Seroprevalence of Bovine Leptospirosis in Odisha, India

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Abstract: In this study, a total of 120 non purposive serum samples (Cows-68; Bullocks-32; Bulls-20) randomly collected from different breeds of bovine (Cross breed- 41; Indigenous- 61 and Non-descript-18) in six districts (Bargarh-40; Angul-15, Koraput-22, Boudh-14, Nayagarh-11, Jagatsinghpur-18) of Odisha by field veterinary officers during surveys from April to May 2013 were included. These samples were tested at 1:100 dilution in microscopic agglutination test (MAT) using live antigens of 14 reference leptospiral serovars in order to investigate the seroprevalence of bovine leptospirosis. The overall seroprevalence of 42.5% (51/120=CI: 95% 34.0 to 51.4) with 48.5% in cows, 28.1% in bullocks and 45.0% in bulls was observed. The overall analysis of chi-square test revealed that seroprevalance in bovine are associated with age (χ^2 =5.78, p<0.10), whereas not associated with breed type and health status. Among the targeted districts, high prevalence was observed in Nayagarh (81.8%) followed by Bargarh (47.5%), Jagatsinghpur (44.4 %) and Boudh (42.8%). In general, the prevalence across the sample regions was significant (χ^2 =12.45, p<0.05) indicating the prevalalence was associated with regions. Out of 51 reacted sera, 13 samples showed reactivity with more than one serovars representing 25.5%. The predominant leptospiral antibodies determined against frequency distribution of the serovars were: Australis (50.9%); Hardjo (23.5%); Canicola, Tarassovi and Kaup (7.8%); Pomona, Hurstbridge (5.9%); Bankinang, Javanica, Hebdomadis and Pyrogenes (3.9%); and Icterohaemorrhagiae, Grippotyphosa and Shermani (2.0%). This study supports that bovines may have a role in maintaining Australis serovar apart from being a well known reservoir for Hardjo serovar in Odisha state, India

Keywords: Leptospirosis, Seroprevalence, Odisha, Bovines, MAT.

INTRODUCTION

Leptospirosis is among the fastest re-emerging anthropozoonosis causing considerable public health problems in most of the countries of Asia, Africa and Latin America. The leptospirosis situation in India is a cause of concern and it is endemic in coastal areas including Andaman and Nicobar Islands, where high prevalence was recorded both in animals and humans [1-3]. The disease affects a variety of domestic animals (cattle, buffalo, goat, sheep, horse and swine) resulting in heavy economic losses to the farming community on account of reproductive problems [4]. In cattle, leptospirosis causes abortion, infertility, stillbirths, birth of weak calves, reduced milk yield and productivity [5]. In general, cattle are the maintenance host for serovar Hardjo [6] and besides being an important cause of bovine abortion, reduced fertility and agalactia, it also poses a potential zoonotic threat to humans who are exposed to infected cattle [7].

Knowledge of prevalent leptospiral serovar (s) in incidental host/animals and its association with one or more maintenance host(s) that serves as reservoir of infection in any particular geographical region is essential to understand the epidemiology of leptospirosis. Diagnosis of leptospirosis is usually carried out by microscopic agglutination test (MAT), a gold standard serological test. The MAT is a well proven, accepted and widely used test for the detection of leptospiral antibodies in animals and humans [8]. Significant percentages of animals that are actively infected with *Leptospira* spp. serovar Hardjo are shedding leptospires and have antibody titres $\leq$100 are considered to be sero negative. Due to dynamics of IgM and IgG antibodies in leptospiral infection in humans and animals and the persistence of leptospiral agglutinins titers in human sera, the MAT measures either IgM or IgG, the titres of which generally peak after 10 to 20 days but decline within 6 to 12 months and consequently demonstrates either recent infection or late infection [9-11]. Infection with host-adapted serovars has been reported to produce subclinical infection with apparently healthy animals serving as chronic carriers and persistent excretion or shedding of the organism through their urine, body fluid or tissue. They thereby pose a risk and source of infection to humans (livestock owners, farm workers, occupational workers, etc). The local abundance of several species of pathogenic leptospiraes may be an useful indicator for transmission potential of leptospira to animals and humans. Hence, the present study was carried out to determine the seroprevalence of various leptospiral serovar specific antibodies in bovine population in Odisha, India.

