COMPARATIVE EVALUATION OF ELISAS BASED ON TEN RECOMBINANT OR PURIFIED *Leishmania* ANTIGENS FOR THE SERODIAGNOSIS OF MEDITERRANEAN VISCERAL LEISHMANIASIS

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**Abstract.** This study compared a panel of 10 enzyme-linked immunosorbent assays (ELISAs) for the serodiagnosis of Mediterranean visceral leishmaniasis (MVL). The ELISAs were based on either one of the following *Leishmania* antigens: crude soluble *Leishmania* antigens (SLAs), recombinant (r) antigens (namely: rgp63, rK39, gene B protein, r H2A and r H2B histones proteins, rLACK, rPSA-2, r P20) and purified lipophosphoglycan. Most of the test antigens showed good performance (sensitivity > 85%, specificity > 80%). rK39 and SLA-based ELISA gave the best results in terms of sensitivity (100%) and predictive value of the negative (100%). The best specificity (97%) and the best predictive value of the positive (92%) were obtained with rK39. These results show that several *Leishmania* antigens are suitable to design a diagnostic ELISA of MVL. However, recombinant proteins add little to the classic crude SLA, which still represents a very good and less costly alternative.

**INTRODUCTION**

*Leishmania* parasites are flagellated protozoa that infect mammalian macrophages and cause a variety of diseases ranging from self-healing cutaneous lesions to potentially lethal visceral leishmaniasis (VL). Each disease tends to be associated with a specific species of *Leishmania.* In the Mediterranean region, visceral leishmaniasis is due to *L. infantum* and occurs as a sporadic disease mainly in children younger than 5. A similar disease occurs in South America and is caused by the highly related parasite, *L. donovani chagasi* (*L. d. chagasi*). The disease occurs rarely in adults, mainly in immunocompromised patients, especially those suffering from AIDS.

Routine diagnosis of Mediterranean visceral leishmaniasis (MVL) is established by direct observation and/or culture of the intracellular stage of the parasite in bone marrow aspirates. High titers of anti-leishmanial antibodies are developed during the acute disease, and are detected by various serologic tests. Improved sensitivity and specificity may be achieved by enzyme-linked immunosorbent assay (ELISA) using either crude or purified leishmanial antigens. However, few comparative analyses have been made between tests using different *Leishmania* antigen preparations to identify the best antigens. Here we evaluated several ELISAs based on a large panel of different antigens for the diagnosis of MVL. Tests were performed under the same experimental conditions using a common panel of sera and included the following antigens: recombinant (r) gp63, rk39, r gene B protein (rGBP), r histones proteins H2A and H2B, rLACK, r P20, rPSA-2, and purified lipophosphoglycan (LPG). The performance of these ELISAs was compared with that of the more classic ELISA using crude soluble leishmanial antigens (SLA).

**MATERIALS AND METHODS**

**Sera.** Sera from 146 individuals were used in the study. All were living in the Governorate of Kairouan in central Tunisia, a region endemic for MVL where our group conducted an extensive retrospective analysis of the disease from 1984–1997. Since 1997, a prospective surveillance has been actively pursued with local health authorities, and all MVL cases are identified and the national registry notified. Patients and controls were divided into three groups:

**Group I.** Thirty-eight sera were obtained before treatment from children suffering from MVL (36 were ages 1–5 years, one was age 6, and one age 10; mean age of the whole group ±SD 35 months ±21). The diagnosis of MVL was established on clinical criteria (fever, anemia, splenomegaly, weight loss) and on the demonstration of *Leishmania* parasites in Giemsa-stained bone marrow smears and/or culture in biphasic Nicolle-Novy-McNeal medium.

**Group II.** Seventy-five sera were obtained from healthy children living in the same area (age, 7 months to 5 years; mean ±SD 37 months ±17). These children were considered healthy on the basis of clinical examination before blood sampling. None of them developed MVL within 3 years after inclusion in the study.

**Group III.** Thirty-three sera were obtained from children (ages 7 months to 5 years; mean ±SD = 24 months ±13) admitted for various acute infectious diseases (pharyngitis, pneumonia, diarrhea, streptococcal or staphylococcal infections, hydatidosis). These patients have fully recovered after receiving a specific therapy adapted to their individual clinical conditions. None of them has developed MVL on follow-up.

Informed consent was obtained from the subjects’ parents or legal guardians.

