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Genetic diversity of *Ehrlichia canis* strains from naturally infected dogs in Rio de Janeiro, Brazil

Diversidade genética de cepas de *Ehrlichia canis* encontradas em cães naturalmente infectados no Rio de Janeiro, Brasil

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Abstract

The aim of this study was to characterize *Ehrlichia canis* strains from naturally infected dogs in Rio de Janeiro, Brazil. In addition, all the clinical and hematological findings observed in these dogs were reported. PCR targeting the 16S rRNA gene was used for diagnostic purposes, and the *TRP*19 and *TRP*36 genes were sequenced to evaluate the genetic diversity. Fifteen samples were positive for *E. canis*. The polymerase chain reaction for the *TRP*19 gene resulted in 11 amplicons (11/15), which were cloned into the pGEM-T easy vector for sequencing. The complete sequence of *TRP*19 gene was compared to those in the GenBank, revealing high identicalness. Phylogenetic analysis on the *TRP*36 gene sequences demonstrated two distinct strains from two dogs, named 56C and 70C. The 56C strain was grouped with the strain Cuiaba 16, which is a hybrid strain formed by Brazilian and US genogroups; and the 70C strain was grouped with other strains of the US genogroup, thus suggesting that there are at least two genogroups of *E. canis* in Rio de Janeiro (US and Brazilian). Those animals, in which the 70C and 56C strains were isolated, showed distinct clinical and hematological manifestations of [1]the disease. The appearance of different genotypes may express new phenotypes, thus resulting in different forms of presentation of the disease and making its diagnosis more complex.

Keywords: Dogs, genogroups, *TRP19*, *TRP36*, CME.

Resumo

O objetivo deste estudo foi caracterizar as cepas de *Ehrlichia canis* em cães naturalmente infectados no Rio de Janeiro, Brasil. Além disso, os achados clínicos e hematológicos observados nos cães foram relatados. O gene 16S rRNA foi utilizado como alvo da PCR para fins diagnósticos, e os genes *TRP*19 e *TRP*36 para avaliar a diversidade genética. Quinze amostras foram positivas para *E. canis*. PCR para o gene *TRP*19 produziu 11 amplicons (11/15) que foram clonados no pGEM-T easy vector para sequenciamento. A comparação das sequências completas do gene TRP19 com outras sequências depositadas no GenBank revelou uma alta identidade. Duas amostras (56C e 70C) após o ensaio da PCR, tendo como alvo o gene *TRP*36, geraram sequências, e a análise filogenética mostrou que a cepa 56C foi agrupada com a cepa Cuiabá 16, que é uma cepa híbrida, formada pelo genogrupo Brasileiro e o genogrupo US; e a cepa 70C agrupou com as outras cepas do genogrupo US, sugerindo a existência de pelo menos dois genogrupos de *E. canis* no Rio de Janeiro (US e Brasileiro). Esses animais apresentaram manifestações clínicas e hematológicas distintas, e diferentes genótipos podem expressar novos fenótipos, resultando em diferentes formas de apresentação da doença e fazendo com que o diagnóstico seja mais complexo.

Palavras-chave: Cães, genogrupos, *TRP19*, *TRP36*, EMC.

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Introduction

Ehrlichia spp. is an α-proteobacterium belonging to the order Rickettsiales that causes diseases of veterinary importance and is also responsible for emerging life-threatening anthropozoonoses. *E. canis* is a small Gram-negative, obligate intracellular dimorphic bacterium transmitted by the brown dog tick, *Rhipicephalus sanguineus* (RISTIC; HUXSOLL, 1984).

The glycoproteins TRP36 and TRP19 are species-specific major immune reactive proteins and appear to be important targets for the host immune response, attachment to the host cell and other potentially significant roles in the pathobiology of *Ehrlichia*. The genes encoding the TRP36 protein (~ 840 bp) and TRP19 protein (414 bp) have frequently been studied and have been used to identify different variations within the *E. canis* species (CARDÉNAS et al., 2007). The *TRP*19 gene is highly conserved, and thus has great potential for development of vaccines and sensitive serological tests when correlated with this protein (DOYLE et al., 2006). The TRP19 protein has been correlated with the acute (or early) immune response in dogs (McBRIDE et al., 2003) and presents reactivity that is also demonstrated in GP200, another immunoreactive protein present in *E. canis* (CARDÉNAS et al., 2007).

