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Rapid Detection of a Point Mutation in the *parC* Gene Associated with Decreased Susceptibility to Fluoroquinolones in *Mycoplasma bovis*▽

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Comparison of the quinolone resistance-determining regions (QRDRs) in 42 *Mycoplasma bovis* clinical isolates revealed amino acid substitutions at both GyrA (position 83) and ParC (position 84) in 10/11 enrofloxacin-resistant strains. The mutation present in the *parC* QRDR was discriminative for enrofloxacin resistance by *parC* PCR-restriction fragment length polymorphism. Comparison of molecular profiles by insertion sequence typing suggests that the currently prevalent enrofloxacin-resistant *M. bovis* strain evolved by selection under field conditions from one of the susceptible strains.

Fluoroquinolones are broad-spectrum antimicrobials highly effective for treatment of a variety of clinical and veterinary infections, including *Mycoplasma bovis* infection in cattle. Their antibacterial activity is due mainly to inhibition of DNA replication. Resistance can arise spontaneously due to point mutations that result in amino acid substitutions within the quinolone resistance-determining regions (QRDRs) of DNA gyrase subunits GyrA and GyrB and/or topoisomerase IV subunits ParC and ParE (5, 11, 12).

In the present study, 91 *M. bovis* strains isolated in Israel from cattle with pneumonia (82), bovine respiratory disease (3), mastitis (3), or arthritis (3) were examined for enrofloxacin susceptibility. These strains include 34 isolates for which geographic origin, clinical condition, and susceptibility profiles have previously been described (8) and an additional 57 *M. bovis* strains isolated in 2006 (26), 2007 (5), and 2008 (26) that were not characterized for susceptibility.

In vitro susceptibility for enrofloxacin (Vetranal) was determined by the broth microdilution method, according to the guidelines recommended by Hannan (10), as previously described (8). *M. bovis* was considered susceptible to enrofloxacin when the MIC was ≤0.25 μg/ml, intermediately susceptible with a MIC of 0.5 to 1 μg/ml, and resistant when the MIC was ≥2 μg/ml, according to the CLSI criteria for veterinary pathogenic bacteria (other than mycoplasmas) in cattle (19).

M. bovis genomic DNA was extracted from 10-ml logarithmic-phase broth cultures using the DNA isolation kit for cell/tissue (Roche). Amplification of the QRDR encoding regions was done with gene-specific primers designed on the basis of the sequences of the following genes in *M. bovis* strain PG45: *gyrA* (gyrA-F, 5′-GACGAATCATCTAGCGAG-3′, and gyrA-R, 5′-GCCTTCTAGCATCAAAGTAGC-3′); *gyrB* (gyrB-F, 5′-CCTTGTTGCCATTGTGTC-3′, and gyrB-R, 5′-CCATCGACATCAGCATCAGTC-3′); *parE* (parE-F, 5′-GGTACTCCTGAAGCTAAAAGTGC-3′, and parE-R, 5′-GAATATGTGCGCCATCAG-3′); and *parC* (parC-F, 5′-GAGCAACAGTTAAACGATTTG-3′, and parC-R, 5′-GGCATAACAACTGGCTCTT-3′). PCRs were performed in 50 μl ready-mix PCR master mix (ABGene, Surrey, United Kingdom) with 30 pmol/μl of each primer (Sigma) and about 100 ng DNA in an MJ Research PT200 thermocycler (Waltham, MA) as follows: 3 min at 95°C; 30 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 45 s; and 72°C for 10 min. The amplicons of 531 bp, 555 bp, 488 bp, and 502 bp, containing the QRDRs of *gyrA*, *gyrB*, *parC*, and *parE*, respectively, were purified from the gel using a QIAquick gel extraction kit (Qiagen, Germany). Sequencing was performed at the DNA Sequencing Unit, Weizmann Institute (Rehovot, Israel), utilizing the Applied Biosystems DNA sequencer with the ABI BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequence editing, consensus, and alignment construction were performed using Lasergene software, version 5.06/5.51, 2003 (DNAStar, Inc., Madison, WI), and the ExPASy Molecular Biology server (available at http://expasy.org/).

