Journal of Animal Research: v.6 n.4, p. 571-577. August 2016

DOI: 10.5958/2277-940X.2016.00065.6

Serological and Clinicopathological Studies on Leptospirosis Among Sheep

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Received: 01 July, 2015 **Accepted:** 28 May, 2016

ABSTRACT

The present study was carried out to investigate occurrence, serovar distribution and clinicopathological attributes of leptospirosis among sheep from South Gujarat. A total of 41 blood and serum samples were randomly collected from apparently healthy and clinically ailing sheep of different breeds and age of either sex, reared in different flocks. Seropositivity was found to be 12.20% among sheep using the Microscopic Agglutination Test (MAT). Among clinically ailing and apparently healthy sheep, seropositivity was found to be 4.35% (1/23) and 22.22% (4/18), respectively with involvement of serovars Pomona, Canicola and Icterohaemorrhagiae. The hemato-biochemical and urinalyses results showed variation among seropositive and seronegative animals however, these differences were non-significant. The study indicated seropositivity with serovar Pomona as main leptospiral serovar among sheep in South Gujarat, however, the specific conclusion on clinicopathological aspect could not be made. Though for prevention and control of the disease where an obvious alteration in the serovars causing the disease is common further epidemiological study is necessary.

Keywords: Leptospirosis, sheep, microscopic agglutination test, seropositivity

Leptospirosis, a disease caused by pathogenic spirochetes i.e. *Leptospira interrogans*, which produces heavy economic losses in livestock production sector due to decreased milk yield, abortion, stillbirth, weak progeny, weight loss, reproductive complications and occasionally death (Radostits *et al.,* 2000). However, the cases of leptospirosis in domestic animals like sheep usually go unnoticed because of nonspecific symptoms (McBride *et al.,* 2005). Leptospirosis is endemic in South Gujarat where since last decade, outbreaks of human leptospirosis have been reported but a few reports available indicating seropositivity in domestic animals like cattle, buffaloes (Srivastava and Kumar, 2003; Savalia and Pal, 2008; Patel *et al.,* 2014), goats and sheep (Savalia and Pal, 2008). So to congregate baseline information on ovine leptospirosis the present study was undertaken to study seroprevalence and clinico-pathological of ovine leptospirosis.

MATERIALS AND METHODS

Collection of samples

A total of 41 blood samples (Ram-21 Ewe-20) were collected randomly from sheep during year 2012 to 2013 from migratory flocks (n=2) and Panjarapole (n=1) in South Gujarat. No vaccination programme against leptospirosis was carried out in these animals. Data related to clinical signs and history of abortion, if any and others were recorded at the time of sample collection. Blood was collected in sterile 6.0 ml K3 EDTA and 9.0

ml plain vacutainers via jugular venipuncture in the field. Samples were transported under cold chain system to our pathology laboratory. Whole blood samples were used for hematology. To obtain serum, whole blood was kept in slanting position in 9.0 ml plain vacutainers until serum was extracted out. The 9.0 ml plain vacutainers were centrifuged at 7000 rpm for 10 minutes, if needed. The straw coloured serum was collected into two sets of 1.5 ml sterile cryo vials and aliquoted. One set was stored at –20 °C for MAT while the other set was used for analyses of biochemical parameters. Urine samples (n=20) were collected randomly from the same population of sheep during blood collection at the same visit. Midstream urine samples (30 to 50 ml) were collected in plastic containers from each animal. All the urine samples were transported to laboratory as soon as possible.

Microscopic Agglutination Test (MAT)

All the sera were tested for antibodies against live antigens of *Leptospira* sp. Serovars Pyrogenes, Australis, Bankinang, Grippotyphosa, Patoc1, Pomona, Icterohaemorrhagiae, Hebdomadis, Canicola, Hardjo, Bellum, Bataviae, Tarassovi, Shermani, Kaup, Hurstbridge and Javanica by Microscopic agglutination test (Table 1) at Leptospira Reference Laboratory, Government Medical College, Surat using standard procedure (Vijayachari *et al.*, 2001) and National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Indian Council of Agricultural Research, formerly Project Directorate on Animal Disease Monitoring and Surveillance (PD-ADMAS), Hebbal, Bengaluru using standard procedure (Faine, 1982).

