VETERINARSKI ARHIV 81 (4), 443-458, 2011

Isolation, identification and molecular characterization of *Mycoplasma* isolates from goats of Gujarat State, India

Pankaj Kumar[1], Ashish Roy[1], Bharat B. Bhanderi[1]*, and Bhik C. Pal[2]

[1]*Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Anand, Anand Agricultural University, Gujarat, India*

[2]*Department of Veterinary Epidemiology, Veterinary University, Mathura (U. P.), India*

KUMAR, P., A. ROY, B. B. BHANDERI, B. C. PAL: Isolation, identification and molecular characterization of *Mycoplasma* isolates from goats of Gujarat State, India. Vet. arhiv 81, 443-458, 2011.

ABSTRACT

A total of 358 samples [nasal discharges (215), lung tissue (6), pleural fluid (4), joint fluid (5), milk (60), preputial swabs (22), conjunctival swabs (16), ear swabs (5) and vaginal discharges (25)] were subjected for cultural isolation on MBHS-L and MBHS-A media. Of these, a total of 30 isolates (8.35 %) of *Mycoplasma* species, (20 from nasal discharges, 4 from lung tissue, 2 from pleural fluid, 3 from milk and 1 from joint fluid) were isolated and characterized biochemically and identified as *M. mycoides* subsp. *capri* and *M. capricolum* subsp. *capricolum* by PCR and PCR-RFLP. The isolates were identified as the *Mycoplasma* species by their cultural, morphological, biochemical characteristics and this was further confirmed by PCR and PCR-RFLP using different cluster, group and species specific primer pairs and by restriction enzyme respectively. This is a rare report on the isolation, identification and molecular characterization of goat *Mycoplasma* species in Western India. These studies have revealed a high prevalence of *Mycoplasma* species infection in Western India.

Key words: India, mycoplasmas, goats, *M. mycoides* subsp. c*apri, M. capricolum* subsp. *capricolum*

Introduction

Mycoplasmas are the smallest fastidious bacteria, which can cause diseases in major species of animals, including humans. In small ruminants, they are known for respiratory disease, arthritis, eye lesions, genital disease and mastitis (NICHOLAS, 2002; SHARIF and MUHAMMAD, 2009). Most of the members of the *Mycoplasma mycoides* cluster group are important pathogens for small ruminants. This group comprises six species and subspecies. Some of these *Mycoplasma* species can cause severe and contagious diseases in goats, with significant economic impact. Of the many *Mycoplasma* diseases, contagious

*Corresponding author:

Bharat B. Bhanderi, Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand-388 001, Gujarat State, India, Phone: +91 92283 09371; E-mail: bbbhanderi@yahoo.co.in

ISSN 0372-5480
Printed in Croatia

caprine pleuropneumonia (CCPP) is a highly fatal disease that occurs in Eastern Europe, the Middle East, Africa and Asia (KOPCHA, 2005).

The *M. mycoides* cluster members are closely interrelated both serologically as well as genetically and include*: M. mycoides* subsp. *mycoides* small colony type (MmmSC), *M. mycoides* subsp. *capri* (Mmc), *M. capricolum* subsp. *capricolum* (Mcc), *M. capricolum* subsp. *capripneumoniae* (Mccp) and *M. leachii* (MANSO-SILVÁN et al., 2009). Apart from MmmSC and Mccp, the etiological agents of CBPP and CCPP respectively, and *M. leachii* which causes bovine arthritis and mastitis, other members have been implicated in the MAKePS syndrome (Mastitis, Arthritis, Keratoconjunctivitis, Pneumonia, Septicaemia) along with *M. agalactiae* and *M. putrifaciens* in goats (THIAUCOURT and BOLSKE, 1996).

Looking at the paucity of the literature in India, the present work was carried out to isolate, identify, and undertake molecular characterization of *Mycoplasma* from caprine infection in Gujarat State, India.

Materials and methods

Sample collection. During this study, all 358 samples (comprising nasal discharges (215), lung tissue (6), pleural fluid (4), joints fluid (5), milk (60), preputial swabs (22), conjunctival swabs (16), ear swabs (5) and vaginal discharges (25)) were collected aseptically on Modified Hank's Balanced Salt Solution Liquid Media (MBHS-L) and transported to the laboratory in a cold chain.

Processing of samples. Samples were placed directly in MBHS-L medium and kept at 37 °C for 1 hour and then cultured by inoculating 0.2 mL amounts of sample into 3 mL of fresh MBHS-L media. The inoculated liquid medium was incubated at 37 °C up to 10 days and was observed daily for signs of growth. Positive cultures were kept at 4 °C and those with no signs of growth were discarded after 10 days of incubation.

The samples that were positive for growth in MBHS-L broth were inoculated into Modified Hank's Balanced Salt Solution Agar Media (MBHS-A) and incubated anaerobically at 37 ºC for up to 10 days under humid conditions in 5-10% CO_2 tension to obtain optimum growth. All the plates were examined daily with a conventional microscope at 4× magnification and growth and colony morphology were recorded. If growth was sparse (1 to 10 colonies per plate) the plates were flooded with 1.5 mL sterile broth and reincubated for an additional 48 to 72 hours to increase the number of colonies. If no growth occurred after 10 days of incubation, then the plates were discarded.

Purification (cloning) of primary culture. Pure stock cultures were prepared by transferring a small agar block containing a single colony of *Mycoplasma* to MBHS-L broth and incubated at 37 ºC for 3-5 days and stored at 4 ºC. The subculturing of each isolate from the liquid broth culture was done every 30th day.

Examination of the isolated colonies. To study the morphology of the suspected colonies of *Mycoplasma,* they were stained by Diene's method, Giemsa's and acridine orange stain as described by GUPTA (2005).

