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Original Article

Novel Quinolone Nonsusceptible *Streptococcus canis* Strains with Point Mutations in Quinolone Resistance-determining Regions and Their Related Factors

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SUMMARY: This study investigated quinolone nonsusceptible *Streptococcus canis* with point mutations in quinolone resistance-determining regions (QRDRs). After selecting targets from 185 isolates, we conducted antimicrobial susceptibility testing using levofloxacin, ciprofloxacin, norfloxacin, and moxifloxacin. We also determined amino acid sequences of QRDRs in *gyrA/gyrB/parC/parE* genes and their point mutations. Finally, we performed *S. canis*-derived M-like protein (SCM) allele typing, multilocus sequence typing, and antimicrobial resistance genotyping. Correlations between nonsusceptible strains and their related factors were examined. We found 13 (7.0%) nonsusceptible isolates consisting of two classes, high-level minimum inhibitory concentrations (MICs) ($n = 7, 3.8\%$), and low-level MICs ($n = 6, 3.2\%$). The mutations Ser81Phe/Ser81Tyr/Glu85Lys in *gyrA*, Ser67Phe/Ser67Tyr/Asp71Tyr in *parC*, Asp438Asn in *parE*, and Gly408Asp in *gyrB* were observed in these nonsusceptible strains. The common mutations were Ser81 and Ser67/Asp71, whereas we found one strain each with Glu85, Asp438 and Gly408 mutations. There was a significant correlation between the nonsusceptible isolates and presence of SCM allele type 2, sequence type 46, tetracycline-resistance genes, and macrolide/lincosamide-resistance genes. These results could be used in the future by veterinarians while treating companion animals with clinical symptoms of streptococcal infections.
INTRODUCTION

*Streptococcus canis*, first reported in 1986 (1), forms large gray/white-colored smooth colonies with β-hemolysis on sheep blood agar plates. Lancefield grouping classifies *S. canis* as group G streptococci based on the composition of carbohydrate antigens in the cell wall. In healthy dogs, *S. canis* is a part of the resident microflora of the oropharynx, skin, genitourinary tract, and anus (2). This microorganism is an emerging zoonotic pathogen and can cause self-limiting dermatitis. In some cases, it leads to severe diseases, including arthritis, streptococcal toxic shock syndrome, necrotizing fasciitis, septicemia, and pneumonia in companion animals (3,4). *S. canis* can also infect humans who have been in close contact with animals and cause either local or systemic diseases (5,6). Clinicians should be aware of the possibility that *S. canis*-related invasive zoonotic infections might be underdiagnosed, as species-level identification is not commonly performed.

Fluoroquinolones, in particular levofloxacin (LVX), have now become the first/second-line treatment modality for many infectious diseases (respiratory and urinary tract infections) in patients with definite or suspected penicillin allergy. Several quinolones target the bacterial DNA gyrase subunits A and B, encoded by *gyrA* and *gyrB*, which are responsible for ATP-dependent DNA supercoiling (7) and therefore inhibit DNA synthesis, triggering bacterial death (8). Previous research on *E. coli* identified the quinolone resistance amino acid (AA) substitutions in regions of *gyrA/gyrB*, which were termed quinolone resistance-determining regions (QRDRs). Additionally, during development of high-level resistance, *gyrA/gyrB* point mutations follow those observed in the associated enzyme DNA topoisomerase IV. It has two subunits A/B encoded by *parC/parE*, which are essential for chromosome partitioning (9). Since the mid 1990s, there have been reports describing strains of *S. pneumoniae* (10), *S. pyogenes* (11), and *S. agalactiae* (12) that were resistant to quinolones, with shared point mutations in the QRDRs of *gyrA/parC*. Surprisingly, the
quinolone resistance conferred by the mutations in gyrB/parE was rarely observed (13).

*Streptococcus canis*-derived M-like protein (SCM), one of the virulence factors, can bind to plasminogen and immunoglobulin G and facilitate anti-phagocytic activity (14,15). Timoney et al. (16) documented four SCM alleles in *S. canis* isolated from diseased and healthy cats and argued that the type 1 allele was predominant in diseased cats. Multilocus sequence typing (MLST) was performed to evaluate the clonal spread of isolates with genetic similarities. It determines the sequence type (ST) based on sequence variations and the allelic combination of seven housekeeping genes (gki–gtr–murI–mutS–recP–xpt–yqiZ) according to the pubMLST website (https://pubmlst.org/scanis/). Pinho et al. (17) have also proposed the use of novel primers (gki_Sc_fwd/gki_Sc_rev, gtr_Sc_fwd/gtr_Sc_rev, murI_Sc_fwd/murI_Sc_rev, recP_Sc_rev, and yqiZ_Sc_rev), since designing the primer sequences were optimized on the basis of the genome sequences to improve the polymerase chain reaction (PCR) amplification and sequencing for some of the loci. Furthermore, considering that significant correlation between tetracycline (TET)-resistance genotypes and open pus/skin-derived β-hemolytic streptococci (mainly *S. canis*) from diseased companion animals has been previously found (18), in this study TET-resistance was also investigated.

