1 Relationship between Antimicrobial Susceptibility and Multilocus Sequence Type of
2 *Mycoplasma bovis* Isolates and Development of a Method for Rapid Detection of Point
3 Mutations Involved in Decreased Susceptibility to Macrolides, Lincosamides,
4 Tetracyclines, and Spectinomycin.

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15 Running Head: Antimicrobial-Low-Susceptible *M. bovis* in Japan

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21 ## ABSTRACT

22 *Mycoplasma bovis* isolates belonging to the sequence type 5 (ST5) group, the dominant
23 group in Japan since 1999, were low susceptible to 16-membered macrolides and
24 tetracyclines and were confirmed to have a guanine-to-adenine transition mutation at
25 position 748 in the 23S rRNA gene (*rrl*) and adenine-to-thymine transversion mutations
26 at positions 965 and 967 in the 16S rRNA gene (*rrs*) (Escherichia coli numbering).
27 Moreover, isolates of ST93 and ST155, members of the ST5 group, were low susceptible
28 to lincosamides and azithromycin and showed an adenine-to-guanine transition mutation
29 at position 2059 of *rrl*. Isolates of ST93 were additionally low susceptible to
30 spectinomycin and showed a cytosine-to-adenine transversion mutation at position 1192
31 of *rrs*. Strains of the ST5 group seem to spread to Japan and Europe from North America
32 with imported cows, while strains of ST93 and ST155 originated in Japan. Melting curve
33 analysis using hybridization probes revealed the existence of point mutations involved in
34 decreased susceptibility to macrolides, lincosamides, and spectinomycin, as demonstrated
35 by changes in the melting curve shape and/or decreases in the melting peak temperature,
36 so the susceptibility to these antimicrobials can be assessed on the same day. For
37 decreased susceptibility to fluoroquinolones to exist, nonsynonymous mutations in the
38 DNA gyrase gene (*gyrA*) and topoisomerase IV gene (*parC*) had to coexist. The
39 combination of amino acid substitutions of serine at position 83 in *gyrA* and serine at
40 position 80 in *parC* resulted in particularly low susceptibility to fluoroquinolones.

IMPORTANCE

Mycoplasma bovis is the main causal species of bovine mycoplasmal disease and leads to significant economic losses because of its severe symptoms, strong infectivity, and refractoriness. As for mastitis, culling cows with intramammary infections is a general countermeasure to prevent spreading. The conventional antimicrobial susceptibility test for mycoplasma is time-consuming and troublesome, but no quick and easy method for grasping the antimicrobial susceptibility of the causal strain exists at present. Treatment without antimicrobial susceptibility information may be one reason why *M. bovis* infection is refractory. Detecting a mutation involved in decreased susceptibility to antimicrobial agents of the causal strain makes it possible to easily select suitable antimicrobials for treatment, and this technique will help improve the cure rate and prevent the overuse of ineffective antimicrobial agents. In this study, we developed a technique to quickly and easily assess antimicrobial susceptibility based on the genetic characteristics of *M. bovis* strains in Japan.

KEYWORDS: *Mycoplasma bovis*, antimicrobial resistance, multilocus sequence typing, hybridization probe

59 **INTRODUCTION**

60 Bovine disease caused by *Mycoplasma bovis*, e.g., calf pneumonia, arthritis, genital
61 disease, mastitis, and otitis, is a problem all over the world and occasionally results in
62 massive outbreaks (1, 2). In Japan, mastitis due to *M. bovis* is recognized as the main
63 mycoplasmal disease on dairy farms and in some cases has led to significant economic
64 losses because of severe symptoms, strong infectivity, and refractoriness (2). No effective
65 vaccine is available for the control of *M. bovis* infection, so antimicrobial treatment is the
66 major therapeutic tool, and bacterial examination of bulk milk is the typical preventive
67 method (2).

68 Although the antimicrobials that are potentially effective against bovine mycoplasmal
69 infection are mostly protein synthesis inhibitors (e.g., aminoglycosides, macrolides,
70 lincosamides, tetracyclines, phenicols, and pleuromutilins) and nucleic acid synthesis
71 inhibitors (e.g., fluoroquinolones), mycoplasmal strains with low susceptibility to some
72 of these antimicrobials have recently been emerging, and the spread of these strains may
73 make antimicrobial therapies more difficult (3–5). Proper prescription of antimicrobial
74 agents will lead not only to improvement of the cure rate but also to prevention of the
75 overuse of ineffective antimicrobials; however, the traditional antimicrobial susceptibility
76 test for mycoplasmas is time-consuming and labor-intensive, so it is not helpful at
77 emergency clinics (6, 7). Hence, it is desirable to develop a method for quick and easy
78 assessment of the antimicrobial susceptibility of the causal strain. In *M. bovis* or
79 *Mycoplasma californicum*, specific mutations located in the 16S rRNA gene (*rrs*), 23S
80 rRNA gene (*rrl*), DNA gyrase gene (*gyrA*), and topoisomerase IV gene (*parC*) were found
81 in strains with low susceptibility to various antimicrobial agents (7–9). Reconfirming the
82 universality of the roles of these mutations and exploiting them may lead to the
83 development of a method that can compensate for the drawbacks of conventional methods

84 (7). Melting curve analysis using hybridization probes is a useful method for detecting
85 target single nucleotide polymorphisms (SNPs); high sensitivity and rapidity are virtues
86 of this method (7, 10).
87 Multilocus sequence typing (MLST) is a genotyping method that has excellent universal
88 comparability and is useful for characterizing the genetic background and for
89 phylogenetic and epidemiological study (11). In addition, we can assess the
90 bacteriological characteristics or epidemic of each sequence type (ST) by annotating the
91 various characteristics of each strain (possession of pathogenic genes, origin, isolating
92 date, isolated host, geographical area, etc.). By combining genotyping by MLST with
93 information regarding antimicrobial susceptibility, we may assess the reasons for the
94 emergence, invasion, or spread of various strains with low susceptibility.
95 In this study, we investigated the change in genotypes by MLST of *M. bovis* strains
96 isolated in Japan over a period of 26 years and clarified the effect of their changes on the
97 susceptibility to various antimicrobial agents. We investigated a variety of mutation points
98 that are suggested to be involved in decreased susceptibility to antimicrobial agents in
99 Japan and reconfirmed their roles in antimicrobial susceptibility. Subsequently, we
100 attempted to establish a probe-based genetic method for the rapid detection of these
101 mutations (9).
102

103 ## RESULTS

104 **Evolutionary relationships and population structure of *M. bovis* field isolates in**
105 **Japan.** The 203 isolates analyzed gave a total of 52 different STs that were divided into
106 three ST groups (i.e., the ST5 group, ST17 group, and ST68 group) using Based Upon
107 Related Sequence Types (BURST) and minimum spanning tree (MST) analyses (Fig. 1A).
108 There were five subcentral STs (i.e., ST10, ST19, ST29, ST73, and ST76) in the ST5
109 group, which was the largest ST group (Fig.1A). Of 52 STs,
110 42werespecifictoJapaneseisolates (Fig. 1A). As for the foreign distribution of each ST
111 group or ST subgroup that was confirmed in Japan, the ST5 group, including the ST19,
112 ST29, ST73, and ST76 subgroups, was mainly confirmed in strains from the United States
113 and Europe. However, most of the strains from Australia, China, and Israel belonged to
114 the ST10 subgroup. Strains belonging to the ST68 group were isolated from the United
115 States, Israel, and Europe. Most strains belonging to the ST17 group were isolated from
116 the United States and Europe before 1998 (Fig. 1A). As for the chronological change of
117 each ST group or ST subgroup in Japan, most of the isolates showed STs belonging to
118 the ST17 group until 1998, but the dominant ST group shifted completely to the ST5
119 group after 1999 (Fig. 1B; see also Table S1 in the supplemental material). Regarding the
120 appearance of subgroups in the ST5 group, the ST29 subgroup was dominant until 2005,
121 but the appearance of isolates other than the ST29 subgroup has been increasing since
122 then (Fig. 1B and Table S1). Isolates belonging to the ST10 subgroup first emerged in
123 2014, and their appearance has been increasing (Fig. 1B and Table S1). Isolates belonging
124 to the ST68 group were sporadically confirmed from 2002 to 2011 (Fig. 1B). Strains of
125 ST5, ST14, ST17, and ST29 were already isolated in the United States before their first
126 confirmation in Japan, and strains of ST10 were also isolated in the United States, China,
127 Israel, and Australia before their first confirmation in Japan.

