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 Rakhsani 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 is pneumonia/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
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 subcluster, 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.

<table>
<thead>
<tr>
<th>Mycoplasma species</th>
<th>Primers</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mycoplasma mycoides</em> Cluster¹</td>
<td>F. MC323</td>
<td>TAG AGG TAC TTT AGA TAC TCA AGG</td>
</tr>
<tr>
<td></td>
<td>R. MC358</td>
<td>GAT ATC TAA AGG TGA TGTT</td>
</tr>
<tr>
<td><em>Mycoplasma mycoides</em> sub-cluster²</td>
<td>F. MM450</td>
<td>GTA TTT TCC TTT CTA ATT TG</td>
</tr>
<tr>
<td></td>
<td>R. MM451</td>
<td>AAA TCA AAT TAA TAA GTT TG</td>
</tr>
<tr>
<td><em>Mycoplasma capricolum</em> subspecies</td>
<td>F. Mccp-spe-F</td>
<td>ATC ATT TTT AAT CCC TTC AAG</td>
</tr>
<tr>
<td><em>capripneumoniae</em> (Mccp)³</td>
<td>R. Mccp-spe-R</td>
<td>TAC TAT GAG TAA TTA TAA TAT ATG CAA</td>
</tr>
<tr>
<td><em>Mycoplasma putrefaciens</em>⁴</td>
<td>F. SSF1</td>
<td>GCG GCA TGC CTA ATA CAT GC</td>
</tr>
<tr>
<td></td>
<td>R. SSR1</td>
<td>AGC TGC GGC GCT GAG TTC A</td>
</tr>
</tbody>
</table>

F, forward; R, reverse.
¹²Bashiruddin et al., 1994; ³Woubit et al., 2004; ⁴Shankster 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 putrefaciens*(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
been reported on the prevalence of respiratory Mycoplasma species among sheep in Balochistan. The present study describes the prevalence of Mmc, Mcc, Mcop 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 capri pneumoniae (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.

REFERENCES


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