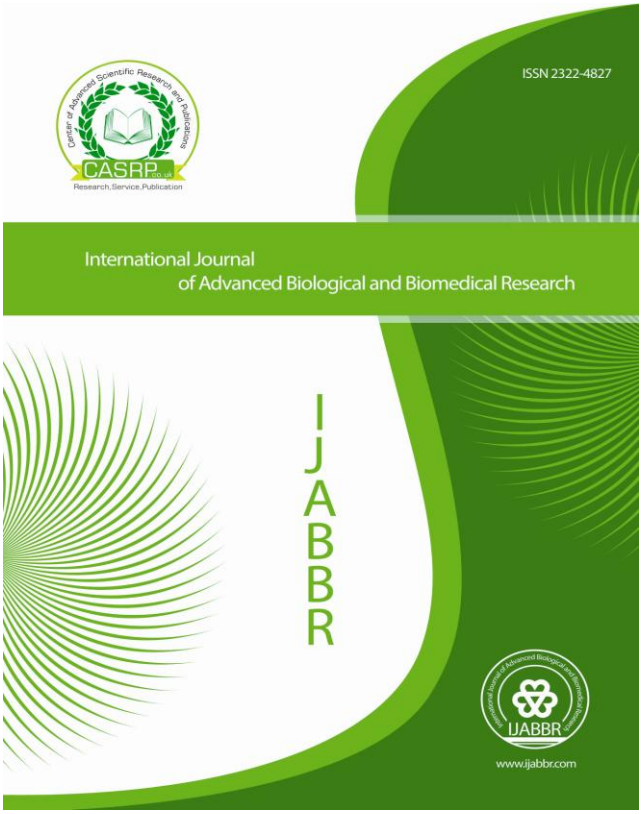

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Original Article Open Access

Bimolecular identification of *M.ovinpeumoniae* from nasal swab samples of sheep from various districts of Balochistan, Pakistan

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Abstract

Mycoplasma ovipneumoniae is an important respiratory pathogen of sheep across the world. In the present study nasal swab samples (n=778) were processed and 51isolates of *M. ovipneumoniae* were identified through biochemical tests and PCR. Difference in the isolation rate among districts (n=12), age groups (n=3), breads (n=6) was assessed. Similarly the isolate rate among healthy (n=513) and respiratory distressed (n=265) sheep were also compared. The results for the district-wise isolation of *Mycoplasma ovipneumoniae* in sheep showed non-significant variations among districts of Balochistan (χ^2= 1.65, *P*=0.9990). Statistically significant difference (χ^2 = 7.698, *P*< 0.02) in the isolation of *Mycoplasma ovipneumoniae* among various age groups was recorded, while highest isolation was seen in sheep of < 1 year.Statistically highly significant difference (χ^2 = 16.6, *P <0.0050)* in the isolation of *Mycoplasma ovipneumoniae* among various breeds of sheep in Balochistan was seen, whereas highest isolation rate (3.70%) was observed in Harnai breed for sheep Furthermore, statistically highly significant difference (Fisher's exact test *P≤0.0001*) in the isolation of *Mycoplasma ovipneumoniae* between healthy and respiratory distressed sheep breeds in Balochistan was found.

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1. Introduction

The livestock sector plays a vital role in the day to day living of the human population (FAO, 2002).During 2009-10, contribution of livestock sector to the national gross domestic product (GDP) was 11.4 %. Balochistan is the largest province of Pakistan with 47% of the total land area of the country (Khan, 2006). Forty eight percent (12.8 millions) of the sheep population of Pakistan is reared in Balochistan (Anonymous, 2006).The majority of sheep population in Balochistan is affected by various infectious and non-infectious diseases. *Mycoplasmas* are associated with serious diseases of sheep and goats (Gilmour et al., 1979). Diseases caused by *Mycoplasma* in the small ruminant lead to significant economic losses (Nicholas, 2002).

Mycoplasma ovipneumoniae was reported first time in New Zealand during 1974 (Alley et al., 1999). *M. ovipneumoniae* can cause respiratory disease alone in small ruminants and also act as predisposing factor for the attack of other microorganisms (Sheehan et al., 2007). Itcauses pulmonary abscess, pleurisy and fibrinous pneumonia under the harsh condition. *Mycoplasma ovipneumoniae* has been isolated from the respiratory tract of healthy sheep (Goltz et al., 1986). Pathogenicity of *M.ovipneumoniae* is experimentally proven as well. It produces atypical non progressive pneumonia in ovine. This organism is associated with increased number of out breaks in small ruminants (Gilmour et al., 1979) and causes long term coughing syndrome with rectal prolapse (Niang et al., 1998) and pneumonia in young sheep (Alley et al., 1999).