128 **Relationship between genotypes by MLST and susceptibility to antimicrobial**
129 **agents.** The MIC distributions of the 16 tested antimicrobial agents against *M. bovis* field
130 isolates, the MIC_{50} and MIC_{90} values, and the MICs for strain PG45^T (MIC_{PG45^T}) (a
131 quality control reference isolate) are shown in Table 1 and Fig. 2. The antimicrobial to
132 which the isolates exhibited the highest susceptibility was valnemulin, with MIC_{50} and
133 MIC_{90} values of 0.008 μg/ml and 0.016 μg/ml, respectively (12). The antimicrobial to
134 which the isolates exhibited the highest susceptibility after valnemulin was tiamulin, with
135 MIC_{50} and MIC_{90} values of 0.125 μg/ml and 0.5 μg/ml, respectively. The MICs of
136 florfenicol, tiamulin, and valnemulin exhibited normal distributions, and the MIC for
137 strain PG45^T also fit into each MIC range, so all of the *M. bovis* isolates were considered
138 to maintain their susceptibility to these antimicrobial agents. On the other hand, *M. bovis*
139 is considered to have natural resistance to erythromycin (a 14-membered macrolide) and
140 flumequine, and the MICs for strain PG45^T (MICs, 256 and 32 μg/ml) and the MIC_{50}
141 (MICs, 256 and 64 μg/ml) and MIC_{90} (MICs, 512 and 128 μg/ml) of the field isolates
142 were extremely high. M. bovis isolates that show low susceptibility to tetracyclines seem
143 to have spread since the 1990s in Japan. The MICs of oxytetracycline and
144 chlortetracycline for strain PG45^T were 0.5 and 1 μg/ml, respectively; however, their MIC
145 ranges for field isolates were higher values (MICs, 2 to 32 μg/ml), and the MIC_{50} and
146 MIC_{90} values were 16 and 32 μg/ml, respectively. Moreover, the MICs of 16-membered
147 macrolides (e.g., tylosin and tilmicosin) and fluoroquinolones other than flumequine
148 exhibited clear bimodal distributions. Isolates highly susceptible to tylosin and tilmicosin
149 with MIC values of ≤ 8 μg/ml are members of ST14 and the ST17 group, and isolates
150 with low susceptibility such that their MICs for tylosin and tilmicosin are ≥16 μg/ml are
151 members of ST68 and the ST5 group (Tables S1 and S2). As for fluoroquinolones other
152 than flumequine, there was no clear correlation between MIC and genotypes by MLST,

153 and both the high MIC groups and low MIC groups consisted of various STs (Tables S1
154 and S2). Most of the isolates exhibited high susceptibility to azithromycin (a 15-
155 membered macrolide), lincosamides, and spectinomycin; however, isolates with low
156 susceptibility were sporadically confirmed. All three isolates of ST93 were less
157 susceptible to azithromycin (MIC, 1,024 μg/ml), lincosamides (MICs, 128 to 512 μg/ml),
158 spectinomycin (MICs, 512 to 1,024 μg/ml), and fluoroquinolones (MICs, 8 to 32 μg/ml)
159 than most of the remaining isolates except for isolates of ST155 (Tables S1 and S2). Both
160 isolates of ST155 were less susceptible to azithromycin (MICs, 128 to 512 μg/ml) and
161 lincosamides (MICs, 128 to 256 μg/ml) than all of the remaining isolates except for
162 isolates of ST93 (Tables S1 and S2). Other than these isolates, two isolates of ST103 and
163 ST29 were less susceptible to spectinomycin (MICs, 256 to 512 μg/ml) than all of the
164 remaining isolates except for isolates of ST93 (Tables S1 and S2).

165 **Relationship between mutations in target genes and susceptibility to**
166 **antimicrobial agents.** The SNPs in target genes (i.e., *rrs*, *rrl*, *gyrA*, and *parC*) that were
167 confirmed in *M. bovis* field isolates in Japan are shown in Fig. 3. The adenines at positions
168 965 and 967 (A965 and A967, respectively) in helix 31 of both *rrs* genes were thymines
169 (A965T and A967T) in all isolates belonging to the ST5 group and isolates of ST68 and
170 ST103 (Table S1). Moreover, both *rrs* A967T were confirmed in all three isolates of ST14,
171 and both *rrs* genes had cytosine (A967C) in 18 isolates belonging to the ST17 group
172 (Table S1). The guanine at position 1058 (G1058) in helix 34 of either or both *rrs* genes
173 was adenine (G1058A) in two isolates of ST5 and one isolate of ST67 (Table S1). It has
174 been suggested that SNPs at these positions are involved in decreased susceptibility to
175 tetracyclines (oxytetracycline MICs, from 2 to 4 μg/ml [normal isolates] to 4 to 32 μg/ml
176 [isolates with A965T and/or A967Y]; chlortetracycline MICs, from 2 to 8 μg/ml [normal
177 isolates] to 4 to 32 μg/ml [isolates with A965T and/or A967Y]) (8). Overall, isolates in

178 which the presence of either combination of SNPs was confirmed showed a significantly
179 low susceptibility to oxytetracycline and chlortetracycline; however, some isolates
180 possessing SNPs showed the same MIC values as normal isolates (Fig. 4). Moreover,
181 there were no clear results as to whether the presence of *rrs* G1058A would affect the
182 decreased susceptibility to tetracyclines (Fig. 4).

183 The cytosine at position 1192 (C1192) in helix 34 of either or both *rrs* genes was
184 adenine (C1192A) in one isolate of ST29, one isolate of ST103, and all three isolates of
185 ST93 (Table S1). These five isolates which possessed *rrs* C1192A showed significantly
186 low susceptibility to spectinomycin ($P < 0.0001$) (spectinomycin MICs, from 0.5 to 8
187 μg/ml [normal isolates] to 256 to 1,024 μg/ml [isolates with C1192A]) (Fig. 4) (9).

188 The guanine at position 748 (G748) in hairpin-loop 35 of either or both *rrl* genes was
189 adenine (G748A) in all isolates belonging to the ST5 group and one isolate each of ST17
190 and ST68 (Table S1). The adenine at position 2059 (A2059) in the peptidyl transferase
191 center circle of either or both *rrl* genes was guanine (A2059G) in all five isolates of ST93
192 and ST155 (Table S1). It is suggested that the SNP at *rrl* G748 is involved in decreased
193 susceptibility to 16-membered macrolides, and the SNP at *rrl* A2059 is involved in
194 decreased susceptibility to macrolides and lincosamides (7, 9). Significantly low
195 susceptibility to tylosin and tilmicosin was observed in the 173 isolates in which *rrl*
196 G748A was confirmed ($P < 0.0001$) (tylosin MICs, from 0.25 to 8 μg/ml [normal isolates]
197 to 8 to 256 μg/ml [isolates with G748A]; tilmicosin MICs, from 0.5 to 8 μg/ml [normal
198 isolates] to 8 to 512 μg/ml [isolates with G748A]), and an additional decrease in
199 susceptibility was observed in five isolates in which the coexistence of rrl A2059G was
200 confirmed ($P < 0.0001$ or < 0.05) (tylosin and tilmicosin MICs, 256 to 1,024 μg/ml) (Fig.
201 4). On the other hand, isolates in which rrl G748A was confirmed did not show a
202 significant change in susceptibility to azithromycin or lincosamides (azithromycin MICs,

