Research Note

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Detection of *Leishmania infantum* in *Lutzomyia longipalpis* captured in Campo Grande, MS

Detecção de *Leishmania infantum* em *Lutzomyia longipalpis* capturados em Campo Grande, MS

Rodrigo Casquero Cunha[1]; Renato Andreotti[2]*; Marlon Cezar Cominetti[3]; Elaine Araújo Silva[4]

[1]Programa de Pós-graduação em Biotecnologia, Universidade Federal de Pelotas – UFPel, Pelotas, RS, Brasil

[2]Embrapa Gado de Corte, Campo Grande, MS, Brasil

[3]Programa de Pós-graduação em Doenças Infecciosas e Parasitárias, Universidade Federal de Mato Grosso do Sul – UFMS, Campo Grande, RS, Brasil

[4]Centro de Controle de Zoonoses – CCZ, Secretaria Municipal de Saúde, Campo Grande, MS, Brasil

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Abstract

Leishmaniasis is a zoonotic disease caused by protozoa of the genus *Leishmania* (Ross, 1903) and is the focus of considerable attention in human and veterinary medicine. In the city of Campo Grande, MS, the causative agent of visceral leishmaniasis is *Leishmania infantum* (= *L. chagasi*) primary vector, comprising approximately 92.9% of the local sandfly population, is *Lutzomyia longipalpis*. The aim of this work was to compare real-time PCR with PCR as a tool for the detection of the kinetoplast DNA (kDNA) of *L. infantum* in sandflies. Sandflies of this species were caught, and a total of 38 samples with 1-4 individuals in each sample were obtained; these were distributed across 13 districts and divided between seven urban areas of the city of Campo Grande, MS. Three positive samples were found by PCR and, when using real-time PCR, this was able to detect the presence of this agent in 6 of the 13 districts sampled, which were all located on the outskirts of the city, where indicates the greater enzootic potential of these regions, as they are closer to natural forest reserves. We conclude that real-time PCR can be used for epidemiological studies of *L. infantum*.

Keywords: PCR, real-time PCR, phlebotomines, epidemiology.

Resumo

A Leishmaniose é uma zoonose causada por protozoários do gênero *Leishmania* (Ross 1903), objetos de considerável atenção em medicina humana e veterinária. Na cidade de Campo Grande – MS, o agente etiológico da Leishmaniose Visceral é *Leishmania infantum* (= *L. chagasi*), e o principal vetor é a espécie *Lutzomyia longipalpis*, que representa cerca de 92,9% da população de flebotomíneos. Este trabalho teve como objetivo avaliar a PCR em tempo real como ferramenta para a detecção de kDNA de *L. infantum* em flebotomíneos, comparando-se com PCR convencional. Flebotomíneos dessa espécie foram capturados, somando 38 amostras de 1 a 4 espécimens cada, distribuídas em 13 bairros, divididos entre as 7 regiões urbanas da cidade de Campo Grande – MS, e armazenados a –70 °C até a extração de ADN e amplificação por PCR e PCR em tempo real. Das 38 amostras testadas, foram encontradas 3 amostras positivas pela PCR convencional e 11 pela PCR em Tempo Real. Na otimização da PCR em tempo real, a temperatura de dissociação do amplificado foi de 82, 89 °C. Neste estudo, utilizando-se a técnica da PCR em tempo real, foi possível detectar a presença desse agente em 6 dos 13 bairros amostrados, todos na periferia da cidade, indicando o maior potencial enzoótico dessas regiões, que têm maior proximidade com reservas de matas naturais. Conclui-se que a PCR em tempo real pode ser utilizada para estudo epidemiológico de *L. infantum*.

Palavras-chave: PCR, PCR em tempo real, flebotomíneos, epidemiologia.

