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ASSESSMENT OF PCR IN THE DETECTION OF *Leishmania* spp IN EXPERIMENTALLY INFECTED INDIVIDUAL PHLEBOTOMINE SANDFLIES (DIPTERA: PSYCHODIDAE: PHLEBOTOMINAE)

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SUMMARY

DNA amplification by the polymerase chain reaction (PCR) was applied in the investigation of the presence of *Leishmania* (Kinetoplastida: Trypanosomatidae) parasites in single phlebotomine sandflies. Three phlebotomine/parasite pairs were used: *Lutzomyia longipalpis/Leishmania chagasi, Lutzomyia migonei/Leishmania amazonensis* and *Lutzomyia migonei/Leishmania braziliensis*, all of them incriminated in the transmission of visceral or cutaneous leishmaniasis. DNA extraction was performed with whole insects, with no need of previous digestive tract dissection or pooling specimens. The presence of either mouse blood in the digestive tract of the sandflies or the digestive tract itself did not interfere in the PCR. Infection by as few as 10 *Leishmania* sp. per individual were sufficient for DNA amplification with genus-specific primers. Using primers for *L. braziliensis* and *L. mexicana* complexes, respectively, it was possible to discriminate between *L. braziliensis* and *L. amazonensis* in experimentally infected vectors (*L. migonei*).

KEYWORDS: *Lutzomyia; Leishmania;* PCR; Leishmaniasis.

INTRODUCTION

Leishmaniasis are a group of enzootic and zoonotic diseases caused by protozoan parasites of the genus *Leishmania* (Kinetoplastida:Trypanosomatidae) displaying varying degrees of specificity for the host[5]. They are widely distributed in the American continent from the United States to Northern Argentina, under two distinct clinical forms: visceral and cutaneous. The transmission of leishmaniasis occurs upon biting by several species of phlebotomine vectors, whose source of infection comprises different mammal species.

In Brazil, *Lutzomyia longipalpis* is the natural vector of *Leishmania chagasi*, agent of visceral leishmaniasis. On the other hand, the cutaneous form of leishmaniasis can be caused by several *Leishmania* species; among them *Leishmania braziliensis* and *Leishmania amazonensis,* belonging to *L. braziliensis* and *L. mexicana* complexes, respectively, are the most well known. *L. braziliensis* is spread over several Brazilian regions, whereas *L. amazonensis* was described in the northern South America. Both species of *Leishmania* may infect *Lutzomyia migonei*[4].

The rate of naturally infected sandflies in endemic areas and the correct identification of the infecting *Leishmania* in a determined phlebotomine species are of prime importance in vectorial and epidemiological studies of leishmaniasis. The most commonly used method for that investigation is the laborious and time-consuming search for the parasite *in loco*, after dissection of the digestive tract of the insect. Besides, in putative positive cases, the infection has to be confirmed by *in vitro* culture of the *Leishmania*, often susceptible to contamination, or by inoculation into laboratory animals, as other non-identified flagellates are commonly found in the digestive tract of those insects[13,15].

In the last ten years, molecular methods have been developed for the identification of certain species of *Leishmania*, either isolated from cultures or from patients[13] as well as in the detection of the parasite in a pool of phlebotomine specimens[14]. The main advantages of these methods are their sensitivity and specificity, independently of the number, stage and localization of the *Leishmania* in the digestive tract of the vector[10]. In the last few years, it has been successfully applied to field studies on the vectorial competence of phlebotomine sandflies[1,12], even in areas with low rates of infection[9,14].

Our aim in the present study was to assess the use of PCR in the detection of *Leishmania* sp. in individual sandflies (*L. longipalpis* and *L. chagasi)* experimentally infected, using the whole insect for the DNA extraction, without any dissection step. The specificity of the method was also tested after infection of *L. migonei* with *L. braziliensis* and *L. amazonensis*, to which complex-specific primers are available[6,8].

MATERIAL AND METHODS

***Leishmania* parasites** - Strains of *L. chagasi* (MHOM/BR/74/PP/75), *L. braziliensis* (MHOM/BR/75/M2903) and *L. amazonensis* (IPLA/

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BR/67/PH8) were cultured in M199 medium containing 5% fetal calf serum. *L. chagasi* amastigotes were obtained by *in vitro* transformation[2] whereas the other two strains were periodically inoculated into the rear paw of hamster (*Mesocricetus auratus*).

