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Detection of *Theileria* and *Babesia* in brown brocket deer (*Mazama gouazoubira*) and marsh deer (*Blastocerus dichotomus*) in the State of Minas Gerais, Brazil

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ABSTRACT

Intraerythrocytic protozoan species of the genera *Theileria* and *Babesia* are known to infect both wild and domestic animals, and both are transmitted by hard-ticks of the family Ixodidae. The prevalences of hemoprotozoa and ectoparasites in 15 free-living *Mazama gouazoubira*, two captive *M. gouazoubira* and four captive *Blastocerus dichotomus* from the State of Minas Gerais, Brazil, have been determined through the examination of blood smears and the use of nested polymerase chain reaction (nPCR). The cervid population was inspected for the presence of ticks and any specimens encountered were identified alive under the stereomicroscope. Blood samples were collected from all 21 animals, following which blood smears were prepared, subjected to quick Romanowsky staining and examined under the optical microscope. DNA was extracted with the aid of commercial kits from cervid blood samples and from tick salivary glands. The nPCR assay comprised two amplification reactions: the first was conducted using primers specific for a 1700 bp segment of the 18S rRNA gene of *Babesia* and *Theileria* species, whilst the second employed primers designed to amplify a common 420 bp *Babesia* 18S rRNA fragment identified by aligning sequences from *Babesia* spp. available at GenBank. The ticks *Amblyomma cajennense*, *Rhipicephalus microplus* and *Dermacentor nitens* were identified in various of the cervids examined. Of the animals investigated, 71.4% (15/21) were infected with hemoprotozoa, including *Theileria cervi* (47.6%), *Theileria* sp. (14.3%), *Babesia bovis* (4.8%) and *Babesia bigemina* (4.8%). However, only one of the infected wild cervids exhibited accentuated anaemia (PCV = 17%). This is first report concerning the occurrence of *Theileria* spp. in Brazilian cervids.

1. Introduction

The identification of infectious agents in wild animals is not only crucial for the preservation of species but also provides valuable information regarding the epidemiological chain of diseases. This is particularly important with regard to members of the Cervidae, for example, whose natural habitat has been transformed significantly as a result of intense deforestation driven by the needs of farmers and cattle breeders. One consequence of such changes is that many cervids have started living in close proximity with domestic ruminants, hence favouring the interchange of infectious microorganisms between the populations. Additionally, knowledge relating to infectious microorganisms is important for cervids maintained in captivity, since it has significant application in animal management, particularly with respect to the transit of animals between zoos and breeding stations and the inadvertent introduction of exotic pathogens into such locations.

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Intraerythrocytic protozoan species of the genera *Theileria* and *Babesia* are known to infect both wild and domestic animals, and both are transmitted by hard-ticks of the family Ixodidae (Ristic and Kreier, 1981). Species of *Theileria* are cosmopolitan parasites (Chae et al., 1999) that have been detected in wild ruminants in Japan (Inokuma et al., 2004), Germany (Höfle et al., 2004) and South Korea (Han et al., 2009). In the United States, the occurrence of *Theileria cervi* has been reported in white-tailed deer (*Odocoileus virginianus*) (Kocan and Kocan, 1991), elk (*Cervus canadensis*), mule deer (*Odocoileus hemonius*), Axis deer (*Axis axis*) and sika deer (*Cerves nippon*), with the distribution of the parasite being associated with the geographic distribution of the vector, namely, the tick *Amblyomma americanum* (Laird et al., 1988; Waldrup et al., 1989; Kocan and Kocan, 1991). Infection with *T. cervi* is considered benign, although some clinical symptoms have been observed in cervids that have been weakened by other parasites, or are undernourished or stressed (Kocan and Kocan, 1991; Fowler, 1993; Yasbley et al., 2005). There are, however, no reports of the presence of *Theileria* spp. in South American cervids.

The hemoparasites *Babesia bigemina* (Smith and Kilborne, 1893) and *B. bovis* (Babes, 1888) have been detected by indirect immunofluorescence (IFAT) and nested polymerase chain reaction (nPCR) assays in free white-tailed deer in northern Mexico (Cantu et al., 2007). The presence of anti-*Babesia odocoilei* antibodies has also been described in this cervid (Waldrup et al., 1989, 1992). Although the actual impact of such parasite on wild populations is not known, the occurrence of clinical manifestations has been reported in an immunosuppressed cervid (Perry et al., 1985).

