Investigation of the prevalence of *Mycoplasma ovipneumoniae* in Southern Xinjiang, China

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Abstract

Introduction: It is very important to monitor the infection of *Mycoplasma ovipneumoniae* as a potential threat to the sheep industry. Southern Xinjiang is a major sheep breeding base in China, however, there is no relevant information concerning the infection of the region's ovine stock with this bacteria at present. This study aimed to address this knowledge gap. **Material and Methods:** A total of 824 nasal swabs and the lungs of six sheep that died of pneumonia were collected in four regions between 2018 and 2020. Primers specific for *M. ovipneumoniae* and universal ones for the genus were used for PCR. Sequencing was undertaken of 159 universal primer-positive samples (153 nasal swabs and 6 lungs) and of 84 specific primer-positive samples (80 nasal swabs, 20 per region; and 4 lungs, 1 per region). The lungs were also sampled for the isolation of *M. ovipneumoniae*. A phylogenetic tree based on partial sequences of the *Mycoplasma* 16S rRNA gene was built. **Results:** The overall nasal swab positive rate for *M. ovipneumoniae* was 40.78%; the rate of animals older than 12 months was significantly different to those of younger sheep (< 3 months, 53.39%; 3 – 12 months, 46.01%; >12 months, 31.76%). Four strains of *M. ovipneumoniae* were isolated from six lungs. Phylogenetic analysis indicated their origin outside southern Xinjiang. Two other species were also detected: *M. arginine* and *M. conjunctivae*. **Conclusion:** Our survey indicated that a high level of *M. ovipneumoniae* asymptomatic colonisation in sheep, especially in lambs, affects southern Xinjiang and also confirmed the existence of *M. conjunctivae* and *M. arginine*. Our results showed that the health of sheep in southern Xinjiang is facing a great threat, and relevant prevention and control measures should be strengthened.

Keywords: molecular investigation, *Mycoplasma ovipneumoniae*, sheep, southern Xinjiang, China.

Introduction

Mycoplasma ovipneumoniae is one of the major respiratory pathogens causing pneumonia in sheep and goats with high morbidity and mortality and characterised by coughing, panting, progressive wasting and chronic proliferative interstitial pneumonia (2, 4, 5, 6, 13). This pathogen was first isolated from domestic sheep in England (14) and officially named *M. ovipneumoniae* in 1972 (7). Over the past few decades, its presence has been confirmed in many countries and has caused great losses to the sheep industry (1, 2, 10, 12, 15). The current view on its pathogenic mechanism is that when transmitted through air droplets, *M. ovipneumoniae* can invade the respiratory tract and colonise tracheal epithelial cells, then produce the toxic metabolite hydrogen peroxide which can damage the function of cilia clearance and lead to serious mixed infection (3, 18). Because of its genetic heterogeneity, so far there is no effective commercial vaccine for the prevention and control of the disease caused by *M. ovipneumoniae*.

Polymerase chain reaction is a commonly used detection method in laboratories. McAuliffe *et al.* (17) designed a pair of specific primers to amplify *M. ovipneumoniae* 16S rRNA gene sequences and they are often used by researchers (8, 19). The universal primers designed by Vankuppeveld *et al.* (21) can be

used to detect the *Mycoplasma* genus and are widely used in the identification of cell contamination. Because of its unique growth characteristics, it is difficult to isolate and culture *Mycoplasma*, but it is still the gold standard method for diagnosing of disease caused by these bacteria. Showing a difference from other *Mycoplasma* colonies, *M. ovipneumoniae* colonies isolated using modified Hayflick's medium are transparent, round, with the center deeply invaginated and have no central umbilical.

With an area of 1.63 million square kilometres and accounting for approximately one sixth of the total area in China, Xinjiang is one of the major sheep raising provinces, producing a large part of the 30 million total sheep in China recorded by the end of 2018. Xinjiang is divided into south and north regions with Tianshan as the boundary. The climates in the south and north are quite different, which is mainly reflected in the difference of precipitation and temperature, southern Xinjiang being drier than northern Xinjiang. Cheng *et al.* (8) investigated *M. ovipneumoniae* infection in sheep in northern Xinjiang and confirmed it; however, there are no corresponding data in southern Xinjiang. In this study, we estimated the prevalence of *M. ovipneumoniae* in sheep in this area. In total, 830 samples comprising 824 nasal swabs and 6 lungs were collected in four regions of southern Xinjiang between 2018 and 2020. All samples were analysed by PCR, and the lungs were also used for the isolation of *M. ovipneumoniae*. This research fills the gaps in local epidemiological knowledge of this bacterium.

