Mycoplasma agalactiae Subsp. bovis in Pneumonia and Arthritis of the Bovine

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ABSTRACT

The pneumonic lungs of 42 cattle from 26 feedlots were examined for the presence of mycoplasma, pathogenic bacteria and viruses. Four animals representative of two lots failed to yield mycoplasma. One of these yielded the virus of infectious bovine rhinotracheitis and Pasteurella hemolytica, the other yielded only P. multocida. Nine animals in eight lots yielded Mycoplasma sp.: five of these were M. bovirhinis, two were M. arginini and two were untypable. All of these animals yielded one or more of P. hemolytica, P. multocida, infectious bovine rhinotracheitis virus or bovine virus diarrhea virus. Twenty-five of 29 animals in 16 lots yielded M. agalactiae subsp. bovis from lung tissues. The same organism was recovered from the arthritic joints of 12 of these animals. Eight of the 25 animals yielded no other pathogen and all of these had not received any treatment. Nine of the 25 M. agalactiae subsp. bovis positive animals also yielded one or more of P. hemolytica, P. multocida, Corynebacterium pyogenes or infectious bovine rhinotracheitis virus. Bacteriological and virological studies were not completed for the remaining eight of the 25 positive animals.

In five lots of cattle which had not received medication for pneumonia and for arthritis only M. agalactiae subsp. bovis was recovered. Twenty-five grossly normal lungs obtained from normal cattle at the time of slaughter were cultured and all were negative. The possible role of M. agalactiae subsp. bovis in pneumonia and arthritis was discussed.

RÉSUMÉ

Cette étude visait à rechercher des mycoplasmes, des bactéries pathogènes et des virus, dans des cas de pneumonie affectant 42 bovins qui provenaient de 26 parcs d'engraissement. Quatre sujets représentatifs de deux lots s'avérèrent exempts de mycoplasmes; l'un d'eux recelait cependant à la fois le virus de la rhino-trachéite infectieuse bovine et Pasteurella hemolytica, tandis qu'on isola seulement P. multocida d'un autre de ces sujets. Neuf bovins provenant de huit parcs d'engraissement recelaient des mycoplasmes: dans cinq cas, il s'agissait de Mycoplasma bovirhinis; dans deux autres, il s'agissait de M. arginini; on ne réussit pas à identifier les mycoplasmes isolés des deux autres animaux. Tous ces sujets recelaient, seuls ou non, P. hemolytica, P. multocida, ainsi que les virus de la rhino-trachéite infectieuse et de la diarrhée à virus bovines. Les poumons de 25 des 29 sujets provenant de 16 parcs d'engraissement recelaient M. agalactiae subsp. bovis; on isola aussi le même mycoplasme des articulations de 12 de ces animaux qui souffraient également d'arthrite. On n'isola aucun autre agent pathogène de huit de ces 25 sujets auxquels on n'avait administré aucun traitement. Neuf des 25 animaux dont on isola M. agalactiae subsp. bovis recelaient aussi, seuls ou non, P. hemolytica, P. multocida, Corynebacterium pyogenes et le virus de la rhino-trachéite infectieuse bovine. On ne compléta pas les examens bactériologiques et virologiques relatifs aux huit derniers sujets de ce groupe.

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On ne recouvra que M. agalactiae subsp. bovis des sujets de cinq lots qui n'avaient reçu aucun traitement pour la pneumonie ou l'arthrite. La culture de 25 poumons d'apparence saine, obtenus lors de l'abattage de bovins normaux, donna des résultats négatifs. L'auteur commente le rôle possible de M. agalactiae subsp. bovis dans les cas de pneumonie et d'arthrite.

The reports of *M. agalactiae* subsp. *bovis* associated with pneumonia and arthritis in cattle and the sporadic recoveries of this organism from arthritis in calves in Western Canada during the period 1969-1971 led to this study on the incidence of *Mycoplasma* sp. in feedlot pneumonia and/or arthritis.