The TRP36 antigen is an acute-phase glycoprotein that is exposed on the bacterial surface and is secreted into the host cytoplasm (McBRIDE et al., 2003). It is an orthologue of the protein TRP47 of *E. chaffeensis* and has been used to differentiate between the variations that exist in the specie *E. canis* (DOYLE et al., 2005). Differences in the *TRP*36 gene have been reported, thus indicating some degree of *E. canis* diversity in nature, and studies have suggested that TRP36 is useful for genotyping *E. canis* strains based on differences in tandem repeat number or sequences (HSIEH et al., 2010; KAMANI et al., 2013).

In Brazil, the gene encoding the protein TRP36 has been used for bacterial typing, and new genogroups have emerged along with evidence of genetic recombination between different strains of *E. canis* (AGUIAR et al., 2013).

The aim of this study was to characterize *E. canis* strains from naturally infected dogs in Rio de Janeiro, Brazil, using a polymerase chain reaction (PCR) to target the *TRP*19 and *TRP*36 genes, and after sequencing, to perform bacterial typing. In addition, the clinical and hematological findings observed in dogs infected with *E. canis* strains from this study were reported.

Materials and Methods

Blood collection

A total of 300 blood specimens from client-owned dogs (146 male and 154 female) were obtained from veterinary clinics and home visits. These specimens were obtained from rural areas (100 each from the municipalities of Cachoeiras de Macacu and Maricá) and urban areas (100 from the municipality of Duque de Caxias) in the state of Rio de Janeiro (Figure 1) and were named according to the identification number of the animal followed by the initial letter of the municipality where it was collected (C = Duque de Caxias; M = Maricá; CM = Cachoeiras de Macacu).

All the dogs were recruited at private veterinary clinics. Animals previously subjected to antibiotic therapy during the past four weeks were excluded from this study. Most samples (245/300; 81.1%) were from dogs more than one year old. The population

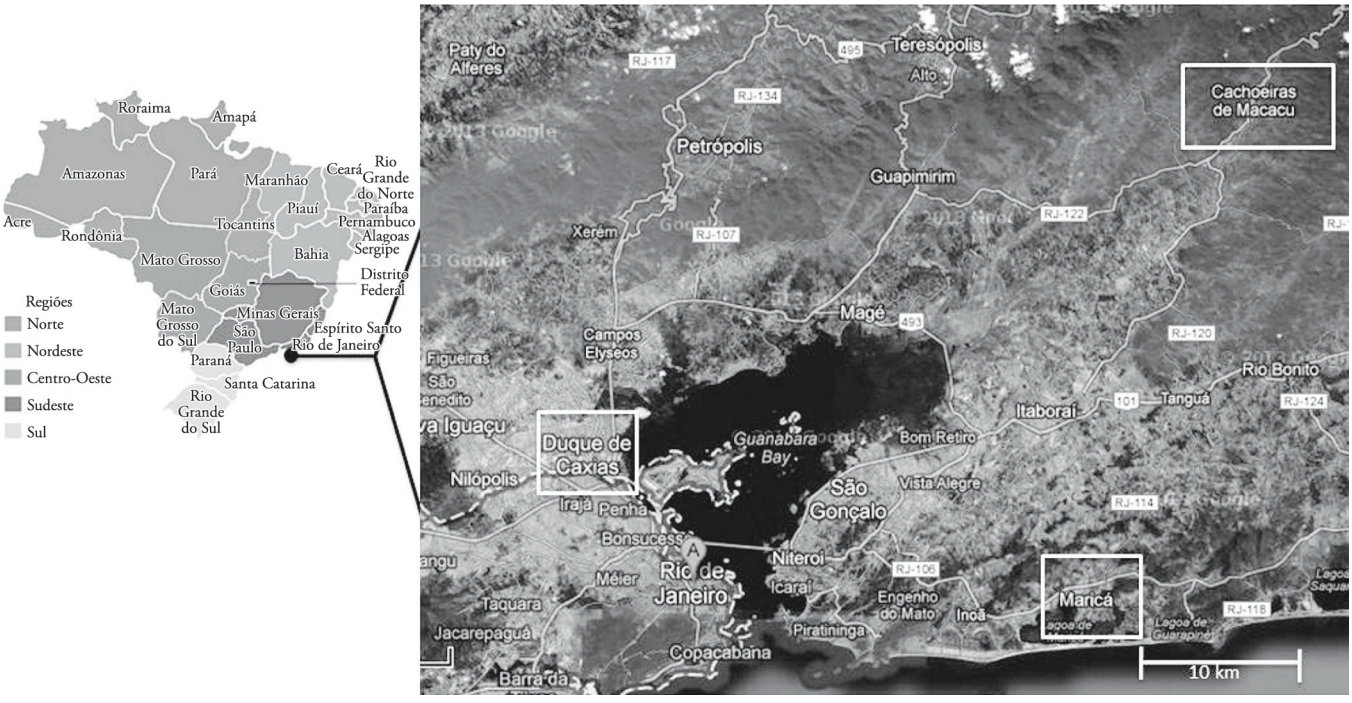

Figure 1. Map showing the regions of the state of Rio de Janeiro where the dogs of the present study were sampled. The squares indicate: Cachoeiras de Macacu, Duque de Caxias and Maricá, and highland and metropolitan regions, respectively.