Introduction

Leishmaniasis is a serious and potentially fatal disease. The *Centro de Controle de Zoonoses* (CCZ) of Campo Grande, MS has been working since 2005 to control this and other diseases that affect dogs and endanger the health of both dogs and humans. Since 2007, health workers have performed house-to-house surveys to collect blood from animals to test for the presence of leishmaniasis. Different protocols are followed based on the test results. The domestic dog is considered epidemiologically to be the most important reservoir for visceral leishmaniasis, which is transmitted by the bite of the sandfly (straw). To combat this

*Corresponding author: Renato Andreotti
Embrapa Gado de Corte, Av. Rádio Maia, 830, Zona Rural, CEP 79106-550, Campo Grande, MS, Brasil
e-mail: renato.andreotti@embrapa.br

disease, it is necessary to control the sandfly population and to monitor its domestic reservoir, the dog (SILVA et al., 2008).

The disease is contracted after a bite by an infected sandfly. When the parasite reaches the blood, it begins multiplying, and the animal can be considered infected. At this point, infected dogs become a source of the parasite for sandflies, which can subsequently infect other dogs and humans. The primary symptoms in humans are weight loss, anemia, paleness, weakness, breathing problems (e.g., coughing), diarrhea, abdominal growth, and an irregular fever over an extended period of time. The disease primarily attacks the elderly and children under ten years of age. In dogs, the primary symptoms are weight loss, skin flaking, hair loss, lesions around the eyes, tearing, injuries to the tips of the ears, overgrowth of the nails, and the muzzle and paws becoming quiet and still (apathy). A dog may be positive for canine visceral leishmaniasis, and not manifest any symptoms of the disease. In addition, studies show that up to 21.3% of asymptomatic animals have the potential to infect humans (MOLINA et al., 1994; GUARGA et al., 2000; COSTA-VAL et al., 2007).

Leishmaniasis is traditionally divided into visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL), as these diseases are caused by different *Leishmania* species. Visceral leishmaniasis is a serious chronic disease that is potentially fatal to humans, and mortality can reach 10% when not adequately treated (GONTIJO; MELO, 2004). In the city of Campo Grande, MS, the causative agent of visceral leishmaniasis is *Leishmania infantum* (= *L. chagasi*), and the primary vector, comprising approximately 92.9% of the local population of sandflies, is *Lutzomyia longipalpis* (Psychodidae: Phlebotominae) (SILVA et al., 2008).

The aim of this study was to test the efficacy of real-time PCR compared to PCR when detecting the kinetoplast DNA (kDNA) of *L. infantum* and to evaluate the presence of *L. infantum* in sandflies based on the real-time PCR technique.

Materials and Methods

Study area and capture

The county of Campo Grande occupies 8.096 km^2 in the central portion of Mato Grosso do Sul, near the watershed divide of the Paraná and Paraguay basins. The coordinates of its central milestone are 20°26'34" S, 54°38'47" W. Altitudes range from 500 to 675 m (CAMPO GRANDE, 2013). The population in 2006 was estimated to be 765,247 (IBGE, 2006). The climate is predominantly Aw (Köppen's classification), that is, tropical humid with wet summers and dry winters. The precipitation is heaviest from October to March, which is the period when mean temperatures are approximately 24 °C. June, July, and August are the driest months (SILVA et al., 2008).

The capture method and sites were performed as described elsewhere (SILVA et al., 2008). Briefly, 16 neighborhoods were chosen in the seven urban regions that comprise the entire county. Traps were placed outdoors and in households in neighborhoods with a higher prevalence of leishmaniasis and those in which human cases had been reported. In the Segredo region, the neighborhood chosen was Vila Nasser; in the Prosa region, Vila Margarida and Jardim Veraneio; in the Bandeira region, Maria Aparecida Pedrossian and Moreninhas; in the Central region, Amambai; in the Anhanduizinho region, UFMS, Guanandi, Jardim Aero-Rancho, Jardim Centenário, and Jardim Centro-Oeste; in the Lagoa region, Jardim Caiobá, Jardim Tarumã, and Jardim São Conrado; and in the Imbirussu region, Jardim Santo Antonio and Jardim Panamá. Captures were carried out from 4 pm to 7 am for four consecutive days each month from October 2005 to September 2006 using CDC-type light traps (8 traps per neighborhood for a total of 128 traps per month) (SILVA et al., 2008).

