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Molecular diagnosis of caprine mycoplasmosis in Cauvery delta region of Tamil Nadu

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Abstract
Goat farming is one of the enterprise which has been practiced by a large section of human population specially in developing world and it also plays an immensely important role in employment generation, capital storage and improving house hold nutrition. The goat population in our country is frequently exposed to ravages of infectious diseases. Pneumonia has been noticed as one of the most frequently encountered condition and is responsible for around 28.7% mortality. Amongst various infections, mycoplasmosis is one of the most dreaded diseases of goats. *Mycoplasma* causes a number of important disease viz., CBPP, CCPP, CRD, arthritis, contagious agalactiae and mastitis in livestock and poultry. The present study was undertaken to detect the caprine mycoplasma infection in Cauvery delta region of Tamil Nadu from the tissue materials viz., lungs, lymph nodes, spleen and liver from the clinically suspected goats. All the tissue materials were subjected to isolation and PCR assay with *Mycoplasma group specific* primers. The findings indicate that the PCR assay is very simple and useful method for detecting the mycoplasma infection directly from the tissue materials in a very short span.

Keywords: *Mycoplasma*, PCR, infection, detection and tissue materials

Introduction
Goat farming is one of the enterprises which have been practiced by a large section of human population specially in developing world and it also plays an immensely important role in employment generation, capital storage and improving house hold nutrition. In spite of the enormous technological and developmental efforts in Livestock improvement sector in the country, the status of goats has largely remained as resource for poor farmers. A number of factors are responsible for economic losses to the goat industry, among them the health of goats is utmost important. The goat population in our country is frequently exposed to ravages of infectious diseases. Pneumonia has been noticed as one of the most frequently encountered condition and is responsible for around 28.7% mortality [1]. Amongst various infections, Mycoplasmosis is one of the most dreaded diseases of goats [2, 3, 4]. It is now an emerging threat and transboundary epidemiological disease posing a worldwide regulation on goat productions and hence huge economic constraints for farmers.

Mycoplasma causes a number of important diseases viz., CBPP, CCPP, CRD, arthritis, contagious agalatiae and mastitis. Amongst them, Contagious Caprine Pleuro Pneumonia (CCPP) has been a major cause of economic losses to goat industry and still exists at least in 33 countries of Africa and Asia. However in India, the CCPP was first reported by steel [5] from Bombay and since a number of outbreaks have been observed in different parts of the country. The one of the causative agent *Mycoplasma mycoides* subsp. *capri* was first isolated by [6] in 1949 in India and later on by [7] and then [8] from the kids and goats. To diagnose the CCPP, various serological tests viz., Slide agglutination test (SAT), Latex agglutination test (LAT), Double immunodiffusion (DID), Indirect haemagglutination (IHA), Enzyme linked immunosorbent assay and Complement fixation test (CFT) have been used routinely to diagnose the mycoplasma antibodes in livestock and poultry [9, 10, 11, 12]. However, the conventional method of isolation and identification of the causative agents of *Mycoplasma* diseases are generally employed in conjunction with the serology which have the limitation of cross reactivity with other *Mycoplasma* and poor growth of the organisms. To overcome these problems, the PCR assay which is rapid, sensitive and specific was used in the present study to detect the Caprine *Mycoplasma* infection directly from the tissue materials in the Cauvery delta region of Tamil Nadu.

Materials and Methods

Clinical picture and sample collection

A total of 60 samples were collected from suspected cases of caprine mycoplasmosis from clinically suspected goats of different age groups brought to Veterinary College and Research Institute, Orathanadu, Thanjavur. Suspected animals showed clinical signs like pyrexia (41-43 °C), reduced feed intake, reduced water intake, nasal discharge with mucopurulent in nature, frequent coughing, closure of one or both eyes and respiratory distress. , Mortality 5% and morbidity of 50% was reported. All ages and both sexes were susceptible and prone to infections. Postmortem was carried out from dead animals and the samples include swab from conjunctiva, nasal swab and tracheal swab from live animals as well as heart blood swab, trachea, lungs, liver, spleen and mesenteric lymph nodes tissue samples from dead animals. These samples were collected in Pleuro Pneumonia like Organism (PPLO) transport medium.

