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16S rRNA Gene Mutations Associated with Decreased Susceptibility to Tetracycline in *Mycoplasma bovis*

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***Mycoplasma bovis* isolates with decreased susceptibilities to tetracyclines are increasingly reported worldwide. The acquired molecular mechanisms associated with this phenomenon were investigated in 70 clinical isolates of *M. bovis*. Sequence analysis of the two 16S rRNA-encoding genes (*rrs3* and *rrs4* alleles) containing the primary binding pocket for tetracycline (Tet-1 site) was performed on isolates with tetracycline hydrochloride MICs of 0.125 to 16 μg/ml. Mutations at positions A965T, A967T/C (*Escherichia coli* numbering) of helix 31, U1199C of helix 34, and G1058A/C were identified. Decreased susceptibilities to tetracycline (MICs, ≥2 μg/ml) were associated with mutations present at two (A965 and A967) or three positions (A965, A967, and G1058) of the two *rrs* alleles. No *tet*(M), *tet*(O), or *tet*(L) determinants were found in the genome of any of the 70 *M. bovis* isolates. The data presented correlate ($P < 0.0001$) the mutations identified in the Tet-1 site of clinical isolates of *M. bovis* with decreased susceptibility to tetracycline.**

The bacterial pathogen *Mycoplasma bovis* causes a variety of clinical manifestations in cattle, including respiratory disease, mastitis, arthritis, and otitis, which result in substantial economic losses (1). The tetracyclines are among the few important antimicrobial agents that may be used to treat *M. bovis* infections (2).

Tetracyclines are broad-spectrum antimicrobials that have been widely used in human and veterinary medicine (3). They inhibit protein synthesis by binding to the 30S ribosomal subunit and blocking an attachment of aminoacyl-tRNA to the A site (3). Resistance to tetracyclines is common in many bacterial species and may be achieved by (i) an energy-dependent efflux of the drug across the cell membrane mediated by efflux pumps, (ii) the presence of ribosomal protection proteins that confer tetracycline resistance, either by a reduction of the affinity of ribosomes to tetracyclines or by releasing the bound antimicrobial from the ribosome, (iii) the enzymatic inactivation of the drug, or (iv) the mutations in the 16S rRNA genes that affect the binding sites of tetracyclines (4).

In *Mollicutes*, two mechanisms of resistance to tetracyclines have been identified so far, both of which are in *Mollicutes* species that infect humans. These include ribosomal protection by *tet*(M) determinants, described in naturally tetracycline-resistant strains of *Mycoplasma hominis* and *Ureaplasma* spp. (5, 6), as well as target modification with point mutation(s) in the 16S rRNA genes of *in vitro* obtained mutants of *Mycoplasma pneumoniae* (positions 968 and 1193) and *M. hominis* (positions 346, 965 to 967, and 1054) (7).

Even though high MICs to tetracyclines have been identified in many *Mycoplasma* spp. of veterinary importance (reviewed in reference 8), including *M. bovis* (9–15), the genetic background for decreased susceptibility has not been elucidated in either field isolates or mutants selected *in vitro*. *M. bovis* contains one or two rRNA operons (*rrn*). Indeed, analysis of genome sequences of *M. bovis* type strain PG45 and two field strains, isolated in China, revealed that two out of the three annotated genomes (type strain PG45 and HB0801) (16, 18) contained two tandem *rrn* alleles (*rrn3* and *rrn4*), both of which consisted of 16S rRNA (*rrs3* [gene ID MBOPG45 0956] and *rrs4* [MBOPG45 0958]) and 23S rRNA (*rrl3* [MBOPG45 0957] and *rrl4* [MBOPG45 0959]), while in strain Hubei-1, only one *rrn* was identified (17). No putative *tet*(M) determinant was found in the annotated genomes of *M. bovis*.

The aim of this study was to investigate the mechanisms associated with acquired decreased susceptibilities to tetracyclines in *M. bovis* isolates.

