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Isolation and Identification of *Mycoplasma agalactiae* subsp. *bovis* from Arthritic Cattle in Iowa and Nebraska

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Two strains of *Mycoplasma* were isolated from synovial fluids of arthritic feeder cattle and were identified as *Mycoplasma agalactiae* subsp. *bovis* by growth inhibition and fluorescent antibody tests. The strains (Iowa 1136 and Nebraska 2) could not be distinguished from known strains (Donetta and California 01) by immunoelectrophoresis or by agar gel precipitation.

In 1971, Singh et al. (13) reported the first isolations of *M. agalactiae* var. *bovis* from cases of arthritis in calves in Canada. In 1972, polyarthritis and synovitis in yearling feedlot cattle in California were attributed to the same microorganism (8). During studies on severe outbreaks of polyarthritis in cattle on pasture in Iowa and Nebraska, mycoplasmas were isolated from the synovial fluids of affected joints. This report concerns the isolation, characterization, and identification of two strains from arthritic cattle. Strain Iowa 1136 of *M. agalactiae* var. *bovis* caused severe arthritis when given to calves and cows. The results of these studies are reported separately (16).

MATERIALS AND METHODS

Isolation. Samples (0.1 ml) of carpal synovial fluid from heifer 1136 were inoculated into duplicate tubes of mycoplasma growth medium (10) and spread on the surface of plates of solidified medium. The inoculated plates were placed in a desiccator jar; plates and tubes were incubated at 37 C. At 2 and 7 days, samples from each inoculated tube were streaked on agar plates for the detection of mycoplasmas (15). Samples of synovial fluid were also examined for T mycoplasmas by inoculation into Hayflick medium (7) as modified by Livingston (12). The isolate from Iowa heifer 1136 was maintained in liquid medium by subcultures at 3- to 4-day intervals and by storage at −70 C.

Characterization. Strains Iowa 1136 and Nebraska 2 (supplied by E. L. Stair, Jr., University of Nebraska, Lincoln) from synovia of arthritic cattle were purified by filter-clone techniques. A culture in liquid medium was passed through a cellulose filter (0.45-mm pores) and serial 10-fold dilutions were inoculated on plates on solidified medium (10). After 3 days of incubation at 37 C, a single, well-isolated colony was removed with a sterile needle and streaked across the surface of another plate medium. The resulting colonies were inoculated into a tube of broth medium with some assurance that the cultures were the progeny of a single colony-forming unit (CFU).

Strains Iowa 1136 and Nebraska 2 were tested for fermentation of glucose in medium (10) supplemented with glucose (0.5%) and phenol red (1:2,000); controls of medium without glucose and noninoculated medium were used. Reversion to bacterial morphology was tested by three subcultures in liquid medium made without thallium acetate or penicillin. To test for the requirement of sterol for growth, the strains were inoculated into medium made without horse serum and, after incubation for 3 days, were streaked on agar medium lacking horse serum. Strains were also tested for inhibition of growth by digitonin (4), hydrolysis of arginine (1), filterability (20), hemolysis (3), and adsorption of erythrocytes (14) or sperm (19).

Identification. Cultures of mycoplasmas were identified by growth inhibition tests with antisera prepared against the following species of *Mycoplasma*: *M. pneumoniae*, *M. pulmonis*, *M. spumans*, *M. iners*, *M. fermentans*, *M. neurolyticum*, *M. salivarum*, *M. meleagridis*, *M. gallinarum*, *M. hyorhinis*, *M. arginini*, *M. canis*, and *M. orale* types 1, 2, and 3 (supplied by J. G. Tully, National Institutes of Health, Bethesda, Md.). Antisera were made in horses against *M. bovigenitalium*, *M. agalactiae* subsp. *bovis* strain Donetta, *M. bovirhinis*, *M. conjunctivae*, *M. alkalescens*, *M. dispar*, *M. bovoculi*, *Acholeplasma laidlawii*, and *A. modicum*. These cultures were obtained from World Health Organization Reference Laboratory (supplied by J. G. Tully, National Institutes of Health, Bethesda, Md.) and grown in liquid medium (10) to concentrations of 10^6 to 10^9 CFU/ml. One liter of each culture was centrifuged at 27,000 × *g* for 1 h. The sedimented organisms were suspended in 0.85% NaCl solution at $^1/_{100}$ the original volume and stored at 5 C until used to immunize horses. Each horse received from 10 to 12 injections (1 ml) of the same antigen at weekly intervals. The first three injections were of antigen mixed with equal volumes of Freund complete adjuvant given intramuscularly. The other injections were into the blood of the jugular vein. Antisera were produced in rabbits to strain Iowa 1136, Nebraska 2, and California 01 (supplied by D. E. Jas-

