Molecular analysis on protozoa in wild mammals run over in southern Rio Grande do Sul, Brazil

Análise molecular de protozoários em mamíferos silvestres atropelados no Sul do Rio Grande do Sul

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ABSTRACT
The phylum Apicomplexa is divided into four main groups, which include important genera such as Babesia, Theileria and Neospora, which are responsible for diseases that can affect domestic and wild animals. The objective of this study was to ascertain occurrences of Neospora caninum, Babesia sp., Babesia microti, Piroplasmida sp. and Theileria equi in wild mammals in the southern region of the state of Rio Grande do Sul, Brazil. Twenty-two wild mammals that were found dead as roadkill in this region were necropsied, and organ fragments...
were collected. The spleen was used for DNA extraction and for parasite detection via PCR. Studies carried out through molecular analysis contribute towards identifying the elements that cause new cases of disease and the emergence of potential reservoirs and vectors. Thus, through these molecular studies, the following were recorded for the first time in Brazil: *Neospora caninum* and *Babesia microti* in *Cavia aperea*, *Babesia* sp., *Theileria equi* and *Piroplasmida* sp. in *Procyon cancrivorus*; and *T. equi* in *Galictis cuja*.

**Keywords:** wild mammals, *Neospora caninum*, *Babesia microti*, *Babesia* sp., *Theileria equi*, *Piroplasmida* sp.

**RESUMO**

O filo Apicomplexa está dividido em quatro grupos principais, que incluem gêneros importantes como Babesia, Theileria e Neospora, que são responsáveis por doenças que podem afetar animais domésticos e selvagens. O objetivo deste estudo foi verificar as ocorrências de *Neospora caninum*, *Babesia* sp., *Babesia microti*, *Piroplasmida* sp. e *Theileria equi* em mamíferos selvagens na região sul do estado do Rio Grande do Sul, Brasil. Vinte e dois mamíferos selvagens que foram encontrados mortos na estrada nesta região foram necropsiados, e fragmentos de órgãos foram coletados. O baço foi usado para extração de DNA e para a detecção de parasitas via PCR. Estudos realizados através de análise molecular contribuem para identificar os elementos que causam novos casos de doença e o surgimento de potenciais reservatórios e vetores. Assim, através destes estudos moleculares, foram registrados, pela primeira vez no Brasil, os seguintes *Neospora caninum* e *Babesia microti* em *Cavia aperea*, *Babesia* sp., *Theileria equi* e *Piroplasmida* sp. em *Procyon cancrivorus*; e *T. equi* em *Galictis cuja*.

**Palavras-chave:** mamíferos selvagens, *Neospora caninum*, *Babesia microti*, *Babesia* sp., *Theileria equi*, *Piroplasmida* sp.

**1 INTRODUCTION**

The phylum Apicomplexa is divided into four main groups: Coccidia (coccids), Gregarinasina (gregarines), Haemospororida (haemosporidians) and Pyroplasmorida (piroplasms) (ADL et al., 2012). Pyroplasmorida encompasses two important genera, Babesia and Theileria, with wide geographical distribution, which are responsible for diseases that can affect domestic and wild animals and also cause numerous economic losses (CONRAD et al., 2006; GRAY & WEISS, 2008; HERWALD et al., 2003; UILENBERG, 2006).

*Babesia* spp. is considered to be the second most common protozoon found in mammalian blood (KAKOMA & MEHLHORN, 1994; TELFORD et al., 1993). It has a heteroxenic cycle, i.e. it needs both a vertebrate host and an invertebrate host to complete its life cycle. *Theileria* spp. is transmitted by ixodid ticks of the genera Amblyomma, Haemaphysalis, Hyalomma and Rhipicephalus.

*Neospora caninum* Dubey, Carpenter, Speer, Topper & Uggla, 1988 (Coccidia) has wide geographical distribution and a variety of hosts, such as dogs, cats, cattle, horses, pigs, rodents
and coyotes, among others (DUBEY et al., 2007; FERROGLIO et al., 2007; HUANG et al., 2004). It has great importance in veterinary healthcare, given that it can cause neosporosis, which can lead to spontaneous abortion in cattle rearing (DUBEY et al., 2007).

Wildlife hit-and-run accidents are the second largest cause of biodiversity loss, only behind the reduction of natural environments (CHEREM et al., 2007). According to the National Department of Highways/Military Institute of Engineering (DNER/IME, 2001), 475 million animals are run over per year in Brazil. These animals form a source of data for diagnostic, epidemiological, dietary and parasitic fauna studies.

Therefore, the objective of the present study was to ascertain occurrences of Neospora caninum, Babesia sp., Babesia microti, Piroplasmida sp. and Theileria equi in wild mammals that were run over in the southern region of the state of Rio Grande do Sul, Brazil.

