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Brief reports

Fluoroquinolone resistance in *Mycoplasma gallisepticum*: DNA gyrase as primary target of enrofloxacin and impact of mutations in topoisomerases on resistance level

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Resistant mutants of *Mycoplasma gallisepticum* were selected *in vitro* by passaging strains 10 times in increasing concentrations of enrofloxacin. The regions of *gyrA*/*gyrB* and *parC*/*parE*, encoding the quinolone resistance-determining regions (QRDRs) of DNA gyrase and DNA topoisomerase IV, respectively, of the mutants obtained during different passages were sequenced. Several mutations were found in the four fluoroquinolone targets. Substitution of Ser-83→Arg in GyrA and Ser-80→Leu or Trp in ParC QRDRs seem to have the greatest impact on resistance to fluoroquinolones. The results obtained also suggest that the preferential target of enrofloxacin in *M. gallisepticum* is DNA gyrase.

Introduction

Mycoplasma gallisepticum, a major poultry pathogen, causes chronic respiratory disease in chickens and infectious sinusitis in turkeys,[1] both of which can lead to condemnation at slaughter. Fluoroquinolones can be used to control mycoplasmosis. However, under field conditions, fluoroquinolone treatment cannot eradicate infection. These therapeutic failures may be due to the selection of resistant mutants. In order to study the mechanisms involved in this process, *in vitro* selection of enrofloxacin (the veterinary fluoroquinolone most similar to ciprofloxacin)-resistant *M. gallisepticum* mutants has been performed. In a previous report,[2] our analysis of resistant mutants obtained following 10 passages in escalating concentrations of enrofloxacin revealed a large variety of mutations in the genes encoding the four major fluoroquinolone targets, GyrA, GyrB, ParC and ParE. Our current study describes the enrofloxacin susceptibility levels and the sequences of the quinolone resistance-determining regions (QRDRs) of *gyrA*, *gyrB*, *parC* and *parE* of mutants obtained after each of the 10 passages, revealing the order in which each mutation appeared, and the effect of each on resistance.

Materials and methods

Two strains of *M. gallisepticum*, MG ATCC 15302, a reference strain, and MG 41-91, a field strain,[2] were used for the selection of enrofloxacin-resistant mutants by serial passage. Enrofloxacin was provided by Bayer Pharma (Puteaux, France). For each passage, Frey broth[3] cultures (1 mL) supplemented with penicillin (50 mg/L) and containing ~10^4 colour-changing units were set up in microtubes without enrofloxacin, or with one of seven different enrofloxacin concentrations. The concentrations used during the first passage were the MIC of enrofloxacin for the wild-type organism, and three doublings above and below this value. Cultures were incubated at 37°C until the culture growing in the absence of enrofloxacin developed an acid colour change. At this point the culture containing the highest concentration of enrofloxacin that also showed an acid colour change was retained. The remaining cultures were further incubated at 37°C, for up

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to a total of 5 days, and the culture containing the highest concentration of enrofloxacin that showed an acid colour change was retained. Using this method, two mutants were potentially obtained during each passage. The cultures retained from one passage were used to seed the next, with the range of enrofloxacin concentrations used being increased to reflect any decreased susceptibility observed in the previous passage. The enrofloxacin concentration in which the culture was able to grow during the previous passage was used, together with three doublings above and below this value. A total of 10 passages was completed. Selection experiments were repeated three to four times for each strain.

Following each passage, aliquots of the two cultures retained were plated on to Frey agar to obtain single colonies prior to MIC determinations and genetic analyses. MICs were determined by a dilution method[4] on Frey agar. The MIC was defined as the lowest concentration for which no visible growth was observed. A strain was considered susceptible to enrofloxacin when the MIC was ≤0.5 mg/L and resistant when the MIC was >2 mg/L, according to the breakpoints given for this antibiotic.[5] For genetic analysis, chromosomal DNA from all strains was prepared by cellular lysis according to the method of Kellog & Kwok.[6] Amplification of *gyrA*, *gyrB*, *parC* and *parE* QRDRs was performed and all purified PCR products were sequenced directly on both strands as described previously.[2]

Results and discussion

During the selection process, mutants were generally able to grow in increasing concentrations of enrofloxacin at each passage. However, the emergence of mutants able to grow at increased enrofloxacin concentrations did not occur at every passage. For example, for the mutant ATm6 derived from MG ATCC 15302 (Table 1), the ability to grow at higher concentrations of enrofloxacin was only observed at passages 2, 6, 7, 8 and 9. When this phenomenon was observed, the mutants derived were selected in order to determine their susceptibility to enrofloxacin and to analyse the QRDRs of their four fluoroquinolone targets.

