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Genetic variability of *Leishmania infantum* in naturally infected dogs in the state of Bahia, Brazil

Variabilidade genética de *Leishmania infantum* em cães naturalmente infectados no estado da Bahia, Brasil

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Abstract

In Brazil, *Leishmania infantum* mainly affects humans and dogs. The state of Bahia presents many dogs that are positive for this parasite. Despite the importance of epidemiology in this region, there are still very few studies that have assessed the genetic characteristics of *L. infantum*. The aim of this study is to investigate the genetic variability of *L. infantum* isolated identified in naturally infected dogs, in order to verify occurrence of subpopulation of this parasite in the different biomes existing in the state of Bahia. Thirty-two samples of *L. infantum* were analyzed, which were obtained isolated in dogs from the Mata Atlântica (rainforest), Caatinga (semi-arid scrub forest), and Cerrado (a vast tropical savannah eco-region) Bahia municipalities' biomes. All animals presented with clinical changes suggestive of *Leishmania* spp. and they exhibited positive reactions to serological tests. kDNA analysis with RFLP markers revealed the presence of genetic variability and gene flow in subpopulations of *L. infantum*; samples from the Mata Atlântica areas were genetically more similar to those from the areas of Caatinga and they were less likely to resemble those of the Cerrado. This data may be used to investigate the dissemination of parasite in the canine population of state of Bahia.

Keywords: Canine Leishmaniasis, PCR-RFLP, populations, enzyme, zoonosis.

Resumo

No Brasil a *Leishmania infantum* afeta principalmente o homem e os cães. O estado da Bahia apresenta elevado número de cães positivos por este parasito. Apesar da importância epidemiologia para a região, ainda há poucos estudos que avaliam as características genéticas de *L. infantum.* Objetivou-se com este estudo investigar a variabilidade genética de cepas de *L. infantum* identificadas em cães naturalmente infectados, a fim de verificar a ocorrência de sobpopulações do parasito nos diferentes biomas existentes no estado da Bahia. Foram analisadas 32 amostras de *L. infantum* isoladas em cães de municípios baianos distribuídos nos biomas Mata Atlântica, Caatinga e Cerrado. Todos os animais apresentavam alterações clínicas sugestivas de *Leishmania* spp. e reação positiva em exames sorológicos. A análise do kDNA com marcadores RFLP revelaram a presença de variabilidade genética e fluxo gênico nas subpopulações de *L. infantum* sendo que as amostras das áreas de Mata Atlântica foram geneticamente mais semelhantes as das áreas de Caatinga e foram mais distantes daquelas oriundas do Cerrado. Estas informações podem auxiliar em investigações de dispersão do parasito na população canino do Estado da Bahia.

Palavras-chave: Leishmaniose canina, PCR-RFLP, populações, enzima, zoonose.

Introduction

In Brazil, *Leishmania infantum* is responsible for cases of visceral leishmaniasis (VL) and it affects thousands of people every year (WHO, 2015; DANTAS-TORRES & OTRANTO, 2014). The number of infected dogs is also high and can vary according to the region studied (LEÇA et al., 2015; CARVALHO et al., 2015; OLIVEIRA et al., 2016). These infected dogs, when infected by *L. infantum*, pose numerous risks to public health (DANTAS-TORRES, 2007). However, there are few studies about the genetic profile of this parasite, and occurrence of subpopulations in the differents ecosystems of states of Bahia.

The use of molecular techniques represents an alternative for the early and accurate identification of the *Leishmania* spp.

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present in animals, and they still facilitate studies of their genetic structure (BANETH et al., 2008; MIRÓ et al., 2008). Different regions of the genetic code of the parasite can be analyzed by using several different genetic markers. Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis techniques have allowed the identification and evaluation of the genetic structure of populations and strains of *Leishmania* spp., revealing the presence of different genotypes and polymorphisms in strains isolated in Brazil and in Europe (CORTES et al., 2006; ALONSO et al., 2010). These factors, may be attributed to known genetic events, as well as, external environmental pressures (LAINSON & SHAW, 1987).

The obtaining epidemiological information as much as genetics are essential to understanding of parasite's biology, and dinamic of disease, as well as to stimulate the realize of new studies and methodological development which allow a greater control of dissemination of parasite, in animals and humans for competent public authorities.

The aim of this study was to investigate the genetic variability of *L. infantum* isolated present in naturally infected dogs by using the PCR-RFLP technique, in order to verify occurrence of subpopulation of parasite in the different biomes existing in the state of Bahia.