203 from 0.125 to 2 μg/ml [normal isolates] to 0.25 to 16 μg/ml [isolates with G748A];
204 lincomycin MICs, from 0.125 to 4 μg/ml [normal isolates] to 0.25 to 8 μg/ml [isolates
205 with G748A]; pirlimycin MICs, from 0.125 to 4 μg/ml [normal isolates] to 0.125 to 4
206 μg/ml [isolates with G748A]), but five isolates in which rrl A2059G coexists showed
207 significantly low susceptibility to azithromycin and lincosamides ($P < 0.0001$)
208 (azithromycin MICs, 128 to 1,024 μg/ml; lincomycin MICs, 256 to 512 μg/ml; pirlimycin
209 MICs, 128 to 256 μg/ml) (Fig. 4).
210 These results suggested that a nonsynonymous mutation in the quinolone resistance-
211 determining region (QRDR) of *gyrA* or *parC* is often confirmed in isolates with low
212 susceptibility to fluoroquinolones (9). In *gyrA* of 109 isolates, nonsynonymous mutations
213 (A227T, G241A, C248W, T250C, G259M, A260T, andA281T) resulting in amino acid
214 substitutions (Lys76Met, Gly81Ser, Ser83Phe, Ser83Tyr, Ser84Pro, Glu87Lys, Glu87Gln,
215 Glu87Val, and Gln94Leu) were observed (Table S1). Of these nonsynonymous mutations,
216 SNP C248W, which causes an amino acid substitution of serine at position 83 (Ser83),
217 was observed in 90 isolates and was the most frequent SNP in *gyrA* (Table S1). Moreover,
218 SNP T240C, a silent mutation located between these nonsynonymous mutations, was
219 observed in two isolates (Table S1). In *parC* of 47 isolates, nonsynonymous mutations
220 (G239T, T240A, T241C, G250H, A251K, and G253C) resulting in amino acid
221 substitutions (Ser80Ile, Ser80Arg, Ser81Pro, Asp84Tyr, Asp84His, Asp84Asn, Asp84Val,
222 Asp84Gly, and Ala85Pro) were observed (Table S1). Of these nonsynonymous mutations,
223 SNPs G239T and T240A, which cause an amino acid substitution of serine at position 80
224 (Ser80), were observed in 24 isolates and 4 isolates, respectively. G239T was the most
225 frequent nonsynonymous mutation in *parC* (Table S1). Moreover, SNP C252T, a silent
226 mutation located between these nonsynonymous mutations, was observed in 159 isolates
227 (Table S1). In 66 isolates in which a nonsynonymous mutation was confirmed in only the

228 QRDR of *gyrA*, significantly low susceptibility to fluoroquinolones was observed (P <
229 0.0001), but isolates showing MICs overlapping the MICs of normal strains were also
230 observed (enrofloxacin MICs, 0.063 to 0.5 μg/ml [normal isolates] to 0.125 to 1 μg/ml
231 [isolates with a nonsynonymous mutation in the QRDR of *gyrA*]; danofloxacin MICs,
232 0.125 to 0.5 μg/ml [normal isolates] to 0.125 to 2 μg/ml [isolates with a nonsynonymous
233 mutation in the QRDR of *gyrA*]; marbofloxacin MICs, 0.063 to 0.5 μg/ml [normal
234 isolates] to 0.125 to 2 μg/ml [isolates with a nonsynonymous mutation in the QRDR of
235 *gyrA*]) (Fig. 4). Moreover, four isolates in which nonsynonymous mutations were
236 confirmed in only the QRDR of *parC* did not show significantly low susceptibility to
237 enrofloxacin or danofloxacin (enrofloxacin MICs, 0.125 to 0.5 μg/ml; danofloxacin MICs,
238 0.125 to 0.5 μg/ml; marbofloxacin MICs, 0.25 to 1 μg/ml) (Fig. 4). On the other hand, 43
239 isolates in which nonsynonymous mutations were confirmed in the QRDRs of both *gyrA*
240 and *parC* showed obvious and significantly low susceptibility to fluoroquinolones (P <
241 0.0001) (enrofloxacin MICs, 1 to 16 μg/ml; danofloxacin MICs, 2 to 16 μg/ml;
242 marbofloxacin MICs, 2 to 64 μg/ml) (Fig. 4). In particular, the coexistence of amino acid
243 substitutions at Ser83 in *gyrA* and Ser80 in *parC* resulted in significantly lower
244 susceptibility to fluoroquinolones than did other combinations of amino acid substitutions
245 (P < 0.0001) (enrofloxacin MICs, 8 to 16 μg/ml; danofloxacin MICs, 8 to 16 μg/ml;
246 marbofloxacin MICs, 8 to 64 μg/ml) (Fig. 4).
247 **Rapid detection of mutations associated with decreased susceptibility to
248 antimicrobials by melting curve analysis using hybridization probes.** The
249 hybridization probe is a useful tool for detecting SNPs, but it simultaneously detects
250 meaningless SNPs, such as silent mutations. For five SNPs without silent mutations
251 around the target SNPs, i.e., *rrs* A965T and/or A967Y, *rrs* C1192A, *rrl* G748A, and *rrl*
252 A2059G, a detection method using hybridization probes was examined. On the other hand,

253 detection by DNA sequencing was investigated as a way to detect target SNPs in the
254 QRDRs of *gyrA* and *parC* with silent mutations around the target SNPs. The primers
255 designed in this study sufficiently amplified three DNA regions of *M. bovis* field isolates
256 and strain PG45^T containing five target SNPs involved in low susceptibility to
257 tetracyclines, spectinomycin, macrolides, and lincosamides (Table 2). The primers for the
258 amplification of the QRDRs of *gyrA* and *parC* also sufficiently amplified these DNA
259 regions of *M. bovis* field isolates and strain PG45^T. On the other hand, these DNA regions
260 of other non-*M. bovis* organisms (eight mycoplasma-type strains, two acholeplasma-type
261 strains, and nine bacterial strains that cause bovine mastitis) were not amplified by PCR
262 using these primers. Moreover, the mixing of these other microbials did not affect the
263 melting curve analysis using hybridization probes or DNA sequencing of the QRDR. In
264 this study, the detection limits of the PCR and DNA extraction method were suggested to
265 be 126 CFU/ml for the milk samples with somatic cell counts (SCCs) of 20×10^3 and $716\times$
266 10^3 cell/ml, respectively, but decreased to 1,260 CFU/ml for the milk samples with SCCs
267 of $1{,}600 \times 10^3$ and $3{,}000 \times 10^3$ cell/ml, respectively. Storage at -20°C or 4°C for 7 days
268 resulted in a further reduction in the detection limit of each sample to 1/10.
269 The numbers of *rrs*-*rrl* operons vary among microbial species, but there are usually
270 two operons in the genome of *M. bovis* as with many bovine mycoplasma (GenBank
271 accession no. CP002188) (13). In the melting curve analysis using hybridization probes,
272 a single melting peak at a lower-than-normal temperature is observed for the case in which
273 the mutations occur together at target SNPs in both *rrs*-*rrl* genes. In this case, the
274 temperature of the melting peaks detecting each SNP decreased from 53.53 ± 0.12°C to
275 50.86 ± 0.09°C, 48.45 ± 0.1°C, and 43.05 ± 0.32°C (SNP at *rrs* A965 and/or A967), from
276 55.46 ± 0.40°C to 48.39 ± 0.08°C (SNP at *rrs* C1192), from 56.04 ± 0.13°C to 50.99 ±
277 0.17°C (SNP at *rrl* G748), and from 55.79 ± 0.62°C to 47.58 ± 0.06°C (SNP at *rrl* A2059)

278 (Fig. 5). However, the melting curve changed to a bimodal curve if the mutation occurred
279 at the target SNP in either *rrs-rrl* gene (Fig. 5). These changes in the melting curve were
280 automatically detected by the analytical software of the real-time PCR machine (Fig. 5).

281 ## DISCUSSION

282 Mutations associated with decreased susceptibility to many antimicrobial families
283 were previously investigated in field isolates or laboratory-derived mutants of various
284 mycoplasmal species (5, 7, 9, 14–16). Mutations *rrs* A965T and A967T, *rrs* C1192A, *rrl*
285 G748A, and *rrl* A2059G were confirmed in Japanese *M. bovis* isolates that showed low
286 susceptibility to tetracyclines, spectinomycin, macrolides, and lincosamides, and these
287 mutations were also confirmed in Hungarian isolates which showed low susceptibility to
288 these antimicrobial agents (5, 9). Moreover, mutations *rrs* A965T and A967T, *rrl* G748A,
289 and *rrl* A2059G were confirmed in the genome data from Chinese isolates that belong to
290 the ST10 subgroup (i.e., Hubei-1, 08M, CQ-W70, HB0801, NM2012, and Ningxia-1). Of
291 these mutations, *rrl* G748A was almost entirely absent in isolates belonging to the ST17
292 group that show high susceptibility to 16-membered macrolides; however, members of
293 the ST17 group have only rarely been isolated. On the other hand, the mutation *rrl* G748A
294 was confirmed in all isolates belonging to the ST5 group, the members of which show
295 low susceptibility to 16-membered macrolides. Mutations involved in decreased
296 susceptibility to tetracyclines, *rrs* A965T and A967T (8), are also genetic characteristics
297 of the ST5 group. According to the MLST database, the ST5 group is currently confirmed
298 everywhere in the world. The ST5 group became the dominant group worldwide in the
299 2000s. Tetracyclines and macrolides had been used as first-line antimicrobial agents for
300 the treatment of bovine mycoplasmal disease, so the low susceptibility to these
301 antimicrobial agents caused by mutations *rrs* A965T and A967T and *rrl* G748A may be
302 one reason that the ST5 group became the dominant group in the world. It is speculated
303 that strains belonging to the ST5 group with these mutations originate from North
304 America. Strains of ST29 and ST5, the main STs of the ST5 group in Japan, were first
305 confirmed in the United States in 1994 and 2000, respectively, prior to being confirmed

