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Alterations in the Quinolone Resistance-Determining Regions and Fluoroquinolone Resistance in Clinical Isolates and Laboratory-Derived Mutants of *Mycoplasma bovis*: Not All Genotypes May Be Equal

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***Mycoplasma bovis* is considered a major contributor to respiratory diseases in young cattle. Resistant *M. bovis* isolates have increasingly been reported worldwide due to extensive use of antimicrobials to treat bovine pneumonia. The frequency of isolates resistant to fluoroquinolones varies considerably from one country to another. The MICs of isolates collected in France have only increased from "very low" to "low." The present study was conducted to investigate whether alterations in the quinolone resistance-determining regions (QRDRs) could account for this slight modification in susceptibility. No correlation between QRDR alterations and increased MICs was evidenced in clinical isolates. In addition, all clinical isolates were subtyped, and the tendencies of the different sequence types to develop resistance through mutations in QRDRs under selective pressure *in vitro* were examined. *In vitro*, 3 hot spots for mutations in QRDRs (position 83 in GyrA and positions 80 and 84 in ParC) were associated with a high level of resistance when cumulated. We showed that the point mutations in the QRDRs observed *in vitro* were different (in location and selection rapidity) between the different subtypes. Our *in vitro* observations were corroborated by the recent detection of a clinical isolate highly resistant to fluoroquinolones (MIC ≥ 16 μg/ml) and belonging to the subtype which easily accumulates QRDR alterations *in vitro*. The current increased prevalence of this subtype in clinical isolates highlights the urgent need to control fluoroquinolone usage in veterinary medicine.**

Mycoplasma bovis is a wall-less bacterium responsible for severe infections in cattle, including pneumonia, mastitis, arthritis, and otitis (1). In young cattle, it is now recognized as a major contributor to economic losses associated with bovine respiratory diseases (BRD). The infection pressure of BRD usually peaks 2 to 3 weeks after calves are mingled in fattening units following transportation from their respective birth farms (2). Since no efficient vaccines are available and licensed for use outside the United States (3), efforts to control *M. bovis* infections often rely on antimicrobial treatments, administered either prophylactically or in the early stages of the disease. Antimicrobials used for the treatment or prevention of BRD usually include broad-spectrum cephalosporins (cefquinome and ceftiofur), extended-spectrum fluoroquinolones (enrofloxacin, danofloxacin, and marbofloxacin), florfenicol, and long-lasting macrolides (tulathromycin, gamithromycin, and tildipirosin) (4). This extensive use of antibiotics has predictably resulted in an increase in resistant isolates over time. In France, it was recently shown that contemporary *M. bovis* strains had become significantly less susceptible than archival strains to 9 of the 12 antimicrobials tested (5). With regard to fluoroquinolones, the decrease in susceptibility was limited (only 1 dilution of the MIC), and no highly resistant isolates were observed in a set of more than 90 strains. This was unexpected, since one of the two major medical indications of injectable extended-spectrum fluoroquinolones for cattle is BRD (6). However, in contrast to other antimicrobials, such as tulathromycin, tilmicosin, ceftiofur, and florfenicol, expanded-spectrum fluoroquinolones are not approved for metaphylaxis or prevention in herds (2, 7). This restricted use could have limited the acquisition of resistance in France. Moreover, we recently demonstrated that recent *M. bovis* isolates from France belong to the same unique subtype and suggested that this monoclonal spread on a country-wide scale could be linked to the acquisition and selection of multiresistance through therapeutic practices and prevention strategies (8). The reason for fluoroquinolones being spared in this selection process has yet to be clarified.

Elsewhere in the world, results for *M. bovis* susceptibility to fluoroquinolones are very varied. Three studies reported highly resistant isolates, with MICs of >8 μg/ml, in the United Kingdom, Japan, and Belgium (9–11), while several others indicated a low to no increase in resistance, except for a few isolates, in Japan, the United States, and several European countries (6, 12–15). Some of these studies involved isolates collected before 2005 (11, 13), but the majority referred to recent ones (6, 9, 10, 12, 14, 15), suggesting a recent phenomenon. In the United Kingdom, a shift of resistance from an MIC_{90} of 1 μg/ml to one of 32 μg/ml occurred during the last decade (9, 16). This significant increase over a

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relatively short period is indicative of highly efficient resistance mechanisms. Quinolones are synthetic bactericidal agents that are able to eliminate actively dividing bacteria by inhibiting the topoisomerases II and IV required for DNA replication. In mycoplasmas, resistance to fluoroquinolones generally results from several alterations in the so-called quinolone resistance-determining regions (QRDRs) of the genes encoding topoisomerases, namely, *gyrA*, *gyrB*, *parC*, and *parE* (17). No alterations related to resistance to fluoroquinolones have been found so far in *gyrB* or *parE* in any clinical isolates of mycoplasmas (17). In contrast, two hot spots for mutations, in ParC and GyrA, were described for *M. bovis* isolates and were associated with large increases of the MICs (2.5 to 16 μg/ml). These were the Asp84Asn substitution in ParC associated or not with the Ser83Phe mutation in GyrA (10, 12). Although target mutations are the main mechanisms conferring resistance to fluoroquinolones, an active efflux system has been described for the human pathogen *M. hominis* and suggested for another ruminant mycoplasma, *M. mycoides* subsp. *capri*, but has so far not been reported for *M. bovis* (18–20).