Investigations of the infection of cervids by hemoparasites in Brazil are somewhat scarce. However, a high prevalence of *Babesia* spp. was reported in pampas deer (*Ozotocerus bezoarticus*) from the Brazilian Pantanal (Villas-Boas et al., 2009). Additionally, Machado and Müller (1996) reported that the frequencies of *B. bovis* and *B. bigemina* in wild pampas deer from the State of Goiás were, respectively, 8.3 and 29.7%. According to serological tests, however, the prevalences of these two parasites in marsh deer (*Blastocerus dichotomus*) from the Porto Primavera Hydroelectric Power Station located in Paraná River (State of Paraná, Brazil) were considerably higher, at 88.2 and 92%, respectively (Duarte, 2007). Experimental inoculation of the grey brocket deer (*Mazama gouazoubira*; also known as brown brocket deer or bush deer) with *B. bovis* or *B. bigemina* revealed that the former parasite is more pathogenic than the latter (Duarte, 2006). Interestingly, antibodies against *B. bovis*, *B. bigemina* or *B. odocoilei* were not present in wild specimens of *M. gouazoubira* from the Gran Chaco in Bolivia (Deem et al., 2004).

In contrast to the above, the occurrence of ectoparasites in Brazilian cervids has been widely reported. Ticks of the species *Rhipicephalus microplus*, *Dermacentor nitens*, *Amblyomma cajennense*, *Amblyomma mantiqueirense*, *Haemaphysalis kohlsi*, *Ixodes luciae* and *Ixodes aragoi* have been variously found in the cervids *M. gouazoubira*, *B. dichotomus* and *O. bezoarticus* (Aragão and Fonseca, 1961; Serra-Freire and Teixeira, 1993; Szabó et al., 2003; Duarte, 1997; Cançado et al., 2009).

The aim of the present study was to evaluate the occurrences of intraerythrocytic protozoa and ticks in free-living and captive specimens of the cervids *M. gouazoubira* and *B. dichotomus* from the State of Minas Gerais, through the analysis of blood smears and by nPCR assay.

2. Materials and methods

The study, which was carried out during the period June 2007 and September 2009, was approved by the Ethical Committee on Animal Experimentation (CETEA/UFMG, Belo Horizonte, MG, Brazil) under protocol no. 142/08, and by the Brazilian Institute for Environment and Natural Renewable Resources (IBAMA, Belo Horizonte, MG, Brazil) under licence no. 16064-1.

2.1. Animal population

The animal population (Table 1) comprised free-living specimens of *M. gouazoubira* ($n = 15$) and captive specimens of *M. gouazoubira* ($n = 2$) and *B. dichotomus* ($n = 4$). The free-living animals had recently been captured by the Forestry Police and conveyed either to IBAMA ($n = 13$) or to the conservation station Fazenda Engenho d'Água (Ouro Preto, MG, Brazil; $n = 2$). The captive animals, some of which had been born in captivity and others captured from the wild, had been maintained for a number of years in the Fundação Zoobotânica de Belo Horizonte.

2.2. Blood sampling

Blood from all 21 animals was collected by puncture of the jugular vein and samples were transferred immediately to vials containing EDTA. In the case of free-living *M. gouazoubira*, sampling was performed within two days of their original capture from the wild. Blood smears were prepared, subjected to quick Romanowsky staining (Panótico Rápido; Laborclin, Pinhais, PR, Brazil) and examined under the optical microscope at 100× magnification. For each sample, at least 40 microscopic fields were observed. Packed cell volume (PCV) was determined using the microhematocrit method (Jain, 1993). Further aliquots of blood samples were frozen and stored for subsequent DNA extraction.

2.3. Tick collection

Animals were inspected for the presence of ticks and all specimens collected were examined alive under an Olympus (Tokyo, Japan) stereomicroscope model SZ 40 and identified according to the criteria of Aragão and Fonseca (1961). Ticks were placed in a biochemical oxygen demand (BOD) chamber and maintained at 26 °C and 80% relative humidity until moulting occurred. After moulting, ticks remained in the chamber for 50 days, after which they were transferred to a rubber (ethylene vinyl acetate) chamber, which was sealed with cotton fabric fixed in place by universal contact adhesive (Brascoplast; Brascola, São Bernardo do Campo, SP, Brazil), and allowed to feed on a calf

Table 1
Characterization of the cervids examined, namely, *Mazama gouazoubira* and *Blastocerus dichotomus*, with respect to sex/age, origin, sampling date, blood smears results, molecular sequencing results (*Theileria* sp., *T. cervi*, *Babesia bovis* and *B. bigemina*) and infestation by ticks (*Rhipicephalus microplus*, *Dermacentor nitens* and *Amblyomma cajennense*).

