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REVIEW

Genomic and Molecular Epidemiological Analyses and Antimicrobial Susceptibility of Bovine Mycoplasmas in Japan

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Abstract

Mycoplasma bovis and *M. californicum* cause a variety of bovine diseases. Spread of these mycoplasmas is difficult to control because it occurs predominantly through asymptomatically infected individuals according to the results of molecular epidemiological analyses. After the 2000s, *M. bovis* strains having low susceptibility to 16-membered macrolides and tetracyclines have spread in Japan. With the spread of these low-susceptibility *M. bovis* strains seeming to be involved in the refractoriness to antimicrobial therapy often found in cases of *M. bovis* infection, it is speculated that these *M. bovis* strains entered Japan through the importation of bovine biological resources, such as breeding dairy cows, semen, and fertilized eggs. Additionally, appearance of *M. bovis* strains having low susceptibility to fluoroquinolone is suggested to have relevance to the increase of the amount of fluoroquinolone purchased, being as it is a frequent characteristic of beef cattle-derived strains. The identification of mutations involved in antimicrobial susceptibility and the development of methods to directly detect such mutations have significantly reduced the time required to understand a causal strain's antimicrobial susceptibility. Selection of antimicrobial agents by this method is expected not only to improve the cure rate but also to greatly contribute to the reduction of antibiotic usage.

Discipline: Animal Science
Additional key words: mycoplasma, genome, epidemiology, MLVA, PFGE, antimicrobial susceptibility, hybridization probe

Introduction

Bovine mycoplasmas cause a variety of bovine diseases, such as arthritis, mastitis, otitis, pneumonia, and urogenital diseases, and often cause disease of a chronic and persistent nature. Some mycoplasma species appear to have strong pathogenicity and infectivity, thereby causing huge economic losses to farms, but some bovine mycoplasma species are regarded as commensal bacteria. Mycoplasmas are characterized by their small genome sizes and are thought to have undergone reductive evolution, a process by which they lose many genes that other bacteria possess. The lack of genes involved in cell wall synthesis is characteristic of Mollicutes, so these microorganisms are not equipped with a rigid cell wall (Razin et al. 1998). Hence, all antimicrobial agents whose mechanisms of action are the inhibition of the cell wall synthesis pathway and the destruction of cell wall structures are not useful for mycoplasma infection (Gautier-Bouchardon 2018). In addition, the emergence of strains with low susceptibility to antimicrobials in recent years may be making treatment more difficult (Gautier-Bouchardon 2018). Presumably, mycoplasmas can evolve reduced genomes, as they have survived in such a way as to acquire essential products from their hosts *in vivo*. Therefore, many species show high host specificity. Currently, a total of 13 bovine mycoplasma species are known. *Mycoplasma bovis*, which causes a variety of bovine diseases, is the most pathogenic bovine mycoplasma species except for *Mycoplasma mycoides* subsp. *mycoides* (Mmm), a causative species of contagious bovine pleuropneumonia (CBPP). *M. bovigenitalium* and

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M. californicum being pathogenic species that often cause mastitis and urogenital disease. Slow growth is a bacteriological characteristic of mycoplasmas (Razin et al. 1998), and colony formation usually takes 2-7 days. In as many conventional examination methods for mycoplasmas being based on culturing, the following methods are time-consuming and thus cannot be applied in pressing clinical settings. In contrast, examination methods without culturing such as direct detection and typing of bacterial DNA from specimens can provide clear results quickly and easily. This review outlines current knowledge related to bovine mycoplasmas, especially the genomics, epidemiology, and antimicrobial resistance of *M. bovis* and *M. californicum*, and discusses the development of genetic analysis methods.

Genome research

The largest genome among bovine mycoplasma species is the 1.2-Mb genome of Mmm strains; approximately 1,000 genes are coded, but the comparative genomics data revealed that mycoplasmas lack significant numbers of genes. Many genes involved in the biosynthesis pathway for amino acids, fatty acids, and nucleotides are absent in the genomes of mycoplasmas. Another characteristic is the lack of many energy-yielding systems. The genes, leading to these species depending mostly on glycolysis to synthesize ATP, with most nonfermentative species and some fermentative species possessing the arginine dihydrolase pathway to synthesize ATP (Razin et al. 1998), involved in the tricarboxylic acid cycle and electron transport system are also incomplete in mycoplasmas.

In contrast to other pathogenic bacteria, no typical primary virulence genes have been found on the genomes of bovine mycoplasma species. Hydrogen peroxide and superoxide radicals as toxic by-products of mycoplasma metabolism are known to cause oxidative damage to host cell membranes (Pilo et al. 2007, Razin et al. 1998). The genes involved in the production of these by-products are fully equipped in the genomes of Mmm and *M. leachii* (Wise et al. 2012). They are also found in the genome of *M. bovirhinis*, recognized as a commensal mycoplasma (Hata et al. 2017b), but some of these genes are absent in the genomes of many pathogenic mycoplasma, such as *M. bovis* (Wise et al. 2011), *M. bovigenitalium* (Hata et al. 2017a), and *M. californicum* (Hata & Murakami 2014), so their importance as virulence factors in bovine disease seems questionable. Molecules located on the outer surface or in the plasma membrane of Mycoplasma species, such as lipoproteins, capsular polysaccharides, and biofilms composed mainly of fibrillar polycarbohydrates surrounding cells, are generally assumed to protect the pathogen from the bactericidal activity of complement and other host defense functions. Moreover, these microbe-derived constituents act as triggers of the inflammatory process in the infected host. Capsular or secreted polysaccharide that Mmm produces is the main virulence factor for CBPP (Pilo et al. 2007), and Bertin et al. (2015) reported a series of constitutive enzymes for its production pathway. The genes encoding these enzymes are fully equipped in the genomes of *M. bovis* (Wise et al. 2011), *M. bovigenitalium* (Hata et al. 2017a), *M. californicum* (Hata & Murakami 2014), *M. bovoculi* (Calcutt & Foecking 2014), and *M. leachii* (Wise et al. 2012), and these species are involved in various bovine diseases.

