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Research Article

# Molecular Survey on the Prevalence of *Caprine mycoplasma* in the Goats of Quetta City of Pakistan

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**ABSTRACT**

Mycoplasmosis represents the most important group of infectious diseases in goats. There are several and well recognized species of *Mycoplasma* causing high morbidity and mortality to adult goats and kids. In the present study a total of 385 nasal swab samples of randomly selected goats were collected from Quetta abattoir for molecular detection of different *Mycoplasma* species. The DNA was extracted from the nasal swab samples by using genomic DNA purification kit (Gentra-Puregene, USA). The polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLP) were performed for the molecular detection of different species of *Mycoplasma*. The highest prevalence for *Mycoplasma mycoides* cluster members 35(9%) was observed, followed by *Mycoplasma mycoides* sub-cluster members 29(7.5%) and *Mycoplasma putrefaciens* 19(5%) from all nasal swab samples (n=385) processed for the molecular detection of Mycoplasma species, while the lowest prevalence was detected for *Mycoplasma capricolum* sub sp.*capricolum* 6(6%). None of the DNA sample was found positive for *Mycoplasma capricolum* sub sp. *capripneumoniae*. The presence of *Caprine Mycoplasma* species detected by PCR seems promising as this technique is rapid, reproducible, and efficacious as compared to classical tools used in the diagnosis of *Mycoplasma* induced diseases in goats.

**Key words:** Caprine Mycoplasma, PCRs and RFLP, Extraction, Detection, DNA

## INTRODUCTION

Mycoplasmas are ubiquitous pathogens in the animal as well as in plant kingdom. They are Prokaryotes but without a cell wall (Dybvig, 1990). The diseases caused by Mycoplasmas are collectively known as Mycoplasmosis. Most of the diseases inflicted are per acute, acute and chronic in nature. They cause infections in urogenital and respiratory tracts because they have a high affinity for mucosal surfaces (Fehri *et al*., 2005). They are also responsible for infections in mammary gland and serous membranes, particularly in caprine (*Al-Momani and Nicholas, 2006).* The infections lead to high morbidity and in certain cases high mortality (McMartin *et al.*, 1980). The clinical manifestations include fever, coughing, sneezing, pneumonia, hydrothorax, painful respiration, arthritis, conjunctivitis and abortions (Kusiluka and Kambarage, 1996). Affected animals continue to graze and move like healthy animals until their body temperatures rise up to 41ºC. Gradually, their respiration becomes laborious, with violent coughing (Thiaucourt and Bolske, 1996). In the final stage, the animals show inability to move, stiffed neck, protruded tongue and continuous dripping of saliva from mouth (McMartin *et al.*, 1980). *Mycoplasma* infections exist worldwide and lead to sizable economical losses by significant reduction in milk, meat, skins and hair in small ruminants (*Nicholas et al., 2006)*.

The Mycoplasmas belong to *Mycoplasma mycoides* cluster which are important pathogenic agents of caprine. This group comprises five species and sub species (Manso Silvan *et al.*, 2009). In this cluster *Mycoplasma mycoide*

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sub species *capri* (*Mmc*), *Mycoplasma capricolum* sub sp.*capricolum* (*Mcc*) and *Mycoplasma capricolum* sub sp. *capripneumoniae (Mccp*) cause septicemia, arthritis, mastitis and pneumonia in goats and sheep. *Mmc* is considered as a main cause of pneumonia in goats (*Nicholas et al.*,2006).The countries in which small ruminates are farmed, *Mmc* is considered as one of the most wide spread pathogens. In case of outbreak of any disease caused by *Mmc,* mortality can reach up to 90% (Rodriguez *et al*., 1995). Most of the countries are unable to diagnose *Mycoplasma* infections, so its impact on live stock is probably under estimated (Angelo *et al.*, 2010).

Quetta is the capital of Balochistan, a minor but still sizable proportion of the rural population is engaged in livestock rearing activities. Sheep and goats are reared for domestic as well as commercial purposes. Goats are brought in Quetta from different parts of the province of Balochistan which suffer from many respiratory diseases due to cold weather and poor husbandry practices (Tariq, 1980).

