Acta vet. scand. 1979, *20*, 51—59.

From the State Veterinary Serum Laboratory, Copenhagen, Denmark.

ISOLATION OF MYCOPLASMA BOVOCULI FROM CASES OF INFECTIOUS BOVINE KERATOCONJUNCTIVITIS

By

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FRIIS, N. F. and K. B. PEDERSEN: *Isolation of Mycoplasma bovoculi from cases of infectious bovine keratoconjunctivitis.* Acta vet. scand. 1979, *20*, 51—59. — Five outbreaks of infectious bovine keratoconjunctivitis were examined for bacteria and mycoplasmas. Mycoplasma bovoculi was demonstrated in four of the five outbreaks. Other mycoplasmatales were represented by ureaplasma in one sample. Moraxella bovis and Neisseria ovis were found in all the outbreaks, the former being present in the vast majority of the animals. Transmission experiments with Mycoplasma bovoculi and Moraxella bovis in combination were carried out on four young, colostrum-deprived calves. Mycoplasma bovoculi appeared to have an enhancing effect on the pathogenicity of Moraxella bovis.

infectious bovine keratoconjunctivitis; Mycoplasma bovoculi; Moraxella bovis.

Many different microorganisms have been incriminated as etiological agents in infectious bovine keratoconjunctivitis. Moraxella bovis (M. bovis) is the organism isolated most regularly from diseased animals, but also other organisms have been found. Attempts to reproduce the typical lesions with pure cultures of M. bovis have generally given inconsistent results, while it has been possible to reproduce the disease with great regularity by additional treatment with ultraviolet light. Mycoplasma bovoculi (Myc. bovoc.) (*Langford & Leach* 1973) is a newly recognized organism which appears to be frequently involved in the keratoconjunctivitis syndrome, but its pathogenicity remains to be ascertained (*Nayar & Saunders* 1975, *Nicolet et al.* 1976).

The isolation of M. bovis has been reported in previous Danish studies on the etiology of infectious bovine keratoconjunctivitis (*Pedersen* 1973). The purpose of the present work was to exa-

mine if also Myc. bovoc. could be isolated from field cases of this disease. Furthermore, by inoculation of a few calves with both microorganisms attempts were made to reproduce the disease experimentally.

MATERIAL AND METHODS

Field cases

During 1973 through 1977, five outbreaks (Nos. I to V) of infectious bovine keratoconjunctivitis were examined*. From each of a few animals in each herd a sample was obtained from one diseased eye and cultured for bacteria and mycoplasmas. Samples from Herds II and III (Jutland) were sent to the institute by ordinary mail whereas samples from Herds I, IV and V (islands of Lolland and Zealand) were brought in by car.

Outbreak I occurred among 30 animals on pasture. Twenty-two of them were newly purchased Jersey cows. Outbreak II occurred among 650 young bulls purchased successively at six months of age and kept indoors for fattening. Outbreak III occurred among a large flock of fattening animals pastured on a moor. Outbreak IV occurred among 50 Hereford cattle (adults and young stock) on pasture. Outbreak V occurred among 50 calves purchased successively at 10 days of age and kept indoors for fattening. Outbreaks I and IV were visited personally by the authors.

Microbiological procedures

Isolation and identification of M. bovis and Neisseria ovis (N. ovis) were carried out as described by *Pedersen* (1973). For cultivation of Myc. bovoc. the procedures for propagation of Mycoplasma suipneumoniae were followed (*Friis* 1975), and for cultivation of Mycoplasma dispar and ureaplasma the procedures indicated by *Bitsch et al.* (1976). For serological identification of members of the genus mycoplasma the disc growth inhibition test (DGI) was used, and in the case of Myc. bovoc. sometimes also indirect epi-immunofluorescence on colonies (IMF) (*Rosendal & Black* 1972). Antisera for the type strains of Myc. dispar (NCTC 10125) and Myc. bovoc. (NCTC 10141) were used. Exam-

* The authors are greatly indebted to the Institute of Internal Medicine, Royal Veterinary and Agricultural University, Copenhagen, for valuable cooperation.

ination for chlamydia was performed as four passages of one week in the yolk sacs of embryonated hens' eggs. Examination for virus* was performed as two passages of 14 days on bovine turbinate cells (NADC, Ames, Iowa) and on primary bovine kidney or testis cells.

Reproduction of the disease

Experimental animals. The animals used were four colostrum-deprived male calves of the Jersey breed originating from two farms where eye disease had never been noticed. They were isolated immediately after birth and brought to the institute, where they were fed with commercial whole milk for the first four days, whereafter the milk was gradually replaced by a milk substitute (Trumf®, Kemovit A/S, Copenhagen) made mainly of soybean meal. From three weeks of age access to hay and rolled barley or oats was allowed. Ampicillin (Penbritin Vet®, A/S Ferrosan, Copenhagen) was given on days 2—6 in daily doses of 10 mg/kg i.m., sulphadoxin/trimethoprim (Duoprim Vet®, Wellcome) on the following three days in daily doses of resp. 13 and 3 mg/kg i.m. If the calves got diarrhea, which two of them did, the milk was replaced by a dietary salt-glucose solution (Diætan®, Kemovit A/S, Copenhagen) for three days, during which period dimetridazol (Emtryl Vet® 40 %, Pharma Rhodia) was administered orally in daily doses of 1.6 g. The animals were housed in rooms with subdued light, direct sunshine being excluded. If necessary, flies were eliminated with pyrethrum spray (Calves III and IV).

