

## Serological investigation and PCR in detection of pathogenic leptospires in snakes<sup>1</sup>

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**ABSTRACT.**- Biscola N.P., Fornazari F., Saad E., Richini-Pereira V.B., Campagner M.V., Langoni H., Barraviera B. & Ferreira Junior R.S. 2011. **Serological investigation and PCR in detection of pathogenic leptospires in snakes.** *Pesquisa Veterinária Brasileira* 31(9):806-811. Centro de Estudos de Venenos e Animais Peçonhentos, Universidade Estadual Paulista, Fazenda Experimental Lageado, Rua José Barbosa de Barros 1780, Botucatu, SP 18610-307, Brazil. E-mail: [rseabra@cevap.org.br](mailto:rseabra@cevap.org.br)

Detection of *Leptospira* by PCR had not yet been described in snakes. This study investigated, by microscopic agglutination test (MAT) and PCR, the presence of antibodies to *Leptospira* spp. and *Leptospira* spp., respectively, in venomous and non-venomous wildlife and captivity snakes. All snakes were divided into three groups to be compared: Group 1 (wildlife snakes - WS); Group 2 (snakes in intensive captivity - IC), and Group 3 (collective semi-extensive captivity -CC). Of the 147 snakes studied, 52 (35.4%) were positive for leptospirosis by MAT, 8 (15.4%) belonging to Group 1 (WS), 34 (65.4%) to Group 2 (IC) and 10 (19.2%) to Group 3 (CC). Jararaca (*Bothrops jararaca*) presented the highest average titer (66.7%, N=22/33) among the three group studied, and Hardjo prajitino was the most prevalent serovar (88.5%, N=46/52), with titers varying from 100 to 3200. *Leptospira interrogans* was revealed by PCR in kidney and liver of caíçaca (*Bothrops moojeni*) and jararaca-pintada (*Bothrops pauloensis*), showing 100% and 93% identity respectively. Future studies should be carried out for better understanding of the role of snakes as a reservoir of *Leptospira* in nature.

INDEX TERMS: PCR, MAT, *Leptospira* spp., snakes, *Bothrops jararaca*, *Bothrops moojeni*, Hardjo prajitino.

**RESUMO.- [Investigação sorológica e PCR na detecção de leptospiros patogênicas em serpentes.]** A detecção de *Leptospira* pela técnica de PCR não havia sido descrita em serpentes. Este estudo investigou pelo teste de aglutinação microscópica (MAT) e PCR, a presença de anticorpos anti-*Leptospira* spp. e *Leptospira* spp., respectivamente, em serpentes peçonhentas e não peçonhentas de vida livre e de cativeiro. As serpentes foram divididas em três grupos para comparação: Grupo 1 (serpentes recém-chegadas da natureza - WS); Grupo 2 (serpentes em regime de cativeiro intensivo - IC) e Grupo 3 (serpentes em regime de cativeiro coletivo semi-

extensivo - CC). Do total de 147 serpentes estudadas, 52 (35,4%) foram positivas para leptospirose pelo MAT, as quais 8 (15,4%) pertenciam ao Grupo 1 (WS), 34 (65,4%) ao Grupo 2 (IC) e 10 (19,2%) ao Grupo 3 (CC). Das espécies estudadas, a jararaca (*Bothrops jararaca*) apresentou maior soropositividade (66,7%, N=22/33). O sorovar mais prevalente foi o Hardjo prajitino (88,5%, N=46/52) e os títulos variaram de 100 a 3200. *Leptospira interrogans* foi revelada por PCR nos rins e no fígado de caíçaca (*Bothrops moojeni*) e de jararaca-pintada (*Bothrops pauloensis*), mostrando 100% e 93% de identidade, respectivamente. Futuros estudos devem ser realizados para melhor compreensão do papel das serpentes como reservatório de leptospiros na natureza.

TERMOS DE INDEXAÇÃO: PCR, MAT, *Leptospira* spp., serpentes, *Bothrops jararaca*, *Bothrops moojeni*, Hardjo prajitino.

### INTRODUCTION

Leptospirosis is a worldwide zoonotic disease with high occurrence in tropical countries. Rats are considered the most significant reservoirs. In tropical countries other animals may

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also be important sources of infection, such as cattle, horses, swine, dogs and wildlife (Weekes et al. 1997, Blancou 2005, Aguiar et al. 2006, Ghneim et al. 2007, Hashimoto et al. 2007). Among wildlife animals, venomous and non-venomous snakes may be considered natural reservoirs, mainly due to their diet, which is based on rodents (Hyakutake et al. 1980).

