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# Molecular detection and isolation of Mycoplasma conjunctivae from goats with infectious keratoconjunctivitis in Sichuan province, southwest China

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*Short Communication*

# Molecular detection and isolation of *Mycoplasma conjunctivae* from goats with infectious keratoconjunctivitis in Sichuan province, southwest China

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***Abstract***

Infectious keratoconjunctivitis (IKC) is a common contagious ocular disease in ruminants worldwide characterized with keratitis and conjunctivitis and, in severe cases, blindness. Recently two outbreaks of infectious keratoconjunctivitis (IKC) occurred in goats in Sichuan Province, China. To identify the causative agent responsible for these outbreaks, ocular swabs were then taken from 40 goats with IKC and 22 goats without IKC for isolation and molecular detection of mycoplasma. *Mycoplasma conjunctivae*-specific nested-PCR was 85.0% positive among samples from goats with IKC and 40.9% positive from goats without IKC. Nine PCR products were sequenced and showed that there was 99.6-100% sequence homology among themselves and 99.6-100% with NCBI *M. conjunctivae* DNA sequences from strain Goat 655, HRC/583 and HRC/581T. A total of 6 strains of *M. conjunctivae* were also successfully isolated. In this study, we present conclusive evidence of the presence of *M. conjunctivae* in Sichuan Province, southwest China. This is the first report of *M. conjunctivae* infection outbreaks in small ruminants in mainland of China and will be helpful for vaccine development and control of IKC in goats in this region.

**Keywords:** *Mycoplasma conjunctivae*, goat, molecular detection, isolation, China

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## Introduction

Infectious keratoconjunctivitis (IKC) is a common ocular infection in ruminants (Arnal *et al*., 2013; Fernández-Aguilar *et al*., 2017; Giacometti *et al*., 2002; Williams *et al*., 2019) and is characterized by inflammation of the conjunctiva and cornea, often leading to clinical complications due to corneal opacity, anterior uveitis, with occasional chronic fungal infections and blindness, which causes economic consequences.

In sheep, goats and other wild caprine species, *M. conjunctivae* is believed to be the major and primary etiologic agent of IKC which has been demonstrated by both experimental infection and frequent isolation of the organism in affected animals (Bass *et al*., 1997; Dagnall., 1993; Giacometti *et al*., 1998; Handeland *et al*., 2020; Marco *et al*., 2009; ter Laak *et al*., 1988; 1998;). IKC-*M conjunctivae* infection in small ruminants has been reported in North America, Europe, South Africa and New Zealand (Akerstedt *et al*., 2004; Giacometti *et al*., 2002; Giangaspero *et al*., 2010; Jansen *et al*., 2006; Marco *et al*., 2009; Motha *et al*., 2003; Tschopp *et al*., 2005; van Halderen *et al*., 1994). In Asia, a molecular survey in Central Karakoram, Pakistan showed 33.3% and 50% positive rates in sheep and goats with keratoconjunctivitis, respectively (Fernández-Aguilar *et al*., 2017). Hsu *et al*., (2017), reported an IKC outbreak in an indoor dairy goat barn on the Taiwan island of China; *M. conjunctivae* was detected from the diseased goats by using PCR. So far, there have been no reports from other Asian countries and regions, including the mainland of China. Hence, this study was carried out to investigate the presence of *M. conjunctivae* in goats.

## Materials and Methods

During a surveillance concerned with mycoplasma diseases in goats in Sichuan province, southwest China, IKC cases were noticed in two goat herds. These herds were 400 kilometers apart, with no obvious connectivity for the pathogen transmission in-between. The morbidity of IKC was 17.1% (72/420) in herd 1 and 11.9% (32/268) in herd 2. The diseased animals showed typical IKC clinical signs, including bilateral lacrimation, palpebral edema, severe scleral congestion, corneal ulceration/edema (cloudy or opaque), conjunctival edema, anterior uveitis and profuse muco-purulent ocular discharge (Fig. 1A). Most cases were self-limiting and resolved within 4 weeks. Reported veterinary treatments included ofloxacin (eye drops), tylosin, oxytetracyclin and amoxicillin for herd 1 with tylosin, oxytetracyclin, florfenicol and tiamulin for herd 2.

To identify the causative agent responsible for these outbreaks in the goats, ocular swabs were taken from 62 goats including 23 with IKC and 14 without IKC in herd 1; 17 with IKC and 8 without IKC in herd 2 (Table 1). The swabs were pre-moistened with modified Hayfflick's medium (Thiaucourt *et al*., 1996) containing 100U/ml penicillin and then inserted and gently rolled in the medial conjunctival sac deep beneath the third eyelid. The swabs were then immediately inoculated into 5ml of modified Hayfflick's medium and shipped to the laboratory within 12 hours for testing. Upon arrival, 1 ml of each sample was used for molecular detection and characterization while the remaining were used for mycoplasma isolation.

