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Prevalence of *Mycoplasma* Infections in Sheep and Goats in Jiangsu, China

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ABSTRACT

***Background*:** *Mycoplasma* infections are widespread globally, causing respiratory and extrapulmonary diseases in animals, particularly in sheep and goats. Symptoms include coughing, nasal discharge, respiratory distress, and pneumonia. Several *Mycoplasma* species like *Mycoplasma ovipneumoniae* (MO), *Mycoplasma capricolum* ssp. *capripneumoniae* (MCCP), *Mycoplasma mycoides* ssp. *capri* (MMC), and *Mycoplasma arginini* (MA) are identified as causes. MA was first identified in sheep with respiratory diseases in 1972, and its presence has been consistently reported. In China, MA isolation from respiratory samples was achieved in 1991, correlating with pulmonary lesions. Sheep and goats are economic resources in China. In recent years, China encourages the sheep and goat breeding model to convert into scale. Nevertheless, sheep and goats farming are threatened by *Mycoplasma*, which greatly slowed down the production. Currently, there is a scarcity of data and reports regarding *Mycoplasma* infection in sheep and goat farming in Jiangsu province. This study aims to determine the prevalence and species of *Mycoplasma* in sheep and goats in Jiangsu province. The results may contribute to the prevention and control of *Mycoplasma* infection in Jiangsu region, and will also expand our understanding of the distribution of these respiratory diseases in sheep and goats.

***Materials, Methods & Results*:** The study collected 920 nasal swab samples from sheep and goats in six cities in Jiangsu province. Following sample processing, DNA extraction was carried out using the lysis boiling method for both clinical samples and reference strains. Subsequently, PCR amplification was conducted utilizing universal and specific primers to detect and identify *Mycoplasma* species. Finally, the positive PCR products underwent sequencing and were compared for phylogenetic analysis. The results show that through using PCR specific primers to amplify genes associated with the *Mycoplasma*, 109 (11.85%) were confirmed to be *Mycoplasma* positive out of 920 samples that were collected from clinically diseased (470) and healthy (450) sheep and goats from Jiangsu province, China. 109 positive samples were obtained and classified as MO in 78.9% and MA in 21.1%. No MCCP and MMC were detected, and there was no evidence of mixed Mycoplasma infection. The phylogenetic analysis of the partial sequences of positive samples based on the MO p113 gene and MA ADI gene showed a high degree of similarity with some sequences of *Mycoplasma* in GenBank.

***Discussion*:** Among clinically healthy animals, 28 out of 450 samples (6.22%) tested positive, while among those with respiratory diseases, 81 out of 470 samples (17.23%) were positive, indicating a higher risk of respiratory disease in infected sheep and goats. *Mycoplasma* was found even in asymptomatic sheep and goats, suggesting it may not be the sole cause of respiratory symptoms. Immunized animals had lower infection rates, indicating some effectiveness of current vaccines, though overall efficacy remains modest. Regional variations in infection rates may stem from differences in vaccination and hygiene practices. Out of 109 positive samples, 86 were identified as MO infection and 23 as MA infection, while no infections of MCCP and MMC were detected. In this study, MO isolates lacked a central umbilicus in colonies, while MA isolates resembled "fried egg like" morphology. MO P113 gene sequencing revealed lower homology (86.0%) with certain strains compared to ATCC 29419, GZ-QX1, and NCTC 10151. This discrepancy could be ascribed to genetic mutations or disparities in pathogenic strains. MA ADI gene sequencing showed high homology (> 99.1%) with ATCC 23838, NCTC 10129, and HAZ145 strains. This indicates that prevalent Mycoplasma linked to respiratory diseases in Jiangsu province are MO and MA.

Keywords: ovine, caprine, small ruminants, *Mycoplasma*, epidemiology, sequencing, hylogenic analysis.

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INTRODUCTION

Mycoplasma infections are widely prevalent globally and are known to cause respiratory and extra-pulmonary body sites diseases in animals [5,15]. Particularly in sheep and goats, *Mycoplasma* is a prominent pathogen associated with respiratory diseases [14]. Multiple *Mycoplasma* species, such as *Mycoplasma ovipneumoniae* (MO), *Mycoplasma capricolum* ssp. *capripneumoniae* (MCCP), *Mycoplasma mycoides* ssp. *capri* (MMC) and *Mycoplasma arginini* (MA) have been identified as potential causes of respiratory diseases in sheep and goats [6]. Clinical manifestations commonly observed in sheep and goats infected with respiratory diseases include elevated body temperature, coughing, respiratory distress, weight loss, and the presence of serous and fibrinous inflammation in the chest and pleura [12,18]. The manifestation of these respiratory diseases can occur either acutely or chronically [9]. In 1972, MA was first identified in sheep experiencing respiratory diseases [1]. Since then, multiple researchers have consistently reported the occurrence of MA in the sheep with respiratory diseases [3,10,19]. In China, the successful isolation of MA was achieved for the first time in 1991 [20].

