Evaluation of *Leishmania* Species Reactivity in Human Serologic Diagnosis of Leishmaniasis

Ricardo Silvestre,† Nuno Santarém,† Lúcia Teixeira, Joana Cunha, Henk Schallig, and Anabela Cordeiro-da-Silva*

Parasite Disease Group, IBMC—Instituto de Biologia Molecular e Celular, Universidade do Porto, Portugal; Departamento de Bioquímica, Faculdade de Farmácia, Universidade do Porto, Portugal; KIT (Koninklijk Instituut voor de Tropen/Royal Tropical Institute), KIT Biomedical Research, Amsterdam, The Netherlands

**Abstract.** The sensitivities and specificities of IgG-ELISA and IgG flow cytometry based techniques using different *Leishmania* species were determined using sera collected from 40 cutaneous or visceral leishmaniasis patients. The flow cytometry technique, using promastigote parasite forms, performed better than total soluble extract IgG-ELISA. At the species level, the use of *Leishmania amazonensis* and *Leishmania major* as antigens in enzyme linked immunosorbent assay (ELISA) decreased the overall sensitivity. To assess the specificity of these tests, sera from malaria, toxoplasmosis, amoebiasis, schistosomiasis, and leprosy patients were used. We also included sera from *Leishmania* non-infected endemic individuals. The cutaneous species showed a decreased specificity in both assays. Although more sensitive, flow cytometry using promastigote parasite forms generally presented lower levels of specificity when compared with total extract of IgG-ELISA. Overall, the results of the study show the potential of IgG flow cytometry for the diagnosis of leishmaniasis. Although highly sensitive, a refinement of the flow cytometry method should be performed to improve the overall specificity.

**INTRODUCTION**

Leishmaniasis is one of the major communicable diseases of the world, being the third most important vectorborne disease after malaria and sleeping sickness. Leishmaniasis is traditionally classified into three major clinical manifestations: cutaneous (CL), mucocutaneous (MCL), and visceral leishmaniasis (VL). These distinct entities are characterized by a broad spectrum of non-pathognomonic clinical manifestations. Leishmaniasis symptoms can mimic diseases of other etiologies rendering the clinical diagnosis to be difficult. Furthermore, other pathogens with similar clinical manifestations (leprosy, tuberculosis, skin cancers, cutaneous mycoses for CL and malaria, African trypanosomiasis, brucellosis, toxoplasmosis, amoebiasis or schistosomiasis for VL) are common in *Leishmania* endemic areas. Hence, a differential diagnosis is essential for confirming the clinical suspicion.

Ideally, all cases of leishmaniasis should be confirmed by direct detection and identification of infecting parasite species observation. Clinical specimens examined are usually infected skin biopsies (for CL and MCL) or aspirates from spleen, bone marrow or lymph nodes (for VL). The definitive diagnosis is achieved when parasites are detected by direct observation in stained microscopic preparations, in culture medium or in animal inoculation. Although parasitologic diagnosis still remains the method of choice to confirm leishmaniasis, it is time-consuming and not feasible under field conditions. Moreover, the sensitivity of this technique can be low in sub-clinical infections depending on the clinical material, the sampling procedure and on the skills of the technical staff. Recently, the use of molecular biology techniques (polymerase chain reaction [PCR]-based) is becoming increasingly relevant to the diagnosis of leishmaniasis because it is capable of detecting DNA or RNA unique to the parasite, with a high degree of specificity and sensitivity just a few weeks after the appearance of the first clinical symptoms. Although molecular biology techniques can be used in the confirmation of all leishmaniasis cases, these methodologies are frequently the most powerful diagnosis tests producing positive results in CL, MCL and human immunodeficiency virus (HIV)/VL co-infected patients in which the humoral response is generally weak. Nevertheless, the use of PCR as a routine diagnostic method requires high equipment and working costs, which limits the feasibility of PCR diagnosis in developing countries.

