Jpn. J. Infect. Dis., 70, 394–398, 2017

Original Article

Identification of Group G Streptococcal Isolates from Companion Animals in Japan and Their Antimicrobial Resistance Patterns

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SUMMARY: In this study, we conducted a species-level identification of group G streptococcal (GGS) isolates from companion animals in Japan and analyzed antimicrobial resistance (AMR) patterns. Strains were isolated from sterile and non-sterile specimens collected from 72 animals with clinical signs or symptoms in April–May, 2015. We identified the strain by 16S rRNA sequencing, mass spectrometry (MS), and an automated method based on their biochemical properties. Antimicrobial susceptibility was determined using the broth microdilution method and E-test. AMR determinants (*erm*(A), *erm*(B), *mef*(A), *tet*(M), *tet*(O), *tet*(K), *tet*(L), and *tet*(S)) in corresponding resistant isolates were amplified by PCR. The 16S rRNA sequencing identified the GGS species as *Streptococcus canis* (n = 68), *Streptococcus dysgalactiae* subsp. *equisimilis* (n = 3), and *S. dysgalactiae* subsp. *dysgalactiae* (n = 1). However, there were discrepancies between the sequencing data and both the MS and automated identification data. MS and the automated biochemical technique identified 18 and 37 of the 68 sequencing-identified *S. canis* strains, respectively. The AMR rates were 20.8% for tetracycline and 5.6% for clarithromycin, with minimum inhibitory concentrations $(MIC)_{50}$ –MIC_{90} of 2–64 and ≤ 0.12–0.25 μg/mL, respectively. AMR genotyping showed single or combined genotypes: *erm*(B) or *tet*(M)-*tet*(O)-*tet*(S). Our findings show the unique characteristics of GGS isolates from companion animals in Japan in terms of species-level identification and AMR patterns.

INTRODUCTION

The emergence of streptococcal toxic shock syndrome (STSS) caused by β-hemolytic Lancefield group G streptococci (GGS) has been reported in companion animals, such as dogs and cats. Miller et al. (1) reported the clinical, pathological, and bacteriological findings in dogs (n = 7) that developed severe invasive infections with GGS over a 6-month period in southern Ontario. Three dogs with STSS without necrotizing fasciitis (NF) died or were euthanized within 48 h after admission, whereas 4 dogs with both STSS and NF survived after surgical debridement, supportive medical treatment, and antibiotic administration. In addition, 3 independent fatal outbreaks of GGS infection in shelter cats were reported within a 2-year period (2). The earlier outbreaks, in shelters 1 and 2, manifested as skin ulcerations and chronic respiratory infections that progressed to necrotizing sinusitis and meningitis. The later outbreak in shelter 3, progressed rapidly from NF with skin ulceration to STSS, sepsis, and death. In most cases, *Streptococcus canis* (SC) was the sole pathogen identified. Recently, pathogenic β-hemolytic streptococcal infections, multiple documented pyogenic syndromes, were reported in cats (n = 234) at an institutional boarding facility, and SC was the only species associated with these pyogenic infections (3). This pathogen was also the apparent zoonotic source of human infections in several case reports (4–6). SC-related invasive zoonotic infections may have been underdiagnosed because of inadequate species-level identification of GGS isolates.

Streptococcus dysgalactiae subsp. *equisimilis* (SDSE) isolates with β-hemolytic activity and group G or C antigens are increasingly being recovered from patients with severe invasive infections, including STSS, NF, meningitis, infectious endocarditis, sepsis, septic arthritis, osteomyelitis, etc., worldwide (7). These SDSE strains also cause zoonotic infections in humans and animals, including house pets and horses (8,9).

Anhalt and Fenselau (10) first used mass spectrometry (MS) to identify bacteria. Since then, matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS devices for routine use have become commercially available. This technique can be used to generate fingerprint signatures of ribosomal subunit proteins derived from whole bacterial cells (11). Thus, bacteria can be rapidly identified by comparing the fingerprints to a database of reference spectra using various algorithms. The first trial to evaluate the performance of MALDI-TOF MS for identifying bacterial isolates from clinical specimens demonstrated that using this method, 84.1% of 1,660 strains could be identified to the species-level (12).