In Brazil, *Leptospira* in snakes were first investigated in 1976 by Hyakutake et al. that verified remarkable predominance of serovar Andamana (80.6%). Abdulla & Karstad (1962) showed that captive snakes may acquire leptospirosis due to their food and water intake and also by direct contact with each other.

*Leptospira* is detectable through urine or tissues by culture, dark field microscopy, immuno-staining or polymerase chain reaction (PCR) (Adler and Moctezuma 2010). The most widely used microscopic agglutination test (MAT) has the advantage of being specific for serovars and serogroups. Several PCR protocols for detection of *Leptospira* DNA in clinical material have been developed since the 1990s and most of them reported a high sensitivity. Compared with dark field microscopy and immuno-staining, polymerase chain reaction (PCR) has several advantages as rapidity, simplicity and low-cost (Romero & Yasuda 2006, Adler & Moctezuma 2010). However, no protocol for *Leptospira* detection in snakes by PCR has been described up to this moment.

The aim of this study was to investigate the presence of *Leptospira* spp. and *Leptospira* spp. antibodies in venomous and non-venomous snakes using MAT and PCR methods to understand the role of wildlife and captive snakes as reservoirs in nature.

**MATERIALS AND METHODS**

Blood samples (n=147) were collected from the caudal vein of 127 venomous and 20 non-venomous snakes distributed into three groups: Group 1 (G1): 35 wildlife snakes (WS) including six jararacas (*Bothrops jararaca*), three jararacas-pintada (*Bothrops pauloensis*), 20 cascavéis (*Crotalus durissus terrificus*), four jibóias (*Boa constrictor amarali*), one jararaca-da-seca (*Bothrops leucurus*), and one caíçaca (*Bothrops moojeni*). Group 2 (G2): 64 snakes in intensive captivity (IC) kept in individual plastic boxes adapted to their size under controlled temperature and humidity. The boxes were cleaned daily, water was given “ad libitum” and the snakes were fed with one mouse every 30 days. This group comprised 15 caíçacas (*Bothrops moojeni*), 15 jararacas (*Bothrops jararaca*), 14 jararacas-pintada (*Bothrops pauloensis*) and 20 cascavéis (*Crotalus durissus terrificus*). Group 3 (G3): 48 snakes kept in semi-extensive collective captivity (CC) in heated stalls with running water and solarium. This group comprised 12 jibóias (*Boa constrictor amarali*), 12 jararacas (*Bothrops jararaca*), 20 cascavéis (*Crotalus durissus terrificus*) and four caninanas (*Spilotes pullatus*). The G2 and G3 snakes were maintained in captivity for more than one year until tests were carried out.

Blood samples were collected and centrifuged at 1000G for 10 min. Serum was stored in 1.5 mL microtubes at -20°C for batch testing. This study was developed after receiving authorization from the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) and Animal Research Ethics Committee (CEEA) number 778/2010.

Samples were analyzed using MAT as recommended by the World Organization for Animal Health Animal (OIE) (Faine 1982; Office World Organization for Animal Health Animal 2009). Samples

**Table 1. Species, serogroups and respective serological variants for *Leptospira* diagnosis using MAT**

| Species                          | Serogroup           | Serovar                         | Strain                       |
|----------------------------------|---------------------|---------------------------------|------------------------------|
| <i>Leptospira interrogans</i>    | Australis           | Australis                       | Ballico                      |
| <i>Leptospira interrogans</i>    | Autumnalis          | Bratislava                      | Jez-bratislava               |
| <i>Leptospira interrogans</i>    |                     | Autumnalis                      | Akiyami A                    |
| <i>Leptospira kirshneri</i>      | Ballum              | Butembo                         | Butembo                      |
| <i>Leptospira interrogans</i>    |                     | Castellonis                     | Castellón                    |
| <i>Leptospira interrogans</i>    | Bataviae            | Bataviae                        | Van Tienen                   |
| <i>Leptospira santarosai</i>     |                     | Brasiliensis                    | An 776                       |
| <i>Leptospira interrogans</i>    | Canicola            | Canicola                        | Hond Utrecht IV              |
| <i>Leptospira weilli</i>         | Celledoni           | Whitcombi                       | Whitcombi                    |
| <i>Leptospira kirshneri</i>      | Cynopteri           | Cynopteri                       | 3552 C                       |
| <i>Leptospira interrogans</i>    | Djasiman            | Djasiman                        | Djasiman                     |
| <i>Leptospira kirshneri</i>      |                     | Sentot                          | Sentot                       |
| <i>Leptospira interrogans</i>    | Grippotyphosa       | Grippotyphosa                   | Moska V                      |
| <i>Leptospira interrogans</i>    | Hebdomadis          | Hebdomadis                      | Hebdomadis                   |
| <i>Leptospira interrogans</i>    | Icterohaemorrhagiae | Copenhageni Itero-haemorrhagiae | M 20 RGA                     |
| <i>Leptospira borgpetersenii</i> | Javanica            | Javanica                        | Veldrat Batavia 46/RA 94     |
| <i>Leptospira nogochii</i>       | Panama              | Panama                          | CZ 214 K                     |
| <i>Leptospira interrogans</i>    | Pomona              | Pomona                          | Pomona                       |
| <i>Leptospira interrogans</i>    | Pyrogenes           | Pyrogenes                       | Salinem                      |
| <i>Leptospira interrogans</i>    | Sejroe              | Hardjo                          |                              |
|                                  |                     | Hardjo prajitno                 |                              |
|                                  |                     | Hardjo mini                     |                              |
|                                  |                     | Hardjo ctg                      |                              |
|                                  |                     | Wolfii                          |                              |
| <i>Leptospira santarosai</i>     | Shermani            | Shermani                        | LT 821                       |
| <i>Leptospira borgpetersenii</i> |                     | Tarassovi                       | Perepelicin ou Mitis Johnson |
| <i>Leptospira biflexa</i>        | Andamana            | Andamana                        | CH 11                        |
| <i>Leptospira biflexa</i>        | Semarang            | Patoc                           | Patoc I                      |