MATERIALS AND METHODS

***Mycoplasma bovis* isolates and growth conditions.** A total of 70 *M. bovis* field isolates from Israel ($n = 33$; 1995 to 2011), the United Kingdom ($n = 11$; 2000 to 2009), Germany ($n = 11$; 1978 to 1991), Spain ($n = 2$; 1993), Australia ($n = 3$; 2006), Hungary ($n = 6$; 2006 to 2010), Lithuania ($n = 3$; 2007 to 2010), and Cuba ($n = 1$; 1980 [19]) were tested in this study, and their details are given in Table 1. Each isolate originated from different farms, was selected at random, and had no epidemiological link to other isolates unless indicated otherwise. The reference type strain *M. bovis* PG45 was obtained from the National Collection of Type Cultures, United Kingdom (strain NCTC10131, corresponding to ATCC 25523).

All the isolates were propagated at 37°C in standard *M. bovis* broth medium (19) supplemented with 0.5% (wt/vol) sodium pyruvate and 0.005% (wt/vol) phenol red (pH 7.8) (20). Isolates of *M. bovis* were identified by immunofluorescence of colonies using species-specific conjugated antiserum. Aliquots of cultures were stored at −80°C until used. An aliquot was then thawed, and the number of CFU per ml was determined by performing serial 10-fold dilutions in broth, plating each dilution on

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TABLE 1 Molecular characterization of the primary binding pocket for tetracycline in *rrs* genes of *M. bovis* field isolates with different susceptibilities to tetracycline

No.	Strain[a]	Yr	Country	Clinical sign	MIC (μg/ml)	Mutation in the Tet-1 site of:					
						rrs3			*rrs4*		
						A965	A967	G1058	A965	A967	G1058
	PG45	1962	USA	Mastitis	0.06						
1	422	1980	Cuba	Respiratory	0.125						
2	330-2	2005	Israel	Mastitis	0.125						
3	KS6	1995	Israel	Respiratory	0.125						
4	357/81	1981	Germany	Mastitis	0.125		T				
5	213	2008	Israel	Respiratory	0.25				NR[b]	NR	NR
6	212/81	1981	Germany	Respiratory	0.25						
7	997/79	1979	Germany	Respiratory	0.25						
8	362/88	1991	Germany	Mastitis	0.25				NR	NR	NR
9	2D[A]	2006	Australia[c]	Respiratory	0.25						
10	2E[A]	2006	Australia[c]	Respiratory	0.25				T	T	
11	018/91	1981	Germany	Mastitis	0.25		T				
12	780/78	1978	Germany	Mastitis	0.5						
13	002/91	1991	Germany	Respiratory	0.5						
14	589/78	1978	Germany	Respiratory	0.5						
15	097/81	1981	Germany	Respiratory	0.5						
16	277/83	1983	Germany	Mastitis	0.5				NR	NR	NR
17	268B07	2007	United Kingdom	Arthritis	0.5		T				
18	100/91	1991	Germany	Respiratory	0.5		T		NR	NR	NR
19	7028	2007	Israel	Respiratory	0.5	T					
20	742B08	2008	United Kingdom	Mastitis	0.5		T				
21	99B08	2008	United Kingdom	Respiratory	0.5		T				
22	57R	2000	United Kingdom	Respiratory	0.5		T			T	
23	155	1999	Israel	Respiratory	0.5		T			C	
24	H[A]	2006	Australia[c]	Arthritis	0.5				T	T	
25	116/93	1993	Spain	Mastitis	1		C			C	
26	145/93	1993	Spain	Mastitis	1		C			C	
27	393B08	2008	United Kingdom	Mastitis	2	T	T		T	T	
28	341B09	2009	United Kingdom	Mastitis	2	T	T		T	T	
29	346B09	2009	United Kingdom	Mastitis	2	T	T		T	T	
30	KS3	1995	Israel	Respiratory	2	T	T		T	T	
31	144B08	2008	United Kingdom	Respiratory	2	T	T		T	T	
32	6099	2007	Israel	Mastitis	2	T	T		T	T	
33	345B09	2009	United Kingdom	Mastitis	2	T	T		T	T	
34	5180[B]	2006	Israel	Respiratory	2	T	T		T	T	
35	68891	2010	Israel	Respiratory	2	T	T		T	T	
36	9088	2008	Israel	Respiratory	2	T	T		T	T	
37	510	2011	Israel	Mastitis	2	T	T		T	T	
38	F92	2010	Hungary[c]	Respiratory	2	T	T		T	T	
39	5028[C]	2008	Israel	Respiratory	4	T	T		T	T	
40	4426	2008	Israel	Respiratory	4	T	T		T	T	
41	5936[D]	2008	Israel	Respiratory	4	T	T		T	T	
42	1590[E]	2010	Israel	Mastitis	4	T	T		T	T	
43	72084[E]	2010	Israel	Genital	4	T	T		T	T	
44	7227	2008	Lithuania[c]	Respiratory	4	T	T		T	T	
45	72242[E]	2010	Israel	Respiratory	4	T	T		T	T	
46	71931	2010	Lithuania[c]	Respiratory	4	T	T		T	T	
47	6866	2008	Hungary[c]	Respiratory	4	T	T		T	T	
48	1366[E]	2010	Israel	Respiratory	4	T	T		T	T	
49	8830	2006	Hungary[c]	Respiratory	4	T	T	A	T	T	A
50	293B09	2009	United Kingdom	Respiratory	4	T	T	A	T	T	A
51	3374	2006	Hungary[c]	Respiratory	4	T	T	A	T	T	A
52	18R	2002	United Kingdom	Respiratory	4	T	T	A	T	T	A

