

Parasitology

Comparison of polymerase chain reaction with other laboratory methods for the diagnosis of American cutaneous leishmaniasis

Diagnosis of cutaneous leishmaniasis by polymerase chain reaction

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Abstract

An evaluation of 5 laboratory methods for diagnosing American cutaneous leishmaniasis (ACL) was carried out on patients from an endemic area of Brazil. From 164 patients presenting cutaneous lesions, and suspected to have ACL, 133 (81.1%) were confirmed for the disease by Montenegro skin test (MST) and/or parasitologic examination (PE). In both groups of patients, the positivity of polymerase chain reaction (PCR) was similar to that of immunofluorescence assay and enzyme-linked immunosorbent assay, and higher than that of MST and PE ($P < .05$). In the group of patients suspected to have ACL, PCR presented the same positivity as PE and MST together. No correlation between positivity of the laboratory methods and clinical or epidemiologic aspects was observed. Our data confirmed the value of PCR as an alternative laboratory method for diagnosing ACL, especially for those patients with negative PE and MST.

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1. Introduction

American cutaneous leishmaniasis (ACL) is a parasitic disease caused by several species of the protozoa *Leishmania*, from the subgenera *Viannia* and *Leishmania*, and is endemic from the Yucatán peninsula to northern Argentina (WHO, 1990; Dedet, 1999; Lawn et al., 2004). ACL is therefore considered a great public health problem, with social and economic reflexes, and in most cases characterized as an occupational disease because it affects mainly rural workers. Recently, the disease has been reported to also present some characteristics of domiciliary and

periurban transmissions (Marzochi and Marzochi, 1994; Passos et al., 2000; Weigle et al., 2002; Castro et al., 2005). The annual incidence of cutaneous leishmaniasis in the world is estimated at 1.0–1.5 million cases (WHO, 1990). More than 90% of the cutaneous cases occur in Afghanistan, Saudi Arabia, Algeria, Brazil, Iran, Iraq, Syria, and Sudan (Singh and Sivakumar, 2003). In Brazil, the advance of ACL can be assessed by the disease detection rate, which increased from 10.45 in 1985 to 21.88/100 000 inhabitants in 2001 (BRASIL/MS/FUNASA/CENEPI, 2002).

The diagnostic methods available at present are mostly based on clinical and epidemiologic features, parasite detection (stained smears, culture, and histopathology), and immunologic methods (Rodrigues et al., 2002). Up to now, no single laboratory method has been accepted as the gold standard for diagnosing ACL. Parasitologic tests of a skin biopsy specimen are not always conclusive in patients

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with a clinical diagnosis of cutaneous leishmaniasis (Faber et al., 2003). Therefore, the association of immunologic tests with parasitologic examination has been used in the laboratory routine. The Montenegro skin test (MST) has been the immunologic method of choice followed by immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA) (Marzochi, 1992; Passos et al., 2000). Although serologic tests for ACL diagnosis present some limitations (Ulrich et al., 1988; Chiaramonte et al., 1999), they may contribute to the early diagnosis of mucosal lesions or extensive and multiple cutaneous lesions (Chiari et al., 1973a, 1973b; Mendonça et al., 1988).

The direct microscopic examination and MST, even when associated, are not sufficient to diagnose all ACL cases (Lopez et al., 1993). Thus, polymerase chain reaction (PCR) has recently been used for ACL diagnosis. PCR has been able to detect *Leishmania* DNA in human lesions and in other animals suspected to bear the infection (Rodgers et al., 1990; De Brujin et al., 1993; Lopez et al., 1993; Rodriguez et al., 1994; Pirmez et al., 1999; Marques et al., 2001; Weigle et al., 2002; Lawn et al., 2004).

The present study compares the positivity of the 5 most used laboratory methods for diagnosing ACL and reports clinical and epidemiologic aspects of the disease in the endemic region of Minas Gerais, Brazil.