The purpose of this study was to investigate *S. canis* isolates resistant/nonsusceptible to quinolone and to define the relationship between the resistant/nonsusceptible isolates and their related factors (SCM allele type, ST and mobile resistance genotype).

**MATERIALS AND METHODS**

**Selection of *S. canis* strains:** During previous studies (18,19), we collected 68 and 117 *S. canis* isolates in 2015 and 2017, respectively, from diseased companion animals along, with the animal information. We identified the *S. canis* strains at the species level based on 16S rRNA sequencing data (18,19). We also validated the accuracy of the identification through
PCR amplification of a \textit{S. canis} \textit{cfg} gene\textsuperscript{(20,21)}. All \textit{S. canis} strains (one isolate per companion animal) were stored at –70 °C to –80 °C for further analyses.

Minimum inhibitory concentrations (MICs, $\mu$g/mL) of LVX were determined using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines for $\beta$-hemolytic streptococci\textsuperscript{(22)}. We selected all isolates with LVX MICs $\geq 1$, since there was a possibility that point mutations are inserted into QRDRs at the low MICs. The isolates with the MIC $\leq 0.25$ were randomly chosen as control strains. We also included National Collection of Type Cultures (NCTC) 12191(T) strain of \textit{S. canis} as a control and two isolates (TA4 and OT1) from human cases of bacteremia with and without a dog bite\textsuperscript{(21,23)}. All three strains were subjected to whole genome sequencing analysis.

**Antimicrobial susceptibility testing of quinolones using Etest:** We conducted antimicrobial susceptibility testing (AST) by Etest (SYSMEX bioMérieux Inc.) of four quinolones including LVX, ciprofloxacin (CIP), norfloxacin (NOR), and moxifloxacin (MXF) according to manufacturer’s instructions, to confirm the nonsusceptibility to these quinolones\textsuperscript{(12)}. Since the low-level MICs (e.g., LVX 2–3) as well as the high-level MICs (LVX $> 32$) by the Etest, with mutations in \textit{gyrA} and \textit{parC}, were documented in six resistant isolates of \textit{S. pyogenes} in Spain\textsuperscript{(24)}, these four quinolones were selected. The MICs of the four antimicrobials by Etest were measured independently by two investigators.

The thresholds for nonsusceptibility to quinolones using Etest were defined as LVX MIC $> 1$ $\mu$g/mL and CIP MIC $\geq 2$ $\mu$g/mL, similar to values reported by Lin et al.\textsuperscript{(25)} used to define the quinolone nonsusceptible \textit{S. pyogenes} isolates.

**Amplifying and sequencing of QRDRs to confirm their point mutations:** To amplify and sequence the QRDRs of \textit{gyrA}, \textit{gyrB}, \textit{parC}, and \textit{parE} from the isolates, we used the primer sets (\textbf{Table 1}) originally used to detect the QRDRs of \textit{S. pyogenes}-specific \textit{gyrA}, \textit{parC},\textsuperscript{(26)} and \textit{parE}\textsuperscript{(27)}, since there was a maximum of 2 nucleotide mismatches between the specific primer sequences and the corresponding genomic sequences in the \textit{S. canis} type strain. We
found a maximum of 4 nucleotide mismatches between the specific primer sequences for 
*gyrB* in *S. pyogenes* (27) and the corresponding genomic sequences in the type strain, and thus, synthesized a new primer set (Table 1) to detect the QRDR of *S. canis*-specific *gyrB*.

The three strains (FU1/FU97/FU129) exhibiting the high and low capabilities of biofilm formations and cell invasion abilities were subjected to the whole genome sequencing by MiSeq (Illumina Inc.), to find the specific sequences leading to the characteristic phenotypes. The targeted sequences of the four QRDRs were extracted from the draft whole genome sequences (WGSs), instead of PCR amplification.

To confirm the determined AA positions in the QRDRs of *gyrA/*gyrB/*parC/*parE*, we performed multiple alignments using the AA sequences of the QRDRs from our strains and other species including susceptible-*S. pneumoniae* (strain R6), nonsusceptible-*S. pyogenes* (NIH-R01-GAS), and nonsusceptible-*S. agalactiae* (GTC 1966). The alignments were generated using ClustalW implemented in MEGA X (version 10.0.5) (28).

