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Infectious keratoconjunctivitis and occurrence of *Mycoplasma conjunctivae* and Chlamydiaceae in small domestic ruminants from Central Karakoram, Pakistan

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Infectious keratoconjunctivitis (IKC) is a contagious eye disease primarily caused by *Mycoplasma conjunctivae* in domestic and wild Caprinae. *Chlamydophila* species have also been detected in ruminants with IKC. The objectives of this study are to investigate the ocular infection of *M. conjunctivae* and Chlamydiaceae and assess its interaction in relation to IKC in sheep and goats from remote communities around the Central Karakoram National Park in Pakistan, performing a combination of cross-sectional and case–control study design. Mostly asymptomatic and endemic infections of *M. conjunctivae* and Chlamydiaceae were found in sheep (19.3 per cent and 4.5 per cent, respectively) and goats (9.5 per cent and 1.9 per cent, respectively) from all communities, assessed by qPCR. Prevalence significantly differed between species only for *M. conjunctivae* ($P=0.0184$), which was also more prevalent in younger sheep ($P<0.01$). *Chlamydophila pecorum* was identified by sequencing and was related with IKC only when coinfection with *M. conjunctivae* occurred, which suggest a synergic interaction. Cluster analysis of *M. conjunctivae* strains revealed higher diversity of strains than expected, evidenced interspecific transmission and suggested a higher local livestock trade than previously assumed. These results highlight the widespread occurrence of *M. conjunctivae* in sheep worldwide and its implications for wildlife should be assessed from a conservation perspective.

Infectious keratoconjunctivitis (IKC) is a common contagious ocular disease among ruminants.¹ In small domestic ruminants, IKC is usually bilateral and produces ocular discharge, epiphora, mild conjunctivitis and/or corneal opacity, causing transitory blindness in most of the cases. However, IKC outbreaks may course with more severe clinical signs, including affection of the cornea leading to ulceration and perforation of the eye if no treatment is applied.²

Mycoplasma conjunctivae has been associated with most of the IKC outbreaks reported in small domestic ruminants and wild Caprinae worldwide and is considered the primary pathogen of this condition.^{1, 3-5} However, *M. conjunctivae* is also commonly detected in the eyes of asymptomatic sheep and is eventually endemic in sheep flocks throughout Europe.⁶⁻⁸ *M. conjunctivae* infection is therefore not consistent with IKC in sheep, indicating that other factors may determine the development of clinical

signs in non-epizootic conditions.⁸ Other infectious agents such as *Moraxella (Branhamella) ovis*, *Chlamydophila* species and *Listeria monocytogenes* have been isolated from the eyes of clinically affected sheep and may act opportunistically as secondary invaders and contribute to the onset of IKC.^{7 9} However, *Chlamydophila* ocular infections have been occasionally associated with ocular disease in small domestic ruminants¹⁰⁻¹³ and wild ruminants,^{14 15} and therefore their potential role as primary pathogens for IKC has been discussed.^{12 13} Although chlamydial infections are frequent in the eyes of diseased and asymptomatic sheep and goats,^{16 17} ocular coinfections with *M. conjunctivae* have not always been addressed in IKC control-case studies to properly assess a causal relationship. Neither did the finding of both infectious agents in clinical cases described in some studies provide conclusive aetiological information.¹⁸⁻²⁰

In mountain ecosystems, sheep and goats share alpine pastures with other IKC-susceptible wild species such as chamois (*Rupicapra* species), Alpine ibex (*Capra ibex*) or Himalayan tahr

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(*Hemitragus jemlahicus*).^{1 21} IKC outbreaks in wild free-ranging ruminants can cause significant mortality and demographic impact on affected herds.^{20 22} Although host specificity of certain *M. conjunctivae* strains or genotypes has been suggested,²³ interspecific transmission from domestic to wild ruminants can occur,²⁴ and domestic sheep may play a key role as an *M. conjunctivae* reservoir host for wild ruminants.^{1 6 8} Therefore, asymptomatic or mildly symptomatic small domestic ruminants can be at the origin of IKC outbreaks in wild ruminants, particularly

if the latter have not previously been in contact with *M. conjunctivae*.