Lutzomyia longipalpis females were grouped in pools of 1 to 4 specimens depending on the neighborhood, capture site (indoors or outdoors), and month of capture and were frozen at –70 °C until DNA extraction.

DNA extraction and PCR

The DNA extraction was performed using a DNAzol kit (Invitrogen, Karlsruhe, Germany), as follows: homogenization of the sample material (1 pool of 1 to 4 specimens) with 1 mL DNAzol, 10 min of centrifugation at 16,000 × *g*, collection of the supernatant and precipitation with 500 µL ethanol. After 10 min of centrifugation at 16,000 × *g*, the supernatant was discarded, and the precipitate was diluted with 30 µL of 8 mM NaOH and stored at –70 °C. The concentration and purity of the DNA were not determined (SILVA et al., 2008).

All samples were subjected to conventional PCR to confirm that the DNA was appropriately preserved. PCR was performed using RV1/RV2 primers from Lachaud et al. (2002), according to Cunha et al. (2012), with some modifications. A 25 µL reaction was prepared containing 1 µL of DNA, 1x buffer, 0.22 mM dNTPs, 1.5 mM MgCl, 0.16 pmol of each primer, and 2 U of Taq polymerase, and pure water was added until the final volume was reached. The mixture was subjected to 94 °C for 4 min, followed by 40 cycles of denaturation (94 °C; 30 s), annealing (59 °C; 30 s), and extension (72 °C; 20 s), with a final extension at 72 °C for 3 min. These primers yielded 145 bp amplicons, which were visualized in 4% agarose gel by ethidium bromide staining (0.5 mg/mL) using a UV transluminator (CUNHA et al., 2012).

The reactions were standardized using control DNA from *L. infantum* (MHOM/BR/74/PP/75) provided by the Laboratory of Leishmaniasis of the Centro de Pesquisas René Rachou, Fiocruz (Belo Horizonte, Brazil).

Real time PCR

We developed a real-time PCR protocol for the detection of *L. infantum* DNA in phlebotomines. Kinetoplast DNA was chosen as the target to achieve high sensitivity. To accomplish this, the RV1 and RV2 primers were used. Amplifications were performed using an Applied Biosystems 7300 thermocycler, and each reaction was run in 12.5 µL of PCR mix composed of 6.25 µL of SYBR Green ROX Plus mix (Taq-Star DNA polymerase, reaction buffer, dNTPs, SYBR Green I, and ROX), 0.5 µL of each 10 pmol primer, 4.75 µL of water, and 1 µL of DNA or 1 µL water as a negative control. Cycling began with 15 sec at 95 °C, followed by 35 cycles of 15 seconds at 95 °C, 15 seconds at 55 °C, and 15 seconds at 72 °C. A standard curve was obtained

and a dilution series of standard DNA from *L. infantum* (MHOM/BR/74/PP/75) was used to evaluate the limit of detection of the reaction. The standard curve was established using *L. infantum* DNA extracted from 5×10^6 parasites from cell culture. Then, 1 µL aliquots of serial dilutions, ranging from 10 ng to 0.0001 ng of parasite DNA, were used to obtain the standard curve; these dilutions corresponded to 10,000 to 0.01 parasites, respectively. The accuracy of the assay was verified by performing real-time PCR experiments with 1 µL of water instead of DNA. The detection limit (parasites / sample pool of phlebotomine) was calculated by considering the amount of DNA in the reaction (1 µL) and the elution volume of the extracted DNA (30 µL).

