Detection of *Plasmodium* sp. in capybara

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Abstract

In the present study, we have microscopically and molecularly surveyed blood samples from 11 captive capybaras (*Hydrochaeris hydrochaeris*) from the Sanctuary Zoo for *Plasmodium* sp. infection. One animal presented positive on blood smear by light microscopy. Polymerase chain reaction was carried out accordingly using a nested genus specific protocol, which uses oligonucleotides from conserved sequences flanking a variable sequence region in the small subunit ribosomal RNA (ssrRNA) of all *Plasmodium* organisms. This revealed three positive animals. Products from two samples were purified and sequenced. The results showed less than 1% divergence between the two capybara sequences. When compared with GenBank sequences, a 55% similarity was obtained to *Toxoplasma gondii* and a higher similarity (73–77.2%) was found to ssrRNAs from *Plasmodium* species that infect reptile, avian, rodents, and human beings. The most similar *Plasmodium* sequence was from *Plasmodium mexicanum* that infects lizards of North America, where around 78% identity was found. This work is the first report of *Plasmodium* in capybaras, and due to the low similarity with other *Plasmodium* species, we suggest it is a new species, which, in the future could be denominated ‘‘*Plasmodium hydrochaeri’’*.

Keywords: *Plasmodium*, Capybaras, PCR, Brazil.

1. Introduction

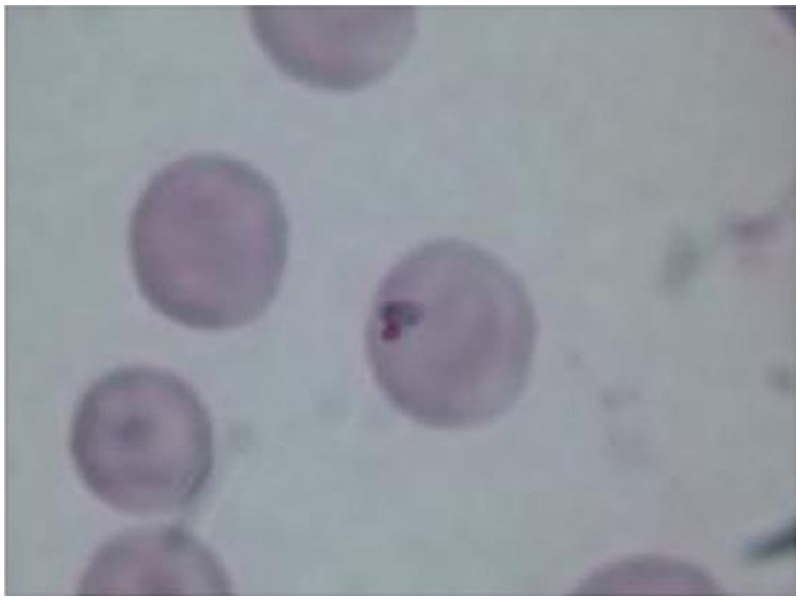
The causative agent of malaria belongs to the genus *Plasmodium*, a protozoan of the Apicomplex phylum, which includes more than 100 species and infects a great number of vertebrates, including humans, primates, rodents, birds and reptiles (Warrel, 2002). Rodent *Plasmodium* species are extensively used as a model to study malaria in experimentally infected laboratory mice (Landau and Gautret, 1998). Naturally occurring disease in rodents by *Plasmodium* sp. has only been reported in the African rodents, thicket rats of the genera *Thamnomys*, *Grammomys* and *Praomys* and the brush-tailed porcupine *Atherurus africanus* (Perkins et al., 2007). Capybaras (*Hydrochaeris hydrochaeris*) are the biggest rodents on earth and naturally occur in humid and densely vegetated areas of South America. Their semi-aquatic habitats (Oliveira and Bonvicino, 2006) overlap geographically with those of several mosquitoes that transmit disease (Forattini, 2002). Although this exposure may potentially suggest natural *Plasmodium* infection in capybara, to the author's knowledge there are no reports to date of capybara infected by *Plasmodium* species, either naturally or experimentally.

2. Case report

In March 2007, a wounded free-range capybara was captured and brought to the Veterinary Hospital of the Bela Vista Biological Sanctuary (BVBS) of Itaipu Binational, Southern Brazil. A clinical exam was performed and no other clinical signs found, however, during a routine cell blood count (CBC) at the Environmental Laboratory, an inclusion-like *Plasmodium* trophozoite was found in a blood smear (Fig. 1). The rodent presented CBC within normal range at that time, and was immediately released after wound treatment with no further exam. However, close contact of free-range capybaras with captive ones from the BVBS raised

animal health concerns. Considering that the use of polymerase chain reaction increases the sensitivity of detection of malaria parasites in blood samples compared to microscopy (Conway, 2007), in the present study we have microscopically and molecularly surveyed blood samples of captive capybaras for *Plasmodium* sp. infection.

