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IMMUNE RESPONSES TO *MYCOPLASMA CONJUNCTIVAE* IN ALPINE IBEX, ALPINE CHAMOIS, AND DOMESTIC SHEEP IN SWITZERLAND

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ABSTRACT: The humoral immune response of three alpine chamois (*Rupicapra rupicapra rupicapra*), two alpine ibex (*Capra ibex ibex*) and three domestic sheep naturally affected with infectious keratoconjunctivitis (IKC), and four ibex and two sheep experimentally infected with *Mycoplasma conjunctivae* was analysed. In addition, the local immune response to *M. conjunctivae* was analysed using conjunctival washes from chamois and sheep. Immunoblot analysis of sera using whole cell antigens of *M. conjunctivae* revealed the major immunogenic proteins which had molecular masses of 175, 83, 68, 60, 50, 42, 36, and 33 kDa. Major antigens were found at 83, 68, 60, and 42 kDa in both sera and conjunctival washes from naturally infected animals of all three Caprinae species. In experimentally infected animals, antibodies to the 68 and 60 kDa antigens were dominant. Naturally infected animals showed much stronger immune reactions than those experimentally infected, and specific antibodies appeared 2 to 4 wk after experimental infection. To evaluate possible cross-reactions, whole cell antigen of *M. conjunctivae* was analysed by immunoblot against hyperimmune sera of closely related *Mycoplasma* spp. Antibodies to the 175, 73, 68, 60, and 33 kDa antigens appeared to be specific to *M. conjunctivae*. Cross-reactions mainly with 83, 50, and 42 kDa antigens were detected, in particular with *M. ovipneumoniae* and *M. bovoculi* hyperimmune sera, but also with antisera against *M. capricolum capricolum* and *M. putrefaciens*.

Key words: *Capra ibex ibex*, chamois, domestic sheep, ibex, immune response, infectious keratoconjunctivitis, *Mycoplasma conjunctivae*, *Rupicapra rupicapra rupicapra*.

INTRODUCTION

Infectious keratoconjunctivitis (IKC) commonly affects both domestic ruminants (Wilcox, 1968; Jones, 1991) and wild Caprinae species (Daniel and Christie, 1963; Gauthier, 1991). *Mycoplasma conjunctivae* has often been isolated from the eyes of affected domestic sheep (Nicolet et al., 1974; Jones, 1991), domestic goats (Trotter et al., 1977), chamois (*Rupicapra rupicapra rupicapra*) (Nicolet and Freundt, 1975; Giacometti et al., 1997), and ibex (*Capra ibex ibex*) (Mayer et al., 1997). Experimental infections have demonstrated the pathogenicity of this organism in small domestic ruminants (Jones et al., 1976; Trotter et al., 1977) and in alpine ibex (Giacometti et al., 1998). Phylogenetically, *M. conjunctivae* belongs to the *Mycoplasma neurolyticum* cluster of the hominis group, and this agent is closely related

to *M. bovoculi* and *M. ovipneumoniae* (Pettersson et al., 1996).

Mycoplasma conjunctivae infections are endemic in sheep in the eastern Swiss Alps (Giacometti et al., 1997), and transmission of IKC among sheep and wild Caprinae is suggested on alpine meadows (Daniel and Christie, 1963; Nicolet and Freundt, 1975). No vaccine against infections with *M. conjunctivae* exists. Although antibody titers to *M. conjunctivae* have been detected in the serum and lachrymal fluid of experimentally infected goats (Trotter et al., 1977) by use of the metabolic inhibition test as described by Taylor-Robinson et al. (1966), only very limited information about antibody responses of sheep, goats, and wild ruminants to infections with *M. conjunctivae* is available at present. The dynamics of the immune response has not been described, and neither the *M. conjunctivae* antigens involved in the response

TABLE 1. Animals sampled for detection of *M. conjunctivae* antibodies in 1994–1998.

