)
***erm*A-R**	reverse	CCCTGTTTACCCATTTATAAACG	23		
***erm*B-F**	forward	TGGTATTCCAAATGCGTAATG	21	745	
***erm*B-R**	reverse	CTGTGGTATGGCGGGTAAGT	20		
***mef*A-F**	forward	CAATATGGGCAGGGCAAG	18	317	
***mef*A-R**	reverse	AAGCTGTTCCAATGCTACGG	20		
***tet*M-F**	forward	GTGGACAAAGGTACAACGAG	20	406	
***tet*M-R**	reverse	CGGTAAAGTTCGTCACACAC	20		
***tet*O-F**	forward	AACTTAGGCATTCTGGCTCAC	21	515	
***tet*O-R**	reverse	TCCCACTGTTCCATATCGTCA	21		
***tet*K-F**	forward	GATCAATTGTAGCTTTAGGTGAAGG	25	155	
***tet*K-R**	reverse	TTTTGTTGATTTACCAGGTACCATT	25		
***tet*L-F**	forward	TGGTGGAATGATAGCCCATT	20	229	
***tet*L-R**	reverse	CAGGAATGACAGCACGCTAA	20		
***tet*S-F**	forward	TCCGATAGTGATCCCCTTCT	20	445	
***tet*S-R**	reverse	GGAAATCTGCTGGCGTACTG	20		

Table 7. Reaction mixture composition and cycling conditions for antimicrobial-resistant genes

Reagents	Volume
10x Ex-Taq Buffer	2.5 μL
10mM dNTP mix	2 μL
primer F (5 μM)	2 μL
primer R (5 μM)	2 μL
Template	1 μL
Ex-Taq HS DNA polymerase (TaKaRa)	0.125 μL
MiliQ	15.375 μL
Total	25μL

94°C	1 min	
94°C	30 sec	35 cycles
62°C	90 sec	
72°C	90 sec	
72°C	10 min	

Table 8. Animal backgrounds and sample sources of β-hemolytic streptococci-Part 1

Sample ID.	Prefecture	Species	Age	Sex	Sample source
KU1	Tokyo	dog	ND	M	Urine
KU2	Miyagi	dog	8	F	Pyometra
KU3	Tokyo	dog	12	F	Uterine contents
KU4	Tokyo	dog	10	M	Eye discharge (OS)
KU5	Tokyo	dog	13	F	Pus
KU6	Tokyo	dog	10	M	Cornea (OS)
KU7	Tochigi	dog	13	M	Pus
KU8	Tokyo	dog	9	F	Urine
KU9	Kanagawa	dog	11	F	Ear discharge
KU10	Tokyo	dog	13	M	Tympanic bulla wash fluid (AD)
KU11	Fukuoka	dog	12	M	Anal sac fluid
KU12	Saitama	dog	13	M	Ear discharge
KU13	Tokyo	dog	15	M	Pus
KU14	Ibaraki	dog	13	F	Urine
KU15	Tokyo	cat	3	F	Subcutaneous abscess
KU16	Chiba	cat	ND	M	Pus
KU17	Chiba	dog	17	M	Nasal discharge
KU18	Fukui	dog	13	F	Pus
KU19	Chiba	dog	12	M	Pus
KU20	Kanagawa	dog	14	M	Eye discharge
KU21	Tokyo	dog	9	F	Upper right third premolar
KU23	Chiba	cat	13	M	Pus
KU24	Aichi	dog	13	F	Urine
KU25	Aichi	dog	12	F	Urine
KU26	Kanagawa	dog	8	F	Nasal discharge
KU27	Tokyo	dog	12	F	Urine
KU28	Tokyo	dog	8	F	Urine
KU29	Saitama	cat	12	F	Pus
KU30	Tokyo	dog	2	F	Uterine contents
KU31	Tokyo	dog	ND	F	Pus
KU32	Saitama	dog	13	F	Ascites
KU33	Saitama	dog	4	F	Pus
KU34	Tokyo	cat	2	F	Nasal dischargepus
KU35	Kanagawa	cat	6	M	Pus
KU36	Aichi	dog	12	F	Urine
KU37	Aichi	dog	13	F	Urine
KU38	Tokyo	dog	12	F	Urine
KU39	Tokyo	dog	8	F	Urine
KU40	Saitama	cat	12	F	Pus

Continued on the next page

Table 9. Animal backgrounds and sample sources of β-hemolytic streptococci-Part 2

Sample ID.	Prefecture	Species	Age	Sex	Sample source
KU41	Tokyo	cat	14	F	Pus
KU42	Saitama	dog	3	M	Ear discharge
KU43	Gifu	dog	14	F	Pus
KU44	Tokyo	dog	7	M	Cornea (OS)
KU45	Chiba	dog	ND	F	Pus
KU46	Chiba	dog	8	M	Urine
KU47	Chiba	dog	10	F	Ear discharge
KU48	Gifu	dog	11	M	Pus
KU49	Kanagawa	dog	ND	M	Anal sac fluid
KU50	Tokyo	dog	15	F	Urine
KU51	Tokyo	dog	14	F	Pus
KU52	Tokyo	dog	10	F	Pus
KU53	Chiba	dog	12	M	Urine
KU54	Chiba	dog	13	M	Ear discharge
KU55	Chiba	dog	ND	F	Pus
KU56	Aichi	dog	ND	M	Pus (ear discharge)
KU57	Tokyo	dog	6	M	Cornea (OS)
KU58	Tokyo	dog	2	F	Cornea (OS)
KU59	Tokyo	dog	13	F	Uterine contents
KU60	Saitama	dog	ND	F	Uterine contents
KU61	Aichi	dog	9	F	Urine
KU62	Tokyo	dog	11	F	Pus
KU63	Tokyo	cat	15	F	Ear discharge
KU64	Tokyo	dog	13	M	Urine
KU65	Tokyo	dog	ND	M	Pus
KU66	Kanagawa	dog	13	M	Eye discharge (OS)
KU67	Kanagawa	dog	2	M	Eye discharge
KU68	Tokyo	dog	14	F	Ear discharge
KU69	Tokyo	dog	9	F	Fluid in ear canal (AD)
KU70	Tokyo	dog	14	F	Ear discharge
KU71	Chiba	dog	13	M	Cornea
KU72	Chiba	dog	9	F	Lochia
KU73	Tokyo	dog	13	M	Ear discharge
KU74	Saitama	dog	ND	M	Pus
KU75	Chiba	dog	16	F	Lochia
KU76	Chiba	dog	ND	M	Ear discharge
KU77	Chiba	dog	17	F	Ear discharge
KU78	Tokyo	dog	9	M	Pus
KU79	Shizuoka	dog	13	F	Pus
KU81	Tokyo	dog	11	M	Pus

Continued on the next page

Table 10. Animal backgrounds and sample sources of β-hemolytic streptococci-Part 3

Sample ID.	Prefecture	Species	Age	Sex	Sample source
KU82	Tokyo	dog	15	F	Ear discharge
KU83	Tochigi	dog	ND	M	Urine
KU84	Tokyo	dog	12	F	Pus (uterine stump)
KU85	Tokyo	dog	10	M	Pus
KU86	Chiba	dog	16	M	Urine
KU87	Saitama	dog	1	F	Pus
KU88	Chiba	dog	13	F	Ear discharge
KU89	Chiba	dog	13	M	Ear discharge
KU90	Saitama	dog	ND	F	Ear discharge（AD）
KU91	Aichi	dog	8	F	Ear discharge
KU92	Nagasaki	dog	ND	M	Urine
KU93	Tokyo	dog	9	M	Ear discharge
KU94	Chiba	dog	9	F	Pus
KU95	Aichi	dog	11	F	Pus (uterine contents)
KU96	Tokyo	cat	13	F	Pus
KU97	Ibaraki	dog	4	F	Ear discharge
KU98	Kanagawa	dog	ND	F	Pus
KU99	Ibaraki	dog	9	M	Ear discharge
KU100	Saitama	dog	11	M	Pus
KU101	Chiba	dog	15	M	Pus
KU102	Chiba	dog	13	M	Urine
KU103	Tokyo	dog	7	M	Ear discharge（AD）
KU104	Chiba	dog	13	M	Pus
KU105	Tokyo	dog	14	M	Urine
KU106	Aichi	dog	10	M	Urine
KU107	Chiba	dog	14	M	Ear discharge
KU108	Miyagi	dog	10	M	Pus
KU109	Iwate	cat	1	F	Pus (subcutaneous abscess)
KU110	Ibaraki	cat	17	M	Nasal discharge
KU111	Kanagawa	dog	17	F	Skin decubitus

ND: No data, M: Male, F: Female

Table 11. Species/subspecies identification of β-hemolytic streptococci based on 16S rRNA sequencing data according to the cell wall carbohydrate antigenicity

Lancefield group	Identification data of the streptococci isolated in 2021	Identification data of the streptococci isolated in 2017
G	*S. canis* (*n* = 102, 93.6%), *S. dysgalactiae* subsp. *equisimilis* (*n* = 1, 0.9%)	*S. canis* (89.3%), *S. dysgalactiae* subsp. *equisimilis* (1.5%)
C	*S. dysgalactiae* subsp. *equisimilis* (*n* = 3, 2.8%)	*S. dysgalactiae* subsp. *equisimilis* (1.5%), *S. dysgalactiae* subsp.*dysgalactiae* (0.8%), *S. equi* subsp. *zooepidemicus* (0.8%)
B	*S. agalactiae* (*n* = 2, 1.8%)	*S. agalactiae* (5.3%)
A	*S. dysgalactiae* subsp. *dysgalactiae* (*n* = 1, 0.9%)	*S. dysgalactiae* subsp. *equisimilis* (0.8%)

Table 12. Species identification of β-hemolytic streptococci-Part 1

Sample ID.	Species	Reference strain	Concordance Rate (%)	Species-specific gene
KU1	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU2	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU3	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU4	*S. canis*	ATCC43496(T)	99.7	*cfg*
KU5	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU6	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU7	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU8	*S. canis*	ATCC43496(T)	99.9	*cfg*
KU9	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU10	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU11	*S. canis*	ATCC43496(T)	99.9	*cfg*
KU12	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU13	*S. canis*	ATCC43496(T)	99.3	*cfg*
KU14	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU15	*S. canis*	ATCC43496(T)	99.7	*cfg*
KU16	*S. canis*	ATCC43496(T)	99.7	*cfg*
KU17	SDSE	NCTC13762(T)	100.0	*emm* (*stG840.0*)
KU18	*S. canis*	AATCC43496(T)	100.0	*cfg*
KU19	SDSD	AATCC43078(T)	99.3	*emm* (*stC46.2*)[1)]
KU20	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU21	*S. canis*	ATCC43496(T)	99.9	*cfg*
KU23	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU24	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU25	*S. canis*	ATCC43496(T)	99.9	*cfg*
KU26	*S. canis*	ATCC43496(T)	99.9	*cfg*
KU27	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU28	*S. canis*	ATCC43496(T)	99.9	*cfg*
KU29	*S. canis*	ATCC43496(T)	99.9	*cfg*
KU30	*S. canis*	ATCC43496(T)	99.9	*cfg*
KU31	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU32	*S. canis*	ATCC43496(T)	99.7	*cfg*
KU33	*S. canis*	ATCC43496(T)	99.7	*cfg*
KU34	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU35	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU36	*S. canis*	ATCC43496(T)	99.9	*cfg*
KU37	*S. canis*	ATCC43496(T)	99.7	*cfg*
KU38	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU39	*S. canis*	ATCC43496(T)	99.7	*cfg*
KU40	*S. canis*	ATCC43496(T)	99.9	*cfg*

