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Short Communication

Seroprevalence of *Mycoplasma ovipneumoniae* among Sheep from Different Districts of Baluchistan, Pakistan

Shahid Amin[1*], Sajjad Ur Rahman[1], Iftikhar Hussain[1] and Ghulam Muhammad[2]
[1]*Institute of Microbiology, Faculty of Veterinary Science, University of Agriculture, Faisalabad-38040, Pakistan*
[2]*Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad-38040, Pakistan*
*For correspondence: libra061@gmail.com

Abstract

In this study, seroprevalence of *Mycoplasma ovipneumoniae* in sheep was determined in Baluchistan, Pakistan. A total of 1047 sera samples from 139 flocks of sheep were tested using indirect haemagglutination assay (IHA). An overall prevalence of *M. ovipneumoniae* in sheep in Baluchistan was 16.53%. There was no difference in the prevalence among various districts of Baluchistan. The cumulative mean titter (CMT) of 20.32, 50.86 and 50.90 was found in < 1 year, 1-2 year and > 3 years of sheep, respectively. Age of sheep significantly influenced the prevalence being highest in 1-2 years old sheep followed by < 1 and > 3 year old sheep. Likewise, an association was also found between the prevalence and different breeds of sheep highest (70%) in Balochi followed by Harnai and Mengali. The use of IHA was found reproducible, inexpensive and effective tool to monitor the antibodies against *M. ovipneumoniae* in sheep in Baluchistan.

Keywords: *Mycoplasma ovipneumoniae*; Seroprevalence; IHA; Sheep; Baluchistan

Introduction

Production of sheep in the world is facing the challenge of respiratory diseases and results in high mortality and morbidity (Bergonier *et al*., 1997; Nicholas, 2002). *Mycoplasma ovipneumoniae* has been linked to the rapid spread of atypical pneumonia in sheep; therefore, rapid diagnostic procedures are helpful for early diagnosis (Ruffin, 2001). Several serological tests such as complement fixation test (CFT), indirect haemagglutination test (IHA), enzyme-linked immune-sorbent assays (ELISA) and Latex agglutination test (LAT) are reported for the diagnosis of Mycoplasma infection (March *et al*., 2003). IHA test has increasingly been used to demonstrate antibodies (Armstrong, *et al*., 1983; Mekuria *et al*., 2008; Rahman *et al*., 2003) in *Mycoplasma* diseases while many modifications in the test are also reported (Krogsgaard-Jensen, 1971). The present study was carried out to determine seroprevalence of *M. ovipneumoniae* in different districts of Baluchistan, a densely sheep populated province of Pakistan.

Materials and Methods

Study Locales and Samples Collection

This study was conducted in 6 divisions (Quetta, Loralai, Naseerabad, Sibi, Kalat and Mekran) of Baluchistan. The sampling 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. In total 1047 blood samples were collected aseptically from the jugular vein of sheep (12 districts of Baluchistan). Serum was separated from all the clotted blood samples to use in the IHA test for the seroprevalence of *M. ovipneumoniae*.

Standardization of IHA

The method reported by Cho *et al.* (1976) was used with little modifications in the selection of growth medium used for *M. ovipneumoniae* and application of bicinchoninic acid (BCA) protein quantification kit.

Preparation of *M. ovipneumoniae* Antigen for IHA

The freeze-dried culture of *M. ovipneumoniae* (obtained from CASVAB, University of Baluchistan) was reactivated in Eaton's broth medium at 37°C for 72 h (Nicholas and Baker, 1998). The *Mycoplasma* cells were washed 4X with autoclaved normal saline and re-suspended in normal saline (1% bacterial cell suspension). The bacterial cell suspension was sonicated (Ultra sonicator) for a total of 40 min (2 min of sonification and 2 min cooling interval). The supernatant was used as an antigen for IHA. The protein concentration of the supernatant was determined using bicinchoninic acid (BCA) protein assay kit (Bio-World, USA). The protein concentration (10 mg/mL) was adjusted with Phosphate Buffer Saline (PBS).

Glutaraldehyde was used to fix the sheep RBC by following the method as reported by Cho *et al.* (1976). The glutaraldehyde fixed sheep RBC were washed with sterile saline and re-suspended (10% suspension). Sodium azide @ 0.1% was also added into this suspension and kept at 4°C.

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Glutaraldehyde fixed sheep RBC were sensitization with *M. ovipneumoniae* antigen. The antigen sensitized glutaraldehyde fixed SRBC were washed 3X with PBS at 1500 rpm for 10 min. The washed SRBC were re-suspended (20%) in PBS and stored in refrigerator. Two per cent suspension of antigen sensitized glutaraldehyde fixed SRBC in PBS was used for IHA.

