INTRODUCTION

Pathogenesis of bacterial infections is initiated with the adhesion of bacteria to the host cells. Some bacteria have evolved a specialized strategy to survive and escape the immune system by entering the host cells (referred to as “intracellular invasion” or “internalization”) (1). Invasive pathogens are localized to membrane-bound phagosomes. Some pathogens can evade the cytoplasm within a few hours after destroying the phagosome membrane, whereas others remain entrapped in the compartments (1). In the former situation, the pathogens can intracellularly survive, multiply, and eventually spread to adjacent cells. In the latter condition, phagosome fusion with vacuoles produces intracellular degradation environments, leading to the lysis of microorganisms (1). However, to the best of our knowledge, there are no descriptions concerning the intracellular invasion of Streptococcus canis to date.

Streptococcus pyogenes has evolved a variety of surface-bound and extracellular factors that can modify host inflammatory responses and impair phagocytic clearance (2). There is a biochemical level to colonize the host environment and to escape from the immune system using similar or different strategies as compared with those in other streptococci. Whole genome sequences (WGSs) of S. canis isolates included the coding regions encoding phage-derived proteins that were highly homologous with phage and phage-associated proteins present in S. pyogenes (3,4). Using the seven WGS data from the isolates (FU1, FU6, FU29, FU53, FU93, FU97, and FU129), the presence or absence of the fibronectin-binding proteins (FBPs) gene was searched on a web-based pipeline (5). Remarkably similar FBP amino acid (AA) sequences were found in all seven WGS data, and these sequences were similar to those in S. pyogenes.

S. canis, first reported in 1986 (6), forms large, smooth gray/white colonies with β-hemolysis on sheep blood agar plates. The Lancefield grouping classifies S. canis as group G streptococci based on the cell wall carbohydrate antigenicity. In healthy dogs, S. canis forms part of the resident microflora of the oropharynx,
skin, genitourinary tract, and anus (7). This bacterium is an emerging zoonotic pathogen that can cause self-limiting dermatitis. In some cases, this bacterial infection leads to severe diseases, including arthritis, streptococcal toxic shock syndrome, necrotizing fasciitis, septicemia, and pneumonia in companion animals (8,9). *S. canis* can infect humans who have been in contact with animals and cause either local or systemic diseases (10).

*S. canis* M-like protein (SCM), a virulence factor, can bind to plasminogen and immunoglobulin G and facilitate anti-phagocytic activity (11,12). Timoney et al. (13) reported four SCM alleles in *S. canis* isolated from diseased and healthy cats and suggested that allele type 1 was predominant in ill cats. Nine SCM alleles (belonging to group I) were also determined among isolates from 2015 to 2017 in Japan (14). Thereafter, novel alleles (belonging to group II) were identified, and the association of their alleles were elucidated using sequence types (STs) by multilocus sequence typing (MLST) (15). A significant difference has been observed in the distribution of STs between groups I and II SCM populations. STs have been documented in a case series of dogs with ulcerative keratitis caused by *S. canis* (16). The overall antimicrobial resistance (AMR) rates were reported to be as follows: 39.7% for minocycline, 19.8% for erythromycin, and 17.6% for clindamycin, with minimum inhibitory concentrations (MICs) of >4 μg/mL, >2 μg/mL, and >1 μg/mL, respectively, in β-hemolytic streptococci (mainly *S. canis*) isolated from dogs and cats (17). Recently, quinolone nonsusceptible *S. canis* isolates (n = 13, 7.0%) have been documented, along with point mutations in quinolone resistance-determining regions (18).

This study aimed to evaluate human lineage cell invasion ability (CIA) of the isolates, and to clarify the relationship between the CIA population and its microbiological characteristics, including hemolytic activity (HA), SCM allele type, ST, and AMR phenotype/genotype. An important aspect of this investigation was to find the interaction between companion animal-origin *S. canis* and human cells in the CIA assay.

**MATERIALS AND METHODS**

**Selection of *S. canis* isolates and host information:** One-hundred seventeen *S. canis* isolates were collected in 2017, along with information regarding companion animals, including that of dogs and cats (animal species, sex, age, clinical specimen, collection date, and isolated location) (17). The isolates were identified at the species level, based on the 16S rRNA sequencing data and polymerase chain reaction (PCR) amplification of *S. canis*-specific cef encoding the CAMP-factor (17,19,20). All *S. canis* isolates (one isolate per companion animal) were stored at −70°C to −80°C for further analyses. Forty isolates (with the host information) were assigned to 117 samples by random sampling in order.

