RESEARCH ARTICLE

Isolation and Molecular Detection of *Mycoplasma ovipneumoniae* and *Mycoplasma arginini* in Sheep and Goats in Karnataka, India

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Abstract

Mycoplasmosis is one of the economically important diseases in small ruminants. Mycoplasma affects respiratory system predominantly apart from synovial joints, udder and mucus membrane bearing surface. In the current study, a total of 514 samples consisting of 465 nasal swabs, 18 lung tissues, 16 milk, 11 synovial fluids, 3 ear swabs, and one fetal fluid were subjected for isolation of Mycoplasma in PPLO broth and PPLO agar. Inoculated samples were incubated at 37°C for 24-48 h under 5% CO_2, until turbidity and colour change was observed. Broth cultures were also subjected for Mycoplasma genus specific and species specific PCR. Of the 514 samples that were cultured, 32 (6.22%) produced characteristic 'fried egg' microcolonies on the agar surface. Of the 32 isolates, 21 were *M. ovipneumoniae,* 7 were *M. arginini* and 4 unidentified mycoplasma species. Seventy five (14.59%) samples were positive for genus specific PCR. By species specific PCR, 21 (28%) samples were identified as *M. ovipneumoniae,* 13 (17.33%) *M. arginini,* whereas 41 (54.66) samples did not amplify with either of the clusters specific primers or other species specific primers and remain unidentified.

Key words: Karnataka, Mycoplasma isolation, *M. arginini, M. ovipneumoniae,* PCR, Sheep and goats

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Introduction

Mycoplasmas are smallest prokaryotes that are wall-less bacteria belonging to the class Mollicutes. These are normal inhabitants of surfaces of mucus membranes and are the root cause of some of the most economically significant diseases in sheep and goats. These include illnesses recognized by the World Organisation for Animal Health (WOAH, Founded as OIE), such as contagious caprine pleuropneumonia (CCPP) and contagious agalactia (CA), as well as chronic respiratory and arthritic syndrome (CRAS) and atypical pneumonia (AP), both of which are widespread on all continents where small ruminants are raised for food (Dudek *et al.,* 2022). Among various diseases caused by Mycoplasma, CCPP and CA are WOAH list B notifiable diseases.

Mycoplasma infections are difficult to diagnose. Currently, only molecular techniques can definitively identify an isolate, thus, faster, more sophisticated procedures like polymerase chain reaction (PCR) came into focus. The molecular epidemiology of Mycoplasma infections has been established using molecular techniques including PCR and nucleotide sequencing. The PCR assays are exceedingly sensitive and often utilized in various laboratories. Because of its speed and dependability, PCR (and sequencing) has now surpassed all previous methods for identifying Mycoplasma species. To avoid contamination, PCR reactions must be carried out carefully (OIE, 2021). This study was focused on isolation and molecular detection of *M. ovipneumoniae* and *M. arginini* in sheep and goats in Karnataka, India.

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Materials and Methods

Selection of Animals

Sheep and goats with and without clinical signs of mycoplasmosis were chosen, including respiratory distress, a respiratory grunt, an increase in rectal temperature, nasal discharges, coughing, sneezing, ocular discharges, mastitis,

otitis, synovitis, arching of the back, etc. Healthy animals that were in contact with diseased were also included. Post-mortem samples were also collected (Fig. 1). Sheep and goats from four geographical divisions of Karnataka state namely, Bengaluru, Mysuru, Belagavi, and Kalburgi, divisions were included in the study.

Collection and Processing of Samples

The samples were collected in PPLO broth that was prepared as per Reji *et al.* (2018) for isolation of Mycoplasma. Samples were incubated at 37°C for 24-48 h under 5 % CO_2. Samples from sheep (n=251) and goats (n=263), including nasal swabs, tissues, milk, synovial fluids, fetal fluid and ear swabs were collected.

Isolation and DNA Extraction of Mycoplasma

Isolation was carried out as described by Mousa *et al.* (2021). The remaining portion of broth after plating was subjected for the DNA extraction. The boiling method described by Fan *et al.* (1995) was employed.

Polymerase Chain Reaction

A spectrum of primers of common mycoplasmas was used to identify the mycoplasma at genus to species level. These included Mycoplasma genus, Mycoides cluster specific, *M. arginini* and *M. ovipneumoniae* with appropriate thermal cycling conditions as described by Reji *et al.* (2018), Kumar *et al.* (2011), Valsala *et al.* (2017) and Santhiya *et al.* (2021) (Table 1, 2, respectively).

