PERSISTENCE OF *MYCOPLASMA SYNOVIAE* IN HENS AFTER TWO ENROFLOXACIN TREATMENTS

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Introduction

Mycoplasma synoviae (MS) infection most frequently occurs as a subclinical upper respiratory infection and is sometimes responsible for infectious synovitis in chickens and turkey (1). The economic consequences may be important because of decreased egg production, retarded growth, reduced weight gains, and condemnation at slaughter due arthritis lesions. MS may be transmitted either vertically, through the eggs, or laterally, by direct contact or via the environment (1, 2). MS is susceptible to several antibiotics *in vitro*, including tetracyclines, macrolides (except erythromycin) and fluoroquinolones (3, 4). However, even if antibiotic treatments decrease the symptoms, they do not eliminate MS infections (1). These therapeutic failures may be due to the development of resistant strains, as shown *in vitro* (5).

In the present study, we report the effect of two enrofloxacin treatments on the persistence of *M. synoviae* in experimentally infected hens and on the emergence of resistant mycoplasmas.

Material and Methods

On day 0, thirty eleven-week-old mycoplasma-free hens were infected by aerosol with 500 mL of a culture of the MS 317 strain containing approximately 10^6 colony forming units (CFU) per milliliter. This MS strain was susceptible to enrofloxacin (minimum inhibitory concentration (MIC)= 0.25 µg/mL) (3).

Hens were randomly assigned to two separate animal rooms, containing 20 and 10 birds.

Three weeks after infection, the first medication with enrofloxacin (Baytril 10%) at the therapeutic dose (TD= 10 mg/kg of body weight) was given for five consecutive days (from day 23 to day 28) in the drinking water of the first animal room. A second treatment was administered one week after the end of the first medication (from day 36 to day 41). Hens of the second animal room were kept untreated.

Before and after each treatment, five hens of the first animal room were sacrificed and examined postmortem for gross lesions. Tracheal swabs were collected and cultured for MS recovery. Five infected untreated hens were also sacrificed after each treatment.

All tracheal swabs were placed in 2 mL of transport medium (2% buffered peptone water containing glycerin (1.2% v/v), amphotericin B: 2.5 µg/mL, ampicillin: 2 units/mL and colistin: 7.5 µg/mL). Mycoplasmas were directly cultured from tracheal swabs by diluting 100 µL of transport medium from each swab in 900 µL of FM4 broth (6). Serial ten-fold dilutions up to 10^{-4} were prepared and incubated at 37°C until the culture developed an acid color change or up to 30 days. When a color change of the broth medium was observed, the uncloned cultures were aliquoted and stored at -70°C before MIC determination.

As these cultures could contain mixtures of wild and mutant cells, some cultures, obtained before or after the first treatment or after the second treatment, were cloned, aliquoted and stored at -70°C.

The enrofloxacin MIC of the different positive cultures and of the clones were determined by the metabolic inhibition method performed with 96-well microtiter plates in Frey broth medium (7) as previously described (8). Enrofloxacin concentrations ranged from 0.03 to 32 µg/ml.

The SAS software was used to compare MIC results with the Student-Newman-Keuls test. Differences were estimated significant when $p < 0.05$.

Results

After infection and before the first treatment, the mycoplasmal infection was confirmed by culture. One hundred percent of the hens were positive at the beginning of the first treatment at the therapeutic dose, and 100% of the birds were still positive at the end of the two successive treatments (Table 1). Furthermore, no difference in the number of mycoplasmas was observed between the cultures from tracheal swabs collected before and after the treatments, or between the cultures from treated and untreated birds (data not shown).

The results of MIC determination did not show any significant difference between the susceptibility to enrofloxacin of the MS uncloned cultures recovered after the first treatment or before the second treatment and the wild-type strain (Table 1). However, a significant increase of the resistance level to enrofloxacin of the reisolated mycoplasma cultures obtained from the treated birds was observed after the second treatment ($p < 0.05$).

Table 1: re-isolation of M. synoviae *and susceptibility levels of the MS uncloned cultures before and after two successive treatments with enrofloxacin.*

	Percentage of positive hens		MIC_{ENRO}[c] for MS cultures (µg/mL)	
	Treated	Untreated	Treated	Untreated
Before T1[a] (Day 23)	100	ND	0.25-0.5[d] 0.46[e]	ND
After T1 (Day 28)	100	100	0.25-1 0.50	0.25-0.5 0.46
Before T2[b] (Day 36)	100	ND	0.25-0.5 0.42	ND
After T2 (Day 41)	100	100	1-2 1.18*	0.25-1 0.53

[a]: first treatment; [b]: second treatment
[c]: minimum inhibitory concentration of enrofloxacin
[d]: MIC range; [e]: MIC geometric mean
*: significantly different from the five other groups of cultures ($p < 0.05$).

Enrofloxacin MICs were also determined on clones of some MS cultures obtained before and after the first and second treatment (Table 2).

As for the uncloned suspensions from the positive cultures, these results clearly point out an increase in the MICs of *M. synoviae* after the second treatment for most

of the clones, with values ranging from 0.5 to 4 µg/mL of enrofloxacin.

Table 2: Susceptibility levels of clones of M. synoviae *before and after two successive treatments with enrofloxacin.*

	Number of clones isolated	MIC_{ENRO}[c] (µg/mL)
Before T1[a]	5	0.25 0.25 0.5 0.5 0.25
After T1 and before T2[b]	5	0.5 0.5 0.5 0.5 0.25
After T2	6	1 0.5 1 4 2 1-2

[a]: first treatment; [b]: second treatment
[c]: minimum inhibitory concentration of enrofloxacin

Discussion-Conclusion

Two successive treatments at the therapeutic dose of enrofloxacin did not have any influence on the MS recovery from tracheal swabs, with 100% of the hens still positive after the second treatment. This persistence phenomenon has already been described for *M. gallisepticum* (MG) in chickens (9), but successive treatments at the therapeutic dose reduced the percentage of MG-infected birds. Hypotheses to explain the mycoplasma persistence in treated birds include possible development of resistance mechanisms, survival on materials in the animal room environment and subsequent natural re-infection of birds, the ability to invade host cells for long periods and persist inside the cell in a fluoroquinolone-insensitive state, as described for *M. penetrans* (10). Mycoplasmas might also persist in tissues or subcellular fractions where enrofloxacin cannot diffuse.

The development of resistance in *M. synoviae* and *M. gallisepticum* has already been described *in vitro* (5). However, this is the first time that such an increase of resistance is described after several *in vivo* treatments at the therapeutic dose: despite the re-isolation of mycoplasmas, no significant MIC change was previously observed in *M. gallisepticum* clones after several treatments (9).

Further studies should be conducted to determine if the increase of resistance can be attributed to the development of resistance mechanisms, especially in the genes coding for DNA-gyrase or topoisomerase IV, as described for mutants of *M. gallisepticum* selected *in vitro* (11, 12)

In conclusion, results showed that, under these experimental conditions, oral enrofloxacin treatments were not effective for the eradication of *M. synoviae*. This persistence could be associated to a decrease of the susceptibility level of some reisolated clones.

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