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The identification of *Mycoplasma conjunctivae* as an aetiological agent of infectious keratoconjunctivitis of sheep in South Africa

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ABSTRACT

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Ovine keratoconjunctivitis was successfully reproduced in lambs under 1 year of age, in four separate transmission trials, by the use of mycoplasma isolates obtained from field outbreaks of ovine infectious keratoconjunctivitis. Mycoplasma isolates used in one of these trials, were identified as *M. conjunctivae* by means of immunofluorescence. Mycoplasma was isolated from approximately 87 % of field cases examined. *Branhamella ovis* was isolated from 22 % of field cases examined. No *Chlamydia* sp. or viruses were isolated from any of the outbreaks.

INTRODUCTION

Ovine infectious keratoconjunctivitis (OIKC) is an economically important disease of small ruminants, with a world-wide distribution (Greig 1989; Egwu, Faull, Bradbury & Clarkson 1989; Egwu 1991). It occurs throughout South Africa, including the western and southern Cape Province (Stellenbosch Regional Veterinary Laboratory, unpublished data). This condition was first described in South Africa in 1931 by Coles, who proposed *Rickettsia (= Colesiota) conjunctivae* as the causal agent, basing this on his observation of cytoplasmic inclusion bodies in conjunctival cells. OIKC is characterized by conjunctivitis, increased lachrymation, blepharospasm and photophobia, with keratitis and corneal ulceration in advanced cases (Cottew 1979; Hosie 1988; Greig 1989; Egwu 1991). The morbidity in a flock is usually high, and the disease appears to become endemic, once present. In South African sheep, as in sheep from elsewhere, the disease has been described in a number of breeds. Sheep of all ages may be affected, although it is more common in younger animals. In older sheep, however, the condition tends to be more severe, as noted by Greig (1989). Relapses are common, even with antibiotic treatment.

Outbreaks involved sheep kept on grazing varying from dry Karoo veld to lush kikuyu pastures. OIKC has a very seasonal occurrence, with all outbreaks in the western and southern Cape occurring between October and April (dry, warm period).

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MATERIALS AND METHODS

Field surveys

Twenty-five field outbreaks of ovine infectious kerato-conjunctivitis have been investigated in eight magisterial districts in the western and southern Cape since November 1988. A total of 521 affected eyes were swabbed (in cases of bilateral OIKC, both eyes were swabbed). As far as possible, the samples were collected from early, untreated cases. Specimens were collected for the isolation of bacteria, mycoplasma organisms, Chlamydia and viruses *(vide infra)*.

Transmission trials

Various transmission trials were conducted.

Viruses

Conjunctival swabs collected from field outbreaks of OIKC were suspended in phosphate-buffered saline (PBS) overnight. The PBS was filtered through 220-nm millipore filters, and drops of the filtered PBS were instilled in the left eyes of ten previously unexposed 6-month-old Dormer and Mutton Merino lambs.

Bacteria

Branhamella ovis isolates cultured from field outbreaks of OIKC were instilled in the left eyes of six previously unexposed 6-month-old Mutton Merino-cross lambs. Dry swabs were used to remove the bacterial cultures from the medium; these were then rubbed onto the conjunctiva of the eyelid and conjunctival sac.

Mycoplasma

Four separate trials were conducted. Mycoplasma isolates cultured from field outbreaks of OIKC were instilled in the left eyes of ten, seven, ten and four previously unexposed lambs under one year of age. Dry swabs were used to scrape the mycoplasma colonies from the agar surface, and these were then rubbed onto the conjunctiva of the left eye. There was no prior scarification of the conjunctiva or exposure to ultra-violet radiation. Conjunctival swabs were collected from experimental sheep, prior to instillation of the cultures for bacterial and mycoplasma isolation *(vide infra)*. After instillation of the cultures these lambs were kept in isolation out of doors on grass pastures and observed for clinical signs of OIKC.

Sampling techniques

Sterile, dry cottonwool swabs used for sampling were rubbed under the upper eyelid and under the third eyelid. Separate swabs were used for bacterial, mycoplasma, chlamydia and virus isolation. Where sampling was done on farms, and immediate processing was not possible, appropriate transport medium was used to keep the swabs moist after collection of conjunctival material. The swabs were then kept in cooler bags until they could be processed at the laboratory.