Fig. 1. *Plasmodium* trophozoite found in a free-range capybara blood smear.



3. Materials and methods

3.1. Sampling and CBC

Eleven healthy captive capybaras from the BVBS Zoo, three males and eight females with ages ranging from 1 to 4 years, were surveyed in this study. Rodents were manually restrained and later anesthetized using 2% xilazine chloridrate (0.5 mg/kg) and 10% ketamine chloridrate (7 mg/kg). Blood samples were obtained from a femoral vein using vacuum tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant. Thin and thick smears

were immediately made. A blood aliquot was immediately submitted to CBC, and another was frozen for DNA processing. CBC was manually performed using a hemocytometer chamber; other parameters were obtained under standard protocols, as previously described (Santos, 1999). Both thin and thick blood smears were stained with Wright and examined by high magnification light microscopy (1000x). CBC results were compared between infected and non-infected animals through Mann–Whitney U test and P-values <0.05 were considered significant. Statistical analysis was performed using Stata 10.0 (College Station, TX, USA) software.

3.2. DNA extraction and PCR amplification

Thawed samples were centrifuged and the red blood pellet was used for the DNA extraction procedure. Initial lyses were made with 1% saponin, followed by two washes of pellets in ultra pure water and submitted to DNA extraction following manufacturer's protocol (GFX^{TM} Genomic Blood DNA Purification Kit, Amersham Biosciences, Piscataway, NJ, USA). Polymerase chain reaction was carried out according to previously described nested genus-specific protocol (Singh et al., 1999), which use oligonucleotides in conserved sequences flanking a variable sequence region in the small subunit ribosomal RNA (ssrRNA) of all *Plasmodium* organisms. The first amplification reaction was modified in order to increase the sensitivity, considering samples with low parasitemia. Therefore, although original protocol describes primers rPLU1 and rPLU5 in nest 1, we have used primer rPLU6 (Snounou et al., 1993) as reverse primer (named here as rPLU6R: 50-CGT TTT AAC TGC AAC AAT TTT AA-30) instead of rPLU5, with a final fragment of ~600 bp instead of ~1.7 kb. The nest 2 amplification was performed in order to obtain a ~240 bp product (Singh et al., 1999). PCR

products were submitted to electrophoresis in 1% agarose gel and stained with ethidium bromide.

3.3. Sequencing and data analysis of sequences

Amplified products were purified from agarose gels and sequenced using a commercial kit (BigDyeTM terminator mix, Applied Biosystems, Foster City CA, USA) in both directions with rPLU3 and rPLU4 primers. Final consensus sequences were submitted to GenBank using the BLASTN program version 2.2.18+ (Altschul et al., 1997). Alignment with some of the sequences from GenBank (Table 1) was performed using ClustalX version 1.81 (Thompson et al., 1997). Maximum parsimony analysis was performed using the default options of PAUP 4.0b10 (Swofford, 1998), and similarity matrix using Point Replacer v2.0 (http://www.geocities.com/alvesjmp/software.html).

4. Results and discussion

Normal range capybara hematology is still very scarce, with few reports to date. In the present study we used a Portuguese indexed paper on captive capybaras from Southern Brazil (Arouca et al., 2000). The CBC results were mostly within normal range. Differences were observed in the packed cell volume (43.64 ± 2.4%), mean corpuscular volume (123.00 ± 13.23 fl) and mean corpuscular hemoglobin (34.48 ± 5 pg) values, which were above the reference range (45–52%, 126.8–137.5 fl and 39.8–42.9 pg, respectively). There were also differences in neutrophil (53.09% ± 9.72) and eosinophil (0.74% ± 0.45) numbers (reference range 39–52% and 2–5%, respectively). However, reference range literature was also based on a limited number of healthy animals (11). No significant differences were found on CBC

