A Mycoplasma Isolated from Cattle with Infectious Bovine Keratoconjunctivitis

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SUMMARY

A mycoplasma has been recovered from the eyes of calves in two naturally-occurring outbreaks of infectious bovine keratoconjunctivitis; also from a third group of calves accidentally exposed to an animal which had ocular exudates from one of the outbreaks instilled into its eyes.

The severity of the ocular lesions in infectious bovine keratoconjunctivis outbreaks may be related to a mixed infection with the mycoplasma and Moraxella bovis.

Preliminary typing studies indicate the mycoplasma is not serologically related to any known bovine mycoplasma.

RÉSUMÉ

Dans deux troupeaux différents, on a isolé un mycoplasme du produit de raclage oculaire de veaux atteints de kérato-conjonctivité. Le même organisme on été retrouvé dans un groupe de veaux entrés accidentellement en contact avec un animal d'expérience. Celui-ci présentait un exsudat oculaire causé par l'inoculation de grattages conjonctivaux prélevés dans l'un de ces deux troupeaux.

On pense que la gravité des lésions oculaires seraient due à l'association du mycoplase et de Moraxella bovis.

Il a été impossible jusqu'ici d'identifier sérologiquement ce mycoplasme qui ne correspond à aucun type bovin connu.

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INTRODUCTION

Mycoplasma have been isolated from many tissues in cattle and their role in several diseases has been clearly demonstrated. The literature has been reviewed by Adler (1) and Morton (14), and other more recent publications record the isolation of mycoplasma from bovine lungs, nasal swabs, mammary glands, vagina, prepuce, semen, aborted foetus, endometrial discharges and bulk tank milk (2, 6, 7, 8, 9, 10, 11, 12, 15).

Al-Aubaid (2), in two attempts, failed to isolate the organism from the bovine eye. Wilcox (17), in his comprehensive review of the literature on infectious bovine keratoconjunctivitis (IBK), refers to rickettsia or rickettsia-like organisms (RLO) in the eyes of sheep and cattle but does not suggest the presence of a mycoplasma in the bovine eye or ocular exudates.

Pleuropneumonia-like organisms (PPLO) have been recovered from cats with conjunctivitis (3, 5) and from the conjunctiva and nasal mucosa of mice (16). The presence of RLO in the conjunctiva of cattle and the recovery of PPLO from the conjunctiva of cats and mice suggested the possibility that a mycoplasma might be found in the eyes of cattle with IBK.

Gourlay and Thomas (9) recently reported on the isolation of several strains of mycoplasma from the eyes of cattle suffering from IBK in Great Britain. These isolates were T-mycoplasmas, *Mycoplasma bovirhinis, Mycoplasma laidlawii* and an unidentified strain from four out of 20 cases.

In this study we attempted to isolate mycoplasma from the eyes of normal cattle and those with IBK.

MATERIALS AND METHODS

ANIMALS

Four groups of animals were examined.

(1) Herd A: This group of 14 cattle was indirectly exposed to ocular discharges from the eyes of naturally infected animals in herd C. Four of the animals contracted IBK ten days later.

(2) Herd C and R: Some of the animals in these herds were naturally infected cases of IBK.

(3) Herd W: This was a closed herd which was under almost daily observation and in which clinical symptoms of IBK had not been observed over many years.

(4) A group of three 11-months old normal calves from herd W, which had been raised in isolation was used for preliminary transmission studies. A mixture of a field strain of *Moraxella bovis* and a mycoplasma was instilled into the right eye of each animal.

SWABS

Conjunctival swabs were taken from the animals under study from March to December, 1968. These swabs were immersed and rotated in 5.0 ml of brain heart infusion broth[1] and sealed in either screw cap tubes or plastic capped vials. The sealed tubes were stored at –70°C in a mechanical freezer for periods of two to 26 weeks. When required the tubes were thawed at room temperature and approximately 0.5 ml of broth suspension was added to 5.0 ml of appropriate broth culture media.

