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Mycoplasma arginini: high frequency involvement in goat pneumonia

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Abstract: Mycoplasmas are an important class of bacteria associated with caprine pneumonia. Some of the main species involved are *M. mycoides* subsp. *capri*, *M. capricolum* subsp. *capricolum*, and *M. capricolum* subsp. *capripneumoniae*. In addition, there are frequent reports of *M. arginini* involvement. In the present study an attempt was made to see the association of various mollicutes in caprine pneumonia. In total 244 pneumonic goat lung samples collected over a 10-year period (2003–2012) were screened for the isolation and identification of associated mollicutes. The identification was done on the basis of biochemical tests, growth inhibition test, PCR, and sequencing. The most frequent isolates were *Acholeplasma* (20/244, i.e. 8.19%) and *Mycoplasma arginini* (13/244, i.e. 5.3%), followed by *M. mycoides* subsp. *capri* (1/244) and *M. agalactiae* (1/244). Seven representative isolates of *M. arginini* were subjected to partial sequencing of the rpoB gene. On phylogenic analysis, *M. arginini* isolates were found to be highly identical, exhibiting >98% identity with that of the standard *M. arginini* (ATCC 23243) strain. The results encourage exploration of the role of *M. arginini* in goat pneumonia in much detail, due to its high frequency involvement.

Key words: *Mycoplasma arginini*, goat, pneumonia, identification

1. Introduction

Mycoplasmas are the smallest self-replicating prokaryotes. They are ubiquitously present throughout the animal kingdom and have been identified from a wide range of mammals, birds, reptiles, amphibians, and fish. While many of them are considered to be of minor epidemiological relevance, some are responsible for a number of important diseases in livestock and poultry. However, over the last few decades there have been reports of many Mycoplasma species gaining importance as emerging pathogens in humans and animals, especially in immunocompromised individuals.

In small ruminants, mycoplasmas are considered an important class of bacteria responsible for respiratory and other systemic infections. Heavy mortality in susceptible goats and sheep herds affected by mycoplasma results in significant economic losses in tropical areas (1). Among these, *M. capricolum* subsp. *capripneumoniae*, the agent responsible for contagious caprine pleuropneumonia (CCPP), is the most important. Difficulty in isolation of the agent and lack of expertise are the main lacunae for arriving at a definite and conclusive diagnosis. *M. mycoides* subsp. *capri* also produce pleuropneumonia lesions in addition to other manifestations such as mastitis, arthritis, keratitis, keratoconjunctivitis, and abortions (2).

Other mycoplasmal agents isolated from pneumopathies include *M. ovipneumoniae*, *M. arginini*, *M. agalactiae*, etc. *M. ovipneumoniae* produces chronic, nonprogressive atypical pneumonia in sheep (3). It can act as a predisposing factor for infection with other bacterial and viral agents. *M. arginini* is frequently isolated along with *M. ovipneumoniae* from cases of atypical pneumonia in sheep (4). In an outbreak in Portugal among goats, *M. ovipneumoniae*, *M. arginini*, *Mannheimia haemolytica*, and *Pasteurella multocida* were isolated from pneumonic lung lesions (5). *M. agalactiae* is considered the classical etiological agent of contagious agalactia, the OIE listed disease characterized by MAKePS syndrome (6). Although it is isolated from pneumonic cases, it is more often associated with mastitis and abortion cases.

M. arginini has been isolated from cattle, camel, sheep, and goats with various disease conditions such as pneumonia, keratoconjunctivitis, mastitis, and arthritis (7,8). Higher incidence of *M. arginini* in pneumonic as compared to normal sheep (9) is suggestive of its pathogenic potential. In Egypt, it was isolated from cases of granular vulvovaginitis and balanoposthitis in sheep and cattle (10). The organism has also been isolated from the pneumonic lungs of camels at a frequency of 8.8% (7). In India, *M. arginini* etiology of keratoconjunctivitis cases

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from goats has been proven (8). Moreover, *M. arginini* is routinely recovered along with *M. ovipneumoniae* from young lambs with respiratory disease (11). Although earlier it was not considered a primary pathogen, several recent case reports suggest its pathogenic role as a human zoonosis (12,13). Multilocus sequence typing (MLST) based on 5 housekeeping genes was developed for assessing genetic diversity among *M. arginini* isolates (14), which demonstrated high genetic variability among isolates.

