

Comparative Susceptibilities of Various Animal-Pathogenic Mycoplasmas to Fluoroquinolones

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The in vitro activities of six antimicrobial agents were tested against 162 mycoplasma strains of eight species isolated from poultry and livestock at different geographic sites. Tiamulin was most active (MICs at which 90% of the isolates were inhibited [MIC₉₀s], 0.025 to 0.25 µg/ml); enrofloxacin and danofloxacin had near equivalent activities (MIC₉₀s, 0.05 to 1.0 µg/ml), but were much more active than flumequine (MIC₉₀s, 1 to 50 µg/ml). The MIC₉₀s of tylosin and oxytetracycline were 0.25 to >100 µg/ml and 0.25 to 100 µg/ml, respectively.

Mycoplasmas are of considerable veterinary importance, causing infections of the respiratory and urogenital tracts, mammary glands, joints, or eyes of various poultry and livestock species. For poultry the predominant mycoplasmal pathogens are *Mycoplasma gallisepticum*, *M. synoviae*, and *M. iowae* (11); for swine the main *Mycoplasma* species are *M. hyopneumoniae*, the etiological agent of mycoplasmal pneumonia of swine, *M. hyosynoviae*, and *M. hyorhinis* (28); for cattle *M. bovis* is one of the most pathogenic and exists worldwide (8); and for sheep and goats *M. agalactiae* causes contagious agalactia, which is of serious significance particularly in Mediterranean countries (5).

Control of mycoplasma infections by vaccination is limited to some specific infections because only a few vaccines are available (22). Control of these infections by chemotherapy is the most practical way to minimize economic losses due to them. Many antimicrobial agents, such as macrolides and lincosamides (e.g., tylosin, spiramycin, lincomycin, and clindamycin), tetracyclines, tiamulin, and fluoroquinolones (e.g., enrofloxacin and danofloxacin), have been shown to possess in vitro activity against various veterinary mycoplasmas (2, 4, 9, 13, 25). Although macrolides and tetracyclines have been known as effective antibiotics against *Mycoplasma* species, resistance to some of these compounds has been encountered (19, 26). Tiamulin is the most effective agent against various mycoplasmas (13, 16), but it has a narrow spectrum of activity against the bacteria that regularly occur as secondary infectious agents in mycoplasma infections and is normally used only in swine. Although much information exists about the antibacterial effects of fluoroquinolones (29), their effects against veterinary *Mycoplasma* species are less well documented. Comparative studies are scarce, and most investigations have centered on *Mycoplasma* species from a single host species from one geographical site. MICs have been determined by different methods with little standardization among laboratories, making comparison of results difficult. Therefore, this study examined the in vitro activities of commonly used quinolones and other antimicrobial drugs against a wide range of mycoplasma field isolates gathered worldwide from chickens, turkeys, pigs, cat-

tle, goats, and sheep by using one culture medium and a standardized MIC procedure.

Seventeen to twenty-eight field isolates of eight *Mycoplasma* species (162 isolates) and one type strain per species were used. For each species except *M. agalactiae*, five strains were isolated in the United States, five were isolated in Japan (except *M. iowae*), five were isolated in the United Kingdom, and the remainder were isolated in France, Germany, or Denmark. *M. agalactiae* strains were isolated predominately from goats in France ($n = 10$) and four Mediterranean countries. Field strains were kindly supplied by J. M. Bradbury, University of Liverpool; N. F. Friis, National Veterinary Laboratory, Copenhagen, Denmark; K. H. Hinz and H. Kirchhoff, School of Veterinary Medicine, Hannover, Germany; S. Kleven, University of Georgia; R. Nicholas, Central Veterinary Laboratory, Addlestone, United Kingdom; R. F. Ross, Iowa State University; P. Whittlestone, University of Cambridge; T. Yoghishi, Nippon Institute for Biological Science, Tokyo, Japan; and K. Yamamoto, National Institute of Animal Health, Aomori, Japan.

