



# Development of a Multiplexed Assay for Detection of *Leishmania donovani* and *Leishmania infantum* Protein Biomarkers in Urine Samples of Patients with Visceral Leishmaniasis

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**ABSTRACT** Visceral leishmaniasis (VL) is a serious and fatal disease caused by the parasites *Leishmania infantum* and *Leishmania donovani*. The gold standard diagnostic test for VL is the demonstration of parasites or their DNA in spleen, lymph node, or bone marrow aspirates. Serological tests exist but cannot distinguish active VL from either prior exposure to the parasites or previously treated VL disease. Using mass spectroscopy, we have previously identified three *L. infantum* protein biomarkers (*Li-isd1*, *Li-txn1*, and *Li-ntf2*) in the urine of VL patients and developed a sensitive and specific urine-based antigen detection assay for the diagnosis of VL that occurs in Brazil (where VL is caused by *L. infantum*). However, unpublished observations from our laboratory at DetectoGen showed that these biomarkers were detected in only 55% to 60% of VL patients from India and Kenya, where the disease is caused by *L. donovani*. Here, we report the discovery and characterization of two new biomarkers of *L. donovani* (*Ld-mao1* and *Ld-ppi1*) present in the urine of VL patients from these two countries. Capture enzyme-linked immunosorbent assays using specific rabbit IgG and chicken IgY were developed, and the assays had sensitivities of 44.4% and 28.8% for the detection of *Ld-mao1* and *Ld-ppi1*, respectively. In contrast, a multiplexed assay designed to simultaneously detect all five leishmanial biomarkers markedly increased the assay sensitivity to 82.2%. These results validate the utility of leishmanial protein biomarkers found in the urine of VL patients as powerful tools for the development of an accurate diagnostic test for this disease.

**KEYWORDS** antigen detection test, antigenuria, *Leishmania donovani*, *Leishmania infantum*, visceral leishmaniasis

Visceral leishmaniasis (VL), or kala-azar, is a systemic parasitic disease that is endemic in 75 countries, with more than 500 million people being at risk of infection. It is estimated to affect 50,000 to 90,000 people each year, with 90% of the cases occurring in Brazil, Ethiopia, India, Kenya, Somalia, South Sudan, and Sudan (1). VL is a fatal disease if it is not treated (1, 2). VL has historically been divided into two artificial geographical entities: New World (primary South American) VL, which is caused by

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*Leishmania infantum*, and Old World (primarily Indian) VL, which is caused by *Leishmania donovani*. However, it is now known that, in addition to South America, *L. infantum* is the agent of VL that occurs in the Mediterranean, Middle Eastern, and Central Asian regions of the world. Moreover, *L. donovani* is the agent of VL in East African countries, in addition to the Indian subcontinent (2). Notwithstanding the fact that the geographical division of VL is incorrect, it is a classical concept that is still commonly and loosely used.

The definitive diagnosis of active VL relies primarily on direct observation of *Leishmania* parasites in smears or cultures of either spleen, bone marrow, or lymph node aspirates, the collection of which requires invasive and risky sampling procedures. In addition, the sensitivity of these tests is, in general, modest and varies enormously (3–7). Although nucleic acid amplification tests are relatively more sensitive than microscopy and culture (8–10), they are complicated and expensive and are restricted to referral hospitals and advanced research centers (11).

Conventional serological tests, designed to detect an antibody response to parasite antigens, are employed in the field for the diagnosis of VL. However, these tests need to be interpreted in the context of the region of endemicity in a patient who presents with the clinical manifestations of VL, i.e., fever for at least 2 weeks, weakness, weight loss, and enlargement of the spleen and liver. The limitations of a diagnostic test based on detection of an antibody response are as follows: (i) positive serology is present in patients with both asymptomatic and active VL (12–16); (ii) serum anti-*Leishmania* antibodies remain in the circulation for several years after cure, which complicates the diagnosis of relapsed VL (17, 18); (iii) some individuals from areas where VL is endemic with no history of VL (asymptomatic or active disease) may have cross-reactive antibodies, which is a major hindrance to the specificities of these tests (19); and (iv) the sensitivity of serological tests in VL- and HIV-coinfected patients is poor, particularly if VL occurs after HIV infection (20, 21).

An test detecting leishmanial carbohydrate antigens in the urine of VL patients with active disease was developed approximately 2 decades ago (22). The sensitivity/specificity of the test varied widely, probably due to the heterogeneity of the carbohydrate antigens (23–27). As an alternative, we have previously shown that an assay that uses three defined *Leishmania infantum* protein antigens circumvented these restrictions for the diagnosis of VL that occurs in Brazil (28, 29). However, unpublished observations from our laboratory showed that these antigens or biomarkers were detected in only 55% to 60% of patients with VL caused by *Leishmania donovani* (in India and Kenya).

These results were somewhat unexpected because the discovered *L. infantum* antigens share almost 100% homology with those produced by *L. donovani* (28–30). We postulated that these results could be explained on the grounds of the different serological, pathological, and clinical manifestations that exist between the VLs that occur in different geographical areas of the world. For example, conventional serological diagnostic tests for VL using antigens such as K39 are highly sensitive in South America (31, 32) but are less sensitive for diagnosis of the disease in the Mediterranean region (33) and in East Africa (34). In addition, VL caused by *L. donovani* is primarily an anthroponotic disease, and dogs are rarely infected by this parasite species (35, 36). In contrast, VL caused by *L. infantum* is clearly a zoonotic disease and dogs and canids in general constitute a major reservoir of this parasite (37). Moreover, a substantial number of VL patients from the Indian subcontinent and East Africa who recover from treatment develop a dermatosis commonly known as post-kala-azar dermal leishmaniasis (PKDL) (38). As many as 10% of Indian cases and 50% of Sudanese cases develop PKDL after successful treatment of VL. In contrast, PKDL is extremely rare in patients with New World VL (38, 39). Consequently, we hypothesized that the leishmanial protein biomarkers eliminated in the urine of *L. donovani*-infected patients may also differ from the biomarkers found in *L. infantum*-infected patients.

