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Specificity of *Mycoplasma conjunctivae* strains for alpine chamois *Rupicapra r. rupicapra*

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Infectious keratoconjunctivitis (IKC) is worldwide known as a common infection by *Mycoplasma conjunctivae* affecting the eyes of domestic sheep and goats where it generally leads to moderate symptoms known as 'pink eye'. IKC also occurs in severe outbreaks in free-ranging alpine chamois *Rupicapra r. rupicapra* and alpine ibex *Capra i. ibex*. It is characterised by grave clinical symptoms resulting in blindness and perforations of the cornea of the affected animals, and finally leads to their death. Transmission of *M. conjunctivae* from domestic sheep to wild Caprinae has been demonstrated by molecular epidemiological studies of the infectious agent. The relatively high prevalence of *M. conjunctivae* in sheep populations that share pastures in the Alps with wild Caprinae, in which outbreaks occur infrequently but with high virulence, raised an important question concerning the role of host-specificity of various strains of *M. conjunctivae*. Investigations on genetic variations of *M. conjunctivae* strains allowed subtyping of *M. conjunctivae* isolates using a molecular genetic method that can be directly applied to samples taken by eye swabs. Our studies revealed that sheep with IKC are frequently infected simultaneously with up to four different strains of *M. conjunctivae*. In contrast, affected chamois only showed infections with a single strain. Furthermore, among the different strains of *M. conjunctivae* that have been determined until now, 36 strains are repeatedly found in sheep, while only a few different strains, all belonging to a phylogenetically related cluster, were found in chamois with IKC. These few strains were responsible for all severe outbreaks of IKC in chamois during the last four years in Switzerland, Austria and Italy. Our studies indicate that a particular cluster of strains of *M. conjunctivae* has a host predilection for chamois where they cause severe infections.

Key words: infectious keratoconjunctivitis, inter-species transmission, molecular epidemiology, wild Caprinae

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Infectious keratoconjunctivitis (IKC) caused by the bacterium *Mycoplasma conjunctivae* is a contagious ocular infection which is common in domestic sheep and goats (Jones 1991, Motha et al. 2003). In the European Alps outbreaks of IKC also occur in free-ranging alpine chamois *Rupicapra r. rupicapra* and alpine ibex *Capra i. ibex* (Giacometti et al. 1998, Grattarola et al. 1999, Giacometti et al. 2000, 2002a; Giacometti et al. 2002b). IKC in domestic sheep and goat, known as 'pink eye', is manifested by hyperaemic conjunctiva and congestion of conjunctival vessels associated with serous mucous effusions. Untreated cases may progress to mucopurulent conjunctivitis and corneal ulceration that results in opaque corneas and transient blindness. Outbreaks of IKC in sheep with severe symptoms might have occurred when infected animals were introduced to immunologically naive herds (Naglic et al. 2000). However, the impact of IKC on sheep health is generally relatively low. In adult sheep symptoms of *M. conjunctivae* infections are moderate or even absent. In lambs *M. conjunctivae* seems to be weakly pathogenic. In contrast, the consequences of *M. conjunctivae* infections in wildlife are generally fatal. IKC in wild Caprinae is often manifested by perforation of the cornea leading to irreversible blindness. Blind chamois and ibex face particularly insidious circumstances in steep and rocky areas, where affected animals may fall from cliffs and die. Another consequence of blindness in wild Caprinae is starvation. In certain situations IKC leads to a mortality rate of up to 30% and thus has a marked population impact (Giacometti et al. 2002a).

In a stratified random sample analysis of 674 sheep of 123 herds from 25 out of 26 Swiss cantons the sero-prevalence for *M. conjunctivae* in sheep in Switzerland was determined for adult animals to be 53% at the individual level and 89% at herd level. Sero-prevalence in 2-6 month old lambs was 50.5%, indicating that IKC is endemic and self-maintained in the domestic sheep population (Janovsky et al. 2001). In contrast, sero-prevalence is relatively low

in alpine chamois. In subpopulations with previous IKC outbreaks sero-prevalence has been shown to be low (8%) and in subpopulations with recent IKC outbreaks sero-prevalence was even lower (3%). Most cases of IKC in alpine chamois occur in summer and autumn when sheep are grazing at alpine pastures (Giacometti et al. 2002b). These observations led to the conclusion that *M. conjunctivae* infections were not self-maintained in alpine chamois and that the infectious agent might originate from domestic sheep sharing the same alpine pastures with chamois during summer (Giacometti et al. 2002b).