between *Plasmodium* infected and non-infected capybaras. Out of eleven capybaras samples, only the capybara number nine (CAP9) presented positive under light microscopy, with two trophozoites found in the thin blood smear (data not shown). The PCR analysis revealed three positive animals (CAP4, CAP7 and CAP9), including the positive for light microscopy. Our data confirmed the presence of *Plasmodium* in capybara from Parana State, Southern Brazil, and to the author's knowledge this is the first report of this parasite in this rodent. Sequence analysis was performed on PCR products from two positive capybaras: CAP4 (GenBank accession number EU623445) and CAP7 (GenBank EU623446) (for the alignment, see Fig. 3). The results showed less than 1% divergence between the two capybara sequences, an expected value of intraspecific polymorphism in this fragment. We had first analyzed the similarity to *Toxoplasma gondii*, already described in Brazilian capybaras (Yai et al., 2008) and only 55% similarity was obtained with the sequence GenBank EF472967, the most similar ssrRNA sequence found in *T. gondii*. A higher degree of similarity was found to the ssrRNAs of *Plasmodium* species, based on comparison of sequences available to date. Comparison showed 73.8% similarity with species infecting primates, including human beings (*Plasmodium falciparum, Plasmodium malariae, Plasmodium vivax, Plasmodium ovale, Plasmodium reichenowi, Plasmodium fragile, Plasmodium knowlesi, Plasmodium cynomolgi* and *Plasmodium simium*), 73% with species infecting rodents (*Plasmodium berghei, Plasmodium yoelii*, and *Plasmodium chabaudi*), 75% with species infecting birds (*Plasmodium gallinaceum, Plasmodium lophurae*, and *Plasmodium cathemerium*) and 77.2% with species infecting lizards (*Plasmodium mexicanum* and *Plasmodium floridense*) (Fig. 2 and Table 2). The highest similarities of the capybara *Plasmodium* sequences (approximately 78.57 and 77.98%) were to *P. mexicanum*, found in a lizard from North America (GenBank L11716) (Table 3). In mammals, malaria is well documented in murine rodents and experimentally infected primates. However, apart from African rats, porcupines (Perkins et

al., 2007) and primates in South America, Asia and Africa (Gysin, 1998), there are few reports of Old World ungulates, bats and squirrels as natural *Plasmodium* hosts (Garnham and Kuttler, 1980). The last paper reporting *Plasmodium* infection in a non-rodent or non-primate mammal was described in the white-tailed deer from Texas, USA. Therein, the authors state that *Plasmodium odocoilei* was the only species described in ungulates in the New World (Garnham and Kuttler, 1980). More recently, *Plasmodium* sp. was encountered in a small rodent from Africa (Opara and Fagbemi, 2008), but there are no records of malaria in rodents from the New World. As there are no *Plasmodium* species described in the rodent genus *Hydrochaeris*, and since we found low similarity in the ssrRNA fragment sequenced of *Plasmodium* from capybara when compared to many other *Plasmodium* species available sequences, we suggest that the *Plasmodium* sp. occurring in the capybaras is a new species that, in the future could be denominated as ‘‘*Plasmodium hydrochaeri*’’. Further epidemiological, morphological, life cycle and genetic studies should be conducted using material from naturally infected capybaras to confirm this.

Fig. 2. Phylogenetic relationship between 17 *Plasmodium* species (see Table 1) and two isolates obtained from capybaras inferred from the ssrRNA fragment. The tree is derived by the maximum parsimony method from the similarity matrix shown in Table 3.

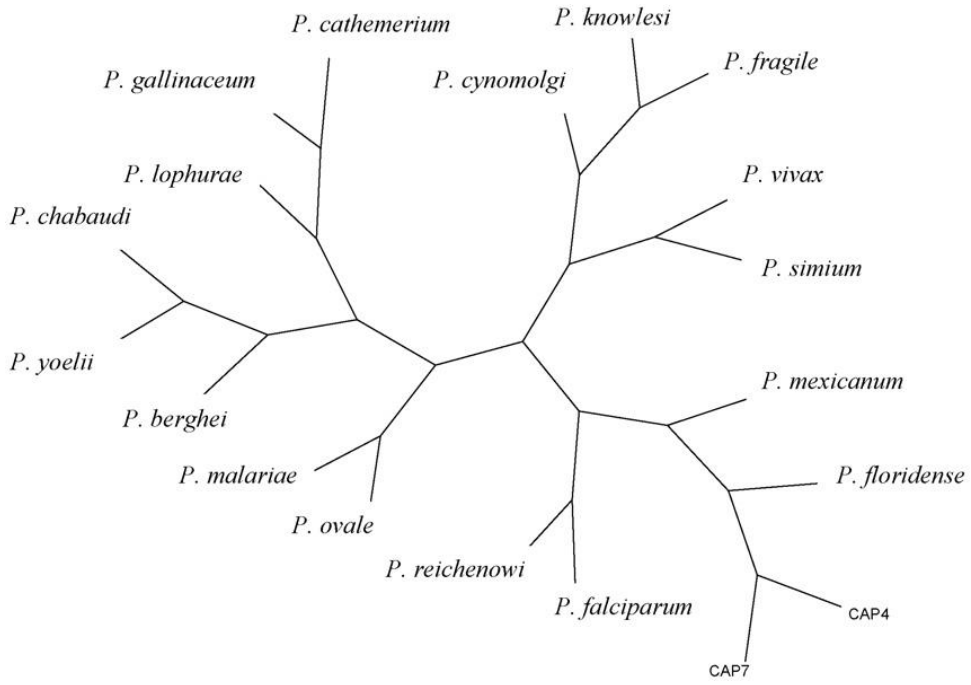


Table 1

GenBank accession numbers of the ssrRNA sequences from different *Plasmodium* species used in the alignment with the capybara sequences.

