The In Vitro Susceptibility of Moraxella Bovis to Selected Antibiotics and Sulfonamides

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Technical Bulletin T-84

June 1960

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Bovine infectious keratitis is an important cause of economic losses in cattle in the United States. The losses are due primarily to lowered milk production in dairy cattle and loss of weight in beef cattle.

At present there is no completely effective method of immunizing animals against infectious keratitis and the recommended therapeutic treatments have not been wholly successful.

This infectious disease in cattle in the United States is reputed to be caused by a pathogenic bacterium, *Moraxella bovis*. There has been some disagreement among previous workers as to its exact classification and etiological role in the disease. However, at the present time, it is generally accepted as being the causative agent of bovine infectious keratitis. The name *Moraxella bovis*, will be used in this paper in accordance with the terminology presented in Bergeys Manual of determinative Bacteriology 7th ed.

Barner (1952) was able to isolate *Moraxella bovis* from 92 of 95 cattle infected with acute keratitis. He was able to reproduce an inflammatory eye condition typical of field cases of "pinkeye" in four experimental calves using a pure culture of *Moraxella bovis*. In addition, he tested the sensitivity of four cultures of *M. bovis* to five antibiotics using antibiotic discs placed on an inoculated seed layer surface. Under these conditions chloramphenicol and penicillin gave the largest zones of growth inhibition. The five antibiotics tested were bacitracin, chloramphenicol, penicillin, dihydrostreptomycin and terramycin.

During an outbreak of infectious keratitis, Rastegaeva *et al.* (1947) found the lesions yielded a penicillin sensitive hemolytic diplococcus morphologically resembling the pneumococcus.

Faull *et al.* (1954) using sensitivity tablets found the organism sensitive *in vitro* to 0.5 units of penicillin, 20 ug of streptomycin and 40 ug of chloromycetin.

The present study was instigated to determine the susceptibility of *M. bovis* to a number of different antibiotics and sulfonamides, and to see if there was any significant difference in susceptibility among different isolates of the organism.

Materials and Methods

The organism was isolated from 21 clinical cases of keratitis by swabbing the eyes with a sterile cotton swab and streaking on tryptose blood agar to which 10 per cent citrated bovine blood had been added. After 24 hours incubation a colony of *M. bovis* was selected and transferred to a blood agar slant. Following a 24 hour incubation period the bacteria were harvested using a small amount of sterile bovine serum. A small aliquot of the bovine serum with the organisms in suspension was then lyophilized.

The cultures were lyophilized as they were collected since it was not feasible to test each culture when it was isolated. This procedure standardized the length of time each culture had grown on artificial media prior to testing its sensitivity to the antibiotics.

Cultures were collected from very young calves, year-old steers, and older brood cows. Lyophilized cultures were also obtained from Kansas State University, Lederle Laboratories, and the American Type Culture Collection.

When ready to be tested the lyophilized cultures were opened and reconstituted with sterile distilled water. They were then streaked on blood agar and incubated 24 hours to obtain a typical colony which was transferred to a blood agar slant. This slant was incubated 24 hours and harvested with distilled water to obtain the inoculum used in the seed layer.

Assay cups containing 0.1 ml of antibiotic solution were used to produce zones of growth inhibition. By using the assay cup method one can determine the exact concentration of antibiotic desired. Different concentrations were prepared to insure a zone of growth inhibition for each antibiotic.

Large pyrex plates (13.5 X 8.75 inches) were used instead of petri dishes. By employing plates this size it was possible to use 21 assay cups per plate which greatly facilitated pouring the base layer and seed layer. This method also helped to prevent contamination as it enabled one to inoculate 50 ml of seed media with a standardized dilution of inoculum at one time. The base layer was an enriched agar using yeast extract (3 gm), peptone (10 gm), beef extract (3 gm), sodium chloride (5 gm), agar (17 gm) and distilled water (1000 ml). A 200 ml quantity of this agar was used to cover the bottom of the plate. The seed layer used was a tryptose blood agar base (Difco) containing 0.3 per cent yeast extract to which 10 per cent citrated bovine blood was added. A 50 ml quantity of seed agar was used per plate. When using this amount of media the depths of the media were virtually the same as the petri dish method using 20 ml of base medium and 5 ml of seed media. Tests were made to compare the petri dish and the large plate methods. These tests produced identical results.

The base layer was poured and allowed to stand for at least one hour. The seed agar was then melted and cooled to 50° C. Five ml of citrated blood and 1 ml of a suspension of *M. bovis* were added and the agar poured on the base layer. The suspension of *M. bovis* was prepared by harvesting the growth on a 24 hour blood agar slant using sterile distilled water. This suspension was put in a screw cap tube with glass beads and agitated vigorously. The organisms were then diluted to 60 per cent transmittance through a 13 mm tube using a photolometer set at 525 mu.

