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Occurrence of *Ehrlichia canis* in free-living primates of the genus *Callithrix*

Ocorrência de *Ehrlichia canis* em primatas de vida livre pertencentes ao gênero *Callithrix*

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Abstract

Bacteria of the genus *Ehrlichia* are Gram-negative and coccoid-shaped microorganisms that cause ehrlichiosis – a serious infectious disease that often leads to death. These bacteria present a strong zoonotic potential and primates may act as reservoir hosts. This study involved a molecular analysis to detect these microorganisms in blood samples collected from nineteen primates of the genus *Callithrix* living free in an Atlantic Forest fragment in the municipality of Viçosa, state of Minas Gerais, Brazil. One of the 19 primates was found to be infected with *Ehrlichia canis*. This finding points to a new wild host of *E. canis* with a strong potential for transmission to humans because of its increasing contact with people. This is the first report of *Ehrlichia* spp. in primate of the genus *Callithrix*.

Keywords: *Ehrlichia*, *Callithrix*, marmoset, ticks.

Resumo

Bactérias do gênero *Ehrlichia* são gram-negativas em forma de cocos. Provocam uma doença infecciosa grave denominada erliquiose que, muitas vezes, causa morte. Essas bactérias apresentam grande potencial zoonótico, e os primatas podem agir como reservatórios. Este estudo objetivou a detecção molecular desse microorganismo em amostras de sangue coletadas de dezenove primatas de vida livre num fragmento de Mata Atlântica pertencentes ao gênero *Callithrix*, no município de Viçosa, Minas Gerais, Brasil. Entre os 19 espécimes de sagui, um estava infectado com *Ehrlichia canis*. Esse achado aponta para um novo hospedeiro selvagem para *E. canis* com grande potencial de transmissão ao homem, devido ao seu crescente contato com pessoas. Este é o primeiro relato de primata do gênero *Callithrix* infectado com *Ehrlichia* spp.

Palavras-chave: *Ehrlichia*, *Callithrix*, sagui, carrapatos.

Ehrlichiosis is transmitted to humans and animals by tick bites and is considered an emerging health problem. *Ehrlichia* bacteria are preserved in nature through the infection of mammals.

Classified as a Gram-negative α -proteobacterium, *Ehrlichia* is an obligate intracellular parasite currently found throughout the world, with ten species described so far: *E. canis*, *E. chaffeensis*, *E. ewingii*, *E. ruminantium*, *E. muris*, *E. equi*, *E. phagocytophila*, *E. risticii*, *E. ruminantium* and *E. sennetsu*. Among these ten species, the first three and *E. mineirensis* (non-validated species) have been reported in Brazil, all with zoonotic potential (CRUZ et al., 2012; COWDRY, 1925; DUMLER et al., 2001;

FOGGIE, 1949; HOLLAND et al., 1985; LEWIS et al., 1975; VIEIRA et al., 2011; WEN et al., 1995).

The increasingly close contact between humans and wildlife populations has facilitated the spread of many infectious and parasitic agents to new hosts and environments, establishing new host-parasite relationships and new ecological niches in the chain of transmission of diseases (POGGIANI & OLIVEIRA, 1998). Among the factors that have led to this situation is the expansion of agriculture and animal husbandry to “conservation areas,” favoring the contact of domestic animals and humans with wild animals. Marmosets of the genus *Callithrix* are a diverse group of platyrrhine primates that are part of the Callitrichidae family. These small primates, who live in forest fragments adjacent to urbanized areas, have come into increasing contact with humans and domestic animals, creating a permanent conflict between

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human needs and wildlife habitats and health. This situation has been aggravated by the involvement of wild animals in emerging and unknown epidemiological chains, showing a potential risk to public health (FOWLER, 1986). To date, the literature contains no report about the occurrence of *Ehrlichia* infection in marmosets. Therefore, this paper describes and characterizes the first detection of *E. canis* in free-living primates of the genus *Callithrix*.

This study, which was conducted from March 2010 to January 2012, involved animals living in an urbanized area called Vila Gianetti, in a small preserved natural fragment of the Atlantic Forest biome ($-20^{\circ} 45' 16.59''$, $-42^{\circ} 52' 19.80''$) located on the campus of the Federal University of Viçosa, in the municipality of Viçosa, state of Minas Gerais, in southeastern Brazil ($20^{\circ} 45' S$, $42^{\circ} 52' W$).

