SERODIAGNOSIS OF LEISHMANIASIS WITH RECOMBINANT ORFF ANTGEN

V. SAMUEL RAJ, ANIRBAN GHOSH, VANDANA S. DOLE, RENTALA MADHUBALA, PETER J. MYLER, AND KENNETH D. STUART

School of Life Sciences, Jawaharlal Nehru University, New Delhi, India; Seattle Biomedical Research Institute, and Department of Pathobiology, University of Washington, Seattle, Washington

Abstract. The serodiagnostic potential of recombinant ORFF protein (rORFF) from Leishmania infantum was assessed by ELISA. Of 49 sera from confirmed cases of visceral leishmaniasis (VL), all were seropositive using 5 ng of rORFF and serum diluted 1:20, while only 38 were positive with 500 ng of soluble antigen (SA) and 44 were positive by a direct agglutination test. There was also a positive correlation between spleen size and level of seropositivity with rORFF or SA. The reciprocal endpoint titer with rORFF was 1,280 for sera from VL patients, but < 20 with sera from malaria, filariasis, and tuberculosis patients, as well as with sera from healthy individuals from endemic and non-endemic areas. Sera from 10 confirmed cutaneous leishmaniasis cases from Turkey were negative or only weakly positive with rORFF, although 9 were positive with SA. Thus, rORFF protein appears useful as a sensitive reagent for the differential diagnosis of VL caused by the Leishmania donovani complex.

Protozoan parasites of the genus Leishmania cause a spectrum of human disease ranging from self-limiting cutaneous leishmaniasis (CL) to the lethal visceral leishmaniasis (VL). There is a general correlation between the species of Leishmania and the type of disease, with the Leishmania donovani complex causing VL or kala-azar. Routine diagnosis of VL has long been based on the microscopic detection of parasites in smears of lymph node, bone marrow, or splenic aspirates or culturing the parasite from patient material. These surgical procedures for diagnosis are painful and dangerous; thus, there is an urgent need for safe and reliable tests for specific diagnosis of leishmaniasis.

Serologic tests that have been developed and evaluated clinically include an immunofluorescent antibody test (IFAT), an ELISA with either whole parasites or purified antigens, a dot-ELISA, immunoblot analysis, and the direct agglutination test (DAT). The DAT using microtiter plates for the diagnosis of VL and American CL gives satisfactory results and is easy to perform. It is a simple technique with high sensitivity and specificity. One of its major drawbacks is that it has no prognostic value for evaluating the progression of the disease. The DAT is used as a routine serologic test for VL in India, and the parameters of the test have been established under local conditions with very little false positivity.

In this paper, we report the serodiagnostic potential for Indian VL of recombinant ORFF protein (rORFF) that is encoded in the LD1 locus of chromosome 35 of L. infantum. The rORFF appears to be specifically recognized by sera from patients with VL compared with those with CL. Furthermore, the sensitivity of detection appears to be greater than with total L. donovani promastigote soluble antigen, suggesting the utility of rORFF protein as a sensitive and differential diagnostic reagent.

MATERIALS AND METHODS

Patient sera. Sera were collected from 49 patients with clinically confirmed VL in the Sahibganj and Godda districts of Bihar, India, where kala-azar is endemic. Sera were also collected from 22 suspected VL cases, four patients who were intermittently treated for VL, and three uninfected individuals from the same region. Diagnosis of VL patients was based on the clinical symptoms such as persistent fever, splenomegaly, anemia, and weight loss. The patients were predominantly males and less than 10 years of age. Clinically diagnosed cases were also assessed by the DAT, which shows a very high degree of specificity and sensitivity for VL. The DAT endpoint titers from Indian VL patient sera had titers > 1:500, which are generally considered positive for all leishmanial infections. Sera were collected from 10 patients in Turkey with CL, presumably due to infection with L. major or L. tropica, and from 7 healthy individuals from non-endemic areas of India with no history of exposure to Leishmania. Sera were also collected from 18 Indian patients who were diagnosed with malaria, tuberculosis, or filarial infection by standard serologic and microscopic methods. Informed consent was obtained from patients or their relatives before sample collection, in accordance with the guidelines indicated by the Jawaharlal Nehru University Ethical Review Committee.