2 MATERIALS AND METHODS

Twenty-two wild mammals that had been run over on highways in Rio Grande do Sul were collected (10 specimens of Cavia aperea, 8 of Procyon cancrivorus and 4 of Galictis cuja), in accordance with a license granted by ICMBio/SISBIO (no. 38913-5). These specimens were found in the municipalities of Capão do Leão (31° 46′ 3″ South; 52° 26′ 55″ West), Pelotas (31° 46′ 34″ South; 52° 21′ 34″ West) and Arroio Grande (32° 14′ 19″ South; 53° 5′ 27″ West). The carcasses were taken to the Laboratório de Parasitologia de Animais Silvestres (LAPASIL) of the Universidade Federal de Pelotas, where they were frozen at -20 °C until processing. Tissue collection was performed during the necropsies on the animals.

For DNA extraction, the mammalian spleen was macerated and placed in tubes with a proteinase K solution (20 mg/ml) for digestion in a water bath at 55 °C for 2 hours. DNA extraction was performed using the Invisorb® spin tissue kit, following the manufacturer's instructions. The DNA was quantified and its purity was ascertained through spectrophotometry. For quality control of DNA extraction, strains of known lineages were used as positive controls and samples consisting of water and reagents were used as negative controls.

To diagnose Neospora caninum through PCR, the primers used were NSP6PLUS (5´-CTGGCCAGTCAACCTACGTCTTCT-3′) and NSP21PLUS (5´-CCCAGTGCGTCCAATCTGTAA-3′) (Romano et al., 2009), for the 18S DNA region. The amplification conditions consisted of an initial cycle of denaturation at 95 °C for 5 min; followed by 40 cycles at 95 °C for 1 min, 63 °C for 1 min and 72 °C for 1 min; and a final extension at 72 °C for 5 min, followed by cooling to 4 °C.
To diagnose Babesia spp., Piroplasmida sp. and Theileria spp., semi-nested PCR was performed. In the first reaction, the primers RLB-F2 (5'-GACACAGGGAGGTAGTGACAAAG-3') and RLB-R2 (5'-CTAAGAATTTACCTGACAGT-3') (GUBBELS et al., 1999) were used. The amplification included a denaturation step of 5 min at 95 °C; followed by 25 repetitions of 30 s at 95 °C, 45 s at 50 °C and 1.5 min at 72 °C; and a final extension at 72 °C for 10 min. For the second PCR reaction, 1 μl of the PCR product from the first reaction and the internal primer RLB-FINT (5'-GACAAGAAATAACACRGGC-3') together with RLB-R2 were used. The reaction mixture was performed in a final volume of 25 μl, using Promega PCR Master Mix (Promega Corporation, WI, USA), 20 pM of each primer and 100 ng of DNA. The cycling programs were identical to the direct amplification of RLB-F2 / RLB-R2, but the number of cycles increased to 40, while the annealing temperature was 50 °C and 55 °C in the first and second PCR, respectively. Positive and negative control samples were included in the reaction.

The amplification products were analyzed by means of electrophoresis on agarose gel (2%) with blue green loading dye I (LGC Bio) staining, in 0.5X TBE solution (0.4 M tris-base, 0.20 M boric acid and 0.5 M EDTA solution; pH 8.0). The products were viewed using a transilluminator.

The positive amplicons were purified using the Mebep Bioscience product purification kit and were subjected to sequencing at the Genome Laboratory of the Biotechnology Center of the Federal University of Pelotas. The resulting sequences were compared with records in the GenBank database using the BLAST local alignment search tool. The Clustal X software (http://www-igbmc.u-strasbg.fr/BioInfo) was used to build multiple sequence alignments.

3 RESULTS

Among the total of 22 mammal specimens that were analyzed, at least one specimen in each group was positive for one genus of each of the protozoa studied, as shown in Table 1.

Table 1 - Prevalence of Neospora caninum, Babesia sp., Babesia microti, Piroplasmida sp. and Theileria equi in the spleen of mammals that were run over in the southern region of the state of Rio Grande do Sul, Brazil.

<table>
<thead>
<tr>
<th>Hosts</th>
<th>Protozoa</th>
<th>Nº de Hosts positives</th>
<th>Theileria equi</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cavia aperea Erxleben, 1777</td>
<td>Neospora 1 Babesia 1</td>
<td>Negatives</td>
<td>Negatives 20%</td>
<td></td>
</tr>
<tr>
<td>Procyon cancrivorus (Cuvier, 1798)</td>
<td>Piroplasmida sp. Negatives 3</td>
<td>Negatives</td>
<td>1 87.50%</td>
<td></td>
</tr>
<tr>
<td>Galictis cuja (Molina, 1782)</td>
<td>Theileria equi Negatives 1</td>
<td>Negatives</td>
<td>25%</td>
<td></td>
</tr>
</tbody>
</table>
Neospora caninum in C. aperea, presented 100% similarity with four sequences (GU300774.1; KX683873.1; KF649848.1; MG973172.1) that were obtained through the BLAST search. Babesia microti in C. aperea presented 93.26% similarity with two sequences (AB242176.1; AB190287.1).

Theileria equi in G. cuja was found to present 100% similarity with seven sequences (MG052902.1; MF510479.1; KY952226.1; KX722520.1; KY464024.1; KX227629.1; KX227623.1).