Isolation and Identification of *Mycoplasma*

Samples were processed for *Mycoplasma* isolation by standard procedures. For Mycoplasma isolation, samples were initially enriched in PPLO broth (Himedia) supplemented with 15% heat inactivated horse serum (Invitrogen), 10% yeast extract (HiMedia), 10% glucose (HiMedia), 1% NAD (HiMedia), 2.5% thallium acetate (HiMedia), benzyl penicillin sodium salt (100000 IU) and 0.5% phenol red, for a period of 3-7 days or till the media changes colour from red to yellow at 37 °C under 5% CO2 tension. Broth cultures are sub cultured on PPLO agar plate and kept at 37 °C under 5% CO2 tension for 5-10 days. The processed samples were inoculated into 2 ml of PPLO broth and incubated at 37 °C with 5% CO_2 tension for one week. After active growth, it was sub cultured in 5 ml of PPLO broth and again incubated at 37 °C with 5% CO_2 tension for one week to 21 days with intermittent shaking until the appearance of sharp yellow colour change indicating the optimum growth. The cultures showing the growth were sub cultured in PPLO agar plate and incubated at 37 °C in incubator with 5% CO_2 tension. The plates were viewed under stereomicroscope for presence of fried egg appearance colony.

Molecular detection

Cultures suspected for *Mycoplasma* were subjected to PCR. Broth culture (1ml) was centrifuged at 12,000 rpm for 10 min in a microfuge tube to pellet the organism. The pellet was washed twice with phosphate-buffered saline (PBS).Nuclease-free water (NFW) 50 µL was used to dissolve the pellet and the contents were boiled at 95 °C for 10 min followed by immediate cooling at − 20 °C for 5 min. Finally, the tubes were centrifuged at 12,000 rpm for 5 min and the supernatant was used as the template DNA for the PCR. Briefly, the reaction mixture was prepared by adding the following components; 12.5 µl of Red dye master mix (Ampliqon) containing 1.5mM MgCl2, 1µl each of forward and reverse primers (10 pmol/µl). Appropriate quantity of DNA was added and final volume of 25 µl was made with nuclease free water. Positive and negative controls were included during amplification.

Mycoplasma group specific PCR

Initially, the extracted DNA was screened by PCR using *Mycoplasma group specific* primers (GPO- 1 [5'-ACT CCT ACG GGA GGC AGC AGT A-3'] and MGSO [5'-TGC ACC ATC TGT CAC TCT GTT AAC CTC -3']) [13].

Mycoides cluster specific PCR

Cluster specific amplification was carried out using the primers MC 323 (5'- TAG AGG TAC TTT AGA TAC TCA AGG-3') and MC 358(5'-GAT ATC TAA AGG TGA TGG T-3') derived from the sequence of CAP- 21. The temperature of cycling was consisted of initial denaturation of 94 °C for 1 min and final extension at 72 ºC for 5 min with 30 intervening cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 2 min [14].

Mycoides group specific PCR

Mycoides group specific PCR was performed using primers MM 450 (5'-GTA TTT TCC CTA ATT TG-3') and MM 451 (5'- AAA TCA AAT TAA TAA GTT TG-3'). The reaction mixture and temperature cycling were similar to the cluster specific PCR.

Mycoplasma capri specific PCR

M. capri specific PCR was performed using primers P4 (5 '-ACT GAG CAA TTC CTC TT-3') and P6 (5'- TTA ATA AGT CTC TAT ATG AAT- 3'). The reaction mixture and temperature cycling were similar to the cluster specific PCR except that the annealing temperature of the primer was 46 °C.

Detection of PCR products

The PCR products were separated by agarose gel electrophoresis (Broviga, India) using 1.5% agarose gel in1X tris-acetic acid-EDTA buffer at constant voltage of 80 volts. The ethidium bromide (10 mg/ml) stained gel was visualized under UV and recorded in Gel Documentation system (Universal Hood II, Bio-Rad, USA).