Furthermore, IKC outbreaks in livestock can cause occasional economic losses for farmers, as well as a detrimental impact on animal welfare.²⁵ In developing countries, livestock production is an important economic income in rural areas. In Pakistan, there are 29.1 million sheep and 66.6 million goats. In 2013-2014, livestock production represented 55.9 per cent of the agriculture and 11.8 per cent of the Gross Domestic Product.²⁶

The objectives of this study are to investigate the presence of *M. conjunctivae* and Chlamydiaceae in the eyes of sheep and goats from two isolated valleys in the buffer zone of the Central Karakoram National Park (CKNP) in Pakistan and to evaluate its health significance in relation to IKC. Factors affecting prevalence are explored and cluster analysis of the *M. conjunctivae* strains are also performed to establish epidemiological associations and evaluate strain diversity in this remote area.

Materials and methods

Study design and sample collection

This study was based on a combination of cross-sectional sampling strategy to estimate the apparent prevalences of *M. conjunctivae* and Chlamydiaceae and a case-control design to evaluate their influence on the clinical condition of IKC. From March 2013 to April 2014, eye swabs were collected from 334 small domestic ruminants (176 sheep and 158 goats) belonging to six communities at the boundaries of the CKNP (Fig 1). The minimum sample size required for pathogen prevalence estimation was 196, calculated with the WinEpiScope V.2.0 software²⁷ with an expected prevalence of 15 per cent (95 per cent CI, 5 per cent accepted error), in unknown total population,⁸ and was achieved depending on the availability for sampling. In particular, a team of veterinarians (including three of the authors) and livestock assistants either operated at a congregation point where local sheep and goat owners had been invited via Muezzin's announcement or freely moved across a village looking for owners' consent to sampling. All individuals in available flocks were sampled.

CKNP is the largest protected area of Pakistan and one of the largest worldwide, covering over 10,000 km² in the Gilgit-Baltistan district (Fig 1). The sampled communities are located in two of the main valleys in the area, namely the Hunza Nagar Valley in the northwest (36°15' 4.25' N, 74°32' 9.89' E) and the Hushe Valley in the southeast (35°27' 51.64' N, 76°21' 4.23' E), at elevations ranging between 1900 m and 3500 m (Fig 1). Local climate is dry (rainfall < 200 mm/year), with a relatively greater precipitation in winter and spring, arid continental climate in summer and sudden onsets of cold weather in early autumn. Winter snowfalls are not abundant but temperatures frequently reach -15°C.

Based on data provided by official veterinarians at the local Gilgit-Baltistan Livestock Department, there was a census of 7173 sheep and 6167 goats in the sampled communities at the beginning of the last decade. Typically, sheep and goats are kept in small (10-30 heads) mixed household flocks, which are seasonally merged into larger summer flocks of few hundred individuals.

In the investigated area, small domestic ruminants share habitat with approximately 6000 cattle (cows, yaks and hybrids) and a limited number of horses and donkeys. Two wild Caprinae species are also found, the relatively common Asian ibex (*Capra sibirica*), and the rare and localised Flare-horned Markhor (*Capra falconeri*). Seasonal contacts between wild and domestic Caprinae occur mainly in summer, as shown to authors by members of the sampled

communities. Sheep and goats in this study were sampled while kept in the surroundings of the investigated communities, before or just after the traditional summer transhumance to the high pastures.

Swabs were collected under the third eyelid from both eyes, transported refrigerated and stored frozen at -20°C until analysed. Ocular clinical signs were recorded at sampling considering the clinical condition of IKC whenever signs of ocular damage, inflammation or discharge occurred. Species, age, sex and body condition score (BCS) were also recorded.²⁸ Age was determined in 141 of the 158 goats and in 166 of the 176 sheep by definitive incisor teeth eruption.²⁹ All applicable institutional and/or national guidelines for the care and use of animals were followed.

Mycoplasma conjunctivae detection and LPPS sequencing

At the laboratory, eye swabs were placed into sterile tubes with 0.5 ml of lysis buffer (100mM Tris-HCl, pH 8.5, 0.05 Tween 20, 0.24 mg/ml proteinase K). After mixing with a vortex, cells were lysed for 60 minutes at 60°C and then heated to 97°C for 15 minutes in order to inactivate proteinase K.