ID	Sex and age	Year	Municipality and canton	Infection	Clinical signs	<i>M. conjunctivae</i>		
						By culture	By PCR	Serum Ab
<i>chamois</i>								
c-3	Female, 5 yr	1998	Boltigen, BE	Nat. Inf.	Severe	ND ^a	+	+
c-4	Female, 11 yr	1998	Boltigen, BE	Nat. Inf.	Severe	ND	+	+
c-8	Female, 12 yr	1998	Plaffeien, FR	Nat. Inf.	Severe	ND	+	+
c-14	Female, 7 yr	1998	Alterswil, FR	Neg. Contr.	Healthy	ND	–	–
<i>ibex</i>								
i-6	Male, 4 yr	1994	Matt, GL	Nat. Inf.	Mild	+	ND	+
i-11	Male, 10 yr	1994	Flims, GR	Nat. Inf.	Mild	+	ND	+
i-20	Male, 2 yr	1994	Sta. Maria, GR	Neg. Contr.	Healthy	–	ND	–
i-71	Male, 1 yr	1996	Utzenstorf, BE	Exp. Inf.	Mild	+	+	+
i-73	Female, 1 yr	1996	Utzenstorf, BE	Exp. Inf.	Mild	+	+	+
i-74	Male, 1 yr	1996	Utzenstorf, BE	Exp. Inf.	Mild	+	+	+
i-75	Male, 1 yr	1996	Utzenstorf, BE	Exp. Inf.	Mild	+	+	+
<i>sheep</i>								
s-2	Female, adult	1998	Gysenstein, BE	Nat. Inf.	Mild	ND	+	+
s-3	Female, adult	1998	Gysenstein, BE	Nat. Inf.	Mild	ND	+	+
s-6	Male, subadult	1998	Kemptthal, ZH	Nat. Inf.	Mild	ND	+	+
s-G	Male, 1 yr	1996	Berne, BE	Exp. Inf.	Healthy	–	–	+
s-M	Male, 1 yr	1996	Berne, BE	Exp. Inf.	Healthy	–	–	+
s-K	Male, 1 yr	1996	Berne, BE	Exp. Inf.	Healthy	–	–	+
s-H	Female, adult	1998	Basel, BS	Neg. Contr.	Healthy	ND	–	–

^a ND, not done.

nor cross-reactions with closely related mycoplasmas have been studied. In the present study, the conjunctival and humoral immune responses against antigens of *M. conjunctivae* in experimentally and naturally infected sheep, ibex, and chamois are described. Furthermore, serologic responses to various antigens of *M. conjunctivae* and to antigens of closely related *Mycoplasma* spp. are compared to evaluate possible cross-reactions.

MATERIALS AND METHODS

Alpine chamois, alpine ibex, and domestic sheep were sampled in different regions of Switzerland (45°49' to 47°49'N, 5°58' to 10°29'E) from 1994 to 1998 (Table 1). Sera and conjunctival swabs were collected from three free-ranging chamois with severe IKC shot by state game-keepers as well as from two free-ranging ibex and three domestic White Mountain breed sheep with mild IKC. Ibex were immobilized using 125–250 mg xylazine hydrochloride + 100–200 mg ketamine hydrochloride per animal which was remote injected using a dart projector.

Four captive ibexes and three domestic Heidschnucke breed sheep were purchased from herds free of clinical IKC for at least 10 yr. They were placed in pens away from other ruminants and fed daily with hay and mixed grain. Experimental animals were tested and declared free from infections caused by *M. conjunctivae* and *Chlamydia* spp. *Mycoplasma conjunctivae* was detected as described below. A direct fluorescent antibody technique was used for detection of chlamydial antigens (including *C. psittaci*) in ocular smears (Chlamydia Direct IF, BioMérieux, Marcy-l'Etoile, France). Three ibex and three domestic sheep were experimentally infected with field strain G 140 of *M. conjunctivae* isolated in 1995 from a naturally infected sheep in Brigels (Grisons, Switzerland) (Giacometti et al., 1998), and one ibex served as contact animal. Sera were collected before inoculation and on days 29 and 38 postinfection (PI) in ibex and 59 days PI in sheep respectively. Conjunctival washes were collected from sheep after they recovered from conjunctivitis.

Sera from a chamois, an ibex and a domestic Heidschnucke breed sheep from herds where IKC had not been reported for at least 10 yr served as negative controls.