Kimura et al. (13) reported the prevalence and antimicrobial drug susceptibility of *Streptococcus* spp. isolates from bacterially infected dogs and cats at a veterinary hospital in 2006–2013. Of the 96 strains tested, 79 (82.3%) were identified as GGS. However, few inves-

Received August 24, 2016. Accepted November 18, 2016. J-STAGE Advance Publication December 22, 2016. DOI: 10.7883/yoken.JJID.2016.375

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tigators have reported species-level identification data for GGS isolates from companion animals in Japan and their antimicrobial resistance (AMR) genes. The purpose of this investigation was to conduct a species-level identification and evaluation of the AMR patterns of GGS isolates recovered from dogs and cats throughout Japan in 2015. We also evaluated the species identification data from MALDI-TOF MS, since this method is currently being used for the identification of clinical isolates.

MATERIALS AND METHODS

Study design, collection of bacterial isolates, and host information: Human pet owners brought their pets to a veterinary clinic or hospital because they observed poor activity, appetite loss, chills, respiratory abnormalities (i.e., tachypnea), ear discharge, and open pus in their pet. To determine the causative agents, clinical specimens were submitted by veterinary practitioners to the Sanritsu Zelkova Veterinary Laboratory, together with request sheets including host information nationwide. These specimens were obtained from companion animals (dogs and cats), showing significant symptoms or signs that were observed by their owners or veterinary clinicians, that visited either clinic or hospital from April 1 through May 25, 2015 (approximately a 2-month period). The specimens were obtained from either sterile samples (blood, joint fluid, and others) or non-sterile samples (ear discharge, open pus, and others). Each specimen was used to inoculate a sheep blood agar plate, which was incubated at 35°C in 5% CO_2 for 24 h. Gray-white colonies with β-hemolytic activity were subjected to latex agglutination testing with antisera specific for the classification of Lancefield carbohydrate antigens (Seroiden Strepto Kit; Eiken, Tokyo, Japan). All the isolates (one per animal) were stored at −70°C to −80°C until processing. The host information (animal species, sex, age, clinical specimen type, date collected, and the Japanese prefecture in which the veterinary practitioners worked) was obtained from the request sheets.

Species identification: We identified the GGS strains to the species level using 16S rRNA sequencing data, automated identification based on biochemical properties, and MALDI-TOF MS data, as previously described (14). The automated identification based on biochemical properties was performed using the Vitek 2 system with the Gram positive (GP) ID Card (SYSMEX bioMérieux, Tokyo, Japan). The criteria used to accept the identification data determined by 16S rRNA sequencing or the Vitek 2 system were that the isolate was identified as the only choice with either ≥ 98.7% similarity to the 16S rRNA sequence of the type strain or ≥ 90% probability, respectively. The quality of the Vitek 2 system results was controlled by periodically testing with a range of American Type Culture Collection (ATCC) strains (ATCC12394, ATCC29212, ATCC49619, ATCC29213, ATCC25923, and ATCC12344).

For the MALDI-TOF MS identification, we used a Microflex LT instrument (Bruker Daltonics, Bremen, Germany) and FlexControl software (ver. 3.4; Bruker Daltonics). Automated analysis of the raw spectral data was performed with MALDI BioTyper software (ver. 3.1; Bruker Daltonics) and a library of 5,989 spectra (database update September 2015). In brief, the isolates were identified by touching the colony surface with a sterile pipette tip and directly applying a small amount of the obtained sample onto a polished or ground steel MSP 96 target plate (Bruker Daltonics). The deposited bacteria were overlaid with 1 μL of CHCA matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid) and air dried at room temperature to allow cocrystallization. The spectra were acquired by the mass spectrometer and were compared to reference spectra in the latest MS database. The results were considered valid (probable identification to the species level) when the score was ≥ 2.000, as the scores are divided as follows: highly probable species identification (2.300–3.000), probable species identification (2.000–2.299), probable genus identification (1.700–1.999), and unreliable identification (< 1.700).