Preparation of Leishmania antigens. Soluble antigen was prepared from promastigotes of L. donovani AG83 (MHOM/IN/83/AG83). The cells were harvested, washed three times with phosphate-buffered saline (PBS), pH 7.2, at 4°C, followed by sonication. The lysate was centrifuged at 12,000 × g (Sorvall RC5c centrifuge, SS34 rotor; Dupont, Newtown, CT) for 15 min at 4°C. The supernatant was collected and the protein concentration was estimated by the Lowry method. This protein was used as soluble antigen (SA). Recombinant ORFF antigen from L. infantum (rORFF) was prepared essentially as described previously. The 858-basepair ORFF gene was amplified by the polymerase chain reaction from clone pB30 and cloned into Bam HI-digested pET17b (Novagen, Inc., Madison, WI). The recombinant protein was expressed in Escherichia coli BL21 cells (Novagen) after induction with isopropyl-p-D-thiogalactopyranoside. The bacterial pellet was dissolved sequentially in 2 M, 4 M, 6 M, and 8 M urea. The rORFF (34 kD) dissolved only in the 8 M fraction, where it constituted the vast majority of protein visible by staining with Coomassie brilliant blue after separation by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The 8 M fraction was dialyzed several times against PBS to remove urea and protein was concentrated by precipitation with (NH₄)₂SO₄. The precipitated rORFF was dissolved in PBS and protein concentration was determined as above.
Serodiagnostic assays. The DAT was carried out essentially as described. Antigen for the test was prepared from trypsin-treated, Coomassie brilliant blue–stained, formalin-preserved promastigotes of *L. donovani* AG83. Agglutination was carried out using a 1:500 dilution of patient sera in PBS containing 1% fetal bovine serum. The test was read visually after overnight incubation at 22 °C. The ELISAs were carried out in polystyrene microtiter plates essentially as described earlier. The optimal concentrations of rORFF and SA to be used for coating the plates and optimal dilution of the patient sera were standardized by performing initial ELISAs with various concentrations of the antigen and dilutions of sera. Subsequently, ELISA plates were coated with 5 ng of rORFF or 500 ng of SA (optimal concentrations) in 50 μl of PBS, pH 7.5. The wells were blocked with 200 μl of 3% bovine serum albumin (BSA) in PBS for 1–2 hr at 37 °C. After the wells had been washed three times with PBS, 0.1% Tween 20, they were incubated with 50 μl of serum diluted in PBS plus 1% BSA for 2 hr at 37 °C. Serum dilutions were usually 1:20 for rORFF and 1:100 for SA, except for endpoint titrations where two-fold dilutions of sera starting at 1:20 were used. The wells were then washed three times with PBS, 0.1% Tween 20, incubated with 50 μl of goat anti-human-IgG conjugated with alkaline phosphatase (Sigma, St. Louis, MO) diluted 1:2,000 in PBS plus 1% BSA for 1–2 hr at 37 °C, and rinsed three times with PBS, 0.1% Tween 20. After incubation for 30 min at room temperature with 50 μl of p-nitrophenylphosphate (Sigma), the reaction was stopped with 50 μl of 3N NaOH. The optical density at 405 nm (OD_{405}) of each well was determined using ELISA plate reader (7520 ELISA Reader; Cambridge Technology, Inc., Watertown, MA). Each serum sample was assayed in duplicate. Serum samples giving OD_{405} values greater than the mean OD_{405} plus three standard deviations for noninfected, non-endemic controls (0.429 or 0.633 for ORFF; depending on the set of sera from healthy individuals, and 0.445 or 0.735 for SA) were considered seropositive.