Although transposons and prophages are often present as mobile genetic elements (MGEs) in bovine mycoplasma genomes, these MGEs seldom contain genes that change bacterial characteristics. However, a highly transmissible gene cluster that confers resistance against aminoglycosides such as kanamycin, neomycin, and nourseothricin was found in a 54-kb prophage-like region in the genome of *M. bovirhinis* isolated in Japan, and this gene cluster had been found earlier in the genomes of other bacteria (Hata et al. 2017, Lysnyansky & Borovok 2021). An antimicrobial susceptibility survey of *M. bovirhinis* isolates from Japanese homebred cattle revealed emerging low-susceptibility isolates against kanamycin (Uemura et al. 2010), so the spread of field strains carrying this gene cluster is expected. Most of the decreased susceptibility to antimicrobial agents in mycoplasma are caused by point mutations in the target genes (Gautier-Bouchardon 2018), and the acquisition of antimicrobial resistance with the insertion of MGE is a very rare in mycoplasma.

Epidemiological research

Identification of infection sources and important control points by epidemiological analysis is essential for controlling infectious diseases, and the genotyping of isolates is a great help in conducting epidemiological analysis.

Pulsed-field gel electrophoresis (PFGE) was first reported as a method for the structural analysis of chromosomal DNA (Schwartz & Cantor 1984) but was later applied to molecular epidemiological analysis methods and is now widely used as the gold standard method for molecular epidemiological analysis of various bacterial species (Tenover et al. 1995). The restriction enzymes used for the fragmentation of genomic DNA differ depending on the bacterial species. *Sma*I and *Mlu*I are usually used for PFGE of *M. bovis* (Arcangioli et al. 2012), and *Bam*HI is used for PFGE of *M. californicum* (Hata et al. 2014). PFGE genotyping results suggest that mycoplasma organisms inhabiting a mucosal surface of a body site or milk cause respiratory disease or mastitis, and these strains spread between herds along with the movements of the carriers. These causal strains persist in asymptomatic carriers within herds after the clinical signs have disappeared, and these asymptomatic carriers, which are hard to detect, become the next infectious sources. Because these causal strains remain in herds for such long periods, carriers with clinical signs often recur at intervals of several months (Arcangioli et al. 2012, Hata et al. 2014, Punyapornwithaya et al. 2010).

The advantages of multilocus sequence typing (MLST) are that its results are unambiguous, portable, reproducible, and scalable, with MLST being a genotyping method based on the typing of multiple loci, using DNA sequences of internal fragments of multiple housekeeping genes to characterize isolates. MLST data can be used to investigate evolutionary relationships among isolates, providing superior discrimination power to differentiate isolates. Combined with other analysis results, various characteristics of isolates of each genotype can be clarified. Register et al. (2015) developed an MLST method for *M. bovis* and established the PubMLST website to accumulate genotyping results, which they have revised thereafter (Register et al. 2020). The minimum spanning tree of sequence types (STs) based on the revised MLST method for *M. bovis* is shown in Figure 1. *M. bovis* isolates are broadly classified into three ST groups: ST12 group, ST21 group, and ST180 group. Isolates belonging to ST21 group are further classified into seven ST subgroup: ST19 subgroup, ST29 subgroup, ST52 subgroup, ST60 subgroup, ST100 subgroup, ST111 subgroup, and ST122 subgroup. Isolates collected up to 2000 are mostly classified as ST12 group, as is PG45^{T}, a type strain of *M. bovis* isolated in 1961. ST12 group was once the world's major ST group, but is now rarely detected. Next, most of the isolates collected after 2000 are classified as ST21, and this ST group is still predominant worldwide (Fig. 1B). After 2000, the ST subgroup recognized as the first predominant genotype in Japan was ST111 subgroup, and the rates of other ST subgroups increased after 2006 (Fig. 2). As for differences among geographical areas, ST21 and STs closely related to it have been the major STs in Japan, North America, and some European countries since 2006. After the spread of ST21, it is speculated that the ST19 and ST100 subgroups derived from ST21 in Japan (Fig. 1A, Fig. 2). ST52 subgroup is the predominant subgroup in Australia, China, and Israel, and all registered Australian isolates are ST52. On the other hand, the ST180 group and the ST29 subgroup are mainly confirmed in Mediterranean countries. In addition, the ST60 subgroup has been found primarily in North American countries (Fig. 1A). The international distribution of biological resources is believed to be deeply involved in the international spread of pathogens. Indeed, there is a report of bovine semen contamination by *M. bovis* (Haapala et al. 2018). Japan, for example has imported breeding cattle, bovine semen, and fertilized bovine eggs mainly from North American countries (Fig. 3). ST12 and ST21 were all detected in North American countries before they became widespread in Japan. The importation of breeding cattle from North American countries to Japan was stopped in 2004 after an outbreak of Bovine spongiform encephalopathy; breeding cattle have been imported to Japan only from Australia thereafter (Animal Quarantine Service 1980-2019) (Fig. 3). This is presumed to be one of the reasons for the recent increase in the detection of the ST52 subgroup, the only known genotype in Australia, in Japan (Fig. 2). These findings remind us of the importance of quarantining biological resources to prevent the international spread of pathogenic microorganisms.

As for risk factors associated with *M. bovis* infection at the farm management level, cooperative-type farms, the purchase of cattle from other farms, and larger herd size were identified as risk factors on farms in Japan. These factors seem to be reflected in the higher frequencies of moving carriers and fomites to and from other farms, which increase the risk of pathogen introduction (Murai & Higuchi 2019).

Antimicrobial susceptibility

Mycoplasmas are naturally resistant to antimicrobial agents that act on the cell wall. Moreover, polymyxin, sulfonamides/trimethoprim, rifampicin, and first-generation quinolones are useless against mycoplasma infections (Taylor-Robinson & Bébéar 1997). Due to the lack of a cell wall synthesis pathway is the natural resistance against antimicrobial agents that act on the cell wall, such as β-lactams, glycopeptides, and fosfomycin, as mentioned above (Gautier-Bouchardon 2018). Similarly, because mycoplasma lacks lipopolysaccharides and folate synthesis pathways, polymyxin and sulfonamides/trimethoprim, which inhibit these pathways, also do not help against mycoplasma infection (McCormack 1993, Olaitan et al. 2014). The mechanism of resistance to rifampicin is the inhibition of binding by natural mutation in the *rpoB* gene, which codes the RNA polymerase β subunit, the target of rifampicin (Gaurivaud et al. 1996, Bébéar & Bébéar 2002). Moreover, many