were screened at 1:100 dilution using a battery of 29 live antigen serovar strains (Table 1).

Three animals died of natural causes during the study. Fragments of the heart, spleen, pancreas, intestine, liver and kidney were collected and processed for DNA extraction from these snakes by PCR: one jararaca-pintada (*Bothrops pauloensis*) from G1 and two caíçacas (*Bothrops moojeni*) from G2.

Genomic DNA was extracted from tissue samples using a commercially available DNA extraction (Ilustra Tissue & Cells genomic Prep Mini Spin® kit, GE Healthcare) and evaluated in spectrophotometer (NanoVue®, GE Healthcare).

The amplification of *Leptospira* spp. DNA was performed utilizing primers LEP-1 (5'-GGCGGCGGTCTTAAACATG-3') and LEP-2 (5'-TTCCCCCATTGAGCAAGATT-3') resulting in 330 pb (Mérein 1992). The process carried out in a 25 µL solution containing 1·Taq polymerase buffer (10mM Tris HCl pH 8.0, 50mM KCl), 1.5mM MgCl<sub>2</sub>, 200 µM dNTP, 10 pmol of each primer (LEP-1 and LEP-2), 10 ng of purified DNA and 0.2 U/µl of Taq Platinum DNA polimerase system (Invitrogen). A MasterCycler gradient (Eppendorf) with initial denaturation at 94°C for 3 min, followed

by 30 cycles at 94°C for 1 min, then at 63°C for 1 min and 30 sec and a final extension at 72°C for 2 min was used.

Aliquots of the PCR products, along with a 100 bp DNA ladder (Invitrogen), were loaded into 2% agarose gel (Invitrogen) stained with SYBR® safe (Invitrogen) and submitted to electrophoresis in TBE buffer (0.09 M Tris-HCl, 0.09 M boric acid, 2mM EDTA, pH 8.3) for 60 min at 80V (Electrophoresis Power Supply Model EPS 301; GE Healthcare). The amplified DNA fragments were observed through GelDoc-IT™ Imaging System, using Vision Works® LS Software.

The amplicons were purified by using ExoSap (USB) and the sequencing reactions were carried out on both strands in a MegaBace™1000 (GE-Healthcare). Sequences were analyzed using Chromas 2.3 and Mega 4 software and compared with NCBI database by using BLAST search in GenBank (National Center for Biotechnology Information, Washington, D.C., <http://www.ncbi.nlm.nih.gov/BLAST>).

Tukey test was employed to compare proportions with  $\alpha = 0.05$  (Zar 1999) and to study epidemiological variables and serological test for leptospirosis.

## RESULTS

Of the 147 snakes studied, 52 (35.4%) were seropositive for leptospirosis by MAT, with eight (15.4%) in G1 (WS), 34 (65.4%) in G2 (IC) and ten (19.2%) in G3 (CC), according Table 2. Table 3 shows the number of snakes positive for *Leptospira* using MAT according to group, specie, serovar and titers. Titers varied from 100 to 3200 and Hardjo prajitino was the most prevalent serovar (88.5%, N=46/52), as shown in Table 3. Jararaca (*Bothrops jararaca*) (66.7%, N=22/33) showed the highest average titer among the three groups.