The present study was conducted to examine the presence of potential alterations in the QRDRs of *M. bovis* clinical isolates collected in France and whether these could account for the previously observed decrease in susceptibility to fluoroquinolones. In addition, clinical isolates were subtyped by single-locus sequence typing (8) and multilocus sequence typing (MLST) (21), and their ability to become resistant to fluoroquinolones through mutations of their QRDRs was explored *in vitro*. The results were analyzed in order to determine whether the process and rapidity of developing resistance to fluoroquinolones under selective pressure may differ by subtype.

MATERIALS AND METHODS

Mycoplasma isolates, growth, identification, and subtyping. Fifty-two French *M. bovis* clinical isolates were included in this study (see Table S1 in the supplemental material). They originated from a collection maintained at ANSES Lyon and mostly derived from the French national surveillance network for mycoplasmoses of ruminants (VIGIMYC) (22). They were identified by membrane filtration dot-immunobinding tests (23) and by a species-specific PCR assay targeting the *polC* gene (24). Strain PG45^T and two isolates from neighboring Switzerland (25) were added as controls. Isolates were grown in PPLO broth, modified as previously described (26), at 37°C in 5% CO_2. Each isolate was subtyped by single-locus sequence typing (using a 486-bp region of the *polC* gene), which has been shown to provide relevant typing results (8). Clustering of the isolates was confirmed by applying the MLST scheme of Register et al. (21). In brief, the *adh-1*, *gltX*, *gpsA*, *gyrB*, *pta-2*, *tdk*, and *tkt* loci were amplified by PCR and sequenced. A polyfasta file comprising the 7 alleles was generated for each isolate and uploaded into a newly created MLST database (http://pubmlst.org/mbovis/) to be assigned a subtype. Individual subtypes were compared to those already present in the database.

MIC assays. The MICs of enrofloxacin (ENR), danofloxacin (DAN), and marbofloxacin (MAR), all purchased from Sigma, were determined using the agar dilution method on modified PPLO agar as previously described (5, 27). In brief, 1-μl aliquots of each strain, diluted to 10^4 to 10^5 CFU/ml, were spotted onto agar plates containing serial 2-fold dilutions of each fluoroquinolone (0.125 to 64 μg/ml). MIC assays were performed at least twice for each isolate. The MIC was defined as the lowest fluoroquinolone concentration causing 100% inhibition of growth at 37°C in 5% CO_2 for 72 h. The number of CFU per milliliter was determined by plating 2-μl aliquots of serial 10-fold broth dilutions onto agar plates. After incubation for 3 days, the colonies for several dilutions were counted using a stereomicroscope, and the mean final cell concentration was determined.

Selection of spontaneously ENR-resistant *M. bovis* variants. ENR-resistant clones were selected by plating 50-μl aliquots of parental cultures at 10^7 to 10^8 CFU/ml onto agar medium containing increasing inhibitory concentrations of enrofloxacin as described elsewhere (10, 28), with minor modifications. Each selection experiment involved four steps, using ENR concentrations of 1, 2, 4, and 8 times the MIC for the respective parent isolate (see Fig. 1 for details). At each selection step, resistant colonies were picked from the agar plates after incubation for 7 days and recultured in broth medium containing an equivalent amount of enrofloxacin before being inoculated onto another plate with twice that antimicrobial concentration. The recovery frequency was determined as the number of colonies appearing on the plate in the presence of enrofloxacin divided by the number of colonies contained in the inoculum. Several individual colonies were picked from the agar plate at each step in order to (i) analyze their QRDRs and (ii) determine their MICs.

PCR amplification and sequence analysis of *gyrA*, *gyrB*, *parE*, and *parC* QRDRs. Genomic DNAs were extracted from 20-ml logarithmic-phase broth cultures of *M. bovis* by using the phenol-chloroform method (29). QRDRs were amplified using previously described specific primers (12). For *parE*, the hybridization temperature in the original publication was modified from 56°C to 54°C. PCR products were sequenced using an external facility at Beckman Coulter Genomics. Sequence editing, consensus, and alignment construction were performed using Seaview software (http://doua.prabi.fr/software/seaview). For convenience, the amino acid numbering refers to the *Escherichia coli* numbering and is based on the *E. coli* K-12 sequences for GyrA (AAC75291.1), GyrB (AAT48201.1), ParC (AAC76055.1), and ParE (AAA69198.1).

RESULTS

An initial set of 31 clinical isolates of *M. bovis*, either old (1978 to 1983) or recent (2009 to 2012), was randomly chosen from our collection. These isolates were then subtyped by sequencing of the *polC* locus as previously described (8). As expected, all old strains and PG45^T were homogeneously grouped into subtype 1 (ST1), while most (19/20 strains) of the recent strains were subtype 2 (ST2), which diverges from ST1 by one single nucleotide polymorphism (SNP) in the 486-bp *polC* locus. One of the recent isolates (strain 15527) belonged to ST3, an uncommon subtype characterized by 16 SNPs (see Table S1 in the supplemental material) (8).

