Mycoplasma and Associated Bacteria Isolated from Ovine Pink-eye

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SUMMARY

A mycoplasma was recovered from the untreated conjunctival membranes of nine sheep affected by Pink-eye. It was neither isolated from the conjunctiva of treated animals which were affected nor from the conjunctiva of normal animals either in contact or not in contact with affected animals.

Bacteria found on normal conjunctival membranes were Neisseria ovis, Escherichia coli, Staphylococcus epidermididis, Streptococcus **and** Bacillus **spp.**

Bacteria found in clinical cases of Pink-eye were N. ovis, E. coli, **a** Streptococcus **and** Pseudomonas **spp.**

RÉSUMÉ

On isola un mycoplasma des conjonctives non traitées de neuf moutons atteints de "Pink-eye". On ne réussit pas à l'isoler de la conjonctive d'animaux affectés mais traités, ni de celle d'animaux sains, en contact ou non avec des animaux malades. Neisseria ovis, Escherichia coli, Staphylococcus epidermididis, Streptococcus **et** Bacillus SPP **furent isolés des conjonctures normales.** N. ovis, E. coli **ainsi qu'un** Streptococcus **et des espèces de** Pseudomonas **furent isolés dans les cas cliniques de "Pink-Eye".**

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INTRODUCTION

Mycoplasma have been associated with mastitis, arthritis, edema of the periarticular tissues, cellulitis, and conjunctivitis in sheep and goats (2,4,5,9,10). The conjunctival isolates were *Mycoplasma mycoides var capri* from a goat in Connecticut (5) and isolates tentatively named *Mycoplasma conjunctivae var ovis* recovered from sheep with Pink-eye in Australia (9). The latter isolations were made during a clinical and cytological study of the changes occurring in Pink-eye. Transmission studies showed the organism could colonize on the conjunctiva of sheep and cause a mild conjunctivitis.

MATERIALS AND METHODS

ANIMALS

Four groups of animals were examined from two flocks which were almost equal in size between Nov. 7th and Dec. 10th, 1968. Two flocks consisted of approximately 300 purebred and good grade animals belonging to the same owner but ranged about a mile apart. In addition, a flock of 60 grade animals maintained in isolation at the A.D. R.I.(W) was examined during the autumn of 1970.

Group A — These animals were from two flocks which at no time had clinical signs of Pink-eye as described by Surman (9).

Group BN — These animals were classified

as normal in-contact animals on the basis of presence in an infected herd and the lack of clinical signs.

Group BT — These were classified as affected, treated animals on the basis of clinical signs and known treatment[1] by the insufflation of a powder consisting of tyrothricin, di-phenthane-70, acriflavine neutral, sulfanilamide, sulfathiazole, sulfisoxazole, and boric acid onto the affected eyes.

Group BI — These were classified as untreated affected animals since the samples were taken before treatment was started.

Swabs

Conjunctival swabs were taken either by the owner or by veterinarians and immediately transported to the laboratory without artificial refrigeration since the ambient temperature on the days of collection was approximately 3-5°C. On arrival, usually within three hours of sampling, they were processed for examination and storage as described previously (6).

Media

The media used, method of examination and criteria for classification of isolates as *Mycoplasma* spp. have been described previously (6).

Primary plating for bacteria other than mycoplasma was made onto MacConkey agar plates and tryptose agar plates with 5% bovine blood added.

Brain heart infusion broth was inoculated and used as an enrichment broth. After 24 hours incubation the broths were also plated onto MacConkey and tryptose blood agar plates.

Biochemical reactions were determined using Bacto prepared media[2]. Fermentation studies were carried out using phenol red broth base plus 0.5% of the appropriate carbohydrate.

Microscopical Studies

Ocular impression slides were prepared from five clinically affected sheep in groups BT and BI and stained with Giemsa. The morphological and staining characteristics of the mycoplasma isolates were studied as described previously (6).

[1]Suloptic — Allied Laboratories (Canada) Ltd., Guelph, Ont.

[2]Difco Laboratories, Detroit, Mich.

RESULTS

The initial isolations were made from swabs taken by the owner from 12 clinically affected untreated cases, after four days incubation at 37°C aerobically and also in the presence of 5% CO_2. The colonies were typical of mycoplasma but somewhat granular in appearance. These isolates did not grow on serum-free media at 37°C or on media containing 20% serum at 22°C. The initial isolates were cloned three times and serially transferred ten times on media devoid of inhibitor without altering the colonial or morphological characteristics of the isolates. Glucose was fermented, urea and arginine were not hydrolysed and 2,3,5 triphenyl tetrazolium chloride was reduced. The minimum requirement of serum for growth was 7.5%. The mycoplasma isolates failed to grow on 5% tryptose blood agar plates either on initial isolation or after serial passages in non-inhibitor medium during 96 hours incubation at 37°C. A second isolate differed from the above in that it did not ferment glucose, hydrolyse urea nor reduce 2,3,5 triphenyl tetrazolium chloride. Arginine was hydrolysed.

Representative isolates were forwarded to Dr. R. H. Leach of the Mycoplsma Reference Laboratory, London, England. His report substantiated the biochemical studies of the author. He also stated that no reaction occurred in growth inhibition tests using antisera prepared against *Mycoplasma laidlawii*, or mycoplasma of bovine and ovine origin.