Here we report the identification of unique *L. donovani* biomarkers in the urine of VL patients from Kenya and India, which were assumed to be infected with this parasite

species. In addition, we present the initial clinical validation of two of these new markers, which should help the development of a sensitive and accurate assay to diagnose active VL from both the New and Old Worlds.

## MATERIALS AND METHODS

**Clinical specimens.** A total of 24 urine samples from patients with New World VL (Brazil), assumed to be infected with *L. infantum*, and 55 of patients with Old World VL (Kenya,  $n = 45$ ; India,  $n = 10$ ), assumed to be infected with *L. donovani*, were evaluated in this study. These samples were collected before the initiation of therapy and were from patients (age range, 2 to 65 years) diagnosed with VL based on the following criteria: a clinical course consistent with VL (e.g., fever, anemia, hepatosplenomegaly), a confirmatory laboratory finding (identification of *Leishmania* in spleen or bone marrow aspirates), and a positive serological test. None of the patients had any clinical symptoms or laboratory findings compatible with renal or urinary tract abnormalities, nor were any of them receiving anti-*Leishmania* therapy at the time of urine collection. All samples from Brazil were obtained from the University Hospital Clemente of Farias (Montes Claros, Minas Gerais State, Brazil). Approval to use the samples was obtained from the Human Research Ethics Committee (COEP; CAAE-00842112.2.0000.5149) of the Federal University of Minas Gerais. The samples from Kenya were obtained from Kacheliba County Hospital (West Pokot County) and from the Kimalel Health Center (Baringo County). Approval to use these samples was obtained from the KEMRI Scientific and Ethics Review Unit (KEMRI/SERU/CCR/0011/3120). The samples from India were obtained from the Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. Informed consent and the protocol were approved by the Ethics Committee of the Institute of Medical Sciences, Banaras Hindu University. In addition, 40 urine samples were obtained from healthy control subjects living in the same geographical areas as the VL patients. Their serological tests for VL were negative. All samples were anonymized.

**Mass spectroscopy analysis.** Frozen individual urine samples (15 ml) from six patients with confirmed VL from Kenya and nine from patients from India were thawed, centrifuged, and filtered over 0.2- $\mu$ m-pore-size filters. The urine samples were then concentrated to  $\sim 200$  to 300  $\mu$ l using Centricon P3 filters (cutoff, 3 kDa). Equal volumes of concentrated urine samples were mixed with electrophoresis sample buffer and then submitted to SDS-PAGE followed by Coomassie staining. Bands ranging from  $\sim 5$  kDa to  $\sim 75$  kDa were excised from the gel and submitted for mass spectroscopy (MS) analysis at the Taplin Mass Spectrometry Facility, Harvard Medical School, Boston, MA. For each urine sample, 8 to 10 bands were cut from the gel. Each band was then independently submitted to MS runs. Gel bands were trypsin digested into peptides. The peptides were analyzed by nanoscale liquid chromatography coupled to a tandem mass spectrometer. Eluted peptides first had their molecular masses measured and were then fragmented, and finally, the fragment masses were measured. A computer search of the specific fragmentation pattern against predicted tryptic peptides from all known proteins from genome sequencing projects of humans and *Leishmania* protozoa was then performed. The power of the technique is in its redundancy. Because many peptides are generated from the initial gel band, multiple matches to the protein of interest are detected. In this way, the protein identity is completely unambiguous. Peptide score cutoff values were chosen at a cross correlation (XCorr) value of 1.8 for singly charged ions, 2.5 for doubly charged ions, and 3.0 for triply charged ions, along with delta correlation (DelCn) values of 0.1, and rank of Sequest preliminary score (RSP) values of 1. The cross-correlation values chosen for each peptide ensure a high-confidence match for the different charge states, while the DelCn cutoff ensures the uniqueness of the peptide hit. The RSP value of 1 ensured that the peptide matched the top hit in the preliminary scoring and that the peptide fragment file matched only one protein hit.

**Cloning of *Leishmania donovani* genes and protein expression and purification.** The sequences of DNA coding for the discovered *L. donovani* antigens were codon optimized for expression in *Escherichia coli*. The genes were synthesized by Blue Heron (Bothell, WA). To allow subcloning, the sequences of the restriction enzymes NdeI and BamHI were included at the 5' and 3' ends, respectively, of the optimized DNA fragment. The synthetic genes were digested with the restriction enzymes and subcloned into a pET-14b expression vector, which was similarly digested for directional cloning. Proteins expressed in the pET-14b expression vector contain a 6-residue histidine tag at the N terminus of the molecule, which facilitates purification by affinity chromatography on a QIAexpress Ni-nitrilotriacetic acid (NTA) agarose matrix (Qiagen, Valencia, CA). Recombinant proteins were obtained and purified from 100 ml of IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside)-induced batch cultures by affinity chromatography using the QIAexpress Ni-NTA agarose matrix (Qiagen, Chatsworth, CA) as described previously (40). The yields of recombinant proteins were 10 to 20 mg per liter of induced bacterial culture, and purity was assessed by SDS-PAGE followed by Coomassie blue staining.