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MATERIALS AND METHODS

Serum Samples

A total of 120 bovine serum samples (Cows-68; Bullocks-32; Bulls-20) randomly collected from different breeds of bovine (Cross breed- 41; Indigenous- 61 and Non-descript-18) in different districts (Bargarh-40; Angul-15, Koraput-22, Boudh-14, Nayagarh-11, Jagatsinghpur-18) of Odisha by field veterinary officers during surveys from April to May 2013 were included (Table **1**). The serum samples collected from the villages/ Ghosalas (are an Indian form of protective shelters for cows) that had the history of abortions and other reproductive problems/disorders. These samples were submitted to Project Directorate on Animal Disease Monitoring and surveillance (PD_ADMAS) for serodiagnosis of Brucellosis and Infectious Bovine Rhinotracheaitis (IBR) and other reproductive animal diseases by AICRP (All India Coordinated Research Project) on ADMAS Centre, Animal Disease Research Institute, Phulnakhara, Cuttack, Odisha. Hence, these samples were used as non purposive samples for screening of bovine leptospirosis.

Leptospira Media and Cultures

Ellinghausen McCullough Johnson and Harris (EMJH) liquid medium prepared as per the standard protocols using the EMJH base and Enrichment media (BD Difco™ USA) with 200 µg of 5-fluorouracil per ml of medium was used for the propagation of leptospira cultures. MAT was performed on all sera using the 14 reference serovars obtained from WHO National reference Laboratory, Regional Medical Research Centre (RMRC), Port Blair, India (Table **2**). The serovars selected cause disease in animals or may be of use as sentinel serovars to measure the potential spread and prevalence.

Microscopic Agglutination Test (MAT)

Leptospira live antigens required for MAT were grown in EMJH medium in Leptospira Laboratory, PD_ADMAS, Bangalore. For this purpose, serovars were incubated at 30°C for 7 days in EMJH medium. Five to seven-day-old cultures at a concentration of 1-2x10^8 organisms/ml were used as live antigen for MAT. The test was performed as per OIE procedures and sera were tested for the presence of specific antibodies against leptospira by MAT as described earlier [12, 13].

First to determine positive reactor, all serum samples were initially diluted 1:50 {100µl of the serum sample in 4.9 ml of Phosphate buffered saline (PBS-pH 7.4)}. After that, 100µl of 1:50 diluted serum samples was dispensed into the 96 wells of the U bottom microtitre plates, and the same amount of live antigen was added (final dilution is 1:100). This procedure was performed with 14 different serovars separately. After incubation at 30°C for 2-3 h, a loopful of each mixture was examined under dark field microscope for the presence of agglutination and lysis. Negative and

Table 1: Details of the Samples Collected During Survey in Bovine Population

District/Area/Human Population*/ Forest area/ Average Rainfall/ Bovine population#	Village	Cows	Bulls	Bullocks	Total
Bargrh/ 5,832 Km²/ 1481,255/ 1,216.13Km^2/1,527 mm/899,253	Bargarh	33	7	-	40
Angul/6,232 Km^2/ 1481,255/ 2,909.542 Km^2/1,421 mm/714,380	Nilapasi	3	3	9	15
Nayagarh (coastal)/ 3,890 Km^2/ 962,789/ 2,080.97Km^2/ 1,449.1 mm/ 395,412	Patharchakada	5	5	1	11
Koraput/ 8,379 Km^2/ 1,180,637/16,131 Km^2/ 1,522 mm/823,844	Kadaamba	1	-	1	2
	Tukum	2	-	2	4
	Nandigaon	3	1	12	16
Jagatsinghpur (coastal)/ 1,759 Km^2/ 1136,971/132.92 Km^2/1,501.3 mm/312,924	Bamara	14	4	-	18
Boudh/ 3,098 km^2/ 441,162/ 1,277 km^2/ 1,623.1mm/284,717	Lambakani	7	-	7	14
Total		68	20	32	120

*****2011 census; Office of the registrar general & census; Ministry of Home Affairs, Government of India (http://censusindia.gov.in).
#18th livestock census 2007; Department of Animal Husbandry, Dairying and Fishery, Ministry of Agriculture, Government of India (http://www.dahd.nic.in).