(Continued on following page)

TABLE 1 (Continued)

No.	Strain[a]	Yr	Country	Clinical sign	MIC (μg/ml)	Mutation in the Tet-1 site of:					
						rrs3			*rrs4*		
						A965	A967	G1058	A965	A967	G1058
53	92279[B]	2010	Israel	Respiratory	4	T	C	A	T	C	A
54	2029	2006	Hungary[c]	Respiratory	4	T	T	A	T	T	A
55	1716	2007	Israel	Respiratory	4	T	T	A	T	T	A
56	13	2008	Israel	None	4	T	T	A	T	T	A
57	3036-1	2007	Hungary[c]	Respiratory	4	T	T	A	T	T	A
58	3181/3[C]	2006	Israel	Respiratory	4	T	T	A	T	T	A
59	2670	2005	Israel	Respiratory	8	T	T	A	T	T	A
60	9603	2007	Lithuania[c]	Respiratory	8	T	T	A	T	T	A
61	88127	2010	Israel	Respiratory	8	T	T		NR	NR	NR
62	6512	2008	Israel	Respiratory	8	T	T		T	T	A
63	869[D]	2008	Israel	Respiratory	8	T	T		T	T	A
64	432	2008	Israel	Mastitis	8	T	T	C	T	T	
65	8934[F]	2005	Israel	Respiratory	8	T	T	A	T	T	A
66	170[F]	2005	Israel	Respiratory	8	T	T	A	T	T	A
67	3222	2005	Israel	Respiratory	8	T	T	A	T	T	A
68	94724	2011	Israel	Respiratory	8	T	C	A	T	C	A
69	861	2005	Israel	Respiratory	8	T	C	A	T	C	A
70	8998-2	2007	Israel	Respiratory	16	T	T	A	T	T	A

[a] The same superscript uppercase letter (A to F) indicates that *M. bovis* strains were isolated from the same shipment or on the same farm.
[b] NR, not relevant, *M. bovis* isolate with one *rrn*.
[c] *M. bovis* isolates isolated in Israel from imported calves in quarantine stations.

agar, incubating for 96 h, and then counting the colonies as described previously (21).

Tetracycline susceptibility. The susceptibilities of *M. bovis* isolates to tetracycline hydrochloride (≥95% active substance; Sigma, Rehovot, Israel) were tested by the agar dilution method as described previously (12) using *M. bovis* agar plates (19). Briefly, 2-fold dilutions of tetracycline from 0.03 to 32 μg/ml were incorporated onto the agar plates. Five microliters of each isolate, containing 1×10^5 to 1×10^6 CFU/ml, was spotted onto the agar plates. Plates were incubated at 37°C with 5% CO_2 for 4 days. The NCTC *M. bovis* type strain PG45, which is regularly tested in the laboratory, was used as a control to ensure that the results were consistent with results obtained previously (22). The procedure was repeated independently three times for the reference strain PG45 and for 50% of the isolates, selected at random, with the same results obtained within a single 2-fold dilution (data not shown).

