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Molecular Prevalence of *Mycoplasma ovipneumoniae* in Small Ruminants in Tamil Nadu, India

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Mycoplasma ovipneumoniae (M. ovipneumoniae) causes atypical, non-progressive pneumonia in sheep and goats, leading to significant economic losses in the global small-ruminant industry. In the current study, we assessed the molecular prevalence of *M. ovipneumoniae* infections in sheep and goats showing respiratory Clinical signs in Tamil Nadu, India.During the period from January 2023 to March 2024, a total of 200 samples (186 nasal swabs and 14 lung tissue samples) were collected from sheep and goats exhibited typical respiratory signs reared in various districts of Tamil Nadu. The growth of mycoplasma on cultures indicated by a color change in Mycoplasma experience liquid media, were used for Polymerase Chain Reaction (PCR) assay targeting the 16S rRNA gene of the *Mycoplasma* genus and *M. ovipneumoniae* specifically. The results showed that 78 out of 200 samples (39%) tested positive in the 16S rRNA Mycoplasma genus-specific PCR test, with an overall positivity rate of 30% for *M. ovipneumoniae.*This study revealed that *M. ovipneumoniae* is the most common Mycoplasma species found in sheep and goats with respiratory infections in Tamil Nadu.

Keywords: Small ruminants; Mycoplasma ovipneumoniae; polymerase chain reaction; Tamil Nadu.

1. INTRODUCTION

"Small ruminants, particularly sheep and goats, significantly contribute to the Indian farming economy through milk and meat production. As a result, they are valuable assets to farmers. According to the Food and Agriculture Organization, Asia had the largest sheep and goat populations in the world, accounting for 43.6% and 55.4% of the global sheep and goat populations, respectively" [1]. In India, the 20th livestock census (2019) reported a populations of 74.26 million sheep and 148.88 million goats.

Among all diseases that affect small ruminants, respiratory diseases represent 5.6% [2]. These infections lead to poor body weight gain and high mortality in sheep and goat flocks [3]. Respiratory diseases in these animals have multifactorial causes involving numerous etiological agents [4].

Mycoplasmosis is an emerging and transboundary disease in small ruminants, causing significant economic losses for farmers [5]. "Mycoplasmas are the smallest self-replicating prokaryotic, organisms ranging from spherical (0.3 to 0.9 µm in diameter) to filamentous (up to 1.0 µm long). Unlike other bacteria, they lack a rigid cell wall but have a flexible three-layered cytoplasmic membrane, allowing them to pass through bacterial membrane filters with pore sizes of 0.22 µm to 0.45 µm. They replicate by binary fission or by breaking up elongated forms and are known for forming fried-egg microcolonies on agar plates. The *Mycoplasmataceae* family requires sterols for growth, and their genome size ranges from 540 to 1300 kb" [6].

Common Mycoplasma pathogens causing respiratory infections in small ruminants include *M. mycoides* subsp*. capri* (goats), *M. capricolum* subsp. *capripneumoniae* (goats), *M. capricolum* subsp. *capricolum* (goats), and *M. ovipneumoniae* (sheep and goats) [7,8]. *M. ovipneumoniae*, is considered the cause of non-progressive pneumonia. In 1963, *M. ovipneumoniae* was first isolated from the lung of a diseased sheep in Scotland [9] and then identified in Australia in 1972 [10]. Along with other pathogenic mycoplasmas, *M. ovipneumoniae* can cause variations in morbidity and mortality within the same animal [11,12,13]. Diagnosis based on clinical and post- mortem lesions is not definitive, as symptoms overlap with other infections. Mycoplasma is highly fastidious and difficult to isolate in artificial media. However, molecular techniques, such as PCR tests using specific oligonucleotide primers, enable rapid and specific detection of *M. ovipneumoniae*.

The present study is aimed to assess the prevalence *Mycoplasma ovipneumoniae* by molecular methods in the samples collected from sheep and goats with respiratory signs from various parts of Tamil Nadu.

2. MATERIALS AND METHODS

2.1 Sample Collection

Nasal swabs were collected from sheep and goats that showed respiratory signs , from

various organized and private farms in Salem, Kallakurichi, Namakkal, Chengalpet and Nilgiri districts of Tamil Nadu. Additionally, samples were collected from ailing sheep and goats brought to the Veterinary Clinical Complex, Veterinary College and Research Institute, Salem. The samples were aseptically collected. A total of 200 samples were collected, that include 186 nasal swabs (102 from sheep and 84 from goats) and 14 lung tissue samples (2 from sheep and 12 from goats). These samples were then inoculated in Mycoplasma experience liquid media (M/s. Mycoplasma Experience Private limited,UK).

