



## Article/Artigo

# Evaluation of *Leishmania (Leishmania) chagasi* strains isolated from dogs originating from two visceral leishmaniasis-endemic areas in Brazil using multilocus enzyme electrophoresis

Avaliação de amostras de *Leishmania (Leishmania) chagasi* isoladas de cães oriundos de duas áreas endêmicas de leishmaniose visceral no Brasil através da eletroforese de isoenzimas

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### ABSTRACT

**Introduction:** Domestic dogs are the most important reservoir in the peridomestic transmission cycle of *Leishmania (Leishmania) chagasi*. The genetic variability of subpopulations of this parasite circulating in dogs has not been thoroughly analyzed in Brazil, even though this knowledge has important implications in the clinical-epidemiological context. **Methods:** The objective of this study was to evaluate and compare the phenotypic variability of 153 *L. chagasi* strains isolated from dogs originating from the municipalities of Rio de Janeiro (n = 57) and Belo Horizonte (n = 96), where the disease is endemic. Strains isolated only from intact skin were selected and analyzed by multilocus enzyme electrophoresis using nine enzyme systems (6PG, GPI, NH<sub>1</sub> and NH<sub>2</sub>, G6P, PGM, MDH, ME, and IDHNADP). **Results:** The electrophoretic profile was identical for all isolates analyzed and was the same as that of the *L. chagasi* reference strain (MHOM/BR/74/PP75). Phenetic analysis showed a similarity index of one for all strains, with the isolates sharing 100% of the characteristics analyzed. **Conclusions:** The results demonstrate that the *L. chagasi* populations circulating in dogs from Rio de Janeiro and Belo Horizonte belong to a single zymodeme.

**Keywords:** Dog. *Leishmania chagasi*. Genetic variability. MLEE.

### RESUMO

**Introdução:** Cães domésticos são considerados os reservatórios mais importantes no ciclo peridoméstico de transmissão de *Leishmania (Leishmania) chagasi*. No entanto, a variabilidade genética de sub-populações que circulam neste hospedeiro é ainda pouco explorada no Brasil, sendo tal conhecimento de grande importância no contexto clínico-epidemiológico. **Métodos:** O objetivo deste estudo foi avaliar e comparar a variabilidade fenotípica de 153 amostras de *L. chagasi* isoladas de cães oriundos dos municípios do Rio de Janeiro (n = 57) e Belo Horizonte (n = 96), onde a doença é endêmica. Foram selecionadas somente amostras isoladas de pele íntegra e analisadas por eletroforese de isoenzimas (MLEE) empregando nove sistemas enzimáticos (6PG, GPI, NH<sub>1</sub> e NH<sub>2</sub>, G6P, PGM, MDH, ME, IDHNADP). **Resultados:** Todas as amostras analisadas apresentaram perfil eletroforético idêntico entre si e com a amostra de *L. chagasi* utilizada como referência neste estudo (MHOM/BR/74/PP75). A análise fenética demonstrou índice de similaridade igual a um para todas as amostras, revelando um compartilhamento de 100% dos caracteres avaliados. **Conclusões:** A partir desses resultados, podemos inferir que as populações de *L. chagasi* que estão circulando nos cães do Rio de Janeiro e Belo Horizonte podem ser agrupadas em um único zimodema.

**Palavras-chaves:** Cão. *Leishmania chagasi*. Variabilidade genética. MLEE.

### INTRODUCTION

Leishmaniasis are diseases caused by different species of the genus *Leishmania*, including the subgenera *Viannia* and *Leishmania*<sup>1</sup>. Clinically, leishmaniasis can be divided into visceral leishmaniasis and tegumentary leishmaniasis, with both diseases presenting a broad spectrum of manifestations<sup>2,3</sup>.

In Brazil, *Leishmania (Leishmania) chagasi* [syn. *Leishmania infantum*] is the only agent that causes visceral leishmaniasis and is associated with the vector *Lutzomyia longipalpis* in most regions. Recently, *Leishmania (Leishmania) chagasi* has been detected in natural canine infections in areas unaffected by visceral leishmaniasis<sup>4</sup>. The role of the dog as a domestic reservoir in the transmission cycle of visceral leishmaniasis has been well established. The disease progresses slowly in this animal but shows an insidious onset and a variable spectrum of clinical manifestations. During more advanced stages of the disease, onychogryphosis, splenomegaly, lymphadenopathy, alopecia, dermatitis, skin ulcers, keratoconjunctivitis, a runny nose, anemia, apathy, diarrhea, intestinal hemorrhage, paw edema, vomiting, and hyperkeratosis are frequent findings<sup>5</sup>. However, the disease may remain latent in some cases and may even resolve spontaneously<sup>6</sup>.