MEDIA

The liquid medium consisted of the following:

Bacto PPLO broth W/O CV[1]	2.1% W/V
Bacto Autolysed Yeast	1.0% W/V
Thallium acetate[2]	0.25% W/V
Penicillin G Sodium[3]	2000 IU/ml
Horse serum Seitz-filtered and inactivated at 56° C for 30 minutes	20% V/V
Distilled deionized water to make	100 ml

The weighed amounts of PPLO broth medium and Autolysed Yeast were added to 70 ml of warm water in an Erlenmeyer flask. The mixture was warmed and agitated on a heated magnetic stirrer until the solids had dissolved. Preliminary clarification was carried out by the addition of 1.0 gm of Celite[4] which was thoroughly mixed into the medium and the suspension was passed through a Whatman No. 1 filter under slight negative pressure. The filtrate was brought up to 80 ml by the addition of deionized distilled water. Fresh (less than three weeks old) sterile inactivated horse serum, thallium acetate and penicillin were added and the pH adjusted to 7.8 at 25°C. Initially, the medium was prefiltered and sterilized through disposable filters[5] of 0.45/μ and 0.2 μ porosity. More recently, a microfibre glass prefilter[6] and a filter disc of 0.22 μ have been used at 10 PSI pressure. The sterile liquid medium was tubed aseptically in 5.0 ml quantities and stored at 4°C until required.

Agar plates were prepared from Bacto PPLO agar according to the manufacturer's directions and autoclaved for 20 minutes at 15 lbs pressure. Horse serum to give a final concentration of 20%, thallium acetate and penicillin in a sterile solution were added when the medium had cooled to approximately 54°C. Plastic petri dishes[7] 60 × 15 mm were used throughout this study.

PPLO broth and agar plates without inhibitors (thallium acetate and penicillin) were also prepared. These were used to compare the growth characteristics of the isolates in the presence and absence of the inhibitors.

PPLO agar plates were prepared with 0, 5, 10, 15 and 20% v/v fresh sterile inactivated horse serum to determine the serum requirements of the isolates.

All isolations were inoculated onto tryptic soy agar with 5% bovine blood added and incubated at 37°C aerobically, anaerobically and in 5% CO_2 and examined daily to determine if they would grow on conventional solid bacteriological media.

Early in the study the inoculated media were incubated at 37°C and 22°C aerobically, anaerobically and in the presence of 5% CO_2; after the initial isolations were made, incubation was maintained at 37°C in an atmosphere of 5% CO_2 and air.

[1]Difco Laboratories, Detroit, Mich.
[2]Fisher Scientific, Montreal, P.Q.
[3]E. R. Squibb and Sons Ltd., Montreal, P.Q.
[4]Johns Manville (Fisher Scientific, Montreal, P.Q.).
[5]Nalge Co., Rochester, N.Y.
[6]Millipore Ltd., Montreal 9, P.Q.
[7]Falcon Plastics, Los Angeles, Calif.

TABLE I. Isolation of a Mycoplasma from Normal and IBK Affected Animals

	Number of Animals Examined	Positive	Negative
Herd W (closed herd)	119	0	119
Herd C (animals with IBK)	4	1	3
Herd R (animals with IBK)	20	9	11
Herd R (normal contacts)	10	1	9
Totals	153	11	142

The morphological and staining characteristics of the mycoplasma were studied by centrifuging broth cultures at 5,400 X g for ten minutes. The supernatant was removed by pipetting and the sediment was resuspended in the drop or two of broth left in the tube. The broth-sediment mixture was spread on a slide, air dried, fixed in methyl alcohol for three to five minutes and stained with Giemsa using a 1/50 dilution of the stock solution for 80 minutes.

RESULTS

The first isolations were made from swabs, taken from natural cases of IBK and incubated at 37°C in 5% CO_2. Duplicate plates cultured aerobically, anaerobically and at 22°C were negative.