SDSE: *S. dysgalactiae* subsp. *equisimilis*
SDSD: *S. dysgalactiae* subsp. *dysgalactiae*

1) GenBank accession number: LC649931

Continued on the next page

Table 13. Species identification of β-hemolytic streptococci-Part 2

Sample ID.	Species	Reference strain	Concordance Rate (%)	Species-specific gene
KU41	*S. canis*	ATCC43496(T)	99.7	*cfg*
KU42	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU43	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU44	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU45	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU46	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU47	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU48	*S. canis*	ATCC43496(T)	99.9	*cfg*
KU49	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU50	*S. canis*	ATCC43496(T)	99.9	*cfg*
KU51	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU52	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU53	*S. canis*	ATCC43496(T)	99.9	*cfg*
KU54	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU55	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU56	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU57	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU58	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU59	*S. canis*	ATCC43496(T)	99.9	*cfg*
KU60	*S. canis*	ATCC43496(T)	99.9	*cfg*
KU61	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU62	*S. canis*	ATCC43496(T)	99.7	*cfg*
KU63	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU64	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU65	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU66	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU67	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU68	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU69	*S. canis*	ATCC43496(T)	99.9	*cfg*
KU70	*S. canis*	ATCC43496(T)	99.9	*cfg*
KU71	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU72	*S. canis*	ATCC43496(T)	99.9	*cfg*
KU73	SDSE	NCTC13762(T)	99.6	*emm* (*stC9431.0*)
KU74	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU75	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU76	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU77	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU78	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU79	*S. canis*	ATCC43496(T)	99.9	*cfg*

Continued on the next page

Table 14. Species identification of β-hemolytic streptococci-Part 3

Sample ID.	Species	Reference strain	Concordance Rate (%)	Species-specific gene
KU81	*S. canis*	ATCC43496(T)	99.3	*cfg*
KU82	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU83	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU84	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU85	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU86	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU87	*S. canis*	ATCC43496(T)	99.5	*cfg*
KU88	*S. canis*	ATCC43496(T)	99.8	*cfg*
KU89	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU90	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU91	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU92	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU93	*S. canis*	ATCC43496(T)	99.7	*cfg*
KU94	*S. agalactiae*	ATCC13813(T)	100.0	*dltS* (Ia)
KU95	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU96	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU97	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU98	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU99	SDSE	NCTC13762(T)	99.4	*emm* (*stC37.0*)
KU100	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU101	*S. canis*	ATCC43496(T)	99.5	*cfg*
KU102	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU103	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU104	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU105	*S. canis*	ATCC43496(T)	100.0	*cfg*
KU106	*S. canis*	ATCC43496(T)	99.8	*cfg*
KU107	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU108	SDSE	NCTC13762(T)	100.0	*emm* (*stL1929.1*)
KU109	*S. canis*	ATCC43496(T)	99.6	*cfg*
KU110	*S. canis*	ATCC43496(T)	99.5	*cfg*
KU111	*S. agalactiae*	ATCC13813(T)	100.0	*dltS* (Ⅴ)

Table 15. AMR phenotypes among β-hemolytic streptococci in 2021 and 2017

Resistance phenotype	Percentage of 2021(*n* = 109)	Percentage of 2017(*n* = 131)
Minocycline	34.9 (*n* = 38)	39.7 (*n* = 52)
Erythromycin	22.0 (*n* = 24)	19.8 (*n* = 26)
Azithromycin	22.9 (*n* = 25)	19.8 (*n* = 26)
Clindamycin	21.1 (*n* = 23)	17.6 (*n* = 23)
Levofloxacin	10.1 (*n* = 11)	4.6 (*n* = 6)

Table 16. MIC_{50} and MIC_{90} of selected antimicrobials against *S. canis*

Antimicrobials	MIC range (μg/mL)	MIC_{50}	MIC_{90}
Minocycline	≦ 0.5-> 4	1	> 4
Erythromycin	≦ 0.12-> 1	≦ 0.12	> 2
Azythromycin	≦ 0.25-> 4	≦ 0.25	> 4
Clindamycin	≦ 0.12-> 1	≦ 0.12	> 1
Levofloxacin	≦ 0.25-> 8	0.5	8

Table 17. AMR genotypes among β-hemolytic streptococci in 2021 and 2017

Resistance genotype	Percentage of 2021(*n* = 109)	Percentage of 2017(*n* = 131)
***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)

Table 18. Primer sets for amplifying VAGs and PCR-based amplicon size

VAG (encoding protein)	Primer	Direction	Sequence (5'→3')	Length (kmer)	Melting temperature	Expected amplicon size (bp)	Positive control strain
inl	inlF1	Forward	ACACCCAATTTCCTTTGCTG	20	51	223	NCTC 12191(T)
(Internalin)	inlR1	Reverse	ACCGAGTTTCCCATCTTGTG	20	52		
sagA	sagF1	Forward	GCTACTAGCGTAGCCGAAACA	21	57	118	NCTC 12191(T)
(Streptolysin S)	sagR1	Reverse	AGCTACCGCTACCACCTTGA	20	55		
slo	sloF1	Forward	AAATAGACCATGCGGAGTCG	20	53	233	NCTC 12191(T)
(Streptolysin O)	sloR1	Reverse	TTGAAGGGCACCTGGATAAG	20	52		
scp	scpF1	Forward	GGAATGGCTCCTAATGCTCA	20	53	248	FU6
(C5a peptidase)	scpR1	Reverse	GCACCGTCATTACCAATTCC	20	51		
lbp	lbpF1	Forward	GATTCAATCCAGAGCGGGTA	20	52	233	NCTC 12191(T)
(Lamininbinding protein)	lbpR1	Reverse	CCGGGTCAATACCATCTGTT	20	51		
fbp	fbpF1	Forward	TGGTGGCAATGTGATTGACT	20	51	248	TA4
(Fibronectinbinding protein)	fbpR1	Reverse	CGTCAGTTTGTCTTGTATCCTCA	23	54		
gbp	gbp_q_F	Forward	TTGCTTGGGGTGGAACAGTT	20	53	291	NCTC 12191(T)
(Glucanbinding protein)	gbp_q_R	Reverse	GGTTGGTCTCTGACGCAGAA	20	54		
ap1	ap1F1	Forward	GAAGGTTTGCCAACTGGGTA	20	51	249	FU53
(Pilus ancillary protein 1)	ap1R1	Reverse	ACTAGCGAAAAGCCAAACGA	20	54		
fp1	fp1F2	Forward	AACAGTTGGCGCAGAAGTTT	20	53	693	FU53
(Fimbrial protein)	fp1R1	Reverse	GAGGGTCTGGATTTCGATCA	20	52		
brp	brpF1	Forward	TGGTGCTGAAGCCAAACTGA	20	54	837	NCTC 12191(T)
(Biofilm regulatory protein)	brpR1	Reverse	GCTGGAACAACAGGAGTCGT	20	54		

Melting temperatures were calculated using nearest neighbor method.

Table 19. Reaction mixture composition for VAG amplification

Reagents	Volume
10x Ex-Taq Buffer	5 μL
10 mM dNTP mix	4 μL
primer F (5 μM)	4 μL
primer R (5 μM)	4 μL
Template	2 μL
Ex-Taq HS DNA polymerase (TaKaRa)	0.25 μL
MiliQ	30.75 μL
Total	50 μL

Table 20. PCR cycling conditions for each VAG

For *sag*A*, slo, scp, ap1* & *fbp a*mplification

94°C	3 min	
94°C	1 min	30 cycles
60°C	1 min	
72°C	1 min	
72°C	10 min	

For *inl, lbp, fp1,* & *brp a*mplification

94°C	3 min	
94°C	1 min	30 cycles
64°C	1 min	
72°C	1 min	
72°C	10 min	

For *gbp* amplification

94°C	3 min	
94°C	1 min	30 cycles
62°C	1 min	
72°C	1 min	
72°C	10 min	

Table 21. Primer set for *scm* amplification

Gene	Primer	Sequence5' → 3'	Length (mer)	Ref
scm	Sc_Mprot_F1	GGTGAAGACAAGCTTTTTAGC	21	(69)
scm	Sc_Mprot_R1	CGGTGTCATTCATGTACTTAG		

Table 22. Reaction mixture composition for *scm* amplification

Reagents	Volume
10x Ex-Taq Buffer	5 μL
10 mM dNTP mix	4 μL
primer F (5 μM)	4 μL
primer R (5 μM)	4 μL
Template	2 μL
Ex-Taq HS DNA polymerase (TaKaRa)	0.25 μL
MiliQ	30.75 μL
Total	50 μL

Table 23. PCR cycling conditions for *scm*

94°C	1 min	
94°C	25 sec	30 cycles
48°C	40 sec	
72°C	1 min 25sec	
72°C	10 min	

Table 24. Primer sets used for the analysis of the MLST

Target genes	Primers	Direction	Sequence (5'→3')	Length (mer)	Sequence length (bp)	Ref.
gki	gki_Sc_fwd	forward	GCTGATTTCGTAGGTATTGGTATGG	25	498	(68, 69)
	gki_Sc_rev	reverse	GTGAGCGTAGAAATTCTCCTGCTG	24		
gtr	gtr_Sc_fwd	forward	GGAATTGATTTAGACATCATGCCAGGAG	28	450	
	gtr_Sc_rev	reverse	TACAATAACCCCACCATCCATG	22		
murI	murI_Sc_fwd	forward	TTACGGACCAAGGCCAGCTGAGC	23	438	
	murI_Sc_rev	reverse	TTTCAGGACTTGCTGTCGTATAAAA	25		
mutS	mutS_fwd	forward	AGGTCAGATGTTAGAGGCTAGG	22	405	
	mutS_Sc_rev	reverse	CCTAATTCATCAAATAAAATGAGC	24		
recP	recP_fwd	forward	TGTCCGCACCCTATCAATGGAT	22	459	
	recP_Sc_rev	reverse	CGTCTTTCACAAGAATGTGTTGCC	24		
***xpt*(M13)**	xpt-fwd-M13F	forward	TGTAAAACGACGGCCAGTATGCAGTTACTTGAAGAACGCATCTTAAC	47	450	
	xpt-fwd-M13R	reverse	CAGGAAACAGCTATGACGCCTCCAAGAAGTTTAGATTACCA	41		
yqiZ	yqiz_fwd	forward	CAGATGCTTTTAACAATTACCACATGG	27	434	
	yqiZ_Sc_rev	reverse	ACCATTCACATTGACGATATCAGG	24		

Table 25. Reaction mixture composition for MLST analysis

Reagents	Volume
10x Ex-Taq Buffer	5 μL
10mM dNTP mix	4 μL
primer F (5 μM)	2 μL
primer R (5 μM)	2 μL
Template	2 μL
Ex-Taq HS DNA polymerase (TaKaRa)	0.1 μL
MiliQ	34.9 μL
Total	50 μL

Table 26. PCR cycling conditions for MLST analysis

94℃	4 min	
94℃	1 min	30 cycles
50℃	1 min	
72℃	1 min	
72℃	5 min	

94℃	4 min	
94℃	1 min	30 cycles
46℃	1 min	
72℃	1 min	
72℃	5 min	

The annealing temperature was set at 46℃ for isolates with poor amplification in the PCR conditions.