306 in Japan in 1996 and 2006, respectively; the United States and Canada were the main
307 countries from which live cows were imported at that time (Fig. S1) (17). Rosales et al.
308 suggested that the extensive livestock trade led to international expansion of specific *M.*
309 *bovis* strains (11). Hence, the genetic background research and antimicrobial
310 susceptibility data for *M. bovis* suggested that tetracyclines and 16-membered macrolides
311 may already be inappropriate to use as first-line antimicrobial agents for treating *M. bovis*
312 infection (5, 9, 18). On the other hand, *Mycoplasma bovigenitalium* or *Mycoplasma*
313 *californicum* showed adequate susceptibility to tetracyclines and 16-membered
314 macrolides (4, 7). The mutation *rrl* A2059G caused decreased susceptibility to macrolides
315 and lincosamides and additionally caused significantly lower susceptibility to 16-
316 membered macrolides than did *rrl* G748A (5, 9, 19). It has been observed in many
317 mycoplasma species that SNPs located at *rrl* A2058 and *rrl* A2062 also cause similar
318 susceptibility changes (5, 7, 9, 14–16). In Mycoplasma pneumoniae, mutations at these
319 positions have harmful effects on growth (20). This effect is presumed to be one reason
320 why *M. bovis* of ST93 and ST155 with the mutation *rrl* A2059G did not spread widely.
321 The mutation *rrs* C1192A caused decreased susceptibility to spectinomycin and has only
322 been confirmed in five isolates of ST29, ST93, and ST103 in Japan. *M. bovis* cases
323 showing low susceptibility to spectinomycin are rare in Japan, but they are widespread in
324 Europe, North America, and Israel (2, 3, 9, 18). The use of spectinomycin against *M. bovis*
325 was widespread in these countries, and this seems to have led to the current situation,
326 whereas spectinomycin is generally used to treat poultry diseases and not bovine diseases
327 in Japan. *M. bovis* cases showing low susceptibility to fluoroquinolones were sporadically
328 observed in Japan. Most of the STs in these cases were previously confirmed to be
329 susceptible prior to the isolation of an isolate with low susceptibility, or they were specific
330 to Japan. Hence, these strains with low susceptibility to fluoroquinolones were speculated

331 to emerge with the use of fluoroquinolone agents for the treatment of cattle in Japan. In
332 *M. bovis*, essential mutations leading to decreased susceptibility to fluoroquinolones were
333 located in the QRDR of *gyrA* or *parC*, and mutations in *gyrB* or *ParE* appear to be
334 irrelevant to changes in susceptibility (21). Furthermore, the coexistence of
335 nonsynonymous mutations in both *gyrA* and *parC* led to more greatly decreased
336 susceptibility than mutations in either *gyrA* or *parC* alone. In particular, amino acid
337 substitutions at Ser83 in *gyrA* and Ser80 in *parC* form a combination that most effectively
338 leads to decreased susceptibility, and mutations at these positions seem to be observed
339 frequently in field isolates (9). The coexistence of amino acid substitutions at Ser83 in
340 *gyrA* and Ser80 in *parC* was also confirmed in the genome data from a Chinese strain,
341 Ninxia-1. These SNPs could be easily reproduced through fewer than 10 passages in a
342 medium supplied with the respective antimicrobial agent (9).
343 Florfenicol and pleuromutilins are speculated to be useful antimicrobial agents for the
344 treatment of *M. bovis* infection, not only in Japan but also in many other countries (9, 18).
345 Although it is suggested that SNPs associated with decreased susceptibility to florfenicol
346 and pleuromutilins were located at several positions in *rrl* genes, i.e., *rrl* C2035, *rrl*
347 G2448, *rrl* C2500, and *rrl* G2506 (9), these SNPs have not been observed in Japanese
348 field isolates. In Japan, florfenicol is applied to bovine pneumonia and porcine diseases,
349 but pleuromutilins are commonly used only for porcine diseases. The formation and
350 cleavage of base pairs between positions *rrl* A2057 and *rrl* C2611 are crucial factors in
351 the change in susceptibilities to erythromycin and lincosamides (7). Many bovine
352 mycoplasmas which do not form base pairs between these positions show natural
353 resistance to erythromycin and susceptibility to lincosamides. Conversely, mycoplasma
354 species forming a base pair between these positions are sensitive to erythromycin and
355 show natural resistance to lincosamides, i.e., *M. pneumoniae*, *Mycoplasma gallisepticum*,

356 *Ureaplasma parvum*, and *Ureaplasma urealyticum* (22–24). Flumequine also appears to
357 be ineffective for the treatment of *M. bovis* infection, but its resistance mechanism is
358 unknown. Unlike *M. bovis* and *M. californicum*, *Mycoplasma bovirhinis*, an indigenous
359 mycoplasma in the bovine nasal cavity, has adequate susceptibility to flumequine, and so
360 it may be useful for the isolation of *M. bovis* or *M. californicum* from bovine nasal
361 samples containing huge quantities of *M. bovirhinis* (7).

362 Hybridization probes are typically used in SNP analysis, and a real-time PCR assay
363 can accurately differentiate mutations of target DNA regions by measuring the melting
364 temperature of the probe-amplicon hybrid, even if there are few nucleotide differences or
365 only one such difference (10). The method presented in this study successfully detected
366 target mutations, and all mutations were shown as shifts in the melting-peak temperature
367 or changes in the shape of the melting curve. If possible, DNA sequencing of the mutated
368 domain is recommended for more accurate judgment (10). The total time required for this
369 method was only 3h, and the cost of the reagent was $2.40 per sample. On the other hand,
370 mutations involved in fluoroquinolone resistance were detected by direct DNA
371 sequencing of the QRDR of *gyrA* and *parC* (21). These SNPs are scattered in the QRDR
372 of *gyrA* and *parC*, and some of them are silent mutations (9). In such cases, even if DNA
373 sequencing of the target genes is more time-consuming than SNP analysis using
374 hybridization probes, it may be easier than the traditional method. In experimental
375 intramammary infections caused by *M. bovis*, *M. bovis* persisted in milk for
376 approximately 2 weeks, and its concentration in udder tissue remained very high (10^3 to
377 10^8 CFU/ml) (25). If a fresh milk sample was used, *M. bovis* was usually detectable by
378 this method (detection limit, 126 to 1,260 CFU/ml). Storage of the milk sample led to a
379 decrease in the sensitivity of this method, and the survivability of the mycoplasma was
380 also greatly reduced by storage (26). When it is impossible to test immediately, culture

381 samples that can be expected to contain a large number of *M. bovis* bacteria should be
382 prepared at the same time (26). In the present study, we established a rapid and easy
383 method for possibly predicting the reduced susceptibility of tetracyclines, spectinomycin,
384 macrolides, and lincosamides against *M. bovis*. Guidelines for the effective and safe use
385 of spectinomycin, lincosamides, and pleuromutilins, which are currently impossible to
386 use in bovine mastitis treatment in Japan, are still to be developed. The route and dosage
387 need to be examined, as does the severity of mastitis that can be successfully treated with
388 antimicrobial therapy. In particular, the treatment of infected but asymptomatic cows,
389 which are common in outbreaks of bovine mycoplasmal mastitis, is an important issue
390 that must be addressed in order to avoid culling and the consequent economic losses (27).
391 Macrolides and tetracyclines are recognized as first-line antimicrobial agents for the
392 treatment of bovine mycoplasmal disease in Japan, but *M. bovis* strains of the ST5 group,
393 the members of which show low susceptibility to these antimicrobial agents, have been
394 spreading in Japan since 1999. In addition, *M. bovis* strains which show low susceptibility
395 to lincosamides, spectinomycin, or fluoroquinolones sporadically emerged. Specific
396 SNPs were confirmed in isolates with low susceptibility to tetracyclines, macrolides,
397 lincosamides, and spectinomycin, and a melting curve analysis method using a
398 hybridization probe was established for the quick and easy detection of these SNPs. This
399 technique will help in the selection of useful antimicrobials for the treatment of bovine
400 mastitis caused by *M. bovis*.
401
402 ## MATERIALS AND METHODS
403 **Mycoplasmal isolates, antimicrobial agents, and susceptibility testing.** A total of 203
404 *M. bovis* isolates were collected from bovine samples obtained between 1993 and 2018
405 in Japan. The methods used to isolate and identify these isolates have been described

