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Source: Journal of Wildlife Diseases, 38(2) : 297-304

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-38.2.297

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Journal of Wildlife Diseases, 38(2), 2002, pp. 297–304
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MYCOPLASMA CONJUNCTIVAE INFECTION IS NOT MAINTAINED IN ALPINE CHAMOIS IN EASTERN SWITZERLAND

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ABSTRACT: The occurrence of infectious keratoconjunctivitis (IKC) was assessed in alpine chamois (*Rupicapra rupicapra rupicapra*) in Grisons (Switzerland) from 1950 to 1999. The first IKC outbreaks were reported in the 1950's. Since then, the number of affected subpopulations constantly increased and, by 1999, IKC outbreaks were reported in 39 of 51 (77%) chamois subpopulations. From 1992-99, a total of 243 chamois which died of the consequences of IKC were recorded. The number of cases differed between years, and a distinct seasonal trend was observed. Infectious keratoconjunctivitis was more common during summer and autumn, with 48% of the cases recorded in August–October. Juveniles (<4 yr of age) were mostly represented. To verify the presence of *Mycoplasma conjunctivae* in chamois we analyzed conjunctival swabs taken from animals affected with IKC. Among a sample of 28 affected chamois, *M. conjunctivae* was identified 14 times (50%). An indirect enzyme-linked immunosorbent assay (ELISA) was developed to detect specific *M. conjunctivae* antibodies in sera of alpine chamois with IKC. We performed a serologic investigation to assess whether *M. conjunctivae* infection is self-maintained in the chamois population in Grisons. In subpopulations with IKC oubreaks, seroprevalence was low (8%). Seroprevalence was even lower in subpopulations with recent IKC outbreaks (3%). We concluded that the *M. conjunctivae* infection is not self-maintained in alpine chamois in Grisons. The agent may originate in domestic sheep living in proximity to chamois during summer. Control of IKC in chamois should consider immunoprophylaxis in sheep or limiting interspecific transmission of *M. conjunctivae*.

Key words: Alpine chamois, ELISA, epidemiology, infectious keratoconjunctivitis, *Mycoplasma conjunctivae, Rupicapra rupicapra rupicapra*, serodiagnosis, Switzerland.

INTRODUCTION

Persistence of infections in animal populations is determined mainly by the potential of the pathogen to maintain its life cycle in the host and by host population characteristics such as number of individuals, percentage of susceptible animals within the population, and interaction between animals (Thrusfield, 1995). Examples of infectious diseases self-maintained in wildlife are foot-and-mouth disease in African buffalo (*Syncerus caffer*) in South Africa (Thomson, 1994), tuberculosis (*Mycobacterium bovis*) in both the European badger (*Meles meles*) in United Kingdom and in the brush-tailed possum (*Trichosurus vulpecula*) in New Zealand (Morris et al., 1994), and brucellosis in bison (*Bison bison*) in Yellowstone National Park (USA) (Dobson and Meagher, 1996). In different circumstances however, wildlife infections may be only apparently "self-maintained," and common occurrence may be due to the sporadic unrecognized introduction of pathogens from another host living in proximity (Daszak et al., 2000). Prerequisites to evaluate whether or not a particular wild animal population acts as a true reservoir for a specific infectious agent include long term studies on a population scale and availability of methods for detecting the presence of the disease. In addition to these methodical aspects, the model requires that only sproadic spill-over of the infectious agent occurs from domestic animals to avoid a masked persistence of the infectious agent in wildlife.

Infectious keratoconjunctivitis (IKC) caused by *Mycoplasma conjunctivae* is a

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highly contagious ocular infection of particular interest for both domestic and wild Caprinae species in the European Alps (Giacometti et al. 2000). In the Swiss domestic sheep population, mycoplasmal IKC is endemic (Janovsky et al., 2001). The disease may be a nuisance to farmers since animals require treatment and some nursing in order to reduce the period of blindness. Blind chamois and alpine ibex (*Capra ibex ibex*) face particularly treacherous circumstances in steep rocky areas, and in these species mortality can reach 30% (Degiorgis et al., 2000a). However, the role of wild Caprinae in the maintenance of *M. conjunctivae* infection is controversial. In Vanoise National Park (France), IKC was assumed to be endemic in chamois and ibex populations (Gauthier, 1991). In contrast, no chamois affected by IKC were observed after outbreaks in the Bauges National Reserve (France) in 1977 (Loison et al., 1996) and in Simmental-Gruyéres (Switzerland) in 1997–99 (Degiorgis et al., 2000a) suggesting disappearance of the infection in these study sites. The uncertainty is due to the lack of long-term studies on the occurrence of IKC in wild Caprinae populations and to technical difficulties in detecting *M. conjunctivae* infections.

