Veterinary Microbiology 229 (2019) 81–89

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Mutations associated with change of susceptibility to lincosamides and/or macrolides in field and laboratory-derived *Mycoplasma californicum* strains in Japan, and development of a rapid detection method for these mutations

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ARTICLE INFO

Keywords:
Mycoplasma californicum
Antibiotic resistance
Bovine mastitis
Hybridization probe

ABSTRACT

Five mutations involved in changing of susceptibility to lincosamides and/or macrolides were investigated in field isolates of *Mycoplasma californicum* in Japan, and reconfirmed in laboratory-derived mutants. In addition, a quick and easy detection method for these mutations was established. Guanine at position 748 (*Escherichia coli* numbering) of the 23S rRNA gene (*rrl*) was shown to be involved with decreased susceptibility to 16-membered macrolides, and adenines at positions 2059 and 2062 of *rrl* were involved with decreased susceptibility to both lincosamides and macrolides. Both guanine at position 2576, and change from cytosine to thymine at position 2611 of *rrl* were found to be involved with decreased susceptibility to lincosamides, and the latter mutation also increased the susceptibility to erythromycin. These mutations were easily induced by several to approximately 30 passages in a medium containing the respective antimicrobial, but they did not return after their initial appearance. The melting curve analysis using hybridization probes revealed the existence of these mutations by the change in the melting curve shape and/or decrease in the melting peak temperature. The detection limit in milk samples with a somatic cell count up to 716×10^3 cell/mL was 133 cfu/mL, but an excessive increase in the cell count in milk or storage of the milk sample at chilling or freezing temperature decreased the sensitivity. This method requires only a few hours, so field veterinarians can make a same-day determination of susceptibility to macrolides and lincosamides, which are first-line antibiotics for bovine mycoplasmal mastitis.

1. Introduction

Mycoplasma californicum is both a commensal bacterium for cattle and a causal bacterium of bovine mastitis, arthritis, and pneumonia (Hewicker-Trautwein et al., 2002; Hata et al., 2014). In Japan, mastitis due to *M. californicum* has been increasing in recent years, and the incidence of latent intramammary infections with *M. californicum* has increased over the same period, making the detection and prevention of bovine infections difficult (Hata et al., 2014). These bovine mycoplasmal diseases spread quickly in herds, and often lead to severe and refractory cases, so culling of infected cows in the early stage is recommended rather than treatment as a countermeasure. As a result, the economic losses from mycoplasmal diseases can be very significant.

The potentially effective antimicrobials against bovine mycoplasmal infection consist mainly of protein synthesis inhibitors (i.e., aminoglycosides, macrolides, lincosamides, tetracyclines, phenicols, and pleuromutilins) and nucleic acid synthesis inhibitors (i.e., fluoroquinolones). Macrolides, lincosamides, and tetracyclines are often used as first-line antibiotics for bovine mycoplasmal disease in Japan, with fluoroquinolones being recognized as second-line antibiotics. On the other hand, decreased susceptibility to some of these antibiotics has recently been confirmed in multiple mycoplasmal species, and spreading of strains with low susceptibility may make antimicrobial therapies more difficult (Kawai et al., 2014; Lerner et al., 2014). Determining the antimicrobial susceptibility of causal strains is thus indispensable for the proper prescription of antibiotics at treatment, and would be expected to both improve the cure rate and reduce overuse of ineffective antimicrobials.

For many pathogenic bacteria, antimicrobial susceptibility can be checked quickly and easily in a clinical setting by means of an antimicrobial disk diffusion test, and the breakpoint for resistant bacteria has been clearly established (CLSI, 2015). However, no quick and easy

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https://doi.org/10.1016/j.vetmic.2018.12.017
Received 7 November 2018; Received in revised form 13 December 2018; Accepted 16 December 2018

method has been established to test the antimicrobial susceptibility of bovine mycoplasmas, and traditional methods such as the broth microdilution or agar dilution method are time-consuming (requiring 2~3 weeks) and labor-intensive (Hannan, 2000) due to the culture characteristics of mycoplasmas. Hence, these traditional methods are not helpful at emergency clinics. As an alternative approach, methods for the rapid detection of susceptibility-associated mutations may be useful for judging the antimicrobial susceptibility of specific mycoplasmal strains. Genetic mutations associated with decreased susceptibility to various antimicrobials have been examined in field strains and/or resistant mutants of various mycoplasmal species other than *M. californicum*; some of these were found to be universal, while others varied among mycoplasmal species or specific regions (Kobayashi et al., 2005; Lysnyansky et al., 2015; Pereyre et al., 2004). For example, many of the mutation points on *M. bovis*, an important mycoplasma of bovine infectious diseases, are located on specific genes, such as 16S and 23S ribosomal RNA (*rrs* and *rrl*), DNA gyrase (*gyrA*), and topoisomerase IV (*parC*) (Sulyok et al., 2017). The number of *rrs-rrl* operons varies among microbial species, but there are usually two operons in the genome of *M. californicum* as with many bovine mycoplasmas (Hata and Murakami, 2014).

In this study, we investigated the antimicrobial susceptibility of *M. californicum* isolates in Japan, and identified mutation points associated with changing susceptibility to antimicrobials in low-susceptibility field isolates and laboratory-derived low-susceptibility mutants. Based on our results, we established a method for the rapid detection of these mutation points.

2. Materials and methods

2.1. Mycoplasmal isolates, antimicrobial agents, and susceptibility testing

A total of 184 *M. californicum* isolates were collected from bovine quarter milk and bulk tank milk between 2005 and 2017 in Japan. The methods used to isolate and identify these isolates have been described previously (Hata et al., 2014). The susceptibility of the *M. californicum* isolates to fifteen antimicrobials approved for therapeutic applications in veterinary use in Japan were examined. These consisted of an aminoglycoside (spectinomycin), macrolides (erythromycin, azithromycin, tylosin, and tilmicosin), lincosamides (lincomycin and pirlimycin), tetracyclines (oxytetracycline and chlortetracycline), a phenicol (florfenicol), fluoroquinolones (enrofloxacin, danofloxacin, and flumequine), and pleuromutilins (tiamulin and valnemulin). Two type strains (*M. californicum* ST-6^T and *M. bovis* PG45^T) were also added to this study for quality control of the test and for later evaluation, and the final minimum inhibitory concentration (MIC) values of antimicrobial agents against each isolate were determined by the agar microdilution method according to the recommendation method of Hannnan et al. (2000). Furthermore, MIC values of erythromycin and flumequine against six type strains (*M. alkalescens* PG51^T, *M. arginini* G230^T, *M. bovigenitalium* PG11^T, *M. bovirhinis* PG43^T, *M. bovoculi* M165/89^T, and *M. canadense* 275C^T) were simultaneously investigated.