The results of attempted isolation of mycoplasma from four groups of animals in herds W, C and R are given in Table I.

TABLE II. The Isolation of Mycoplasma Correlated with the Stage of Infection

	No. Examined	Positive	Negative
Normal contacts	10	1	9
Acute stage IBK	10	8	2
Chronic stage IBK	14	2	12
Total	34	11	23

The results of the bacteriological examination of eye swabs from animals in the affected herds C and R are recorded in Table II on the basis of apparently normal contacts, acute stage IBK (lacrimination and slight irritation) and chronic stage (ulceration and corneal opacity).

Table III correlates the results of bacteriological examinations with clinical symptoms of IBK in animals from herd A. Animals 1, 2, 3 and 5 showed symptoms on day 1 and a mycoplasma was isolated from two of these on the first attempt along with *Moraxella bovis*. Although animals 3 and 5 showed a slight conjunctivitis, ocular swabs were negative on day one.

Ocular swabs from animals 2 and 3 yielded mixed cultures on four and three occasions, respectively, over a period of ten days and the eyes of these animals were the most seriously affected; a mixed culture was recovered from animal 5 on two occasions and it was the next most seriously affected. Animals 1 and 4 yielded a mixed culture on a single occasion. The former had IBK but recovered in seven days while the latter never showed symptoms of the disease. Animal 6 yielded the mycoplasma only on two occasions ten days apart and never showed symptoms.

TABLE III. The Bacteriological Results Correlated with Clinical Data in Exposed Animals of Herd A

Days Following Initial Symptoms	1			5			7			10			13			17		
Animal No.	Isol. M	Isol. MB	CS	Isol. M	Isol. MB	CS	Isol. M	Isol. MB	CS	Isol. M	Isol. MB	CS	Isol. M	Isol. MB	CS	Isol. M	Isol. MB	CS
1	+	+	A	−	+	C	−	+	R	+	−	R	−	ND	R	−	ND	R
2	+	+	A	+	+	C	+	+	C	+	+	C	−	ND	C	−	ND	C
3	−	−	A	+	+	A	+	+	C	+	+	C	−	ND	C	−	ND	C
4	−	+	N	−	+	N	−	+	N	+	+	N	−	ND	N	−	ND	N
5	−	−	A	+	+	C	+	+	C	−	+	C	−	ND	C	−	ND	R
6	ND	ND	N	−	−	N	+	−	N	−	−	N	−	ND	N	+	ND	N

Abbreviations: Isol. = Isolations; CS = Clinical signs; M = Mycoplasma; MB = *Moraxella bovis*; A = Acute (inflammation and lacrimation); C = Chronic (corneal opacity and/or ulceration); R = Recovering (cornea clearing); ND = Not done; N = Normal; in contact

The three normal animals from herd W which were inoculated with a mixture of mycoplasma and *Moraxella bovis* were examined daily. Mycoplasma were not recovered from the left (uninoculated) eye of one of these animals. The mycoplasma was recovered from the right eye of only one animal on days 1, 3, 4, 7, 10, 14 and 17. *M. bovis* was recovered from the inoculated eyes on days 1, 3 and 4. No animal showed clinical symptoms of IBK at any time.

Growth and Morphological Characteristics

The colonial morphology of the isolated mycoplasma is typical for the genus and is illustrated in Figs. 1 and 2. The colonies are 30 to 150 μ in diameter. A large and small colonial variant has been observed on the same plate but each type has yielded both large and small colonies on subculture.

Examination of the stained slides at 1200 X magnification revealed a very pleomorphic organism which had coccobacillary, crescent, horseshoe and signet ring shapes. Bipolar staining was frequently observed in the coccobacillary forms. The organism was gram negative but stained faintly.