Amplification of the *M. bovis* primary tetracycline binding site. Sequence analysis of the primary binding pocket for tetracycline (the Tet-1 site) was performed for all 70 *M. bovis* field isolates tested in this study. Genomic DNA was extracted from 400 μl of logarithmic-phase broth culture using the Maxwell DNA isolation kit for cells/tissues and the Maxwell 16 apparatus (Promega) according to the manufacturer's instructions. The 16S rRNA-encoding gene (*rrs3* and *rrs4* alleles) containing the Tet-1 site, formed by residues 1054 to 1056 and 1196 to 1200 of helix 34 and residues 964 to 967 of helix 31 (numbers corresponding to *Escherichia coli* 16S rRNA), was amplified in *M. bovis* isolates using the primers listed in Table 2. The primer sets MB-282-F–MB-tet3/4-R and MB-rrs-3F–MB-287-R allowed the amplification of *rrs3* and *rrs4*, respectively (Table 2). The primers were developed and commercially synthesized (Sigma, Rehovot, Israel) based on the nucleotide sequence of *M. bovis* type strain PG45 (NCBI reference sequence NC_014760.1 [16]) and strain HB0801 (NC_009497.1) (18).

PCRs were carried out in 50-μl volumes containing 250 ng of template DNA, 1 μl of Phire Hot Start II DNA polymerase (Thermo Scientific, Waltham, MA, USA), 10 μl of 5× Phire reaction buffer, 1 μl of 10 mM deoxynucleoside triphosphate (dNTP), and 1 μl of 20 μM each primer. PCR amplifications were carried out in a C1000 series thermocycler (Bio-Rad, Hercules, CA, USA). The conditions for the PCRs are specified in Table 2.

The amplicons were then extracted and purified from the gel using the MEGAquick-spin PCR and agarose gel DNA extraction system (iNtRON

TABLE 2 Primers and PCR amplification programs used in this study

Primer designation	Primer source	Primer sequence (5′ to 3′)	Target amplified	Amplification protocol	Size of the amplified product (bp)[a]
MB-282-F	MBOPG45 0282	GGATATCTAACGCCGTGTCT	MBOPG45 0282 (3′ end), *rrs3*	98°C for 30 s; 40 cycles of 98°C for 5 s, 60°C for 5 s, 72°C for 1 min; a final stage at 72°C for 3 min	1,857
MB-tet3/4-R	*rrs3* and *rrs4*	CGTTCTCGTAGGGATACCT			
MB-rrs-3F	*rrs3* and *rrs4*	CGAGAGTTTGATCCTGGCTC	*rrs4* (3′ end), MBOPG45 0287 (5′ end)	98°C for 30 s; 40 cycles at 98°C for 5 s, 64°C for 5 s, 72°C for 2 min; a final stage at 72°C for 3 min	5,294
MB-287-R	MBOPG45 0287	CTAATTCCAAGTGCCACTAGCG			

[a] The length of the PCR products is according to *M. bovis* type strain PG45.

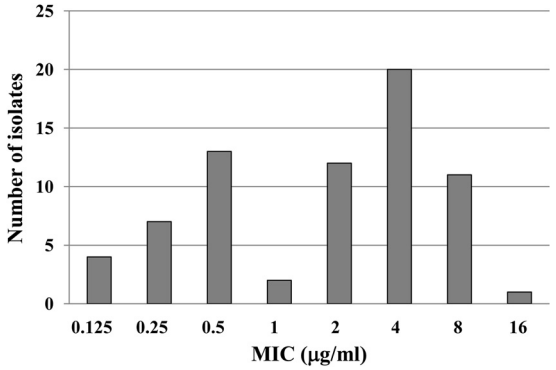

FIG 1 Distribution of *Mycoplasma bovis* isolate MICs to tetracycline. MICs of tetracycline tested in this study are shown on the *x* axis; the number of *M. bovis* isolates corresponding to each MIC is shown on the *y* axis.

Biotechnology, South Korea). Sequencing was performed at the DNA Sequencing Unit at the Weizmann Institute (Rehovot, Israel). Sequence editing, consensus, and alignment construction were performed using DNASTAR software, version 5.06/5.51 (Lasergene, Inc., Madison, WI, USA) and BioEdit (Ibis Biosciences [23]). The numbering of each nucleotide was based on the respective 16S rRNA gene of *E. coli* unless indicated otherwise.

