RESEARCH ARTICLE

Isolation, Identification and Molecular Characterisation of Mycoplasma Isolates from Small Ruminant Mycoplsmosis of Karnataka State, India

Sumathi B. Raju*, Saritha N. Sannegowda, Sanganagouda Koppad, Amitha Reena Gomes, Kavitha Govindaiah, Chandranaik B. Marinaik, Sreevatsava Venkataramana

Abstract

Mycoplasmosis, the diseases caused by mycoplasma, are one of the challenging and continuous threats to small ruminant farming resulting in high morbidity and mortality and huge economic loss. In India there are only few published reports of mycoplasmosis manifestations in small ruminants. In the present study, attempts have been undertaken for the isolation, identification and molecular characterization of Mycoplasma isolates from suspected respiratory manifestations and mastitis in small ruminants of Karnataka state, India. A total of 300 samples were collected from 180 goats and 80 sheep from different districts of Karnataka, India; 110 nasal swabs, 35 lungs and bronchial tissue, 50 lacrymal secretions from respiratory manifestations, 90 milk samples from contagious mastitis and 15 samples from joints abscess aspiration fluid were cultured on pleuropneumonia-like organisms (PPLOs) media for cultivation of *Mycoplasma* species. The genus and species were confirmed by genus and species-specific PCR. Further, sequencing and phylogenetic analysis was adopted to identify and classify the isolates. A total of 14 *Mycoplasma* isolates were isolated using PPLO broth and agar media with slight modifications in the composition, identified by growth pattern and microcolony morphology. Among these 7 isolates were confirmed as *Mycoplasma capri, 4* isolates as *M. agalectiae*, and 3 isolates as *M. arginine* by genus and species-specific PCR. A purified isolates of *M. capri, M. agalectiae* and *M. arginini* was sequenced and phylogenetic analysis was carried out. The study revealed the prevalent species of Mycoplasma associated with respiratory manifestation, mastitis and arthritis**.**

Key words: Arthritis, Mastitis, *Mycoplasma*, Pneumonia, Polymerase chain reaction, Small ruminants.

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Introduction

In many regions of India, sheep and goats are recognised as mobile ATM, where rearing of these animals has tremendous significance in rural economy. Different species of Mycoplasma are associated with many pathological problems in human beings and small ruminants mainly, contagious caprine pleuropneumonia (mostly in goats), contagious agalactia (both sheep and goats), atypical pneumonia (usually sheep), besides arthritis, mastitis, seminal vesiculitis, ampullitis, epididymitis, orchitis, urethritis, conjunctivitis and meningitis either alone or as classical syndromes (Cetinkaya *et al.,* 2009; Vasala *et al.,* 2017; Mounier *et al.,* 2019). Mycoplasma infections lead to high morbidity and mortality rates in sheep and goats populations in African countries including Egypt, European countries and India (Sandip *et al.,* 2014).

Mycoplasmas are the smallest fastidious organisms belonging to a group of Mollicutes with the size of 0.3 to 0.8 µm. The common mycoplasma includes Mycoplasma mycoides subspecies mycoides, *M. mycoides subsp. capri, M. capricolum subsp. capripneumoniae, M. capricolum subsp. capricolum, M. agalactiae, M. bovis* and *M. conjunctivae etc*. Most of the mycoplasmas produce a conglomerate of clinical signs hampering diagnosis. Common clinical symptoms associated with the infection include fever, anorexia, lethargy, and unwillingness to follow the herd, followed by clinical symptoms depending upon the involvement of various organs such as mammary glands, lungs, genitalia, joints, and conjunctiva (Kumar *et al*., 2013). The contagious agalactia mainly known to be caused by *M. agalectiae* is characterised by high morbidity (sometimes up to 50 and 90% of the lactating female sheep and goats, respectively), drastic reduction of milk production and, in 90% of cases, the most visible sign is interstitial mastitis (Loria and Nicholas, 2013). The present study was carried out to isolate, identify and

Institute of Animal Health and Veterinary Biologicals, KVAFSU, Hebbal, Bengaluru-560024, Karnataka, India

Corresponding Author: Dr. Sumathi, B.R., Institute of Animal Health and Veterinary Biologicals, KVAFSU, Hebbal, Bengaluru-560024, Karnataka, India. e-mail: sumathibr81127@gmail.com

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characterize Mycoplasma isolates from suspected respiratory manifestations and mastitis cases of small ruminants of Karnataka.