Serologic approaches are also sensitive and often applied as indirect diagnostic methods. They are more commonly used in the diagnosis of VL than in CL or MCL, because the latter are in most cases associated with low levels of *Leishmania* circulating antibodies. Among the serologic methods, the agglutination tests (direct agglutination test [DAT] and fast agglutination screening test [FAST]) are specific and sensitive tests, suitable for both laboratory and field use. These tests use whole trypsinized, coomassie stained promastigotes either as a suspension or in a freeze-dried form. Other serologic methods, such as indirect fluorescent antibody test (IFAT), immunoblotting, and enzyme-linked immunosorbent assay (ELISA) are classic methods used in the detection of *Leishmania*-specific antibodies. With the exception of ELISA, the latter are methods normally limited to laboratory conditions and require technical expertise. In contrast, ELISA-based techniques are valuable tools in the serodiagnosis of leishmaniasis and allow high throughput screening of a large number of samples. Most commonly used in ELISA are total crude soluble antigens derived from the promastigote stage of different *Leishmania* species. These ELISA showed sensitivities and specificities ranging from 80% to 100% and 85% to 95%, respectively. More recently, alternative methodologies have been proposed to increase overall sensitivity and specificity of conventional serologic approaches. One such approach is IgG flow cytometry to detect anti-live *Leishmania braziliensis* antibodies in sera of active cutaneous leishmaniasis patients. In theory, the flow cytometry technique can specifically detect the antibodies recognizing the *Leishmania* surface antigens, therefore helping to restrain the potential cross-reactivity against more conserved intracellular structures. In addition, flow cytometry advantageously allows the analysis of thousands of parasites per assay. Therefore, it is generally accepted that

---

*Address correspondence to Anabela Cordeiro-da-Silva, Parasite Disease Group, Biology of Infection and Immunology, Instituto de Biologia Molecular e Celular da Universidade do Porto, Rua do Campo Alegre, 823, 4150-180 Porto, Portugal. E-mail: cordeiro@ibmc.up.pt
†These authors contributed equally to this work.
while the diagnostic sensitivity depends on the test and the serologic procedure used, the specificity will depend strictly on the antigen rather than the methodology itself.10

The objective of this study was to evaluate *Leishmania* spp.-specific recognition using both *Leishmania* promastigote total soluble extract IgG-ELISA and *Leishmania* promastigote IgG flow cytometry techniques.

**MATERIAL AND METHODS**

**Enzyme linked immunosorbent assay (ELISA) for immunoglobulin.** The soluble promastigote extracts and the ELISA technique were performed adapting the protocols described elsewhere with minor modifications.11,12 Briefly, after blocking with milk buffer (3% of low-fat milk in phosphate buffered saline pH 7.4 [PBS]), 50 μL of an optimized 1/100 dilution in PBS/Tween 20 0.05% of each serum was incubated for 30 min at 37°C in wells previously coated with 10 μg/mL of each extract. A peroxidase labeled goat anti-human IgG (Sigma, St. Louis, MO) secondary antibody diluted 1/5000 was added for a similar period of time, and the plates were developed with 0.5 mg/mL o-phenylenediamine dihydrochloride (OPD, Sigma) in citrate buffer.

**Immunofluorescence by flow cytometry.** The flow cytometry analyses with intact live *Leishmania* spp. promastigotes were performed as follows. The parasites were recovered from stationary-phase (5 days) cultures. After three washing steps with PBS, 5 × 10^6 promastigotes were incubated in 96-well U-bottom polystyrene plates (BD Falcon, San Jose, CA) with 25 μL of each serum diluted 1/50 in PBS/bovine serum albumin (BSA) 1% for 30 min at 4°C. Then, 10 μL of mouse fluorescein (FITC) labeled anti-human IgG (BD Pharmingen, San Diego, CA) were incubated in each well for 30 min at 37°C. After three washing steps, *Leishmania* spp. promastigotes were re-suspended in 300 μL of PBS/BSA 1% and analyzed by flow cytometry in a fluorescence-activated cell sorting (FACS) scan equipped with CellQuest Pro software (BD Bioscences, San Jose, CA). In all experiments we included positive and negative controls as previously described.11 Parasites were identified on the basis of forward/side scatter values and a total of 10,000 events were acquired. For each experiment, on the basis of the histogram representing the binding of parasites incubated in the absence of human serum but in the presence of FITC conjugated mouse anti-human, an area was chosen to contain a maximum of 2.0% of positive fluorescent parasites (PPF). This area allowed for the measurement of the % PPF in all data analyzed.