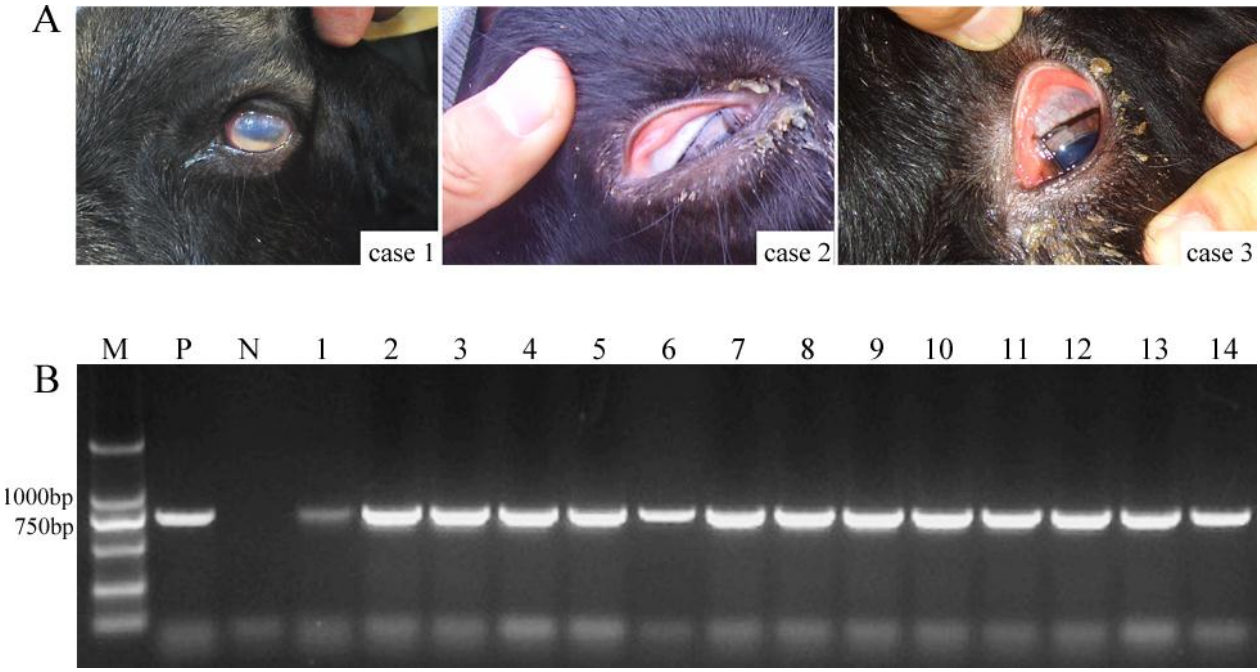


**Figure 1** Clinical signs and molecular confirmation of *Mycoplasma conjunctivae* associated IKC in China. **A**) Clinical signs during outbreaks in Sichuan province, China. Case 1, palebral edema and corneal edema (cloudy or opaque); case 2, conjunctival edema and muco-purulent ocular discharge; case 3, scleral congestion with edema of conjunctiva and the third eyelid. **B**) Detection of *M. conjunctivae* DNA amplicons from second step of nested PCR. M, molecular ladder; P, positive control using DNA from *M. conjunctivae* strain HRC/581; N, negative control, no DNA template, Lanes 1-7 and 8-14, DNA from conjunctival swab samples from goats in herd 1 and 2, respectively.

**Table 1** Detection and isolation of *Mycoplasma conjunctivae* from goats

| | | Goats with eye symptoms | Goats without eye symptoms |
|---|---|---|---|
| *M. conjunctivae* specific nested PCR | Herd 1 | 86.9%(20/23) | 50.0%(7/14) |
| | Herd 2 | 82.4%(14/17) | 25.0%(2/8) |
| | total | 85.0%(34/40) | 40.9%(9/22) |
| *M. conjunctivae* isolation | Herd 1 | 13.0%(3/23) | 7.1%(1/14) |
| | Herd 2 | 11.8%(2/17) | 0.0%(0/8) |

To identify *M. conjunctivae* and other *Mycoplasma* spp. present in the ocular swabs collected, DNA was extracted from 1 ml aliquot as mentioned above using a commercial Bacterial DNA Kit (Omega, USA) following the manufacturer's instructions. For detection of *M. conjunctivae*, the nested PCR were performed as previously described by Giacometti *et al.*(1999), with the mycoplasma universal primers MOLIGEN1-L (5'-ACTCCTACGGGAGGCAGCA-3') and 16SUNI-R (5'-GTGTGACGGGCGGTGTGTAC-3') in the first PCR step and *M. conjunctivae*-specific primers McoR1 (5'-CAGCGTGCAGGATGAAATC CCTC-3') and McoF1(5'-GTATCTTTAGAGTCCTCGT CTTTCAC-3') in the second (nested) PCR step. Subsequently PCR products were randomly selected and sequenced at the Beijing Genomics Institute in Shenzhen, China. All samples were also screened for the existence of *Mycoplasma ovipeumoniae*, *Mycoplasma arginini* and members of Mycoplasma mycoides cluster using corresponding specific PCR as previously described (Weiser *et al*., 2012; Hotzel *et al*., 1996).