Table 2: Panel of Leptospiral Reference Serovars Used in the Microscopic Agglutination Test

Species	Serovar	Strain	Serogroup
L. interrogans	Australis	Ballico	Australis
L. interrogans	Bankinang	Bankinang 1	Autumnalis
L. interrogans	Canicola	Hond Utrech IV	Canicola
L. kirschneri	Grippotyphosa	MoskvaV	Grippotyphosa
L. interrogans	Hardjo	Hardjoprajitno	Sejroe
L. interrogans	Hebdomadis	Hebdomadis	Hebdomadis
L. fainei	Hurstbridge	BUT 6	Hurstbridge
L. interrogans	Icterohaemorrhagiae	RGA	Icterohaemorrhagiae
L. brogpetersenii	Javanica	Poi	Javanica
L. inadai	Kaup	LT-64-68	Tarassovi
L. interrogans	Pomona	Pomona	Pomona
L. santarosai	Shermani	1342K	Shermani
L. interrogans	Pyrogenes	Salinem	Pyrogenes
L. borgpetersenii	Tarassovi	Perepelicin	Tarassovi

positive sera determined previously by MAT were included in each plate as controls.

Serum sample causing 50% of leptospires to agglutinate and /or lyse were considered as positive reactor. If the serum sample agglutinate the leptospires between 50-75% and > 75%, then it is considered as moderate and strong positive reactors, respectively. Each serum sample which gave positive reaction was further examined by diluting it 2-fold starting from 1:100 to 1:3200 in order to determine the end point MAT antibody titres. For this, the diluted 1:100 serum sample was dispensed in the first well and a serial dilution (1:100, 1:200, 1:400, 1:800, 1:1600, to 1:3200) was made using PBS and test was performed as described earlier. The end point titre is the highest titre in which 50% agglutination was occurred with the live antigen serovar(s). A MAT titre of 1:100 or above is taken as positive reactor as per WHO/OIE manual for leptospirosis [8, 14].

Statistical Analysis

The estimation of apparent prevalence with 95% confidence interval and data analysis were carried out using Statistical Analysis System (SAS) version 9.2 package (SAS India Ltd., Mumbai). Chi-square test was used as per standard statistical method [15] for testing the association of seroprevalence of leptospira across age groups, breed and across the sample regions for statistical inference to determine significant difference.

RESULTS AND DISCUSSION

Leptospirosis has been known to occur in India since early parts of the 20th century. The disease is common, in cattle in virtually all the states of India [4]. Many leptospirosis cases in animals and humans in India have been reported in the rainy season and after flooding and heavy rainfall. Seasonal outbreak has also been reported in coastal areas especially in Southern Peninsular region, Maharashtra, Gujarat, Odisha and Andaman Nicobar Islands. Knowledge of the prevailing serovars is important for understanding the epidemiology of leptospirosis and establishing public health policies aimed at its prompt diagnosis and control measures. Studies of bovine leptospirosis in different parts of the world indicate that serovars responsible for reproductive losses vary depending on type of serovars that are locally endemic. Since leptospiral antibodies may be present in the serum for a considerable period of time after infection, the seroreactivity may indicate the present or past exposure to leptospiral antigens. Since MAT is relatively serovar / serogroup specific test, it is a choice for seroepidemiologic studies.

In this study, all the serum samples were tested at 1:100 using 14 reference serovars initially. The test results of serum samples age-, breed-, and district-wise are shown in the Table **3**. Out of a total 120 sera tested, 51 samples at 1:100 dilution reacted in MAT representing the 42.5% seroprevalence. Among these

Table 3: Statistical Analysis of Results of Samples Screened for Prevalence of Bovine Leptospirosis