The role of *M. arginini* in pneumonia etiology/association particularly in goats and sheep is still uncertain. However, on several occasions (7,15,16) its high frequency of isolation from clinical pneumonia cases has been observed from different livestock species. Therefore, considering the pathogenic significance of *M. arginini*, the present study was carried out to determine its involvement along with other mollicutes in goat pneumonia.

2. Materials and methods

2.1. Sample collection and processing

Lung samples (n: 244) over a period of 10 years (2003–2012) from pneumonic goats (Table 1) were collected from an abattoir in Bareilly district of U.P. as well as from the postmortem house, IVRI, Izatnagar, Bareilly. Samples received from different states, i.e. Uttar Pradesh, Jammu & Kashmir, Andhra Pradesh, Kerala, Rajasthan, and Uttarakhand, were also screened exclusively for isolation and identification of mycoplasmas only. Before processing, the samples were washed in sterile phosphate buffered saline (PBS, pH 7.4) and small pieces of the samples were triturated in modified PPLO broth of pH 7.6. Serial tenfold dilution was prepared and 10^{-3} dilutions of the triturated suspension were inoculated into liquid and solid modified PPLO media. Inoculated media were incubated at 37 °C in a bacteriological incubator at 5%–10% CO_2. The media were observed regularly for growth up to 1 week before discard. A mild turbidity in liquid medium and appearance of fried egg colonies on solid media are indicative of suspected mollicute growth. The obtained colonies were further cloned 3 times in modified PPLO (containing procaine penicillin @1000 IU/mL), with one reverse passage in PPLO (without procaine penicillin) and repassage in antibiotic containing PPLO broth.

2.2. Biochemical characterization

Out of the 244 lung samples, 15 exhibited a fried egg appearance on PPLO agar. All the colonies were first individually subjected to the digitonin sensitivity test (17) to differentiate between mycoplasmas and acholeplasmas. Later a glucose fermentation test, arginine hydrolysis, liquefaction of coagulated serum, and phosphatase reduction tests were performed for the isolates found sensitive to digitonin (18). Based on the results of biochemical tests, the isolates were further characterized by growth inhibition test (19) using a battery of standard antisera (available with Reference Laboratory on Mycoplasma, IVRI, Izatnagar, Bareilly, U.P., India).

2.3. Molecular identification

To conduct PCR, the DNA of all the isolates was extracted from the bacterial culture (20). The extracted DNA of good quality (Absorbance 260/280 values ≥1.6–1.8) was kept at –20 °C for PCR confirmation. For group-specific confirmation of mollicutes (Mycoplasma, Acholeplasma, Spiroplasma, and Ureaplasma genera) the PCR was performed as described previously (21). Primers based on the 16SrRNA gene,

Table 1. Details of pneumonic goat lung samples processed over the 10-year period 2003–2012.

	Jan	Feb	March	April	May	June	July	August	Sep	Oct	Nov	Dec	Total
2003	10	11	8	-	-	-	-	-	-	-	-	-	29
2004	15	6	13	2	-	-	-	-	-	-	-	-	36
2005	10	-	22	-	-	-	-	-	-	-	-	4	36
2006	7	6	5	3	-	1	1	-	-	-	-	-	23
2007	4	-	1	-	-	-	-	10	7	2	1	1	26
2008	2	3	-	1	7	1	1	-	-	-	1	2	17
2009	1	2	1	2	-	-	-	-	1	2	2	2	13
2010	3	5	5	-	3	-	2	4	-	-	2	1	25
2011	2	1	-	-	-	1	-	4	6	2	8	3	27
2012	4	3	-	-	1	1	-	-	1	-	1	1	12
Total: 244													

i.e. GPO-3 (5'-ACTCCTACGGGAGGCAGCACTA-3') and MGSO (5'-TGCACCATCTGTCACTCTGT TAACCTC-3'), were used.