**Amplifying and sequencing scm nucleotide sequences and SCM allele typing:** We amplified and sequenced full-length *scm* nucleotide sequences as previously reported (17,29). Based on the variations in AA sequences, the SCM allele typing was performed (17,29).

In the 3 strains where the WGSs were obtained, the full-length *scm* nucleotide sequences were extracted from the contig data.

**MLST analysis:** We performed MLST analysis on all isolates enrolled in this study according to protocols reported by Pinho et al. (17). To amplify the *xpt* gene, *xpt*-fwd-M13F including the M13F universal sequencing primer and *xpt*-rev-M13R-pUC including the M13R universal sequencing primer were used, since the sequences of the previous primer set (*Xptgc-up/Xptgc-dn*) were too close to the *xpt* allele-determining sequences. STs were grouped into clonal complexes (CCs), whereby related STs were classified as single locus variants differing in only one housekeeping gene.

In the 3 strains with WGS data, MLST analysis was performed using a web-based
application of MLST (https://cge.cbs.dtu.dk/services/MLST/, Center for Genomic Epidemiology) on which the contig data were processed.

**Characterization of antimicrobial resistance genes:** The presence of antimicrobial resistance (AMR) genes, including macrolide/lincosamide (ML)-class resistance genes, \(ern(A)\), \(ern(B)\), and \(mef(A)\), in addition to TET-class resistance genes, \(tet(M)\), \(tet(O)\), \(tet(K)\), \(tet(L)\), and \(tet(S)\) in all \(\beta\)-hemolytic streptococcal isolates, was determined by PCR (18).

In the 3 strains that were subjected to the whole genome sequencing, the presence of antimicrobial resistance genes was determined using web-based application ResFinder3.0 (https://cge.cbs.dtu.dk/services/ResFinder/, Center for Genomic Epidemiology) on which the contig data were processed.

**Animal ethics statement:** The Ethics Committee of the Sanritsu Zelkova Veterinary Laboratory approved the study design (approval number SZ20190723) to maintain privacy of the affected animals.

**Statistical analysis:** We used Fisher's exact probability test (two-sided) to analyze significant relationships between the nonsusceptible isolates with mutations and their related factors. A \(p\) value of \(< 0.05\) indicated statistical significance.

**RESULTS**

**S. canis strains enrolled in the study:** Backgrounds of the strains enrolled are shown in Table 2. Sixteen isolates with LVX MICs \(\geq 1\) and the eight isolates with MIC \(\leq 0.25\) were chosen. Of the sixteen, seven were resistant to LVX, and one showed intermediate susceptibility according to the CLSI criteria. The controls [NCTC 12191(T), TA4, and OT1] showed an MIC value of 0.5.

The eight isolates resistant/intermediate-susceptible to LVX were recovered from five prefectures of Tokyo \((n = 3)\), Chiba \((n = 2)\), and Aichi/Kanagawa/Nara \((n = 1)\). Five were
isolated in 2017 and three in 2015. All the isolates were obtained from non-sterile sites (open pus, ear discharge, and urine) of dogs. The dogs’ demographics were as follows: mean age, 10.0 years; age range, 4–14 years; sex, 6 males and 2 females.

**MICs of four quinolones by Etest:** MIC values of the four drugs by Etest are shown in Table 3. In addition to the eight isolates resistant/intermediate-susceptible to LVX using broth microdilution, the other strains ($n = 5$) were shown to be nonsusceptible according to the Etest definitions of LVX/CIP. The strains indicated the LVX MIC of 2 measured by broth microdilution. These isolates were recovered from the prefectures of Chiba, Ishikawa, Fukui, Tokyo, and Okinawa. Three were isolated in 2017 and two in 2015. All the isolates were obtained from non-sterile sites (open pus, ear discharge, and urine) of dogs ($n = 4$) and a cat. The companion animal demographics were as follows: mean age, 9.0 years; age range, 7–13 years; sex, 3 males and 2 females.

On the other hand, the three that had the MIC value of 1 by broth microdilution and all the controls (clinical isolates and the type strain) revealed susceptibility based on the Etest definitions.

**Determination of AA sequences of the QRDRs and their point mutations:** AA sequences of the QRDRs and substitutions different from those in NCTC 12191(T) are shown in Table 3. Multiple alignments of AA sequences in the QRDRs revealed substitutions at positions 81/85 in gyrA, 57/67/71/95 in parC, 438 in parE, and 408 in gyrB. Based on Etest data from our isolates, the mutations Ser81Phe/Ser81Tyr/Glu85Lys in gyrA, Ser67Phe/Ser67Tyr/Asp71Tyr in parC, Asp438Asn in parE, and Gly408Asp in gyrB were observed in the nonsusceptible strains.