Isolation and identification is most commonly employed technique for the diagnostic purpose of ovine Mycoplosmosis (Ruffin, 2001). Several biochemical testssuch as digitonin sensitivity, glucose fermentation, tetrazolium reduction, casein digestion, arginine decarboxylation, inspissated serum liquefaction, phosphatase activity and film and spot are generally used for bio-characterization of *Mycoplasma* species (Poveda and Nicholas, 1998). All *Mycoplasma* species produce typical fried egg colonies, whereas the *Mycoplasma ovipneumoniae* showed centre less colonies on the pleuropneumonia like organism (PPLO) medium (DaMassa et al., 1992).

Traditional diagnostic procedures like culture and biochemical test procedure for identification of *M. ovipneumoniae* infections in sheep are time consuming. *M. ovipneumoniae* is involved in the rapid spread of atypical pneumonia in sheep, so specific, sensitive and rapid diagnostic procedures are needed for its early diagnosis (Ruffin, 2001). Polymerase Chain Reaction (PCR) is a sensitive and rapid diagnostic procedure for the early diagnosis of *Mycoplasma* infection. *Mycoplasma ovipneumoniae*has been isolated and detected through PCR from the nasal swab samples (Ongor et al., 2011) and from pneumonic sheep lungs (Bochlisch et al., 1989).

2. Materials and methods

2.1. Study locales and settings

This study was conducted in 6 divisions (Quetta, Loralai, Naseerabad, Sibi, Kalat and Mekran) of Balochistan. All the selection was carried out randomly based on the estimated sample size with regards to the sheep population in the respective district as reported in Livestock Census (Anonymous, 2006).

2.2. Sample collection and mycoplasma isolation

Sheep nasal swabs samples (n=778) were collected between 2009 and 2011 from sheep flocks in Balochistan randomly from healthy as well as from respiratory distressed sheep respectively for the isolation of *Mycoplasma*. All the nasal swabs were directly inoculated into *Mycoplasma* media prior to transportation to the laboratory.

The Eaton's broth and agar medium (Nicholas and Baker, 1998) were used for the isolation of *Mycoplasma* spp. Cultured plates and tubes were incubated at 37^0C in a humidified CO_2 incubator and were monitored daily for *Mycoplasma* growth. Any cloudiness in broth and fried egg colonies in agar indicated growth.

Tubes having no growth after 14 days of incubation were discarded. Once the fried egg colonies were seen, they were purified and cloned three times (triple cloned) by filtration of broth culture through a membrane filter

of 0.45um (Nicholas and Baker, 1998). All the purified and triple cloned *Mycoplasma* suspected culture were kept at -80^{o}C for further identification studies.

2.3. Biochemical characterization of *Mycoplasma* species

Classical biochemical tests were carried out for each of the isolate by following the standard method (Poveda, 1998). All the identified *Mycoplasma* isolates stored at -80^{o}C and were later used for the molecular characterization of *Mycoplasma species* by the PCR.

2.4. Molecular detection

All the *Mycoplasma* preserved at -80^{0}C were reactivated in Eaton's broth as mentioned in above section. The DNA was extracted from each of the isolated *Mycoplasma* from cattle lungs samples by using PUREGENE genomic DNA extraction kit (Gentra System, USA). The purified genomic DNA samples were stored in micro tubes (1.5 ml) at -20^{0}C until used in specific PCR for the *Mycoplasma* species.

Polymerase chain reaction (PCR) tests for the detection of *Mycoplasma ovipneumoniae* (McAuliffe et al., 2003) was performed on DNA samples purified from the cultures of the *Mycoplasma* isolates. *Mycoplasma ovipneumoniae* was amplified using primer (Gene-Link USA) pair LMFI (5'- TGAACGGAATATGTTAGCTT-3') (forward) and LMRI (5'- GACTTCATCCTGCACTCTGT-3') (Reverse) as described previously (McAuliffe et al., 2003).