For isolation of mycoplasma, the ocular swabs were filtered with a 45µm bacterial filter and four serial dilutions of 1:10 were prepared with modified Hayfflick's and then incubated at 37°C with 5% $CO_2$ for a total of 14 days. When an obvious colour change was observed, DNA was then extracted from the cultures and identified using the *M. conjunctivae*-specific nested-PCR as described above.

## *Results and Discussion*

By using *M. conjunctivae*-specific nested-PCR, it was found that 85.0% of eye swabs collected from 40 goats with IKC (20 out of 23 in herd1 and 14 out of 17 in herd2, respectively) were positive for *M. conjunctivae* while 40.9% of eye swabs from 22 goats without IKC (7 out of 14 in herd1 and 2 out of 8 in herd2, respectively) were positive (Table 1 and Fig. 1B). No other *Mycoplasma* spp. were detected by PCR methods. To confirm that the PCR products were *M. conjunctivae*-positive, nine PCR products were then randomly selected from these *M. conjunctivae*- positive samples (designated CD1-CD6 from herd 1 and CD7-CD9 from herd 2) and then sequenced. The results demonstrated that these nine *M. conjunctivae* sequences examined shared 99.6-100% sequence homology. When compared with NCBI *M. conjunctivae* DNA sequences, there were 99.7-100% sequence homology with strain Goat 655 (accession FJ226571.1, Volokhov D., 2008, unpublished, USA), 99.6-100% with HRC/583 (accession NR_074135.1, sheep isolate, Calderon-Copete SP *et al*.,, 2013, unpublished, Switzerland) and 99.6-100% with strain HRC/581T (complete genome, accession FM864216.2, sheep isolate, Calderon-Copete SP *et al*., 2008, unpublished, Switzerland) (Fig. 2). These results confirmed the prevalence of *M. conjunctivae* in the goat herds affected by IKC and strongly associated with the occurrence of IKC among goats in southwest China. To the best of our knowledge, this is the first time *M. conjunctivae* has been identified in goats with IKC from mainland of China. Furthermore, 9 out of 22 samples collected from goats with no clinical signs at the time of sampling were also positive for the presence of *M. conjunctivae* by *M. conjunctivae*-specific nested-PCR methods, while 8 of them failed to isolate *M. conjunctivae*. There is a possibility that *M. conjunctivae* presents in the eyes of asymptomatic goats with a low number from which it is difficult to be isolated due to low sensitivity of the mycoplasma isolation method. This finding is consistent with other reports (Fernández-Aguilar *et al*., 2017; Motha *et al*., 2003). This also suggests that those animals are likely to become asymptomatic carriers and an important source of infection. Furthermore, the findings of this study and other studies suggest the possibility that *M. conjunctivae,* like many other mycoplasma species, is a conditional pathogen in the eye. When the corneal epithelium is irritated by "risk factors" such as dust, flies, trauma, thick-stemmed hay and ultraviolet light or when a host is affected by physical stresses or microbial infection, leading to hypoimmunity, massive proliferation of *M. conjunctivae* can take place and cause IKC. This problem deserves further study.

