Parasitology

Mucocutaneous leishmaniasis: accuracy and molecular validation of noninvasive procedures in a L. (V.) braziliensis–endemic area

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ABSTRACT

The aim of this study was to evaluate the effectiveness of polymerase chain reaction (PCR) using Kinetoplastid DNA (kDNA) from nasal swabs (NSs), saliva, and oral filter paper imprints (OFPI) in diagnosing mucocutaneous leishmaniasis (ML) and cutaneous leishmaniasis (CL). Seventeen patients with ML, 19 patients with CL, and 33 controls were evaluated. In patients with ML, PCR from NS showed an 86% diagnostic accuracy (95% confidence interval [CI] = 73.81–93.05), followed by saliva 74% (95% CI = 60.45–84.13) and OFPI 68% (95% CI = 54.19–79.24). The highest sensitivity was reached by using the NS 58.82% (95% CI = 36.01–78.39), followed by saliva 23.53% (95% CI = 9.56–47.26) and OFPI 5.88% (95% CI = 1.05–26.98). The specificities of the tests were complete. The NS and OFPI were positive in 2 cases of CL. Mucous membrane samples exhibited a higher specificity compared to the Montenegro skin test and indirect immunofluorescence. NS sensitivity was higher than that of parasitological examinations.

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1. Introduction

The accurate diagnosis of American tegumentary leishmaniasis (ATL) is difficult, and no established gold standard currently exists (Reithinger et al., 2007; Rodríguez-Cortés et al., 2010; Souza et al., 2012). Therefore, studies of the diagnosis of ATL are based on a combination of clinical and laboratory criteria (Reis et al., 2008).

Current methods for recognizing leishmaniasis include the detection of an immune response using the leishmanin Montenegro skin test (MST) and serological tests as well as detection of the parasites in culture, smears, histopathologic examinations (HE), and polymerase chain reactions (PCRs) (Reis et al., 2008). Studies have shown that PCR enhances the accuracy of Leishmania identification (Oliveira et al., 2005).

Recently, positive PCR results using mucosal swabs were demonstrated for mucocutaneous leishmaniasis (ML) and cutaneous leishmaniasis (CL) (Boggild et al., 2011b; Figueroa et al., 2009). Similarly, a single case report showed that PCR was positive from DNA of the saliva of a patient with CL (Corvalan et al., 2011). Controlled studies are needed to verify the utility of these techniques.

The aim of this study was to evaluate the effectiveness of PCR using DNA taken from nasal swabs (NSs) and to develop new techniques for collecting saliva and oral filter paper imprints (OFPIs) in the diagnosis of ML among a hospital-based population. We also evaluated the ability of these techniques to positively identify CL.

2. Materials and methods

2.1. Study location

This study was conducted from January 1, 2012 to June 30, 2013, at the Hospital Universitário de Brasilia, Brasilia, Brazil, to which patients are referred by primary care facilities for the diagnostic confirmation of suspected cases of ATL. PCR experiments were performed at the Laboratory of Dermatology, Hospital das Clínicas, University of São Paulo, Ribeirão Preto, Brazil.

2.2. Study population

All patients who had clinical signs consistent with ATL were consecutively included. After the application of the allocation criteria, they were divided into 3 groups: 1) ML, 2) CL, and 3) controls (Fig. 1).
Noninvasive samples can be collected because ATL is a mucocutaneous disease. In a field research, the use of minimally invasive samples aims to replace any form of ATL also raises the suspicion of mucosal involvement. In this group, patients with mucosal or skin lesions were not separated.

2.3. Tests for allocation (composite reference standard)

Oral and nasal cavities of patients with suspected leishmaniasis were clinically examined. In patients with evidence of mucosal injury, a video nasofibroscopy study was conducted.

For all patients, the following tests were performed in parallel: MST, indirect immunofluorescence (IIF), HE (hematoxylin and eosin, Giemsa), culture of aspirate from the lesion (Neal, Novy, Nicolle), smears (Giemsa), and PCR using filter paper imprints of lesion biopsy (FPILs) (Boggild et al., 2010; Boggild et al., 2011a). IIF was considered positive if the titers were greater than or equal to 1:40.

