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An Assessment of the Genetic Diversity of *Leishmania infantum* Isolates from Infected Dogs in Brazil

Luís Fábio da S. Batista, Marcela Segatto, Carlos Eduardo S. Guedes, Rosana S. Sousa, Cleusa A. T. Rodrigues, Júlia Cristina M. Brazuna, Joselli S. Silva, Silvana O. Santos, Daniela Larangeira, Andréa Mara Macedo, Albert Schriefer, and Patrícia S. T. Veras*

Laboratório de Patologia e Biointervenção do CPqGM, FIOCRUZ, Bahia, Brazil; Laboratório de Genética Bioquímica, Departamento de Bioquímica e Imunologia ICB-UFMG, Minas Gerais, Brazil; Serviço de Imunologia, Hospital Universitário Professor Edgard Santos, UFBa, Bahia, Brazil; Universidade Federal de Mato Grosso do Sul, Departamento de Morfofisiologia, Campo Grande, Brazil; Prefeitura Municipal de Campo Grande, Secretaria Municipal de Saúde Pública Sesau, Centro de Controle de Zoonoses, Mato Grosso do Sul, Brazil; Departamento de Biointeração, Instituto de Ciências da Saúde, UFBa, Bahia, Brazil; Instituto Nacional de Ciência e Tecnologia em Doenças Tropicais (INCT-DT)

Abstract. Correlations between the genetic diversity of *Leishmania infantum* (syn. *L. chagasi*) isolates and their respective geographic origins support the theoretic assumption that visceral leishmaniasis probably originated in the Old World. Because dogs are widely considered to be the main reservoir of this disease, the present study aimed to investigate the degree of genetic divergence among 44 leishmanial canine isolates from two Brazilian cities, Jequié and Campo Grande, located approximately 2,028 km from each other. We hypothesized that a low degree of genetic divergence would be observed among these isolates. In fact, statistical analyses found no significant differences between the isolates using both random amplified polymorphic DNA and multilocus microsatellite typing genotyping techniques with three and seven markers, respectively. These findings provide support for the recent introduction of *L. infantum* into the New World.

INTRODUCTION

Leishmaniases are zoonoses that are listed among the six most prevalent neglected tropical diseases because of symptom severity, wide geographical distribution, and high prevalence.[1] Visceral leishmaniasis is unevenly distributed throughout tropical and subtropical zones in 88 countries, with almost 90% of all cases concentrated in 5 countries: Bangladesh, India, Nepal, Sudan, and Brazil.[2] In Brazil, visceral leishmaniasis is currently found in the urban and outlying areas of several large cities, including São Paulo, Rio de Janeiro, Salvador, Belo Horizonte, Campo Grande, Natal, Fortaleza, Teresina, São Luis, and Santarém.[2–5] The zoonotic cycle of visceral leishmaniasis includes the participation of humans as well as wild and domesticated animals. The evidence indicates that dogs are the main reservoir of visceral leishmaniasis, because human cases have often been preceded by canine cases, and the extensive presence of parasites in canine skins suggests that these animals are the main source of sandfly infection.[6,7] *Leishmania infantum* (syn. *L. chagasi*)[8] is one of the main parasites that causes visceral leishmaniasis in Europe, North Africa, South America, and Central America.[2] Studies on the genetic diversity of *L. infantum* strains in the Old World have clarified epidemiological issues regarding visceral leishmaniasis, including the geographical distribution of different genotypes and the associations between specific genotypes and the severity of disease.[9–13] In addition, these studies have made it possible to formulate hypotheses about the evolution of the genus *Leishmania*.[10,11] Correlations between the genetic diversity of *L. infantum* isolates from the Old World and their diverse geographical origins have been previously described.[6] Genotypic data indicate that the *L. donovani* complex originated 3–4 million years ago, and these correlations are consistent with the degree of evolution observed in the complex throughout Europe, Asia, and Africa.[11,13,14]

Until recently, the degree of genetic diversity of Brazilian *L. infantum* isolates has not been thoroughly investigated. Two studies identified a low degree of genetic variability among human *L. infantum* isolates from distinct geographic regions in Brazil.[15,16] In addition, a recent study performed a continent-wide analysis of New World *L. infantum* population structure and showed that Brazilian *L. infantum* strains belonged to the Old World MON-1 zymodeme, indicating a recent introduction of this parasite into the New World.[17] However, only few canine isolates have been analyzed in these previous studies.[11,12,17] To further investigate the degree of genetic diversity among *L. infantum* canine isolates in the New World, we compared the degree of genetic divergence among isolates from Jequié (Bahia) and Campo Grande (Mato Grosso do Sul), two geographically distinct regions in Brazil located 2,028 km apart from each other. If *L. infantum* was indeed recently introduced into the New World, we hypothesized that the parasite isolates from these two distant locations would show no significant degree of divergence. Genotyping was performed using both random amplified polymorphic DNA (RAPD) and multilocus microsatellite typing (MLMT) methods, and a high degree of genetic homogeneity among the canine isolates has been found, supporting the notion of a recent introduction of *L. infantum* into the New World.