Materials and Methods

Thirty-two isolated of *L. infantum* identified in dogs in municipalities of the state of Bahia were studied and they were found to present different geo-climatic characteristics. A total of 5 mL of blood was obtained from dogs presenting with the clinical signs for *L. infantum*, and their serological reactions in enzyme-linked immunosorbent assay (ELISA) and PCR tests were positive for infection with this parasite. The genomic DNA was obtained from 500 µL of whole blood with added extraction buffer (20 mM Tris, 50 mM EDTA, 5 µg/mL of K, and sodium dodecyl sulfate [SDS] proteinase 1%), and it was kept at 60°C for 80 min. The samples were purified with phenol: chloroform: active amyl alcohol at a ratio of 25:24:1 (Invitrogen®), precipitated with 100% ethanol and 5 M ammonium acetate (SAMBROOK et al., 1989). The samples were quantified in NanoDrop2000 (Thermo Fisher Scientific, Waltham, MA, USA), standardized and amplified with 10 pmoles of specific primers MC1 5' - GTTAGCCGATGGTGGTCTTG-3´ and MC2 5' - CACCCATTTTTCCGATTTTG-3' (CORTES et al., 2006), 2 mM of $MgCl_2$, 1.5 U of Taq DNA polimerase (Ivintrogen®), 2.5% of dimethyl sulfoxide (DMSO), and 100 ng of the genomic DNA in a final volume of 50 µL submitted to 35 cycles and held at a annealing temperature of 60°C. Then, the amplicons obtained were purified with a Purelink™ kit (Invitrogen®) according to the manufacturer's protocol. Subsequently, they were subjected to digestion with enzymes BgL II RsaI (AflI), DdeI, VspI (AseI), Bme1390 (BssKI), HapII, and HaeIII. For the digestion reaction buffer at 1X, 2 U of enzyme and approximately 100 ng of amplicon were used. The results were viewed in 3.5% agarose gel and stained with Syber® Safe DNA Gel Stain. The results were analyzed with the LabImage® ID program. The control samples of *L. infantum* were provided by the *Leishmania* Collection of the Instituto Oswaldo Cruz (CLIOC) and Universidade Federal de Minas Gerais (UFMG) (Table 1). The obtained restriction patterns were analyzed with the TFPGA program version 1.3 (MILLER, 1997) by adopting Nei's coefficient (NEI, 1972). The Neighbor-joining method, with 10,000 bootstrap replications, was used in the cluster analysis. This study was approved by the Ethics Committee of Animal Use - CEUA/UESC under protocol number 018/12 and followed the guidelines established by the Brazilian College of Animal Experimentation (COBEA), Federal Law 11.794.

Results and Discussion

The samples that were positive for *L. infantum* were distributed in the Mata Atlântica (28.12%), Caatinga (56.25%), and Cerrado (15.62%) biomes. The isolated were identical when examined with the BgLII, HapII, and HaeIII enzymes. However, during the analysis with the DdeI, AflI, BsspK, and AseI enzymes, different electrophoretic patterns were identified (Table 1).

The subpopulations of *L. infantum* that were studied in this investigation presented with 17 polymorphic loci in all 21 that were analyzed (Table 2). Eleven genotypic profiles were identified, the most frequent of which were classified as Brazil A (BrA = 57.57%), Brazil B (BrB = 12.12%), and Brazil C (BrC = 12.12%). Some of these genotypes profiles are present in all of the analyzed biomes, while others were exclusive to certain biomes. The Caatinga and Cerrado biomes, presented greatest genotype diversity compared to areas of Mata Atlântica. Some genotypes observed in samples of *L. infantum*, present in areas of Caatinga has already been reported in isolated parasite, studied in others regions (CORTES et al., 2006; ALVARENGA, 2007; ALONSO et al., 2010).

When analyzing the behavior of the samples of *L. infantum* in relation to the studied biomes, one notes intense gene flow

Table 1. References isolated of *Leishmania infantum* provided by Instituto Oswaldo Cruz and Universidade Federal de Minas Gerais.