2. Materials and methods

2.1. Patients

From 1997 to 2000, 164 patients with cutaneous but not with mucosal lesions were detected on clinical examination at the Leishmaniasis outpatient clinic in the city of Caratinga, Minas Gerais, Brazil. These patients were from rural regions of Vale do Rio Doce. A study was carried out with the purpose of describing clinical, epidemiologic, and laboratory aspects related to ACL. A questionnaire regarding the subject was submitted to all patients who answered the following questions about clinical and epidemiologic variables: lesion's time, number, site, and diameter; and patient's age, sex, and skin color. A differential diagnosis with other similar lesions was considered. Patients who presented typical cutaneous ulcers and a positive parasitologic examination (PE) and/or MST tests were defined as ACL clinical cases. Informed consent for blood collection and biopsies was obtained from all patients. The project was previously approved by the Ethical Committee for Human Research of Santa Casa de Misericórdia Hospital in Belo Horizonte, State of Minas Gerais, Brazil (CEPH # 007/98).

2.2. Parasitologic examination

After lesion asepsis, 0.5–1.0 mL of 2% xylocaine was subcutaneously injected nearby. A scalpel with a disposable blade was used for the biopsy. A wedged skin fragment (5 mm length) from the border of the lesion of each patient

was obtained. After removing the blood excess from the biopsy fragment, 12 imprints were performed for each of 3 microscopic slides. After air drying, slides were fixed in methanol, stained with Giemsa, and observed via optical microscopy (magnification $\times 1000$) for the presence of amastigote forms of the parasite. The results were obtained after a thorough examination of the 3 slides.

2.3. Montenegro skin test

This test was used to assess the patients' cellular immune response in vivo. A promastigote antigen from *Leishmania* (*Leishmania*) *amazonensis* (clone PH8-1 IIIId) forms was used (da Costa et al., 1996). The antigen was produced by Biobrás (Montes Claros, Minas Gerais, Brazil) under good manufacturing practice conditions and registered as Montenegro antigen C-40[®]. MST was carried out as described by Melo et al. (1977). The result was considered positive when an induration of ≥ 5 mm in diameter could be observed after 48 h of the antigen injection.

2.4. Polymerase chain reaction

The same tissue biopsy fragments used for slide imprints were further used for extracting *Leishmania* DNA. The DNA extraction from samples was performed with 100 μ L buffer solution (10 mmol/L Tris-HCl and 1 mmol/L ethylenediaminetetraacetic acid, pH 8.0) and 100 μ g/mL proteinase K (final concentration), incubated at 56°C for 3 h, and homogenized from time to time. The digestion was stopped by proteinase K inactivation by boiling it for 15 min. Samples were centrifuged, and the supernatant was used as the *Leishmania* template DNA source for the PCR reaction (Belli et al., 1998; Passos et al., 1999). Reaction mixtures contained 200 μ mol/L each of dUTP, dATP, dCTP, and dGTP, 1 μ mol/L of each primer, buffer (10 mmol/L Tris-HCl, 50 mmol/L KCl, pH 8.0), 1.5 mmol/L MgCl₂, 0.75 U of Taq DNA polymerase (PhN, Belo Horizonte, Minas Gerais, Brazil), and 1 μ L of DNA sample in a final volume of 10 μ L. Twenty microliters of mineral oil was poured over the reaction mixture to avoid evaporation. PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 29 cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 30 s, and a final extension at 72 °C for 5 min. Primers directed to amplify the conserved region of the *Leishmania* kDNA minicircle were: forward 5'-GGG (G/T)AG GGG CGT TCT (G/C)CG AA-3' and reverse 5'-(G/C)(G/C)(G/C) (A/T)CT AT(A/T) TTA CAC CAA CCC C-3', and the PCR product obtained was 120 nucleotide bp long (Degraeve et al., 1994; Passos et al., 1996).

DNA standards were obtained from *Leishmania* (*Viania*) *braziliensis* strains MHOM/BR/1975/M2903 and MCAN/BR/1973/BH 348 (Mayrink et al., 1979), from a reference cryobank at the Laboratório de Leishmaniose, Departamento de Parasitologia, Universidade Federal de Minas Gerais, Brazil. *Leishmania* DNA standards were obtained from promastigote forms cultivated in liver infusion

tryptose medium and collected at the exponential growth phase. Cultures were washed 3 times in sterile phosphate-buffered saline solution, pH 7.2, at 4 °C, centrifuged at $500 \times g$ for 15 min, and DNA was extracted as described previously. DNA samples were also obtained from skin biopsies of 26 individuals without lesions or other lesions than ACL. These patients were diagnosed by clinical and parasitologic examinations at the Clínica Dermatológica (a dermatological clinic) of the Santa Casa de Misericórdia Hospital in Belo Horizonte, Brazil, and were used as controls.