MATERIALS AND METHODS

Animals. Stray dogs were collected from the streets of Jequié and Campo Grande and held in kennels for serological testing and clinical evaluation. To detect the presence of anti-*Leishmania* antibodies in sera of the captured dogs, an adapted enzyme-linked immunosorbent assay (ELISA)[18] was used in Jequié, whereas the indirect immunofluorescence antibody test (IFAT)[19] was used in Campo Grande. A clinical evaluation was performed for each animal according to a standardized protocol.[20] Stray dogs were usually kept at the kennel for 1 week or less, which is insufficient time for serological

*Address correspondence to Patrícia S. T. Veras, Laboratório de Patologia e Biointervenção, CPqGM, FIOCRUZ, RuaWaldemarFalcão, 121, Candeal, Salvador/BA, Brazil. E-mail: pveras@bahia.fiocruz.br

conversion and disease manifestation even if dog to dog transmission were to occur.

Endemic areas. The Municipality of Jequié covers an area of 3,035 km^2 and had a population of 151,921 in 2010, with 91.79% of the population living in urban areas and 8.21% living in rural areas (Brazilian Census Bureau [IBGE], 2010). Jequié is located at 11°10′50″ N latitude and 40°31′06″ W longitude at an altitude of 463 m above sea level. The Municipality of Campo Grande covers an area of 8,096 km^2 and had a population of 787,240 in 2010, with 98.65% residing in urban areas and 1.34% residing in rural areas (IBGE, 2010). Campo Grande is located at 20°26′34″ S latitude and 54°38′47″ W longitude at an altitude of 695 m above sea level. Jequié and Campo Grande were selected for the present study because of increases in the incidence of canine visceral leishmaniasis over the last 15 years.[2,21,22] Jequié is located in the Brazilian northeast in the state of Bahia (BA). Cases of canine visceral leishmaniasis have been reported in this locale since the 1960s,[23] and the number of human cases of visceral leishmaniasis has recently increased, with 118 cases reported between 2004 and 2008.[22] Campo Grande, the capital of Mato Grosso do Sul (MS), is located in southwestern Brazil. The first canine case in Campo Grande was recorded in 1998,[24] and 813 cases of human visceral leishmaniasis were reported between 2001 and 2008.[25]

Culturing of *Leishmania* isolates. *L. infantum* amastigotes were isolated from 20 spleen aspirates of infected dogs in Jequié, whereas bone marrow aspirates were isolated from 24 dogs in Campo Grande. At the time of use, all frozen amastigotes were thawed, and stationary-phase promastigotes were intraperitoneally inoculated in hamsters. After 60 days, the parasites were harvested from the spleens of infected hamsters as previously described.[26] To transform the isolated amastigotes into their promastigote forms, the isolated parasites were cultured in Novy–MacNeal–Nicolle (NNN) biphasic medium and Schneider's complete medium consisting of Schneider's insect medium (Sigma Chemical Co., St. Louis, MO) supplemented with 20% fetal bovine serum (FBS; Gibco BRL, New York, NY) and 100 μg/mL gentamicin (Sigma Chemical Co.). After reaching the stationary growth phase, the parasites were transferred to Schneider's complete medium at a concentration of 5×10^6 parasites/mL. After two, three, or four passages, an aliquot containing 1.4×10^7 to 1.4×10^8 parasites was used for parasitic genomic DNA extraction.[27]

***Leishmania* genomic DNA extraction.** *L. infantum* promastigotes in a stationary growth phase were lysed, and DNA was extracted using a phenol-chloroform method. After precipitation, the DNA samples were dried and dissolved in Tris (ethylenedinitrilo)tetraacetic acid (EDTA) buffer and then subsequently stored at −20°C until use as previously described.[27] The extracted DNA samples were quantified using a Nanodrop Spectrophotometer (Thermo, Wilmington, DE), and all concentrations were adjusted to 5 ng/μL in DNAse-free water. Aliquots containing the DNA samples, each with the required volume for either RAPD or MLMT reactions, were stored at −20°C until use.