Material and Methods

A total of 824 nasal swabs were sampled by stratified random sampling on eleven sheep farms in four regions in southern Xinjiang (Hotan, Kashgar, Aksu, and Bazhou). Respiratory symptoms of severe coughing and rhinorrhea were evident in 32 sheep, but the other animals had no perceptible symptoms. Stratification of the samples was based on animal age (<3months, 3 months – 12 months and >12 months), and the sheep were all local breeds (385 were Kazak breed and 439 Duolang). In addition, the lungs of six dead sheep were also collected. This tissue originated from four regions: Hotan (1 animal), Kashgar (2 animals), Aksu (2 animals), and Bazhou (1 animal); the cause of death as confirmed by autopsy was pneumonia and the lung lesions were pulmonary consolidation, pleural effusion and pleural adhesions. The nasal swabs were conserved in tubes

with modified Hayflick medium. All samples were transported under low temperature to a veterinary microbiology laboratory within 48 h. Total DNA was extracted from the samples using a DNA Extraction Kit (Tiangen, Beijing, China) following the manufacturer's instructions. Table 1 details the primers used in this study. The PCR Master Mix was prepared in a total volume of 25 μL per sample, which contained 0.125 μL of Taq DNA polymerase (5 U/μL), 1.5 μL of 25 mmol/L MgCl$_2$, 2.5 μL of 10× PCR buffer, and 2 μL of 2.5 mmol/L dNTP. Extracted DNA in a 2 μL volume was added to the mixture as the template and the reaction volume was brought up to 25 μL with ddH$_2$O. PCR products were visualised by ethidium bromide staining after electrophoresis through a 1% agarose gel. For the specific primer PCR-positive products of the four regions, partial products of each region were randomly selected for sequencing, and the universal primer PCR-positive products were all selected. Then the selected products were cloned into a pGM-Simple-T Fast vector (Tiangen, Beijing, China) for sequencing, which was completed by Sangon Biotech (Shanghai, China). The resultant sequences were compared to the published 16S rRNA genes of *Mycoplasma* on the NCBI web site and a phylogenetic tree was constructed using the neighbour-joining method by MEGA 6.0 (20).

The isolation and culture of *M. ovipneumoniae* were only conducted on lung tissue. Modified Hayflick medium comprised of pleuropneumonia-like organism (PPLO) broth (BD Diagnostics, Oxford, UK), 21 g/L; pyruvic acid, sodium salt (VWR, Chicago, IL, USA), 10 g/L; glucose (Sun, Tianjin, China), 10 g/L; fresh yeast extract (Angel, Beijing, China), 100 mL/L; heat inactivated horse serum (Gibco, Australia), 200 mL/L; penicillin (200,000 IU mL/L), 1 mL/L; and 1% phenol red (Solarbio, Beijing, China), 3 mL/L was used for the growth and maintenance of all strains. The lung tissues were cut into sections 1 cm^3 in size and ground in 2 mL of physiological saline in a sterile mortar. A 500 μL volume of the ground tissue was inoculated into the liquid medium (4.5 mL). Inoculated cultures were incubated at 37°C in a humidified atmosphere with 5% CO$_2$ for 5 to 7 days. The liquid cultures were streaked onto solid medium and cultured for 5 to 7 days for colony formation. Colony morphology and size were observed under a stereo microscope, and suspected colonies were extracted and purified. Universal primers and specific primers were used for the analysis. For lung samples, PCR-positive products of the universal and specific primers were all sequenced and compared with the results of nasal swab samples.

Table 1. Primers used in this study

Species	Primer name	Primer sequence	Annealing temperature	Amplicon size (bp)	Reference
Mycoplasma genus	MGSO RNA5	TGCACCATCTGTCACTCTGTTAACCTC AGAGTTTGATCCTGGGCTCAGGA	58°C	1021	(21)
M. ovipneumoniae	P1 P2	GACTTCATCCTGCACTCTGT TGAACGGAATATGTTAGCTT	55°C	361	(17)

Results

Because of the low isolate content of nasal swabs, in this study the isolation and culture of *M. ovipneumoniae* was only conducted on lung tissue. Four strains of *M. ovipneumoniae* were isolated from six lungs. Among the 824 tested nasal swabs, 336 samples were PCR-positive for *M. ovipneumoniae* detected using the *M. ovipneumoniae*-specific primers, and the positive rate was 40.78% (Table 2).