Materials and Methods

The study was conducted on sheep and goat farms in Karnataka (Sira, Ballary, Ramanagar, Mandya, Tumkur, and Kolar) suspected of Mycoplasma infections during a disease investigation between February 2020 and April 2021. Various manifestations of Mycoplasma infections are illustrated in Fig. 1.

Clinical Examination and Sample Collection

The suspected sheep and goats were examined for signs of respiratory manifestations, including high fever, depression, respiratory distress, nasal and ocular secretions, coughing, abdominal breathing, joint swelling, and severe mastitis. A total of 300 samples were collected from 180 goats and 80 sheep of different ages, sexes, and breeds exhibiting respiratory infections, conjunctivitis, and mastitis. These samples included 145 nasal swabs, 50 lacrimal secretions, 90 milk samples, and 15 aspirated fluid samples from joint abscesses. All samples were collected aseptically in PPLO broth and transported to the laboratory under cold chain conditions.

Isolation and Identification of Different Mycoplasma Species

Approximately 0.5 to 1.0 mL of all suspected samples in transport medium were inoculated into 5 mL of PPLO broth and incubated at 37 °C for 2-4 days and monitored for pH changes towards acidity or bacterial contamination. The growth was determined with change in color of media from reddish to reddish yellow. Samples showing growth in PPLO broth were inoculated into PPLO agar by running drop technique and incubated at 37 °C for up to 7 days in 5-10% CO_2 to optimize growth (WOAH, 2021). Plates were examined daily under 4x magnification for growth and microcolony morphology (Cetinkaya *et al*., 2009; Vasala *et al*., 2017).

Growth was confirmed by a color change in the broth from reddish to reddish yellow, increased turbidity and the appearance of fried egg-like microcolonies on PPLO agar. The changes in turbidity and color of the PPLO broth, as well as the growth patterns on PPLO agar from day 1 to day 7, are shown in Figs. 2 and 3, respectively.

Purification of Primary Culture

Pure stock cultures were prepared by transferring a small agar block containing a single Mycoplasma microcolony to PPLO broth, incubating at 37 °C for 3-5 days, and then storing at 4°C. Subculturing of each isolate from the liquid broth culture was performed regularly.

Molecular Characterisation

There were 14 isolates showing fried egg colonies on PPLO agar and turbidity with yellowish tinge in PPLO broth. The DNA extraction was performed using Qiagen kit (extraction protocol provided with the kit was followed) from all the 14 samples and subjected to group and species-specific PCR (Table 1).

PCR amplifications were carried out in 25 µL reaction volume containing 12.5 µL of master mix (ampliqon), 1 µL of each primer (10 pmol/µL), 5 µL genomic DNA template, and 5.5 µL deionized water. The amplification programme for both genus and species-specific PCR followed was: initial denaturation at 95 ^{o}C for 2 min; followed by 40 cycles of denaturation, annealing, extension (95 ^{o}C for 30 sec, 55 ^{o}C for 1 min, 72 ^{o}C for 1 min respectively) and final extension at 72 ^{o}C for 5 min followed by 4 ^{o}C hold at infinity. All reactions were run on a Mini amp plus – Applied Biosystems thermal cycler.