RAPD. Forty-four DNA samples were amplified using the RAPD technique. Under standardized conditions, amplification was performed as previously described using the following primers: ABI-18 5′-CCACAGCAGT-3′, B-01 5′-GTTTCGCTCC-3′, and B-10 5′-CTGCTGGGAC-3′.[28] The reaction products were separated in a 1.3% agarose gel followed by gel staining in a 0.5 μg/mL ethidium bromide solution (Sigma) as previously described.[29] The Lb13500 strain of *L. braziliensis*, isolated from a Brazilian patient with mucocutaneous leishmaniasis, as well as the La483 strain of *L. amazonensis*, isolated from a Brazilian patient with localized cutaneous leishmaniasis, were used as outgroups. The reference strain *L. infantum* MHOM/BR/00/MER02, isolated from a Brazilian patient with visceral leishmaniasis, was previously typed using Multilocus Enzyme Electrophoresis (MLEE) as MON-1. Each RAPD reaction was repeated four times and run in duplicate.

Microsatellite typing. Seven microsatellite markers were selected for the present study: Li22-35, Li23-41, Li45-24, Li71-33, Lm2TG, Lm4TA, and TubCA.[30] These markers were previously developed for the *L. donovani* complex and showed polymorphism within the MON-1 zymodeme, the predominant *L. infantum* zymodeme in the Old World and New World.[17,30] Each polymerase chain reaction (PCR) was performed using fluorescent primers, 3 ng DNA target, and a stepdown protocol as described earlier.[16] The allele sizes were determined in a 6% denaturing polyacrylamide gel using an Automatic Laser Fluorescent (ALF) sequencer (GE Healthcare, Milwaukee, WI). Images were processed using the AlleleLocator software (GE Healthcare, Milwaukee, WI).

DNA fingerprinting analysis and estimation of genetic diversity. RAPD profiles of the 44 isolates were visually scored according to the presence (one) or absence (zero) of bands to construct a binary matrix. Only the reproducible bands that were present in three of four replicates were considered. Dendrograms were generated using the Phylogenetic Analysis Using Parsimony (PAUP) software package (Sinauer Associated, Inc., Sunderland, MA) based on neighbor-joining tree algorithms. Bootstrap analysis was performed using 100,000 iterations (95% confidence interval [CI]).

The MLMT data from 41 of 44 isolates were submitted to population genetic analysis using two approaches: a Bayesian model-based clustering method and a distance-based method. Genetic distances Dps (proportion of shared alleles) were calculated for the repeat numbers at each microsatellite locus using MICROSAT software.[31] Dps follows the infinite allele mutation (IAM) model in which every new mutation is assumed to give rise to a new allele.[32] A neighbor-joining tree was constructed based on these distances using the PHYLIP package v. 3.67[33] and visualized with TreeView v.1.6.6.[34]

To investigate population structure, a Bayesian model-based clustering method was used using STRUCTURE v.2.2 software.[35] The number of possible subpopulations derived from each isolate genotype dataset was determined by theoretically dividing the isolate populations into K subpopulations, with K ranging from one to six. To determine the probabilistic number of subpopulations, the Markov chain Monte Carlo method was used with searches consisting of a burn-in length of 50,000 iterations followed by a run of 250,000 replications for each setting of K (with 10 replicate runs of each). The estimated number of subpopulations in our samples was determined by comparison of the log-likelihood values for K = 1–6 and calculation of ΔK.[36]

To measure intra- and interpopulation diversity, the 41 of 44 isolates were segregated into two groups in accordance with geographic origin: Jequié (Group J; $N = 20$) and Campo Grande (Group CG; $N = 21$). The MLMT datasets were analyzed using Arlequin 3.5 software[37] to generate a series of parameters: (1) the mean observed and expected heterozygosity

(H_O and H_E, respectively) at Hardy–Weinberg equilibrium[38]; (2) the inbreeding coefficients calculated per locus (F_{IS}) as well as for each group (F_{ST}); (3) the proportion of polymorphic loci (P); and (4) the mean number of alleles per locus (MNA). F_{ST} values higher than 0.25 indicate strong genetic differentiation.

Ethics statement. All procedures involving animals were conducted in accordance with Brazilian Federal Laws pertaining to Animal Experimentation (Law 11794[39]; http://www.planalto.gov.br/ccivil_03/_ato20072010/2008/lei/l11794.htm). The procedures followed the Oswaldo Cruz Foundation guidelines for animal research (http://sistemas.cpqam.fiocruz.br/ceua/hiceuaw000.aspx) as well as the manual for the surveillance and control of visceral leishmaniasis.[22] This study was approved by the ethics committee for the use of animals in research (CPqGM-FIOCRUZ, CEUA, protocol 129 N.040/2005).