Statistical analysis

The level of agreement between the results of the PCR and real-time PCR was measured using Cohen's kappa method at a 5% level of significance. The relationship between sensitivity and specificity was analyzed by constructing a receiver operator characteristic (ROC) curve.

To minimize possible errors and quantify the *Leishmania* infection/insect, a minimal infection rate (MR) was estimated using the formula MR=number of positive groups (pools) × 100 / number of total insects (PAIVA et al., 2006).

Results and Discussion

We obtained 38 samples of 1 to 4 individuals each of female *L. longipalpis*. The samples were obtained from 13 of the 16 neighborhoods in which collection was performed, which were divided between 7 urban regions in the county of Campo Grande (SILVA et al., 2008).

PCR using the RV1/RV2 primers allows for the detection of fewer than one *L. infantum* per sample. This set of primers does not amplify *L. amazonensis* and *L. braziliensis*, which are causative agents of Leishmaniasis in Brazil (GOMES et al., 2007) and in Mato Grosso do Sul (LIMA Jr et al., 2009; DORVAL et al., 2013; NUNES et al., 1995). The PCR reaction was able to detect 3 positive pooled samples (7.9%), two from Tarumã, which are samples that have previously been discussed (SILVA et al., 2008), and one from Centro-Oeste, which was not previously detected. This last sample was a single-specimen pool, which means that changes in the PCR protocol resulted in an increase in the ability of the technique to detect kDNA. Additionally, this finding means that the extracted kDNA maintained its integrity over time and could be utilized for real-time PCR. These two neighborhoods are in the southern region of Campo Grande, which may mean that there is a higher prevalence of female *L. longipalpis* infected with *L. infantum* in this region.

The analytical sensitivity of the real-time PCR technique was tested using serial dilutions of parasite DNA extracted from a known number of parasites. The kDNA of *L. infantum* was detected at a level corresponding to at least 0.01 parasites per reaction tube. Figure 1a presents the standard curve, slope, and detection level of the real-time PCR with a high coefficient of determination ($R^2 = 0.995$) and amplification efficiency (Eff% = 114.2). A slope of –3.3 corresponds to an efficiency of 100.0%, indicating that the number of amplified molecules doubles with each cycle of