**Antigens.** A *L. d. infantum* strain isolated from a Tunisian patient suffering from MVL (strain MHOM/TN/87/KA412 zymodeme MON-1) was used in this study. Promastigotes were grown at 26°C in RPMI 1640 medium (Sigma, St Louis) containing 2 mM L-glutamine, 100 U/mL penicillin, 100 µg streptomycin, and 10% heat-inactivated fetal calf serum. SLA was prepared using Scott’s method. The protein concentration was determined by the Lowry protein assay.

The recombinant protein K39 (rK39) is a repeated 39-amino acid sequence derived from a gene cloned from *L. chagasi* and expressed in *E. coli.* This protein was provided by S.G. Reed, Corixa Inc., Seattle.

Gene B protein (rGBP) is a recombinant protein corresponding to an abundant stage-regulated protein that is ex-
pressed on the surface of infective metacyclic promastigote and amastigote stages of *L. major*. This protein is a hydrophilic molecule containing extensive proline-rich amino acid repetitive sequences constituting 45% of the total protein.\(^{16,28-30}\) GPB is one of a family of proteins, now named the hydrophilic acylated surface proteins, that are expressed in all *Leishmania* species studied to date. rGBP was provided by D.F. Smith, London.

Recombinant gp63 (rgp63) protein, the dominant membrane glycoprotein of *L. major*,\(^{14}\) was provided by R. McMaster, Seattle.

PSA-2 is the promastigote surface antigen-2, which belongs to a family of three related polypeptides encoded by genes of the same family as gp46/M2.\(^{21,22}\) Three forms of this protein were tested in this study: rPSA-2-GST, rPSA-2-MBP, and rPSA-2-TR1-GST; rPSA-2-GST is a fusion protein containing the *L. major* amastigote form of PSA-2 and the *Schistosoma japonicum* glutathione S-transferase (GST); rPSA-2-MBP is the full-length amastigote PSA-2 linked to the maltose binding protein (MBP); rPSA-2-TR1-GST is a genomic clone for which there is no evidence of expression in the parasites; however, it has significant homology to both the amastigote PSA-2 and to gp46/M2 of *S. japonicum*. This is the water-soluble fragment without the hydrophobic GPI-signal sequence, linked to GST.

GST, the *S. japonicum* glutathione S-transferase, was used as a control of the rPSA-2-GST and rTR1-GST fusion proteins.

Lipophosphoglycan (LPG) is the dominant glycolipid of *Leishmania* parasites, present on the surface of promastigotes. It consists of repeated disaccharide units attached to a GPI-lipid anchor.\(^{23,31-33}\)

rPSA-2-GST, rPSA-2-MBP, rPSA-2-TR1-GST, GST, and purified LPG of *L. donovani* were provided by E. Handman, Melbourne.

Histones are structural proteins that play an important role in the organization and function of DNA within the eukaryotic nucleus.\(^{34}\) *Leishmania* histone H2A is a 14-kDa protein that is expressed during the promastigote stage. It is more abundant in promastigotes growing in logarithmic phase than in the same cells in stationary cultures.\(^{17,18,34}\) *Leishmania* H2B mRNAs were present in both the promastigote and amastigote forms; however, their levels are several times higher in promastigotes. The molecular mass of this protein is 12 kDa.\(^{19,34}\)

The genes coding for the *L. infantum* histone proteins H2A and H2B were cloned after screening of *L. infantum* cDNA expression library constructed in the AMOSLeox vector with polyclonal antibodies to a P16 *L. infantum* antigen. The full-length H2A and H2B cDNAs were amplified by polymerase chain reaction (PCR) from the initial clones, inserted into a pET-22b vector (Novagen, Fontenay-sous-Bois, France), and used to transform *E. coli* BL21 (Novagen).

The recombinant proteins H2A and H2B linked to a polyhistidine tail were isolated by dissolving inclusion bodies in 6M guanidine HCl and passing over a nickel-containing column according to the manufacturer’s recommendations (Amersham-Pharmacia, Biotech, Grylada, Greece). After dialysis to remove Guanidine HCl, purified proteins H2A and H2B were checked for purity by SDS 18% polyacrylamide gel and Coomassie blue staining.

The LACK protein is the *Leishmania* homologue of receptors for activated C kinase. This 36-kDa protein is highly conserved among related *Leishmania* species and is expressed in both promastigote and amastigote forms of the parasite.\(^{35-37}\) The LACK gene was amplified by reverse transcriptase (RT)-PCR from *L. major* (MHOM/TN/95/GLC94 zymodeme MON-25) amastigote total RNA and finally ligated to pET-22b(+). This construct encodes the LACK antigen fused with a stretch of six residues of histidine to allow its chromatographic purification on histaplug columns (Amersham-Pharmacia).