The laboratory guidelines of the National Committee for Clinical Laboratory Standards do not provide information for the susceptibility testing of *Mycoplasma* species or recommendations for standard culture media (20). For the culture of organisms and MIC testing we used a commercially available M broth (Mycoplasma Experience Ltd., Reigate, Surrey, United Kingdom) containing 0.004% (wt/vol) phenol red for all species. The M broth for the glucose-fermenting species (*M. gallisepticum*, *M. synoviae*, *M. iowae*, *M. hyopneumoniae*, and *M. hyorhinis*) also contained 0.1% (wt/vol) glucose, and the pH was adjusted to 7.6. For tests with *M. bovis* and *M. agalactiae*, 0.5% (wt/vol) pyruvate was added to the M glucose broth (pH 7.6). For tests with *M. hyosynoviae*, 0.1% (wt/vol) arginine instead of glucose was added to M broth and the pH was adjusted to 7.0. Mycoplasmas were grown in liquid medium until the color changed from pink to orange-yellow for glucose- or pyruvate-fermenting species or from orange-yellow to pink for arginine-fermenting species. The incubation period required to produce adequate numbers of mycoplasmas for MIC tests was established by thawing a sample of each mycoplasma culture, diluting the culture in 4 ml of the broth medium, and incubating it at 36°C until the color changed. Viable counts were then made to establish the dilution necessary to produce inocula containing 10^3 to 10^5 color-changing units per ml. This incubation period and dilution procedure were also used to

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produce inocula for MIC tests. For these tests the following antimicrobial agents were used: enrofloxacin (Bayer AG, Leverkusen, Germany), danofloxacin (extracted from commercially available injectable solution), flumequine (Solvay Duphar, Weesp, The Netherlands), tiamulin hydrogen fumarate (Sandoz Ltd., Camberley, United Kingdom), and tylosin tartrate and oxytetracycline (both from Sigma Chemical Co., Poole, United Kingdom). Stock solutions (1,000 µg/ml) were sterilized by filtration and stored at -20°C or used immediately. For determination of the MICs, the solutions were further diluted with M broth to achieve the inocula required for the microtitre plates.

MIC tests were carried out in blank microtitre plates by a modification of the method described by Tanner and Wu (24). A volume of 0.1 ml of drug dilution was mixed with 0.1-ml volumes of the challenge inocula in microtitre wells. Each plate contained uninoculated medium as a sterility control, medium at pH 6.8 or pH 7.5 as an end point control, and drug-free inoculated growth controls. Plates were sealed with adhesive tape and incubated at 36°C until the color in the growth well matched that of the end point control. Readings were then taken; the MIC was the lowest drug concentration to show no color change in the medium. The values obtained correspond to the initial MICs described by some authors (2, 7, 25), who took more than one reading when determining MICs. MICs were assessed in a single test; if the MIC results differed to a large extent from the MIC for the type strain, the test was repeated. When the MIC of enrofloxacin was ≤ 0.5 µg/ml, the strain was considered susceptible; when the MIC was 1 µg/ml, it was considered intermediately susceptible; and when the MIC was ≥ 2 µg/ml, the strain was considered resistant (20). The corresponding values were ≤ 4 , 8, and ≥ 16 µg/ml, respectively, for oxytetracycline (20), ≤ 4 , 8, and ≥ 16 µg/ml, respectively, for flumequine (3), ≤ 1 , 2, and ≥ 4 µg/ml, respectively, for tylosin (27), and ≤ 8 (susceptibility) and ≥ 16 µg/ml (resistance) for tiamulin (reference 20, no intermediate susceptible zone given). These breakpoints are based on guidelines for testing the susceptibility of bacteria that affect humans or companion animals; none of these values have been established for mycoplasmal pathogens. Criteria for danofloxacin are not published.

Results for the mycoplasma type and field strains are presented in Table 1 as MIC ranges, MICs at which 50% of the isolates are inhibited (MIC₅₀s), and MIC₉₀s. Tiamulin was the most active agent against the type strains, except the *M. synoviae* type strain, with MICs ranging from 0.0025 µg/ml (*M. gallisepticum*) to 0.125 µg/ml (*M. agalactiae*). The fluoroquinolones, enrofloxacin and danofloxacin, also showed high activities against the type strains and were severalfold more active than flumequine. As a rule, tylosin had activity similar to the activities of the fluoroquinolones but was considerably less active against *M. iowae*. Overall, the activity of oxytetracycline against the type strains was intermediate, with MICs from 0.05 to 0.5 µg/ml. The testing of the type strains allowed a direct comparison to be made between the MICs found in this study and those found in other studies using the same strains and various formulations of solid or liquid culture media. Our results are in general agreement with those obtained in some earlier studies involving avian (2, 9), porcine (7, 9, 10, 16, 25), and bovine (7, 9) type strains but differ slightly from those obtained in other studies (1, 12, 13, 18, 26), which found MICs up to ten times higher than those found in the present study. These differences could not be attributed to any particular factor since tests carried out with liquid (1, 2, 7, 10, 16, 25, 30) or solid (9, 12, 13, 16) media with various levels of inocula and different incubation times yielded results either similar to or

divergent from those obtained in the present study. The in vitro susceptibilities of the field strains were similar to those of the type strains, except for the lower susceptibilities of field isolates from some geographical areas, especially their lower susceptibilities to tylosin.