A search for inhibitors was performed in samples that showed PCR-negative results to assess possible PCR failures to detect *Leishmania* DNA. In the same PCR tube, 1 μ L of DNA preparation from a negative sample and 1 μ L of *Leishmania* DNA standard sample at 10 fg/ μ L were added as template. A positive result means no presence of inhibitors in the tested sample. The biologic materials were coded before the laboratory tests to avoid any bias on the interpretation of the results.

2.5. Serology

The IFA and ELISA were used for detecting anti-*Leishmania* total IgG in sera of 164 patients with cutaneous lesions who were suspected to have ACL. IFA was evaluated using total anti-IgG conjugates labeled with fluorescein isothiocyanate, specific for humans and diluted at 1:100 (Bio-Manguinhos/FIOCRUZ, Rio de Janeiro, Brazil). The antigen was obtained from promastigote forms from *L. (L.) amazonensis* MHOM/BR/1960/BH6 strain fixed with 1.0% formalin in saline. Titer $\geq 1:40$ was considered positive (da Costa et al., 1991). ELISA was performed using total anti-IgG conjugates, labeled with peroxidase, specific for humans, and diluted at 1:3000 (Sigma Chemical Company, St. Louis, MO). Soluble antigen of *L. (V.) braziliensis* obtained from the MHOM/BR/1975/M2903 strain was used. Sera from 40 individuals from Caratinga, Minas Gerais, with no history of ACL or cutaneous lesions and negative IFA were diluted at 1:80 and used to determine the cutoff point for ELISA. The cutoff point was established as the average absorbance at 492 nm of those sera plus 2 SD (Abs ≥ 0.125).

2.6. Treatment

Patients defined as ACL clinical cases received antimicrobial therapy (Glucantime®, Rhodia, Brazil) at the dosage of 17 mg Sb^v/(kg day) i.m. (Mayrink et al., 1976), for 10 consecutive days, alternated with a 10 day-interval without treatment. Patients were submitted to treatment cycles until the lesions were healed.

2.7. Statistics

A correlation between clinical and epidemiologic parameters was determined by Pearson's coefficient. A comparative analysis of positivity between the laboratory methods was performed using χ^2 test.

3. Results

3.1. Casuistic description

Of 164 suspected patients, 133 (81.1%) were confirmed as ACL clinical cases according to the case history (individuals exposed to risk of acquiring the disease), clinical examination of lesions, MST, and/or PE (Fig. 1). Most ACL patients were white males, aged 2–89 years (mean, 29 years; median, 32 years), and 63.9% aged 10.5–40.5 years.

3.2. Clinical characteristics of lesions

The main clinical characteristics of ACL patients' lesions were a single lesion (66.2%), with an average of 14.5 mm, median of 12.0 mm, and diameter range of 5.0–50.0 mm, on the lower limbs (47.6%) and a developing time of ≤ 60 days (66.9%). Pearson's index values have shown no correlation between clinical and epidemiologic parameters (lesions' developing time, number, site, and diameter; and patients' age, sex, and skin color).

3.3. Quantitative analysis of MST, IFA, and ELISA according to the developing time of lesions

One hundred patients positive to MST, 109 to IFA, and 112 to ELISA were evaluated (Table 1). The induration of MST ranged from 8 to 45 mm, with a mean of 24.3 mm and median of 25.0 mm. The titer of IFA ranged from 1:40 to 1:640, with mean of 1:116 and median of 1:80. The absorbance of ELISA ranged from 0.129 to 0.504, with a mean of 0.248 and median of 0.228. Median was used as cutoff point to separate samples in the laboratory tests. Quantitative analyses show no significant differences for