The susceptibilities of the isolates to 3 fluoroquinolones, namely, enrofloxacin (ENR), danofloxacin (DAN), and marbofloxacin (MAR), were determined according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI), using the agar dilution method for MIC estimation (27). *M. bovis* PG45^T was used as a control. Moderate increases in the MICs, i.e., 2-fold for ENR and 4-fold for DAN and MAR, were observed for all recent isolates in comparison to PG45^T and the old isolates (Table 1). In contrast, 2 recent isolates from Switzerland were shown to be susceptible. These results are consistent with those of a previous study (5) and suggest an ongoing shift of isolates from the *M. bovis* population in France toward a low-level, non-clinically relevant quinolone resistance phenotype.

In order to determine whether this shift was associated with mutations in the quinolone resistance-determining regions (QRDRs), we then sequenced the *gyrA*, *gyrB*, *parC*, and *parE* QRDRs. Apart from a silent mutation in ParC (position 84; GAC → GAT; Asp), all recent isolates harbored a single amino acid substitution in GyrB (Asp362Asn) that was absent from all old strains and PG45^T. This mutation had already been described and

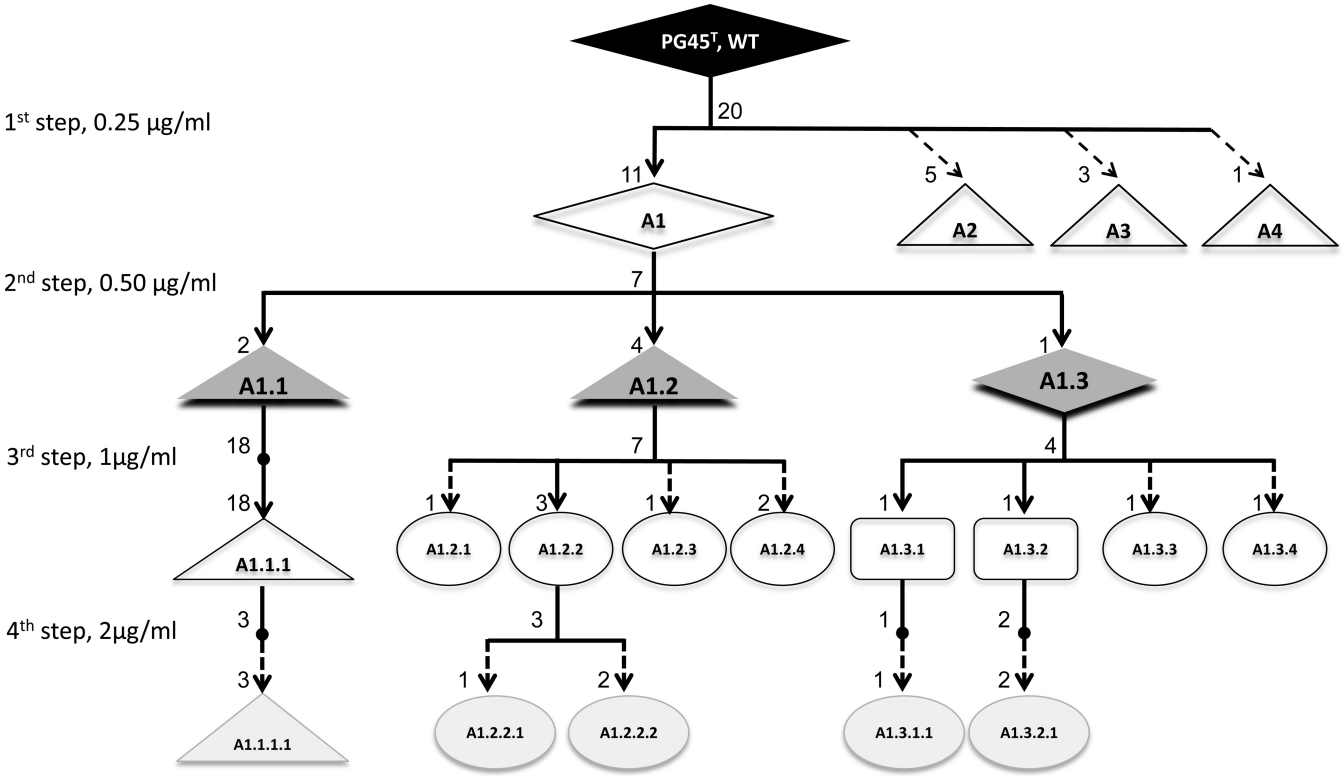

FIG 1 Experimental design of *in vitro* selection assays with enrofloxacin, using PG45^T as an example. The selected clones are named by a letter followed by incremental numbers corresponding to successive experimental steps. The diamond symbols represent clones with no mutation, while clones with alterations in ParC, GyrA, and both loci are shown as triangles, squares, and circles, respectively. The numbers at nodes and arrow extremities indicate the number of clones isolated for each step and their distribution within the different genotypes. The enrofloxacin concentration for each step is indicated on the left. MIC assays were performed for all PG45^T-derived clones except A2, A3, and A4. Dotted arrows indicate experimental steps with no further selection passages.