INTRODUCTION

Since Carter (2) made the first isolation of a mycoplasma from pneumonic cattle in Canada in 1954 many other workers have reported the isolation of *Mycoplasma* sp. from either the normal or diseased respiratory system of cattle.

Leach (14) collected representative strains of bovine mycoplasma recovered in laboratories throughout the world. Of the eight groups he characterized, five were associated with the bovine respiratory tract: *Mycoplasma mycoides* var. *mycoides* (19), *Mycoplasma bovirhinis* (8), *Acholeplasma laidlawii* (17) and Group 6 (12) and Group 8 (9). The last two now have been characterized and named *Acholeplasma modicum* and *Mycoplasma alkalescens*, respectively, by Leach (16). Other mycoplasma which have been recovered from the bovine respiratory tract are *M. bovigenitalium* (3) and *Mycoplasma* sp. Leach Group 7 (3), *M. agalactiae* subsp. *bovis* (11, 25), *M. arginini* (15), *M. dispar* (7) and T-strain mycoplasma (21). Fabricant (5) has reviewed the respiratory pathogenicity of these organisms.

The following mycoplasma frequently have been isolated from arthritis and/or synovitis: *M. mycoides* subsp. *mycoides*, vaccine strain (18), *M. agalactiae* subsp. *bovis* (10, 24, 25), Group VII of Leach (3).

Bronchopneumonia with lymphoreticular hyperplasia and peribronchiolar lymphoid hyperplasia has been related to several members of the order Mycoplasmatales: *M. bovirhinis, M. dispar, M. agalactiae* subsp. *bovis, Ureaplasma* sp. and *A. laidlawii* (1, 7, 25). However, experimental attempts to reproduce pneumonia and histological changes using these organisms have failed with the exception of T-strain (6) and *M. agalactiae* subsp. *bovis* (25). The experimental production of arthritis by *M. agalactiae* subsp. *bovis* has been reported by Stalheim (23).

MATERIALS AND METHODS

MEDIA

All media were prepared using distilled deionized water. Broth media were sterilized by filtration through stacked Millipore[1] filters consisting of a prefilter and filters of the following porosity in the order listed: 100 nm, 85 nm, 45 nm and 22 nm. Liquid components added to solid media were sterilized in a similar manner.

The composition of the media were as follows:

Broth medium IHSB	
Bacto PPLO base[2]	2.1 gm
Water	70 ml
Inactivated horse serum	20 ml
Fresh yeast extract 25% (w/v)	10 ml
D.N.A. calf thymus[3] 0.2% (w/v)	1.2 ml
Potassium penicillin G	1000 μ/ml
Thallium acetate	250 μ gm per ml
pH adjusted to 7.7.	
Dispensed in 1.8 ml quantities into 12 x 75 mm snap cap tubes[4]	

Solid medium IHSA	
Bacto PPLO agar	3.5 gm
Water	70 ml
Autoclave 15 lbs for 15 min	
Cool to 56°C	
Add aseptically the following presterilized fluid mixture at 56°C	
Inactivated horse serum	20 ml
Fresh yeast extract 25% (w/v)	10 ml
D.N.A. calf thymus	1.2 ml
Potassium penicillin G	1000 in per ml
Thallium acetate	250 μ gm per ml
Mix and dispense 6 ml per 15 x 60 mm Petri dish[5]	

[1]Millipore Corporation, Bedford, Massachusetts.

[2]Difco Laboratories, Detroit, Michigan.

[3]Calbiochem, LaJolla, California.

[4]Falcon Plastics, Oxnard, California.

[5]Falcon Plastics, Oxnard, California.

Media for the cultivation of *Ureaplasma* sp. was prepared as described by Shepard (22) and media for the cultivation of *M. dispar* was prepared as described by Gourlay (7). Bacterial isolations were made on tryptose agar plates[6] with 5% bovine blood.