RESULTS

Dog collection and clinical classification. A total of 64 stray dogs were collected from the streets of Jequié in 2006, and 78 dogs were collected in 2008. Of these dogs, 128 animals tested serologically positive for anti-*Leishmania* antibodies. Parasite cultures from 20 samples of splenic aspirates tested positive. In Campo Grande, 100 stray dogs were captured, and 65 dogs tested serologically positive for anti-*Leishmania* antibodies. Parasite cultures from the bone marrow aspirates of 24 animals tested positive. The serologically positive dogs were euthanized in accordance with the Brazilian Ministry of Health's guidelines for visceral leishmaniasis control. Clinical examination data revealed that 34 of 44 parasitologically positive dogs presented symptoms consistent with visceral leishmaniasis. Of these dogs, 23 dogs were found to be polysymptomatic, 11 dogs were oligosymptomatic (with no more than three symptoms of visceral leishmaniasis), and 10 dogs were classified as asymptomatic. Table 1 lists *Leishmania* isolate identification and code information, year of parasite isolation, and location.

Table 1

Leishmania isolates obtained from stray dogs collected in two endemic areas of visceral leishmaniasis in Brazil

Code	Year	Origin
1J*	2006	Jequié
2J*	2006	Jequié
3J*	2006	Jequié
4J*	2006	Jequié
5J*	2006	Jequié
6J*	2006	Jequié
7J*	2006	Jequié
8J*	2008	Jequié
10J*	2008	Jequié
11J*	2008	Jequié
12J*	2008	Jequié
13J*	2008	Jequié
14J*	2008	Jequié
15J*	2008	Jequié
16J*	2008	Jequié
17J*	2008	Jequié
18J*	2008	Jequié
19J*	2008	Jequié
20J*	2008	Jequié
21J*	2008	Jequié
1CG*	2006	Campo Grande
2CG*	2006	Campo Grande
3CG	2006	Campo Grande
4CG*	2006	Campo Grande
5CG	2006	Campo Grande
6CG*	2006	Campo Grande
7CG	2006	Campo Grande
8CG*	2006	Campo Grande
9CG*	2006	Campo Grande
10CG*	2006	Campo Grande
11CG*	2006	Campo Grande
12CG*	2006	Campo Grande
13CG*	2008	Campo Grande
14CG*	2008	Campo Grande
15CG*	2008	Campo Grande
16CG*	2008	Campo Grande
17CG*	2008	Campo Grande
18CG*	2008	Campo Grande
19CG*	2008	Campo Grande
20CG*	2008	Campo Grande
21CG*	2008	Campo Grande
23CG*	2008	Campo Grande
24CG*	2008	Campo Grande
25CG*	2008	Campo Grande

*Isolates were also used in the MLMT analysis.
The above isolates were used for RAPD analysis.

Assessment of genetic diversity among *L. infantum* canine isolates in Jequié and Campo Grande using the RAPD technique. DNA samples were extracted from the 44 leishmanial isolates and amplified using the RAPD technique, which generated a total of 748 bands ranging from ~450 to ~2,080 base pairs that could not be scored as alleles at specific loci. Monomorphic bands comprised 29.4% of the analyzed bands, and 70.6% were absent for at least one isolate. DNA amplification using the B-01 primer produced polymorphic bands ~1,250 and ~2,100 base pairs in length (Figure 1). The ABI-18 primer produced polymorphic bands ~800, ~900, and ~1,500 base pairs (data not shown) in length, and the B-10 primer generated polymorphic bands ranging from ~900 to ~1,450 base pairs in length (data not shown).

The neighbor-joining tree (Figure 2) illustrates the distribution of *Leishmania* isolates according to degree of genetic similarity. *L. infantum* was used as a reference strain, whereas *L. amazonensis* and *L. braziliensis* were used as outgroups. A comparison of the RAPD profiles identified 24 genotypes among the 44 isolates (Figure 2). The genetic variance among these isolates ranged from 17.39% to 3.44%. Most of the neighbor-joining branches contained a mix of isolates from both Jequié and Campo Grande. Only two branches, A and B, were composed primarily of isolates from Campo Grande or Jequié, respectively. Bootstrap analysis showed low values (below 50%) for most of the branches. Only the branch connecting the outgroup strains (*L. amazonensis* and *L. braziliensis*) with the 44 *L. infantum* isolates presented significant bootstrap values (86%) (Figure 2). In sum, the tree revealed a predominantly mixed pattern of distribution of the isolates in relation to the geographical origin and also did not present any correlation with the year of parasite isolation (not shown).

Assessment of genetic diversity among *L. infantum* canine isolates in Jequié and Campo Grande using the MLMT technique. Genetic diversity was also assessed using MLMT. Amplification reactions were successfully performed for 41 of 44 isolates using seven microsatellite markers. None of these markers amplified DNA samples from *L. amazonensis* or *L. braziliensis*. The Li22-35, Li23-41, Li45-24, Li71-33, and TubCA loci were shown to be monomorphic, with isolates sharing identical alleles at each locus. However, the Lm4TA and Lm2TG loci were found to be polymorphic, with four and five allelic variants detected, respectively (Table 2).

