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Isolation and molecular identification of mycoplasma in small ruminants in Bengaluru division of Karnataka state

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Abstract
Mycoplasmosis is one of the economically important diseases among small ruminants having direct impact on sheep and goat rearing farmers. An attempt was made to isolate and identify Mycoplasma from sheep and goats. A total of 121 samples (62 from sheep and 59 from goats) comprising of nasal discharges, milk, lung tissues and synovial fluids were subjected for isolation of Mycoplasma organisms in PPLO broth and PPLO agar. Inoculated samples were incubated at 37 °C for 24-48 hrs in CO_2 incubator with 5% CO_2, until turbidity and colour change was seen. Twenty-two samples produced characteristic fried egg micro colonies suggestive of Mycoplasma on PPLO agar. Mycoplasma isolates were further confirmed by PCR using Genus and *M. arginini* species specific primers. Two isolates were identified as *M. arginini* by PCR. Isolation of Mycoplasma as the gold standard test, along with PCR would give a rapid identification technique for these bacteria.

Keywords: Mycoplasma, isolation, PCR, *M. arginini*, sheep and goats, Karnataka

1. Introduction
Sheep and goats are two of the first animals domesticated by humans. The domestication of goats might have occurred between 6,000 and 7,000 BCE and the domestication of sheep was even earlier, between 11,000 and 9,000 BCE (Aldridge *et al.*, 2018) [3]. Sheep have been raised for thousands of years before cows. The initial purposes for raising these animals were for their meat, milk and skin. Initially, most goat and sheep breeds were developed in Southwest Asia (Chessa *et al.*, 2009) [8]. Though sheep and goats are considered in one group as small ruminants, they differ in their chromosome numbers (sheep have 54 chromosomes, while goats have 60 chromosomes) and behavior (Mitra and Brian, 2020) [21]. According to the Food and Agriculture Organization (FAO) database, Asia had the largest sheep and goat population in the world compared to other continents which included 43.6 and 55.4% of world sheep and goat population, respectively (FAO, 2018) [11].
In sheep and goats, some diseases are of economic importance, which are caused by Mycoplasma, small wall-less bacteria of the class *Mollicutes*. These diseases include World Organization for Animal Health (OIE) listed, such as Contagious caprine pleuropneumonia (CCPP) and Contagious agalactia (CA), as well as chronic respiratory and arthritic syndrome (CRAS) and atypical pneumonia, both are worldwide distributed wherever small ruminants are being reared.
Mycoplasma affecting ruminants are grouped under '*Mycoplasma mycoides cluster*' (*M. mycoides cluster*). Apart from *Mycoplasma mycoides* subsp. *mycoides* (Mmm) that causes Contagious bovine pleuropneumonia (CBPP) and *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp) that causes CCPP, other mycoplasmas included under '*M. mycoides cluster*' are bovine pathogen *M. leachii* and the small ruminant pathogens *M. mycoides* subsp. *capri* (Mmc) and *M. capricolum* subsp. *capricolum* (Mcc) (Fischer *et al*., 2012) [12]. Ayling *et al.* (2007) [4] reported *Mycoplasma ovipneumoniae* (*M. ovipneumoniae*) and *Mycoplasam arginini* (*M. arginini*) being frequently isolated from atypical cases of pneumoniae from sheep. Mycoplasma belongs to the class Mollicutes with eight genera in the class. Five genera of these are seen in animals *viz*., *Mycoplasma*, *Ureaplasma*, *Acholeplasma*, *Anaeroplasma*, and *Asteroplasma*. Only *Mycoplasma* and *Ureaplasma* are more pathogenic to animals (Ruffin, 2001) [27].

Mycoplasmas are the smallest prokaryotic cells capable of self-replication, are pleomorphic organisms ranging from spherical (0.3 to 0.9 µm in diameter) to filamentous (up to 1.0 µm long). They do not have rigid cell wall unlike other bacteria, but have flexible three-layered cytoplasmic membrane. Their flexibility allows them to pass through bacterial membrane filters of pore sizes from 0.22 µm to 0.45µm. They either replicates by binary fission or by breaking up of elongated form. These bacteria are known for their ability to form fried-egg micro colonies on agar plates. The members of the family Mycoplasmataceae require sterol for their growth. The size of their genome is about 540 to 1300 kb containing around 500-1000 genes with a G+C content 23-41 mol% (Benedetti *et. al.*, 2020) [5].

When examined microscopically at low magnification, unstained microcolonies of *Mycoplasma* species are of diameter 0.1 to 0.6 mm and give 'fried-egg' appearance. Colonies of certain Mycoplasma species are up to 1.5 mm in diameter and are visible without magnification (Quinn *et al.*, 1994) [25]. Various techniques have been described for the detection of Mycoplasmas with advantages and disadvantages (Freshney *et al.*, 2005) [13]. They include Isolation, Polymerase reaction (PCR), Enzyme linked immunosorbent assay (ELISA), Autoradiography and immunostaining etc. Isolation of Mycoplasma, the gold standard method, is laborious and time consuming. Rapid and sensitive method for identification of Mycoplasma is PCR.