The lysates obtained were tested for the presence of *M. conjunctivae* DNA with a TaqMan qPCR, using the primers LPPSTM-L, LPPS-TM-R and the probe LPPS-TM-FT as described.³⁰

For cluster analysis, a subtyping of the *M. conjunctivae* strains based on the *lppS* gene was attempted with a selection of 26 samples which showed the lowest Ct values at the TaqMan qPCR. DNA was amplified by nested PCR according to the method described²⁴ with minor modifications of the primers (online supplementary table s1). All PCR products were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Rotkreuz, Switzerland) for subsequent DNA sequence analysis. DNA sequence determination was performed using the BigDye termination cycle sequencing kit (Applied Biosystems, Foster City, California, USA) with the sequencing primers Ser_start2, Ser_start0 and Ser_end0 (online supplementary table s1). The sequences obtained were trimmed to contain the variable part of gene *lppS* and flanking regions corresponding to the nucleotides 3935-5035 of *lppS* of the type strain HRC/581 of *M. conjunctivae* (accession number AJ318939). Cluster relationships between strains were assessed by generation of phylogenetic trees, based on the sequence of using the MEGA 6 software with the following parameters: gap opening penalty 15; gap extension penalty 6.6, DNA weight matrix IUB, transition weight 0.5 including the corresponding DNA sequence data from *lppS* of HRC/581 for comparison.

Chlamydiaceae detection and identification

For the detection of Chlamydiaceae species in the eye swabs lysates, an SYBR green-based qPCR assay was performed using the primers Chuni-1F and Chuni-2R.³¹ Each reaction consisted of 2.5 μl of DNA sample, 12.5 μl of SYBRGreen PCR Master Mix 2x (Applied Biosystems, Warrington, UK), 400nM of each forward and reverse primer and nuclease-free water to a total volume of 25 μl . PCR was performed following reported cycling conditions.³¹ Samples were assayed per duplicate and were assayed with an exogenous Internal Positive Control (IPC; Applied Biosystems, USA) to detect eventual PCR inhibitors.

The positive samples were analysed further with a PCR that targets the *Chlamydiales* specific 298 bp signature of the 16S rRNA

gene using the primers 16S1GR and 16S1GF.³² Each reaction consisted of 2.5 μ l of test sample, 25 μ l of AmpliTaq Gold 360 Master Mix (Applied Biosystems, UK), 400nM of each primer and nuclease-free water up to 50 μ l. Amplifications were performed starting with an initial denaturation at 95°C for 10 minutes, 40 cycles that consisted in denaturation at 95°C for 30 seconds, annealing at 51°C for 30 seconds and extension at 72°C for 30 seconds, followed with a final extension step at 72°C for 7 minutes. All PCR reactions were run on an ABI 7500 instrument (Applied Biosystems, USA). Purified amplicons (Minelute Gel Extraction Kit, Qiagen, Hilden, Germany) were sequenced for its identification with Big Dye Terminator V.3.1 Kit and the ABI 3130xl Genetic Analyzer (Applied Biosystems, UK). The sequences obtained were introduced in the BLAST server from the National Centre for Biotechnology website (<http://www.ncbi.nlm.nih.gov/blast/>) to compare with the sequences available in GenBank.

Statistical analyses

A tree modelling approach was performed in order to identify factors that drive the *M. conjunctivae* and Chlamydiaceae infection. Conditional inference trees estimate relationship by binary recursive partitioning in which associations between variables are defined by P values. It is a robust statistical tool capable of dealing with variables of different nature and are suitable for complex epidemiological data.^{33 34} *M. conjunctivae* and Chlamydiaceae infection were considered as response variables (Bernoulli distribution) in two independent classification trees, whereas host (sex, age and BCS) and population variables (community and valley) were included as explanatory variables in each tree. The analyses were performed separately for sheep and goats. A conditional inference tree approach was also used to identify risk factors for IKC, including the occurrence of *M. conjunctivae* and Chlamydiaceae infection (ie, discrete nominal variables with two categories positive/negative) and individual host factors (sex, age and BCS) as explanatory variables. These analyses were also performed separately for each ruminant species. Differences of Ct values of the *M. conjunctivae* qPCR between asymptomatic and clinical sheep and goats were assessed by Wilcoxon signed-rank test. Prevalences of *M. conjunctivae* and Chlamydiaceae were compared between species and communities using tests of proportions and setting statistical significance at 0.05. Statistical analyses were performed with R software,³⁵ using the 'party' package for the trees and the 'EpiR' package to calculate the prevalence estimates.³⁶

