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Susceptibilities of *Mycoplasma bovis*, *Mycoplasma dispar*, and *Ureaplasma diversum* Strains to Antimicrobial Agents In Vitro

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The purpose of this study was to determine the susceptibility of various strains of *Mycoplasma bovis*, *Mycoplasma dispar*, and *Ureaplasma diversum*, which are prevalent causes of pneumonia in calves, to 16 antimicrobial agents in vitro. The MICs of the antimicrobial agents were determined by a serial broth dilution method for 16 field strains and the type strain of *M. bovis*, for 19 field strains and the type strain of *M. dispar*, and for 17 field strains of *U. diversum*. Final MICs for *M. bovis* and *M. dispar* were read after 7 days and final MICs for *U. diversum* after 1 to 2 days. All strains tested were susceptible to tylosin, kitasamycin, and tiamulin but were resistant to nifuroquine and streptomycin. Most strains of *U. diversum* were intermediately susceptible to oxytetracycline but fully susceptible to chlortetracycline; most strains of *M. bovis* and *M. dispar*, however, were resistant to both agents. Strains of *M. dispar* and *U. diversum* were susceptible to doxycycline and minocycline, but strains of *M. bovis* were only intermediately susceptible. Susceptibility or resistance to chloramphenicol, spiramycin, spectinomycin, lincomycin, or enrofloxacin depended on the species but was not equal for the three species. The type strains of *M. bovis* and *M. dispar* were more susceptible to various antimicrobial agents, including tetracyclines, than the field strains. This finding might indicate that *M. bovis* and *M. dispar* strains are becoming resistant to these agents. Antimicrobial agents that are effective in vitro against all three mycoplasma species can be considered for treating mycoplasma infections in pneumonic calves. Therefore, tylosin, kitasamycin, and tiamulin may be preferred over oxytetracycline and chlortetracycline.

Several mycoplasma species, of the many that have been isolated from cattle, are pathogenic. *Mycoplasma bovis* is regarded as the most pathogenic, with the exception of *Mycoplasma mycoides* subsp. *mycoides* small-colony biotype, the agent of contagious bovine pleuropneumonia. *M. bovis* causes mastitis in dairy cattle, respiratory tract infections in calves, and arthritis in all age groups of cattle, and it is prevalent worldwide (5). In addition, *Mycoplasma dispar* and *Ureaplasma diversum* have frequently been isolated from the respiratory tracts of pneumonic calves, and their prevalence is assumed to be worldwide also (16). Their pathogenic significance in calf respiratory disease has been proven (7). *U. diversum* can also be isolated from the genital tracts of cows and bulls. *M. bovis*, *M. dispar*, and *U. diversum* are prevalent in the Netherlands (23, 25).

Because bacteria and mycoplasmas are involved in calf pneumonia, calves are often treated with antimicrobial agents. The massive and timely use of macrolide antibiotics, singly or in combination with other drugs, contributed to the recovery of more than 90% of calves affected by pneumonia (12). Although antimicrobial agents cause calves to recover from the clinical signs of respiratory disease (12), they do not eliminate mycoplasmas from the herd. Although the susceptibility of *M. bovis* and *U. diversum* to antimicrobial agents has been studied in vitro (4, 8–11, 13, 14, 20, 22, 28), little is known about the susceptibility of *M. dispar* (9, 11).

In the present study, we examined the susceptibility of numerous strains of these three bovine pathogens to 16 antimicrobial agents by using a serial broth dilution method.

MATERIALS AND METHODS

Mycoplasma strains. Type strain Donetta of *M. bovis* and type strain 462/2 of *M. dispar* were obtained from E. A. Freundt of the former FAO/WHO Collaborating Centre for Animal Mycoplasmas, Institute of Medical Microbiology, University of Aarhus, Århus, Denmark. The type strain of *U. diversum* was not available to us during the study. From 1983 to 1988, field strains of *M. bovis*, *M. dispar*, and *U. diversum* were isolated and identified as described earlier (23). Strains of *M. bovis* were cultured in modified Edward media containing 0.4% tetrazolium chloride, strains of *M. dispar* were cultured in Friis NHS20 broth, and strains of *U. diversum* were cultured in Friis NHU pH 6.0 broth (23).