Currently, there is a scarcity of data and reports regarding *Mycoplasma* infection in sheep and goat farming in Jiangsu province. This study aims to determine the prevalence and species of *Mycoplasma* in sheep and goats in Jiangsu province. The results will contribute to the prevention and control of *Mycoplasma* infection in Jiangsu region, as well as, will expand our understanding of the distribution of these respiratory diseases in sheep and goats.

MATERIALS AND METHODS

Reference strains, Chemicals & Samples collection

Reference strains: The reference strains of *Mycoplasma* ovipneumoniae Y98, *Mycoplasma capricolum* ssp. *capripneumoniae* F38, and *Mycoplasma mycoides* ssp. capri PG3 were graciously supplied by Professor Yuefeng Yu from Lanzhou Veterinary Research Institute and Professor Maojun Liu from the Chinese Academy of Agricultural Sciences. *Escherichia coli* (ATCC25922), *Salmonella* (ATCC36400), *Staphylococcus aureus* (ATCC25923), clinical isolates of *Clostridium perfringens* type E, and *Mycoplasma arginini* isolate gy1 are all preserved isolates in the Microbiology Teaching and Research Department of the College of Veterinary Medicine at Yangzhou University (YZU).

- Chemicals: *Mycoplasma* broth medium1, *Mycoplasma* agar medium[1], Premix Taq[2], DL2000 Marker[2], pMD 19-T2, DH5α Competent Cells[3], Axy-Prep DNA Gel Extraction Kit[4].

- Samples Collection: From December 2020 to May 2021, a comprehensive collection of 920 nasal swab samples from sheep and goats was conducted across six cities in Jiangsu, including Nantong, Yangzhou, Taizhou, Xuzhou, Lianyungang, and Suqian (Figure 1). These nasal swabs were meticulously placed in centrifuge tube containing 2 mL of physiological saline, accurately labelled, and subsequently stored in a controlled temperature incubator until they were transported to the laboratory for subsequent analysis. Before testing, the nasal swabs were mixed extensively with physiological saline and subjected to low-speed centrifugation at 2000 *g* for 2 min. From the resulting supernatant, 0.5 mL was collected and subsequently filtered through a 0.22 μm PVDF membrane under sterile conditions to eliminate bacteria. The filtered liquid was then introduced into 4.5 mL of *Mycoplasma* broth medium and incubated at 37°C in a biochemical incubator. The monitoring of the color change in the liquid medium was conducted, and in the event that the bacterial liquid exhibited an orange-yellow hue without any signs of turbidity, subculturing was carried out. Conversely, if the bacterial liquid displayed turbidity, it was subjected to sterile filtration through a 0.22 μm PVDF membrane, and this process was repeated for three times utilizing the aforementioned methodology.

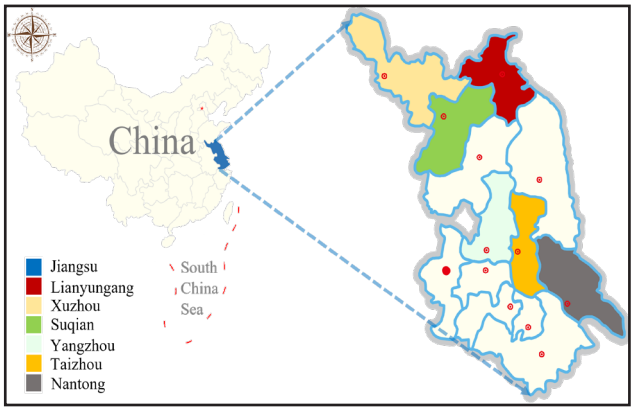

Figure 1. Distribution map of sampling districts of 6 cities in Jiangsu of China.

