Novel Antigen Detection Assay to Monitor Therapeutic Efficacy of Visceral Leishmaniasis

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Abstract. Visceral leishmaniasis (VL) diagnosis is routinely performed by invasive liver, spleen, bone marrow, or lymph node biopsies, followed by microscopic identification of the parasites. Conventional serological tests cannot distinguish active disease from asymptomatic VL or from cured infection. Here, we report the initial validation of an enzyme-linked immunosorbent assay (ELISA) assembled to detect the *Leishmania infantum/donovani* antigens iron superoxide dismutase 1 (*Li-isd1*), trypanothione 1 (*Li-trx1*), and nuclear transport factor 2 (*Li-ntf2*) as a tool to monitor therapeutic efficacy of VL. The assembled ELISA detected the antigens in the urine samples from seven VL patients before initiation of therapy. Importantly, the antigens were no longer detected in all patients after completion of the treatment. These preliminary observations point to a promising tool to follow treatment efficacy of VL.

An antigen detection test for visceral leishmaniasis (VL) was developed approximately 10 years ago as a tool for the diagnosis of active cases of this disease.¹ This assay is a latex agglutination test (KAtex) based on the detection of leishmanial carbohydrate complex antigens using latex beads adsorbed with specific polyclonal rabbit antibody.² KAtex has been used intermittently for VL diagnosis as well as a tool for monitoring disappearance of leishmanial antigens after successful treatment of VL.^{3–8} Recent studies involving VL patients coinfecting with human immunodeficiency virus (HIV) have shown that the sensitivity of KAtex varied from 47.7% to 85.7% and specificity from 96% to 98.7%.⁹ These conflicting results can be explained on the grounds of the uncontrolled specificity and sensitivity (affinity/avidity) of the heterogeneous anti-carbohydrate antibodies used in the test.² Recently, two protein antigen detection tests have been reported for the diagnosis of VL.¹⁰ Both methods used preparations of whole *Leishmania donovani* promastigotes to generate the anti-whole parasite promastigote antibodies used in the tests.

However, defined and purified recombinant microbial proteins are an interesting alternative to carbohydrates and to whole parasite antigens because they circumvent the above restrictions (sensitivity and specificity). We have recently described this test strategy using urine samples from VL patients from the New World.^{11,12} These studies successfully identified three *Leishmania infantum* antigens, iron superoxide dismutase 1 (*Li-isd1*), trypanothione 1 (*Li-trx1*), and nuclear transport factor 2 (*Li-ntf2*). These antigens or biomarkers were used in the assembly of a capture enzyme-linked immunosorbent assay (ELISA) diagnostic test that identified 20/20 VL patients from Brazil. The specificity was also 100% based on testing of patients with cutaneous leishmaniasis, Chagas' disease, schistosomiasis, and tuberculosis.^{11,12} Here, we report a pilot study designed to investigate the use of this ELISA as a tool to monitor the therapeutic efficacy of VL treatment.

A capture ELISA using purified specific anti-*Li-isd1*, anti-*Li-trx1*, and anti-*Li-ntf2* produced in rabbits, or IgY produced in chickens, has been described in detail.^{11,12} The