**Generation of specific polyclonal antibodies.** The purified recombinant proteins (250  $\mu$ g) were emulsified with an equal volume of incomplete Freund's adjuvant and injected at multiple subcutaneous (s.c.) sites into female New Zealand rabbits or New Hampshire Red chickens (Capralogics Inc., Hardwick, MA). The animals were given two s.c. boosters (250  $\mu$ g of antigen in incomplete Freund's adjuvant) 3 weeks apart. At 1 week after the final boost, the animals were bled and sera were collected. Immunizations and bleeding procedures followed the National Institutes of Health guidelines and were approved by the Capralogics Institutional Animal Care and Use Committee (protocol 16-699R for the rabbits and 17-723C for the chickens). IgG (from rabbits) and IgY (from chickens) were purified by standard affinity chromatography or by use of the antigens immobilized on Sepharose 4B resin (CNBr-activated Sepharose 4B; GE Healthcare). A portion of the rabbit IgG was biotinylated by use of an EZ-Link-Sulfo-NHS- $\text{LC}$  biotinylation kit from Thermo Fisher Scientific (Pittsburgh, PA) according to the

manufacturer's instructions. Between 1 and 2.5 molecules of biotin per IgG molecule were invariably obtained for all IgG batches.

**Western blotting.** Purified recombinant proteins (100 ng) and whole-lysate extract from *L. donovani* and *L. infantum* promastigotes were fractionated by SDS-PAGE (4% to 20% gradient gel) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Medford, MA). Whole lysates of *L. donovani* and *L. infantum* were prepared from promastigote parasites cultured for 7 to 10 days in complete Schneider's medium at 26°C. The blots were blocked overnight at 4°C with Tris-buffered saline with 0.1% Tween 20 (TBS-T) containing 1% bovine serum albumin (BSA) and subsequently probed with antigen-specific rabbit antiserum or preimmune rabbit serum. After several rinses with TBS-T, goat anti-rabbit IgG labeled with horseradish peroxidase (Thermo Scientific Pierce, Rockford, IL) was added. After additional washings, bound conjugates were detected using an ECL enhanced chemiluminescence system (Amersham/GE Healthcare, Piscataway, NJ) and proteins were visualized on a ChemiDocTouch imaging system from Bio-Rad, Hercules, CA.

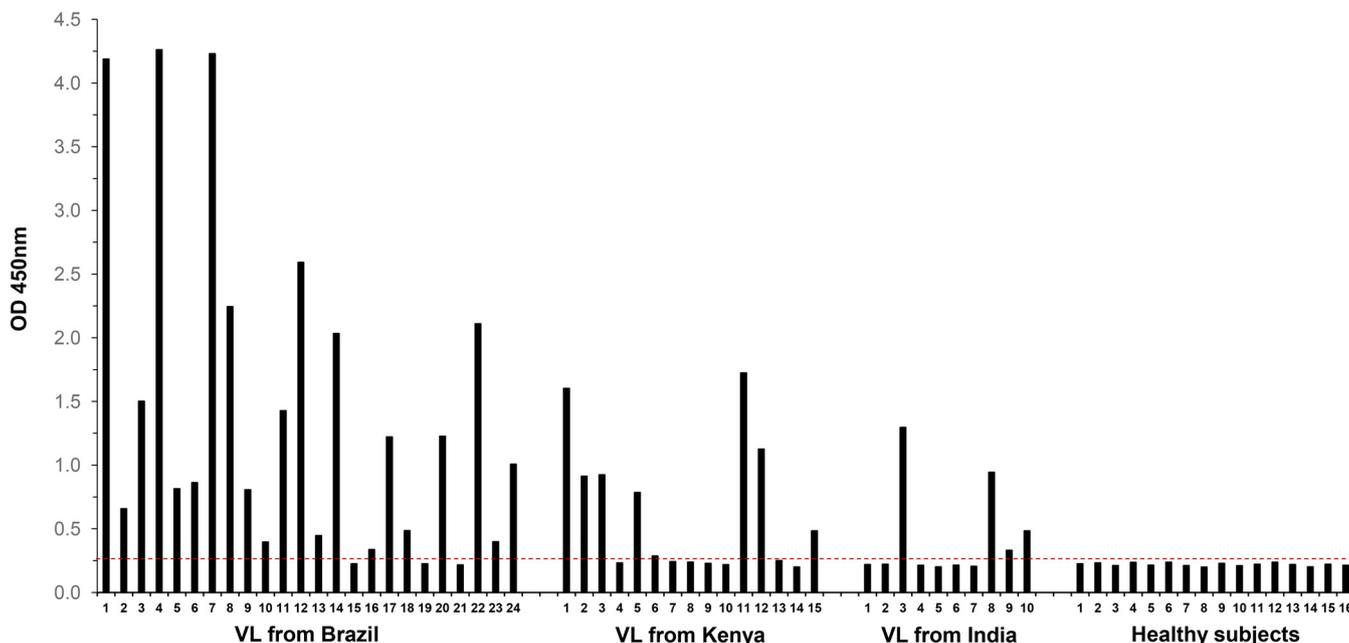
**ELISA.** A capture enzyme-linked immunosorbent assay (ELISA) for antigen detection was developed using purified IgG anti-*L. donovani* recombinant antigens obtained from antisera produced in two different rabbits or IgY produced in chickens. Briefly, the wells of 96-well ELISA plates (EIA/RIA plate, high binding; Corning International, Corning, NY) were coated overnight at 4°C with 0.2 µg of purified IgY diluted in bicarbonate buffer (pH 9.0). The wells were washed with phosphate-buffered saline (PBS)–0.1% Tween 20 (Sigma Chemical Co., St. Louis, MO) and blocked at room temperature with PBS–1% BSA–0.1% Tween 20 (PBS-BSA-Tween) for 2 h. After washing, human urine samples were added and the mixture was incubated overnight at 4°C. The plates were washed, followed by incubation for 1 h with biotin-labeled rabbit IgG at 1/2,000, a dilution previously determined by conventional sandwich ELISA. Following several rinses in PBS-BSA-Tween, peroxidase-labeled streptavidin at a 1:2,000 dilution (BD Bioscience, Franklin Lakes, NJ) was added for 30 min. The plates were then washed, and the reactions were developed with tetramethylbenzidine (TMB) substrate and read at 450 nm.

