

**MOLECULAR CHARACTERIZATION OF CONTAGIOUS *CAPRINE*  
PLEUROPNEUMONIA (CCPP) IN SMALL RUMINANTS OF KHYBER  
PAKHTUNKHWA, PAKISTAN**

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**ABSTRACT**

This study was conducted to determine the molecular identification of Contagious *Caprine* Pleuropneumonia in field outbreak. The study included isolation and identification of *Mycoplasma* species from field outbreaks by usage of a selective differentiating hay flick medium, growth inhibition test and a Polymerase Chain Reaction (PCR) test. Out of 120 inoculated samples, 30% and 22.5% were positive on culture from lungs and pleura. Isolates were identified as *Mycoplasma mycoides* subspecies *Capri* by a growth inhibition test and PCR. Similarly, tissue samples that were negative on culture were also subjected to PCR analysis. Out of 120 samples 62.5% and 54.16% from lungs and pleura, respectively, were positive. On statistical analysis, a significant difference ( $P < 0.05$ ) was found between results of PCR and culture. This difference reflects that the PCR technique is more sensitive than the culture method. Based upon these findings, disease was prevalent in almost all selected regions of province associated with *Mycoplasma mycoides* subspecies *Capri*.

**Key words:** Contagious *Caprine* pleuropneumonia, growth inhibition test, PCR, *Mycoplasma mycoides capri*, goat

**INTRODUCTION**

Among livestock sector goat population plays a prominent role as it contributed largest number of 58.3 million to the total animal population of 146.6 million (Economic survey, 2008). Goats play important role to fulfill requirements like milk, meat, skin and leather. Livestock holders belong to lower socioeconomic class and their livelihood depends on income generation from animals. Goat is also called poor man cow. However, Goat population faces various challenges in the form of intense harsh climatic condition, poor management, scarcity of fodder and disease. Among various diseases, contagious *Caprine* pleuropneumonia (CCPP) is major threat to goat population. CCPP is wide spread in Pakistan and causing heavy losses to goat population (Awan, 1985, Rahman *et al.*, 2003 and Awan *et al.*, 2009). This disease is cosmopolitan in distribution particularly in countries having extensive goat farming (Rurangirwa and Kinyili, 2000). It is common in African countries (Macowan, 1976 and Mekuria *et al.*, 2008), Middle East (Lefevre *et al.*, 1987, Jones and wood, 1988), Europe (Pettersson *et al.*, 1996) and subcontinent (Kumar *et al.*, 1995 and Mondal *et al.*, 2004). It is enlisted by the Office of International Epizootic as list B disease.

Causative agent of contagious *Caprine* pleuropneumonia in goat is a bacteria *Mycoplasma*. it

consist of a group of species and sub species called “*M. mycoides* clusters” that includes *M. Capricolum* subspecies *Capricolum*, *M. Capricolum* subspecies *Capripneumoniae*, *M. mycoides* subspecies *Capri*, *M. mycoides*, sub species *mycoides* large colony, *M. mycoides* subspecies *Mycooides* small colony and *Mycoplasma* species Bovine group 7. Among these some species have common antigenic and genomic properties (Cottew *et al.*, 1987). In subcontinent the disease is caused by *Mycoplasma mycoides* subspecies *Capri* which is one of the major causes of contagious *Caprine* pleuropneumonia (Awan, 1985 and Mondal *et al.*, 2004, Ingle *et al.*, 2008).

An accurate and reliable diagnostic technique is essentially required for rapid detection and confirmation of infected animals (Rurangirwa *et al.*, 1987). Confirmation of the disease is difficult for reasons that, from clinical point of view, CCPP can not be differentiated from a number of diseases presenting similar respiratory signs in small ruminants, such as Peste Des Petits Ruminants and Pasteurellosis. Secondly being a fastidious organism it is very difficult to isolate *Mycoplasma* on ordinary media in vitro. Consequently, isolation trials are usually failed. It has been observed that a negative result of cultivation of *Mycoplasma* does not indicate the absence of infection (Thiaucourt and Bölske, 1996). To overcome on these constraints among different species, DNA amplification techniques provide

an accurate identification of *M. mycoides* cluster members.