Sequencing and Phylogenetic Analysis

The PCR products were sequenced at Eurofins®, Bengaluru. To study the evolutionary relationships sequence alignment, trimming and reverse complementation was done employing the BioEdit 7.2.5.0 tool. The family tree of organisms was made using MEGA11 program as described by Kumar *et al.* (2018) by Neighbor-Joining method with 1000 bootstrap replicates by using MEGA X.

Results and Discussion

Presumptive Mycoplasma microcolonies on PPLO agar had a characteristic "fried egg" appearance. Micro-colonies of different morphologies with 1-1.5 mm in diameter with raised centers with irregular borders were noticed and some lacking centrally raised colonies and irregular border and raised with umbonate and flat (Fig. 2 A-H).

Out of 514 samples cultured, 32 (6.22%) produced characteristic 'fried egg' micro-colonies on the agar surface.

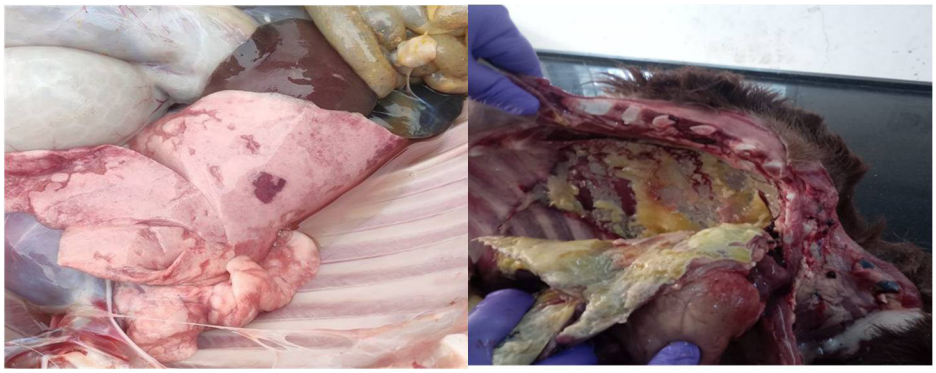


Fig. 1: Lung showing consolidation (L) and extensive adhesions (R) in thoracic cavity

Table 1: Oligonucleotide sequences for PCR

Sl No	Primer Name		Nucleotide Sequences	Size (bp)	Reference
1	Mycoplasma Genus	GPO3F	5′-TGGGGAGCAAACAGGATTAGATACC-3′	280	Reji *et al.* (2018)
		MGSOR	5′-TGCACCATCTGTCACTCTGTTAACCTC-3′		
2	Mycoides cluster specific	CA-F	5′-CGAAAGCGGCTTACTGGCTTGTT-3′	548	Kumar *et al.* (2011)
		CA-R	5′-TTGAGATTAGCTCCCCTTCACAG-3′		
3	*M. arginini*	F	5′-TTGACGGGGTTGTAACATACGT-3′	885	Valsala *et al.* (2017)
		R	5′-CAGCTAATCCTAGGTGTAATTCGAG-3′		
4	*M. ovipneumoniae*	MOVPF	5′-GTTGGTGGCAAAAGTCACTAG-3′	418	Santhiya *et al.* (2021)
		MOVPR	5′-CTTGACATCACTGTTTCGCTG-3′		

Table 2: PCR thermal cycling conditions

Sl No	Species	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
			No of cycle: Mycoides cluster -30, 35 for rest			
1	Genus	94°C -2mins	94°C-15S	59.3°C-15S	72°C- 15S	72°C-10mins
2	Mycoides cluster	94°C -5mins	95°C-1min	53°C-1min	72°C-2min	72 °C-10mins
3	*M. arginini*	95°C -5mins	94°C-1min	61°C-1min	72°C-1min	72°C-10mins
4	*M. ovipneumoniae*	94°C -1mins	94°C-1min	61.5°C-1min	72°C-2min	72°C-5mins

