

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

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Introduction

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. Confronting antimicrobial-resistant bacteria is one of the critical challenges of this approach. Recently, there has been an increase in AMR rates of bacteria isolated from dogs and cats in Japan¹⁾. Furthermore, several papers have warned about the transmission of bacteria, including drug-resistant strains, between companion animals and human²⁾. To address these situations, it is essential for healthcare professionals, including veterinarians, to have a thorough understanding of bacterial infections and antimicrobial resistance (AMR). This knowledge is crucial for providing appropriate prevention and treatment strategies.

β -hemolytic streptococci are among the most crucial bacteria in both human and veterinary medicine. Within this group, *Streptococcus canis*, first reported in 1986³⁾, is the most prevalent species in dogs and cats.

S. canis is commonly isolated from various clinical specimens like pus, ear discharge, corneal swabs, sterile samples like blood, and ascites of dogs and cats, and causes lesions in various tissues, including the skin, cornea, genitourinary tract, and other visceral organs. *S. canis* is sometimes involved in severe conditions such as streptococcal toxic shock syndrome and sepsis. Moreover, it is closely associated with outbreaks of pyogenic diseases in cat colonies and shelters⁴⁾.

Additionally, this zoonotic bacterium has been associated with various human lesions, posing significant concerns in human medicine⁴⁾.

Given these circumstances, the applicant surveyed the isolation status of β -hemolytic streptococci from diseased companion animals. Moreover, the focus was on isolates from the ophthalmology field, which showed high rates of antimicrobial resistance, aiming to explore the relationship between the

resistance and other factors.

Purpose

The applicant conducted the following study to elucidate the clinical significance of β -hemolytic streptococci in companion animal practice and provide information for the appropriate use of antibiotics.

1. Survey of the isolation status of β -hemolytic streptococci and their antimicrobial-resistance profile from diseased companion animals.
2. Hemolytic activity (HA) measurement and virulence-associated gene (VAG) profiling of *S. canis* from the canine eyes.
3. Analysis of clonal complexes in *scm* gene and Multi Locus Sequence Typing (MLST) of the eye-origin isolates.
4. Investigation of the antimicrobial susceptibility profile of the eye-origin isolates.

Materials and methods

1. Collection of the specimen

The study used β -hemolytic streptococci isolated from diseased dogs and cats at the Sanritsu Zelkova Veterinary Laboratory from April 1st to May 31st, 2021⁵⁾. Among these isolates, we selected the eye-origin strains of *S. canis* and also chose ear-origin strains as controls. Additionally, 13 eye-origin isolates from another study⁶⁾ (2017-eye) and the National Collection of Type Cultures (NCTC) 12191(T) were used as controls.

2. HA measurement

Isolates were cultivated in Todd-Hewitt broth and yeast extract overnight. After centrifuging the culture solution, the supernatant was transferred to 96-well plates with sheep red blood cells and incubated at 37°C. 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.

3. VAG profiling

The PCR-primer sets to confirm the isolates' VAG profiles were constructed. These sets included primers for *inl*, *sagA*, *slo*, *scp*, *lbp*, *fbp*,

gbp, *ap1*, *fp1*, and *brp*, which encode proteins involved in various virulence factors. In addition, as positive controls, NCTC 12191(T), FU6, TA4, FU53, and FU97, with whole genome sequences, were included. For some VAG-positive isolates, direct sequencing further confirmed the amplified VAG sequences.

4. SCM allele typing

For analysis of the *scm*, the study used specific primers for PCR amplification, resulting in amplicons with sizes ranging from 1,700 to 2,100 bp^{7, 8)}. Following the nucleotide sequencing, an unrooted phylogenetic tree was constructed using the Neighbor-Joining method based on the deduced amino acid sequences. The tree was scaled by the Poisson correction method and branch lengths, which reflected the same units as distances. Finally, we conducted allele typing based on variable or conserved amino acid sequences in the phylogenetic tree, with all analyses performed using MEGA X software (version 10.0.5).

5. MLST

MLST analysis was conducted on all enrolled isolates with the already published protocol⁸⁾. 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.