Primary target of enrofloxacin

In two mutants derived from the reference strain MG ATCC 15302, the first mutation was a Ser→Arg substitution at position 83 in the GyrA QRDR (mutants ATm6-2 and ATm3/4-3: Table 1). For two other mutants (ATm7-4 and ATm1-9), two mutations appeared during the same passage. The mutant ATm7-4 showed Glu-87→Gln and Cys-467→Phe substitu-

Table 1. Mutations that appeared during the selection of mutants derived from MG ATCC 15302 and their influence on enrofloxacin resistance

			Sequence of the QRDRs of[a]									
			GyrA				GyrB	ParC		ParE		
Strain	[Enro][b] (mg/L)	MIC Enro (mg/L)	81	83	84	87	464	80	84	458	467	MIC increase (fold)
MG ATCC 15302	–	0.03	Gly	Ser	Ala	Glu	Asn	Ser	Glu	Asp	Cys	–
ATm1-9[c]	0.5	0.5	–	–	–	**Gly**	–	–	**Gly**	–	–	ND
ATM1	1	2	–	–	–	Gly	**Asp**	–	Gly	–	–	4
ATm3/4-3	0.25	1	–	**Arg**	–	–	–	–	–	–	–	32
ATm3/4-4	4	4	–	Arg	–	–	–	–	**Lys**	–	–	4
ATm3-5	32	8	**Ala**	Arg	–	–	–	–	Lys	–	–	4
ATm4-5	8	16	–	Arg	**Pro**	–	–	–	Lys	–	–	2
ATm6-2	0.25	1	–	**Arg**	–	–	–	–	–	–	–	32
ATm6-6	1	1	–	Arg	–	–	–	–	–	**Asn**	–	1
ATm6-7	4	4	–	Arg	–	–	–	–	**Lys**	Asn	–	4
ATm6-8	8	8–16	–	Arg	–	–	–	–	Lys	Asn	–	2–4
ATm6-9	32	32	–	Arg	**Pro**	–	–	–	Lys	Asn	–	2–4
ATm7-4	0.125	0.25	–	–	–	**Gln**	–	–	–	–	**Phe**	ND
ATm7-8	0.5	2	–	–	–	**Lys**	–	**Leu**	–	–	Phe	ND
ATM7	8	8	–	–	–	Lys	–	Leu	–	–	Phe	4

[a]*E. coli* numbering.
[b]Concentration of enrofloxacin (Enro) that gave rise to the different mutants during individual passages.
[c]ATM1 corresponds to mutant 1 obtained from strain MG ATCC 15302 at the end of the selection (10th passage); ATm1-9 corresponds to mutant 1 obtained from strain MG ATCC 15302 during the ninth passage of the selection; ATm3-5 and ATm4-5, mutants derived from mutant ATm3/4-4.
–, no difference from the parental strain; ND, not determined because of the appearance of two mutations during the same passage; in bold type, mutations appeared during that passage.

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Table 2. Mutations that appeared during the selection of mutants derived from MG 41-91 and their influence on enrofloxacin resistance

			Sequence of the QRDRs of[a]									
				GyrB		ParC				ParE		
Strain	[Enro][b] (mg/L)	MIC Enro (mg/L)	GyrA 83	426	465	64	80	81	84	420	463	MIC increase (fold)
MG 41-91	–	0.06	Ser	Asp	Glu	Ala	Ser	Ser	Glu	Asp	Ser	–
41m8-4[c]	0.06	0.125	–	**Asn**	–	–	–	–	–	–	–	2
41m8-5	0.125	0.5	–	Asn	–	–	–	–	–	–	**Leu**	4
41m8-9	1	1	**Asn**	Asn	–	–	–	–	–	–	Leu	2
41M8	16	2	Asn	Asn	–	–	–	–	**Gln**	–	Leu	2
41m10-3	0.125	0.125	–	**Asn**	–	–	–	–	–	–	–	2
41m10-4	0.5	0.5	–	Asn	–	–	–	–	–	–	**Leu**	4
41m10-7	1	1	**Ile**	Asn	–	–	–	–	–	–	Leu	2
41m10-9	2	4	Ile	Asn	–	**Ser**	–	–	–	–	Leu	4
41M10	2	16	Ile	Asn	–	Ser	–	–	**Gly**	–	Leu	4
41m15/18-4	0.125	0.125	–	**Asn**	–	–	–	–	–	–	–	2
41m15/18-5	0.5	0.25–0.5	–	Asn	–	–	–	**Pro**	–	–	–	2–4
41m15/18-8	1	2	**Ile**	Asn	–	–	–	Pro	–	–	–	2–4
41m15-9	8	32	Ile	Asn	–	–	**Trp**	Pro	–	–	–	16
41m18-9	64	16	Ile	Asn	–	–	**Leu**	Pro	–	–	–	8
41m16/19-6	0.5	1	**Arg**	–	–	–	–	–	–	–	–	16
41m16/19-7	1	2	Arg	–	–	–	–	–	–	**Asn**	–	2
41m16/19-8	4	16	Arg	–	–	–	**Leu**	–	–	Asn	–	8
41m16-9	16	32	Arg	–	**Gly**	–	Leu	–	–	Asn	–	2
41m19-9	32	16	Arg	–	**Lys**	–	Leu	–	–	Asn	–	1

[a]*E. coli* numbering.
[b]Concentration of enrofloxacin that gave rise to the different mutants during individual passages.
[c]41M8 corresponds to mutant 8 obtained from strain MG 41-91 at the end of the selection (10th passage); 41m8-4 corresponds to mutant 8 obtained from strain MG 41-91 during the fourth passage of the selection; 41m15-9 and 41m18-9, mutants derived from mutant 41m15/18-8.
–, no difference from the parental strain; in bold type, mutations appeared during that passage.

tions in GyrA and ParE, respectively, at the fourth passage, and the mutant ATm1-9 showed Glu-87→Gly and Glu-84→Gly changes in GyrA and ParC, respectively, during the ninth passage (Table 1). The observation of two mutations occurring during the same passage cannot be due to a mixture of populations in the culture since individual colonies were used for genetic analysis.