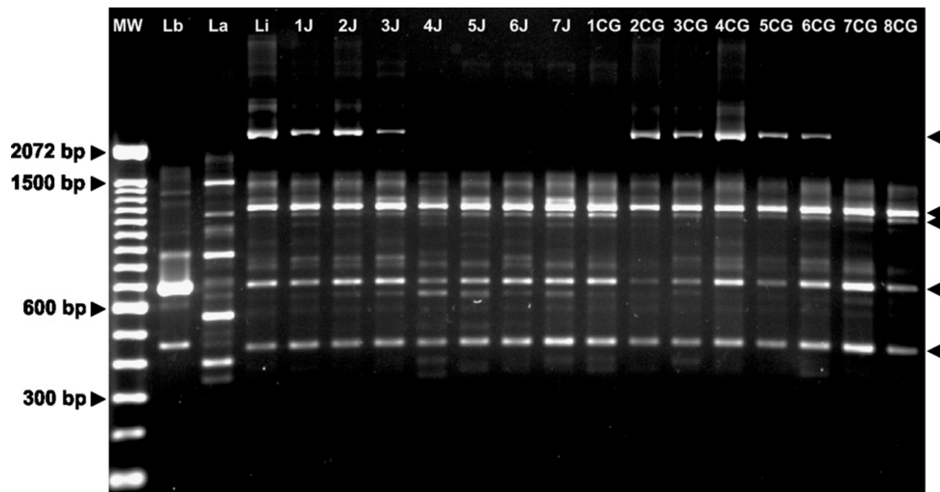

FIGURE 1. Genotypic profiles generated from RAPD reactions (primer B-01) using Jequié and Campo Grande isolates. Reaction products were separated by electrophoresis in an agarose gel; images were captured under ultraviolet light. The Lb13500 strain of *L. braziliensis* (Lb) and La483 strain of *L. amazonensis* (La) were used as outgroups, and the MHOM/BR/00/MER02 strain was used as a reference for *L. infantum* (Li) species. Arrows indicate polymorphic bands. CG = Campo Grande isolate; J = Jequié isolate; MW = molecular weight.

Nevertheless, the polymorphic loci exhibited the predominance of a single allele. Among the 41 canine *L. infantum* isolates evaluated, 11 distinct but closely related genotypes were identified. Most isolates had homozygous profiles with respect to all seven markers considered. Isolate H_O values were very low (Table 3), and F_{IS} values, which evaluate homozygosity at a given locus,[40] were determined to be positive (data not shown). Moreover, three or more peaks, which are suggestive of aneuploidy or mixed heterozygous strains, were not observed. Taken together, these findings indicate the predominance of homozygosity among these parasites.

Population diversity parameters for each group (Jequié and Campo Grande) are shown in Table 3. The mean expected heterozygosity (H_E) values and the MNA per locus were low for each group, and similar values were observed between the two groups. The proportion of polymorphic loci (P) was found to be identical within the two populations (0.286), and the F_{ST} values per population (Jequié = 0.116, Campo Grande = 0.111) and pair-wise value (0.113) remained low, indicating that no genetic differentiation was detected between these two groups.

MLMT datasets were used to calculate the genetic distances among isolates as well as construct a neighbor-joining tree (Figure 3). As observed in the RAPD-based tree, most branches contained a mix of isolates from both Jequié and Campo Grande, and only two branches were formed by isolates from the same geographic origin: one was exclusively composed of seven isolates from Campo Grande (cluster A) and another was composed of eight isolates belonging to Jequié (cluster B).

To further evaluate the degree of genetic differentiation with respect to geographical location, the MLMT profiles of all 41 isolates were analyzed using STRUCTURE[35] software to infer the population structure, with K ranging from one to six. Two subpopulations were identified in the datasets as determined by ΔK[36] (Figure 3). Subpopulation 1 consisted of 21 isolates (8 from Jequié and 13 from Campo Grande), whereas subpopulation 2 contained 20 isolates (12 from Jequié and 8 from Campo Grande). These findings support the notion that the two groups of *L. infantum* studied herein were not genetically structured in accordance to their geographic origin.

DISCUSSION

Using two distinct genotyping techniques, RAPD and MLMT, we have shown that the genetic profiles of canine *L. infantum* isolates from Jequié and Campo Grande are very similar regardless of their point of geographic origin. Although reproducibility of the RAPD technique has been called into question by some works,[41,42] it is an efficient and inexpensive technique that generates a substantial amount of information, and it is widely used as a tool in studies concerning the genetic diversity of *Leishmania* parasites.[11,13,29,43] Moreover, the reproducibility of RAPD can be improved by selecting optimized experimental designs,[29] such as those designs used in the present study.