linked to the recent *M. bovis* subtype ST2 (8). However, this mutation was also present in recent susceptible isolates from Switzerland and has never been described for highly resistant strains, so it is unlikely to be associated with resistance. No mutations in GyrA and ParE were evidenced, and only three isolates showed single mutations in ParC, either in codon 80 (Ser → Ile) or in codon 84 (Asp → Tyr), but without any marked difference in their MICs. Hence, the observed shift in fluoroquinolone susceptibility was not associated with mutations in QRDRs.

We also investigated whether clinical isolates were equally able to achieve higher, clinically relevant MICs through the acquisition of mutations in their QRDRs *in vitro*. The following three isolates, representing different genomic backgrounds (subtypes and QRDR genotypes), were chosen for the *in vitro* selection process: PG45^T

TABLE 1 Relationships between MIC values and mutations in *gyrA*, *gyrB*, *parC*, and *parE* QRDRs of *M. bovis* clinical isolates

Isolate name or characteristic	Origin, no. of isolates, yr of isolation	MIC (value or range) (μg/ml)[a]			QRDR-encoded substitution[b]				
					No. of isolates with phenotype/total no. of isolates	GyrA	GyrB	ParC	
		ENR	DAN	MAR		Ser83	Asp362	Ser80	Asp84
PG45^T (control)	USA, 1, 1962	0.25	0.25	0.5	1/1				
Old	France, 11, 1978–1983	0.125–0.25	0.125–0.25	0.25–1	11/11				
Recent	France, 20, 2009–2012	0.25–0.5	0.5–1	0.5–2	17/20		Asn		
		0.5	1	2	1/20		Asn	Ile[d]	
		0.5–1	0.5–1	2[c]	2/20		Asn		Tyr[d]
Contemporary[e]	France, 21, 2013–2014	0.25–0.5	0.5–1	0.5–2	20/21		Asn		
		16	**16**	**>64**	**1/21**	**Phe[d]**	**Asn**	**Ile[d]**	
Control	Switzerland, 2, 2010–2011	0.125[c]	0.25[c]	0.5[c]	2/2		Asn		

[a] ENR, enrofloxacin; DAN, danofloxacin; MAR, marbofloxacin.
[b] The genotype of PG45^T was used as a reference, and *E. coli* amino acid numbering was used. There were no mutations in ParE.
[c] Same MIC values for both strains.
[d] Mutation already described for *M. bovis* isolates in other countries (10, 12).
[e] Data in bold refer to the recently detected clinical isolate with high-level resistance to fluoroquinolones.

for subtype ST1, 15762 for ST2, and 15527 for ST3 (Table 2). In addition, two isolates belonging to ST2 and showing native alterations in ParC compared to 15762 were also selected (15488 and 15875). Laboratory-generated mutants were obtained by stepwise exposure to increasing concentrations of ENR, as illustrated in Fig. 1 for PG45^T.

The first selection step consisted of plating the wild-type (WT) isolate onto agar medium containing ENR at a concentration corresponding to its MIC. Table 2 shows the frequencies at which ENR-resistant clones were recovered and the distribution of randomly selected colonies between the different types of QRDR mutants. In general, the recovery frequency of ENR-resistant clones was low (6×10^{-7} to 2×10^{-5}) regardless of the parental subtype. Eight different amino acid substitutions were identified as being encoded in the QRDRs of mutants: five in GyrA, three in ParC, and none in GyrB. No ParE sequences were analyzed, since previous studies had shown that they were seldom altered and that the rare mutations obtained were not associated with fluoroquinolone resistance (10, 12, 17). Two mutations (Ser83Phe in GyrA and Asp84Asn/Gly in ParC) recurred in several mutants derived from different parental isolates. The lowest rate of mutation acquisition was observed for isolate 15762 (ST2), with no mutation at all during the 1st selection step, and also for PG45^T (ST1), for which 45% of the selected colonies were mutated (clones A2, A3, and A4 in Fig. 1). Another ST2 isolate, 9072, was also included in the first step of ENR selection but failed to grow under antibiotic pressure, despite repeated attempts. Since the 2nd step of *in vitro* selection was carried out before sequences were available, many of the generated ENR-resistant colonies were not put into broth culture and hence were not available for susceptibility tests. However, several of the selected ENR-resistant colonies showed 2- to 32-fold increases in the fluoroquinolone MICs (Table 2). Resistant colonies that had not acquired any mutations, such as strains PG45^T (clone A1 in Fig. 1) and 15762, showed at most a 2-fold increase in the MIC. In contrast, mutants with alterations in both GyrA and ParC, the latter either inherited from parental strains (isolates 15488 and 15875) or acquired during the selection process (isolate 15527 and clones of genotype 2 [GT2] and GT3), attained MICs as high as 64 μg/ml.