Sixteen field strains of *M. bovis* were isolated from the lungs of pneumonic calves (B16, F1, Q19, AN13b, AZ25, BD25, BG32, BY47b, DY20b, EA17, and EB14), from mastitic milk (AX20 and DL19), or from synovia collected from arthritic joints (DR40, EO11, and EU46). The strains were isolated from cattle from 14 farms. Nineteen field strains of *M. dispar* (E6, Q26, V24, Y15, AA9, AF7, AF18d, AS15d, AS19, AZ11, BA20, BD5, BD8, BI35, BM10, BS18, BX43, BY47d, and DT10) were isolated from the lungs of pneumonic calves from 16 farms. Seventeen field strains of *U. diversum* were isolated from the lungs (B20, E5, G10, Y9, Y22, V25, AA9, AB13, AF18u, AN13u, AS15u, AT5, BS19, BY48, and DT8) or noses (B15, Q22) of pneumonic calves from 15 farms. Strains B15, B20, E5, G10, AN13u, AT5, and BY48 had been identified as *U. diversum* serogroup A; strains Y9 and AA9 had been identified as serogroup B. The other *Ureaplasma* strains had not been serotyped. The farms from which the three mycoplasma species were isolated were located in various parts of the Netherlands.

Primary cultures of *M. bovis* strains were purified three times by using a Pasteur pipette to suction an agar plug

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bearing one colony (6). Cultures of *M. dispar* and *U. diversum* cannot be purified in this way because no viable cultures are obtained. Therefore, primary cultures of *M. dispar* and *U. diversum* were purified three times by conventional filtration cloning techniques, by using a 450-nm-pore-size membrane filter (26). After the first filtration step, the culture was serially diluted 10-fold in eight tubes with culture broth. Immediately thereafter, the contents of each tube was transferred to eight wells (200 μl per well) of a microtiter plate that was sealed with adhesive tape and incubated at 37°C for 7 (*U. diversum*) or 14 (*M. dispar*) days. The contents of the well containing the highest dilution of viable culture was serially diluted 10-fold in four tubes of broth medium. The tube containing the highest viable dilution was used for the second and third filtration steps.

Antimicrobial agents. The following antimicrobial agents were used: oxytetracycline, chloramphenicol, streptomycin, ampicillin, and benzylpenicillin potassium (Gist-Brocades, Delft, the Netherlands); chlortetracycline and minocycline (Cyanamid, Lederle, the Netherlands); doxycycline (Pfizer, Rotterdam, the Netherlands); spiramycin (Rhône-Mérieux, Toulouse, France); tylosin (Elanco, Nieuwegein, the Netherlands); kitasamycin (Inffa, Houten, the Netherlands); spectinomycin and lincomycin (Upjohn, Ede, the Netherlands); tiamulin (Coopers, Haarlem, the Netherlands); enrofloxacin (Bayer, Mijdrecht, the Netherlands); and nifuroquine (quinaldofur) (Duphar, Amsterdam, the Netherlands).

Tylosin and tiamulin are used only in veterinary medicine. Tylosin is an antibiotic with a structure similar to that of erythromycin. Tiamulin is a semisynthetic antimicrobial agent that does not belong to a particular group. Kitasamycin is a macrolide antibiotic that is used in veterinary medicine. In Japan it has been used successfully in human medicine (15). The three agents have a spectrum similar to the spectrum of the macrolides. Nifuroquine is a quinoline derivative that is used in therapy of bovine mastitis.