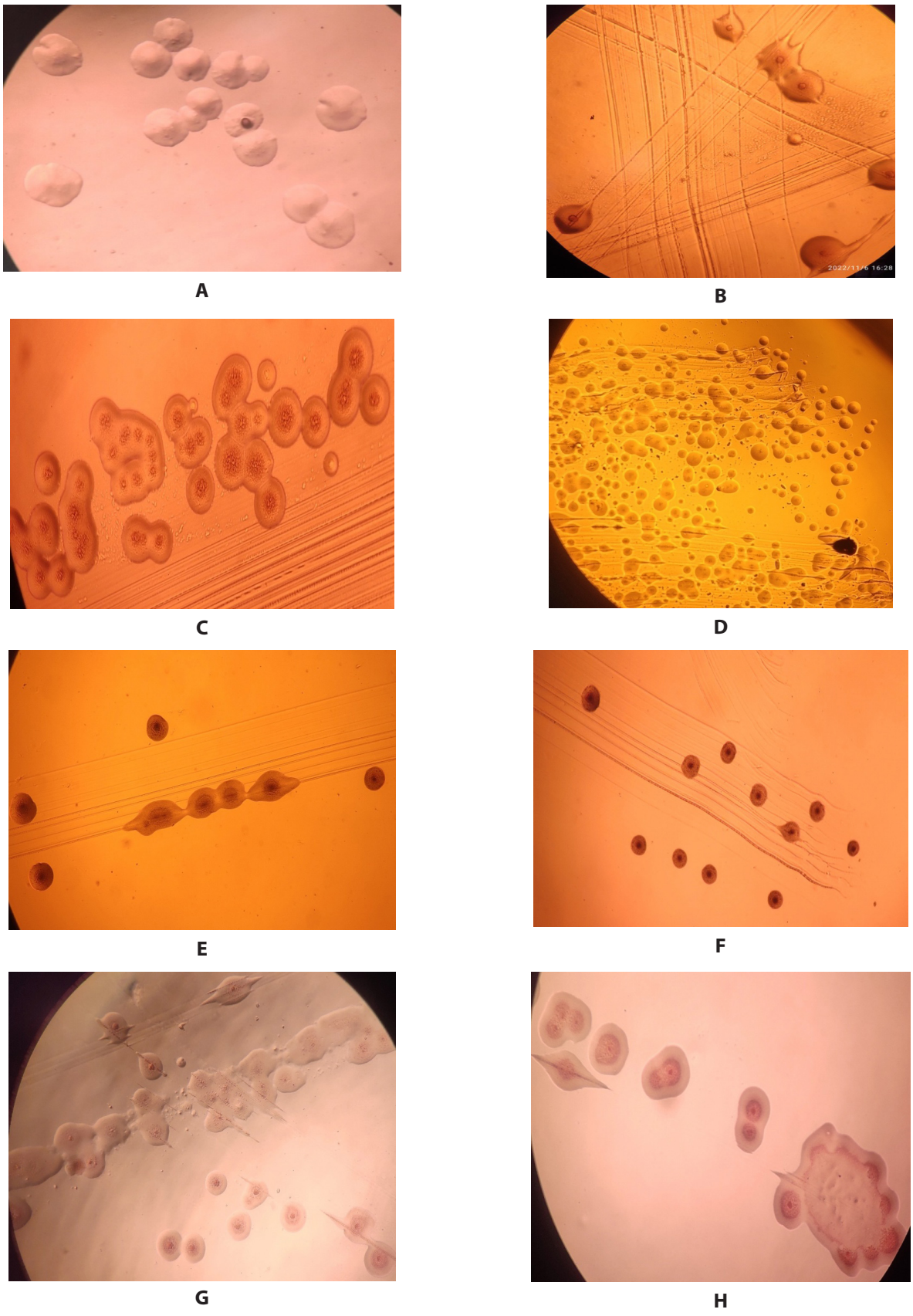
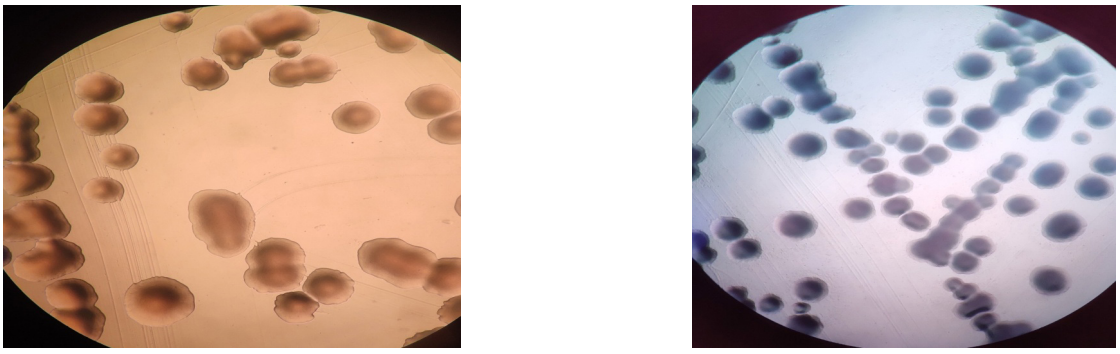