Molecular epidemiological studies have shown that the same strains of *M. conjunctivae* can be isolated from chamois affected by IKC and from sheep grazing on the same alpine pasture during summer (Belloy et al. 2003a). Based on these molecular results and on the observations made in the field on close contacts between chamois and sheep (Degiorgis et al. 1999, Rüttimann 2008) it appeared likely that chamois in the Alps do become infected by spill over of *M. conjunctivae* from domestic sheep sharing the same summer pastures. This occurs presumably via aerosols and insects that have eye contacts. In view of the high prevalence of *M. conjunctivae* in sheep and the relatively low incidents of IKC outbreaks that occur within the alpine chamois population, though with grievous losses, we performed a detailed molecular epidemiological study on *M. conjunctivae* found in sheep and chamois in alpine areas where IKC in chamois was observed. The molecular epidemiological method for subtyping of *M. conjunctivae* strains is based on variations found in a segment of the adhesion lipoprotein LppS of *M. conjunctivae* (Belloy et al. 2003b). This method allows the amplification by PCR of the lppS gene directly from eye swabs of animals infected by *M. conjunctivae* followed by DNA sequence analysis of the variable segment, either directly or after subcloning of the lppS gene fragments contained in the sample with multiple *M. conjunctivae* strains.

Material and methods

Clinical samples

Clinical samples from chamois were obtained from animals that were shot by gamekeepers after observation of visible signs of conjunctivitis and keratitis or signs of blindness as indicated by abnormal behaviour such as stumbling, circling, uncertain gait or inability to climb. A few samples were from chamois that were found dead with signs of conjunctivitis or keratitis. Samples from sheep were selected from individuals with pink eye showing swollen eyelids and seromucous ocular effusion. These animals were chosen from areas where IKC was diagnosed in populations of wild Caprinae. Our sampling period covered the four years between 2000 and 2004, when several severe outbreaks of IKC occurred in chamois in the Alps. Conjunctival swabs were taken from behind the third eyelid using standard cotton swabs, which were subsequently kept in tubes without transport medium and stored at -20°C . In total, we analysed 110 eye swabs samples, 74 from sheep and 36 from chamois, collected over the 4-year period (2000-2004) from alpine areas in Switzerland, Austria and Italy, where IKC was diagnosed in chamois (Fig. 1).

Microbiological analysis

After thawing the samples, the swabs were placed into Eppendorf microcentrifuge tubes containing

0.5 ml lysis buffer (100 mM Tris-HCl, pH 8.5, 0.05% Tween 20, 0.24 mg/ml Proteinase K) and mixed thoroughly for one minute at room temperature. Then the cotton swabs were removed and the buffer was incubated for 60 minutes at 60°C and then for 15 minutes at 97°C . Aliquots of $1\ \mu\text{l}$ lysate were analysed by a specific 16S rRNA gene rrs based PCR method to detect *M. conjunctivae* as described previously (Giacometti et al. 1999).

Genetic subtyping of *M. conjunctivae*

Subtyping of *M. conjunctivae* strains for molecular epidemiological analysis was performed by PCR amplification of the variable segment encoding the poly-serin domain on the third part of the lppS gene and subsequent DNA sequence analysis (Belloy et al. 2003a). PCR was carried out in a DNA thermal cycler (9800 fast thermal cycler; Perkin Elmer Cetus, Norwalk CT, USA) in $50\ \mu\text{l}$ containing $10\ \mu\text{M}$ of each primer Ser_start1 (GCT CAA GAG CAA ACT GAC C) and Ser_end (GCA GCA ACT GCT GAA AGT C) matching nucleotide (nt) positions 3927-3945 and 4746-4728 respectively of GenBank/EMBL nucleotide sequence accession number MCO318939 (Belloy et al. 2003a), 1 mM dNTPs, Taq buffer and 1.25 U Taq polymerase, 1.5 mM MgCl_2 with $1\ \mu\text{l}$ lysate as template. Cycling conditions were denaturation at 94°C for three minutes, followed by 35 cycles at 94°C for 30 seconds, 49°C for 60 seconds, 72°C for one minute followed by a final extension step at 72°C for seven minutes (Belloy et al. 2003a). PCR amplification on template DNA of *M. conjunctivae* type strain HRC/581^T resulted in a fragment of 819 bp. The sizes of PCR fragments amplified from ocular swabs of the field samples in this study were between 750 and 830 bp depending on the number of serine codons in the lppS gene of the corresponding strain. PCR products were purified with the HighPure PCR purification kit (Roche Diagnostics, Rotkreuz, Switzerland) and 20 ng PCR product were used for DNA sequence determination using the BigDye termination cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with the sequencing primers Ser_start1 and Ser_end.