The antimicrobial agents were diluted in distilled water to prepare stock solutions, except for the four tetracyclines, which were diluted in 10% methanol, and chloramphenicol and spiramycin, which were diluted in 0.5% *N,N*-dimethylformamide (no. 3034; E. Merck AG, Darmstadt, Germany). Concentrations were calculated as pure substances to prepare stock solutions. The activity of spiramycin was equivalent to 4,468 IU/mg, that is, 1.4 times the activity of the World Health Organization standard. The activity of penicillin was equivalent to 1,592 U/mg. Stock solutions were sterilized by filtration through a 200-nm-pore-size membrane filter and used immediately or stored at 4°C overnight.

Serial broth dilution method for determining MICs. The serial broth dilution method was recommended by an ad hoc working group of the International Research Program on Comparative Mycoplasmology (IRPCM), part of the International Organization for Mycoplasmology, as the most useful and reproducible assay (18). Each antimicrobial agent was serially diluted twofold in culture broth in 10 wells of a microtiter plate; each well contained 25 μl. A standard number of organisms grown in broth without bacterial inhibitors was used. An amount of 175 μl, containing 1.7×10^3 to 1.7×10^4 color-changing units, was added to each well. The susceptibility or resistance of *M. dispar* strains to the antimicrobial agent was indicated by whether the culture was able to metabolize glucose in the presence of one of the concentrations of the antimicrobial agent. Strains of *U. diversum* were tested similarly, but urea instead of glucose was the substrate to be metabolized. Metabolism of glucose was demonstrated by a change of the phenol red indicator from red to yellow; metabolism of urea was demonstrated by a change from yellow to red. The susceptibility or resistance of *M. bovis* strains to the antimicrobial agent was indicated by whether the culture was able to reduce the colorless 2,3,5-triphenyltetrazolium chloride to the red formazan in the presence of one of the concentrations of the antimicrobial agent. Strains of *M. bovis* also produced a film layer on top of the broth.

On the day of inoculation, the required numbers of color-changing units were prepared from stock cultures with a known number of cells, which had been stored at −70°C. Organisms were counted again to verify the actual numbers of organisms in the system. The microtiter plates were sealed with adhesive tape and incubated aerobically at 37°C in the dark to prevent spontaneous reduction of the tetrazolium chloride. The MIC of the antimicrobial agent was determined as the lowest concentration at which the medium did not change color or produce a film layer. The color changes were read several times for 7 days. Because the film developed more slowly than color change, it was read only at day 7. Initial MICs were recorded as soon as the inoculum controls (without the antimicrobial agent) changed color in comparison with the color of the culture medium controls. This was on day 1 to 2 for *U. diversum*, day 2 to 3 for *M. bovis*, and day 2 to 4 for *M. dispar*. Final MICs for *M. bovis* and *M. dispar* were read when color changes or film production had stopped for 1 to 2 days, that is, day 7. Because *Ureaplasma* species grow rapidly, final MICs for *U. diversum* were read after only 24 to 48 h. These time points for reading MICs were in agreement with the recommendations of the IRPCM working group, which recommended that MICs for *Mycoplasma* species be read from 48 h to 7 days and that MICs for *Ureaplasma* species be read after 24 h (18). All tests were performed in duplicate; when MICs differed by no more than a factor of two, the higher concentration of the two was recorded as the MIC.

Interpretation of MICs. When the MIC of the tetracycline group was ≤1 μg/ml, the strain was considered susceptible; when the MIC was 2 or 4 μg/ml, it was considered intermediately susceptible; and when the MIC was ≥8 μg/ml, the strain was considered resistant. These values were ≤4, 8, and ≥16 μg/ml for chloramphenicol; ≤4, 8 or 16, and ≥32 μg/ml for streptomycin; ≤2, ≥4 to ≤16, and ≥32 μg/ml for ampicillin; and ≤0.25, ≥0.5 to ≤4, and ≥8 μg/ml for penicillin (27). These MICs were based on guidelines for testing the susceptibility of bacteria that affect humans. These criteria were used because criteria for animals are not generally available. Criteria for the other antimicrobial agents were not available.