## RESULTS

**Discovery and characterization of unique *L. donovani* protein antigens present in the urine of patients with VL from Kenya and India.** Our previously described antigen discovery strategy initially reported the identification of three *L. infantum* antigens in the urine of VL patients from Brazil (28, 29). These antigens, *L. infantum* iron superoxide dismutase 1 (*Li-isd1*), *L. infantum* trypanothione 1 (*Li-tnx1*), and *L. infantum* nuclear transport factor 2 (*Li-ntf2*), were used to generate antibody reagents for the assembly of a diagnostic capture ELISA. This assay detected VL in ~90% of New World VL patients with a specificity of 100%. Specificity was confirmed by using urine samples from healthy control subjects as well as from patients with cutaneous leishmaniasis, Chagas disease, schistosomiasis, and tuberculosis. The results were very promising and prompted us to test the assay using urine samples from patients with VL caused by *L. donovani*. Fifteen urine samples from VL patients from Kenya and 10 urine samples from patients from India (Old World VL) were tested using a capture ELISA assembled in a multiplexed format to detect the three *L. infantum* biomarkers (*Li-isd1*, *Li-tnx1*, and *Li-ntf2*) (30). Surprisingly, only 45% to 50% of Kenyan samples and 30% to 40% of the Indian samples were positive (Fig. 1). As expected, 85% to 90% of the Brazilian samples were positive.

To resolve this assay limitation, we used our proven antigen discovery strategy to identify unique *L. donovani* protein antigens in the urine of VL patients from Kenya and India. For antigen discovery we used the same protocol previously employed for the identification of leishmanial proteins in the urine of VL patients from Brazil (28, 29). Six urine samples from patients from Kenya and nine urine samples from patients from India were analyzed. Urine bands were excised from the PAGE gels and submitted for mass spectroscopy analysis, which revealed that several urine samples contained putative *L. donovani* proteins (Table 1). In addition, the *L. donovani* homologues of *Li-isd1*, *Li-tnx1*, and *Li-ntf2* were also identified in several urine samples from VL patients from both Kenya and India (Table 1, shaded rows). In this study, we use the same abbreviations as those used when we reported the discovery of these three biomarkers in urine from Brazilian patients with VL (29, 30).

The *L. donovani* peptide donor proteins for the four new biomarkers, *Ld-mad1*, *Ld-clp1*, *Ld-mao1*, and *Ld-ppi1*, and the three homologues of biomarkers that we formerly found in urine samples from patients with New World VL were unambiguously identified because between 2 and 4 peptides spanning the entire sequence of each peptide donor protein (Fig. 2) were found in multiple urine samples from the VL



**FIG 1** Multiplexed ELISA for the detection of *Li-isd1*, *Li-txn1*, and *Li-ntf2* (New World VL markers) in urine samples from patients in Brazil and from patients with Old World VL (Kenya and India). The wells of the ELISA plates were coated with a pool of antigen affinity-purified IgY anti-*Li-isd1* antibody, antigen affinity-purified rabbit anti-*Li-txn1* antibody, and purified rabbit IgG anti-*Li-ntf2* antibody. After addition of patient and control urine samples followed by washes, the wells were incubated with a pool of biotinylated rabbit IgG specific for the three antigens. Reactions were then revealed with streptavidin-labeled horseradish peroxidase plus TMB. Samples were from VL patients from Brazil ( $n = 24$ ), India ( $n = 10$ ), and Kenya ( $n = 15$ ). Healthy control subjects ( $n = 12$ ) were also from these three countries. The red dashed line represents the cutoff value (OD = 0.255), which is the average OD for the samples from the healthy control subjects plus 3 SD. The results presented here are representative of those from at least three experiments performed at different times with the same urine samples.

patients from Kenya and India. Importantly, the XCorr for most individual peptides was highly significant, with an average of  $>3$  (Table 1). In addition, none of the peptides had homology with human protein sequences (not shown).

**Purification and prioritization of *L. donovani* biomarkers.** The genes encoding the full length of each protein (except for *Ld-clp1*, which was produced in two halves of ~45 kDa) were codon optimized for expression in *E. coli*, synthesized, and cloned in competent *E. coli* DH5 $\alpha$  cells, followed by subcloning into the pET-14b expression vector and transformation into competent *E. coli* BL21 (DE3)pLysS cells. This vector generates a recombinant protein containing 6 histidine residues at the N terminus to facilitate protein purification. Recombinant proteins for *Ld-mao1* and *Ld-ppi1* were readily obtained and purified using Ni-NTA agarose resin, and purity was assessed and confirmed by SDS-PAGE with Coomassie blue staining and Western blot analysis (Fig. 3). More than 20 mg of each pure recombinant protein was produced in order to generate antibody reagents. Although *Ld-mad1* and the two halves of *Ld-clp1* were also successfully expressed, they could not be purified in a soluble form. Many different attempts were undertaken to circumvent this problem, but unfortunately, no workable concentrations of the recombinant proteins were obtained. Therefore, these two proteins were deprioritized in the present study.