RESULTS

Recombinant ORFF antigen from *L. infantum* (rORFF) is a sensitive VL serodiagnostic using ELISA. A 1:10 dilution of serum from a parasitologically confirmed VL patient reacted strongly with rORFF at concentrations as low as 10 ng/well (Figure 1). In contrast, there was little signal over background with less than 100 ng of total SA from *L. donovani* promastigotes. Thus, the serodiagnostic assay using rORFF is at least 10 times more sensitive than that using SA. A series of ELISAs using different concentrations of rORFF or SA with various dilutions of patient sera led to optimization of the assays using 5 ng of rORFF with a 1:20 dilution of sera or 500 ng of SA with a 1:100 dilution of sera. These conditions were used for subsequent analyses.

Serum samples from 49 clinically confirmed VL patients in Bihar, India were compared using the DAT and the SA- and rORFF-based ELISAs (Table 1). Forty-four of 49 sera (90%) tested positive by the DAT, based on an agglutination titer > 1:500. Only thirty-eight of the 49 sera (77%) tested positive by the ELISA using 500 ng of SA per well and a 1:100 dilution of sera. When 500 ng of SA was used with a 1:20 dilution of sera, significant false-positive results were obtained with sera from malaria patients and from healthy individuals from endemic or nonendemic areas. In contrast, all (100%) of the 49 sera were positive by the ELISA using 5 ng of rORFF per well and a 1:20 dilution of sera. Sera from noninfected individuals from nonendemic areas (negative controls) gave the lowest ELISA readings (mean ± SD OD_{405} = 0.256 ± 0.058). Sera from the three uninfected individuals from the endemic area showed a mean OD_{405} value of 0.438 ± 0.051, which is slightly above the cut-off value. This may reflect an undocumented history of *Leishmania* infection in these patients. All five of the confirmed VL sera that were negative by the DAT were positive by the rORFF-based ELISA. In these cases, the OD_{405} values were low (between 0.438 and 0.679), suggesting a low level of antibody and perhaps, by extension, a low parasitemia, although their spleen sizes (5–6 cm), were indicative of significant infection.

Sera from 22 patients with suspected VL and 4 that were intermittently treated for VL were also tested by the DAT.
FIGURE 2. Spleen size and antibody levels in patients with visceral leishmaniasis (VL). Sera from 49 clinically confirmed patients with VL (spleen sizes \( \geq 5 \text{ cm} \)), 22 clinically suspected cases (spleen sizes \( = 3-4 \text{ cm} \)), 3 uninfected endemic normal (healthy) individuals, and 5 uninfected non-endemic controls were assayed by ELISA. A, 5 ng of recombinant ORFF (rORFF) antigen using a 1:20 dilution of sera. B, 500 ng of soluble antigen (SA) using a 1:100 dilution of sera. The horizontal line indicates the cut-off value (mean optical density at 405 nm \([\text{OD}_{405}] \pm 3 \text{ SD for five healthy controls from non-endemic areas}\) used to determine a positive result. The horizontal bars indicate the mean \([\text{OD}_{405}]\) values for each range of spleen sizes or control group. Those means that are significantly different from the uninfected endemic controls (using the Student’s \(t\)-test) are indicated by ** \((P < 0.001)\), * \((P < 0.01)\), + \((P < 0.02)\), or # \((P < 0.05)\).

and the SA- and rORFF-based ELISAs (Table 1). Twenty (91%) of the former and two (50%) of the latter tested positive by the DAT, while 19 (86%) and two (50%), respectively, tested positive using the SA-based ELISA. In contrast, all but one (95%) of the suspected VL cases and all of the intermittently treated patients were positive by the rORFF-based ELISA. Indeed, the mean \([\text{OD}_{405}]\) value for the latter group (1.282 \(\pm\) 0.437) was as high as that for the confirmed VL cases (1.201 \(\pm\) 0.461).