406 previously (27). The susceptibilities of the *M. bovis* isolates to 16 antimicrobials approved
407 for therapeutic applications in veterinary use in Japan were examined. These consisted of
408 an aminoglycoside (spectinomycin), macrolides (erythromycin, azithromycin, tylosin,
409 and tilmicosin), lincosamides (lincomycin and pirlimycin), tetracyclines (oxytetracycline
410 and chlortetracycline), a phenicol (florfenicol), fluoroquinolones (enrofloxacin,
411 danofloxacin, marbofloxacin, and flumequine), and pleuromutilins (tiamulin and
412 valnemulin). For quality control of the test and for later evaluation, *M. bovis* PG45^T was
413 also added to this study, and the MIC values of the antimicrobial agents against each
414 isolate were determined by the agar microdilution method according to the method
415 recommended by Hannan (6).

416 **MLST, phylogenetic analysis, and analysis of mutations involved in decreased
417 susceptibility to antimicrobial agents.** *M. bovis* genomic DNAs were prepared from
418 logarithmic-phase broth cultures by using an InstaGene matrix (Bio-Rad Laboratories,
419 Hercules, CA), according to the manufacturer's instructions. All PCR amplifications were
420 conducted using PrimeSTAR GXL DNA polymerase (TaKaRa Bio, Inc., Otsu, Japan),
421 and the products were purified using a LaboPass PCR purification kit (Cosmo Genetech,
422 Seoul, South Korea) and sequenced on a 3130 Genetic Analyzer (Applied Biosystems)
423 using a BigDye Terminator (version 3.1) ready reaction cycle sequencing kit (Applied
424 Biosystems). MLST was performed with the oligonucleotide primers described
425 previously (28), and these primers have also been posted on the MLST website
426 (https://pubmlst.org/mbovis/). The allele number and ST were determined using the
427 MLST website, and the central and subcentral STs of each ST group were determined by
428 using BURST in the MLST website
429 (https://pubmlst.org/bigsdb?db=pubmlst_mbovis_isolates&page=query). The possible
430 evolutionary relationship between the isolates and *M. bovis* population structure was

431 determined using the BioNumerics software version 7.5 (Applied Maths, Sint-Martens-
432 Latem, Belgium) and was evaluated by a minimum spanning tree (MST) analysis. The
433 MST was created based on a database of the isolates used in this study and isolates
434 registered on the MLST website.

435 Oligonucleotide primers for the PCR amplification and sequencing of target genes (i.e.,
436 the 16S and 23S rRNA genes [*rrs* and *rrl*]) were designed from the *M. bovis* PG45^T
437 genome (GenBank accession no. CP002188) (13). Oligonucleotide primers for the QRDR
438 of *gyrA* and *parC* were used as reported in a previous study (21). The sequences of the
439 oligonucleotide primers are shown in Table S3. Sequence editing, consensus, and
440 alignment were performed using GENETYX version 13 (Tokyo, Japan). The numbering
441 of the nucleotide positions and the amino acid positions throughout the article is based on
442 the *rrs*, *rrl*, *gyrA*, and *parC* genes of *Escherichia coli* (21, 29, 30), unless otherwise
443 indicated.

444 **Rapid detection of mutations associated with changed susceptibility to**
445 **antimicrobials by melting curve analysis using hybridization probes.** Extraction of
446 DNA from milk samples was described previously (7). For the detection of target
447 mutations, a 50μl amplicon was prepared using PrimeSTAR GXL DNA polymerase with
448 1.5 μl of DNA template from the milk sample, or at least 10ng of genomic DNA. The
449 PCR primers for amplification of the target regions were designed by comparative
450 analysis of the *rrs* and rrl sequences between M. bovis (GenBank accession numbers
451 CP002188, CP002058, CP002513, CP005933, CP011348, CP019639, and CP023663)
452 and various Mollicutes bacteria (GenBank accession numbers AB182581,
453 AMWK01000000, AORH00000000, AP013353, AP014631, AP014657, AP017902,
454 AP018135, AUAL01000000, BX293980, CP000896, CP002107, CP002108, CP007154,
455 CP007229, CP007521, CP009770, CP011096, FP236530, FR668087, FUXF00000000,

456 JFDP00000000, JNJU00000000, NC_000908, NC_000912, NC_011374, NR_076192,
457 and X68421). The primer sequences, annealing temperatures, and amplicon sizes are
458 shown in Table 2. The PCR amplification conditions consisted of 35 cycles of 98°C for
459 10s, the annealing temperature for 15s, and 68°C for 60s/kb of amplicon size. The melting
460 curve analysis assay for the detection of target mutations was carried out in a 20-µl
461 solution of the 2-fold-diluted PCR product containing 0.2 µM each hybridization probe.
462 A total of four hybridization probe sets against target mutations were also designed from
463 the *M. bovis* PG45^T genome (GenBank accession number CP002188) (13). These
464 consisted of a donor probe whose 3' end was labeled with fluorescein isothiocyanate
465 (FITC) and an acceptor probe whose 5' end was labeled with LC Red640. The
466 hybridization probe sequences are also shown in Table 2. The protocol for the melting
467 curve analysis using a LightCycler 480 system II (Roche Diagnostics GmbH, Mannheim,
468 Germany) consisted of a single cycle of 95°C for 60s (20°C/s), 35°C for 60s (20°C/s),
469 and 70°C for 0s (0.05°C/s). The analytical results were calculated using the analytical
470 software included with the real-time PCR machine (operated in automatic mode), i.e.,
471 LightCycler3 480 SW 1.5.1 and Exor4 for XDMS_R (Roche Diagnostics GmbH). The
472 species specificity of this method was confirmed on genomic DNA samples of nine bovine
473 mycoplasmal strains (*M. alkalescens* PG51^T, *M. arginini* G230^T, *M. bovigenitalium*
474 PG11^T, *M. bovirhinis* PG43^T, *M. bovoculi* M165/89^T, *M. canadense* 275C^T, *M.*
475 *californicum* ST-6^T, *M. dispar* 462/2^T, and PG45^T), two acholeplasmal strains
476 (*Acholeplasma axanthum* S743^T and *Acholeplasma laidlawii* PG8^T), and nine strains
477 belonging to bacterial species that can cause bovine mastitis (*E. coli* ATCC 12810,
478 *Enterococcus faecalis* ATCC 19433, *Enterococcus faecium* NCTC 7171, *Staphylococcus*
479 *aureus* ATCC 12600, *Staphylococcus epidermidis* ATCC 146, *Streptococcus agalactiae*
480 NCTC 11360, *Streptococcus dysgalactiae* NCDO 2023, *Streptococcus uberis* ATCC

481 19436, and *Streptococcus parauberis* DSM 6631). The SCCs in the milk samples were
482 checked using a DeLaval cell counter (Cardiff, UK). Bovine milk samples with various
483 SCCs (20×10^3, 716×10^3, $1{,}600 \times 10^3$, and $3{,}000 \times 10^3$ cell/ml) and serial dilutions of
484 strain PG45T (1.26×100 to 1.26×10^7 CFU/ml) were prepared to confirm the sensitivity
485 of this method, and the effects of two milk-sample storage conditions were also evaluated
486 (-20°C or 4°C for 7days).
487

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496 bovine samples from mycoplasmal diseases. Moreover, pirlimycin was kindly supplied
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499 approved the manuscript.