Assay sensitivity - 3- to 4-day-old laboratory reared females of *L. longipalpis* were dissected and to their digestive tracts were added serial dilutions of *L. chagasi* from *in vitro* cultures, containing 10, 100 or 1,000 promastigotes in 10 µl of PBS, followed by 10 µl of PBS or mouse blood. Controls containing *L. chagasi* culture dilutions only were prepared.

Blind assay - 3- to 4-day-old laboratory reared females of *L. longipalpis* were dissected and to their digestive tracts were added or not 10 µl of *L. chagasi* cultures. After codified identification, twenty samples were submitted to DNA extraction and amplification with genus-specific primers, as described.

Experimental infection - Groups of 130 4-day-old laboratory-reared females of *L. longipalpis* were experimentally infected by artificial feeding[15] on heparinized mouse blood, containing 2 x 10^7 parasites/ml of *L. chagasi*. The same procedure was used for infection of *L. migonei* with either *L. amazonensis* or *L. braziliensis*. Individual test samples were prepared with whole insects, without any dissection, 7 days after the artificial feeding, as follows: 1- Thirty samples of *L. longipalpis* after meal on blood infected with *L. chagasi;* 2- Thirty samples of *L. migonei* after meal on blood infected with *L. braziliensis*; 3- Thirty samples of *L. migonei* after feeding on blood infected with *L. amazonensis*. All samples were stored in 10 µl of PBS at –80 ºC. Each sample was submitted to DNA extraction and amplification, as described.

DNA extraction - DNA was extracted by maceration in a microtube using a plastic pestle, followed by addition of 35 µl of lysis buffer (100 mM TRIS-HCl, 100 mM NaCl, 25 mM EDTA, 0.5% SDS, pH 8.0) and another maceration step. The test samples were digested overnight at 37 ºC by proteinase K (1.25 µl of a 10 mg/ml solution) and the DNA extracted by phenol-chloroform[3]. The DNA pellet was ressuspended in 20 µl of TE (10 mM TRIS-HCl pH 8.0, 1 mM EDTA). Ten µl were used to estimate the DNA concentration and purity at 280 and 260 nm in a spectrophotometer, then discarded. The remaining 10 µl were stored at –20 ºC until use.

DNA amplification - Extracted DNA was amplified by hot start PCR in a thermocycler (Perkin-Elmer 2400). The reaction mixture was prepared as follows: 10 µl of PCR buffer (100 mM TRIS-HCl, 500 mM KCl, 15 mM $MgCl_2$, pH 9.0), 5 µl dNTPs (2 mM each), 2 µl each of primers (200 ng/µl), 0.5 µl of Taq DNA polymerase (2.5 U/µl) and 26.5 µl of ultrapure water. Two µl of DNA (10 ng/µl) were added per reaction and DNA amplification was performed as described, using primers previously designed for *Leishmania* spp.[7]: 5' GGG GAG GGG CGT TCT GCG AA 3'; 5'CCG CCC CTA TTT TAC ACC AAC CCC 3'; 5'GGC CCA CTA TAT TAC ACC AAC CCC 3'. In the case of amplification with specific primers, B1 (5' GGG GTT GGT GTA ATA TAG TGG 3') and B2 (5' CTA ATT GTG CAC GGG GAG G 3') for *L. braziliensis*[6] complex or M1(5'CCA GTT TCG AGC CCC GGA G3') and M2 (5'GGT GTA AAA TAG GGG CGG ATG CTC TG 3') for *L. mexicana*[8] complex, the following volumes were used: 5 µl of PCR buffer, 8 µl of dNTPs, 0.5 µl of each primer (20 pmol/µl), 2 µl of DNA (10 ng/µl), 0.5 µl de Taq DNA polymerase (2.5 U/µl) and 33.5 µl of ultrapure water. DNA amplification was performed for 35 cycles under the temperatures established before[6]. Negative (no DNA) and positive (*L. braziliensis* or *L. amazonensis* DNA) controls were used in all experiments.

DNA electrophoresis - 15 µl of the amplification products were analysed by 2% (genus level) or 1% (complex level) agarose gels (6.4 cm x 10.0 cm) in TBE buffer (0.089 M TRIS-HCl, 0.089 M boric acid, 0.02 M EDTA) containing 0.75% ethidium bromide for 30 min at 100 V, after addition of orange G as the tracking dye in the samples. ΦX174RF/*Hae*III or pUC19/*Hpa*II DNA fragments were used as size markers. The amplification products were visualyzed under UV light and the gels documented by an Eagle Eye System (Stratagene, La Jolla, USA).