Initially, molecular characterization of the QRDRs of GyrA, GyrB, ParC, and ParE was performed for 34 previously described (8) clinical isolates of *M. bovis* differing in the levels of susceptibility to enrofloxacin (Table 1, numbers 1 to 34). Most nucleotide (nt) substitutions within the *gyrA*-amplified products were synonymous with only one substitution (C to T), resulting in an amino acid change from Ser to Phe at position 83 (*Escherichia coli* numbering). With two exceptions, this substitution appeared in all intermediate and resistant *M. bovis* strains tested (Table 1, numbers 24 to 38 and 40 to 42). However, three *M. bovis* isolates with MICs in the susceptible range (0.32 μg/ml) possessed Phe, and one strain with an intermediate MIC (0.63 μg/ml) contained Ser at position 83 (Table 1, numbers 20 to 22 and 23, respectively).

The predicted amino acid sequence of the GyrB QRDR revealed one nonsynonymous substitution (position 440, Val/Ile) in four susceptible *M. bovis* strains: 861, 8998, 2670, and 111-2 (data not shown). In addition, some diversity in the non-QRDR region of the *gyrB* amplicon, unrelated to suscep-

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TABLE 1. MIC and molecular characterization of the QRDR in clinical isolates of *M. bovis*

Isolate no.	Strain	MIC (μg/ml)[a]	Amino acid change in QRDRs[b]		IS major type (IS*Mbov3*/IS*Mbov4*)[d]
			GyrA (position 83)	ParC (position 84)	
	PG45	0.16	Ser (S)	Asp (D)	NT
1	330	0.08	Ser (S)	Asp (D)	I (A/1)
2	861	0.08	Ser (S)	Asp (D)	II (B/2)
3	8998	0.08	Ser (S)	Asp (D)	NT
4	2670	0.08	Ser (S)	Asp (D)	III (C/3)
5	111-2	0.16	Ser (S)	Asp (D)	IV (D/4)
6	3222	0.16	Ser(S)	Asp (D)	V (E/5)
7	4554	0.16	Ser (S)	Asp (D)	VI (A/6)
8	5180	0.16	Ser (S)	Asp (D)	NT
9	K	0.16	Ser (S)	Asp (D)	NT
10	2E	0.16	Ser (S)	Asp (D)	NT
11	2D	0.16	Ser (S)	Asp (D)	NT
12	H	0.16	Ser (S)	Asp (D)	NT
13	9249	0.16	Ser (S)	Asp (D)	VII (F/2)
14	6099	0.16	Ser (S)	Asp (D)	NT
15	9603	0.16	Ser (S)	Asp (D)	VIII (G/2)
16	476-2	0.16	Ser (S)	Asp (D)	NT
17	6226	0.32	Ser (S)	Asp (D)	NT
18	8934	0.32	Ser (S)	Asp (D)	NT
19	7910	0.32	Ser (S)	Asp (D)	IX (F/7)
20	1416	0.32	**Phe (F)**	Asp (D)	X (H/2)
21	7043	0.32	**Phe (F)**	Asp (D)	X (H/2)
22	2029	0.32	**Phe (F)**	Asp (D)	X (H/2)
23	170	0.63	Ser (S)	Asp (D)	NT
24	77	0.63	**Phe (F)**	Asp (D)	X (H/2)
25	3179	0.63	**Phe (F)**	Asp (D)	X (H/2)
26	2643	1.25	**Phe (F)**	Asp (D)	X (H/2)
27	5848	1.25	**Phe (F)**	Asp (D)	X (H/2)
28	2458	1.25	**Phe (F)**	Asp (D)	X (H/2)
29	3036	1.25	**Phe (F)**	Asp (D)	X (H/2)
30	3374	1.25	**Phe (F)**	Asp (D)	X (H/2)
31	1306	1.25	**Phe (F)**	Asp (D)	X (H/2)
32	1972	2.5	**Phe (F)**	**Asn (N)**	XI (H/8)
33	3181	2.5	**Phe (F)**	**Asn (N)**	X (H/2)
34	8830	5	**Phe (F)**	**Asn (N)**	X (H/2)
35	4243[c]	2.5	**Phe (F)**	**Asn (N)**	VII (F/2)
36	1665[c]	2.5	**Phe (F)**	**Asn (N)**	XII (A/4)
37	4241[c]	2.5	**Phe (F)**	**Asn (N)**	VII (F/2)
38	0523[c]	2.5	**Phe (F)**	**Asn (N)**	VII (F/2)
39	4925[c]	2.5	Ser (S)	**Asn (N)**	XIII (A/7)
40	9771[c]	5	**Phe (F)**	**Asn (N)**	X (H/2)
41	0962[c]	5	**Phe (F)**	**Asn (N)**	XIV (I/7)
42	4879[c]	5	**Phe (F)**	**Asn (N)**	XV (I/4)

[a] MIC was obtained by broth microdilution method.
[b] *E. coli* numbering. Amino acid (nt) changes from Ser to Phe at position 83 in the GyrA QRDRs and from Asn to Asp at position 84 in the ParC QRDRs are shown in bold.
[c] Enrofloxacin-resistant *M. bovis* strains identified by *parC* PCR-RFLP analysis.
[d] NT, not tested.

tibility, was determined (data not shown). No amino acid substitutions were found within the QRDR of ParE.