Positive amplifications of predictive amplicons of *Leptospira* spp. were detected in the kidney of one jararaca-pintada (*B. pauloensis*) (G1) with titer of 200 Hardjo prajitino and two caïçacas (*B. moojeni*) (G2) with titers of 200 and 1600 Hardjo prajitino, as shown in Fig. 1. When submitted to BLASTn analysis, all the purified and sequenced amplicons revealed 100% identity with *Leptospira interrogans* sequences deposited in the GenBank, producing unambiguous fragments

**Table 2. Comparison of antibodies to *Leptospira* seropositivity in the three groups and in the different species studied**

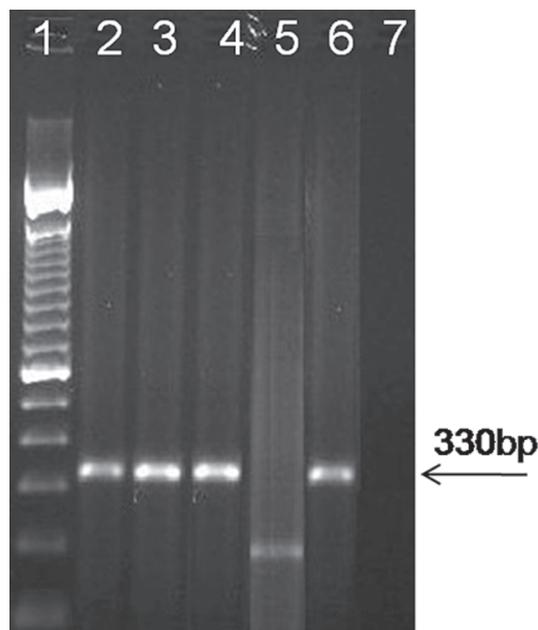
| Group  | N    | Species                             | Non reactive | Reactive | Total |
|--------|------|-------------------------------------|--------------|----------|-------|
| 1 (WS) | 35 b | <i>Bothrops jararaca</i>            | 4            | 2 A      | 6     |
|        |      | <i>Bothrops pauloensis</i>          | 1            | 2 A      | 3     |
|        |      | <i>Crotalus durissus terrificus</i> | 17           | 3 B      | 20    |
|        |      | <i>Bothrops moojeni</i>             | 1            | 0 B      | 1     |
|        |      | <i>Bothrops leucurus</i>            | 0            | 1 B      | 1     |
|        |      | <i>Boa constrictor amarali</i>      | 4            | 0 B      | 4     |
| 2 (IC) | 64 a | <i>Bothrops jararaca</i>            | 4            | 11 A     | 15    |
|        |      | <i>Bothrops moojeni</i>             | 2            | 13 A     | 15    |
|        |      | <i>Bothrops pauloensis</i>          | 5            | 9 A      | 14    |
|        |      | <i>Crotalus durissus terrificus</i> | 19           | 1 B      | 20    |
|        |      | <i>Boa constrictor amarali</i>      | 11           | 1 B      | 12    |
| 3 (CC) | 48 b | <i>Bothrops jararaca</i>            | 3            | 9 A      | 12    |
|        |      | <i>Crotalus durissus terrificus</i> | 20           | 0 B      | 20    |
|        |      | <i>Spilotes pullatus</i>            | 4            | 0 B      | 4     |
|        |      | Total                               | 86           | 52       | 147   |

$\chi^2 = 0.10$ .  $P \leq 0.05$ , lowercase letters indicate a statistical analysis among the 3 groups, while capital letters indicate a statistical analysis within each group. Same letters do not differ statistically. WS: wild snakes; IC = intensive captivity; CC = collective captivity; N = number of snakes by group.

**Table 3. Number of snakes positive for *Leptospira* using MAT according to group, species, serovar and titers**

| Group  | N  | Serovar                   | Titer (1/) |       |      |      |      |      |
|--------|----|---------------------------|------------|-------|------|------|------|------|
|        |    |                           | 100        | 200   | 400  | 800  | 1600 | 3200 |
| 1 (WS) | 34 | Hardjo prajitino          |            | 2 Bp  | 1 Bl |      | 2 Bj |      |
|        |    | Sentot                    | 2 Cdt      |       |      |      |      |      |
|        |    | Andamana                  | 1 Cdt      |       |      |      |      |      |
| 2 (IC) | 64 | Hardjo prajitino          | 4 Bj       | 5 Bj  | 3 Bm | 1 Bj | 3 Bm |      |
|        |    |                           | 1 Bm       | 3Bm   |      | 3Bm  |      |      |
|        |    |                           | 4 Bp       | 4Bp   |      | 1Bp  |      |      |
|        |    |                           |            | 1Cdt  |      |      |      |      |
|        |    | Hardjo mini Patoc         | 2 Bj       | 3 Bj  | 1 Bj |      |      |      |
|        |    | Gryppo-thyphosa Pyrogenes |            | 1 Bj  |      |      | 1 Bm |      |
| 3 (CC) | 48 | Hardjo prajitino          | 1 Bj       | 1 Bj  | 3 Bj |      | 1 Bj | 2 Bj |
|        |    | Panama                    |            | 1 Bca |      |      |      |      |
|        |    |                           |            |       |      |      |      |      |