Characterization of Mycoplasma. To determine the biochemical activity of *Mycoplasma* isolates, tests such as catabolism of glucose, hydrolysis of arginine, phosphatase activity, tetrazolium reduction, serum digestion, digitoin sensitivity test and film and spot formation were carried out as per the technique adopted by GUPTA (2005).

Molecular characterization. DNA extraction. The template DNA from the broth culture was prepared according to the method of JOHANSSON et al. (1998) with slight modifications. About 1 mL of the broth culture of each isolate was centrifuged at 12000 rpm in a microcentrifuge at 4 °C for 10 minutes and the pellet washed in 0.5 mL of PBS twice. The pellets were resuspended in 100 µL of 0.3X TE and treated with proteinase K (Genei, Cat no. 105973) at 60 °C for 30 minutes and finally kept at 97 °C for 10 minute. Cell debris was removed by centrifugation and 5 µL of the supernatant was used as a DNA template in the PCR reaction mixture.

Identification of Mycoplasma mycoides 'Cluster'. PCR was performed using the *M. mycoides* cluster specific primers MC 323 (TAG AGG TAC TTT AGA TAC TCA AGG) and MC 358 (GAT ATC TAA AGG TGA TGG T) for an expected amplified product of 1.5 kb long sequence homologous to the CAP-21 probe of the *M. mycoides* cluster members (BASHIRUDDIN et al., 1994). The amplification was carried out using the following programme: initial denaturation at 94 °C for 1 min followed by 30 cycles of denaturation, annealing and extension (94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min), and final extension at 72 °C for 5 min followed by 10 °C hold at infinity.

Similarly PCR was performed using the *M. mycoides* cluster specific primers CA (F) (CGA AAG CGG CTT ACT GGC TTG TT) and CA (R) (TTG AGA TTA GCT CCC CTT CAC AG) for an expected amplified product of 548 bp long sequence of the 16S rRNA gene of the the *M. mycoides* cluster members (BOLSKE et al., 1996). The amplification was carried out using the following programme: initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation, annealing and extension (95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min), and final extension at 72 °C for 10 min followed by 10 °C hold at infinity.

Identification of mycoides group members. Mycoides group specific PCR was performed using the primers MM 450 (GTA TTT TCC TTT CTA ATT TG) and MM 451 (AAA TCA AAT TAA TAA GTT TG) for an expected amplified product of 574 bp of the CAP-21 sequence of the 16S rRNA gene of the *Mycoides* group members MmmSC, Mmc and MmmLC (BASHIRUDDIN et al., 1994). The amplification was carried out using the following programme: initial denaturation at 94 °C for 1 min followed by 30 cycles of

denaturation, annealing and extension (94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min), and final extension at 72 °C for 5 min followed by 10 °C hold at infinity.

Identification of Mycoplasma isolates up to species level. PCR using the *Mycoplasma capricolum* subsp. *capricolum* specific primers P4 (ACT GAG CAA TTC CTC TT) and P8 (GTA AAC CGT GTA TAT CAA AT) for an expected amplified product of 192 bp long sequence of the 16S rRNA gene of the Mcc (HERNANDEZ et al., 2006). The amplification was carried out using the following programme: initial denaturation at 94 °C for 1 min followed by 30 cycles of denaturation, annealing and extension (94 °C for 30 sec, 56 °C for 1 min, 72 °C for 1.5 min), and final extension at 72 °C for 5 min followed by 10 °C hold at infinity.

PCR using the *Mycoplasma mycoides* subsp. *capri* specific primers P4 (ACT GAG CAA TTC CTC TT) and P6 (TTA ATA AGT CTC TAT ATG AAT) for an expected amplified product 194 bp long sequence of the 16S rRNA gene of the the Mmc (HERNANDEZ et al., 2006). The amplification was carried out using the following programme: initial denaturation at 94 °C for 1 min followed by 30 cycles of denaturation, annealing and extension (94 °C for 1 min, 46 °C for 1 min, 72 °C for 2 min), and final extension at 72 °C for 5 min followed by hold at infinity.

PCR using *Mycoplasma agalactia*e specific primers [Mga F (CCT TTT AGA TTG GGA TAG CGG ATG / MgaR (CCG TCA AGG TAG CGT CAT TTC CTA C)] and *M. putrefaciens* specific primers [SSF1(GCG GCA TGC CTA ATA CAT GC) / SSR1 (AGC TGC GGC GCT GAG TTC A)] for an expected amplified product of 360 bp (DE AZEVEDO et al., 2006) and 540 bp (SHANKSTER et al., 2002) long amplified product of the 16S rRNA gene of the respectively.

To confirm the targeted PCR amplification, 5 µL of PCR product from each tube was mixed with 1 µL of 6X gel loading buffer from each tube and electrophoresed on 1.5 % agarose gel along with 100 bp DNA Ladder (GeneRuler- Fermentas) and stained with ethidium bromide (1 % solution at the rate of 5 µL/100 mL) at a constant 80 V for 30 minutes in 0.5X TBE buffer. The amplified product was visualized as a single compact band of the expected size under UV light and documented by the gel documentation system (Syn Gene, Gene genius bioImaging System, UK).

PCR-RFLP analysis. Restriction digestion of the PCR products of 16S rRNA gene was carried out to confirm the identity of PCR products by using the restriction enzymes *PstI* (BOLSKE et al., 1996) and *MboII.* The restriction enzymes were selected from a restriction map created using the sequences available in GenBank and NEBcutter V2.0 software. A reaction mixture was prepared in a 200 µL capacity PCR tube for 16S rRNA gene using *MboII* and incubated in a water bath or heat block for 1 hour at 37 °C and inactivated the *MboII* by incubation at 65 °C for 20 minute. For *Pst I* (Fast Digest) reaction mixture was

prepared in a 200 µL capacity PCR tube and incubated in a water bath or heat block for 30 minutes at 37 °C.