In some of the field outbreaks, conjunctival scrapings for electron microscopy were collected by scraping a glass slide over the conjunctiva of the upper and lower eyelid, and suspending the collected material in PBS.

Paired blood samples for chlamydia serology were collected from the jugular vein of ten sheep in one outbreak. The animals were bled within 2 d of the onset of clinical signs; a second sample was collected from the same sheep 14 d later.

Culture techniques

Mycoplasma

Hayflick's agar and broth and Oxoid mycoplasma agar was used for outbreaks 1–11. From outbreak 12–25 Chalquest's agar medium was used. After the swabs had been inoculated, they were incubated at 37 °C in 95 % $C0_2$ and 5 % N_2 for 5 d.

Bacteria

Swabs were inoculated onto blood tryptose agar (BTA) prepared with bovine blood and incubated at 37 °C in normal atmospheric conditions for 2–3 d.

Viruses

Conjunctival material was inoculated onto monolayers of low-passage, primary calf-foetal-kidney (CFK) cells and incubated at 37 °C. Subcultures of all samples were made weekly for 3 weeks. The cells were checked for cytopathic effects (CPE).

Chlamydia

Conjunctival material collected from the first ten outbreaks was inoculated into the yolk sacs of 8-day-old embryonated eggs. Samples collected from outbreaks 19 and 26 were inoculated onto vero-cell monolayers in TRAC bottles and, after 5 d of incubation, the coverslips were stained by means of the Giminez method to check for the presence of chlamydia.

RESULTS

Field surveys

The results of the various isolations from field outbreaks are given in Table 1. Where Chalquest's agar medium was used, mycoplasma spp. were isolated from 88,6 % of field cases investigated, whereas no growth was obtained on Hayflick's agar medium or broth. *Branhamella ovis* was isolated from 22,2 % of cases investigated. No viruses nor chlamydia were isolated from any of the cases examined.

TABLE 1 Isolations per outbreak investigated

Outbreak no.	Magisterial district	Month	Mycoplasma	Bacteria	Chlamydia	Viruses
1	Wellington	Nov.	–	2/20 *Branhamella ovis*	0/20	0/20
2	Wellington	Nov.	0/20	0/20	–	–
3	Malmesbury	Jan.	0/16	7/25 *B. ovis*	0/25	0/25
4	Riversdale	Feb.	0/18	1/18 *B. ovis* 1/18 *Staph. aureus*	0/18	–
5	Malmesbury	Feb.	0/23	9/23 *B. ovis* 1/23 *Staph. aureus*	0/23	0/23
6	Wellington	March	0/27	7/27 *B. ovis*	0/25	0/26
7	Riversdale	March	0/41	16/46 *B. ovis* 4/46 *Staph. aureus*	0/40	0/40
8	Stellenbosch	Sept.	0/24	10/24 *B. ovis* 5/24 *Staph. aureus*	0/24	–
9	Malmesbury	Oct.	0/3	0/3	0/3	–
10	Ladismith	Oct.	0/15	2/15 *B. ovis* 6/15 *Staph. aureus*	0/15	–
11	Stellenbosch	Oct.	0/24	–	–	–
12	Malmesbury	Nov.	4/17	–	–	–
13	Stellenbosch	Nov.	11/17	–	–	–
14	Malmesbury	Nov.	13/20	–	–	–
15	Stellenbosch	March	5/6	–	–	–
16	Tulbagh	March	40/60	8/40 *B. ovis*	–	–
17	Caledon	March	8/8	0/8	–	–
18	Malmesbury	March	50/50	–	–	–
19	Malmesbury	Dec.	33/34	21/34 *B. ovis*	0/34	–
20	Malmesbury	Dec.	17/17	–	–	–
21	Malmesbury	Feb.	36/36	–	–	–
22	Worcester	Oct.	21/21	0/21	–	–
23	Malmesbury	Oct.	29/40[a]	–	–	–
24	Malmesbury	Nov.	26/26	0/26	–	–
25	Stellenbosch	April	26/28	0/28	0/28	–

[a] No mycoplasma growth was obtained from 11 samples, owing to bacterial contamination of medium

– = Not collected

Conjunctival scrapings submitted for electron microscopy revealed the presence of numerous bacteria and mycoplasmas.