500

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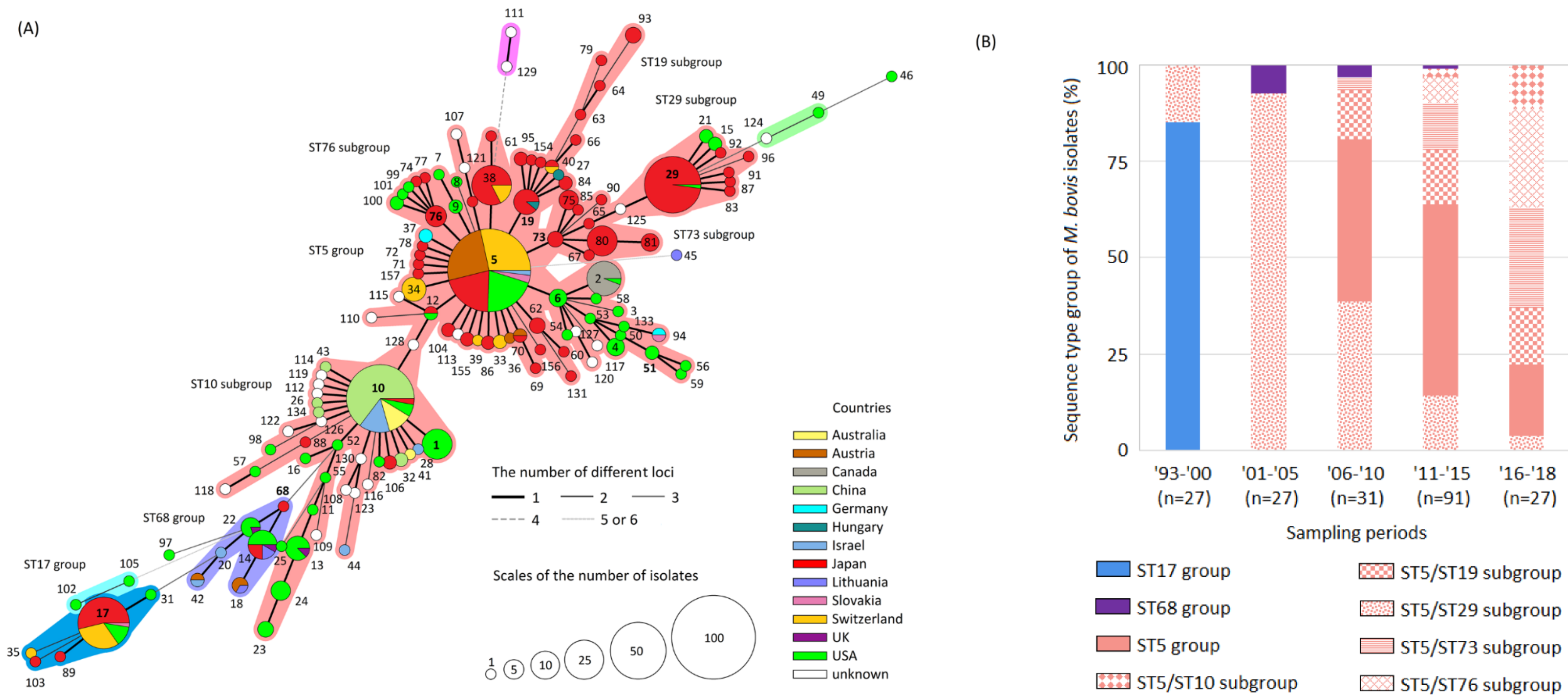
604

605 **FIGURE**



606

607 **FIG 1** (A) Minimum spanning tree representing the evolutionary relationship between *M. bovis* sequence types (STs) by multilocus sequence typing. Numbers
608 indicate STs, and the central ST and subcentral STs of each ST group are indicated in bold. A group of STs in which two or fewer loci differ from each other is
609 considered an ST group and is surrounded by a specific color. The size of the circles represents the population size, and the circular chart in each circle indicates
610 the proportion of countries of origin in each ST. The thickness and dotting of the lines indicate the number of different loci between the STs; a thicker line denotes
611 a closer distance than a thin line, and a thin line denotes a closer distance than a dotted line. Among the whole-genome-analyzed strains, strain PG45^T was

612 identified as a member of ST17, and all strains from China were members of the ST10 subgroup (Hubei-1, 08M, CQ-W70, HB0801, NM2012, and Ningxia-1).
613 (B) Chronological change of genotypes by MLST of *M. bovis* isolates in Japan. Numbers on the x axis represent years, with the last two digits shown for each
614 year.

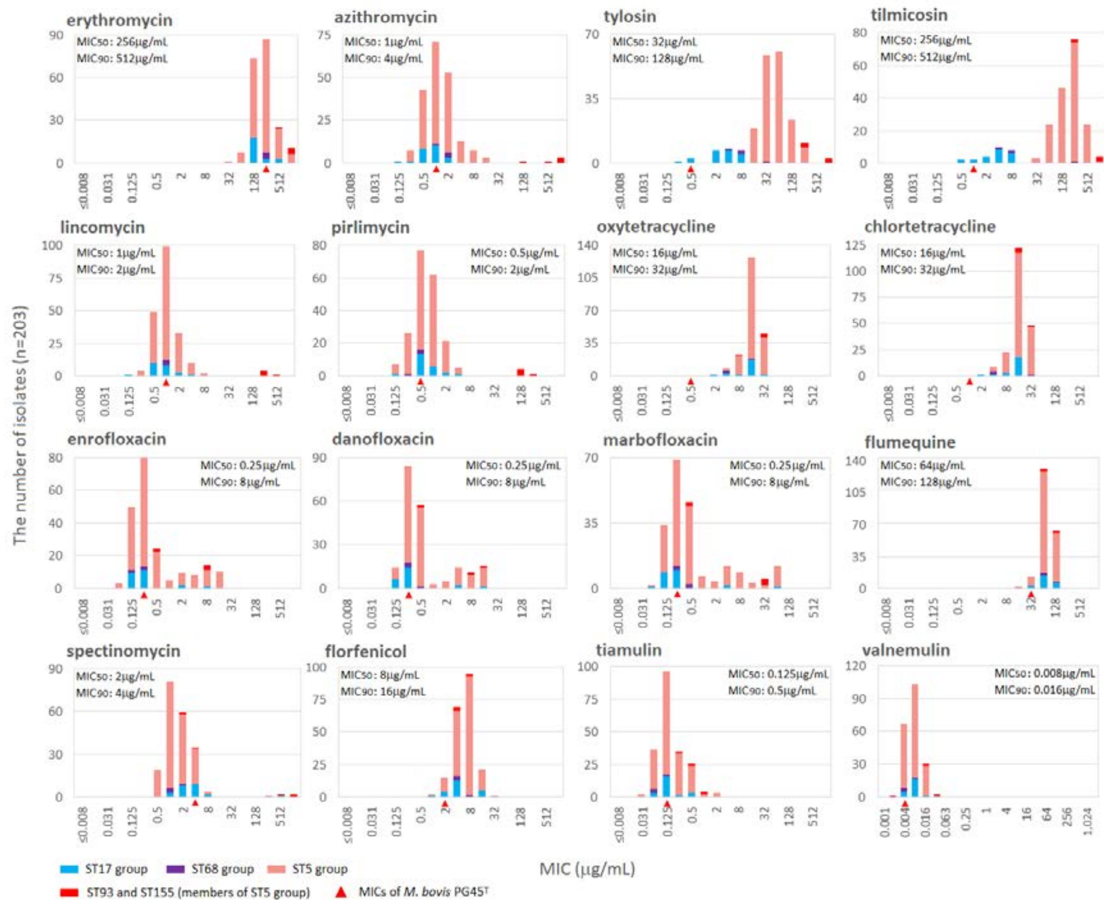
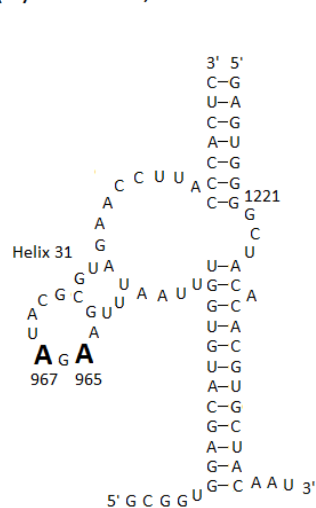
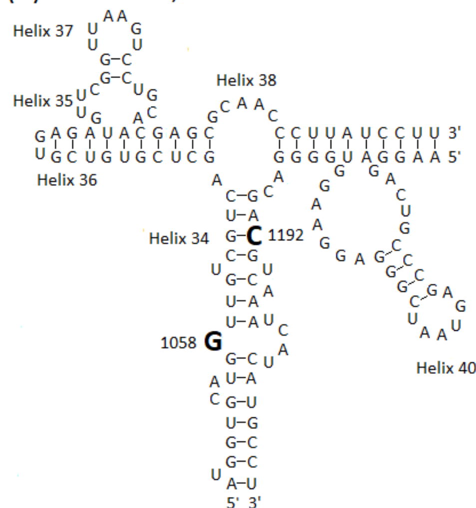
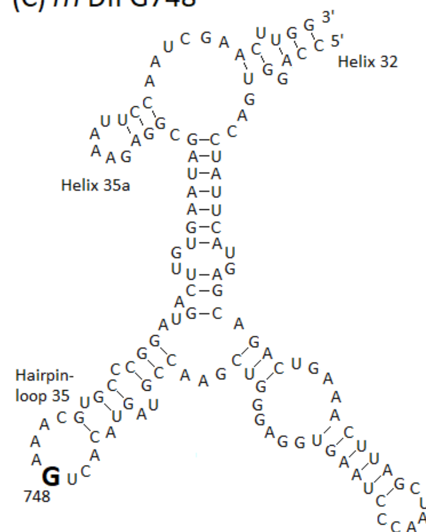
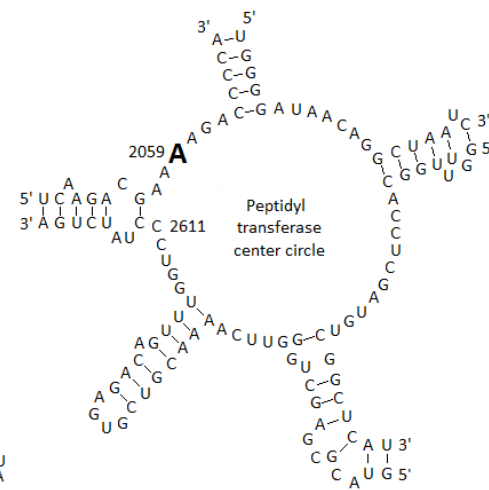