When the seed layer had hardened the assay cups were placed on the media with sterile forceps. The bottom of the pyrex plates had been previously marked with glass ink so that when the plate was held over an illuminated brucella testing box the marks were plainly visible. This procedure facilitated placing the assay cups the proper distance (approximately 5 cms) apart on the media.

To obtain the required concentration of antibiotic 100 mg of antibiotic were weighed out on an analytical balance and sufficient sterile double distilled water was added to make the correct dilution. Erythromycin and carbomycin were not easily dissolved in water so they were first dissolved in 0.5 ml of methyl alcohol and the water then added. This small amount of methyl alcohol did not affect the size of the zones of inhibition. The assay cups used were stainless steel cups with an outside diameter of 0.80 cms.

After 24 hours the antibiotic solutions had been absorbed by the media and the zones of growth inhibition which appeared on the plates were read at that time. To read the zones of inhibition a low power microscope (10X) was used. It was necessary to use a microscope to distinguish the true zone of inhibition when studying this hemolytic organism to avoid confusing the zone of hemolysis and the zone of inhibition. The zones were measured using a caliper with sharpened ends. The caliper points were placed at opposite edges of the zone and the distance between the two caliper points was then measured using a millimeter rule. All tests were run in duplicate and the average of the two recorded as the zone of inhibition.

Results

The susceptibility of *M. bovis,* to the antibiotics, as measured by the diameter of the zone of growth inhibition produced using 24 isolates and thirteen antibiotics is summarized in table I.

Table I—The Average Zone of Inhibition for 24 Isolates Using 13 Antibiotics

Antibiotic	Micrograms per 0.1 ml solution	Average diameter* of the zones of inhibition (cms)
Achromycin	0.5	1.18
Achromycin	1.0	1.55
Albamycin	1.0	1.25
Aureomycin	0.5	1.24
Aureomycin	1.0	1.57
Bacitracin	10.0	0.94
Bacitracin	20.0	1.22
Bacitracin	40.0	1.43
Carbomycin	5.0	1.61
Chloroamphenicol	1.0	1.42
Erythromycin	1.0	1.58
Magnamycin	30.0	0.80
Magnamycin	60.0	0.83
Magnamycin	90.0	0.98
Neomycin	10.0	1.35
Penicillin	0.15	2.85
Penicillin	0.30	3.77
Polymyxin B Sulfate	20.0	0.95
Polymyxin B Sulfate	40.0	1.14
Polymyxin B Sulfate	60.0	1.33
Streptomycin	5.0	1.65
Terramycin	1.0	0.97
Terramycin	2.0	1.28

*The diameter of the zone includes the width of the assay cup which is 0.80 cms.

Table I lists the antibiotics used, the concentration of antibiotic used per 0.1 ml of antibiotic solution and the average diameters (cm) of the zones of inhibition for the 24 isolates studied.

Penicillin was used at the lowest concentration (0.15 ug) and produced the largest zones of inhibition. It produced a zone with an average diameter of 2.85 cm when used at this concentration. Achromycin, albamycin, aureomycin, chloramphenicol, erythromycin, and terramycin were all tested at concentrations of 1.00 ug. These antibiotics with the exceptions of terramycin and albamycin all had average diameters of approximately 1.5 cms. Terramycin at this concentration had zones which averaged 0.97 cms in diameter while albamycin had an average zone of 1.25 cms. Carbomycin and streptomycin were tested at a concentration of 5.00 ug. At this concentration carbomycin and streptomycin produced average zones of 1.61 cm and 1.65 cm respectively. Bacitracin, polymyxin B sulfate, and magnamycin were all tested at concentrations of 10.00 ug or greater and produced zones much smaller than the rest of the antibiotics.

Table II—The Average Zone of Inhibition for 24 Isolates Using 5 Sulfonamides

Antibiotic	Micrograms per 0.1 ml solution	Average diameter* of the zones of inhibition (cms)
Sulfanilamide	30	0.81
Sulfanilamide	60	0.82
Sulfanilamide	90	0.86
Sulfanilamide	120	1.03
Sulfanilamide	250	1.30
Sulfadiazine	30	0.83
Sulfadiazine	60	0.89
Sulfadiazine	90	1.09
Sulfadiazine	250	1.41
Sulfamerizine	30	0.83
Sulfamerizine	60	0.91
Sulfamerizine	90	1.09
Sulfamerizine	250	1.41
Sulfapyridine	30	0.84
Sulfapyridine	60	0.88
Sulfapyridine	90	1.14
Sulfapyridine	250	1.56
Sulfathiazole	30	0.86
Sulfathiazole	60	0.95
Sulfathiazole	90	1.36
Sulfathiazole	250	1.81

*The diameter of the zone includes the width of the assay cup which is 0.80 cms.