RESULTS

MICs of antimicrobial agents were determined for 50% of the strains tested (MIC_{50}) and for 90% of the strains tested (MIC_{90}). Table 1 shows the MIC_{50}s, the MIC_{90}s, and the MIC range of 15 antimicrobial agents for 16 field strains of *M. bovis*, read at day 7; Table 1 also shows the MICs for type strain Donetta of *M. bovis*. The MIC_{90} of doxycycline was larger at day 7 than at day 4 by a factor of 8, and the MIC_{90} of minocycline was larger by a factor of 16. MIC_{90}s of the other antimicrobial agents were larger than, by a factor of 2 to 4, or equal to the MIC_{90}s read at day 4.

Table 2 shows the MIC_{50}s, the MIC_{90}s, and the MIC range of 16 antimicrobial agents for 19 field strains of *M. dispar*, read at day 7; Table 2 also shows the MICs for type strain

TABLE 1. MICs of antimicrobial agents used against field strains and type strain Donetta of *M. bovis*, determined by a serial broth dilution method[a]

Antimicrobial agent	MIC (μg/ml) for:			
	Field strains (n = 16)			Type strain Donetta
	50%	90%	Range	
Oxytetracycline	16	32	8–>64	4
Chlortetracycline	16	32	8–32	4
Doxycycline	1	4	0.25–8	0.125
Minocycline	2	8	0.5–8	0.5
Chloramphenicol	16	32	8–64	8
Spiramycin	0.5	1	0.25–16	0.25
Tylosin	0.25	0.5	0.06–4	0.125
Kitasamycin	2	2	0.5–8	2
Spectinomycin	2	4	1–4	4
Tiamulin	0.03	0.06	≤0.015–0.5	≤0.015
Lincomycin	0.5	1	0.25–1	0.25
Enrofloxacin	1	1	0.5–2	1
Nifuroquine	32	>64	1–>64	4
Streptomycin	16	>64	8–>64	>64
Penicillin	>64	>64	>64	>64

[a] MICs were read when color changes had stopped for 1 to 2 days, that is, day 7 after inoculation (final MICs).

462/2 of *M. dispar*. MICs were generally larger by a factor of 2 to 4 at day 7 than at day 5.

Table 3 shows the MIC_{50}s, the MIC_{90}s, and the MIC range of 16 antimicrobial agents for 17 field strains of *U. diversum*, read at day 1 to 2.

DISCUSSION

The purpose of the study was to determine the in vitro susceptibilities of strains of *M. bovis*, *M. dispar*, and *U. diversum*, which are prevalent causes of pneumonia in calves, to antimicrobial agents. In vitro susceptibility testing of mycoplasmas presents several problems that make standardization of methods difficult (3). Because no single medium is suitable for in vitro testing of all species, we had to use a different medium for each of the three species. Because broth cultures of mycoplasmas develop only faint turbidity, alternative methods must be used to measure mycoplasmal growth, for example, metabolism of glucose, arginine, or urea. A pH indicator such as phenol red visualizes the pH shift due to biochemical activities of multiplying mycoplasmas. Because *M. bovis* has none of these biochemical activities, however, various methods have been used for in vitro susceptibility testing, including acid production (because *M. bovis* may slightly acidify the medium) (8), hydrolysis of Tween 80 (4), an agar diffusion method (9, 28), a nephelometric method, and a test for phosphatase (14). We used tetrazolium reduction as an indicator for growth, as suggested by the IRPCM working group (18). Because the formazan that was produced stained only slightly red and this color often disappeared after 1 to 2 days, results were confirmed by observing the film layer that developed slowly and could be read only after 7 days of incubation. Furthermore, the growth rate of the various mycoplasma species differs. For example, *U. diversum* reaches its stationary growth phase within 24 to 48 h, whereas *M. dispar* reaches this stage after 4 days. This can cause instability of antimicrobial agents in media. A loss of antimycoplasmal activity of tetracyclines during prolonged incubation, in fact, is a well-known phenomenon (3). Although this may be the reason for the resistance of the *Mycoplasma* strains to oxytetracycline and chlortetracycline in this study, strains of other species were found to be susceptible after an incubation period of 7 days in an earlier study that used identical methods (24).