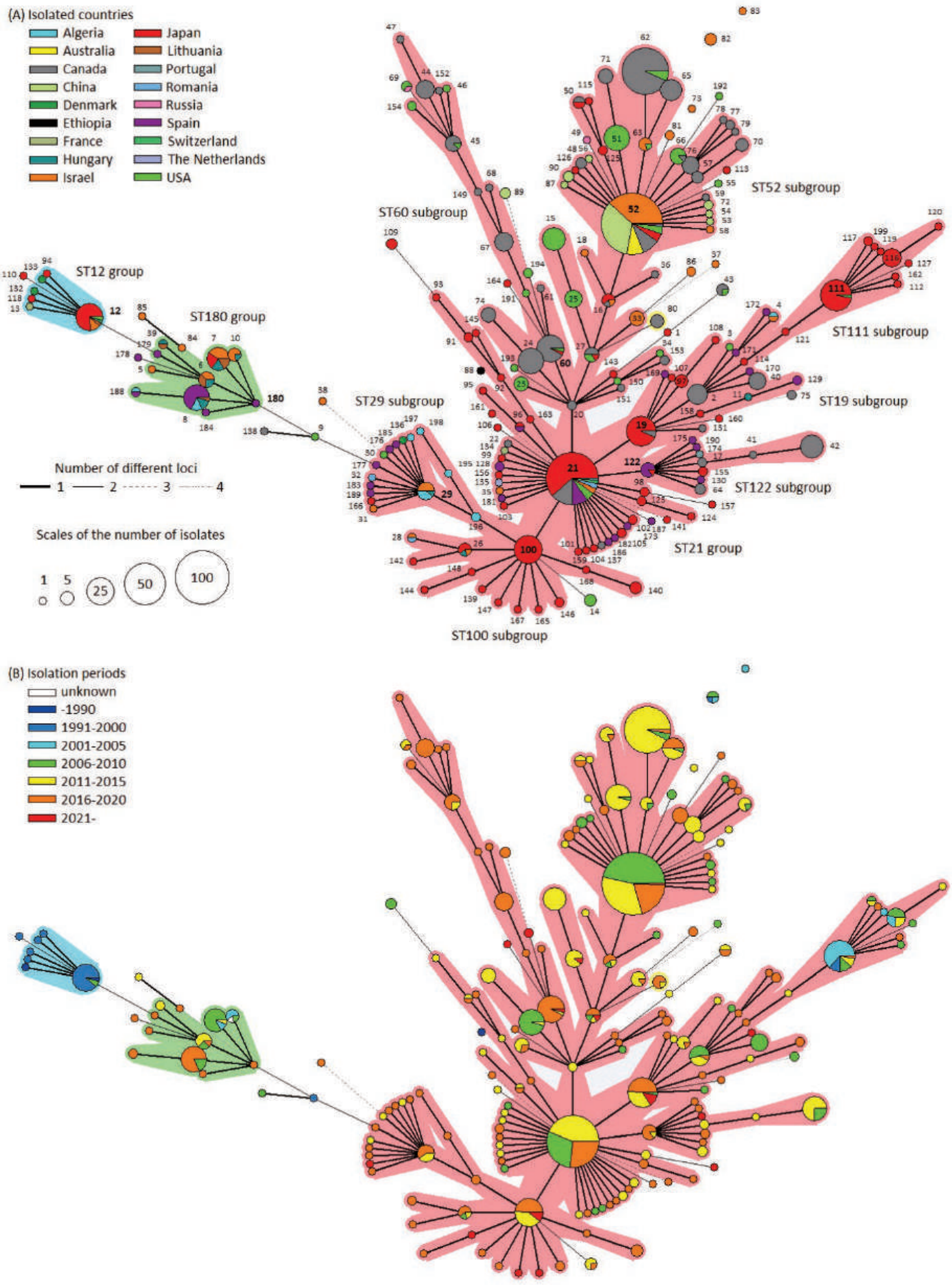

Fig. 1. Minimum spanning tree representing the evolutionary relationship between *M. bovis* sequence types (STs) by multilocus sequence typing

Numbers indicate STs, and the central ST and subcentral STs of each ST group are indicated in bold. The size of each circle represents the population size, and the circular chart in each circle indicates the proportion of (A) countries of origin and (B) isolated periods in each ST. These figures are based on the data as of February 10, 2022.

species of bovine mycoplasmas, such as *M. bovis*, *M. bovigenitalium*, *M. californicum*, and *M. synoviae*, are naturally resistant to erythromycin, a 14-membered macrolide. In these mycoplasma species, the nucleotide at position 2057 of the 23S ribosomal RNA (rRNA), which is located in the peptidyl transferase center circle of the 23S rRNA (i.e., the target of macrolides), are adenines (A2057). Hence, A2057 cannot form a base pair with

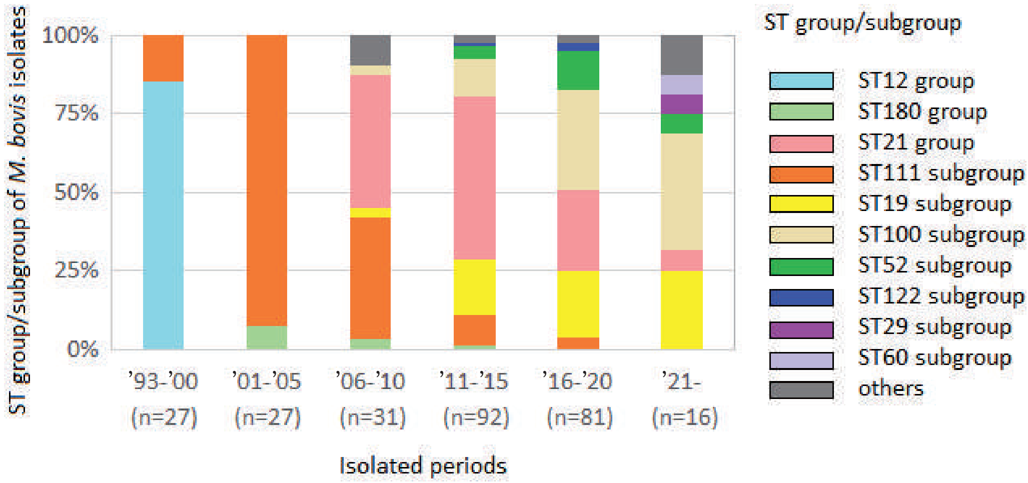

Fig. 2. Chronological change of MLST-based genotypes of *M. bovis* isolates in Japan