The P20 protein is a novel 20-kDa membrane-associated *L. infantum* antigen that was identified in our laboratory by immunoscreening of *L. infantum* promastigote cDNA expression library with an acute-phase MVL patient serum (Chenik M and others, unpublished data). P20 gene is present in one copy in the *L. major* and *L. infantum* genome.

The partial-length p20 open reading frame (408 bp) was subcloned into the pET-22b expression vector (Novagen, Fontenay-sous-Bois, France). The recombinant P20-(His)_6 was then purified by affinity chromatography (Amersham-Pharmacia), and purity was demonstrated by SDS-PAGE.

Detection of antibodies to *Leishmania* antigens by ELISA. The ELISAs developed for this project, based on a panel of 10 *Leishmania* antigens, were optimized as follows: In preliminary checkerboard titration, antigens in increasing protein concentration (0.25 to 10 μg/mL) were titrated against serial dilutions of known positive and negative sera (1:50 to 1:3,200). The optimal combination of antigen concentration/anti-serum dilution that consistently gave the best discrimination between positive and negative sera was then used throughout the study.

We determined that the use of the optimal antigen concentration and a single dilution of sera at 1:100 allows clear separation between positive and negative sera.

After optimization, all antigens were diluted in carbonate/bicarbonate buffer 0.1 M, pH 9.6 at a concentration of 0.25 μg/mL for LPG; 0.5 μg/mL for rgp63, rGBP, and rK39; 5 μg/mL for SLA, rH2A, and rH2B; and 10 μg/mL for rPSA-2-GST, rTR1-GST, rPSA-2-MBP, rP20, and rLACK. Diluted antigens were used (50 μL/well) to sensitize microassay plates (Nunc Maxisorp Immuno Plates, Poly Labo, Strasbourg, France) by incubation for 1h at 37°C and then overnight at 4°C. The plates were washed three times with 0.1 M PBS containing 0.1% Tween 20 (PBS-T). To avoid non-specific binding of protein sera, wells were further blocked by 200 μL of PBS containing 0.1% Tween 20 and 0.5% gelatin for 1h at 37°C. After washing with PBS-T, sera diluted 1:100 in PBS-T were added (50μL/well) and incubated for 2h at 37°C. Wells were then washed five times with PBS-T, followed by the addition of 100 μL per well of anti-IgG (Fc specific) horseradish peroxidase (Sigma) diluted 1:2,000 with PBS-T and further incubation for 1h at 37°C. Substrate solution (0.1 M sodium citrate, pH 5, 0.1% OPD (Sigma) and 0.03% H₂O₂) was added at 100 μL/well. The reaction was developed at room temperature and stopped with 50 μL of 4N sulphuric acid (H₂SO₄), and the absorbance was measured at 492 nm. All sera were tested in duplicate, and the mean value was recorded. Sera that gave borderline values were tested twice.

Statistical analysis. The cutoff value for each ELISA was defined as the mean optical density (OD) plus two standard deviations of the values obtained with sera from healthy controls. The proportions of true positives and negatives for each
test among patients and controls permitted us to calculate the sensitivity and specificity, respectively. Likewise, predictive values of positive and negative were calculated by assessing the proportions of true positives and negatives for each test among total positives and negatives, as described elsewhere.38,39

RESULTS

Figure 1 shows the absorbance values of the optimized ELISAs using a panel of 10 antigens tested with 146 sera from MVL patients and controls. The spectrum of absorbance values differs from one antigen to another. The widest spectrum was obtained with rk39, rH2A, rH2B, and SLA antigens with 81%, 71%, 65%, and 47% of MVL sera yielding to an OD higher than 1, respectively, allowing easier interpretation of the result. Rk39, rH2A, rH2B, and SLA antigens allowed the best discrimination between positive and negative sera. Lower OD values were obtained with the other antigens used (Figure 1 and Table 1).

The cutoff value of reactivity with each antigen was defined as the mean OD + 2 SDs obtained with sera of group II (the control group of healthy children). It was equal to: 0.194, 0.144, 0.138, 0.177, 0.102, 0.111, 0.120, 0.156, 0.592, 0.383, 0.554, and 0.926, for SLA, rk39, rH2A, rH2B, rGBP, rgp63, rLACK, LPG, rP20, rPSA-2-MBP, rPSA-2-TR1-GST and rPSA-2-GST, respectively. These cutoff values allowed us to identify positive and negative sera and consequently to estimate the performance parameters of the ELISAs (Table 2).