Results and Discussion

Clinical signs

Pyrexia (41-43 ^{0}C), reduced feed intake and water intake, there was nasal discharge with catarrhal in nature at the beginning and becomes mucopurulent in the later stage of disease (Fig.1), respiration is accelerated and painful and accompanied by frequent coughing, closure of one or both eyes, respiratory distress, Mortality 5% and morbidity of 50% was reported. All ages and both sexes were susceptible and prone to infections. Our findings were in accordance with *Mycoplasma* mycoides *capri* (*Mmc*) infection along with the classical signs of respiratory system was also observed by [15, 16, 17].

Necropsy findings

Detailed necropsy was conducted from dead animals on 15 adult goats and 5 kids that died in and around Cauvery delta region of Tamil Nadu during the period of study and gross lesions were recorded. Internal examination revealed profuse frothy exudates in tracheal lumen extending up to the bronchi and lungs. Thoracic cavity contained accumulation of serosanguinous fluid; lung appeared with lung congestion, fibrinous pleuropneumonia (Fig.2), yellow coloured fluid accumulation in the lung (Fig.3) and bilateral lung adhesion. Upon incision, red frothy exudates oozed out from the parenchyma and the consolidated areas were hard to incise with thickening of septa. Similar types of gross lesions were also reported by [18, 19, 20] in an outbreak of CCPP caused by *Mycoplasma capricolum* subsp.*capripneumoniae* (Mccp).

Isolation of Mycoplasma

The processed samples were inoculated into 2 ml of PPLO broth and incubated at 37 °C with 5% CO_2 tension for one week. After active growth, it was sub cultured in 5 ml of PPLO broth and again incubated at 37 °C with 5% CO_2 tension for one week to 21 days with intermittent shaking until the appearance of sharp yellow colour change indicating the optimum growth. The cultures showing the growth were sub cultured in PPLO agar plate and incubated at 37 °C in incubator with 5% CO_2 tension. The plates were viewed under stereomicroscope for presence of fried egg appearance colony (Fig. 4). Similar isolation findings were also reported by [18, 21, 3]. Our results were in accordance with [22] isolated, identified and molecularly characterized mycoplasma isolates from goats of Gujarat State in India and found *M. mycoides* subsp. *capri*, *M. agalactiae*, *M. capricolum* subsp. *capricolum* (Mcc) as the main isolates. Similar isolation studies were also done with [23] on Contagious caprine pleuropneumonia. By culture isolation, a 8.3% (5/60) Caprine mycoplasmosis was detected in goats affected with disease in Cauvery delta region of Tamil Nadu.

Molecular detection

Caprine mycoplasmosis detected by culture isolation method was subjected to PCR. The clinical materials viz., lungs, lymphnodes, spleen and liver were subjected to PCR. By PCR, a 13.3% (8/60) Caprine mycoplasmosis was detected in goats affected with disease in Cauvery delta region of Tamil Nadu.Initially with *Mycoplasma group specific* primers GPO-1 and MGSO yielded 715 bp (Fig.5). Similar findings of 715 bp observed by [24] on Isolation and molecular characterization of *Mycoplasma* spp in sheep and goats in Egypt. Cluster specific primers MC 323 and MC 358 yielded 1.5 kbp long products (Fig.6). Similar findings of 1.5 kbp developed by [25] to differentiate the strains of *Mmm* SC from closely related *Mmm* LC strains and other members of the *mycoides cluster*. Further, it was also characterized by using *mycoides group specific* primers (MM 450 and MM 451) and *M. capri* specific primers (P4 and P6) which gave an amplicons of 574 bp (Fig.7) and 195 bp (Fig.8) products, respectively. Our findings was in accordance with [26] stated that the amplified 574 bp fragment from *M. mycoides* group for differentiation of mycoides group from the capricolum group of the clusters. Similar observation was also made by [27] found that the PCR for *Mycoplasma* detection proved to be rapid and sensitive method. Similar findings was also made by [28] on clinical and laboratory diagnosis of Contagious caprine pleuropneumonia in Qassim region of Saudi Arabia and [22] was also observed the similar results on Contagious caprine pleuropneumonia. Earlier reports also recommended molecular diagnostic assays for easy, early and accurate diagnosis of *M. capri* by PCR assay [4].