Genetic analysis of identification accuracy: To assess the accuracy of the identification by 16S rRNA sequencing, we included PCR amplification of: i) a species-specific gene (*cfg*) encoding a co-hemolysin (CAMP-factor) produced by SC and ii) the *emm* gene, encoding a virulence factor (M protein) in SDSE that is involved in its attachment to the host epithelium, as previously described (15,16). All *emm* genotyping was based on the US Centers for Disease Control and Prevention database <http://www2a.cdc.gov/ncidod/biotech/strepblast.asp>.

Antimicrobial susceptibility testing and determination of resistance genes: The minimum inhibitory concentrations (MICs) of various antimicrobial agents, including penicillin G, ampicillin, cefepime, cefotaxime, ceftriaxone, meropenem, tetracycline (TET), erythromycin, clarithromycin (CLR), clindamycin, levofloxacin, vancomycin, and chloramphenicol, were determined by the broth microdilution method (MICroFAST Panel Type 5J; Beckman Coulter, Brea, CA, USA), according to the Clinical and Laboratory Standards Institute guidelines for β-hemolytic streptococci (17). The quality of the susceptibility testing was controlled by using 2 ATCC strains (ATCC29212 and ATCC49619). The high-level MICs of isolates resistant to antibiotics of the TET and/or macrolide/lincosamide (ML) classes were measured by Etest using TET and/or CLR (SYSMEX bioMérieux) because the MICs could not be determined by the broth microdilution method.

The presence of the ML-resistance determinants, *erm*(A), *erm*(B), and *mef*(A), and the TET-resistance determinants *tet*(M), *tet*(O), *tet*(K), *tet*(L), and *tet*(S), in ML- and TET-resistant strains, respectively, was confirmed by PCR, as previously described (18,19). We also confirmed the sequences of the resistance determinants of several isolates that were positive for the targeted genes.

Animal ethics committee approval: To ensure the privacy of the affected animals, the Ethics Committee of the Sanritsu Zelkova Veterinary Laboratory examined and approved the study design before starting it.

RESULTS

Collection of GGS isolates and host information: During the approximately 2-month period from April 1 to May 25, 2015, 72 GGS strains were collected from

the various areas (18 prefectures). The most common prefectures sampled were Tokyo (n = 19), Chiba (n = 13), Aichi (n = 9), Saitama (n = 6), Gifu (n = 4), Ibaraki (n = 4), Nara (n = 3), and Niigata (n = 3). These isolates were recovered from ear discharge (n = 23), open pus (n = 22), urine (n = 10), skin-derived samples (n = 5), and others (n = 12), including blood, joint fluid, and uterine specimens from dogs (n = 63) and cats (n = 9). We obtained information on the host animals (mean age, 9.5 years; sex, 45 males, 26 females, and 1 unknown).

Species identification: Species identification by 16S rRNA sequencing revealed that the GGS isolates were SC (n = 68, 94.4%), SDSE (n = 3, 4.2%), and *Streptococcus dysgalactiae* subsp. *dysgalactiae* (SDSD; n = 1, 1.4%). We compared the species or subspecies identification data determined by 16S rRNA sequencing with data obtained by MALDI-TOF MS and the automated biochemical identification method (Table 1). There were discrepancies between the 16S rRNA sequencing data and the identification data from both MALDI-TOF MS and the automated biochemical method. The 16S rRNA sequencing identified 68 strains as SC, whereas MALDI-TOF MS identified only 18 (26.5 %) of these as SC, and the automated biochemical method identified 37 (54.4 %) of them as SC. One SC strain was misidentified as *Staphylococcus aureus* in the MS database.