ELISAs based on rk39, rH2A, rH2B, and SLA had the best sensitivity (100%). The best specificity was obtained with rk39 (97%), with no reactivity among 33 sera from children with other infectious diseases (group III) and only three false-positive sera from 75 healthy controls (group II). The predictive values of the positive sera were 92%, 86%, 80%, and 82% for rk39, SLA, rH2A, and rH2B, respectively (Table 2). In addition, our results indicate that 97%, 97%, 86%, and 92% of the sera from children with MVL contain significant levels of antibodies to rGBP, rLACK, rgp63, and LPG, respectively. Finally, only ELISAs based on PSA-2 or P20 proteins showed rather poor performance.

The ELISAs based on rPSA-2 antigens deserve special comment. This protein was used as fusion protein with either MBP or GST. In the latter case, two forms of the protein were tested. rPSA-2-GST represents the full-length protein expressed by L. major amastigotes. It gave a very high background with the two control groups, II and III, with a cutoff value of about 1 OD. The second fusion protein, rPSA-2-TR1-GST, represents the water-soluble fragment of PSA-2 protein without the hydrophobic GPI-signal sequence. The reactivity with control group III was very high, decreasing the global performance of the test. In addition, when the partner fusion protein GST was checked separately as a control antigen, it appeared that most MVL patients react strongly with GST. Quite unexpectedly, a sensitivity of 92% and a specificity of 94% were obtained when using this unrelated antigen. Finally, the performance of ELISA based on rPSA-2 fused to an alternative protein MBP also was poor because of a high background reactivity which decreased the sensitivity of the test to 57%.

Since children composing control groups II and III were living in an area endemic for MVL, a positive serology to Leishmania antigens may indicate either a false positivity, an asymptomatic infection, or a smoldering disease that may precede the development of full-blown MVL.40 MVL sera reacted consistently (38/38) with at least four antigens (SLA, rk39, rH2A, and rH2B). In addition to the latter antigens, 37, 37, 35, and 33 sera reacted with rGBP, rLACK, LPG, and rgp63, respectively. These data show that individual MVL sera usually react with a large panel of Leishmania antigens. In contrast, the occasional sera from 108 control individuals (groups II and III) that were found positive with either one of the ELISAs reacted most frequently with one or few antigens whereas they were unreactive with the majority. Only two, four, and three control sera were reactive with six, four, or three antigens, respectively. In addition, the OD was usually weak, just above the cutoff value of the ELISA test. Strong reactivity with either SLA, rk39, rH2A, rH2B, rLACK, or rP20 was observed with only one serum each time. These results suggest that the occasional seroreactivities detected with control sera are unlikely to indicate a Leishmania infection but rather represent false positivities. All control children who tested positive were individually checked, and none has developed MVL within 3 years of inclusion in the study.

Finally, it appears that a judicious combination of two ELISAs based on rk39 and SLA slightly improves the specificity of the assay. Accordingly, a serum was considered positive if it was screened positive by SLA ELISA and then by rK39 ELISA. Sensitivity and specificity of 100% and 99%, respectively, were obtained when using the combined tests.

DISCUSSION

In this study and for the first time, different ELISAs were compared using a common panel of sera and a large variety of Leishmania antigens tested under the same experimental conditions. Several of these antigens were previously described in the literature as suitable for ELISA to detect anti-Leishmania antibodies in patients with visceral leishmaniasis but were not comparatively evaluated.

The panel of sera was obtained from 38 patients with parasitologically proven MVL. Healthy children living in the same area (n = 75) were used as controls. An additional control group consisted of 33 children suffering from various infectious diseases. This group was investigated to allow an evaluation of the performance of each antigen in the context of symptoms of infectious diseases that may be presenting symptoms of MVL. Actually, on admission, most MVL patients may present, in addition to classic symptoms of MVL, symptoms of pneumonia, diarrhea, otitis, and urinary tract infection.23 The 3-year time interval during which no control children developed MVL is long enough to exclude the possibility that some control individuals may have preclinical MVL. Actually, it has been reported that the incubation period is short and that full-blown disease develops within a few months after infection.40–42