Code	International code	Country	State	Zimodem*
2301H CE	MHOM/BO/1997/LP-0017	Bolívia	La Paz	MON 1
2664C CE	MCAN/BR/2002/LVV-135	Brasil	Mato Grosso do Sul	MON 1
2926C MT	MCAN/BR/2006/CP-67	Brasil	Espírito Santo	MON 1
3107H MT	MHOM/BR/2009/HU-UFS02	Brasil	Sergipe	NI
3482C MT	MCAN/BR/2013/LPBI CÃO	Brasil	Bahia	NI
BK6 CE	BK6-UFMG	Brasil	Minas Gerais	NI

* NI - no information.

Table 2. Fragment patterns obtained after enzymatic digestion of amplicom (MC1/MC2) in *Leishmania infantum*. The results are expressed in cut patterns obtained for each enzyme and its respective identified loci.

Enzymes	BglII	HapII	DdeI			RsaI (AflI)		Bme1390I (BssKI)		VspI (AseI)		Hae III
	I	I	I	II	III	I	II	I	II	I	II	I
Fragments in base pairs	450	450	450	320 100 30	240 180 30	450	260 190	450	290 120 40	450	310 140	390 70

and little genetic structuring in subpopulations with a value of FST = 0.0122 (Figure 1).

In Brazil *L. infantum* is commonly identified in areas of dry climate and with relief composed of valleys and mountains, although it has already been registered the presence of these parasites in large urban centers and coastal areas in the northeast (COURA-VITAL et al., 2011; DIAS et al., 2011; BRASIL, 2014). It was possible to verify the existence of a unique *L. infantum* population with genetic variability and gene flow in the *Leishmania* isolated identified in dogs of different Bahia biomes. These data differ from those found by Alonso et al. (2010), that using PCR-RFLP identified homogeneous standards and reduced genetic variability in strains of *L. infantum* obtained in Teresina, located in the Caatinga region. Factors such as carrying out transference of cultures, the scientific methodology adopted, the region where the DNA was obtained, and the genetic marker used can contribute to such divergent responses.

With respect to *Leishmania* spp., genetic isoenzymatic markers (multilocus enzyme electrophoresis [MLEE]) are commonly used, and although they share a co-dominant character, they feature limitations as they are influenced by intrinsic cellular processes involved in the production of enzymes and cellular proteins; thus, they may not accurately reflect the specific genetic sequence (BAÑULS et al., 1999; QUISPE TINTAYA et al., 2004).

Recently, other co-dominant markers, such as PCR-RFLP and microsatellites, have enabled the identification of genetic variability in the genome of strains of *L. infantum*, even in those strains grouped in the same zymodeme and isolated in the same region (BAÑULS et al., 1999; CORTES et al., 2006; KUHLS et al., 2008; ALVARENGA et al., 2012).

The cluster analysis performed here revealed a group that tested positive for genetically similar strains of *L. infantum*, while in others strains the occurrence of genetic variability is observed. Samples obtained from the Mata Atlântica regions grouped with greater affinity than those obtained in the Caatinga biome. This may reflect the closeness between these biomes and the fact that these transition areas interact, which can contribute to the circulation of vectors and parasites, while maintaining the genetic flow among the strains of *L. infantum*

In other groupings, there is the approximation of strains of *L. infantum* that were derived from the Caatinga and Cerrado regions. These biomes also share common areas and characteristics (such as temperature and similar rainfalls), which may favor the presence of the parasites and their vectors.

The presence of the Caatinga biome in the northeastern region of Brazil promotes greater geographic distance between strains that are present in coastal areas with those in the Cerrado regions.

