ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1982, p. 554–557
0066-4804/82/040554-04$02.00/0

Vol. 21, No. 4

Antimicrobial Susceptibility of *Moraxella bovis* Determined by Agar Disk Diffusion and Broth Microdilution

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The antimicrobial susceptibility of 84 isolates of *Moraxella bovis* was evaluated by the standard agar disk diffusion and broth microdilution procedures. All isolates were resistant to cloxacillin by disk diffusion, with 97% of isolates having a minimal inhibitory concentration of ≥2 μg/ml. Of the hemolytic isolates, 68% were resistant to streptomycin. A high frequency of susceptibility was recorded for all other antimicrobial agents tested. Quantitative data supported the use of sulfonamides, but not tylosin, for parenteral infectious bovine keratoconjunctivitis therapy.

A wide range of astringents, antiseptics, and antimicrobial agents have been used for the treatment of infectious bovine keratoconjunctivitis (IBK) (8, 9, 12, 21, 26, 27). *Moraxella bovis* is considered to be the most important infectious agent involved in the etiology of IBK (4, 29).

Antimicrobial susceptibility, as measured by the standard agar disk diffusion procedure, has been reported for *M. bovis* (2, 24). Of 276 *M. bovis* isolates tested by Arora and Killinger (1) and Arora et al. (2), 9.6% of the hemolytic and 1.7% of the nonhemolytic isolates were resistant to streptomycin. Isolates were collected from different herds on a single farm over a period of time. A greater frequency of resistance was seen in 160 isolates of *M. bovis* collected by Pugh and McDonald from 30 epizootics of IBK (25). More than 60% of the isolates were resistant to nalidixic acid, lincomycin, and sulfamethoxypyridazine. The minimal inhibitory concentration (MIC) of chloramphenicol, neomycin, penicillin, sulfonamides, and tetracycline for 10 isolates of *M. bovis* has been reported to be ≤0.8 μg/ml in each case (20, 25).

The purposes of this study were to determine (i) whether there was any significant antimicrobial resistance to drugs commonly used in ophthalmic preparations for the treatment of IBK in *M. bovis* field isolates and (ii) whether, when standard techniques were used, qualitative (disk diffusion) and quantitative (MIC) differences occurred in the antimicrobial susceptibility of *M. bovis* field isolates obtained over a broad geographic area in Missouri in different years.

(This communication is part of a dissertation by J.J.W. submitted to the Graduate School, University of Missouri, Columbia, in partial fulfillment of the requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Bacterial organisms. A total of 84 isolates of *M. bovis* were used in the study; 71 were cultured from cattle in 54 epizootics of IBK in Missouri from 1978 to 1981. In addition, G. W. Pugh (National Animal Disease Center, Ames, Iowa) kindly supplied 12 isolates of *M. bovis* and 1 each of *M. liquefaciens* and *M. nonliquefaciens*. Strain ATCC 10900 of *M. bovis* was obtained from the American Type Culture Collection, Rockville, Md. Identification of all isolates was done on the basis of standard biochemical reactions (16, 24) and confirmed by fluorescence microscopy (23).

Disk diffusion susceptibility. The modified, standardized single high-potency agar disk diffusion method for antimicrobial susceptibility testing was used (7). This method is currently recommended by the U.S. Food and Drug Administration (10, 11). The antimicrobial disks used and their contents are listed in Table 1. A disk containing 5 μg of kanamycin was used instead of a disk containing 30 μg, which differs from standard methods (10, 11).

Five isolated colonies of 24-h-old cultures on brain heart infusion agar (Difco Laboratories, Detroit, Mich.), containing 5% defibrinated bovine blood, were used to inoculate 4-ml brain heart infusion broth (Difco Laboratories) tubes. These were incubated for 4 to 6 h at 35°C in a shaker bath until the turbidity reached that of a 0.5 McFarland nephelometer standard. This suspension, containing approximately 1.5×10^8 colony-forming units per ml, served as the inoculum for the agar disk diffusion and MIC determinations, which were performed simultaneously. Mueller-Hinton agar (BBL Microbiological Systems, Cockeysville, Md.) was used for the agar disk diffusion procedure.

MIC. The MIC was determined by a broth microdilution procedure (13, 14, 19) with V-bottom 96-well microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.). The antimicrobial agents used and the range of their dilutions tested are listed in Table 1.