Screening of *M. bovis* isolates for the presence of tetracycline resistance determinants. To check for the presence of *tet*(M), *tet*(O), and *tet*(L) determinants, all 70 *M. bovis* isolates and the PG45 type strain were subjected to DNA dot blot analysis. Briefly, for each isolate, approximately 1 μg of *M. bovis* genomic DNA was spotted onto a positively charged nylon membrane. The *tet*(M)-, *tet*(O)-, and *tet*(L)-related probes were amplified using genomic DNA of beta-hemolytic streptococci group G (GGS) containing these determinants (obtained from a collection of the Department of Clinical Microbiology and Infectious Diseases, Hadassah-Hebrew University Medical Center, Jerusalem, Israel). The primers and PCR conditions used for *tet*(M), *tet*(O), and *tet*(L) amplifications were as previously described (24–26). The obtained *tet*(M), *tet*(O), and *tet*(L) PCR amplicons were purified from the gel as described above and sequenced, and their nucleotide sequences were compared to the data present in the NCBI Nucleotide Database (http://blast.ncbi.nlm.nih.gov).

The *tet*(M), *tet*(O), and *tet*(L) amplicons were subsequently labeled by digoxigenin (DIG) following the manufacturer's protocol (Roche Diagnostics GmbH, Mannheim, Germany). Spotted DNA was then hybridized with DIG-labeled probes, washed, incubated with alkaline phosphatase (AP)-tagged anti-DIG antibody, and detected as previously described by Lysnyansky et al. (27). Chemiluminescence detection and imaging were performed using a G:BOX Chemi XR5 scanner (Syngene, Cambridge, United Kingdom). Genomic DNAs of the GGS isolates containing *tet*(M), *tet*(O), and *tet*(L) determinants were used as positive controls.

In addition, PCR amplification of *tet*(M) was performed on genomic DNA of all 70 *M. bovis* isolates using the primers (tetMF and tetMR) and PCR conditions previously described by Blanchard et al. (28).

Statistical analysis. The significance of observed associations between the presence of mutations within the 16S rRNA gene(s) and decreased susceptibility to Tet was analyzed using Fisher's exact test. Two-tailed *P* values were calculated using the GraphPad QuickCalc website (http://graphpad.com/quickcalcs/contingency1/). $P < 0.05$ was considered statistically significant.

RESULTS

DNA sequence analysis of the 16S rRNA-encoding genes of *M. bovis* isolates with different susceptibilities to tetracycline. The MICs of the 70 *M. bovis* field isolates ranged from 0.125 to 16 μg/ml, as shown in Fig. 1 and Table 1. A bimodal distribution of the MICs was identified, indicating decreased susceptibilities in isolates in the higher range of MICs (≥2 μg/ml) (Fig. 1). Indeed, 44/70 (63%) isolates had MICs of 2 to 16 μg/ml. At least two isolates per defined MIC were identified for further analyses, except at the MIC of 16 μg/ml, as only one isolate was found (Fig. 1, Table 1).

The sequence analysis of the Tet-1 site of 16S rRNA-encoding genes (*rrs3* and *rrs4* alleles) showed that 5/70 *M. bovis* isolates had one *rrs* allele (two isolates with an MIC of 0.25 μg/ml, two with an MIC of 0.5 μg/ml, and one with an MIC of 8 μg/ml). The distribution of mutations in the Tet-1 site relative to the tetracycline MICs is shown in Table 1. Two cohorts of isolates were identified.

The first cohort contained 26 *M. bovis* isolates with MICs of ≤1 μg/ml. In 13/26 isolates, no mutations were identified at positions 964 to 967 of helix 31 or at positions 1054 to 1056 and 1196 to 1200 of helix 34 in either *rrs* allele. However, a single mutation at position A965T or A967T of the *rrs3* allele was found in seven isolates, two isolates contained the A965T and A967T mutations in *rrs4*, and four isolates had mutations at position A967T/C in *rrs3* and *rrs4* alleles (Table 1, isolate numbers 1 to 26).

The second cohort contained 44 *M. bovis* isolates with MICs of ≥2 μg/ml. Twenty-two out of 44 isolates possessed the double mutations AGA 965 to 967 TGT in the two *rrs* alleles, 18 isolates contained triple mutations (AGA 965 to 967 TGT/C and G1058A) in the two *rrs* alleles, and 3 isolates contained the double mutations AGA 965 to 967 TGT in the two *rrs* alleles and a G1058C mutation in *rrs3* (one isolate) or a G1058A mutation in *rrs4* (two isolates). In addition, one isolate (with only one *rrn*) contained the double mutations AGA 965 to 967 TGT (Table 1, isolate numbers 27 to 70).