DNA Sequencing and Phylogenetic Analysis

DNA was extracted from isolates in the late exponential growth phase using the QIAamp DNA Mini Kit (Qiagen Kit). The pure amplified PCR products were sequenced in the forward and/or reverse directions. Initially BLAST® analysis (Basic Local Alignment Search Tool) was performed to find out sequence identity to gene bank accessions and the nearest homology of the organism. A comparative analysis of sequences was performed using the Clustal W multiple

Table 1: Primers used for genus and species-specific PCR

Primer	Genus/ Species	Sequence	Target gene	Fragment length	Reference
Genus specific primers					
GPO- 1(F) MGSO ®	Mycoplasma	5′-ACTCCTACGGGAGGCAGCAGTA-3′ 5′-GTTGCAGAAGAAAGTCCAATCA-3′	16S rRNA	715 bp	Van Kuppeveld *et al.* (1992)
Species specific primers					
P4/P6	*M. mycoides capri*	5′-ACTGAGCAATTCCTCTT-3′ 5′-TTAATAAGTCTCTATATGAAT-3′	16S rRNA/ CAP-21	195 bp	Kumar *et al*. (2011)
FS1/FS2	*M. agalectiae*	5′-AAAGGTGCTTGAGAAATGCC-3′ 5′-GTTGCAGAAGAAAGTCCAATCA-3′	P80 lipoprotein gene	375 bp	Santos *et al*. (2018)
M. arginini F/R	*M. arginini*	5′ -TTTGACGGGGTTGTAACATACGT-3′ 5′ -CAGCTAATCCTAGGTGTAATTCGAG-3′	B subunit rpoB gene	885 bp	Sillo *et al*. (2012)

sequence alignment program and Mega align module. Phylogenetic analyses were done using maximum likelihood neighbour-joining and maximum parsimony in MEGA11 software to identify homology between different strains (Koichiro *et al.,* 2021).

Results and Discussion

The suspected sheep and goats, which suffered from different signs of respiratory manifestations including fever 40-42 °C with depression, nasal and ocular discharges, cough, abnormal respiration, mastitis, arthritis, corneal opacity (Fig. 1) were examined and these clinical samples were collected and further subjected for analysis.

Out of 300 different clinical samples obtained from 13 outbreaks from six places containing 180 goats and 80 sheep with clinical signs high fever, respiratory distress, coughing, nasal and ocular discharge, joint swelling, mastitis processed, 14 samples showed fine turbidity and pH change towards acidic, imparting a reddish yellow colour to the broth medium within 3 to 10 days (Fig. 2) and on further inoculation into PPLO agar, the samples yielded microcolonies of 1 to 2 mm size exhibiting typical fried egg appearance, indicating the mycoplasma growth (Fig. 3). The species identified were *M. mycoides subsp. Capri* (7), *M. arginine* (3) and *M. agalectiae* (4) The present study revealed isolation rate of 4.67%, which was found to be in agreement with Ikhloea *et al.* (2004), who obtained similar results of 3.7 to 11%.

Fig. 1: (a) Goat with severe respiratory infection, nasal and lachrymal discharge (b) Goat with respiratory distress, with abdominal respiration (c) & (d) Goats with pneumonia and corneal opacity (e) Sheep with sever mastitis and respiratory distress (f) Goat with arthritis, limping with lachrymal and nasal discharges