2.2 Sample Inoculation

Nasal swabs and lung tissue samples from sheep and goats suspected of Mycoplasmosis were inoculated into 2ml of Mycoplasma Experience Liquid Medium (Code No. ML50, Ms. Mycoplasma Experience Pvt Ltd, UK) and incubated at 37°C for 2 to 3 days. The inoculated tubes were examined daily for the growth by means of mass turbidity and color change, (color shift from red to slight yellow). The positive cultures, shown color change were subjected to DNA extraction and PCR assay. The samples positive by PCR assay have been subjected to isolation of *Mycplasma species* in solid media. A loopful of liquid culture was inoculated in the agar plates (Mycoplasma Solid Agar Media, Code No. MS5, Ms. Mycoplasma Experience Pvt Ltd, UK) and incubated at 37°C in a candle jar for 10-15 days.

2.3 DNA Extraction

The boiling method [14] was followed for DNA extraction from the positive broth cultures. The broth suspension was centrifuged at 10,000 rpm for 10 minutes, followed by two washing with PBS, and then the pellet was re-suspended in Nuclease free water. This suspension was boiled at 96°C for 10 minutes, then snap cooled in deep freezer and centrifuged at 10,000 rpm for 2 minutes. The supernatant was collected and stored at -20°C for use in PCR assays.

2.4 Polymerase Chain Reaction

PCR assay for the detection of the 16S rRNA gene of the *Mycoplasma* genus and *Mycoplasma ovipneumonia* was performed [15,11]. The PCR amplification was performed in a total volume of 20 µL in 200 µL PCR tubes. Each reaction contained 6 µL of molecular biology grade nuclease-free water, 10 µL of 2X PCR master mix, 1 µL (10 pM) of each forward and reverse primer and 2 µL of DNA. The reaction mixture without template DNA was included as negative control to monitor for contamination. The contents of the tubes were gently mixed, briefly spun, and then placed in a thermal cycler for amplification (M/s BioRad, Germany).

2.4.1 Identification of 16S rRNA gene specific to genus Mycoplasma

“A Mycoplasma genus-specific PCR was performed using the primers targeting the 16S rRNA gene. The primers used were GPO3F (5’ TGG GGA GCA AAC AGG ATT AGA TAC C 3’) and MGSO (5’ TGC ACC ATC TGT CAC TCT GTT AAC CTC 3’), which are expected to amplify a 280 bp product” [15]. The thermal cycling conditions for PCR amplification were as follows: initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 15 seconds, annealing at 59.3°C for 15 seconds, extension at 72°C for 15 seconds, and a final extension at 72°C for 5 minutes.

2.4.2 Identification of *Mycoplasma ovipneumoniae*

“PCR was carried out using *M. ovipneumoniae*-specific primers MOVPF (5’ GTT GGT GGC AAA AGT CAC TAG 3’) and MOVPR (5’ CTT GAC ATC ACT GTT TCG CTG 3’), designed to amplify a 418 bp product” [11]. The thermal cycling conditions for PCR amplification were as follows: initial denaturation at 94°C for 1 minute, followed by 35 cycles of denaturation at 94°C for 60 seconds, annealing at 61.5°C for 60 seconds, extension at 72°C for 120 seconds, and a final extension at 72°C for 5 minutes.

2.5 Agarose Gel Electrophoresis

Amplified products of Mycoplasma genus and *Mycoplasma ovipneumoniae* targeting the 16S rRNA gene were analyzed by agarose gel electrophoresis in 1.5% agarose gel in TAE buffer containing ethidium bromide , along with 100bp DNA molecular weight marker at 80 V for 50 minutes.. The gels were viewed under a UV transilluminator and documented using a gel documentation system.

3. RESULTS

In the present study, on culturing 200 samples in Mycoplasma experience liquid medium 120 samples (60%) showed colour changes (Fig. 1). The genus-specific 16S rRNA gene of

Mycoplasma was amplified by PCR assay in 78 samples out of 200 samples (Table 1) revealing a 280 bp amplicon (Fig. 2) confirmed a prevalence of 39% Mycoplasma infection among small ruminants in Tamil Nadu.