In addition, three isolates with MICs of 2 to 4 μg/ml tetracycline had a U1199C mutation in *rrs3*, and 20 *M. bovis* isolates with MICs of 2 to 8 μg/ml tetracycline contained the C1192A mutation in one *rrs* (12 isolates) or in both *rrs* alleles (8 isolates). These mutations were not identified in *M. bovis* isolates with MICs of 0.125 to 1 μg/ml (data not shown). It should be noted that other mutations/nucleotide substitutions were also identified within the *rrs3* allele of some isolates (at positions 1005 [2 strains, with MICs of 0.125 and 0.5 μg/ml], 1013 [24, with MICs of 0.125 to 8 μg/ml], 1281 [6, with MICs of 0.125 to 8 μg/ml], and 1331 [1, with an MIC of 0.5 μg/ml]) and/or within the *rrs4* allele (at positions 1005 [2, with MICs of 0.125 and 0.5 μg/ml), 1013 [37, with MICs of 0.25 to 16 μg/ml], 1153 [61, with MICs of 0.125 to 16 μg/ml], 1184 [4, with MICs of 0.25 to 0.5 μg/ml], 1189 [1, with an MIC of 0.125 μg/ml], 1268 [59, with MICs of 0.125 to 16 μg/ml], and 1331 [1, with an MIC of 4 μg/ml]). None of these positions is located within or close to the Tet-1 site, and nucleotide substitution at position 1013 (A to G in *rrs3* and G to A in *rrs4*) appeared to represent intra-*rrs* variability.

Screening of the *M. bovis* genomic DNA for the presence of *tet*(M), *tet*(O), and *tet*(L) determinants. Genomic DNA samples of 70 *M. bovis* isolates with tetracycline MICs of 0.25 to 16 μg/ml were screened by dot blot analysis for the presence of *tet*(M), *tet*(O), and *tet*(L) genes as described in Materials and Methods. No positive signals were identified by any of the DIG-labeled probes (data not shown). In contrast, strong signals were detected for GGS-*tet*(M), *tet*(O), and *tet*(L) determinants used as positive controls. In addition, no PCR product was amplified using genomic DNA of the 70 *M. bovis* isolates and the PCR system developed by Blanchard et al. (28).

DISCUSSION

Tetracyclines are often used for the treatment of *M. bovis*-related infections, and thus tetracycline resistance in this organism is of increasing concern. In this study, the molecular mechanisms associated with *M. bovis*-decreased susceptibility to tetracycline were investigated. Our results showed that an increase of MICs to tetracycline (≥2 μg/ml) is correlated with the number of mutated nucleotide positions within the Tet-1 site of *M. bovis* field isolates. Indeed, 43/44 *M. bovis* isolates with MICs of ≥2 μg/ml contained two (A965T and A967T/C) or three (A965T, A967T/C, and G1058A/C) mutations in both the *rrs3* and *rrs4* alleles (from 4 to 6 mutated sites in total; $P < 0.0001$). The additional isolate had only one *rrs* allele, which contained A965T and A967T mutations (Table 1, number 61). In contrast, only single- or double-base-pair mutations (maximum of two mutated positions) were identified in 13/26 *M. bovis* isolates with MICs of ≤1 μg/ml tetracycline (Table 1, numbers 1 to 26). It is likely that the impact of single- or double-base-pair mutations on the susceptibility to tetracycline of *M. bovis* isolates containing two *rrs* alleles is minor. This can be explained by the fact that a nonmutated *rrs* copy may cover the function of the mutant copy. Indeed, from 12 out of 13 isolates with two *rrs* alleles, 10 were heterozygotes with MICs of ≤0.5 μg/ml. Two additional isolates (116/93 and 145/93) were homozygous for the single mutation A967C and had an MIC of 1 μg/ml tetracycline. More homozygotes for a single mutation should be tested to clarify whether one mutated position present in both of these *rrs* alleles may account for a slight increase in MIC (1 μg/ml). In addition, it will be of interest to test the correlation between the presence of single- or double-base-pair mutations and MICs of tetracycline in a cohort of *M. bovis* isolates with one *rrs* allele (unfortunately, only 5 isolates with one *rrs* allele were identified in our study [Table 1, numbers 5, 8, 16, 18, and 61]).