For identification of *M. arginini*, the PCR was performed with species-specific primers targeting the rpoB gene (F 5' TTTGACGGGGTTGTAACATACGT 3' and R 5' CAGCTAATCCTAGGTGTAATTC GAG 3') as described by Sillo et al. (22).

For identification of *M. mycoides* subsp. *capri* and *M. agalactiae* the PCR tests were performed as per Hernandez et al. (23) and Tola et al.(24) respectively.

2.4. Sequencing and phylogenetic analysis of M. arginini isolates

Amplified PCR products of *M. arginini* (from 7 representative Indian isolates) were purified using a Qiaquick PCR purification kit (Qiagen, USA). Purified PCR products were sequenced (Eurofins, Bangalore, India) in both directions. Based on the sequencing results, contigs were constructed using DNA Baser software. Assembled DNA sequences were subjected to BLAST analysis. After aligning the sequences using the Clustal W algorithm method, phylogenetic analysis was done by neighbor-joining method using MEGA 6.0 software. The confirmed sequences were submitted to NCBI GenBank.

3. Results

In the present study, 244 pneumonic goat lung samples were processed for isolation and identification of associated mycoplasma. Out of these, 35 mollicute strains were isolated. These mollicutes were identified by digitonin sensitivity, growth inhibition test, biochemical tests, PCR, and sequencing. Twenty of the mollicutes isolates were not sensitive to digitonin, which were identified as *Acholeplasma* by biochemical tests. The results of the biochemical characterization are given in Table 2.

The growth inhibition test was performed with a battery of standard antiserum available with the repository of the Referral Laboratory on Mycoplasmas, at IVRI, Izatnagar. The isolates of *M. arginini*, *M. mycoides* subsp. *capri*, and *M. agalactiae* exhibited zones of inhibition of around 2–3 mm with their respective antiserum disc. Molecular identification by species-specific PCR was carried out for all the digitonin-sensitive isolates.

The group-specific PCR for all the isolates showed positive results with amplicon size of 270 bp (Figure 1).

The single isolate each of *M. agalactiae* and *M. mycoides* subsp. *capri* gave amplified products of size 194 bp and 375 bp, respectively, and hence were confirmed.

All 13 isolates of *M. arginini* on PCR exhibited amplicon of size 885 bp, showing confirmation (Figure 2).

As the association of *M. arginini* in goat lung was of more concern to us, amplified PCR products from seven representative isolates were subjected to sequencing analysis of the purified PCR products by Sanger's dideoxy method and the sequences were submitted to NCBI GenBank (Table 3: Accession numbers KP685369.1–KP685375.1).

A phylogenetic tree was constructed based on the rpoB sequence of seven Indian *M. arginini* isolates and standard strain (Figure 3). The isolates 01/11 T2B, 01/11 T6H, 01/11E (from Ladakh, Jammu & Kashmir), and VP 3B/05 (from Bareilly slaughterhouse) showed 100% sequence identity with VP 3A/05 (from Bareilly slaughterhouse). Except 26/10, all Indian isolates showed divergence of 1.9 from the ATCC sequence (Figure 4). Sequencing of the rpoB gene from *M. arginini* isolates revealed that there was no significant difference among them. All the isolates clustered together, indicating high degree of sequence identity, separate from the sequences of standard strain (ATCC 23243). Moreover, sequences of all these isolates showed >98% identity with that of the standard strain.

4. Discussion

Different Mycoplasma species have been implicated in a number of important diseases affecting goats. Major symptoms associated with mycoplasmoses include pneumonia, arthritis, conjunctivitis, mastitis, and abortion. *Mycoplasma mycoides* subsp. *capri* (*Mmc*), *Mycoplasma capricolum* subsp. *capripneumoniae* (*Mccp*), *Mycoplasma capricolum* subsp. *capricolum* (*Mcc*), and *M. arginini* are the major mycoplasmal agents isolated from caprine ailment cases. Although *M. arginini* was not previously considered a primary pathogen, several case reports (25) are suggestive of its suspected pathogenic role in animal species as well as in humans.