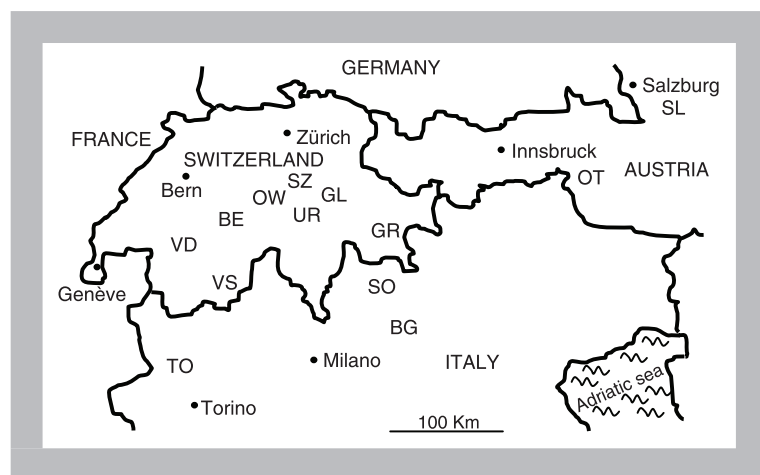


Figure 1. Area of sampling. The different countries or districts where the animals were sampled as referenced in Figure 2, are given with two-letter abbreviations: BE = canton of Bern; BG = Bergamo; GR = Grisons; OT = Ost-Tirol/Eastern Tirolia; OW = Obwalden; SL = Salzburg Land; SO = Sondrio; SZ = Schwyz; TO = Torino; UR = Uri; VD = Vaud; VS = Valais. Countries are given in capital letters. The lines represent the country borders. A few major cities are indicated.

Sequencing products were analysed on a ABI Prism 3100 genetic analyser (Applied Biosystems) and edited using the DNA sequence analysis software Sequencher (GeneCodes, Ann Arbor, MI, USA). Overlapping peaks in the chromatogram of DNA sequence determinations revealed the presence of more than one strain of *M. conjunctivae* in the same sample. For the analysis of such samples, the PCR product that was obtained by amplification with the oligonucleotide primers Ser_start1 and Ser_end was cloned on plasmid T-easy (Promega Corp., Madison, WI, USA) to differentiate the individual fragments. This was obtained by ligation of the PCR amplification product onto cloning vector T-easy and subsequent transformation of *Escherichia coli* strain XL-1blueMRF' (Stratagene, La Jolla, CA, USA) with the ligation product using standard gene cloning methods (Ausubel et al. 1999). Plasmid DNA was then extracted from the individual clones by alkaline lysis using Miniprep Kits (Qiagen AG, Basel, Switzerland). The cloned DNA segment from each of the individual clones was then sequenced as described above but using the oligonucleotide primers Turbo L (CCA GCT GGC GAA AGG GGG ATG TGC TGC) and Turbo R (CCA GGC TTT ACA CTT TAT GCT TCC GGC TCG) matching both ends flanking the cloning sites on vector T-easy. For each sample with multiple strains, 20 individual cloned lppS segments on plasmids were sequenced to ensure recovery of the major strains present.