Fig. 2: Different colony morphologies (A-H) observed on agar surface. Flat, centrally raised, umbonate, oil paint type, small diameter and large diameter microcolonies (x40)

The isolates were from 28 nasal swabs and 4 tissue samples, where 13 (40.62%) and 19 (59.37%) isolates were from sheep and goats, respectively. Other samples did not reveal any suggestive growth of mycoplasma microcolonies which were observed up to 10 days. Similar isolation procedures were reported by Monika *et al.* (2020). In another study, Jain *et al.* (2015) isolated Mycoplasma from 68 samples with change of colour of the medium from red to yellow indicating mycoplasma growth and presence of characteristic "fried egg" micro-colonies under microscope. The culture plates showing typical micro-colonies were also stained by using Diene's stain where micro-colonies were intensely stained blue at centre and lighter periphery was noticed (Fig. 3 and Fig. 4 A-D). Similarly, Reji *et al.* (2018) confirmed the Mycoplasma isolates from goat samples on agar plates using Diene's staining. Of the 32 isolates, 21 were identified as *M. ovipneumoniae*, 7 were *M. arginini* and 4 unidentified mycoplasma species by species specific PCR.

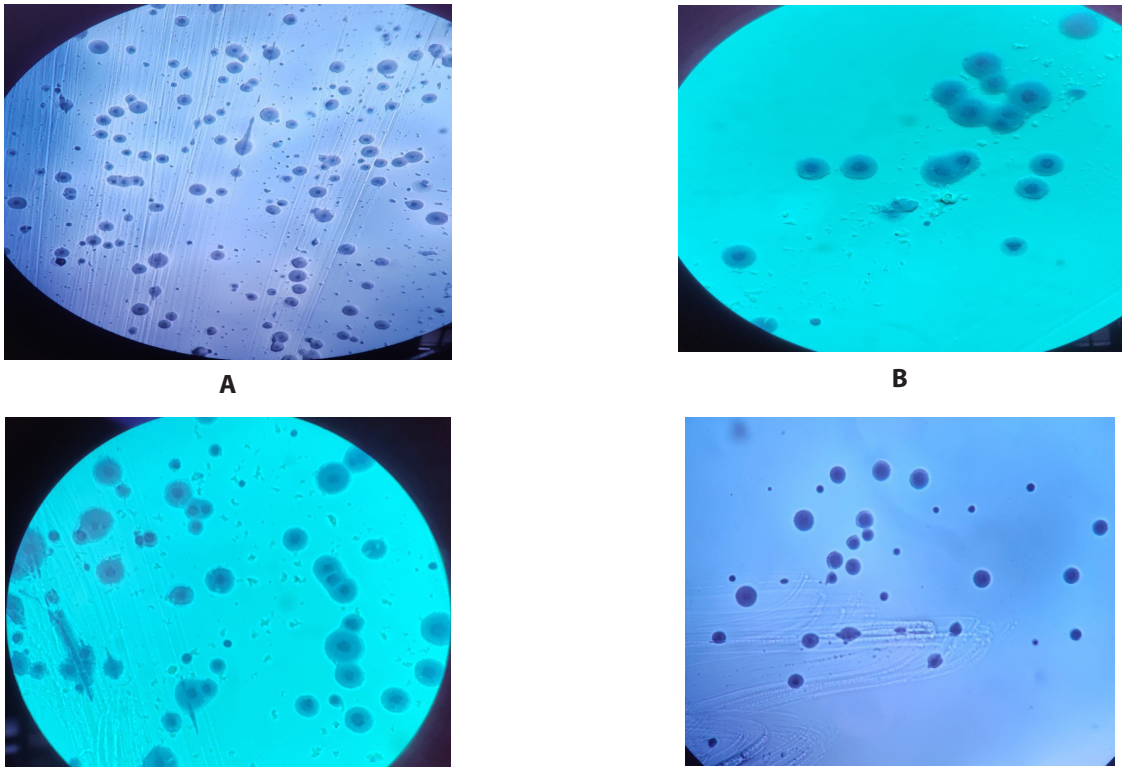
In this study, extracted DNA from 514 samples was screened by Mycoplasma genus specific PCR using 16S rRNA gene. Of these, 75 (14.59%) samples were identified as Mycoplasma organism where 278 bp size amplicon was produced (Fig. 5).