The sensitivity and specificity of ELISAs using each of the recombinant proteins to detect specific anti-leishmanial antibodies were compared with those obtained with the more classic antigen SLA (Table 2). The latter represents the standard antigen used in many endemic areas for the serodiagnosis of MVL. Its main inconvenience is that as a crude antigen,
Figure 1. Enzyme-linked immunosorbent assay (ELISA) reactivity of sera from patients with Mediterranean visceral leishmaniasis (MVL), healthy controls (C1), and children with other infectious diseases (C2) with soluble *Leishmania* antigens (SLA, 5 μg/mL), recombinant *Leishmania* proteins: rK39 (0.5 μg/mL), rH2A (5 μg/mL), rH2B (5 μg/mL), rGBP (0.5 μg/mL), rLACK (10 μg/mL), rgp63 (0.5 μg/mL), rP20 (10 μg/mL), rPSA-2-GST (10 μg/mL), rPSA-2-TR1-GST (10 μg/mL), rPSA-2-MBP (10 μg/mL), and purified *Leishmania* LPG (0.25 μg/mL). Bars show the cutoff value for each ELISA that is defined as the mean optical density plus two standard deviations of the values obtained with sera from healthy controls.
FIGURE 1. (Continued).
Enzyme-linked immunosorbent assay (ELISA) reactivity of sera from patients with Mediterranean visceral leishmaniasis (group I), healthy controls (group II), and children with other infectious diseases (group III) with soluble Leishmania antigens (SLAs), recombinant Leishmania proteins: rK39, rH2A, rH2B, rGBP, rLACK, rgp63, r P20, rPSA-2-GST, rPSA-2-TR1-GST, and rPSA-2-MBP; purified Leishmania lipophosphoglycan (LPG); and purified glutathione S-transferase (GST) of Schistosoma japonicum. The range of reactivity of patients' sera (min and max) and mean optical density (OD) are shown as well as the mean OD of control sera (groups II and III). The cutoff value of the ELISA was defined as the mean OD of group II plus two standard deviations.

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Range of OD</th>
<th>Mean OD (SD) × 10^3</th>
<th>Cutoff value</th>
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<tbody>
<tr>
<td></td>
<td>Patients: I</td>
<td>Control individuals</td>
<td>Group II</td>
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<tr>
<td>Crude SLA</td>
<td>320 3318</td>
<td>1352 (888)</td>
<td>76 (59)</td>
</tr>
<tr>
<td>rk39</td>
<td>175 3470</td>
<td>2245 (1111)</td>
<td>30 (57)</td>
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<tr>
<td>rH2A</td>
<td>160 3209</td>
<td>1752 (1003)</td>
<td>50 (44)</td>
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<tr>
<td>rH2B</td>
<td>185 2777</td>
<td>1695 (987)</td>
<td>73 (52)</td>
</tr>
<tr>
<td>rGBP</td>
<td>0.5 24 2735</td>
<td>729 (585)</td>
<td>42 (30)</td>
</tr>
<tr>
<td>rLACK</td>
<td>10 112 3263</td>
<td>842 (749)</td>
<td>66 (27)</td>
</tr>
<tr>
<td>rgp63</td>
<td>0.5 24 2735</td>
<td>335 (281)</td>
<td>53 (29)</td>
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<tr>
<td>rP20</td>
<td>10 270 2972</td>
<td>1037 (692)</td>
<td>288 (152)</td>
</tr>
<tr>
<td>rPSA-2-GST</td>
<td>10 298 3105</td>
<td>967 (626)</td>
<td>438 (244)</td>
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<tr>
<td>rPSA-2-TR1-GST</td>
<td>10 61 2731</td>
<td>666 (672)</td>
<td>204 (175)</td>
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<tr>
<td>rPSA-2-MBP</td>
<td>10 124 2831</td>
<td>548 (518)</td>
<td>191 (96)</td>
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<tr>
<td>Purified LPG</td>
<td>0.25 52 1977</td>
<td>580 (423)</td>
<td>66 (45)</td>
</tr>
<tr>
<td>Purified GST</td>
<td>10 54 1307</td>
<td>429 (328)</td>
<td>50 (28)</td>
</tr>
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</table>

it may share common epitopes with other microbial antigens and may react with sera from other infectious diseases, especially Chagas' disease.9,12,43 In this study, we demonstrate that SLA antigen constitutes a good and reliable marker for MVL in the Mediterranean region where trypanosomiasis is absent. In fact, a specificity of 94% and a sensitivity of 100% were obtained with an SLA-based ELISA.

