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Molecular detection of leptospirosis in captive sloth bears (*Melursus ursinus*)

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Abstract
Leptospirosis is a zoonotic disease of domestic and wild mammals that poses a great threat to the public and personnel involved in their management. Knowledge of prevalence of Leptospira in wild and captive carnivores like sloth bears supports the implementation of effective control programmes and thus reduces the transmission risks. The present study aims to screen serologically for the evidence of antibodies against leptospirosis using Microscopic Agglutination Test (MAT) and Latex Agglutination Test (LAT) using LipL32 antigen. Fifteen serum samples were collected from captive sloth bears maintained in Nehru Zoological Park, Hyderabad, irrespective of sex and age were subject to testing. Antileptospiral agglutinins were detected in 3 bears by MAT and 4 bears LAT out of the 15 samples. Results from this study revealed that the predominant serogroups were Leptospira interogans serovar Pomona and serovar Pyrogenes. A titre value of 1:200 for serovar Pomona and a titre value of 1:100 for serovar Pyrogenes were recorded in this study. Further studies using larger number of samples from wild fauna and are required, in order to obtain the detailed information on the incidence of leptospirosis.

Keywords: Sloth bear, leptospirosis, microscopic agglutination test, latex agglutination test

1. Introduction
Bears are large, impressive, extremely popular zoo animals and the subject of considerable folklore [1]. Among them sloth bears (*Melursus ursinus*) are widely distributed on the Indian subcontinent and on the Islands of Sri Lanka [2]. The health and disease related information in captive sloth bears is still in a state of infancy in India. Understanding the health related parameters and evidence of diseases in captive-reared bears will be significantly helpful towards enriching the management of captive bears in India. Here, attempts were also made to rule out specific disease of leptospirosis in sloth bears using molecular techniques.

Leptospirosis is a zoonotic disease of domestic and wild mammals that poses a great threat to the public and personnel involved in their management. The disease is caused by serovars belonging to the spirochete Leptospira interrogans. The organisms are maintained in nature by chronic renal infection of carrier mammals, which excrete the organisms in their urine [3]. Humans become infected through direct exposure to infected animals or their urine or through indirect contact via contaminated water or soil [4]. Leptospirosis has been recognized as an emerging infectious disease and timely diagnosis of leptospirosis is essential because prompt and specific treatment, as early as possible, is important in ensuring a favourable clinical outcome.

Very little information is, however available on leptospirosis in ursids although this disease has been extensively studied in other species of carnivores. Forty two European brown bears from both captive and free living animals in Croatia by microscopic agglutination test were screened and concluded that the prevalence of antibodies in captive bears were higher than in free-living and free born animals [5]. In captivity three bear cubs tested positive for leptospiral antibodies and all the three had antibody titres to *Copenhageni* ranging from 1:100 to 1:3,200 and 2 of the 3 bears had titres to *Mankarso* ranging from 1:200 to 1:1,600 [6]. The current study aims to screen serologically for the evidence of antibodies against leptospirosis using Microscopic agglutination test (MAT) [5, 7] and Latex agglutination test (LAT) [8].

2. Materials and Methods
A total of 15 blood samples were collected from captive sloth bears maintained in Nehru Zoological Park, Hyderabad, irrespective of sex and age.

Collection was done after restraining the animal with the help of chemical immobilization using the combination of ketamine hydrochloride and xylazine hydrochloride at the dose rate of 5 mg/kg body weight and 2 mg/kg body weight, respectively using a blow pipe. Serum was separated by centrifugation at 2000 rpm for 20 min and stored at -20 °C until used. The stored sera samples from captive sloth bears were screened for antibodies against Leptospirosis using Microscopic agglutination test (MAT) and Latex agglutination test (LAT).