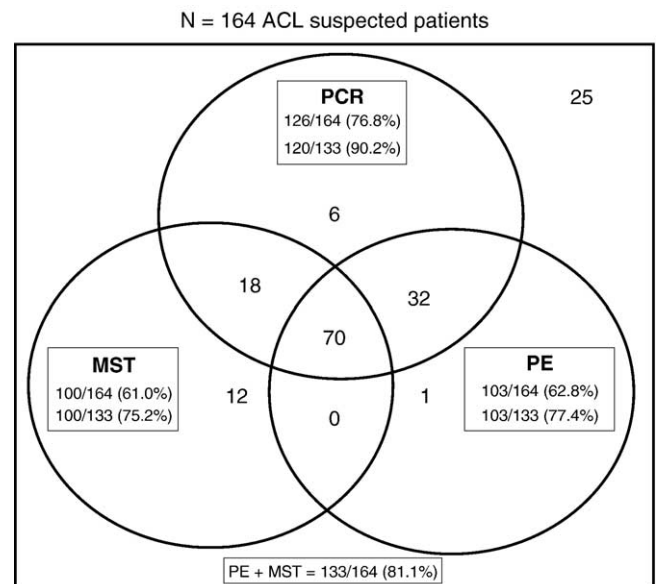


Fig. 1. Venn diagram with data about PCR, PE, and MST positivity regarding ACL-suspected patients ($n = 164$; PE + MST = PCR > PE = MST, $P < .05$) and confirmed ones ($n = 133$; PE + MST > PCR > PE = MST, $P < .05$).

Table 1

Quantitative analysis of positive patients to MST, IFA, and ELISA according to the developing time of lesions

Developing time of lesions (days)	Patient proportion (%) ^a (n = 164)					
	MST induration diameter (mm) (n = 100)		IFA titer (n = 109)		ELISA absorbance (n = 112)	
	≤25.0 ^a (n = 60)	>25.0 ^a (n = 40)	≤1:80 ^a (n = 70)	>1:80 (n = 39)	≤0.228 ^a (n = 57)	>0.228 (n = 55)
≤60 ^a	67.0	75.0	64.0	59.0	68.0	60.0
>60	33.0	25.0	36.0	41.0	32.0	40.0

^a Cut-off represents the median of the parameters (developing time of lesions, MST diameter, indirect IFA titer, and ELISA absorbance).

* P > .05.

MST, IFA, and ELISA regarding the developing time of lesions ($P > 0.05$).

3.4. Comparative analysis of ACL diagnosis by different laboratory methods

Fig. 1 and Table 2 show the positivity of 5 laboratory methods for diagnosing ACL in groups of suspected and confirmed patients. Patients were considered suspected based on suggestive clinical and epidemiologic aspects only or when they were negative for PE and MST. On the other hand, patients were confirmed with ACL when, besides the suggestive clinical and epidemiologic aspects, they were also positive for PE and/or MST. Analysis of positivity for MST, PCR, and PE of patients suspected of ACL ($n = 164$) shows that 12, 6, and 1 patients were positive exclusively in each one of the methods, respectively. Furthermore, 70 patients were positive and 25 negative in the 3 methods. The positivity of the 5 methods was higher in patients of the ACL-confirmed group than those from the suspected one ($P < .05$). In both groups of patients, the positivity of PCR was the same as that of IFA and ELISA (Table 2) and higher than that of MST and PE (Fig. 1; $P < .05$). In the group of patients suspected to have ACL, PCR presented the same positivity as PE and MST together (Fig. 1). PE and MST were used as standards to calculate the positive predictive value (PPV) and the negative predictive value (NPV) of diagnostic methods (Table 2); therefore, these parameters were not applicable

Table 2

Positivity and predictive values of different laboratory methods for diagnosing ACL in groups of suspected and confirmed ACL patients

Group of patients	Positivity of the laboratory method, ^a n (%)			P
	PCR	IFA	ELISA	
PE+ (n = 103)	102 (99.0)	97 (94.2)	98 (95.1)	.16
PE-/MST+ (n = 30)	18 (60.0)	10 (33.3)	14 (46.7)	.11
ACL confirmed (n = 133)	120 (90.2)	107 (80.4)	112 (84.2)	.10
ACL-suspected (n = 164)	126 (76.8)	109 (66.5)	112 (68.4)	.08
PE-/MST- (n = 31)	6 (19.4)	2 (6.5)	0 (0.0)	.02*
Predictive value				
PPV	(95.2)	(98.2)	(100.0)	
NPV	(65.8)	(52.7)	(59.6)	

^a The positivity of the methods was higher in the ACL patient confirmed group than in the suspected one ($P < .05$).* $P < .05$, statistically significant.

for both. Thus, the following percentages regarding PCR, IFA, and ELISA, respectively, were found: 95.2%, 98.2%, and 100.0% for PPV, and 65.8%, 52.7%, and 59.6 for NPV. Emphasis should be given to the fact that in the group of ACL-suspected patients, PCR was positive in 6 of 31 individuals (19.3%) who were negative for PE and MST (Table 2). Of those 6 patients, 3 returned to the clinic and were then submitted to new tests (PE and MST), being confirmed as ACL cases. They were treated and their lesions healed.