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TABLE 1. Antimicrobial agents used for *M. bovis* susceptibility testing[a]

Antimicrobial agent	Disk potency	Serial dilution range for MIC (per ml)
Ampicillin	10 μg	128–0.12 μg
Bacitracin	10 U	128–0.12 U
Chloramphenicol .	30 μg	128–0.12 μg
Cloxacillin	1 μg	128–0.12 μg
Gentamicin	10 μg	128–0.12 μg
Kanamycin	5 μg	128–0.12 μg
Neomycin	30 μg	128–0.12 μg
Nitrofurazone	100 μg	16–0.01 μg
Oxytetracycline ..	30 μg	128–0.12 μg
Penicillin	10 U	128–0.12 U
Polymyxin B	300 U	128–0.12 U
Streptomycin.....	10 μg	128–0.12 μg
Triple sulfa	300 μg	512–1.00 μg
Tylosin.....	ND[b]	128–0.12 μg

[a] Susceptibility testing by agar disk diffusion and broth microdilution procedures.
[b] ND, Not done; no disks available.

Serial two-fold dilutions of the antimicrobial agents were prepared in the microtiter plates with an automatic diluting apparatus (Titertek Medi-Mixer, Flow Laboratories, Inc., Rockville, Md.). The inocula used for the disk diffusion procedure were further diluted to contain 2×10^6 colony-forming units per ml; these suspensions were then inoculated into the microtiter plates with disposable polystyrene multi-inoculators (Dynatech Laboratories), which deposited 0.01 ml of inoculum in each well of the plate. Plates were incubated at 35°C, and the results were read after 18 to 24 h. The endpoint (MIC) was taken as the lowest concentration of antimicrobial agent at which the tested organism did not show growth.

Quality control. Three reference strains (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853) for which there are published standards for disk diffusion zone size and broth microdilution procedures (6, 13) were tested, using the same procedures, media, antimicrobial disks, and antimicrobial dilution plates. Results were considered acceptable if disk diffusion zone sizes fell within the published acceptable limits for the three reference strains and if MIC values were within 1 logarithm dilution of published standards.

RESULTS

Agar disk diffusion. All strains of *M. bovis* tested were considered to be resistant to cloxacillin, as growth was not inhibited with the disks containing 1 μg of the drug; 68% of the hemolytic isolates were resistant to streptomycin, whereas all of the nonhemolytic isolates were susceptible to streptomycin. One hemolytic isolate was resistant to triple sulfonamides. In all other instances, the *M. bovis* isolates were susceptible to the antimicrobial agents listed in Table 1 (Table 2).

MIC. The MIC values of cloxacillin, gentamicin, penicillin, and streptomycin were significantly ($P < 0.01$) higher for hemolytic isolates than for nonhemolytic isolates. The geometric mean (5) and the range of MIC values for hemolytic and nonhemolytic isolates are shown in Table 3. The apparent uniform resistance to cloxacillin by disk diffusion was supported in the MIC determination; 97% of the hemolytic isolates tested had MIC values of ≥2 μg/ml, and 3% had an MIC of 1 μg/ml (Table 4). Of isolates found resistant to streptomycin by disk diffusion, 87% had streptomycin MIC values of ≥128 μg/ml. The isolates that were susceptible to streptomycin by disk diffusion had corresponding MIC values of ≤0.5 μg/ml (Table 4).

TABLE 2. In vitro susceptibility of 84 *M. bovis* isolates determined by agar disk diffusion

M. bovis isolate	No. of strains tested	% of isolates resistant to:		
		Cloxacillin	Streptomycin	Other antimicrobial agents
Hemolytic	66	100	68	1.5[a]
Nonhemolytic	18	100	0	0[b]

[a] One isolate was resistant to triple sulfonamides.
[b] All isolates were susceptible to all of the other antimicrobial agents listed in Table 1.

There was no significant difference in antimicrobial susceptibility among *M. bovis* isolates obtained from different areas in Missouri. There likewise was no difference between isolates obtained in Missouri and those obtained from G. W. Pugh, with one exception: one of the latter group of isolates was resistant to sulfonamides, as determined by disk diffusion.

M. nonliquefaciens was susceptible to all antimicrobial agents by disk diffusion and MIC, whereas *M. liquefaciens* was resistant only to cloxacillin by disk diffusion (cloxacillin MIC, 2 μg/ml).

Contrary to the findings of LeGoffic and Martel (17), no *Moraxella* isolates were resistant to kanamycin.