The predominant organism recovered from all of the initial swabs on primary plating was a Gram-negative diplococcus. The colonies of this organism were grey, convex with regular borders and a marked zone of *beta*-hemolysis. They were catalase and oxidase positive and reduced nitrates. They did not produce indol or hydrogen sulfide and did not grow on MacConkey agar. Growth occurred without liquefaction in gelatin stabs and on inspissated bovine serum slopes. No acid or gas was produced after five days incubation at 37°C from dextrose, maltose, mannitol, rhamnose, raffinose or trehalose. Litmus milk was unchanged after five days incubation. On the basis of these results the organism was considered to be *Neisseria ovis*, as described by Fairlie (3), Lindquist (7), and Spradbrow (8). The bacteria isolated are recorded in Table I.

TABLE I. Recovery of Mycoplasma and Bacteria from Pink-eye in Sheep

Group	Animals Examined	Mycoplasma Isolations/Samples	Bacterial Isolations	Samples
A[a]	24	0/48	**E. coli**	2/48
			Streptococcus	1/48
			N. ovis	3/48
			Bacillus	2/48
			Staphylococcus epidermididis	2/48
BN[b]	4	0/8	**E. coli**	1/8
			N. ovis	4/8
BT[c]	2	0/4	Pseudomonas	1/4
			N. ovis	2/4
BI[d]	9	15/18	Streptococcus	3/18
			E. coli	4/18
			Pseudomonas	1/18
			N. ovis	12/18

[a]A — normal, not in contact
[b]BN — normal, in contact
[c]BT — infected, treated
[d]BI — infected, not treated

Giemsa stained impression smears from the conjunctiva of five clinically affected sheep in groups BT and BI revealed the presence of extracellular and intracytoplasmic cocco-bacillary and ring-shaped bodies which morphologically resembled mycoplasma in three of the nine slides examined. A mycoplasma was isolated from swabs taken from two of the three eyes which were microscopically positive. Clumping of the cocco-bacillary bodies, as reported by Surman (9), to form trachoma-like inclusion bodies was not observed. Epithelial cells predominated over mononuclear type cells in the smears examined from the advanced clinical cases. The intracellular cytoplasmic inclusions which resembled mycoplasma were present in the epithelial cells.

Since *N. ovis* was the most frequently recovered bacteria, it was considered possible that the apparent mycoplasma might be an L-form of this organism induced by the inhibitors in the mycoplasma media. Therefore, all isolates of *N. ovis* which had been associated with a mycoplasma as the flora of infected eyes were transferred to a broth mycoplasma medium with inhibitors. Four serial transfers were made in this medium and at each transfer, cultures were made to blood agar and mycoplasma plates.

Rickettsia-like organisms have been observed previously (1), and were presumed to be the causative agent of Pink-eye in sheep, even though cultural and isolation attempts were unsuccessful. Material from infected eyes in this study was forwarded to Dr. G. Bannister who inoculated embryonated chicken eggs and mice and checked their tissues for inclusion bodies as found in psittacosis or a rickettsial infection; results were negative. Guinea pigs were inoculated and their sera checked by complement-fixation tests with psittacosis-lymphogranuloma and Q-fever antigens and results were negative.

DISCUSSION

The recovered organism was considered to be a mycoplasma on the basis of characteristics given previously (6). It is not thought to be an L-form of bacteria as repeated cultures on blood agar from mycoplasma broth media devoid of inhibitors during several successive transfers were negative. No L-forms were induced by the use of inhibitors on cultures of the bacteria most prevalent in the affected eyes but these tests do not rule out the possibility as more sophisticated techniques might have induced them.

Surman (9) suggests that the use of plastic petri dishes may be deleterious to the recovery of mycoplasma. In contrast to

this, we have used this type of plate exclusively and have not observed an inhibitory effect on the growth of this or other mycoplasma isolated.

The recovery by Jonas (5) of *M. mycoides var capri* from fluids taken from a goat with edema of the periorbital tissue and a mild conjunctivitis at least indicates that this organism may also invade tissue and produce clinical signs of disease.

Surman (9) was apparently the first to culture, characterize and associate a mycoplasma with Pink-eye in sheep. He was also successful in transmitting the organism to healthy sheep, in reproducing a mild form of the disease, and in recovering the mycoplasma, thus fulfilling Koch's postulates. He suggests that all sheep may harbor conjunctival mycoplasma in a latent form and that activation occurs as a result of trauma or under conditions of stress. It is quite possible that trauma may have been a predisposing cause in this outbreak as the flock involved was ranging on a stubble field and the frequent high winds common to the area caused dust clouds. Flies and other insects were not observed around either flock at the time of the outbreak. The coldness of the season would preclude their presence in any numbers and they were not therefore considered as a possible transmitter of the conditions either mechanically or otherwise at this time. The failure to recover this organism from a limited number of normal animals in both the healthy and the affected flock would suggest that probably a few, rather than many animals, as suggested by Surman (9), act as latent carriers. Intracellular forms of the organism, if present, might also be more difficult to culture than those which are extracellular.

The recovery of the organism from nine untreated affected animals supports the findings of Surman (9), and indicates that a mycoplasma is associated with Pink-eye in sheep. It therefore seems apparent that further studies are needed to determine the incidence and type or types of mycoplasma present on both the normal and diseased conjunctival membranes of sheep by a variety of immunological and other techniques.

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