WS = wild snakes; IC = intensive captivity; CC = collective captivity; N = number of snakes per group. Cdt = *Crotalus durissus terrificus*; Bm = *Bothrops moojeni*; Bj = *Bothrops jararaca*; Bp = *Bothrops pauloensis*; Bl = *Bothrops leucurus*; Bca = *Boa constrictor amarali*.



**Fig.1.** Electrophoresis of the PCR products using primers Lep1 and Lep2 with products of 331 pb in samples of kidney and liver of *Bothrops pauloensis* and *Bothrops moojeni*. Lane 1-7. 1) 100bp DNA ladder (Invitrogen), 2) kidney *Bothrops moojeni* (Group 2), 3) kidney *Bothrops moojeni* (Group 2), 4) kidney *Bothrops pauloensis* (Group 1), 5) liver *Bothrops pauloensis* (Group 1), 6) positive control; 7) negative control.

from 364 bases (Table 4). Liver samples from jararaca-pintada (*B. pauloensis*- G1) showed one lower band than expected and 93% sequence identity with *Leptospira* spp. (Table 4).

## DISCUSSION

Many animals are *Leptospira* spp. hosts, and each serotype has one or more hosts with different adaptation degrees. The

**Table 4. Nucleotide sequence alignment and percentage of identity on liver and kidney samples of *Bothrops pauloensis* and *Bothrops moojeni***

| Sample | Species/<br>Group                | Nucleotide sequence alignment                                                                                                                                                                                                                                                                                                                                                                                                            | % identity/<br>GenBank access |
|--------|----------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------|
| Liver  | <i>Bothrops pauloensis</i><br>G1 | CGGAAAGGCTCAGACAGCGACTACTGTCCAG<br>GGGTGGGACTGCGCGGTGATGTAACATCT<br>GTGGCGGATAAAGCAATGGCCGACATGAGTC<br>TCTGGGACTAACTTTCCAGTAACGGTGAAGCG<br>TAACTACATGGATTGGTCCCGTAGGAGAGTCA<br>ATAAGGATTTTTCGGGTAAAGATTTATTGCTC<br>GGAGATGAGCCCGCGTCCGATTAGCTAGCTTG<br>GTGATGTGTAACAGGCTCACCAAGGCGACGA<br>TCGGTAGCCTGTGCCTGAGAGTGGTGTTCGGC<br>CACAATGGAAGTGGAGACAGGTCCTACTCCT<br>ACGGCGAGGCATGCAGTTAAGAATCTTGCTCA<br>ATGGGGGAAACATGG                     | 93% (237/265)<br>AY461867.1   |
| Kidney | <i>Bothrops pauloensis</i><br>G1 | CCCTTCTTGGCGGCGCTCTTAAACATGCAA<br>GTCAAGCGGAGTAGCAATACTCAGCGGCGA<br>ACGGGTGAGTAACACAGTGGGTAATCTTCCTC<br>TGAGTCTGGGATAACTTTCCGAAAGGGAAGC<br>TAATACTGGATGGTCCGAGAGATCATAAGA<br>TTTTTCGGGTAAAGATTTATTGCTCGGAGAT<br>GAGCCCGCTCCGATTAGCTAGTTGGTGAGG<br>TAAAGGCTCACCAAGGCGACGATCGGTAGC<br>CGGCCTGAGAGGGTGTTCGGCCACAATGGA<br>ACTGAGACACGGTCCATACTCCTACGGGAGG<br>CAGCAGTTAAGAATCTTGCTCAATGGGGGGA<br>ACCCTGAAGCAGCGACGCCGCTGAACGAT<br>GAA         | 100% (364/364)<br>AE010300.2  |
| Kidney | <i>Bothrops moojeni</i><br>G2    | CTTCTTGGCGGCGCTCTTAAACATGCAAGT<br>CAAGCGGAGTAGCAATACTCAGCGGCGAAC<br>GGGTGAGTAACACAGTGGGTAATCTTCCTCTG<br>AGTCTGGGATAACTTTCCGAAAGGGAAGCTA<br>ATACTGGATGGTCCCGAGAGATCATAAGATT<br>TTTCGGGTAAAGATTTATTGCTCGGAGATGA<br>GCCCCTGAGAGGGTGTTCGGCCACAATGGAAGT<br>AAGGCTCACCAAGGCGACGATCGGTAGCCG<br>GCCTGAGAGGGTGTTCGGCCACAATGGAAGT<br>GAGACACGGTCCATACTCCTACGGGAGGCA<br>GCAGTTAAGAATCTTGCTCAATGGGGGGAAC<br>CCTGAAGCAGCGACGCCGCTGAACGATGA<br>AATTCCT | 100% (364/364)<br>AE010300.2  |
| Kidney | <i>Bothrops moojeni</i><br>G2    | CTTCTTGGCGGCGCTCTTAAACATGCAAGT<br>CAAGCGGAGTAGCAATACTCAGCGGCGAAC<br>GGGTGAGTAACACAGTGGGTAATCTTCCTCTG<br>AGTCTGGGATAACTTTCCGAAAGGGAAGCTA<br>ATACTGGATGGTCCCGAGAGATCATAAGATT<br>TTTCGGGTAAAGATTTATTGCTCGGAGATGA<br>GCCCCTGAGAGGGTGTTCGGCCACAATGGAAGT<br>AAGGCTCACCAAGGCGACGATCGGTAGCCG<br>GCCTGAGAGGGTGTTCGGCCACAATGGAAGT<br>GAGACACGGTCCATACTCCTACGGGAGGCA<br>GCAGTTAAGAATCTTGCTCAATGGGGGGAAC<br>CCTGAAGCAGCGACGCCGCTGAACGATGA<br>AATTCCT | 100% (364/364)<br>AE010300.2  |