The IHA was performed as described by Cho *et al*. (1976) with little modification. All the sheep test sera were heat inactivated at 56°C for 30 min. Test sera from the sheep were diluted (twofold) to a volume of 50 µL with PBS. Finally, 50 µL of antigen coated glutaraldehyde fixed SRBC (2%) were added into all the wells having two fold diluted test sera from sheep. The plate was incubated at 37°C for one h. The negative, positive and antigen controls were also made along with the test sera. The antibody titter against *M. ovipneumoniae* was read as reciprocal of the highest dilution of sheep test sera showing agglutination of the antigen sensitized glutaraldehyde fixed SRBC.

Production of Hyper-Immune Sera against *M. ovipneumoniae*

The hyper immune serum was raised in Albino rabbits (n=4) following Clyde (1964) with slight modification at CASVAB, University of Baluchistan, Quetta. Serologically negative rabbits (from IHA) were injected with 5 injections of 0.2, 0.4, 0.6, 0.8 and 1 mL of formaldehyde inactivated *M. ovipneumoniae* culture (diluted in PBS) intravenously at 2 days interval. Finally, 1 mL of the same inactivated antigen with an equal volume of Montanide (Cipic, France) adjuvant was injected to the rabbits hind quarter (subcutaneously) and foot pads (intramuscularly). The animals were bled after the last injection. The serum was separated, decomplemented (56°C for 30 min) and tested by slide agglutination test.

Seroprevalence of *M. ovipneumoniae* through IHA

The indirect haemagglutination assay (Cho *et al.,* 1976) with little modification was used to quantify the antibodies against *Mycoplasma ovipneumoniae* from randomly selected sheep from various districts of Baluchistan.

Statistical Analysis

The proportions were used to calculate the seroprevalence of *M. 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).

Results

District Wise Prevalence of *M. ovipneumoniae* in Sheep

Results revealed that serum antibodies against *M. ovipneumoniae* prevailed in sheep from selected districts of Baluchistan. The lowest prevalence was noted in Panjgury (8.33%), followed by Jaffarabad (8.89%) and Naseerabad (9.38%). Conversely, highest prevalence was found of in Killa Saifullah (20.80%) followed by Kohlu (19.08%), Killa Abdullah (18.33%) and Kalat (18%) districts. Overall prevalence in all the districts was 16.53%. There was, however, no difference ($P \leq 0.59$) in the prevalence of *M. ovipneumoniae* in sheep among various districts of Baluchistan (Table 1).

Sero-kinetics Studies of *M. ovipneumoniae* in Sheep

The results of sero-kinetics studies showed highest Geo Mean Titters (GMT) (119.4) against *M. ovipneumoniae* in sheep of 1-2 years age group in district Kohlu followed by 104.0 GMT 78.8 68.6 in the age group of > than 3 years in Killa Saifullah, Zhob and Kohlu districts (Table 2).

The cumulative mean titter (CMT) of 20.32 was found in sheep of < 1 year of age; whereas, CMT of 50.86 and 50.90 were recorded in the sheep of the 1-2 year and >3 years of age, respectively (Fig. 1).

Age-wise Prevalence of *M. ovipneumoniae* in Sheep

The highest prevalence of 8.88% was seen in sheep of 1-2 years of age, while the lowest prevalence of 3.82% in sheep of <1 year and >3 years, of age was recorded. Statistically highly significant difference ($\chi^2_= 34.37$) in the prevalence of *M. ovipneumoniae* among different age groups was observed (Table 3).

Breed Wise Prevalence of *M. ovipneumoniae* in Sheep

The highest prevalence (70%) was seen in the Balochi breed of sheep followed by in Harnai (64.71%) and Mengali (53.85%) breed of sheep. In contrast, the lowest prevalence was noticed in Beverigh (22.22%) followed by Shinwari (25.00%) and Rakhshani (26.09%) breeds of sheep. Statistically highly significant difference ($\chi^2= 16.6$) in the prevalence of *M. ovipneumoniae* among different breeds of sheep in Baluchistan was observed (Table 4).

Discussion

The present study described the IHA based seroprevalence of *M. ovipneumoniae* infection in sheep from Baluchistan, Pakistan. IHA test was selected based on its simplicity, sensitivity, specificity, reproducibility and cost effectiveness. The cut-off value for positive titre in IHA was assumed as titre ≥1:2, because no cross reactivity was previously reported with members of *M. mycoides* cluster and non-cluster organisms.