The sulfonamides were tested at concentrations ranging from 30.00 to 250.00 ug. It was observed that the phenomenon of partial inhibition was produced by some of the isolates. In the zones of partial inhibition,

colonies were present but were so small they could be easily overlooked. Only the zones of complete inhibition for the sulfonamides were recorded in table II.

Table II shows that sulfapyridine and sulfathiazole produced zones whose average diameter was slightly greater than the other three sulfonamides.

To get a more complete inhibition curve for each antibiotic a culture of *M. bovis* (ATCC) was tested using each antibiotic at the following concentrations: 0.0625, 0.125, 0.25, 0.50, 1.0, 2.0, 4.0, 10.0, 20.0, 30.0, 40.0, and 50.0 ug.

The curves of inhibition produced by the following antibiotics: penicillin, erythromycin, achromycin, streptomycin, albamycin, magnamycin and bacitracin are shown in figure 1.

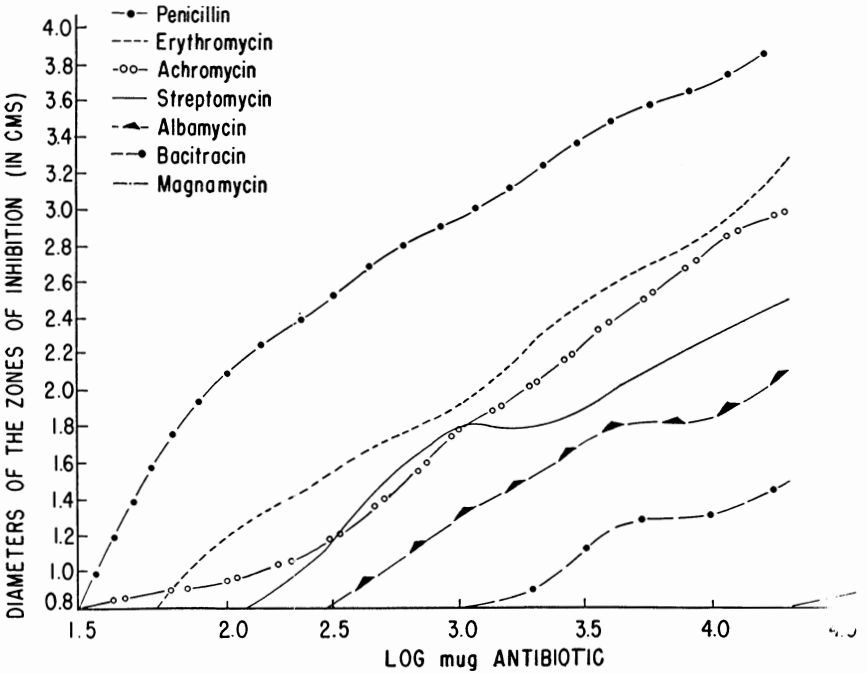

Figure 1. Growth Inhibition Curves for M. Bovis (ATCC) Using Penicillin, Erythromycin, Achromycin, Streptomycin, Albamycin, Magnamycin, and Bacitracin.

The curves of inhibition produced by the following antibiotics: chloramphenicol, aureomycin, neomycin, carbomycin, terramycin and polymyxin B sulfate are shown in figure 2.

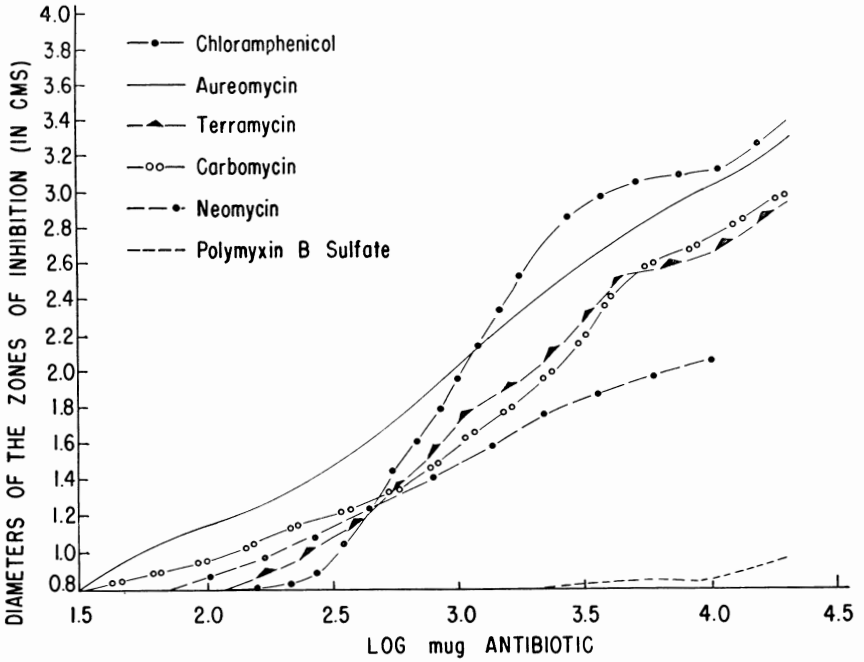

Figure 2. Growth Inhibition Curves for M. bovis (ATCC) Using Chloramphenicol, Aureomycin, Terramycin, Carbomycin, Neomycin, and Polymyxin B Sulfate.