Examination of the VL patients showed a correlation between spleen size and serum antibody levels as determined by the rORFF- and SA-based ELISAS. The \([\text{OD}_{405}]\) values in the rORFF ELISA ranged between 0.540 and 2.181 for sera from different VL patients, with spleen sizes ranging from 3 to 12 cm. Mean \([\text{OD}_{405}]\) values for each spleen size were significantly higher than both non-endemic and endemic controls in all cases, except for patients with 6-cm spleens tested using the SA-based ELISA (Figure 2). There was a clear linear relationship between \([\text{OD}_{405}]\) and spleen size (\(r^2 = 0.34, P < 0.001; \text{Figure 2A}\)). A similar correlation was obtained using the SA-based ELISA when 500 ng of SA and a 1:100 dilution of sera was used, although the linear relationship was less pronounced (\(r^2 = 0.17, P < 0.001; \text{Figure 2B}\)). Analysis using the nonparametric Spearman rank order correlation test generated similar statistics (\(r = 0.650, P < 0.001\) for the rORFF ELISA and \(r = 0.403, P < 0.001\) for the SA-based ELISA).

The rORFF-based ELISA showed specificity for VL compared with other endemic diseases. The endpoint titers of sera from 15 VL patients ranged from 1:80 to 1:1,280 for VL sera, but were less than 1:20 with sera from uninfected individuals from endemic and non-endemic areas, as well as from patients infected with malaria, filaria, and tuberculosis (Figure 3). The rORFF-based ELISA was also able to discriminate between patients with VL and CL. Of the 10 sera (diluted 1:20) from parasitologically confirmed CL patients (presumably infected with \(L. major\) or \(L. tropica\)), four showed only a marginal reaction with rORFF (mean \([\text{OD}_{405}] = 0.763 \pm 0.024\) versus a cut-off value of 0.633 for sera from healthy individuals) (Figure 4). The remaining sera had \([\text{OD}_{405}]\) values below the cut-off value and thus showed no significant reaction with rORFF. In contrast, 9 of the 10 sera (diluted 1:100) showed a positive reaction with \(L. donovani\) SA, although two were only slightly higher than the cut-off value (0.735). The mean \([\text{OD}_{405}]\) value (2.156 \(\pm\) 0.759) for these sera was significantly greater (\(P < 0.001\)) than that for the control sera using the SA-based ELISA, but not for the rORFF-based ELISA.

**DISCUSSION**

This study shows that rORFF has substantial potential as an antigen for the serodiagnosis of \(L. donovani\) infection. An ELISA proved to be sensitive with as little as 5 ng of
FIGURE 3. Specificity of the recombinant ORFF (rORFF) ELISA for Leishmania infection. Reciprocal endpoint titers of sera from patients with visceral leishmaniasis (n = 15), malaria (n = 6), filariasis (n = 6), tuberculosis (n = 6), or uninfected individuals from non-endemic (n = 8) or endemic (n = 3) regions were determined by ELISA using 5 ng of rORFF.

rORFF and serum diluted 1:100 or greater resulting in a positive reading. In addition, for confirmed VL patients, there was a positive correlation between OD$_{405}$ value (presumably reflecting antibody levels) and spleen size. This level of sensitivity suggests that the antigen can be readily adapted to other assay formats. Endpoint titers using 5 ng of rORFF were only 2–3-fold less than using 100 ng of SA (1:80–1:1280 versus 1:160–1:5,120). Since ORFF represents only ~0.16% (1/600) of the total cellular protein in L. donovani AG83 (Ghosh A and others, unpublished data), this suggests that antibodies against ORFF represent a substantial fraction of the anti-parasite immune response in VL.