Although the working group of the IRPCM standardized most procedures for testing the susceptibility of mycoplasmas (18), the length of incubation time has yet to be standardized. The working group recommended that final MICs should be read when the color has stopped changing after a defined period of incubation. In an earlier study, we concluded that this period should not exceed 2 days (24). In the present study, however, cultures sometimes continued to change color, depending on species, strain, or antimicro-

TABLE 2. MICs of antimicrobial agents used against field strains and type strain 462/2 of *M. dispar*, determined by a serial broth dilution method[a]

Antimicrobial agent	MIC (μg/ml) for:			
	Field strains (n = 19)			Type strain 462/2
	50%	90%	Range	
Oxytetracycline	16	>64	2–>64	2
Chlortetracycline	16	64	4–64	2
Doxycycline	0.5	4	0.06–4	0.125
Minocycline	0.25	1	≤0.015–1	0.03
Chloramphenicol	2	4	1–8	4
Spiramycin	0.5	2	0.25–4	1
Tylosin	0.06	0.25	0.03–0.25	0.25
Kitasamycin	1	2	0.5–2	1
Spectinomycin	1	2	0.5–1	2
Tiamulin	0.125	0.25	0.06–0.25	0.125
Lincomycin	0.5	1	0.25–1	0.5
Enrofloxacin	0.25	0.5	0.125–2	0.25
Nifuroquine	64	>64	16–>64	64
Streptomycin	64	>64	32–>64	>64
Ampicillin	>64	>64	>64	>64
Penicillin	>64	>64	≥64	>64

[a] MICs were read when color changes had stopped for 1 to 2 days, that is, day 7 after inoculation (final MICs).

TABLE 3. MICs of 16 antimicrobial agents for 17 field strains of *U. diversum*, determined by a serial broth dilution method[a]

Antimicrobial agent	MIC (μg/ml)		
	50%	90%	Range
Oxytetracycline	4	16	1–32
Chlortetracycline	0.25	2	≤0.125–4
Doxycycline	0.25	0.5	0.06–2
Minocycline	0.125	0.5	0.06–2
Chloramphenicol	8	16	2–16
Spiramycin	16	16	1–>16
Tylosin	0.5	1	0.03–2
Kitasamycin	0.25	0.5	0.03–0.5
Spectinomycin	8	32	4–32
Tiamulin	0.125	0.5	≤0.015–0.5
Lincomycin	>64	>64	16–>64
Enrofloxacin	4	8	2–8
Nifuroquine	64	>64	4–>64
Streptomycin	>64	>64	4–>64
Ampicillin	>64	>64	>64
Penicillin	>64	>64	>64

[a] MICs were read when color changes of the inoculum controls were maximal, that is, day 1 to 2 after inoculation (final MICs).

bial agent used. Therefore, final MICs for the *Mycoplasma* strains were read when most strains had stopped changing color for 1 or 2 days. Another useful suggestion is to read final MICs after a period of time twice as long as that required for the control to change color (17).

In several *U. diversum* tests, especially those with chlortetracycline, color continuously changed during the observation period of 7 days. Probably, urea continued to be degraded by the enzyme urease in the inhibited organisms. MICs could not be read for one particular strain of *U. diversum* after 2 days of incubation because an alkaline color change had developed in all inoculated wells. Bloomster and Lynn (2) demonstrated that residual urease activity from dead organisms considerably influenced the dynamics of color changes in *Ureaplasma urealyticum* cultures. This color change could cause errors in evaluating the susceptibility of ureaplasmas to antimicrobial agents. Reading MICs for *U. urealyticum* strains after incubation periods of different lengths is the main cause for the variation in the MICs published for a particular antimicrobial agent (21). Therefore, and because *Ureaplasma* species grow rapidly, the working group of the IRPCM recommended that final MICs of *Ureaplasma* cultures can generally be read after 24 h (18).