There were no significant increases in chlamydia-complement-fixation-test (CFT) titres in the ten paired blood samples collected 14 d apart.

Transmission trials

Virus-transmission trial

No IKC could be reproduced in the ten experimental lambs inoculated with the filtered material obtained from clinical cases of IKC.

Branhamella ovis trial

No IKC could be reproduced in the six experimental lambs that had had *B. ovis* cultures instilled on their conjunctivas, although *B. ovis* could still be cultured from five out of six of these eyes on day 7.

Mycoplasma trials

See Tables 2–5.

DISCUSSION

Mycoplasma spp. were isolated from 319 out of 360 swabs (88,6 %) collected from field outbreaks of ovine IKC, when Chalquest's medium was used. No growth was obtained on Hayflick's agar or broth. Once present in a flock, the infection was very persistent and relapses were common, as noted by

TABLE 2 Trial 1: Primary mycoplasma isolates cultured from field outbreak 19, instilled in left eye of 10 lambs

Lamb no.	Day 0	Day 2	Day 3
1	0 L (MB)	0 L (M)	L (M)
2	0 L (MB)	0 (–)	++ L (M)
3	0 L (M)	0 (–)	0 (M)
4	0 L (MB)	0 (–)	0 (M)
5	0 L (MB)	0 (–)	0 (M)
6	0 L (B)	+ L (M)	++ L (M)
7	0 L (B)	0 (–)	0 (M)
8	0 L (B)	0 (–)	+ L (M)
9	0 L (B)	0 (–)	0 (M)
10	0 L (B)	+ LM	++ L (M)

L = Left eye
R = Right eye
(M) = Mycoplasma isolated
(B) = *Branhamella ovis* isolated
(–) = No isolation
0 = Normal
\+ = Mild conjunctivitis with lachrymation
++ = Moderate conjunctivitis with lachrymation and blepharospasm
+++ = Severe conjunctivitis with lachrymation, blepharospasm and corneal opacity

TABLE 3 Trial 2: Primary mycoplasma cultures obtained from field outbreak 20 and instilled on the conjunctiva of the left eye of seven previously unexposed experimental lambs

Lamb no.	Day 0	Day 3	Day 5	Day 6	Day 10	Day 11
1	0 L (MB)	+ L (M)	+ L	0	0	0
2	0 L (MB)	++ L (M) ++ R (M)	+ L & R	+ L & R	0	0
3	0 L (M)	++ L & R (M)	+++ L & R	+ L & R	0	0
4	0 L (M)	++ L & R (MB)	+ L & R	0	0	0
5	0 L (M)	+ L (M)	+ L	0	0	0
6	0 (–)	+ L (M)	++ L & R	+ L & R	0	0
7	0 (–)	0 (M)	+++ L	++ L & R	+++ L & R	++ L & R

Lambs 1–6 treated daily with penicillin/dihydrostreptomycin ointment intraocularly from day 3 onward
Sheep 7 treated daily from day 5 onward

TABLE 4 Trial 3: Primary mycoplasma isolates obtained from field outbreak 22 and instilled on conjunctiva of right eye of ten previously unexposed lambs (± 6 months of age)