Four ENR-resistant clones with different genotypes (subtypes and QRDRs) were selected during the 1st step for further selection. These were clones of GT1 from PG45^T (ST1), GT4 from 15762 (ST2), and GT2 and GT3 from 15527 (ST3) (Table 2).

Additional point mutations were identified in GyrA and ParC during the successive selection steps (Table 3; Fig. 1). The substitutions varied, but their positions in the QRDRs were always the same. The most frequently altered codons were those for Ser83 in GyrA and Asp84 in ParC, and their concomitant mutation was systematically associated with high MICs (Table 3). Another mutation in ParC, namely, Ser80Ile, was associated with a high MIC for PG45 mutants (ST1). The Gly81Asp/Asn mutation in GyrA was observed after the 3rd and 4th selection steps, and only in PG45 mutants. Interestingly, the frequencies of recovery of ENR-resistant clones varied only slightly (10^{-6} to 10^{-4}) for PG45^T (ST1) and 15762 (ST2), regardless of the selection step, but varied a lot (10^{-7} to 1) for clones derived from 15527 (ST3). More specifically, when the selection pressure was lower than the MIC attained in the 1st selection step, no new mutation was acquired and, as expected, the recovery frequency reached about 1. Furthermore, once the two key mutations (GyrA Ser83Phe and ParC Asp84Asn) had been acquired, the enrofloxacin concentrations could be increased rapidly without influencing the mutant's ability to grow. The switch from Asn to Lys at position 84 of ParC, which corresponds to a change of the amino acid R-group charge from neutral to positive, contributed to further increasing the MIC. In contrast, for isolates belonging to the ST2 subtype, it was either impossible (9072) (data not shown) or difficult (15762) to maintain viable mycoplasma cells under increasing selective pressure, and no mutation in ParC was acquired over the whole selection process.

Because the ability to develop resistance to fluoroquinolones through mutations in the QRDRs under selective pressure *in vitro* was different for the different subtypes, we investigated the evolution of clinical isolates by screening a more recent set of isolates. The susceptibilities to fluoroquinolones and the subtypes of 21 clinical isolates collected in 2013 and 2014 were determined (Table 1; see Table S1 in the supplemental material). The poorly abundant subtype ST3, which represented only 5% of the recent (2009 to 2012) *M. bovis* population, was found to have become more prevalent (20%) in the contemporary (2013 and 2014) *M. bovis* population. The MIC distributions were identical to those for the 2009-2012 isolates, except for one isolate, namely, 8428, that was shown to be highly resistant to all 3 fluoroquinolones (MICs of ≥16 μg/ml). These high MIC values were associated with the Ser83Phe mutation in GyrA and the Ser80Ile mutation in ParC, i.e., two of the mutations that had been observed in mutants selected *in vitro*. Furthermore, this isolate was demonstrated to be of the ST3 molecular subtype (see Table S1).

The clustering of isolates into different subtypes based on single-gene polymorphism (*polC*) was further confirmed using the MLST scheme proposed by Register et al. (21). The *polC*-defined ST1, ST2, and ST3 subtypes were grouped under ST17, ST18, and ST5, respectively, in the MLST tree of Register et al., with ST17 and ST18 belonging to the same clade, thereby confirming the distribution of isolates between different genotypes. Moreover, another isolate from the ST3 subtype, namely, 8619, was randomly chosen to confirm our *in vitro* selection results obtained with isolate 15527, originally chosen as representative of ST3. Twenty percent of the isolate 8619 colonies selected in the 1st step were shown to have gained a mutation in GyrA (Ser83Phe) (Table 2) associated with a moderate increase in MICs. In the 3rd selection step, as soon as some of the ParC codons had been altered (Ser80Ile or Asp84Gly/Asn/Tyr), the MICs reached very high values. This result confirms the ease with which ST3 isolates acquire and select key point mutations in QRDRs that lead to a high level of resistance to fluoroquinolones.

DISCUSSION

Most often, a low level of resistance to fluoroquinolones, associated with a narrow MIC distribution in the range of 0.25 to 4 μg/ml, is considered to represent a single homogeneous population of isolates (6). However, in the French *M. bovis* population, the MIC distribution of enrofloxacin, in the range of 0.125 to 1 μg/ml, was previously shown to be bimodal, with a very moderate shift of MICs between old and recent isolates (5). This shift was further associated with a modification of the molecular subtype from ST1 (old isolates) to the predominant subtype ST2 (recent isolates) (8). In the present work, we confirmed the existence of two groups of clinical isolates with different MIC levels. Old strains, isolated between 1978 and 1983, were highly susceptible

TABLE 2 Characterization of the 1st-step enrofloxacin-resistant clones selected *in vitro*