Table 27. Animal backgrounds and sample sources of enrolled isolates

Population	Isolate ID no.	Prefecture	Age	Breed	Tetracyclin resistance gene
2021-eye	**KU4**	Tokyo	10	ND	Eye discharge
	KU6	Tokyo	10	ND	Cornea
	KU20	Kanagawa	14	ND	Eye discharge
	KU44	Tokyo	7	ND	Cornea
	KU57	Tokyo	6	ND	Cornea
	KU58	Tokyo	2	ND	Cornea
	KU66	Kanagawa	13	ND	Eye discharge
	KU67	Kanagawa	2	ND	Eye discharge
	KU71	Chiba	13	ND	Cornea
2021-ear	**KU9**	Kanagawa	11	ND	Ear discharge
	KU12	Saitama	13	ND	Ear discharge
	KU42	Saitama	3	ND	Ear discharge
	KU47	Chiba	10	ND	Ear discharge
	KU54	Chiba	13	ND	Ear discharge
	KU56	Aichi	ND	ND	Ear discharge
	KU68	Tokyo	14	ND	Ear discharge
	KU69	Tokyo	9	ND	Fluid in ear canal
	KU70	Tokyo	14	ND	Ear discharge
	KU76	Chiba	ND	ND	Ear discharge
	KU77	Chiba	17	ND	Ear discharge
	KU82	Tokyo	15	ND	Ear discharge
	KU88	Chiba	13	ND	Ear discharge
	KU89	Chiba	13	ND	Ear discharge
	KU90	Saitama	ND	ND	Ear discharge
	KU91	Aichi	8	ND	Ear discharge
	KU93	Tokyo	9	ND	Ear discharge
	KU97	Ibaraki	4	ND	Ear discharge
	KU103	Tokyo	7	ND	Ear discharge
	KU107	Chiba	14	ND	Ear discharge
2017-eye	**FU14**	Tokyo	11	Shih Tzu	Cornea
	FU21	Chiba	14	Toy Poodle	Conjunctiva
	FU39	Ibaraki	ND	ND	Cornea
	FU49	Tokyo	ND	ND	Eye discharge
	FU59	Chiba	ND	Dalmatian	Cornea
	FU67	Chiba	ND	French Bulldog	Eye discharge
	FU70	Tokyo	2	Shih Tzu	Conjunctiva
	FU76	Tokyo	5	Yorkshire Terrier	Conjunctiva
	FU83	Kanagawa	ND	Boston Terrier	Cornea
	FU96	Aichi	ND	Shih Tzu	Eye discharge
	FU104	Tokyo	6	Shih Tzu	Conjunctiva
	FU123	Okayama	1	Boston Terrier	Cornea
	FU131	Tokyo	10	Shih Tzu	Cornea

ND: No data

Table 28. HA values of the 2021-eye

Isolate ID no.	Isolation source	HA value (mean ± standard deviation)
KU4	Eye discharge	0.34 ± 0.01
KU6	Cornea	0.36 ± 0.02
KU20	Eye discharge	0.30 ± 0.01
KU44	Cornea	0.36 ± 0.02
KU57	Cornea	0.33 ± 0.01
KU58	Cornea	0.37 ± 0.01
KU66	Eye discharge	0.35 ± 0.01
KU67	Eye discharge	0.31 ± 0.01
KU71	Cornea	0.29 ± 0.02

Table 29. HA values of the 2017-eye

Isolate ID no.	Isolation source	HA value (mean ± standard deviation)
FU14	Cornea	0.36 ± 0.02
FU21	Conjunctiva	0.47 ± 0.03
FU39	Cornea	0.35 ± 0.01
FU49	Eye discharge	0.37 ± 0.01
FU59	Cornea	0.50 ± 0.02
FU67	Eye discharge	0.46 ± 0.01
FU70	Conjunctiva	0.37 ± 0.01
FU76	Conjunctiva	0.41 ± 0.01
FU83	Cornea	0.42 ± 0.01
FU96	Eye discharge	0.34 ± 0.03
FU104	Conjunctiva	0.32 ± 0.01
FU123	Cornea	0.42 ± 0.03
FU131	Cornea	0.31 ± 0.01

A red-filled cell indicates a high value.

Table 30. HA values of the 2021-ear

Isolate ID no.	Isolation source	HA value (mean ± standard deviation)
KU9	Ear discharge	0.37 ± 0.01
KU12	Ear discharge	0.36 ± 0.01
KU42	Ear discharge	0.32 ± 0.01
KU47	Ear discharge	0.31 ± 0.01
KU54	Ear discharge	0.46 ± 0.02
KU56	Ear discharge	0.43 ± 0.02
KU68	Ear discharge	0.41 ± 0.01
KU69	Fluid in ear canal	0.34 ± 0.02
KU70	Ear discharge	0.33 ± 0.01
KU76	Ear discharge	0.44 ± 0.03
KU77	Ear discharge	0.35 ± 0.01
KU82	Ear discharge	0.48 ± 0.02
KU88	Ear discharge	0.42 ± 0.01
KU89	Ear discharge	0.44 ± 0.04
KU90	Ear discharge	0.43 ± 0.03
KU91	Ear discharge	0.36 ± 0.01
KU93	Ear discharge	0.36 ± 0.01
KU97	Ear discharge	0.41 ± 0.02
KU103	Ear discharge	0.47 ± 0.03
KU107	Ear discharge	0.37 ± 0.02

Table 31. VAG profile of the 2021-eye

Isolate ID no.	**Isolation source**	***inl***	***sagA***	***slo***	***scp***	***lbp***	***fbp***	***gbp***	***apl***	***fpl***	***brp***
KU4	Eye discharge	+	+	-	+	+	-	+	-	-	+
KU6	Cornea	+	+	+	+	+	+	+	-	-	+
KU20	Eye discharge	+	+	+	+	+	+	+	-	-	+
KU44	Cornea	+	+	+	+	+	+	+	-	-	+
KU57	Cornea	+	+	+	+	+	+	+	-	-	+
KU58	Cornea	+	+	+	+	+	+	+	-	-	+
KU66	Eye discharge	+	+	+	+	+	+	+	-	-	+
KU67	Eye discharge	+	+	+	+	+	+	+	-	-	+
KU71	Cornea	+	+	+	+	+	+	+	-	-	+

+: Detected, -: Not detected

Table 32. VAG profile of the 2017-eye

Isolate ID no.	Isolation source	*inl*	*sagA*	*slo*	*scp*	*lbp*	*fbp*	*gbp*	*apl*	*fpl*	*brp*
FU14	Cornea	+	+	+	+	+	+	+	-	-	+
FU21	Conjunctiva	+	+	+	+	+	+	-	-	-	+
FU39	Cornea	+	+	+	+	+	+	+	-	-	+
FU49	Eye discharge	+	+	+	+	+	+	-	-	-	+
FU59	Cornea	+	+	+	+	+	+	+	-	-	+
FU67	Eye discharge	+	+	+	+	+	+	+	-	-	+
FU70	Conjunctiva	+	+	+	+	+	+	-	+	-	-
FU76	Conjunctiva	+	+	+	+	+	+	+	-	-	+
FU83	Cornea	+	+	+	+	+	+	+	-	-	+
FU96	Eye discharge	+	+	+	+	+	+	+	-	-	+
FU104	Conjunctiva	+	+	+	+	+	+	+	-	-	+
FU123	Cornea	+	+	+	+	+	+	+	-	-	+
FU131	Cornea	+	+	+	+	+	+	+	-	-	+

+: Detected, -: Not detected

Table 33. VAG profile of the 2021-ear

Isolate ID no.	Isolation source	*inl*	*sagA*	*slo*	*scp*	*lbp*	*fbp*	*gbp*	*apl*	*fpl*	*brp*
KU9	Ear discharge	+	+	+	+	+	+	+	-	-	+
KU12	Ear discharge	+	+	+	+	+	+	+	-	-	+
KU42	Ear discharge	+	+	+	+	+	+	+	-	-	+
KU47	Ear discharge	+	+	+	+	+	+	+	-	-	+
KU54	Ear discharge	+	+	+	+	+	-	+	-	-	+
KU56	Ear discharge	+	+	+	+	+	+	+	-	-	+
KU68	Ear discharge	+	+	+	+	+	+	-	-	-	+
KU69	Fluid in ear canal	+	+	+	+	+	+	+	-	-	+
KU70	Ear discharge	+	+	+	+	+	+	+	-	-	+
KU76	Ear discharge	+	+	+	+	+	+	+	-	-	+
KU77	Ear discharge	+	+	+	+	+	+	+	-	-	+
KU82	Ear discharge	+	+	+	+	+	+	-	-	-	+
KU88	Ear discharge	+	+	+	+	+	+	+	-	+	+
KU89	Ear discharge	+	+	+	+	+	+	+	-	-	+
KU90	Ear discharge	+	+	+	+	+	+	+	-	-	+
KU91	Ear discharge	+	+	+	+	+	+	+	-	-	+
KU93	Ear discharge	+	+	+	+	+	+	+	-	+	+
KU97	Ear discharge	+	+	+	+	+	+	+	+	-	+
KU103	Ear discharge	+	+	+	+	+	+	+	-	-	+
KU107	Ear discharge	+	+	+	+	+	+	+	-	-	+

+: Detected, -: Not detected

Table 34. Prevalence of VAGs in each group

Genotypic VAG traits	Prevalence among the 2021-eye (*n* = 9)	Prevalence among the 2021-ear (*n* = 20)	Prevalence among the 2017-eye (*n* = 13)
inl	100%	100%	100%
sagA	100%	100%	100%
slo	88.9%	100%	100%
scp	100%	100%	100%
lbp	100%	100%	100%
fbp	88.9%	95.0%	100%
gbp	100%	90.0%	76.9%
ap1	0%	5.0%	7.7%
fp1	0%	10.0%	0%
brp	100%	100%	92.3%

Table 35. *scm* allele profile and accession numbers in DDBJ

Group	Isolate ID no.	M-like protein allele	Accession no.
2021-eye	**KU4**	Allele 13	LC662017
	KU6	Allele 10	LC662018
	KU20	Allele 2	LC662021
	KU44	Allele 2	LC662023
	KU57	Allele 2	LC662027
	KU58	Allele 1	LC662028
	KU66	Allele 2	LC662030
	KU67	Allele 2	LC662031
	KU71	Allele 2	LC662035
2021-ear	**KU9**	Allele 1	LC662019
	KU12	Allele 2	LC662020
	KU42	Allele 2	LC662022
	KU47	Allele 2	LC662024
	KU54	Allele 1	LC662025
	KU56	Allele 1	LC662026
	KU68	Allele 1	LC662032
	KU69	Allele 9	LC662033
	KU70	Truncated variant	LC662034
	KU76	Allele 1	LC662036
	KU77	Allele 2	LC662037
	KU82	Allele 6	LC662038
	KU88	Allele 11	LC662039
	KU89	Allele 1	LC662040
	KU90	Allele 1	LC662041
	KU91	Allele 2	LC662042
	KU93	Allele 11	LC662043
	KU97	Allele 10	LC662044
	KU103	Allele 1	LC662045
	KU107	Allele 2	LC662046
2017-eye	**FU14**	Allele 1	LC363841
	FU21	Allele 2	LC363847
	FU39	Allele 1	LC363777
	FU49	Allele 1	LC363795
	FU59	Allele 4	LC363812
	FU67	Allele 9	LC363787
	FU70	Allele 1	LC363800
	FU76	Allele 2	LC363822
	FU83	Allele 7	LC363801
	FU96	Allele 4	LC363806
	FU104	Allele 9	LC363827
	FU123	Allele 2	LC363760
	FU131	Allele 4	LC363765

Table 36. Consolidated data of *scm* allele types

Allele type	2021-eye (n = 9)	2021-ear (n = 20)	2017-eye (n = 13)
Allele type 1 (n = 13)	11.1% (n = 1)	40.0% (n = 8)	30.8% (n = 4)
Allele type 2 (n = 15)	66.7% (n = 6)	30.0% (n = 6)	23.1% (n = 3)
Allele type 4 (n = 3)	0%	0%	23.1% (n = 3)
Allele type 6 (n = 1)	0%	5.0% (n = 1)	0%
Allele type 7 (n = 1)	0%	0%	7.7% (n = 1)
Allele type 9 (n = 3)	0%	5.0% (n = 1)	15.4% (n = 2)
Allele type 10 (n = 2)	11.1% (n = 1)	5.0% (n = 1)	0%
Allele type 11 (n = 2)	0%	10.0% (n = 2)	0%
Allele type 13 (n = 1)	11.1% (n = 1)	0%	0%
Truncated variant (n = 1)	0%	5.0% (n = 1)	0%