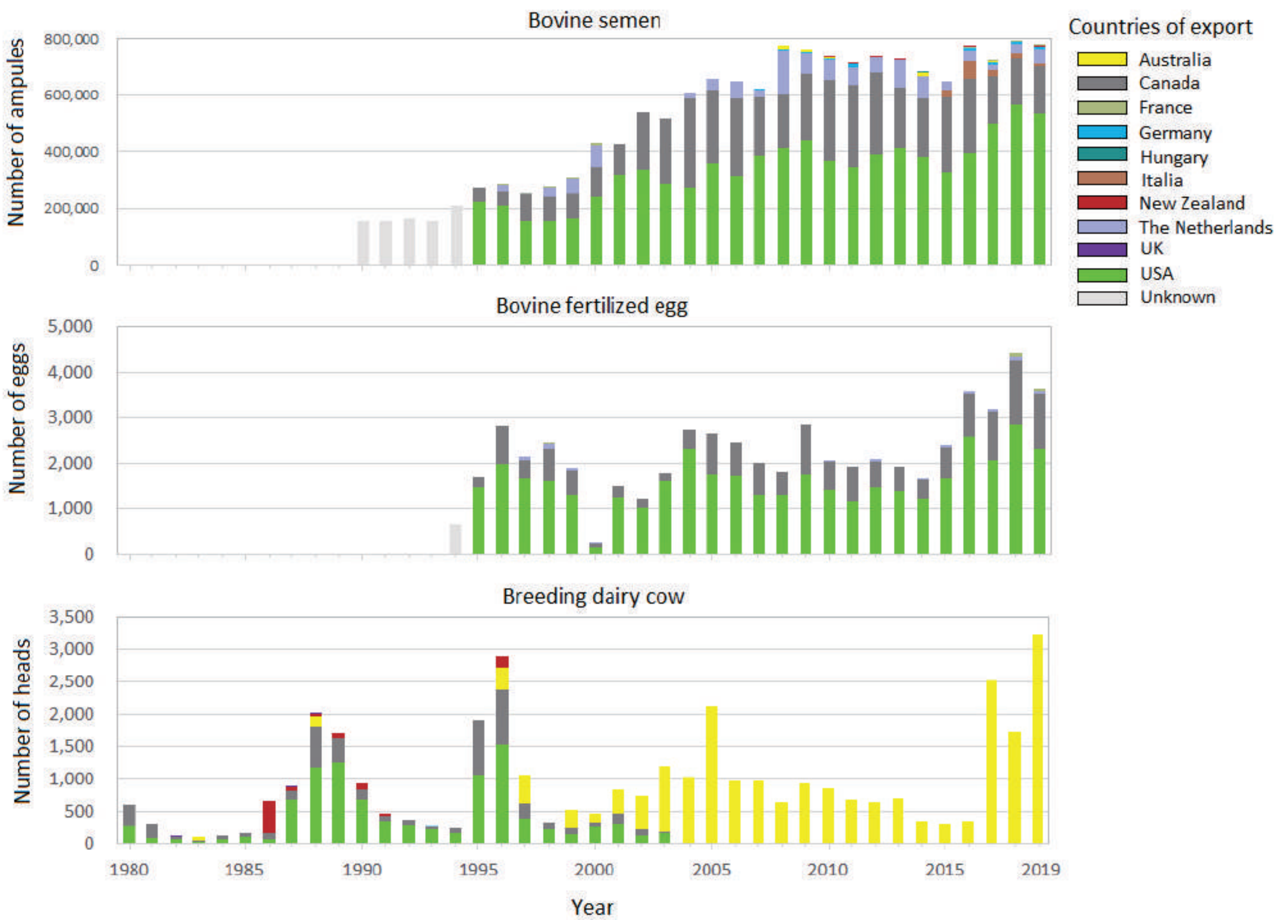

Fig. 3. Import status of bovine biological resources in Japan

C2611 in the 23S rRNA (Fig. 4). On the other hand, in species susceptible to erythromycin, such as *M. gallisepticum*, *M. genitalium*, *M. pneumoniae*, and Ureaplasma, the nucleotide at position 2057 of the 23S rRNA are guanines, so they can form base pairs with C2611 in the 23S rRNA (Gautier-Bouchardon 2018, McCormack 1993). The conformational change of the peptidyl transferase center circle associated with this natural mutation is speculated to affect binding between erythromycin and target regions (Hata et al. 2019a,b).

Tetracyclines, macrolides, lincosamides, fluoroquinolones, and some aminoglycosides are recognized as the primary antimicrobial agents for the treatment of mycoplasmal infections in animals (Aarestrup & Kempf 2006, Bébéar & Kempf 2005). However, recent *M. bovis* isolates tend to be less susceptible against these antibiotics (Gautier-Bouchardon 2018, Hata et al. 2019b, Uemura et al. 2010). In addition, susceptibility to aminoglycosides varies significantly among mycoplasma species. Interestingly, susceptibility to 16-membered macrolides and tetracyclines is closely associated with genotypes of *M. bovis* isolates, and isolates belonging to the ST21 group, which have been predominant worldwide since 2001, are less susceptible