Cervids	Identification	Sex/age	Date	Blood smear	*Theileria* sp.	*T. cervi*	*B. bovis*	*B. bigemina*	*R. microplus*	*D. nitens*	*A. cajennense*
Mazama gouazoubira	MGI1	Adult male	13/6/2007		X						
	MGI2	Adult female	15/6/2007			X			X		X
	MGI3	Adult male	20/6/2007			X			X		X
	MGI4	Adult female	26/6/2007								
	MGI5	Adult female	5/11/2007	X		X					
	MGI6	Adult female	8/9/2008			X					
	MGI7	Fawn female	21/1/2009								
	MGI8	Adult female	13/4/2009	X		X				X	
	MGI9	Fawn female	16/6/2009		X				X	X	
	MGI10	Adult female	10/6/2009								
	MGI11	Adult female	15/7/2009	X		X			X	X	X
	MGI12	Adult female	17/7/2009	X		X			X	X	X
	MGI13	Fawn female	9/9/2009		X						
	MGE1	Adult male	17/5/2008	X		X					
	MGE2	Adult male	14/6/2008	X				X		X	
	MGZBH1	Adult female	30/9/2008			X					
	MGZBH2	Adult male	7/11/2008								
Blastocerus dichotomus	BDZBH1	Adult female	14/7/2008								
	BDZBH2	Adult male	14/8/2008								
	BDZBH3	Adult male	23/9/2008			X					
	BDZBH4	Adult male	10/7/2009				X				

for three days. Nymphs and adults were dissected, the salivary glands collected and immersed in a cell lysis solution for subsequent DNA extraction.

2.4. DNA extraction and PCR amplification

DNA was extracted from cervid blood samples (300 μL) with the aid of a Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) employed according to the manufacturer's instructions. In order to extract DNA from tick salivary glands, the same commercial kit was used following the manufacturer's instructions designated for the extraction of DNA from tissue cultures.

The nPCR assay of genomic DNA involved two separate amplification reactions. The first reaction was carried out using the primers RIB-19 (5′CGGGATCCAACCTGGTTGATCCTGC3′) and RIB-20 (5′CCGAATTCCTTGTTACGACTTCTC3′) that are specific for a 1700 bp segment of the 18S rRNA gene from *Babesia* and *Theileria* spp. (Zahler et al., 2000). The reaction mixture comprised 1.2 μL of dNTPs (0.2 mM), 0.15 μL of Taq polymerase (0.05 U), 1.5 μL which buffer (1×), 0.6 μL of a solution containing the mixed primers (10 μM) and sufficient sterile ultra-pure water to give a final volume of 15 μL. A 1.5 μL aliquot of the DNA template was added to the reaction mixture, and amplification was performed using an Eppendorf (São Paulo, SP, Brazil) Mastercycler® thermocycler programmed as follows: 94 °C for 5 min (initial denaturation step), 30 cycles each comprising 92 °C for 1 min (denaturation), 54 °C for 1 min (annealing) and 72 °C for 2 min (extension), and a final extension step at 72 °C for 8 min. Following amplification, reaction mixtures were maintained at 12 °C. PCR amplicons were separated by electrophoresis on 1% agarose gel (40 min, 100 V), stained with ethidium bromide and visualised under ultraviolet light.

The second reaction was carried out using primers BabRumF (5′ACCTCACCAGGTCCAGACAG3′) and BabRumR (5′GTACAAAGGGCAGGGACGTA3′) that were designed to amplify a common 420 bp *Babesia* 18S rRNA fragment identified by aligning sequences from *Babesia* spp. available at GenBank (http://www.ncbi.nlm.nih.gov), namely, *B. bigemina* (X59607), *B. odocoilei* (U16369), *Babesia divergens* (U07885) and *B. bovis* (L31922). The reaction mixture comprised 2.0 μL of dNTPs (0.2 mM), 0.25 μL of Taq polymerase (0.05 U), 2.5 μL which buffer (1×), 1.0 μL of a solution containing the mixed primers (10 μM) and sufficient sterile ultra-pure water to give a final volume of 25 μL. An aliquot (2.5 μL) of amplicon obtained in the first reaction were added to the reaction mixture and amplification was carried out under the conditions described above. Products were separated by electrophoresis and visualised as described above, and subsequently purified with the aid of QIAquick PCR Purification Kit (Qiagen Biotecnologia Brasil, São Paulo, SP, Brazil) used according to the recommendations of the manufacturer. Sequencing of the purified amplicons was performed on a MegaBACE 1000 DNA Analysis System (GE Healthcare, Waukesha, WI, USA) using the BabRum primers. Sequences were aligned, edited and analysed at the URL http://asparagin.cenargen.embrapa.br/phph/ using MEGA