Hematological analyses

Blood samples were subjected to various haematological analyses i.e. hemoglobin estimation (Hb), packed cell volume (PCV), total erythrocyte count (TEC), mean corpuscular volume(MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total leucocyte count (TLC) and were analyzed using Automatic Whole Blood Analyzer (Medonic CA 620/530 VET, Boule Medical AB, Sweeden) while Differential Leucocyte Count (DLC) on Giemsa stained blood smears was performed manually.

Serum Biochemical analyses

Within 24 hours of sample collection, serum samples were analyzed for various serum biochemical test i.e. total protein, albumin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine, blood urea nitrogen (BUN) using Randox Kits (M/S Randox Laboratory Limited., 55 Diamond road, Crumlin, co. Antrim, BT 29 4 QY, United Kingdom) in Auto Serum Analyzer (M/S Chemwell Awareness Technology, INC.).

Table 1: Panel of *Leptospira* serogroups and serovars used in MAT

Sr. No.	Sero groups	Serovars
I	Pyrogenes	Pyrogenes
II	Australis	Australis
III	Autumnalis	Bankinang
IV	Grippotyphosa	Grippotyphosa
V	Semeranga	Patoc1
VI	Pomona	Pomona
VII	Icterohaemorrhagiae	Icterohaemorrhagiae
VIII	Hebdomadis	Hebdomadis
IX	Canicola	Canicola
X	Sejroe	Hardjo
XI	Bellum	Bellum
XII	Bataviae	Bataviae
XIII	Tarassovi	Tarassovi
XIV	Shermani	Shermani
XV	Tarassovi	Kaup
XVI	Hurstbridge	Hurstbridge
XVII	Javanica	Javanica

Urinalyses

Urine samples were subjected to various tests to determine different parameters like leukocytes, nitrite, urobilinogen, protein, pH, blood, specific gravity, ketone bodies, bilirubin and glucose using Reagent Strips (10P) (M/S Beacon Diagnostic Pvt. Ltd., Kabilpore, Navsari). In addition, Dark Field Microscopy was performed on urine sediment obtained post filtration (0.45 micrometer pore size filters, Pall life science) and centrifugation at 7800 rpm for 10 minutes to detect the presence of *Leptospira* organism, if any.

To investigate clinicopathological changes if any related details of seropositive and seronegative sheep were compared and here MAT was used as screening test to know status of animal and then animals were grouped into two groups viz. seropositive and seronegative.

Table 2: Seroprevalence of leptospirosis in sheep using MAT

Attributes	Number Tested	Number Positive	Per cent Positive
Region			
South Gujarat	41	5	12.20
Sexwiseseroprevalence			
Male	21	3	14.29
Female	20	2	10.00
Total	41	5	12.20
	$\chi^2 = 0.177^{NS}$		
Breedwiseseroprevalence			
Marwari	13	2	15.38
Patanwadi	20	3	15.00
Non-descript	8	0	0
Total	41	5	12.20
	$\chi^2 = 1.385^{NS}$		
Agewiseseroprevalence			
< 1 year	4	0	**0**
1-3 years	20	1	5
> 3 years	17	4	23.53
Total	**41**	**5**	**12.20**
	$\chi^2 = 3.560^{NS}$		

Note: ^{NS}-Non significant at level P<0.05

Statistical analysis

The hematobiochemical and urinalyses data were analysed by Student's T-Test using Statistical Packages for Social Science (SPSS) software (version 17) whereas seroepidemilogical data were analysed by Chi-square test using Web Argi Stat Package (WASP) software developed by Jangam and Wadekar, Indian Council of Agricultural Research (ICAR), Goa, India (Jangam and Wadekar, 2012). Results were considered to be significant at <0.05.

RESULTS AND DISCUSSION

Out of a total 41 sera examined using MAT, antileptospiral antibodies were found in 5 out of 41 sheep (Table 2). Details of sexwise, breedwise and agewise seroprevalence are presented in Table 2. In MAT, only three serovars i.e. Pomona, Icterohaemorrhagiae and Canicola reacted with sera. Presently 3 sheep reacted with one serovar, 1 reacted with two serovars and 1 reacted with three serovars (Table 3).

