Survey of *Leptospira* spp in pampas deer (*Ozotoceros bezoarticus*) in the Pantanal wetlands of the state of Mato Grosso do Sul, Brazil by serology and polymerase chain reaction


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This work reports a survey of *Leptospira* spp in pampas deer (*Ozotoceros bezoarticus*) in the Pantanal wetlands of the state of Mato Grosso do Sul, Brazil by serology and polymerase chain reaction (PCR). Seventy pampas deer were captured in the dry season and surveyed using PCR, microscopic agglutination test (MAT) (n = 51) and by both techniques (n = 47). PCR detected infections in two pampas deer and MAT detected infections in three. Through sequencing and phylogenetic analyses, the PCR-amplified fragment detected in deer was identified as *Leptospira* interroga. Serovars Pomona and Butembo were detected using MAT and the highest titre was 200 for serovar Pomona. Epidemiological aspects of the findings are discussed.

Key words: pampas deer - *Leptospira* spp - MAT - PCR - Pantanal

Leptospirosis is a worldwide disease (Scarcelli et al. 2003) responsible for important reproductive losses in cattle and severe zoonosis in man and domestic animals. Reports of leptospirosis in wildlife that share habitats with cattle are increasing (Mathias et al. 1999, Bengis et al. 2002, Girão et al. 2003, Scarcelli et al. 2003, Deem et al. 2004). Because of the expansion of cattle ranching into areas traditionally occupied by wildlife, such as the Pantanal wetlands in the state of Mato Grosso do Sul (MS), Brazil, interest in pathogens that affect native and livestock populations simultaneously has become more important.

The cervids belong to the order Artiodactyla, family Cervidae (Emmons & Feer 1990, Eisenberg & Redford 1999). In the Pantanal wetlands there are four species of cervids, *Blastocerus dichotomus* (marsh deer), *Ozotoceros bezoarticus* (pampas deer), *Mazama americana* (red brocket deer) and *Mazama gouazoubira* (brown brocket deer), which have distinct habitats (Vaughan 1986, Rodrigues et al. 2002, Tomás et al. 2004, Tiepolo & Tomás 2006).

Populations of pampas deer are distributed all over the Pantanal, with higher densities in the fields and ebbs (Mourão et al. 2000, Tomás et al. 2001, 2004). There are more pampas deer in the Pantanal than in the Cerrado biome; these two populations are sympatric (Rodrigues et al. 2002). The region of Nhecolândia has the highest population density of pampas deer in the Pantanal, estimated to be approximately 60 thousand individuals (Mourão et al. 2000).

In the Pantanal, ecological conditions are highly suitable for *Leptospira* spp, mainly because of the high temperatures and pluviometric index (1,200 mm/year) (Garcia & Castro 1986, Lins et al. 1986).

Leptospirosis is diagnosed by the detection of specific antibodies using microscopic agglutination test (MAT) and the isolation of the *Leptospira* pathogen in culture media. However, both techniques are laborious and time consuming. MAT requires the use of live antigens and the culture technique demands freshly collected samples (Faine et al. 1999, Cerequeira & Picardeu 2009).

Polymerase chain reaction (PCR) has been used for the diagnosis of leptospirosis in humans and domestic animals, demonstrating high accuracy and sensitivity when compared with MAT (Van Eys et al. 1989, Gerritsen et al. 1991, Radstone & Woodward 1996).

The objectives of this work were to estimate the prevalence of pampas deer infected with *Leptospira* spp in the Pantanal wetlands of MS, to identify the infective serovars and to evaluate a PCR method to detect these pathogens, assessing the analytic sensitivity. The study was conducted in four contiguous ranches located in the central area of the Pantanal, known as Nhecolândia (18°59′11.5″S 56°37′63″N), over an area of approximately 40,000 ha. The captures were performed from May-November 2006. Nhecolândia has the highest density population of pampas deer in the Pantanal; its population is estimated to be approximately 60,000
animals (Mourão et al. 2000). The surveyed area included private beef cattle ranches with land areas between 4,000-20,000 ha. In these ranches, cow-calv operations were based on extensive breeding on natural pastures.

