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Molecular Study on the Prevalence of Respiratory *Mycoplasma* Species in Small Ruminants of Kuchlak, District Quetta and Khanozai, District Pishin, Balochistan

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Abstract.-Respiratory diseases of small ruminants are among the most important problems throughout the world as well as in Balochistan, Pakistan. Various *Mycoplasma* species lead to pneumonia and other respiratory diseases in sheep and goats and inflict heavy economic losses in Balochistan. The aim of present study was to highlight the prevalence of respiratory *Mycoplasma* species in nasal swab samples of sheep and goats through polymerase chain reaction (PCR) and further validation through Restriction Fragment Length Polymorphism (RFLP). In total, 240 nasal swab samples of Rakhshani breed of sheep and 200 nasal swab samples of Khurasani breed of goats were collected in 2011 from randomly selected sheep from Khanozai district, Pishin and goats from Kuchlak, district Quetta respectively. The extracted DNA samples were analyzed using the PCR for *Mycoplasma mycoides* cluster group, *Mycoplasma mycoides* sub-cluster group, *Mycoplasma capricolum* subspecies *capripneumoniae* (*Mccp*), *Mycoplasma capricolum* subsp *capricolum* (*Mcc*) and *Mycoplasma putrefaciens* (*Mp*). The highest prevalence of 7.5% (n=18) was observed for *Mycoplasma mycoides* cluster members , followed by 6.25% (n=15) for *Mycoplasma mycoides* sub-cluster members, 5% (n=12) for *Mp* and 1.25% (n=3) for *Mcc* .Further none of the prevalence was seen for *Mccp.* The present PCR results for the *Mycoplasma mycoides* sub-cluster members were further validated by the RFLP, with the yield of three fragments (230, 178, and 153bps) specific for *Mmc.* Furthermore comparable results for various *Mycoplasma* species using PCR were also observed in goats. The PCR based prevalence of different mycoplasma species in sheep and goats in the study area is alarming and needs attention to contain the mycoplasmosis using efficacious mycoplasma vaccines.

Key words: Respiratory diseases, *Mycoplasma*, PCR, RFLP.

INTRODUCTION

Mycoplasma organisms belonging to the class *Mollicutes* are the smallest known free-living life forms. Members of the class *Mollicutes* inflict a wide range of diseases in animals and humans and are generally associated with clinical manifestations, *viz.*, pneumonia, conjunctivitis, arthritis, abortion and infertility (Nicholas, 2002). Most of the members of *Mycoplasma mycoides* cluster group are the significant pathogens for small ruminants. This group comprises 5 species and subspecies (Awan *et al.*, 2009). Many mycoplasma such as *Mycoplasma capricolum* subsp. *capripneumoniae*, *M. mycoides* subsp. *capri* and *M. capricolum* subsp. *capricolum* (*Mcc*) can infect lungs of small ruminants and induce respiratory disease. Moreover, *M. agalactiae* and *M. putrefaciens* can cause mastitis, arthritis and ocular disease (Al-Momani and Nicholas, 2006). *Mycoplasma* infections cause indirect economic losses as a result of emaciation, delayed market weight and infertility, owing to the sub-acute or chronic pneumonia especially in small ruminants, which are of great importance in rural development. A major health problem of small ruminants ispneumonia/pleuropneumonia, which may be caused by *Mycoplasma* species alone or in conjunction with other microbes (Adehan *et al.*, 2006).

In certain infectious diseases, the companion animals may play a key role in the transmission of infection across the susceptible animal population. Knowledge of their mechanism of virulence is still scarce and the activation of the immune system of

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the host probably plays a major role in the pathogenesis of mycoplasmoses. In general, mycoplasmas are not highly virulent but rather induce chronic diseases (Radostitis *et al*., 2007).

Classical method of isolation and identification for mycoplasmas is laborious and time consuming and are complicated by serological cross reaction between the closely related organisms. Limited studies have been reported on the use of molecular biological test such as PCR for the diagnosis of ovine and caprine mycoplasmosis in Balochistan (Awan *et al.,* 2009). In the present study, PCR technique was used on account of its rapidity, sensitivity and specificity as compared to the isolation and identification to detect the prevalence of respiratory *Mycoplasma* species.