Isolate (ST)	Resistant colonies[a]			QRDR mutation at indicated position[b]							Genotype	MIC (μg/ml)[c]		
				GyrA			GyrB	ParC						
	ENR concn (μg/ml)	Recovery frequency (no. of clones)	Mutation distribution (no. of clones with mutation/total no. of clones)	81	83	87	362	80	84	98		ENR	DAN	MAR
PG45 (ST1)				Gly	Ser	Glu	Asp	Ser	Asp	Thr	WT	0.25	0.25	0.5
	0.25	2E−05 (20)	11/20								GT1	0.25	1	0.5
	0.25		5/20						Gly			NA	NA	NA
	0.25		3/20						Asn			NA	NA	NA
	0.25		1/20						Asn	Arg		NA	NA	NA
15762 (ST2)				Gly	Ser	Glu	**Asn**	Ser	Asp	Thr	WT	0.25	0.5	1
	0.25	3E−06 (20)	20/20								GT4	0.25	0.5	1
15488 (ST2)				Gly	Ser	Glu	**Asn**	**Ile**	Asp	Thr	WT	0.5	1	2
	2	6E−07 (5)	3/5		Phe							16	16	>64
	2		1/5		Tyr							NA	NA	NA
	2		1/5			Gly						NA	NA	NA
15875 (ST2)				Gly	Ser	Glu	**Asn**	Ser	**Tyr**	Thr	WT	1	1	2
	2	4E−07 (4)	3/4			Lys						4	8	8
	2		1/4		Phe							4	8	8
15527 (ST3)				Gly	Ser	Glu	**Asn**	Ser	Asp	Thr	WT	0.5	0.5	2
	0.5	7E−05 (12)	1/12									NA	NA	NA
	0.5		6/12			Val						NA	NA	NA
	0.5		1/12			Val			Gly			NA	NA	NA
	0.5		2/12		Phe							NA	NA	NA
	0.5		1/12		Phe				Asn		GT2	4	4	8
	0.5		1/12		Phe				Asn	Arg	GT3	4	2	8
8619 (ST3)				Gly	Ser	Glu	**Asn**	Ser	Asp	Thr	WT	0.25	0.5	0.5
	0.25	2E−06 (10)	8/10									NA	NA	NA
	0.25		2/10		Phe						GT5	1	2	1

[a] Resistant colonies were selected on plates containing enrofloxacin at the indicated concentrations. The recovery frequency was determined as the number of colonies appearing on the plate in the presence of enrofloxacin divided by the number of colonies contained in the inoculum.

[b] Point mutations detected in QRDRs of *gyrA*, *parC*, and *gyrB* are presented as the corresponding amino acid substitutions in individual clones. For each isolate, the WT phenotype is shown first. The GT1, GT2, GT3, and GT4 genotypes were used to perform further selection steps. When the phenotype of an isolate differs from that of PG45^T, the mutation is indicated in bold. Newly acquired mutations are underlined.

[c] MIC of an ENR-resistant mutant representing the genotype. NA, not applicable (mutant not available in broth culture).

TABLE 3 Characteristics of ENR-resistant clones obtained *in vitro* under increased selective pressure, point mutations in QRDRs, and MIC values

Clone[a]	ENR concn (μg/ml)[b]	Mutation distribution[c]			Mutation at indicated position						MIC (μg/ml)[d]		
					GyrA			ParC					
		2nd step	3rd step	4th step	Gly81	Ser83	Glu87	Ser80	Asp84	Thr98	ENR	DAN	MAR
PG45 (GT1 [ST1], GyrB Asp362, 0.25)	0.5	2/7						Ile			4	8	8
	1		18/18					Ile			4	8	16
	2			3/3				Ile			8	8	16
	0.5	4/7							Tyr		1	2	2
	1		1/7			Tyr			Tyr		4	8	8
	1		2/7			Lys			Tyr		4	8	4
	1		1/7			Phe			Tyr		4	8	8
	1		3/7*		Asp				Tyr		4	8	4
	2			1/3	Asn				Tyr		4	8	4
	2			2/3	Asp				Tyr		4	4	4
	0.5	1/7									1	2	1
	1		1/4*			Tyr					2	2	4
	1		1/4*			Phe					1	2	2
	1		1/4			Tyr			Tyr		4	8	8
	1		1/4			Phe			Tyr		4	8	8
	2			1/1		Tyr		Ile			16	16	16
	2			2/2		Phe			Tyr		16	16	16
15762 (GT4 [ST2], GyrB Asn362, 0.25)	0.5	2/7					Lys				ND	ND	ND
	0.5	1/7									0.5	1	2
	0.5	4/7*									0.5	1	2
	1		5/8			Tyr					ND	ND	ND
	1		1/8			Phe					ND	ND	ND
	1		1/8				Lys				ND	ND	ND
	1		1/8								ND	ND	ND
15527 (GT2 [ST3], GyrB Asn362, 4)	1	12/12				Phe			Asn		8	8	16
	2		36/36			Phe			Asn		ND	ND	ND
	8–16			5/6		Phe			Asn		16	8	16
	8–16			1/6		Phe			Lys		32	8	16
15527 (GT3 [ST3], GyrB Asn362, 4)	1	12/12				Phe			Asn	Arg	8	8	16
	2		36/36			Phe			Asn	Arg	ND	ND	ND
	8–16			1/2		Phe			Asn	Arg	16	8	16
	8–16			1/2		Phe			Lys	Arg	16	16	32
8619 (GT5 [ST3], GyrB Asn362, 1)[e]	0.5	22/22				Phe					1	2	2
	1		3/4			Phe					ND	ND	ND
	1		1/4*			Phe		Ile			32	16	>64
	2			2/2		Phe		Ile			32	16	>64
	0.5	22/22				Phe					1	2	2
	1		12/28			Phe					ND	ND	ND
	1		2/28			Phe			Gly		8	8	16
	1		4/28*			Phe			Asn		8	8	16
	1		10/28*			Phe			Tyr		8	8	16
	2			2/2		Phe			Asn		8	8	16
	2			2/2		Phe			Tyr		8	8	16