Genetic assessment of the accuracy of identification: All SC isolates contained the *cfg* gene, and this gene was not detected in the SDSE strains collected from the animals. Three *emm* genotypes were detected in the SDSE isolates: *stC1929.1*, *stC9431.0*, and *stG6792.3*. However, we did not detect *emm* in the SC strains, and the SDSD isolates also did not contain *cfg* or *emm*.

Antimicrobial susceptibility and resistance genes: The antimicrobial activities of oral and parenteral antibiotics against the collected GGS strains are shown in Table 2. The overall resistance rate to TET was 20.8% (n = 15), and that to CLR was 5.6% (n = 4), with MIC values defined as the lowest concentration of the antibiotic

Table 1. Identification of group G streptococci from dogs and cats by mass spectrometry and automated identification by biochemical properties, as compared with that by 16S rRNA sequencing

16S rRNA sequencing	Mass spectrometry by MALDI BioTyper[1)]		No. (%)		Automated identification by Vitek 2 GP ID Card		No. (%)	
	Identified		Not reliably identified	Misidentified	Identified		Not reliably identified	Misidentified
	Matching at the genus	Matching at the species			Matching at the genus	Matching at the species		
S. canis (n = 68)	46 (67.6)	18 (26.5)	3 (4.4)	1 (1.5)[2)]	16 (23.5)	37 (54.4)	15 (22.1)	
	Non-matched species: *S. dysgalactiae* *S. pyogenes* *S. castoreus*				Non-matched species: *S. agalactiae* *S. dysgalactiae* subsp. *equisimilis* *S. dysgalactiae* subsp. *dysgalactiae* *S. constellatus* subsp. *constellatus*			
S. dysgalactiae subsp. *equisimilis* (n = 3)	1 (33.3)	2 (66.7)			2 (66.7)		1 (33.3)	
	Non-matched species: *S. canis*				Non-matched species: *S. agalactiae*			
S. dysgalactiae subsp. *dysgalactiae* (n = 1)			1 (100)			1 (100)		

[1)]: Score values are divided into 4 descriptions: highly probable species identification (2.300 − 3.000), probable species identification (2.000 − 2.299), probable genus identification (1.700 − 1.999), and identification not reliable (< 1.700).
[2)]: One isolate was misidentified as *Staphylococcus aureus*.

Table 2. Antimicrobial activities of oral and parenteral antibiotics against group G streptococcal isolates from dogs and cats

Antibiotic	*S. canis* (n = 68)			*S. dysgalactiae* (n = 4)		
	MIC range (μg/mL)	MIC_{50}	MIC_{90}	MIC range (μg/mL)	MIC_{50}	MIC_{90}
penicillin G	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03
ampicillin	≤0.06	≤ 0.06	≤ 0.06	≤ 0.06	≤ 0.06	≤ 0.06
cefepime	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5	≤ 0.5
cefotaxime	≤ 0.06	≤ 0.06	≤ 0.06	≤ 0.06	≤ 0.06	≤ 0.06
ceftriaxone	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12
meropenem	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12	≤ 0.12
tetracycline[1)]	≤ 0.5 − 96	4	64	≤ 0.5 − 4	≤ 0.5	4
erythromycin	≤ 0.12 − > 1	≤ 0.12	0.25	≤ 0.12	≤ 0.12	≤ 0.12
clarithromycin[1)]	≤ 0.12 − > 256	≤ 0.12	0.25	≤ 0.12	≤ 0.12	≤ 0.12
clindamycin	≤ 0.12 − > 1	≤ 0.12	0.5	≤ 0.12	≤ 0.12	≤ 0.12
levofloxacin	≤ 0.25 − 8	0.5	1	0.5	0.5	0.5
vancomycin	≤ 0.12 − 0.5	0.25	0.5	≤ 0.12 − 0.5	0.5	0.5
chloramphenicol	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4	≤ 4

MIC, minimum inhibitory concentration.
[1)]: The whole resistance rates to tetracycline and clarithromycin were 20.8% (n = 15) and 5.6% (n = 4), respectively.

at which 50% (MIC_{50})–90% (MIC_{90}) of the strains were inhibited, 2–64 and ≤ 0.12–0.25 μg/mL, respectively. All the TET and/or CLR-resistant strains were SC (n = 16). There were 4 (5.6%) and 2 (2.8%) SC isolates that were resistant to clindamycin and levofloxacin, respectively. No strains were resistant to β-lactams.