There are 12.8 million sheep and 11.8 million goats in Balochistan. The aim of the present study is to underline the prevalence of different *Mycoplasma* species in sheep and goats through various PCR tests and further validation by RFLP. It is anticipated that the present study would be helpful in designing strategies in order to control the respiratory *Mycoplasma* diseases in Balochistan

MATERIALS AND METHODS

Nasal swab samples of sheep (n=240) and goats (n=200) were collected from field sheep of Khanozai district Pishin and field goats of Kuchlak, district Quetta. Samples were taken randomly from apparently healthy flocks of sheep and goats irrespective of breed, gender or age. The DNA was extracted from each of the nasal swab samples (n=440) by using genomic DNA purification kit (Gentra-Puregene, USA) by following the method as described in the Instruction Manual for body fluids protocol with little modification. Briefly each of the nasal swab sample was swirled in 2 ml PBS, and 100µl suspension was used for the extraction of DNA. All the primers used in the present study are shown in Table I.

The PCR master mix for *Mycoplasma mycoides* cluster and sub-cluster was prepared by following the procedure as described by Bashiruddin *et al*. (1994). The PCR (Thermal cycler, Model # 2720, Applied Biosystem) cycling conditions for *Mycoplasma mycoides* cluster and sub-cluster were similar (Bashiruddin *et al.,* 1994). Further the PCR master mix for *Mycoplasma capricolum* subspecies *capripneumoniae (Mccp)* and *Mycoplasma putrefaciens (Mp)* was prepared by following the method as described by Woubit *et al.* (2004) and Shankster *et al.* (2002), respectively. Agarose (Vivantis-USA) gel (2%) was used for gel electrophoresis (35 min at 100 Volts). The gel slab was observed for PCR product (band) under the gel documentation system (Dolphin-View, Wealtec-USA).

The presence of *Mcc* organisms in all the DNA samples collected from nasal swab samples of the sheep and goats was based on the results of DNA samples positive in *Mycoplasma mycoides* cluster PCR, negative in *Mycoplasma mycoides* sub-cluster, *Mccp* and *Mp* PCR tests.

The RFLP test using *Vsp1* restriction endonuclease was used for the validation of *Mycoplasma mycoides* sub-cluster PCR product following Bashiruddin *et al.* (1994). Furthermore no DNA sample extracted from nasal swabs of goats was not positive *Mccp* specific PCR, no its RFLP was performed unit.

RESULTS

PCR products of *Mycoplasma* cluster, *Mycoplasma* sub-cluster and *Mycoplasma putrefaciens* (Fig. 1) were identified as 1500, 574 and 800 bp bands, respectively (Fig. 1). None of the PCR product for *Mccp* was found positive in nasal swab samples of sheep and goats. The RFLP results for the PCR product (574bps) of *Mycoplasma mycoides* sub-cluster members (*Mmc* and *Mmm* SC) yielded three bands (fragments) of 230, 178, and 153bps specifically for *Mmc* when digested with *Vsp1* (Fig. 2). None of the PCR product was observed with two bands of 379bp and 178bp specific for *Mmm* SC in RFLP. The results obtained for the prevalence of *Mycoplasma* species by PCR in the nasal swabs of sheep and goats are shown in Table II. Of all the total nasal swab samples (n=240) from sheep, 20% prevalence (n=48) was observed. On the other hand, 23% (n=46) of the total nasal swab samples of goats (n=200) were found positive for *Mycoplasma* species.