2. Materials and Methods

The present study was carried out in Bengaluru division of Karnataka state, India. For isolation of Mycoplasma, nasal discharges, milk, tissues and synovial fluids were collected from sheep and goats showing respiratory symptoms. The samples were directly collected aseptically in PPLO broth.

A total of 121 samples (62 from sheep and 59 from goats) comprising of 98 nasal swabs (56 from sheep and 42 from goats), 16 milk from goats, six lung tissue from sheep and one synovial fluid from goat were collected. Samples were subjected for isolation in PPLO broth.

2.1 For agar and broth culture method

PPLO agar plates and broths prepared from Mycoplasma broth (Difco: 1002914812). Broth and agar were supplemented with 20% swine serum, 2.5% yeast extract (HiMedia RM), 0.5% sodium pyruvate, 0.05% Thallium acetate, Penicillin (3,60,000 units). PPLO broth and agar base were autoclaved and were enriched by adding filtered supplements. Supplement was prepared in double distilled water with 0.2% arginine and 1.0% DNA. Agar was prepared with PPLO agar base w/o crystal violet (Himedia) and similar enrichments to that of broth.

Serum: Swine serum was decomplemented at 55 °C for 30 mins. Periodically, serum was mixed every 2-3 minutes during the decomplementation procedure.

Samples were collected in the broth and brought to laboratory in ice pack, on reaching the laboratory; the samples were incubated at 37 °C under 5% CO_2. The growth was determined with change in color of media from red to yellow. The broths were diluted 1 in 10 and sub-cultured into fresh broth once turbidity was noticed. Similarly, 3-4 subcultures were made from each sample prior to plating. A loop full of such sub cultures was used to inoculate on agar plates and the remaining portion of broth was used for DNA extraction for PCR assay. Inoculated plates were incubated at 37 °C for 24-48 hrs in CO_2 incubator with 5% CO_2.

DNA extraction: The boiling method described by Fan *et al.* (1995) [10] was employed. The broth suspension was centrifuged at 10,000 rpm for 10 mins followed by two washes in PBS and re-suspending pellet in PBS. Suspension was boiled at 96 °C for 10 minutes and allowed to cool in refrigeration for a brief period and centrifuged at 20,000g for 2 minutes. The supernatant was collected and stored at -20 °C for further use in PCR assays.

For PCR: *Mycoplasma* 16S rRNA genus specific primers published by Botes *et al.* (2005) [6] was used. Primers were synthesized at Eurofins Genomics Pvt Ltd. Bengaluru. The 2XMaster mix was obtained from Eurofins. *Mycoplasma* 16S rRNA genus specific primer pair used were; GPO3(F) 5'-TGGGGAGCAAACAGGATTAGATACC-3'and MGSO(R) 5'-TGCACCATCTGTCACTCTGTTAACCTC-3' with an amplicon size of 278bp. The thermal cycling conditions for PCR amplification were: initial denaturation at 94 °C for 2 mins followed by 35 cycles of denaturation at 94 °C for 15 sec, annealing at 59.3 °C for15 sec, extension at 72 °C for 5 mins and final extension at 72 °C for 10 mins (Botes *et. al.*, 2005) [6].

For identification of *M. arginini*, the PCR was performed with species-specific primers targeting the *rpo*B gene; F-5'TTTGACGGGGTTGTAACATACGT 3' and R-5' CAGCTAATCCTAGGTGTAATTCGAG3'asdescribed by Sillo *et al.* (2012) [28] and Valsala *et al.* (2017) [31]. The thermal cycling conditions for PCR amplification were: initial denaturation at 94 °C for 5 mins followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 61 °C for 1min, extension at 72 °C for 1min and final extension at 72 °C for 10 mins. Annealing temperature was determined following gradient PCR between temperatures 49 °C to 64 °C.

The Mycoplsama reference isolates, *Mycoplasma capri and M. arginini* obtained from Indian Veterinary Research Institute, Izatnagar, Utter Pradesh, India, were used as positive control.

3. Results and Discussion

In the present study, Mycoplasma organisms were isolated from 22 samples out of 121 samples screened for isolation in PPLO broth and agar by producing characteristic "fried egg microcolonies". All the Mycoplasma isolates were further subjected to genus specific PCR for confirmation.

Samples were sub cultured until a characteristic whirlpool formation was noticed on slight agitation of broth or a deposit being formed at bottom of tube (Fig. 1). Similar observation of characteristic whirlpool formation in broth was also reported by Monika *et al.* (2020) [22].