Results

An outbreak of respiratory disease occurred in a herd of 1000 goats in the State of Gujarat, India, in March, 2009. The first cases were seen in March, but the number of affected animals peaked during April. Initially, 300 animals died over a period of 10 days and, after 1 month, the flock mortality had reached 80%. Animals of all ages were affected, although they were mostly adults. Kids usually died in less than 1 week after the onset of clinical signs, whereas adults survived longer, up to 2 weeks.

The clinical picture was characterized by high fever, bilateral nasal discharge, difficult breathing, conjunctivitis, prostration, ear drop, and very low milk production. Arthritis was observed in some animals. Six animals on necropsy showed grey, red areas of consolidation in the lungs, affecting more than one lobe and animals showed marked pleuritis and pleural effusion. The pleural fluid was thick and yellowish. The cut surface of some affected lungs revealed a fine granular texture with hepatization.

Isolation. Modified Hank's Balanced Salt Solution Liquid (MBHS-L) medium and Modified Hank's Balanced Salt Solution Agar **(**MBHS-A) medium was used as a primary broth and solid medium respectively for isolation of *Mycoplasma* organisms. The typical fried egg appearance of colonies of Mycoplasmas was observed on solid media

Morphological and staining character of isolates. The morphology of the organism was observed after Giemsa's staining. The organisms were of varying shape, highly pleomorphic revealing spherical, ovoid, pear shapes and short filamentous.

Microscopic colonies character of the isolates. To study the morphology of the suspected colonies of *Mycoplasma,* they were stained by Diene's and Acridine orange method. The light blue periphery of the *Mycoplasma* colonies with a deep blue central nipple was observed under a low power (4x) microscope after the dienes staining procedure, while *Mycoplasma* colonies stained with acridine orange showed a light orange periphery and deep orange central growth under a low power microscope.

Digitonin sensitivity. To specifically identify and differentiate isolates of *Mycoplasma* from *Acholeplasma*, digitonin sensitivity test was done. All the isolates were found to be sensitive for digitonin, revealing a zone of inhibition of more than 2 mm, thus indicating that they belong to the *Mycoplasma* species.

Biochemical characterization of Mycoplasmal isolates. All the 30 isolates obtained on cultural isolation and on preliminary screening by morphological and colony characteristics as well as digitonin sensitivity tests, primarily identified as Mycoplasmas, were subjected to various biochemical tests.

Table 1. Overall biochemical reactions of goat *Mycoplasma* isolates

Groups	Strains examined	Glucose hydrolysis	Arginine catabolism	Serum digestion	Phosphatase activity	Tetrazolium reduction	Films and spot	No. isolated	Percentage	Species designation
I	11	+	+	+	_	+	_	11/30	36.66	Mcc
II	19	+	_	+	_	+	v	19/30	63.33	Mmc

(+) positive, (-) negative, (v) variable (All the isolates were negative for film and spot except ML 25 and ML 27); Mcc (*Mycoplasma capricolum* subsp. *capricolum*); Mmc (*Mycoplasma mycoides* subsp. *capri*)

Table 2. Result of PCR using various primers for detection of mycoides cluster, mycoides group and species identification of *Mycoplasma* species CA (F)/CA(R), MC323/MC358, MM450/MM451, P4/P8, P4/P6, Maga(F)/Maga(R) and SSF1/SSR1)

Sr. No.	Name of isolates	Gene (16S rRNA gene) and Primers							Species identified
		Cluster specific primers		Group specific primer	Species specific primers				
		MC 323/ MC 358	CA (F)/ CA (R)	MM 450/ MM 451	P4/ P8	P4/ P6	Maga(F)/ Maga(R)	SSF1/ SSR1	
1	MN1	+	+	-	+	-	-	-	Mcc
2	MN2	+	+	-	+	-	-	-	Mcc
3	MN3	+	+	-	+	-	-	-	Mcc
4	MN4	+	+	-	+	-	-	-	Mcc
5	MN5	+	+	-	+	-	-	-	Mcc
6	MN6	+	+	-	+	-	-	-	Mcc
7	MN7	+	+	-	+	-	-	-	Mcc
8	MN8	+	+	-	+	-	-	-	Mcc
9	MN9	+	+	+	-	+	-	-	Mmc
10	MN10	+	+	+	-	+	-	-	Mmc
11	MN11	+	+	+	-	+	-	-	Mmc
12	MN12	+	+	+	-	+	-	-	Mmc
13	MN13	+	+	+	-	+	-	-	Mmc
14	MN14	+	+	+	-	+	-	-	Mmc
15	MN15	+	+	+	-	+	-	-	Mmc
16	MN16	+	+	+	-	+	-	-	Mmc

Table 2. Result of PCR using various primers for detection of mycoides cluster, mycoides group and species identification of *Mycoplasma* species CA (F)/CA(R), MC323/MC358, MM450/MM451, P4/P8, P4/P6, Maga(F)/Maga(R) and SSF1/SSR1) (Continued)