The neighbor-joining tree generated from RAPD profiles illustrates the distances among the *Leishmania* isolates, the reference strain, and outgroups. All 44 isolates were found to belong to the same branch as the *L. infantum* reference strain (Figure 2), which indicates that all Jequié and Campo Grande isolates are *L. infantum*. By contrast, none of the isolates were found to be associated with the outgroup strains *L. amazonensis* and *L. braziliensis*.

In addition to RAPD, the MLMT technique was also used, because this approach allows for the differentiation of strains belonging to the *L. donovani* complex as well as within the complex itself (i.e., among *L. infantum*).[23,24] As observed in the RAPD analysis, the MLMT-based tree (Figure 3) depicted several clusters with short genetic distances between them. These clusters were composed of a mix of isolates from both geographic locations (cluster C) as well as two clusters that contained isolates exclusively from one of either of the two regions (clusters A and B). Moreover, the measurements of population genetic diversity were low, and geographic structure was not observed in the populations, which reinforces the notion that the canine *L. infantum* isolates studied belong to a homogeneous population. Similarly to the present study,

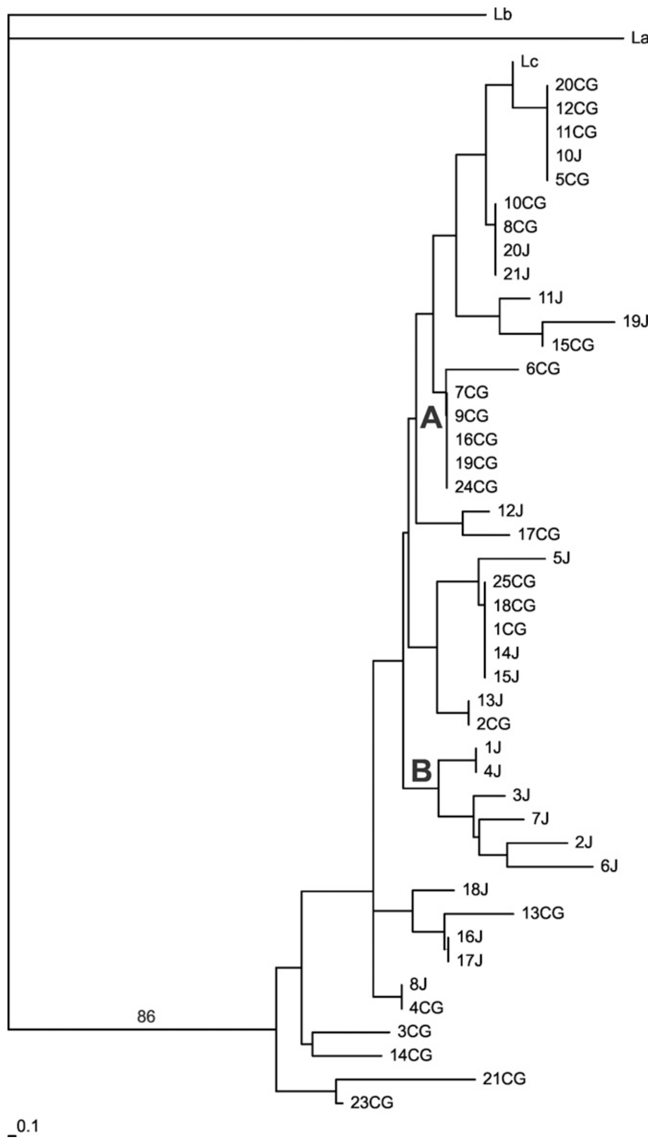

FIGURE 2. Neighbor-joining dendrogram generated from RAPD profiles illustrates the genetic distances between the *L. infantum* isolates. The bootstrap value is indicated in bold. A = cluster containing isolates only from Campo Grande; B = cluster containing isolates only from Jequié; bar size = genetic distance among isolates; CG = Campo Grande isolate; J = Jequié isolate.