Results

M. conjunctivae had a 14.7 per cent prevalence (CI₉₅ 11.3 to 18.9, 49/334) in the sampled domestic ruminants. Prevalence was significantly (P=0.01842) higher in sheep (19.3 per cent, CI₉₅ 14.2 to 25.8, 34/176) than in goats (9.5 per cent, CI₉₅ 5.8 to 15.1, 15/158), both overall and for each sampling site (Tables 1 and 2). Chlamydiaceae prevalence was lower than *M. conjunctivae*, both overall (3.3 per cent, CI₉₅ 1.8 to 5.8, 11/334) and for each species separately (sheep 4.5 per cent, CI₉₅ 2.3 to 8.7, 8/176 and goats 1.9 per cent, CI₉₅ 0.6 to 5.4, 3/158). Conversely to *M. conjunctivae*, the prevalence of Chlamydiaceae did not significantly differ between species. *M. conjunctivae* was detected in all investigated communities in sheep, and in four of them in goats, whereas Chlamydiaceae were detected in five communities in sheep and in three communities in goats (Tables 1 and 2).

Among the 34 *M. conjunctivae*-positive sheep, 4 (11.8 per cent) had KC with ocular discharge, whereas the remaining 30 sheep

(88.2 per cent) had no clinical signs at the time of sampling (Table 1). One of the 15 *M. conjunctivae*-positive goats showed severe signs of KC (bilateral cornea perforation) associated with the ocular presence of *M. conjunctivae* (Fig 2), whereas the remaining 14 (93.3 per cent) *M. conjunctivae*-positive goats did not show any clinical signs. Median Ct values of the *M. conjunctivae* qPCR showed no statistical differences in sheep with IKC (median 30.2) and without (median 31.7), and in goats with IKC (26.3 in the only IKC case) and without (31.3). On the other hand, eight sheep (4.5 per cent, 8/176) and one goat (0.6 per cent, 1/158) with ocular clinical signs tested negative to the *M. conjunctivae* qPCR (Table 1). Also, 3 sheep of the 11 domestic small ruminants (8 sheep and 3 goats) positive to Chlamydiaceae were also positive to *M. conjunctivae*. KC clinical signs in the Chlamydiaceae-positive small ruminants were observed only when coinfection with *M. conjunctivae* occurred, in two out of these three sheep.

M. conjunctivae Chlamydiaceae

n Positive Prevalence (CI95) Positive Prevalence (CI95)

Sheep

With KC 12 4 33.3% (13.8 to 60.9) 2* 16.7% (4.7 to 44.8)

Without KC 164 30 18.3% (13.1 to 24.9) 6 3.7% (1.7 to 7.7)

Total 176 34 19.3% (14.2 to 25.8) 8 4.5% (2.3 to 8.7)

Goats

With KC 2 1 50.0% (2.6 to 97.4) 0 0% (0 to 65.8)

Without KC 156 14 9.0% (5.4 to 14.5) 3 1.9% (0.6 to 5.5)

Total 158 15 9.5% (5.8 to 15.1) 3 1.9% (0.6 to 5.4)

Total 334 49 14.7% (11.3 to 18.9) 11 3.3% (1.8 to 5.8)

Summary of samples analysed (n), positives to qPCR (Positive) and prevalence (%) of *M. conjunctivae* and Chlamydiaceae in eye swabs of small domestic ruminants from the Central

Karakoram National Park area, showed by ruminant species and by ocular clinical signs (KC)

*These two sheep were also positive to *M. conjunctivae*

KC, keratoconjunctivitis

Decision tree analyses indicated that *M. conjunctivae* prevalence was significantly ($P < 0.01$) higher in sheep younger than one year than in older sheep (Table 3). Differences by age category were not observed in goats and no other factors considered in the tree analysis were identified to influence significantly on *M. conjunctivae* prevalence. No age-related differences were observed in both hosts for Chlamydiaceae. On the other hand, no risk factors for IKC were identified among the explanatory variables considered in the tree, including *M. conjunctivae* and Chlamydiaceae occurrence in the eyes of sheep and goats.