Experimental rates of infection - Seven days after the artificial feeding, 30 specimens per group were dissected and examined microscopically for the presence of *Leishmania* parasites. Experimental rates of infection per group were determined as the percentage of infected individuals among the total examined.

RESULTS

Our initial step was to test the sensitivity of the PCR assay by using variable amounts of *Leishmania* parasites for DNA extraction. In the range tested, the amplified product characteristic of the genus *Leishmania* (120bp) was observed in samples containing as few as 10 promastigotes (Fig. 1A). Presence of digestive tract of the insect, with or without additon of mamalian blood, did not interfere with the assay (Fig. 1A). Hundred per cent of positivity was observed in the PCR when a blind assay was performed (Table 1).

Using experimentally infected sandflies, DNA amplification with genus-specific primers gave the results shown in Table 2, using whole *L. longipalpis* insects without any dissection step. The 120bp-fragment (Fig. 1B) was present in 87% of the test samples, respectively. The possibility of false-negative results due to the presence of any inhibitory contamination in the amplification reaction was checked by arbitrary addition of *Leishmania* DNA and new PCR. All negative results were confirmed (data not shown).

Similar assays using specific primers for *L. braziliensis* or *L. mexicana* complexes gave 40% and 36% positivity for *L. migonei*, respectively, indicated by the amplification of a 700-800bp fragment in both cases (Fig. 2A and 2B). The percentages of infection were further confirmed by amplification with genus-specific primers for *Leishmania.*

DISCUSSION

PCR has been extensively used in molecular diagnosis of human diseases and epidemiological studies due to its high sensitivity[11]. Theoretically, the presence of one parasite per sandfly should be enough to be detected by PCR[6]. In practice, purified DNA from 5 *L. (V.) braziliensis* parasites from serially diluted cultures were shown to be the minimum required for amplification[13]. However, this number has not been confirmed when field specimens were used for DNA extraction. The fact was attributed to a probable interference of digestive tract contents of the sandfly[1]. A semi-nested PCR was then developed which was able to detect a minimum of 3 parasites per sandfly[1]. Even though, the need of dissection was emphasized.