2.1 Microscopic agglutination test (MAT)

The leptospiral cutures of 5-8 days old, having concentration of ~2 X 10^8 leptospires/ml were used in microscope agglutination test (MAT). A panel of 6 *Leptospira interrogans* serovars Hebdomadis, Hardjo, Javanica, Pomona, Icterohaemorrhagiae and Pyrogenes were used for microscope agglutination test (MAT). Microscopic agglutination test was conducted as per the method of Cole *et al.* [9] with modifications as per Govindarajan *et al*. [5]. The test was conducted in microtitre plates. In the initial screening phase, the serum, samples were diluted to 1:50 and 100 µl volume was added in a 96 well "U" bottom plate and an equal volume of cultures in the panel were mixed. The plates were incubated for 2 hrs at 37 °C. Samples showing more than 50% agglutination were considered as positive serum samples. Those found positive in the initial screening using six leptospiral strains were further subjected to quantitative MAT to determine the titre against that particular strain of leptospira. The serum samples (50µl) in 1/50 dilution, were serially two fold diluted in phosphate buffered saline (PBS) starting from 1:100 to 1:1,600 and an equal volume of the reacting leptospiral culture was added to all the wells and incubated for 2 hrs. Ten µl of the mixture of antigen and serum were placed on a clean grease free slide and examined under dark field microscope. The end titre was assessed as reciprocal dilution of serum which gave 50% agglutination or reduction in the concentration of leptospires in comparison with the respective negative control.

2.2 Latex agglutination test (LAT)

Latex beads, 0.08 µm (Sigma, USA) were sensitized with recombinant LipL32 antigen. Latex bead suspension (10%) was washed twice by centrifugation at 6700 X g for 3 min each time in carbonate – bicarbonate buffer (pH 9.6). Finally, the latex beads were made into 2% suspension with carbonate- bicarbonate buffer, which was later mixed with an equal volume of recombinant LipL32 antigen diluted in the same buffer. The mixture was incubated at 37 °C for 6 hrs with constant shaking at 200 rpm. The sensitized beads were centrifuged for 3 min and the pellet was resuspended as a 2% suspension in phosphate buffered saline (PBS) containing 5mg/ml (w/v) of bovine serum albumin (BSA). The latex beads were left at 37 °C in a water bath overnight. Finally, the beads were centrifuged as before and the pellet was resuspended in PBS containing 0.5 mg/ml BSA and 0.1% sodium azide as a 0.5% suspension. Sensitized latex beads were stored at 4 °C until use.

The LAT was performed on a 12-well glass slide by mixing equal volumes (20µl) of serum sample and sensitized latex beads. The slide was rocked gently for 2-5 min. PBS and normal rabbit serum were used as negative controls and rabbit-leptospiral hyperimmune sera was used as a positive control. In the latex agglutination test the positive results were read on the scale of +1 to +4 depending on the extent of agglutination and the time taken for the development of agglutination. The serum samples were considered negative if there was no agglutination within 5min. The test was conducted as per the methods of Dey *et al.* [10].

3. Results

The study revealed that amongst 15 sloth bears, antileptospiral agglutinins were detected in 3 bears by MAT and 4 bears LAT out of the 15 samples. Results from this study revealed that the predominant serogroups were Leptospira interogans serovar Pomona and serovar Pyrogenes.

3.1 Microscopic agglutination test

The study revealed that among 15 sloth bears, *Leptospira interrogans* serovar Pomona antibodies were observed in two sera with titre value of 1:200 and the *Leptospira interrogans* serovar Pyrogenes antibodies were present in one sera with the titre value of 1:100 (Table 1).