The National Collection of Type Cultures (NCTC) 12191(T) of *S. canis* harboring WGS data, as an internal control, was used. Two isolates (TA4 and OT1) harboring the WGS data (3.4) from human cases of bacteremia with and without a dog bite (20,21) were enrolled to confirm the interaction between human-origin isolates and human cells in the CIA assay.

**Measuring CIA:** Human cell-line colon carcinoma epithelium Caco-2 (HTB-37, American Type Culture Collection [ATCC]) was used in this study. The CIA assay was performed according to a previously described protocol (22), with the following modifications: the multiplicity of infection used was 10 for *S. canis* in the mid-log growth phase per host cell; no centrifugation was performed; the isolates were added onto semi-confluent Caco-2 cells (2–4 × 10^5 cells/well) grown in a 12-well culture plate with minimum essential medium Eagle with Earles salts, L-glutamine, and non-essential AAs supplemented with 15% fetal bovine serum. After a 1-h incubation at 37°C, cells were washed with phosphate-buffered saline (PBS) to remove the bacteria outside. The monolayers were maintained in media containing penicillin (10 U/mL) and gentamicin (100 μg/mL) for 1 h. Whole cells were trypsinized using 0.05% trypsin (containing 0.53% EDTA). The trypsinized cells were vortexed and disrupted with sterile distilled water to obtain the cell extracts. Cell lysates were serially diluted with Dulbecco’s phosphate buffered saline without calcium and magnesium to appropriate concentrations and plated on agar plates produced from Todd-Hewitt broth, supplemented with yeast extract (THY), to determine the internalized bacterial load (colony-forming units [CFU]). ATCC 12394 *S. dysgalactiae* subsp. *equisimilis* (SDSE), which is genetically close to *S. canis*, was used as an external control, with the mean value (CFU/100 cells) of ATCC 12394 calculated in four wells of every CIA assay. Data are expressed as mean (fold value/mean value of ATCC strain) ± standard deviation (SD) of four wells by one isolate.

To confirm the similarity of CIA data as previously described (23), a human cell-line keratinocyte HaCaT was used in this assay. Although almost all the experimental procedures using HaCaT were similar to those using Caco-2, the following reagent with serum concentration was different: Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum.

**Measuring HA:** The isolates were cultured in THY broth overnight at 37°C. After the culture solutions were centrifuged at 2,000 × g for 5 min, 100 μL of the supernatant was transferred to 96-well plates. A total of 100 μL of 5% sheep red blood cells in PBS (containing 0.5% bovine serum albumin) was added to each well (24). After incubation for 1 h at 37°C, the plates were centrifuged at 13,000 × g for 30 s. The supernatants were transferred to new 96-well plates. Data are expressed as the supernatant absorbance at 545 nm (mean ± SD, n = 5/one isolate).

**SCM allele typing:** PCR-based amplification of *scm* was performed using the primer set Sc_Mprot_gF1/Sc_Mprot_gR1 (25) and M-SCAF2/M-SCAR3 or M-SCAF2/M-SCAR4 (13,14). After directly sequencing the purified PCR product, a phylogenetic unrooted tree of deduced SCM AA sequences was constructed based on the determined nucleotide sequences, using the neighbor-joining method (26). Allele typing was carried out based on different or similar positions produced by the variable and conserved AA sequences in the constructed phylogenetic tree. Alleles 1–9 were
designated as group I and allele 10–15 as group II, respectively (15). The allele types in the seven isolates (FU1, FU6, FU29, FU53, FU93, FU97, and FU129) harboring the WGS data were also included (5).

**MLST:** MLST was conducted on all isolates according to the protocols reported (18, 25). STs were grouped into clonal complexes (CCs), whereby related STs were classified as single locus variants differing in only one housekeeping gene. The STs were included in the seven isolates harboring WGS data (5).