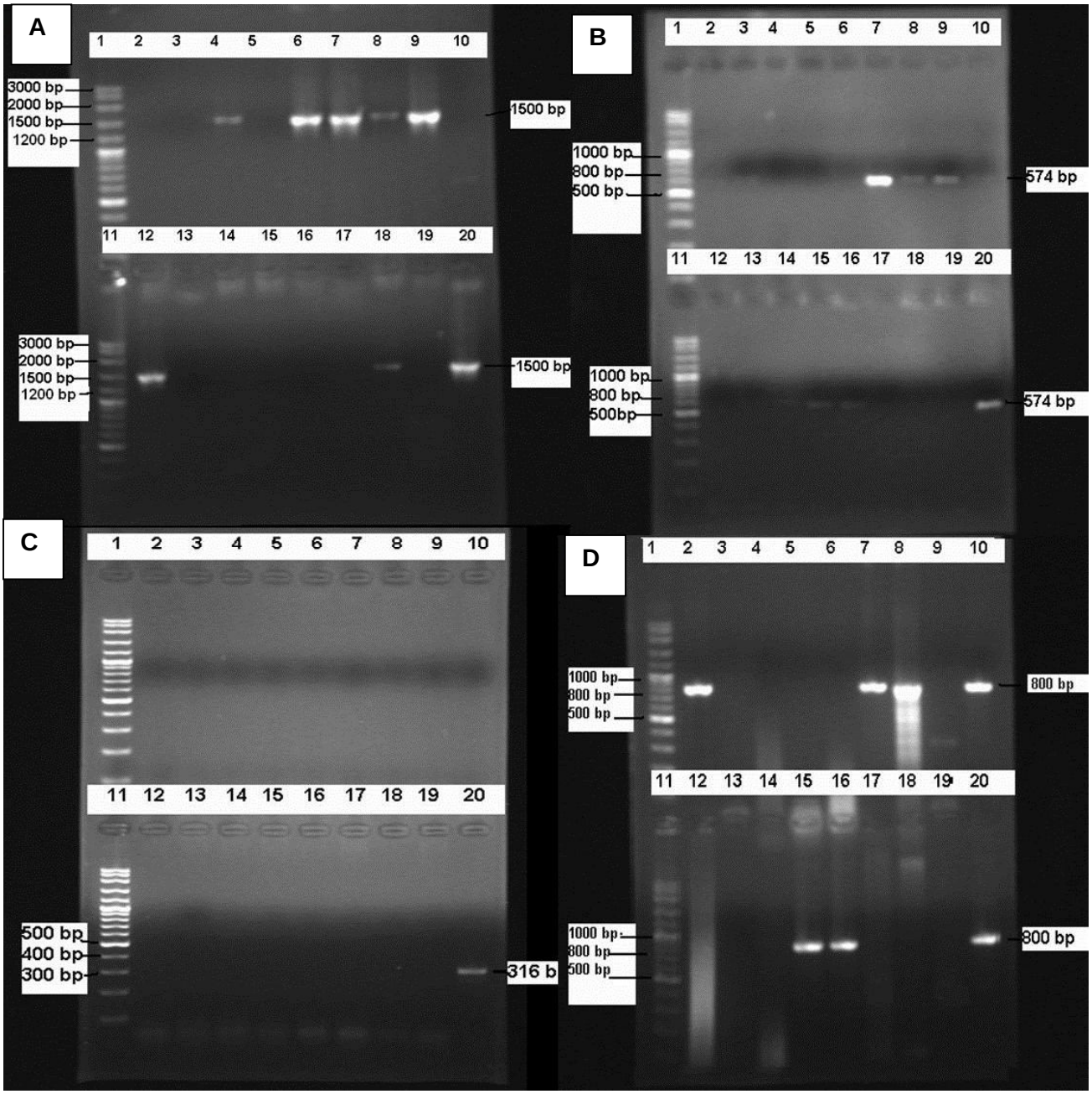

Fig. 1. Molecular detection (PCR and RFLP) of *Mycoplasma* species from sheep in Khanozai, district Pishin, Balochistan. A, *M. mycoides*cluster showing an amplicon size of 1500 bp; B, members of *M. mycoides* sub cluster showing an amplicon size of 574bp; C, *Mycoplasma capricolum* subsp *capripneumoniae.*showing an amplicon size of 316bp; D,:*Mycoplasma putrifaciens*(*Mp*) showing an amplicon size of 800bp.

DISCUSSION

Mycoplasma respiratory disease has a special place in veterinary medicine (Stalheim, 1983). Sheep and goats are the multifaceted animals for milk and meat. Economic losses associated with the disease are often the result of the combined effect of infection, poor management and environmental

Table I. Sequence of primers (oligonucleotides) used in PCRs for the identification of *Mycoplasma* species.