History

All of the cattle were raised on ranches in Alberta or Saskatchewan. They were transported from the ranches to sales yards and from there to the feedlot for fattening. Usually the outbreak of pneumonia involving 10 to 30% of the animals, occurred within two to three weeks of placement in the feedlot but occasional cases developed four to eight weeks after placement. Arthritis was observed in 12 of the 26 pneumonic herds, usually as a sequela to the pneumonia but rarely arthritis occurred in individual animals with no prior pneumonic signs. The size of the herds ranged from 75 to over 2000 head. Treatment with penicillin, streptomycin and tetracyclines by intramuscular, intravenous or intra-articular routes were of little benefit.

Specimens

The specimens examined consisted of portions of pneumonic lungs, pleural and joint fluids, fibrinous pleural and pericardial tissue taken at necropsy from animals which died as a result of pneumonia or from animals with severe pneumonia and euthanized for necropsy. Two specimens were submitted by practicing veterinarians and the remainder were collected by laboratory personnel. A few synovial samples taken by paracentesis were submitted from live arthritic animals after they were observed to be suffering from pneumonia.

Twenty-five grossly normal lungs from clinically normal animals were collected from packing plants at the time of slaughter and examined for mycoplasma.

Sample Processing

Approximately 0.5 grams of lung was added to 4.5 ml of IHSB media less inhibitors in a Ten Broek tissue grinder. After thorough maceration, 0.2 ml of the suspension was added to 1.8 ml each of Gourlay's dispar medium, Shepard's T strain broth and IHSB. The suspension was inoculated onto 5% bovine blood tryptose agar plates.[7] Subsequent cultural techniques have been previously described (13).

Antisera Production

The hyperimmune antisera used for typing isolates in this study were in part prepared by a method previously described (14) or by the following procedure. *Mycoplasma* sp. and *Acholeplasma* sp. were grown in broth media in which the horse serum had been replaced by 20% and 5% rabbit serum respectively. Incubation was at 37°C in a gyratory incubator and the cultures were passed through 5 ml, 25 ml and 2000 ml volumes at 18-24 hour intervals. The organisms were harvested by centrifugation of the culture at 14,000 RCF for 60 minutes in the GS3 rotor of a Sorvall RC2B[8] refrigerated centrifuge. The sediment was resuspended and washed with 70 ml of 0.15 molar phosphate-buffered saline pH 7.2. Centrifugation, resuspension and washing was repeated twice more. The mycoplasma were lysed according to the method of Rottem and Razin (19) and lysis was continued until there was no further decrease in optical density as measured by Spectronic 20[9] using a wavelength of 500 nm. The lysed cells were centrifuged at 1,000 RCF in Sorvall centrifuge rotor SS34. The supernatant was collected and centrifuged at 35,000 RCF for 30 minutes in the SS34 Sorvall rotor. The supernatant was discarded and the pellet was resuspended in 5 ml of 0.25 molar NaCl. The protein concentration of the antigen was determined by the biuret method and adjusted to 2 mg protein per ml using 0.25 molar naCl in water. Antigen (1.5 ml) was mixed with 1.5 ml of Freund's incomplete adjuvant in a Sorvall mixer until a drop placed on water did not spread. The prepared antigen was injected into a pretested negative rabbit as follows: two 0.5 ml doses intramuscularly, two 0.5 ml doses, one into each hind foot pad and a total of 1 ml into five or more sites subcutaneously. The first bleeding was made ten to 14 days postinjection to determine if any response had taken place and the rabbit was reinjected intramuscularly using the same antigen adjuvant mixture as above (two doses

[6]Difco Laboratories, Detroit, Michigan.

[7]Difco Laboratories, Detroit, Michigan.

[8]Ivan Sorvall Inc., Norwalk, Connecticut.

[9]Bausch and Lomb, Rochester, New York.