Of the several methods adopted for the diagnosis, molecular detection methods like polymerase chain reaction (PCR) for *Mccp* (Woubit *et al*., 2004) and restriction fragment length polymorphisms (RFLP) for *Mycoplasma mycoides* sub cluster (Bashiruddin *et al.*, 1994) are widely being used. The use of PCR and RFLP are better over the classical diagnostic techniques, because of their higher sensitivity and specificity and the results are obtained with in few hours (Rawdi and Dussurget, 1995; Nicholas *et al*., 2003; Grand *et al*., 2004). Previously limited work was conducted on goat mycoplasmosis in Pakistan, especially in Balochistan (Tariq, 1980), but recently an extensive study was conducted using PCR–RFLP for the survey of caprine mycoplasmosis in Balochistan (Awan *et al.,* 2010). In that study 56.67% lung samples were found positive for the molecular prevalence of *Mcc, Mccp* and *Mp*. In total the molecular prevalence was observed as 17.65% for *Mccp*, 70.59% for Mcc and 11.76% for *Mp*. The RFLP profile has also validated the PCR results of *Mccp* by yielding two bands of 190 and 126 bp. The results of PCR-RFLP strongly indicated the prevalence of CCPP in this part of world (Awan *et al.,* 2010).

The present molecular based study was therefore aimed to highlight the prevalence of *Mycoplasma* associated diseases particularly due to the *Mmc*, *Mycoplasma putrefaciens* (*Mp*), *Mcc* and *Mccp* organisms in goats. It is expected that this research would be helpful: in the early diagnosis of *Mycoplasma* diseases, to design a strategy to start an early treatment and to adopt effective immunoprophylactic measures.

## MATERIALS AND METHODS

### Sample collection

Nasal swabs from 385 randomly selected goats from Quetta abattoirs were collected by the use of sterile cotton swabs and processed for extraction of DNA and molecular detection of caprine Mycoplasmas in the Center for Advanced Studies in Vaccinology and Biotechnology (CASVAB), University of Balochistan, Quetta.

### Sample processing and DNA extraction

The nasal swabs were swirled in 3ml normal saline, and 1.5ml suspension was used for the extraction of DNA. The DNA was extracted by genomic DNA purification kit (Gentra-Puregene, USA) by following the method as described in the instructions manual with little modification. The purified DNA samples were stored in micro tubes at -20 ºC until tested.

### Molecular detection of Caprine Mycoplasma

PCR for the molecular detection of *Mycoplasma mycoides* cluster members, *Mycoplasma mycoides* sub-cluster (*Mmm*SC and *Mmc*) members, *Mccp* and *Mycoplasma putrefaciens (Mp*) were performed on DNA samples purified from the nasal swabs of goats and the PCR results were further confirmed by restriction fragment length polymorphism (RFLP). All the primers (Table I) used in this study were synthesized from Gene-Link USA.

### PCRs for the detection of Caprine Mycoplasma

All the DNA samples (n=385) extracted from the nasal swabs of goats were subjected to the PCRs by following the method of Bashiruddin *et al.,* (1994) for the detection of *Mycoplasma mycoides* cluster members and *Mycoplasma mycoides* sub-cluster members. The method of Woubit *et al.,* (2004) was adopted for the *Mccp* and for the specific detection o*f Mp* the method of Shankster *et al.*, (2002) was used. All PCR products were checked by agarose gel electrophoresis and viewed in gel documentation system (Dolphin view-Wealtec, USA).

### Restriction fragment length polymorphism (RFLP) analysis of PCR products of Mycoplasma mycoides sub- cluster

The RFLP analysis of PCR products of *Mycoplasma mycoides* sub-clusterwas proceeded by following the method as described by Bashiruddin *et al.,* (1994). The reaction mixture was prepared by mixing 5ul PCR product with 2ul of *Vsp1* restriction endonuclease, 2ul PCR grade water and 1ul enzyme buffer (10x) and then incubated at 37ºC in water bath for 30 minutes. Finally the restriction fragment length polymorphisms (RFLP) products were electrophoresed (100V for 35min) in 3% agarose gel and viewed in gel documentation system (Dolphin view-Wealtec, USA).