Lamb no.	Day 0	Day 3	Day 6	Day 7	Day 8	Day 9	Day 10	Day 13	Day 15	Day 20
1 L	0 (–)	+	+++ (M)	+++	++	+	0	++ (M)	+	0
R	0 (B)	+	++ (M)	++	+	0	0	++ (M)	+	0
2 L	0 (–)	+	++ (M)	+	+	0	0	+ (M)	0	+
R	0 (B)	0	+++ (M)	++	+	0	0	+ (M)	0	0
3 L	0 (–)	0	0 (MB)	0	0	0	0	+ (M)	+	0
R	0 (–)	0	0 (MB)	+ (M)	+	0	0	+ (M)	0	+
4 L	0 (–)	0	0 (–)	+ (M)	+	0	0	+ (M)	0	0
R	0 (B)	0	0 (MB)	+ (M)	0	0	0	+ (M)	0	+
5 L	0 (–)	+	+++ (M)	+++	++	+	+	++ (M)	+	0
R	0 (–)	0	++ (M)	++	+	+	0	++ (M)	+	0
6 L	0 (–)	0	+ (M)	+	0	++ (M)	+	+ (M)	0	0
R	0 (B)	0	+ (M)	+	0	+ (M)	+	+ (M)	0	0
7 L	0 (B)	0	+ (M)	0	0	0	0	++ (M)	++	+
R	0 (B)	0	0 (–)	0	0	0	0	++ (M)	++	+
8 L	0 (–)	0	+ (M)	+++	+	+	0	++ (M)	+	+
R	0 (B)	0	0 (MB)	0	0	0	0	++ (M)	+	+
9 L	0 (B)	+	+++ (M)	++	+	0	0	+ (M)	+	0
R	0 (B)	0	++ (MB)	+	0	0	0	0 (M)	0	0
10 L	0 (–)	0	0 (–)	0 (M)	0	0	0	0 (M)	0	0
R	0 (–)	0	0 (–)	+ (M)	0	0	0	++ (M)	+	0

Affected lambs were treated daily from day 6–10 with topical penicillin/dihydrostreptomycin ointment. On day 13 all lambs were given long-acting oxytetracycline intramuscularly because of a flare-up of IKC. Long-acting tetracycline was repeated on day 27 because of another flare-up. Relapses continued, despite treatment, until day 90

TABLE 5 Trial 4: Primary mycoplasma cultures obtained from field outbreak 25, instilled on the conjunctiva of the left eye of four previously unexposed 9-month-old lambs

Lamb no.	Day 0	Day 4	Day 5	Day 6	Day 7	Day 8	Day 11	Day 49
1 L	0 (–)	0	0 (M)	0 (M)	0 (M)	0 (M)	+ (M)	0 (M)
R	0 (–)	0	0 (M)	0 (M)	0 (M)	+ (M)	0 (M)	0 (M)
2 L	0 (–)	+++	+++ (M)	++ (M)	++ (M)	+++ (M)	++ (M)	0 (M)
R	0 (–)	0	++ (M)	++ (M)	++ (M)	+++ (M)	+ (M)	0 (M)
3 L	0 (–)	++	++ (M)	++ (M)	++ (M)	+++ (M)	+ (M)	0 (M)
R	0 (–)	0	0 (M)	++ (M)	++ (M)	+++ (M)	++ (M)	0 (M)
4 L	0 (–)	0	0 (M)	0 (M)	0 (M)	0 (M)	++ (M)	0 (M)
R	0 (–)	0	0 (M)	0 (M)	0 (M)	+ (M)	++ (M)	0 (M)

Swabs were not collected on day 4, owing to problems experienced with the Chalquest's medium prepared. From day 8 onward, affected lambs were treated daily with intramuscular quinolone antibiotic injection. Six primary cultures obtained from the eyes on day 5 and day 8 were referred to the Mycoplasma Reference Unit of the Central Public Health Laboratory, London, United Kingdom, where positive colony immunofluorescence with antiserum to *Mycoplasma conjunctivae* was obtained in all six cases. Negative immunofluorescence was obtained with *M. arginini* and *M. ovipneumoniae* antiserum

König (1983), Ter Laak, Schreuder, Kimman & Houwers (1988a), Ter Laak, Schreuder & Smith-Buys (1988b), and Egwu (1991). Older animals were generally more severely affected, as has been described by Egwu *et al.* (1989).

IKC was successfully reproduced in four separate transmission trials, where cultures were instilled on the conjunctiva without prior exposure of eyes to ultraviolet radiation or scarification of the conjunctiva.

The incubation period before the appearance of clinical signs varied from 2–4 d, with a mean of 3 d. This corresponds with the 3–4 d documented by Jones (1976), and is considerably shorter than the incubation period of 11–21 d and 6–16 d noted in two trials conducted by Ter Laak *et al.* (1988a). Clinical signs varied from mild conjunctivitis with increased lachrymation, to severe keratitis. In most cases the condition was bilateral.