Table 2. Biochemical tests of various mollicute isolates.

Isolates	Glucose fermentation	Arginine hydrolysis	Coagulated serum liquefaction	Phosphatase reduction
M. arginini	–	+	–	–
M. mycoides subsp. *capri*	+	–	+	–
M. agalactiae	–	–	–	+
A. laidlawii	+	–	–	–

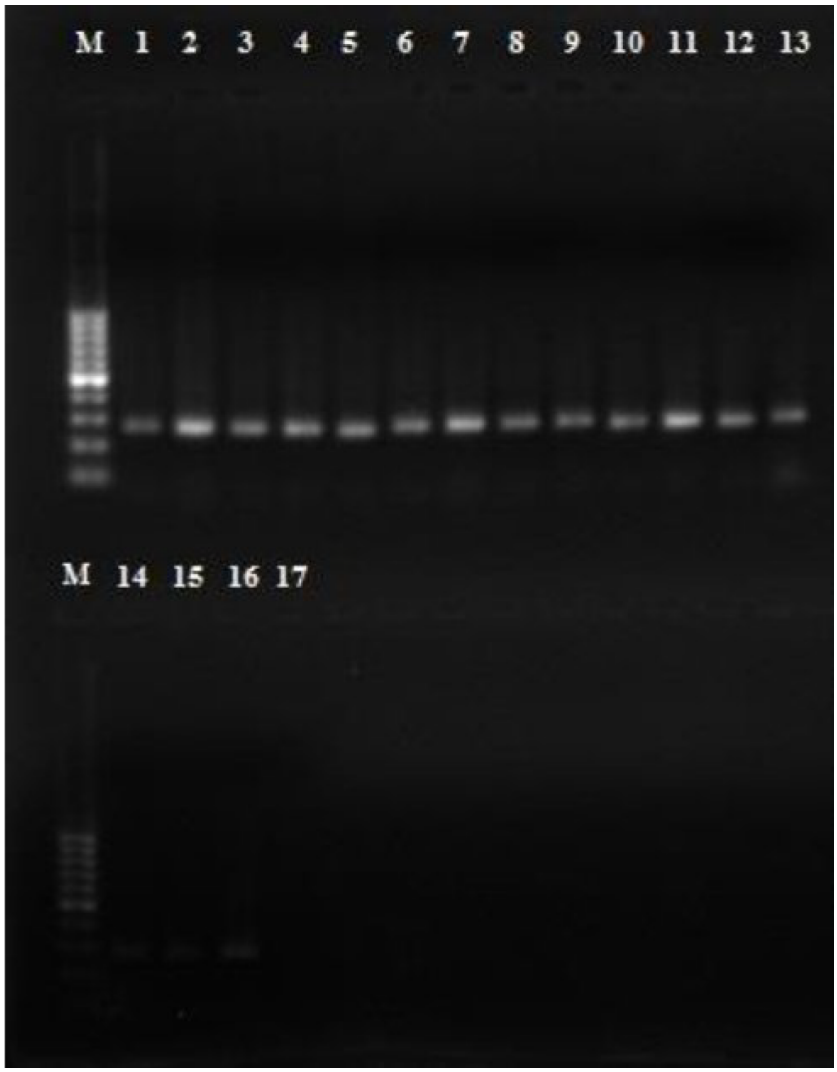

Figure 1. Group-specific PCR of Mycoplasma isolates.
Lane M: 100 bp marker, Lane 1: Positive control, Lanes 2–14: *M. arginini*, Lane 15: *M. agalactiae*, Lane 16: *M. mycoides* subsp. *capri*, Lane 17: Negative control.

In the present study, 244 pneumonic goat lung samples collected over a period of 10 years from 2003 to 2012 were screened for the identification of associated mollicutes. Digitonin resistant acholeplasmas were identified in 20 samples. As the pathogenicity of acholeplasmas in animals is not established, they were not subjected to molecular identification. The most frequent mycoplasma isolated was *M. arginini* (5.3%). There are many reports of *M. arginini* isolation from caprine pneumonia cases. A similar study was conducted for isolation of *Mycoplasma* spp. and *Acholeplasma* spp. from pneumonic lungs of goats and sheep (26). In most cases, the isolate was confirmed to be *M. arginini*, followed by *A. laidlawii.* An isolation rate of 35.29% was reported for *M. arginini* from goat lung and nasal swab samples obtained from different areas of Cairo, Egypt (27).