Table 3: Details of MAT reaction with single and multiple serovars

Sr. No.	No. of antigen/ Serovars	Name of Serovars	No. of reactive animals
I	One	Pomona	3
II	Two	Pomona and Canicola	1
III	Three	Pomona, Icterohaemorrhagiae and Canicola	1
		Total	5

In present study, 12.20% (5/41) seroprevalence was reported among sheep in south Gujarat which supported the findings of earlier workers from Gujarat (Savalia and Pal, 2008). Similarly serological evidence of leptospirosis, with varied rate among sheep, have also reported by many workers in different states in India (Srivastava and Kumar, 2003; Balakrishnan *et al.,* 2008). Himani *et al.* (2013) observed on the base of works carried out at Indian Veterinary Research Institute among different species of animals during 1975-90, the highest seroprevalence was noted in sheep. Based on the various serological surveys data, author reported that prevalence rate varies from region to region or country to country (Bashirua *et al.,* 2013). This variation may be due to the environmental factors of the region which shown to have effects on development of leptospiral infection in animals (Haji Hajikolaei *et al.,* 2007). Present result pointed out the prevalence of leptospirosis among sheep in South Gujarat and can be correlated with environment factors (temperature, high humidity, alkalinity of soil and high rainfall) of the region which favor growth of leptospires.

Genderwise seropositivity did not reveled statistically significant difference (Table 2). Presently higher seropositivity was noted in male sheep than female sheep, however in contrast to these finding, previous reports indicated higher seropositivity in female sheep (Agunloye, 2002; Bashirua *et al.,* 2013). It was also noted that hardly

any reliable evidence exists to support sex bias in sheep i.e. both sexes have equal sensitivity to leptospirosis Agunloye (2002).

Breedwise seropositivity was found statistically non-significant ($p<0.05$) (Table 2). To the best of author's knowledge, till date breedwise seroprevalence of leptospirosis in sheep in India has not been reported. Patanwadi and Marwari breeds are actually reared at North Gujarat region and Rajasthan state, respectively. But every year during scarcity of fodder, the migratory flocks move towards South Gujarat as this region has lush green grass and bushy areas. So seropositivity in these breeds indicates exposure of animals to contaminated environment and there may be a chance of spreading the disease to humans or other domestic animals in the same or different regions. Thus, control measures are necessary for limiting leptospira infection.

Agewise seropositivity was found statistically non-significant ($p<0.05$) (Table 2). Presently reported higher seropositivity in sheep above 3 years age supported the findings of earlier workers in Iran (Haji Hajikolaei *et al.*, 2007; Hassanpour *et al.*, 2011). Contrary to these, earlier reports indicated higher prevalence in sheep less than 2 years of age (Bashirua *et al.*, 2013) and sheep between 2-3 years of age (Agunloye, 2002). Presently noted higher seropositivity in sheep above 3 years age might be due to increased chances of exposure to contaminated environment as the animal ages.

Presently serovars Pomona, Canicola and Icterohaemorrhagiae were reported in sheep (Table 3). These supported the findings of earlier workers in India (Koteeswaran, 2006; Balakrishnan *et al.*, 2008) and abroad (Haji Hajikolaei *et al.*, 2007; Hassanpour *et al.*, 2011). In addition to this, serovars Pomona, Hardjo, Canicola and Andamana were also reported in sheep of Gujarat state (Savalia and Pal, 2008). The difference in serovars distribution in earlier (Savalia and Pal, 2008) and present report indicate sporadic foci of several serovars exist in this region. Moreover the variation in serovars could be due to frequency of the samples tested or changes in the serovars by the matter of time. So that large scale seroprevalence study and isolation of local isolates would be helpful in developing suitable vaccine to control the disease in this area.

Presently serovar Pomona was reported to be the predominant one. This supported the findings of many workers (Agunloye, 2002; Savalia and Pal, 2008). The predominant leptospiral serovar in serological reaction varies between different countries or regions as reported by many authors (Haji Hajikolaei *et al.*, 2007; Hassanpour *et al.*, 2011; Bashirua *et al.*, 2013). There is a need of extensive surveys for leptospirosis in this endemic region time to time, since host - parasite relationship may change depending on the environmental conditions prevailing at a different time period in the region and susceptibility of the animal species to certain serovar.