The possibility that secondary bacteria had aggravated the condition, has been suggested by certain authors (Greig 1989; Egwu *et al.* 1989; Wilsmore, Dagnall & Woodland 1990). In our investigations *Branhamella ovis* was isolated from only 84 out of the 378 swabs (22,2 %) collected from field outbreaks, and in several of the outbreaks, no *B. ovis* was isolated from any of the animals sampled.

No chlamydia were isolated from any of the field outbreaks investigated, and in one of the outbreaks investigated, there was no significant chlamydia-titre increase. Wilsmore *et al.* (1990) successfully reproduced ovine IKC in sheep by subconjunctival inoculation of a chlamydia isolate obtained from a field outbreak of IKC. Certain authors differentiate between a non-follicular infectious keratoconjunctivitis caused by *M. conjunctivae* (Jones 1976) and a follicular keratoconjunctivitis caused by chlamydia (Bogaard 1984; Egwu 1991). The cytoplasmic-inclusion bodies, described by Coles in 1931, in the conjunctival cells of sheep, are now considered by certain authors (Bogaard 1984) to be reticulate bodies of *Chlamydia psittaci* (= *Colesiota conjunctivae*), although other authors assert that these are, in fact, extracellular mycoplasmas (Egwu 1991).

The primary aetiological role of *M. conjunctivae* in non-follicular infectious keratoconjunctivitis was confirmed in our investigations and transmission trials. This is supported by the investigations of Jones (1976), Hosie (1988), Ter Laak *et al.* (1988a; 1988b) and Egwu *et al.* (1989). Unfortunately we have, to date, been unsuccessful in sub-culturing or freeze-drying the primary isolates; this has hampered further investigation.

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REFERENCES

BOGAARD, A.E.J.M. 1984. Inclusion keratoconjunctivitis ("pink eye") in sheep. A proposal for a new name for chlamydial keratoconjunctivitis in sheep and comment on recent clinical trials. *The Veterinary Quarterly,* 6:229–235.

COLES, J.D.W.A. 1931. An unknown intracellular organism of the conjunctival epithelium of sheep. Preliminary reports: *17th Report of the Director of Veterinary Services and Animal Industry,* Onderstepoort, Union of South Africa: 187–189.

COTTEW, G.S. 1979. Caprine-ovine mycoplasmas, in *The Mycoplasmas,* edited by J.G. Tully & R.F. Whitcomb. New York: Academic Press: 103–132.

EGWU, G.O., FAULL, W.B., BRADBURY, J.M. & CLARKSON, M.J. 1989. Ovine infectious keratoconjunctivitis: A microbiological study of clinically unaffected and affected sheep's *(sic)* eyes with special reference to *Mycoplasma conjunctivae. Veterinary Record*, 125:253–256.

EGWU, G.O. 1991. Ovine infectious keratoconjunctivitis: an update. *Veterinary Bulletin,* 61:547–559.

GREIG, A. 1989. Ovine keratoconjunctivitis. An update. *In Practice*, 11:110–113.

HOSIE, B.D. 1988. Keratoconjunctivitis in a hill sheep flock. *Veterinary Record,* 122:40–43.

JONES, G.E. 1976. Mycoplasmas and ovine keratoconjunctivitis. *Veterinary Record,* 99:137–141.

KÖNIG, C.D.W. 1983. "Pink eye" or "zere oogjes" or Keratoconjunctivitis *Infectiosa ovis* (KIO). *The Veterinary Quarterly,* 5: 122–127.

TER LAAK, E.A., SCHREUDER, B.E.C., KIMMAN, T.G. & HOUWERS, D.J. 1988a. Ovine keratoconjunctivitis experimentally induced by instillation of *Mycoplasma conjunctivae. The Veterinary Quarterly,* 10:217–224.

TER LAAK, E.A., SCHREUDER, B.E.C. & SMITH-BUYS, C.M.C. 1988b. The occurrence of *Mycoplasma conjunctivae* in the Netherlands and its association with infectious keratoconjunctivitis in sheep and goats. *The Veterinary Quarterly,* 10:73–83.

WILSMORE, A.J., DAGNALL, G.J.R. & WOODLAND, R.M. 1990. Experimental conjunctival infection of lambs with a strain of *Chlamydia psittaci* isolated from the eyes of a sheep naturally affected with keratoconjunctivitis. *Veterinary Record,* 127:229–231.