FIG 2 Relationship between antimicrobial susceptibility distribution and genotypes by MLST of *M. bovis* field isolates in Japan. MIC values of strain PG45^T are indicated with a triangle under the bar graph. *M. bovis* showed natural resistance to erythromycin and flumequine. Isolates of ST93 (a member of the ST5 group) showed low susceptibility to spectinomycin, macrolides, lincosamides, tetracyclines, and fluoroquinolones. Moreover, isolates of ST155 (a member of the ST5 group) showed low susceptibility to macrolides, lincosamides, and tetracyclines. Although ST14 (a member of the ST68 group) and isolates in the ST17 group (oldcomer) showed high susceptibility to 16-membered macrolides, ST68 and isolates in the ST5 group (newcomer) showed low susceptibility to these agents. Susceptibility to tetracyclines was on a declining trend overall. Isolates that showed low susceptibility to fluoroquinolones emerged sporadically, and their ST groups were varied. On the other hand, susceptibility to pleuromutilins was high, and no isolate with low susceptibility was found.









(E) *gyrA*

GAT GTT TTA GGT AAG TAT CAC CCT CAT GGT GAT TCT TCG GTT TAT GAA GCA ATG GTG CGT ATG GCT CAA GAT TTT TCA ATG AGA TAT CCT TTA

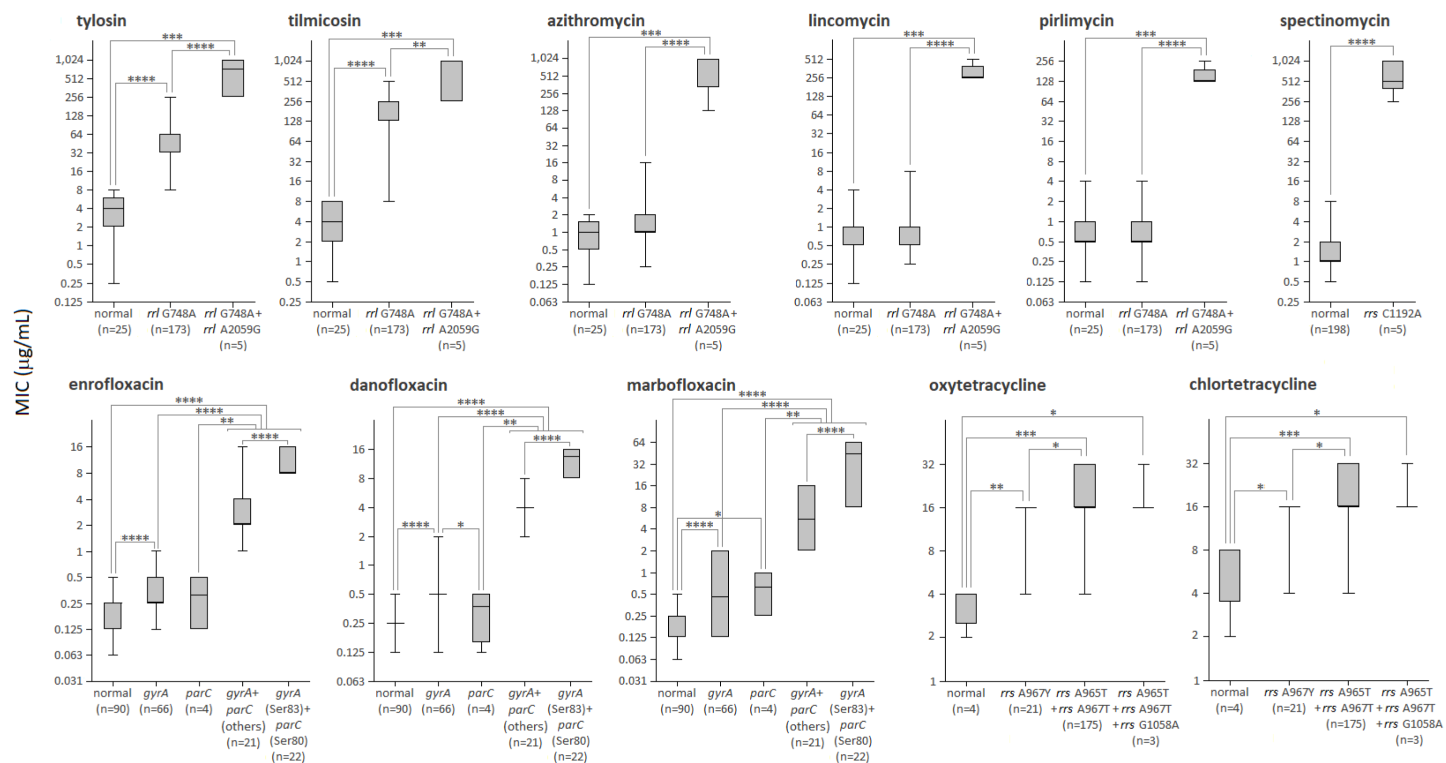
227 (A, Lys76); 240 (T); 241 (G, Gly81); 248 (C, Ser83); 250 (T, Ser84); 259, 260 (GA, Glu87); 281 (A, Gln94)

(F) *parC*

TAT CAC CCG CAT GGT GAT AGT TCT ATT TAT GAC GCA ATG GTT AGG ATG GGG CAA GAG TGA AAA ATG GGG CAT ACA TTA GTA GAA ATG CAT

234 (T); 239, 240 (GT, Ser80); 241 (T, Ser81); 250, 251, 252 (GAC, Asp84); 253 (G, Ala85); 270 (G); 288 (G); 300 (A)

FIG 3 Single nucleotide polymorphisms (SNPs) in target genes that were confirmed in *M. bovis* field isolates in Japan. (A and B) The two-dimensional structure of helix 31 and helix 34 of 16S rRNA (*rrs*). (C and D) Hairpin-loop 35 in domain II and the peptidyl transferase center circle in domain V of 23S rRNA (*rrl*) of *M. bovis* PG45^T (GenBank accession no. CP002188). Each SNP is indicated in large font and bold. (E and F) The DNA sequences of the quinolone resistance determining region of the DNA gyrase gene (*gyrA*) (E) and topoisomerase IV gene (*parC*) (F) of *M. bovis* PG45^T. Of the SNPs indicated in large font, the SNPs which cause nonsynonymous mutations are in bold, and the original amino acids are shown under the triplet. The nucleotides and amino acids are numbered on the basis of the *E. coli* sequence (NCBI RefSeq accession no. NC_002655).