TABLE I. *M. agalactiae* subsp. *bovis* from Cattle Affected with Pneumonia (arthritis)

Herds	Herds Treated	Animals Cultured	Animals Yielding Bacteria	Animals Yielding Viral Agents	Animals Yielding *M. agalactiae* subsp. *bovis*
5	3	10	0	0	8
3	1	5	0	N.D.[a]	5[b]
2	2	5	N.D.	N.D.	3
3	3	5	*Pasteurella haemolytica* 5	IER 2	5
2	2	3	*Pasteurella multocida* 3	IBR 1	3
1	1	1	*Pasteurella sp.* 1 and *Corynebacterium sp. 1*	IER 1	1
16	12	29	9	4	25

[a]N.D. — not done
[b]Two herds had mixed infections: in addition to *M. agalactiae* subsp. *bovis* one yielded *M. bovigenitalium*, the other yielded *A. laidlawii*

1 ml). The second bleeding was ten to 14 days after the second injection. At this time the hyperimmune serum usually gave a zone of 2-3 mm or greater on the growth inhibition test, indirect hemagglutination titre as high as 1/5,000, metabolic inhibition titre as high as 1/1,000 and definite lines in the growth precipitation test.

Preparation of *A. laidlawii* hyperimmune antisera was similar but lysing was attained by suspending the cells in sterile distilled water. All other procedures were the same.

Typing

The methods used in typing either the isolates or the strains submitted have been described previously (13).

Virus Isolation

Lung tissue for virus isolation was ground in a Ten Broek grinder with approximately 10 ml of Eagles MEM with antibiotics (4). Monolayered bovine foetal kidney cells were used in attempts to demonstrate cytopathic viruses in the lung suspension.

Histopathology

Representative portions of lungs collected at necropsy were fixed in buffered formol saline. Paraffin sections were stained with haematoxylin and eosin.

RESULTS

The recoveries of *M. agalactiae* subsp. *bovis* along with the bacteriological and virological results are given in Table I. Twenty-five of 29 animals listed in this table yielded *M. agalactiae* subsp. *bovis* from the lungs. In eight animals, five of which were untreated, the isolates were in pure culture and both virological and other bacteriological studies were negative. In five animals, three of which were untreated, from three herds, *M. agalactiae* subsp. *bovis* was recovered. Three of these five animals also yielded other members of the order Mycoplasmatales, *M. bovigenitalium* and *A. laidlawii*. Virological examinations were not carried out on these animals. Three treated animals of five in another two herds also yielded strains of *M. agalactiae* subsp. *bovis*. However, virological and other tests were not conducted. Treated animals from the remainder of the herds (six) all had mixed flora of mycoplasma, other bacteria and viruses. Lameness was observed by the clinicians and ranchers in many animals of the 16 herds and *M. agalactiae* subsp. *bovis* was recovered from joint aspirates of 12 animals in nine of these herds. *M. agalactiae* subsp. *bovis* was easily recovered from joint aspirates taken when signs of lameness were first observed. However, after 14-21 days of joint involvement the incidence of recovery was lower. Preliminary studies indicate antibodies are present in the joint fluids (unpublished data).

Tissues examined from 13 animals representing ten herds from which *M. agalactiae* subsp. *bovis* was not recovered are listed in Table II. Ten of the 13 animals yielded *Mycoplasma* sp. and in all instances other bacteria and/or viruses recovered as indicated. Neither arthritis nor lameness was observed in these groups of animals. The lower trachea and lungs of the normal animals failed to yield any species of mycoplasma.

TABLE II. Viruses, Bacteria and Mycoplasma other than *M. agalactiae* subsp. *bovis* Isolated from Pneumonic Lungs

Herds	Herds Treated	No. of Specimens	Animals Yielding Pasteurella	Animals Yielding Viral Agents	Animals Yielding Mycoplasma
1	1	1	*haemolytica* 1	BVD 1	*bovirhinis* 1
1	1	1	*haemolytica* 1	N.D.[a]	*bovirhinis* 1
2	2	3	*multocida* 2	N.D.	*bovirhinis* 3
1	1	1	*multocida* and *haemolytica* 1	0	*arginini* 1
1	1	1	0	IBR 1	*arginini* 1
2	2	3	*haemolytica* 2	IBR 3	Not typeable 2
2	0	3	*multocida* 1	0	0
10	8	13	8	5	9

[a]N.D. — not done

Histological examination of the lungs from three animals which failed to yield other pathogens had acute bronchiolitis, bronchopneumonia, excessive inflammatory exudates in the bronchi and bronchioles, fibrinous exudate on the pleural surface of the lungs. Peribronchial lymphoid hyperplasia was observed in two animals.