G1= Group 1 (Wild snakes); G2= Group 2 (Intensive Captivity).

studied snakes came from farms and pastures, where they were probably infected by urine of affected cattle. According to Correa et al. (1965/1967) the predominant serovar in bovines is Hardjo prajitino. The seroprevalence of Hardjo serovar is observed in slaughterhouses and farm employees, suggesting that the exposure risk is related to direct and indirect contact with infected animals (Santa Rosa et al. 1980). Seroagglutination tests showed that bovines reacted positively (45.6%) to serovar Hardjo according to Langoni et al. (2008).

Homem et al. (2001), researching humans and other animals in Amazonia, observed that serovar Hardjo was the most prevalent. Bovine leptospirosis is endemic in Brazil and may cause abortions, infertility, anorexia, jaundice, hemoglobinuria, mastitis and even death (Santa Rosa et al. 1972, Silva 1976, Simpson 2002, Langoni et al. 2004). Out of four pregnant jararacas (*Bothrops*) included in this study, three laid atresic eggs suggesting a possible influence of *Leptospira* on them. These findings indicate that egg atresia may be related to seropositivity to serovar Hardjo, however experimental studies

and isolation of the agent are needed to corroborate this hypothesis.

Several studies have been carried out to determine the occurrence of *Leptospira* spp. and antibodies to *Leptospira* spp. in wild animals, but few include venomous or non venomous snakes. In Brazil this research was first performed by Hyakutake et al. (1976), in which agglutination tests detected Icterohaemorrhagiae, Ballum, Canicola, Guaicurus, Brasiliensis, Andamana and Javanica serovars. The predominant serovar founded by this authors was Andamana (80.6%, N=25/31) and do not corroborate with our results that shown Hardjo prajitno as serovar predominant (88.5%). This observation can be due the snakes came from different locations, and also the inclusion of new serovars, but few studies have been conducted until now.

Calle et al. (2001) found low-level *Leptospira* titers in free-ranging Venezuelan anacondas (*Eunectes murinus*) and showed that this may represent previous infection, antibody responses to *Leptospira* antigens present in ingested prey, or antibodies to *Leptospira* serovars that were not included in the serologic panel.

Stanchi et al. (1986) analyzed 18 urutus-cruzeiro (*Bothrops alternatus*) for the presence of antileptospiral antibodies using a microscopic agglutination test, and observed 72.2% (N=13/18) of positive results to Patoc, Andamana, Wolffi, Tarassovi, Pomona, Pyrogenes and Shermani serovars.

The high prevalence of positive isolates in G2, when snakes were kept in individual boxes, may be explained by Abdulla & Karstad (1962). These authors showed that snakes, both free-ranging and captive, may be infected by ingesting rodents and contaminated water. They also concluded that snakes might transmit leptospirosis by direct contact. The same authors, working with experimental serovar Pomona infection, found spirochetes in the kidneys of these animals six months after inoculation; interstitial nephritis was observed in one of them. Therefore, snakes in captivity may be infected by consuming infected mice or other food or water contaminated with their urine. This fact is demonstrated by the statistical difference between this group and the other G2, even in the collective captivity group, which has access to sunlight, water and more space per animal, as previously indicated by Ferreira Junior et al. (2010).