4.0 software. The identity of each sequence was confirmed by comparison with other sequences available at GenBank using BLAST software.

3. Results

Blood smears and PCV could be evaluated for just 12 blood samples (nine *M. gouazoubira* and three *B. dichotomus*) since the remaining nine presented haemolysis during storage in the fridge. Seven out of the 12 blood smears (58.3%) presented erythrocytes infected with protozoa in the form of small trophozoites (<2 μm). The positives blood smears were the free-living *M. gouazoubira*.

However, the infected animals presented low parasitemia, which varied in the range 0.0125–0.200%. The mean PCV value for *M. gouazoubira* was 30.6% (interval 17–50%), whilst the mean value for *B. dichotomus* was 27%.

According to the nPCR assays, 15 (71.4%) of the 21 blood samples (13 from *M. gouazoubira* and two from *B. dichotomus*) were infected with hemoprotozoa. BLAST analysis of the amplicon sequences showed that the protozoan DNA extracted from one *B. dichotomus* and nine *M. gouazoubira* samples presented high similarity with *T. cervi* DNA (AY735135.1), namely, MGI12 (accession number HM466922) (99%), MGI2 (accession number HM466923), MGI5 (accession number HM466928), MGI6 (accession number HM466929), MGE1 (accession number HM466930), MGZBH1 (accession number HM466926) and BDZBH3 (accession number HM466927) (98%), MGI3 (accession number HM466923) (97%), MGI8 (accession number HM466925) (96%) and MGI11 (accession number HM466920) (91%). Amplicon sequences from a further three *M. gouazoubira* samples, namely, MGI1, MGI13 and MGI9 (accession number HM466921), exhibited 97 to 98% similarity with *Theileria* sp. (FJ668374.1), whilst that from *M. gouazoubira* sample MGE2 (accession number HM466918) presented 99% similarity with *B. bigemina* (EF458206.1). The amplicon sequence from *B. dichotomus* sample BDZBH4 (accession number HM466919) exhibited 96% similarity with *Babesia bovis* (EF458215.1).

Nested PCR assays of the pools of tick salivary glands showed negative although the control was positive.

Although the nested PCR primers had been designed based on *Babesia* sequences, the sequencing from the amplified products showed that all these sequences share some homology, and by the blast search it was shown, undoubtedly, that these sequences came from different organisms. Actually, these results were serendipity founds, as we were searching for *Babesia* species. After the products had been identified as *Theileira*, the sequences between *Babesia* and *Theileiria* were aligned, showing that they present homology to the primers region.

There was general concordance between the results of the nPCR assays and those of blood smears, in that the nPCR-positive samples MGI5, MGI8, MGI11, MGI12, MGE1 and MGE2 were also positive for the presence of hemoprotozoa in the blood smears. In the case of the adult female *M. gouazoubira* MGI11, for example, the blood smear revealed the presence of trophozoites and the animal also exhibited accentuated anaemia (PCV = 17%). As expected, the nPCR-negative samples MGI7, MGZBH2, BDZBH1 and BDZBH2 were also negative for the presence of trophozoites in the blood smear.

Regarding the occurrence of ectoparasites, the majority of the free-living animals were infested with ticks of the species *R. microplus* (5/15; samples MGI2, MGI3, MGI9, MGI11, MGI12), *D. nitens* (5/15; samples MGI8, MGI9, MGI11, MGI12, MGE1) and *A. cajennense* (4/15; samples MGI2, MGI3, MGI11, MGI12). Indeed, from one free-living *M. gouazoubira* presenting intraerythrocytic trophozoites in the blood smear, a total of seven larvae and three engorged nymphs of *Amblyomma* sp. were collected. In contrast, the captive animals maintained at the Fundação Zoobotânica de Belo Horizonte were tick-free.