Clinical sample detecting

DNA templates were prepared from the aforementioned clinical sample cultures and reference bacterial strains utilizing a DNA lysis boiling. The experimental process is as follows: a volume of 1 mL of the bacterial suspension was transferred to a 1.5 mL centrifuge tube and subjected to centrifugation. The resulting supernatant was discarded, and the pellet was reconstituted in 0.8 mL of distilled water, followed by another centrifugation at 10,000 *g* for 10 min. The supernatant was discarded, and the previous step was repeated iteratively until the supernatant appeared colourless. Subsequently, 0.2 mL of distilled water was added, the pellet was resuspended, and the mixture was incubated in a 100°C metal bath for 10 min. Next, the mixture was cooled on ice for 5 min and then subjected to centrifugation at 4°C and 10,000 *g* for 5 min. After the DNA concentration was determined using a NanoDrop 2000, the supernatant was stored at a temperature of -20°C.

A pair of universal primers (Table 1) were synthesized in accordance with previously published literature to detect clinical sample [18]. The PCR Master Mix was prepared in a total volume of 25 uL per sample, which contained 12.5 μL of Premix Taq and 1.0 μL of each upstream and downstream primers (10 mmol/L). Extracted DNA in a 1 uL volume was added to the mixture as the template and the reaction volume was brought up to 25 uL with ddH_2O. The universal primers cycling parameters can be found in Table 2. Following the completion of the reaction, 10 μL of the second round PCR product was loaded onto a 1% agarose gel prepared with TAE buffer. Electrophoresis was conducted at 120 V for approximately 30 min, and the gel was subsequently visualized and photographed using a UV gel imaging system[5]. DL 2000 DNA Marker was employed as a reference. Following the completion of the reaction, 10 μL of the second round PCR product was loaded onto a 1% agarose gel prepared with TAE buffer. Electrophoresis was conducted at 120 V for approximately 30 min, and the gel was subsequently visualized and photographed using a UV gel imaging system[5]. DL 2000 DNA Marker was employed as a reference.

Table 1. Primer sequences of four species of *Mycoplasma.*

Primers		Sequence	Amplified product
Universal Primers	MGSO	TGCACCATCTGTCACTCTGTTAACCTC	1021 bp
	RNA5	AGAGTTTGATCCTGGGCTCAGGA	
Specific Primers	P113-F	TCTCCCAGATGATGCTAACC	287 bp
	P113-R	GCTGAAAATCAACTGGTCTAA	
	Spe-F	ATCATTTTTAATCCCTTCAAG	316 bp
	Spe-R	TACTATGAGTAATTATAATATATGCAA	
	3740-F	GTGATAAAGTTATTGACTTTATTAG	435 bp
	3740-R	TTATTGTACTGTATTGTGTTCTTC	
	ADI-F	GATGGCAGGGATCACAAAATACGA	806 bp
	ADI-R	ACATACAACGAGCGTTACCCATACC	

Table 2. Cycle parameters of different primers.

Primers	Cycling Parameters
Universal Primers	95°C 5 min; 94°C 30 s, 58°C 30 s, 72°C 30 s, 35 cycles
P113	95°C 5 min; 94°C 30 s, 60°C 30 s, 72°C 30 s, 30 cycles
Spe	94°C 5 min; 94°C 30 s, 57°C 45 s, 72°C 45 s, 35 cycles
3740	95°C 5 min; 95°C 30 s, 50°C 20 s, 72°C 30 s, 35 cycles
ADI	94°C 5 min; 94°C 30 s, 52°C 30 s, 72°C 30 s, 35 cycles

Specific identification of Mycoplasma *species*

Four pairs of specific primers (Table 1) were synthesized in accordance with previously published literature to identify different species of *Mycoplasma* [8,21,25]. Primer P113 was designed for the specific amplification of MO, primer Spe for the specific amplification of MCCP, primer 3740 for the specific amplification of MMC, and primer ADI for the specific amplification of MA. The PCR Master Mix was prepared in a total volume of 25 μL per sample, which contained 12.5 μL of Premix Taq and 1.0 μL of each upstream and downstream primers (10 mmol/L). Extracted DNA in a 1 μL volume was added to the mixture as the template and the reaction volume was brought up to 25 μL with ddH_2O. The specific primers cycling parameters can be found in Table 2. Following the completion of the reaction, 10 μL of the second round PCR product was loaded onto a 1% agarose gel prepared with TAE buffer. Electrophoresis was conducted at 120 V for approximately 30 min, and the gel was subsequently visualized and photographed using a UV gel imaging system[5]. DL 2000 DNA Marker was employed as a reference.