## RESULTS

Out of the total nasal swab samples (n=385) of goats, the highest prevalence was observed for *Mycoplasma mycoides* cluster members in 35 (9%) goats, followed by *Mycoplasma mycoides* sub-cluster members and *Mp* in 29 (7.5%) and 19 (5%) goats respectively, by PCRs. The lowest prevalence was observed for *Mcc* in 6 (1.6%) goats in Quetta district Table. II and Fig 1). None of the DNA sample from nasal swabs of the goats was found positive for *Mccp* specific PCR (Table. II, III and Fig 1, 2).

For *Mycoplasma mycoides* sub-cluster organisms (*Mmc* and *Mmm*SC), the PCR products were of 574bp (Fig. 3). When the PCR products positive for *Mycoplasma mycoides* sub-cluster organisms (*Mmc* and *Mmm*SC) were digested with *Vsp1* during RFLP, those specifically yielded three bands of 230, 178 & 153bps specific for *Mmc* (Fig.4).

**Table 1:** Set of primer used in different PCRs.

| Mycoplasma species | Primers | Sequence (5'-3') | Orientation |
|---|---|---|---|
| *Mycoplasma mycoides cluster*[1] | MC323 | TAG AGG TAC TTT AGA TAC TCA AGG | Forward |
| | MC358 | GAT ATC TAA AGG TGA TGG T | Reverse |
| *Mycoplasma mycoides sub-cluster*[11] | MM450 | GTA TTT TCC TTT CTA ATT TG | Forward |
| | MM451 | AAA TCA AAT TAA TAA GTT TG | Reverse |
| *Mycoplasma capricolum subspecies capripneumoniae* (Mccp)[2] | Mccp-spe-F | ATC ATT TTT AAT CCC TTC AAG TAC TAT | Forward |
| | Mccp-spe-R | GAG TAA TTA TAA TAT ATG CAA | Reverse |
| *Mycoplasma putrefaciens (Mp)*[3] | SSF1 | GCG GCA TGC CTA ATA CAT GC | Forward |
| | SSR1 | AGC TGC GGC GCT GAG TTC A | Reverse |

[1] Bashiruddin *et al*., 1994. [2]Woubit *et al*., 2004, [3]Shankster *et al*., 2002.

**Table. 2:** PCR based detection of *Mycoplasma* species from nasal swab samples of goats from abattoir of Quetta district.

| *Mycoplasma* Species/detection by | Number of Nasal Swab Samples* | Samples positive in PCR | RFLP | Positive Samples (%) |
|---|---|---|---|---|
| *Mycoplasma* mycoides cluster /PCR | 385 | 35 | NP | 9 |
| *M. mycoides* sub-cluster/ PCR-RFLP[a] | 385 | 29 | 29 | 7.5 |
| *Mp* /specific PCR | 385 | 19 | NP | 5 |
| *Mcc**/PCR* | 385 | 6 | NP | 1.6 |
| *Mccp* / specific PCR[b] | 385 | 0 | NP | 0 |

*: Nasal swabs samples were collected from randomly selected goats from Quetta abattoir; ** :Samples were identified as *Mcc* as these were found positive for *Mycoplasma mycoides* cluster PCR, negative for *Mycoplasma mycoides* sub-cluster, *Mccp*, and *Mp* PCRs; [a]: RFLP yielded 3 bands specific for *Mycoplasma mycoides* sub species *capri* (*Mmc*); [b]: RFLP was not carried out as none of the sample was positive in *Mccp* specific PCR; NP: Not performed; RFLP: Restriction fragment polymorphism.