Validation of *Ld-mao1* and *Ld-ppi1* as genuine leishmanial molecules was then performed by Western blot analysis using the specific IgG antibodies and whole-cell extracts from both *L. donovani* and *L. infantum* promastigotes. Rabbits and chicken immunized with *Ld-mao1* and *Ld-ppi1* produced high titers ( $>1/81,000$ ) of specific IgG and IgY (not shown). Western blot analysis demonstrated that the specific antibodies recognized the corresponding native *Ld-mao1* and *Ld-ppi1* proteins in both leishmanial lysates and, as expected, the two recombinant proteins (Fig. 4). These results confirm that *Ld-mao1* and *Ld-ppi1* are produced and are present in both *L. donovani* and *L. infantum* organisms.

**TABLE 1** *Leishmania donovani* peptides identified by MS in individual urine samples from patients with VL from Kenya and India

Biomarker	Peptides identified in patient urine samples	XCorr	<i>L. donovani</i> donor protein (putative)	GenBank accession no.	MW (kDa) of donor protein	Frequency <sup>a</sup> in patients' samples (country)
<i>Ld-mad1</i>	R.VAVLGAAGGIGQPLSLLK.N	4.79	Malate dehydrogenase	XP_003864180.1	33.28	3/6 (Kenya), 6/9 (India)
	R.DDLFNTNASIVR.D	3.51				
	R.LFGVTTLDVVR.A	3.14				
	R.IQFGGDEVVK.A	2.50				
<i>Ld-clp1</i>	K.NNPVLIGEPGVGK.T	3.14	Clp protease (HSP78)	XP_003857963.1	90.77	6/9 (India)
	K.TAIVEGLAQR.I	3.63				
	R.EKLIHMDAELKK.T	2.84				
<i>Ld-mao1</i>	K.EMPGPGTVYLSQNLRF	2.8	Maoc family dehydratase	XP_003858460.1	16.97	2/6 (Kenya), 2/9 India
	K.GLISLSNIVQK.T	3.34				
	K.TLQFEGESEWSVTK	3.28				
<i>Ld-ppi1</i>	R.RTGQPTTISYEEAVTELQK.W	4.27	Peptidyl-prolyl isomerase	XP_003858557	12.62	2/6 Kenya, 1/9 (India)
	R.VTFEEAAR.Q	1.92				
<i>Li-isd1</i>	K.LNAAAESNSGLASK.S	4.66	Iron superoxide dismutase	XM_001467829	21.53	5/6 (Kenya), 1/9 (India)
	R.GGGEPSPGLASAIVDSFGSFASF.K	5.00				
<i>Li-txn1</i>	K.HLGDVVK.L	2.51	Tryparedoxin	XM_001466605	16.7	5/6 (Kenya), 5/9 (India)
	K.QNDMVDMSLSGK.T	3.21				
	K.LVEFYEK.H	2.40				
	K.MPWLSIPFEK.R	2.77				
	R.NVVEALK.Q	2.56				
	K.QYKVESIPTLIGLNADTGDVTTR.A	4.63				
	R.HALTQDPEGEQFPWRDE.-	4.05				
<i>Li-ntf2</i>	R.DQLAGIYRPNTLLTWQK.E	3.04	Nuclear transport factor 2	XM_001463701	13.89	4/6 (Kenya), 4/9 (India)
	K.EQVQGVDAIMAR.F	3.27				
	R.FANLGFTEAAFK.Q	4.11				

<sup>a</sup>Frequencies are presented as the number of patients with the peptide/total number of patients tested. The shaded area highlights the three biomarkers that were also found in urine samples of VL patients from Brazil.

**Optimization of capture ELISA for *Ld-mao1* and *Ld-ppi1*.** Various concentrations of the developed antibodies were initially used to assemble a capture ELISA in order to obtain the lowest limit of detection (LOD) to identify the *Leishmania* antigens in human urine. Purified rabbit and chicken immunoglobulin antibodies were titrated, and those antibodies that provided optical density (OD) readings arbitrarily higher than 0.5 above the background at the lowest concentrations (<50 ng/ml) were selected for further evaluation. The purified antibodies were then titrated in an antigen detection ELISA. The plates were coated with different concentrations of the antibodies, followed by incubation with a fixed concentration of the corresponding antigens (5 ng/ml). Biotinylated antibody (purified rabbit IgG) specific for each antigen was then added, followed by the addition of peroxidase-labeled streptavidin and TMB. The concentration of antibody required to provide the highest OD signals above the background for both assays was 2  $\mu$ g/ml (not shown).