For the specific primer PCR positive products of the six 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 pMD 19-T vector for sequencing[6]. 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 neighbourjoining method by MEGA 7.0.

Mycoplasma *separation & purification*

The samples that yielded positive results for *Mycoplasma* through PCR analysis were subjected to culturing in *Mycoplasma* broth medium, followed by three subsequent subcultures. Afterwards, the samples were streaked onto *Mycoplasma* agar medium and incubated in a biochemical incubator set at 37°C for 3 to 5 days. Colonies exhibiting suspicion were carefully selected under low magnification microscopy and subsequently transferred to fresh *Mycoplasma* agar medium for further cultivation. The aforementioned procedure of colony picking and subculturing was iterated thrice in succession, with suspected individual colonies being employed for the inoculation of liquid culture medium. The resultant inoculum derived from a solitary colony was utilized for the preparation of DNA templates. Universal primers and specific primers were used for the analysis.

RESULTS

Clinical sample testing

PCR testing of 920 clinical nasal swab samples yielded a positive result for *Mycoplasma* infection in 109 samples, accounting for 11.85% of the total samples (Table 3).

Among sheep and goats classified as clinically healthy, 28 samples (6.22%) yielded positive results, whereas among sheep and goats afflicted with respiratory diseases, 81 samples (17.23%) exhibited positive outcomes. Although the group immunized with the contagious pleuropneumonia vaccine exhibited a lower positivity rate for *Mycoplasma* than the nonimmunized group (Table 3), it is important to note that the vaccine does not confer absolute protection against *Mycoplasma* infection in the respiratory tract. The findings of the study indicate that there is significant variation in *Mycoplasma* positivity rates across different regions (Table 4). Notably, the highest rate of *Mycoplasma* infection was observed in Lianyungang, where the positivity rate among sheep and goats with respiratory diseases reached 35%. Furthermore, even among clinically healthy sheep and goats in Lianyungang, the infection rate was found to be as high as 13.75%. In contrast, Yangzhou exhibited comparatively lower rates of *Mycoplasma* infection among sheep and goats, with a positivity rate of 6.67% among those with respiratory diseases and 3.33% among clinically healthy sheep and goats.

Specific PCR identification of Mycoplasma *species*

Species specific PCR testing was conducted on 109 nasal swab samples that yielded positive results in the universal PCR assay (Table 5) for identifying *Mycoplasma* species in lamb infections. The findings revealed that out of these samples, 86 were identified as MO infection and 23 as MA infection, while no infections of MCCP and MMC were detected. Furthermore, the occurrence of mixed *Mycoplasma* infections was not observed.

Morphological observation & PCR identification of colonies

When grown on *Mycoplasma* agar medium, *Mycoplasma* exhibits the formation of semi-transparent colonies that are centrally elevated, nearly circular, and of pinpoint size. Microscopic examination reveals that MO colonies do not possess central umbilication,

while MA colonies exhibit a characteristic "fried egg like" morphology (Figure 2). Colonies suspected to be *Mycoplasma* were selected and cultured in liquid medium, and their DNA templates were extracted for identification using universal primer PCR. A total of 19 isolates of *Mycoplasma* were identified. Based on specific primers classification was conducted on a total of 19 *Mycoplasma* isolates obtained from sheep and goats. The results indicated that 10 isolates were identified as MO, while 9 isolates were classified as MA.

Analysis of gene sequences

A phylogenetic tree was constructed using MEGA 7, and phylogenetic analysis indicated that the positive samples in this study cluster on the same evolutionary branch as MO or MA. In the analysis of MO P113 gene sequences, the results revealed that the homology among the positive samples ranged from 87.8% to 99.7%. When compared to reference strains ATCC 29419, GZ-QX1, and NCTC 10151, the homology ranges from 86% to 96.8%, with some positive samples showing lower homology to the reference strains. In the analysis of MA ADI gene sequences, the results revealed that the homology among the positive samples ranged from 99% to 100%. When compared to reference strains ATCC 23838, NCTC 10129, and HAZ145, the homology ranges from 99.1% to 99.5%, indicating a higher degree of homology (Figure 3).

Table 3. Detection results of *Mycoplasma* infection.

Samples	No. of sample	No. of positive	% of positive
Clinically Healthy	450	28	6.22
Respiratory Diseases	470	81	17.23
Vaccine unimmune	380	56	14.74
Vaccine immune	540	53	9.81
Total	920	109	11.85

Table 4. *Mycoplasma* infection in different sheep and goats.