**Patients.** We recurred to both techniques to measure the levels of anti-*Leishmania* immunoglobulins in the sera of 10 Surinamese cutaneous leishmaniasis patients caused by *Leishmania* (Viannia) guyanensis (cutaneous leishmaniasis New World [CLN])13, which presented one to three lesions on their extremities and 10 Afghan cutaneous leishmaniasis patients, with chronic single to few lesions on extremities caused by *Leishmania major* (cutaneous leishmaniasis Old World [CLO]). In addition, we included 10 Indian visceral leishmaniasis patients, caused by *Leishmania donovani* (VL [Ld]) and 10 Brazilian visceral leishmaniasis patients, caused by *Leishmania infantum* (=*Leishmania chagasi*)14 [VL (Li)], all patients with clinical symptoms. Written informed consent was obtained from all patients included in this study.

**Parasites.** The soluble antigens and the live parasites were obtained from four distinct *Leishmania* species: *L. major* (MHOM/MA/81/LEM 265)15 and *Leishmania amazonensis* (originated from the strain collection of WHO; MHOM/BR/76/LTB-012), the main ethiologic agents of cutaneous leishmaniasis in the Old World and in the New World, respectively; and *L. donovani* (reference strain MHOM/IN/83/DD8)16 and *L. infantum* (= *L. chagasi*) (reference strain MHOM/MA/67/ITMAP-263), both agents of visceral leishmaniasis in the Old World. Promastigotes of all *Leishmania* species were cultured as previously described.17

**RESULTS**

**Cut-off determination for the ELISA assays.** The ELISA cut-off values for the soluble extract of each *Leishmania* spp. were defined based on the receiver operating curves (ROC)18 using sera obtained from non-endemic healthy individuals (Dutch volunteers) (N = 10) as negative controls and the reactivity of all leishmaniasis patients, both CL and VL, as positive values (Figure 1). Thus, we established as ELISA cut-off points the optical density (OD) reactivity at 492 nm values of 0.416 for *L. donovani*, 0.389 for *L. infantum*, 0.396 for *L. amazonensis*, and 0.486 for *L. major*. According to Swets, all of the *Leishmania* soluble extracts tested presented similar accuracies since proximal values were obtained for the area

![Figure 1](image_url). **ROC curves obtained for the ELISA assay.** (A) ROC curves for the visceral *L. donovani* (thick solid line) and *L. infantum* (thin solid line) species. (B) ROC curves for the cutaneous *L. amazonensis* (thick dotted line) and *L. major* (thin dotted line) species. The dotted grey line represents the reference line.
under (AUC) each ROC curve: *L. donovani* AUC = 0.96; 95% confidence interval (CI): 0.92–1.0; *L. infantum* AUC = 0.98; 95% CI: 0.95–1.0; *L. amazonensis* AUC = 0.96; 95% CI: 0.91–1.0; and *L. major* AUC = 0.95; 95% CI: 0.89–1.0. 19

VL species present better overall sensitivity in the ELISA technique. Values of overall sensitivity varied from 87.5% to 95%, depending on the *Leishmania* spp. evaluated (Table 1). Among all groups of leishmaniasis patients, higher *Leishmania* IgG recognition was observed for VL patients, regardless of the antigen used. Indeed, all 10 VL sera were found positive for *Leishmania* spp-specific IgG, except when *L. major* antigen was used; in that case one serum sample was found below cut-off value (Figure 2). However, the serologic diagnosis of CL remains problematic because of a lack of sensitivity and specificity when compared with their visceral counterparts 9 and an evident correlation between species-specific diagnosis and pathology could not be found in the present study.