Fig 1: Bilateral mucopurulent discharge

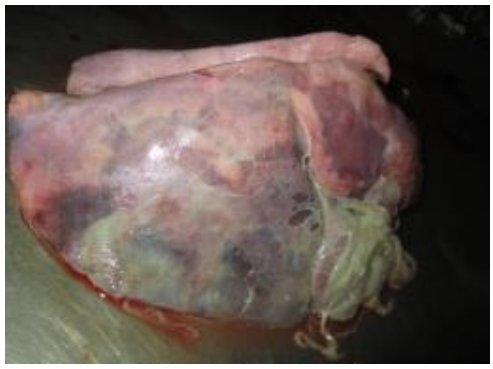

Fig 2: Fibrinous pleuropneumonia

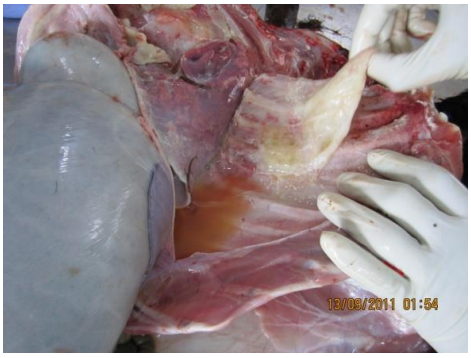

Fig 3: Yellow coloured fluid accumulation in the lung

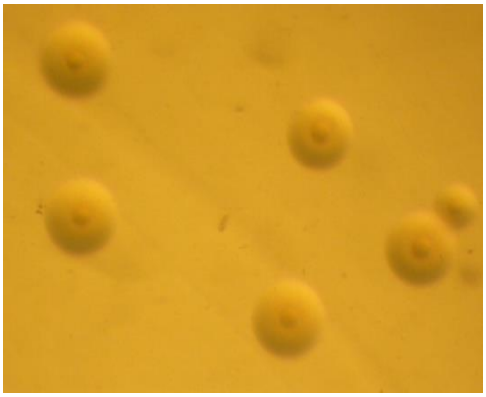

Fig 4: Fried egg appearance (10 X)

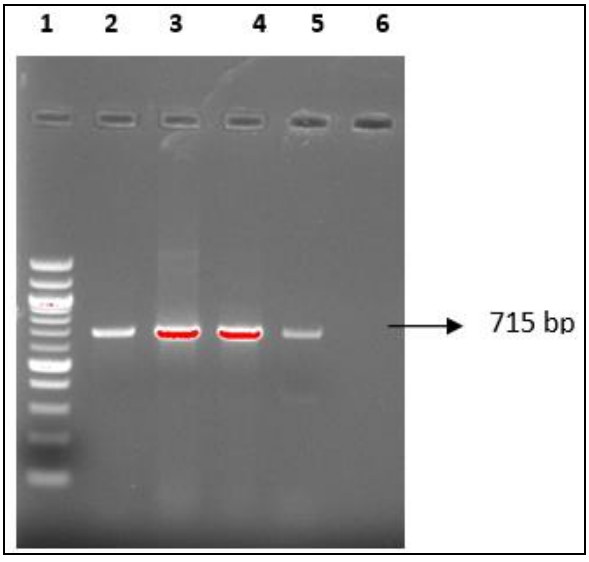

Fig 5: *Mycoplasma group* specific PCR Lane 1-100 bp marker, Lane-1+vecontrol, Lane 2-5 clinical samples, Lane-6-Ve control