Accession no.	Species	Group	(host)	Geographic distribution
AL929354	*P. falciparum*	A	(Human)	Tropics worldwide
AF488000	*P. malariae*	A	(Human)	Tropics worldwide
DQ660817	*P. vivax*	A	(Human)	Tropics worldwide
AB182493	*P. ovale*	A	(Human)	African tropics and Pacific
Z25819	*P. reichenowi*	A	(Chimpanzee)	African tropics
M61722	*P. fragile*	A	(Monkey)	Asian tropics
AY580317	*P. knowlesi*	A	(Monkey)	Asian tropics
L07559	*P. cynomolgi*	A	(Monkey)	Asian tropics
PSU69605	*P. simium*	A	(Monkey)	New World tropics
M14599	*P. berghei*	C	(Rodent)	African tropics
AF180727	*P. yoelii*	C	(Rodent)	Africa
DQ241815	*P. chabaudi*	C	(Rodent)	Africa
M61723	*P. gallinaceum*	E	(Bird)	Asian
X13706	*P. lophurae*	E	(Bird)	Old World
AY625607	*P. cathemerium*	E	(Bird)	Between Asia and Australia
L11716	*P. mexicanum*	D	(Lizard)	North America
L11717	*P. floridense*	D	(Lizard)	North and Central America

A, primates (including human beings); C, rodents; D, reptiles and E, avian.

Table 2

Similarity percentage between the ssrRNAs sequences of *Plasmodium* species found in different groups of hosts.

Group of hosts	A	B	C	D	E
A	89.4				
B	73.8	99.4			
C	82.5	73	95.9		
D	73.9	77.2	76.9	73.8	
E	81.2	75	84.5	75.9	92.7

A, primates (including humans beings); B, capybaras; C, rodents; D, reptiles and E, avian.

Table 3

Similarity percentage between the ssrRNAs sequences of *Plasmodium* species described in Table 1.

	Psi	Pvi	Pkn	Pfr	Pcy	CAP4	CAP7	Pov	Pma	Pyo	Pbe	Pch	Pme	Pfl	Pga	Pca	Plo	Pfa
Psi																		
Pvi	98.84																	
Pkn	85.55	86.63																
Pfr	84.57	85.63	94.77															
Pcy	82.66	83.72	92.31	91.28														
CAP4	68.54	69.49	74.71	73.45	73.84													
CAP7	67.98	68.93	74.14	72.88	73.26	99.37												
Pov	80.35	81.40	84.62	84.30	84.43	75.15	74.56											
Pma	81.25	82.29	84.21	82.29	84.12	77.06	76.47	89.29										
Pyo	78.86	79.89	80.70	79.89	81.07	73.53	72.94	85.03	85.80									
Pbe	78.86	79.89	81.29	81.61	82.25	74.12	73.53	86.83	86.39	98.16								
Pch	76.57	77.59	80.12	79.89	80.47	72.35	71.76	83.83	84.02	95.09	94.48							
Pme	73.03	74.01	79.19	76.84	78.49	78.57	77.98	78.82	83.43	82.35	82.94	80.59						
Pfl	63.89	64.80	68.75	67.04	68.79	76.47	75.88	70.35	73.26	71.51	71.51	72.67	73.84					
Pga	76.84	77.84	79.19	76.70	78.95	75.88	75.29	81.07	84.62	86.31	85.71	83.93	82.94	73.26				
Pca	78.98	80.00	80.23	79.43	81.18	73.53	72.94	82.74	84.52	84.52	85.12	83.33	80.00	69.19	93.45			
Plo	78.53	79.55	79.77	78.98	80.12	76.47	75.88	83.43	85.21	83.93	84.52	82.74	80.00	69.77	92.31	92.26		
Pfa	77.71	78.74	84.80	83.91	84.62	77.19	76.61	87.43	85.80	86.83	87.43	86.23	80.70	74.42	85.21	83.93	84.02	
Pre	78.86	79.89	86.55	85.63	85.80	77.78	77.19	89.22	85.80	85.63	86.23	85.63	81.29	73.84	84.02	83.93	83.43	97.59

Fig. 3. Alignment of the ssrRNA sequences from *Plasmodium* species described in Table 1.