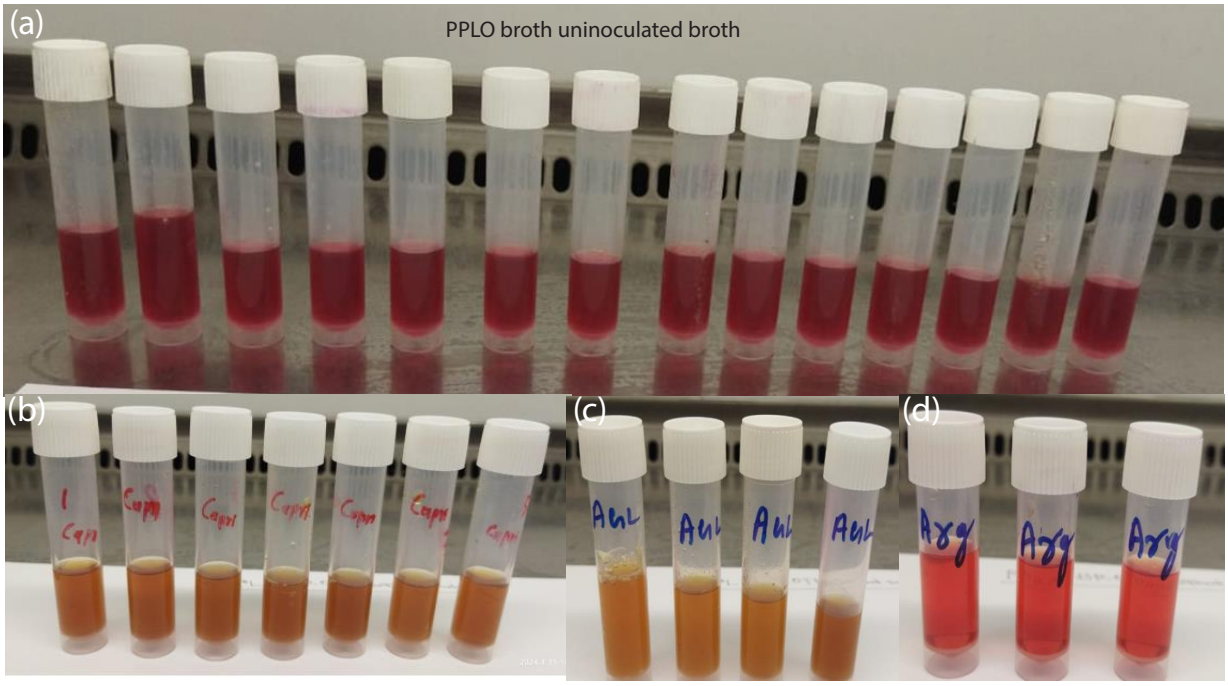

Fig. 2: (a) PPLO uninoculated broth neutral pH. (b) Yellowish culture due to pH change towards acidic, growth of Mycoplasma mycoides capri (c) Yellowish culture due to pH change towards acidic, growth of Mycoplasma agalactiae (d) Reddish yellow culture indicating Mycoplasma arginini growth

A total of 14 isolates were obtained, of which 7 were confirmed as *Mycoplasma capri,* 4 isolates as *M. agalectiae* and 3 isolates as *M. arginine* by genus and species-specific PCR. Among these isolates, five *M. capri* and all the three *M. arginine* isolates were obtained from respiratory manifestations. The 2 *M. capri* and 4 isolates as *M. agalectiae* were obtained from milk samples. A number of mycoplasmas have been reported as cause of mycoplasmosis in sheep and goats in India causing various diseases (Kumar *et al*., 2013; Jain *et al*., 2015). Mycoplasma mycoides cluster pathogens are important aetiological agents of respiratory manifestations in small ruminants. Present study confirms the association of *M. capri* with pneumonia in goats. The involvement of *M. capri* has also been reported by Roy *et al.* (2010), who detected antibodies of *Mycoplasma capri* in goats of Gujarat state, in respiratory manifestations.

After inoculation, isolates produced colonies in three to four days on Mycoplasma agar. The microcolony morphology and growth pattern of various Mycoplasma isolates on petri plates were observed under 4X eye piece of microscope (Fig. 3). Most *Mycoplasma spp.* grow best at 37°C, increased humidity, and CO_2 tension in the atmosphere to enhance growth of Mycoplasma (WOAH, 2018). The growth pattern and microcolony morphology are in accordance with Kabir and Bari (2015).

PCR remains the most valuable, rapid and sensitive method for detecting specific species of Mycoplasma (Amores *et al.,* 2010; Awan *et al.,* 2012). The isolates were confirmed as Mycoplasma species by Genus specific PCR as per the method described by van Kuppeveld *et al.* (1992). All the 14 isolates were confirmed as Mycoplasma species by amplification of 16s rRNA, specific band of molecular size 715 bp. Further, confirmed upto species level as *M. capri, M. agalectiae* and *M. arginine* targeting 16S rRNA (195 bp), P80 lipoprotein gene (375 bp) and rpoB (885 bp) as per the method described by Kumar *et al*. (2011), Santos *et al.* (2018) and Sillo *et al*. (2012), respectively (Fig. 4, 5 & 6).