After primary isolation all isolates were grown for three to six successive passages in PPLO broth and agar media devoid of inhibitors. There was no change in either the colonial characteristics or the morphology of the stained organisms under these conditions. All tryptic soy blood agar plates failed to show any growth after four days incubation.

The concentration of serum required for growth of several isolates has been studied. The organism will neither grow in the absence of serum nor in media containing 5% or 7.5% serum. Growth occurs in media containing 10% serum and good growth requires 15% to 20% serum.

Giemsa-stained impression smears of ocular exudates from a few infected eyes have been examined. Pleomorphic bodies which resembled mycoplasma were observed in two of the smears and a swab from one of the eyes yielded a mycoplasma when cultured.

Representative isolates have been submitted for typing to R. H. Leach of the Mycoplasma Reference Laboratories, London, England. Preliminary studies by him have confirmed that they are mycoplasmas and indicate that they are not serologically relaated to any known bovine mycoplasma (13). Further studies are required to determine whether the isolate is a known Group V mycoplasma or a new isolate which will be placed in this group.

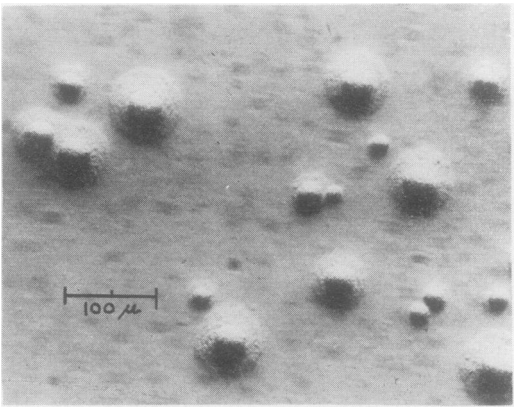

Fig. 1. Transillumination and directional lighting of colonies on PPLO agar media after 72 hours of incubation. Note the raised centre and dense shadow cast by same.

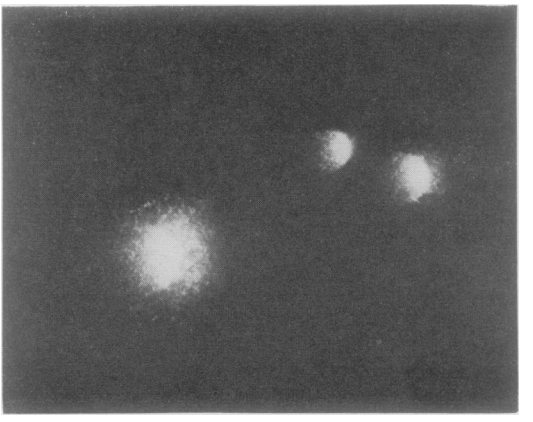

Fig. 2. Transillumination modified to give a dark ground effect for added contrast. Note the irregular periphery of the colony.

DISCUSSION

The unidentified mycoplasma was placed in the family Mycoplasmataceae on the basis of (a) morphology and size, (b) the reasonably good staining with metachromatic dyes (Giemsa) and the very faint Gram-negative staining, (c) the ability to grow in the presence of thallium acetate and penicillin and (d) the serum requirements for growth.

Several successive isolations were made at 37°C and 5% CO_2 tension when at the

same time the duplicate anaerobic and aerobic cultures were negative. It was concluded that the organism would only grow in the presence of 5% CO_2 and at 37°C. For several months all isolations were made under these conditions until Dr. R. H. Leach stated that isolates sent to him were growing aerobically. After further studies, initial isolations have been made aerobically and in the presence of 5% CO_2 but in our experience CO_2 increases the frequency of isolation of the organism.

The observations in Table III suggest that the severity of IBK may be accentuated when both the mycoplasma and *Moraxella bovis* are present. However, the recovery of several different mycoplasma by Gourlay (8) might also indicate that these organisms are only opportunists invading an already damaged tissue. It is also possible that the unidentified mycoplasma may be the stable L-form of a bacteria such as a *Moraxella bovis* and studies similar to those reported by Cohen *et al* (4) should be carried out to clarify this possibility.