The sensitivity of the rORFF-based ELISA was attested by its ability to correctly predict the infection status of all 49 cases with clinically confirmed VL, while the DAT and SA-based ELISA showed 1 and 11 false-negative results, respectively. In addition, there was very good agreement between the rORFF-based ELISA and the DAT in predicting that most (95% and 91%, respectively) patients with suspected VL are indeed infected. In contrast, the SA-based ELISA appeared less reliable (86% positive). The false-negative results in the DAT showed low OD$_{405}$ values in the rORFF ELISA (reflecting low antibody levels), but were nonetheless positive. Thus, the rORFF-based ELISA appears more sensitive than the DAT, which is known for a high degree of specificity and sensitivity for VL. The slightly elevated OD$_{405}$ values for noninfected individuals from endemic regions compared with those from non-endemic regions may reflect subclinical infection. Since the assay has not yet been fully optimized with respect to antigen amount and serum dilution, it is conceivable that an assay can be developed that will accurately predict the parasitologic status of all VL patients.
The correlation between spleen size in VL patient sera (potentially reflecting parasite burden) and OD405 value in the rORFF ELISA provides further support for the importance of ORFF in the immune response to L. donovani infection. However, all four patients who had been intermittently treated for VL were strongly positive in the rORFF ELISA, even though their spleen sizes were normal (2 cm). This suggests that the OD405 value in the rORFF ELISA likely reflects antibody levels, rather than parasite levels per se, and may remain elevated even after spleen size has been reduced following treatment.

The rORFF-based ELISA showed considerable specificity for infection with L. donovani. Sera from VL patients exhibited high endpoint titers with 5 ng of rORFF, while sera from malaria, filariasis and tuberculosis patients showed diminished high endpoint titers with 5 ng of ORFF, and sera from healthy (presumed noninfected) non-endemic and endemic endpoint titers that were essentially indistinguishable from malaria, filariasis and tuberculosis patients showed.

This suggests that the OD405 value in the rORFF ELISA likely reflects antibody levels, rather than parasite levels per se, and may remain elevated even after spleen size has been reduced following treatment.

The rORFF-based ELISA showed considerable specificity for infection with L. donovani. Sera from VL patients exhibited high endpoint titers with 5 ng of rORFF, while sera from malaria, filariasis and tuberculosis patients showed endpoint titers that were essentially indistinguishable from healthy (presumed noninfected) non-endemic and endemic controls. In addition, only 4 of 10 sera from patients from Turkey with VL and confirmed to be infected with Leishmania (presumably L. major or L. tropica) tested positive in the rORFF ELISA and then only marginally so. In contrast, 9 were positive with the less sensitive SA-based ELISA. Thus, it seems likely that ORFF sequence divergence and/or difference in expression between species allows species-specific discrimination using the rORFF ELISA. The ORFF is encoded in the LD1 locus chromosome 35 (~2.2 Mb) in L. infantum and this sequence is present all Leishmania species. There are only 23 amino acid differences (7%) between ORFF from L. infantum and L. major (Myler PJ, unpublished data), most of which represent conservative replacements. However, there is evidence for different ORFF expression profiles between L. donovani and L. major (Ghosh A and others, unpublished data). The ORFF is amplified on small (200–450 kb) linear chromosomes, circular episomes, or a small gene conversion into the rRNA locus in about 15% of the Leishmania isolates examined. These gene amplifications result in increased production of ORFF protein (Ghosh A and others, unpublished data), possibly enhancing the use of rORFF as a serodiagnostic antigen.

While these studies demonstrate the sensitivity of rORFF antigen for the serodiagnosis of VL in the laboratory, further work is needed to test its utility in the field. In addition, the nature and duration of the immune response against ORFF needs to be more fully explored to determine whether the rORFF ELISA can distinguish between patients with active versus inactive VL.

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Authors’ addresses: V. Samuel Raj, Anirban Ghosh, Vandana S. Dole, and Rentala Madhubala, School of Life Sciences, Jawaharlal Nehru University, New Delhi. India. Peter J. Myler and Kenneth D. Stuart, Seattle Biomedical Research Institute, 4 Nickerson Street, Seattle, WA, 98109-1651.