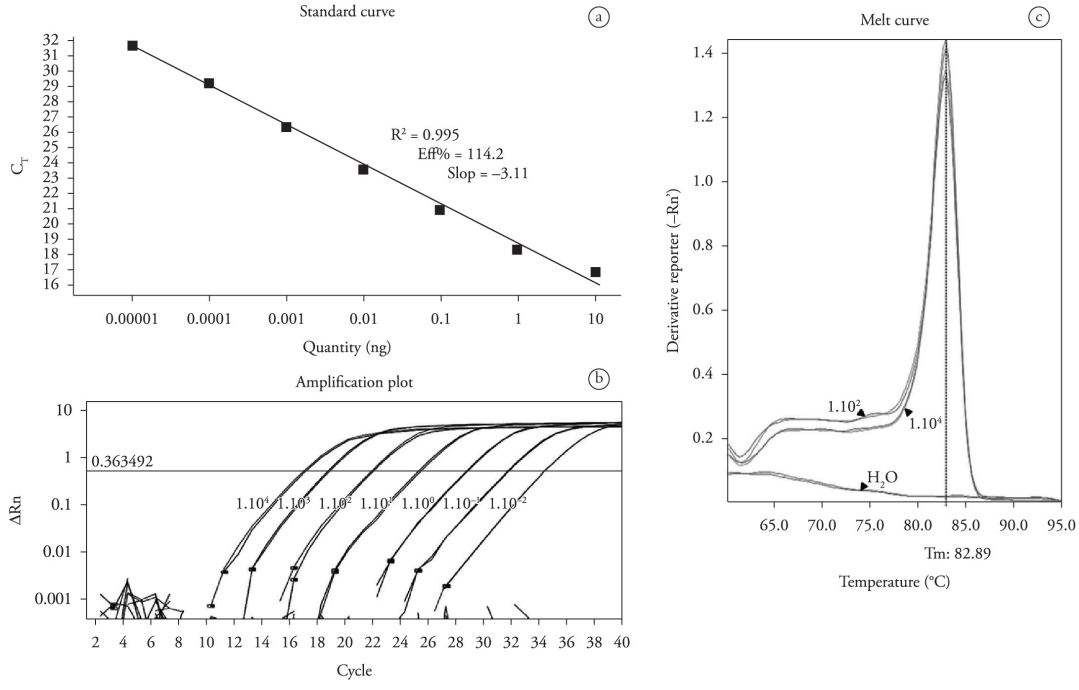

Figure 1. Real-time PCR optimization. a) Standard curve constructed using serial dilutions of *L. infantum* DNA expressed as the amount of DNA per reaction tube from 10 ng to 0.0001 ng, corresponding to from 10,000 to 0.01 parasites, respectively. Each point was tested in duplicate. Slope, –3.11; PCR efficacy, 114.2%; R^2, 0.995. b) Amplification curves for different dilutions of parasite DNA, and the threshold (0.363492). c) Dissociation curves of the amplification products from two *L. infantum* DNA dilutions (1×10^4 and 2×10^{-2} parasites per reaction) and from one negative reaction.

real-time PCR. The slope of our real-time PCR was –3.11, which demonstrates that real-time PCR was highly efficient. Figure 1b demonstrates the amplification plot for each serial dilution with its respective baseline start and threshold (0.363492). Figure 1c shows the dissociation curve for the kDNA amplicon yielded by the RV1/RV2 primers with a Tm of 82.89 °C. We can conclude from these results that real-time PCR was performed successfully and can be utilized for *L. infantum* kDNA detection.

Minicircle DNA was amplified from 11 samples (28.9%) by real-time PCR, generating a product of approximately 145 bp, which was observed by electrophoresis on 4% agarose gels. This product corresponded to the expected size. Positive samples were from Jardim Centro-Oeste (2/2), Maria Aparecida Pedrossian (1/4), Jardim Santo Antônio (1/3), Jardim São Conrado (4/5), Jardim Tarumã (2/3), and Vila Nasser (1/5). We did not detect the presence of the parasite in vectors captured in the neighborhoods of Jardim Aero-Rancho (1 sample), Jardim Caiobá (2 samples), Jardim Centenário (3 samples), Guanandi (1 sample), Vila Margarida (1 sample), Moreninhas (1 sample), and Jardim Pamaná (7 samples) by either PCR or qPCR. No phlebotomine females were captured from the neighborhoods of Jardim Veraneio, Amanbai and UFMS (Figure 2).

The detection limit was 0.026 parasite / *L. longipalpis*, taking into account the number of phlebotomines in each pooled sample. Table 1 shows the results for each technique and the agreement between the techniques. Discordance between the tests occurred only when the sample was positive by real-time PCR and negative by PCR. This can be explained by the higher sensitivity of real-time PCR compared with PCR.

The MIR of 100 specimens found by Silva et al. (2008) using PCR was 1.9%. In this work, when using PCR, the MIR was 2.9%, and when using real-time PCR, the MIR was 10.7%. *L. infantum* was detected in 6 of the 13 neighborhoods studied, all of which were on the outskirts of the city. This indicates the greater enzootic potential of these regions, which are closer to natural forest reserves. Furthermore, many of these neighborhoods are close to conservation areas or less-urbanized areas. However, these areas constantly suffer anthropic pressure, forcing wild animals (which are a food source for sandflies) to withdraw from the area to make room for human occupation. Once a house is built, it is common that domestic animals then serve as a source of food for sandflies, creating a link between the sylvatic and domiciliary environments. Additionally, wild areas near houses serve as a primary source for the maintenance of the parasite-vector-host cycle.