tested consisted of mixed-breed dogs (128/300; 42.7%) and purebred dogs (180/300; 60%).

The physical examination consisted of assessment of temperature, mucous appearance, presence of ticks, hepatomegaly and/or splenomegaly. The result from the physical examination was registered in a medical record, as well as any clinical signs reported by the owner. This trial was licensed by our institution's Ethics Committee for Animal Research (PROPPi/UFF - CEPA/NAL) under registration number 0087/2011, and dogs were included in the study only after written consent had been received from their owners.

Collection and processing of blood samples, microscopic examination and DNA extraction

All blood samples were collected from each dog before any treatment was started, by means of venipuncture using either the cephalic or the jugular vein. After collection, the samples were stored in commercial sterilized tubes with anticoagulant (EDTA, ethylenediaminetetraacetic acid) for hematological analysis and were kept at –20 °C until extraction and use in PCR reactions.

Diff-quick blood smears were examined under an optical microscope (E-200 Nikon®) by observing 100 random microscopic fields (Magnification: 1000x) to check for the presence of *E. canis* morulae. A cell blood count (CBC) was performed by means of an automated cell counter (Coulter T900®), in accordance with the manufacturer's instructions. The reference values for blood counts used in this study were: hematocrit 37% to 55%; overall leukocyte count 6,000/µL to 17,000/µL; and platelet count 200,000/µL to 500,000/µL (MEINKOTH; CLINKENBEARD, 2000).

Total DNA extraction and polymerase chain reaction (PCR)

DNA was extracted from blood samples in EDTA solution by using the Ilustra Blood Genomic Prep Mini Spin Kit (GE Healthcare, São Paulo, Brazil), in accordance with the manufacturer's instructions. After purification, the DNA was kept at –20 °C, until use.

The samples were initially subjected to PCR using a single pair of primers for species of the family Anaplasmataceae (EHR16SD/ EHR16SR) that amplify a 345 bp fragment of the 16S rRNA gene. These primers amplify several species, including *E. canis, E. chaffeensis, E. muris, E. ruminantium, Anaplasma phagocytophilum, A. platys, A. marginale, A. centrale, Wolbachia pipientis, Neorickettsia sennetsu, N. risticii* and *N. helminthoeca* (INOKUMA et al., 2001).

Samples that were positive with the EHR16SD/ EHR16SR primer pair were then subjected to a second conventional PCR assay using the primers ECAN/HE3, which amplify a 389 bp fragment of the 16S rRNA *E. canis* gene (adapted from Murphy et al. (1998)). In all the reactions, a positive control of *E. canis* DNA isolated from a dog that had previously been infected was used (accession number: JX392985). Milli-Q® water was used as a negative control.

For *TRP*19 gene characterization, a pair of primers (EC19F/ EC19R) was used to amplify a 414 bp fragment (HSIEH et al., 2010). Another pair of primers (TRP36F and TRP36R) was used to amplify the *TRP*36 gene (DOYLE et al., 2005), for amplification of the following three regions: region 1 (a N-terminal region with 429 bp); region 2 (a tandem repeat region with variable numbers of the 27 bp repeat unit, depending on the isolate); and region 3 (a C-terminal region with 87 bp). The DNA obtained from DH82 infected with *E. canis* (accession number: DQ146154) was used as a positive control. Milli-Q water was used as a negative control.

Cloning and DNA sequencing analysis

The amplicons obtained from PCR by using primers designed for the *TRP*19, *TRP*36 and 16S genes were column-purified using the Pure Link® Quick Gel extraction purification kit (Invitrogen™) and then the PCR-amplified fragments were cloned into the pGEM-T easy vector (Promega), in accordance with the manufacturer's instructions. The fragments were ligated into the p-GEM-T easy vector and transformed by means of electroporation into *Escherichia coli* Bl21 electrocompetent cells. The transconjugants were selected in LB agar containing X-Gal (50 ug/mL) and ampicillin (100 ug/mL) after incubation overnight at 37 °C. White colonies were chosen and grown in LB broth with 100 ug/mL ampicillin under agitation (250 rpm) at 37 °C. The plasmid vector containing the insert was extracted using the alkaline lysis extraction method (EHRT; SCHNAPPINGER, 2003) and was sequenced with primer pairs targeting the *TRP*19 and *TRP*36 genes to confirm the cloned DNA fragment.