**AMR phenotyping/genotyping:** MICs of various antimicrobial agents (penicillin G, ampicillin, cefepime, ceftaxime, ceftriaxone, cefozopran, meropenem, minocycline, erythromycin, azithromycin, clindamycin, levofloxacin, vancomycin, and chloramphenicol) were determined using the broth microdilution method (MICroFAST Panel Type 7J, Beckman Coulter Inc., Tokyo, Japan), according to the Clinical and Laboratory Standards Institute (CLSI) guidelines for β-hemolytic streptococci (CLSI document M100-S25 released in 2015). For susceptibility testing to minocycline, streptococci (CLSI document M100-S25 released in 2015). For susceptibility testing to minocycline, the seven isolates harboring the WGS data (5).

Presence of AMR genes, including macrolide/lincosamide-class resistance genes, *erm*(A), *erm*(B), and *mef*(A), in addition to tetracycline-class resistance genes, *tet*(A), *tet*(O), *tet*(K), *tet*(L), and *tet*(S) in all the isolates, was determined by PCR (17). The sequences of these AMR genotypes from some positive isolates were confirmed by PCR and direct sequencing. AMR genotypes were included in the seven isolates harboring the WGS data (5).

**Animal ethics statement:** The Ethics Committee of the Sanritsu Zelkova Veterinary Laboratory approved this study design (approval number SZ20200220-1). The CIA fold value per baseline value of SDSE was found in the high-/low-frequency CIA between the ST21/ST41 populations. High-frequency CIA and the ST21/ST41 populations. High-frequency CIA and the ST21/ST41 populations. No association was observed between low-frequency CIA and allele type 1. On the other hand, a significantly higher CIA was observed in the allele type 10/type 11 than in other type populations (p < 0.01). The prevalence of SCM allele types (including allele groups I and II among the isolates) is shown in Table 2 on the last page. Of the 43 isolates, group I had 24 isolates including prevalent allele type 1 (n = 16), and group II had 19 isolates, including prevalent allele type 10 (n = 9) and allele type 11 (n = 4). A significant difference was found (p < 0.05) in the high-/low-frequency CIA between the ST21/ST41 populations. No association was observed between low-frequency CIA and allele type 1. On the other hand, a significantly higher CIA was observed in the allele type 10/type 11 than in other type populations (p < 0.01). ST/CC: STs/CCs distribution is indicated in Table 2 on the last page. ST9 (n = 9), ST3 (n = 2), and ST30 (n = 1) that were grouped into CC9 (n = 12) were observed in addition to the distribution of ST21 (n = 9)/ST41 (n = 4). A significant difference (p < 0.01) was observed in the high-/low-frequency CIA between the ST21/ST41 populations and others. No association was found between low-frequency CIA and the CC9 population.

**AMR phenotype/genotype:** Data regarding the AMR phenotype/genotype among the isolates is shown in Table 2 on the last page. The total rate of AMR to either class of antibiotics (tetracycline-macrolide-lincosamide) was 39.5%, and the detection rate of the AMR genotype was 48.8%. None of the isolates were resistant to levofloxacin and β-lactams. No significant difference was found in the high-/low-frequency CIA between the presence and absence of the AMR phenotype/genotype.

**RESULTS**

**Host information of isolates:** A total of 43 isolates included in this study and the host information are shown in Table 1. Except for the type strain and two human isolates, the common prefectures sampled were Chiba (n = 18), Tokyo (n = 7), Aichi (n = 3), Okayama (n = 3), Fukui, Gunma, Ishikawa, Kanagawa, Kyoto, Nagasaki, Niigata, Saitama, and Tottori (n = 1 for each). These isolates were recovered from open pus/wound specimens (n = 16), ear/nose/throat-origin specimens (n = 13), urogenital tracts-origin specimens (n = 6), uterine specimens (n = 2), eye-origin specimens (n = 2), and anal glandular fluid (n = 1) from dogs (n = 30) and cats (n = 10). The animal demographics were as follows: mean age, 10.7 years; age range, 1–19 years; sex ratio, 3:2 (24 males and 16 females).

**CIA:** The CIA fold value per baseline value of SDSE ATCC 12394 is shown for each isolate in Fig. 1. A value ≥ 200 was designated as high-frequency CIA and that < 200 as low-frequency CIA. Nineteen isolates harbored high-frequency CIA and 24 isolates harbored low-frequency CIA.