Sr. No.	Name of isolates	Gene (16S rRNA gene) and Primers							Species identified
		Cluster specific primers		Group specific primer	Species specific primers				
		MC 323/ MC 358	CA (F)/ CA (R)	MM 450/ MM 451	P4/ P8	P4/ P6	Maga(F)/ Maga(R)	SSF1/ SSR1	
17	MN17	+	+	+	-	+	-	-	Mmc
18	MN18	+	+	+	-	+	-	-	Mmc
19	MN19	+	+	+	-	+	-	-	Mmc
20	MN20	+	+	+	-	+	-	-	Mmc
21	ML21	+	+	+	-	+	-	-	Mmc
22	ML22	+	+	+	-	+	-	-	Mmc
23	ML23	+	+	+	-	+	-	-	Mmc
24	ML24	+	+	+	-	+	-	-	Mmc
25	MP25	+	+	+	-	+	-	-	Mmc
26	MP26	+	+	+	-	+	-	-	Mmc
27	MJ27	+	+	+	-	+	-	-	Mmc
28	MM28	+	+	-	+	-	-	-	Mcc
29	MM29	+	+	-	+	-	-	-	Mcc
30	MM30	+	+	-	+	-	-	-	Mcc

Mmc *(Mycoplasma mycoides* subsp. *capri),* Mcc *(Mycoplasma capricolum* subsp. *capricolum)*

The isolates were preliminary characterized by using different biochemical tests: catabolism of glucose, hydrolysis of arginine, phosphatase activity, tetrazolium reduction, serum digestion and film and spot formation, for preliminary identification of the species

Preliminary identification of species. On the basis of the above findings, the 30 isolates of *Mycoplasma* obtained by cultural isolation could be divided into two groups as follows: The details are given in Table 1.

Group I. Consists of 11 isolates comprising: 8 isolates (nasal discharges) from goats with a clinical history of respiratory distress and 3 isolates (milk) from apparently healthy goats. They hydrolyzed glucose, catabolized arginine, did not form a film and spot, reduced tetrazolium, digested serum and did not produce phosphatase. These isolates were preliminarily identified as *Mycoplasma capricolum* subsp. *capricolum* (Mcc).

Group II. Consists of 19 isolates comprising: 12 isolates (nasal discharges), 4 isolates(lungs), 2 isolates (pleural fluid), one isolate (joints) from goats with a clinical history of respiratory distress, arthritis, conjunctivitis and heavy mortality. Isolated *Mycoplasma* strains hydrolyzed glucose, did not catabolized arginine, did not form a film and spot (except 2 isolates of lung tissue, ML25 and ML 27 were positive), reduced tetrazolium, digested serum and did not produce phosphatase. These isolates were preliminary identified as *Mycoplasma mycoides* subsp. *capri* (Mmc).

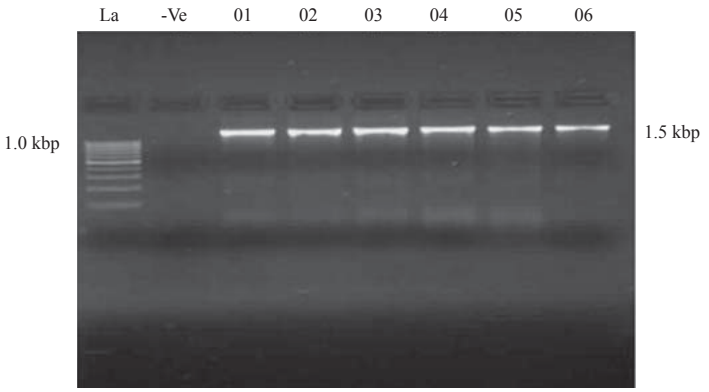

Fig. 1. Agarose gel showing PCR ampilified product for CAP-21 probe of mycoides cluster using primer pair MC 323/ MC 358. La: DNA molecular weight ladder 100 bp; Ve: control; 01-06: field samples.

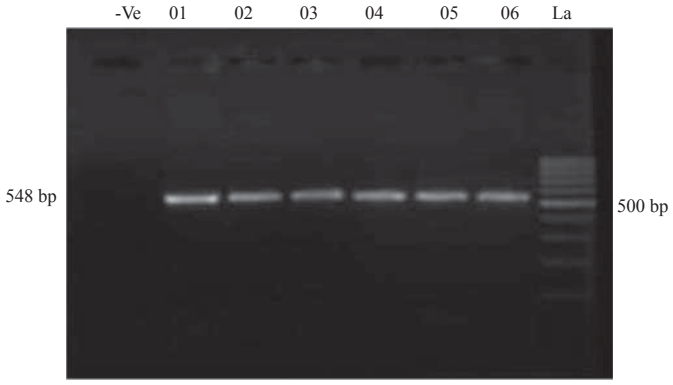

Fig. 2. Agarose gel showing PCR ampilified product (548 bp) for 16S rRNA gene of mycoides cluster using primer pair CA (F)/ CA (R). La: DNA molecular weight ladder 100 bp; -Ve: negative control; 01-06: field samples.

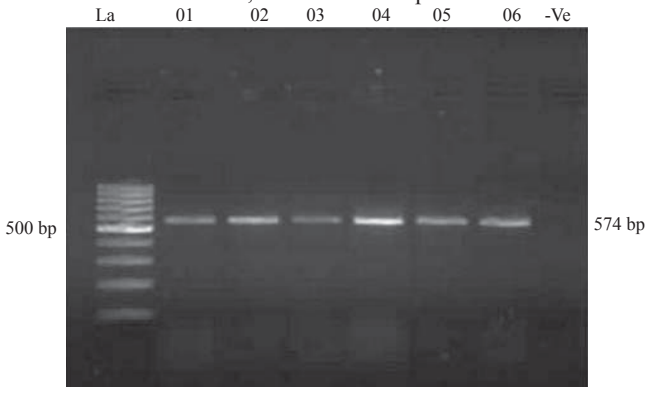

Fig. 3. Agarose gel showing PCR amplification product (574 bp) for CAP-21 probe using primer pair MM 450 / MM 451 for mycoides group members. La: DNA molecular weight ladder 100 bp; -Ve: negative control; 01-06: field samples (isolates).