For the sequencing analysis, clones were purified using GFX PCR DNA and the Gel Band purification kit (GE Healthcare) and were subjected to direct sequencing using the BigDye terminator v. 1.1 cycle sequencing kit (Applied Biosystems, Los Angeles, CA, USA). Both strands of each amplicon were sequenced and the alignments were retrieved and analyzed by means of the Bio Edit sequence alignment editor v. 7.1.11 (http://mbio.ncsu.edu/BioEdit/bioedit.html). The primers were removed and the sequences were compared with other genotypes of *E. canis* available at GenBank (www.ncbi.nlm.nih.gov/genbank). All sequences derived from the Brazilian *E. canis* genotypes generated in this study were deposited in GenBank

Nucleotide identicalness with the GenBank database was assessed using the BLAST tool (http://www.ncbi.nlm.nih.gov/blast). A phylogenetic tree was constructed by means of the neighbor-joining method with 1,000 boot-strap replicates, within the MEGA 5.1 software program (Tamura et al., 2011), using three gene regions based on the divergent *TRP*36 N-terminal, tandem repeat region and C-terminal sequences (DOYLE et al., 2006)

Detection of other vector-borne pathogens

In order to investigate cases of coinfection with *A. platys*, the 15 samples that were positive for E. canis were subjected to another PCR reaction using the primer Ehr16SR/platys (INOKUMA et al., 2001). This did not show any amplification. *Babesia* sp. was also investigated in blood smears, but was not observed.

Results

A total of 15/300 samples (5.0%) were positive for *E. canis* using the ECAN/HE3 primer pair. All the amplicons were subjected to sequence analysis. Partial sequencing of the 16S rRNA gene was performed and the sequences from the present study shared higher nucleotide identicalness (97% to 99%) than did sequences from *E. canis* strains detected in different geographical areas worldwide. These sequences were deposited in the GenBank database (KJ995830, KJ995831, KJ995832, KJ995833, KJ995834, KJ995835, KJ995836, KJ995837, KJ995838, KJ995839, KJ995840, KJ995841, KJ995842, KJ995843, KJ995844).

Eleven of these 15 samples generated amplicons after PCR with the EC19F/EC19R primers and were then subjected to cloning and sequence analysis. For the other four samples, sequencing was not performed due to difficulties in recovering the genetic material or exhaustion of the sample. Comparison of the 11 complete *TRP*19 gene sequences of Brazilian *E. canis* obtained in this study with other strains previously deposited from Brazil and Taiwan in the GenBank database showed that the present samples had higher nucleotide identicalness. Despite the high similarity found, a silent mutation was observed at nucleotide 312 (Figure 2) in three samples: 39C (KF233417), 56C (KF233418) and 49CM (KF233425).

Two out of the 15 samples generated amplicons after PCR with the GP36F/GP36R primers and were subjected to cloning and sequence analysis: 56C (KF233413) and 70C (KF233414). For the other 13 samples, sequencing was not performed due to difficulties in recovering the genetic material or exhaustion of the sample.

Sequence analysis on a 429 bp fragment encoding the TRP36 protein (N-terminal region) revealed that the strain 70C shared the highest nucleotide identicalness with the isolates described in the US genogroup: São Paulo (DQ146154; 100%); Presidente Prudente (JX312076; 99%), Petrolina (JX312078; 99%), Jake (DQ085427; 99%), Florida (DQ146152; 99%), Oklahoma (DQ085428; 99%), Demon (DQ085429; 99%), Louisiana (DQ146151; 99%), North Carolina (DQ146153; 99%) and Cameroon 71 (DQ146155; 100%).

The sequence obtained from animal 56C shared high nucleotide identicalness with four other *E. canis* isolates described in the Brazilian genogroup: Cuiabá 1 (JX31207; 100%); Belém (JX429924; 100%); Londrina (JX312080; 100%) and Monte Negro (15; 97%).

The number of tandem repeat sequences (TEDSVSAPA) ranged from five copies (from animal 70C) to eight copies (from animal 56C). As a result, the entire gene encoding the protein TRP36 showed variations in the number of nucleotides, ranging from 662 bp to 790 bp. The amino acid sequences of the related samples 56C and 70C of the *TRP*36 gene are described in Table 1.