All the 75 (14.59%) samples that were positive for genus specific PCR were further screened for species identification using Mycoplasma mycoides cluster-specific and species-specific PCR. None of the genus-positive samples were PCR-positive for either mycoides



A B

Fig. 3: Mycoplasma colonies on agar surface (A) and Dienes staining (B) of microcolonies (x40)



A B C D

Fig. 4: Micro colonies stained with Diene's stain (A-D) showing fried egg appearance of different diameters (x40)

or capricolum group-specific. By species specific PCR, 21 (28%) samples were identified as *M. ovipneumoniae* (Fig. 6), 13 (17.33%) *M. arginini* (Fig. 7), Whereas 41 (54.66) samples did not amplify with either of the clusters specific primers or other species specific primers, except for genus and so remain unidentified. *M. ovipneumoniae* and *M. arginini* were found in 13 and 8 sheep and goats, respectively, and in 5 and 2 cases, respectively. Four of the mycoplasma samples from goats, however, could not be distinguished by any species-specific PCR assays and remained as unknown mycoplasma species. These findings are in accordance with Öztürkler and Otlu (2020) who reported Mycoplasma isolation 10.4 % (26) from 250 pneumonic lungs and found *M. ovipneumoniae* (12) and *M. arginini* (4) to be major pathogens in Kars region of Turkey.

According to Otlu (1996), *M. arginini* was the predominant species (70.5%) followed by *M. ovipneumoniae* (29.5%) in sheep pneumonia. *Mycoplasma arginini* has been identified in pneumonic conditions by many workers (Abdel-Halium *et al.*, 2019). The present findings are in accordance with Manlove *et al.* (2019) and Mousa *et al.* (2021).

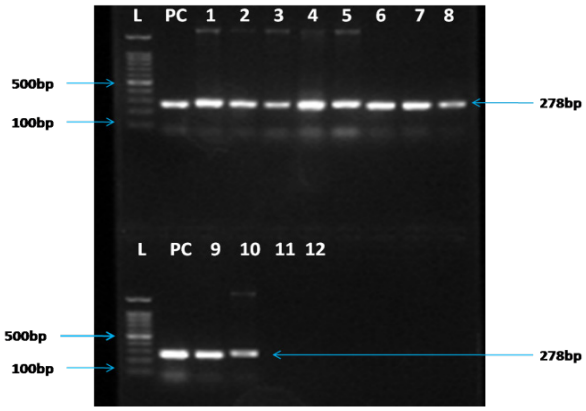


Fig. 5: PCR amplification of Mycoplasma genus 16S rRNA gene (278 bp). L: 100 bp DNA ladder; Lane PC: Positive Control (*Mycoplasma capri* from IVRI); Lanes 1 to 10: Samples positive for Mycoplasma genus; Lane 11: Negative control (Staphylococcus), Lane 12: No template control

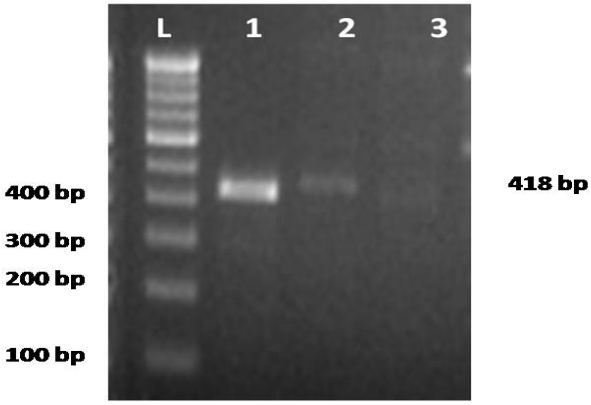


Fig. 6: PCR amplification of *Mycoplasma ovipneumoniae* (418bp). L: 100 bp DNA ladder; Lanes 1 & 2: Samples positive for *Mycoplasma ovipneumoniae*; Lane 3: Negative