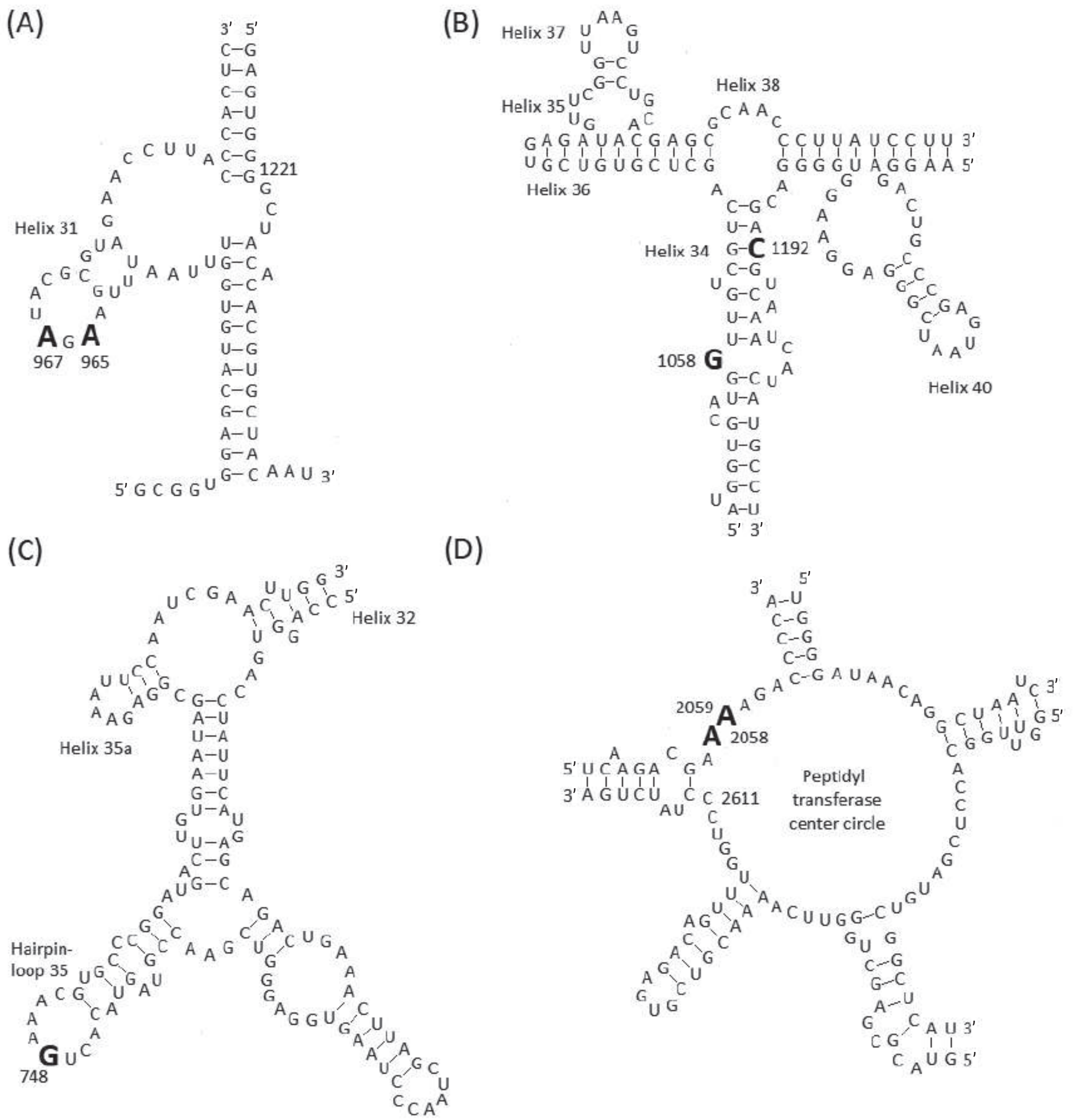

Fig. 4. Mutations in rRNA involved in decreased susceptibility against antimicrobial agents that were confirmed in *M. bovis* field isolates in Japan
(A and B) The two-dimensional structures of helix 31 and helix 34 of 16S rRNA
(C and D) Hairpin-loop 35 in domain II and the peptidyl transferase center circle in domain V of 23S rRNA of *M. bovis* PG45^T

to these agents. In isolates belonging to the ST21 group, 748 of 23S rRNA mutated to adenines (G748A), and this point mutation was found to be responsible for the decreased susceptibility against 16-membered macrolides. This mutation is not confirmed in isolates belonging to ST12 group. Similarly, the mutation at the 967th adenine (A967) of the 16S rRNA that was confirmed in most isolates other than some in ST12 group was involved in decreased susceptibility against tetracyclines (Fig. 4, Fig. 5). Although 16-membered macrolides and tetracyclines were once the most widely used antimicrobial agents against bovine mycoplasma infection, the worldwide spread of ST21 groups has seemed to make it hard to cure *M. bovis* infection by using these antimicrobials. There have also been sporadic emergences of low-susceptibility *M. bovis* isolates against 15-membered macrolides and lincosamides, or spectinomycin. Point mutations at the 2058th and 2059th adenines of 23S rRNA, or at the 1192nd cytocine of 16S rRNA, were found to be responsible for decreased susceptibility against macrolides and lincosamides, or spectinomycin, respectively (Fig. 4). Of note, the mutations at the 2058th and 2059th adenines of 23S rRNA led to less susceptibility to 16-membered macrolides than did the mutation at 748th guanine of 23S rRNA (Hata et al. 2019a,b). In addition, low-susceptibility *M. bovis* strains against fluoroquinolones have also been confirmed in Japan. The target genes involved in decreased susceptibility to quinolones are the *gyrA* and *gyrB* genes, which encode the subunits of DNA gyrase, and the *parC* and *parE* genes, which encode the subunits of DNA topoisomerase IV, but the missense mutations in *gyrB* and *parE* appear to have little to do with the decreased susceptibility to fluoroquinolone in *M. bovis* (Lysnyansky et al. 2009). In addition, where on the one hand the coexistence of missense mutations in both *gyrA* and *parC* is associated with a significant decrease in susceptibility (Hata et al. 2019b), a missense mutation in either *gyrA* or *parC* did not result in a clear decrease in susceptibility. In particular, the coexistence of amino-acid substitutions at the 83rd serine in GyrA and at the 80th serine in ParC, a combination of hot spots, results in significantly lower susceptibility than do other combinations of amino-acid substitutions (Hata et al. 2019b). In Japan, *M. bovis* isolates in which missense mutations coexist in both *gyrA* and *parC*, including the above combination of mutations, are isolated more frequently from beef cattle than dairy cows (Fig. 6). The appearance of a low-susceptibility strain is speculated to