Based on a study performed in Switzerland we hypothesized that the chamois population is not acting as a reservoir of *M. conjunctivae* infections (Giacometti et al., 2000). The objectives of this study were therefore to assess occurrence of *M. conjunctivae* in chamois in Grisons (Switzerland) from 1950–1999, to develop and validate an indirect enzyme-linked immunosorbent assay (ELISA) for detection of specific *M. conjunctivae* antibodies in chamois sera, and to evaluated if *M. conjunctivae* is maintained in the chamois population in the eastern Swiss Alps.

MATERIALS AND METHODS

We studied IKC in alpine chamois in Grisons (46°10′ to 47°03′N, 8°46′ to 10°20′E), a Canton in the eastern Swiss Alps. In Grisons (7,106 km^2), alpine chamois live at elevations between 400 and 3,000 m. Chamois habitat is steep and rocky, and north slopes are colonized as well as other aspects. Open shrubby heaths, clearings, and alpine meadows bordering forests, or rocky areas that provide cover, are preferred foraging sites (Giacometti, 1997). In Grisons, the chamois population was estimated at 25,000 animals at the end of winter. Based on geomorphological criteria, Grisons was subdivided into 51 subpopulation units. Other free-ranging ungulates present are alpine ibex, roe deer (*Capreolus capreolus*), and red deer (*Cervus elaphus*). Domestic sheep, domestic goat, and cattle partially use alpine meadows during the summer grazing period.

In Grisons, state game-keepers have systematically recorded chamois found dead of the consequences of IKC for 50 yr. Live individuals with IKC either presenting ocular lesions regarded to be irreversible or in poor general condition and/or injured were shot. Chamois were considered to be affected by IKC if at least one of the following lesions were detected: bilateral serous or mucopurulent ocular exudate, bilateral corneal opacity, and/or perforation (Mayer et al., 1997). The animals were aged by counting horn rings (Habermehl, 1985), and age-classes were established according to sexual maturity and social behavior. Descriptive data were used to describe IKC occurrence at the subpopulation level in the decades following 1950. In 1992-99, data on dead chamois collected by 62 state game-keepers were centralized and stored in a computerized system.

We analyzed chamois affected with IKC shot by state game-keepers. Sterile cotton swabs were taken from behind the third eyelid of one eye in 1994–2000. The swabs were dipped into transport medium (Transwab, Medical Wire and Equipment, Corsham, UK) and processed within 24 hr after collection. *Mycoplasma conjunctivae* was cultured in standard mycoplasma PPLO broth medium (Difco Laboratories, Detroit, Michigan, USA) enriched with 20% horse serum, 2.5% yeast extract, and 1% glucose (Bannerman and Nicolet, 1971). For detection of *M. conjunctivae* RNA by polymerase chain reaction (PCR), conjunctival swabs were dipped in tubes without transport medium and stored at −18 C until analysis. Nested PCR was based on the 16S rRNA gene (Giacometti et al., 1999).

An immunoblot technique was utilized to detect specific antibodies against *M. conjunctivae* in the sera of two chamois using whole cell antigen of *M. conjunctivae* type strain HRC/581^T (Barile et al., 1972) separated by SDS-PAGE 5–15% gradient gels (Degiorgis et al., 2000b).

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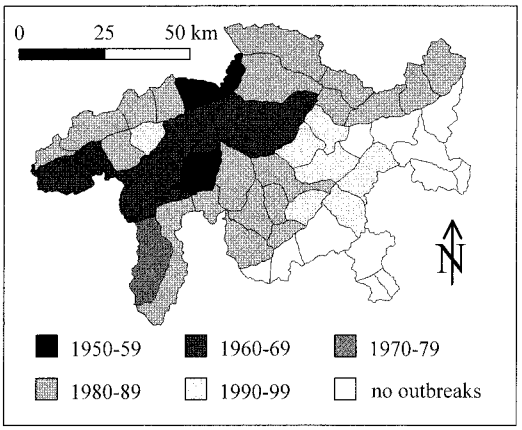


FIGURE 1. Occurrence of the first infectious keratoconjunctivitis outbreaks in chamois in Grisons (Switzerland) in 1950–1999. Units are the 51 chamois subpopulations.