**SCM allele typing based on variations in the AA sequences:** Distribution and prevalence of the SCM allele types are shown in Table 3. We observed type 1 ($n = 3$, 23.1%), type 2 ($n = 7$, 53.8%), type 4 ($n = 2$, 15.4%), and type 10 ($n = 1$, 7.7%) in the 13 nonsusceptible isolates with mutations. However, type 1 ($n = 7$, 50.0%), type 2 ($n = 1$, 7.1%),
type 4 \((n = 2, 14.3\%)\), type 10 \((n = 3, 21.4\%)\), and type 11 \((n = 1, 7.1\%)\) was found in the 14 susceptible isolates without mutations.

There was a significant correlation between the nonsusceptible isolates and the type 2 SCM allele \((p < 0.05)\).

**MLST analysis:** STs (allele profile) obtained by MLST are shown in Table 3. We mainly found the ST46 \((n = 6, 46.2\%)\) and ST2 \((n = 2, 15.4\%)\), that were grouped into CC46, in the nonsusceptible isolates with the mutations. On the other hand, mainly ST9 \((n = 5, 35.7\%)\) and ST3 \((n = 2, 14.3\%)\), that were grouped into CC9, were present in the susceptible isolates without the mutations.

There was a significant correlation between the nonsusceptible strains and the ST46 \((p < 0.01)\) and CC46 \((p < 0.01)\).

**Detection of AMR genotypes:** Distribution and prevalence of the AMR genotypes are shown in Table 3. Our study included mixed TET+ML resistance genotypes \((n = 7, 53.8\%)\) and only TET resistance genotypes \((n = 3, 23.1\%)\) in the nonsusceptible isolates with mutations. The mixed resistance genes were \(erm(B)+tet(O)\) \((n = 5)\), \(erm(B)+tet(M)+tet(O)\) \((n = 1)\), and \(erm(B)+mef(A)+tet(O)\) \((n = 1)\), whereas the only TET resistance genes were \(tet(M)/tet(O)/tet(S)\) \((n =1)\). On the other hand, there were mixed TET+ML resistance genotypes \((n = 1, 7.1\%)\) including \(erm(B)+tet(O)\), only TET resistance genotypes \((n = 2, 14.3\%)\) including \(tet(O)\), and only ML resistance genotypes \((n = 1, 7.1\%)\) including \(erm(B)\), in the susceptible isolates without mutations.

There was a significant correlation between the nonsusceptible isolates and the TET resistance genes \((p < 0.01)\) and the ML resistance genes \((p < 0.05)\).

**DISCUSSION**

In agreement with previous findings (24), we observed a concordance of variations from...
low MICs to the high MICs, for four drugs, in the thirteen nonsusceptible isolates (Table 3).

Our 13 nonsusceptible isolates were from non-sterile sites (open pus, ear discharge, and urine). However, we should pay special attention to the emergence of nonsusceptible isolates from sterile sites (blood, cerebrospinal fluid, joint fluid, pleural effusion, ascites, and deep-sided closed pus). The prevalence of nonsusceptible isolates in 2015 and 2017 was 7.4% (5/68) and 6.8% (8/117), respectively. Therefore, nonsusceptible strains should be monitored throughout the country systematically and additional nonsusceptible isolates need to be characterized in the future.

We confirmed the mutations of Ser81/Glu85 in gyrA, Ser67/Asp71 (corresponding to the positions 79/83 in \textit{S. pneumoniae} R6) in parC, Asp438 (corresponding to the position 435 in \textit{S. pneumoniae} R6) in parE, and Gly408 (corresponding to the position 406 in \textit{S. pneumoniae} R6) in gyrB in our nonsusceptible strains. The common mutations were Ser81 and Ser67/Asp71 (26). However, we found Glu85, Asp438, and Gly408 in only one strain each, although the substitutions Glu85 and Asp438 have already been reported in resistant \textit{S. pyogenes} (30) and \textit{S. pneumoniae} (31). To elucidate whether these three mutations might contribute to nonsusceptibility, we need to perform an \textit{in vitro} induction of nonsusceptibility using our \textit{S. canis} strains, as previously reported (26,32) and establish these nonsusceptible mutants in the future.

The acquired mutations at Ser67/Asp71 in parC and/or Ser81 in gyrA, along with the increasing MICs of 4 quinolones by Etest are indicated in Table 3. Of the nonsusceptible isolates, some strains harboring the low MICs showed the Ser67/Asp71 in parC alone, whereas with the elevation of MICs, some strains indicated the Ser81 in gyrA, except for the MICs using SA15. These observations suggest the acquired mutations might contribute to the increase in MICs among the novel-generation drug (MXF) as well as old-generation one (NOR).