Ferris et al. (1961) isolated *Leptospira interrogans* serovar Ballum in an Eastern Hognose Snake (*Heterodon platyrhinus*). According to Hyakutake et al. (1976) the isolation of serovar Andamana in jararaca (*Bothrops pradoi*) may contribute to the transmission of leptospirosis to humans and other animals. Andrews et al. (1965) observed the presence of *Leptospira ballum* agglutinins in the serum of snakes of Southern Illinois, USA, as well as in other areas of the country. They concluded that these findings demonstrate the feeding habits of these animals.

According to Valentina & Fratini (2005), there are no data of reptiles as source of infection of *Leptospira* to humans, though they may have direct contact with spirochetes in the environment. In the present study, there were animals with high titers such as 3200, but without any clinical signs. Reptiles rarely show clinical disease when infected with *Lep-*

*tospira* spirochetes, although seropositive snakes of various species have been reported with over 6400 titers (Santa Rosa et al. 1972, Cordeiro et al.1981).

MAT showed that snakes came into contact with the pathogen, with titers ranging from 100 to 3200, PCR demonstrated that these animals harbored *Leptospira interrogans*. Several studies have been conducted employing the use of PCR for detection of *Leptospira* spp. in urine, serum and tissue of animals and humans (Gravekamp et al. 1993, Wagenaar et al. 2000, Bonfim et al. 2008). However, PCR had never been used to detect *Leptospira* spp. in snake tissues. Moreover, the present work showed that both wildlife and captivity snakes may maintain the pathogen without clinical signs.

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## REFERENCES

- Abdulla P.K. & Karstad L. 1962. Experimental infections with *Leptospira pomona* in snakes and turtles. Zoonoses Res. 1:295-306.
- Adler B. & Moctezuma A.P. 2010. Leptospira and leptospirosis. Vet. Microbiol. 140:287-296.
- Aguiar D.M., Gennari S.M., Cavalcante G.T., Labruna M.B., Vasconcellos S.A., Rodrigues A.A.R., Moraes Z.M. & Camargo L.M.A. 2006. Seroprevalence of *Leptospira* spp. in cattle from Monte Negro municipality, western Amazon. Pesq. Vet. Bras. 26:102-104.
- Andrews R.D., Reilly J.R., Ferris D.H. & Hanson L.E. 1965. Leptospiral agglutinins in sera from southern Illinois herpetofauna. Bull. Wildl. Dis. Assoc. 1:55-59.
- Bonfim M.R.Q., Barbosa-Stancioli E.F. & Koury M.C. 2008. Detection of pathogenic leptospires in urine from naturally infected cattle by nested PCR. Vet. J. 178:251-256.
- Blancou J., Chomel B.B., Belotto A. & Meslin F.X. 2005. Emerging or re-emerging bacterial zoonoses: factors of emergence, surveillance and control. Vet. Res. 36:507-522
- Calle P.P., Rivas J., Muñoz M., Thorbjarnarson J., Holmstrom W. & Karesh W.B. 2001. Infectious disease serologic survey in free-ranging Venezuelan anacondas (*Eunectes murinus*). J. Zoo Wildl. Med. 32:320-323.
- Cordeiro F., Sulzer C.R. & Ramos A.A. 1981. *Leptospira interrogans* in several wildlife species in Southeast Brazil. Pesq. Agropec. Bras., Sér. Vet. 1:19-29.
- Correa M.O.A., Hyakutake S., Natale V., Galvão P.A. & Aguiar H.A. 1965/1967. Estudos sobre a *Leptospira wolffi* em São Paulo. Revta Inst. Adolfo Lutz, São Paulo, 25/27:11-25.
- Faine S. 1982. Guideline for the Control of Leptospirosis. 2<sup>nd</sup> ed. WHO, Geneva, Offset Publication 67:171.
- Ferreira Junior R.S., Biscola N.P., Campagner M.V. & Barraviera B. 2010. How to raise snakes in captivity? Vet. Microbiol. 141:189.
- Ferris D.H., Rhoades H.E., Hanson L.E., Galton M. & Mansfield M.E. 1961. Research into the nidality of *Leptospira ballum* in campestral hosts including the hog-nosed snake (*Heterodon platyrhinus*). Cornell Vet. 51:405-419.
- Ghneim G.S., Viers J.H., Chomel B.B., Kass P.H., Descollonges D.A. & Johnson M.L. 2007. Use of a case-control study and geographic information systems to determine environmental and demographic risk factors for canine leptospirosis. Vet. Res. 38:37-50
- Gravekamp C., Van de Kemp H., Franzen D., Carrington D., Schoone G.J., Van Eys G.J.J.M., Everard C.O.R., Hartskeerl R.A. & Terpstra W.J. 1993. Detection of seven species of pathogenic leptospires by PCR using two sets of primers. J. Gen. Microbiol. 139:1691-1700.
- Hashimoto V.Y., Gonçalves D.D., Silva F.G., Oliveira R.C., Alves L.A., Reichmann P., Muller E.E. & Freitas J.C. 2007. Occurrence of antibodies against *Lep-*