The immunoblot technique was performed according standard protocols (Ausubel et al., 1990). Sera were diluted 1:50 and bound antibodies were detected using monoclonal antibody (Mab) to goat/sheep-IgG (Sigma Chemicals, Saint Louis, Missouri, USA, product #A-8062) diluted 1:2000.

Chamois blood was collected in 50ml plastic tubes fitted with screw caps by hunters from the thoracic cavity within 15 min after shooting. Samples were stored at −18 C. Samples were then thawed, centrifuged, and the supernatants stored at −18 C until analysis. An indirect ELISA method for detection of specific *M. conjunctivae* antibodies in chamois sera was developed based on Tween 20 extracted membrane protein fraction of *M. conjunctivae* strain HRC/581^T. This test was based on an ELISA previously developed for use in domestic sheep (Belloy et al., 2001). Serum was diluted 1:10 and applied to the plates. For chamois sera, analysis was performed using anti-sheep-anti-goat-IgG, a monoclonal antibody directed against sheep and goat IgG conjugated to horseradish peroxidase (Sigma Chemicals, A-9452) diluted 1:125. Optical densities were measured with a photometer at a wavelength of 405 nm. The value of each sample was expressed as a percentage of a positive reference standard, by taking a negative reference serum as the zero value according to approved standardization methods (Nicolet and Martel, 1996). For ELISA validation in chamois, we compared data of chamois showing IKC signs with results from sheep sera analysed using the ELISA test as described in Belloy et al. (2001).

To assess seroprevalence, we sampled serum of chamois shot in Grisons in the September 1999 hunting season and analysed them using ELISA. To evaluate if *M. conjunctivae* infection is maintained in populations, we considered chamois subpopulations where clinical IKC had been reported in 1997–99 as category A (positive controls), and subpopulations, where clinical IKC had been reported in 1992–97, but not in 1998-99 as category B. We assumed that seroprevalence in category B subpopulations would be comparable to the category A subpopulations if *M. conjunctivae* persisted within chamois populations.

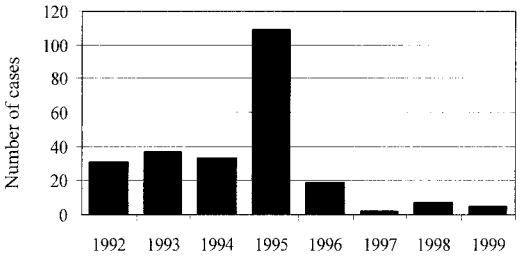


FIGURE 2. Yearly distribution of the number of recorded chamois that died of the consequences of infectious keratoconjunctivitis in Grisons (Switzerland) in 1992–99 (n = 243).

RESULTS

The first IKC outbreaks in alpine chamois were reported in Grisons during the 1950's in two subpopulations in the central and northern part of the canton (Fig. 1). Since then, the number of affected subpopulations has constantly increased. In the decades 1960s and 1980s, nine and 20 subpopulations, respectively, were affected for the first time. By 1999, IKC outbreaks were reported in 39 of 51 (77%) chamois subpopulations. Infectious keratoconjunctivitis-free subpopulations were restricted to the south-eastern part of Grisons.

In 1992–99, a total of 243 chamois that died of consequences of IKC were recorded. The number of cases differed between years (Fig. 2). Most chamois died in 1995, when 109 cased were recorded. In the years 1997-99, mean number of chamois found dead per year was low ($\bar{x}$ = 4.7). A distinct seasonal trend was observed. Infectious keratoconjunctivitis was more common during summer and autumn (Fig. 3), with 48% of the cases recorded in August–October. Juveniles (<4 yr of age)

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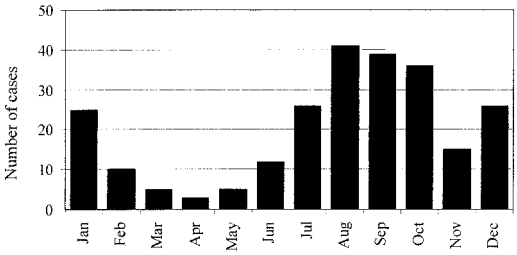


FIGURE 3. Monthly distribution of the number of recorded chamois that died of the consequences of infectious keratoconjunctivitis in Grisons (Switzerland) in 1992–99 (n = 243).