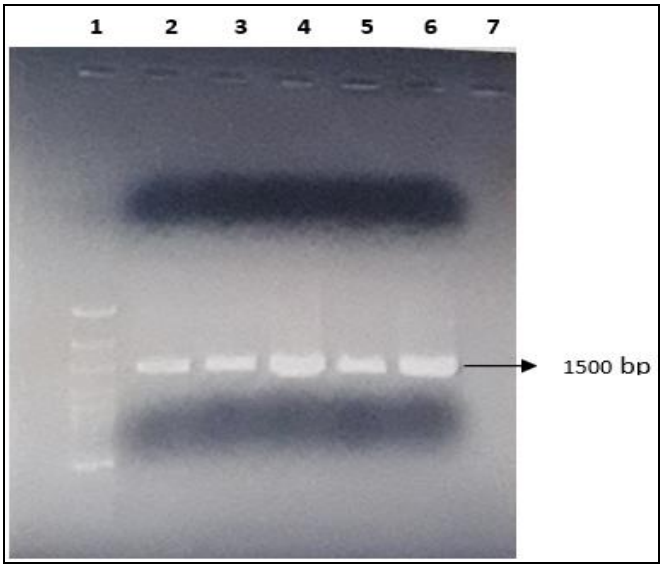

Fig 6: *Mycoplasma cluster specific* PCR Lane1-100 bp, Lane2+Ve control, Lane 3-6- samples,Lane-7-Ve control

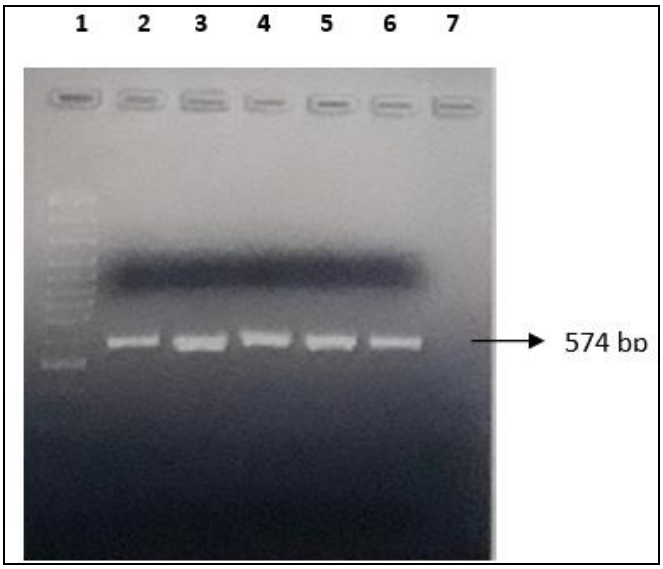

Fig 7: *Mycoplasma mycoides group specific* PCR Lane1-100 bp,Lane2+Ve control, Lane 3-6- samples,Lane-7-Ve control

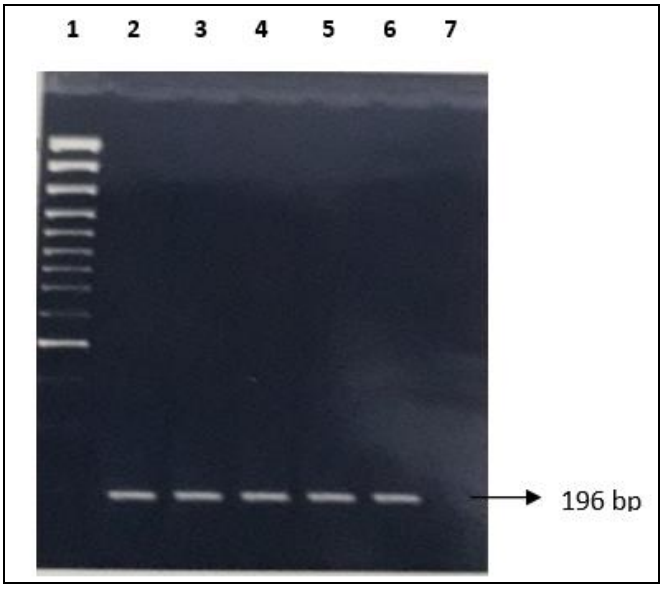

Fig 8: *Mycoplasma capri* specific PCR Lane1-100 bp, Lane2+Ve control, Lane 3-6- samples,Lane-7-Ve control

Conclusion

It is concluded that PCR, a reliable tool for early detection of the disease condition and molecular diagnosis is more rapid and accurate for early identification of Caprine mycoplasmosis from clinical specimens than conventional culture methods in a very short span.

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