- Leptospira* spp. in horses of the urban area of Londrina, Paraná, Brazil. *Revta. Inst. Med. Trop.* 49:327-330.
- Homem V.S.F., Heinemann M.B., Moraes Z.M., Vasconcellos S.A., Ferreira F. & Ferreira Neto J.S. 2001. Estudo epidemiológico da leptospirose bovina e humana na Amazônia oriental brasileira. *Revta. Inst. Med. Trop.* 34:173-180.
- Hyakutake S., Biasi P.D., Santa Rosa C.A. & Belluomini H.E. 1976. Contribuição ao estudo epidemiológico das leptospiroses em serpentes do Brasil. *Rev. Inst. Med. Trop.* 18:10-16.
- Hyakutake S., Biasi P.D., Belluomini H.E. & Santa Rosa C.A. 1980. Leptospiroses in Brazilian snakes. *Int. J. Zoonoses* 7:73-77.
- Langoni H., Da Silva A.V., Pezerico S.B. & De Lima V.Y. 2004. Anti-leptospire agglutinins in equine sera, from São Paulo, Goiás, and Mato Grosso do Sul, Brazil. 1996-2001. *J. Venom. Anim. Toxins incl. Trop. Dis.* 10:207-218.
- Langoni H., Souza L.C., Da Silva A.V., Cunha E.L.P. & Silva R.C. 2008. Epidemiological aspects in leptospirosis: Research of anti-*Leptospira* spp. antibodies, isolation and biomolecular research in bovines, rodents and workers in rural properties from Botucatu, SP, Brazil. *Braz. J. Vet. Res. Anim. Sci.* 45:190-199.
- Mérien F., Amouriaux P., Perolat P., Baranton G. & Saint Girons I. 1992. Polymerase chain reaction for detection of *Leptospira* spp. in clinical samples. *J. Clin. Microbiol.* 30:2219-2224.
- Romero E.C., Yasuda P.H. 2006. Molecular characterization of *Leptospira* sp. strains isolated from human subjects in São Paulo, Brazil using a polymerase chain reaction-based assay: A public health tool. *Mem. Inst. Oswaldo Cruz* 101:373-378.
- Santa Rosa C.A., Sulzer C.R. & Castro A.F.P. 1972. A new leptospiral serotype in the Bataviae group, isolated in São Paulo, Brazil. *Am. J. Vet. Res.* 33:1719-1721.
- Santa Rosa C.A., Sulzer C.R., Castro A.F.P., Yanaguita R.M. & Giorgi W. 1980. Two new serovars in the hebdomadis group isolated from cattle in Brazil. *Int. J. Zoonoses* 7:158-163.
- Silva I. 1976. A new leptospiral serotype isolated in Salvador, Bahia State. *Revta Microbiol.* São Paulo, 73:5-37.
- Simpson V.R. 2002. Wild animals as reservoirs of infectious diseases in the UK. *Vet. J.* 163:128-146.
- Stanchi N.O., Grisolia C.S., Martino P.E. & Peluso F.O. 1986. Presence of antileptospira antibodies in ophidia in Argentina. *Revta Argent. Microbiol.* 18:127-130.
- Valentina V.E. & Fratini F. 2005. Bacterial zoonoses among domestic reptiles. Zoonosi batteriche trasmesse da rettili domestici. *Ann. Fac. Med. Vet.* 58:85-91.
- Wagenaar J.A., Zuerner R.L., Alt D. & Bolin C.A. 2000. Comparison of polymerase chain reaction assays with bacteriologic culture, immunofluorescence, and nucleic acid hybridization for detection of *Leptospira borgpetersenii* serovar hardjo in urine of cattle. *Am. J. Vet. Res.* 61:316-320.
- Weekes C.C., Everard C.O.R. & Levett P.N. 1997. Seroepidemiology of canine leptospirosis on the island of Barbados. *Vet. Microbiol.* 51:215-222.
- World Organization for Animal Health Animal, OIE. Downloaded in April 2010 from <http://www.oie.int/>.
- Zar J.H. 1999. *Bioestatistical Analysis*. 4<sup>th</sup> ed. Prentice Hall, New Jersey. 663p.