Growth on plates was observed daily for 4-5 days. The colonies would appear as tiny dew drops all along the streak line by 3rd day (Fig.2). Characteristic fried egg microcolonies were noticed (Fig.3). Further confirmation was carried out using dienes staining as described by Koneman (2005) [19]. By microscopic examination it was observed that raised centre of colonies were wider in some colonies and smaller in few (Fig.4).

Genus specific PCR for Mycoplasma revealed 278bp amplicon (Fig.5) which were in accordance with Kumar *et al.* (2011) [20]. All the genus positive DNA samples were subjected for *M. arginini* specific PCR. An amplicon of 885 bp (Fig.6) was observed in samples. Two of Mycoplasma isolates gave amplicon of 885bp.

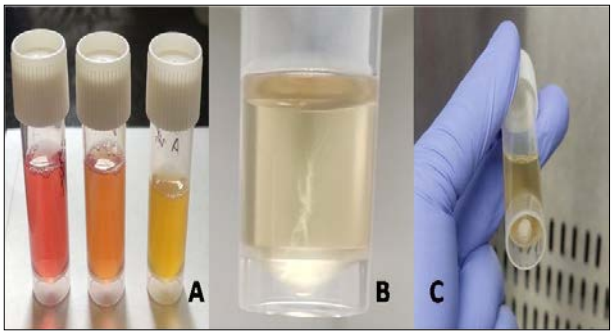


Fig 1: PPLO broth: A; Un-inoculated (red), Inoculated orange (24 hrs), Yellow (48 hrs). B; Characteristic whirlpool formation in broth C; Button formation at bottom of tube

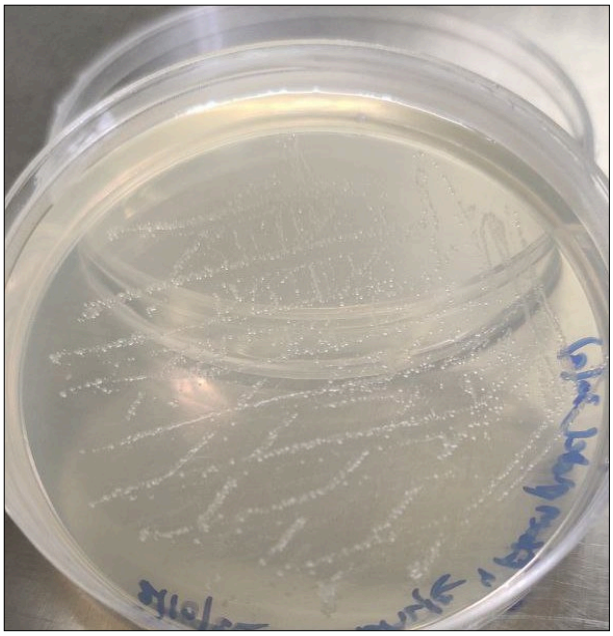


Fig 2: Mycoplasma colonies on PPLO agar showing dew drop appearance

Fig 3: Mycoplasma colony on PPLO agar showing fried egg micro colonies (40x)

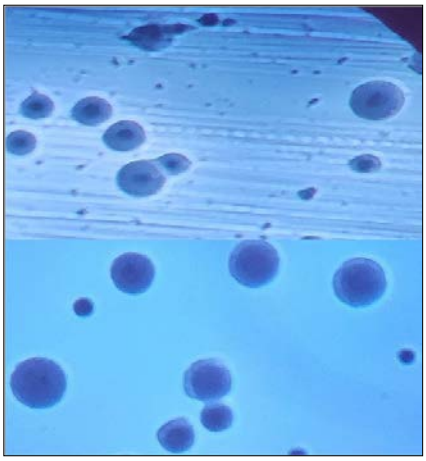
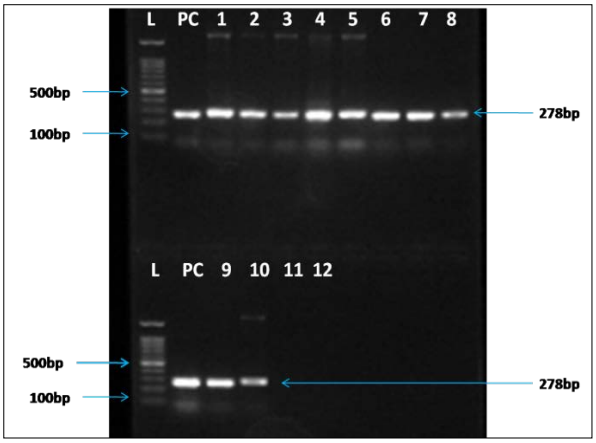
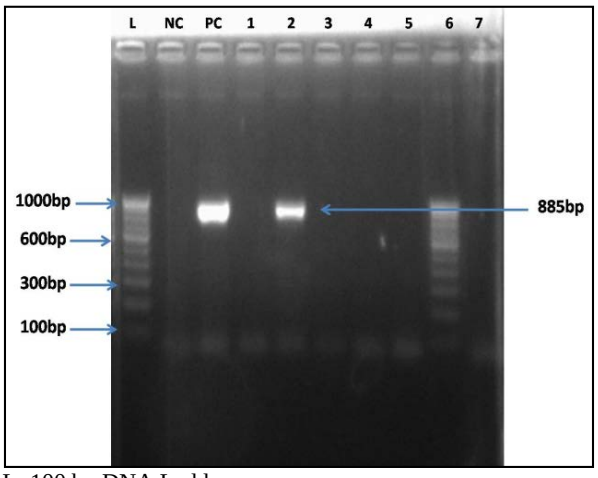