Confirmation of mycoplasma isolates by PCR using various sets of primer and PCR-RFLP. Two sets of cluster specific primers, MC 323 / MC 358 and CA(F) / CA(R), were used to differentiate the *M. mycoides* cluster members from non cluster *Mycoplasmas* by PCR in which all 30 isolates of *Mycoplasma* yielded amplified products of 1.5 Kbp and 548 bp respectively. Further, the members of the *mycoides* group(*M. mycoides* subsp. *capri*) were differentiated from those of the *capricolum* group (*M. capricolum* subsp. *capricolum*) by *mycoides* group specific PCR, using the primers MM 450 and MM 451 yielded 574 bp long amplicons were only observed in the *mycoides* group members (19 isolates of *M. mycoides* subsp. *capri*) (Figs. 1, 2 and 3).

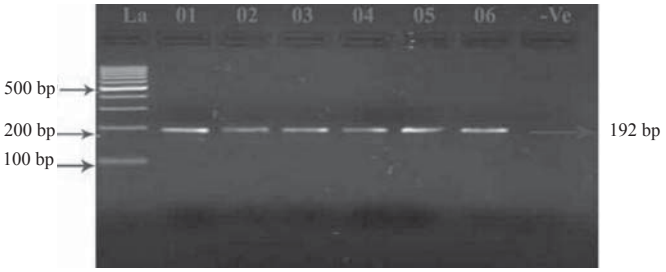

Fig. 4. Agarose gel showing PCR amplification product (129 bp) for 16 S rRNA gene using primer pair P4 and P8 of *M. capricolum* subsp. *capricolum*. La: DNA molecular weight ladder 100 bp; -Ve: negative control; 01-06: field samples (isolates).

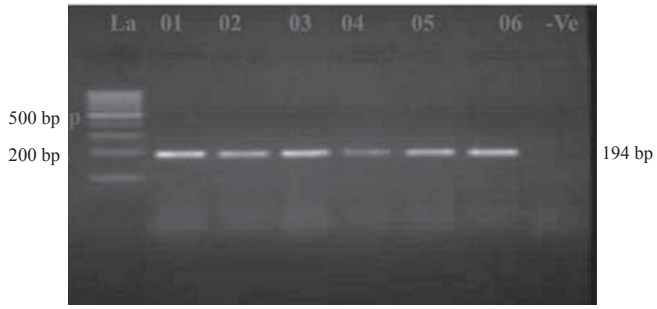

Fig. 5. Agarose gel showing PCR amplification product (194 bp) for 16 S rRNA gene using primer pair P4 and P6 of *M. mycoides* subsp. *capri*. La: DNA molecular weight ladder 100 bp; -Ve: negative control; 01-06: field samples (isplates).

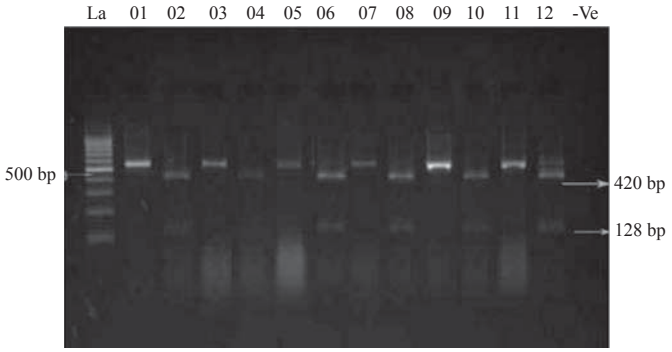

Fig. 6. Agarose gel showing restriction fragmentation pattern of the mycoides cluster by using the enzyme *Pst* I on 548 bp amplicons (specific PCR product amplifield with primer CA (F)/ CA (R). La: DNA molecular weight ladder 100 bp; -Ve: negative control; Lane 01, 03, 05, 07, 08, 11: field samples (548 bp); Lane 02,04,06,08,10,12 are their restriction fragments (420 bp and 128 bp) after digestion with *Pst* I.

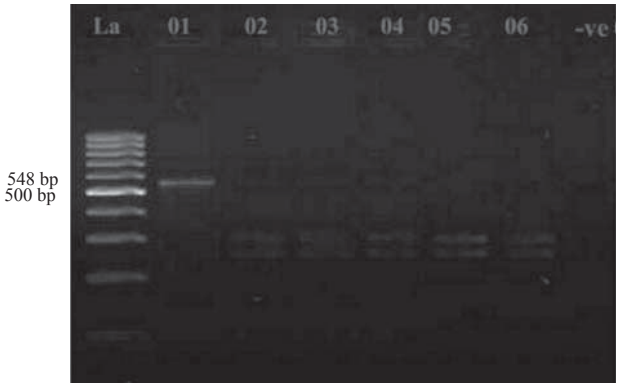

Fig. 7. Agarose gel showing restriction fragmentation pattern of the mycoides cluster by using the enzyme *Mbo* II on 548 bp amplicons (specific PCR product amplifield with primer CA (F)/ CA (R). La: DNA molecular weight ladder 100 bp; -Ve: negative control; Lane 01: 548 bp field samples; lane 02 - 06: restriction fragments (295 bp and 253 bp) after digestion with *Mbo* II

PCR using the *Mycoplasma capricolum* subsp. *capricolum* specific primers P4 and P8 resulted in an approximately 192 bp long amplified product for 11 isolates, identified as *M. capricolum* subsp. *capricolum*, while none of the 19 isolates identified as *M. mycoides* subsp. *capri* yielded any amplified products (Fig. 4).

PCR using the *Mycoplasma mycoides* subsp. *capri* specific primers P4 and P6 resulted in an approximately 194 bp long amplified product of the16S rRNA gene for all the 19 isolates identified as *M. mycoides* subsp. *capri,* but none of the 11 isolates were identified as *M. capricolum* subsp. *capricolum* (Fig. 5). The overall results are given in Table 2.