Diagnosis of CCPP is practiced in Pakistan by common conventional serological and biochemical tests and no efforts have been made so far on its molecular diagnosis. To have an insight on accurate and rapid identification, present work was planned, to isolate and culture *Mycoplasma* sub spp (*M. Mycoides Capri*) from samples of CCPP- natural out breaks in goats and to confirm its identification using serological and molecular techniques.

## MATERIALS & METHODS

**Sample collection:** A total of 120 samples were collected from goats exhibiting clinical signs of pneumonia suspected for CCPP during disease out break in field in different regions (northern, central and southern) of Khyber Pukhtoon Khwa, Pakistan. All physical and clinical parameters of animals were examined and detailed observations were documented. Clinical parameters including temperature, coughing, nasal discharge, lacremation, breathing, diarrhea and posture of animals were recorded. Postmortem was performed on dead animals during disease out break and lesions in different visceral organs were noted. Animals in serious conditions were purchased, sacrificed and lesions in different visceral organs were recorded. Samples for bacterial isolation were collected from pleural fluids and lung at interface between the consolidated and unconsolidated healthy tissues according to standard procedure of O I E (2008). Samples for histopathological study were taken from trachea, lungs, heart, liver, kidney, spleen and intestine and preserved in 10% buffered formalin. Tissue samples collected were packed, labeled, and transported to laboratories in departments of Pathology and Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan.

**Isolation and identification of *Mycoplasma*:** Modified Hay Flick culture media was used for isolation of *Mycoplasma*. Standard procedure of Lefevre et al., (1987) and OIE, (2008) were followed. Broth and agar media were prepared following the instruction of manufacturer. Inoculation of samples in broth and solid media was carried out according to the standard procedure of OIE (2008). Inoculation was made in biological safety cabinet. Samples from lungs were taken by touching the wire loop at the interface of healthy and diseased incised tissues. The loopful of sample was then inserted into a broth medium in a test tube. The test tubes were capped, labeled and incubated at 37°C in 5% carbon dioxide and 95% humid atmosphere. The cultures were examined daily for the growth of *Mycoplasma* by observing change in color from red to yellow and whirl formation. After confirmation of growth in broth

medium, isolates were further streaked on agar plate medium with the help of culture loop in biological safety cabinet. Some plates were cultured by pouring broth media on surface of the agar plate. The plates were sealed, labeled, wrapped in aluminum foil and placed in an incubator at 37°C in a 5% carbon dioxide and humid atmosphere. Agar plates were examined under stereomicroscope daily over 6 days of incubation for growth of typical of *Mycoplasma* colonies. The isolated colonies were subculture three times to obtain pure cultures of the isolates.

Identification of isolate was made by appearance of specific colony types grown on solid medium as described by Mondal et al., (2004). Typical characteristic colonies having a fried egg-like appearance, tinny, smooth and 0.1-1 mm in diameter with dense elevated centers embedded in the medium were suggestive of *Mycoplasma* species. Isolated *Mycoplasma* was identified by a Growth Inhibition Test (Clyde, 1983; Laura et al., 2006). Hyperimmune serum was produced in three rabbits against *Mycoplasma mycoides* subspecies *Capri* (PG3) following a standard protocol using *Mycoplasma* procured from the Veterinary Research Institute, Lahore.

**Polymerase Chain Reaction:** *Mycoplasma* isolates were further identified and confirmed by PCR using the protocol of Hotzel et al., (1996). Two sets of primers were used for the amplification of the template DNA of isolates as used by Hotzel et al., (1996).

1. PI 5-TAT ATG GAG TAA AAA GAC-3'
- P2 5-AAT GCA TCA TAA ATA ATT G-3'
2. P4 5-ACT' GAG CAA TTC CTC TT-3'
- P6 5-TTA AAT AAG TTT GTA TAT GAA T-3'

DNA from culture was extracted according to the procedure described by (Miserez et al., 1997). DNA from pleural fluid and lung tissues was extracted with the help of Fermentas life sciences Pure Extreme TM, EU DNA Purification Kit, after the following instruction. It consisted of first step of an initial denaturation of 2 min at 94 °C, followed by a program of 35 cycles of 30 s at 94 0C, 15 sec at 46 0C and 15 sec at 72 0C and a final extension step of 5 min at 72 0C (Hotzel et al., 1996). The isolated DNA from cell culture and tissues was analyzed by agarose gel electrophoresis as described by (Hotzel et al., 1996) with some modifications to confirm the presence of DNA.