The greatest inhibition activity, per microgram of antibiotic, occurred at concentrations from 0.0 to 5.0 ug with all but three of the antibiotics; therefore, the graphs were plotted using the log ug antibiotic for concentrations from 0.0 to 20.0 ug.

The sulfonamides were tested at the following concentrations: 25, 50, 100, 150, 200 and 250 ug using the same test organism that was used to test the antibiotics. The curves of inhibition produced by the sulfonamides are shown in figure 3.

Discussion

There was some variation in the size of the zones of inhibition for the different isolates using the same antibiotic but the difference was not great. There was a significant difference in the size of the zones of inhibition induced by the selected antibiotics.

When a more complete inhibition curve for each antibiotic was obtained, penicillin, erythromycin, achromycin, chloramphenicol and

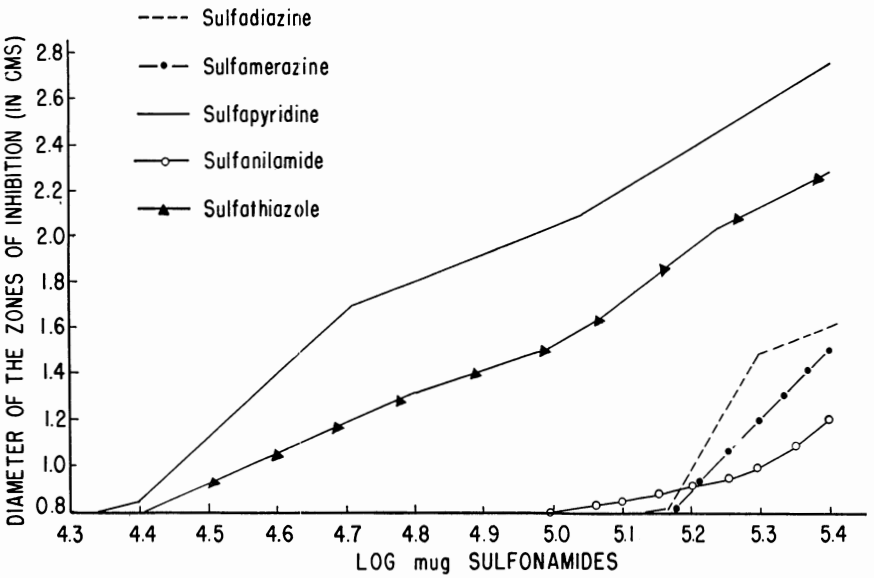

Figure 3. Growth Inhibition Curves for M. Bovis (ATCC) Using Sulfadiazine, Sulfamerizine, Sulfaypridine, Sulfamide, and Sulfathiazole.

aureomycin had the largest zones of inhibition. These antibiotics at the 50 ug level all had zones of inhibition with a diameter of 3.6 cm or larger.

The next group consisting of carbomycin, terramycin, neomycin, streptomycin and albamycin had zones which ranged in diameter from approximately 2.7 cm to slightly over 3.2 cm.

The third or lowest group ranged from practically no zone for magnamcyin to 1.28 cm for polymyxin B sulfate and 1.8 cm for bacitracin.

No tests were performed to determine the rate of diffusion of the selected antibiotics in the agar used. Therefore one can not arbitrarily say that the antibiotic producing the largest zone of inhibition is the most active. However, the size of the zone must be regarded as an indication of the susceptibility of the organism to the antibiotic.

Two of the sulfa drugs, sulfapyridine and sulfathiazole, had zones of inhibition measuring 2.75 and 2.25 cm respectively at concentrations of 250 ug. Sulfadiazine, sulfamerizine and sulfanilamide had zones of inhibition of 1.6 cm, 1.5 cm, and 1.37 cm respectively at the 250 ug.

The phenomenon of partial inhibition was observed with some of the isolates when testing the sulfonamides. This phenomenon was not observed with any of the antibiotics.

Summary

The susceptibility of 24 isolates of *Moraxella bovis* was tested *in-vitro* to thirteen antibiotics and five sulfonamides. The susceptibility of the organism was measured by ascertaining the diameters of the zones of growth inhibition produced when employing the assay cup method on seeded blood agar. There was no great variation of susceptibility between the isolates in the tests. The differences in susceptibility of the organism to the selected test drugs were significant.

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