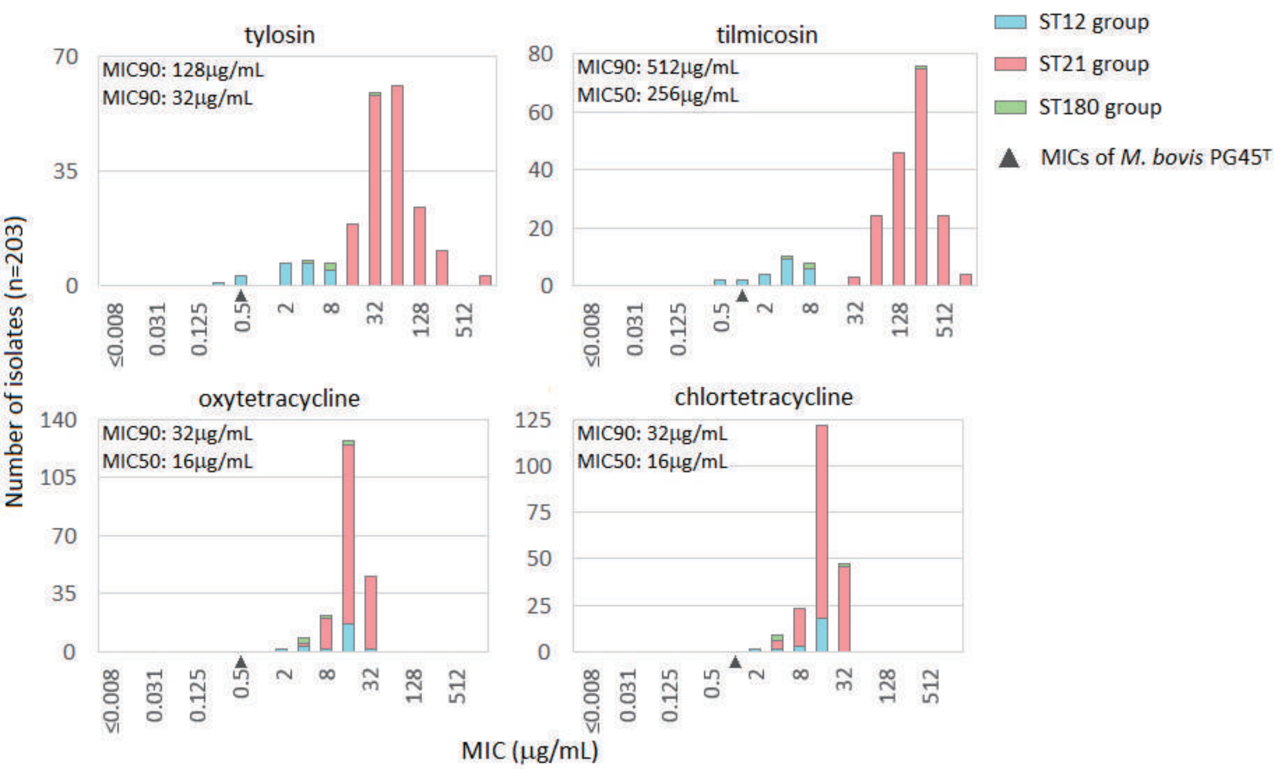

Fig. 5. Relationship between antimicrobial susceptibility distribution and MLST-based genotype of *M. bovis* isolates in Japan

Tylosin & tilmicosin: 16-membered macrolides; oxytetracycline & chlortetracycline: tetracyclines be affected by the amounts of fluoroquinolones consumed, and in fact, the amount of fluoroquinolones purchased for beef cattle treatment has increased since 2012 (Fig. 7) (National Veterinary Assay Laboratory, 2005-2019). Amphenicols and pleuromutilins also effectively treat mycoplasma infections (Gautier-Bouchardon 2018). However, in contrast to the minimum inhibitory concentration (MIC)s of *M. bovis* and *M. californicum* against florfenicol, which is an amphenicol, being high, the MICs of Japanese isolates against valnemulin, which is a pleuromutilin, are clearly lower than in other

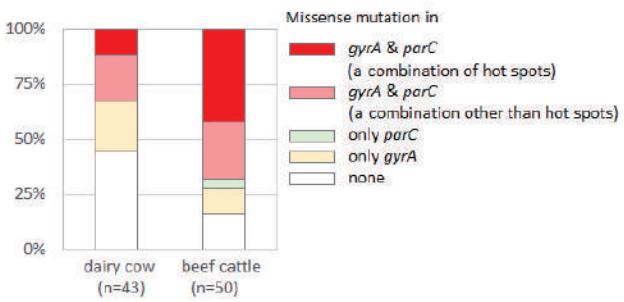

Fig. 6. Detection status of missense mutations involved in decreased susceptibility to fluoroquinolones in *M. bovis* strains isolated in Japan after 2012

antibacterial agents, such as macrolides, lincosamides, tetracyclines, fluoroquinolones, amphenicols, aminoglycosides, and tiamulin (Hata et al. 2019a,b; Sulyok et al. 2017). Indeed, the high therapeutic effect of valnemulin was demonstrated in clinical trials of *M. bovis* calves with respiratory infections (Stipkovits et al. 2005). The absence of a tendency toward decreased susceptibility to amphenicols and pleuromutilins in field isolates also supports the usefulness of these agents (Gautier-Bouchardon 2018, Hata et al. 2019a,b). Valnemulin seems to be the most effective antimicrobial agent against bovine mycoplasmal infection, but unfortunately it is not approved for use in beef cattle and dairy cows in Japan. Unlike *M. bovis*, *M. californicum* and *M. bovigenitalium* appear to generally maintain their susceptibility to the antibiotics mentioned so far (Kawai et al. 2014), so it is unlikely that their infections become refractory. However, mutations that decrease susceptibility to various antimicrobial agents may occur even in these mycoplasma species as anticipated by genetic analyses of laboratory-derived low-susceptibility strains (Hata et al. 2019a, Sulyok et al. 2017). As the results of such analyses of laboratory-derived low-susceptibility strains generally correspond to those of analyses of field isolates, this speculation would be very helpful for grasping decreased susceptibility mechanisms.