2.2. Selection of M. californicum mutants with low susceptibility to antibiotics

Selection of mutants with reduced susceptibility to antibiotics was performed on the ST-6^T type strain and five field isolates (HAZ44, HAZ106, HAZ160, HAZ175, and HAZ517). Moreover, selection of mutants with low erythromycin susceptibility was performed on two field isolates and five laboratory-derived mutants which show low susceptibility against lincosamides (HAZ683, HAZ700, HAZ44$_{lincomycin}$, HAZ106$_{lincomycin}$, HAZ160$_{lincomycin}$, HAZ175$_{lincomycin}$, and HAZ517$_{lincomycin}$). The selection was carried out by serial passages of the isolates in modified Hayflick broth mediums containing a sub-inhibitory concentration of each of the examined antibiotics (Pereyre et al., 2004). The culture containing the highest antibiotic concentration with detectable growth (red to yellow color shift) was used to inoculate the broths containing the next dilution of antibiotic in the series (Pereyre et al., 2004). Passages in broth medium containing antimicrobials were performed 40 times, or until the antimicrobial concentration reached 512 μg/mL. Passages in broth medium containing erythromycin were performed until the antimicrobial concentration reached that of the original strain, or until it reached 512 μg/mL. Laboratory-derived mutants and field isolates that show low susceptibility to lincosamides and/or macrolides were passaged through an antibiotic-free medium more than 30 times. The MICs of antimicrobials for these mutants were reconfirmed at the end of the passages to check whether the phenotype was stable without selection pressure.

2.3. Analysis of mutations associated with change of susceptibility to lincosamides and/or macrolides

M. californicum genomic DNAs were prepared from logarithmic-phase broth cultures by using an InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Oligonucleotide primers for the PCR amplification and sequencing of 23S rRNA genes (*rrl3, rrl4*), ribosomal protein L4 gene (*rplD*), and ribosomal protein L22 gene (*rplV*) were designed from *M. californicum* HAZ160_1 genome (accession no. AP013353) (Hata and Murakami, 2014). The sequences of the oligonucleotide primers are shown in the Supplemental Materials section. Each gene was amplified using PrimeSTAR® GXL DNA Polymerase (Takara Bio Inc., Otsu, Japan), and each amplicon was continuously purified using a LaboPass™ PCR Purification Kit (Cosmo Genetech, Seoul, South Korea) and sequenced on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. Sequence editing, consensus, and alignment were performed using GENETYX® software ver. 13 (GENETYX, Tokyo). In addition, whole-genome sequence analysis of HAZ106 and four laboratory-derived mutants (HAZ106$_{lincomycin}$, HAZ106$_{erythromycin}$, HAZ160$_{lincomycin}$, and HAZ160$_{erythromycin}$) was performed as described previously (Hata and Murakami, 2014).

The numbering of nucleotide positions of *rrl* throughout the article is based on those of *E. coli* (Petrov et al., 2013), unless otherwise indicated. The numbering of the amino acid positions of RplD and RplV throughout the article is based on those of *M. californicum* HAZ160_1, unless otherwise indicated (Hata and Murakami, 2014).

2.4. Rapid detection of mutations associated with change of susceptibility to lincosamides and/or macrolides by melting curve analysis using a hybridization probes

DNA from milk samples was extracted using a DNeasy Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany). First, a sample solution was produced by mixing 500 μL of milk with 500 μL of buffer ATL (the accompanying lysis buffer in the DNA extraction kit) and 60 μL of proteinase K (20 mg/mL), and the mixture was incubated with gentle shaking at 70 °C until completely lysed. A lysate solution was produced by mixing 500 μL of buffer AL to sample solution, and the mixture was incubated at 56 °C for 10 min, subsequently mixed 500 μL of ethanol (96–100%) to lysate solution. Total amount of lysate solution was pipeted into DNeasy mini spin column placed in a 2 mL collection tube, centrifuged at ≥ 6000 × *g* till completely filtered, and flow-through was discarded. For DNA washing, 500μL of Buffer AW1 was added into spin column placed in a new 2 mL collection tube, centrifuged for 1 min at ≥6000 × *g*, and flow-through was discarded. Furthermore, 500μL of Buffer AW2 was added into spin column placed in a new 2 mL collection tube, centrifuged for 3 min at 20,000 × g, and flow-through was discarded. The spin column was transferred to new 1.5 mL of microcentrifuge tube, 20μL of Buffer AE was dropped on the center of the spin column membrane, and incubated for 1 min at room temperature for

Table 1
The primers and probes used in melting-curve genotyping analysis by hybridization probe for detecting mutations involved in abnormal susceptibility against macrolides and/or lincosamides.

target mutations	primers and probes	sequence (from 5' to 3')	annealing temp. (°C)	amplicon size (bp)
rrl DII G748	Forward primer	ACG TTT GGG AAG ACG TAG	60	838
	Reverse primer	CGT TAA ATT ATT GGC GCA GGG		
	Hybridization probe	ACC AAG TGG AGG GCC GAA CC-FITC		
		LC Red640-AGT ACG CTG AAA AGT GCC CG-phosphate		
rrl DV A2059-2062	Forward primer	GGC GTT AGC TGA ATT AGT TG	54	658
	Reverse primer	ACC CGA TTA TGA TGG CAG		
	Hybridization probe	AAA CGC TGG GTA CCC GCA TC-FITC		
		LC Red640- AGA CGA AAA GAC CCC ATG GA-phosphate		
rrl DV G2576	Forward primer	AAC CTG CCA TCA TAA TCG	58	617
	Reverse primer	CCT TGT AGT CTT CAA GGA ATC		
	Hybridization probe	GGG TTG GGC TGT TCG CCC AT-FITC		
		LC Red640- AAG CGG TAC GCG AGC TGG GTT-phosphate		
rrl DV C2611	Forward primer	same as *rrl* DV G2576	58	
	Reverse primer	same as *rrl* DV G2576		
	Hybridization probe	CTG GGT TCA GAA CGT CGT GA-FITC		
		LC Red640- CAG TTC GGT CCC TAT CTG AT-phosphate		

elution of DNA. Finally, DNA solution was collected by centrifuge for 1 min at ≥6000 × *g*. Buffer ATL, buffer AL, buffer AW1, buffer AW2, buffer AE, 2 mL collection tube, and DNeasy mini spin column are included in the DNA extraction kit. For the detection of target mutations, a 50 μL amplicon was prepared using PrimeSTAR® GXL DNA polymerase with 1.5 μL of DNA template from the milk sample, or at least 10 ng of genomic DNA. The PCR primers for amplification of the target region were designed by comparative analysis of the *rrl* sequences between *M. californicum* (accession nos. AP013353 and CP007521) and various Mollicutes bacteria (accession nos. AB182581, AMWK01000000, AORH00000000, AP014631, AP014657, AP017902, AP018135, AUAL01000000, BX293980, CP000896, CP002107, CP002108, CP002188, CP002513, CP007154, CP007229, CP009770, CP011096, FP236530, FR668087, FUXF00000000, JFDP00000000, JNJU00000000, NC_000908, NC_000912, NC_011374, NR_076192, and X68421). The primer sequences, annealing temperatures, and amplicon sizes are shown in Table 1. The PCR amplification conditions consisted of 35 cycles of 98 °C for 10 s, annealing temperature for 15 s, and 68 °C for 60 s/1 kb of amplicon size. The melting curve analysis assay for the detection of target mutations was carried out in a 20 μL solution of the two-fold diluted PCR product containing 0.2 μM of each hybridization probe. A total of four hybridization probe sets against target mutations were also designed from the *M. californicum* HAZ160_1 genome (Hata and Murakami, 2014). These consisted of a donor probe whose 3′ end was labeled with fluorescein isothiocyanate (FITC), and an acceptor probe whose 5′ end was labeled with LC Red640. The hybridization probe sequences are also shown in Table 1. The protocol for the melting curve analysis using a LightCycler®480 System II (Roche Diagnostics GmbH, Manheim, Germany) consisted of a single cycle of 95 °C for 60 s (20 °C/s), 40 °C for 60 s (20 °C/s), and 70 °C for 0 s (0.1 °C/s). Analytical results were calculated using the analytical software included with the real-time PCR machine (operated in automatic mode), i.e., LightCycler3 480 SW 1.5.1 and Exor4 for XDMS_R (Roche Diagnostics GmbH). The species-specificity of this method was confirmed on genomic DNA samples of nine bovine mycoplasmal strains (*M. alkalescens* PG51^T, *M. arginini* G230^T, *M. bovigenitalium* PG11^T, *M. bovirhinis* PG43^T, *M. bovoculi* M165/89^T, *M. canadense* 275C^T, *M. dispar* 462/2^T, *M. bovis* PG45^T, and *M. californicum* ST-6^T), two acholeplasmal strains (*A. axanthum* S743^T and *A. laidlawii* PG8^T), and nine strains belonging to bacterial species that can cause bovine mastitis (*E. coli* ATCC 12810, *Enterococcus faecalis* ATCC 19433, *Ent. faecium* NCTC 7171, *Staphylococcus aureus* ATCC 12600, *Sta. epidermidis* ATCC 146, *Streptococcus agalactiae* NCTC 11360, *Str. dysgalactiae* NCDO 2023, *Str. uberis* ATCC19436, and *Str. parauberis* DSM6631). The somatic cell count (SCC) in milk sample was checked using a DeLaval cell counter (DCC) (DelLaval, Cardiff, UK). Bovine milk samples with various SCCs (20×10^3, 716×10^3, 1600×10^3, and 3000×10^3 cell/mL) and serial dilutions of ST-6^T (1.33×10^0 to 1.33×10^7 cfu/mL) were prepared to confirm the sensitivity of this method, and the effects of two milk-sample storage conditions were also evaluated (-20 °C or 4 °C for 7 days).