Digestion of the 548 bp product yielded by using the primer pair CA(F) and CA(R) amplicons obtained from all the 30 *Mycoplasma* isolates, with the enzyme *Pst* I, resulted in 2 fragments only, 420 bp and 128 bp, which is a characteristic pattern of *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *capri.* Digestion of the 548 bp amplicons obtained from all the 30 *Mycoplasma* isolates with the enzyme *Mbo* II resulted in only 2 fragments of 295 bp and 253 bp for all the 30 *Mycoplasma* isolates (Figs. 6 and 7).

Discussion

Out of the 358 different samples processed, a total of 30 *Mycoplasma* isolates were obtained. Among the isolates, 11 were *Mycoplasma capricolum* subsp. *capricolum* and 19 were *M. mycoides* subsp. *capri*, identified by various biochemical tests and by the PCR method. This indicates and confirms the presence of *Mycoplasma* infection in Western India.

Ten samples (6 lung tissue and 4 pleural fluids) after processing yielded 6 (60%) *Mycoplasma* isolates (4 from lungs, 2 from pleural fluid), and were identified as *M. mycoides* subsp. *capri*. Similar reports regarding isolation have been documented by IKHELOA et al. (2004), RODRIGUEZ et al. (1995) and ADEHAN et al. (2006).

215 nasal discharges yielded 20 (9.30 %) *Mycoplasma* (8 *Mycoplasma capricolum* subsp. *capricolum* and 12 *M. mycoides* subsp. *capri*) isolates. Isolation from nasal discharges has been reported earlier by GUPTA et al. (1988), AL-MOMANI et al. (2006) and AWAN et al. (2009).

Three out of 60 milk samples (5.0%) were found positive for *Mycoplasma capricolum* subsp. *capricolum* isolates. Similar reports with a higher percentage of recovery have been reported by AL-MOMANI et al. (2006) and DELAFE et al. (2009).

5 samples processed from an outbreak of *Mycoplasmosis* in goats, resulted in isolation of one *Mycoplasma* isolate (*Mycoplasma mycoides* subsp. *capri*) from the animals showing typical symptoms of lameness. SINGH et al. (2004) also isolated *Mycoplasma mycoides* from cases of caprine arthritis.

The first group consisting of 11 isolates of *Mycoplasma,* were identified as *Mycoplasma capricolum* subsp. *capricolum* based on the biochemical activity, that is they hydrolyzed glucose, catabolized arginine, did not form a film and spot, reduced tetrazolium, digested serum and did not produce phosphatase. These results are in agreement with the findings of ADEHAN et al. (2006) and DAMASSA et al. (1992) although, IKHELOA et al. (2004) reported similar findings but found that *Mycoplasma capricolum* subsp. *capricolum* isolates showed phosphatase activity.

The second group consists of 19 isolates of *Mycoplasma.* They hydrolyzed glucose, did not catabolize arginine, did not form a film and spot (except two isolates of lung tissue- ML 25 and ML 27), reduced tetrazolium, digested serum and did not produce phosphatase. These isolates were preliminarily identified as *Mycoplasma mycoides* subsp. *capri* (Mmc). These results agree with DAMASSA et al. (1992), AJUWAPE et al. (2003) and NICHOLAS (2002), who reported that isolates of *Mycoplasma* were characterized biochemically as *M. mycoides* subsp. *capri* on the basis of fermentation of glucose, reduction of tetrazolium and serum digestion.

PCR was performed using genomic DNA of all the 30 isolates of *Mycoplasma* using the *M. mycoides* cluster specific primers MC 323 and MC 358, and yielded as expected a 1.5 kbp long sequence, homologous to the CAP-21 analogues from the *M. mycoides* cluster members for all the isolates. This finding was in agreement with that of TAYLOR et al. (1992) and BASHIRUDDIN et al. (1994), who designed these oligos complementary to each end of the sequence of the CAP-21 probe.

A second set of primers CA (F) and CA (R) was also used for all the 30 isolates, and yielded an amplified product of approximately 548 bp long sequence of the 16S rRNA gene of the *M. mycoides* cluster members for all the isolates. This finding was in agreement with that of BOLSKE et al. (1996) and PETTERSSON et al. (1996), who developed a CCPP specific PCR using primers amplifying a 548 bp sequence of the 16S rRNA genes from all the six members of the cluster.

Digestion of the 548 bp amplicons (the product yielded by using primer pair CA(F) and CA(R)) were obtained from all 30 *Mycoplasma* isolates with the enzyme *Pst* I resulting in 2 fragments only, 420 bp and 128 bp, which is the characteristic pattern of *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *capri*. This finding was in agreement with BOLSKE et al. (1996) and HERNADEZ et al. (2006). Digestion of the 548 bp amplicons obtained from all the 30 *Mycoplasma* isolates with the enzyme *Mbo* II, resulted in only 2 fragments of 295 bp and 253 bp for all the 30 *Mycoplasma* isolates.

PCR using the *Mycoides* group specific primers MM 450 and MM 451, resulted in an approximately 574 bp long amplified product of the CAP-21 probe of the *Mycoides* group members *M. mycoides* subsp. *capri* (19 isolates), this finding was in agreement with that of BASHIRUDDIN et al. (1994).

PCR using the *Mycoplasma capricolum* subsp. *capricolum* specific primers P4 and P8 resulted in an approximately 192 bp long amplified product of the16S rRNA gene for 11 isolates, identified as *M. capricolum* subsp. *capricolum*, while none of the 19 isolates identified as *M. mycoides* subsp. *capri* yielded any amplified products. This finding was in agreement with that of HOTZEL et al. (1996), KUMAR (2000) and HERNANDEZ et al. (2006). They used the Mcc specific primer P8 in combination with another primer P4, which was common to all cluster members except MmmSC, to amplify approximately 192 bp product from only Mcc strains.