**Table 3:** Molecular prevalence (PCR) of *Mycoplasma* species from the nasal swabs of goats from Quetta abattoir

| *Mycoplasma* species/ detected by | Samples positive in PCR | PCR Positive %* |
|---|---|---|
| *M. mycoides* sub-cluster/ PCR-RFLP | 29 | 53.7 |
| *Mp/* specific PCR | 19 | 35.2 |
| *Mcc/PCR* | 6 | 11.11 |
| *Mccp* /specific PCR | 0 | 0 |

*:PCR Positive percentage (%) was calculated from the total positive samples of *Mycoplasma mycodies* sub cluster, *Mycoplasma puterfaciens (Mp)* and *Mycoplasma capricolum* sub sp. *Capricolum (Mcc).*

None of the PCR product was observed with two bands of 379bps and 178bps, specific for *Mycoplasma mycoides* sub sp. *Mycoides* Small Colony (*Mmm* SC) in RFLP (Fig. 5). PCR profile of *Mp* yielded a product of band size of 800bp shown that those samples obtained from the nasal swab samples of goats n, were positive for *Mp* (Fig. 6).

Molecular detection of Mycoplasma species by PCR and RFLP has shown prevalence of Mycoplasma species in in goats from Quetta abattoir in Balochistan.

## DISCUSSION

Contagious caprine pleuropneumonia (CCPP) is considered to be the world's most devastating goat disease. This classic disease is caused solely by *Mccp*. Despite the extensive use of vaccines and antibacterial drugs, this disease still prevails in Balochistan, Pakistan (Awan *et al*., 2009). In past, mostly classical biochemical and growth inhibition tests (Poveda and Nicholas, 1998) were mostly used for the detection and diagnosis of the infectious diseases including mycoplasmosis, were time consuming, non-specific and laborious. The use of PCR and RFLP are better than the classic diagnostic techniques, because of their higher sensitivity andspecificity and results obtained within few hours (Rawdi and Dussurget, 1995; Nicholas *et al*., 2003; Grand *et al*., 2004).

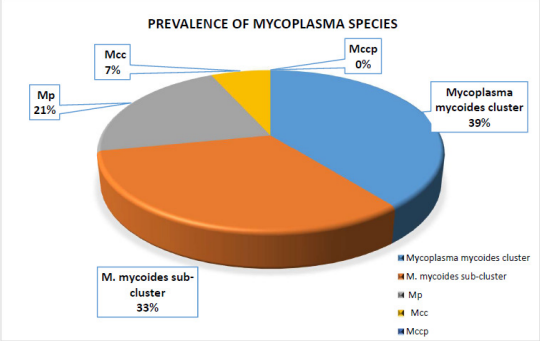


**Fig 1:** PCR based detection of *Mycoplasma* species from nasal swab samples of goats from abattoir of Quetta district.

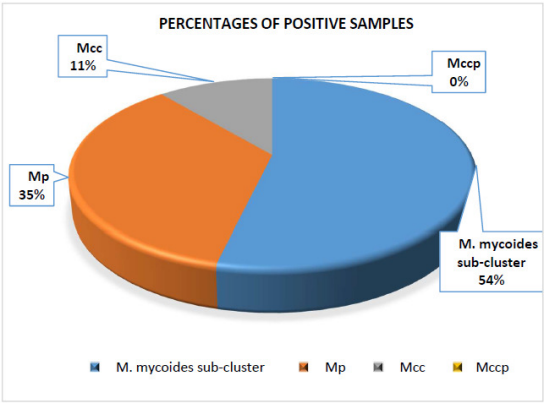


**Fig 2:** Percentages of *Mycoplasma* species positive samples detected by PCR from the nasal swabs of goats from Quetta abattoir

In the present study DNA was directly extracted from the nasal swab samples of goats, rather than from culture or media. In this way more rapid result were obtained than the traditional methods of culture, isolation, and identification using biochemical and serological techniques. The PCR and RFLP profiles obtained in the

present study were in line to those as reported previously (Awan *et al.,* 2009). In the present study the comparatively higher prevalence of *Mmc* and *Mp* was found in the nasal swabs from the randomly selected goats of Quetta abattoir, while prevalence of *Mccp* was found to be zero (Table 2). The higher prevalence of *Mmc* in the goats is also alarming in goats' population of Balochistan. As the majority of the goats in Quetta city are not raised locally, but are brought from different parts of Balochistan, as well as from border areas of Afghanistan. As a result there is a consistent influx of healthy and diseased goats from Afghanistan, and this is the root cause of presence of caprine pleuropneumonia in goats in Quetta city (Awan *et al.,* 2012: Tariq, 1980).