3. Results

3.1. Antimicrobial susceptibility of M. californicum field isolates

The MIC distributions of the tested 15 antimicrobial agents against *M. californicum* field isolates, the MIC_{50} and MIC_{90} values, and the MICs against ST-6^T and PG45^T are shown in Fig. 1. No clear bimodal MIC distributions were confirmed for any of the antimicrobial agents, and the differences between the MIC values against ST-6^T and the MIC_{50} values against field isolates were also within the range of one-eighth to two-fold. The antibiotic to which the isolates exhibited the highest susceptibility was valnemulin, with MIC_{90} and MIC_{50} values of 0.008 μg/mL and 0.002 μg/mL, respectively. The *M. californicum* field isolates also showed high susceptibility to tylosin, tilmicosin, pirlimycin, and tiamulin, with MIC_{90} and MIC_{50} values in the ranges of 0.125-0.063 μg/mL and 0.063-0.031 μg/mL, respectively. On the other hand, *M. californicum* showed extremely low susceptibility to erythromycin, with MIC_{90} and MIC_{50} values of 128 μg/mL and 64 μg/mL, and the MIC against ST-6^T was a similarly low 32 μg/mL. Many of the type strains of bovine mycoplasmas showed susceptibilities to erythromycin as low as those of ST-6^T; the MICs of erythromycin against these strains were as follows: PG51^T, 512 μg/mL; G230^T, 512 μg/mL; PG11^T, 128 μg/mL; PG43^T, 512 μg/mL; PG45^T, 256 μg/mL; M165/89^T, 8 μg/mL; and 275C^T, 256 μg/mL. The susceptibility of *M. californicum* field isolates to flumequine, which is a first-generation drug of the fluoroquinolone family, was also extremely low, unlike the susceptibilities to enrofloxacin and danofloxacin, for which the MIC_{90} and MIC_{50} values were 32 μg/mL and 16 μg/mL, and the MIC against ST-6^T was a similarly high 16 μg/mL. Moreover, the susceptibility of the bovine mycoplasmas to flumequine differed widely among the type strains, with MICs as follows: PG51^T, 128 μg/mL; G230^T, 256 μg/mL; PG11^T, 64 μg/mL; PG43^T, 1 μg/mL; PG45^T, 32 μg/mL; M165/89^T, 0.5 μg/mL; and 275C^T, 16 μg/mL.

Macrolides (azithromycin, tylosin, and tilmicosin) and/or lincosamides (lincomycin and pirlimycin) showed extremely high MIC values against four field isolates (HAZ371, HAZ505, HAZ683, and HAZ700). Four field isolates (HAZ371, HAZ505, HAZ683, and HAZ700) showed extremely high MIC values against macrolides (azithromycin, tylosin, and tilmicosin) and/or lincosamides (lincomycin and pirlimycin); these strains were thus appeared to be resistant to these antimicrobial agents (Fig. 1, Table 2). The MIC values of tylosin and tilmicosin against

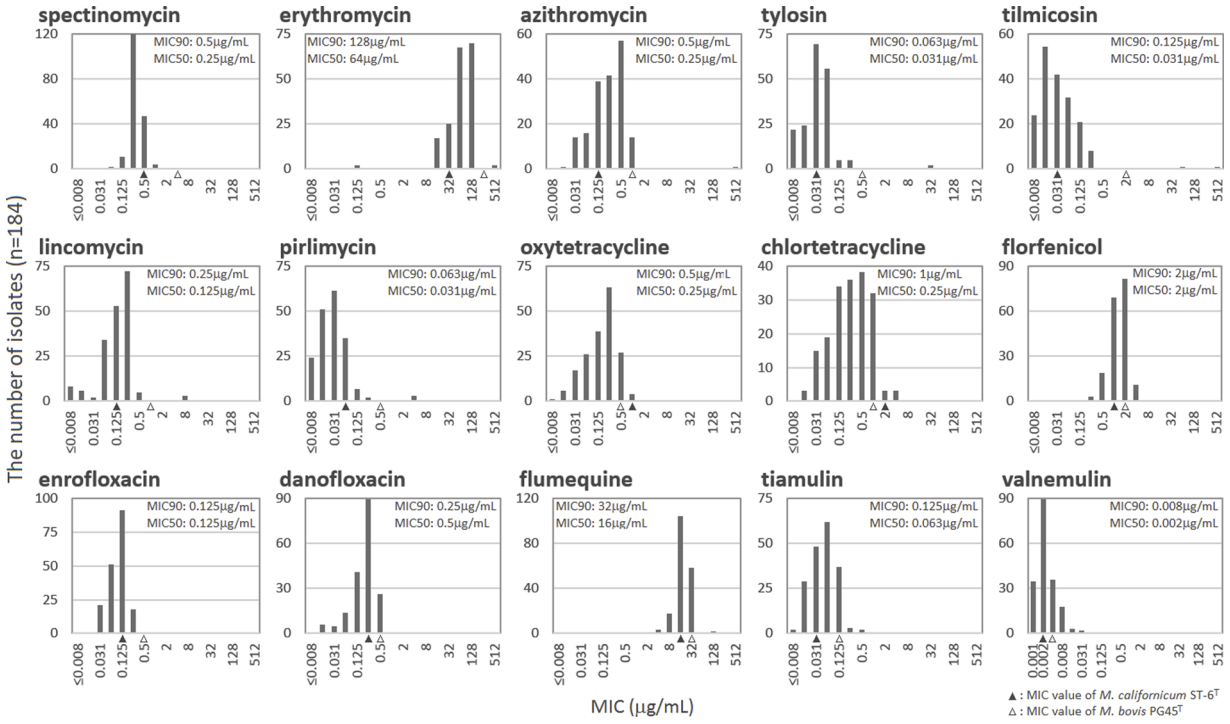

Fig. 1. Antimicrobial-susceptibility distribution of *M. californicum* field isolates in Japan. MIC values of *M. californicum* ST-6^T and *M. bovis* PG45^T are indicated by black and white triangles under the bar-graph, respectively. Although *M. californicum* is insusceptible to erythromycin in nature, two isolates that show low MIC values (0.125 μg/mL) were confirmed.