Pampas deer were captured using anaesthetic darts shot with CO2 guns with adjustable pressure (Distinjet, model 35) or a blowpipe of 2 m length x 11 mm diameter (Zootech). Chemical anaesthesia with tiletamine/zolazepam or zolazepam/xylazine was administered to 42 animals (Piovezan et al. 2006) and a combination of zolazepam, tiletamine, xylazine and atropine was administered to 60 animals (Lima-Borges et al. 2006).

The environmental licenses for captures were provided by Brazilian Institute of Environment and Renewable Natural Resources (032/2005-0214.0022008/05-00 and 005/2007-02014.000382/2007-22). Blood samples were taken from the jugular vein into tubes with or without anticoagulant ethylenediamine tetracetic acid. After centrifugation, serum and erythrocyte layers were used for MAT and PCR, respectively.

Antibodies against Leptospira spp were detected by MAT according to Cole et al. (1973) using a routine panel of 19 live antigens of Leptospira spp and nine field isolates from domestic and wild animals. The panel represented the following serovars and strains of isolates from domestic and wild animals. The panel of field isolates used included Patoc (Patoc 1). The panel of field isolates used included Shermani (1342 K), Tarassovi (Perpelitsin), Sentinel (Hardjoprajitno), Wolffi (3705), Hardjo (Hardjobovis), 214), Pomona (Pomona), Pyrogenes (Salinem), Hardjo (Hardjo), Autumnalis (Akiyami A), Castellonis (Castellon), L./Otsoni (3522-C), Grippotyphosa (Moskva V), Hebdomadis (Hond Utrecht IV), Whitcombi (Whitcombi), Cynopteris (Cerdocyum), Grippophytophas (Moskva V), Hebdomadis (Hond Utrecht IV), Whitcombi (Whitcombi), Cynopteris (Cerdocyum), Grippophytophas (Moskva V), Hebdomadis (Hond Utrecht IV), Whitcombi (Whitcombi), Cynopteris (Cerdocyum), Grippophytophas (Moskva V), Hebdomadis (Hond Utrecht IV), Whitcombi (Whitcombi), Cynopteris (Cerdocyum), Grippophytophas (Moskva V), Hebdomadis (Hond Utrecht IV), Whitcombi (Whitcombi), Cynopteris (Cerdocyum), Grippophytophas (Moskva V), Hebdomadis (Hond Utrecht IV), Whitcombi (Whitcombi), Cynopteris (Cerdocyum), Grippophytophas (Moskva V), Hebdomadis (Hond Utrecht IV), Whitcombi (Whitcombi), Cynopteris 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A 5.9% frequency of *Leptospira* antibodies (CI 95%, 0.0141-0.1654) was found using MAT testing and the serovar Pomona was the most frequently detected. These results differ from those reported by Mathias et al. (1999), who found a seroprevalence of 24% with the Hardjo serovar being the most frequent in the same region. According to several authors, Hardjo is the most frequent serovar found in deer and cattle both in the Pantanal and in MS (Madruga et al. 1980, Pellegrin et al. 1999, Favero et al. 2001). Pellegrin et al. (1999) screened 756 cattle sera from the same cattle ranches where deer live against a reference panel of 17 leptospiral serovars using MAT and reported a frequency of 38% positive sera. The most prevalent serovars were Hardjo, Wolffi and Sejroe and only three sera reacted to serovar Pomona. Tomich et al. (2007) screened 282 samples of cattle serum and detected 143 (50.71%) positive samples with MAT and confirmed that the most prevalent serovars were Hardjo (genotypes Hardjoprajitno and Hardjobovis) and Wolffi. In the same work, the authors found that 57.09% of the sera were positive for IgG in a recombinant LipL32 enzyme-linked immunosorbent assay. Differences in the prevalence of *Leptospira* spp infections in different regions may reflect true variations or may result from differences in the study designs, including the method of sampling, panel of serovars or MAT cut-off values. One study indicated that the earliest agglutination to serovar Pomona in white-tailed deer appeared six days after inoculation and titres of 10 could be detected from sera were positive for IgG in a recombinant LipL32 enzyme-linked immunosorbent assay. By sequencing the PCR products detected in pampas deer during capture. However, there was some disagreement between the results presented here and those obtained by Girio et al. (2003), who found a high frequency of reactions to serovar Wolffi. In the present study, there was an increased frequency of reactions to serovar Pomona maintained by pigs under natural conditions.