Blood was collected from the jugular vein of chemically immobilized ibex and of sheep. From chamois that were killed, blood was collected either from the thorax cage or from the external iliac vein within 15 min after shooting. The conjunctival sacs were sampled with sterile cotton swabs for bacteriologic examination. The swabs were either dipped into Transwab^R-transport medium (Medical Wire and Equipment Co. Ltd., Corsham, UK) for culture of *M. conjunctivae*, or were kept without transport medium for detection by PCR (Giacometti et al., 1999). For conjunctival washes, the eyes of chamois and sheep were rinsed with 2 ml of 0.9% NaCl-solution. The rinsing solution was collected with a plastic pipette. Samples were processed directly or stored at -20 C until used.

Mycoplasma conjunctivae was detected either by culture in a 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) or with a specific polymerase chain reaction (PCR) assay directly from samples of the conjunctival sacs (Giacometti et al., 1999). Hyperimmune sera from rabbits infected with the respective type strains of *M. conjunctivae*, *M. bovoculi*, *M. ovipneumoniae*, *M. capricolum capricolum*, *M. agalactiae*, *M. putrefaciens*, *M. mycoides* subsp. *mycoides* LC, *M. capricolum capripneumoniae*, *M. mycoides capri* and *M. alkalescens* were prepared according to the method of Bannerman and Nicolet (1971).

Mycoplasma conjunctivae type strain HRC/581^T (ATCC 25834) (Barile et al., 1972) was used as antigen on western blots. It was cultured in a modified standard *Mycoplasma* PPLO broth medium (as described above) at 37 C until late logarithmic phase was reached. Cells were harvested by centrifugation at 12,000 × *g* for 15 min, washed three times in TES buffer (10 mM Tris HCl, 1 mM EDTA, 0.85% NaCl, pH 7.5), and then concentrated by resuspending in TES buffer corresponding to 1% of the original culture volume. Whole cell antigen *M. conjunctivae* strain HRC/581^T was prepared for separation by SDS polyacrylamide gel electrophoresis (SDS-PAGE) by mixing the concentrated cells with one volume of SDS sample buffer (0.06 M Tris-HCl, pH 6.8, 2% SDS, 10% Glycerol, 2% β-mercaptoethanol, 0.025% Bromophenol blue) and boiling for 5 min. The antigens were separated by SDS-PAGE 5–15% gradient gels as described above, and blotted onto nitro-cellulose membrane with pore size of 0.2 μm (BioRad, Hercules, California, USA). The membrane was then blocked with milk buffer (100 mM Tris HCl,

pH 7.5, 150 mM NaCl, 0.5% Tween 20, 1% skimmed dry milk) for 1 hr at room temperature (RT), dried, and cut into strips. The strips were incubated for 1 hr at RT with the serum samples diluted 1:50 in milk buffer or with conjunctival washes diluted 1:1 in milk buffer, and washed three times with TBS (100 mM Tris HCl, pH 7.5, 150 mM NaCl) for 5 min. The strips were then incubated 1 hr at RT with alkaline phosphatase labelled conjugates, which were diluted in milk buffer. The conjugates used were i) monoclonal antibody (Mab) to goat/sheep-IgG (Sigma Chemicals, Saint Louis, Missouri, USA) diluted 1:2,000, or ii) Protein G (ZYMED, San Francisco, California, USA) diluted 1:1,000, or iii) polyclonal anti-rabbit IgG antibodies (Kirkegaard and Perry Gaithersburg, Maryland, USA) diluted 1:2,000. All strips were washed three times with TBS buffer. Colour reaction was initiated by 0.3 mg/ml nitroblue tetrazolium (Boehringer Mannheim, Mannheim, Germany) with 0.15 mg 5-bromo-4chloro-3indolyl phosphate (BCIP) (Boehringer Mannheim) in alkaline buffer, and stopped with distilled water as described in Ausubel et al. (1990).