Table 2
Susceptibility to macrolides and lincosamides of *M. californicum* field isolates, and those before and after selection of laboratory-derived mutants.

strain[a]	no. of passages	MIC (μg/mL)					
		erythromycin	azithromycin	tylosin	tilmicosin	lincomycin	pirlimycin
field isolates with abnormal MICs against macrolides and/or lincosamides							
HAZ371		512	1	32	512	0.5	0.125
HAZ505		512	512	32	64	8	4
HAZ683		0.125	0.063	0.031	0.063	8	4
HAZ700		0.125	0.031	0.063	0.031	8	4
original strains							
ST-6^T		32	0.125	0.016	0.031	0.125	0.031
HAZ44		32	0.031	0.016	0.016	0.125	0.031
HAZ106		128	1	0.063	0.063	0.25	0.016
HAZ160		128	0.5	0.063	0.031	0.25	0.063
HAZ175		32	0.5	0.031	0.031	0.25	0.125
HAZ517		64	0.5	0.031	0.031	0.25	0.063
laboratory-derived mutants							
ST-6$^T_{tylosin}$	26	64	0.25	512	512	0.25	0.031
HAZ44 $_{tylosin}$	28	64	0.063	64	128	8	2
HAZ106 $_{tylosin}$	22	512 <	16	512 <	512 <	8	2
HAZ160 $_{tylosin}$	37	512 <	0.25	512 <	256	4	2
HAZ175 $_{tylosin}$	25	32	0.25	32	32	0.5	0.125
HAZ517 $_{tylosin}$	26	512 <	16	512 <	512 <	512 <	32
ST-6$^T_{lincomycin}$	14	512 <	32	32	64	512 <	16
HAZ44 $_{lincomycin}$	26	0.25	0.063	0.063	0.031	8	4
HAZ106 $_{lincomycin}$	27	0.125	0.031	0.031	0.016	32	2
HAZ160 $_{lincomycin}$	13	1	1	0.063	0.031	16	2
HAZ175 $_{lincomycin}$	28	0.25	0.25	0.031	0.016	256	16
HAZ517 $_{lincomycin}$	24	0.063	0.031	0.031	0.031	16	2
HAZ44 $_{erythromycin}$	8	32	0.063	0.031	0.031	8	4
HAZ106 $_{erythromycin}$	14	128	0.5	0.031	0.016	0.063	0.031
HAZ160 $_{erythromycin}$	16	512	0.5	0.063	0.063	16	4
HAZ175 $_{erythromycin}$	21	32	0.5	0.063	0.031	256	16
HAZ517 $_{erythromycin}$	16	512	0.5	0.031	0.031	0.25	0.063
HAZ683 $_{erythromycin}$	10	64	0.063	0.031	0.031	0.125	0.063
HAZ700 $_{erythromycin}$	11	64	0.063	0.063	0.063	0.25	0.125

[a] The name of the laboratory-derived mutant was combined with the name of the original strain and the antimicrobial agent used for selecting the laboratory-derived mutant. The selection of seven mutants with low-susceptibility to erythromycin has been performed on five lincomycin laboratory-derived mutants and two field isolates (i.e. HAZ44 $_{lincomycin}$, HAZ106 $_{lincomycin}$, HAZ160 $_{lincomycin}$, HAZ175 $_{lincomycin}$, HAZ517 $_{lincomycin}$, HAZ683, and HAZ700).

HAZ371 were 32 μg/mL and 512 μg/mL. MIC values of azithromycin, tylosin, tilmicosin, lincomycin, and pirlimycin against HAZ505 were 512 μg/mL, 32 μg/mL, 64 μg/mL, 8 μg/mL, and 4 μg/mL, respectively. MIC values of lincomycin and pirlimycin against HAZ683 and HAZ700 were 8 μg/mL and 4 μg/mL. Moreover, HAZ683 and HAZ700 were susceptible to erythromycin, unlike many other field isolates and ST-6^{T}, and the MIC values of erythromycin against both these isolates was 0.125 μg/mL (Fig. 1, Table 2).

3.2. Analysis of mutations associated with change of susceptibility to lincosamides and/or macrolides

We confirmed the sporadic emergence of *M. californicum* field isolates which show abnormal susceptibility to lincosamides and/or macrolides. Many bacteria, including mycoplasmas have been reported to have various mutations in *rrl*, *rplD*, and *rplV* associated with changes in the susceptibility to lincosamides and/or macrolides (Pereyre et al., 2004), so we checked for variations in these genes in ST-6^{T} and all field isolates. Guanines at position 748 of hairpin-loop 35 in domain II (*rrl* DII) of both *rrl* changed to adenines (G748 A) in HAZ371, the adenines at position 2059 of the peptidyl transferase center circle in domain V (*rrl* DV) of both *rrl* changed to guanines (A2059 G) in HAZ505, and the cytosine at position 2611 of *rrl* DV of *rrl3* changed to thymine (C2611 T) in HAZ683 and HAZ700 (Fig. 2). On the other hand, no amino acid substitutions were observed in RplD or RplV in any of the field isolates (Table 3).