Al-Aubaid (2) failed to isolate mycoplasma from the bovine eye possibly because he cultured ocular exudates from normal animals. We have failed to recover the organism from 128 of 129 clinically normal animals and the only isolate from this group was from an animal in contact with affected calves. In contrast, ocular swabs from ten of 24 animals with clinical symptoms of IBK yielded a mycoplasma. The low recovery rate of mycoplasma in the chronic cases of IBK (Table III) was also interesting, as the ease of isolation of the mycoplasma decreased as the apparent number of *Moraxella bovis* in the ocular exudates increased.

There can be no doubt that the organism is able to propagate in the bovine eye, as evidenced by its recovery on seven occasions and up to 17 days after ocular instillation from one of three exposed animals but the aetiological role requires further study.

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REFERENCES

1. **ADLER, H. E.** Mycoplasmosis in animals. Adv. vet. Sci. **10**: 205-244. 1965.
2. **AL-AUBAID, J. M. and J. F. FABRICANT.** Technics for the isolation of mycoplasma from cattle. Cornell Vet. **58**: 555-571. 1967.
3. **CELLO, R. M.** Association of pleuropneumonia-like organisms with conjunctivitis of cats. Am. J. Ophthalmology **43**: 296-297. 1957.
4. **COHEN, L. R., R. G. WITTLER and J. E. FABER.** Modified biochemical tests for characterization of L-phase variants of bacteria. Appl. Microbiol. **16**: 1655-1662. 1968.
5. **COLE, B. C., L. GOLIGHTLY and J. R. WARD.** Characterization of mycoplasma strains from cats. J. Bact. **94**: 1451-1458. 1967.
6. **DAVIES, G.** Isolation of mycoplasma from calf lungs. J. comp. Path. **77**: 353-357. 1967.
7. **ERN, H., W. N. PLASTRIDGE and M. E. TOURTELLOTE.** Mycoplasma: Isolation from prepuce and semen of bulls. Acta vet. scand. **8**: 123-135. 1967.
8. **GOURLAY, R. N.** The isolation of T-strains of mycoplasma from pneumonic calf lungs. Res. vet. Sci. **9**: 376-378. 1968.
9. **GOURLAY, R. N. and L. H. THOMAS.** The isolation of large colony and T.-strain mycoplasmas from cases of bovine kerato-conjunctivitis. Vet. Rec. **84**: 416-417. 1969.
10. **HAROURNE, J. F., D. HUNTER and R. H. LEACH.** The isolation of mycoplasma from bovine lungs and nasal swabs. Res. vet. Sci. **6**: 178-188. 1965.
11. **JAIN, N. C., D. E. JASPER and J. D. DELLINGER.** Cultural characters and serological relationships of some mycoplasmas isolated from bovine sources. J. gen. Microbiol. **49**: 401-410. 1967.
12. **LEACH, R. H.** Comparative studies of mycoplasma of bovine origin. Ann. N. Y. Acad. Sci. **143**: 305-316. 1967.
13. **LEACH, R. H.** Personal communication. 1969.
14. **MORTON, H. E.** The prevalence of pleuropneumonia-like organisms. Ann. N. Y. Acad. Sci. **79**: 369-370. 1960.
15. **O'BERRY, P. A., J. H. BRYNER and A. H. FRANK.** Isolation of mycoplasma from an aborted bovine foetus and vaginal mucus. Am. J. vet. Res. **27**: 677-681. 1966.
16. **SABIN, A. B.** Mice as carriers of pathogenic pleuropneumonia-like micro-organisms. Science **90**: 18-19. 1939.
17. **WILCOX, G. E.** Infectious bovine kerato-conjunctivitis. A review. Vet. Bull. **38**: 349-360. 1968.