Species/ Age	Cow				Bull				Bullock				Over all			
Years	T	R	P	CI	T	R	P	CI	T	R	P	CI	T	R	P	CI
Age wise analysis																
< 2	12	9	75.0	46.7 to 91.1	13	6	46.2	23.2 to 70.8	-	-	-	-	25	15	60.0	40.7 to 76.6
3 to 5	21	11	52.4	32.3 to 71.6	6	2	33.3	9.6 to 70.0	7	3	42.9	15.8 to 74.9	34	16	47.0	31.5 to 63.3
> 5	35	13	37.1	23.1 to 53.6	1	1	100	21.6 to 100.0	25	6	24.0	11.5 to 43.3	61	20	32.7	22.3 to 45.3
Total	68	33	48.5	37.1 to 60.1	20	9	45.0	25.8 to 65.7	32	9	28.1	15.5 to 45.3	120	51	42.5	34.0 to 51.4
χ^2 Value	5.31*				1.66^{NS}				0.96^{NS}				5.78*			
Breed wise analysis																
Cross breed	35	18	51.4	35.5 to 67.0	6	3	50.0	18.7 to 81.2	-	-	-	-	41	21	51.2	36.5 to 65.8
Indigenous	19	9	47.4	27.3 to 68.3	10	4	40.0	16.8 to 68.7	32	9	28.1	15.5 to 45.3	61	22	36.0	25.1 to 48.6
Non descript	14	6	42.9	21.4 to 67.4	4	2	50.0	15.0 to 85.0	-	-	-	-	18	8	44.4	24.6 to 66.3
Total	68	33	48.5	37.1 to 60.2	20	9	45.0	25.8 to 68.7	32	9	28.1	15.5 to 45.3	120	51	42.5	34.0 to 51.4
χ^2 Value	0.42 ^{NS}				0.20 ^{NS}								2.34 ^{NS}			
Districts																
Bargarh	33	16	48.5	32.5 to 64.7	7	3	42.9	15.8 to 74.9	-	-	-	-	40	19	47.5	32.9 to 62.5
Angul	3	2	66.7	20.7 to 93.8	3	1	33.3	6.1 to 79.2	9	1	11.1	1.9 to 43.5	15	4	26.7	10.9 to 51.9
Nayagarh	5	5	100.0	56.5 to 100.0	5	3	60.0	23.0 to 88.2	1	1	100.0	20.6 to 100.0	11	9	81.8	52.3 to 94.8
Koraput	6	2	33.3	9.6 to 70.0	1	0	0.00	0.00 to 79.3	15	3	20.0	7.0 to 45.1	22	5	22.7	10.1 to 43.4
Jgatsinghpur	14	6	42.9	21.3 to 67.4	4	2	50.0	15.0 to 85.0	-	-	-	-	18	8	44.4	24.6 to 66.2
Boudh	7	2	28.6	8.2 to 64.1	-	-	-	-	7	4	57.1	25.0 to 84.1	14	6	42.8	21.4 to 67.4
Total	68	33	48.5	37.0 to 60.1	20	9	45.0	25.8 to 65.7	32	9	28.1	15.6 to 45.3	120	51	42.5	34.0 to 51.4
χ^2 Value	7.55 ^{NS}				1.49 ^{NS}				7.24*				12.45**			

T-number of serum samples tested; R- positive reactor in Microscopic Agglutination Test (MAT); CI- Confidence interval at 95% level; P-Per cent seroprevalence (%);** and * significant at 5% and10%; NS: not significant.

51 reactors, 19 and 8 samples were found to be moderate and strong positive reactors, respectively. A MAT titre of ≥ 1:100 (WHO/OIE recommenced titre) was taken as positive [14], which indicates the animals either previously exposed to the leptospira or in the carrier status or suffering from the disease. However, due to high endemicity of the disease in Odisha, in order to know the recently infected animals, next dilution of the MAT titre (ie. ≥ 1:200) was considered for reactor. At this titre (1:200), there were 13 samples reacted and were considered or might represented the recently infected animals. The remaining 38 sera showed only the titre of 1:100, which indicates that these animals might have been exposed to infection in past and in the chronic carrier stage. Because of absence of paired serum samples and definite clinical signs pertaining to leptospirosis in bovines, the highly positive reactors of 1:400 (n=13); 1:800 (n=5); 1:1600 (n=3) and 1:3200 (n=1), clearly conclude the active recent infection [14, 8].

Antibodies against different serovars have been reported from Karnataka (4.6%) [16], Andaman (24.2%), Tamil Nadu (51.4%), Andhra Pradesh (10.5) {Cattle-12.2% and buffaloes (11.1%) mainly to Patoc, Icterohaemorrhagiae [2]}, Maharashtra (7.3%) and Uttar Pradesh (4-8%) during surveys [2]. Earlier investigations conducted in India have revealed that since 1995 the various percentage of seroprevalence

of leptospirosis in different species (5.4% in buffaloes, 7.5% in cattle, 12.5% in sheep, 14.6% in horses and 15.9% in dogs) in various states [4].

In this study, among the bovines, the seroprevelence of 48.5% in cows (33/68), 28.1% in bullocks (9/32) and 45% in bulls (9/20) was observed. This high seroprevalence may be due to abortion or other reproductive disorders prevailing in the bovine population in the sampled village/Ghosalas. In general, infected animals are reported to shed the organism in their urine, aborted material discharge, body fluid and tissues. The possibility therefore exists that these apparently healthy seropositive animals may be shedding leptospira and they serve as source of infection to other animals and humans.