Organisms for inoculation. Myc. bovoc., strain Mk274, was isolated from the eye of a calf in Outbreak III. The calf was suffering from acute keratoconjunctivitis. After isolation, the strain was passed through a membrane filter (Gelman, TCM, pore size 0.45 μm), and cloned once on solid medium. When used for inoculation in the seventh or eighth passage, it represented a 10^{22}- to 10^{25}-fold dilution of the original material. It was identified by DGI and IMF. The M. bovis strain used for inoculation was the previously described strain No. 9 (*Pedersen et al.* 1972). As judged by colony morphology the inoculum consisted of richly fimbriated bacterial cells.

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* For the virological work the authors are indebted to Dr. V. Bitsch, of this institution.

Experimental design. Conjunctival and nasal preinoculation swabs were examined for bacteria and mycoplasmas. Calf I was inoculated in both eyes with Myc. bovoc. when 22 days old, and four days later in both eyes with M. bovis. Calves II, III and IV were inoculated with Myc. bovoc. in the right eye when aged resp. 29, 24 and 30 days, and five days later with M. bovis in both eyes. The inoculation was performed by dropwise instillation of approx. 0.5 ml of culture into the ventral conjunctival sac followed by gentle massage of both eyelids. The titers of the mycoplasma inocula were estimated at 10^7—10^8 color-changing units. The calves were observed daily, and if disease appeared, samples for microbiological examination were taken from both eyes two to three times a week, otherwise approximately once a week. During the acute phase of disease one sample was taken for viral and chlamydial examination.

RESULTS

Field cases

Outbreaks I—IV, which occurred during the period August through October, were typical of infectious bovine keratoconjunctivitis, in that a great number of animals were suffering from serous conjunctivitis and/or keratitis at various stages. Outbreak V occurred in November, and just a moderate number of animals were attacked by the disease, which appeared to be secondary to a respiratory disease problem.

The results of the microbiological examinations are given in Table 1. It appears that M. bovis and N. ovis were involved in

Table 1. Microbiological examination of conjunctival swabs from animals in five outbreaks of infectious bovine keratoconjunctivitis.

Outbreak No.	Number of animals examined	Number of animals giving growth of			
		M. bovis*	N. ovis**	Myc. bovoc.***	other mycoplasmas
I	3	3	1	3	0
II	3	3	1	0	0
III	4	4	1	2	0
IV	4	2	3	3	0
V	2	2	1	2	1****

Material from Outbreaks II and III was forwarded to the institute by ordinary mail.

* Moraxella bovis, ** Neisseria ovis, *** Mycoplasma bovoculi, **** ureaplasma.

all the outbreaks. Except for two animals in Outbreak IV, one of which displayed just a serous conjunctivitis, M. bovis was recovered from all the animals examined. Myc. bovoc. was recovered from the majority of animals examined. The rate of recovery was notably lower from samples sent in by mail than from samples brought in directly to the laboratory.

Transmission experiments

The results of the experiments are given in Table 2. Calf I was inoculated in both eyes with M. bovis four days after the inoculation with Myc. bovoc. Serous conjunctivitis developed in both eyes. After two weeks keratitis was observed in the left eye. The condition improved somewhat after a week but was aggravated again as keratitis appeared in the right eye. The signs observed were lacrimation and corneal opacity and vascularisation. Central corneal opacity was still visible after a month. Myc. bovoc. and M. bovis were re-isolated from both eyes.

Table 2. Experimental reproduction of infectious keratoconjunctivitis by conjunctival inoculation of Mycoplasma bovoculi and Moraxella bovis on colostrum-deprived Jersey calves of about one month of age.

Calf No.		Day of inoculation with		First day with appearance of	
		Mycoplasma bovoculi	Moraxella bovis	conjunctivitis	keratitis
I	r	—4	0	3	27
	l	—4	0	3	15
II	r	—5	0	1	2
	l	nd*	0	nl	nl
III	r	—5	0	1	2
	l	nd**	0	2	1
IV	r	—5	0	nl	nl
	l	nd***	0	20	33

Figures indicate days before and after the day of inoculation with M. bovis.
r and l = right and left eye, resp.
nd = not done.
nl = no lesions.
*, **, *** found spontaneously infected with Myc. bovoc. when examined on days 2, 0 and 20, resp.

Calf II was inoculated with M. bovis in both eyes five days after inoculation with Myc. bovoc. in the right eye. After 24 hrs. the right eye showed conjunctivitis, and after a further 24 hrs. keratitis developed. Lacrimation and corneal opacity and vascularisation were the signs observed. The condition improved after three weeks and had almost disappeared after four weeks. Although the left eye was found spontaneously infected by Myc. bovoc. already two days after the inoculation with M. bovis, it remained clinically unaffected. During the period of disease in the right eye, both organisms were recovered from both eyes.