Table 1: Level of leptospiral antibody titre in sera of sloth bears by microscopic agglutination (MAT) test

Sl. No	Positive sera number	Positive titre value	
		Pomona	Pyrogenes
1	05	-	100
2	10	200	-
3	15	200	-

3.2 Latex agglutination test

Out of the 15 samples, 4 samples were positive for leptospirosis. On an agglutination grading scale of +1 to + 4, 2 samples gave a reading of +2 and 2 others gave a reading of +1 (Table 2)

Table 2: Latex agglutination test (LAT) grading using recombinant LipL 32 antigen in sloth bear positive samples

Positive sera number	Grade *			
	+1	+2	+3	+4
05	-	✓	-	-
10	✓	-	-	-
11	-	✓	-	-
15	✓	-	-	-

* +1, mild agglutination in 5 min, +2, moderate agglutination in 3 min, +3, heavy agglutination in 2-3 min, +4, very heavy agglutination in 1 min.

4. Discussions

Leptospirosis is a bacterial zoonosis affecting domestic and wild animals as well as humans.Carnivores are at the top of the feeding chain, thus being exposed to pathogens through their preys [11]. The occurrence of leptospirosis among sloth bears was in agreement with Wallach and Boever [12] who reported leptospirosis as one of the major infectious disease in ursids. Results showed, 2 samples were positive for serovar Pomona which was supported by Fairly *et al.* [13] who reported *L. interrogans* serovar Pomona in red deer calves, Mikaelian [14] in racoons and Shophet [15] in cats. In this study, serovar Pyrogenes was recorded in a sloth bear whereas Govindarajan *et al.* [7] recorded serovar Pyrogenes from dog. However, Modric and Huber [5]. Observed the serovars Austraulis, Sejroe, Canicola and Icterohaemorrhagiae in European brown bears.

In this study a titre value of 1: 200 for serovar Pomona and a titre value of 1:100 for serovar Pyrogenes were recorded. The

MAT titre of 1:100 and above could be considered to be of diagnostic significance and this was supported by Modric and Huber [5], Smits *et al.* [16] and Dey *et al.,* [10] that MAT was more specific for serovar identification. The MAT was a difficult test to perform since it required live cultures and technical skill.

The highest titre found in this study of sloth bears were 1 : 200 and this was suggestive of early infection, since Fairly *et al.* [12] reported that the titre of 1:200 for serovar Hardjo in red deer calves were due to early infection. Normally paired sera samples are required to assess the infectious status of the animals. Since, single sera samples were collected and analysed by MAT the disease status could not be determined.

In Latex agglutination test (LAT) using LipL 32 revealed out of 15 samples, 4 samples were found positive for leptospirosis. Smits *et al*. [16] reported that the latex agglutination test had good sensitivity, specificity and acceptable predictive values. Here, the results were concordant with that of the MAT. This finding was supported by Dey *et al.* [10] who reported that the LAT was sensitive, specific and accurate when compared to the standard microscopic agglutination test.

In this study, the results indicated that serovar Pomona and serovar Pyrogenes were the most prevalent among sloth bears. At the time of sampling none of the animals showed any signs of the disease. The source of infection in sloth bears was not determined, but could be other bears or free living rats acting as reservoirs. This was in agreement with Levett [4] who also stated that rodents could be the reservoir host for leptospiral serogroups. Adinarayanan and James [17] reported that the climate, rainfall, topography and other factors might be the cause for the spread of leptospirosis.

However, mere detection of antibodies alone in this study cannot be considered as confirmation of the infection and further studies using larger number of samples from wild fauna, isolation of leptospires, disease ecology are required, in order to obtain the detailed information on the incidence of leptospirosis.

5. Conclusion

In this study, out of the 15 serum samples investigated for leptospirosis, 3 samples were positive for the presence of *L. interrogans* serovar Pomona and Pyrogenes using microscopic agglutination test and 4 were positive using latex agglutination test. Even though microscopic agglutination test and latex agglutination test showed similar results, latex agglutination test made rapidity and simplicity and also more suitable as a rapid screening test. At the time of sampling none of the animals showed any signs of the disease. The source of infection in sloth bears was not determined, but could be other bears or free living rats acting as reservoirs. However further studies using larger number of samples from wild fauna, isolation of leptospires, disease ecology are required, in order to obtain the detailed information on the incidence of leptospirosis.

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