It is evident from the Table **3** that the seroprevalance of leptospira in cows is associated with age (χ^2 = 5.31, p<0.10), where as it is not associated with age in bulls (χ^2 = 1.66, p>0.10), and bullocks (χ^2 = 0.96, p>0.10). The results showed that seroprevalance of leptospira in cows (χ^2 =0.42, p >0.10), and bulls (χ^2 =0.20, p >0.10) are not associated with the breed. The prevalence in bullocks was significant (χ^2 = 7.24, p<0.10) across the sample regions implying the prevalance was associated with the regions whereas, it was not significant for bulls (χ^2 = 1.49, p>0.10) and cows (χ^2 = 7.55, p>0.10) indicating non association of prevalence with regions for these animals. This significance in bullocks may be due to the fact that these animals might have been used for ploughing in paddy (rice) fields in particular region.

Among two coastal areas, Nayagarh district showed the highest prevalence of 81.8% (9/11) followed by Jagatsinghpur 44.4% (8/18). Among the non-coastal districts, Bargarh showed the highest prevalence with 47.5% (19/40) followed by Boudh 42.8% (6/14), Angul 26.7% (4/15) and Koraput 22.7% (5/22). The most prevalent serovars among the reactive samples were Australis (50.9%) and Hardjo (23.5%). However, 13 samples from 51 reactive sera showed reaction with multiple serovars representing 25.5% prevalence rate, of which the samples have shown to reacted with Australis (n=7) and Hardjo (n=6). Only three sera showed highest antibody titers of 1:1600 against Australis serovars. At higher dilution (≥1:200) the reacting of the positive serum samples with Australis and Hardjo serovars remain same, which may be considered as highly infective serovars prevalent in that particular geographical location. The leptospiral antibodies determined by frequency distribution of the serovars are summarized in Table **4**. Cow calf showed exposure to Australis and Grippotyphosa while Bull calf exposed to Australis, Canicola, Hardjo, Icterohaemorragiae, Pomona, Kaup, Tarassovi and Hursbridge serovars.

It is well known fact that, Hardjo serovar is common in cattle [17] and the present study also showed similar results. In addition to this, the prevalence of serovar

Table 4: Frequency Distribution of the Leptospira Serovars

Serovars	No. of samples reacted (at 1:100 dilution)	% Positive against the total No. of samples tested	% Frequency against the total No. of positives
Australis	26	21.67	50.9
Bankinang	2	1.67	3.9
Canicola	4	3.33	7.8
Grippotyphosa	1	0.83	2.0
Hardjo	12	10.00	23.5
Hebdomadis	2	1.67	3.9
Hurstbridge	3	2.50	5.9
Icterohaemorrhagiae	1	0.83	2.0
Javanica	2	1.67	3.9
Kaup	4	3.33	7.8
Pomona	3	2.50	5.9
Shermani	1	2.00	2.0
Pyrogenes	2	3.90	3.9
Tarassovi	4	7.80	7.8

Australis was highest (50.9%) among the serovars tested, indicating the importance of this serovar in bovine as reported earlier in Maharashtra state [18]. However, the high antibody titres against these two serovars indicate that the population is affected recently with these infective serovars. Although direct transmission through skin abrasions occurs frequently, it is evident from the habitat of bovines that water bodies or contaminated environment for drinking or wallowing or washing, plays an important role in its survival and spread. Subsequently the animals might have been exposed to the leptospira through contaminated urine of other maintenance or reservoir animals like rodents or other infected animals shedding the leptospira in the water bodies. Similarly, Shivraj *et al.* [19] reported presence of antibodies against *Leptospira* serovar Hardjo and Australis in bovine sera which might be due to the cattle grazing in nearby areas of the water bodies.

In general, the major reactive serovars in these regions were Australis, Hardjo, Canicola, Tarassovi, Kaup. The reactive *Leptospira* intermediate species serovars namely Kaup (n=4), Tarassovi (n=4), Hurstbridge (n=3) may of significance in sero-epidemiology, as the prevalence of these *Leptospira* intermediate species has been recently reported in India [20]. However, the most of the other serovars reacted samples also showed multiple reactions with either of the foresaid major serovars. Hence, further systematic purposive random screening of the samples from different regions of Odisha will determine the exact prevalence of major serovars. It will enable to select the required or prevalent panel of serovars to be used for MAT in the diagnostic laboratory for providing early diagnosis in humans as well as in animals and to undertake prompt treatment to reduce the extent of problem caused by the leptospira and the economical losses associated with it in animals.

In conclusion, high prevalence of bovine leptospirosis in Odisha indicates its significance as endemic state. Its prevalence in apparently healthy bovine indicates the presence of this agent in the environment, which may be a potential zoonotic risk to humans. This study also determines the need for continuous monitoring of leptospira burden in animals and humans in close proximity to each other to combat this zoonotic infection.

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