The sequencing of the 16S rRNA (195 bp), P80 lipoprotein gene (375 bp) and rpoB (885 bp) gene was carried out according to the Sanger method using a 16-capillary 3130XL genetic analyzer sequencer (Applied Biosystems).

Sequencing and Phylogenetic Analysis:

The present study revealed the high-frequency rate of isolation of *M. capri* and *M. arginini* from sheep and goats suffering from respiratory manifestations and pneumonic lungs and also involvement of *M. agalactia* from the cases of mastitis. To study further, amplified purified PCR product of one isolate was subjected to sequencing analysis and the sequence then was submitted to NCBI GenBank.

The PCR product from *M. capri* was sequenced and analyzed by BLAST comparison, which showed 98.91% with *Mycoplasma mycoides subsp. capri* strain GM12 30S ribosomal protein S7 gene, (accession number: OP352532.1). Similarly, PCR

product sequence of *M. agalectiae* showed 99.43% similarity with the *Mycoplasma agalactiae* strain RAD1 putative P80 lipoprotein gene (GenBank accession number: JQ745048.1) and *M. arginine* showed 98.92% similarity with *Mycoplasma arginini* strain G230 RNA polymerase beta subunit gene (GenBank accession number: JN935848.1) (Fig. 7a, b, c).

Out of 160 samples (110 nasal swabs, 35 lung tissue and 15 joint aspirate) processed, 7 yielded Mycoplasma isolates and were identified as *M. mycoides mycoides subsp. capri.* Similar reports regarding isolation of *M. capri* have been documented by Ikheloa *et al.* (2004), Adehan *et al.* (2006) and Kumar *et al.* (2011) from respiratory manifestations. Many samples from respiratory manifestations also revealed *M. arginine* and this high-frequency rate of isolation of *M. arginini* from different respiratory manifestations of small ruminants, especially from pneumonia cases is in accordance with Ammar *et al.* (2008), Fernandez *et al.* (2016), Valsala *et al.* (2017) and Mounier *et al.* (2019).