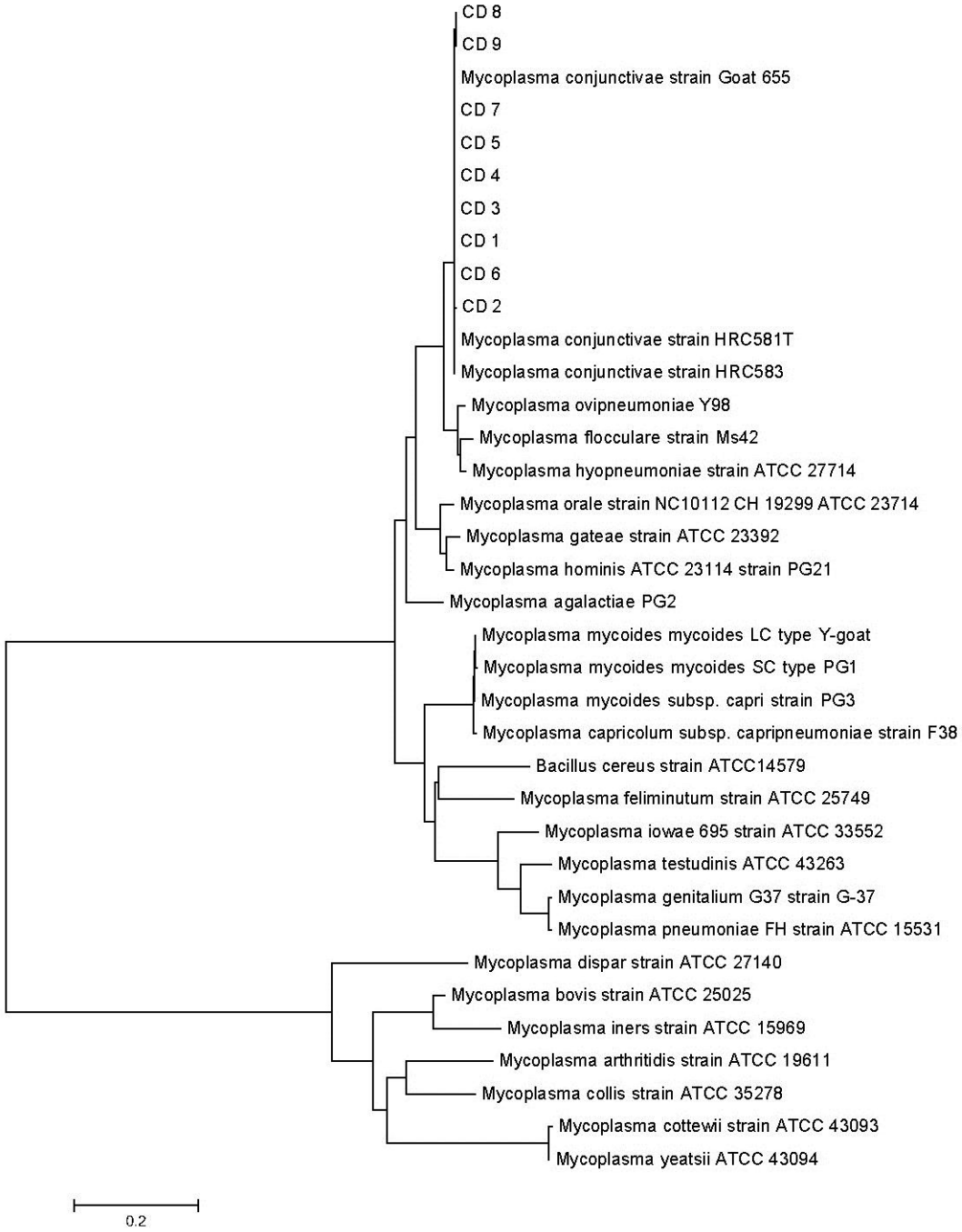


**Figure 2** 16S rRNA gene based phylogenetic analysis for 9 cases (nested PCR amplicons from IKC samples, designated CD1-9) in China with respect to other closely related *Mycoplasma* spp. sequences from GenBank including reference ATCC type strain collections. Neighbor-joining method; MEGA software version 6.0.

To further confirm the existence of *M. conjunctivae* in goats, isolation of mycoplasma was performed on ocular samples with modified Hayfflick's. A total of 6 strains of *M. conjunctivae* was successfully isolated and identified from the ocular samples examined (Table 1), including 4 strains from herd 1 (3 goats with IKC and 1 goat without IKC) and 2 strains from herd 2 (2 goats with IKC), which further confirmed the presence of *M. conjunctivae* in goats in southwest China. However, compared to the high detection rate of PCR methods, the isolation rate was very low. This was probably because these affected goats were treated with antibiotics before sampling, which could cause failure of recovery of *M. conjunctivae* and similar results were also reported in previous studies (Motha *et al*., 2003; Mayer, *et al*., 1996), suggesting isolation of *M. conjunctivae* from eye swabs is difficult and hard to succeed. For successful isolation of *M. conjunctivae*, it might be better to collect samples before antibiotic treatment and from animals at different clinical stages of the disease.

In summary, we reported the occurrence of caprine infectious keratoconjunctivitis in Sichuan, China, for which we present conclusive evidence that it was associated with infection with *M. conjunctivae*. This is the first report of outbreaks caused by *M. conjunctivae* infection in small ruminants in the mainland of China. The presence of highly related strains based on 16S from two distant outbreak locations indicates that this pathogen has been likely underdiagnosed and

circulating in small ruminants in China. For surveillance and controlled epidemiological purposes, the molecular detection of *M conjunctivae* via nested PCR of clinical specimens is a viable alternative method to the culture protocols currently available for this microorganism.

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