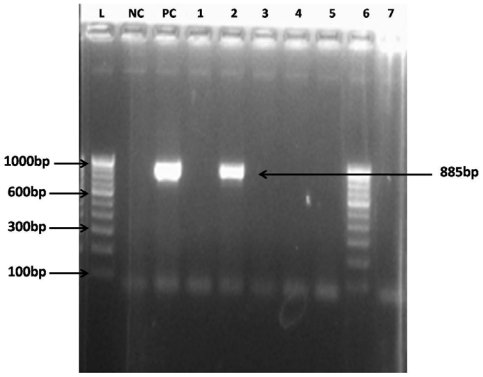


Fig. 7: PCR amplification of *rpo*B gene of *Mycoplasma arginini* (885bp). L & Lane 6: 100 bp DNA ladder; NC: Negative Control (*Staphylococcus aureas*); PC: Positive Control (*Mycoplasma arginini* from IVRI); Lanes 1-5 & 7: Samples positive for *Mycoplasma arginini*

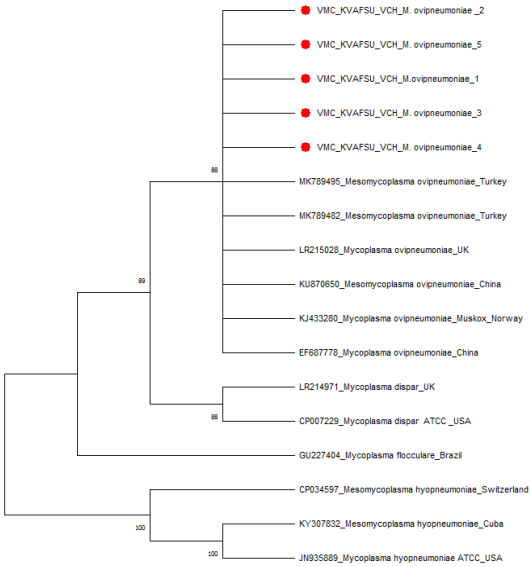


Fig. 8: Phylogenetic tree for the *Mycoplasma ovipneumoniae* isolates

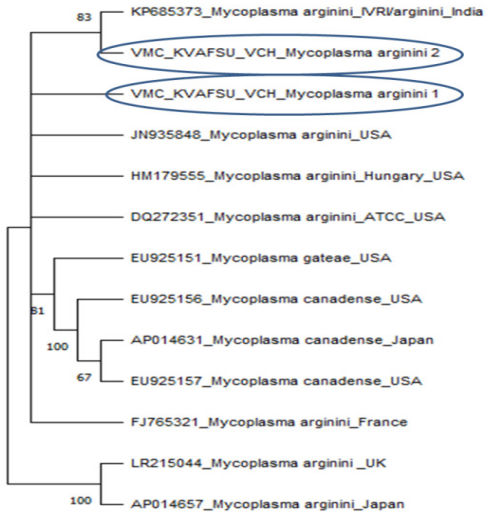


Fig.9: Phylogenetic tree for the *Mycoplasma arginini* isolates

The purified partial gene PCR products of both, *M arginini,* and *M. ovipneumoniae,* were sequenced. A comparative analysis of nucleotide sequences was performed using the CLUSTAL W multiple sequence alignment and phylogenetic analyses were done using neighbour-joining in MEGA X with bootstrap of 1000 replications. The phylogenetic analysis of five *M. ovipneumoniae* isolates of the study revealed that they were clustered with *M. ovipneumoniae* of Turkey, UK and ATCC strain with query cover of 97 % and 100 % identity from BLAST analysis (Fig. 8). Four sequences from *M. ovipneumoniae* were submitted to GenBank with accession numbers of OP801624, OP801625, OP801626 and OP801627. The phylogenetic analysis of two of *M. arginini* isolates were found to be identical, exhibiting >98 % identities with a 100 % query cover that of the standard *M. arginini* (ATCC 23243) strain (Fig. 9). Similar results have been reported by Volokhov *et al.* (2012) for *rpo*B gene sequence of *M. ovipneumoniae* that clustered with hyopneumoniae group. Similarly, Valsala *et al.* (2017) had partially sequenced *rpo*B gene of *M. arginini* isolates and the Phylogenetic trees was drawn to find out more than 90 % homology with that of ATCC strain.

Conclusion

Mycoplasmas are one of the common respiratory pathogens affecting sheep and goats. Different species of mycoplasma are involved in affecting sheep and goats. *Mycoplasma ovipneumoniae* and *M. arginini* are prevalent in sheep and goats. Though isolation of Mycoplasma is considered gold standard, but indeed is time consuming and laborious. In situations where isolation is not possible or that no growth is seen, the PCR assay can be done. The sensitivity of PCR over isolation with rapidity makes this as an important diagnostic tool.

Acknowledgements

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