The master mix for PCR was prepared by mixing the following ingredients. Molecular grade water (29µl), 10X reaction buffer (5µl),50mM $MgCl_2$1.5 µl, 10mM dNTPs 2µl, Forward and reverse primers 5µl of each and Taq polymerase 0.5µl.Finally 2µl of purified DNA was added to the PCR master mix. A total volume of 50 µl was used for PCR. PCR reactions were carried out in thermo cycler (Model # 2720, Applied Bio system) with an initial, DNA denaturation /enzyme activation step of 94^{o}C for 5 minutes, followed by 25 cycles of 94^{o}C for 30s, 55^{o}C for 30s, and 72^{o}C for 30s. A final extension of 72^{o}C for 7 min was included and kept the PCR product at 4^{o}C until further used. *Mycoplasma ovipneumoniae* yield a band of 361bp in 2% agarose gel (Vivantis, USA) (McAuliffe et al., 2003). PCR resulting products were resolved by electrophoresis on 2% agarose gels, stained with ethidium bromide and visualised under UV light. Briefly Mini gel (size 10 x 7 cm) horizontal electrophoresis unit (Elite-300 Wealtec, USA) was used to perform gel electrophoresis.

2.5. Statistical analysis

The proportions were used to calculate the seroprevalence of *Mycoplasma ovipneumoniae*. The chi-square test was used to obtain the difference in seroprevalence among various districts. Chi-square results for the difference were considered statistically significant if p-value was observed less than 0.5 (95% confidence interval).

3. Results

Of the total nasal swab samples (n=778), 51 samples (6.6%) were positive for *M. ovipneumoniae*. The distinct colony characteristics were frequently observed after 96 hours of incubation at 37^{0}C on solid medium in the presence of 5% Co_2. The colonies observed were small, medium, large without particular order, round in shape, raised, and centre less with small size (Fig. 1).The digitonin sensitivity test and growth of *Mycoplasma* cultures on the Eaton's medium without serum was used for preliminary identification of *Mycoplasma.*

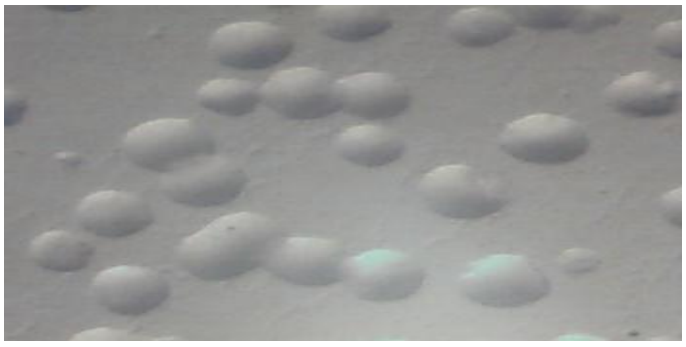

Fig. 1. Centre-less colonies of *Mycoplasma ovipneumoniae* on Eaton's agar medium isolated from nasal swab samples of sheep from Balochistan (35x).

3.1. Biochemical identification of mycoplasma *ovinpneumoniae*

Mycoplasma *ovinpneumoniae* was initially characterized through biochemical tests (Table 1). Of the total 778nasal swab sample processed from different district of Balochistan in the present study 51 (6.6 %)*Mycoplasma ovipneumoniae* were identified through biochemical and PCR tests (Table 1). The results for the district-wise isolation of *Mycoplasma ovipneumoniae* in sheep showed non-significant variations among 12 districts of Balochistan as determined through chi-square test (χ^2= 1.65) (Table 2). The highest isolation rate of 8.82% was seen in sheep from Killah Abdullah followed by Kalat (7.50%), Killah Saifullah and Panjgur (7.14%). Conversely, the lowest isolation rate was (4%) in Jaffarabad, followed by Pishin (5.13%), Naseerabad (5.56%) and Zhob (6.36%).

The results for the isolation of *Mycoplasma ovipneumoniae* in different age groups are shown in Table 3. Of the total nasal swabs samples (n=778) tested, 6.56 % (n=51) showed growth of *Mycoplasma ovipneumoniae*. The highest isolation of 3.21% (n=25) was seen in sheep of 1-2 years of age, followed by 2.19% in sheep of >3 years, while the lowest isolation rate of 1.16% in sheep of <1 year was observed. A statistically significant difference (χ^2 = 7.698) in the isolation of *Mycoplasma ovipneumoniae* among various age groups was noted (Table 3).