Samples from patients with a positive PCR result have shown a unique migration band of the expected size for *Leishmania* (120 bp) after polyacrylamide gel electrophoresis. It was exactly in the same position as for the positive controls at 0.01 and 1.0 pg of *L. (V.) braziliensis* DNA (data not shown). The 26 patients without lesions or with skin lesions other than ACL have shown no specific PCR products for *Leishmania* sp. PCR-negative samples presented no inhibitors in their DNA preparations.

The Pearson's index values have shown no correlation between positivity of the laboratory methods (PE, MST, IFA, ELISA, and PCR) and clinical and epidemiologic parameters (lesions' developing time, number, site, and diameter; patients' age, sex, and skin color).

3.5. Treatment

ACL patients needed an average of 3.54 treatment cycles (71 days) for the complete healing of the lesions. The 133 ACL patients were treated and cured. No side effects requiring treatment discontinuation were observed.

4. Discussion

The advantages of PCR for diagnosing leishmaniasis should be considered in the context of the diagnostic services and according to epidemiologic and clinical characteristics of the disease (Weigle et al. 1993, 2002; Lawn et al., 2004). In the present study, clinical and epidemiologic aspects of ACL in the endemic region of Minas Gerais, Brazil, were reported. Although most ACL patients still come from rural areas, the regular attendance at the Leishmaniasis outpatient clinic, the awareness of the population about the health care facilities and services of the counties around the endemic region have changed the profile of the ACL patients over the last 30 years. A study undertaken by Chiari et al. (1973a) in the same region

showed that 62.2% of the ACL cases presented skin lesions with a developing time of >60 days. At present, the rate decreased to 33.1% suggesting that the population has acquired more knowledge about the disease and has looked for medical assistance more rapidly.

The ACL transmission pattern has changed in the recent years with outbreaks occurring in long-established rural settlements and urban areas (Lainson, 1989; Gontijo et al., 2002; Castro et al., 2005). However, our data have shown that the ACL patients were from rural regions and that 63.9% were between 10.5 and 40.5 years old. At these ages, individuals are more involved in agricultural activities and, as a consequence, they are more at risk for infection, suggesting that ACL transmission in that region still occurs predominantly outside the dwellings. These data corroborate with those of Machado-Coelho et al. (1999) on the occupational character of ACL in the region of Vale do Rio Doce.

In the present study, it was observed that most patients presented a single lesion (66.2%), with an average of 14.5 mm in diameter, on the lower limbs (47.6%), and a developing time of ≤ 60 days (66.9%, $P < .05$). The duration of these patients' lesions differed from that of other regions, which varied between 0.5 and 168 months (Saravia et al., 1989; de Brujin et al., 1993; Aviles et al., 1999; Passos et al., 2000). On the other hand, the predominance of a unique lesion on the lower limbs has been the most frequent finding (Cuba-Cuba et al., 1984; Mendonça et al., 1988; Marzochi, 1992; Convit et al., 1993; Passos et al., 2000). Numerous primary and secondary skin diseases and conditions are frequently misdiagnosed as early lesions of cutaneous leishmaniasis (Singh and Sivakumar, 2003). In that sense, a differential diagnosis with other similar lesions, such as varicose ulcer, impetigo, tuberculosis, leprosy, syphilis, blastomycosis, sporotrichosis, skin cancer, and other cutaneous lesions, was considered. However, other diagnostic methods are required to confirm the clinical suspicion (Herwaldt, 1999).