In P. cancrivorus, Piroplasmida sp. presented the following percentage similarities with GenBank sequences: 98.12% with EF057099.1, 98.17% with EF057099.1 and 97.72% with EF057099.1. Babesia sp. presented 99.66% with MG682489.1, 99.74% with MG682489.1 and 100% with MG682492.1. Theileria equi presented 99.76% similarity with seven sequences (MG052902.1; MF510479.1; KY952226.1; KX722520.1; KY464024.1; KX227629.1; KX227623.1).

4 DISCUSSION

Neospora caninum has a life cycle involving canids as definitive hosts, namely, the domestic dog and Australian dingo (Canis familiaris), grey wolf (Canis lupus) and coyote (Canis latrans), and many other species as intermediate hosts (MCALLISTER et al., 1998; BASSO et al., 2001; DUBEY et al., 2011). Through seroprevalence studies, presence of N. caninum has been reported in several countries and in groups of wild animals such as dogs, cats, mustelids, deer and birds (DUBEY et al., 2008; SOBRINO et al., 2008; MILLÁN et al., 2009; STIEVE et al., 2010).

In Brazil, Yai et al. (2008) investigated the presence of anti-N. caninum antibodies in Hydrochoerus hydrochaeris (Caviidae: Rodentia) (n = 213 serum samples) from 11 locations in the state of São Paulo, by means of the indirect immunofluorescence reaction (IIFR), and found antibodies in 20 (9.4%) of these rodents. In the state of Pará, Truppel et al. (2010) found through molecular amplification of the Ne5 or ITS1 region that 23% (6/26) of their samples were positive for N. caninum DNA. This protozoan was found in the lymphatic glands, heart, liver and blood in H. hydrochaeris.

Although the present study reported the presence of N. caninum in another host and in other tissues, there were similarities in the occurrence data relating to this protozoan (Table 1). It is important to emphasize that this is the first report of N. caninum in C. aperea. Dubey et al. (2014) reported on occurrence of N. caninum in the brain of C. lupus (n = 2) in the United
States, through a molecular study. Through the BLAST search, it can be concluded that presented 100% similarity with the present study.

In Brazil, through molecular studies, Braga et al. (2017) recorded occurrences of T. equi in donkeys, horses and mules on São Luís Island, state of Maranhão, in 2/10 (20%), 15/39 (38.5%) and 13/90 (14.4%), respectively. Although their study was conducted on a group of hosts that differed from those of the present study, similarities can be observed regarding the prevalence data obtained (Table 1). Through the BLAST search, 100% similarity with the sequences obtained was ascertained. With regard to wild animals, especially G. cuja and P. cancrivorus, the present report provides the first record through molecular analysis on tissues.

In a study conducted in Japan on 247 rodents (162 specimens of Apodemus speciosisus: Muridae; 63 of Apodemus argenteus; 11 of Eothenomys andersoni: Cricetidae; 3 of Eothenomys smithii; 4 of Microtus montebelli: Cricetidae; 3 of Clethrionomys rufocanis: Cricetidae; and 1 of Urotrichus talpoides: Talpidae), Saito-Ito et al. (2007) reported that Babesia microti was present in 36 rodents (24 A. speciosisus, 7 A. argenteus and 5 E. andersoni), through PCR detection. Among these 36 rodents, 27 were confirmed as positive through blood smears. Their report presents 93.26% similarity with the sequences of the present study. It is important to note that B. microti is the infectious agent responsible for human babesiosis, which is a growing health problem in the northeastern United States, transmitted through the bite of the tick Ixodes scapularis (GRAY et al., 2010; HOMER et., 2000). The United States is a country where introduction of wild animals as pets is a very common act among the population (SOUZA, 2011).

There are many reports of on piroplasmids in Procyon lotor Linnaeus, 1758 (Procyonidae: Carnivora) in North America and Japan (TELFORD JR. & FORRESTER, 1991; BIRKENHEUER et al., 2006; JINNAI et al., 2009). It has been reported that Babesia spp. has high prevalence in the population of this mammal. In Uruguay, Thompson et al. (2018) reported finding Babesia sp. in blood and different tissues (spleen, liver and muscle) in 5 specimens of P. cancrivorus (n = 13). Corroborating their study, there are similarities in the present study regarding prevalence data (Table 1). The isolate of P. cancrivorus had 100% similarity with the sequences obtained through the BLAST analysis. In Brazil, no reports have diagnosed Babesia spp. in this species of carnivore.

5 CONCLUSION

Wild animals act as important hosts for a diversity of microorganisms. Studies carried out through molecular analysis contribute towards identifying the elements that cause new cases
of disease and the emergence of potential reservoirs and vectors. Through the molecular analyses carried out here, it was possible to record the following for the first time in Brazil: Neospora caninum and Babesia microti in Cavia aperea; Babesia sp., Theileria equi and Piroplasmida sp. in Procyon cancrivorus; and T. equi in Galictis cuja.

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