4. Discussion

Although various species of *Theileria* are known to infect domestic and wild animals, *Theileria* (*Babesia*) *equi* is the only hemoparasite of this description to have been reported in Brazil so far. Moreover, whilst the occurrence of *T. cervi* in North American cervids has been widely reported (Kreier et al., 1962; Laird et al., 1988; Waldrup et al., 1989; Kocan and Kocan, 1991), the present study constitutes the first evidence of *T. cervi* infection in South American cervids. Indeed, since 47.6% (10/21) of the study population exhibited nPCR-positive samples, it is probable that the incidence of infection by *T. cervi* amongst the populations of *M. gouazoubira* and *B. dichotomus* is high. In this context, it is important to know if the cervid population are hemoparasite carriers since, when exposed to stress, the animals may become immunosuppressed thus favouring the emergence of the clinical signs of parasitism. The incidence of anaemia (PCV of 17%) in one of the animals with parasitemia indicates that clinical manifestations of *T. cervi* infection could occur after capture and handling of *M. gouazoubira* and *B. dichotomus* cervids.

The vector for *T. cervi* in cervids of North America is *A. americanum*, a species of tick whose presence has not been reported in South America. One of the key research objectives of our laboratory is to identify hemoparasites in the salivary glands of ticks that infest wild animals, and especially cervids, that may represent sources of disease for ruminants of economical importance. In the present study, therefore, DNA was extracted from the salivary glands of nymphs and adults of *A. cajennense*, a tick that is commonly found amongst wild Brazilian cervids, in order to assay for the presence of *Theileria* sp. Although all samples derived from *A. cajennense* were negative for *Theileria* sp., the tick cannot be rejected as a transmission vector for the hemoparasite since only a small number of samples were examined and the detection of *Theileria* sp. DNA was subject to various technical limitations. Studies employing molecular and morphological techniques are currently taking place in our laboratory in order to caste more light onto this subject and to establish the epidemiology of *Theileria* infection.

South America is considered to be an endemic area for *Babesia* spp., and especially the ruminant infecting species *B. bigemina* and *B. bovis* that cause high morbidity and mortality in cattle. However, the number of reports involving cervids affected by babesiosis in this region is somewhat

small (Deem et al., 2004; Duarte, 2006; Villas-Boas et al., 2009) in comparison with North America where the occurrence of the disease is considerably higher (Emerson and Wright, 1968; Waldrup et al., 1989, 1992; Holman et al., 2000; Cantu et al., 2007, 2009). The present study revealed that only two of the animals studied (9.5%) were nPCR-positive for *B. bigemina* or *B. bovis*, although 23.8% of the population were infested by *R. microplus*. The incidence of parasitic infection reported here is lower than values reported previously for Brazilian cervids (Machado and Müller, 1996; Duarte, 2007; Villas-Boas et al., 2009). Although our sample population was rather small, the results are relevant because of the close proximity between domestic and wild ruminants (Duarte, 2006). According to Duarte (2007), the prevalence of hemoparasites in cervids that inhabit conservation areas (implying an absence of contact with domestic ruminants) reflects the real sanitary situation of the wild population, whilst the occurrence of hemoparasites in cervids that live close to farms may be influenced by the presence of infectious agents that affect cattle.

Although diagnosis of infection is normally achieved through the examination of blood smears, this method shows poor sensitivity owing to the low level of parasitemia in animals infected with *T. cervi*. Furthermore, the differential diagnosis between *T. cervi* and *B. bovis* by direct blood examination is not facile even though these hemoparasites do present distinctive morphological characteristics, for example, the chromatin in *Theileria* trophozoites appears in the form of a cap or demilune covering the pole and extending down the sides, whereas in *Babesia* trophozoites it is normally rounded or extending down one side only (Kreier, 1977). Generally, therefore, the direct method is not reliable enough for distinguishing between these hemoparasites, and most especially when both species occur together in endemic areas. In the present study, nPCR was shown to be very sensitive and should, therefore, be employed in the laboratory analysis of blood derived from wild animals. This type of procedure will provide more consistent data for mapping the distribution of hemoparasites that affect the wild fauna of Brazil.

5. Conclusion

In a population of wild and captive cervids, 71.4% of the animals were infected with hemoprotozoa, including *T. cervi* (47.6%), *Theileria* sp. (14.3%), *B. bovis* (4.8%) and *B. bigemina* (4.8%). Only one of the wild infected cervids exhibited accentuated anaemia (PCV = 17%). This is first report of the occurrence of *Theileria* spp. in Brazilian cervids.

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