Overall prevalence (16.53%) of *M. ovipneumoniae* in sheep was considered high in different districts of Baluchistan. There was no statistical difference in prevalence among different districts, which indicated that geoclimatic conditions of Baluchistan were conducive for

Table 1: District wise prevalence of *M. ovipneumoniae* in sheep from 12 districts of Baluchistan

Districts	Total population	Flocks (n)	Serum samples collected (n)	Sero +ve[1] (n)	%
Dera Bughti	506095	08	65	8	12.31
Jaffarabad	241444	06	45	4	8.89
Kalat	1239499	18	150	27	18.00
Khuzdar	1105410	15	130	21	16.15
Killa Abdullah	325020	07	60	11	18.33
Killa Saifullah	1066690	19	125	26	20.80
Kohlu	1306734	21	152	29	19.08
Naseerabad	148501	05	32	3	9.38
Pishin	837233	17	104	14	13.46
Panjgur	91032	04	24	2	8.33
Turbat	64693	03	18	2	11.11
Zhob	1174735	16	142	26	18.31
Total	8107086	139	1047	173	16.53

[1]χ^2=9.279 (P≤0.59)

Table 2: Sero-kinetics of *Mycoplasma ovipneumoniae* prevalent among various age groups of sheep in Baluchistan

District	Total serum samples collected (n)	Age groups	Sero +ve Samples (n)	IHA titter[1] (Range)	GMT*
Dara Bughti	65	<1 year	1	(1:8)	8.0
		1-2 year	4	(1:8-1:32)	16.0
		>3 year	3	(1:16-1.64)	39.4
Jaffarabad	45	<1 year	1	(1:8)	8.0
		1-2 year	2	(1:16-1.64)	32.0
		>3 year	1	(1:8-1.32)	16.0
Kalat	152	<1 year	5	(1:8-1:64)	27.9
		1-2 year	14	(1:16-1:128)	64.0
		>3 year	8	(1:16-1:32)	22.6
Khuzdar	130	<1 year	6	(1:8-1:32)	16.0
		1-2 year	13	(1:8-1:64)	24.3
		>3 year	2	(1:32-1:64)	48.5
Killa Abdullah	60	<1 year	3	(1:8-1:32)	27.9
		1-2 year	5	(1:8-64)	42.2
		>3 year	3	(1:8-128)	16.0
Killa Saifullah	125	<1 year	6	(1:8-1:32)	22.6
		1-2 year	16	(1:8-1:64)	48.5
		>3 year	4	(1:64-1:128)	104.0
Kohlu	150	<1 year	5	(1:8-1:32)	27.9
		1-2 year	15	(1:8-128)	119.4
		>3 year	9	(1:32-128)	68.6
Naseerabad	32	<1 year	2	(1:8-1:64)	22.6
		1-2 year	1	(1:64)	64
		>3 year	-	-	-
Pishin	104	<1 year	3	(1:8-1:64)	32
		1-2 year	7	(1:32-1:64)	45.3
		>3 year	4	(1:32-1:126)	64.2
Punjgur	24	<1 year	-	-	-
		1-2 year	2	(1:32-1:64)	45.3
		>3 year	-	-	-
Turbat	18	<1 year	1	(1:8)	8.0
		1-2 year	1	(1:64)	64
		>3 year	-	-	-
Zhob	142	<1 year	7	(1:8-1:32)	22.6
		1-2 year	13	(1:32-1:64)	45.3
		>3 year	6	(1:32-1:128)	78.8
Total	1047		173		

the propagation of *M. ovipneumoniae.* As far as district-wise isolation of *M. ovipneumoniae* is concerned, though the highest isolation (8.82%) was seen in sheep from Killa Abdullah district; whereas, the lowest isolation (4%) was noted in sheep from Jaffarabad district. An indirect haemagglutination test on sera from 251 Dall sheep has been reported. All the tested animals were found sero-negative as they were not probably exposed to *M. ovipneumoniae* (Zarnke and Rosendal, 1989). Similarly the use of IHA has been reported in the serodiagnosis of *Mmc* infection in goats in Baluchistan (Awan, 2010).