There are certain pathogenicity reports of *Mycoplasma arginini* in goat kids where the isolate caused transient elevations in rectal temperature, circulating monocytes, circulating neutrophils, and blood fibrinogen. *Mycoplasma arginini* was infective and immunogenic for all inoculated animals and showed a particular affinity for the tonsils (28).

In order to assess the relationship between *M. arginini* isolates of different origins, seven representative isolates were subjected to partial sequencing of the rpoB gene, which showed >98% identity with that of ATCC 23243 *M. arginini* strain. Phylogenetic analysis of rpoB sequences demonstrated higher sequence identity between Indian isolates irrespective of geographical origin in contrast to remarkable genetic variability among Victorian isolates by MLST (14).

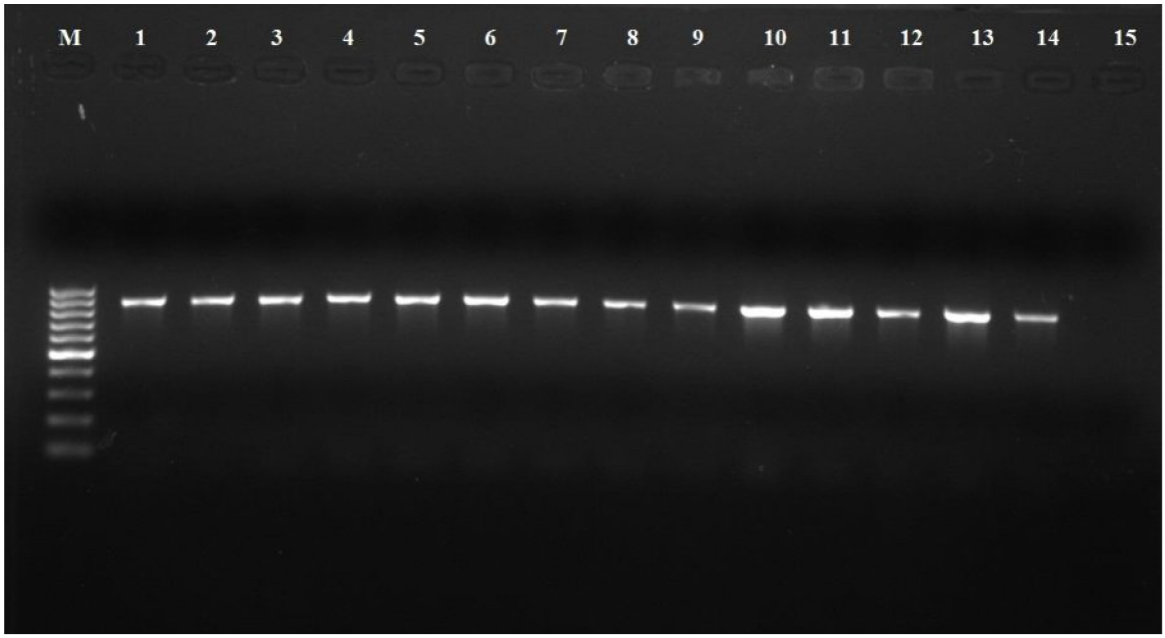

Figure 2. *Mycoplasma arginini*-specific PCR.
Lane M: 100 bp marker, Lane 1: Positive control, Lane 2: 3/03, Lane 3: 03A/05, Lane 4: 03B/05, Lane 5: 03C/05, Lane 6: 03E/05, Lane 7: 26/10, Lane 8: RLM 01/11 T1, Lane 9: RLM 01/11 T2, Lane 10: RLM 01/11 T3, Lane 11: RLM 01/11 T4, Lane 12: RLM 01/11 T5, Lane 13: RLM 01/11 T6, Lane 14: RLM 01/11 T15, Lane 15: Negative control.