Mycoplasma species	Primers	Sequence (5' -3')
*Mycoplasma mycoides*Cluster[1]	F. MC323	TAG AGG TAC TTT AGA TAC TCA AGG
	R. MC358	GAT ATC TAA AGG TGA TGGT
Mycoplasma mycoides sub-cluster[2]	F. MM450	GTA TTT TCC TTT CTA ATT TG
	R. MM451	AAA TCA AAT TAA TAA GTT TG
Mycoplasma capricolum subspecies	F. *Mccp*-spe-F	ATC ATT TTT AAT CCC TTC AAG
capripneumoniae (Mccp)[3]	R. *Mccp*-spe-R	TAC TAT GAG TAA TTA TAA TAT ATG CAA
Mycoplasma putrefaciens[4]	F. SSF1	GCG GCA TGC CTA ATA CAT GC
	R. SSR1	AGC TGC GGC GCT GAG TTC A

F, forward; R, reverse.
[1,2]Bashiruddin *et al.*, 1994; [3]Woubit *et al*., 2004; [4]Shankster *et al*., 2002.

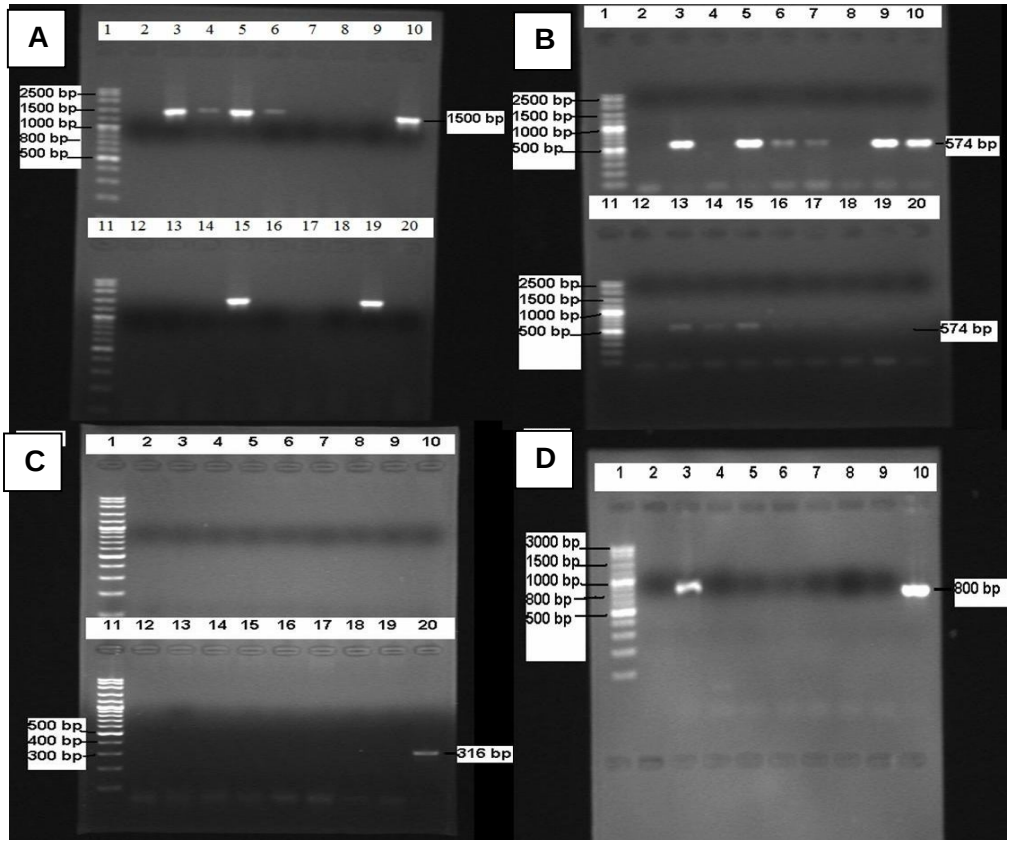

Fig. 2. Molecular detection (PCR and RFLP) of *Mycoplasma* species from goats in Kuchlak, district Quetta, Balochistan; A, *M. mycoides* cluster showing an amplicon size of 1500 bp; D, *M. mycoides* sub-cluster showing an amplicon size of 574bp; C, *Mycoplasma capricolum* subsp *capripneumoniae.* showing an amplicon size of 316bp; D, *Mycoplasma putrifaciens*(*Mp*) showing an amplicon size of 800bp.

condition (Ezzi *et al.*, 2007). The respiratory system is the site of infection of *Mycoplasma* spp. and it is capable of destroying cilia of the epithelial cells of bronchioles which later predispose these to bacterial invasion generally with *Pasteurella* spp. (Quinn *et al.*, 1994). Respiratory diseases in sheep and goats are common in Balochistan. Prevalence of *Mycoplasma* species in small ruminants are abundantly reported by some previous authors (Awan *et al.*, 2009, 2010). Limited studies have

Table II.- Results of molecular detection of *Mycoplasma* species from sheep and goats in Khanozai, district of Pishin, Balochistan.