TABLE 3
Genetic diversity parameters from MLMT analysis of *L. infantum* isolates

Population	N	P	MNA	H_O	H_E	F_{ST}	
						F_{ST}	Pair-wise F_{ST}
Jequié	20	0.286	1.571	0.064	0.146	0.116*	0.113*
Campo Grande	21	0.286	2.000	0.020	0.182	0.111*	0.113*

* $P < 0.05$.
F_{ST} = fixation index; H_E = expected heterozygosity; H_O = observed heterozygosity; MNA = mean number of alleles; N = number of strains; P = proportion of polymorphic loci.

another recently published report found minimal differences between genotype profiles among a large number of human *L. infantum* isolates from distinct geographic origins in Brazil using both RAPD and MLMT techniques.[16]

None of the seven microsatellite markers designed for the MLMT analysis amplified any DNA samples from *L. amazonensis* or *L. braziliensis*, which confirms the specificity of these primers to the *L. donovani* complex. Five of these seven markers were shown to be monomorphic. Using the same seven microsatellite markers, other works have detected locus polymorphism among *L. donovani* complex isolates from several locations in the Old World (India, East Africa, the Mediterranean Basin,[30] Portugal, Spain, and Greece[44]) and in strains from two regions of the New World (the Caribbean and Brazil).[17]

The work by Alonso and others[15] also found a high degree of homogeneity among New World *L. infantum* strains when using MLMT and DNA sequencing of the internal transcribed spacer 1 region. The findings in the work by Alonso and others[15] are consistent with the results of the present study in relation to the genomic DNA of *L. infantum* strains from a single endemic region in Brazil.[15] Similarly, the work by Segatto and others[16] also described a low degree of polymorphism among human *L. infantum* isolates from different geographical regions in Brazil when employing both RAPD and MLMT techniques.

However, when employing PCR-restriction fragment length polymorphism (RFLP) analysis of kinetoplastid DNA (kDNA) minicircles, the work by Alonso and others[15] found a significant level of genetic heterogeneity among New World *L. infantum* isolates. Although genotypes based on the kDNA minicircle have been used extensively throughout the literature for the purpose of strain identification[45–48] by extreme variations, conducting this type of procedure is an exceptionally detailed process, which limits its use for parasite genotyping.[49]

TABLE 2
MLMT genotypes of the *L. infantum* canine isolates based on the number of repeats of the microsatellite markers

Genotype	Number of isolates	Number of repeat units						
		Li45-24	Lm4TA	Li22-35	Li23-41	Li71-33	Lm2TG	TubCA
1	13	14	13	16	18	12	26	8
2	8	14	12/13	16	18	12	25	8
3	4	14	13	16	18	12	27	8
4	4	14	12	16	18	12	28	8
5	4	14	13	16	18	12	25	8
6	3	14	12	16	18	12	29	8
7	2	14	12/13	16	18	12	27	8
8	1	14	14	16	18	12	26	8
9	1	14	12/13	16	18	12	26	8
10	1	14	12	16	18	12	26	8
11	1	14	14/15	16	18	12	26	8
Number of alleles		1	4	1	1	1	5	1

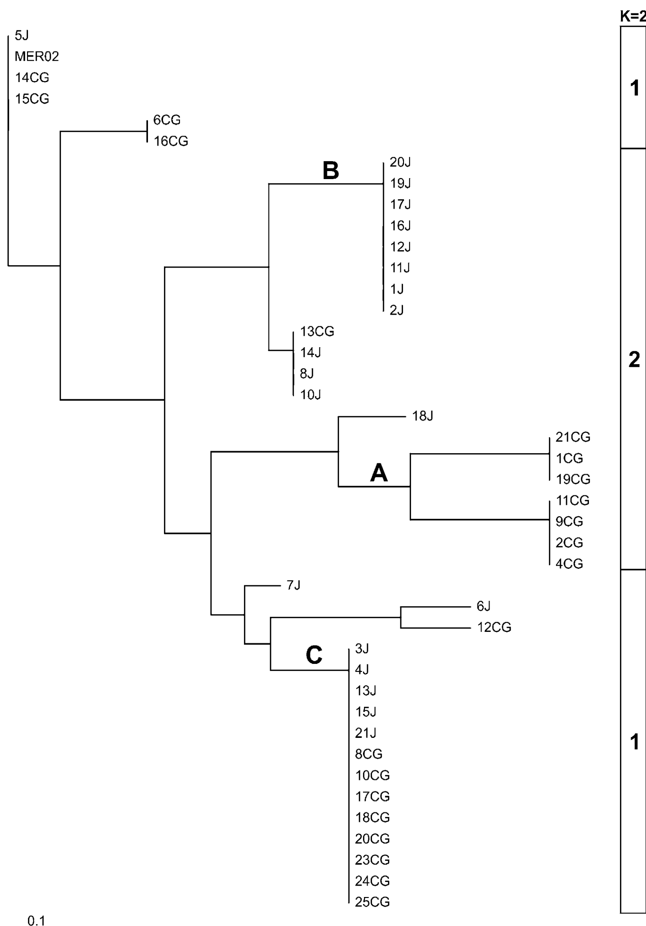

FIGURE 3. The neighbor-joining dendrogram built from MLMT profiles illustrating the genetic distances between the *L. infantum* isolates. MER02 is the *L. infantum* reference strain (MHOM/BR/00/MER02). A = cluster containing isolates only from Campo Grande; B = cluster containing isolates only from Jequié; C = cluster containing isolates from Campo Grande and Jequié; CG, isolates from Campo Grande; J, isolates from Jequié. The bars at the right show the estimated population structure as inferred by STRUCTURE.