RESULTS

Eye swabs taken from three free-ranging chamois affected with IKC all contained *M. conjunctivae* as confirmed by PCR (Table 1). Immunoblots of sera from these animals are shown in Figure 1. Immunoblots showed stronger signals with Mab to goat/sheep-IgG than with protein G. Hence, Mab to goat/sheep-IgG was used throughout this work. The sera of these three animals showed strong reactions to about 25 different protein bands on the immunoblot containing *M. conjunctivae* strain HRC/581^T. With serum from chamois and ibex, the same patterns were obtained when strain G 140 was used (results not shown). Strongly marked bands were found at 175, 83, 73, 68, 60, 50, 42, 36, and 33 kDa. Immunoblots made with conjunctival washes from the same animals had fewer and less intensive bands. Major antigens present in both serum and conjunctival washes from all animals were found at 83, 68, 60, and 42 kDa. Interestingly, the conjunctival washes showed no, or in one case only, a very weak reaction to the 33 kDa antigen, and

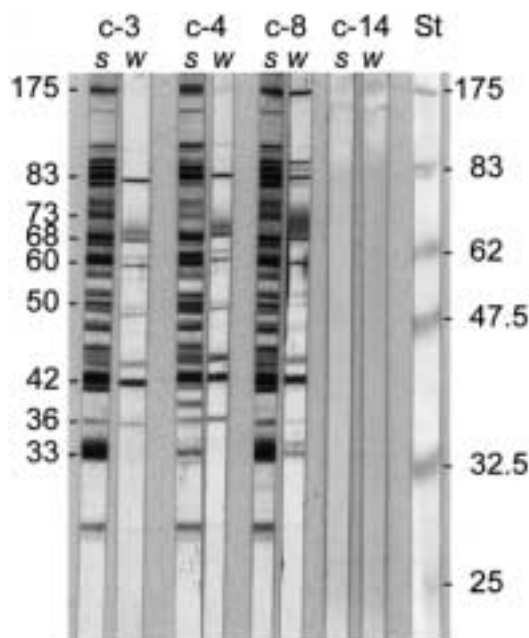


FIGURE 1. Immunoblot of sera (s) and conjunctival washes (w) to whole cell antigens of *Mycoplasma conjunctivae* samples from three chamois with severe keratoconjunctivitis (c-3, c-4, and c-8) and one healthy chamois (c-14). The molecular size markers are given in kDa on the right side. Molecular masses of the immunodominant *M. conjunctivae* antigens are indicated in kDa on the left side.

the reaction to the 175 kDa antigen was clearly present in one animal (c-8), absent in one animal (c-3), and weak in the other animal (c-4, Fig. 1). Serum and conjunctival washes from a healthy free-ranging control animal from the same age group, which was free of *M. conjunctivae*, showed no reactions at all on the immunoblots.

The sera of two free-ranging ibex from which *M. conjunctivae* had been isolated by culture in eye swabs were examined by immunoblot. The sera of these animals (Fig. 2) produced a similar pattern as sera from infected chamois. In contrast to the sera of chamois, the 73 and 33 kDa antigens were hardly visible in sera of the ibex. Furthermore, ibex showed a strong reaction with a 36 kDa antigen which was faint discrete in sera from chamois. Serum from the healthy ibex showed no reactions.

In experimentally inoculated ibex (i-73,

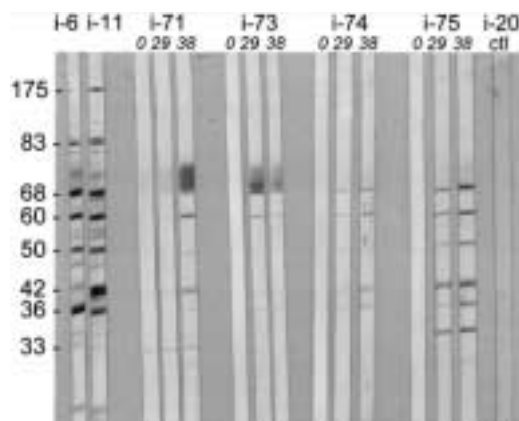


FIGURE 2. Immunoblot of sera (s) and conjunctival washes (w) to whole cell antigens of *Mycoplasma conjunctivae* samples from two ibex with conjunctivitis (I-6, I-11), four experimentally infected ibexes (I-73, I-74, and I-75 were inoculated on day 0, I-71 was exposed to infection by cohabitation) and one healthy ibex (I-20). Molecular masses of the immunodominant *M. conjunctivae* antigens are indicated in kDa on the left side.