*L. major* and *L. amazonensis* are both causative agents of CL, we anticipated a higher sensitivity for autologous antigens. Nonetheless, for the CL patients sera a better sensitivity was obtained when the visceral species, *L. donovani* and *L. infantum* (90%) were used as antigen, compared with their cutaneous counterparts, *L. amazonensis* and *L. major* (80%) in ELISA assay. Although surprising, there are several possible explanations for these results. First, we must consider that the reduced number of patients evaluated might contribute to an apparent lack of species-specific diagnosis. Second, the IgG reactivity of CL patients was found not to be significantly different in these two species, *L. amazonensis* and *L. major*, than when using visceral ones. The sera reactivity at 492 nm of CLO and CLN patients was 0.48 ± 0.22 and 0.78 ± 0.27, respectively, using *L. amazonensis* as antigen and 0.57 ± 0.23 and 0.99 ± 0.36, respectively, using *L. major* (Figure 2). When visceral species were used as antigen in the ELISA technique, these values were 0.62 ± 0.30

### Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th><em>L. donovani</em></th>
<th><em>L. infantum</em></th>
<th><em>L. amazonensis</em></th>
<th><em>L. major</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>CLO (N = 10)</td>
<td>90</td>
<td>90</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>CLN (N = 10)</td>
<td>90</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>VL <em>L. donovani</em> (N = 10)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>VL <em>L. infantum</em> (N = 10)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>Total (N = 40)</td>
<td>95</td>
<td>95</td>
<td>90</td>
<td>87.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Specificity (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Malaria (N = 10)</td>
<td>50</td>
<td>60</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Dermal pathologies</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(N = 5)†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other pathologies</td>
<td>91.7</td>
<td>100</td>
<td>91.7</td>
<td>91.7</td>
</tr>
<tr>
<td>(N = 12)†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endemic negatives</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>90</td>
</tr>
<tr>
<td>(N = 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-endemic negatives</td>
<td>100</td>
<td>100</td>
<td>90</td>
<td>85</td>
</tr>
<tr>
<td>(N = 20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (N = 57)</td>
<td>85.9</td>
<td>89.5</td>
<td>82.4</td>
<td>82.4</td>
</tr>
</tbody>
</table>

* CLO = cutaneous leishmaniasis Old World; CLN = cutaneous leishmaniasis New World; VL = visceral leishmaniasis.
† Dermal pathologies include leprosy (N = 5).
‡ Other pathologies include toxoplasmosis (N = 5), amoebiasis (N = 5), and schistosomiasis (N = 2).

VL species present better overall sensitivity in the ELISA technique. Values of overall sensitivity varied from 87.5% to 95%, depending on the *Leishmania* spp. evaluated (Table 1). Among all groups of leishmaniasis patients, higher *Leishmania* IgG recognition was observed for VL patients, regardless of the antigen used. Indeed, all 10 VL sera were found positive for *Leishmania* spp-specific IgG, except when *L. major* antigen was used; in that case one serum sample was found below cut-off value (Figure 2). However, the serologic diagnosis of CL remains problematic because of a lack of sensitivity and specificity when compared with their visceral counterparts and an evident correlation between species-specific diagnosis and pathology could not be found in the present study.

*L. major* and *L. amazonensis* are both causative agents of CL, we anticipated a higher sensitivity for autologous antigens. Nonetheless, for the CL patients sera a better sensitivity was obtained when the visceral species, *L. donovani* and *L. infantum* (90%) were used as antigen, compared with their cutaneous counterparts, *L. amazonensis* and *L. major* (80%) in ELISA assay. Although surprising, there are several possible explanations for these results. First, we must consider that the reduced number of patients evaluated might contribute to an apparent lack of species-specific diagnosis. Second, the IgG reactivity of CL patients was found not to be significantly different in these two species, *L. amazonensis* and *L. major*, than when using visceral ones. The sera reactivity at 492 nm of CLO and CLN patients was 0.48 ± 0.22 and 0.78 ± 0.27, respectively, using *L. amazonensis* as antigen and 0.57 ± 0.23 and 0.99 ± 0.36, respectively, using *L. major* (Figure 2). When visceral species were used as antigen in the ELISA technique, these values were 0.62 ± 0.30