Table 37. Results of MLST analysis among three groups

Group	Isolate ID no.	Allele profile gki-gtr-murI-mutS-recP-xpt-yqiZ	Sequence Type	Clonal Complex
2021-eye	KU4	6-11-1-6-4-10-6	ST51	
	KU6	2-2-2-2-2-1-2	ST2	CC46
	KU20	2-2-2-2-2-3-2	ST46	CC46
	KU44	2-2-2-2-2-3-2	ST46	CC46
	KU57	2-2-2-2-2-3-2	ST46	CC46
	KU58	3-3-3-3-1-2-3	ST9	CC9
	KU66	2-2-2-2-2-3-2	ST46	CC46
	KU67	2-2-2-2-2-3-2	ST46	CC46
	KU71	2-2-2-2-2-3-2	ST46	CC46
2021-ear	KU9	15-5-9-3-1-2-3	ST48	
	KU12	2-2-2-2-2-3-2	ST46	CC46
	KU42	2-2-2-2-2-1-2	ST2	CC46
	KU47	2-2-2-2-2-1-2	ST2	CC46
	KU54	3-5-3-8-1-2-3	ST29	CC9
	KU56	3-3-3-3-1-2-3	ST9	CC9
	KU68	3-3-3-3-1-2-3	ST3	CC9
	KU69	3-2-4-15-12-7-10	ST56	
	KU70	2-4-15-12-7-10	ST68*	
	KU76	3-5-3-3-1-2-2	ST66*	
	KU77	2-2-2-2-2-1-2	ST2	CC46
	KU82	3-2-6-4-5-3-4	ST27	
	KU88	7-2-3-12-4-2-3	ST41	
	KU89	15-5-9-3-1-2-3	ST48	
	KU90	3-3-3-3-1-2-3	ST9	CC9
	KU91	2-2-2-2-2-3-14	ST69*	CC46
	KU93	7-2-3-12-4-2-3	ST41	
	KU97	3-2-4-3-4-5-1	ST64*	
	KU103	3-3-3-3-1-2-3	ST3	CC9
	KU107	2-2-2-2-2-3-2	ST46	CC46
2017-eye	FU14	3-3-3-3-1-2-3	ST9	CC9
	FU21	2-2-2-2-2-3-2	ST46	CC46
	FU39	15-5-9-3-1-2-3	ST48	
	FU49	3-3-3-3-1-2-3	ST9	CC9
	FU59	4-2-4-4-6-3-4	ST14	
	FU67	3-2-4-15-12-7-10	ST56	CC56
	FU70	3-3-3-3-1-2-3	ST9	CC9
	FU76	2-2-2-2-2-1-2	ST2	CC46
	FU83	4-2-4-18-15-7-13	ST67*	
	FU96	4-2-4-4-6-3-4	ST14	
	FU104	3-2-4-15-12-7-10	ST56	CC56
	FU123	2-2-2-2-2-3-2	ST46	CC46
	FU131	4-2-4-4-6-3-4	ST14	

*represents new type

Table 38. Results of MLST analysis
in the 2021 non-eye non-ear isolates

Isolate ID no.	Sample source	Allele profile gki-gtr-murI-mutS-recP-xpt-yqiZ	Sequence Type	Clonal Complex
KU1	urogenital tract	3-5-3-3-1-2-3	ST9	CC9
KU3	uterine content	3-5-3-3-1-2-3	ST9	CC9
KU7	open pus	3-5-3-3-1-2-3	ST9	CC9
KU11	anal glandular fluid	4-2-4-4-6-3-4	ST14	
KU18	open pus	15-5-9-3-1-2-3	ST48	
KU24	urogenital tract	3-5-3-3-1-2-3	ST9	CC9
KU27	urogenital tract	4-3-3-3-1-2-3	ST63	
KU28	urogenital tract	7-2-3-12-4-7-3	ST41	
KU38	urogenital tract	3-3-3-3-1-2-3	ST3	CC9
KU46	urogenital tract	15-5-9-3-1-2-3	ST48	CC48
KU48	open pus	3-5-3-3-1-2-3	ST9	CC9
KU51	open pus	3-5-3-3-1-2-3	ST9	CC9
KU52	open pus	3-3-3-3-1-2-3	ST3	CC9
KU60	uterine content	4-2-4-4-6-3-4	ST14	
KU75	urogenital tract	3-5-3-3-1-2-3	ST9	CC9
KU100	open pus	2-2-2-2-2-1-2	ST2	CC46
KU106	urogenital tract	9-14-3-17-14-7-5	ST65	

Table 39. Summarized results of MLST analysis

Clonal complex	Sequence Types	2021-eye (n = 9)	2021-ear (n = 20)	2017-eye (n = 13)
CC9 (n = 10)	ST9, ST3, ST29, and ST66	11.1% (n = 1)	30.0% (n = 6)	23.1% (n = 3)
CC46 (n = 16)	ST46, ST2, and ST69	77.8% (n = 7)	30.0% (n = 6)	23.1% (n = 3)
CC56 (n = 4)	ST56 and ST68	0%	10.0% (n = 2)	15.4% (n = 2)
Singleton (n = 12)	ST14, ST27, ST41, ST48,ST51, ST64, and ST67	11.1% (n = 1)	30.0% (n = 6)	38.5% (n = 5)

Red lines indicate statistically significant (p <0.05) pairs

Table 40. AMR phenotypes of the 2021-eye

Isolate ID no.	PCG	AMPC	CFPM	CTX	CTRX	CZOP	MEPM	MINO	EM	AZM	CLDM	LVFX	VCM	CP
KU4	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU6	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	**>4**	0.25	≦0.25	**>1**	0.5	0.5	≦4
KU20	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	**>4**	**>2**	**>4**	**>1**	**>8**	0.5	≦4
KU44	0.06	0.12	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	**>4**	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU57	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	**>4**	**>2**	**>4**	**>1**	**>8**	0.5	≦4
KU58	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU66	0.06	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	**>4**	**>2**	**>4**	**>1**	**>8**	0.5	≦4
KU67	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	**>4**	**>2**	**>4**	**>1**	**>8**	0.5	≦4
KU71	0.06	0.12	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	**>4**	**>2**	**>4**	**>1**	1	0.5	≦4

PCG: Penicillin G, AMPC: Ampicillin, CFPM: CFPM: Cefepime, CTX: Cefotaxime, CTRX: Ceftriaxone, CZOP: Cefozopran, MEPM: Meropenem, MINO: Minocycline,
EM: erythromycin, AZM: Azithromycin, CLDM: Clindamycin, LVFX: Levofloxacin, VCM: Vancomycin, CO: Chloramphenicol
Black: Susceptible, Blue: Intermediate, Red: Resistant

Table 41. AMR phenotypes of the 2017-eye

Isolate ID no.	PCG	AMPC	CFPM	CTX	CTRX	CZOP	MEPM	MINO	EM	AZM	CLDM	LVFX	VCM	CP
FU14	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	**>4**	**>2**	**>4**	**>1**	0.5	0.25	≦4
FU21	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	**>4**	**>2**	**>4**	**>1**	0.5	0.25	≦4
FU39	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.25	≦4
FU49	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.25	≦4
FU59	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	1	0.25	≦4
FU67	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	1	0.5	≦4
FU70	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
FU76	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.25	≦4
FU83	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.25	≦4
FU96	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.25	≦4
FU104	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.25	≦4
FU123	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.25	≦4
FU131	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	**>4**	**>2**	**>4**	**>1**	0.5	0.5	≦4

PCG: Penicillin G, AMPC: Ampicillin, CFPM: CFPM: Cefepime, CTX: Cefotaxime, CTRX: Ceftriaxone, CZOP: Cefozopran, MEPM: Meropenem, MINO: Minocycline,
EM: erythromycin, AZM: Azithromycin, CLDM: Clindamycin, LVFX: Levofloxacin, VCM: Vancomycin, CO: Chloramphenicol
Black: Susceptible, Blue: Intermediate, Red: Resistant

Table 42. AMR phenotypes of the 2021-ear

Isolate ID no.	PCG	AMPC	CFPM	CTX	CTRX	CZOP	MEPM	MINO	EM	AZM	CLDM	LVFX	VCM	CP
KU9	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	>4	>2	>4	>1	0.5	0.5	≦4
KU12	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	>4	2	>4	0.25	>8	1	≦4
KU42	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU47	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	2	0.25	≦4
KU54	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU56	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU68	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	2	0.5	≦4
KU69	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	1	0.5	≦4
KU70	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	1	0.5	≦4
KU76	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU77	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	2	0.5	≦4
KU82	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	1	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU88	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	2	≦0.12	≦0.25	≦0.12	1	0.5	≦4
KU89	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	1	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU90	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	1	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU91	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	>4	>2	>4	>1	>8	0.5	≦4
KU93	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	1	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU97	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU103	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	>4	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU107	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	>4	>2	>4	>1	>8	0.5	≦4

PCG: Penicillin G, AMPC: Ampicillin, CFPM: CFPM: Cefepime, CTX: Cefotaxime, CTRX: Ceftriaxone, CZOP: Cefozopran, MEPM: Meropenem, MINO: Minocycline,
EM: erythromycin, AZM: Azithromycin, CLDM: Clindamycin, LVFX: Levofloxacin, VCM: Vancomycin, CO: Chloramphenicol
Black: Susceptible, Blue: Intermediate, Red: Resistant

Table 43. AMR phenotypes of the 2021 non-eye non-ear isolates

Isolate ID no.	PCG	AMPC	CFPM	CTX	CTRX	CZOP	MEPM	MINO	EM	AZM	CLDM	LVFX	VCM	CP
KU1	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU3	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU7	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.25	0.5	≦4
KU11	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	1	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU18	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	**>4**	**>2**	**>4**	**>1**	0.5	0.5	≦4
KU24	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU27	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	**4**	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU28	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	1	≦0.12	≦0.25	≦0.12	1	0.5	≦4
KU38	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	**>4**	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU46	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	**>4**	**>2**	**>4**	**>1**	0.5	0.5	≦4
KU48	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU51	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU52	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	**>4**	**0.5**	**4**	**>1**	2	0.5	≦4
KU60	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU75	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	**>4**	**>2**	**>4**	**>1**	0.5	0.5	≦4
KU100	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	≦0.5	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4
KU106	≦0.03	≦0.06	≦0.5	≦0.12	≦0.12	≦0.12	≦0.12	1	≦0.12	≦0.25	≦0.12	0.5	0.5	≦4

PCG: Penicillin G, AMPC: Ampicillin, CFPM: CFPM: Cefepime, CTX: Cefotaxime, CTRX: Ceftriaxone, CZOP: Cefozopran, MEPM: Meropenem, MINO: Minocycline,
EM: erythromycin, AZM: Azithromycin, CLDM: Clindamycin, LVFX: Levofloxacin, VCM: Vancomycin, CO: Chloramphenicol
Black: Susceptible, Blue: Intermediate, Red: Resistant

Table 44. Prevalence of AMR phenotypes among three groups

	2021-ear(%)	2021-eye(%)	2017-eye(%)
Presence of AMR phenotype	25.0	77.8	23.1
AMR to Minocycline	25.0	77.8	23.1
AMR to Erythromycin/ Azithromycin	20.0	55.6	23.1
AMR to Clindamycin	15.0	66.7	23.1
AMR to Levofloxacin	15.0	44.4	0