The identity of the etiological agent of visceral leishmaniasis in the Americas has been the matter of numerous discussions<sup>7-9</sup>. For a long time, *L. chagasi* has been considered to be an autochthonous agent in the New World, and this hypothesis directed studies that genetically compared *L. chagasi* and *L. infantum* using isolates obtained from different hosts and by different methods<sup>10-13</sup>. In all studies cited, *L. chagasi* and *L. infantum* presented similar genetic profiles and were considered to be the same species. It is currently assumed that this parasite was introduced to South America during European colonization through dogs infected with *L. infantum*, the predominant species in Mediterranean countries, and that *L. infantum* adapted to new vectors and hosts<sup>14,15</sup>.

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The correct classification of *Leishmania* parasites is fundamental for the establishment of control measures for the diseases caused by these organisms, and numerous tools are currently applied for this purpose. In this respect, multilocus enzyme electrophoresis (MLEE) is the reference standard for the identification of species of the genus *Leishmania* and is one of the methods most used for the characterization of species of this genus<sup>16-21</sup>. The characterization of isolates is important, especially in epidemiological surveillance studies, permitting the mapping of *Leishmania* species and variants that circulate in humans and animals in a given region. In addition, this characterization contributes to a better understanding of the factors related to these phenotypic variations, such as clinical characteristics, drug resistance, parasite virulence, preference for certain hosts, and association with certain immunosuppressive diseases.

In view of the importance of the domestic dog in the transmission cycle of *L. chagasi* and the importance of studies investigating the genetic variability of isolates circulating in this host, the objective of the present investigation was to evaluate and compare the phenotypic characteristics of 153 *Leishmania* strains isolated from dogs originating from two endemic areas in Brazil.

## METHODS

The 153 isolates studied were obtained from the strain collection of the Laboratory of Leishmaniasis Surveillance (VigiLeish, IPEC/FIOCRUZ). The strains isolated from dogs were obtained during epidemiological surveys carried out in the municipalities of Rio de Janeiro (*Campo Grande, Guaratiba, Barra de Guaratiba, Barra da Tijuca, Jacarepaguá, Realengo* and *Bangu*) during the period from 2000 to 2008 (RJ; n= 57) and Belo Horizonte (*Barreiro, Centro Sul, Leste, Nordeste, Noroeste, Norte, Oeste, Pampulha* and *Lagoa de Pampulha*) in 2004 (BH; n = 96). Both regions are located in southeastern Brazil. Only strains isolated from the intact skin of dogs presenting different clinical conditions were selected. First, the isolates were recovered from liquid nitrogen storage and expanded in Schneider's medium (Sigma) supplemented with 10% fetal bovine serum (Cultilab) and antibiotics (200 units/mL penicillin G potassium and 100 units/mL streptomycin) to obtain approximately 10<sup>9</sup> parasites. When in the exponential phase, the culture was pelleted by centrifugation, resuspended in appropriate buffer, and stored at -196°C until isoenzyme electrophoresis.

Multilocus enzyme electrophoresis was performed on 1% gels according to previously described procedures<sup>17,22</sup>. Nine enzyme systems were used: glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), glucose phosphate isomerase (GPI), nucleoside hydrolase (NH<sub>1</sub> and NH<sub>2</sub>), phosphoglucomutase (PGM), malate dehydrogenase (MDH), malic enzyme (ME), and isocitrate dehydrogenase (IDH). The gels were analyzed by determining the electrophoretic mobility of the bands based on the standard positions of the following reference strains: *Leishmania (Leishmania) chagasi* (MHOM/BR/74/PP75), *Leishmania (Viannia) braziliensis* (MHOM/BR/75/M2903), and *Leishmania (Leishmania) amazonensis* (IFLA/BR/67/PH8).

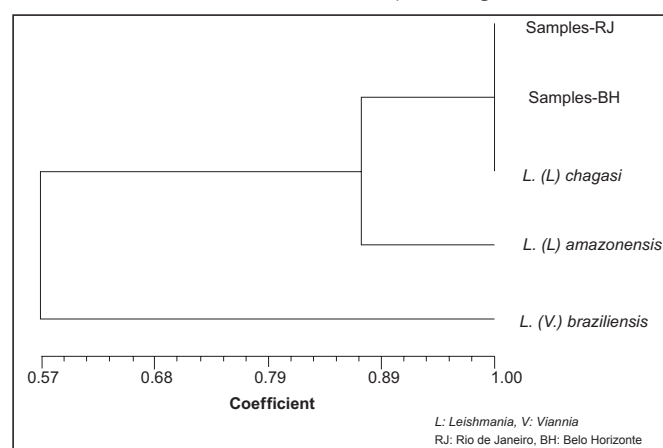
The MLEE bands were compared using the simple matching coefficient of similarity, and the matrix was transformed into a dendrogram using the UPGMA algorithm<sup>23</sup>. Phenetic analysis was performed with the NTSYS-pc program, version 2.02 (Exeter Software, Setauket, NY, USA).