## RESULT

**Isolation of *Mycoplasma*:** Out of 120 inoculated samples, 36 (30.0%) and 27 (22.5%) were positive on culture from lungs and pleura, respectively, in modified hay flick medium. Area-wise positive percentages of CCPP were 37.5%, 30.0%, and 22.9% for lungs and 27.5%, 22.5%, and 17.5% for pleura, in northern, central and southern regions, respectively (Table 1.). Typical

nipple- or poached egg-shaped colonies were developed on day 8 post-inoculation in hay flick medium. The colonies reached maximum numbers and sizes on day 15 post-inoculation. The isolated *Mycoplasma* was identified on the basis of its colony characteristics and by the whirling movement of growth in broth medium. The fitted model indicates that none of the main effect and interaction is significant. Only the odds of the positive response are 1.788 times greater for the northern region as compared to southern region, but still not significant at alpha level 0.10.

**Growth inhibition test:** Isolates were identified by growth inhibition tests. Out of 36 and 27 culture samples from lungs and pleura, respectively, 30 (83.3%) and 22 (78.6%) were identified as *Mycoplasma mycoides* subspecies *Capri*. Area-wise positive percentages of *Mycoplasma mycoides* subspecies *Capri* were 86.6%, 83.3%, and 77.8% for lungs and 81.8%, 77.7%, and 85.7% for pleura in northern, central and southern regions, respectively. A clear zone of inhibition of growth was found around impregnated discs. Statistically analysis of data shows no significance difference in the positive cases found in three regions, two organs or even their interaction at ( $P < 0.10$ ). Graphic presentation is at (Fig 1.)

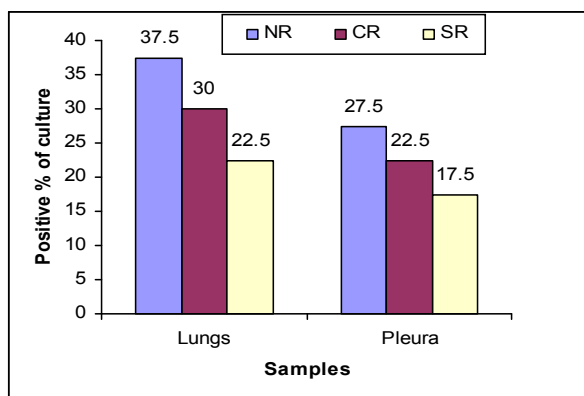
Though the positive responses in northern region are 2 times higher as compared to southern region, still the responses are not statistically significant. Similarly, no significant variation is observed among organs and its interaction with regions.

**Polymerase Chain Reaction:** The identification of isolates was further confirmed by PCR analysis using two sets of primers. The primer pair 1 called universal primers targeting the entire members of *Mycoplasma* clusters yielded a 548-bp band specific for *Mycoplasma*

cluster (Plate.1). The second set of primers was species-specific and resulted in a 194-bp band specific for *Mycoplasma mycoides* subspecies *Capri* (Plate.2). Out of 36 and 27 culture samples from lungs and pleura, respectively, 34 (94.4%) and 24 (88.9%) were identified as *Mycoplasma mycoides* subspecies *Capri*. Area-wise positive percentages of *Mycoplasma mycoides* subspecies *Capri* were 93.3%, 100%, and 88.9% for lungs and 90.9%, 77.8%, and 100% for pleura in northern, central and southern regions, respectively (Table 2). Although, the cases were identified by PCR does varies organ-wise and region-wise but they could not achieve statistical significance. This situation is evident from the relevant fitted logistic regression model given in table 3. The odds of the positive samples are insignificantly lower in northern and central region as compared to southern region. Further, though the odds of the positive samples are 2.133 times greater for the lungs as compared to Pleural Fluid, but still insignificant.