Calf III was inoculated with M. bovis in both eyes five days after inoculation with Myc. bovoc. in the right eye. After 24 hrs. the right eye displayed serous conjunctivitis and after a further 24 hrs. keratitis appeared. Seven days later some regression was noted, but an obvious aggravation of the lesions occurred after another seven days. Profuse lacrimation and heavy corneal opacity and vascularisation were noted. The corneal lesion protruded distinctly above the normal convexity of the cornea and had a small central depression. After four weeks the lesions had disappeared except for a small corneal opacity, which was still persisting after another month. The left eye was found spontaneously infected with Myc. bovoc. on the day of inoculation with M. bovis. Although a beginning corneal lesion was observed 24 hrs. later, conjunctivitis was not evident until after another 24 hrs. The lesions of the left eye were of the same nature as those of the right eye, but never reached the same intensity. Both organisms were recovered from both eyes during the period of disease.

Calf IV was inoculated with M. bovis in both eyes five days after inoculation with Myc. bovoc. in the right eye. Although both organisms became established in the right eye no lesions appeared. In the left eye the inoculation with M. bovis did not result in infection. However, after 20 days the left eye was found spontaneously infected with both organisms, and during the following days a serous conjunctivitis developed. After a further 13 days a moderate keratitis appeared. Lacrimation, opacity and vascularisation were noted. This condition lasted two to three weeks, during which period both organisms were found in great numbers.

The cultivation of the preinoculation swabs revealed the presence of Myc. dispar in the nostrils of Calves I and II. Apart

from this, neither mycoplasmas nor significant bacteria were recovered. No mycoplasmas other than Myc. bovoc. were found in the conjunctivae, notwithstanding the demonstration of Myc. dispar in the nostrils of Calves I and II, and apart from M. bovis no significant bacteria were found. Chlamydia and virus could not be demonstrated.

DISCUSSION

Material from five natural outbreaks of infectious bovine keratoconjunctivitis was examined for bacteria and mycoplasmas. Myc. bovoc. was found in most animals, though not at all in one of the outbreaks, probably because of deterioration of the material. No other species of the order mycoplasmatales appeared to be regularly involved in these outbreaks, the only other finding of such organisms being a ureaplasma in one case. M. bovis and N. ovis were demonstrated in all outbreaks, and M. bovis in almost all of the animals. Although the material examined in this work is rather small, the demonstrated high rate of recovery of Myc. bovoc. corresponds well with the results of similar investigations carried out by other authors (*Langford & Dorward* 1969, *Nicolet et al.* 1976).

Transmission experiments showed that keratoconjunctivitis could be produced in naturally-born, colostrum-deprived calves by the combined use of Myc. bovoc. and M. bovis. The animals were kept indoors and ultraviolet irradiation was not applied. In two of the calves (Nos. II and III) keratitis appeared very rapidly upon inoculation, in the two others (Nos. I and IV) not until after two-four weeks. Only two eyes did not show signs of disease, namely the right eye of Calf IV, inoculated with both organisms, and the left eye of Calf II, which was found infected with both organisms two days after the inoculation with M. bovis.

The regular reproduction of typical lesions of keratoconjunctivitis by means of pure culture of M. bovis has usually been dependent on irradiation of the eyes by ultraviolet light, but in the present experiments it appeared that the effect of irradiation could be substituted for by the effect of Myc. bovoc. Previous attempts to produce keratoconjunctivitis by combining the pathogenic effect of Myc. bovoc. with that of M. bovis (*Langford & Dorward, Nayar & Saunders* 1975) have been unsuccessful, and the discrepancy between these results and those of the present experiments may seem difficult to explain. It is possible, how-

ever, that young, colostrum-deprived calves as used in this study are particularly susceptible, but the explanation is perhaps more likely to be found in the length of the interval between the two inoculations. If the effect of the two organisms is regarded not as a simple additive one, but more in the sense of Myc. bovoc. "paving the way" for the real pathogen, i.e. M. bovis, an interval of five days as chosen in the present work will be appropriate, because it will allow Myc. bovoc. to become well established without as yet being influenced by antibody. The experimental findings in Calves II and III, but not in Calf IV, support this hypothesis.

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SAMMENDRAG

Isolation af Mycoplasma bovoculi fra udbrud af infektiøs bovin keratokonjunctivitis i Danmark.

Fem udbrud af infektiøs bovin keratokonjunctivitis blev undersøgt for forekomst af bakterier og mykoplasmer. Mycoplasma bovoculi blev påvist i 4 af udbruddene. Af andre mykoplasmer fandtes ureaplasma i én prøve. Moraxella bovis og Neisseria ovis isoleredes fra samtlige udbrud og Morexella bovis fra næsten alle undersøgte dyr. Forsøg på reproduktion af lidelsen blev udført som kombineret podning med Mycoplasma bovoculi og Morexella bovis på 4 kalve, der ikke havde fået kolostrum. Resultaterne kunne tyde på, at Mycoplasma bovoculi har en forstærkende effekt på patogeniteten af Morexella bovis.

(Received July 27, 1978).

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