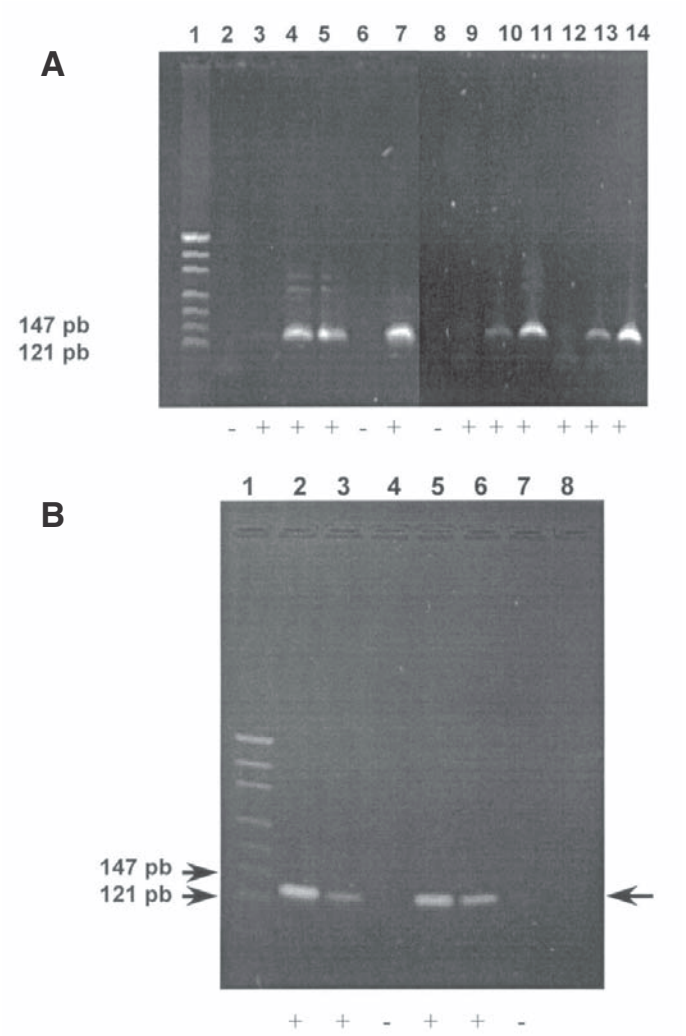

Fig. 1 - Electrophoresis on 2% agarose gel of PCR products after DNA amplification of test samples with genus-specific primers. A. DNA from cultured *Leishmania*. 1- pUC19/*Hpa*II DNA marker, 2- Whole mouse blood; 3, 4 and 5- 10, 100 or 1,000 parasites, respectively; 6- Negative control; 7- Positive control (*L. braziliensis* DNA); 8- Digestive tract of *L. longipalpis*; 9, 10, 11- 10, 100 or 1,000 parasites, respectively, in the presence of digestive tract of *L. longipalpis*; 12, 13, 14- 10, 100 or 1,000 parasites, respectively, in the presence of whole mouse blood and non-infected digestive tract of *L. longipalpis.* B. DNA from *Leishmania* infected sandflies. 1- pUC19/*Hpa* II DNA marker; 2- Positive control (*L. braziliensis* DNA); 3 to 7- Test samples in blind assay (dissected digestive tract of *L. longipalpis* with or without addition of *L. chagasi* parasites). 8- Negative control. Plus (+) or minus (-) at the bottom of the figures indicate the presence or absence of *Leishmania* in the test samples.

In our case, using regular PCR, the genus-specific fragment was obtained in the presence of as few as 10 parasites per sandfly when infection was simulated by adding a known number of parasites to the test sample. In a blind assay, the presence of the parasite was confirmed by PCR results in 100% of cases. The presence of digestive tract of *Lutzomyia* or any content of it including mouse blood, that could inhibit or interfere in the amplification reaction, did not interfere in the assay or its sensitivity.

Satisfactory results were obtained with whole sandflies, suggesting no need of dissection or pooling insects for application in field surveys. The percentages of positive samples by PCR, after experimental infection, were similar to the rates of infection determined by insect dissection. In the latter case, rates below 100% are usually obtained after sandflies feeding on mouse blood due mainly to no feeding at all or a natural failure in the establishment of infection.

Table 1
Detection of PCR products of *Leishmania* in a blind assay using dissected intestinal tract of *Lutzomyia longipalpis* in the presence or absence of *Leishmania chagasi* parasites as test samples*

Sample identification	Presence of genus-specific fragment after PCR	Parasite addition
1	+	Yes
2	-	No
3	+	Yes
4	+	Yes
5	-	No
6	-	No
7	+	Yes
8	+	Yes
9	+	Yes
10	-	No
11	-	No
12	-	No
13	-	No
14	+	Yes
15	+	Yes
16	+	Yes
17	-	No
18	+	Yes
19	+	Yes
20	-	No

* Test samples preparation: 3- to 4-day-old laboratory reared females of *L. longipalpis* were dissected and their digestive tracts added or not by 10 µl of *L. chagasi* cultures. After codified identification, twenty samples were submitted to DNA extraction and amplification with genus-specific primers, as described under Material and Methods.

Use of PCR for diagnosis of leishmaniasis presents many advantages, among which the possibility of precise identification of the infecting parasite in insect vectors as described here for *L. braziliensis*[6] or *L. amazonensis*[8], as long as appropriate primers are available without need of further hybridization[11,13]. The method is simple and fast enough to be used for screening a large number of insects.

RESUMO

Avaliação do PCR na investigação de *Leishmania* spp em flebotomíneos experimentalmente infectados (Diptera: Psychodidae: Phlebotominae)

Neste trabalho avaliamos o uso da reação em cadeia da polimerase (PCR) na investigação da presença de parasitas *Leishmania* (Kinetoplastida:Trypanosomatidae) em flebotomíneos individuais. Para isso, foram utilizados três pares flebotomíneo/parasita: *Lutzomyia*

Table 2
Comparison of *Leishmania* spp infection in *Lutzomyia* spp* by microscopic dissection or whole insect PCR using genus- and complex-specific primers

Phlebotomine Parasite	*L. longipalpis* *L. chagasi*	*L. migonei* *L. amazonensis*	*L. migonei* *L. braziliensis*
Size of amplified fragments	120 bp	760 bp	760 bp
Number of sandflies examined	30	30	30
Rate of experimental infection by microscopic examination (%)	70	40	50/55**
Rate of positivity by PCR (%)	87	33	40