To determine possible interference of urine in the assay LOD, several concentrations of the recombinant antigens were spiked in human urine samples obtained from normal and healthy subjects and tested comparatively with antigens diluted in Tris buffer with 1% BSA. As can be seen in Fig. 5, approximately 15 to 45 pg/ml of *Ld-mao1* could be detected in the presence of buffer. In the presence of control urine, the limit of detection was slightly higher (45 to 137 pg/ml). The limit of detection for *Ld-ppi1* was between 15 and 45 pg/ml in the presence of either buffer or control urine. Hence, the results indicated that both

**Ld-mad1**

MRRSQACFF**RVAVLGAAGGIGQPLSLLLKNN**KYVKELKLYDVKGAPGAAADLSHCAPAKVTGYTKDDL  
 SRAVEGVVVV I PAGI PRKPGMT**RDDL FNTNASIVRD**LAIAVGT HAPKAI VGI ITNPVNSTVPVAAEAL  
 KKVGVYDPA**RLFGVTTLDVVR**ARTFVGEALGKSPLELDI PVVGGHSGETIVPLLSGFP SLSEEQVRQLT  
**HRIQFGGDEVVKA**KDGAGSATLSMAFAGNEWTTAVLRALSGEKGVVVCTYVQSTVEPSCAFFSSPVLG  
 NSGVEKIYPVPM LNAYEEKLMAKCLEGLQGNIKKGI AFGNK

**Ld-clp1**

MLRRRLVQASTALRCGSTAALAAPFGPFPGCGGPRKTAVAAVACSPLASSTA AHTLRTSCRWQSTGVTP  
 WNSSGNSGGGGNGGQQGR TWVNPNAVPPGEFLKKYARDLTEEARMGR LDP I VGREEVIRRTIQVVSRR  
**TKNNPVLIGEPGVGKTAIVEGLAQR**IVKGEVPESIKDRQVYALDMGTLVAGAKFRGEFEERLKGVLKDT  
 IQSHGKVI LFDLHTLVGAGSSGDGSM DAANLLKPSLARGELHCIGATTLDEYRQHIEKDAALARRFQ  
 SVLVTEPTVEETISILRGIKEKYEAAHGLIKDEALVYAAVNSHRYLSERRLPKAIDLIDEAASRLRL  
 QQESKPEALDLIERELIRLKEAEAVKKDKDEVGKEKLENLYEQISQKQKEYDALEERWKKEKNLFQTI  
 KQRTEELDVLRRHLEQAMNSGDFAKAGEIQHSQIPNLLKQIEKDKHLASSKNFMVHDSVTSKDIAEVIA  
 RATGIPVAQLM TSE**REKLIHMDAELKKT**IMGQDAAIESITNVVRSRAGLHSHKRPLG SFLFLGPTGVG  
 KTEVCKSLAKFLFDD E S FICRIDMSEYMERHSVHRLIGAPPGYVGYEEGGELTESVRRRPYQIVL FDEF  
 EKAHPSVSNILLQVLDEGLTDSHGRRVDFKNTIIILTSNIGADV IARLPEGEPSIAMPVMEQVRQRL  
 APEFINRLDDIIMFNRLSREDIR SIVEILFAQVQKMLTEHNI TLEESTEVDWLSVNGYSAVYGARPLK  
 RL VQSELLNQLALMLLDGRIREGELVRLSVQDGHVVVANHALSPEKGP TDARLLDQ

**Ld-mao1**

MSSETAPLVIRVGS RATRTVHISQENVNTFGDVIDDHNP IHS DPEVAKAAGFP TTICY GMYAGSLFSGL  
 MA**KEMPGPGTVYLSQNLRF**TAPV FVGDDLDVIVEVREFR K**GLISLSNIVQKT**DPSTGKTTVCVEGSA  
 VVMN**KTLQFEGESEWSVTK**

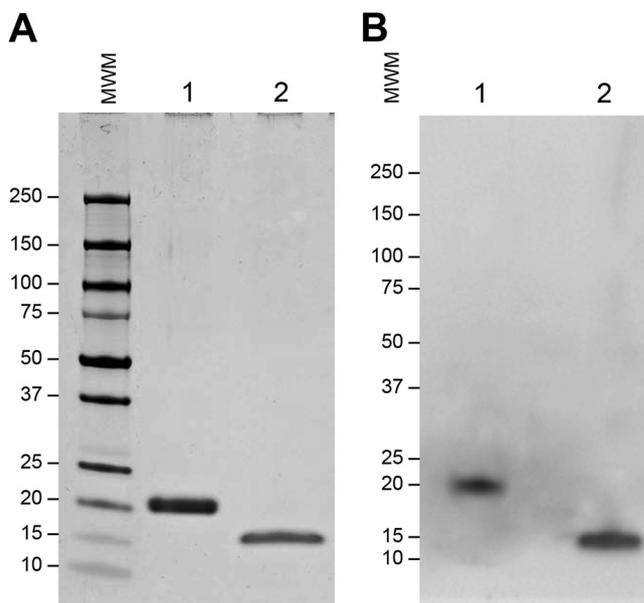
**Ld-ppi1**

MPTSSWQA A HLLIKHSGSRNPVSR**RTGQPTTISYEEAVTELQKWR**QSIEEG**RVTFEEAARQ**RSDCSSYA  
 RGGDLGVFGPGEMMKPFEDATKSLEVGHVSGIVV TDSGVHIIKRIA