In this survey, data for sex and age are presented, but no analysis was performed because only five deer were positive by MAT or PCR: two females and three males. Ferris and Verts (1964) performed a survey of 319 hunted white-tailed deer during the 1959 and 1960 deer hunting seasons in Illinois (USA) and reported 32 reactions by MAT and evidence that these findings seem to be unrelated to the sex and age of the animals.

PCR was able to amplify up to 280 fg/µL of DNA from the positive control. A visible product could be detected on a 1% agarose gel stained with SYBR Gold up to a 1:10^4 dilution and the Lepl and Lep2 primers amplified all the serovars utilised in this study for MAT. The two PCR products obtained from the positive samples were cloned and sequenced. In a BLASTn search, the DNA sequence from sample 2 showed 100% identity and an e-value of 10^-172 with the closest match, *L. interrogans* serovar Australis, accession FJ154557.1. Sample 31 showed 99% identity and an e-value of 10^-118 with the best hit, *L. interrogans* serovar Copenhagheni, accession FJ154558.1. The phylogenetic analysis resulted in a tree with *L. biflexa*, *L. illini* and *T. parva* as outgroups (Morey et al. 2006) and another large group of pathogenic leptospires. The samples from pampas deer were grouped in the *L. interrogans* branch.

The results from this study show that leptospirosis occurs in cervids in the Pantanal wetlands of MS. Because *O. bezoarticus* cohabits with cattle, this may represent a risk for livestock and for other wildlife species. By sequencing the PCR products detected in pampas deer from the Pantanal and searching for homology using the BLASTn algorithm, two serovars were detected, Australis and Copenhagheni. Australis has been found in equines, however, without clinical signs (Pinho et al. 2007, Ma- ciel et al. 2008). The reservoirs of serovar Copenhagheni are usually rodents, but this serovar has been isolated in several species, causing clinical alterations in accidental hosts (Rodrigues 2008). Girio et al. (2003) already detected antibodies to the Copenhagheni serovar in cervids in the Pantanal by MAT, consistent with the current findings; however, the findings of this present study need further confirmation, as the primers used amplify a conserved region of the 16S rRNA gene of *Leptospira* spp.

In a survey of 44 cervids in captivity, Silva et al. (2010) detected 19 (43.1%) infections with the following serovars: seven (36.8%) for Autumnalis, six (31.5%) for Andamana, six (31.5%) for Icterohaemorrhagiae, six (31.5%) for Patoc and five (26.3%) for Canicola. In the same work, other species of domestic and wild animals were surveyed and the same serovars were detected. Jorge et al. (2011) carried out work in the Social Service of Commerce Pantanal Reserve with wild carnivores, domestic dogs and wild horses and found the same serovars. In another work, Mathias et al. (1999) did not show the same positive findings found in these two works.

The low frequency of infection obtained in this study can be explained because deer were captured mostly during the dry season, from May-November, when the suitable conditions of flooded habitats do not exist to allow the survival of large numbers of leptospires. Large numbers of leptospires can survive for long periods in water or soil. Infected animals contaminate pastures, yards, drains, soil and surface waters with leptospires from their urine and leptospirosis has a higher preva-

### TABLE

Frequency of pampas deer (*Ozotoceros bezoarticus*) positive for *Leptospira* spp in the Pantanal of the state of Mato Grosso do Sul, Brazil, by microscopic agglutination test (MAT) and polymerase chain reaction (PCR)