***Figure 2.*** IgG antibody levels determined by ELISA against (A) *L. donovani*, (B) *L. infantum*, (C) *L. amazonensis*, and (D) *L. major* soluble extracts in sera of patients with cutaneous (CLO and CLN) or visceral leishmaniasis [VL(Ld) and VL(Li)], malaria (M), *Leishmania*-negative with dermal pathologies (DP), *Leishmania*-negative with other pathologies (OP), healthy endemic (EN), and healthy non-endemic individuals (NEN). Results are expressed as the optical densities at 492 nm. Dotted line represents cut-off values (as determined by the ROC curves) between negative and positive results. Each dot represents an individual serum; bars display median.
and 1.16 ± 0.46, respectively, for *L. donovani* and 0.53 ± 0.24 and 1.10 ± 0.45, respectively, for *L. infantum*. This data may indicate that the sera of CL patients, which possess lower levels of *Leishmania*-specific antibodies, recognize preferably the most immunogenic *Leishmania* proteins, which should be present in higher quantities or differently transcribed/translated in visceral when compared with cutaneous species. For example, the amastigote stage specific A2 protein in CL isolates was lacking most of the nucleotide repeats that constitute the variable region at the 5′ end of the A2 sequences in VL that are responsible for its immunogenicity. Further studies using a higher number of cutaneous disease patients and *Leishmania* species are needed to explore the lack of species-specific diagnosis in CL.

**Determination of IgG ELISA specificity.** The cross-reactivity of the ELISA was assessed using sera from microscopically confirmed anonymized *P. falciparum* cases (malaria; M) (*N* = 10), leprosy (dermal pathologies; DP) (*N* = 5), and other pathologies ([OP; toxoplasmosis *N* = 5, amoebiasis *N* = 5, and schistosomiasis *N* = 2]) that are infections that frequently overlap with endemic areas of leishmaniasis. In addition, we included sera recovered from *Leishmania* non-infected individuals living in endemic areas. High frequency of cross-reactivity was observed from sera of malaria patients (Table 1). However, all sera recovered from patients with leprosy were found to react below the cut-off value for any antigen used. A similar observation was performed for the sera recovered from patients with other pathologies (toxoplasmosis, amoebiasis, and schistosomiasis). Only one serum was found to be positive in all *Leishmania* soluble extracts, with the exception of *L. infantum*. Moreover, a significant cross-reactivity was found for healthy individuals living in endemic areas of leishmaniasis (Table 1). The overall specificity of the ELISA technique was high, varying from 82.4% with both cutaneous species to 85.9% for *L. donovani* and 89.5% for *L. infantum*.

**Flow cytometry cut-off determination.** The flow cytometry approach was initially developed to overcome the limitations of current ELISA diagnostic approaches. The flow cytometry technique has the advantage of using only outside membrane antigens as the epitope antigenic source for IgG binding. Hence, a decrease of cross-reactivity should be expected because the more conserved intra-cytoplasmatic epitope antigens would not be involved in the serologic detection. Similar to ELISA, the flow cytometry cut-off values for each *Leishmania* spp. were defined based on the ROC using sera obtained from non-endemic healthy individuals as negative controls and the reactivity of all leishmaniasis patients, both CL and VL, as positive values (Figure 3). Thus, we established the % PFP values of 14.63 for *L. donovani*, 11.57 for *L. infantum*, 11.12 for *L. amazonensis*, and 10.9 for *L. major* as flow cytometry cut-off values. All of the *Leishmania* species presented excellent accuracies with similar AUC ([*L. donovani* AUC = 0.97; 95% CI: 0.94–1.0]; [*L. infantum* AUC = 0.97; 95% CI: 0.93–1.0]; [*L. amazonensis* AUC = 0.98; 95% CI: 0.93–1.0]; [*L. major* AUC = 0.99; 95% CI: 0.98–1.0]).