2.4. Criteria for diagnosing ML

A diagnosis of ML was made if there was any evidence of mucosal involvement. A confirmatory diagnosis was based on 2 positive immunologic tests (MST and IIF) or on only 1 test if there was a positive culture, HE, smear, or FPIL (Brasil. Ministério da Saúde, 2007). The samples for the parasitologic tests were collected directly from the mucosal lesions. Patients with concomitant cutaneous and mucosal lesions were included in the group of ML cases.

2.5. Criteria for diagnosing CL

A diagnosis of CL was made in patients with no evidence of mucosal damage. The diagnosis was made using the same confirmation criteria noted for cases. However, the samples for the parasitologic tests were collected directly from skin lesions.

2.6. Criteria for allocating patients to the control group

The control group included patients with a differential diagnosis that included ATL. They were allocated to the control group if the confirmation tests were negative or if only 1 of the immunologic tests were positive (MST or IIF) and no parasites were found. A systematic search for a differential diagnosis was then performed (PCR for other infectious agents, cultures, and special stains in HE).

This group, patients with mucosal or skin lesions were not separated because ATL is a mucocutaneous disease. In a Leishmania (Viannia) braziliensis–endemic area, we assumed that the diagnostic hypothesis of any form of ATL also raises the suspicion of mucosal involvement. Furthermore, the use of minimally invasive samples aims to replace techniques that require direct visualization of the mucosa. In remote areas, diagnostic alternatives such as video nasofibroscopy may not be available. Noninvasive samples can be collected even by non-specialist professionals and sent to a reference laboratory. Comparing patients with ML and control patients with diseases that mimic any form of ATL allows a better external validation for field research.

2.7. Exclusion criteria

Patients who were under 18 years of age, who showed any evidence of immunosuppression or who belonged to indigenous communities, were excluded.

2.8. Sampling

Samples were collected in the following sequence and are referred to here as “molecular mucosal samples” (MMS):

- Saliva. Saliva was collected using a 20-ml sterile syringe and then transferred into a 1.5-ml sterile microtubule. The patient was instructed not to ingest foods or liquids or to brush their teeth for 30 minutes prior to saliva collection.
- OPIL. Shaved oral mucosa (tongue, buccal, and palatal) was collected using a single filter paper (Whatman®, Maidstone, England) by rubbing each mucosal surface three times. The OPIL was then stored in a paper envelope.
- Cotton bilateral NS. Sterile cotton swabs were introduced 2 cm into the nasal vestibule (Boggild et al., 2011b; Figueroa et al., 2009) and rotated 360 degrees 5 times with slight pressure against the nasal septum. The procedure was repeated in both nostrils using the same swab. The stem of the swab was cut using a sterile scalpel blade and stored in a 1.5-ml sterile microtubule.
- The airways of the patients were not manipulated at any time prior to sampling. The samples were transported and then stored at −20 °C and at −80 °C.

2.9. Initial tests

To ensure an adequate cost-effectiveness, the comparison of different extraction techniques was performed in an index ML patient with abundant parasites found in the smear. Filter papers, NS, and saliva were eluted in 150 μL of ultrapure water and then mixed and heated (95 °C) for 10 minutes. This method was compared to commercial kits, including the NucleoSpin® Tissue Kit (MACHEREY-NAGEL Gmbh & Co. KG, Düren, Germany) for FPIL, NS, and OPIL and a PSP® SalivaGene DNA Kit (STRATEC Molecular GmbH, Berlin, Germany) for saliva, following the manufacturer’s instructions. Samples on filter paper were processed according to the protocol provided for blood spots.

To quantify genomic DNA, real-time PCR was performed using the C18X 343-bp 5′-GAAAAGTCGAGCCGGGCCC-3′ and 5′-GCTGAAGCCACCGCCATAG-3′ primer pair, specific for human keratin, to quantify genomic DNA (Lugassy et al., 2006). The reaction was performed in a Rotor–Gene Q (Qiagen®, Hilden, Germany) in a final volume of 25 μL containing 12.5 μL of 2× SYBR® Green Master Mix (Qiagen®), 2.5 μL of each primer (2.5 μmol/L), 5.5 μL of ultrapure water, and 2 μL of DNA extract (50 ng/μL). The amplification cycles included an initial denaturation step of 5 minutes at 95 °C, followed by 35 cycles at 95 °C (5 seconds) and 60 °C (10 seconds), with melting curve measurements (60 °C–95 °C). The use of the commercial kits was later defined as our standard protocol.