The phylogenetic analysis of the sequence data was performed using the Neighbour-Joining method of the software BioNumerics, version 4.0 (Applied Maths, Kortrijk, Belgium). The threshold value for definition of a strain was set to 0.6% sequence difference corresponding to 5 nt difference in the variable segment of the lppS gene. The threshold value to differentiate the clusters of strains was set to 35% sequence difference. For the phylogenetic analysis, sequence data from five previously analysed *M. conjunctivae* strains from ibex (Giacometti et al. 2000) were included in this study (Fig. 2).

Results

All eye swab samples from sheep and chamois were found to be positive for *M. conjunctivae* as revealed by the 16S rRNA gene (rrs) based PCR (Giacometti et al. 1999). In 53 out of the 74 samples from sheep, the DNA sequence of the variable part of the lppS gene could be determined directly from the PCR am-

plicons. These 53 samples represented 32 different strains of *M. conjunctivae* as defined by more than 5 nt (0.6%) differences on the variable domain of the gene lppS. Figure 2 shows a phylogenetic tree of the different *M. conjunctivae* strains used in this study. In 21 ovine samples, the DNA sequence analysis of the lppS segment revealed overlapping multiple DNA sequences that could not be determined directly, indicating the presence of more than one strain of *M. conjunctivae*. We randomly selected six of these samples and analysed them further by sub-cloning the PCR amplified lppS DNA fragments. The remaining 15 were not considered further in this study. In the samples of three sheep we were able to identify four different lppS gene sequences in each. These sequences varied significantly among each other indicating the presence of at least four different *M. conjunctivae* strains in an eye simultaneously. For instance, the sequence numbers 202, 203, 205 and 206 (see Fig. 2), which were identified in the eye of a sheep sampled in an outbreak area of IKC in the canton of Uri (UR), turned out to represent four different strains, A23, A1, A14 and C1, belonging to two distantly related clusters (see Fig. 2). In the remaining three samples that resulted in multiple overlapping lppS DNA sequences, one contained two different strains, and two contained three different strains of *M. conjunctivae*.

Analysis of the 36 samples from chamois resulted in direct determination of the DNA sequence of the variable lppS domain, indicating that all chamois were infected by single strains of *M. conjunctivae*. Among the 36 samples a total of 13 different strains A1-A13 (see Fig. 2) were identified. Among them, seven strains (A1-A7) were also found in sheep, while the remaining five strains (A8-A13) have never been reported in sheep. It has to be noted that one of the strains common to sheep and chamois, strain A1, was particularly found in epidemics of IKC in chamois in the eastern Alps of Switzerland (Grisons), where it also appeared in ibex with IKC (Tschopp et al. 2005; see Fig. 1). Strains A9 and A10 from IKC outbreaks in chamois in central Switzerland (OW) and several parts of western Switzerland (VS, VD, BE) were not detected in sheep (see Fig. 2). Strain A5 was found in chamois with severe IKC causing epidemics in Austria, northeastern Italy and eastern Swiss alpine valleys in 2000 and 2001 and was also found at the same time in sheep that were observed to graze close to the chamois. This strain was isolated already in 1994 from ibex with IKC in the eastern Alps of Switzerland where *M.*