[a] Clones are presented by the name of the parental strain, the subtype, the GyrB phenotype, and the enrofloxacin MIC (μg/ml) at the 1st selection step.
[b] Concentration of ENR used to select the different resistant clones during individual passages.
[c] Asterisks indicate clones used to perform the following selection step.
[d] MICs were evaluated on at least one clone of the selected genotype. When no clone was available in broth culture, MIC assays were not performed. ND, not done.
[e] Step 3 was performed using two different clones (same genotype) randomly selected at step 2.

(MIC range of 0.125 to 1 μg/ml), while recent strains, isolated between 2009 and 2014, were moderately susceptible or intermediate to fluoroquinolones (MIC range of 0.25 to 2 μg/ml). However, no mutations in the QRDR region were evidenced that could explain this loss of susceptibility. The only recurrent mutation that was present in all recent strains and absent from old ones was Asp362Asn, encoded in the *gyrB* QRDR. However, alterations in the *gyrB* gene have rarely been associated with a loss of suscepti-

bility to fluoroquinolones, except in *M. gallisepticum*, where the Asp362Asn substitution was detected once in mutants selected *in vitro* (30). Furthermore, two isolates from Switzerland, included in this study as controls, harbored the same mutation but still remained very sensitive to fluoroquinolones (0.125 to 0.25 μg/ml). These findings suggest that the Asp362Asn mutation in *gyrB* is more likely a molecular marker that discriminates old from recent isolates, as already suggested (8), but does not play a role in isolate susceptibility. Two other mutations, in *parC*, were observed in 3 clinical isolates but were associated with MICs that did not differ from the range for the overall isolate population. These *parC* mutations had previously been described for mutants of nonruminant mycoplasmas selected *in vitro* (28, 30, 31) and had been identified in clinical isolates of *M. bovis* (10, 12). However, only in the case of a concomitant alteration of the GyrA codon for position 83 had they been associated with high resistance (4 to 16 μg/ml).

In the absence of associated alterations of the QRDRs, active efflux is the only known alternative mechanism in mycoplasmas that could lead to acquired resistance to fluoroquinolones and might explain the moderate shift in susceptibility in our set of isolates. It has been described for *M. hominis* strains selected on ethidium bromide *in vitro* (19, 20). *M. hominis* is a human urogenital mycoplasma that belongs to the same phylogenetic group as *M. bovis*, the so-called hominis group. Also, *M. hominis* active efflux has been linked to the overexpression of 2 genes, namely, *md1* and *md2*, encoding multidrug resistance ATP-binding cassette (ABC) transporters that are constitutively expressed in the reference strain (20). In another ruminant mycoplasma, *M. mycoides* subsp. *capri*, it was recently shown that orthovanadate, an inhibitor of ABC efflux pumps, was able to induce a 2-fold decrease of the MICs of 3 fluoroquinolones in both clinical and *in vitro* mutants, suggesting once again the contribution of an efflux mechanism to the overall resistance patterns of isolates (18).

The efflux hypothesis for *M. bovis* is consistent with (i) the presence in the genome of PG45^T of genes encoding several ABC transporters with a predicted role in drug resistance and a multidrug efflux transporter of the MatE family; (ii) the moderate increase of the MICs observed between the recent and old *M. bovis* populations, as efflux systems usually confer low levels of resistance (32, 33); and (iii) the slight differences in the MICs of different fluoroquinolones, as efflux system efficiency usually depends on the hydrophobicity profiles and molecular masses of the extruded fluoroquinolones (34, 35). However, in our set of isolates, we failed to experimentally demonstrate the efflux hypothesis, as we were unable to accumulate enrofloxacin inside mycoplasma cells in a reproducible manner.