**The flow cytometry methodology, although less specific, presents a higher sensitivity than ELISA technique efficiently diagnosing CL.** The flow cytometry analysis using promastigote parasite form improved the IgG recognition, with an overall sensitivity of 95% using *L. major* or the visceral species and 97.5% using *L. amazonensis* (Table 2). All sera recovered from the 10 VL patients were found to be positive regardless of the antigen used (Figure 4 and Table 2). Although no significant differences were found in overall sensitivity, a better species specificity was observed during the flow cytometry analysis. The measurement of flow cytometry % PFP using sera from CLO and CLN patients was mean 30.66 ± 13.70 and 34.37 ± 23.26, respectively, using *L. amazonensis* and 33.47 ± 23.69 and 35.86 ± 16.23, respectively, using *L. major*. In contrast to ELISA measurements, the % PFP values were higher than those obtained using as antigen the visceral species, 23.54 ± 8.96 and 33.81 ± 15.00, respectively, using *L. donovani* and 19.70 ± 8.98 and 31.89 ± 16.09, respectively, using *L. infantum*. Similarly, VL patient sera preferentially recognize visceral species (with the % PFP values of 66.82 ± 31.99 and 70.30 ± 23.37 using *L. donovani* and *L. infantum*, respectively) when compared with the cutaneous ones (with the % PFP values of 46.84 ± 24.78 and 39.20 ± 31.23 using *L. amazonensis* and *L. major*, respectively) (two-tailed student’s *t* test, *P* < 0.05 between *L. donovani* or *L. infantum* and *L. amazonensis* for the VL sera).

**DISCUSSION**

Although a large improvement was observed in terms of sensitivity using the flow cytometry methodology, a loss of
specificity was observed, especially with the cutaneous species used as antigen (Table 2). This was mainly caused by cross-reactivity with sera from malaria, amoebiasis and schistosomiasis patients, with no positive reactivity observed with sera from toxoplasmosis patients. In addition, there was one leprosy serum that cross-reacted with \textit{L. major} promastigotes. Interestingly, a high level of cross-reactivity was found against sera recovered from healthy endemic individuals. The observed cross-reactivity can be explained by the presence of several evolutionarily conserved antigens identified in the \textit{Leishmania} genome database.\textsuperscript{22,23}

Therefore, the cross-reactivity observed with sera from malaria, amoebiasis and schistosomiasis patients might be the result of the presence of highly conserved epitopes of immunogenic \textit{Leishmania} proteins present in both visceral and cutaneous forms. In opposition, the cross-reactivity detected with endemic healthy individuals was higher in flow cytometry than ELISA assays. This suggests previous contact(s) of healthy individuals living in \textit{Leishmania} endemic areas with the parasite. Indeed, because not all infected sand-fly bites result in active infection, a transient and inconsequent contact with the parasite might allow the production of antibodies against easy accessible surface antigens. Overall, our results show that the cross-reactivity was higher when cutaneous species were used. Given the ancient evolutionary divergence in \textit{Leishmania} species,\textsuperscript{26} it is not surprising that different \textit{Leishmania} species, in particular visceral versus cutaneous species, are recognized differently by the host immune system. Although the recent genome sequence completion of three \textit{Leishmania} species show a high degree of conservation with less than 1\% of species-specific genes,\textsuperscript{27} it now seems clear that alternate translational control and protein stability will be responsible for the

<table>
<thead>
<tr>
<th>Groups</th>
<th>\textit{L. donovani}</th>
<th>\textit{L. infantum}</th>
<th>\textit{L. amazonensis}</th>
<th>\textit{L. major}</th>
<th>Sensitivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLO (N = 10)</td>
<td>80</td>
<td>80</td>
<td>100</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>CLN (N = 10)</td>
<td>100</td>
<td>100</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>VL \textit{L. donovani} (N = 10)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>VL \textit{L. infantum} (N = 10)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Total (N = 40)</td>
<td>95</td>
<td>95</td>
<td>97.5</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>Specificity (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malaria (N = 10)</td>
<td>80</td>
<td>80</td>
<td>60</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Dermal pathologies (N = 5)†</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Other pathologies (N = 12)‡</td>
<td>83.3</td>
<td>75</td>
<td>66.7</td>
<td>58.3</td>
<td></td>
</tr>
<tr>
<td>Endemic negatives (N = 10)</td>
<td>70</td>
<td>80</td>
<td>60</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Non-endemic negatives (N = 20)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Total (N = 57)</td>
<td>86</td>
<td>84.2</td>
<td>71.9</td>
<td>73.7</td>
<td></td>
</tr>
</tbody>
</table>

* CLO = cutaneous leishmaniasis Old World; CLN = cutaneous leishmaniasis New World; VL = visceral leishmaniasis
† Dermal pathologies include leprosy (N = 5).
‡ Other pathologies include toxoplasmosis (N = 5), amoebiasis (N = 5), and schistosomiasis (N = 2).