Fig 4: Dienes staining of colonies with both small centered (above) and wider central zone (below) 40x



L: 100 bp DNA Ladder
PC: Positive control (*M. capri*, IVRI)
1-10: Samples
11: Negative sample (*Staphylococcus*)
12: NTC

Fig 5: Genus specific PCR for Mycoplasma



L: 100 bp DNA Ladder
PC: Positive control (*M. arginini*, IVRI)
1-4: Samples
5: Negative sample (*Staphylococcus*)
6: 100 bp DNA Ladder
7: NTC

Fig 6: *Mycoplasma arginini* specific PCR

Though the mollicutes are quite a challenge to isolate, with proper and adequate enrichment components in the media, the organism grows well in media. Out of 121 samples screened, 22 samples revealed growth suggestive of mycoplasma on plates by colony morphology. All 22 samples were found to be positive by PCR. The study found the prevalence of Mycoplasma to be 18.18% (22) from 121 samples. This finding is in contrast with authors Kibor (1983) [17] and Reji *et al.* (2018) [26]. The isolation procedure coupled with molecular technique, PCR, provided a rapid mean for specific identification of *Mycoplasma* from sheep and goats of Bengaluru division of Karnataka.

Mycoplasmas cannot proliferate in milk therefore the organism can be easily overgrown by other types of bacteria present as contaminants or part of a mixed infection in the sample causing false negative results (Parker *et al.*, 2016) [24].

In the present study *M arginini* was found to be 1.65%. This is in agreement with Mousa *et al.* (2021) [23] who reported a prevalence of 4.8% for *M. arginini* from sheep lung tissues. Similar findings were also reported by Abdel-Halium *et al.* (2019) [1] in Egypt, who identified both *M. arginini* and *M. ovipneumoniae* from sheep and goats with pneumonic lesions. However a higher prevalence of *M. arginini* (30.8%) was reported in sheep in Nigeria by Ikheloa *et al*. (2004) [16]. In addition, in Benin by Adehan *et al*. (2006) [2], *M. ovipneumoniae* and *M. arginini* were detected with prevalence rates of 44.4% and 11.1%, respectively, in lung tissues of sheep. Also, a study in Turkey by Kilic *et al.* (2013) [18] detected prevalence of35%. *M. arginini.* Valsala *et al.* (2017) [31] in Kerala of India detected only 5.3% prevalence of *M. arginini* in sheep with caprine pneumonia. Our results are in contrary to Elfaki *et al.* (2002) [9], Gagea *et al.* (2006) [15], Chazel *et al.* (2010) [7] who reported a higher prevalence of *M. arginini*. Due to the frequent reporting of *M arginini*, there is a urge to consider this species as pathogenic unlike earlier where it was not frequently isolated from small ruminants. A lower rate of isolation of this species in this study could be attributed to collection of samples from both apparently healthy and clinically ill small ruminants.

Similarly**,** Timenetsky *et al.* (2006) [29] on studying presence of Mycoplasma from 301 cell cultures by both culture and PCR, found positive in 69 (22.9%) and 93 (30.9%) respectively. Hence it is advised to use both PCR along with isolation.

4. Conclusion

For years many researchers have been screening for Mycoplasmas among sheep and goat, where the difficulty in isolation was indicated alike. Though an extensive standardization has been achieved so far, the standard operating procedure and media preparation are constantly modified accordingly in each laboratory. Nonetheless, the isolation being gold standard, more researchers rely on PCR for identification owing to the rapidity and sensitivity. Whereas, some animals those were apparently normal and without any clinical signs were also found positive by PCR, hence it's recommended to couple isolation and PCR as suggested by Uphoff and Drexler (2002). The study also emphasizes the need to have an extensive screening among both clinically ill and apparently healthy animals to have further knowledge on frequency and distribution of other Mycoplasmas.

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