The relationship between the MICs determined by Etest and the TET- and/or CLR-resistance determinants in the SC strains and the origin of the isolate (e.g., animal species, clinical specimen type, and prefecture) are shown in Table 3. AMR genotyping showed single or combined types: *tet*(M), *tet*(O), and *tet*(S) as the TET-resistance determinants and *erm*(B) as the ML-resistance determinant. There was no relationship between the MICs by TET Etest and the profiles of the TET-resistance determinants. We did not detect *erm*(A), *mef*(A), *tet*(K), or *tet*(L) in any resistant isolate. All of the resistant SC strains were isolated from non-sterile samples and were obtained in various prefectures (n = 10).

DISCUSSION

We identified 3 species and subspecies (SC, SDSE, and SDSD). To the best of our knowledge, this is the first large-scale monitoring of GGS isolates from companion animals in Japan. An investigation of streptococcal infections in 393 dogs in the USA was performed to identify the species (20), and the major ones identified were SC (n = 88, 22.4%), SDSE (n = 13, 3.3%), and *Streptococcus equi* subsp. *zooepidemicus* (n = 4, 1.0%). Therefore, we need to examine the proportion of *S. equi* subsp. *zooepidemicus* isolates in future surveys.

The MALDI-TOF MS method is currently being used to identify large-colony β-hemolytic GGS isolates obtained from human blood in clinical settings, and only a small number of SC strains derived from humans have been identified. All 3 of the detected SC strains were correctly identified with this technique (6,21). However, in the present study, the MS results agreed with only 26.5% of the identifications made by 16S rRNA sequencing. Schulthess et al. (22) reported identification data for Gram-positive cocci (n = 156), which included some of the most clinically important genera, using MALDI-TOF MS and a practical algorithm for routine diagnostics. The algorithm showed a limited capacity to discriminate between SC and SDSE/SDSD at the species-level because more than one species had a score value ≥ 2.000. In addition, the invasive SC and SDSE strains were genetically related (23), and there was evidence of genetic exchange between SC and SDSE (24), suggesting that they were closely related species. Furthermore, only 3 SC strains, including the type strain, have been registered in the MS database that we used, as of September 2015. Although MALDI-TOF MS is expected to become a promising tool for the identification of SC, SDSE, and SDSD, our data show that SC identification by MS is not currently reliable. Therefore, more human and veterinary clinical isolates must be tested to improve this tool for future research and clinical use.

emm-like genes have previously been detected in SC isolates. One report described an SC strain with *stG1389* among 5 SC isolates from humans (23). Another study reported *emm*-like genes in 15 (17%) SC strains collected from dogs; 12 were the *stG1389* genotype, 2 were a novel genotype (*stG1451*), and 1 was *stG663* (24). However, these genes were not detected in the SC isolates in our study. Further analysis is required to clarify the association between the presence of this gene and the pathological status of the strain recovered from invasive infections in veterinary clinical settings.

We confirmed AMR to TET (20.8%) and CLR (5.6%). The resistance genotypes were either single or combined, with *tet*(M), *tet*(O), and/or *tet*(S) and/or *erm*(B). Consistent with our findings, a previous study reported resistance rates of 27% for TET and 10.8% for ML among SC strains (n = 37) from dogs collected in 2000–2005 (25). Pinho et al. (24) also demonstrated either *tet*(M) or *tet*(O) as sole determinants or the combined determinants *tet*(M)/*tet*(L), *tet*(L)/*tet*(S), and *tet*(O)/*erm*(B) in TET-resistant SC isolates (n = 23; resistance rate, 27%). Therefore, AMR should be considered when these various antimicrobials are not effective in veteri-