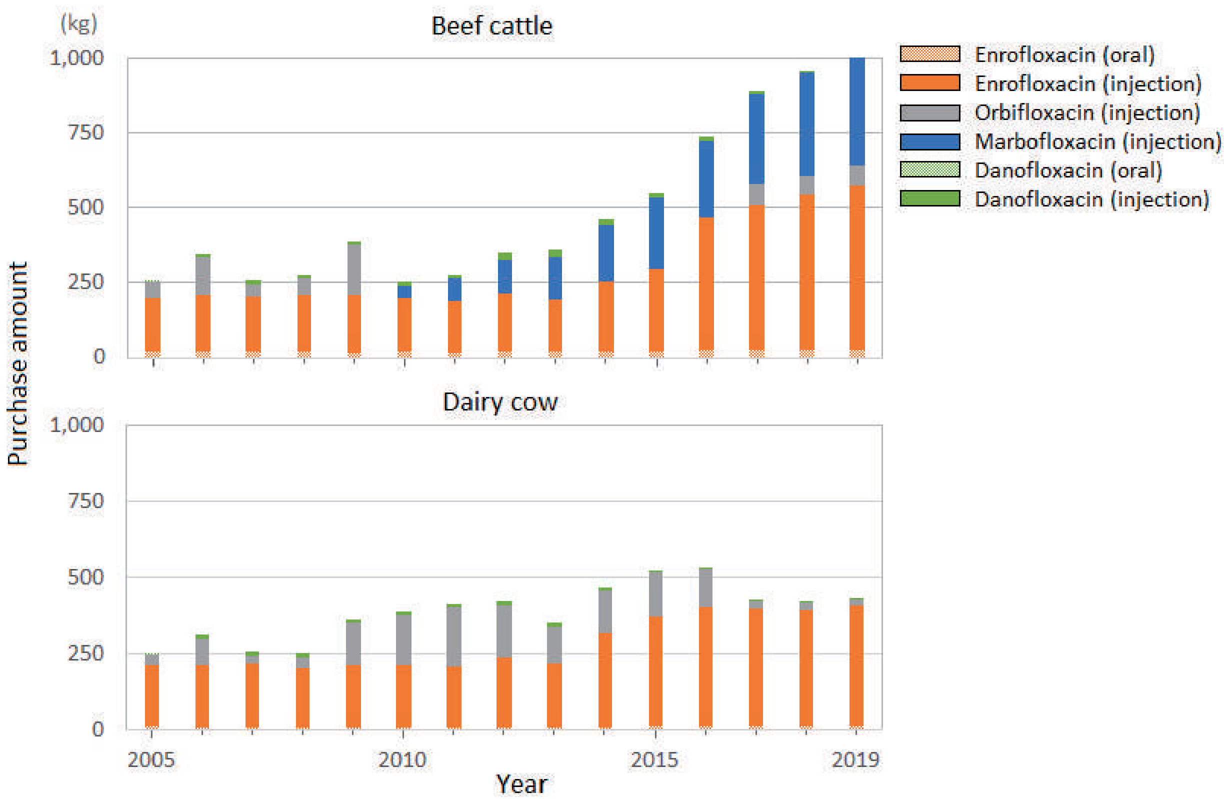

Fig. 7. Chronological changes in total amounts of fluoroquinolones purchased for the treatment of bovine diseases

One of the factors that makes treatment of bovine mycoplasma infections difficult is the limited variety of antibiotics that can be prescribed. Although the current situation is extremely difficult for veterinarians to select appropriate and effective treatment methods for mycoplasma infections, more attention should be paid to antibiotic selection and dosage during treatment to prevent the emergence of low-susceptibility strains.

Establishment of a genetic analysis method

Genotyping is a useful means of epidemiological analysis. High discriminatory power and reproducibility are the most essential factors in epidemiological analysis, and many genotyping methods provide these advantages. The Hunter–Gaston diversity index (HGDI) is used to evaluate the discriminatory power of each typing method. As the power increases, the HGDI score nears 1.0, and a score above 0.90 is desirable if the typing results are to be interpreted with confidence (Hunter & Gaston 1988). Two other essential factors in genotyping are its cost and its ability to easily identify individual genotypes. The author developed a PFGE method and a multiple-locus variable-number tandem-repeat analysis (MLVA) method for *M. californicum*. PFGE is a method in which genomic DNA is cleaved with a specific restriction enzyme and typed based on the electrophoresis pattern of the fragment (Schwartz & Cantor 1984). Therefore, it is necessary to find a restriction enzyme that can provide an electrophoretic image with a clear and polymorphic pattern. *Bam*HI was selected as a restriction enzyme that meets these parameters (Hata et al. 2014). MLVA is a genotyping method that analyzes the polymorphism based on the number of tandemly repeated DNA sequences. The tandem repeats that can be used for MLVA were identified from genomic information of the *M. californicum* HAZ160_1 strain (Hata & Murakami 2014). Ultimately, 4 tandem repeats consisting of 225-bp or 102-bp repeat units were selected for easy identification of typing results (Hata et al. 2014). The reproducibility of PFGE and MLVA was confirmed both *in vivo* and *in vitro* (Hata et al. 2014), and the established genotyping methods were applied to epidemiological analysis of the mycoplasma. The findings of the analyses are described in the "Epidemiological research" section in this review.

Many conventional tests for mycoplasma are based on cultures and thus are time-consuming and labor intensive. As it was almost impossible to select the appropriate antimicrobial agent on treatment according to the test results, the same applies to the traditional antimicrobial susceptibility testing, which usually takes 2-3 weeks to obtain results (Hannan 2000). To solve the shortcomings of the conventional method, the author established a probe-based genetic method to rapidly detect mutations involved in decreased susceptibility. Hybridization probes used in the developed methods are typically used in single nucleotide polymorphism (SNP) analysis, and they can accurately differentiate mutations of target DNA regions by measuring the melting temperature of a probe–amplicon hybrid (Fig. 8), even