We analyzed blood samples drawn from 19 marmosets belonging to four wild native groups. The marmosets were caught using a multiple-entry trap, weighed, and anaesthetized with ketamine hydrochloride (Vetaset, USA) at a dose of 10 mg/kg of body mass, and xylazine hydrochloride (Anasedan, Brazil) at a dose of 0.5 mg/kg of body mass (CARPENTER & MARION, 2013). The skin and fur of all the animals were inspected for ectoparasites using a magnifying glass. After drawing the blood samples, the marmosets were kept in a warm dark room until they recovered from the effects of anesthesia. Four hours later, the animals were released into the same site where they were captured. Blood samples collected with anticoagulant were stored at $-20^{\circ} C$ and the DNA was extracted using QIAmp[®] DNA Mini Kit (Qiagen, USA), according to the manufacturer's instructions. After DNA extraction, the DNA samples were stored at $-20^{\circ} C$ for further analysis. Real time PCR analysis was performed in a step one real-time PCR system (Applied Biosystems, USA), using the primers ER-R1 (5'-GGAGGTAATGCACCAGCC-3') and ER5-3 (5'-GTTAGAGTTCCTTGATGG-3') which identify the specific 16S ribosomal gene for *Ehrlichia* genus organisms (INAYOSHI et al., 2004). *E. canis* DNA and deionized sterile water were used as positive and negative controls, respectively. Ct values above 35 were considered negative. Positive samples were analyzed further by nested PCR to confirm *Ehrlichia* species, also using 16S rRNA gene primers. For the first reaction we used the genus specific primer set ECC (5'-CGTATTACCGCGGCTGCTGGCA-3') and ECB (5'-AGAACGAACGCTGGCGGCAAGCC-3') (DAWSON et al., 1994). For the second reaction we used EC-F (5'-CAATTATTTATAGCCTCTGGCTATAGGAA-3') (YABSLEY et al., 2004) and HE-3 (5'-CAATTGCTTATAACCTTTTGGTTATAAAT-3') (ANDERSON et al., 1992), which are known as specific primers for *E. canis*. The PCR reaction was confirmed by electrophoresis, using 1.5% agarose gel stained with ethidium bromide. To confirm the species in the amplified product, the sample was subjected to sequencing analysis (Macrogen Inc., South Korea), followed by bioinformatics analysis by BLAST (ALTSCHUL et al., 1990), comparing it with *Ehrlichia* DNA sequences deposited in GenBank. The nucleotide sequence obtained was deposited in GenBank under access number KC822950.1, identified by the name *Ehrlichia canis* isolate UFV1. A phylogenetic analysis was performed with the aid of MrBayes 3.2 application, using

Bayesian inference and the Monte Carlo method, with 2,000,000 generations, containing 4 chains and 2 runs (nchains= 4; nrns= 2) (RONQUIST et al., 2012). The sequences were analyzed by comparison, using MEGA5 (TAMURA et al., 2011). ClustalW (THOMPSON et al., 1994) and FigTree v1.3.1 (RAMBAUT, 2008) applications for alignment and visualization of the phylogenetic tree. *Anaplasma bovis* isolate CFT-27 (2009) gene for 16S ribosomal RNA (accession number AB723715.1) was used as the outgroup. To compose the dataset, we selected several *Ehrlichia* 16S ribosomal gene sequences deposited in GenBank, as illustrated in Figure 1.

Only one of the analyzed samples was PCR positive for *Ehrlichia canis* species. The amplified product was sequenced and subjected to BLAST analysis, showing 100% similarity with several *E. canis* sequences stored in GenBank. A Bayesian inference phylogenetic tree was constructed using the nucleotide substitution model GTR + I + G selected by the Akaike Information Criterion (AIC) (Figure 1). Two well-divided clusters were observed in this phylogenetic tree, one of them containing *E. canis* and *E. ovina* sequences, while the other cluster contained *E. ewingii*, *E. muris*, *E. ruminantium* and *E. chaffeensis*, showing 78% of posterior probability.

Monkeys are proven transmitters and hosts of many infectious agents, including a wide range of viruses and bacteria, and are susceptible to some infections common to humans (ANDRADE et al., 1999). The presence of *E. canis* in marmosets of the genus *Callithrix* sp. described here demonstrates the susceptibility of this host, which has not heretofore been listed as a possible accidental host, natural reservoir or even amplifier of *E. canis*. The groups of marmosets of this study have lived for at least three decades in an area of 75 hectares of an urban forest fragment, and have had some level of direct contact with humans through touching, by humans giving them food (bananas and cookies) and by scavenging in garbage. In free-ranging marmosets, patterns of disease acquisition may be influenced by habitat disturbance and fragmentation and by proximity to human settlements.

The phylogenetic analysis revealed that the genotype of *E. canis* found in the marmoset is very similar to other genotypes previously identified in several hosts, such as domestic dogs (*Canis lupus familiaris*), wild cats (*Prionailurus bengalensis euptilurus*), iriomote cats (*Prionailurus iriomotensis*), small spotted cats (*Leopardus tigrinus*), ocelots (*Leopardus pardalis*) and ticks (*Rhipicephalus sanguineus*) in various countries, including Brazil, Tunisia, Thailand, Cape Verde, Taiwan, Italy, Malaysia, Philippines, India and Romania (Figure 1).