2.10. Processing of conventional PCR

The primers were based on a 120-bp sequence in the minicircle kDNA of Leishmania spp.: 5′-GCCATCAAACCCCC-3′ and 5′-GAGGAGGGCGGTCTGCGAAA-3′ (Eurofins MWG Operon®, Huntsville, AL, USA) (Schubach et al.,...
1998). The reactions were performed in a Mastercycler® Pro thermocycler (Eppendorf®, Hamburg, Germany) at a final volume of 23 μL, containing 12.9 μL of ultrapure water, 2 μL of 10× buffer (10 μmol/L Tris-HCl [pH 8.6], 50 μmol/L KCl, 1.5 μmol/L MgCl₂), 2.5 μL of dNTPs (2 mmol/L), 0.2 μL of each primer (40 μmol/L), 0.2 μL of Taq DNA polymerase (5 U/μL) (Invitrogen, São Paulo, Brazil), and 5 μL of DNA extract. The amplification cycles included an initial denaturation step of 3 minutes and 30 seconds at 94 °C, followed by 35 cycles at 93 °C (30 seconds), 60 °C (1 minute), 72 °C (1 minute), a final extension at 72 °C (10 minute), and incubation at 4 °C. All reactions included a negative and a positive control with L. (V.) braziliensis culture lysates.

Two microliters of the amplified product was mixed with 2 μL of a xylene-cyanol preparation (Vetec®, Duque de Caxias, Rio de Janeiro, Brazil) and 1 μL (1:100) of GelRed™ (Biotium®, Hayard, CA, USA) and then loaded onto a 2% agarose gel immersed in 1× Tris base, acetic acid and Ethylenediaminetetraacetic acid (EDTA) buffer. A 100-bp marker was used (Invitrogen®, São Paulo, Brazil). Electrophoresis was performed in a horizontal tank Sub-Cell® GT Cell 170-4403 (BIO-RAD®, Hercules, CA, USA) for 1 hour 30 minutes at 90 V and 400 mAmp. The gel was visualized on an EC3 Imaging System (UVP®, Upland, CA, USA).

MMS results were evaluated by a specialized biomedicist who was masked to the results of the allocation criteria. Real-time PCR was performed using the C18X primers in all samples to ensure DNA extraction and to identify possible PCR inhibitors.

2.11. Subgenus identification

The subgenus was identified using restriction fragment length polymorphism (RFLP). Four microliters of the amplified sample was digested using the enzymes HaeIII and Bsr1 (New England Biolabs® Inc., Ipswich, MA, USA) overnight at 37 °C and 65 °C, respectively. PCR fragments were then visualized using polyacrylamide gel electrophoresis.

2.12. Sample size

The sample size calculation was based on the main outcome defined as the use of NSs. A sensitivity of 95.7% was expected for NS use, and a prevalence of 40.8% of cases of ML for the local hospital-based population was considered according to previous data (Boggild et al., 2011b; Name et al., 2005).

A population of 16 cases and 24 controls was calculated to reach a 95% minimal acceptable lower confidence limit of 0.65, and the probability that this limit is above the minimal acceptable level is 0.95 (Flahault et al., 2005).

2.13. Missing data

A small quantity of tests to determine inclusion was not computed because of difficulty in accessing mucosal lesions for the cultures and smears. Other parallel tests, however, allowed these patients to be included in the study. These losses were ignored when calculating the accuracy of the inclusion tests.

2.14. Statistical analysis

The comparison between patients with ML and controls was defined as the main outcome. Variables related to CL patients were used only for descriptive analysis. The descriptive analysis, chi-square test, Fisher’s exact test, analysis of variance test, and Kruskal-Wallis test were performed using the program SAS® 9.3 (SAS® Institute Inc., Cary, North Carolina, USA). The tests’ properties were evaluated using OpenEpi® version 3.01 (Emory University, Rollins School of Public Health, Atlanta, Georgia, USA) (Sullivan et al., 2009). Quantitative variables were categorized for MST and IIF. Statistical significance was defined as P < 0.05, and confidence intervals (CIs) were set at 95%.