These mutations and changes of antimicrobial susceptibility were reproduced by laboratory-derived mutants. Mutation *rrl* DII G748 A was reproduced in the four laboratory-derived mutants (ST-6$^{T}_{tylosin}$, HAZ44$_{tylosin}$, HAZ106$_{tylosin}$, HAZ175$_{tylosin}$), and MICs of 16-memberd macrolides rose excessively from the MICs against the original strains as with HAZ371 (tylosin MICs: from 0.016-0.063 μg/mL to 32–512 < μg/mL; tilmicosin MICs: from 0.031-0.063 μg/mL to 32–512 < μg/mL) (Fig. 2, Table 2 and 3). The mutation *rrl* DV A2059 G was reproduced in two laboratory-derived mutants (HAZ517$_{tylosin}$ and ST-6$^{T}_{lincomycin}$), and MICs of azithromycin, 16-membered macrolides, and lincosamides rose excessively from the MICs against the original strains as with HAZ505 (azithromycin MICs: from 0.125-0.5 μg/mL to 16-32 < μg/mL; tylosin MICs: from 0.016-0.031 μg/mL to 32–512 < μg/mL; tilmicosin MICs: from 0.031 μg/mL to 64-512 < μg/mL; lincomycin MICs: from 0.125-0.25 μg/mL to 512 < μg/mL; pirlimycin MICs: from 0.031-0.063 μg/mL to 16-32 < μg/mL) (Fig. 2, Table 2 and 3). Moreover, the susceptibility to erythromycin was increasingly decreased in these mutants (erythromycin MICs: from 32 to 64 μg/mL to 512 < μg/mL) (Fig. 2, Table 2 and 3). HAZ106$_{tylosin}$, in which the mutation *rrl* DV A2062C occurred, also showed the same change of susceptibility pattern, suggesting that the mutations *rrl* DV A2059 G and *rrl* DV A2062C may be synonymous (Fig. 2, Table 2 and 3). Meanwhile, HAZ160$_{tylosin}$, in which the mutation *rrl* DV A2062 G occurred, showed increases in the MICs of erythromycin, 16-membered macrolides, and lincosamides, but did not show a rise in the MICs of azithromycin (Fig. 2, Table 2 and 3). Moreover, HAZ106$_{tylosin}$ and HAZ160$_{tylosin}$ showed decreases in the susceptibility to florfenicol (florfenicol MICs: from 2 μg/mL to 128 and 32 μg/mL). Amino acid substitution at the 90th glutamine in RplV occurred in all laboratory-derived mutants induced by tylosin, two of which were also confirmed to show amino acid substitutions at the 89th serine (Table 3). The mutation *rrl* DV C2611 T was reproduced in five laboratory-derived mutants (HAZ44$_{lincomycin}$, HAZ106$_{lincomycin}$, HAZ160$_{lincomycin}$, HAZ175$_{lincomycin}$, and HAZ517$_{lincomycin}$), and these laboratory-derived mutants not only showed an excessive increase in MIC values of lincosamides (lincomycin MICs: from 0.125-0.25 μg/mL to 8–256 μg/mL; pirlimycin MICs: from 0.016-0.125 μg/mL to 2–16 μg/mL), but also an excessive decrease of MIC values against erythromycin as in the case of HAZ683 and HAZ700 (erythromycin MICs: from 32 to 128 μg/mL to 0.063–1 μg/mL) (Fig. 2, Table 2 and 3). To investigate whether the mutation *rrl* DV C2611 T is directly involved in the change of susceptibility to erythromycin, HAZ683, HAZ700 and the five laboratory-derived mutants named above were serial passaged in broth containing erythromycin. After inducing low susceptibility to erythromycin, the mutation *rrl* DV C2611 T re-mutated to the original base in all tested strains (erythromycin MICs: from 0.063 to 1 μg/mL to 32–512 μg/mL) (Table 2). Moreover, amino acid substitutions at the 198th arginine in RplD were also confirmed in all laboratory-derived mutants (Table 2). To search for a universal mutation involved in the

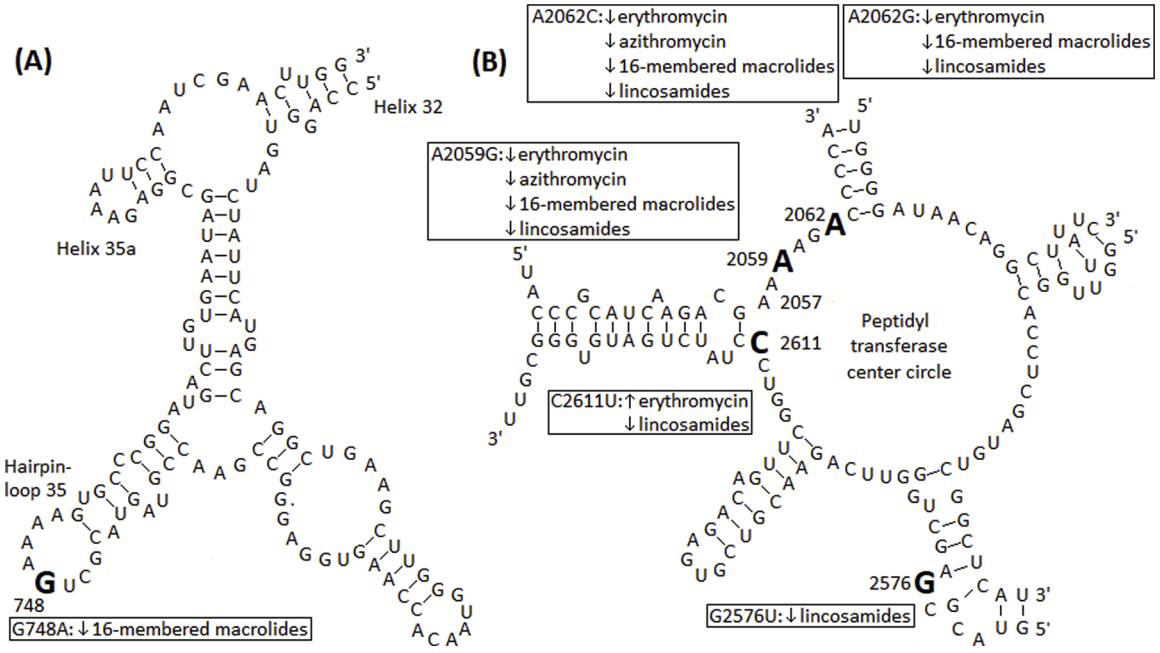

Fig. 2. The two-dimensional structure of the hairpin-loop 35 in domain II of 23S rRNA (A) and the peptidyl transferase center circle in domain V (B) of *M. californicum* HAZ160_1 (accession no. AP013353). The nucleotides are numbered on the basis of the *E. coli* sequence (accession no. NC_002655). Mutations that affect susceptibility to lincosamides and/or macrolides used at therapy of bovine mycoplasmal diseases are highlighted in bold-face, and the change of susceptibility by each identified mutation is shown within the framework. The up-arrow and down-arrow indicate an increase or decrease in susceptibility to each antimicrobial agent.

Table 3
Mutations detected in *rrl*, *rplD* and *rplV* of *M. californicum* field isolates and laboratory-derived mutants[a].