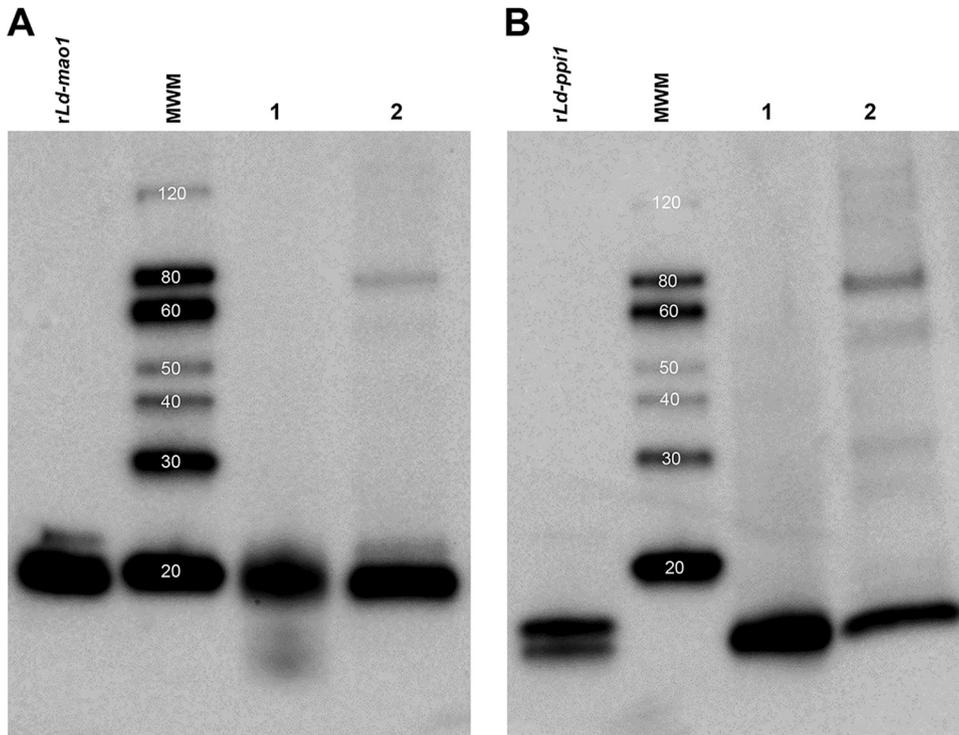
**FIG 2** Amino acid sequence and positioning of discovered peptides within the *L. donovani* protein donor of the peptides. Discovered peptides are highlighted in bold red.

assays are highly sensitive for detection of the antigens and that urine should not interfere with the detection of the antigens in this biological sample from VL patients.

Next, urine samples from VL patients (*n* = 45) and healthy control subjects (*n* = 24) from Kenya were used for the clinical evaluation of the assays. The results showed that

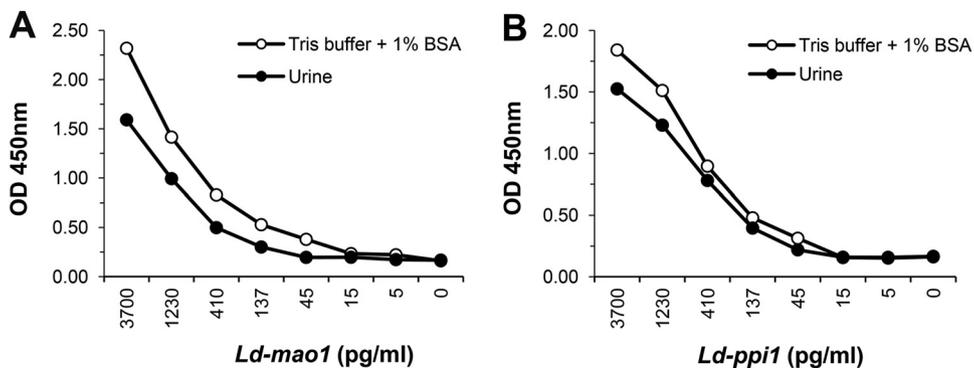


**FIG 3** SDS-PAGE (A) and Western blot (B) analysis of purified recombinant *Ld-mao1* and *Ld-ppi1*. Recombinant proteins containing amino-terminal His-tagged residues were expressed in *E. coli* BL21(DE3)pLysS, followed by purification by affinity chromatography using an Ni-NTA agarose matrix. Purity was evaluated by SDS-PAGE (4 to 20% gradient polyacrylamide gel) and Coomassie blue staining (A) and by Western blotting using anti-His tag MAb (B). Lanes 1, *Ld-mao1*; lanes 2, *Ld-ppi1*; lanes MMW, MW markers (in kilodaltons).