Using all three regions of all Brazilian *E. canis* strains (n = 8) that are available in the GenBank database and 11 others that have been reported worldwide, based on the divergent TRP36 N-terminal, one tandem repeat region and C-terminal sequences, a phylogenetic tree was constructed (Figure 3). The phylogenetic analysis among the Brazilian and other worldwide *E. canis* strains was performed based on the model proposed by Aguiar et al. (2013).

The two strains (56C and 70C) isolated from Rio de Janeiro have the same tandem repeat sequence (TEDSVSAPA) and are grouped in a large clade called US genogroup (A1 and A2; Figure 3), diverging only in the number of tandem repeats (5-8). The 56C sample was segregated to a small branch (A2) consisting of a single hybridizing sample (Cuiabá 16; JX312077), possibly forming a new clade.

Regarding the presentation of the disease in these two animals, it was observed that the 56C strain was collected from a vaccinated and worm-treated nine-year-old male English Cocker Spaniel for which apathy was the main complaint. No fever or coagulation disturbances were reported. Other clinical signs, such as weight loss, pale mucous membranes, hepatomegaly and splenomegaly were also reported. The hematological findings were anemia (12% hematocrit), thrombocytopenia (37,000/μL) and leukocytosis (27,100/μL). This animal had been acquired in the city and no travel history was reported.

On the other hand, the 70C strain was collected from a four-month-old female puppy of mixed breed that had completed the vaccination protocol, undergone worm treated and was tick-free, and which did not have any main complaint. Therefore, no clinical signs were observed and the dog presented only thrombocytopenia (46,000/μL). No travel history was reported.

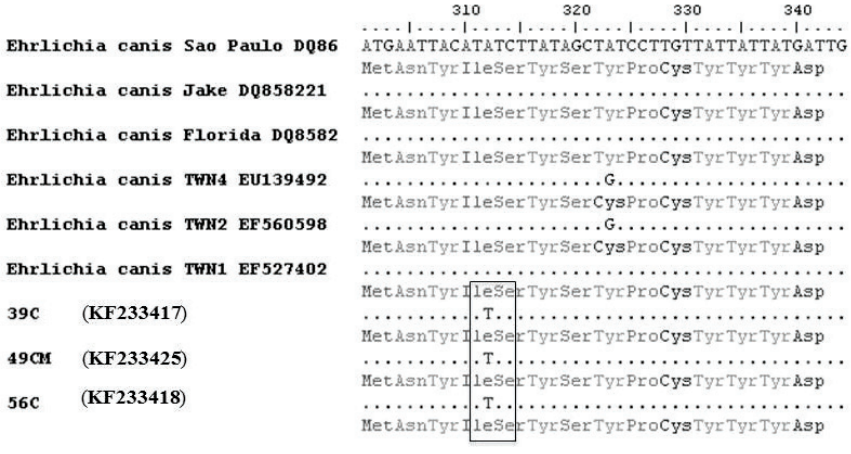

Figure 2. TRP19 amino acid sequences from *Ehrlichia canis* strains from Brazil and worldwide. The square shows a silent mutation in nucleotide 312, where an adenine is replaced by a thymine.

Table 1. TRP36 amino acid sequences from the US genogroup and Brazilian genogroup of *Ehrlichia canis* strains and the two sequences from samples 56C and 70C, from the state of Rio de Janeiro.