following are the characteristics of the purified antibodies used to assemble the ELISA: 1) for *Li-isd1* antigen, affinity purified chicken IgY antibody; for *Li-trx1*, antigen affinity purified rabbit antibody; and for *Li-ntf2*, rabbit IgG antibody (affinity purified using a *Streptococcus* protein G resin). The concentrations of antibody required to provide the highest optical density (OD) signals above the background varied for each antigen/antibody capture ELISA and were 100, 875, and 2,000 ng/well for *Li-isd1*, *Li-trx1*, and *Li-ntf2*, respectively. The capture ELISA was performed as follows: wells of 96-well ELISA plates (EIA/RIA High Bind Microplate; Corning International, Corning, NY) were coated overnight at 4°C with 100 µL of the indicated antibody concentrations diluted in bicarbonate buffer pH 9.0. Wells were washed with PBS + 1% Tween-20 (Sigma Chemical Co., St. Louis, MO) and blocked at room temperature with PBS + 1% BSA + 1% Tween 20 (PBS/BSA/Tween) for 2 hours. After washing, 100 µL of antigen standards or undiluted human urine samples were added and incubated overnight at 4°C. Plates were washed followed by incubation for 1 hour with biotin labeled IgG (obtained from the second immunized rabbit for antigens *Li-trx1* or *Li-ntf2*) or biotin labeled IgY (obtained from a second chicken immunized with *Li-isd1*), at 1/2,000, a dilution previously determined by conventional sandwich ELISA. Following several rinses in PBS/BSA/Tween, peroxidase-labeled streptavidin at 1:2,000 dilution (BD Bioscience, San Jose, CA) was added for 30 minutes. The plates were then washed, and reactions were developed with 3,3',5,5'-tetramethylbenzidine substrate and read at 450 nm. Capture antibodies used to coat the ELISA plates were individual antibodies or a combination of the three antibodies per well at the concentrations indicated above (antigen affinity purified IgY anti-*Li-isd1*, 100 ng/well; antigen affinity purified rabbit anti-*Li-trx1* antibodies, 875 ng/well; and purified rabbit IgG anti-*Li-ntf2* antibody, 2,000 ng/well).

As a follow-up of our promising initial studies with the biomarkers *Li-isd1*, *Li-trx1*, and *Li-ntf2*, we were interested in assessing their possible utility as tools to monitor the treatment of VL. This investigation was performed at Infectious Diseases Research Laboratory of the Department of Medicine, Banaras Hindu University, Varanasi, India, and at its field site Kala-Azar Medical Research Center, Muzaffarpur, Bihar, India. The protocol was approved by the Ethics Committee of the Institute of Medical Sciences, and all subjects provided written informed consent. Seven patients with parasitological

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FIGURE 1. Sensitivity of a multiplexed capture enzyme-linked immunosorbent assay (ELISA) assembled to detect at the same time the *Leishmania* antigens iron superoxide dismutase 1 (*Li-isd1*), trypanothione 1 (*Li-trx1*), and nuclear transport factor 2 (*Li-ntf2*) compared with ELISA assembled to detect each antigen individually. ELISA plates were coated either with an antibody specific for an individual antigen (single) or with a pool of anti-*Li-isd1*, anti-*Li-trx1*, and anti-*Li-ntf2* antibodies (multiplexed). Detection was performed using biotinylated antibody specific for each individual antigen (single) or with a pool of biotinylated antibodies reacting with the three antigens (multiplexed).

confirmed VL were enrolled in the study. Confirmation of VL was done by demonstration of amastigotes in Giemsa-stained smears of splenic aspirate. Control urine samples were from 28 healthy subjects living in the same endemic area for VL from where the patients' samples were collected (Muzaffarpur, Bihar). For both, patients and controls, approximately 50 mL of urine sample were collected by clean catch method and stored refrigerated for less than 24 hours before use. Plain or non-manipulated urine samples were directly used in the capture ELISA, that is, no dilution or concentration of the urine samples were carried out. For the ELISA, 100 μ L of plain patient or control urines was used per well of the ELISA microtiter plate.

Although our initial studies were carried out in Brazil, where VL is caused by *L. infantum*, and the current designed study was performed in India, where VL is caused by *L. donovani*, this etiological consideration is not a hindrance as *Li-isd1*, *Li-trx1*, and *Li-ntf2* share 100% homology between *L. donovani* and *L. infantum*.¹³

The ELISA was performed using urine samples collected before the initiation of therapy (baseline) as well as 30 days later, at the completion of the treatment and discharge. Enrolled patients were treated with amphotericin B deoxycholate infusion in dose of 1 mg/kg on alternate days to a total of 15 infusions.