PCR using the *Mycoplasma mycoides* subsp. *capri* specific primers P4 and P6 resulted in an approximately 194 bp long amplified product of the16S rRNA gene for all the 19 isolates identified as *M. mycoides* subsp. *capri,* but none of the 11 isolates identified as *M. capricolum* subsp. *capricolum*. This finding was in agreement with that of HOTZEL et al. (1996); KUMAR, (2000) and HERNANDEZ et al. (2006), who used Mmc specific primers (P4 and P6) and yielded approximately 194 bp product with Mmc strains.

Conclusions

The analysis of the findings from the present study implies that there is indeed a prevalence of *Mycoplasma* infections in India. Isolation and identification was conducted of Mycoplasmas from goats presenting a range of clinical signs, including respiratory disease, conjunctivitis, arthritis, mastitis and loss in milk production. PCR based detection of *mycoides* cluster, group and species is a rapid and simple method of

detection and identification of the Mycoplasmal organism and can be an effective tool for epidemiological surveys. Restriction enzyme fragmentation assay can be a useful method for differentiation and identification of *Mycoplasmal* isolates.

Acknowledgements

The authors are thankful to the Dean, College of Veterinary Science and Animal Husbandry, Anand (Gujarat), for providing the facilities to pursue this work.

References

ADEHAN, R. K., A. T. P. AJUWAPE, A. I. ADETOSOYE, O. O. ALAKA (2006): Characterization of *Mycoplasmas* isolated from pneumonic lungs of sheep and goats. Small Rumin. Res. 63, 44-49.

AJUWAPE, A. T. P., J. O. IKHELOA, M. D. OYAL, O. O. ALAKA, A. F. ADATESOYE (2003): Biochemical and serological identification of *Mycoplasmas* from pneumonic bovine lungs in Nigeria. Isr. Vet. Med. Ass. 58, 2-3.

AL-MOMANI, W., M. A. HALABLAB, A. ABO-SHEHADA, L. MCAULIFFE,R. A. J. NICHOLAS (2006): Isolation and molecular identification of small ruminant Mycoplasmas in Jordan. Small Rumin. Res. 65, 106-112.

AWAN, M. A., F. ABBAS, M. YASINZAI, R. A. J. NICHOLAS, S. BABAR, R. D. AYLING, M. A. ATTIQUE, Z. AHMED (2009): Prevalence of *Mycoplasma capricolum* subsp. *capricolum* and *Mycoplasma putrefaciens* in goats in Pishin district of Balochistan. Pakistan Vet. J. 29, 179-185.

BASHIRUDDIN, J. B., T. K. TAYLOR, A. R. GOULD (1994): A PCR-based test for the specific identification of *Mycoplasma mycoides* subspecies *mycoides* SC. J. Vet. Diagn. Invest. 6, 428-434.

BOLSKE, G., J. G. MATTSSON, C. R. BASCUNANA, K. BERGSTROM,H. WESONGA, K. E. JOHANSON (1996): Diagnosis of contagious caprine pleuropneumonia by detection and identification of *Mycoplasma capricolum* subsp. *capripneumoniae* by PCR and REA. J. C1in. Microbiol. 34, 785-791.

DAMASSA, A. J., P. S. WAKENELL, D. L. BROOKS (1992): *Mycoplasma* of goats and sheep. J. Vet. Diagn. Invest. 4, 101-113.

DE AZEVEDO, E. O., M. D. B. DE ALCANTARA, E. R. DE NASCIMENTO, I. M. TABOSA, M. L. BARRETO, J. F. DE ALMEIDAO, M. O. ARAUJO, A. R. RODRIGUES, F. R. CORREA, R. S. DE CASTRO (2006): Contagious agalactia by *Mycoplasma agalactiae* in small ruminants in Brazil: first report. Departamento de Microbiologia - ICB II USP Av. Prof. Lineu Prestes, 1374 - Sala 214, Cidade Universitaria.

DELAFE, C., C. SANCHEZ, A. GUTIERREZ, A. CONTRERAS, J. C. CORRALES, P. C. ASSUNÇAO, C. POVEDA, J. B. POVEDA (2009): Effects on goat milk quality of the

presence of *Mycoplasma* spp. in herds without symptoms of contagious agalactia. J. Dairy Res. 76, 562-567.

GUPTA, B. (2005): *Mycoplasma capricolum*: isolation and molecular diagnosis. M. V. Sc. Thesis submitted to the U. P. Pt. Deen Dayal Upadhyaya Pashu-chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura (U. P.). pp. 12-44.

GUPTA, R. K., V. P. SINGH, R. C. PATHAK (1988): Microflora of respiratory tract of sheep and goats during illness. Ind. J. Comp Microbiol. Immunol. Dis. 2, 81-83.

HERNANDEZ, L., J. LOPEZ, M. ST-JACQUES, L. ONTIVEROS, J. ACOSTA, K. HANDEL (2006): *M. mycoides* subsp. *capri* associated with goat respiratory disease and high flock mortality. Can. Vet. J. 47, 366-369.

HOTZEL, H., K. SACHSE, H. PFUTZNER (1996): A PCR scheme for differentiation of organismsbelonging to the *Mycoplasma mycoides* cluster. Vet. Microbiol. 49, 31-43.

IKHELOA, J. O., A. T. P. AJUWAPE, A. I. ADETOSOYE (2004): Biochemical characterization and serological identification of *Mycoplasmas* isolated from pneumonic lungs of goats slaughtered in abattoirs in Northern Nigeria. Small Rumin. Res. 52, 93-97.