Table 3: Age-wise seroprevalence of *M. ovipneumoniae* by indirect haemagglutination based assay (IHA) in serum samples of sheep flocks from 12 different districts of Baluchistan

Districts	Samples collected (n)	Total +ve samples (n)	Age < 1 Year	%	Age 1-2 Years	%	Age > 3 years	%
Dera Bughti	65	8	1	1.54	4	6.15	3	4.62
Jaffarabad	45	4	1	2.22	2	4.44	1	2.22
Kalat	150	27	5	3.33	14	9.33	8	5.33
Khuzdar	130	21	6	4.62	13	10.00	2	1.54
Killa Abdullah	60	11	3	5.00	5	8.33	3	5.00
Killa Saifullah	125	26	6	4.80	16	12.80	4	3.20
Kohlu	152	29	5	3.29	15	9.87	9	5.92
Naseerabad	32	3	2	6.25	1	3.13	0	0.00
Pishin	104	14	3	2.88	7	6.73	4	3.85
Panjgur	24	2	--	0.00	2	8.33	0	0.00
Turbat	18	2	1	5.56	1	5.56	0	0.00
Zhob	142	26	7	4.93	13	9.18	6	4.22
Total	1047	173 (16.52%)	40[b]	3.82	93[a]	8.88	40[b]	3.82

[ab]χ^2=34.37 (P≤0.0001)

Table 4: Comparative seroprevalence of *Mycoplasma ovipneumoniae* in various breeds of sheep samples from 12 different districts of Baluchistan

Breeds	No of flocks examined (n)	No of sero +ve flocks n (%)	Serum samples collected (n)	Sero +ve animals n (%)
Balochi	30	21(70.00)	383	69 (18.02)
Beverigh	09	02(22.22)	47	08 (17.02)
Harnai	17	11(64.71)	102	18 (17.65)
Mengali	52	28(53.8)	312	55(17.63)
Rakhshani	23	06(26.09)	161	17 (10.56)
Shinwari	08	02(25.00)	42	06 (14.29)
Total	139	70[b] (50.36)	1047	173[a] (16.52)

[ab]χ^2 16.6 (P≤0.0053)

The sheep population in Baluchistan belongs to more than six precious breeds, which include Balochi, Beverigh, Harnai, Mengali, Rakhshani and Shinwari. In the present study, Balochi sheep were highest (70%) in prevalence of *M. ovipneumoniae;* whereas, Beverigh had the lowest (22.22%) prevalence indicating susceptibility differences among different breeds of sheep. The other contributing factors for high prevalence could be the cold weather, which prones the Balochi sheep to Mycoplasma infections than the Beverigh breed raised in warm or hot weather.

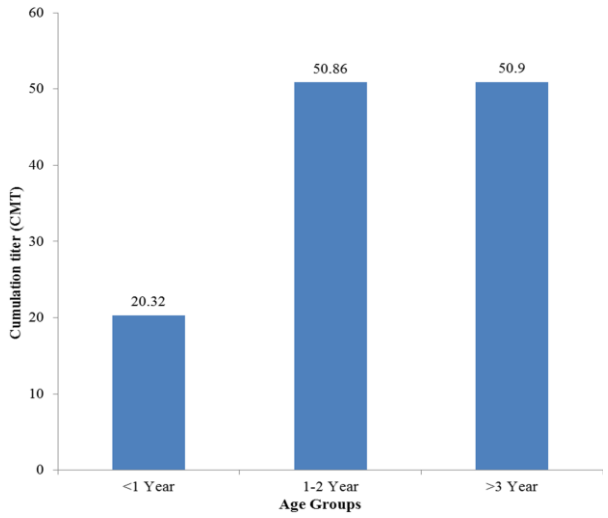

Fig. 1: Highest seroprevalence in sheep with maximum CMT antibodies titre against *M. ovipneumoniae* in 12 districts of Baluchistan

Sheep of all ages were found carrier of *Mycoplasma* spp. In many districts of Baluchistan, the sheep population is very dense and there is constant complaint of respiratory distressed cases. The losses due to respiratory *Mycoplasma* diseases have not been well documented in Baluchistan. But general perception indicated that the direct and indirect losses by the *Mycoplasma* spp. are very high because of huge mortality and morbidity rates in respiratory distressed cases in sheep of Baluchistan. No vaccine is available against ovine *Mycoplasma* species in sheep. Antibacterials are still on top priority for farmers to treat their sheep against the prevailing *Mycoplasma* species. In the present study, seroprevalence of sheep are attempted from fairly extensive region of Baluchistan. Similar investigations are imperative for the entire Pakistan. The present studies, showed that *Mycoplasma* species in general are responsible for the respiratory diseases in sheep, which are common and endemic among the studied districts of Baluchistan.

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