Among the large panel of recombinant and purified antigens, only rK39 introduced significant improvement over the classic SLA. Indeed, rK39 gave the best results in terms of specificity (97%) and predictive value of the positives (92%), with the highest arithmetic mean of OD values obtained with MVL sera and the lowest one with the control groups. With rK39, 81% of MVL sera gave an OD higher than 1, which makes the test easy to interpret. These results confirm the high specificity of rK39 protein in detecting anti-leishmanial antibodies in sera from patients with visceral leishmaniasis.10,11,44,45

Among the other leishmanial antigens, rH2A, rH2B, rGBP, rgp63 rLACK, and LPG also had good performance. To our knowledge, this is the first report of histone protein use for the serodiagnosis of human MVL. In spite of the evolutionary conservation of histone proteins among eukaryotic organisms, sera from children with MVL specifically recognize the L. infantum H2A and H2B histones. In fact, a specificity of 91% and 92% and a sensitivity of 100% were obtained, respectively, with rH2A and rH2B ELISA tests. Our results are in accordance with those obtained by Soto and al.18,19 in canine leishmaniasis. The investigators, using overlapping synthetic peptides covering the complete sequence of these proteins, demonstrated that during the natural infection, the antibodies target antigenic determinants located in the amino and carboxyl terminal regions of protein H2A and the amino terminal region of protein H2B. These regions are highly divergent in sequence relative to the mammalian H2A and H2B histones.18,19

Gp63 is a prominent and highly conserved family of proteins expressed on Leishmania membranes. Okong’-O-odera and others have shown that native gp63 is highly immuno-

genic and is a good diagnostic antigen for VL;13,14 our data confirm these results.

The L. major rGBP, which contains a 28-amino acid repeated motif, was reported to give better results than crude parasite antigen preparations in the diagnosis of cutaneous leishmaniasis.15 Recently, rGBP of L. donovani also was used in ELISA for the serodiagnosis of VL in Sudan, with a sensitivity of 92% and a specificity of 71%.46 The present study showed that L. major rGBP also is suitable for the serodiagnosis of MVL, with a sensitivity of 97% and a specificity of 92%.

rLACK, a molecule previously proposed as a vaccine candidate,35,37 has not been tested for the serodiagnosis of MVL. Our results indicate that this molecule induces a good humoral response in human MVL and we were able to set up a specific ELISA with a sensitivity of 97% and a specificity of 84%.

Although a humoral immune response to LPG antigen was previously reported,42 LPG has never been used to our knowledge for the serodiagnosis of MVL. Using ELISA testing in Kenya, an area endemic for leishmaniasis, Kurtzhals and al. demonstrated that cured kala-azar patients and a proportion of the study population with no history of kala-azar developed a humoral immune response to the purified Leishmania LPG antigen. Antibodies to LPG in individuals with no history of kala-azar could indicate previous subclinical Leishmania infection. The present study, conducted in the Mediterranean region, a geographic area where people are exposed to different hygienic and socioeconomic conditions, shows that LPG could be used as a diagnostic antigen for MVL, with a sensitivity and a specificity of 92%.

Only the rP20 and rPSA-2 antigens had modest performance, since they yielded very high background reactivity. E. coli trace contaminants that escaped the purification steps of the recombinant protein may account for the high background observed with these proteins. However, one should note that the purified recombinant proteins appeared as a single band when tested by SDS-PAGE. Another possible explanation is that the high background may indicate that
some epitopes of these recombinant proteins are shared with other micro-organisms against which individuals frequently develop antibodies.

Unexpectedly, we detected an extensive reactivity of MVL sera with the fusion protein partner GST (Table 1), indicating that a specific antibody response to *Leishmania* GST occurs during MVL, probably due to some cross-antigenicity between GST from *S. japonicum* or *Leishmania* species. This fact precludes the use in serologic tests of leishmaniasis of recombinant proteins fused with GST.

Globally, this study demonstrates that several *Leishmania* antigens can represent suitable reagents for the design of *Leishmania*-specific ELISA for the diagnosis of MVL. SLA, rK39, and histone proteins H2A and H2B allow the best performance, perhaps because they represent *L. infantum* proteins (i.e., homologous parasite) in the present study. For rGBP, rLACK, and rgp63, it is likely that the use of recombinant proteins from *L. infantum* rather than *L. major* as done in the present study (i.e., heterologous parasite) would yield better ELISA performance.

Finally, we believe that the classic crude SLA antigen still represents a very good and less costly alternative to the more-sophisticated recombinant proteins, which may be of interest for developing countries where MVL is prevalent and resources are scarce. However, SLA must be prepared carefully under standardized conditions and checked regularly for performance to avoid the risk of progressive loss of reactivity over time even when stored frozen.

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