There was a significant correlation between the nonsusceptible isolates and the SCM type
2/TET resistance genes/ML resistance genes, suggesting a clonal spread of the isolates in Japan. Other investigators (25,32) have also found the nonsusceptible strains of S. pyogenes to be associated with particular emm types 12/6. Additionally, 80% of the nonsusceptible S. pyogenes clones harboring the emm 12/6/11/1 (n = 30) revealed resistance to both ML due to \textit{erm}(B) and TET due to \textit{tet}(M) (33). Petrelli et al. (34) reported that most of the nonsusceptible S. pyogenes strains belong to emm type 6, even if the other types were present (emm75/89/2), indicating possibility of other types spreading. Therefore, in the future, we should monitor the multiple clonal spreads of the nonsusceptible S. canis isolates in companion animals.

Enrofloxacin and orbifloxacin are used to treat diseased companion animals in Japan (35). According to a recent report from National Veterinary Assay Laboratory, quinolone class antibiotics constituted 7.0% of overall antimicrobials (converted weight in kilograms to bulk powder) used for companion animals in 2016 (36). The limitation of this study was that the detailed therapeutic approaches (especially doses of quinolones administered) were unclear, although we received the animal information. Therefore, the quinolone usage data should be collected from veterinarians in the future investigations.

According to the PubMLST isolate database (https://pubmlst.org/bigsdb?db=pubmlst_scanis_isolates) of S. canis strains (209 total isolates), no isolate was resistant to either LVX or enrofloxacin (as of August 5th, 2019). The novel ST46 observed in the nonsusceptible isolates has recently been registered in the PubMLST database. To the best of our knowledge, this is the first report of S. canis isolates that were resistant/nonsusceptible to quinolones with point mutations in the QRDRs. We also described the relationships between the resistant/nonsusceptible isolates and their related factors. This information could be of use to Japanese veterinary practitioners while treating dogs and cats with clinical symptoms of streptococcal infections.
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Conflict of interest  None to declare.
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| Target (gene)                  | Primer name    | Direction | Sequence (5’→3’)                              | Annealing temperature (°C) | Reference |
|-------------------------------|----------------|-----------|-----------------------------------------------|----------------------------|-----------|
| DNA gyrase subunit A (gyrA)   | gyrA-F         | Forward   | GCAAGATCGAAATTAAATTGACGTC                    | 61 - 36 [55\(^1\)]        | (26)      |
|                               | gyrA-R         | Reverse   | ACTCTCTTGTGTTGTACGTTG                      |                            |           |
| DNA gyrase subunit B (gyrB)   | canis_gyrB_F   | Forward   | TGGCAATTCAAGAGTAGTTAA                       | 47 - 42 [42\(^1\)]        | This study|
|                               | canis_gyrB_R   | Reverse   | TGCTTCTAAAGACTGGTCTCA                      |                            |           |
| DNA topoisomerase IV subunit A (parC) | parC-F     | Forward   | ATGTCAACATTCAAGACATGTCC                    | 61 - 36 [58\(^1\)]        | (26)      |
|                               | parC-R         | Reverse   | AGCCTCGGAAATCCGAGAAG                     |                            |           |
| DNA topoisomerase IV subunit B (parE) | parE-F     | Forward   | GCTCAGATTATCGAGAAGGA                      | 42                         | (27)      |
|                               | parE-R         | Reverse   | CAGCATTGGTCATGATAATA                     |                            |           |

\(^1\) The most frequently applied annealing temperature.
Table 2. Backgrounds of *Streptococcus canis* strains enrolled into this study