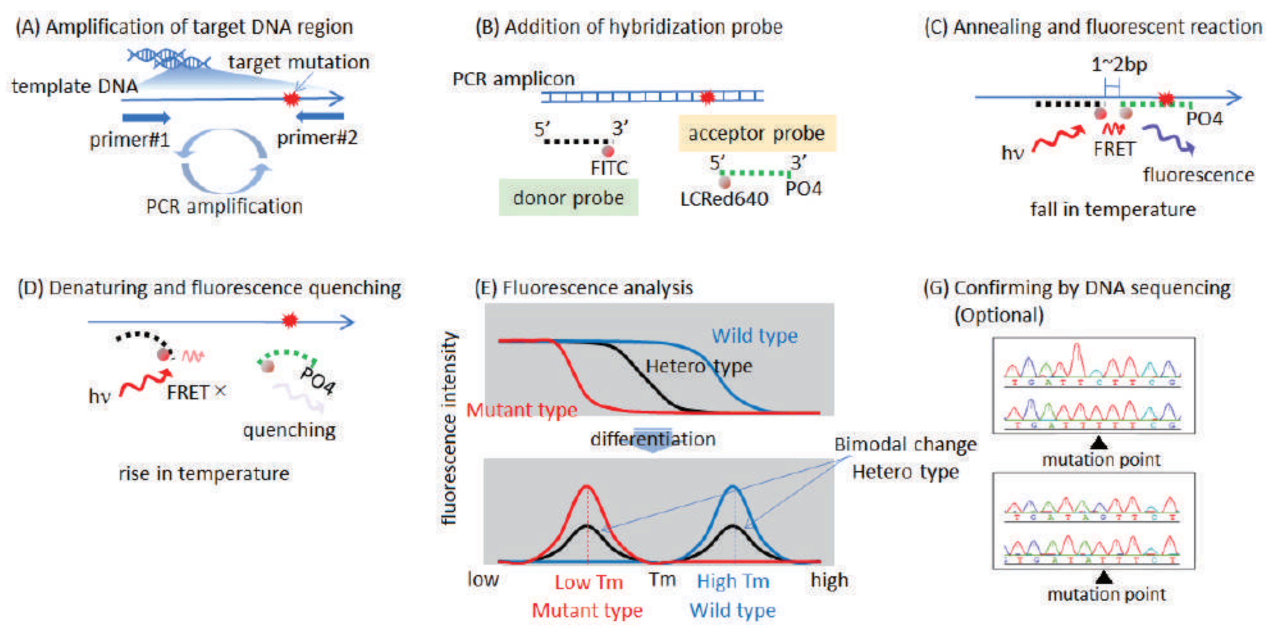

Fig. 8. Detection principle and procedure of target mutation by melt-curve genotyping using hybridization probes
The procedure follows in alphabetical order.

if there are few nucleotide differences or only one such difference (Lyon 2001). Hybridization probes consist of a probe bound to a donor fluorophore (e.g., FITC) and a probe bound to an acceptor fluorophore (e.g., LC Red640) (Fig. 8B). When both probes bind to an amplicon and are close to each other, fluorescence resonance energy transfer (FRET) occurs from the donor fluorophore to the acceptor fluorophore and fluoresces (Fig. 8C). If both probes peel from the amplicon by thermal change, the fluorescence disappears by the annihilation of FRET (Fig. 8D). Table 1 lists the mutations involved in changes in antimicrobial susceptibility detectable by melt-curve genotyping using hybridization probes. The result of the detection of mutations involved in decreased susceptibility to spectinomycin (C1192A in 16S rRNA genes) is shown in Figure 9. The existence of either mutation in multiple-target SNPs (Hetero type) appears as a bimodal change in the melting curve (Fig. 8E). If all target SNPs are mutated (Mutant type), the melting-peak temperature shifted lower than non-mutation (Wild type) (Lyon 2001) (Fig. 8E). This detection method is completed in about 4 hours, so it is possible to find an effective antimicrobial agent in a considerably shorter time than with the conventional method (Hata et al. 2019a,b).

Table 1. Mutations involved in changes in antimicrobial susceptibility detectable by melt-curve genotyping using hybridization probes

Mycoplasmal species	target genes[a]	mutation points	susceptibility	antimicrobial agents
M. bovis	*rrs*	A965, A967	decreased	tetracyclines
M. bovis	*rrs*	C1192	decreased	spectinomycin
M. bovis, M. californicum	*rrl*	G748	decreased	16-membered macrolides
M. bovis, M. californicum	*rrl*	A2058, A2059	decreased	macrolides, lincosamides
M. californicum	*rrl*	G2576	decreased	lincosamides
M. californicum	*rrl*	C2611T	increased	erythromycin
			decreased	lincosamides

[a]*rrs*: 16S rRNA *gene*, *rrl*: 23S rRNA gene

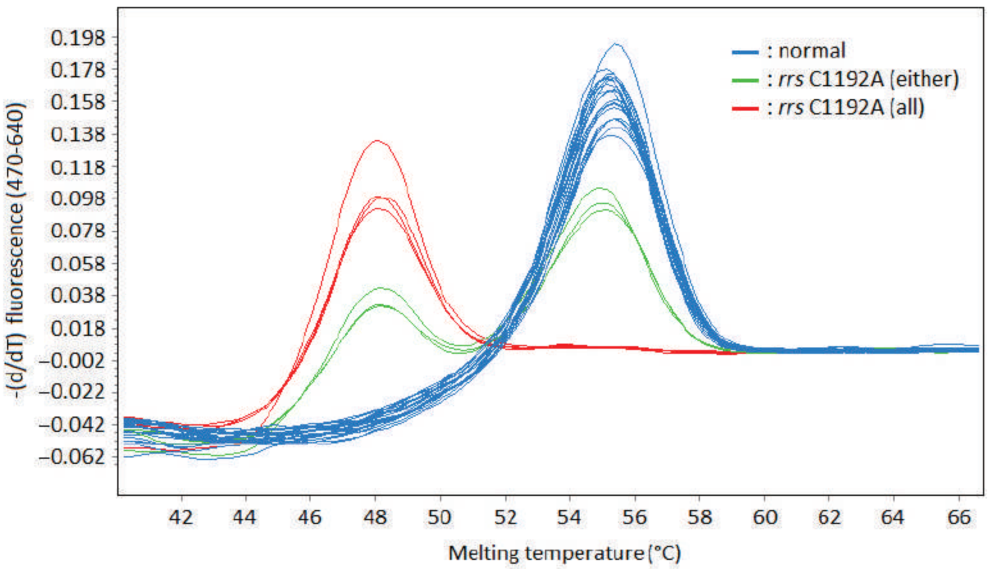

Fig. 9. Results of melt-curve genotyping using a hybridization probe to detect mutation associated with decreased susceptibility to spectinomycin in *M. bovis*
The operons that encode the 16S rRNA gene and the 23S rRNA gene usually exist at two places in *M. bovis* genome. The bimodal curve (green melting curves) indicates the presence of a mutation in either of the copies, and the lower melting temperature (red melting curves) indicates mutation of both copies. Strains carrying the mutation show the similar degree of decreased antimicrobial susceptibility regardless of the number of mutations.

Future developments

Until now, no effective vaccine has been available for bovine mycoplasma infection, so early detection and culling of carrier cows has been the main way to prevent spread. Confirming the effectiveness of antimicrobials selected by the proposed method is the most important as the next step as follows, with the development of a rapid detection method for mutations involved in decreased antimicrobial susceptibility may enhance the effectiveness of antimicrobial treatment. In addition, to improve cure rates, it will be indispensable to find an index that can efficiently identify individuals to be treated. The future goal is to establish a more advanced treatment method that packages these technologies and knowledge.

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We declare that there are no functional or other relationships that might lead to a conflict of interest.

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