i-74, i-75), mild clinical signs began within two days in inoculated ibexes and 20 days later in the contact animal (i-71) (Fig. 2). Sera of the three inoculated ibex produced only weak reactions with few bands at day 29 PI. On day 38 PI, the reactions with the antigens of 68, 60, 50, 42, 36, and 33 kDa became more apparent particularly in one animal (i-75), while only the 68 and 60 kDa antigens were significant in the other two ibex. Clear reactions in the contact animal became apparent on day 38 PI, suggesting a delay of the onset of the infection.

The serological immune responses of three sheep naturally infected with *M. conjunctivae* showed clear response to the 175, 83, 68, 60, and 42 kDa antigens (Fig. 3), with a pattern similar to that of the naturally infected ibex (Fig. 2). However, the 36 kDa antigen, strong in naturally infected ibex, was significant only in one sheep. The immunoblots of conjunctival washes from sheep were similar but weaker than those of sera. Sera taken from experimentally infected sheep 59 days PI, when animals had recovered from conjunctivitis

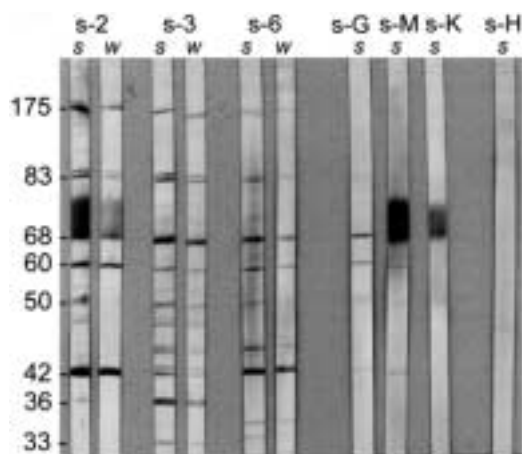


FIGURE 3. Immunoblot of sera (s) and conjunctival washes (w) to whole cell antigens of *Mycoplasma conjunctivae* samples from three sheep affected with conjunctivitis (s-G, s-M, s-M; experimental infection) and one healthy sheep (s-H). Molecular masses of the immunodominant *M. conjunctivae* antigens are indicated in kDa on the left side.

and *M. conjunctivae* infection (Table 1), only showed weak reactions to the 68 and the 60 kDa antigens and faint reactions to the 42 kDa antigens. Serum of the negative control animal showed no reactions.

Results of testing for serological cross-reactions among the ruminant mycoplasmas are shown in Figure 4. Antibodies to the 175, 73, 68, 60, and 33 kDa antigens appeared to be specific to *M. conjunctivae*. Cross-reactions to the 83, 50 and 42 kDa antigens were observed in particular with anti-*M. bovoculi* and anti-*M. ovipneumoniae* hyperimmune serum, but also with antisera against other mycoplasmas. In addition, 62 and 44 kDa antigens, which do not correspond to those of the major antigens of *M. conjunctivae* detected in naturally infected chamois, strongly reacted with antibodies against *M. ovipneumoniae*, *M. capricolum* subsp. *capricolum*, *M. putrefaciens*, *M. mycoides mycoides* LC and *M. capricolum capripneumoniae*.