strain[b]	mutation associated with abnormal MICs (*rrl3-rrl4*)[c]					mutation with no effect (*rrl3-rrl4*)[c]								mutation with unknown effect (*rrl3-rrl4*)[c]									(RplD)[d]	(RplV)[d]	
	748	2059	2062	2576	2611	93	237	342	934	1349	1590	1791	2218	141	687	770	1348	1590	2045	2549	2618	2631	592-594(198)	265-267 (89)	268-270 (90)
original strains																									
ST-6[Ta]	G-G	A-A	A-A	G-G	C-C	A-A	T-C	T-C	C-C	A-A	T-T	A-T	C-T	G-G	C-C	G-G	A-A	A-T	C-C	G-G	G-G	G-G	CGT(Arg)	TCA (Ser)	CAA (Gln)
HAZ44							C-C	T-T			A-A	A-A	T-T												
HAZ106						G-A	C-C	C-T	T-C	G-A	T-A	A-A	T-T												
HAZ160						G-A	C-C	C-T	T-C	G-A	T-A	A-A	T-T												
HAZ175							C-C	T-T			T-A	A-A	T-T												
HAZ517						G-G	C-C	C-C	T-T	G-G		A-A	T-T												
field isolates with abnormal MICs against macrolides and/or lincosamides																									
HAZ371	A-A					G-A	C-C	C-T	T-T	G-G		A-A	T-T												
HAZ505		G-G				G-G	C-C	C-C	T-T	G-G		A-A	T-T												
HAZ683					T-C	G-A	C-C	C-T	T-C	G-A	T-A	A-A	T-T												
HAZ700					T-C	G-A	C-C	C-T	T-C	G-A	T-A	A-A	T-T												
laboratory-derived mutants																									
ST-6$^{T}_{tylosin}$	G-A									A-G				G-A		A-G	A-G								CAC (His)
HAZ44 $_{tylosin}$	G-A			G-T			C-C	T-T			A-A	A-A	T-T											TTA (Leu)	AAA (Lys)
HAZ106 $_{tylosin}$	G-A		A-C			G-A	C-C	C-T	T-C	G-A	T-A	A-A	T-T						A-C						CGA (Arg)
HAZ160 $_{tylosin}$	T-C		G-G			G-A	C-C	C-T	T-C	G-G		A-A	T-T		C-A		A-G	A-T				C-C			AAA (Lys)
HAZ175 $_{tylosin}$	A-G						C-C	T-T			T-A	A-A	T-T											TTA (Leu)	AAA (Lys)
HAZ517 $_{tylosin}$		A-G	C-A			G-G	C-C	C-C	T-T	G-G		A-A	T-T												CAT (His)
ST-6$^{T}_{lincomycin}$		G-G								A-G				G-A			A-G								
HAZ44 $_{lincomycin}$				T-G	T-C		C-C	T-T			A-A	A-A	T-T												
HAZ106 $_{lincomycin}$					T-C	G-A	C-C	C-T	T-C	G-A	T-A	A-A	T-T									G-T			
HAZ160 $_{lincomycin}$				T-G	T-C	G-A	C-C	C-T	T-C	G-A	T-A	A-A	T-T							G-T	G-T				
HAZ175 $_{lincomycin}$				T-T	T-T		C-C	T-T			T-A	A-A	T-T												
HAZ517 $_{lincomycin}$					T-C	G-G	C-C	C-C	T-T	G-G		A-A	T-T												
HAZ44 $_{erythromycin}$				T-G			C-C	T-T			A-A	A-A	T-T										CTT(Leu)		
HAZ106 $_{erythromycin}$						G-A	C-C	C-T	T-C	G-A	T-A	A-A	T-T									G-T	AGT(Ser)		
HAZ160 $_{erythromycin}$				T-G		G-A	C-C	C-T	T-C	G-A	T-A	A-A	T-T							G-T	G-T		CCT(Pro)		
HAZ175 $_{erythromycin}$				T-T			C-C	T-T			T-A	A-A	T-T										CTT(Leu)		
HAZ517 $_{erythromycin}$						G-G	C-C	C-C	T-T	G-G		A-A	T-T										CCT(Pro)		
HAZ683 $_{erythromycin}$						G-A	C-C	C-T	T-C	G-A	T-A	A-A	T-T										CCT(Pro)		
HAZ700 $_{erythromycin}$						G-A	C-C	C-T	T-C	G-A	T-A	A-A	T-T										TTT(Phe)		

[a] All SNPs of each gene were shown in comparison to the bases of *M. californicum* ST6^{T} at the same positions.
[b] The name of the laboratory-derived mutant was combined with the names of the original strain and the antimicrobial agent used for selecting the laboratory-derived mutant. The selection of seven mutants with low-susceptibility to erythromycin has been performed on five lincomycin laboratory-derived mutants and two field isolates (i.e. HAZ44 $_{lincomycin}$, HAZ106 $_{lincomycin}$, HAZ160 $_{lincomycin}$, HAZ175 $_{lincomycin}$, HAZ517 $_{lincomycin}$, HAZ683, and HAZ700).
[c] The numbering follows that for *E. coli* (Petrov et al., 2013).
[d] The numbering follows that for *M. californicum* HAZ160_1 (Hata and Murakami, 2014).

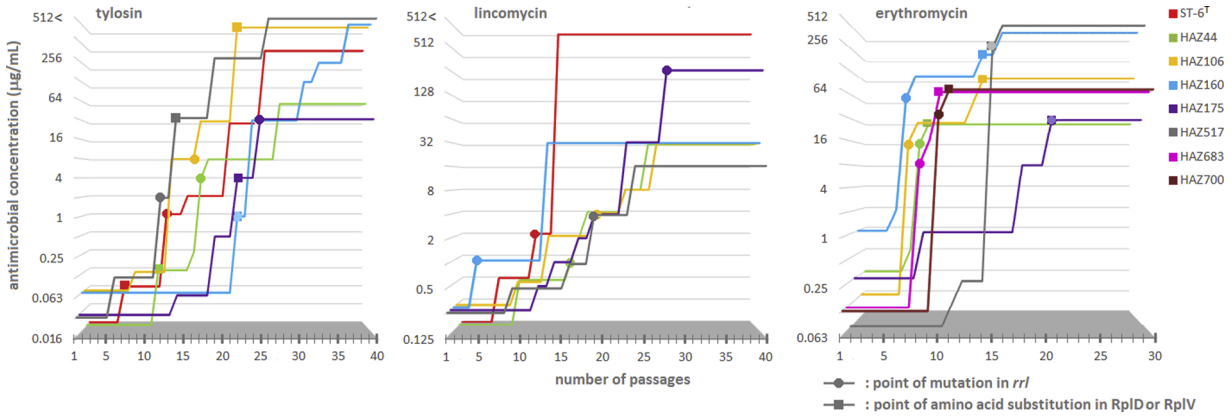

Fig. 3. Change in the MIC of *M. californicum* isolates during serial subculture in Hayflick's broth containing tylosin or lincomycin. Field isolates and laboratory-derived mutants in which the mutation *rrl* DV C2611 T occurred were additionally passaged in Hayflick's broth containing erythromycin. Starting points of mutation in *rrl*, and those of amino acid substitutions in RplD and/or RplV associated with changes of antimicrobial concentration are indicated by circles and squares, respectively.

change of susceptibility to lincomycin and erythromycin other than the mutation *rrl* DV C2611 T or amino acid substitutions at the 198th arginine in RplD, genome analysis was performed for each of HAZ106, $HAZ106_{erythromycin}$, and $HAZ106_{lincomycin}$ (accession nos. AP018940, AP018941, AP018942). Nineteen missense mutations and one frame-shift mutation in seventeen cording sequences (CDSs), and one point mutation in *rrl* genes were further confirmed along with the induction of low susceptibility to lincomycin and erythromycin (Supplemental materials), but these mutations were not universally present in each of HAZ683, HAZ700 and other laboratory-derived mutants induced by lincomycin or erythromycin. Therefore, these mutations appeared not to be involved in the decreased susceptibility to lincomycin and erythromycin. The mutation *rrl* DV G2576 T occurred in four laboratory-derived mutants ($HAZ44_{tylosin}$, $HAZ44_{lincomycin}$, $HAZ160_{lincomycin}$, and $HAZ175_{lincomycin}$), and these laboratory-derived mutants showed excessive rise of MIC values against lincosamides (lincomycin MICs: from 0.125-0.25 μg/mL to 8–256 μg/mL; pirlimycin MICs: from 0.031-0.125 μg/mL to 2–16 μg/mL) (Fig. 2, Tables 2 and 3). Even though the laboratory-derived mutants with mutation *rrl* DV C2611 T returned to the original base as in the cases of $HAZ44_{erythromycin}$, $HAZ160_{erythromycin}$, and $HAZ175_{erythromycin}$, the susceptibility to lincosamides remained low (Fig. 2, Tables 2 and 3). According to the genome analysis for $HAZ160_{erythromycin}$ and $HAZ160_{lincomycin}$ (accession nos. AP018943, AP018944), three missense mutations and one frame-shift mutation in four CDSs, and four point mutations in *rrl* genes including the mutations *rrl* DV C2611 T and *rrl* DV G2576 T, appeared along with the induction of low susceptibility to lincomycin (Table 3, Supplemental materials). Even after the induction of low susceptibility to erythromycin, seven mutations, including *rrl* DV G2576 T, remained, but *rrl* DV C2611 T no longer remained. Six of the seven mutations—i.e., all except for *rrl* DV G2576T—were not confirmed in other isolates or laboratory-derived mutants that show low susceptibility to lincosamides, so these remaining mutations (that is, all except for *rrl* DV G2576 T and *rrl* DV C2611 T) appeared not to contribute to the decreased susceptibility to lincosamides.