2.15. Ethics

Patients were included in the study after signing the informed consent form. The study complies with the 1975 Declaration of Helsinki, as revised in 2013 (Declaration of Helsinki World Medical Association, 2014), and was approved by the Committee for Ethics in Research of the School of Medicine at the University of Brasilia (036/2011).

Fig. 2. Real-time PCR amplification (a) and melting curve analysis (b) of mucous membrane samples collected from the index case. The highest quantity of genomic DNA measured by human keratin real-time PCR was obtained by FPILs, followed by NS, OFPIs, and saliva. OFPI (A), FPIL (B), NS (C), and saliva (D) eluted in ultrapure water; OFPI (E), FPIL (F), NS (G), and saliva (H) utilizing commercial kits; positive control (I); negative control (J).
when compared to patients with CL (45.7 ± 15.1; average age in years was greater in patients with ML (58.3 ± 10.0); these differences were not statistically significant (P = 0.4011) and the rate of recidivism (P = 0.0001) was similar between groups.

3.3. ML patients

For 5 of the 17 patients with ML, only DNA from NS tested positive for *Leishmania*. In 4 patients, DNA from NS and the saliva were positive, and 1 patient tested positive in DNA from both the NS and OPPI. Video nasofibroscopy showed that 11 of the 17 patients with ML presented with isolated lesions in the nasal cavity, 4 presented with contiguous nasal and oropharyngeal lesions, and 2 presented with isolated lesions in the oropharynx (Table 1). All patients who were positive for *Leishmania* spp. based on NS, saliva, or OPPI presented with nasal lesions, whether isolated or not.

3.4. Comparing patients with ML and controls

The most accurate method for the diagnosis of ML was via PCR of the NS samples, which yielded 86% accuracy (95% CI = 73.81–93.05), followed by saliva with 74% (95% CI = 60.45–84.13) and OPPI with 68% (95% CI = 54.19–79.24). The highest sensitivity was reached by using NS 58.82% (95% CI = 36.01–78.39), followed by saliva 23.53% (95% CI = 9.56–47.26) and OPPI 5.88% (95% CI = 1.05–26.98). Specificity values were complete for all tests (Table 2).

When assessing the accuracy of the components of the inclusion tests defined as the composite reference standard, FPIL was the most accurate (88.37, 95% CI = 75.52–94.93), followed by smears (80.95, 95% CI = 66.70–90.02) and culture (77.50, 95% CI = 62.50–87.68) (Table 3). Although the second and third most accurate tests were smear and in vitro culture, these techniques were less sensitive (27.27%, 95% CI = 9.75–56.57 and 10%, 95% CI = 1.79–40.42, respectively). The highest sensitivity was achieved by MST (93.75%, 95% CI = 71.67–98.89) and IIF (68.75, 95% CI = 44.40–85.84). Patients with ML have an average MST of 23.1 ± 11.9, which is significantly higher than the controls (4.9 ± 7.2, P < 0.0001).

3.5. Patients with CL

DNA taken from NSs and OPPIs was positive for *Leishmania* in only 2 patients with CL, and video nasofibroscopy revealed no evidence of mucosal involvement. None of the patients with CL tested positive for *Leishmania* in DNA taken from their saliva, and none represented a recurrence of skin lesions. In 1 patient, we identified the previous use of etanercept and methotrexate as treatment for a diagnosis of psoriatic arthritis. The medication was discontinued 3 months before the sample collection. Exclusion criteria only addressed immunosuppression at the time of the collection of samples, which allowed for the inclusion of this patient in the study. In both patients with CL, no signs of visceral disease were identified using tests such as the bone marrow aspiration (smear, culture, and PCR). The clinical characteristics of these 2 patients are shown in Table 4.

3.6. Subgenus identification

The subgenus *Viannia* was identified in 12 cases of ML and 11 cases of CL. The subgenus *Leishmania* was only identified in 1 patient with CL.

4. Discussion

The early diagnosis of ML is essential because of its social impact (Al-Qahtani et al., 2012). Most parasitological tests have low accuracy.

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**Table 1**

Basic characteristics and positivity of tests conducted on patients with mucosal leishmaniasis (cases).