JOHANSSON, K. E., A. PERSSON, M. PERSSON (1998): Diagnosis of contagious caprine and contagious bovine pleuropneumonia by PCR and restriction enzyme analysis: Towards livestock diseases diagnosis and control using in the 21st century, Proceedings of an International symposium on Diagnosis and Control of Livestock Diseases using Nuclear and Related Techniques, Vienna, Austria, 1998, International Atomic Energy Agency.

KOPCHA, M. (2005): Respiratory diseases of sheep and goats. The Merck Veterinary Manual, Merck & CO., Inc., Whitehouse Station, NJ., USA. 1229-1230.

KUMAR, M. (2000): PCR for detection of *M. mycoides* cluster. M. V. Sc. Thesis, Indian Veterinary Research Institute, Deemed University, Izatnagar, India.

MANSO-SILVÁN, L., E. M. VILEI, K. SACHSE, S. P. DJORDJEVIC, F. THIAUCOURT, J. FREY (2009): *Mycoplasma leachii* sp. nov. as a new species designation for *Mycoplasma* sp. bovine group 7 of Leach, and reclassification of *Mycoplasma mycoides* subsp. *mycoides* LC as a serovar of *Mycoplasma mycoides* subsp. *capri.* Int. J. Syst. Evol. Microbiol. 59, 1353-1358.

NICHOLAS, R. A. J. (2002): Improvements in the diagnosis and control of disease of small ruminants caused by *Mycoplasmas.* Small. Anim. Res. 45, 145-149.

PETTERSSON, B., T. LEITNER, M. RONAGHI, G. BOLSKE, M. UHLEN, K. JOHANSSON (1996): Phylogeny of the *Mycoplasma mycoides* cluster as determined by sequence analysis of the 16S rRNA genes from the two rRNA operons. J. Bacteriol. 178, 4131-4142.

RODRIGUEZ, J. L., J. B. POVEDA, J. OROS, P. HERRAEZ, M. A. SIERRA, A. FERNANDEZ (1995): High mortality in goats associated with the isolation of a strain of *Mycoplasma mycoides* subsp. *mycoides* (Large Colony type). Zentralbl. Veterinärmed. B. 42, 587-593.

SHANKSTER, S., R. D. AYLING, D. LENFANT, P. MERCIER, R. A. J. NICHOLAS (2002): Development and evaluation of a PCR to detect *Mycoplasma putrefaciens* directly from goats. Res. Vet. Sci. 72, 26.

SHARIF, A., G. MUHAMMAD (2009): Mastitis control in dairy animals. Pakistan Vet. J. 29, 145-148.

SINGH, V. P., N. C. SRIVASTAVA, M. KUMAR, M. SUNDER, J. P. JAI VARSHNEY (2004): Isolation and characterisation of an Indian strain of *Mycoplasma mycoides* subsp. *mycoides* type LC from a case of caprine arthritis. Comp. Immunol. Microbiol. Infect. Dis. 27, 273-284.

TAYLOR, T. K., J. B. BASHIRIDDIN, A. R. GOULD (1992): Application of a diagnostic: DNA probe for the differentiation of the two types of *Mycoplasma mycoides* subsp. *mycoides.* Res. Vet. Sci. 53, 154-159.

THIAUCOURT, F., G. BOLSKE (1996): Contagious caprine pleuropneumonia and other pulmonary *Mycoplasmosis* of sheep and goats. Rev. Sci. Tech. 15, 1397-1414.

VILEI, E. M., B. M. KORCZAK, J. FREY (2006): *Mycoplasma mycoides* subsp. *capri* and *Mycoplasma mycoides* subsp. *mycoides* LC can be grouped into a single sub- species. Vet. Res. 37, 779-790.

Received: 10 June 2010
Accepted: 17 March 2011

KUMAR, P., A. ROY, B. B. BHANDERI, B. C. PAL: Izdvajanje, identifikacija i molekularne značajke mikoplazama izdvojenih iz koza u Gujaratu u Indiji. Vet. arhiv 81, 443-458, 2011.

SAŽETAK

Ukupno je 358 uzoraka različitog materijala koza bilo nacijepljeno na hranjive podloge MBHS-L and MBHS-A radi izdvajanja mikoplazama. Pretraženo je bilo 215 uzoraka nosnog iscjetka, šest uzoraka plućnog tkiva, četiri uzorka pleuralne tekućine, pet uzoraka zglobne tekućine, 60 uzoraka mlijeka, 22 uzorka prepucijskog ispirka, 16 uzoraka obriska očne spojnice, pet uzoraka obriska uške i 25 uzoraka vaginalnog iscjetka. Mikoplazme su bile izdvojene iz 30 (8,35%) uzoraka, od čega iz 20 uzoraka nosnog iscjetka, četiri uzorka plućnog tkiva, dva pleuralne tekućine, tri uzorka mlijeka i jednog uzorka zglobne tekućine. Izolati su bili identificirani kao *M. mycoides* subsp. *capri* i *M. capricolum* subsp. *capricolum* i to na osnovi kulturalnih, morfoloških i biokemijskih svojstava te dodatne potvrde lančanom reakcijom polimerazom i PCR-RFLP rabeći različite skupine specifičnih početnica i restrikcijskih enzima. Ovo je rijetko izvješće o izdvajanju, identifikaciji i molekularnim svojstvima mikoplazama u Zapadnoj Indiji, koje ujedno pokazuje da je na tom području velika učestalost mikoplazmalnih zaraza u koza.

Ključne riječi: Indija, mikoplazme, koze, *M. mycoides* subsp. *capri, M. capricolum* subsp. *capricolum*