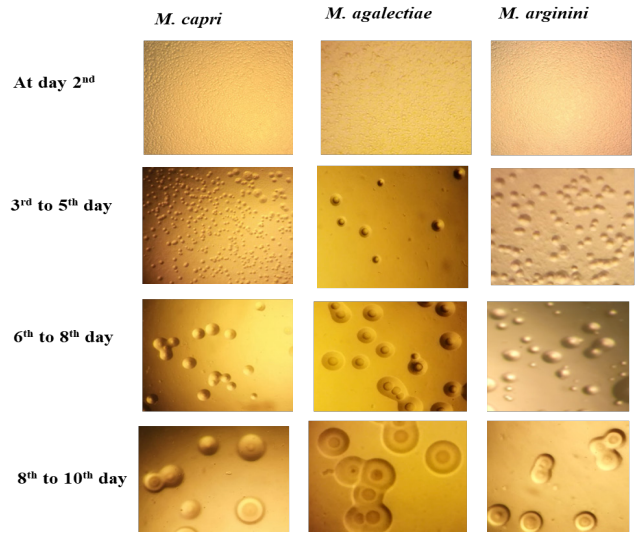

Fig. 3: Microcolony morphology and growth characteristics of Mycoplasma species

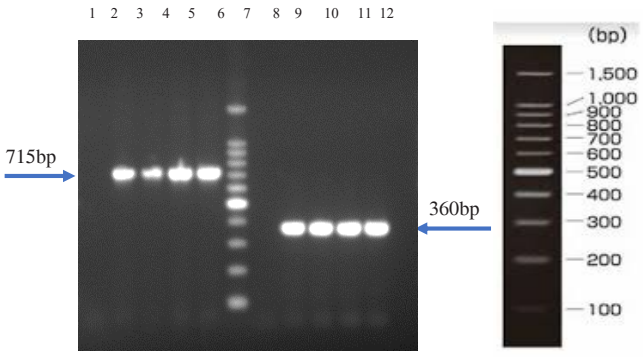

Fig. 5: PCR amplification of gene segments of M. agalactiae isolates (Species specific-360bp and Genus specific-715bp). Lane 1: Negative control for M. agalactiae, Lane 2,3,4 and 5: PCR amplification of M. arginine for different isolates with Group specific primers, Lane 6: 100bp DNA ladder from Takara, Lane 7: Negative control for M. agalactiae, Lane 8, 9, 10 and 11: PCR amplification of M. agalactiae for different isolates with Species specific primers,

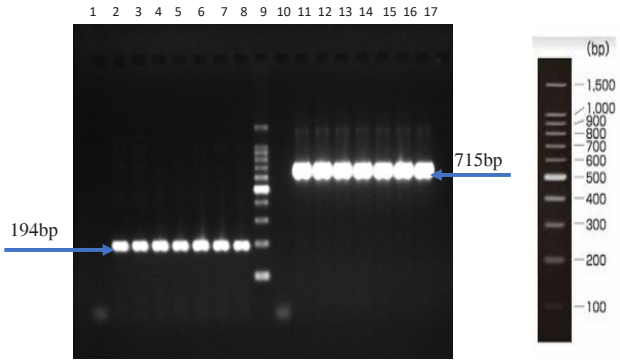

Fig.4: PCR amplification of gene segments of M. capri isolates (Species specific-195bp and Genus specific-715bp). Lane 1: Negative control for M. capri, Lane 2,3,4,5,6,7 and 8: PCR amplification of M. capri for different isolates with Species specific primers, Lane 9: 100bp DNA ladder from Takara, Lane 10: Negative control for M. Capri, Lane 11, 12, 13, 14, 15, 16, and 17: PCR amplification of M. capri for different isolates with Group specific primers.

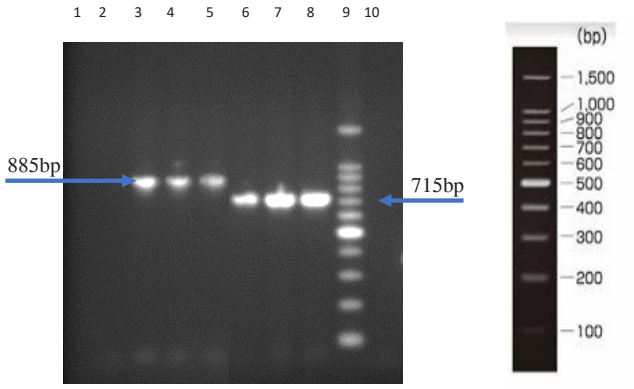

Fig. 6: PCR amplification of gene segments of M. arginine isolates (Species specific-885bp and Genus specific-715bp), Lane 1: Negative control for M. arginini, Lane 2, and 4: PCR amplification of M. arginine for different isolates with Species specific primers, Lane 3, 6 and 7: PCR amplification of M. arginine for different isolates with Group specific primers, Lane 8: 100bp DNA ladder from Takara.