Eleven PCR-amplified fragments of the *lppS* gene of *M. conjunctivae* were sequenced from seven goats and from four sheep taken from four different communities. Sequence analyses allowed to differentiate seven *M. conjunctivae* strains that were all phylogenetically related to *M. conjunctivae* strains from sheep (2820s) and Alpine chamois (*Rupicapra rupicapra*) (2778c, 2784c) that were isolated in the Alps and related to IKC outbreaks in chamois and sheep²⁴ (Fig 3). Among the strains sequenced, only one (P117) was associated with clinical KC in a sheep. In four goats (P119, P124, P122, P136) from Hoper community, a common strain was identified. Furthermore, a common strain was found in a sheep (P98) and a goat (P16) from Minapin and Kandy communities, respectively (Fig 3). The EMBL/GenBank accession numbers for the *lppS* gene fragments of the different strains are also shown in Fig 3.

Sequences of the Chlamydiaceae 16S rRNA were obtained from five Chlamydiaceae-positive samples (four sheep and one goat from two different communities) and all had the highest similarity (96-100) with *Chlamydomphila pecorum* 16S ribosomal RNA complete sequence (accession number NR_121750.1).

Discussion

M. conjunctivae and Chlamydiaceae were detected in asymptomatic and clinically affected eyes of sheep and goats

from the CKNP, Gilgit-Baltistan district of Pakistan. These results agree with the previously reported sporadic IKC cases in sheep flocks with endemic *M. conjunctivae* infections and its capability to establish asymptomatic ocular infections.^{8 37} Reports of non-epidemic IKC in goats are scarce³⁸ and most of the IKC descriptions refer to outbreaks.^{39 40} However, the results obtained in this study suggest that *M. conjunctivae* is endemic in mixed sheep-goat flocks of the CKNP area. Although *M. conjunctivae*

Community

Goats Sheep

S KC *M. conjunctivae* Chlamydiaceae S KC *M. conjunctivae* Chlamydiaceae

Hisper 23 0 0.0% (0) 0.0% (0) 9 0 11.1% (1) 0.0% (0)

Hoper 22 1 22.7% (5) 4.6% (1) 10 2 30.0% (3) 10.0% (1)

Hushe 36 0 5.6% (2) 2.8% (1) 43 5 18.6% (8) 4.6% (2)

Kanday 61 1 11.5% (7) 0.0% (0) 73 5 17.8% (13) 4.1% (3)

Minapin 2 0 0.0% (0) 0.0% (0) 15 0 26.7% (4) 6.7% (1)

Skanderabad 14 0 7.1% (1) 7.1% (1) 26 0 19.2% (5) 3.9% (1)

Total 158 2 9.5% (15) 1.9% (3) 176 12 19.3% (34) 4.5% (8)

Summary of samples (S), clinical signs (KC) and prevalence (percentage and number of positives) of *M. conjunctivae* and Chlamydiaceae bacteria in the eyes of small domestic ruminants

from the Central Karakoram National Park area in Pakistan

* Mean *M. conjunctivae* prevalence was significantly ($P=0.01842$) higher in sheep than in goats both overall and for each sampling community

KC, keratoconjunctivitis

FIG 2: Image of a goat exhibiting a severe stage of infectious keratoconjunctivitis, with bilateral panophthalmitis, lacrymation, hypopyon and perforation of the cornea. *Mycoplasma conjunctivae* was confirmed in eye swabs by qPCR.

has been previously reported in sheep from Pakistan⁴¹ and several other locations worldwide,^{18 42-44} its detection had not been reported in traditionally reared flocks from such remote and isolated communities. This finding confirms that *M. conjunctivae* is probably one of the most common and geographically widespread pathogens found in sheep.