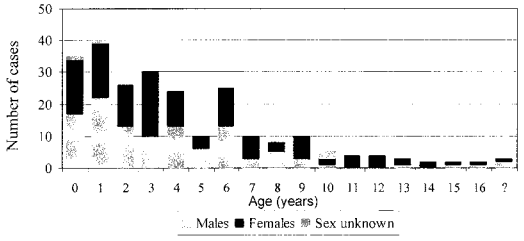


FIGURE 4. Sex and age of chamois that died of the consequences of infectious keratoconjunctivitis in Grisons (Switzerland) in 1992–99 (n = 243).

were most numerous among animals that died of IKC (n = 129, 55%, Fig. 4). Overall, more females than males died of IKC (127 females, 109 males). However, adult males were well represented (47 adult males, 60 adult females).

To verify the presence of *M. conjunctivae* in chamois in Grisons we analyzed conjunctival swabs taken from animals affected with IKC (Table 1). Clinical signs were generally severe and included corneal opacity and ulceration. Among a sample of 28 affected chamois from 13 localities, *M. conjunctivae* was identified 14 times (50%).

Optimal protein concentration of 6.25 μg/ml of *M. conjunctivae* extract produced maximum resolution of a positive reference serum from a chamois affected with IKC on ELISA; this was used as 100% value. Sera from 109 chamois from regions without IKC history for more than 10 yr showed ELISA titers around 10% ($\bar{x}$ = 7.3%, SD = 7.8%). The resulting cut-off value for a negative result on the ELISA was set at $\bar{x}$ + 3 SD = 31% (Fig. 5). Two individuals (1.8%) had ELISA titers that were above the cut-off value, but immunoblot analysis (data not shown) revealed no serum proteins known to be specific for *M. conjunctivae* as reported in Degiorgis et al. (2000b). To validate the ELISA we also analyzed 53 sera of chamois showing IKC signs collected in six regions of the central Alps (Aosta [Italy]; Berne, Fribourg, Glarus, Grisons, and Schwyz [Switzerland]). These sera clearly showed elevated ELISA titers (Fig. 5). Percentage of chamois showing IKC signs with positive sera was 60%, compared to 67% for domestic sheep (n = 84, data not shown). This difference was not statistically significant (Chi square test, $P > 0.05$).

Overall, seroprevalence in chamois in Grisons was 5% (n = 481). We analyzed 16 category A subpopulations (n = 220 samples, $\bar{x}$ = 13.8 samples per subpopulation, SD = 8.1) and 15 category B subpopulations (n = 152 samples, $\bar{x}$ = 10.1

TABLE 1. Identification of *Mycoplasma conjunctivae* from conjunctival swabs by culture or nested PCR in affected alpine chamois (*Rupicapra rupicapra rupicapra*) during natural infectious keratoconjunctivitis outbreaks from 1994 to 2000 in Grisons, Switzerland.

Date	Locality	Number of samples analyzed[a]	Number of positive samples
6/22/1994	Pitasch	1	0
8/9/1994	Nufenen	2	1
9/1/1994	Safien	1	0
12/6–16/1994	Sedrun	9	7
6/19/1995	Pigniu	2	1
7/12/1995	Trin	1	0
7/20/1995	Breil/Brigels	3	2
7/24/1995	Waltensburg	1	0
8/22/1995	Flims	2	1
9/8/1995	Zillis	2	1
8/21/1996	Vals	2	0
10/2/1996	Cinuos-chel	1	0
9/13/2000	Poschiavo	1	1
Total 1994 to 2000	Grisons	28	14

[a] From 1994–96: Swabs were cultured for *Mycoplasma* (method: Bannerman and Nicolet, 1971); in 2000, nested PCR (Giacometti et al., 1999) was used to detect *M. conjunctivae*.