per, University of California, Davis) as described (17). After several injections of antigen, samples of blood were collected and tested for inhibition of growth of the homologous mycoplasma by a modified growth inhibition test (2). Zones of growth inhibition measured 3 to 8 mm.

Procedures used were described for the identification of mycoplasma colonies with fluorescein-conjugated antiserum and incident ultraviolet light (17), for immunoelectrophoresis (17), and for agar gel precipitation tests (17). For the immunoelectrophoresis and agar gel precipitation analyses, each mycoplasma was adjusted to the same nitrogen concentration; micro-Kjeldahl determination was used.

RESULTS

Isolation, characterization, and identification. Carpal joint fluid of heifer 1136 was negative for T mycoplasmas, but numerous colonies of mycoplasmas were observed. Isolate Iowa 1136 grew readily in artificial medium; $\geq 5 \times 10^8$ CFU/ml were determined by plate counts. Strains Iowa 1136 and Nebraska 2 did not ferment glucose or hydrolyze arginine; they did not grow in medium without serum or revert to bacterial form in medium without penicillin or thallium acetate. In the digitonin test, 10-mm zones of inhibition were observed. The strains both hemolyzed erythrocytes from the cow, sheep, and rabbit and also adsorbed erythrocytes from these same species. Bovine sperm were not adsorbed. In a study of filterability characteristics, undiluted culture of strain Iowa 1136 contained 2×10^9 CFU/ml; filtrates yielded 1.6×10^8, 6.5×10^6, and 4×10^3 CFU with 450-, 300-, and 220-nm filters, respectively.

The results of growth inhibition tests revealed close antigenic relatedness between strains Iowa 1136 and Nebraska 2 and *M. agalactiae* subsp. *bovis* strains Donetta and California 01 (Table 1). In the direct fluorescent antibody test in which conjugated horse antiserum to strain Donetta was used, all four strains reacted +3.

The agar gel precipitation patterns of the four mycoplasmas against three rabbit antimycoplasma antisera are shown (Fig. 1). There is a similar spectrum of overlapping contiguous precipitin bands with all four mycoplasmas. Patterns obtained with the Iowa antiserum appear to be more intense than those obtained with the California or Nebraska antiserum. Difficulties in comparing overlapping precipitin bands are better resolved by using immunoelectrophoresis (Fig. 2). In addition to the large number of precipitin bands toward the cathode side of the plate, a band can be seen at the anode side with no. 3 mycoplasma antigen, California and the California antiserum. The Iowa antiserum also shows the same band on the anode side with California antigen (1) and with Nebraska (3) and *M. agalactiae* (4). This anodic precipitin band is not visible in any of the immunoelectrophoresis patterns in which the Nebraska antiserum is used.

DISCUSSION

Outbreaks of severe arthritis in cattle due to *M. agalactiae* subsp. *bovis* occurred in calves confined in barns in Canada and in feedlot cattle in California (8, 13). The present report

TABLE 1. *Results of growth inhibition tests with strain of M. agalactiae subsp. bovis*

Antisera	Zones of growth inhibition (mm) of strains:			
	Donetta	Iowa 1136	Nebraska 2	California 01
Donetta	5	4	5	5
Iowa 1136	5	4	5	4
Nebraska 2	5	4	5	2
California 01	4	3	2	3