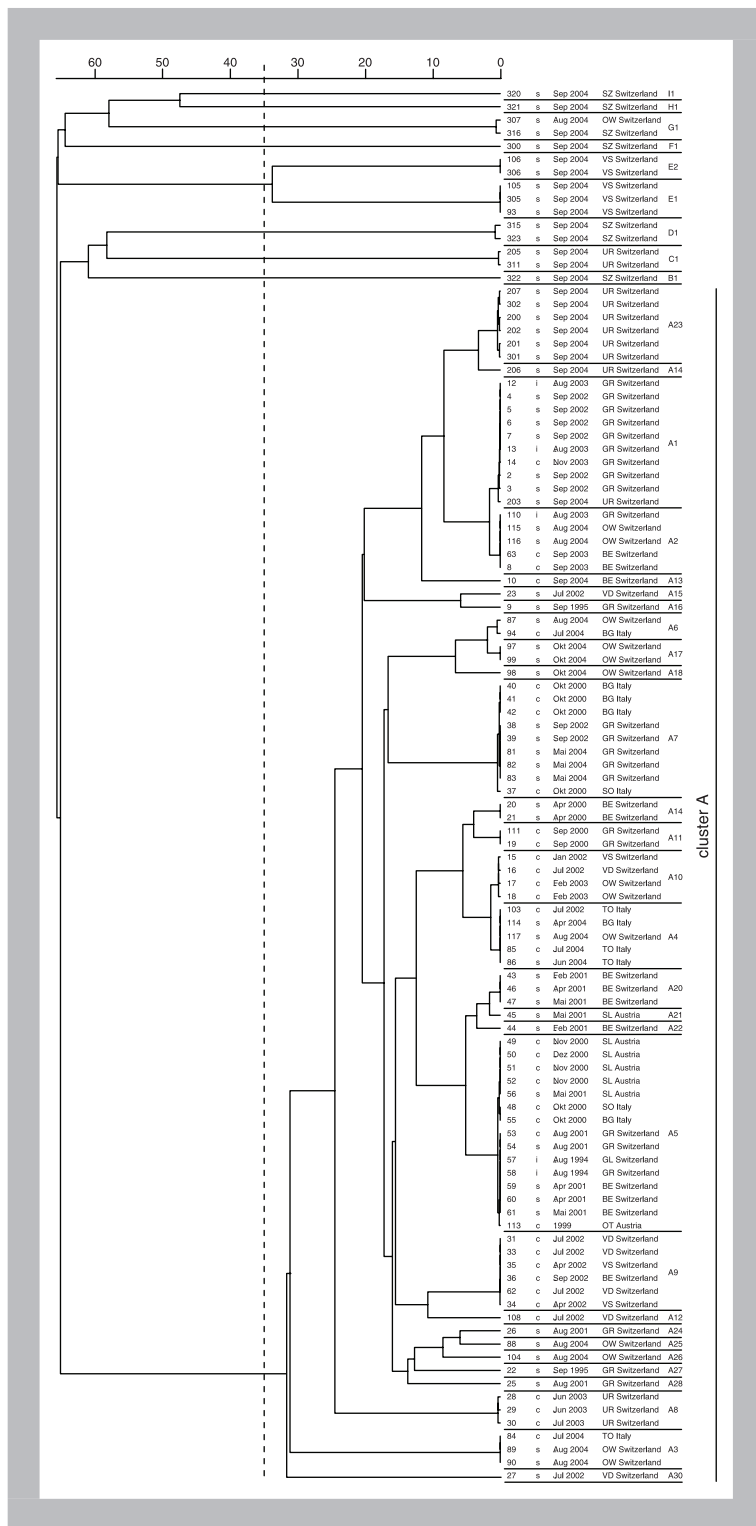


Figure 2. Phylogenetic presentation of all isolates analysed. The dendrogram was built by the neighbour-joining method. The scale on the top indicates divergence in the nucleotide sequence. The dashed vertical line indicates the threshold value differentiating the clusters. Vertical lines on the right part separate the individual strains. Strains belonging to cluster A were found in chamois with IKC and in sheep. Strains of the other clusters (B-I) were solely found in sheep. The rows of numbers indicate the following: first row: number of the individual lppS gene sequence; second row: animal species, i, ibex; c, chamois; s, sheep; third row: date of isolation; fourth row: area where the sample was taken, respectively where the diseased animal was found (see the legend of Figure 1 for a list of the abbreviations); fifth row: cluster and strain designation.

(see Figs. 1 and 2). Strain A7 was found several times in the south-eastern Swiss Alps and the adjacent Italian alpine valleys in chamois and sheep between 2000 and 2004 and seems to be endemic to this area. Two individual strains, which were unique for chamois, each appeared in a single animal. These strains were not found in sheep, which might be due to the fact that only few sheep were screened for *M. conjunctivae* during these outbreaks. Strains A3 and A6 were both found in chamois with IKC in the Aosta valley in Italy and in sheep in central Switzerland in 2004. There is, however, no record on a possible contact between these sheep and chamois. Therefore, in this case the sheep with *M. conjunctivae* infection cannot be considered as a direct source of infection for chamois. The largest number of different individual strains of *M. conjunctivae* (N = 21; A14-A30, plus strains from clusters B-I), were found solely in sheep and were not detected in chamois during this survey, although the sheep were sampled from areas where IKC out-

conjunctivae provoked a severe epidemic of IKC in ibex (Grattarola et al. 1999, Belloy et al. 2003a) and in 1999 in a chamois in neighbouring Austria

breaks in chamois were reported. However, it is important to note that all strains found in chamois belong to cluster A and no strains belonging to the

other clusters (B-I) were isolated from chamois. Strains from clusters B-I, which were exclusively isolated from sheep, are genetically distant from strains of cluster A (see Fig. 2).