We regarded the MICs for *M. bovis* that were read after 7 days of incubation as the final MICs, but other studies have read final MICs after 2 to 3 (4), 3 (20), 4 (14), 5 (28), 6 (9), or 7 (8) days. Only Hannan et al. (9) reported MICs for type strain Donetta of *M. bovis*. Our results generally agree with those reported earlier (4, 8, 14, 20).

Although *M. dispar* grows more slowly than *M. bovis*, final MICs were read after 7 days (Table 2). At day 7, color changes had stopped for only 1 day, in general. Only Hannan et al. (9) reported MICs for type strain 462/2 of *M. dispar*. We found a higher MIC for oxytetracycline (2 μg/ml) than that of Hannan et al. (0.25 μg/ml), but methods also differed. MICs of tylosin and tiamulin were similar in both studies. Matsuoka et al. (11) reported MICs of tylosin for *M. dispar* similar to those we found. No other reports on MICs for *M. dispar* are available.

Andrews et al. (1) reported that *M. dispar* was more frequently isolated when ampicillin in the media was substituted for penicillin that was used in a concentration of 200 IU/ml. The MICs of penicillin had not been determined, however. The highest concentration of penicillin that we tested was 64 μg/ml, which is equivalent to 102 IU/ml. We found a final MIC of this value for three strains, so we confirm that penicillin must not be incorporated in media for *M. dispar*.

Final MICs for *U. diversum* were read after only 1 to 2 days of incubation, and strains were found to be resistant to various antimicrobial agents. Our results generally agree with those reported earlier (10, 13, 20, 22). The resistance of *U. urealyticum* to lincomycin was reported in 1968 (19). We confirmed the resistance of *U. diversum* to lincomycin.

It has been the experience in chemotherapy for decades that two groups of antimicrobial agents, i.e., tetracyclines and macrolides, are of primary importance in treating animal mycoplasma infections. The older tetracyclines, such as oxytetracycline and chlortetracycline, are cheaper than the newer tetracyclines, such as doxycycline and minocycline. The dosage of the newer tetracyclines, however, can be lower, because they are better absorbed after oral administration (3).

However, bovine mycoplasma strains are acquiring resistance to tetracyclines (in particular, to oxytetracycline), as demonstrated by our study. This finding is notable because oxytetracycline is frequently used in calf husbandry in the Netherlands. *M. bovis* and *M. dispar* were less susceptible to doxycycline and minocycline, however, than *U. diversum* was.

Mycoplasma strains are known to be susceptible to tiamulin and tylosin, although strains are generally more susceptible to tiamulin than to tylosin. The MICs of these antimicrobial agents were low for all strains studied; for one *M. bovis* strain, however, the MIC of tylosin was as high as 4 μg/ml. The *U. diversum* strains were generally less susceptible to tylosin than strains of *M. bovis* or *M. dispar*. Because in other countries some strains were less susceptible to tiamulin (*M. bovis*) or tylosin (*M. bovis* and *U. diversum*), these strains may also be acquiring resistance to tylosin (14, 20). Another indication that field strains of *M. bovis* are developing resistance is that the MICs of various antimicrobial agents for type strain Donetta were lower than those for any field strain. The type strain of *M. dispar* was more susceptible to the tetracyclines than most field strains; this indicates that *M. dispar* strains are also acquiring resistance.

Because the three mycoplasma species often occur in pneumonic calves of one herd and even in the respiratory tract of one calf (23), antimicrobial agents that are effective in vitro against all three species can be considered for use in vivo. Therefore, tylosin, kitasamycin, and tiamulin may be preferred over oxytetracycline and chlortetracycline.

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