The *M. ovipneumoniae*-specific 16S rRNA gene with amplicon size of 418 bp were amplified by PCR in 60 samples out of 200 samples (Fig. 3). The molecular assay revealed a prevalence of 30% *M.ovipneuminia* infection in small ruminants in Tamil Nadu.

On culture and isolation, 45 samples showed characteristic fried egg microcolonies on Mycoplasma experience solid media (Fig. 4).

Table 1. Details of samples screened by PCR

Species	No. of samples screened			No. of Positive Samples by *Mycoplasma* genus specific PCR	No. Positive Samples by *Mycoplasma ovipneumoniae* specific PCR	*Mycoplasma ovipneumoniae* Prevalence (%)
	Nasal swabs	lung	Total			
Sheep	102	2	104	50	35	33.65
Goat	84	12	96	28	25	26.04
Total	186	14	200	78	60	30.00

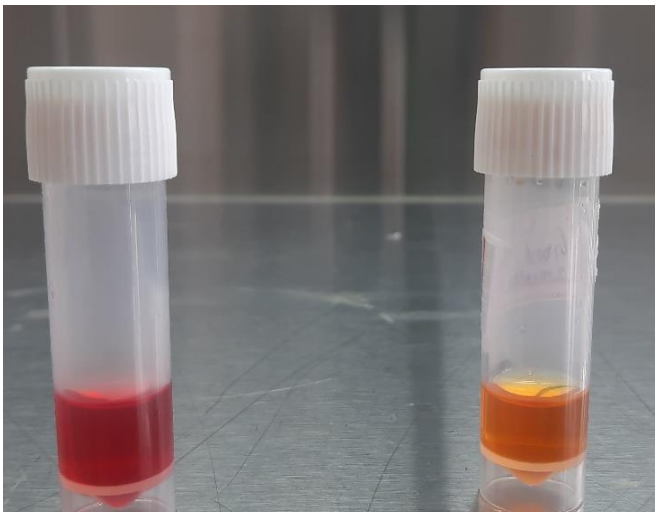

Fig. 1. Colour change in Mycoplasma experience liquid media inoculated with nasal swab after 3 days of incubation