6. Antimicrobial Susceptibility Testing (AST) and AMR genotyping

The study used the broth microdilution method (MICroFAST Panel Type 7J for *Streptococcus spp.*, Beckman Coulter Inc., Tokyo, Japan) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines for β -hemolytic streptococci to examine the minimum inhibitory concentrations (MICs) of 14 antimicrobial agents (penicillin G, ampicillin, cefepime, cefotaxime, ceftriaxone, ceftazidime, meropenem, minocycline, erythromycin, azithromycin, clindamycin, levofloxacin, vancomycin, and chloramphenicol). In addition, the AMR rate and MIC values for the antimicrobial agents were calculated for each β -hemolytic strain.

Furthermore, the presence of resistance 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, and verified by direct sequencing.

Results

1. Isolation rates and AMR status

We obtained 2,112 clinical specimens from dogs ($n = 1,464$) and cats ($n = 648$) from April 1st to May 31st, 2021. Among these, β -hemolytic streptococci were 109 isolates with an isolation rate of 5.2%. The most prevalent species was *S. canis* (93.6%), followed by *S. dysgalactiae* subsp. *equisimilis* (3.7%), *S. agalactiae* (1.8%), and *S. dysgalactiae* subsp. *dysgalactiae* (0.9%). They demonstrated a predominant resistance to tetracyclines; however, there was no significant difference compared to the results of a similar survey in 2017⁶).

Out of these isolates, the 2021-eye-origin *S. canis* (2021-eye) were selected from nine dogs (8.8%), while the 2021-ear-origin isolates (2021-ear) were from ear discharge of 20 dogs (19.6%).

2. HA measurement

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

3. VAG profiling

The detection rates of each VAG in the 2021-eye did not significantly differ from those in the 2021-ear and 2017-eye.

4. SCM allele typing

Of the *scm* sequence in the 2021-eye, allele 2 ($n = 6$, 66.7%) was the most predominant. On the other hand, the 2021-ear and 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 2017-eye ($p = 0.079$).

5. MLST

Among the 2021-eye, CC46 [consisting of ST46 ($n = 6$) and ST2 ($n = 1$)] of MLST was the most prevalent ($n = 7$, 77.8%). 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 2017-eye ($p = 0.027$).

6. AST and AMR genotyping

The 2021-eye showed higher AMR phenotype and genotype levels than the 2021-ear and 2017-eye. 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%), with *tet(O)—erm(B)* being the most common genotype. Statistical analysis revealed a significant association between the 2021-eye and AMR phenotypes/genotypes compared to the 2021-ear ($p = 0.014$) and the 2017-eye ($p = 0.027$).

Discussion

The 2021 survey identified four species/subspecies of β -hemolytic streptococci in diseased dogs and cats in Japan, with *S. canis* being the most commonly isolated and exhibiting a notable resistance to tetracyclines⁵⁾.

The current study narrowed the focus on the antibiotic resistance and clonal relationship of *S. canis* isolated from canine ocular lesions in the 2021 survey. The results showed that the clonal spread of *S. canis* with multiclass antibiotic resistance occurred among the 2021-eye, specifically ST46/allele 2 harboring genotypes of *tet(O)—erm(B)* or *tet(O)* alone. In contrast, there was a smaller number of ST46/allele 2 harboring multiclass antibiotic resistance in the 2021-ear and 2017-eye. In addition, the study revealed a stronger association of the 2021-eye with minocycline resistance compared to the 2021-ear and 2017-eye. Moreover, four isolates were resistant to levofloxacin, while none showed quinolone-nonsusceptibility among the 2017-eye, suggesting the current spread of quinolone-nonsusceptible strain. According to a recent paper on Japanese veterinary practitioners⁹⁾, tetracyclines, including those for human use, are the fourth most commonly used class of antibiotics. Furthermore, fluoroquinolones are the third most frequently used antibiotics as veterinary products⁹⁾, although they are restricted to use only when the first-choice drugs are ineffective.

Therefore, the applicant suggests educating veterinarians about the rational use of antibiotics in practice, emphasizing systemic and local

application, particularly in ophthalmology.

Furthermore, monitoring these isolates continuously and characterizing additional eye-origin isolates is essential.

The study's limitations include insufficient host information and the therapeutic course. In the future, more detailed information is necessary to clarify the association of the features of the eye-origin isolates with their clinical implication.

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

Conclusion

This study first documented an instance of ocular isolates predominantly containing ST46 with multiclass AMR phenotypes and *tet(O)*—*erm(B)* genotypes. In addition, fluoroquinolone resistance was highly prevalent, which will be a significant 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 to better understand the microbiological and epidemiological characteristics of *S. canis*, and the applicant believes the study's results will benefit future veterinary and human clinical settings.

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