Table 4: Prevalence of leptospirosis in clinically ailing and apparently healthy sheep

Sr. No.	Particulars	Total cases	No. of seropositive animals	Serovars reacted
I	Clinically ailing animals	18	4 (22.22 %)	
	Abortion	4	1 (25.00 %)	Pomona and Canicola
	Anorexia	6	1 (16.67 %)	Icterohaemorrhagiae, Pomona and Canicola
	Fever	1	0	—
	Agalactia/ Oligolactia	3	1 (33.33 %)	Pomona
	Mastitis	1	0	—
	Icteric mucous membranes	3	1 (33.33 %)	Pomona
II	Apparently healthy animals	23	**1 (4.35 %)**	Pomona
	Total (1. & 2.)	41	5 (12.20 %)	
			$\chi^2 = 3.018^{NS}$	

Note: ^{NS}-Non significant at level $P<0.05$

The serovar Pomona may be incidental or adapted/ maintained by sheep in this region. As per available literature, sheep is incidental host for Pomona, Canicola and Icterohaemorrhagiae serovars whereas pigs or cattle, dogs and rats are maintenance hosts for serovars Pomona, Canicola and Icterohaemorrhagiae respectively (Radostits *et al.*, 2000). In South Gujarat, among bovine, predominance of serovar Pomona have been reported (Patel *et al.*, 2014). So it may be presumed that Pomona serovar have been transmitted by the infective urine of bovines.

Antibodies against more than one serovar were found in present study. This supported the reports of earlier workers (Haji Hajikolaei *et al.,* 2007; Hassanpour *et al.,* 2011). This may be due to mixed serovar infection or cross reactivity among different serovars.

In seropositive clinically ailing sheep the clinical signs included abortion, anorexia, agalactia or oligolactia and icteric mucous membranes in different combinations and were noted in 25.00, 16.67, 33.33 and 33.33 %, respectively (Table 4). Presently serovars Pomona and Canicola were reported with abortion case and Pomona serovar was reported from oligolactia/agalactia case. Besides the above serovars, Cousin *et al.* (1989) reported serovars Grippotyphosa and Ballum from sheep having reproductive losses. Many workers have reported involvement of serovar Hardjo (Ellis *et al.,* 1986) and Pomona (Davidson and Hirsh, 1980) in cases of abortions/ stillbirths and neonatal/lamb mortalities.

Urinalyses

Among seropositive sheep mean ± SE values of pH and specific gravity were 7.62 ± 0.125 and 1.008 ± 0.001, respectively as compared to seronegative sheep: pH 7.90 ± 0.093 and specific gravity 1.011 ± 0.010. The difference between seropositive and seronegative groups in respect of pH and specific gravity did not differ significantly ($P<0.05$). Other parameters like leukocytes, nitrite, urobilinogen, RBCs, ketone bodies, bilirubin and glucose were found to be negative in seropositive sheep. However, Jamshidi *et al.* (2008) and Sophie Hedberg (2013) noted proteinuria, bilirubinuria, hematuria, pyuria, presence of granular casts and low specific gravity in clinical cases of dogs with severe leptospirosis. Unfortunately we could not locate report on urine analysis among sheep.

Presently, a total 20 urine samples were screened under dark field microscope (DFM) but leptospires could not be detected in any sample. This may be due to discharge of very low number of leptospires in urine samples. This supported the observations made by Shivaraj *et al.* (2009) in India, where they could not detect any leptospires from 60 ovine samples (blood, tissue and urine).

Hematology

Non-significant difference was observed in respect of Hb, PCV, TEC, TLC, MCV, MCH, MCHC and DLC values between seropositive and seronegative sheep (Table 5). Similar finding was reported in experimental induced leptospiral infection in Wistar rats by Tonin *et al.* (2012). Reports on leptospirosis with neutrophilia and leucocytosis in canine (Sophie Hedberg, 2013) and neutrophilia, leucocytosis and decreased MCHC in equine (Melissa *et al.,* 2010) have been documented. Ananda *et al.* (2008) reported higher value of PCV, TLC and lower value of haemoglobin in dog infected with *Leptospira* organism. The recorded minor variations could be due to certain unrelated minor pathological conditions like anemia or day to day variation in physiological status of the animal like estrus, pregnancy, age variation, stress, etc.