Mycoplasma species	Samples tested for specific PCR	Positive samples (%)
Sheep		
Mycoplasma mycoides cluster specific PCR	240	18 (7.5)
Mycoplasma mycoides sub-cluster specific PCR-RFLP	240	15 (6.25)
Mccp specific PCR-RFLP	240	0 (0)
Mp specific PCR	240	12 (5)
Mcc specific PCR	240	3 (1.25)
Total		48 (20)
Goat		
Mycoplasma mycoides cluster specific PCR	200	25 (12.5)
Mycoplasma mycoides sub-cluster specific PCR-RFLP	200	15 (7.5)
Mccp specific PCR-RFLP	200	0 (0)
Mp specific PCR	200	1 (0.5)
Mcc specific PCR	200	5 (2.5
Total		46 (23)

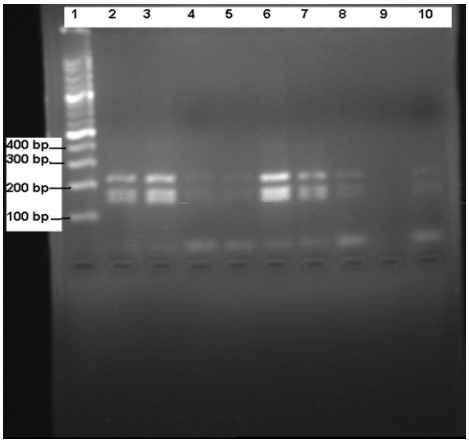

Fig. 3. RFLP using *Vsp1* endonuclease on the *Mycoplasma mycoides* sub-cluster *PCR* product (amplicon) showing three fragments of 230, 178 and 153bp.

been reported on the prevalence of respiratory *Mycoplasma* species among sheep in Balochistan. The present study describes the prevalence of *Mmc*, *Mcc, Mccp* and *Mp* organisms directly from the nasal swab samples from the apparently healthy field sheep and goats from two districts of Balochistan using PCR-RFLP analysis.

The present study shows highest prevalence of *Mycoplasma mycoides* cluster organisms in goats (12.5%) followed by sheep (7.5%). Furthermore, the highest prevalence was observed for *Mycoplasma mycoides* subsp. *P. mycoides* cluster with 7.5 % in goats and 6.25% in sheep, whereas no prevalence was found for *Mycoplasma mycoides* sub-species *capripneumoniae* (*Mccp*) in goats and sheep. *Mccp* is generally reported in goats and not considered a pathogen of sheep. In the present study the absence of *Mccp* in goats and sheep reflects that this pathogen is not prevailing among these animals in the target areas. Disease transmission or prevalence could be enhanced by poor management of animals which includes poor ventilation, sanitary cleanliness, mix flocking, mixing of diseased animals with healthy animals. OIE (2008) reported that in CCPP outbreaks in mixed goat and sheep herds, sheep may also be affected which has been verified by isolation of causative agents as well as detection of antibodies from clinically affected sheep and goats. *Mccp* has also been isolated from healthy sheep and the role of sheep as reservoirs of infection is unclear. *Mycoplasma* cluster group have 5 members and they are more susceptible to be found in apparently healthy flocks of sheep and goats. Present investigation also shows the highest prevalence of *Mp* organisms in sheep (5%) followed by goats (0.5%). This type of *Mycoplasma* species is reported to be present among sheep in many countries of the world including Pakistan (Awan *et al.,* 2009). Moreover, its association in respiratory diseases in sheep and goats in Pakistan needs to be investigated. The specific PCR for the detection of *Mcc* has been reported but in the present study the specific primers could not reveal the specific bands and need further laboratory standardization. For this reason the prevalence of *Mcc* was indirectly based on the positivity of *Mycoplasma mycoides* cluster PCR and negativity of *Mycoplasma mycoides* sub-cluster, *Mp* and *Mccp* PCR.

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