Table 2. *M. ovipneumoniae* PCR detection results in nasal swabs

Region	Examined	Positive	Prevalence (%)
Hotan	200	76	38.00
Kashgar	242	128	52.89
Aksu	232	73	31.47
Bazhou	150	59	39.33
Total	824	336	40.78

The positive rates among Kazak and Duolang sheep were 37.92% and 43.28%, respectively. It is worth noting that the positive rate in sheep older than 12 months was statistically significantly lower than that in younger animals (Table 3). Sequencing results of PCR products isolated with the species-specific primers showed that among the selected nasal swabs and lung samples, those of each of the four regions yielded only one sequence; the sequence results of samples in the same area were consistent. There were some divergences in the sequences between different regions. According to the sequence alignment results, the PCR-positive sample sequences amplified by the specific primers were confirmed at the molecular level as *M. ovipneumoniae*, and the PCR-positive sample sequences amplified by the universal primers were confirmed as three species of *Mycoplasma*: *M. ovipneumoniae*, *M. arginine*, and *M. conjunctivae*. A phylogenetic tree based on 16S rRNA genes indicated that the isolates were clustered in the same branch with the correlating strain. The *M. ovipneumoniae* identified in this study did not cluster in the same branch as the strains isolated in other parts of China, indicating that the genetic distance between them was greater.

Table 3. *M. ovipneumoniae* positive rates in nasal swabs by animal age

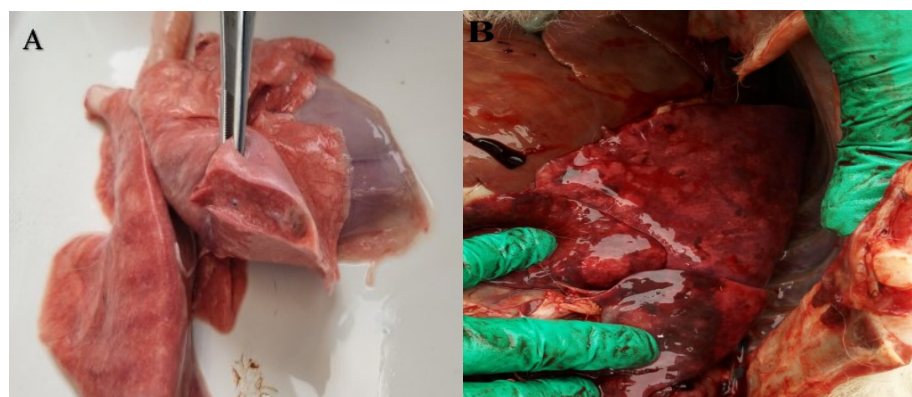| Age (months)	Examined			Positive			Prevalence (%)		
	Kazak	Duolang	Total	Kazak	Duolang	Total	Kazak	Duolang	Total
< 3	106	130	236	59	67	126	55.67	51.54	53.39
3-12	78	85	163	38	37	75	50.67	49.33	46.01
>12	201	224	425	60	75	135	29.85	33.48	31.76
Total	385	439	824	147	189	336	38.10	43.05	40.78

Fig. 1. Pathological changes in the lungs of sheep with suspected *M. ovipneumoniae* infection. A - caseous transformation; B - lung surface consolidation
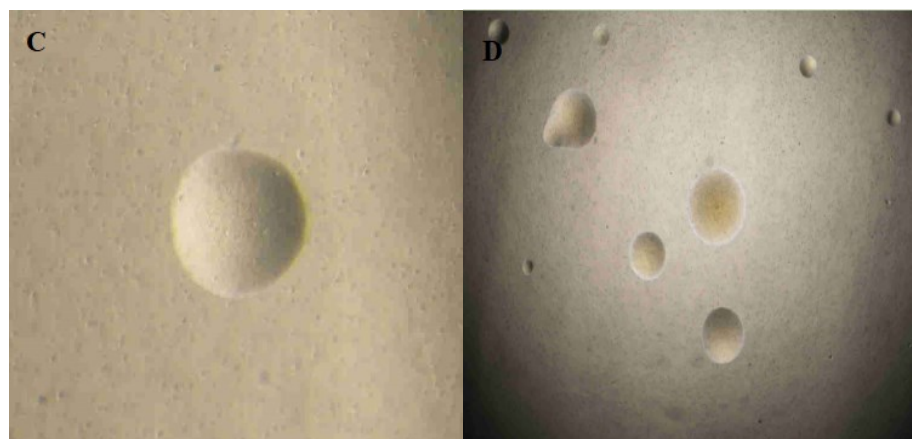