Table 3. MICs by Etests and TET- and/or CLR-resistance determinants in *Streptococcus canis* isolates and the origins

Strain No.	Animal species	Clinical specimen	Prefecture	MIC (μg/mL) by TET E-test	MIC (μg/mL) by CLR E-test	TET- and/or CLR-resistance determinant
SA1	Dog	Ear discharge	Tokyo	64	ND	*tet* (M)
SA3	Dog	Ear discharge	Tokyo	96	> 256	*tet* (O) and *erm* (B)
SA7	Dog	Ear discharge	Chiba	64	ND	*tet* (M), *tet* (O), and *tet* (S)
SA10	Dog	Ear discharge	Ibaraki	ND	> 256	*erm* (B)
SA12	Dog	Ear discharge	Wakayama	64	ND	*tet* (M) and *tet* (O)
SA13	Dog	Open pus	Tokyo	48	ND	*tet* (M) and *tet* (O)
SA20	Dog	Skin-derived sample	Saitama	96	> 256	*tet* (M), *tet* (O), and *erm* (B)
SA21	Dog	Nasal discharge	Osaka	64	ND	*tet* (M), *tet* (O), and *tet* (S)
SA22	Cat	Ear discharge	Tochigi	48	4	*tet* (M), *tet* (O), *tet* (S), and *erm* (B)
SA27	Dog	Open pus	Niigata	48	ND	*tet* (M) and *tet* (O)
SA31	Dog	Urine	Nara	96	ND	*tet* (M) and *tet* (O)
SA35	Dog	Open pus	Tokyo	96	ND	*tet* (O)
SA43	Dog	Open pus	Tokyo	64	ND	*tet* (O)
SA49	Dog	Skin-derived sample	Aichi	96	ND	*tet* (M)
SA54	Dog	Urine	Saitama	64	ND	*tet* (O)
SA67	Dog	Ear discharge	Aichi	96	ND	*tet* (O)

MIC, minimum inhibitory concentration; TET, tetracycline; CLR, clarithromycin; ND, not determined.

nary clinical practice.

Our study had 2 limitations. First, except for blood, joint fluid, and uterine specimens, most clinical specimens were non-sterile (e.g., ear discharge, open pus, and others). Additional investigations with more sterile specimens should be undertaken to explore the current status of invasive infections (i.e., STSS, NF, and others). Secondly, we obtained limited host information (only animal species, sex, age, clinical specimen type, collection date, and collection area). In future studies, more-detailed information should be collected, including the underlying illness, infectious diagnosis, therapeutic approaches (clinical histories of surgical procedures, supportive treatment, and antibiotics for the diseased animals), and outcomes through the future establishment of a Veterinary Streptococcal Disease Working Group in Japan.

In conclusion, our observations of species-level identification of GGS and AMR suggest that GGS isolated from companion animals in Japan (April–May, 2015) have unique characteristics. Therefore, our findings should be useful for veterinary practitioners when examining and treating animals with clinical symptoms or signs of GGS infections, such as ear discharge, open pus, etc. These strains must be monitored throughout the country, and additional GGS isolates need to be characterized using various approaches including MALDI-TOF MS. To improve the reliability of MS identification, the number of GGS-related reference spectra in the MS database should be increased.

Many individuals in Japan, from children to the elderly, like to keep companion animals in their home. In fact, some human hospitals have even introduced animal-assisted therapy as part of human patients' mental care. In addition, the lives of companion animals are completely controlled by the owners. Studies on the possible exchange of bacteria, including GGS, between humans and companion animals should be performed to help them both maintain healthy lives.

Acknowledgment The authors wish to thank Ms. Haruno Yoshida (Laboratory of Infectious Diseases, Kitasato Institute for Life Sciences, Kitasato University, Tokyo, Japan) for her helpful assistance.

Conflict of interest None to declare.

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