Table 1. Frequency distribution and agreement between the results of the two molecular tests (conventional PCR and real-time PCR).

Test		qPCR		Agreement (%)	Kappa (95% CI)	p-Value
		Negative	Positive			
PCR	Negative	27	8	78.95%	0.348 (0.107 to 0.589)	0.005
	Positive	0	3			

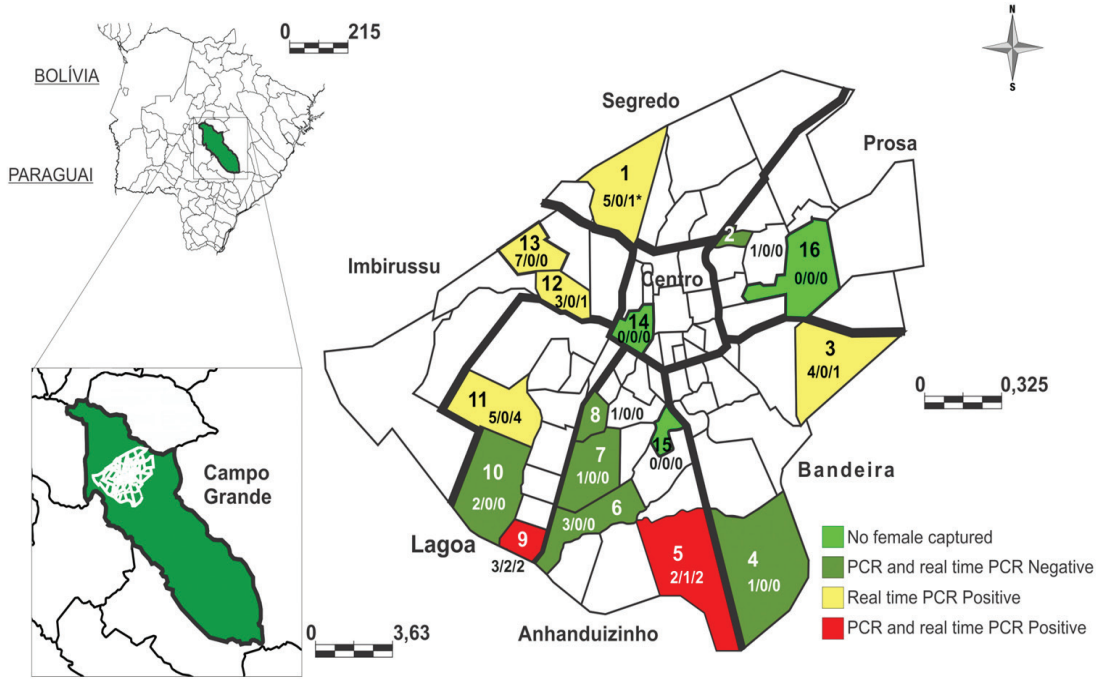

Figure 2. Location and map of Campo Grande, MS, and the distribution of regions and neighborhoods, highlighting the capture location of sandflies and outlining the distribution of samples of sandflies that were positive by real-time-PCR for *L. infantum*. 1. Vila Nasser, 2. Vila Margarida, 3. Maria Aparecida Pedrossian, 4. Moreninha, 5. Jardim Centro Oeste, 6. Jardim Centenário, 7. Jardim Aero Rancho, 8. Guanandi, 9. Jardim Tarumã, 10. Jardim Caiobá, 11. Jardim São Conrado, 12. Jardim Santo Antônio, 13. Jardim Panamá, 14. Amambai, 15. UFMS, 16. Jardim Veraneio. *Phlebotomines captured/PCR/qPCR. Scale in Km.

Based on this study, it can be concluded that real-time PCR can be used not only in epidemiological studies of *L. infantum* but also for other pathogens and their vectors. Additionally, of the methods available, real-time PCR is particularly advantageous because of its efficacy when analyzing samples containing a small number of parasites.

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