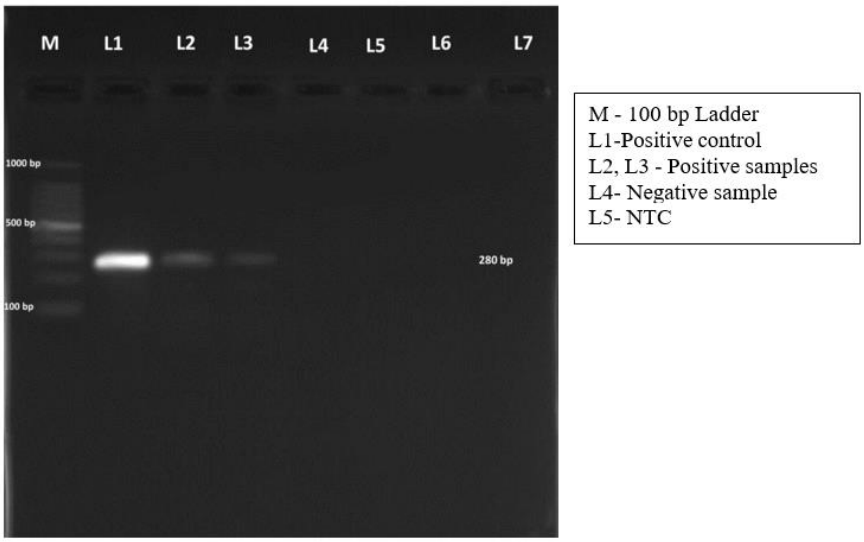

Fig. 2. *Mycoplasma* genus specific PCR product

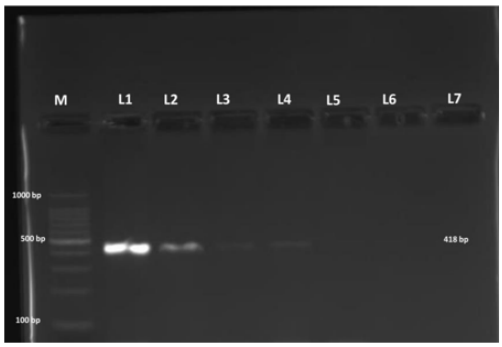

Fig. 3. *Mycoplasma ovipneumoniae* specific PCR

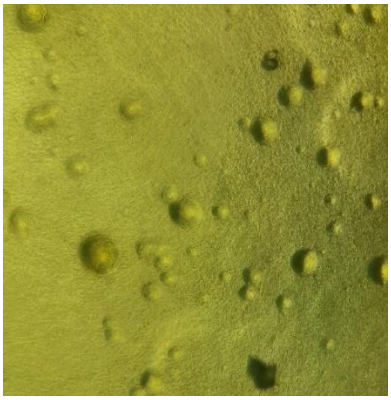

Fig. 4. Fried egg colonies of Mycoplasma in Mycoplasma experience solid media (10X)

4. DISCUSSION

"Respiratory infections cause significant economic losses in small ruminants. While many etiological agents are involved, *Mycoplasma species* are particularly substantial contributors to these infections, having a considerable socioeconomic impact, especially in regions where small ruminants are a key source of milk and meat. Mycoplasma infections are associated with various severe problems, including contagious caprine pleuropneumonia, conjunctivitis, arthritis, mastitis, and mild respiratory distress" [16]. "*M. ovipneumoniae* and *M. arginini* are frequently found in pneumonic lesions among small ruminants" [17]. "*Mycoplasma ovipneumoniae* is a key co-factor in the pathogenesis of chronic, atypical pneumonia in sheep, leading to production losses in domestic lambs and significant deaths in wild sheep populations" [18].

In the present study, nasal swab and lung samples from sheep and goat showing respiratory signs were inoculated in Mycoplasma Experience liquid medium and the positive samples produced mild turbidity with yellow colour change in liquid medium after 3 days of incubation (Fig. 1). Similar kind of observation was also reported by Karthik et al. [19] in Karnataka state of India. DNA extracted from the positive broth were subjected to PCR for confirmation. Out of 120 samples which produced colour changes in Mycoplasma Experience liquid media, 78 samples yielded 280bp long sequence of 16S rRNA gene of *Mycoplasma* (Fig. 2) in PCR and 60 samples yielded 418bp long sequence of 16S rRNA gene of *Mycoplasma ovipneumoniae* (Fig. 3). Overall, 60 out of 200 samples screened across the districts of Tamil Nadu have positive for *Mycoplasma ovipneumoniae*, indicating a molecular prevalence of 30 per cent. The results have suggested that *M. ovipneumoniae* is one among the organisms associated with respiratory tract infections in sheep and goats in Tamil Nadu. Similarly,respiratory disease associated by *M. ovipneumoniae* has been reported in several studies in India [20,21]. In a study conducted in Kerala [22] *M. ovipneumoniae* was identified as the predominant organism associated with respiratory infections in goats, followed by *M. conjunctivae* and *M. agalactiae*. Monika et al. [23] isolated *Mycoplasma ovipneumoniae* from

sheep and goats in Andhra Pradesh and detected the organism molecularly by targeting the 16S rRNA gene. Similarly, Santhiya et al. [15] reported that 68 samples (45.33% of 150 samples) tested positive in *M. ovipneumoniae*-specific PCR test.

A study in Italy by Pavone et al. [24] revealed a high prevalence of respiratory infections from *M. ovipneumoniae* and *M. arginini* in sheep and goats reared in Italian farms. *M. ovipneumoniae* was the most frequently detected Mycoplasma species in goats (45%), whereas a co-infection of *M. ovipneumoniae* and *M. arginini* (57%) was prevalent in sheep. Chen et al. [25] screened nasal swabs and pleural fluid samples from goats with pneumonia in Anhui Province, China, and reported a 27.50% positivity rate for *M. ovipneumoniae* infection. Beyond domesticated goats and sheep, *M. ovipneumoniae* also affects wild ruminants. In Washington, Highland et al. [26] detected *M. ovipneumoniae* in nasal swabs taken from mule deer using PCR.

5. CONCLUSION

M. ovipneumoniae is the most common Mycoplasma species found in sheep and goats with respiratory infections in Tamil Nadu. Further studies are needed to gain a better epidemiological understanding of disease dissemination by Mycoplasma species in small ruminants in Tamil Nadu.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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