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Psi        TACGTCTTGTGAGCAT-GTACTTGTTAAGCCTTTATAAGAAAAAAGTTAA
Pvi        ..................................................
Pkn        .TTA.T.CT....TG.............-..........G.....T
Pfr        .TTA.T.CT....TG.............-..........G......T
Pcy        .TAACT.CT....TG.......C........-..T.............T
CAP4       .TTA...GT...CAG-....T.....--..G----.--........A.CG
CAP7       .TTA...GT...CAG-...CT.....--..G----.--........A.CG
Pov        .TT..G.CT.AC.T.................-..-.....G.......T.
Pma        ..T..T..T....T......T.........-........G......AT.
Pyo        CCG.AG.AA.T.........T.........-............T-..--
Pbe        .TG.AG.AA.T.........T.........-............T-..--
Pch        CCGAGA.A-.TT........T.........-...........TT-..--
Pme        ..AAAA.AA.A.AT......TC.........-....----......A..G
Pfl        GTTAGACA.ATTTACAG...T.....-..GG-..GA..AGG...G..GC.
Pga        AGGTAA.GAA..A..C....T.........-.................-
Pca        AGGT...GAAA.A..C....T.........-.................-
Plo        AGGT...GT...A..C....T.........-.................-
Pfa        .TTT-A.CT.TG.ATAA...T......G...-.................-
Pre        .TTA.A.CT.AG.ATAA..........G...-.................-
```

```
Psi       T-AAC-TT-AAGGAATGATAACAAAGAAGTGACACATAAAAA-GGACTCG
Pvi       .................................................
Pkn       ...............T.............A....G.---..T...T.AT
Pfr       ...............T.............A....G.-G...T...T.AA
Pcy       ...............T.............A....G---T..T...T-.C
CAP4      AC.CAA..C...................ACA....G---T.-TAA.TGAA
CAP7      AC.CAA..C...................ACA....G---T.-TAA.TGAA
Pov       C...............T.............A.....---T..TAAGT.TA
Pma       .T..............-.............A.....---T..TAA.T.TC
Pyo       .T..-...A.......T.............A.....---T..TAT.T.TA
Pbe       .T..-...A.......T.............A.....---T..TAT.T.TA
Pch       .T..-...G.......T.............A.....---T..TAT.T.TA
Pme       .TT.AA..A.......T.............A....G---T..TAA.T.AT
Pfl       .A.TAACAC.....G.............ACA....G---T.-TAA.T-AT
Pga       .T..-T..........T............CA.....---T..TAA.ACTT
Pca       .T..............T............GA.....---T..T...ACTC
Plo       CT.....-A.......T............CA.....---T..TAA.GCT.
Pfa       .T..............T.............A....G---T..TAA.T.TA
Pre       .T..............T.............A....G---T..TAA.T.TA
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```
Psi         TCCC---ATTTTCT-AGTGTGTATCAATCGAGTTTCTGACCTATCAGCTT
Pvi         ..................................................
Pkn         .-.........T.....................................
Pfr         CT.........T.T...................................
Pcy         GT.........T-....................................
CAP4        .AATTT....----...................................
CAP7        .AATTT....----...................................
Pov         C.TT.......---...................................
Pma         G-ATTT.....---...................................
Pyo         .TAT.......---...................................
Pbe         CTAT.......---...................................
Pch         CTAT.......---...................................
Pme         --ATTT....A---...................................
Pfl         .TATTTT...A---...................................
Pga         .GTTTT.....---...................................
Pca         CGTTTC.....---...................................
Plo         CGT.TT.....---...................................
Pfa         .TTT.......---...................................
Pre         .TTT.......---...................................
```

```
Psi        TTGAATGTTA-GGGTAT-GGCCTAACATGGCT
Pvi        ....-...........T.............
Pkn        ....-...........T.............
Pfr        ....-.....C.....T.............
Pcy        ....-...........T.............
CAP4       ....-......A.....T.............
CAP7       ....-......A.....T.............
Pov        ....-...........T.............
Pma        ....-...........T.............
Pyo        ....-...........T.............
Pbe        ....-...........T.............
Pch        ....-...........T.............
Pme        ....-...........T.............
Pfl        ....-...........T.............
Pga        ....-...........T.............
Pca        ....-...........T.............
Plo        ....-...........T.............
Pfa        ....-...........T.............
Pre        ....-...........T.............
```

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