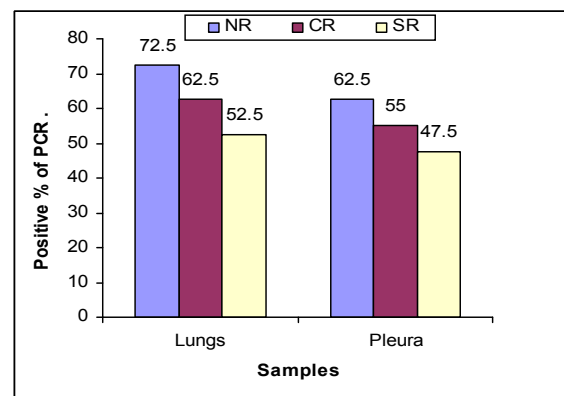
Similarly, tissue samples from lungs and pleura of all animals were also subjected to PCR analysis. Out of 120 samples, 75 (62.5%) from lungs and 65 (54.2%) from pleura were positive, while the remaining yielded no band on PCR analysis. The area-wise percentages were 72.5%, 62.5%, and 52.5% for lungs and 62.5%, 55.0%, 47.5% for pleura in northern, central and southern regions, respectively (Table 4.7). PCR test on the positive sample does shows marked difference region-wise as well as organ-wise. In particular, Northern region, where the odds are 1.84 times higher as compared to Southern. However, these differences could not attain significance even at 0.10 levels (Table 4).

The odds though greater for the northern and central region as compared to southern region, however they were short of being statistically significance. Region-wise comparison reveals the same story.



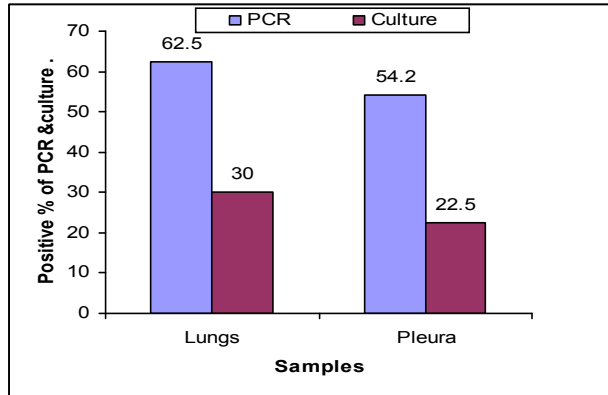
NR: Northern Region, CR: Central region, SR: Southern Region

**Fig.1. Culture-positive results of *Mycoplasma mycoides* subspecies *Capri* on sample collected from different regions of NWFP**



NR: Northern Region, CR: Central region, SR: Southern Region, PCR: Polymerase Chain Reaction

**Fig. 2. PCR result for identification of *Mycoplasma mycoides* *Capri* on culture and tissues samples collected from natural outbreak of CCPP across different regions NWFP**



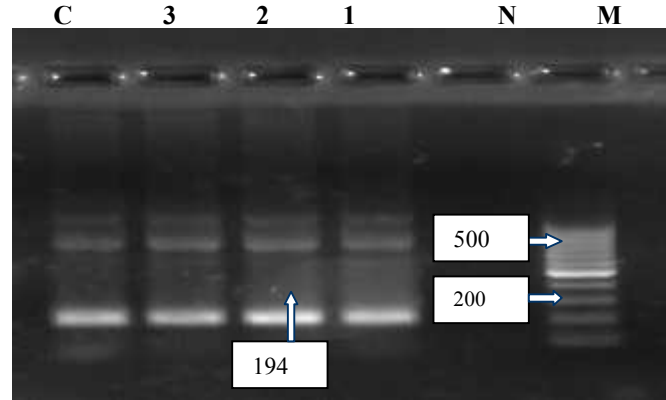
**Fig. 3.** Comparison between PCR and culture technique for detection of *Mycoplasma mycoides Capri* on tissues samples collected from natural outbreak of CCPP.

The results of both culture and PCR were compared and revealed that the positive percentages on cultures were 30.0% and 22.5% and on PCR 62.5% and 54.2% from samples of lungs and pleura, respectively. Logistic Regression Model exposed a highly significant difference (P. value 0.000) between two results. It is revealed that PCR technique is more sensitive as compare to culture (Figure 2)

The relevant logistic regression model indicates significance difference ( $p=0.000$ ) in the two tests regarding sensitivity in detecting the responses. The odds are almost 4 times higher for the PCR in detecting the case as compared to the Culture test.