	N - terminal region											**Tandem repeat region**									**C- terminal region**					
	10	**19**	**24**	**30**	**35**	**37**	**42**	**44**	**46**	**48**	**49**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**5**	**6**	**7**	**8**	**9**	**14**
E. canis Brazil 56C KF233413	K	G	N	S	V	V	Q	E	G	S	G	**T**	**E**	**D**	**S**	**V**	**S**	**A**	**P**	**A**^{8}	F	K	Q	H	H	E
E. canis Brazil 70C KF233413	M	N	H	Q	G	S	E	D	E	H	P	**T**	**E**	**D**	**S**	**V**	**S**	**A**	**P**	**A**^{5}	S	T	T	S	Y	E
E. canis Petrolina JX312078	M	N	H	Q	S	S	E	D	E	H	P	**T**	**E**	**D**	**S**	**V**	**S**	**A**	**P**	**A**^{13}	S	T	T	S	Y	G
E. canis Pres. Prudente 14 JX312076	M	N	H	Q	G	S	E	D	A	H	P	**T**	**E**	**D**	**S**	**V**	**S**	**A**	**P**	**A**^{7}	Q	Q	H	.	I	N
E. canis Jake DQ085427	M	N	H	Q	G	S	E	D	E	H	P	**T**	**E**	**D**	**S**	**V**	**S**	**A**	**P**	**A**^{12}	S	T	T	S	Y	G
E. canis Cuiaba 16 JX312077	M	.	.	.	.	.	.	.	.	.	.	**T**	**E**	**D**	**S**	**V**	**S**	**A**	**P**	**A**^{3}	S	T	T	S	Y	.
E. canis Cuiaba 1 JX312079	M	.	.	.	.	.	.	.	.	.	.	**A**	**S**	**V**	**V**	**P**	**E**	**A**	**E**^{13}							
E. canis Belem JX429924	M	.	.	.	.	.	.	.	.	.	.	**A**	**S**	**V**	**V**	**P**	**E**	**A**	**E**^{6}		Q	.	K	A	.	D
E. canis Londrina JX312080	M	.	.	.	.	.	.	.	.	.	.	**A**	**S**	**V**	**V**	**P**	**E**	**A**	**E**^{15}							
E. canis Monte Negro 15 JX312081	M	.	.	.	.	.	.	.	.	.	.	**A**	**S**	**V**	**V**	**P**	**E**	**A**	**E**^{10}							
E. canis Sao Paulo DQ146154	M	N	H	Q	G	S	E	D	A	H	P	**T**	**E**	**D**	**S**	**V**	**S**	**A**	**P**	**A**^{18}	S	T	T	S	Y	.
E. canis Florida DQ146152	M	N	H	Q	G	S	E	D	E	H	P	**T**	**E**	**D**	**S**	**V**	**S**	**A**	**P**	**A**^{4}	S	T	T	S	Y	G
E. canis TWN1 EF551366	M	.	.	.	.	.	.	.	.	.	.	**T**	**E**	**D**	**S**	**V**	**S**	**A**	**P**	**A**^{13}	S	T	T	S	Y	G
E. canis TWN2 EF560599	M	.	.	.	.	.	.	.	.	.	.	**T**	**E**	**D**	**S**	**V**	**S**	**A**	**P**	**A**^{12}	S	T	T	S	Y	G
E. canis TWN3 EF651794	M	.	.	.	.	.	.	.	.	.	.	**T**	**E**	**D**	**S**	**V**	**S**	**A**	**P**	**A**^{10}	S	T	T	S	Y	G
E. canis TWN4 EU139491	M	.	.	.	.	.	.	.	.	.	.	**T**	**E**	**D**	**S**	**V**	**S**	**A**	**P**	**A**^{14}	S	T	T	S	Y	G
E. canis Oklahoma DQ085428	M	N	H	Q	G	S	E	D	E	H	P	**T**	**E**	**D**	**S**	**V**	**S**	**A**	**P**	**A**^{5}	S	T	T	S	Y	G
E. canis Demon DQ085429	M	N	H	Q	G	S	E	D	E	H	P	**T**	**E**	**D**	**S**	**V**	**S**	**A**	**P**	**A**^{16}	S	T	T	S	Y	G
E. canis Louisiana DQ146151	M	N	H	Q	G	S	E	D	E	H	P	**T**	**E**	**D**	**S**	**V**	**S**	**A**	**P**	**A**^{5}	S	A	L	A	T	S
E. canis DJ (North Carolina) DQ146153	M	N	H	Q	G	S	E	D	E	H	P	**T**	**E**	**D**	**S**	**V**	**S**	**A**	**P**	**A**^{18}	S	T	T	S	Y	G
E. canis Cameroon 71 DQ146155	M	N	H	Q	G	S	E	D	E	H	P	**T**	**E**	**D**	**S**	**V**	**S**	**A**	**P**	**A**^{16}	S	T	T	S	Y	G