Comparison of the ParC QRDRs revealed the presence of an Asn/Asp substitution at position 84, resulting from the change of nt G to A at position 265 of the *parC* amplicon (corresponding to positions 250 and 283 of the *E. coli* and *M. bovis parC* genes, respectively) in all *M. bovis* enrofloxacin-resistant strains and only in these strains (Table 1, numbers 32 to 34). Restriction enzyme site analysis revealed that this G-to-A nt substitution generates the restriction site for PsiI (T/TATAA) at nt 262 to 266. Restriction of the amplicon of resistant strains with PsiI (*parC* PCR-restriction fragment length polymorphism [RFLP]) yielded the predicted 262- and 226-bp fragments that are readily distinguished in a standard agarose gel from the 488-bp nonrestricted amplicon of sensitive *M. bovis* field isolates (data not shown).

Analysis of genomic DNAs of the cohort of *M. bovis* clinical isolates with unknown enrofloxacin susceptibility by *parC* PCR-RFLP resulted in 8/57 strains restricted by *parC* PsiI (Table 1, numbers 35 to 42) and possessing the same amino acid substitutions observed with the three previously identified enrofloxacin-resistant strains (Table 1, numbers 32 to 34). In parallel, preliminary screening of the cohort of 57 *M. bovis* clinical isolates for enrofloxacin resistance was performed on agar containing enrofloxacin at a concentration above the susceptibility cutoff value, according to recommendations for the agar dilution method (10). For the test, 5-μl aliquots of each *M. bovis* culture, containing 10^4 to 10^5 color-changing units/ml, were spotted according to a standard pattern on an agar plate containing 2.5 μg/ml of enrofloxacin or no antibiotic. After 5 days of incubation at 37°C in the presence of CO_2, growth was determined microscopically. Two strains with MICs determined in the microbroth test (strain 8830 with a MIC of 5 μg/ml and strain 330 with a MIC of 0.08 μg/ml) were used as positive and negative controls, respectively. Comparison of growth levels between plates with and without antibiotics identified 8/57 isolates that grew in the presence of 2.5 μg/ml of enrofloxacin; the same isolates were restricted with PsiI in the *parC* PCR-RFLP test (Table 1, numbers 35 to 42, respectively). The MICs of these isolates were 2.5 to 5 μg/ml by the conventional microbroth method. According to criteria of the CLSI (19), all eight *M. bovis* strains containing a PsiI site within the QRDR of the *parC* amplicon were resistant to enrofloxacin (Table 1, numbers 35 to 42).

The genetic variability of *M. bovis* isolates in this study was assessed by insertion sequence (IS) hybridization profiles, as previously described (15, 18). Primers (Mbov-3-F, 5′-GGTGG TTTGATATACAAAACT-3′, and Mbov-3-R, 5′-GGACGAA GAGATAATTTACC-3′; and Mbov-4-F, 5′-CCTAGCACTG GCGAAATA-3′, and Mbov-4-R, 5′-CCTCTAATGAAAGGT CAAC-3′) were used to amplify *M. bovis* PG45 genomic fragments corresponding to IS*Mbov3* and IS*Mbov4* elements (15, 23). The amplification parameters were 95°C for 3 min; 30 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 1 min; and 72°C for 10 min. The IS-specific probes were labeled by digoxigenin-11-dUTP using the PCR DIG probe synthesis kit (Roche) according to the manufacturer's instructions and were used in Southern blot analysis of genomic DNAs digested with 10 U HindIII restriction enzyme (Fermentas). IS pattern analysis was by visual comparison using exposed X-ray film (Fujifilm). Isolates were considered identical if the major band patterns were the same with both probes.