Red lines indicate statistically significant (p <0.05) pairs

Table 45. AMR genotypes among three groups

Population	Isolate ID no.	Tetracyclin resistance gene	Macrolide/ Lincosamide resistance gene
2021-eye	**KU4**		
	KU6	*tet*(M)	*mef*(A)
	KU20	*tet*(O)	*erm*(B)
	KU44	*tet*(O)	
	KU57	*tet*(O)	*erm*(B)
	KU58		
	KU66	*tet*(O)	*erm*(B)
	KU67	*tet*(O)	*erm*(B)
	KU71	*tet*(O)	*erm*(B)
2021-ear	**KU9**	*tet*(O)	*erm*(B)
	KU12	*tet*(O)	*erm*(B)
	KU42		
	KU47		
	KU54		
	KU56		
	KU68		
	KU69		
	KU70		
	KU76		
	KU77		
	KU82		
	KU88		
	KU89		
	KU90		
	KU91	*tet*(O)	*erm*(B)
	KU93		
	KU97		
	KU103	*tet*(O)	
	KU107	*tet*(O)	*erm*(B)
2017-eye	**FU14**		
	FU21	*tet*(O)	*erm*(B)
	FU39		
	FU49		
	FU59		
	FU67		
	FU70		
	FU76		
	FU83		
	FU96		
	FU104		
	FU123	*tet*(O)	*erm*(B)
	FU131	*tet*(O)	

Table 46. Prevalence of AMR genotypes among three groups

	2021-ear(%)	2021-eye(%)	2017-eye(%)
Presence of AMR genotype	25.0	77.8	23.1
***tet*(O)/*tet*(M)**	25.0	77.8	23.1
***erm*(B)/*mef*(A)**	20.0	66.7	15.4

Red lines indicate statistically significant ($p < 0.05$) pairs

Table 47. AMR genotypes in the 2021 non-eye non-ear isolates

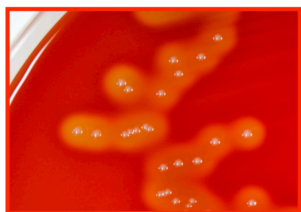
Isolate ID no.	Tetracyclin resistance gene	Macrolide/ Lincosamide resistance gene
KU1		
KU3		
KU7		
KU11		
KU18	*tet*(O)	*erm*(B)
KU24		
KU27	*tet*(O)	
KU28		
KU38	*tet*(O)	
KU46	*tet*(O)	*erm*(B)
KU48		
KU51		
KU52	*tet*(O)	
KU60		
KU75	*tet*(O)	*erm*(B)
KU100		
KU106		

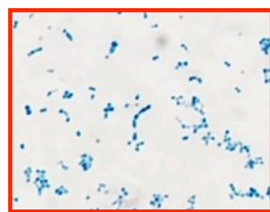
VIII. Figures

Sampling by local veterinary practitioners

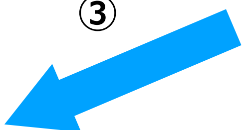
The Sanritsu Zelkova Veterinary Laboratory

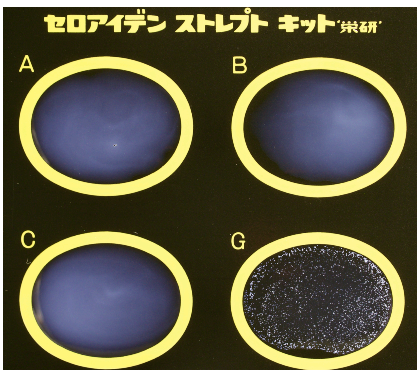
②





- β-hemolysis
- chain-forming cocci





- Classification by Lancefield classification kit (Seroiden Strepto Kit Eiken®; Eiken Chemical Co.)

Minimum inhibitory concentrations(MICs, µg/ mL) of **penicillin G, ampicillin, cefepime, cefotaxime, ceftriaxone, cefozopran, meropenem, minocycline, erythromycin, azithromycin, clindamycin, levofloxacin, vancomycin, chloramphenicol** (MICroFAST Panel Type 7J for Streptococcus spp., Beckman Coulter Inc., Tokyo, Japan) (23)

Among these processes, ② to ④ were conducted in the Sanritsu Zelkova Veterinary Laboratory.