| Strain | Animal species | Sex and age (year-old) | Isolation prefecture | Isolation year | Isolation source | Antimicrobial activity of levofloxacin (µg/mL) |
|--------|----------------|------------------------|----------------------|----------------|-----------------|---------------------------------------------|
| FU47   | Dog            | Male and unknown       | Tokyo                | 2017           | Open pus        | > 8                                         |
| FU71   | Dog            | Male and six           | Chiba                | 2017           | Ear discharge   | > 8                                         |
| FU75   | Dog            | Male and four          | Aichi                | 2017           | Open pus        | > 8                                         |
| FU5    | Dog            | Female and twelve      | Kanagawa             | 2017           | Urine           | > 8                                         |
| SA3    | Dog            | Male and nine          | Tokyo                | 2015           | Ear discharge   | 8                                           |
| SA15   | Dog            | Male and fourteen      | Tokyo                | 2015           | Ear discharge   | 8                                           |
| SA31   | Dog            | Male and thirteen      | Nara                 | 2015           | Urine           | 8                                           |
| FU95   | Dog            | Female and twelve      | Ciba                 | 2017           | Open pus        | 4                                           |
| FU3    | Dog            | Male and unknown       | Chiba                | 2017           | Open pus        | 2                                           |
| FU32   | Dog            | Female and unknown     | Ishikawa             | 2017           | Ear discharge   | 2                                           |
| FU115  | Dog            | Female and seven       | Fukui                | 2017           | Open pus        | 2                                           |
| SA35   | Dog            | Male and thirteen      | Tokyo                | 2015           | Open pus        | 2                                           |
| SA68   | Cat            | Male and seven         | Okinawa              | 2015           | Urine           | 2                                           |
| FU40   | Dog            | Male and fifteen       | Saitama              | 2017           | Subcutaneous fluid aspirated | 1                                        |
| SA16   | Dog            | Male and seventeen     | Aichi                | 2015           | Open pus        | 1                                           |
| SA25   | Dog            | Male and unknown       | Chiba                | 2015           | Earwax          | 1                                           |
| NCTC 12191(T) | Bovine | | | | | 0.5                                      |
| FU1    | Cat            | Male and unknown       | Ciba                 | 2017           | Open pus        | ≤ 0.25                                     |
| FU74   | Dog            | Male and five          | Tokyo                | 2017           | Open pus        | ≤ 0.25                                     |
| FU92   | Dog            | Male and twelve        | Tokyo                | 2017           | Urine           | ≤ 0.25                                     |
| FU97   | Dog            | Male and eleven        | Okinawa              | 2017           | Open pus        | ≤ 0.25                                     |
| FU129  | Dog            | Male and nine          | Niigata              | 2017           | Open pus        | ≤ 0.25                                     |
| SA34   | Dog            | Male and ten           | Osaka                | 2015           | Ear discharge   | ≤ 0.25                                     |
| SA61   | Dog            | Female and unknown     | Tokyo                | 2015           | Ear discharge   | ≤ 0.25                                     |
| FU128  | Dog            | Male and unknown       | Ciba                 | 2017           | Open pus        | ≤ 0.25                                     |
| TA4    | Human          | Male and seventy-one   | Tokyo                | 2016           | Blood           | 0.5                                         |
| OT1    | Human          | Female and ninety-one  | Gifu                 | 2012           | Blood           | 0.5                                         |

1): Antimicrobial activity was determined using broth microdilution method according to the Clinical and Laboratory Standards Institute document M100-S22.
Table 3. Quinolone nonsusceptibility/susceptibility determined and their microbiologically related factors