Table 3. Sequences of *Mycoplasma arginini* used in phylogenic analysis.

Sl. No.		Source	Accession no.
1	ATCC 23243	NCBI	DQ272351.1
2	3/03	RLM, IVRI	KP685370.1
3	3A/05	RLM, IVRI	KP685371.1
4	3B/05	RLM, IVRI	KP685372.1
5	26/10	RLM, IVRI	KP685373.1
6	01/11E	RLM, IVRI	KP685369.1
7	01/11 T2B	RLM, IVRI	KP685374.1
8	01/11 T6H	RLM, IVRI	KP685375.1

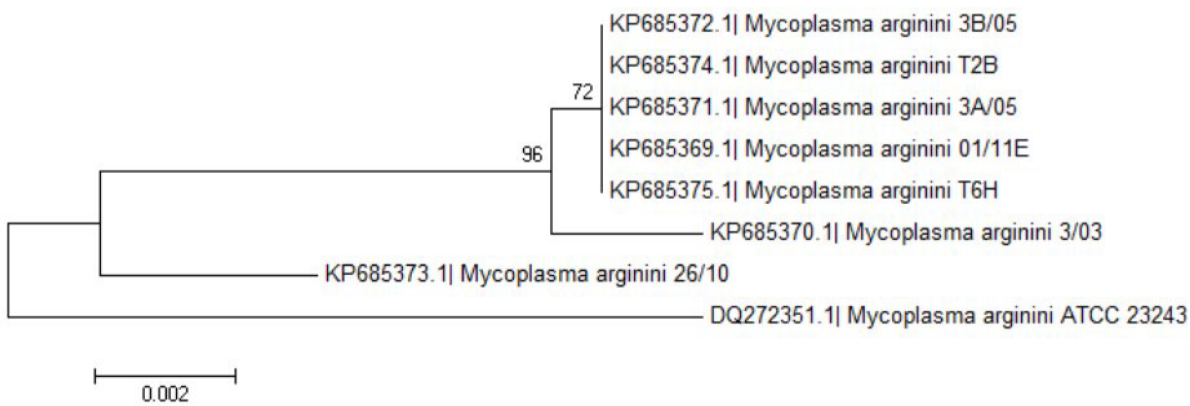

Figure 3. Phylogenic analysis of *M. arginini* isolates based on rpoB gene sequence.

Percent Identity

	1	2	3	4	5	6	7	8		
1		99.7	99.7	99.7	99.7	98.7	99.7	98.1	1	3-03.seq
2	0.3		100.0	100.0	100.0	99.0	100.0	98.1	2	3A-05.seq
3	0.3	0.0		100.0	100.0	99.0	100.0	98.1	3	3B-05.seq
4	0.3	0.0	0.0		100.0	99.0	100.0	98.1	4	T2B.seq
5	0.3	0.0	0.0	0.0		99.0	100.0	98.1	5	T6H.seq
6	1.3	1.0	1.0	1.0	1.0		99.0	98.6	6	26-10.seq
7	0.3	0.0	0.0	0.0	0.0	1.0		98.1	7	01-11E.seq
8	1.9	1.9	1.9	1.9	1.9	1.5	1.9		8	ATCC.seq
	1	2	3	4	5	6	7	8		

Divergence

Figure 4. Sequence identity and divergence between various isolates of *M. arginini*.

Based on biochemical and molecular tests, 5.3% prevalence of *M. arginini* was identified on screening 244 caprine pneumonia lung samples, which is in fact slightly less but surely gives an insight into its seriousness. Considerably higher prevalence of the organism in diseased lung samples signifies their probable role in the pathogenesis of pneumonia, beside other relevant bacterial and viral agents of proven pneumonia etiology. Furthermore, it may also play a predisposing role in disease establishment. As the literature also says about the zoonotic potential of *M. arginini*, further studies are needed on larger goat populations with parallel laboratory work to prove Koch's postulates in the host proper as well as studies on tissue culture and organ culture work in vitro.

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