The results for the age-wise isolation of *Mycoplasma ovipneumoniae* in sheep from various districts of Balochistan are shown in Table 3. The highest isolation of 1.82% (Zhob) followed by 1.67% (Kalat), 1.63% (Kohlu), while lowest isolation of 0.95% (Khuzdar), preceded by 1.02% (Killa Saifullah) and 1.28% (Pishin) was seen in sheep of < 1 year. Likewise, the highest isolation of 7.14% (Panjgur) followed 5.56% (Naseerabad), 5.10% (Killa Saifullah) and 4.44% Dera Bughti, whereas lowest recovery of *Mycoplasma ovipneumoniae* was found in Zhob (1.82%) followed by Kohlu (2.44%) and Pishin (2.56%) in sheep of 1-2 years of age. The highest isolation of 5.88% (Killa Abdullah) followed by 4.0% (Jaffarabad), 2.73% (Zhob), whereas lowest isolation of 1.02% (Killa Saifullah), followed by 1.28% (Pishin) and 1.90% (Khuzdar) was seen in sheep of > 3 years (Table 3).

Results for the breeds' wise isolation of *Mycoplasma ovipneumoniae* in six breeds of sheep from healthy and respiratory distressed animals are shown in Table 4. Among the healthy sheep, *Mycoplasma ovipneumoniae* was isolated from 2.53% (n=13) out of a total of 513 sheep tested. The highest isolation rate of 3.70% was seen in Harnai breed of sheep followed by 2.91% for Mengali and 2.29% from the Balochi breed of sheep was observed. In contrast, the lowest isolation in healthy sheep breeds was seen in Beverigh (2.11%) followed by Rakhsahni (2.27%) and Balochi breed of sheep (2.29%). Statistically highly significant difference (χ^2 = 16.6) in the isolation of *Mycoplasma ovipneumoniae* among different breeds of sheep in Balochistan was seen. Further results for the Breeds wise seroprevalence of *Mycoplasma ovipneumoniae* in six breeds of sheep (n=1047) serum samples were obtained *(*n=173), 16.52 percent showed seropositivity.The highest seroprevalence of 18.02% was observed in Balochi breed (n=173) in serum samples (n=1047) followed by 17.65% (Harnai) and 17.63% (Mengali), while lowest seroprevalence of 10.16% (Rakhshani), preceded by 14.29% (Shinwari) was noted (Table 4).

The results for the breed's wise isolation of *Mycoplasma ovipneumoniae* in six sheep breeds from respiratory distressed are shown in Table 4. Among the respiratory distressed sheep breeds the total isolation of *Mycoplasma ovipneumoniae* was 14.38% (n=38) out of (n=265) respiratory distressed sheep breeds of Balochistan. The highest isolation of 18.75% was seen in Harnai breed of sheep followed by Balochi (17.24%) and Beverigh breed of sheep (14.29%) was observed. In contrast, the lowest isolation from respiratory distressed sheep breeds was seen in Rakhshani (8.47%) followed by Shanwari (13.89%) and Beverigh breed of sheep (14.29%). Statistically highly significant difference (Fisher's exact test P≤0.0001) in the isolation of *Mycoplasma ovipneumoniae* between healthy and respiratory distressed sheep breeds in Balochistan was found (Table 4).

3.2. Results of molecular characterization of *mycoplasmas* isolates recovered from sheep by using polymerase chain reaction (PCR)

All the isolates of *M. ovipneumoniae*were identified through biochemical tests and further confirmed through PCR (Table 1 & Plate 1). All the DNA samples (n=55) were purified from local isolates of the *Mycoplasma* species. Mycoplasma ovipneumoniae (n=51, 6.5 %) was obtained by using specific PCR for *Mycoplasma ovipneumoniae*.