Fig. 2. Morphological identification of *M. ovipneumoniae*. Single colony of *M. ovipneumoniae* (C, 100×); multi-colony of *M. ovipneumoniae* (D, 40×)
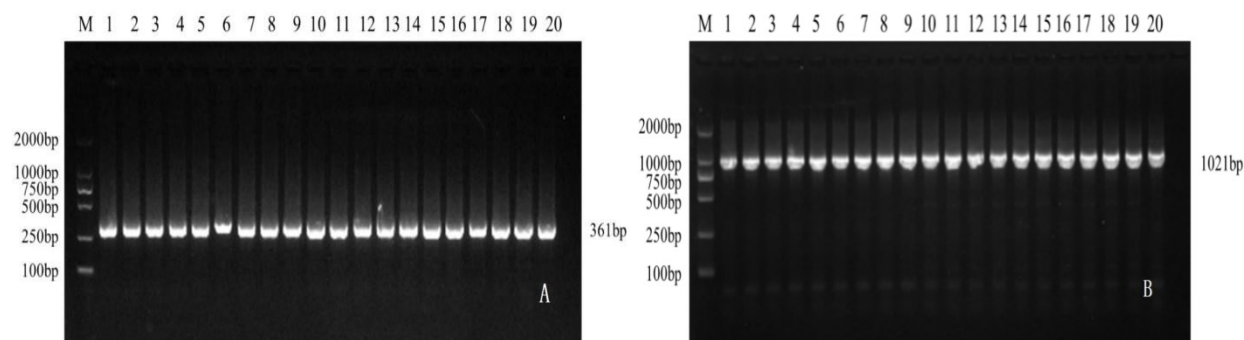
Fig. 3. Detection of *M. ovipneumoniae* and *Mycoplasma* genus in samples from sheep by PCR. M - DNA marker DL-2000 (2000, 1000, 750, 500, 250, 100 bp); A - 1–20 *M. ovipneumoniae* positive samples; B - 1–20 *Mycoplasma* genus positive samples
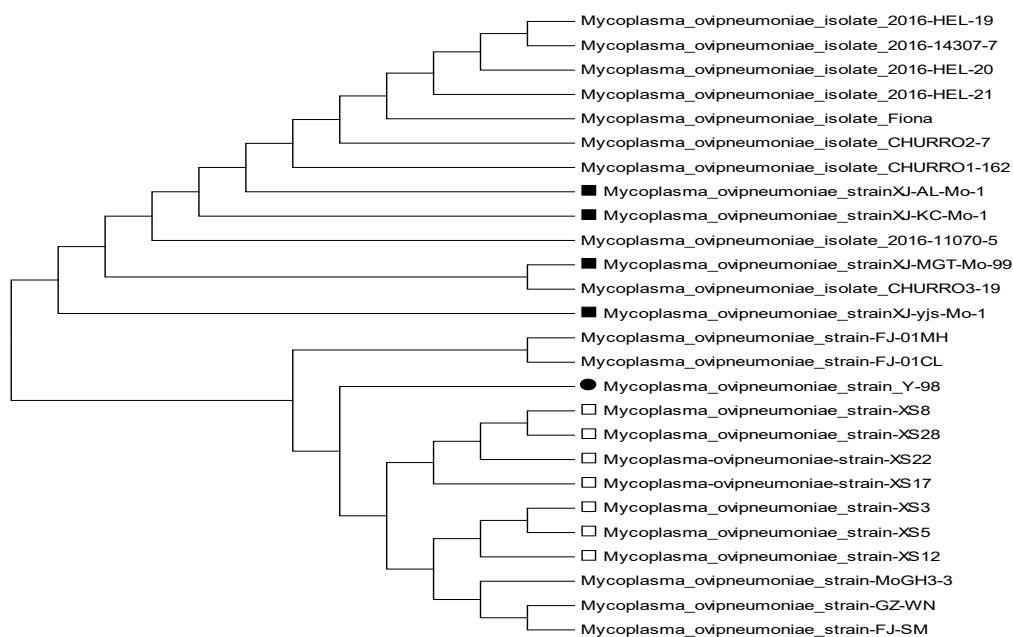