DISCUSSION

The recovery of *M. agalactiae* subsp. *bovis* in pure culture from pneumonic lungs taken at necropsy from eight animals representing five herds indicates that this species by itself may have a role in the pathogenesis of pneumonia. It is possible, however, that some or all of these animals may have had a prior infection with either some other bacteria or viruses capable of inducing an inflammatory response and the mycoplasma invaded the damaged tissue after the initial invader had been controlled by the host defence system and/or treatment. The failure to recover *Mycoplasma* sp. from the normal lungs is further evidence that these organisms should be considered as a possible aetiological agent of pneumonia in cattle.

Ruhnke (21) in her study of mycoplasma in pneumonic lungs of cattle as reported in her Table XIII, showed an increasing incidence of recovery of *M. agalactiae* subsp. *bovis* from animals under one month to over 12 months of age. However, the overall recovery rate reported was seven from 67 animals compared to 25 from 42 in this study. In comparison she reported 16 of 67 lungs positive for *M. bovirhinis* compared to five of 42 animals reported herein. The discrepancies between these two reports could represent a difference in the mycoplasma flora between Central and Western Canada at the time the studies were conducted or a difference in the age of the animals studied since those reported herein were all feedlot animals in excess of six months of age. Ruhnke (personal communication) has indicated that subsequent to her initial study there has been an increase in the frequency of isolation of *M. agalactiae* subsp. *bovis* from pneumonic lungs in cattle in Central Canada.

Thomas *et al* (25) recently reported the isolation of *M. agalactiae* subsp. *bovis* from feeder calves in Great Britain. They also reported the transmission of this organism and the production of pneumonia by endobronchial exposure. This result is similar to the T strain work of Gourlay (6). Stalheim (23, 24) reported the isolation of *M. agalactiae* subsp. *bovis* from arthritic joints and the induction of arthritis by intravenous or intra-articular inoculation of the isolate.

The isolation of *M. agalactiae* subsp. *bovis* from joints and lungs is a confirmation of other studies (11, 21, 23, 24, 25). A diagnosis of infection by *Mycoplasma agalactiae* subsp. *bovis* should be considered when pneumonia and arthritis or synovitis are occurring simultaneously or the latter are sequelae to pneumonia. Recovery of mycoplasma has been more difficult and the incidence of isolation has been lower from joints which had been clinically affected for 14 or more days. This may be due to antibody response either humoral or local as initial studies have revealed the presence of antibodies in joint aspirates. The polyarthritis is similar to the allergic response reported by Piercy (18). Failure to isolate

mycoplasma from joints which have been swollen or arthritic for more than ten days should not be a basis for excluding a diagnosis of mycoplasmal arthritis when arthritis is a sequela to pneumonia.

Further studies are being conducted in an effort to investigate the pathogenicity of *M. agalactiae* subsp. *bovis* and the immunological response of cattle to infection with *M. agalactiae* subsp. *bovis*.