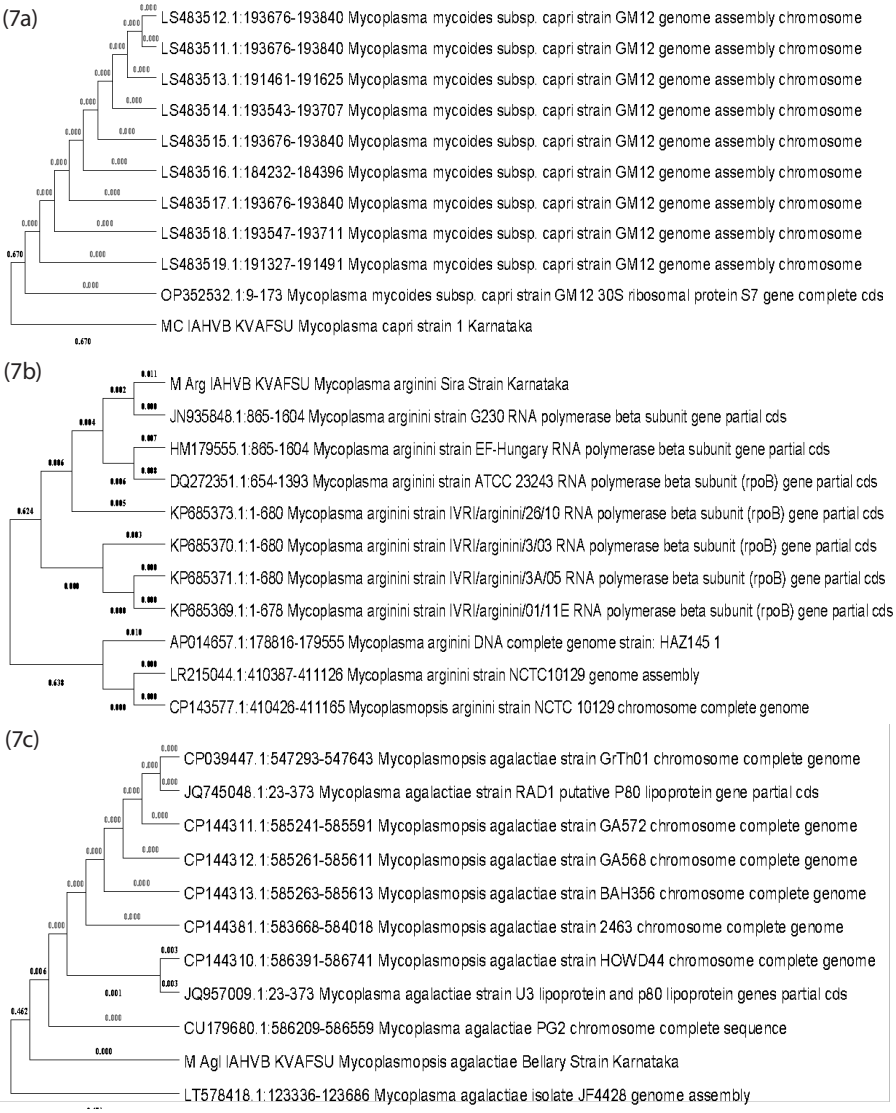

Fig. 7a,b,c: A phylogenetic tree was formed based on species specific gene sequence of one purified strain of each species from the Karnataka for *M. capri* (7a) *M. agalactiae* (7b) and *M. arginini* (7c), isolated from small ruminants (from GenBank). Sequencing of species-specific genes of *M. capri* (16S rRNA, 195bp) *M. agalactiae* (P80 lipoprotein gene, 375bp) and *M. arginini* (rpoB, 885bp) across different districts showed no significant difference between them and as well as the accession nos of other countries.

The present study revealed *M. agalactiae* as prevalent cause of mastitis. Similar reports of earlier studies which accounts for 90% of outbreaks of Contageous agalactia in goats (Garrido *et al.,* 2016) and almost 100% in sheep (Bergonier *et al.,* 1997; Migliore *et al.,* 2021) has been reported, where *M. agalactiae* was the sole cause of classical contagious agalactia.

Conclusion

The present study emphasises the involvement of different species of mycoplasma such as *Mycoplasma capri*, *M. agalectiae* and *M. arginini*, either alone or in combination to cause mycoplasmosis with respiratory manifestations, conjunctivitis, arthritis and mastitis in sheep and goats in Karnataka, India, The study needs to be extended including wide geographical area, covering a greater number of suspected outbreaks to report different species of Mycoplasma known to cause Mycoplasmosis involving different systems in small ruminants. In areas with frequent outbreaks/endemicity, usage of either monovalent or polyvalent autogenous vaccine having prevailing strains of Mycoplasma could be the appropriate option for the control of clinical manifestations of Mycoplasmosis in small ruminants.

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