* Groups of 130 laboratory-reared females of *L. longipalpis* were experimentally infected by artificial feeding on heparinized mouse blood containing 2 x 10^7 parasites/ml of *L. chagasi*. The same procedure was used for infection of *L. migonei* with either *L. amazonensis or L. braziliensis*. Thirty specimens per group were submitted to DNA extraction and amplification for determination of the rate of positivity by PCR. The experimental rates of infection were determined as the percentage of infected individuals among 30 microscopically examined (per group) for the presence of *Leishmania*. See further details under Material and Methods. ** Results of two independent groups.

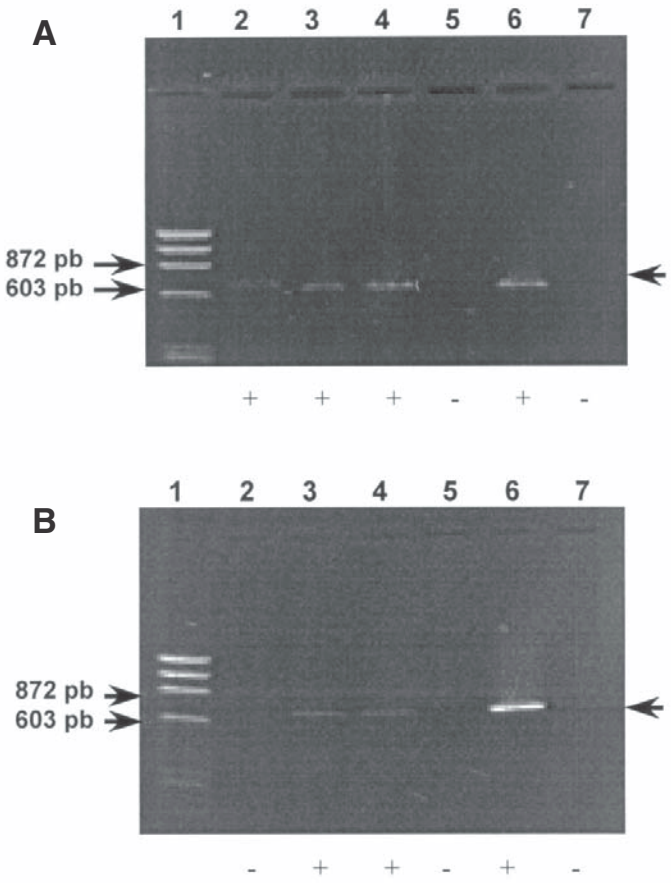

Fig. 2 - Electrophoresis profile on 1% agarose gels of PCR products after DNA amplification of test samples with complex-specific primers. A. DNA from *Leishmania* infected sandflies. 1- ΦX174RF/*Hae*III DNA marker; 2- Positive control (*L. braziliensis* DNA); 3 to 6- *L. migonei* after feeding in mouse blood infected with *L. braziliensis*. 7- Negative control. B. DNA from *Leishmania* infected sandflies. 1-ΦX174RF/*Hae*III DNA marker; 2 to 5- *L. migonei* after feeding in mouse blood infected with *L. amazonensis*; 6- Positive control (*L. amazonensis* DNA); 7- Negative control. The arrow on the right side of both figures indicates the 700 to 800-bp DNA fragment. Plus (+) or minus (-) at the bottom of the figures indicate the presence or absence of *Leishmania* in the test samples.

longipalpis/Leishmania chagasi, Lutzomyia migonei/Leishmania amazonensis e *Lutzomyia migonei/Leishmania braziliensis*, todos eles incriminados na transmissão de leishmaniose cutânea ou visceral. O DNA total a ser amplificado foi extraído de flebotomíneos inteiros, contendo ou não o parasita, sem dissecção prévia do trato digestivo ou combinação de indivíduos. Conteúdos do trato digestivo de flebotomíneos, em especial sangue de camundongo, não interferiram na reação de amplificação. Dez parasitas *Leishmania* sp. por flebotomíneo foram suficientes para detecção com iniciadores gênero-específicos. Com a utilização de iniciadores para os complexos *L. braziliensis* e *L. mexicana*, respectivamente, foi possível discriminar entre *L. braziliensis* e *L. amazonensis,* em flebotomíneos infectados experimentalmente (*L. migonei*).

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