Figure 1. Schematic diagram of sample collection

The photo of the cat is included with permission of the cat owner

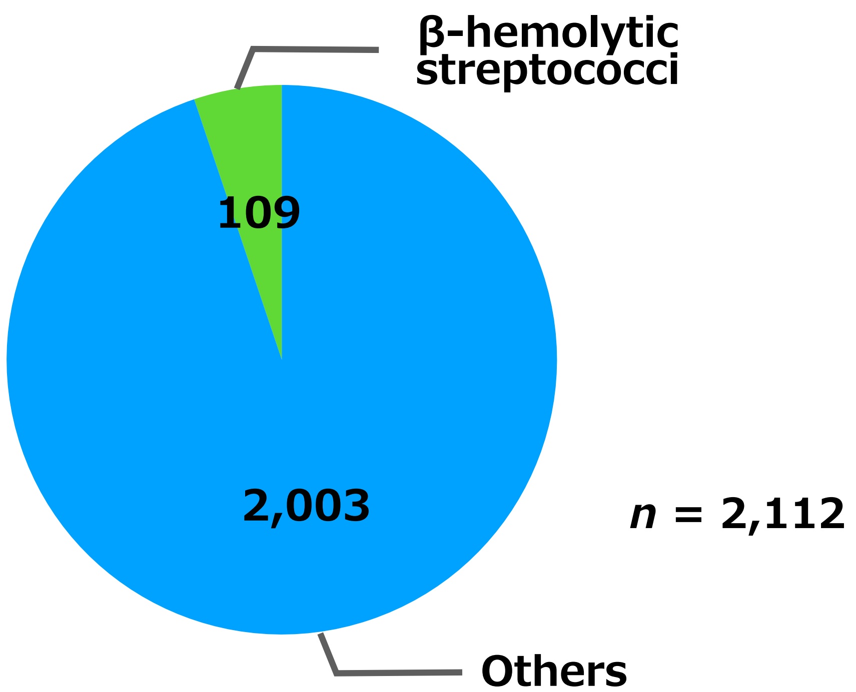


Figure 2. Proportion of β-hemolytic streptococci

n = 2,112

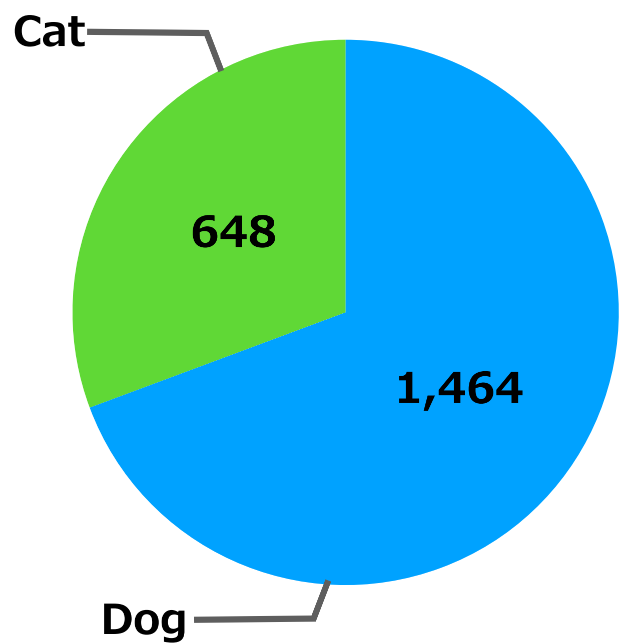


Figure 3. Proportion of dogs and cats

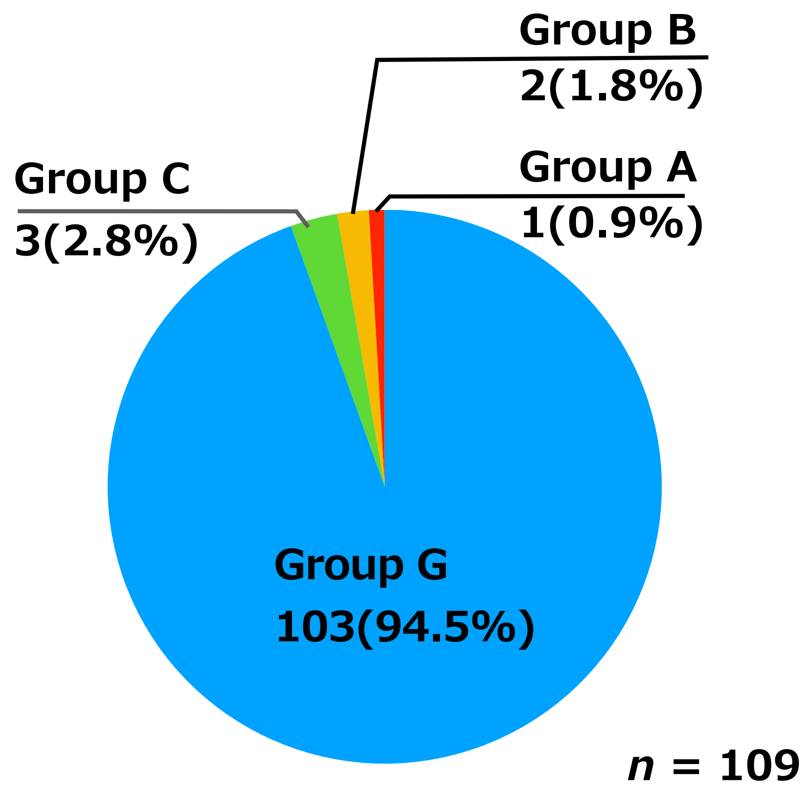


Figure 4. Lancefield grouping of β-hemolytic streptococci

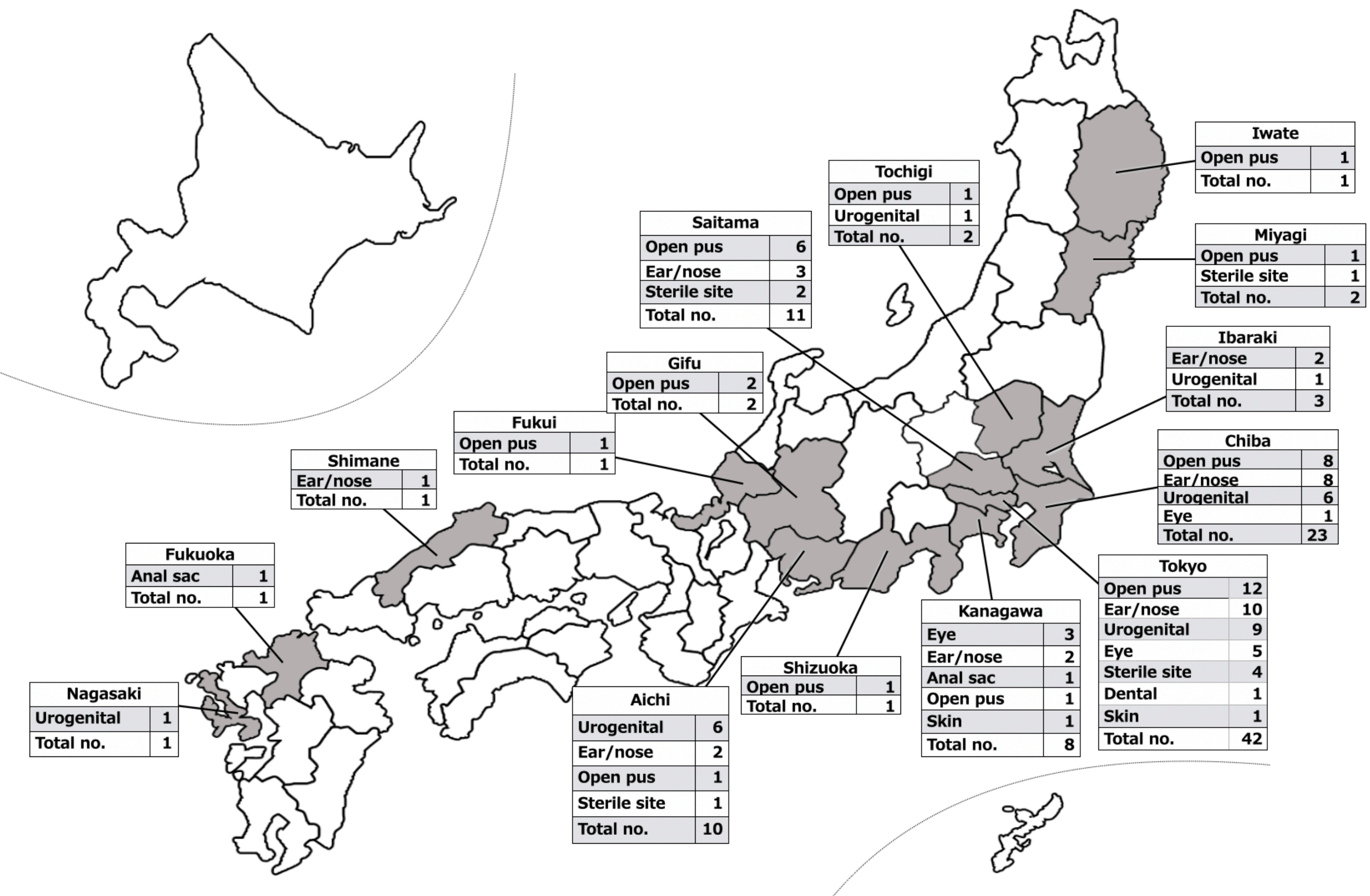


Figure 5. Sample collection locales and collected sources of isolates

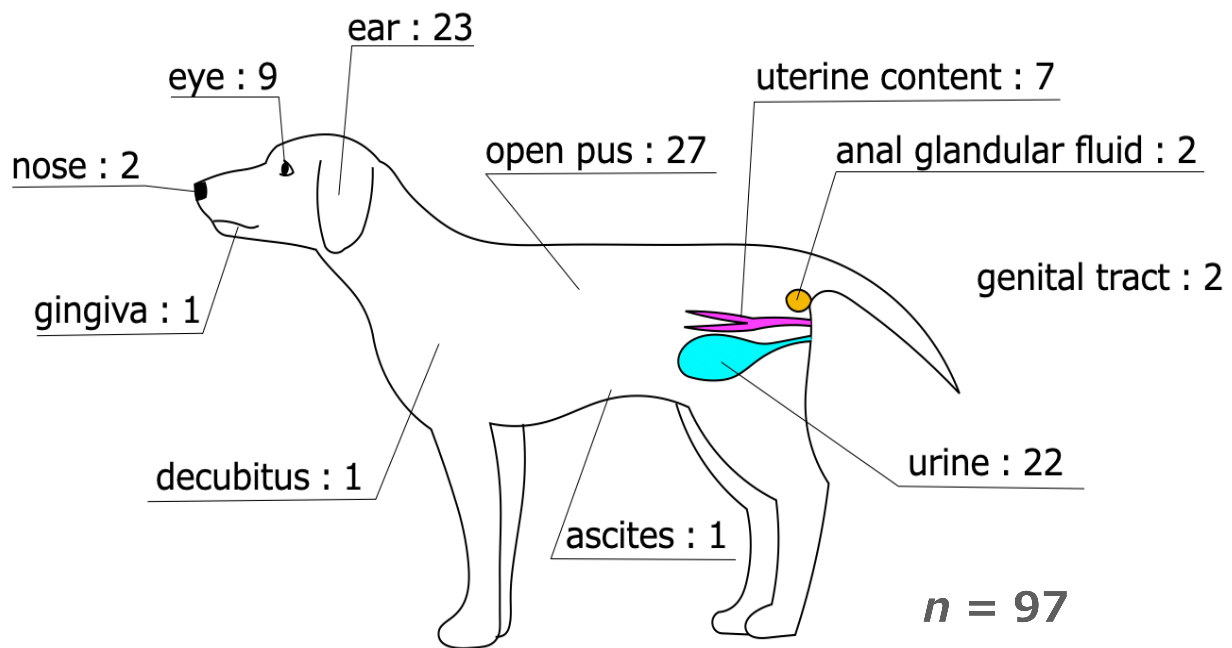


Figure 6. Sample collection site (dogs)

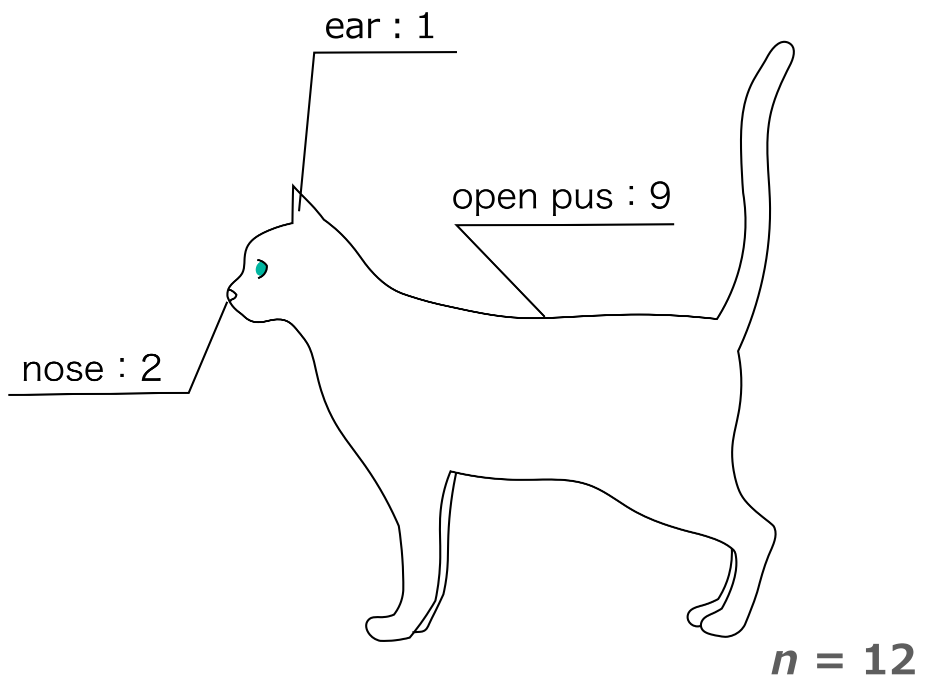


Figure 7. Sample collection site (cats)