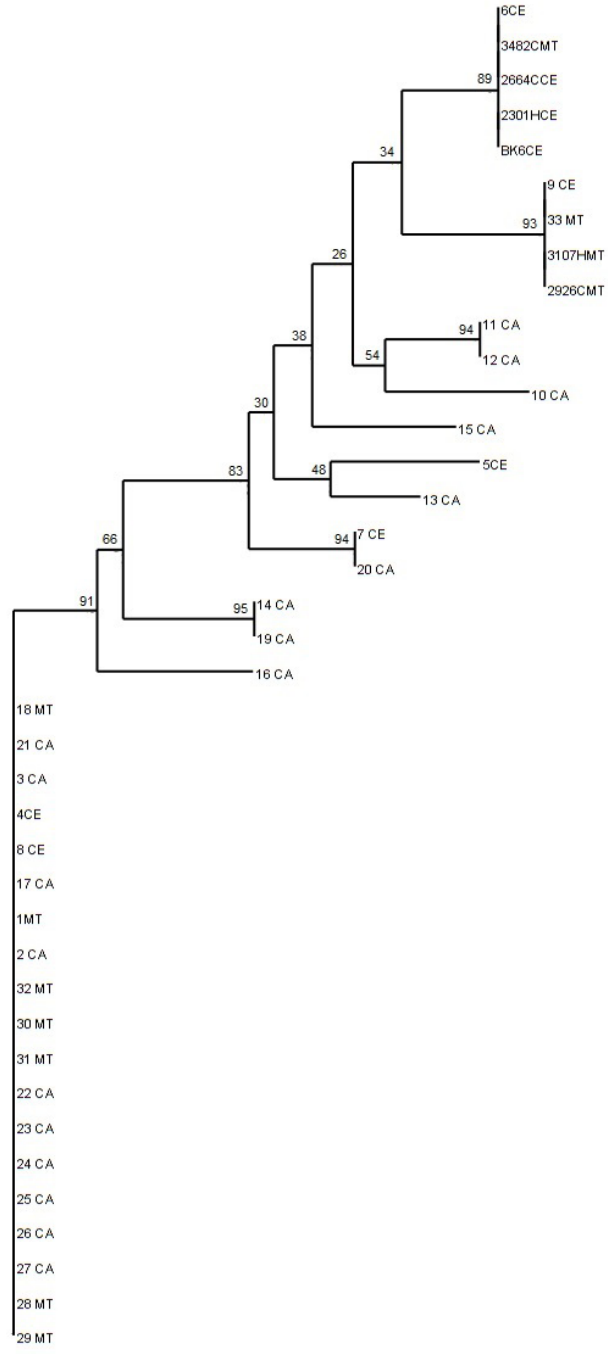

Figure 1. Dendrogram generated from the genotypes identified in the Bahia biomes presenting two main groups and intense correlation of isolated *Leishmania infantum* and studied biomes. MT – Mata Atlântica; CA – Caatinga; EC – Cerrado in the state of Bahia.

Geophysical factors, such as the presence of the São Francisco River and high-altitude areas (such as Chapada Diamantina, which are mainly comprised of the Serra do Sincorá, Barbado, Mangabeira, and Espinhaço), which are present in the Caatinga biome, can act as limiting factors in the dissemination of vectors and strains of *L. infantum*, consequently resulting in greater species distinction and isolation. These factors may be contributing to greater genetic variability, as well as to the increasing number of genotypes identified (Figure 2).

When analyzing the grouping of reference strains, the formation of a homogenous block including samples from the states of Bahia, Sergipe, and Espírito Santo was found. In another group, this finding was evident in samples from Bahia, Minas Gerais, Mato Grosso do Sul, and Bolivia. The genotypic proximity of isolated strains in Brazil and those of Bolivia corroborate the findings of studies that have shown the dissemination of strains of *L. infantum* following construction of the Northwest Railway, the Vitória-Corumbá highway, and the Bolivia Brazil gas pipeline in the 1990s (CARDIM et al., 2013). Spatial and temporal analyses carried out by Antonialli et al. (2007) demonstrate the dissemination of and increases in cases of VL toward the west and east, primarily involving the states of Mato Grosso do Sul and São Paulo. In the regions of Rio de Janeiro and Minas Gerais there is no Caatinga biome and the relationship between the coastline and the areas of Cerrado becomes narrow, which can justify the proximity between some strains isolated in these environments.

Although we didactically suggest the presence of three subpopulations, the value of Fst near to zero shows intense genetic flow among the analyzed samples. This likely occurred due to the clonal profile of strains of *L. infantum*, as well as to the intense traffic of people and dogs, which are possibly infected, between the municipalities of the studied areas. The largest number of genotypes identified in the Caatinga biome (Figure 2) can indicate the center of origin of *L. infantum*, which then disseminated to other areas of Bahia, although the colonization process (which took place more than 500 years ago) began in the Bahia foreshore and in other areas of Brazil's northeast. A bottleneck effect, rare mechanisms of exchange of genetic material, small mutations (TIBAYRENC & AYALA, 1999; LYTHGOE, 2000; VICTOIR & DUJARDIN, 2002), and evidence of sexual reproduction (KREUTZER et al., 1994; AKOPYANTS et al., 2009) can also justify the value of Fst and the variability found in the studied strains.