M. conjunctivae has been traditionally studied as the main aetiological agent in IKC outbreaks characterised by high morbidity affecting all age classes and severe clinical signs.^{25 41 44}

Asymptomatic carriers may have lower *M. conjunctivae* loads,⁴⁵ which altogether with the fastidious nature of *M. conjunctivae* may have complicated its identification, resulting eventually in the underestimation of its frequency by traditional methods of isolation.^{7 40} Similar prevalence to those found in sheep and goats from the CKNP area has been reported in sheep from northern Spain (25.7-29.2 per cent) when assessed by qPCR, with also a similar percentage of asymptomatic *M. conjunctivae*-positive sheep (87.3 per cent).^{8 20} Since *M. conjunctivae*-infected sheep from Pakistan were mostly asymptomatic, no statistically significant relationship could be established between *M. conjunctivae* infection and IKC. In these endemic and mostly subclinical infections, the development of clinical signs may depend on other factors, such as host immunity, strain virulence or concurrent infections.

^{7 8} Ocular clinical signs were however not related with the Ct values of the *M. conjunctivae* qPCR and several asymptomatic infections exhibited low Ct values (ie, inverse to mycoplasma loads), which suggests that a different host-mycoplasma interaction than the previously described in wild Caprinae occurred in sheep and goats.^{5 45 46}

The higher prevalence of *M. conjunctivae* in sheep younger than one year compared with the older ones indicates that acquired immunity may influence the course of the infection in sheep. IKC typically causes more severe clinical signs in adult sheep

Age category

0-1 years >1 year

M. conjunctivae

Goats 16.3% (8/49) 3.3% (3/92)

Sheep 30.0% (24/80)* 10.5% (9/86)*

Total 24.8% (32/129) 6.7% (12/178)

Chlamydiaceae

Goats 2.0% (1/49) 2.2% (2/92)

Sheep 5.0% (4/80) 4.7% (4/86)

Total 3.9% (5/129) 3.4% (6/178)

Results of *M. conjunctivae* and Chlamydiaceae qPCR (prevalence %; positive/tested)

showed by age in 141 goats and 166 sheep in which age was determined

*Statistically significant differences ($P < 0.01$)

FIG 3: Phylogenetic representation of the *M. conjunctivae* strains sequenced from sheep and goats from the Central Karakoram National

National

Park area (number starting with P) in comparison with the type strain of *M. conjunctivae* (HRC/581) and strains from sheep in the Eastern

Swiss Alps (38s and 2820s), from chamois in the Austrian Alps (2778c and 2784c), from sheep in Croatia imported from Australia (My6695)

and from a goat imported to Croatia from the Southern French Alps (My7/96). The species and the community are specified next to the strain reference. Infectious keratoconjunctivitis (IKC) indicates the presence of ocular clinical signs at sampling. The EMBL/GenBank accession numbers for the *lppS* gene fragments of the different strains identified in the study are indicated in bold, whereas the accession

numbers for the reference strains are indicated in plain text.

than lambs.^{2 37} However, *M. conjunctivae* persistence in the eyes do not necessarily have to be related with clinical signs as broadly described in experimental infections.^{6 47} Higher prevalence of *M. conjunctivae* in young animals has been also described in ibex,^{5 45} which suggests that younger animals/lambs may be more important for the *M. conjunctivae* maintenance in the herd/population.

Endemic and mostly asymptomatic *M. conjunctivae* infections had no apparent effect on BCS, which is probably influenced by other factors not assessed in this study. The small sample size and number of clinical cases might also have impaired the identification of risk factors associated with the disease.

The higher prevalence of *M. conjunctivae* in sheep compared with goats corresponds to previous reports from other geographic areas.⁸ This higher prevalence of *M. conjunctivae* in sheep may be related to host specificity of the prevalent local strains, or to a higher density, aggregation and intraspecific contact among sheep than among goats within mixed flocks. Overall, the results of this study and previous descriptions suggest that sheep are better hosts than goats for the endemic and mostly asymptomatic maintenance of *M. conjunctivae*. However, high pathogenic strains of *M. conjunctivae* were isolated during IKC outbreaks in both ruminant species,^{25 39} and goats can also develop severe IKC in endemic infections as observed in this study (Fig 2).