The high susceptibility of avian field mycoplasma isolates to tiamulin, danofloxacin, and enrofloxacin agrees with earlier findings (2, 4, 9, 12, 14). A recent study (15) describes the pharmacokinetic behavior of enrofloxacin and danofloxacin in chickens. It remains to be seen whether isolates for which the danofloxacin MIC is 0.5 µg/ml will be considered susceptible when breakpoints for this compound are available. The MIC results demonstrated the vastly improved antimycoplasmal activities of the newer fluoroquinolones over that of flumequine. None of the *M. synoviae* and *M. iowae* isolates, and only 45% of the *M. gallisepticum* isolates, were susceptible to flumequine. Based on a susceptibility breakpoint of ≤ 1 µg/ml (27), tylosin had no effect on any *M. iowae* isolate. One British and three of five Japanese *M. gallisepticum* isolates and one French as well as all Japanese *M. synoviae* isolates exhibited tylosin resistance. This observation is consistent with reports on Israeli and Japanese *M. gallisepticum* field strains (18, 23). Tylosin resistance, as detected in our *M. iowae* isolates, has been observed in field strains from France and Israel (14, 19) and, to a lesser extent, in field isolates from Britain (12). All strains of *M. gallisepticum* were susceptible to oxytetracycline (MIC₉₀, 0.5 µg/ml), but the oxytetracycline MICs for all Japanese *M. synoviae* isolates (MICs ≥ 100 µg/ml) and some of the *M. iowae* isolates from Europe (MIC range, 0.5 to 5 µg/ml) and the United States (MIC range, 0.5 to 10 µg/ml) increased. All field strains of *M. hyopneumoniae* were fully susceptible to the antimicrobials tested. In contrast to our results, a marked decrease in susceptibility to oxytetracycline for *M. hyopneumoniae* strains isolated in Japan (10) was observed. The high susceptibilities of *M. hyopneumoniae*, *M. hyosynoviae*, and *M. hyorhinis* to tiamulin and enrofloxacin observed in the current study reflects those found for European and Japanese strains (7, 16, 25). Similar findings regarding the susceptibilities of *M. hyopneumoniae* and *M. hyosynoviae* to danofloxacin were reported (4). The MIC₉₀s of enrofloxacin and danofloxacin for *M. hyorhinis* were both 1 µg/ml. *M. hyosynoviae* and *M. hyorhinis* strains exhibited 0 and 15% susceptibility to flumequine, respectively. Sixteen of twenty (80%) *M. hyorhinis* isolates were susceptible to tylosin, and most of the tylosin-resistant isolates were from Japan, which is in agreement with recent investigations (16). Resistance to tylosin for Japanese *M. hyosynoviae* isolates (17) was observed in one of five isolates. Isolation of *Mycoplasma* species from tissues requires successive subculturing, and it has been described that during this process resistance may disappear (16, 17). It can be speculated that the resistance to macrolides in vivo can be higher than described here. The high susceptibility of *M. bovis* to tiamulin, enrofloxacin, and danofloxacin is consistent with literature data (6, 7, 21, 26); high MICs of the two fluoroquinolones were observed for two German isolates. Slightly increased MICs were observed for some recent isolates from Northern Ireland and The Netherlands (1, 26). Some tylosin resistance of *M. bovis* strains from all four geographical regions was noticed; no activity at all was observed for two American strains (>100 µg/ml). The susceptibility pattern of the *M. agalactiae* isolates was similar to that of *M. bovis*; tylosin resistance, however, was not observed. Both mycoplasma species were resistant to flumequine.

In the present study the comparative susceptibilities of various *Mycoplasma* species of veterinary importance were assessed by carrying out MIC tests by a microtiter plate method which allows precise single readings, using a broth medium

TABLE 1. MICs of antimicrobial agents against field and type strains of eight mycoplasma species isolated from poultry, swine, and ruminants from different geographical locations