**DISCUSSION**

Intracellular invasion of *S. pyogenes* is dependent on at least two classes of surface proteins: M proteins (27) and FBPs (28). M proteins (encoded by the *emm* gene) have many functions in the pathogenesis of *S. pyogenes*, including resistance to phagocytosis, adherence, and intracellular invasion (29). The FBP and its allelic variants are adhesins and invasins. This study clarified the relationship between isolates with high-frequency CIA and their microbiological features. Particularly, a higher CIA was found in SCM allele type 10/type 11 than in other type populations. An association was found between high-frequency CIA and the ST21/ST41 populations. High-frequency internalization of host epithelia by a clonal variant of...
M1 (encoded by the \textit{emm1}) \textit{S. pyogenes} is dependent on the M1 protein; the CIA was blocked by the anti-M1 antibody (27). Additionally, Nerlich et al. (30) examined the CIA of M3 \textit{S. pyogenes} into human endothelial cells and identified host signaling factors required to initiate cell entry. Furthermore, the binding of M49 and plasminogen/plasmin promotes integrin-mediated internalization of \textit{S. pyogenes} into keratinocytes (31). \textit{S. agalactiae} ST17 isolates with serotype III \((n = 2)\) differed in their abilities to attach and invade into the decidual cells, whereas the attachment and invasive abilities of the ST19/ST23 isolates with type III \((n = 1)\) for each) were lower than those of the ST17 isolates (32). Further work clarifying the contribution of allele type 10/type 11 or ST21/ST41 to high-frequency CIA at the molecular level is warranted.

FBP is involved in \textit{S. pyogenes} attachment to and ingestion by human laryngeal epithelial cells,

| Isolate | Host | Sex and age (year-old) | Isolation prefecture | Isolation year | Isolation source |
|---------|------|------------------------|----------------------|----------------|-----------------|
| NCTC 12191(T) | Bovine | | | | |
| TA4 | Human | Male and 71 | Tokyo | 2016 | Blood |
| OT1 | Human | Female and 91 | Gifu | 2012 | Blood |
| FU1 | Cat | Male and unknown | Chiba | 2017 | Open pus |
| FU3 | Dog | Male and unknown | Chiba | 2017 | Open pus |
| FU6 | Cat | Male and 6 | Okayama | 2017 | Open pus |
| FU7 | Cat | Male and unknown | Aichi | 2017 | Open pus |
| FU14 | Dog | Female and 11 | Tokyo | 2017 | Cornea |
| FU15 | Dog | Female and 15 | Tottori | 2017 | Ear discharge |
| FU16 | Dog | Male and 18 | Tokyo | 2017 | Nasal discharge |
| FU17 | Dog | Female and unknown | Chiba | 2017 | Anal glandular fluid |
| FU24 | Dog | Male and 9 | Tokyo | 2017 | Ear discharge |
| FU25 | Dog | Male and 9 | Chiba | 2017 | Ear discharge |
| FU29 | Dog | Female and 6 | Kanagawa | 2017 | Vaginal swab |
| FU30 | Cat | Male and unknown | Chiba | 2017 | Nasal discharge |
| FU31 | Cat | Male and 19 | Tokyo | 2017 | Nasal discharge |
| FU32 | Dog | Female and unknown | Ishikawa | 2017 | Ear discharge |
| FU52 | Dog | Female and 10 | Tokyo | 2017 | Open pus |
| FU53 | Cat | Female and unknown | Chiba | 2017 | Nasal discharge |
| FU57 | Dog | Female and 15 | Aichi | 2017 | Uterine content |
| FU58 | Dog | Female and unknown | Chiba | 2017 | Urine |
| FU63 | Dog | Female and 13 | Chiba | 2017 | Open pus |
| FU64 | Dog | Male and 13 | Nagasaki | 2017 | Ear discharge |
| FU65 | Dog | Male and 7 | Gunma | 2017 | Bladder lavage fluid |
| FU66 | Dog | Female and 14 | Chiba | 2017 | Open pus |
| FU67 | Dog | Male and unknown | Chiba | 2017 | Eye discharge |
| FU69 | Cat | Male and unknown | Saitama | 2017 | Open pus |
| FU77 | Dog | Female and unknown | Chiba | 2017 | Urine |
| FU78 | Dog | Male and 7 | Chiba | 2017 | Ear discharge |
| FU90 | Dog | Male and 9 | Chiba | 2017 | Ear wax |
| FU91 | Dog | Male and 9 | Chiba | 2017 | Ear wax |
| FU92 | Dog | Male and 12 | Tokyo | 2017 | Urine |
| FU93 | Dog | Female and 9 | Chiba | 2017 | Open pus |
| FU97 | Dog | Male and 11 | Okayama | 2017 | Open pus |
| FU100 | Cat | Female and 12 | Chiba | 2017 | Open pus |
| FU112 | Dog | Male and 15 | Kyoto | 2017 | Urine |
| FU113 | Cat | Male and unknown | Aichi | 2017 | Open pus |
| FU115 | Dog | Female and 7 | Fukui | 2017 | Open pus |
| FU116 | Dog | Male and unknown | Chiba | 2017 | Throat swab |
| FU120 | Dog | Male and 11 | Tokyo | 2017 | Wound |
| FU121 | Cat | Female and 1 | Okayama | 2017 | Intrauterine pus |
| FU128 | Dog | Male and 11 | Chiba | 2017 | Open pus |
| FU129 | Dog | Male and 9 | Niigata | 2017 | Open pus |