## DISCUSSION

The isolate was grown within 2-3 days in broth media and colony appeared in agar plate on 8<sup>th</sup> day post inoculation in hay flick media. The colonies were reached to maximum in number and sizes on 15<sup>th</sup> day of post inoculation with a characteristic putrefying smell. Such observations were also made by OIE, 2008. Out of 120 inoculated samples 36 (30.00 %) and 27 (22.50 %) were positive on culture from lungs and pleura in modified hay flick media. Area wise positive %age of CCPP by culture from lungs and pleura was 37.5% 30%, 22.9% and 27.50 %, 22.50 %, 17.50 % in northern, central and southern regions respectively This high percentage of disease on culture in the northern region might be due to severe cold climatic condition prevailed in northern hilly tract of the country and nomadic nature of livestock owners. Both these factors contributed to stress eventually prone animals to *Mycoplasma* infections. Secondly the boundary of that region is in close with boundary of Afghanistan and nomads easily cross over and enter the area leads to Tran boundary transmission of disease.



**Plate. 1.** PCR for *Mycoplasma mycoides Capri* from culture showing an amplicon size of 194 bp M = 100bp ladder, N = Negative control, C = Positive control, 1 = Sample 1, 2 = Sample 2, 3= Sample.

In the present study the isolate on culture was identified by growth inhibition test by using known antiserum against *Mmc*. The same test was also used for identification of *Mycoplasma* by, (Thiocourt 1994, Rodriguez *et al.*, 1996, OIE, 2000, Wesonga *et al.*, 2004, Mondal *et al.*, 2004 and Gelagey *et al.*, 2007). Out of 36 and 27 culture samples from lungs and pleura 30 (83.33 %), 22 (78.57 %) were identified as *Mycoplasma mycoides Capri*. As the sensitivity of serological test are poor and not reliable due to the fact that some time it gives false positive result due to cross reactivity of the mixed infections. Therefore, both isolate and tissue samples were subjected to PCR analysis for further confirmation of the species of *Mycoplasma*.

At present, it would not be possible to recognized CCPP by conventional method if it is imported in disease-free countries, Because of difficulty in isolation due to indiscriminate use of antibiotic for treatment and lack of facilities for isolation and growth of *Mycoplasma*. In such cases PCR provide an opportunity and play major role in the surveillance of CCPP as described by (OIE, 2008, Woubit *et al.*, 2004 and Dominique *et al.*, 2004, Hotzel *et al.*, 1996 Bashiruddin *et al.*, 1994, Bascunana *et al.*, 1994 and Taylor *et al.*, 1992). Two sets of primers were used the primers 1 which have amplified the entire *Mycoplasma* clusters give 548 bps band. The second set of primer which was species specific for *Mycoplasma mycoides Capri* has amplified and gives a band of 196 bps. Similar finding were also reported by Laura *et al.*, 2006, Woubit *et al.*, 2004 and Hotzel *et al.*, 1996. The isolation and confirmation of the species of *Mycoplasma mycoides Capri* from the infected lungs of natural out break confirmed the wide spread distribution of this disease in the country. Such wide spread prevalence of CCPP in other parts of the world is also reported by many workers like Rodriguez *et al.*, 1996, DaMassa *et al.*, 1993, Jones, 1989 Gourlay and Haward, 1982.

Out of 36 and 27 culture samples from lungs, pleura 34 (94.44 %), 24 (88.88 %) were identified as *Mycoplasma mycoides Capri*. Similarly tissue samples from lungs and pleura of all animals were also subjected for PCR analysis. Out of 120 samples 75 (62.5 %) from lungs and 65 (54.16 %) from pleura were positive while remaining yielded no band on PCR analysis. Total positive percentage of CCPP on PCR was 62.5%. By comparing the result of culture and PCR a significant difference ( $P < 0.05$ ) was present among two techniques. It was obvious that PCR is more sensitive and accurate as compared to culture. The low percentage of *Mycoplasma* on culture revealed its fastidious nature to grow. Secondly, the stage of disease during sample collection may effect on the growth of this micro organism. The other possible reason is the preservation and transportation of samples from far flung areas of the country. The culture of *Mycoplasma* from clinical material of natural outbreak may not be possible due to indiscriminate use of antibiotics in the treatment. Similar observations were also made by Dominique *et al* 2004, Mondal *et al.*, 2004, Rurangirwa *et al.*, 1997. The remaining samples that were negative on PCR might be due to some other respiratory disease that presenting similar signs symptoms. The other possible reason may be some other species of *Mycoplasma clusters* that causes the disease.

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