634

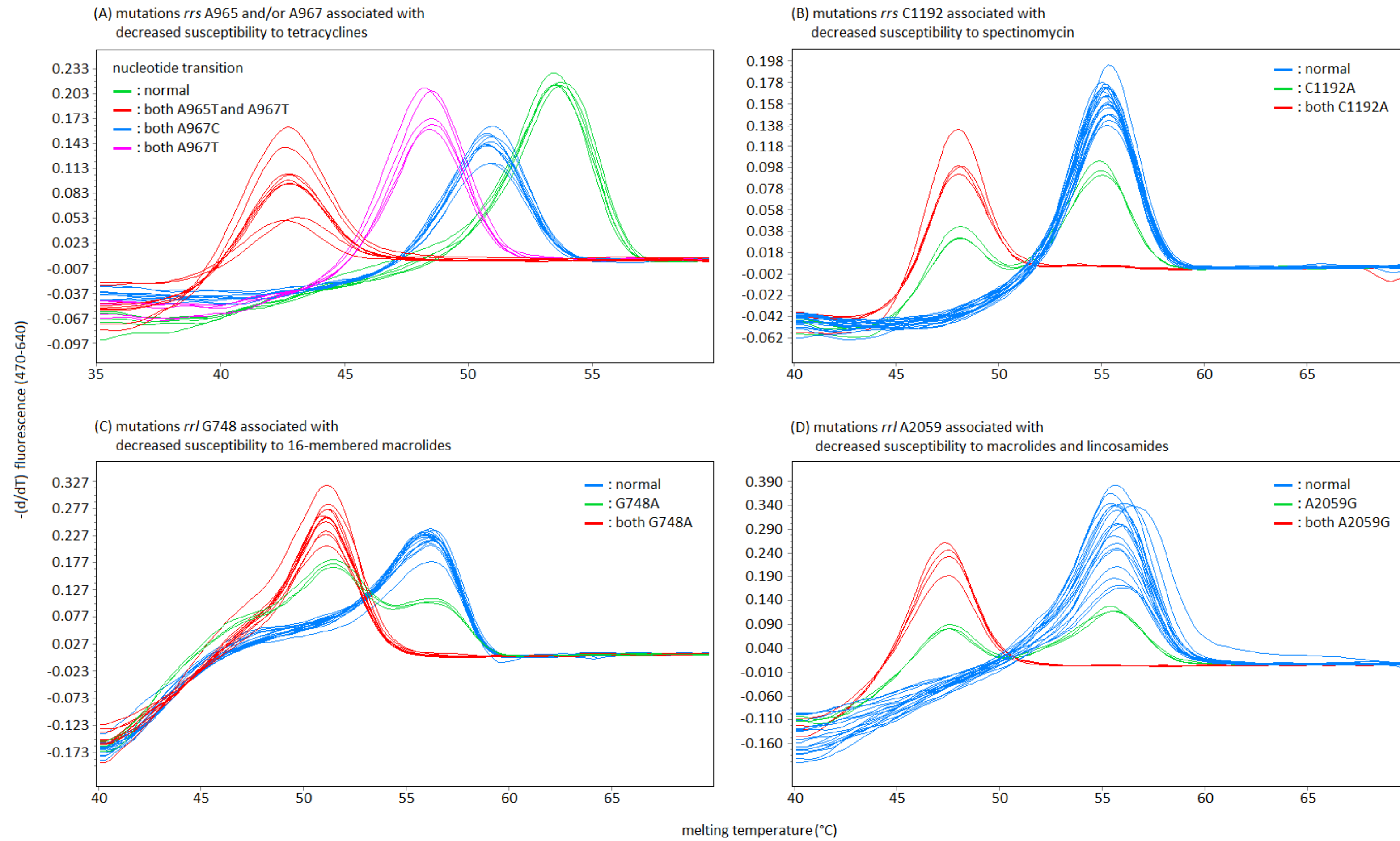
635 **FIG 4** Relationship between the existence of nonsynonymous mutations or SNPs in target genes and MICs to each antimicrobial agent. The box-and-whisker

636 plot indicates the maximum, upper quartile, median, lower quartile, and minimum MIC value. As for fluoroquinolones, *gyrA* (Ser83) + *parC* (Ser80) refers to

637 isolates in which the coexistence of amino acid substitutions at Ser83 in gyrA and Ser80 in parC was confirmed, and *gyrA* + *parC* (others) refers to isolates in

638 which combinations of other amino acid substitutions were confirmed. Significant differences were evaluated by the Mann-Whitney U test. ****, $P < 0.0001$;

639 ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.



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641 **FIG 5** The results of melt-curve genotyping using hybridization probes for detecting mutations associated with decreased susceptibility to tetracyclines,

642 spectinomycin, lincosamides and/or macrolides in *M. bovis*.

TABLE 1 Antimicrobial susceptibility profiles of M. bovis field isolates from 203 cases of bovine infection in Japan from 1993 to 2018

Antimicrobial agents	MIC data (µg/mL)			
	Range	MIC_{50}	MIC_{90}	MIC_{PG45^T}
Erythromycin	32 - 1,024	256	512	256
Azithromycin	0.125 - 1,024	1	4	1
Tylosin	0.25 - 1,024	32	128	0.5
Tilmicosin	0.5 - 1,024	256	512	1
Lincomycin	0.125 - 512	1	2	1
Pirlimycin	0.125 - 256	0.5	2	0.5
Oxytetracycline	2 - 32	16	32	0.5
Chlortetracycline	2 - 32	16	32	1
Enrofloxacin	0.063 - 16	0.25	8	0.25
Danofloxacin	0.125 - 16	0.25	8	0.25
Marbofloxacin	0.063 - 64	0.25	8	0.25
Flumequine	16 - 128	64	128	32
Spectinomycin	0.5 - 1,024	2	4	4
Florfenicol	1 - 32	8	16	2
Tiamulin	0.031 - 2	0.125	0.5	0.125
Valnemulin	0.002 - 0.031	0.008	0.016	0.004

TABLE 2 Primers and probes used in melting curve genotyping analysis performed by hybridization probe or DNA sequencing for detecting mutations involved in reduced susceptibility to tetracyclines, spectinomycin, macrolides, lincosamides, and fluoroquinolones

Target mutation(s)	Primer or probe	Sequence (5' to 3')	Annealing temp. (°C)	Amplicon size (bp)	Reference
rrs A965 and/or A967	Forward primer	GCA TAG GAA ATG ATG CTA CC	61	808	This study
	Reverse primer	TGC TCC ATG TCA CCA CTT C			
	Hybridization probe	CGG TGG AGC ATG TGG TTT AA-FITC			
		LC Red640-TTG AAG ATA CGC GTA GAA CC -phosphate			
rrs C1192	Forward primer	same as *rrs* A965 and/or A967	61		
	Reverse primer	same as *rrs* A965 and/or A967			
	Hybridization probe	CCG AGT AAT CGG GAG GAA GG-FITC			
		LC Red640- GGG GAC GAC GTC AAA TCA TC-phosphate			
rrl DII G748	Forward primer	AGC TTT TGG GAA GAA GCG	65	836	This study
	Reverse primer	TTA CAT TGT CGG CGC AAG G			
	Hybridization probe	CCC TAA GTG GAG GGT CGA AC-FITC			
		LC Red640-GTA GTA CAC TGA AAC GTG CC-phosphate			
rrl DV A2059	Forward primer	GGA CTT TTG TCT GAA TTT GCC	61	609	This study
	Reverse primer	GTT GAC TCC ACT GCT ACT G			
	Hybridization probe	GAA AAC GCT GGG TTC CCG CA-FITC			
		LC Red640-CAA GAC GAA AAG ACC CCA TG-phosphate			
QRDR in *gyrA*	gyrA-F primer	GACGAATCATCTAGCGAG	59	531	(21)
	gyrA-R primer	GCCTTCTAGCATCAAAGTAGC			
QRDR in *parC*	parC-F primer	GAGCAACAGTTAAACGATTTG	59	488	(21)
	parC-R primer	GGCATAACAACTGGCTCTT			