<table>
<thead>
<tr>
<th>Case identification</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Localization</th>
<th>First symptoms (months)</th>
<th>NS</th>
<th>Saliva</th>
<th>OPPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>4/2012</td>
<td>M</td>
<td>64</td>
<td>Oral</td>
<td>12</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>6/2012</td>
<td>M</td>
<td>60</td>
<td>Nasal</td>
<td>180</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>7/2012</td>
<td>M</td>
<td>56</td>
<td>Oral</td>
<td>96</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>8/2012</td>
<td>M</td>
<td>54</td>
<td>Nasal</td>
<td>12</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>12/2012</td>
<td>M</td>
<td>66</td>
<td>Nasal and oral</td>
<td>180</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>15/2012</td>
<td>M</td>
<td>56</td>
<td>Nasal and oral</td>
<td>60</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>31/2012</td>
<td>F</td>
<td>55</td>
<td>Nasal</td>
<td>24</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>32/2012</td>
<td>M</td>
<td>58</td>
<td>Nasal and oral</td>
<td>24</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>34/2012</td>
<td>F</td>
<td>65</td>
<td>Nasal</td>
<td>180</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>40/2012</td>
<td>F</td>
<td>45</td>
<td>Nasal</td>
<td>312</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>42/2012</td>
<td>M</td>
<td>60</td>
<td>Nasal</td>
<td>24</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>43/2012</td>
<td>M</td>
<td>51</td>
<td>Nasal and oral</td>
<td>120</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>45/2012</td>
<td>M</td>
<td>49</td>
<td>Nasal</td>
<td>6</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>48/2012</td>
<td>M</td>
<td>59</td>
<td>Nasal</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>54/2013</td>
<td>F</td>
<td>69</td>
<td>Nasal</td>
<td>24</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>66/2013</td>
<td>M</td>
<td>67</td>
<td>Nasal</td>
<td>48</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>69/2013</td>
<td>F</td>
<td>37</td>
<td>Nasal</td>
<td>37</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

**Table 2**

ML × controls: comparison of non-invasive mucous membrane samples to differentiate between mucosal leishmaniasis and controls.

<table>
<thead>
<tr>
<th>Exam</th>
<th>Sens (%) (95% CI)</th>
<th>Spec (%) (95% CI)</th>
<th>PPV (%) (95% CI)</th>
<th>NPV (%) (95% CI)</th>
<th>Accuracy (%) (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS</td>
<td>58.82 (36.01–78.39)</td>
<td>100 (89.57–100)</td>
<td>100 (72.25–100)</td>
<td>82.5 (68.05–91.25)</td>
<td>86 (73.81–93.05)</td>
</tr>
<tr>
<td>Saliva</td>
<td>23.53 (9.56–47.26)</td>
<td>100 (89.57–100)</td>
<td>100 (51.01–100)</td>
<td>71.74 (57.45–82.68)</td>
<td>74 (60.45–84.13)</td>
</tr>
<tr>
<td>OPPI</td>
<td>5.88 (1.05–26.98)</td>
<td>100 (89.57–100)</td>
<td>100 (20.65–100)</td>
<td>67.35 (53.38–78.79)</td>
<td>68 (54.19–79.24)</td>
</tr>
</tbody>
</table>

Sens = sensitivity; Spec = specificity; PPV = positive predictive value; NPV = negative predictive value. (Wilson score.)
sensitivities, and other diagnostic options, such as MST, have high sensitivities but low specificities (Reithinger et al., 2007). IIF has limited worth but may be useful when parasites are not detected by other methods (Szargiki et al., 2009; Zeyrek et al., 2007). The present study has confirmed these properties (Boggild et al., 2007; Faber et al., 2003; Momeni Boroujeni et al., 2013; Rahman and Bari, 2003; Romero et al., 2001; Sadeghian et al., 2013).

One important question about the reproducibility of PCR for diagnosing ML lies in the lack of standardized inclusion criteria (de Oliveira et al., 2010; Shirian et al., 2012). In our region, we believe that parasitological criteria alone are insufficient (Kline et al., 2013). L. (V.) braziliensis is endemic in midwestern Brazil, and a small number of parasites are all that is necessary to cause ML disease (Brelaz-de-Castro et al., 2012). The use of a composite reference standard addresses the low sensitivity of parasitological tests.