The pathogenic role of *Mmc* and *Mp* is well reported in the literature. In 1987, *Mp* caused a severe outbreak of mastitis and arthritis/polyarthritis in a herd located in the Central Valley of California (DaMassa *et al.,* 1987). Khan *et al*, (1989) very first time isolated *Mmc* in goats of Pakistan and showed that 5.63% positive cases of pneumonia among the affected goats were due to *Mmc*. Another case about the occurrence of *Mycoplasma mycoides*sub sp. *mycoides* Large Colony (*Mmm* LC) in goats of Hungary was reported by Bajmocy *et al.,* (2000). The deaths of five 3-week-old goat kids due to mycoplasmosis with clinical symptoms was documented by Szeredi *et al.,* (2003), during epidemic outbreaks due to *Mmc* which was confirmed by the PCR test. Further *Mmc* was also found as a cause of a CCPP disease in goats by the PCR test (Hernandez *et al*., 2006b). For several years, *Mp* was thought as the major cause of mastitis leading to agalactia in caprine, (Adler *et al.,* 1980). However, in 1987 a herd of 700 goats located in the Central Valley of California, was killed due to a severe outbreak of mastitis, arthritis and polyarthritis caused by *Mp* (DaMassa *et al.,* 1987). Awan *etal.,* (2009) isolated and identified *Mccp* and *Mp* in Pishin district of Balochistan from the goats with clinical signs of cCCPP during 2008 by PCR tests. Similarly, in the present study the prevalence of *Mmc* and *Mp* in goats in Quetta region cannot be ignored.

In the present study out of 385 of nasal swabs only 9% were found positive for *Mycoplasma* species. Of these only 7.5% were found positive for *Mycoplasma mycoides* sub cluster, 5% for *Mp* and 1.6% for *Mcc*, while none of the sample was found positive for *Mccp*. The results obtained in this study have certain similarities and dissimilarities with the results obtained by Awan *et al.,* (2009).They reported zero prevalence of not only *Mccp* but also *Mmc* in goats of Pishine district but in the present study *Mmc* (9%) showed higher prevalence in goats of Quetta city. Similarly they obtained higher prevalence of *Mcc* (40%) as compared to *Mp* (6.7%) in nasal swabs samples of goats but present study showed higher prevalence of *Mp* (5%) than *Mcc* (1.6%).This variation in the results may be due to the difference in husbandry conditions, samples collections methods, time periods and weather conditions.

The present study highlights the causes of prevalence of pleuropneumonia in goats of Quetta city due to *Mmc, Mp* and *Mcc* rather than *Mccp*. An extensive study is required to address the prevalence of other species of *Mycoplasma* in pleuropneumonia suspected caprine diseases in Quetta.

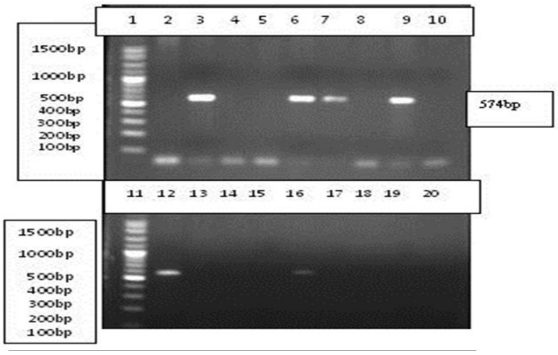


**Fig 3:** PCR profile of *Mycoplasma mycoids* sub-cluster members (band size of 574bp is positive) obtained from the nasal swab samples of goats from Quetta abattoir in Balochistan; Lanes 1 and 11: Molecular ladder (1500bp); Lane 2: Negative control; Lane 3: Positive control; Lanes 6, 7 9, 12 and 16: Positive field samples for *Mycoplasma mycoids* sub-cluster members; Lanes 4, 5, 8, 13-15 and 17-20: Negative field samples for *Mycoplasma mycoids* sub-cluster members.