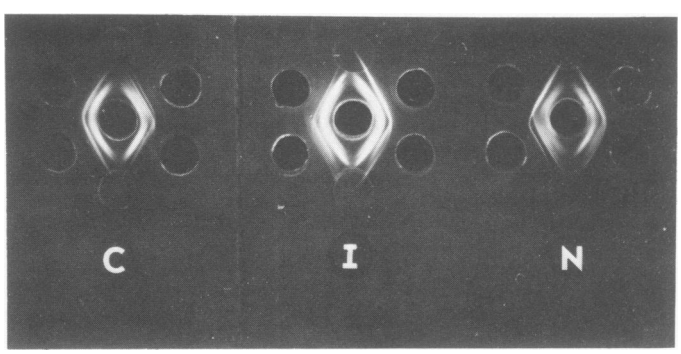

FIG. 1. *Double diffusion precipitin patterns of four strains of M. agalactiae subsp. bovis against three rabbit antimycoplasma sera. Strain California 01 is in cell well at 2 o'clock, strain Iowa 1136 at 4, strain Nebraska 2 at 8, and strain Donetta at 10 in each of the three patterns. C, I, and N represent antiserum against California, Iowa, and Nebraska strains in the central wells.*

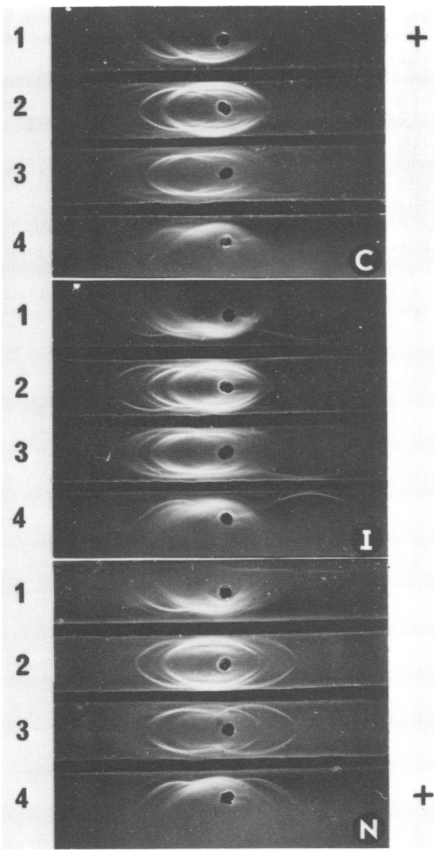

FIG. 2. *Immunoelectrophoretic patterns of four strains of M. agalactiae subsp. bovis in the wells: (1) California 01, Iowa 1136, Nebraska-2, strain Donetta. C, I, and N represent rabbit antisera in the troughs raised against the California 01, Iowa 1136, and Nebraska-2 isolates of M. agalactiae var. bovis.*

relates the isolation and identification of the same organisms from cattle on pastures in the Midwest. Strains Iowa 1136 and Nebraska 2 could not be distinguished from *M. agalactiae* subsp. *bovis* strain Donetta isolated from mastitic milk in Connecticut (6) and strain 01 isolated in California (9). When given to calves or cows, strain Iowa 1136 caused severe arthritis, with clinical signs like those of natural outbreaks (16).

Preparation of standard sera is not without hazard as shown by Fig. 1 and 2. Although the same relative concentrations of mycoplasma protein antigen were used, the immune response varied particularly with the Iowa 1136 antiserum, which had a broader spectrum of antibody than the sera prepared against the California 01 and Nebraska mycoplasma. The overlapping of precipitin bands was to a large degree eliminated in immunoelectrophoresis.

Information on the incidence and economic importance of mycoplasmal arthritis in cattle will become available when clinicians learn to recognize the disease and diagnostic laboratories are able to isolate and identify mycoplasmas from clinical specimens. Diagnostic procedures in animal mycoplasma infections were described by M. L. Frey and other members of the Mycoplasmosis Committee of the American Association of Veterinary Laboratory Diagnosticians (5). Lehmkuhl and Frey (11) recently reported the use of fluorescein-conjugated antisera in a direct immunofluorescence procedure for the identification of mycoplasma colonies grown on microscopic slides overlaid with agar medium. Specific immunofluorescence was easily observed against a dark background with vertical ultraviolet light. We can anticipate, therefore, that the isolation and identification of *M. agalactiae* subsp. *bovis* and other readily cultivated mycoplasmas will shortly be routine in many clinical microbiologic laboratories.

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