Some restriction patterns obtained in this research were identical to the ones found in other studies conducted on *L. infantum* that were already carried out in Brazil and Portugal (CORTES et al., 2006; ALVARENGA, 2007; ALONSO et al., 2010). Three genotypes obtained in the Bahia samples were identical to the ones found in samples of *L. infantum* isolated from dogs in Portugal and in other countries (CORTES et al., 2006). The genotypical profile E (BrJ) shares similarities to the samples from Brazil and Portugal; while G (BrI) was identified in samples of Portugal, Sudan, and Ethiopia; and O (BrF) was found in isolated samples from Malta

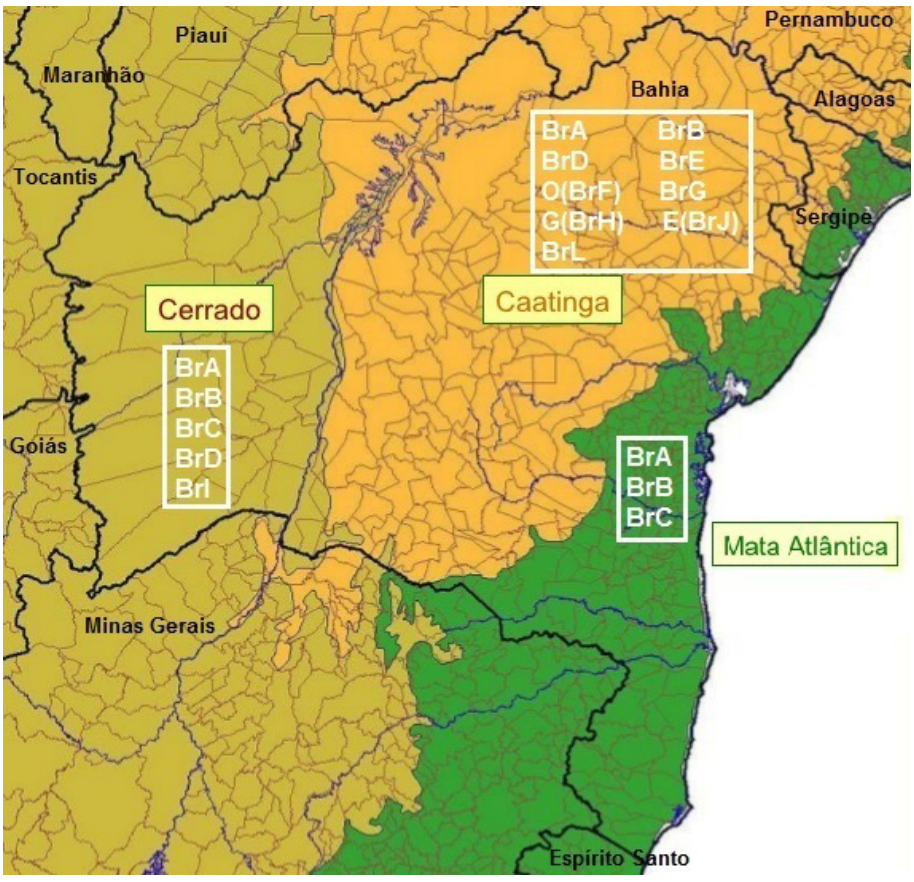

Figure 2. Map of the state of Bahia with their respective biomes and genotypes of *Leishmania infantum* identified.

(CORTES et al., 2006). These data corroborate the previously available data on the dissemination of the parasite among continents (WHO, 2015), and they provide further support for the type of asexual reproduction used by the parasite (TIBAYRENC et al., 1993; TIBAYRENC & AYALA, 1999)

However, the genotypes generated by BssKI, RsaI, and AseI presented with atypical patterns to those found in Portugal and Minas Gerais, Brazil (CORTES et al., 2006; ALVARENGA, 2007). In this study, the HaeIII enzyme was employed for the first time, although it cleaved the DNA sequence of *L. infantum* into two fragments; this revealed a homogeneous pattern between the samples and the controls analyzed. Atypical genotypes may represent important changes in the kDNA molecule of *Leishmania* due to the genetic and population processes already mentioned above. Such changes can promote adaptive advantages to certain individuals or subpopulations of parasites in different biomes.

Conclusion

The *Leishmania infantum* strains that were naturally isolated in dogs showed intense genetic variability and gene flow among the strains, revealing the presence of a single population of parasites, although variations occurred in the kDNA regions studied. It was not possible to determine specific patterns in the strains of *L. infantum* studied herein, which would have enabled correlations to be made with the biomes studied.

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