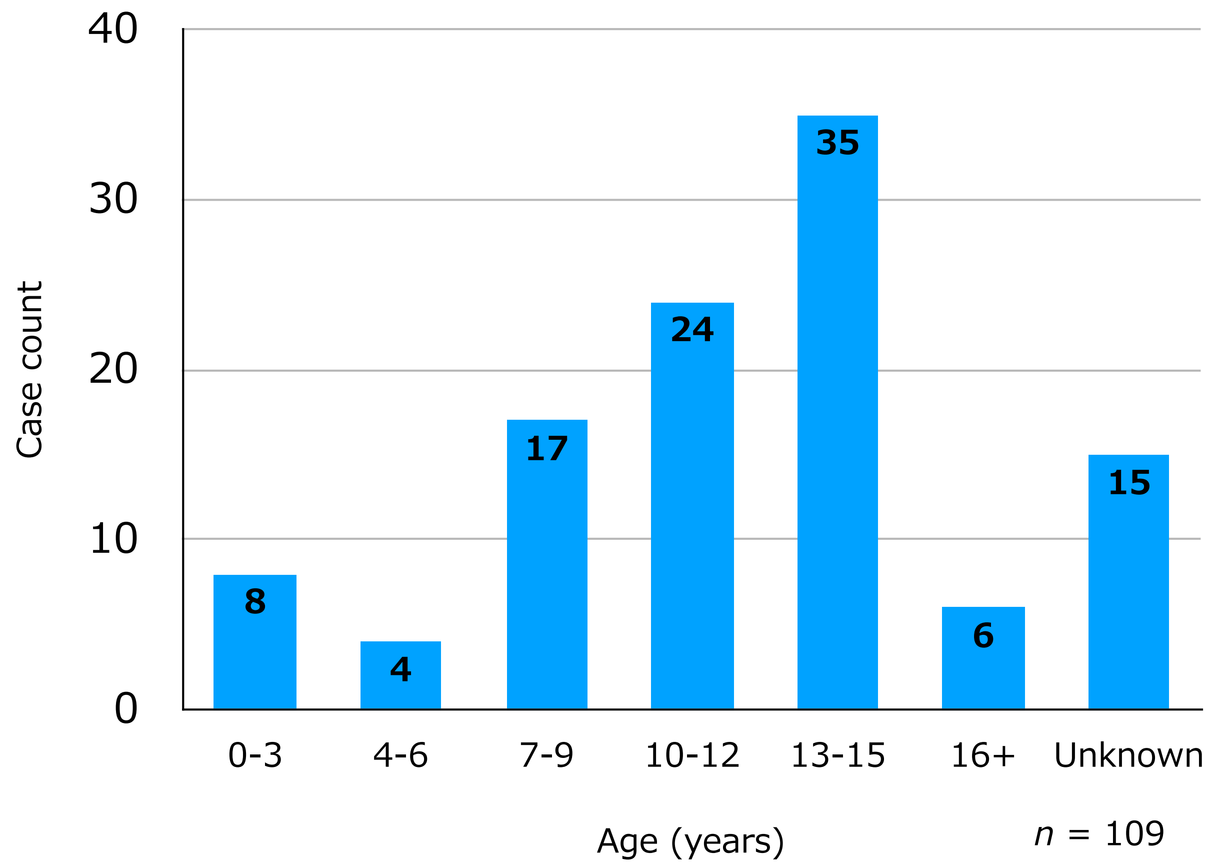


Figure 8. Age distribution across all cases

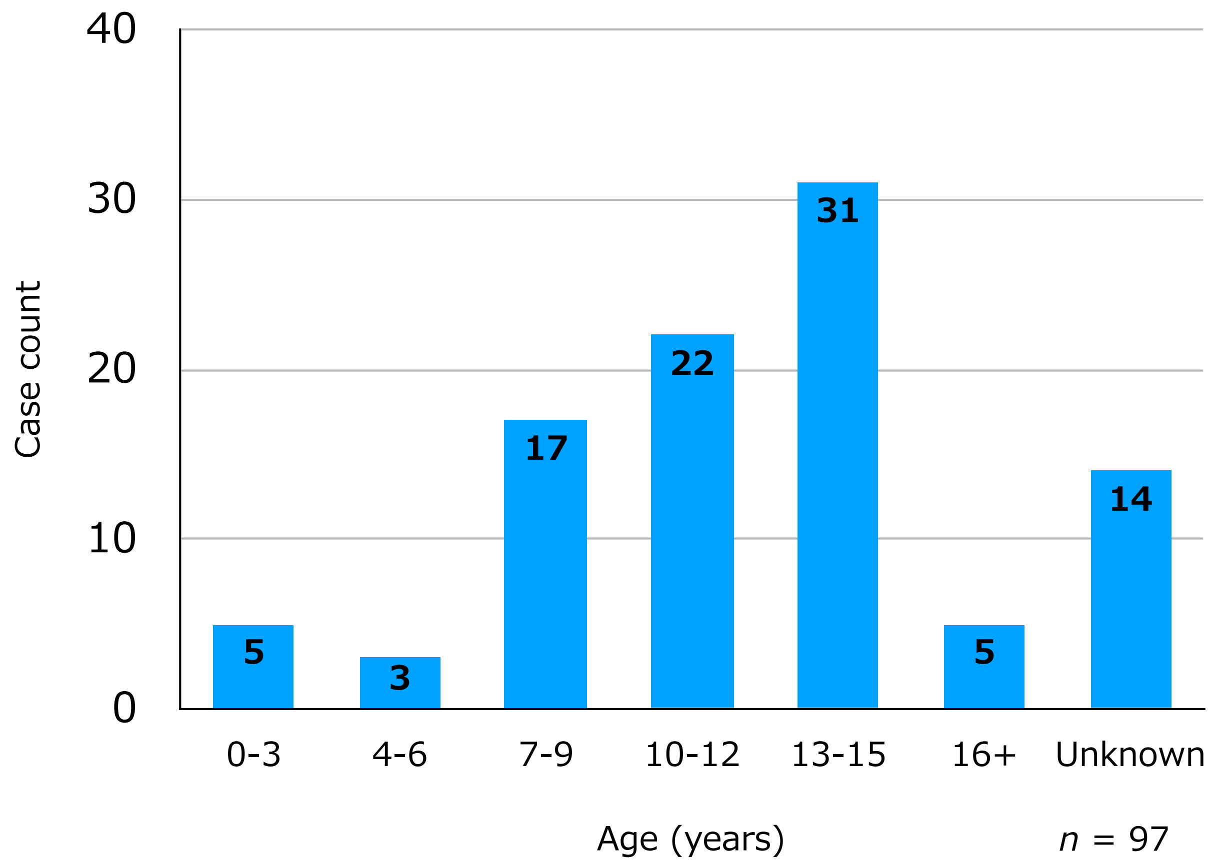


Figure 9. Age distribution in dogs

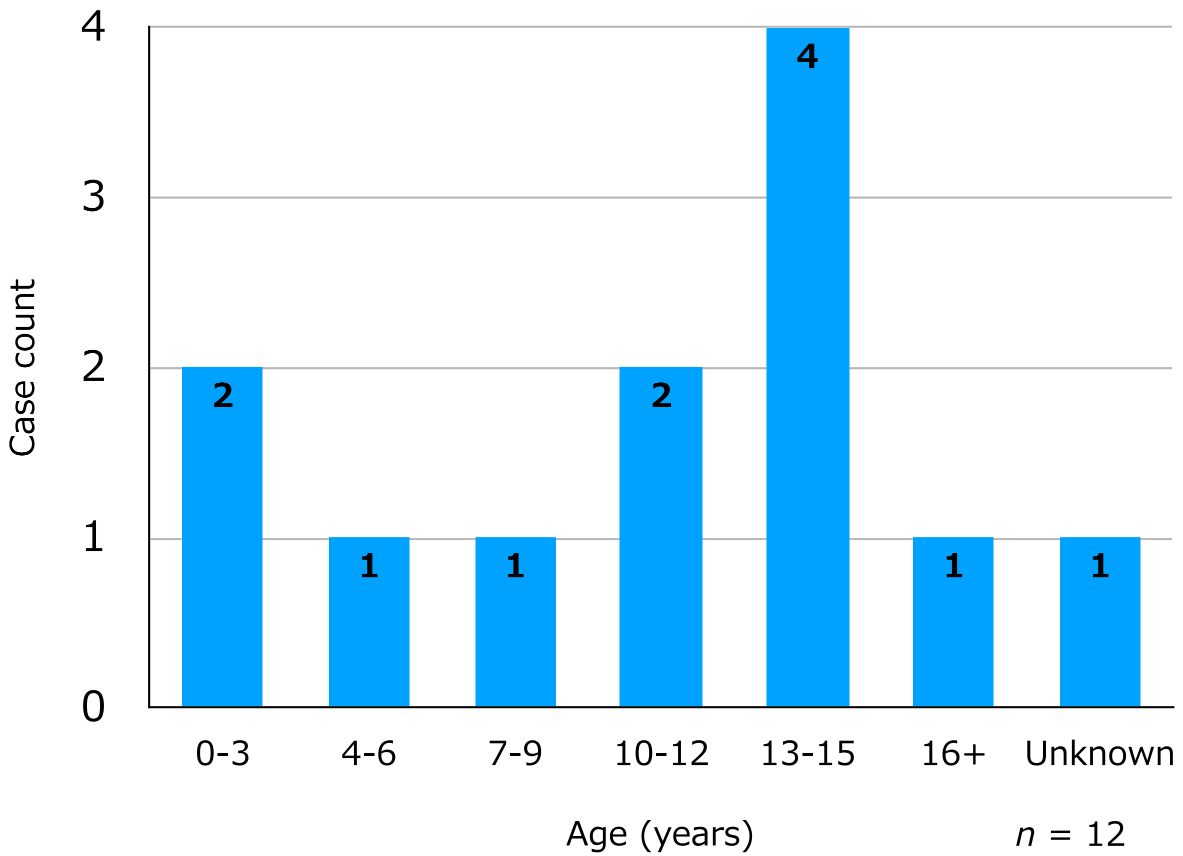


Figure 10. Age distribution in cats

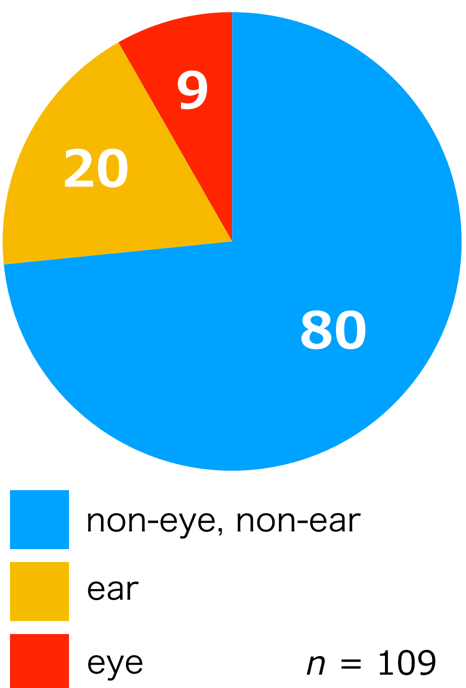


Figure 11. Isolation site of *S canis* in the 2021 study

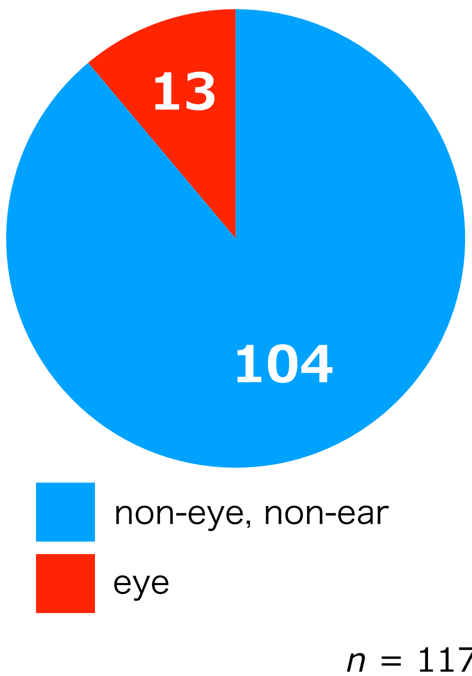


Figure 12. Isolation site of *S canis* in the 2017 study

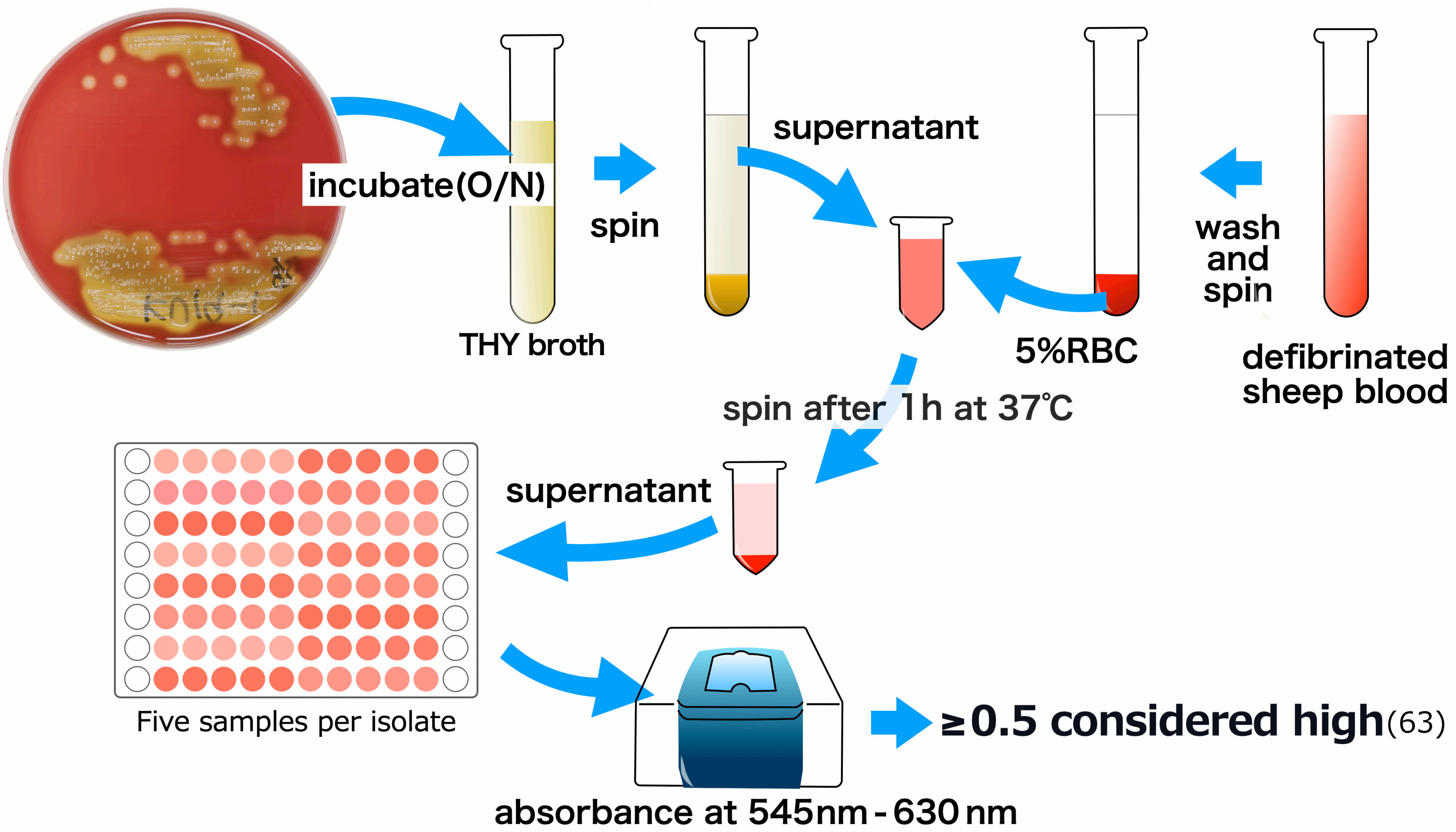


Figure 13. Schematic diagram of HA measurement (64)