Overall, among the 33 representative *M. bovis* field isolates tested, nine IS*Mbov3*-related types, arbitrarily designated A

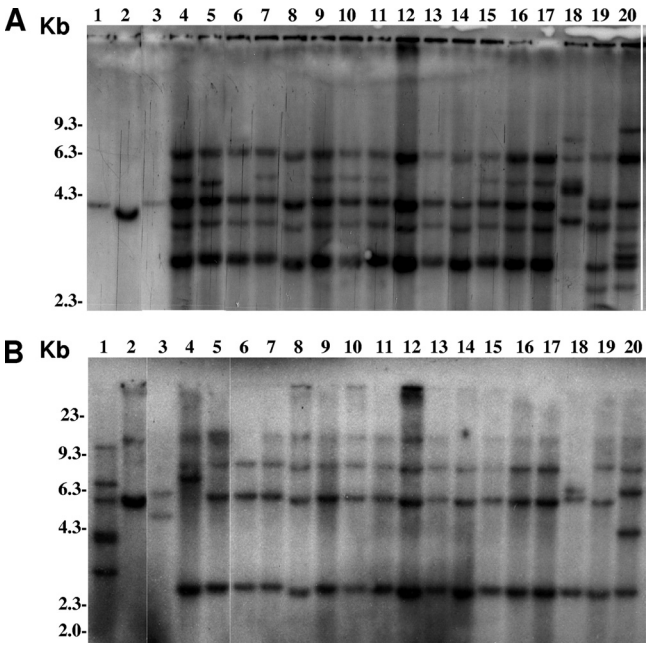

FIG. 1. Representative IS profiles of *M. bovis* enrofloxacin-resistant and -susceptible strains. Hybridization patterns obtained by IS*Mbov3* (A) and IS*Mbov4* (B) elements are presented. Molecular size markers are indicated on the left of each panel. *M. bovis* strains used in lanes 1 to 20 are 330, 111-2, 4554, 1972, 3181, 7043, 5848, 3179, 1306, 8830, 3374, 1416, 2029, 77, 2458, 3036, 2643, 81, 9603, and 7910. The predominant IS hybridization profile X (H/2), found in three, eight, and three *M. bovis* strains that were resistant, intermediately susceptible, and susceptible, respectively, to enrofloxacin, can be seen in lanes 5 to 17.

through I, and eight IS*Mbov3*-related types, designated 1 to 8, were distinguished, respectively (Fig. 1 and Table 1). Fifteen major molecular types (I to XV) were identified, based on the composite of the IS*Mbov3* and IS*Mbov4* patterns (Table 1). Five molecular types (XI to XV) were uniquely present in the cohort of *M. bovis* enrofloxacin-resistant strains. However, the predominant IS hybridization profile was X (H/2), present in 13/33 *M. bovis* strains, including two isolates susceptible to enrofloxacin, eight isolates with intermediate susceptibility, and three resistant strains (Table 1).

The data presented here suggest that a change in GyrA (at position 83, Ser to Phe) is sufficient to achieve an intermediate level of susceptibility to fluoroquinolone, but a concurrent modification in the ParC protein (at position 84, Asp to Asn) is required for resistance. Previous studies have reported hot spots for amino acid substitutions at GyrA Ser-83 and ParC Asp-84 in other bacteria and mycoplasmas (2, 3, 7, 14, 16, 22, 25).

Today, in vitro susceptibility testing of mycoplasma isolates is not routinely performed. Conventional antimicrobial susceptibility testing by broth microdilution, agar dilution, or Etest methods (9, 10) requires isolation of mycoplasmas in pure culture, requiring 2 to 3 weeks. In this context, the development of a rapid genetic assay for detection of mycoplasma resistance may be attractive. Several studies have described the use of different genetic methods, such as PCR-RFLP analysis (1, 6, 13, 20), PCR-oligonucleotide ligation (4), and a real-time PCR assay (21, 24), for rapidly screening key gene mutations associated with fluoroquinolone resistance in various microorganisms. However, the only molecular/genetic methods reported for mycoplasmas are for detection of macrolide resistance, and nothing has been reported for fluoroquinolones (17, 26). The PCR-RFLP described here is a simple and rapid method for the detection of fluoroquinolone-resistant *M. bovis* strains and can be carried out as a routine assay in a diagnostic laboratory.

Results of the molecular typing suggest that the currently prevalent enrofloxacin-resistant *M. bovis* strain evolved by selection under field conditions from one of the susceptible strains. However, this apparently was not a unique occurrence, as evidenced by the presence of resistant strains with different IS molecular patterns.

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