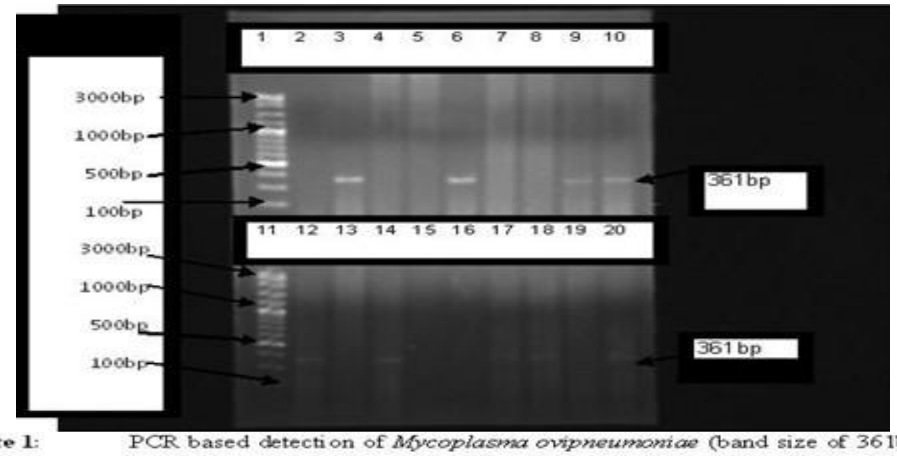

Plate 1: PCR based detection of *Mycoplasma ovipneumoniae* (band size of 361bp is positive) in the nasal swab samples of sheep in Balochistan. Lanes 1 & 11: Molecular ruler; lane 2: –ve control; lane 3: *Mycoplasma ovipneumoniae* + ve control; lanes 6,9-10,12,14,17-18,20 positive field samples for *M. ovipneumoniae;* lanes 4-5,7-8,13,15-16,19 negative field samples for *M. ovipneumoniae*.

Table 1
Biochemical and molecular identification of *Mycoplasma* isolates from sheep in 12 districts of Balochistan.

Total no of nasal swab	Isolation (%)[+]	Digitonin test	Glucose fermentation test	Arginine hydrolysis test	Tetrazolium reduction test	Casein hydrolysis test	Film & spot formation	Biochemical based identification of *Mycoplasma* species	PCR Based identification of *Mycoplasma* species
778	51	Sen	+ve	-ve	+ve	-ve	-ve	*Mo*	*Mo*[1]

Sen = Sensitive, *Mo* = *Mycoplasma ovipneumoniae*, [1]*Mycoplasma ovipneumoniae* specific PCR *(McAullffe* et al., 2003).

Table 2
District wise isolation of *Mycoplasma ovipneumoniae* in sheep from districts of Balochistan.

S/no.	Districts	Total population	Flocks (n)	Samples collected from nasal swabs	Culture positive	%
1.	Dera Bughti	506095	08	45	3	6.67
2.	Jaffarabad	241444	06	25	1	4.00
3.	Kalat	1239499	18	120	9	7.50
4.	Khuzdar	1105410	15	105	7	6.67
5.	Killa Abdullah	325020	07	34	3	8.82
6.	Killa Saifullh	1066690	19	98	7	7.14
7.	Kohlu	1306734	21	123	8	6.50
8.	Naseerabad	148501	05	18	1	5.56
9.	Pishin	837233	17	78	4	5.13
10.	Panjgur	91032	04	14	1	7.14
11.	Turbat	64693	03	8	0	0
12.	Zhob	1174735	16	110	7	6.36
Total		8107086	139	778	51	6.56

X^2 = 1.650(P=0.0.9994).

Table 3
Age wise distribution in the isolation rate of *Mycoplasma ovipneumoniae* from nasal swab samples in sheep flocks samples from 12 different districts of Balochistan.

S. No.	Districts	Samples (n)	*Mycoplasma ovipneumoniae*Isolates(n)	Age < 1 year	%	Age 1-2 year	%	Age > 3 years	%
1.	Dera Bughti	45	3	0	0.00	2	4.44	1	2.22
2.	Jaffarabad	25	1	0	0.00	0	0.00	1	4.00
3.	Kalat	120	9	2	1.67	4	3.33	3	2.50
4.	Khuzdar	105	7	1	0.95	4	3.81	2	1.90
5.	Killa Abdullah	34	3	0	0.00	1	2.94	2	5.88
6.	Killa Saifullah	98	7	1	1.02	5	5.10	1	1.02
7.	Kohlu	123	8	2	1.63	3	2.44	3	2.44
8.	Naseerabad	18	1	0	0.00	1	5.56	0	0.00
9.	Pishin	78	4	1	1.28	2	2.56	1	1.28
10.	Panjgur	14	1	0	0.00	1	7.14	0	0.00
11.	Turbat	8	0	0	0.00	0	0.00	0	0.00
12.	Zhob	110	7	2	1.82	2	1.82	3	2.73
	Total	778	51	9*	1.16	25*	3.21	17*	2.19

*χ^2= 7.698 (P<0.02).

Table 4
Mycoplasma ovipneumoniae isolates recovered from various breeds of sheep from healthy and respiratory distress cases in Balochistan.