Region	Clinically Healthy	No. of positive	Respiratory Diseases	No. of positive
Nantong	100	5	120	14
Lianyungang	80	11	80	28
Yangzhou	60	2	60	4
Xuzhou	60	2	50	7
Suqian	100	5	100	16
Taizhou	50	3	50	12
Total	450	28	470	81

Table 5. Detection and isolation results of *Mycoplasma* in different areas.

Region	Species	No. of positive	% of positive
Nantong	MO	14	6.36
	MA	5	2.27
Lianyungang	MO	31	19.38
	MA	8	5.00
Yangzhou	MO	6	5.00
	MA	0	0
Xuzhou	MO	7	6.36
	MA	2	1.82
Suqian	MO	17	8.50
	MA	4	2.00
Taizhou	MO	11	11.00
	MA	4	4.00
Total		109	11.85

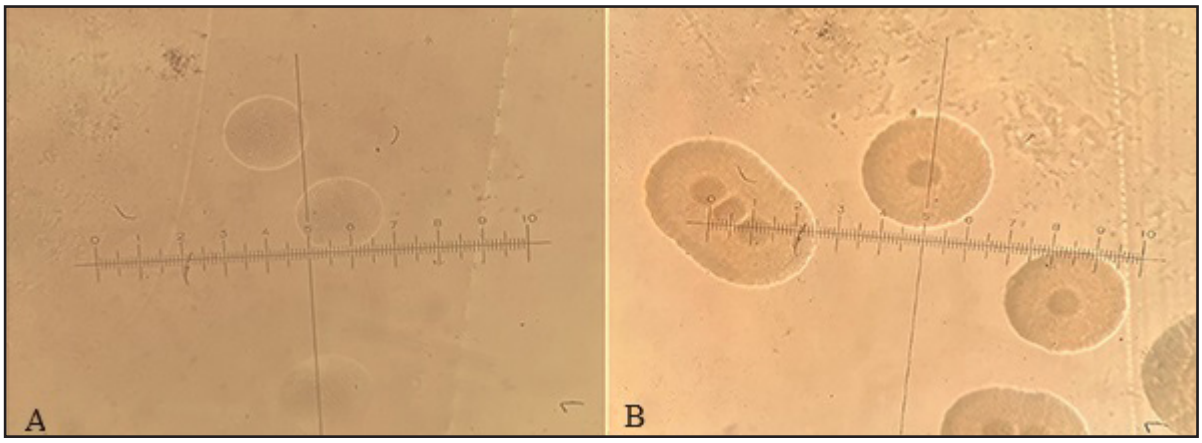

Figure 2. The colonies of *Mycoplasma* on agar medium (×20). A. MO; B. MA.

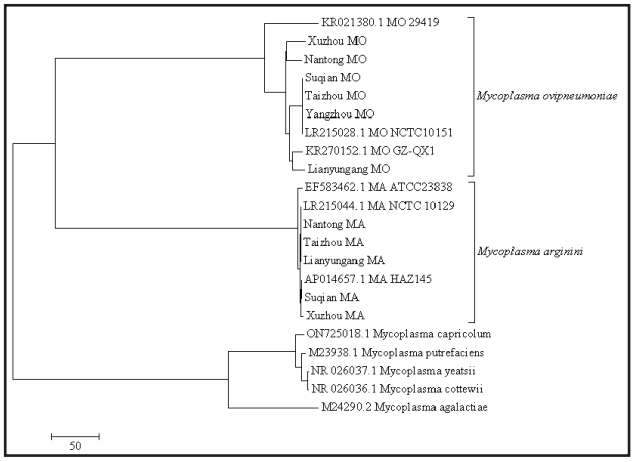

Figure 3. Analysis of Gene Sequences.

DISCUSSION

Mycoplasma was initially discovered in 1898 during research at the Pasteur Institute, where isolated from pleural fluid in diseased cattle [16]. In 1962, the first successful isolation of *Mycoplasma* in artificial culture media was achieved [4]. The occurrence of contagious caprine pleuropneumonia in China has been sporadically reported since the previous century, with an epidemic of contagious caprine pleuropneumonia in goats occurring in Inner Mongolia in 1935 [7]. In 1997, the widespread occurrence of *Mycoplasma* in more than ten provinces and regions of China was reported [2]. In 1998, the instances of *Mycoplasma* infection in sheep and goats in various regions of Sichuan, including Aba and Liangshan, where the infected sheep and goats exhibited typical respiratory symptoms [24]. From 2014 to 2015, the provinces of Inner Mongolia, Hebei, and Jilin observed seropositive rates of 76.80%, 50.00%, and 21.99%, respectively, indicating the presence of *Mycoplasma* antibodies in sheep and goats [22]. These findings and investigations collectively illustrate the significant impact of *Mycoplasma* on China's lamb farming industry.