DISCUSSION

The 84 *M. bovis* isolates tested were susceptible in vitro to all of the antimicrobial agents commonly used in topical ophthalmic preparations. With topical treatment, levels of antimicrobial agent far in excess of the MIC can readily be achieved in the precorneal tear film. However, in the face of profuse lacrimation as seen in acute cases of IBK, it is doubtful whether, with sporadic application, these levels can be sustained for a sufficient length of time (18) to enable the antimicrobial agent to totally eliminate the *M. bovis* carrier state in IBK (22). The mean MICs obtained in our study for triple sulfonamides and tylosin were 7.13 and 6.69 μg/

TABLE 3. MICs[a] of *M. bovis* isolates

Antimicrobial agent	U or μg/ml	Hemolytic *M. bovis* (*n* = 66)		Nonhemolytic *M. bovis* (*n* = 18)	
		Mean[b]	Range	Mean[b]	Range
Ampicillin	μg	0.13	0.12–0.50	0.13	—[c]
Bacitracin	U	1.80	0.12–16.0	3.43	0.25–16.0
Chloramphenicol	μg	0.81	0.25–4.00	0.71	0.25–1.00
Cloxacillin	μg	6.42[d]	1.00–>128	2.94	1.00–8.00
Gentamicin	μg	0.37[d]	0.12–2.00	0.15	0.12–0.50
Kanamycin	μg	0.15	0.12–0.50	0.16	0.12–0.50
Neomycin	μg	0.13	—[c]	0.13	—[c]
Nitrofurazone	μg	0.92	0.25–2.00	0.82	0.25–2.00
Oxytetracycline	μg	0.64	0.12–2.00	0.52	0.12–1.00
Penicillin	U	0.25[d]	0.12–1.00	0.13	0.12–0.25
Polymyxin B	U	0.13	0.12–1.00	0.14	0.12–0.50
Streptomycin	μg	19.74[d]	0.12–>128	0.27	0.12–0.50
Triple sulfonamides	μg	7.13	1.00–512	4.16	2.00–32.0
Tylosin	μg	6.69	1.00–>128	7.41	4.00–128

[a] As determined by broth microdilution.
[b] Geometric mean.
[c] All values were alike; thus, no range could be calculated.
[d] Statistically significant difference in MIC ($P < 0.01$).

ml, respectively. These levels can readily be achieved in cattle, for sulfonamides (21), by a single intravenous dose of sulfadimidine at 100 mg/kg of body weight. The maximum achievable blood level of tylosin is 1 μg/ml after intramuscular administration of 12.5 mg/kg (15). Despite its wide distribution in body tissues and fluids (3), it is doubtful whether concentrations of tylosin can be achieved in the lacrimal glands and nasal and sinus mucosae that approach the mean MIC recorded for the 84 *M. bovis* isolates in this study.

TABLE 4. MICs of *M. bovis* isolates for cloxacillin and streptomycin

MIC (μg/ml)	%[a] of *M. bovis* isolates inhibited by:			
	Cloxacillin		Streptomycin	
	Hemolytic	Nonhemolytic	Hemolytic	Nonhemolytic
>128	100.0	100.0	100.0	100.0
128	97.0	100.0	59.1	100.0
64	97.0	100.0	40.9	100.0
32	97.0	100.0	37.9	100.0
16	93.9	100.0	34.8[b]	100.0
8	87.9	100.0	34.8	100.0
4	42.4	83.8	34.8	100.0
2	13.6	55.6	34.8	100.0
1	3.0	5.6	34.8	100.0
0.5	0	0	31.8[c]	100.0
0.25	0	0	27.2	66.7
0.125	0	0	10.6	22.2

[a] Data are expressed as cumulative percentage of isolates.
[b] All isolates with streptomycin MIC values of ≥16 were resistant by disk diffusion.
[c] All isolates with streptomycin MIC values of ≤0.5 μg/ml were susceptible by disk diffusion.

Antimicrobial mastitis ointments are often used for the topical treatment of IBK (28); hence, cloxacillin was included in the antimicrobial agents selected for in vitro susceptibility testing of *M. bovis*. The uniform resistance of all *M. bovis* isolates to cloxacillin should discourage the use of cloxacillin mastitis preparations for IBK therapy.

In general, our finding that *M. bovis* is susceptible to most antimicrobial agents used in ophthalmic preparations supports the findings of others (1, 20, 23). However, the high frequency of resistance to streptomycin that occurred only in hemolytic isolates of *M. bovis* and the uniform resistance of all *M. bovis* isolates to cloxacillin have not been reported previously. Quantitative data support the systemic use of sulfonamides for IBK therapy. Based on MIC values in this study, it is doubtful that tylosin would be an effective systemic drug for the treatment of *M. bovis* infection, unless *M. bovis* isolates had tylosin MIC values of 1 μg/ml.

ACKNOWLEDGMENTS

This study was supported in part by U.S. Department of Agriculture formula grant no. 800-C-2-5021 and a grant from Grand Laboratories, Freeman, S.D.

We thank Karen McLaughlin and Judy Cavcey for their excellent technical assistance.

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