S/No.	Breeds	Samples collected from healthy animals (n)	Positive sample n (%)	Sample collected from respiratory distressed animals (n)	Positive samples n (%)
1	Balochi	131	03 (2.29)	58	10 (17.24)
2	Beverigh	95	02 (2.11)	35	05 (14.29)
3	Harnai	54	02 (3.70)	32	06 (18.75)
4	Mengali	103	03 (2.91)	45	07 (15.56)
5	Rakhshani	88	02 (2.27)	59	05 (08.47)
6	Shinwari	42	01 (2.38)	36	05 (13.89)
	Total	513	13 (2.53)	265	38 (14.34)

Fisher's exact test (P≤0.00).

4. Discussion

Sheep population is confronted with many infectious and non-infectious diseases; among these, *Mycoplasma* is an important factor which is a serious threat to sheep population in this part of world. In past no study regarding ovine Mycoplasmosis in Balochistan has been conducted and no vaccination is available for this serious threat to the sheep population. The present study highlights the isolation and bio-molecular characterization of *M. ovipneumoniae* a respiratory pathogen of sheep.

Of the total nasal swab samples (n=778), 51 samples (6.6 %) were positive for *M. ovipneumoniae*. It produced a typical centre-less colony. The distinct colony characteristics were frequently observed after 96 hours of incubation at 37^0C on solid medium in the presence of 5% Co_2. The colonies observed were small, medium, large without particular order, round in shape, raised, and centre less.

Mycoplasma ovinpneumoniae was initially characterized through biochemical tests. Statistically highly significant difference in the isolation of *Mycoplasma* species in sheep among various districts of Balochistan was

observed. The present study was in line with the research in which *Mycoplasma ovipneumoniaeand some other Mycoplasma* have been recovered and biochemically identified from sheep (Brogden et al., 1988). Similarly, Bochlisch et al. (1989) isolated *Mycoplasma ovipneumoniae* in pure and in mixed culture with other bacteria from lungs of lambs.

Difference in isolation rate among different districts, age groups, breads was assessed in the present study. Moreover the isolate rate among healthy and disease sheep were also recorded. The results for the district-wise isolation of *Mycoplasma ovipneumoniae* in sheep showed non-significant variations among 12 districts of Balochistan as determined through chi-square test (χ^2= 1.65). Similarly a statistically significant difference (χ^2 = 7.698) in the isolation of *Mycoplasma ovipneumoniae* among various age groups was recorded and a highest isolation was seen in sheep of < 1 year. Statistically highly significant difference (χ^2 = 16.6) in the isolation of this organism among the six breeds of sheep in Balochistan was seen and a highest isolation rate of 3.70% was recorded in Harnai breed.

Among the healthy sheep, *Mycoplasma ovipneumoniae* (2.53%, n=13) was isolated from a total of 513 sheep . The highest isolation rate of 3.70% was seen in Harnai breed of sheep. Statistically highly significant difference (χ^2 = 16.6) in the isolation of *Mycoplasma ovipneumoniae* among different breeds of sheep in Balochistan was seen. Amongthe respiratory distressed sheep breeds the total isolation of *Mycoplasma ovipneumoniae* was 14.38%, n=38) out of respiratory distressed sheep (n=265) breeds of Balochistan. The highest isolation of 18.75% was seen in Harnai breed of sheep. Statistically highly significant difference (Fisher's exact test P≤0.0001) in the isolation of *Mycoplasma ovipneumoniae* between healthyand respiratory distressed sheep breeds in Balochistan was found.

All the isolates of *M. ovipneumoniae* were also confirmed through PCR. All theDNA sampleswere purified from local isolates of the *Mycoplasma* species. *Mycoplasma* ovipneumoniae (n=51) was obtained by using specific PCR for *Mycoplasma ovipneumoniae*. The present findings are consistent with the results reported by Besser et al. (2008) whereas *Mycoplasma ovipneumoniae* was reported to be strongly associated with bronchopneumonia in free-ranging bighorn sheep based on the application of PCR. Likewise, the isolation of *Mycoplasma ovipneumoniae* and other *Mycoplasma* species from nasal swab samples of sheep were reported by Mohkber Dezfouli *et al.* (2011). Recently, the presence of *Mycoplasma ovipneumoniae* and other *Mycoplasmas* are investigated by culture and PCR in the nasal swab samples of 692 goats. Overall the rate of isolation (more than 50%) of *Mycoplasma ovipneumoniae* from sheep was also reported (Ongor et al., 2011).

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