## Ethical considerations

The collection of biological samples from the dogs was approved by the Ethics Committee on the Use of Animals of FIOCRUZ (CEUA/FIOCRUZ), license numbers P-286/06 and L-023/06.

## RESULTS

All 153 canine isolates analyzed by MLEE demonstrated similar electrophoretic profiles, which were similar to that of the *L. chagasi* reference strain (MHOM/BR/74/PP75). No isoenzyme variants were observed among parasites isolated from Rio de Janeiro and Belo Horizonte. All isolates were classified into the same zymodeme, with a similarity index of one, demonstrating that all isolates studied shared 100% of the characteristics analyzed (**Figure 1**).



**FIGURE 1** - MLEE dendrogram showing the similarity among the canine samples obtained from two areas endemic for visceral leishmaniasis and the *Leishmania chagasi* reference strain.

## DISCUSSION

Visceral leishmaniasis is widely distributed in Brazil, with outbreaks occurring in urban areas, a fact highlighting the importance of the domestic dog in this context<sup>2,24,25</sup>. Clinically, dogs infected with *L. chagasi* can show a broad spectrum of manifestations or can remain asymptomatic, suggesting the circulation of distinct subpopulations of the parasite in these hosts. Because of this variation, studies investigating the genetic variability of strains isolated from dogs are extremely important but are scarce in the literature. Therefore, the objective of this study was to evaluate and compare the phenotypic characteristics of *L. chagasi* strains isolated from dogs originating from two endemic areas in Brazil.

In addition to being an effective method for the identification of *Leishmania* isolates, isoenzyme electrophoresis has various applications. One of the disadvantages of this technique is the need for previous isolation of the parasite to subsequently obtain a large culture volume, a fact that has contributed to the use of PCR-based methods in many studies<sup>26,27</sup>. However, the variability of parasites of the genus *Leishmania* has been widely studied by MLEE<sup>11,19,28</sup>.

In the present study, nine enzyme systems were used for the analysis of 153 *L. chagasi* strains isolated from animals originating from two endemic regions. These isolates presented identical electrophoretic profiles. This result demonstrates that the isolates originating from the two endemic regions showed no difference that could be associated with geographic area or host status, indicating

complete homogeneity of this species. This fact has also been reported by other investigators who demonstrated the absence of enzyme polymorphisms in *L. chagasi* isolates<sup>11,29,30</sup>. In this respect, one interesting fact is the large heterogeneity observed among *L. infantum* strains, with the numerous zymodemes circulating naturally in different regions of the Old World<sup>31</sup>. MON-1 is the only zymodeme described for *L. infantum* that shows identity with *L. chagasi* in the Americas. This fact suggests that the introduction of *L. chagasi* to the American continent occurred at a single time point and that all strains in the Americas are derived from this zymodeme or that only this zymodeme found conditions that permitted the adaptation and propagation of the parasite. This fact may also explain the homogeneity of *L. chagasi* observed in this study and reported by other investigators<sup>12</sup>. The homogeneity of this species may also be related to the poor diversity of vertebrate and invertebrate hosts in the transmission cycle or to the fact that all isolates analyzed were obtained from a single anatomical site on the animals (intact skin), somehow standardizing subpopulations that show tropism for this site. Parasitism of intact skin of dogs infected with *L. chagasi*, which was described for the first time in the 1950s<sup>32</sup>, is one of the characteristics that renders the dog an important reservoir in the transmission cycle. This site has been used for the parasitological confirmation of seroreactive dogs, and the isolates for the present study were obtained from this site, demonstrating that the skin is an excellent target for the parasitological confirmation of canine visceral leishmaniasis<sup>33,34</sup>.

The present results demonstrate the low variability of *L. chagasi*. In addition, the findings demonstrate that the population of *Leishmania* parasites circulating in dogs from Rio de Janeiro and Belo Horizonte is extremely homogeneous and shares 100% of characteristics, revealing that the isolates analyzed in this study all belong to a single zymodeme.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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