Under increased antimicrobial pressure, the selected *M. bovis* clones were able to gain resistance and to achieve higher MICs than those of their parental clinical isolates. In most cases, concomitant alterations were observed in the QRDRs, with the exception of some clones derived from PG45^T (1st step) (Table 2) and 15762 (2nd step) (Table 3) that showed slightly higher MICs despite their WT QRDR genotype. The latter observation is also in favor of a native efflux system that can be upregulated to better allow tolerance of increased antimicrobial concentrations. The point mutations observed in the present study were identical to hot spots for resistance to fluoroquinolones previously described for various mycoplasma species (10, 12, 19, 30, 36, 37). These substitutions included 5 in GyrA (2 in codon 83 [Ser83Phe/Tyr] and 3 in codon 87 [Glu87Val/Gly/Lys]) and 4 in ParC (1 in codon 80 [Ser80Ile], 1 in codon 98 [Thr98Arg], and 2 in codon 84 [Asp84Asn/Tyr]). The most frequently observed substitutions were Ser83Phe in GyrA and Asp84Asn/Tyr in ParC, leading to 8- to 16-fold increases in the MICs, consistent with results from previous studies of *M. pneumoniae in vitro* (28) and *M. bovis in vivo* (12). Another combination of mutations was observed less frequently (only 3 of 72 selected clones), namely, Ser83Phe in GyrA and Ser80Ile in ParC, and was associated with 16- to 128-fold increases in the MICs. In contrast to our results, this combination of mutations was reported to occur very frequently *in vitro* for *M. hominis* (28) and for Japanese and Chinese clinical isolates of *M. bovis* (10, 38). Hence, our investigations of *in vitro* resistance selection clearly confirmed the existence of hot spots for mutations conferring high resistance levels and the cumulative effects of mutations in GyrA and ParC on the MICs. We also showed that the sequence in which mutations were accumulated by an isolate could vary as a function of the strain's molecular subtype. The 1st mutation appeared (i) at ParC position 84 for PG45^T (ST1), (ii) at GyrA position 83 for strain 15762 (ST2), (iii) at GyrA when ParC was already mutated in the WT genotype (15488; ST2), and (iv) equally in both genes for strain 15527 or 8619 (both ST3).

Furthermore, the frequency of resistant clone selection was also shown to vary between isolates. The recovery of PG45^T (ST1) and 15762 (ST2) was low during all the selection steps, whereas that of ST3 clones (15527 and 8619) could become high once they had acquired the double mutation in GyrA83 and ParC80/84. ST2 mutants had difficulty in acquiring alterations in QRDRs and failed to mutate in *parC*, and hence to accumulate the *parC* and *gyrA* mutations associated with high-level resistance. In contrast, the ST3 clones easily accumulated mutations leading to high levels of resistance. These results suggest that ST2 isolates are somehow blocked in a configuration which does not facilitate a gain in resistance and could have helped to limit the emergence of resistance in clinical isolates in France, most of which belong to ST2 (8). In contrast, ST3 isolates seemed able to rapidly and efficiently gain a high level of resistance. It is tempting to speculate that ST3 isolates are able to counteract the fitness loss associated with topoisomerase mutations. Whether they have increased mutation rates due to deficient DNA repair systems, which could facilitate the acquisition of compensatory mutations, has yet to be investigated. This hypothesis does not exclude the contribution of efflux systems to the early steps toward resistance.

The recent increased prevalence of ST3 isolates, from 5 to 20%, in the French *M. bovis* population supports this hypothesis. Indeed, the selective pressure imposed by the use of antimicrobials might result in the selection of this subtype that used to be rare (8). Furthermore, the highly resistant clinical isolate detected in 2014 (isolate 8428), with an MIC of 16 μg/ml for ENR and DAN and of >64 μg/ml for MAR, harbored the same mutations as the *in vitro* mutants (Ser83Phe in GyrA and Ser80Ile in ParC) and was of the ST3 subtype. This nicely emphasizes the need to survey the selection and spread of ST3 strains in France. Actually, this subtype was already described in Switzerland and Austria (8) and in the United States, as early as 2000 and 2002 (13, 21). Whether it was imported into France through international cattle trade has yet to be ascertained. Direct importation is unlikely, since according to the history of U.S. cattle trade (http://www.ers.usda.gov/data-products/livestock-meat-international-trade-data.aspx), only approximately

1,000 heads of cattle have been exported from the United States to Europe since 2002, and never to France.

Conclusions. This study attempted to decipher the molecular mechanisms responsible for the moderate decrease in susceptibility of recent French clinical isolates of *M. bovis* to fluoroquinolones. No direct link was established between QRDR polymorphisms and the shift in MIC values between old and recent isolates. Although an efflux system appears to be the most probable mechanism behind the observed phenotypes, our investigations *in vitro* were inconclusive. However, we showed that different clinical isolates, with different initial MICs and different genetic subtypes, were not equal in the ability to gain resistance *in vitro*. Notably, isolates belonging to the subtype 3 cluster were more likely to rapidly accumulate mutations in their QRDRs under selective pressure *in vitro* and hence to become resistant. These results are congruent with *in vivo* observations, as the first resistant clinical isolate reported in France was shown to belong to subtype 3. The relative potential contributions of (i) active efflux pumps and (ii) increased mutation rates leading to compensatory mutations to the process of developing resistance to fluoroquinolones in ST3 have yet to be explored. Lastly, the increasing prevalence of subtype 3 observed in the French population of *M. bovis* isolates strongly indicates an urgent need to control the veterinary usage of fluoroquinolones in France in order to preserve the favorable epidemiological situation there, where the level of resistance to fluoroquinolones is only moderate.

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