In this study, we utilized previously published primers that were specifically designed for the conserved region of the Mycoplasma 16S rRNA gene to conduct expeditious PCR testing on nasal swabs obtained from lamb farms of varying sizes in Jiangsu [17]. This approach facilitated the examination of the overall prevalence of *Mycoplasma* infection in sheep and goats from Jiangsu. Among sheep and goats classified as clinically healthy, 28 out of 450 samples (6.22%) yielded positive results, while among sheep and goats afflicted with respiratory diseases, 81 out of 470 samples (17.23%) exhibited positive outcomes. This finding suggests a significantly higher likelihood of developing respiratory disease in sheep and goats afflicted with *Mycoplasma* than in those without *Mycoplasma*. Furthermore, the findings of this study suggest that *Mycoplasma* can be present in the respiratory tracts of sheep and goats even in the absence of clinical manifestations, thereby indicating that respiratory symptoms in sheep and goats cannot necessarily be attributed solely to *Mycoplasma* infection. The incidence of *Mycoplasma* infection in sheep and goats that have received immunization is significantly lower than that in sheep and goats that have not been immunized, indicating a certain level of effectiveness in the current vaccine-based immunization approach for preventing *Mycoplasma*. However, the overall

efficacy of immunization remains below the desired level. The prevalence of *Mycoplasma* infection in sheep and goats exhibits regional variations, which are likely attributed to differences in local vaccine immunization protocols and daily hygiene practices [13]. A total of 19 *Mycoplasma* isolates were isolated from 109 nasal swab samples that exhibited positive results for *Mycoplasma* (109/920). Following the identification process based on specific primers through PCR, 10 isolates (10/19) were classified as MO, 9 isolates (9/19) as MA, while strains MCCP and MMC were not detected. The findings of this study suggest that the prevailing bacterial strains linked to respiratory diseases infection in sheep and goats from Jiangsu Province are MO and MA.

In the present study, the MO isolates exhibited colonies lacking a central umbilicus, whereas the MA isolates displayed colonies resembling the characteristic "fried egg like" morphology, which aligns with previous findings reported MO and MA isolates from Xinjiang, China. The analysis of MO P113 gene sequencing results indicated that certain MO from Jiangsu displayed a comparatively lower degree of similarity with ATCC 29419, GZ-QX1, and NCTC 10151 strains, with the minimum identity observed at 86.0%. This result aligns with findings of similar homology between P113 gene sequences of four MO isolates from Anhui and ATCC 29419 and GZ-QX1 strains, ranging from 88.6% to 89.3% [11,23]. It was reported that the identity between the P113 gene in GZ-CS1 and GZ-QX1 strains from Guizhou and the prevailing SCO1 strain in Sichuan was merely 90.2% and 89.1%, respectively. This discrepancy could be ascribed to genetic mutations or disparities in pathogenic strains. The analysis of MA ADI gene sequencing results indicated that the MA from Jiangsu displayed a homology of more than 99.1% with the reference strains ATCC 23838, NCTC 10129, and HAZ145. These findings offer a more specific foundation for the prevention of *Mycoplasma* in sheep and goats within Jiangsu province.

CONCLUSION

Sheep and goats afflicted with *Mycoplasma* exhibit a notably heightened susceptibility to respiratory diseases. The predominant bacterial strains associated with *Mycoplasma* infection in sheep and goats from Jiangsu province are MO and MA. In the future, it is necessary to conduct a more comprehensive investigation of *Mycoplasma* in a wider range of regions and sheep and goats with different ages.

MANUFACTURERS

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***Ethical approval*.** All animals were handled in strict accordance with good animal practice as defined by the Animal Ethics Procedures and Guidelines of the People's Republic of China. The study protocol was approved by the Animal Care and Use Committee of the College of Veterinary Medicine, Yangzhou University (Approval ID: SCXK [Su] 2021-0013).

***Declaration of interest*.** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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