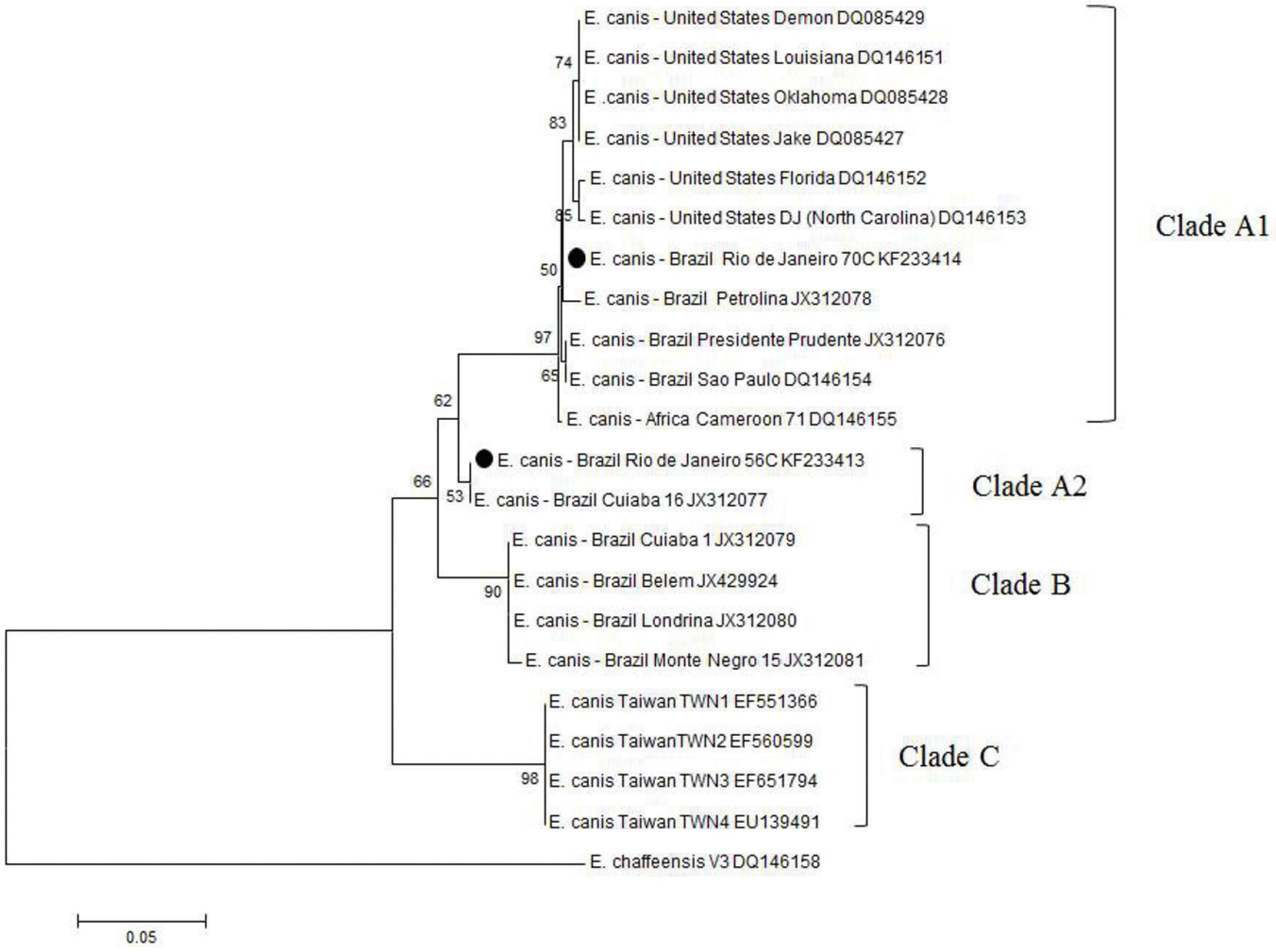

Figure 3. Phylogenetic tree based on the *Ehrlichia canis* TRP36 nucleotide sequences from Brazilian strains isolated in this study and other strains worldwide. The numbers at the nodes represent 50% of 1,000 bootstrap resamplings. Clades A1 and A2 represent the US genogroup, Clade B represents the Brazilian genogroup and Clade C represents the major group of *E. canis* genotypes. Clade A1 shows where the 70C strain is placed and clade A2 shows where the 56C strain is placed. The *E. chaffeensis TRP*47 gene sequence (*TRP*36 gene orthologue) was included as an outgroup.

Discussion

This study presented the first characterization of the *TRP*36 and *TRP*19 genes of *E. canis* from naturally infected dogs in Rio de Janeiro, Brazil and their correlation with the clinical and hematological descriptions shown by the diseased dogs. The results will contribute towards understanding some aspects of the *E. canis* disease and towards elucidating the diversity of *E. canis* strains, despite the limited number of animals in this study.

When the whole sequences of the gene encoding for the surface glycoprotein TRP19 of the 11 samples that were positive for *E. canis* by means of PCR were analyzed and compared with other sequences available in GenBank, a high degree of identicalness was observed. This result was expected, since the gene encoding the TRP19 protein is highly conserved. Doyle et al. (2006) proposed that the TRP19 protein could be a good candidate for creating a serological assay and could be used to develop a vaccine for ehrlichiosis. Even though a silent mutation in nucleotide 312 was detected, the sequences were still highly conserved, thus corroborating this hypothesis.