Upon the reduction in the susceptibilities to lincomycin, tylosin, or erythromycin, the MICs of the original strains started to change from the 4th ($HAZ160_{lincomycin}$) to 22nd passages ($HAZ160_{tylosin}$), and reached the highest values from the 8th ($HAZ44_{erythromycin}$) to 37th passages ($HAZ160_{tylosin}$) (Fig. 3, Table 2). Seven laboratory-derived mutants ($ST\text{-}6^{T}_{tylosin}$, $HAZ44_{tylosin}$, $HAZ160_{tylosin}$, $HAZ160_{lincomycin}$, $HAZ106_{erythromycin}$, $HAZ683erythro_{mycin}$, $HAZ700erythro_{mycin}$) were mutated in *rrl*, *rplD*, or *rplV* at the first MIC change, and in many cases these mutations occurred when MIC increased to some extent (Fig. 3). Even after passaging through antibiotic-free medium more than 30 times, these mutations did not return, and the MIC values of lincosamides and macrolides did not change.

3.3. Rapid detection of mutations associated with change of susceptibility to lincosamide and/or macrolides by melting curve analysis using a hybridization probes

The primers designed in this study could sufficiently amplify three DNA regions containing five target bases (*rrl* DII G748, *rrl* DV A2059, *rrl* DV A2062, *rrl* DV G2576, and *rrl* DV C2611) of all tested *M. californicum* isolates and ST-6^{T}. On the other hand, the target DNA regions of various kinds of other microbials (eight mycoplasmal type strains, two acholeplasmal type strains, and nine bacterial strains that cause bovine mastitis) were not amplified. Moreover, mixing of these other microbials did not affect the melting curve analysis by hybridization probes. In this study, the detection limits of the PCR and DNA extraction method were suggested to be 133 cfu/mL for the milk samples with SCCs of 20×10^3 and 716×10^3 cell/mL, but decreased to 1330 cfu/mL for the milk samples with SCCs of 1600×10^3 and 3000×10^3 cell/mL, respectively. Storage at -20 °C or 4 °C for 7 days resulted in a further reduction in the detection limit of each sample to one tenth.

In the melting curve analysis using a hybridization probes, a single melting peak at a lower-than-normal temperature is observed for the case that the mutation occurs together at target bases in both *rrl* genes, but a bimodal melting curve is observed in theory if the mutation occurs at the target base in only one *rrl* gene. Most target mutations were clearly detectable by the theoretical change of the melting curve (Fig. 4). When mutations *rrl* DV A2059 G and A2062C occurred in *rrl4* and *rrl3* of $HAZ517_{tylosin}$, the melting curve changed to a bimodal one, with melting peak temperatures of 55.7 °C and 44.2 °C (Fig. 4B). On the other hand, even if the mutation *rrl* DV A2062C occurred in *rrl4* of $HAZ106_{tylosin}$, the melting curve did not change to bimodal. Instead, the temperature of the melting peak shifted to 55.7 °C (Fig. 4B). These changes of the melting curve were distinguished automatically by the analytical software of the real-time PCR machine.

4. Discussion

M. californicum and many other bovine mycoplasmas show natural resistance to erythromycin and high susceptibility to lincosamides. The genomes of many bovine mycoplasmas show *rrl* DV A2057 and *rrl* DV C2611 in nature, but when *rrl* DV C2611 T occurs in *M. californicum*, the susceptibility of the mycoplasma to erythromycin is increased and that to lincosamides is decreased. It has been speculated that the formation and cleavage of base pairs between positions 2057 and 2611 in *rrl* is a

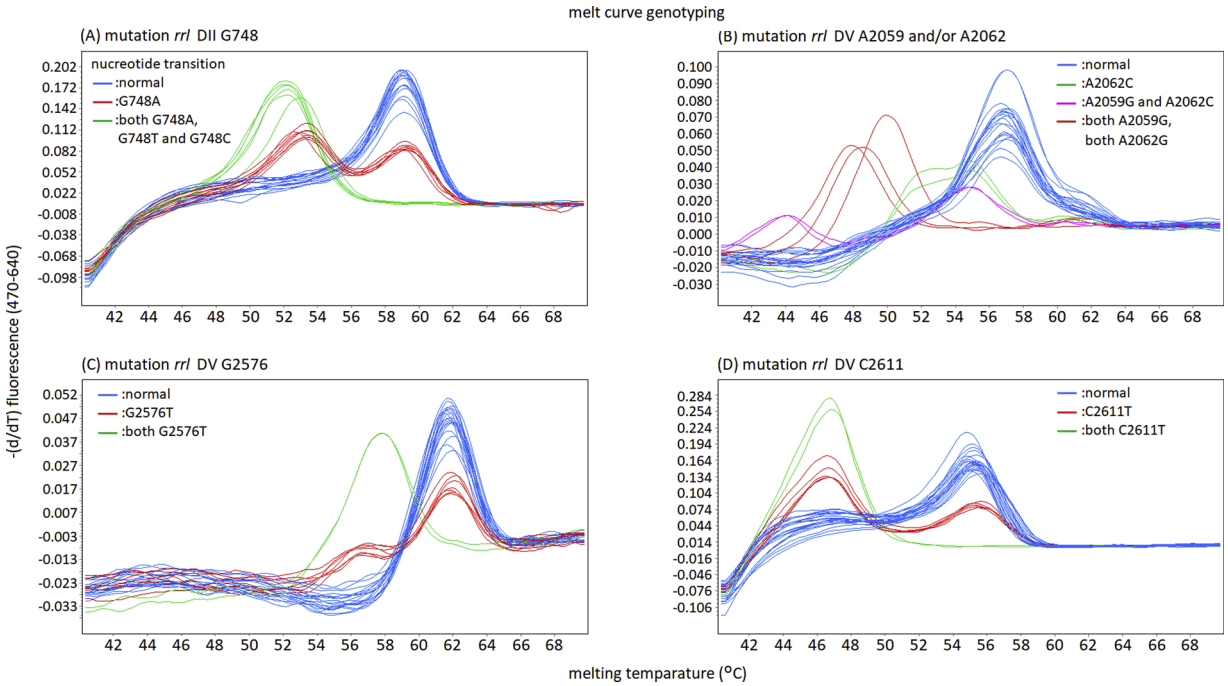

Fig. 4. The results of melt-curve genotyping by hybridization probes for detecting mutations associated with abnormal susceptibility to lincosamides and/or macrolides in *M. californicum*.