PE positivity in ACL-suspected patients (62.8%) reached the upper values reported—33.0% to 63.0% (de Brujin et al., 1993; Rodriguez et al., 1994; Pirmez et al., 1999; Aviles et al., 1999). Along the years, the finding of *Leishmania* by PE at the leishmaniasis outpatient clinic in Caratinga has improved greatly. PE increased from 19.4% (Mayrink et al., 1979) to 62.8% at present. This improvement seems to be due to a better tissue collection, slide preparation, and an exhaustive examination of 12 imprints performed for each of the 3 microscopic slides. No correlation between positivity of the PE and developing time of the lesions could be observed. PE positivity for patients with ≤ 60 -day-old lesions was identical to that of patients with >60-day-old lesions. Nevertheless, there are studies showing a greater difficulty to detect parasites on >90-day-old (Pirmez et al., 1999) and 180-day-old lesions (Weigle et al., 1987; Gutierrez et al., 1991; Marzochi, 1992; Singh and Sivakumar, 2003).

The ease of processing and the short time to obtain MST results (48 h) allowed its use as the main indirect test performed in the laboratory for diagnosing ACL cases in endemic areas (Gontijo et al., 2002; Oliveira et al., 2003). In a study of mucocutaneous leishmaniasis in Três Braços, Bahia, Brazil, Cuba-Cuba et al. (1984) found out that 93.0% of patients with cutaneous lesions were MST positive. They also showed that MST could not be positive up to 6 or more weeks after the emergence of cutaneous lesions. The present work shows that MST presented 61.0% positivity for patients suspected and 75.2% for patients confirmed with ACL. It has been described that MST may remain positive after clinical cure (Furtado, 1980; Cuba-Cuba et al., 1984; Kar, 1995). Moreover, MST has been pointed out as a good marker of cell immune response in leishmaniasis whose negative results may imply in failure in the treatment (Saravia et al., 1990; Passos et al., 2001). Furthermore, MST is not able to differentiate an active from an inactive infection. In our study, MST was associated with PE for diagnosing ACL infection and confirmed the disease in 81.1% of the patients suspected to have ACL.

Although, at present, no single laboratory technique has been accepted as the gold standard for diagnosing *Leishmania* infection, the results obtained by PCR are significant. PCR presented 76.8% positivity for the patients suspected to have ACL and 90.2% for the patients confirmed with ACL. The positivity of the 5 methods used in this work was higher in patients of the confirmed group than in those from the suspected one ($P < .05$). Belli et al. (1998) described that PCR showed 100.0% sensitivity and specificity when compared with direct microscopy. Pirmez et al. (1999) reported in ACL patients a PCR positivity of 96.9% and a PE positivity of 67.4%. Our results have shown that in both groups of patients the positivity of PCR was higher than that of PE and MST alone. PCR appears to be the most sensitive single diagnostic test for cutaneous leishmaniasis (Weigle et al., 2002; Faber et al., 2003; Lawn et al., 2004). As the parasite finding or the isolation and growth have a lower sensitivity, PCR should be added to the lesion healing as criteria of cure for the ACL treatment (WHO, 1990; Guevara et al., 1993; Delgado et al., 1996; Schubach et al., 1998; Passos et al., 2001; Coutinho et al., 2002).

It is worthwhile to note that PCR was positive in 6 of 31 patients (19.4%) who were negative for PE and MST, thus showing the failure of these 2 methods in diagnosing ACL, even when they were associated. Of the 6 PCR-positive patients, 3 returned to the clinic and were further confirmed for ACL by either PE and/or MST. They were treated and cured, therefore demonstrating the PCR value under those circumstances.

Positivities of IFA, ELISA, and PCR were similar for each group of patients. The positivities observed for IFA (66.7%) and ELISA (68.4%) were approximately the same as those reported (Cuba-Cuba et al., 1984; Garcia-Miss et al., 1990; Convit et al., 1993; Passos et al., 2000). Although

there are reports in the literature about the correlation between quantitative data of IFA and ELISA and the number and time of lesions (Chiari et al., 1973a, 1973b; Mendonça et al., 1988; Kar, 1995), we have not observed such correlations. Maybe because the humoral immune response is time-dependent and most of the lesions of our casuistic were recent (≤ 60 days), given insufficient time for the immune response to be elicited. Moreover, quantitative serologic evaluation has been suggested in the follow-up of the treated patients, as there is a reduction of the titers with the therapy (Chiari et al., 1973b; Walton, 1980; Mendonça et al., 1988; Passos et al., 2001).

PCR should be used selectively for chronic cases or when other methods have not detected ACL case. In conclusion, our data confirm the value of PCR as an alternative laboratory method for diagnosing ACL, particularly in those cases where PE and MST had failed to detect the disease.

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