Fig. 4. Phylogenetic tree of *M. ovipneumoniae* based on partial sequences of the *M. ovipneumoniae* 16S rRNA gene amplified with specific primers. The *Mycoplasma* 16S rRNA gene sequences were aligned using MEGA 6.0 software; 1000 bootstrap replicates were used to determine the nucleotide sequence distance. A consensus phylogenetic tree was created using the neighbour-joining method. The black boxes signify the *M. ovipneumoniae* strains XJ-AL-Mo-1, XJ-KC-Mo-1, XJ-MGT-Mo-99 and XJ-yjs-Mo-1 identified in this study. The white boxes signify *M. ovipneumoniae* strains isolated in northern Xinjiang (Chen *et al.*, 2015). MoGH3-3, GZ-WN, FJ-SM, FJ-01MH are strains from inland China, and the black circle signifies the *M. ovipneumoniae* international standard strain Y-98

Discussion

In the present study, *M. ovipneumoniae* were detected in 40.78% (336/824) of the nasal swabs from sheep in southern Xinjiang, China, compared with 10.18% (119/1169) in northern Xinjiang (8). In this study, the positive rate in the Kashgar region was the highest, and the difference was significant compared with other regions (P<0.05). Also, sheep 12 months old and older were statistically significantly less frequently infected than younger animals (P<0.05). In the animals which gave all the positive nasal swab samples, only a small number (32) of individuals had obvious clinical symptoms, and the rest had no abnormal symptoms. The results show that *M. ovipneumoniae* asymptomatic colonisation was at a high level in southern Xinjiang, especially in lambs. The reason for the north–south difference could be the culmination of many factors, including individual traits, natural environment differences, and differences in epidemic strains. In terms of climate, northern Xinjiang has temperate continental arid and semi-arid climates; however, southern Xinjiang is much drier lays claim to a vast area of the Taklimakan Desert, and has a continental arid climate. Fernandez *et al.* (9) investigated the relationship between natural environmental factors and *Mycoplasma* infection rate. The results indicated that higher temperatures and lower relative humidity favour the presence of *Mycoplasma* species. The role which temperature and humidity play in the prevalence of *Mycoplasma* species needs further study. Although northern and southern Xinjiang are geographically linked, the sequence genetic relationship of isolates from

the two parts is not strong. Phylogenetic analysis indicates that the sequences of *M. ovipneumoniae* obtained in this study are closely related to strains from India and the USA, indicating that they did not originate from northern Xinjiang or inland areas of China.

In the sampling process, we found that lamb production is more intensive than that of adult sheep, which may have led to the higher positive rates for lambs than for adult sheep in this study. Despite the high asymptomatic colonisation rate, there was no large-scale morbidity or mortality on the 11 farms that were sampled; with the exception of a few cases of rhinorrhea, the rest of the sheep were performing normally. This indicated that *M. ovipneumoniae* is an opportunistic pathogen, and the incidence could be related to stress factors, as previous studies suggested (22, 23). Although for most individuals there were no obvious respiratory symptoms in this study, there could still be a hidden danger in the carriage of this pathogen by healthy sheep, and the relevant preventive measures should be strengthened.

In this study, *M. arginine* and *M. conjunctivae* were also detected. *Mycoplasma arginine* is often isolated with *M. ovipneumoniae*, but its pathogenicity has not been reported. In contrast, *M. conjunctivae* is the primary agent of infectious keratoconjunctivitis in wild and domestic caprinae species (10). *M. conjunctivae* can invade ocular structures and cause severe clinical signs that can impair vision and eventually lead to the perforation of the cornea (16). In this study, we also confirmed multiple *Mycoplasma* colonisation in a single host, although at the time of writing there was no large morbidity. Further studies are needed to estimate the risk of co-infection of *M. ovipneumoniae* and *M. conjunctivae*, which may increase the mortality rate.

In conclusion, we confirmed the prevalence of the three *Mycoplasma* species *M. ovipneumoniae*, *M. conjunctivae* and *M. arginine* in southern Xinjiang, China, and demonstrated the asymptomatic colonisation of *M. ovipneumoniae* to be at a high level. The reasons for the difference of colonisation rates between the north and south of Xinjiang need further study. Relevant prevention and control measures are necessary to be strengthened. This is the first report of the *M. ovipneumoniae* prevalence in southern Xinjiang, China.

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