Prevalence of Chlamydiaceae was lower than reported in previous studies in small domestic ruminants.^{16 17} The differences could be in part because different sampling and diagnostic methodology were used. Several *Chlamydophila* species, such as *Chlamydophila abortus*, *C. pecorum*, *Chlamydophila psittaci* and *Chlamydia suis* have been identified in the eyes of livestock without correlation with ocular clinical signs.^{12 16 17} On the other hand, *C. pecorum* has been occasionally associated with KC and polyarthrititis in sheep and goats⁴⁸ and IKC was successfully induced experimentally with *C. psittaci* infection (as taxonomically considered at that time).¹¹ Chlamydial KC outbreaks have also been reported both in domestic and wild ruminants.^{10 14} Since Chlamydiaceae and particularly *C. pecorum* infection in sheep and goats from CKNP area were exclusively associated with clinical signs of IKC in case of coinfection with *M. conjunctivae*, it is possible that they acted synergically with *M. conjunctivae* as a secondary infectious agent for the development of clinical disease. However, the few number of coinfection cases found in this study does not allow inferring conclusions on virulence synergism with a statistical approach. The relative aetiological importance of Chlamydiaceae may also rely on other factors, such as the strain pathogenicity.⁴⁸ Similar concurrent infections of *M. conjunctivae* and Chlamydiaceae have been reported in domestic sheep^{12 18} and free-ranging chamois^{19 20} in both diseased and asymptomatic individuals. According to these results, the interaction of *Chlamydophila* species with *M*

conjunctivae should be considered in aetiological investigations of ocular disease in small domestic ruminants, although experimental evidence would probably be necessary to assess whether such coinfections determine the onset of IKC.

The diversity of the *M. conjunctivae* strains identified in the CKNP area suggests that *M. conjunctivae* has been present for a long time in northern Pakistan. However, the relatively close relationship of the *M. conjunctivae* strains found in this study with strains described in sheep and wild Caprinae from the Alps (Central Europe) suggests that *M. conjunctivae* might have been introduced in this area along with its hosts, as it was described in Croatia in the early 1990s.²⁴ Furthermore, the molecular identification of the same strain infecting sheep and goats confirms interspecific transmission and the need to approach the epidemiological study of IKC considering all the susceptible hosts, taking into account the differences in host-pathogen interaction.⁸ Cluster analyses also revealed that the same *M. conjunctivae* strain was present in the communities of Kandy and Minapin, separated by 190-km straight-line distance and mountains ranging 5500–8000 m of altitude (Fig 1). Livestock trade in northern Pakistan is mainly on a local scale, and it can be assumed that trade movements between Kandy and Minapin are limited due to the great physical and cultural distance and the deriving lack of relationships. However, the molecular analyses of *M. conjunctivae* in sheep and goats mirror more complex trade habits and suggest a long endemic presence of *M. conjunctivae* in the small domestic ruminants of the CKNP area. The introduction of *M. conjunctivae* asymptomatic carriers into naive populations supposes a risk of severe IKC outbreaks in sheep flocks.^{25–44} Similarly, interspecific transmission of *M. conjunctivae* from domestic to wild mountain ruminants may result into massive IKC outbreaks in wildlife as demonstrated in Alpine chamois.²⁴ Therefore, domestic sheep and goat populations of the CKNP area, asymptotically infected with *M. conjunctivae*, may represent a potential source of infection and IKC outbreaks in sympatric wild hosts, namely the Asian ibex and the Flarehorned Markhor. To the authors' knowledge, there is currently no robust information about IKC in wild ruminants from the CKNP. Syndromic surveillance of IKC in local flocks however would improve rapid detection of any major risk of spill over of virulent *M. conjunctivae* strains at the livestock-wildlife interface. In conclusion, mostly asymptomatic and endemic infections of *M. conjunctivae* and Chlamydiae were found in sheep and goats populations of the CKNP area in the Gilgit-Baltistan district of Pakistan. Chlamydiae was associated with ocular clinical signs only when coinfection with *M. conjunctivae* occurred, but further studies are needed to better assess the effect of such coinfections in eye disease. Cluster analysis of *M. conjunctivae* strains revealed higher diversity of strains than expected, evidenced interspecific transmission and suggested a higher local livestock trade than previously assumed. *M. conjunctivae* is probably one of the most common pathogens found in sheep worldwide and its implications should be assessed from a conservation perspective if sheep are allowed to share pastures with endangered or vulnerable ruminant species.

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