For most mutants derived from the field strain MG 41-91, the first mutation was an Asp-426→Asn change in the GyrB QRDR (Table 2). However, one mutant (41m16/19-6) harboured a Ser-83→Arg substitution in GyrA, like mutants ATm6-2 and ATm3/4-3, above.

Mutations in ParC or ParE were observed only in mutants bearing at least one mutation in GyrA or GyrB, and exhibiting an increase in the MIC of enrofloxacin (e.g. ATm6-6 and 41m15/18-5 mutants). Furthermore, a study of the sequential appearance of mutations showed that substitutions were observed first in DNA gyrase, then in topoisomerase IV (Tables 1 and 2). This 'ping pong' model has been described previously in *Escherichia coli* mutants by Heisig,[7] who suggested that the appearance of an alteration in the primary target (DNA gyrase) leads to a decrease in its affinity for the quinolone. Then, in turn, topoisomerase IV becomes the primary target. This scenario was observed in our study, when the first mutation appeared in GyrB (e.g. in mutants 41m15/18, 41m10 and 41m8). However, when the first change was a Ser→Arg substitution at position 83 in the GyrA QRDR, the two subsequent mutations were observed in topoisomerase IV [e.g. in mutants ATm6 (passages 6 and 7) and 41m16/19 (passages 7 and 8)]. Arginine carries a larger lateral chain compared with serine, and this steric hindrance could be responsible for a greater change in the GyrA subunit, which might lead to a more pronounced decrease in the quinolone affinity of DNA gyrase.[8] This hypothesis could explain the appearance of two successive mutations in the topoisomerase IV. Taken together, these results suggest that in *M. gallisepticum* the DNA gyrase is the primary target for enrofloxacin, unlike *Mycoplasma hominis*, in which the primary target of ciprofloxacin (similar to enrofloxacin) is topoisomerase IV.[9]

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Influence of mutations on resistance level

Two other mutations were found in the GyrA QRDR at position 83: Ser→Asn or Ile. They were associated with small increases in resistance (two- and two- to four-fold, respectively) unlike the mutation Ser→Arg, which led to a 16-fold increase for mutant 41m16/19-6 (Table 2). Furthermore, this Ser→Arg mutation was responsible for a 32-fold increase in the MIC of enrofloxacin for mutants ATm6-2 and ATm3/4-3, compared with the parental strain (Table 1). The specific effects of different mutations on resistance level could be explained by structural differences between the three amino acids, as suggested by Kim *et al.*[8] Mutations were also found at positions 81, 84 and 87 in the GyrA QRDR in mutants derived from MG ATCC 15302. However, these mutations led to smaller increases in resistance (two- to four-fold) than the Ser-83→Arg mutation (Table 1). Mutation of Ser-80→Leu or Trp in the ParC QRDR also seems to play a role in enrofloxacin resistance. Mutants 41m18-9, 41m16/19-8 and 41m15-9, which carry one of these mutations, exhibited eight- to 16-fold increases in their enrofloxacin MIC (Table 2). As seen for the GyrA QRDR, mutations observed at other positions (64, 81 and 84) led to smaller increases in resistance level (two- to four-fold) (Tables 1 and 2).

Four mutations were found in the GyrB and ParE QRDRs at three and four different positions, respectively (Tables 1 and 2). These mutations had only a slight influence on resistance: the substitutions Asp→Asn, at positions 426 in GyrB and 420 in ParE QRDRs, respectively, were responsible for only a two-fold increase in the MIC of enrofloxacin (Table 2). Mutant ATm7 harboured the same alterations in passage 8 (ATm7-8) and in passage 10 (ATM7), but had different MICs (Table 1). This might be explained by the appearance of alterations elsewhere in the topoisomerases genes (outside the QRDR) or in unrelated gene loci (e.g. drug efflux systems).

In conclusion, DNA gyrase seems to be the primary target of enrofloxacin in *M. gallisepticum*. Our data also suggest that the position of the modified amino acid plays an important role in resistance to quinolones in *M. gallisepticum*, as was observed in *M. hominis*.[10] Furthermore, the nature of the amino acid also has an influence on the resistance level since Ser-83→Arg and Ser-80→Trp observed in the GyrA and ParC QRDRs, respectively, have a greater impact on the resistance level than other mutations described at the same position. It would be interesting to determine whether such mutations could be found in field strains of *M. gallisepticum* with reduced susceptibility or resistance to enrofloxacin.

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