NCTC, National Collection of Type Cultures.
and contributes to the elucidation of the underlying molecular events leading to the CIA of S. pyogenes (28). Internalization of FBP-negative M3 S. pyogenes into primary endothelial cells was characterized in a previous study (30). M3 S. pyogenes lacks these major epithelial invasive molecules, but has a high potential to cause invasive diseases. Therefore, FBP does not seem to play a significant role in CIA induction.

There were limitations to this study. We could not analyze the relationship between high-frequency CIA and veterinary clinical implications as limited demographics of the animals (species, sex, age, isolation source, isolation date, and isolation prefecture) were obtained. More detailed information, including underlying medical conditions, diagnosis of infectious diseases, therapeutic approaches (surgical procedure, supportive treatment, and treatment with antimicrobials), and outcomes (survival/death and related sequelae)
should be collected from Japanese veterinarians to establish the relationship between high-frequency CIA and clinical presentations. Although human cases of local or systemic infections with *S. canis* are not often encountered as compared with the incidence of animal cases, the clinical significance of high-frequency CIA populations in human infections should be clarified. The CIA data combined with clinical relevance may be useful to medical doctors/veterinarians treating human/companion animals with symptoms or signs suggestive of streptococcal infections.

To the best of our knowledge, this is the first report to date that has documented high-frequency CIA among Japanese isolates. Complete WGSs of four isolates from Korean dogs were constructed by our study group (GenBank accession numbers CP053790, CP053792, CP053789, and CP046521) (33). For future studies, CIA data among isolates from other countries need to be considered. In conclusion, our observations suggest that *S. canis* CIA and its related microbiological features (SCM allele type 10/type 11 or ST21/ST41) support the genetic clonality of the high-frequency CIA population. Further studies should focus on delineating the association of high-frequency CIA with host cell characteristics (e.g., signaling factors). Improved and sequential analysis to monitor the variations in these findings would be beneficial.

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**Conflict of interest** None to declare.

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Table 2. Microbiological features of *S. canis* isolates enrolled into this study