Discussion

Mycoplasma conjunctivae was identified as the primary agent of infection, while other common ocular pathogens such as *Mycoplasma bovoculi*, *Moraxella* sp. and *Chlamydia* sp. were not detected in IKC of chamois and ibex (Mayer et al. 1997, 1996, Giacometti et al. 2002a). Outbreaks of IKC in chamois and also in ibex occur sporadically in the Swiss Alps. During such outbreaks, the number of affected animals is significantly higher than in outbreaks related to notifiable diseases of livestock (Tschopp et al. 2005), and can cause mortality that occasionally reaches 30% (Giacometti et al. 2002a). In view of the important role of sheep as a potential reservoir of *M. conjunctivae* (Janovsky et al. 2001) and their potential as sources for infections of chamois and also other wild Caprinae in the Alps, we have analysed the bacterial parameters that might be important for the risk assessment of IKC transmission and subsequent outbreaks of IKC in chamois. The study used molecular typing to differentiate individual strains of *M. conjunctivae* based on variations in the gene *lppS* that encodes the adhesin lipoprotein S (Belloy et al. 2003a,b). It revealed that sheep with symptoms of pink eye often carried several different strains of *M. conjunctivae* belonging to a wide range of distantly related clusters, while in chamois with IKC only single strains of *M. conjunctivae* were detected. Furthermore our study revealed that the sheep population which was sampled in the areas where IKC in chamois was diagnosed carried many more different and distantly related strains of *M. conjunctivae* compared to chamois where only a few specific and closely related strains were identified. With the exception of two strains A9 and A10 (see Fig. 2), all strains found in chamois could also be revealed in sheep that resided in the same areas. On the other hand, several sheep that were sampled in the same areas frequently carried strains that were never found in chamois. Among those, many strains belonged to clusters B-I. Strains of these clusters are phylogenetically distant from cluster A that contains chamois strains and strains that are common to sheep and chamois (see Fig. 2). These data indicate that not all strains of *M. conjunctivae* carried by

sheep are transmitted and cause IKC to chamois, suggesting that only certain strains of *M. conjunctivae* are a threat to chamois. Among the strains that were isolated from chamois with IKC, strain A5 (see Fig. 2) merits particular attention. This strain was found in chamois with IKC both in the northern and southern part of the Alps (Austria, Italy and Switzerland) where it was detected during severe IKC outbreaks in chamois populations in 2001 and 2002 both from chamois and sheep. Interestingly this strain was isolated already in 1994 from ibex with severe IKC. Since IKC is not maintained in alpine chamois (Giacometti et al. 2002b) it is most likely that this strain resided in an ovine reservoir and reappeared several years later during an IKC outbreak in chamois, due to its capacity to infect wild Caprinae. This observation would further support our hypothesis that among the large number of different strains of *M. conjunctivae* that are found within sheep, only a few seem to have the potential to cause symptoms of IKC. This would explain why outbreaks of IKC occur sporadically in chamois, in spite of the relatively high carrying rate of *M. conjunctivae* in sheep. The method used for typing *M. conjunctivae* strains is based on a specific gene involved in adhesion in which the variable poly-serine containing C' terminal segment is supposed to be involved in the binding process, in analogy to the poly-serine-glutamic acid site of the clumping factor Clf of *Staphylococcus aureus* (McDevitt et al. 1994, Belloy et al. 2003b). Hence, the strong clustering of *M. conjunctivae* strains with high potential to cause severe disease in specific animal species indicates that this locus might play a particular role in specificity of adhesion and, in consequence, in host predilection of *M. conjunctivae*. This perception together with knowledge from immunological and behavioural studies will contribute to develop methods for controlling *M. conjunctivae* infections and its devastating consequences in chamois and other susceptible wild Caprinae.

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