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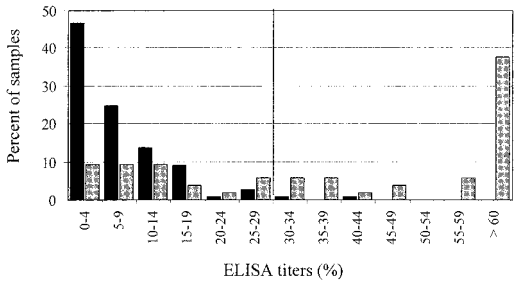


FIGURE 5. Distribution of ELISA results from alpine chamois originating from subpopulations free of infectious keratoconjunctivitis (IKC) and subpopulations with known IKC infections. The bars represent the percent of chamois corresponding to the range of ELISA indicated on the x-axis. Results are given in percentages of a standard position serum which was designated 100%. Black bars represent chamois from subpopulations known to be without IKC for > 10 yr (n = 109). Grey bars represent chamois with signs of IKC from six alpine regions (n = 53). The dark vertical line represents the cut-off value for negative.

samples per subpopulation, SD = 5.3). Seroprevalence in chamois of category B was low (3.3%) compared to seroprevalence in category A (7.7%).

DISCUSSION

In Grisons we commonly identified *M. conjunctivae* from the eyes of alpine chamois showing clinical signs of IKC. *Mycoplasma conjunctivae* has repeatedly been detected in chamois affected with IKC in several alpine regions (Nicolet and Freundt, 1975; Giacometti et al., 1999; Grattarola et al., 1999; Degiorgis et al., 2000a), and this agent is regarded as the major single cause of IKC in domestic and wild Caprinae species (Giacometti et al., 1998; Baker et al., 2001). Using mycoplasmal culture, identification of *M. conjunctivae* succeeded in 50% of the cases. Reasons accounting for the modest recovery rate of *M. conjunctivae* include decrease of mycoplasmal viability due to the storage of samples prior to analysis (Jones et al., 1976) and bacterial contamination of eye swabs (Nicolet and Freundt, 1975). This may explain the negative result in six localities despite presence of clinical IKC signs in sampled chamois. In these cases however, only one to two eye samples were cultured, and some results may have been falsely negative.

In chamois in Grisons, IKC was first described in 1950 (Ratti, 1967). Since then, an increasing number of subpopulations have been affected by the disease, and by 1999, three quarters of the chamois subpopulation in Grisons have experienced IKC outbreaks. Infectious keratoconjunctivitis outbreaks were reported to spread with a mean speed of 15 km/yr (Degiorgis et al., 2000a). Assuming an unhindered spread of IKC in the chamois population, all regions within Grisons would have been exposed within 10 yr. However, spread of IKC was disjointed during 1950-99 suggesting various sources of infection.

Mortality associated with IKC was most common in juveniles. This is similar to earlier observations (Ratti, 1967; Catusse, 1982; Gauthier, 1991; Degiorgis et al. 2000a). However, in contrast to other reports, the number of adult males that died of the consequences of IKC was nearly as high as the number of adult females. In 1990, changes in hunting for chamois were implemented in Grisons (Ratti and Jenny, 2000). These included guidelines for relative protection of adult males. The numbers of adult females compared to adult males that died of the consequences of IKC may therfore reflect a balanced percentage of these sex/age-classes within the chamois population. Compared to mortality observed in sarcoptic mange outbreaks in the eastern Italian Alps (Rossi et al., 1995), the impact of IKC on the chamois subpopulations in Grisons was generally not severe. In *M. conjunctivae* outbreaks mortality is usually low (<5%) and spontaneous recovery is the most common outcome (Gauthier, 1991). In some situations, however, mortality may reach 30% (Degiorgis et al., 2000a). In Grisons we observed particularly high mortality in 1995, when more than 100 chamois died of the consequences of IKC. The question is still open whether differences in mortality dur-

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ing IKC outbreaks are due to differences in virulence of distinct *M. conjunctivae* strains, due to a particular predisposition of hosts (e.g. genotype, health condition, overcrowding), due to secondary infections, or due to environmental predisposing factors (e.g. UV-irradiation; Nicolet, 1985).