| Isolate   | SCM allele type (allele group) | ST (clonal complex) | AMR phenotype\(^1\) | AMR genotype |
|-----------|--------------------------------|---------------------|----------------------|--------------|
| NCTC 12191(T) | 1 (I)                          | 9 (9)               | None                 | None         |
| TA4       | 1 (I)                          | 9 (9)               | None                 | None         |
| OT1       | 1 (I)                          | 9 (9)               | None                 | None         |
| FU1       | 1\(^2\) (II)                   | 41\(^3\)           | None                 | None         |
| FU3       | 4 (I)                          | 14 (14)             | Minocycline, azithromycin | *erm*(B), *tet*(O) |
| FU6       | 1 (I)                          | 9 (9)               | None                 | None         |
| FU7       | 10\(^5\) (II)                 | 21\(^1\)           | Minocycline          | *tet*(O)     |
| FU14      | 2 (I)                          | 46 (46)             | Minocycline, erythromycin, azithromycin, clindamycin | *erm*(B), *tet*(O) |
| FU15      | 1 (I)                          | 9 (9)               | None                 | None         |
| FU16      | 11\(^2\) (II)                 | 41\(^3\)           | None                 | None         |
| FU17      | 11\(^2\) (II)                 | 41\(^3\)           | None                 | None         |
| FU24      | 1 (I)                          | 9 (9)               | None                 | None         |
| FU25      | 12 (II)                        | 47                  | None                 | None         |
| FU29      | 5 (I)                          | 44                  | None                 | None         |
| FU30      | 11\(^2\) (II)                 | 41\(^3\)           | None                 | None         |
| FU31      | 1 (I)                          | 48                  | None                 | *tet*(O)     |
| FU32      | 10\(^5\) (II)                 | 21\(^1\)           | Minocycline          | *tet*(S)     |
| FU52      | 1 (I)                          | 3 (9)               | Minocycline          | *tet*(O)     |
| FU53      | 10\(^5\) (II)                 | 21\(^1\)           | None                 | None         |
| FU57      | 1 (I)                          | 2 (46)              | None                 | None         |
| FU58      | 14 (II)                        | 49                  | None                 | None         |
| FU63      | 2 (I)                          | 46 (46)             | Minocycline, erythromycin, azithromycin, clindamycin | *erm*(B), *tet*(O) |
| FU64      | 3 (I)                          | 13 (14)             | Minocycline          | *tet*(M)     |
| FU65      | 15 (II)                        | 50                  | None                 | None         |
| FU66      | 1 (I)                          | 9 (9)               | None                 | None         |
| FU67      | 1 (I)                          | 9 (9)               | None                 | None         |
| FU69      | 13 (II)                        | 51                  | Minocycline          | *tet*(M)     |
| FU77      | 1 (I)                          | 9 (9)               | None                 | None         |
| FU78      | 10\(^5\) (II)                 | 21\(^1\)           | None                 | *tet*(O)     |
| FU90      | 6 (I)                          | 27                  | Minocycline          | *tet*(O)     |
| FU91      | 10\(^5\) (II)                 | 21\(^1\)           | None                 | *tet*(S)     |
| FU92      | 4 (I)                          | 11                  | None                 | None         |
| FU93      | 10\(^5\) (II)                 | 21\(^1\)           | Minocycline          | *tet*(S)     |
| FU97      | 4 (I)                          | 14 (14)             | Erythromycin, azithromycin, clindamycin | *erm*(B) |
| FU100     | 14 (II)                        | 5                   | None                 | None         |
| FU112     | 1 (I)                          | 48                  | Minocycline, erythromycin, azithromycin, clindamycin | *erm*(B), *tet*(O) |
| FU113     | 10\(^5\) (II)                 | 21\(^1\)           | Minocycline          | *tet*(O)     |
| FU115     | 1 (I)                          | 30 (9)              | None                 | None         |
| FU116     | 14 (II)                        | 49                  | Minocycline          | *tet*(M)     |
| FU120     | 1 (I)                          | 48                  | Minocycline, erythromycin, azithromycin, clindamycin | *erm*(B), *tet*(M) |
| FU121     | 10\(^5\) (II)                 | 21\(^1\)           | None                 | *tet*(O)     |
| FU128     | 1 (I)                          | 3 (9)               | Minocycline          | *mef*(A), *tet*(O) |
| FU129     | 10\(^5\) (II)                 | 21\(^1\)           | Minocycline, erythromycin, azithromycin, clindamycin | *erm*(B), *tet*(O) |

\(^1\): Antimicrobial activity was determined using broth microdilution method according to the Clinical and Laboratory Standards Institute document M100-S25.

\(^2\): A significant association \((p < 0.01)\) between high-frequency cell invasion ability and SCM allele type 10/type 11 populations.

\(^3\): A significant association \((p < 0.01)\) between high-frequency cell invasion ability and ST21/ST41 populations.

SCM, *Streptococcus canis* M-like protein; ST, sequence type; AMR, antimicrobial resistance; NCTC, national collection of type cultures.