The Tet-1 site consists of two domains in 16S rRNA: helix 34 (residues 1054 to 1056 and 1196 to 1200) and the loop next to helix 31 (residues 964 to 967). It lies in a clamp-like pocket at the A site for binding of tRNA, as was previously shown by the crystal structures of *Thermus thermophilus* 30S ribosome-tetracycline complexes (29, 30). Mutations detected in this study were located within this site or close to it (position 1058). It has been shown that low-level resistance to tetracycline in *Helicobacter pylori* resulted from single- and double-base-pair mutations at positions 965 to 967 (31, 32), while high resistance to tetracycline was achieved by triple-base-pair mutation AGA 965 to 967 TTC (33, 34). In another study, three isolates with a single mutation (at positions 966, 967, or 1054), one isolate with a double mutation (at positions 346 and 965), and one isolate with a triple mutation (at positions 346, 965, and 966) were identified in mutants of *M. hominis* type strain PG21, selected *in vitro* using doxycycline (7). In the same study, an *in vitro*-obtained mutant of *M. pneumoniae* type strain FH harboring the single-base-pair mutation G1193A and another mutant with the double-base-pair mutations G1193A and T968C were also selected. It has been acknowledged that compared to the parent *M. hominis* and *M. pneumoniae* strains, the selected mutants showed decreased susceptibilities to tetracyclines; however, no remarkable increase in MICs was observed (only one mutant had an MIC of 8 μg/ml) (7).

In addition, decreased susceptibility to tetracycline acquired by a mutation of G to C at position 1058, located directly adjacent to the Tet-1 site, was previously described in clinical isolates of *Propionibacterium acnes*, *Brachyspira hyodysenteriae*, and *Brachyspira intermedia* (35–37). It has been suggested that a mutation at this position can influence the base pairing G1058 with U1199 and might lead to a conformational change and closing of the Tet-1 binding pocket (29). In our study, two additional mutated positions, C1192A and U1199C, were identified in groups of isolates with MICs of 2 to 8 and 2 to 4 μg/ml, respectively (data not shown). While the mutation U1199C itself may influence the binding of tetracycline to the Tet-1 site, mutation at position 1192 was shown to be associated with resistance to spectinomycin in *E. coli* (38, 39).

It is well documented that tetracycline resistance in *M. hominis* and *Ureaplasma* spp. with high MICs (≥32 μg/ml) is associated with the *tet*(M) determinant (5–7, 28, 40). However, tetracycline-susceptible *Ureaplasma urealyticum* and two *M. hominis* isolates harboring the *tet*(M) gene were recently identified (7, 40). While no mutations in the coding region of the *tet*(M) gene or in the promoter region were found in the tetracycline-susceptible *U. urealyticum* isolate, one of two *M. hominis* isolates had an insertion of insertion-like sequence IS*Mhom1* (IS*30* gene family) upstream to *tet*(M), which possibly caused the lack of *tet*(M) transcription in this isolate (7, 40). In this study, no *tet*(M), *tet*(O), or *tet*(L) determinants were found in any of the 70 *M. bovis* isolates. Several assumptions can explain such results: (i) it may be that tetracycline resistance in *M. bovis* is not acquired via *tet* determinants, and (ii) no *M. bovis* isolates with high MICs were identified and tested in this study, a fact that may have negatively influenced the chance to detect those genes. The existence of *M. bovis* isolates with high MICs (≥64 μg/ml) to different tetracyclines has been shown (summarized in reference [8]); however, no isolates with such high MICs were found in our study, and only 1/70 isolates had an MIC of 16 μg/ml (Table 1). The possible explanation for this discrepancy is that there are many difficulties in comparing results obtained from the different studies due to the lack of standardization in the performance of the susceptibility assays, which includes use of different methods, different control isolates, and different tetracyclines. More isolates with MICs of ≥16 μg/ml should be tested to confirm the results obtained in this study.

In summary, we have identified mutations in the primary binding pocket for tetracycline of clinical isolates of *M. bovis*, which correlated with decreased susceptibilities to tetracycline. Moreover, the data presented here demonstrate that the increase in the MICs for tetracycline (≥2 μg/ml) in *M. bovis* field isolates correlated with the number of nucleotide positions affected within the Tet-1 site of the *rrn3* and *rrn4* alleles.

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We declare no conflicts of interest.

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