Table 5: Details of various hematological parameters studied

Sr. No.	Parameters studied	Seronegative (n= 36)	Seropositive (n= 5)
		Mean ± SE	
I	Hb (gm/dl)	7.66 ± .035	6.84 ± 0.60^{NS}
II	PCV (%)	21.42 ± 0.98	19.40 ± 2.27^{NS}
III	TEC (× 10^6/µl)	7.46 ± 0.27	8.17 ± 0.84^{NS}
IV	TLC (× 10^3/µl)	8.13 ± 0.41	9.56 ± 1.23^{NS}
V	MCV (fl)	30.57 ± 1.94	26.56 ± 7.17^{NS}
VI	MCH (pg)	10.92 ± 0.69	9.13 ± 1.97^{NS}
VII	MCHC (g/dl)	36.02 ± 0.78	35.71 ± 1.61^{NS}
VIII		DLC	
	Neutrophils %	37.63 ± 1.89	29.60 ± 5.74^{NS}
	Lymphocytes %	50.12 ± 2.34	61.0 ± 6.64^{NS}
	Eosinophils %	8.89 ± 1.24	5.60 ± 2.63^{NS}
	Monocytes %	2.52 ± 0.22	3.00 ± 0.44^{NS}
	Basophils %	0.80 ± 0.06	0.80 ± 0.20^{NS}

Note: ^{NS} Non Significant at $P<0.05$as compared to seronegative animals

Biochemistry

Biochemical analyses did not reveled any significantly difference between seropositive (n=5) and seronegative (n=36) groups in respect to ALT, AST, ALP, bilirubin, BUN, creatinine, total protein and albumin values (Table 6). On the same line, Millar *et al.* (1977) could not observe any alteration in liver function in experimentally infected sheep. Contrarily, Tonin *et al.* (2012) reported increased levels of ALP, ALT, urea and creatinine in experimentally

infected Wistar rats. Similar observations were also observed in dogs (Goldstein *et al.* 2006) and horses (Melissa *et al.*, 2010). However minor increase in ALT, AST and total bilirubin activity/value was suggestive of mild liver damage. Among ruminants ALT activity is nonspecific but AST activity is indicative of liver damage. Similarly increase in total bilirubin level and hypoproteinemia also occurs in liver damage. Though these changes indicate mild liver damage, it would be hypothetical to correlate these changes to leptospirosis as a number of nonspecific factors like parasitism, low/poor protein level in feed and hepatic ailment would have been responsible.

Table 6: Details of various biochemical parameters studied

Sr. No.	Parameters studied	Seronegative (n=36)	Seropositive (n=5)
		Mean ± SE	
I	ALT (IU/L)	18.65 ± 2.11	21.32 ± 1.80 ^{NS}
II	AST (IU/L)	65.31 ± 4.05	65.76 ± 10.41 ^{NS}
III	ALP (IU/L)	66.28 ± 7.88	62.38 ± 2.55 ^{NS}
IV	Total Bilirubin (mg/dl)	0.57 ± 0.01	0.66 ± 0.07 ^{NS}
V	BUN (mg/dl)	14.91 ± 0.63	15.59 ± 1.65 ^{NS}
VI	Creatinine (mg/dl)	1.04 ± 0.14	1.06 ± 0.48 ^{NS}
VII	Total Protein (g/dl)	10.03 ± 0.65	6.84 ± 1.10 ^{NS}
VIII	Albumin (g/dl)	2.80 ± 0.11	2.37 ± 0.22 ^{NS}

Note: ^{NS} Non Significant at P<0.05as compared to seronegative animals

In conclusion, the study showed seropositivity in sheep and provided general view on the occurrence of leptospirosis in these species in South Gujarat which indicates that sheep could play a role in the epidemiology of leptospirosis. These findings can be used as baseline data to carry out extensive regional survey. Non-significant clinic-pathological attributes suggest present of leptopsira antibodies without major pathological conditions.

ACKNOWLEDGEMENTS

Authors are very thankful to Professor and Head and In-charge Leptospirosis Reference Laboratory at Government Medical College, Surat and The Director, National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Indian Council of Agricultural Research, formerly Project Directorate on Animal Disease Monitoring and Surveillance (PD-ADMAS), Hebbal, Bengaluru for technical support. We are also thankful to Dr. N. H. Kelawala, Dean & Principal, Vanbandhu College of Veterinary Science & Animal Husbandry, Navsari Agricultural College, Navsari for financial assistance.

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