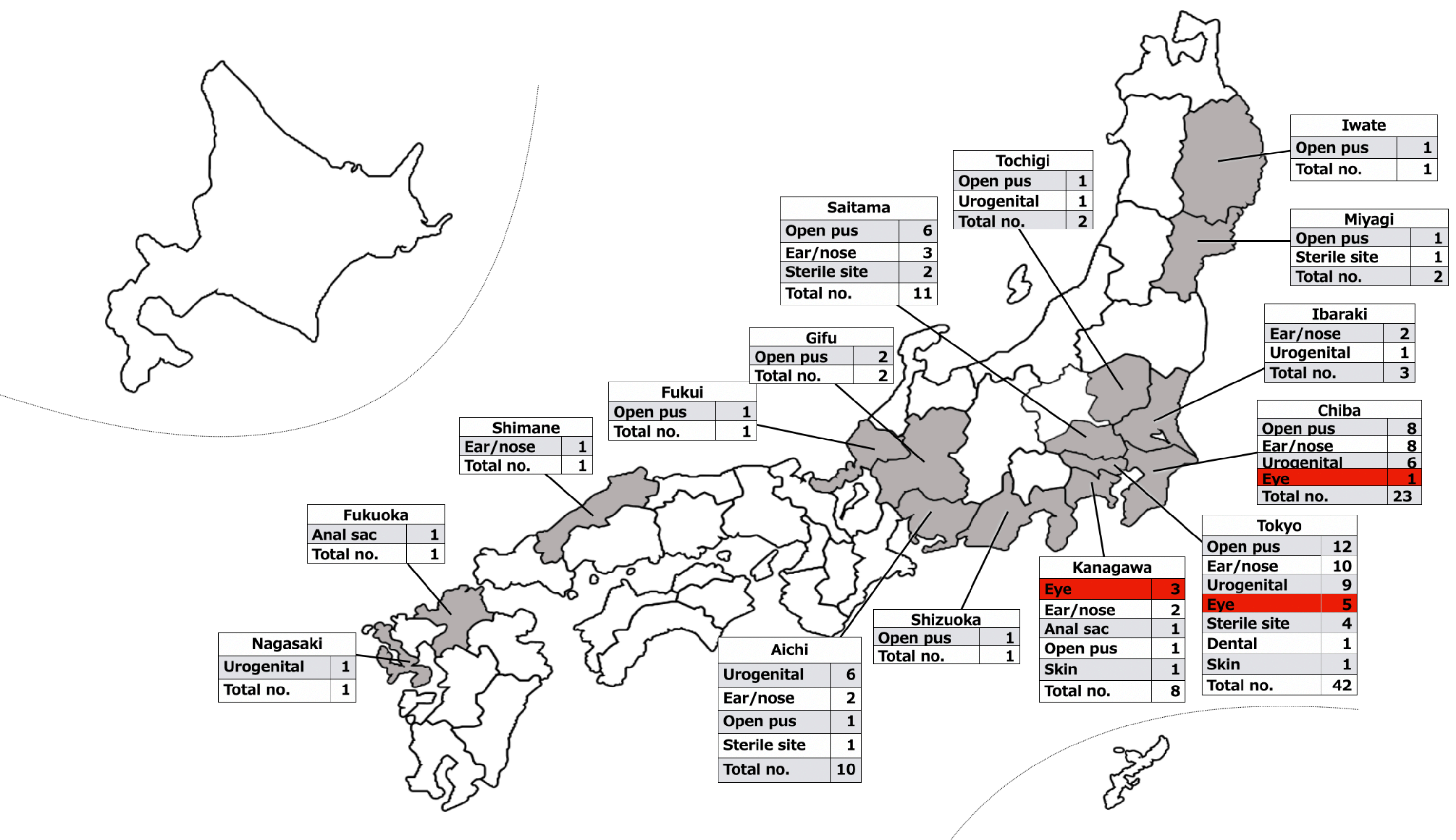
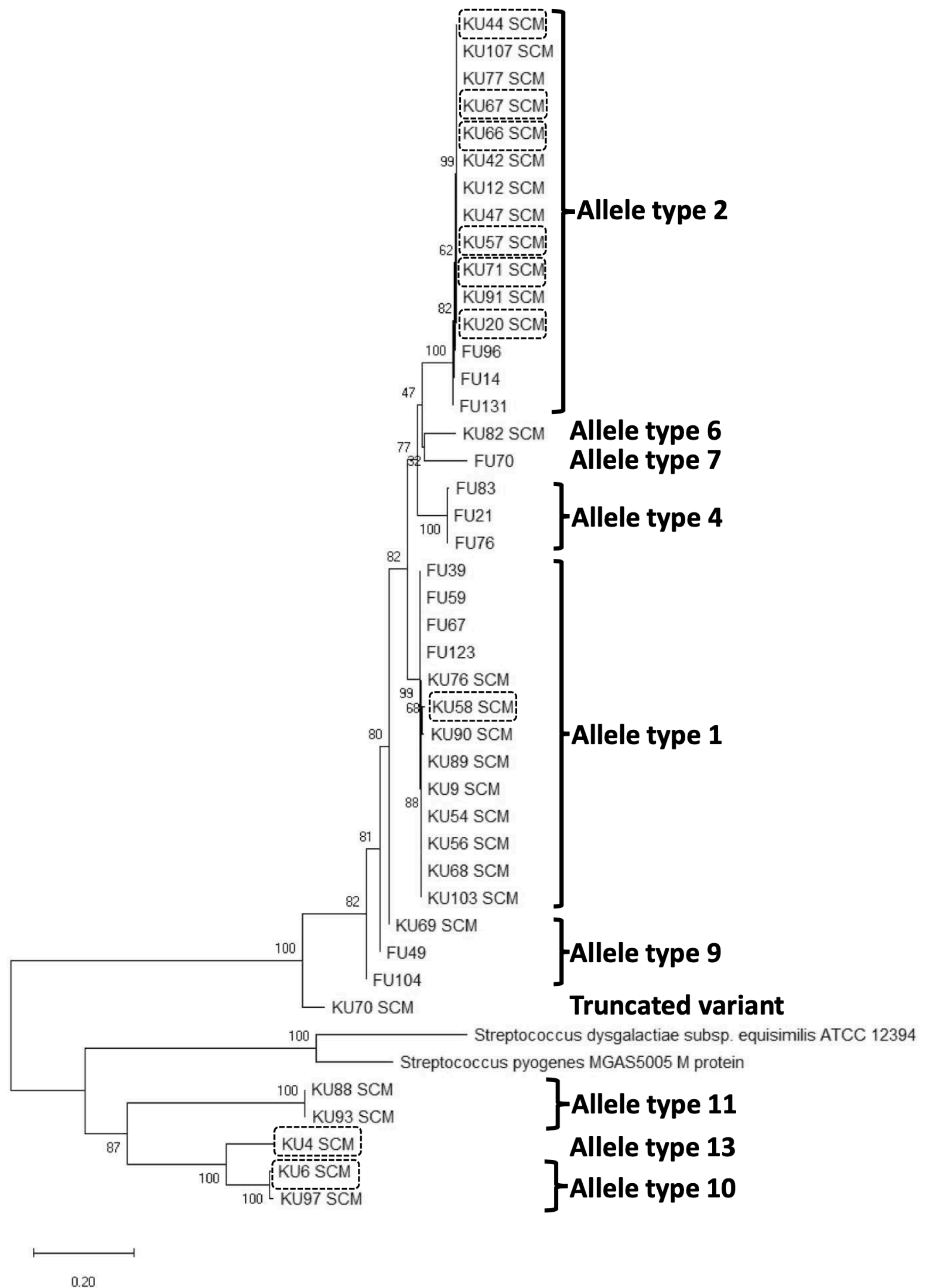


Figure 14. Sample collection locales of the eye origin isolates in 2021 (red-filled cells)



The IDs enclosed by the dashed square represent the 2021-eye.

Figure 15. Phylogenetic tree of deduced SCM protein AA sequences

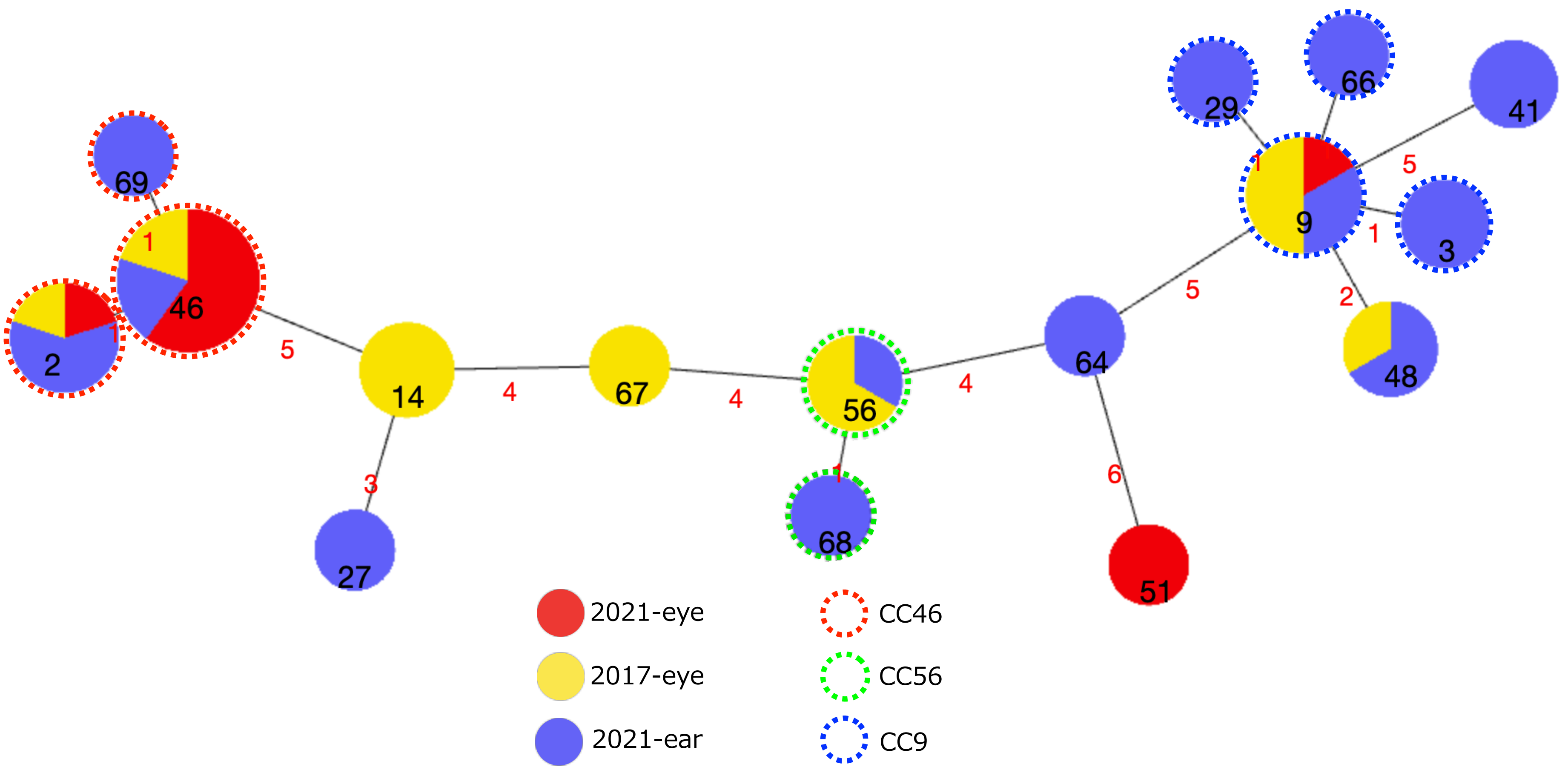


Figure 16. Population structure of CCs by goe-BURST