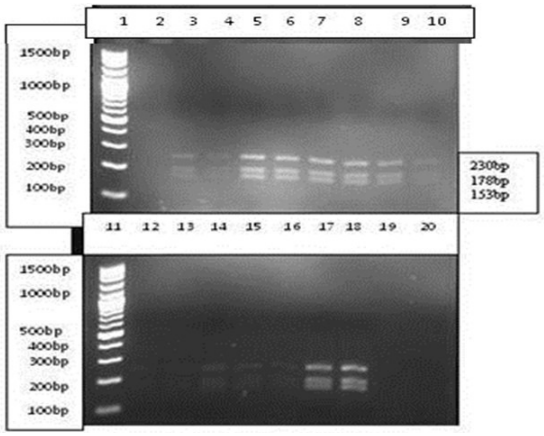


**Fig 4:** RFLP of *Mycoplasma mycoids* sub-cluster membersositive PCR products (fragments of 230, 178 and 153bp are specific for *Mmc* after cleavage of 574bp PCR product with *VspI restriction endonuclease);* Lanes 1 and 11: Molecular ladder; Lane 2: Negative control; Lane 3: Positive control; Lanes 4-10 and 12-18: Positive field samples; Lanes 19 and 20: empty wells (no sample run)

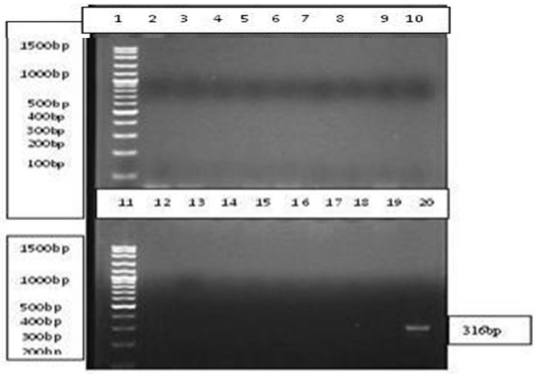


**Fig 5:** PCR profile of *Mccp* band size of 316bp is positive) obtained from the nasal swab samples of goats from Quetta abattoir in Balochistan; Lanes 1 and 11: Molecular ladder; Lane 19: Negative control; Lane 20: Positive control; All test samples are negative for *Mccp*

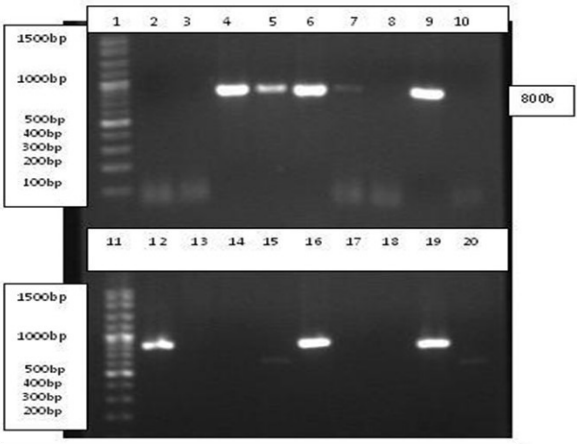


**Fig 6:** PCR profile of *Mp* (band size of 800bp is positive) obtained from the nasal swab samples of goats from Quetta abattoir in Balochistan; Lanes 1 and 11: Molecular ladder (1500bp); Lane 2: Negative control; Lane 19: Positive control; Lanes 4-6, 9, 12 and 16: Positive field samples for *Mp;* Lanes 3, 7-8, 10, 13-15, 17-18 and 20: Negative field samples for *Mp.*

## Conclusion

The present study implies that there is a prevalence of *Mycoplasma* infections in goats of Balochistan and the PCR based detection of *Mycoplasma* species is a rapid and specific method of diagnosis. Similarly restriction enzyme fragmentation (RFLP) assay is useful for the differentiation of *Mycoplasma mycoides* sub species *capri (Mmc*) and *Mycoplasma mycoides* sub sp. *Mycoides* Small Colony (*Mmm* SC). These methods would be helpful in the early diagnosis of *Mycoplasma* diseases, in order to design a strategy to start an early treatment and preparation of an effective *Mycoplasma* vaccine.

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