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REFERENCES

1. **ANONYMOUS.** Mycoplasma in calf pneumonia. Vet. Rec. **92**: Members Information supplement No. 112. 1973.
2. **CARTER, G. R.** Pleuropneumonia-like organisms isolated from bronchopneumonia of cattle. Science **120**: 113. 1954.
3. **COTTEW, G. S.** Mycoplasmas isolated from cattle in Australia. Aust. vet. J. **46**: 378-381. 1970.
4. **DARCEL, C. le Q. and W. J. DORWARD.** Recovery of infectious bovine rhinotracheitis virus following corticosteroid treatment of vaccinated animals. Can. vet. J. **16**: 87-88. 1975.
5. **FABRICANT, J.** The pathogenicity of bovine mycoplasmas. Ann. N.Y. Acad. Sci. **225**: 369-381. 1973.
6. **GOURLAY, R. N. and L. H. THOMAS.** The experimental production of pneumonia in calves by the endobronchial inoculation of T-mycoplasmas. J. comp. Path. **80**: 585-594. 1970.
7. **GOURLAY, R. N. ad R. H. LEACH.** A new Mycoplasma species isolated from pneumonic lungs of calves (**Mycoplasma dispar** sp. nov.). J. med. Microbiol. **3**: 111-123. 1970.
8. **HARBOURNE, J. F., D. HUNTER and R. H. LEACH.** The isolation of mycoplasma from bovine and nasal swabs. Res. vet. Sci. **6**: 178-188. 1965.
9. **HUDSON, J. R. and J. R. ETHERIDGE.** A new type of pleuropneumonia-like organism (PPLO) from the nose of cattle. Aust. vet. J. **39**: 1-5. 1963.
10. **JASPER, D. E., N. C. JAIN and L. H. BRAZIL.** Clinical and laboratory observations on bovine mastitis due to Mycoplasma. J. Am. vet. med. Ass. **148**: 1017-1029. 1966.
11. **JASPER, D. E.** Mycoplasmas: Their role in bovine disease. J. Am. vet. med. Ass. **151**: 1650-1655. 1967.
12. **LANGER, P. H. and L. E. CARMICHAEL.** Identification of pneumo-enteritis isolates from cattle as mycoplasma. Proc. 67 a. Meet. U.S. Livestock Sanit. Ass. pp. 129-137. 1963.
13. **LANGFORD, E. V.** Mycoplasma species recovered from the reproductive tracts of Western Canadian cows. Can. J. comp. Med. **39**: 133-138. 1975.
14. **LEACH, R. H.** Comparative studies of **Mycoplasma** of bovine origin. Ann. N.Y. Acad. Sci. **143**: 305-316. 1967.
15. **LEACH, R. H.** The occurrence of **Mycoplasma arginini** in several animal hosts. Vet. Rec. **87**: 319-320. 1970.
16. **LEACH, R. H.** Further studies on classification of bovine strains of Mycoplasmatales, with proposals for new species, **Acholeplasma modicum** and **Mycoplasma alkalescens.** J. gen. Microbiol. **75**: 135-153. 1973.
17. **OLSON, N. O., W. R. SEYMOUR, A. D. BOOTH and L. DOSZA.** Characteristics of PPLO isolated from the genital and respiratory tract of cattle. Ann. N.Y. Acad. Sci. **79**: 677-685. 1960.
18. **PIERCY, D. W. T.** Reaction to killed **Mycoplasma mycoides** in joints in specifically sensitized calves. J. comp. Path. **82**: 279-290. 1972.
19. **PIERCY, S. E.** Observations on the type species **Mycoplasma mycoides.** Ann. N.Y. Acad. Sci. **79**: 665-669. 1960.
20. **ROTTEM, S. and S. RAZIN.** Isolation of mycoplasma membranes by digitonin. J. Bact. **110**: 699-706. 1972.
21. **RUHNKE, H. L.** Studies of mycoplasma from the bovine respiratory tract. Thesis, University of Guelph. 1972.
22. **SHEPARD, M. C. and C. D. LUNCEFORD.** Urease color test medium U-9 for the detection and identification of 'T' mycoplasmas in clinical material. Appl. Microbiol. **20**: 539-543. 1970.
23. **STALHEIM, O. H. V. and L. A. PAGE.** Naturally occurring and experimentally induced mycoplasmal arthritis of cattle. J. clin. Microbiol. **2**: 165-168. 1975.
24. **STALHEIM, O. H. V. and S. S. STONE.** Isolation and identification of **Mycoplasma agalactiae** subsp. **bovis** from arthritic cattle in Iowa and Nebraska. J. clin. Microbiol. **2**: 169-172. 1975.
25. **THOMAS, L. H., C. J. HOWARD and R. N. GOURLAY.** Isolation of **Mycoplasma agalactiae var bovis** from a calf pneumonia outbreak in the south of England. Vet. Rec. **97**: 55-56. 1975.