<table>
<thead>
<tr>
<th>Test</th>
<th>Animals (n)</th>
<th>Positives (%)</th>
<th>Sex (n)</th>
<th>Age (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>PCR</td>
<td>70</td>
<td>2 (2.04)</td>
<td>31</td>
<td>39</td>
</tr>
<tr>
<td>MAT</td>
<td>51</td>
<td>3 (5.9)</td>
<td>24</td>
<td>27</td>
</tr>
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lence in tropical regions, especially following periods of heavy rainfall (Faine et al. 1999, Haake et al. 2002). Additionally, MAT is a technique of low sensitivity, mainly to the Hardjo serovar in cattle because some renal carriers present no detectable antibody titres or titres less than the current reference cut-off of 1/100 (Ellis et al. 1981). Cut-off values of 1/30 for serovar Hardjo and 1/100 for the other serovars can be used for the screening of leptospirosis in cattle herds (Alonso-Andicoberry et al. 2001). MAT is not standardised for the diagnosis of leptospirosis in wild animals or at lower cut-offs, which would improve its sensitivity in testing cervids (Espí et al. 2000). Sequence-based molecular phylogeny of the fragment obtained by PCR in deer suggested that the infective serovar is a *L. interrogans* genospecies, but this could not confirm because this isolate was not recovered from any surveyed deer. Results from MAT led to the hypothesis that feral pig (*Sus scrofa feral*), which is one of the largest biomasses in the Pantanal (Mourão et al. 2002), could be involved in the transmission of serovar Pomona to pampas deer because surveys conducted in cattle from the Pantanal showed that serovar Pomona is not frequent in cattle (Pellegrin et al. 1999), despite the fact that Girio et al. (2003) found a high frequency of this serovar in buffalo and sheep in the same area. Serovar Pomona has also been reported in other species of deer, such as *Odocoileus virginianus* (Ingebrigtsen et al. 1986, Goyal et al. 1992), *Cervus elaphus hispanicus* (Iberian red deer), and *Dama dama* (fallow deer) (Espí et al. 2010).

Serovar Butembo has been found in cattle and pampas deer in Southern Brazil (Saldanha et al. 2007, Tonin et al. 2011). Serum samples of repeat breeder cows in São Lourenço do Oeste, state of Santa Catarina, were surveyed for leptospiral anti-agglutinins and 100% were positive for serovar Butembo, with titres between 100-800. After treatment with streptomycin sulphate, 92% of the cows recovered from reproductive disorders. In a case report, Tonin et al. (2011) described severe symptoms of clinical leptospirosis in a pampas deer, such as jaundice, weakness and weight loss, and the results of MAT against a panel of eight reference serovars demonstrated seroreactivity for serovars Butembo (1/200), Bratislava (400), Wolffi (200) and Hardjo (400). Cattle and farmed deer in New Zealand are recognised as a maintenance host for serovar Hardjo and pigs for serovar Pomona. However, the interpretation of leptospirosis titres can vary according to the serovar: Hardjobovis and Bratislava generally present lower titres compared with Pomona (Ayanegui-Alcerreca 2006), but Pomona can cross react with Bratislava; therefore, using lower cut-offs in the MAT can lead to an overestimation of the number of infections by serovar Pomona. Wild boar and feral pigs frequently have an adverse impact on agriculture and the environment. Feral pigs are an exotic, invasive pest species in many areas of the world and can introduce diseases to livestock populations (Bengis et al. 2002). In some parts of Europe, wild boar is an increasingly abundant native species (Artois et al. 2002) and can be infected by *Leptospira* spp (Espí et al. 2010).

These results suggest that, for the studied area, the role of cattle in the spread of serovar Hardjo to pampas deer is questionable and that pampas deer may be involved in the disease cycle of serovar Pomona more than for Hardjo. This observation is consistent with the findings of previous studies (Fournier et al. 1986). The source of serovar Pomona for deer and cattle could be other wildlife, in particular feral pigs and wild boar (Espí et al. 2010). These results indicate that pampas deer are exposed to *Leptospira* organisms, but further studies on leptospirosis in pampas deer, feral pigs and cattle are required to explain the relationship between these species in the epidemiology of leptospirosis in the Pantanal.

**REFERENCES**