Organism (no. of isolates) and antimicrobial agent	MIC ($\mu\text{g/ml}$) for ^a :			
	Type strain ^b	Field strains		
		Range	50%	90%
<i>M. gallisepticum</i> (20)				
Enrofloxacin	0.01	0.025–1	0.05	0.1
Danofloxacin	0.005	0.01–0.5	0.05	0.1
Flumequine	0.5	2.5–10	5	10
Tiamulin	0.0025	0.0005–0.25	0.001	0.025
Tylosin	0.01	0.0025–10	0.01	2.5
Oxytetracycline	0.1	0.05–0.5	0.25	0.5
<i>M. synoviae</i> (28)				
Enrofloxacin	0.5	0.05–0.5	0.25	0.5
Danofloxacin	0.5	0.1–0.5	0.25	0.5
Flumequine	10	5–50	25	50
Tiamulin	0.1	0.05–0.5	0.1	0.25
Tylosin	0.025	0.0025–50	0.025	50
Oxytetracycline	0.1	0.025–>100	0.1	100
<i>M. iowae</i> (19)				
Enrofloxacin	0.005	0.005–1	0.025	0.5
Danofloxacin	0.01	0.01–1	0.025	0.5
Flumequine	5	2.5–100	10	25
Tiamulin	0.005	0.005–0.1	0.01	0.1
Tylosin	0.5	10–>100	>100	>100
Oxytetracycline	0.25	0.25–10	1	5
<i>M. hyopneumoniae</i> (20)				
Enrofloxacin	0.05	0.01–0.1	0.025	0.05
Danofloxacin	0.025	0.01–0.05	0.025	0.05
Flumequine	0.5	0.25–1	0.5	1
Tiamulin	0.025	0.01–0.1	0.05	0.05
Tylosin	0.025	0.025–0.25	0.1	0.25
Oxytetracycline	0.25	0.025–1	0.25	1
<i>M. hyosynoviae</i> (18)				
Enrofloxacin	0.25	0.05–0.25	0.1	0.25
Danofloxacin	0.5	0.1–0.5	0.25	0.25
Flumequine	25	5–50	10	25
Tiamulin	0.025	0.0025–0.01	0.005	0.025
Tylosin	0.05	0.025–>10	0.25	1
Oxytetracycline	0.5	0.25–10	0.5	5
<i>M. hyorhinis</i> (20)				
Enrofloxacin	0.5	0.1–1	0.5	1
Danofloxacin	0.25	0.25–1	0.5	1
Flumequine	5	2.5–25	5	10
Tiamulin	0.05	0.025–0.5	0.1	0.25
Tylosin	0.5	0.25–2.5	0.5	2.5
Oxytetracycline	0.05	0.05–10	0.25	2.5
<i>M. bovis</i> (20)				
Enrofloxacin	0.25	0.05–1	0.1	0.25
Danofloxacin	0.25	0.1–2.5	0.25	0.5
Flumequine	10	10–100	25	50
Tiamulin	0.05	0.025–0.125	0.05	0.125
Tylosin	0.05	0.025–>100	1	5
Oxytetracycline	0.1	0.1–10	1	2.5
<i>M. agalactiae</i> (17)				
Enrofloxacin	0.1	0.05–1	0.25	0.25
Danofloxacin	0.25	0.05–2.5	0.25	0.5
Flumequine	25	5–100	25	50
Tiamulin	0.125	0.05–0.25	0.125	0.125
Tylosin	0.25	0.1–1	0.5	0.5
Oxytetracycline	0.25	0.1–0.25	0.1	0.25

^a 50% and 90%, MIC₅₀S and MIC₉₀S, respectively.

^b The *Mycoplasma* type strains are as follows: *M. gallisepticum*, NCTC 10115; *M. synoviae*, NCTC 10124; *M. iowae*, NCTC 10185; *M. hyopneumoniae*, NCTC 10110; *M. hyosynoviae*, NCTC 10167; *M. hyorhinis*, NCTC 10130; *M. bovis*, NCTC 10131; and *M. agalactiae*, NCTC 10123.

capable of growing all of the mycoplasmas included in this study. Because broth cultures of mycoplasmas develop only faint turbidity, alternative methods, such as incorporation of substrates like glucose or arginine, must be used to measure mycoplasma growth. However, certain *Mycoplasma* species, e.g., *M. bovis* and *M. agalactiae*, ferment neither glucose nor arginine, and others may only produce slight color changes in glucose broth. We have found that *M. bovis* and *M. agalactiae* will cause clearly visible acid color changes when pyruvate is added to the glucose-containing medium. Other investigators have used tetrazolium reduction to facilitate color changes with *M. bovis* but have found that the color often disappeared after 1 to 2 days (26). This did not occur with the pyruvate-containing medium.

These results confirm the very good activities of tiamulin and the newer fluoroquinolones against veterinary mycoplasmas. In addition to their broad antibacterial effects, the fact that the fluoroquinolones are mycoplasmaicidal (1, 9) may give them an advantage over other agents with good mycoplasma static activity. The extent of their *in vivo* efficacies will ultimately be determined by their pharmacokinetic properties in different host species. It is interesting to note the apparent widespread emergence of moderate to quite marked resistance in various species of avian, porcine, and bovine mycoplasmas to tylosin and oxytetracycline, agents that have been in veterinary use for many years.

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