| Strain | MIC (μg/mL) of each drug by Etest | Codon (AA) at: SCM allele type | Sequence type (allele profile) | Macrolide/tetracycline resistance gene |
|--------|----------------------------------|-------------------------------|--------------------------------|-----------------------------------|
|        | LVX > 32 CIP > 32 NOR > 256 MXF | Position 67 in parC<sup>13</sup> | Position 71 in parC<sup>13</sup> | Position 81 in gyrA | Other positions in QRDR | |
| FU47   | 32 > 32 > 256 2                | TTC (Phe)                     | TTC (Phe)                        | No AA substitutions            | 2 | 46 (2-2-2-2-2-2-2-2-2-2-8-2-2-2-2) | tetr(B), tet(A), elo(T) |
| FU71   | 32 > 32 > 256 3                | TTC (Phe)                     | TTC (Phe)                        | No AA substitutions            | 2 | 46 (2-2-2-2-2-2-2-2-2-2-8-2-2-2-2) | tetr(B), tet(T) |
| FU75   | 32 > 32 > 256 3                | TTC (Ser)                     | TTC (Ser)                        | No AA substitutions            | 2 | 46 (2-2-2-2-2-2-2-2-2-2-8-2-2-2-2) | tetr(B), tet(O) |
| FU5    | 32 > 32 > 256 3                | TTC (Phe)                     | TTC (Phe)                        | No AA substitutions            | 2 | 46 (2-2-2-2-2-2-2-2-2-2-8-2-2-2-2) | tetr(B), tet(O) |
| SA3    | 32 > 32 > 256 4                | TTC (Phe)                     | TTC (Phe)                        | No AA substitutions            | 2 | 46 (2-2-2-2-2-2-2-2-2-2-8-2-2-2-2) | tetr(B), tet(O) |
| SA15   | 24 4 24 1                      | TTC (Phe)                     | TTC (Phe)                        | AAT (Asp) at position 438 in parC<sup>13</sup> | 2 | 2 (2-2-2-2-1-2-2) | None |
| SA31   | 24 > 32 > 256 3                | TTC (Phe)                     | TTC (Phe)                        | No AA substitutions            | 1 | 46 (2-2-2-2-2-2-2-2-2-2-8-2-2-2-2) | tetr(B), tet(M), tetr(O) |
| FU95   | 3 4 32 1.5                     | TTC (Ser)                     | TTC (Ser)                        | AAT (Asp) at position 85 in gyrA | 4 | 13 (4-2-4-4-5-3-4) | ter(M) |
| FU3    | 3 2 32 0.38                    | TTC (Ser)                     | TTC (Ser)                        | No AA substitutions            | 4 | 14 (4-2-4-4-6-3-4) | tetr(B), tetr(O) |
| FU32   | 3 4 48 0.32                    | TTC (Phe)                     | TTC (Ser)                        | GAC (Asp) at position 408 in gyrB<sup>14</sup> | 10 | 21 (7-4-5-3-4-7-1) | ter(S) |
| FU115  | 3 2 16 0.25                    | TAC (Tyr)                     | TTC (Ser)                        | No AA substitutions            | 1 | 30 (3-8-3-1-1-2-3) | None |
| SA35   | 3 4 96 0.38                    | TTC (Phe)                     | TTC (Ser)                        | No AA substitutions            | 2 | 2 (2-2-2-2-1-2-2) | ter(O) |
| SA68   | 4 8 96 1                       | TTC (Phe)                     | TTC (Ser)                        | No AA substitutions            | 1 | 9 (3-5-3-1-2-3) | None |
| FU40   | 1 2 12 0.25                    | TTC (Ser)                     | TTC (Ser)                        | No AA substitutions            | 1 | 3 (3-3-3-1-2-3) | tetr(O) |
| SA16   | 0.38 0.5 3 0.19                | TTC (Ser)                     | TTC (Ser)                        | No AA substitutions            | 1 | 9 (3-5-3-1-2-3) | None |
| SA25   | 1 1 4 0.19                     | TTC (Ser)                     | TTC (Ser)                        | No AA substitutions            | 10 | 21 (7-4-5-3-4-7-1) | None |
| NCTC 12191(T) | 0.5 0.38 2 0.125 | TTC (Ser)                     | TTC (Ser)                        | No AA substitutions            | 1 | 9 (3-5-3-1-2-3) | None |
| FU1    | 0.38 0.38 2 0.19               | TTC (Ser)                     | TTC (Ser)                        | No AA substitutions            | 11 | 41 (7-2-3-12-4-7-3) | None |
| FU74   | 0.38 0.5 2 0.125               | TTC (Ser)                     | TTC (Ser)                        | No AA substitutions            | 1 | 9 (3-5-3-1-2-3) | None |
| FU92   | 0.38 0.25 1.5 0.125            | TTC (Ser)                     | TTC (Ser)                        | No AA substitutions            | 4 | 11 (2-4-4-4-3-2-2) | None |
| FU97   | 1 1 12 0.25                    | TTC (Ser)                     | TTC (Ser)                        | TTC (Phe) at position 95 in parC<sup>13</sup> | 4 | 14 (4-2-4-4-6-3-4) | tetr(B) |
| FU129  | 0.5 0.5 2 0.19                 | TTC (Ser)                     | TTC (Ser)                        | No AA substitutions            | 10 | 21 (7-4-5-3-4-7-1) | tetr(B), tetr(O) |
| SA34   | 0.38 0.5 2 0.19                | TTC (Ser)                     | TTC (Ser)                        | No AA substitutions            | 10 | 21 (7-4-5-3-4-7-1) | None |
| SA61   | 0.5 0.5 3 0.19                 | TTC (Ser)                     | TTC (Ser)                        | GTC (Val) at position 57 in parC<sup>13</sup> | 2 | 45 (2-2-2-13-2-3-2) | None |
| FU28   | 0.38 0.38 2 0.125              | TTC (Ser)                     | TTC (Ser)                        | No AA substitutions            | 1 | 3 (3-3-3-1-2-3) | tetr(O) |
| TA4    | 0.5 0.5 3 0.19                 | TTC (Ser)                     | TTC (Ser)                        | No AA substitutions            | 1 | 9 (3-5-3-1-2-3) | None |
| OT1    | 0.38 0.38 3 0.19               | TTC (Ser)                     | TTC (Ser)                        | No AA substitutions            | 1 | 9 (3-5-3-1-2-3) | None |

MIC, minimum inhibitory concentration; LVX, levofloxacin; CIP, ciprofloxacin; NOR, norfloxacin; MXF, moxifloxacin; AA, amino acid; QRDR, quinolone resistance-determining region; SCM, S. canis M-like protein.

<sup>1</sup> It corresponds to the AA sequence positions 69, 79, 83 and 107 in S. pneumoniae R6 parC.<br> <sup>2</sup> It corresponds to the AA sequence positions 435 in S. pneumoniae R6 parE.<br> <sup>3</sup> It corresponds to the AA sequence positions 406 in S. pneumoniae R6 gyrB.