This range of hosts demonstrates the complex relationship of the ecology of this bacterium found in different vertebrates and the risk it poses to marmosets, and to humans and domestic animals in close contact with these primates. This close association could facilitate the transmission of ehrlichiosis to humans through brown tick bites, *R. sanguineus*, from domestic dogs, and its parasitism on humans has been reported in South America (GUGLIELMONE et al., 2006). These ticks occasionally infest wild animals (DANTAS-TORRES et al., 2010). However, the brown tick parasitizing marmosets was heretofore unknown. Because ehrlichiosis is a zoonosis (CALIC et al., 2004), greater

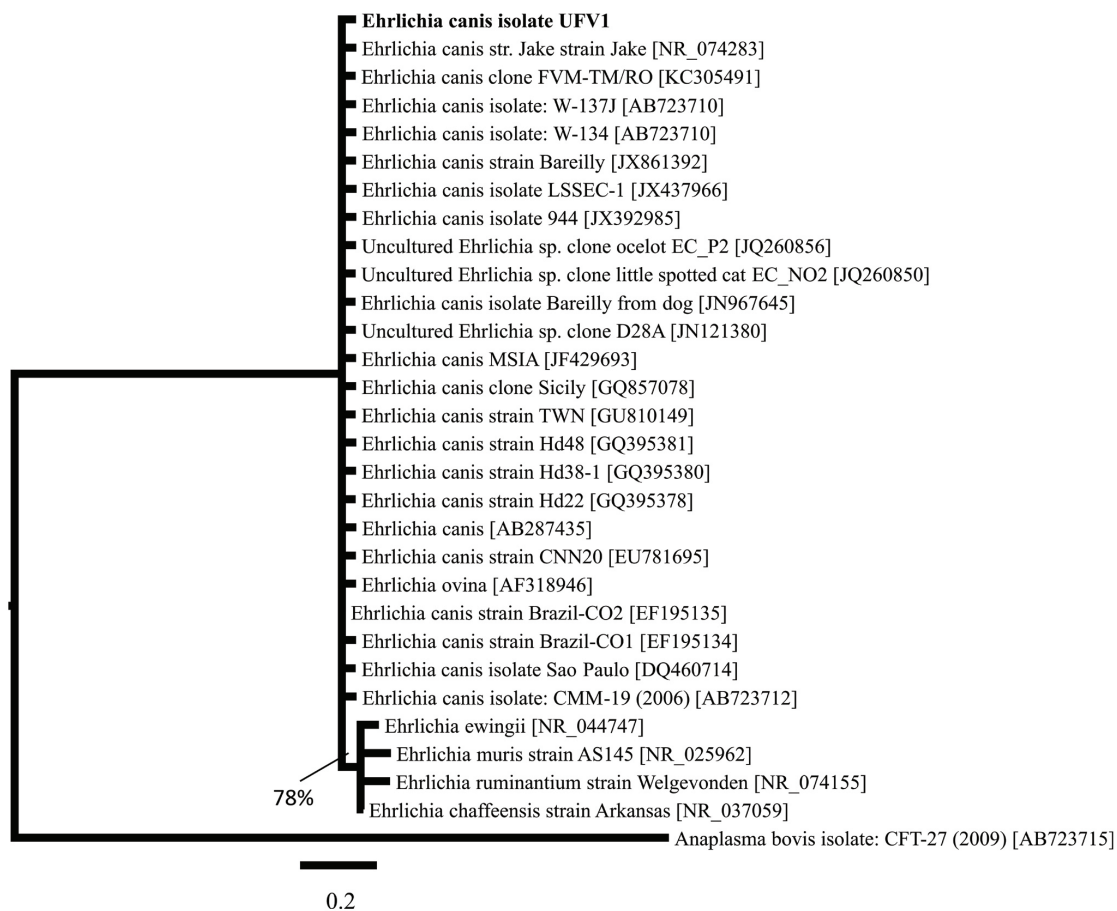


Figure 1. Phylogenetic tree by Bayesian inference for the 16S rRNA gene of *Ehrlichia* bacteria. A 2,000,000 generations were required for convergence using the model GTR + I + G. The discovery in the study sample is bold. The number indicates the percentage posterior probability. The accession numbers are shown in brackets. *Anaplasma bovis* was used as outgroup.

attention and vigilance is needed in areas where these small mammals are distributed, as well as additional studies on the health of wild animals that may participate in the peri-urban or urban cycle of this agent. With regard to the possible vector or vectors of the ehrlichiosis agent involved in the marmoset population under study, it should be noted that no ectoparasites were found during the capture campaigns.

We emphasize the importance for further studies aimed at gaining a better understanding of the natural history of this agent in interactions such as this one, and on determining whether or not *Callithrix* sp. primates are effectively hosts or reservoirs of ehrlichiosis amplifiers. To date, the scientific literature contains no reports about marmosets infected by *Ehrlichia* species. In this paper, we describe the detection and characterization of *E. canis* infection in free-living marmosets of the genus *Callithrix*. In view of these findings, there is an evident need for more studies to evaluate the role of this primate as a possible host and/or amplifier of *Ehrlichia* organisms and related agents in the wild and in peri-domiciliary environments, which pose a risk for domestic and wild animals and for humans.

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