crucial factor in the change of these antimicrobial susceptibilities. In the case of *M. pneumoniae*, which shows *rrl* DV G2057 and *rrl* DV C2611 in nature, so *M. pneumoniae* will form base pairs between them. Hence, *M. pneumoniae* may show the high susceptibility to erythromycin and natural resistance to lincosamides (Bébéar et al., 2011). This phenomenon was reproduced for the first time in this study in laboratory-derived mutants of *M. californicum*. Moreover, mutations at *rrl* DII G748, *rrl* DV A2059, *rrl* DV A2062, and *rrl* DV G2576 have been shown to be crucial for decreasing susceptibility to lincosamides and/or macrolides. In a previous study on other Mycoplasma species, mutations at domain II (positions 748 and 752) were confirmed in strains with low susceptibility to 16-membered macrolides. Mutations at domain V (positions 2058 to 2062) were also confirmed in strains with low susceptibility to lincosamides and/or macrolides (Chrisment et al., 2012; Kobayashi et al., 2005; Kong et al., 2016; Lerner et al., 2014; Lysnyansky et al., 2015; Pereyre et al., 2002, 2004; Sulyok et al., 2017). Mutations at *rrl* DV A 2062 may trigger a decrease in susceptibility to florfenicol, and mutations at this position were also seen in laboratory-derived mutants of *M. bovis* with low susceptibility to florfenicol (Sulyok et al., 2017). The present study was the first to demonstrate that the mutation *rrl* DV G2576T decreases susceptibility against lincosamides in laboratory-derived mutants. The relationship between the conformational change of 23S ribosomal RNA resulting from this mutation and the mechanisms of antimicrobial susceptibility should be resolved as soon as possible.

Amino acid substitutions in ribosomal proteins L4 and L22 were shown to be responsible for the resistance of laboratory-derived *M. pneumoniae* to macrolide and lincosamides, but these mutations were confirmed only in laboratory-derived mutants and not in field isolates with low susceptibility in this study (Pereyre et al., 2004). Therefore, as in the case of *M. bovis,* these mutations may not be essential for the resistance of *M. californicum* to macrolide and lincosamides (Lerner et al., 2014). These mutations occur relatively easily, and appear to be irreversibly maintained, as demonstrated in this study and our previous reports (Kobayashi et al., 2005; Sulyok et al., 2017).

As for *M. bovis*, which is a main causal species for bovine mycoplasmal disease, the mutations *rrl* DII G748 A and *rrl* DV A2058 G were often confirmed in *M. bovis* field isolates with low-susceptibility to lincosamides and/or macrolides in European countries, Australia, China, and Israel after 2005 (Kong et al., 2016; Lerner et al., 2014; Sulyok et al., 2017). Even in Japan, susceptibility of 16-membered macrolides tends to decrease, so the mutation *rrl* DII G748 A may occur in most field strains. On the other hand, isolates with low susceptibility to lincosamides have still not been detected (Kawai et al., 2014). Frequent use of specific antimicrobial agents should be avoided in order to prevent decreasing susceptibility to antimicrobial agents. Moreover, the movement of live cows between nations has led to the worldwide spread of bovine mycoplasmas, so a mycoplasma test in quarantined animals may be the most effective measure for preventing the worldwide spread of strains with low susceptibility (Rosales et al., 2015).

Some single nucleotide polymorphisms (SNPs) are known to cause antimicrobial susceptibility. Hybridization probes are typically used in SNP analysis, and a real-time PCR assay can accurately differentiate mutations of target DNA regions by measuring the melting temperature of the probe-amplicon hybrid, even though there are few nucleotide differences or only one such difference (Lyon, 2001). The method presented in this study successfully detected target mutations, and all mutations were shown as shifts in the melting-peak temperature or changes in the shape of the melting curve. The total time required for this method (including the PCR amplification and melting curve analysis) was only 3 h, and the cost of the reagent was US $2.33 per sample. In addition, the enforcement of the DNA sequencing of the mutational domain afterward is recommended for a more accurate judgment (Lyon, 2001). In *M. bovis*, SNPs involved in abnormal susceptibility to various antimicrobials (i.e., macrolides, lincosamides, tetracyclines, spectinomycin, fenicols, and pleuromutilines) are located in the *rrs-rrl* operons, so these SNPs may be easily detectable by the procedure used in this study (Sulyok et al., 2017). On the other hand, antibiotic-resistance mechanisms based on amino acid substitutions, such as fluoroquinolone resistance, will not be efficiently detectable by this SNP analysis, because this method also detects many silent mutations (Sulyok et al., 2017). In such cases, DNA sequencing of the target genes may be easier method than traditional method, may it will be more time-consuming than SNP analysis using a hybridization probes. The extraction of DNA from milk samples by a modified method of a DNeasy Blood & Tissue Kit is useful in term of cost and sensitivity. The reagent

cost was US $5.83 per sample. The shedding of large quantities of mycoplasma in milk is a general disease symptom of bovine mycoplasmal mastitis. In previous experiments on intramammary infections by *M. californicum*, *M. californicum* was persisted in milk for approximately two weeks, and its concentration in milk remained very high (6,000–1.1 × 10^9 cfu/mL) (Reilly et al., 1993). If a fresh milk sample were used, the limit of detectability for this method would be sufficiently lower than this colony count (133-1330 cfu/mL), so this method is more effective for milk containing low SCC, such as in the case of latent intramammary infections. Milk samples containing high SCC leads to high viscosity at the step of lysate preparation, and lysate solution with high viscosity makes subsequent filtration steps difficult. High viscosity in the lysate solution may also adversely affect subsequent elution step. On the other hand, storage of the milk sample led to a decrease in the sensitivity of this method, and the survivability of the mycoplasma was also greatly reduced by storage (Boonyayatra et al., 2010). Therefore, the use of fresh milk samples is optimal, but in cases where this is not possible, the addition of glycerol or re-culturing of storage samples may be useful (Boonyayatra et al., 2010).

In the present study, we established a rapid and easy method for determining the usefulness of lincosamides or macrolides against *M. californicum*. However, several problems remain to be solved before this method can be applied in a clinical setting. It will be necessary to examine whether the conventional dosage and application can be improved, and the severity of mastitis that can be successfully treated with antibiotic therapy. In particular, the treatment of infected but asymptomatic cows, which are common in outbreaks of bovine mastitis by *M. californicum*, is the most important issue in order avoid the necessity of culling and the consequent economic losses (Hata et al., 2014). Finally, the kind or location of mutations may be different with isolates from each geographical area, so the investigation of them is indispensable to apply this method without anxiety.

5. Conclusion

Lincosamides and/or macrolides are frequently used as first-line antimicrobial agents for bovine mycoplasmal mastitis, but *M. californicum* isolates with low susceptibility to these agents were detected in mastitic milk in this study. Five point mutations that determine the changes in susceptibility to lincosamides and/or macrolides were identified, and a melting curve analysis method using a hybridization probe was established for the quick and easy detection of these mutations. This technique will help in the selection of useful antimicrobials for the treatment of bovine mastitis caused by *M. californicum*.

Funding information

This study was supported by research project 2017–2021 FY of the Ministry of Agriculture, Forestry and Fisheries of Japan: Improving Animal Disease Prevention Technologies to Combat Antimicrobial Resistance.

Conflict of interest

The authors declare that there are no functional or other relationships that might lead to a conflict of interest. All authors have seen and approved the manuscript.

Acknowledgements

The authors would like to thank the staff of the Livestock Hygiene Service Center, the Animal Research Center Hokkaido Research Organization, Rakuno Gakuen University, and the Federation of Hokkaido Agricultural Mutual Aid Associations for their help with the collection of *M. californicum* isolates and bovine mycoplasmal mastitic milk samples.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2018.12.017.

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