The nucleotide and AA substitutions observed in nonsusceptible strains are underlined in bold letters.
Supplementary table. Accession numbers of genes encoding QRDR and genes encoding SCM in *S. canis*

The corresponding sequences in the quinolone resistance-determining regions of *gyrA*, *gyrB*, *parC*, and *parE* have been deposited in DDBJ/EMBL/GenBank as follows.

Additionally, the novel nucleotide sequences encoding *S. canis*-derived M-like protein (SCM) have been deposited in DDBJ/EMBL/GenBank as follows.

| Accession number | EntryID | Accession number | EntryID | Accession number | EntryID |
|------------------|---------|------------------|---------|------------------|---------|
| LC495778         | FU47-gyrA | LC495779         | FU71-gyrA | LC495780         | FU75-gyrA |
| LC495781         | FU5-gyrA  | LC495782         | SA3-gyrA  | LC495783         | SA15-gyrA |
| LC495784         | SA31-gyrA | LC495785         | FU95-gyrA | LC495786         | FU3-gyrA  |
| LC495787         | FU32-gyrA | LC495788         | FU115-gyrA | LC495789         | SA35-gyrA |
| LC495790         | SA68-gyrA | LC495791         | FU40-gyrA | LC495792         | SA16-gyrA |
| LC495793         | SA25-gyrA | LC495794         | FU1-gyrA  | LC495795         | FU74-gyrA |
| LC495796         | FU92-gyrA | LC495797         | FU97-gyrA | LC495798         | FU129-gyrA |
| LC495799         | SA34-gyrA | LC495800         | SA61-gyrA | LC495801         | FU28-gyrA |
| LC495802         | TA4-gyrA  | LC495803         | OT1-gyrA  | LC495804         | FU47-gyrB |
| LC495805         | FU71-gyrB | LC495806         | FU75-gyrB | LC495807         | FU5-gyrB  |
| LC495808         | SA3-gyrB  | LC495809         | SA15-gyrB | LC495810         | SA31-gyrB |
| LC495811         | FU95-gyrB | LC495812         | FU3-gyrB  | LC495813         | FU32-gyrB |
| LC495814         | FU115-gyrB| LC495815         | SA35-gyrB | LC495816         | SA68-gyrB |
| LC495817         | FU40-gyrB | LC495818         | SA16-gyrB | LC495819         | SA25-gyrB |
| LC495820         | FU1-gyrB  | LC495821         | FU74-gyrB | LC495822         | FU92-gyrB |
| LC495823         | FU97-gyrB | LC495824         | FU129-gyrB | LC495825         | SA34-gyrB |
| LC495826         | SA61-gyrB | LC495827         | FU28-gyrB | LC495828         | TA4-gyrB |
| LC495829         | OT1-gyrB  | LC495830         | FU47-parC | LC495831         | FU71-parC |
| LC495832         | FU75-parC | LC495833         | FU5-parC  | LC495834         | SA3-parC  |
| LC495835         | SA15-parC | LC495836         | SA31-parC | LC495837         | FU95-parC |
| LC495838         | FU3-parC  | LC495839         | FU32-parC | LC495840         | FU115-parC|
| LC495841         | SA35-parC | LC495842         | SA68-parC | LC495843         | FU40-parC |
| LC495844         | SA16-parC | LC495845         | SA25-parC | LC495846         | FU1-parC  |
| LC495847         | FU74-parC | LC495848         | FU92-parC | LC495849         | FU97-parC |
| LC495850         | FU129-parC| LC495851         | SA34-parC | LC495852         | SA61-parC |
| LC495853         | FU28-parC | LC495854         | TA4-parC  | LC495855         | OT1-parC  |
| LC495856         | FU47-parE | LC495857         | FU71-parE | LC495858         | FU75-parE |
| LC495859         | FU5-parE  | LC495860         | SA3-parE  | LC495861         | SA15-parE |
| LC495862         | SA31-parE | LC495863         | FU95-parE | LC495864         | FU3-parE  |
| LC495865         | FU32-parE | LC495866         | FU115-parE| LC495867         | SA35-parE |
| LC495868         | SA68-parE | LC495869         | FU40-parE | LC495870         | SA16-parE |
| LC495871         | SA25-parE | LC495872         | FU1-parE  | LC495873         | FU74-parE |
| LC495874         | FU92-parE | LC495875         | FU97-parE | LC495876         | FU129-parE|
| LC495877         | SA34-parE | LC495878         | SA61-parE | LC495879         | FU28-parE |
| LC495880         | TA4-parE  | LC495881         | OT1-parE  | LC500134         | FU1_scm   |
| LC500135         | FU32_scm  | LC500136         | FU129_scm | LC500137         | SA25_scm  |
| LC500138         | SA34_scm  |