![Figure 4](image-url) Figure 4. Flow cytometry analysis of IgG antibodies against (A) \textit{L. donovani}, (B) \textit{L. infantum}, (C) \textit{L. amazonensis}, and (D) \textit{L. major} in the sera of patients with cutaneous (CLO and CLN) or visceral leishmaniasis (VL(Ld) and VL(Li)), malaria (M), \textit{Leishmania}-negative with dermal pathologies (DP), \textit{Leishmania}-negative with other pathologies (OP), healthy endemic (EN), and healthy non-endemic individuals (NEN). Dotted line represents cut-off values (as determined by the ROC curves) between negative and positive results. Each dot represents an individual serum; bars display median.
different phenotypical characteristics, such as *Leishmania* species tropism. Similarly, one may consider that these mechanisms might lead to increased epitope conservation on cutaneous strains, which will explain their increased cross-reactivity observed in this study. Future comparative genetic and proteomic studies will certainly elucidate this point.

Recently, the determination of IgG subclasses by flow cytometry was proposed as an upgrading methodology to obtain overall improved performance. In addition, another reactivity observed in this study. Future comparative genetic cutaneous strains, which will explain their increased cross-mechanisms might lead to increased epitope conservation on all types of leishmaniasis.

In summary, on the basis of our results, we conclude that 1) the IgG flow cytometry methodology offers higher sensitivity than the ELISA technique; 2) promastigote visceral species offer better overall sensitivities in both techniques; 3) the diagnosis of CL, caused by Old and New World species, can be efficiently achieved recurring to flow cytometry; and 4) further studies focusing on IgG subclasses should be performed to improve the unexpected low specificity of flow cytometry.

Received September 3, 2008. Accepted for publication April 20, 2009.

Financial support: R. Silvestre and N. Santarém were supported by fellowships from FCT, POCl 2010, and co-funded by FEDER in the project SFRH/BPD/41476/2007 and POCl/SAU-FTC/59837/2004, respectively. The work was also supported by FCT, POCl 2010, and co-funded by FEDER in the project PTDC/SAU-FTC/67551/2006 and PTDC/CVT/65047/2006.

Disclosure: This study was performed as part of a reviewed and approved protocol by the Medical Ethical Committee of the Academic Medical Center in Amsterdam (MEC 03/228) in 2003.

Authors’ addresses: Ricardo Silvestre, Nuno Santarém, Lúcia Teixeira, Joana Cunha, and Anabela Cordeiro-da-Silva, Parasite Disease Group, Biology of Infection and Immunology, IBMC—Instituto de Biologia Molecular e Celular, Rua do Campo Alegre, 823, 4150-180 Porto, Portugal, Tel: +351-226-074-900, Fax: +351-226-099-157, and Departamento de Bioquímica, Faculdade de Farmácia, Universidade do Porto, Portugal, Rua Aníbal Cunha, 164, 4050-047 Porto, Tel: 00351-222-078-900, Fax: 00351-222-003-977, E-mails: rclea@ibmc.up.pt, nsantar@portugalmail.pt, jcuinha@ibmc.up.pt, luciat@ibmc.up.pt, and cordeiro@ibmc.up.pt. Henk Schallig, KIT (Koninklijk Instituut voor de Tropen/Royal Tropical Institute), KIT Biomedical Research, Meibergdreef 39, 1105 AZ Amsterdam, The Netherlands, Tel: 0031(0)20-5665447, Fax: 0031(0)20-6971841, E-mail: h.schallig@kit.nl.

Reprint requests: Anabela Cordeiro-da-Silva, Parasite Disease Group, Biology of Infection and Immunology, Instituto de Biologia Molecular e Celular da Universidade do Porto, Rua do Campo Alegre, 823, 4150-180 Porto, Portugal, E-mail: cordeiro@ibmc.up.pt.

REFERENCES