DISCUSSION

Presence of antibodies against *M. conjunctivae* and specificity of this immune response were analyzed by immunoblot,

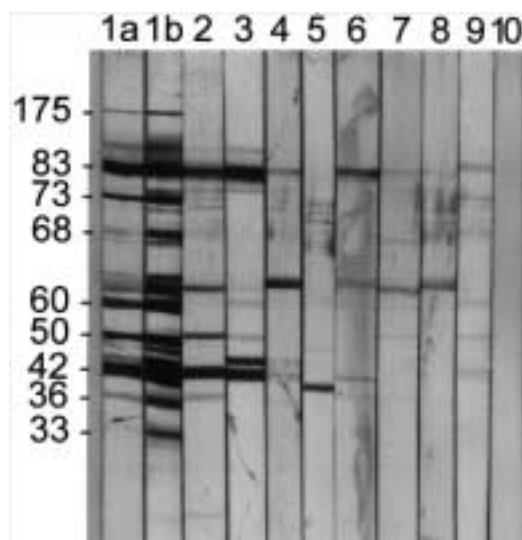


FIGURE 4. Immunoblot of whole cell antigens of *Mycoplasma conjunctivae* strain HRC/581^T. 1a is with serum from a chamois naturally infected with *M. conjunctivae*. 1b is with hyperimmune rabbit serum against *M. conjunctivae*. 2–10 is hyperimmune rabbit serum against type strains of *M. bovoculi* (2), *M. ovipneumoniae* (3), *M. capricolum capricolum* (4), *M. agalactiae* (5), *M. putrefaciens* (6), *M. mycoides mycoides* LC (7), *M. capricolum capripneumoniae* (8), *M. mycoides capri* (9), and *M. alkalescens* (10). Molecular masses of immunodominant antigens against *M. conjunctivae* are given on the left side.

using sera and conjunctival washes of naturally and experimentally infected chamois, ibexes and sheep. Since protein G showed only low affinity to IgG of chamois and ibex, we used Mab to goat/sheep-IgG which gave strong reactions with sheep, chamois, and ibex sera. Immunoblot analysis allowed us to identify the major specific immunogenic proteins of *M. conjunctivae* of 175, 83, 68, 60, 50, 42, 36, and 33 kDa. Of these antigens, only the 83, 68, 60, and 42 kDa reacted also with antibodies in conjunctival washes of all three infected Caprinae species.

Immunoblots reactions in naturally infected ibex and sheep were similar, but in naturally infected chamois more bands were visible on immunoblots and only weak reactions were apparent against the 36 kDa antigen. Clinical signs, which were more severe in the chamois analyzed, or

differences in virulence of *M. conjunctivae*-strains might have influenced immune responses. Trotter et al. (1977) did not observe an increase of serum antibodies in goats experimentally inoculated with *M. conjunctivae*, and suggested that IKC was a localized infection with little, if any, systemic involvement. In the present study, however, the humoral immune response in chamois was stronger than the local one. In sheep, no obvious differences were observed in reactions of sera and conjunctival washes, but the strength of the local immune response was similar to that of chamois. This suggests that the systemic immune response correlated with the severity of the disease.

The humoral immune responses were weaker in experimentally infected animals as compared to those naturally infected; reactions to the 175 and 83 kDa antigens were present only in naturally infected animals. It is possible that cloning and passages of the *M. conjunctivae*-strain used for experimental infection in culture may have caused a loss of virulence. Furthermore, the sheep-strain of *M. conjunctivae* used for experimental infection of ibex might be less virulent than strains naturally occurring in ibex, and therefore these results might not be fully comparable to reactions in naturally infected ibexes.

Reactions in experimentally infected ibex allow evaluation of the dynamic processes of immune response to infections with *M. conjunctivae*. On day 29 PI, only a weak reaction was visible in the three inoculated animals, and essentially no reaction in the contact animal; on day 38 PI, all four animals showed clear immunologic reactions. Thus, it appears that 2 to 4 wk are required for formation of specific antibodies after infection with *M. conjunctivae*. Trotter et al. (1977) and Costa (1986) observed that acquired immunity was insufficient to resist experimental infection, and further investigations are required to assess persistence of specific antibodies.

Mycoplasma conjunctivae shared common antigens with several of the related

mycoplasmas, in particular with *M. bovoculi* and *M. ovipneumoniae* which are phylogenetically most closely related to *M. conjunctivae* (Pettersson et al., 1996). However, we conclude that 175, 73, 68, 60, and 33 kDa antigens are specific for *M. conjunctivae*. However, cross-reactions to *M. ovipneumoniae* antigens must be taken in account in development of serologic tests, because *M. ovipneumoniae* infections are widespread, especially in sheep.

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