For these studies, we used a multiplexed ELISA designed to detect the three biomarkers at the same time, that is, a single assay assembled with the combination of the reagents necessary to detect at the same time the three *Leishmania* biomarkers (*Li-isd1*, *Li-trx1*, and *Li-ntf2*). The assay was initially optimized to have the same sensitivity of the ELISAs assembled to detect each biomarker individually. A pool of purified antibodies specific for each antigen was used to coat the plates, and an equal pool of biotinylated antibodies was used as the detecting reagent. Figure 1 shows the results and clearly indicates that no loss of the assay sensitivity occurs when the multiplexed ELISA is used to detect the individual biomarkers compared with the ELISAs assembled to detect each antigen individually. This test sensitivity is 100 pg/mL, which is 40 times more sensitive than that described for the two recently antigen detection tests (about 4,000 pg of total *Leishmania* antigens per mL of urine).¹⁰ In addition, the discrimination achieved between positive and negative samples of the current test is 9-fold, almost double to that of the

other two tests.¹⁰ These differences may be because the selected biomarkers described here were chosen because they were initially found in abundance in the urine of VL patients. Hence, an assay that targets these previously selected proteins will be by definition more sensitive and specific than an assay that uses antibodies against the whole parasite antigenic repertoire. In other words, a vast number of the parasite proteins that are detected by the latter antibodies are likely not to be present in the patients' urine. Finally, the use of the defined recombinant proteins to generate the antibody reagents offer much higher batch to batch reproducibility to the capture ELISA than antibodies generated against crude extract parasite antigen preparations.

FIGURE 2. Evaluation of the multiplexed enzyme-linked immunosorbent assay (ELISA) that detects *Leishmania infantum* antigens iron superoxide dismutase 1 (*Li-isd1*), trypanothione 1 (*Li-trx1*), and nuclear transport factor 2 (*Li-ntf2*) before and after treatment in patients from a visceral leishmaniasis (VL)-endemic area of India. ELISA plates were coated with a pool of anti-*Li-isd1*, anti-*Li-trx1*, and anti-*Li-ntf2* antibodies. Urine samples from VL patients ($N = 7$) were from the endemic area of Uttar Pradesh, India. Samples were collected before treatment and at 30 days after the completion of treatment. Dashed line represents the cutoff, which was calculated as the mean of the optical density (OD) obtained for urine samples from 20 control subjects +3 standard deviation of the mean.

We next used the multiplexed ELISA to test the unconcentrated urine samples from VL patients before and after treatment. Figure 2 illustrates the findings and confirms that urine samples collected at day zero (before treatment) from all seven enrolled patients were positive for the biomarkers *Li-isd1*, *Li-trxn1*, or *Li-ntf2*. In contrast, and importantly, the ELISA was no longer positive in the urine samples from the same patients collected after the completion of treatment. These results, in spite of the small sample size, provide a proof of principle that a urine-based antigen detection assay to identify leishmanial protein biomarkers *Li-isd1*, *Li-trxn1*, and *Li-ntf2* should be a successful test to measure, in a non-invasive manner, the therapeutic efficacy of VL. We are currently expanding these observations in clinical settings of both India and Brazil.

This capture ELISA test may overcome the significant drawbacks of the carbohydrate antigen detection assay (KAtex) because it detects protein antigens. In addition, its readout is unambiguous, which is a major advantage over the latex agglutination test, which can be subjective. Because antigen detection tests, by definition, are dependent on the release of specific molecules by actively multiplying microorganisms, they are of particular interest in that they are efficacious as markers of disease condition. Therefore, the developed capture ELISA is a very promising tool to follow treatment efficacy of VL, as antigen abundance decreases concomitantly with the elimination of the parasites. Moreover, this simple test can be an interesting alternative to a previously described real-time quantitative polymerase chain reaction that can be used as test of cure after VL treatment.¹⁴ Finally, this antigen detection test should be especially effective for detecting VL in individuals with HIV, who often have low antibody response to the parasite antigens.^{15,16}

Received April 14, 2016. Accepted for publication June 25, 2016.

Published online August 1, 2016.

Financial support: This work was supported by the National Institutes of Health grant R43 AI113992.

Disclosure: Claudia Abeijon is an employee of DetectoGen Inc. and has no ownership or ownership option in the company. Antonio Campos-Neto is a consultant for DetectoGen Inc.

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