In some IKC outbreaks in chamois, intraspecific transmission of *M. conjunctivae* occurs throughout the year (Degiorgis et al., 2000a). However, IKC losses in Grisons in 1992-99 were more frequent during summer and fall. This coincides with the presence of domestic sheep grazing on summer pastures. In Switzerland, mycoplasmal IKC is a very common ocular disease in domestic sheep (Nicolet et al., 1974; Janovsky et al. 2001). Close encounters between individuals of domestic and wild Caprinae are not uncommon in the Alps (Degiorgis, 1998), and flies are possible vectors for interspecific transmission of *M. conjunctivae* (Degiorgis et al., 1999). Therefore, *M. conjunctivae* may occasionally be transmitted from infected sheep and start IKC outbreaks in chamois. Use of molecular methods could characterize *M. conjunctivae* strains involved in simultaneous sheep and chamois outbreaks and verify the hypothesis.

To identify specific *M. conjunctivae* antibodies in sera of chamois we developed and tested an indirect ELISA. Infection of chamois with *M. conjunctivae* induced a strong humoral response which could be detected by immunoblot analysis through the presence of seroreactive proteins of molecular masses of 175, 83, 69, 60, 50, 42, 36, and 33 kDa (Grattarola et al., 1999; Degiorgis et al., 2000b). Extraction of membrane proteins of *M. conjunctivae* using the Tween 20 method (Johansson and Hjerten, 1974) resulted in a protein fraction antigenetically specific for *M. conjunctivae* as shown in sheep sera (Belloy et al., 2001). To test chamois sera we used anti-sheep-anti-goat-IgG monoclonal antibodies instead of anti-ruminant-IgG which were used to test sheep sera (Belloy et al., 2001). The method was validated analyzing a large number of chamois from subpopulations known to be free of IKC for more than 10 yr. Only two animals had a false positive ELISA result. In a large number of chamois with IKC signs, a significant proportion of the animals (60%) had ELISA titers. Animals with ELISA titers below the cut-off value were considered serologically negative and they represented animals at an early stage of the infection. In sheep, specific antibodies appeared 2-4 wk after experimental infection with *M. conjunctivae* (Degiorgis et al., 2000b), whereas clinical signs became visible with a delay of only a few days (Jones et al., 1976). We therefore considered the ELISA a specific and sensitive tool for detection of *M. conjunctivae* antibodies in the sera of chamois and used it to perform a screening of IKC in chamois subpopulations in Grisons.

Overall, seroprevalence in chamois (5%) was low compared to domestic sheep in Switzerland (53%; Janovsky et al., 2001). Seroprevalence also was low in subpopulations with ongoing IKC outbreaks (8%). In chamois, IKC outbreaks spread within herds, and the disease may not occur in neighboring herds despite the absence of natural barriers (Degiorgis et al., 2000a). Therefore, *M. conjunctivae* infection may only affect portions of subpopulations; this may explain the low seroprevalence at the subpopulation level. Seroprevalence was even lower in subpopulations with recent IKC outbreaks (3%). We deduce that the *M. conjunctivae* infection, a disease which is highly contagious within herds, does not persist in alpine chamois populations. This may be due to the limited contact of chamois from different herds and to the fact that persistence of infection in individual animals does not exceed 3-6 mo in sheep (Janovsky et al., 2001).

In conclusion, we consider IKC of alpine chamois a disease sometimes associated with a significant mortality. The *M. conjunctivae* infection is not maintained in the chamois population of the eastern

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Swiss Alps, and transmission of the agent from sheep living in proximity during summer may be the source of epidemics in chamois. Managers should therefore consider measures such as vaccination of sheep or limiting interspecific transmission of *M. conjunctivae* by decreasing contact between the species.

ACKNOWLEDGEMENTS

The authors are grateful to P. Ratti who initiated and supported the study in Grisons. Special thanks go to the state game-keepers in Grisons for their collaborative attitude and for collecting data and samples in the field. Many thanks to the hunters in Grisons for collecting blood samples of chamois in 1999. The authors also acknowledge state game-keepers in Aosta, Bern, Friborg, Glarus, and Schwyz for collecting sera of chamois with symptoms of IKC, D. Mayer for providing some of the ocular swabs of chamois, M. Krawinkler for expert help with isolation, identification and cultivation of mycoplasmas, as well as Dr. Bommeli AG, Liebefeld (Berne, Switzerland), for the ELISA plates. This study was supported by the Fund for Research of Infectious Keratoconjunctivitis, Chur, by Swiss Federal Office for Environment, Forests and Landscape, Berne, and by the Research Fund of the Institute for Veterinary Bacteriology, Berne. This work is part of the COST Action 826 on Ruminant Mycoplasmoses.

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Received for publication 20 July 2001.

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