There is also no standard definition for controls. Diseases such as leprosy and paracoccidioidomycosis are endemic in our region and are often misdiagnosed as ATL. In the control group, we did not segregate patients with mucosal or skin lesions because we wished to enhance the external validity of the data for fieldwork collection. In remote areas, samples can be collected without direct visualization of the mucosa, even by non-specialists.

In the ML group, all patients who presented with positive NSs had nasal lesions, whether isolated or not. This finding indicates that direct contact of the NS with the clinical lesion is important. The nasal septum is composed of delicate mucosa rich in macrophages that can harbor Leishmania (Farias et al., 2013). Lesions in this location are friable, resulting in better cytology samples. This fact is enforced by our real-time PCR results after PCR-RFLP fragmentation in this case reinforces this finding indicates the detection of the species Leishmania peruviana and L. (V.) braziliensis, were identified. In midwestern Brazil, the predominant detectable species are L. (V.) braziliensis and Leishmania (L.) amazonensis. The former is most likely responsible for all ML cases reported here, as shown by our RFLP results and by epidemiology (Brasil. Ministério da Saúde, 2007). Autochthonous cases of ML caused by other species have not been described in our region. L. (V.) braziliensis induces an intense inflammatory reaction and causes clinical manifestations with low levels of parasites according to diagnostic tests (Brelaz-de-Castro et al., 2012; Romero et al., 2001), which may explain the lower sensitivity of the NS collection in the present study. The accuracy of diagnosis using DNA from salivary collection was 74% (95% CI = 60.45–84.13). Interestingly, 3 of the 4 patients positive for Leishmania in the saliva presented with isolated nasal lesions. The presence of post-nasal drip may explain this association.

The accuracy of PCR in diagnosing ML from DNA collected by OFPI was 68% (95% CI = 54.19–79.24), but this test was positive in only 1 patient, who had a contiguous nasal and oral lesion. This indicates that L. (V.) braziliensis is not frequently present in the oral mucosa and that direct contact with the clinical lesion is essential (Motta et al., 2007).

These findings are consistent with data that point to an increased tropism of L. (V.) braziliensis for the anterior nasal septum (Lessa et al., 2012). NSs ensure easy access to this region; however, this technique may generate discomfort. Despite the lower sensitivity, the collection of saliva is more comfortable. To our knowledge, there are no consistent reports in the literature on the use of DNA from saliva and OFPI in diagnosing ML.

For the diagnosis of CL using DNA from NS samples, previous data indicate that several species of Leishmania may be present in the mucosa, despite causing no detectable clinical lesions (Figueredo et al., 2009). Indeed, in the present study, only 2 patients with CL were positive using both the NS and OFPI, but not for saliva.

The previous use of immunosuppressants was reported by one of these patients. Despite this, the patient was included in the study because there was no active immunosuppression at the time of the inclusion, and the half lives of etanercept and methotrexate are relatively rapid (Combe., 2008; Dalrymple et al., 2008). However, a previous reduction in cellular immunity can clearly explain Leishmania dissemination and the positivity for mucosal samples. In the other case, primer dimerization can explain the presence of PCR bands similar to the expected ones in the real infection. The use of primers with low molecular weight enhances this possibility. The absence of visible results after PCR-RFLP fragmentation in this case reinforces this theory. (Table 4)

Interestingly, Figueredo et al. (2009) reported positive test results using PCR on mucosal swabs in 81% of patients with CL (Figueredo et al., 2009). The authors described the detection of the species L. (V.) panamensis in a large majority of patients (Figueredo et al., 2009).

The fact that L. (V.) braziliensis is found less often compared to other species in the mucosa of patients with CL seems to be inconsistent, given that this subspecies is the primary cause of ML (Goto and Lauletta Lindoso., 2012). However, the clinical observation that L. (V.) braziliensis is the most frequent mucosal parasite identified in patients with ATL should be reviewed with caution, as the findings from this and previous studies indicate the contrary (Boggild et al., 2011b; Figueredo et al., 2009). The fact that other species are more frequent and act as silent hosts cannot be excluded. In vitro studies have shown that L. (V.) braziliensis strongly stimulates the formation of granulomas (Maretti-Mira et al., 2011; Weinkopff et al., 2013). Based on the above data, we infer that L. (V.) braziliensis possibly acts as the main species that causes ML but may not be


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Conflict of interest statement.

On behalf of all authors, the corresponding author states that there are no conflicts of interest.

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