In Brazil, two genogroups based on the amino acid sequences of the tandem repeat region are circulating and they have different patterns: the Brazilian genogroup (ASVVPEAE) and the US genogroup (TEDSVSAPA). The Brazilian genogroup has only been described in the northern and southern regions, while the US genogroup has described in the northeastern and southeastern regions. In the central-western region, a hybrid (Cuiabá 16) has been described, possibly created through an association between two strains of different genogroups. The Brazilian genogroup has not been described in the southeastern region (AGUIAR et al., 2013). Since the sequence 56C was grouped with the sequence Cuiabá 16 and the identicalness between the sequences of the N-terminal region was high (100%), these sequences formed a new branch in the US genogroup, thus confirming that in the

state of Rio de Janeiro, the Brazilian genogroup may also be circulating, since the Cuiabá 16 nucleotide sequence and the 56C nucleotide sequence showed 100% identicalness with the N-terminal regions of sequences from the Brazilian genogroup (Cuiabá 1, Belém and Londrina). On the other hand, the animal infected with this strain (56C) did not have any history of travel to any of the regions where the Brazilian genogroup had already been described, which reinforces the idea suggested by Aguiar et al. (2013) that recombination between strains of *E. canis* exists in Brazil, where the prevalence is high.

Regarding the region of tandem repeat sequences of the samples described (56C and 70C), the number of tandem repeats (tedsvsapa) ranged from five copies (70C) to eight copies (56C). As a consequence of this variation, the whole gene that encodes the TRP36 protein presented variation in the numbers of nucleotides and amino acids (CARDÉNAS et al., 2007). One important issue that should be pointed out is the fact that the number of tandem repeats can result in different conformations of the protein, thereby changing its biological function and behavior (ALBERTS et al., 2008).

By combining the clinical and hematological findings presented by the animals with the results from the analyses on the sequences obtained from the samples 56C and 70C, some interesting differences were observed. The animal 70C was infected with a strain that belongs to the US genogroup, which is the oldest genogroup described and the one that is most prevalent on Brazilian territory, and this animal did not show any clinical signs of disease. Regarding hematological changes, only thrombocytopenia (46,000/μL) was observed. When treatment is not administered and the animal is immunodeficient (as in the case of the animal 70C), Canine Monocytic Ehrlichiosis has three phases: acute, subclinical and chronic (WOODY; HOSKINS, 1991). Although this puppy was immunodeficient and a severe acute phase was expected, a subclinical phase was observed.

In general, puppies present the classic form of the disease due to deficiency in their immune response. This implies the onset of clinical signs and hematological findings such as apathy, weight loss, fever, hepatomegaly, splenomegaly, anemia, leukopenia or leukocytosis (WOODY; HOSKINS, 1991, NEER 1998; VIEIRA et al., 2011). This did not happen in this case, given that this animal was brought back to the clinic for reinforcement every 21 days after the first vaccine dose (at the age of 45 days). It could happen due to factors relating to the host's adaptation to bacteria, since the US genogroup seems to be the oldest type (AGUIAR et al., 2013).

The animal 56C, which was infected with a hybrid sample, showed the acute classic form of the disease, as expected, showing clinical signs such as weight loss, pale mucous membranes, splenomegaly and hepatomegaly. The hematological findings were severe anemia (hematocrit 12%), thrombocytopenia (37,000/μL) and leukocytosis (27,100/μL), thus suggesting that these were a means of adaptation by the bacteria, as a response to selective pressure experienced, since *TRP* genes have been associated with functional host-pathogen interactions such as adhesion, internalization, actin nucleation and immune evasion (McBRIDE; WALKER, 2011).

Although no coinfections with *A. platys* and *Babesia* sp. were detected in samples that tested positive for *E. canis* in this study, other vector-borne pathogens associated with hematological changes in dogs, such as *Leishmania* sp. and *Leptospira* sp., should be included in the differential diagnosis in cases of dogs with hematological disorders (VIEIRA et al., 2011).

Conclusion

The present study was the first attempt to evaluate the genetic diversity of *E. canis* using *TRP*19 and *TRP*36 gene targets, in Rio de Janeiro, Brazil. Despite the limited number of samples, we can suggest that at least two distinct genogroups were present. Coexistence of different genogroups needs to be carefully monitored, since this gives rise to diversity in the genes encoding proteins, which is directly related to the host immune response. Different genotypes may express new phenotypes, thereby resulting in different forms of disease presentation.

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