A variety of factors may explain the degree of genetic homogeneity found among Brazilian leishmanial isolates *versus* the heterogeneity observed among the same parasite species in several regions of the Old World.[11,13,17,50] First, the *L. donovani* complex is known to have originated in the Old World.[11,13,14,50] Second, in the Old World, zoonotic visceral leishmaniasis is known to be transmitted by several vectors, including *Phlebotomus perniciosus*, *P. ariasi*, *P. perfiliewi*, and *P. neglectus*. However, in Brazil, *Lutzomyia longipalpis*[51] is known to be mainly responsible for disease transmission, but two other species, *Lu. cruzi*[52] and *Lu. evansi*,[53] have also been implicated. Nonetheless, the role that these vectors play in sustaining genetic diversity among *Leishmania* isolates merits additional study.[44] Finally, *L. infantum* causes pleomorphic diseases in humans with visceral and cutaneous manifestations in the Old World, but there is a predominance of visceral manifestations found in the New World.[28,54] Interestingly, *Leishmania* infection by these parasites in the Caribbean region can result in either type of clinical manifestation.[55,56] However, all parasite strains found in the Caribbean belong to non–MON-1 zymodeme, which is distinct from the zymodeme of the Brazilian MON-1 strains.[17] Perhaps the involvement of other vector species (e.g., *Lu. evansi*),[53] together with other unknown factors, may contribute to the variability seen in clinical manifestations around the world.

The low degree of genetic diversity found among these *L. infantum* isolates provides support for the theory proposed in the work by Momen and others[57] and supported in the work by Lukes and others,[11] which states that this parasite was recently introduced into the New World. Using MLMT, a recent study showed that Brazilian *L. infantum* strains belonged to the Old World MON-1 zymodeme.[17] Additionally, using 14 microsatellite markers, the work by Leblois and others[58] analyzed a large number of strains from the *L. donovani* complex and showed that most of the Central and South American *L. infantum* were organized within the Portuguese *L. infantum* clade. *L. infantum* would have likely diverged from the early *L. donovani* lineage ~1 million years ago in Eurasia. *L. donovani* invaded India and Africa soon thereafter, and the introduction of *L. infantum* in the Americas probably occurred during the European colonization. Probably, a parasite strain had crossed the Atlantic Ocean through infected dogs and reached Central America and Brazil around 500 years ago.[17,57,58] Founder effect creates a genetic bottleneck event that reduces the genetic variability of a new population. This scenario may be plausible with respect to *L. infantum* parasites, which supposedly use a predominantly asexual method of reproduction.[59]

In summary, we conclude that insufficient time has passed since the introduction of *L. infantum* in the New World for this species to evolve into genetically distinct parasite populations. The present study corroborates the theory that *L. infantum* has recently arrived in the Americas[11] and may contribute to future studies that aim to trace the history of visceral leishmaniasis in Brazil.

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Authors' addresses: Luís Fábio da S. Batista, Carlos Eduardo S. Guedes, Joselli S. Silva, Silvana O. Santos, Daniela Larangeira, and Patrícia S. T. Veras, Laboratório de Patologia e Biointervenção, CPqGM, FIOCRUZ, Salvador, BA, Brazil, E-mails: luisfabio.batista@terra.com.br, cesguedes@yahoo.com.br, jssilva@bahia.fiocruz.br, sicavet@yahoo.com.br, danielalarangeira@yahoo.com.br, and pveras@bahia.fiocruz.br. Marcela Segatto and Andréa Mara Macedo, Laboratório de Genética Bioquímica, Departamento de Bioquímica e Imunologia, ICB, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil, E-mails: marcelasegatto@yahoo.com.br and andrea@icb.ufmg.br. Cleusa A. T. Rodrigues, Universidade Federal de Mato Grosso do Sul, Departamento de Morfofisiologia, Campo Grande, MS, Brazil, E-mail: cleusaatr@terra.com.br. Júlia Cristina M. Brazuna, Prefeitura Municipal de Campo Grande, Secretaria Municipal de Saúde Pública Sesau, Centro de Controle de Zoonoses, Campo Grande, MS, Brazil, E-mail: ccz@sesau.capital.ms.gov.br. Rosana S. Sousa and Albert Schriefer, Universidade Federal da Bahia, Hospital Universitário Professor Edgard Santos, Serviço de Imunologia, Salvador, BA, Brazil, E-mails: rosanassousa@ig.com.br and aschriefer@globo.com.

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