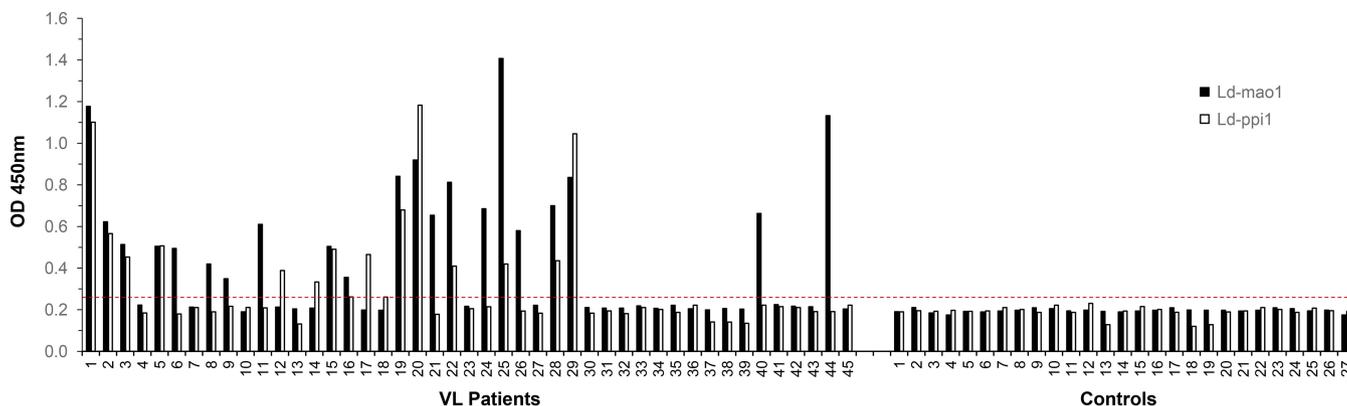


**FIG 4** Immunochemical detection of *Ld-mao1* and *Ld-ppi1* in *L. donovani* and *L. infantum* promastigotes. Validation of *Ld-mao1* and *Ld-ppi1* proteins as genuine molecules produced by *L. donovani* and *L. infantum* was performed by Western blotting using promastigote cell lysates from the parasites and antigen-specific rabbit antisera. (A) Anti-*Ld-mao1*; (B) anti-*Ld-ppi1*. Lanes: *rLd-mao1*, recombinant *Ld-mao1*; *rLd-ppi1*, recombinant *Ld-ppi1*; 1, *L. donovani* crude extract; 2, *L. infantum* crude extract; MWM, MW markers (in kilodaltons). No bands were seen in membranes blotted with the same promastigote cell lysates and probed with rabbit preimmune serum (not shown).

the sensitivities of the assays for *Ld-mao1* and *Ld-ppi1* were 44.4% and 28.8%, respectively (Fig. 6). The cutoff for a positive response was calculated as the mean OD reading for the controls plus 3 standard deviations (SD). Importantly, several urine samples that were positive for *Ld-mao1* (samples 6, 8, 9, 11, 21, 24, 26, 40, and 44) were negative for *Ld-ppi1*. By the same token, samples 12, 14, and 17 were positive for *Ld-ppi1* but were negative for *Ld-mao1*. This complementation is important, in that it yields a combined



**FIG 5** Determination of the limit of detection (LOD) of the capture ELISAs assembled for the proteins *Li-mao1* and *Li-ppi1* spiked in urine samples from normal healthy subjects. Capture antibodies at previously determined concentrations were used to coat the ELISA plates. These concentrations were 2  $\mu$ g/well for antigen affinity-purified rabbit anti *Li-mao1* antibody (A) and antigen affinity-purified rabbit anti-*Li-ppi1* antibody (B). The wells were then incubated with various concentrations of the antigen diluted either in Tris plus 1% BSA or in urine from normal healthy subjects followed by incubation with biotin-labeled IgY anti-antigen secondary antibody. Reactions were developed after addition of streptavidin-labeled peroxidase, substrate ( $H_2O_2$ ), and the chromophore TMB. Results are expressed as the OD read at 450 nm.



**FIG 6** Capture ELISA for detection of *Ld-mao1* and *Ld-ppi1* in urine from VL patients and local healthy controls from Kenya. Urine sample numbers were from VL patients ( $n = 45$ ) and control subjects ( $n = 24$ ). The red dashed line represents the cutoff values ( $OD = 0.222$  [not shown] and  $0.269$  for *Ld-mao1* and *Ld-ppi1* respectively), which were calculated using the average OD obtained for urine from normal healthy control subjects plus 3 SD. The results presented here are representative of those from at least three experiments performed at different times with the same urine samples and by the same capture ELISA.

sensitivity of 53%. These results highlight and confirm our previous observation that a highly sensitive assay would need to include reagents that detect several different biomarkers preferentially assembled in a multiplexed format.

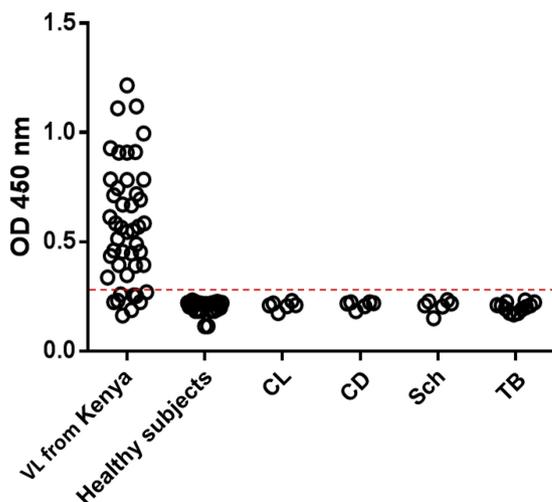
**Configuration and initial clinical validation of a multiplexed assay.** A multiplexed assay offers several advantages over single assays. The most obvious ones are that a multiplexed assay enables the detection of several biomarkers per test, which is crucial to achieving a high degree of sensitivity, and is faster to perform and less expensive to produce and use than several single assays that detect individual biomarkers.

In the current study we used a multiplexed ELISA designed to detect not only *Ld-mao1* and *Ld-ppi1* but also the previously discovered biomarkers *Li-isd1*, *Li-txn1*, and *Li-ntf2* because, as depicted in Fig. 1, approximately 45% to 50% of the Kenyan samples were positive for the last three markers. Hence, a multiplexed assay to detect *Ld-mao1*, *Ld-ppi1*, *Li-isd1*, *Li-txn1*, and *Li-ntf2* was then optimized. A pool of purified antibodies specific for each antigen was used to coat the plates, and a second pool of biotinylated antibodies was used as the detecting reagent. This assay had the same sensitivity as the single ELISAs assembled to detect each biomarker individually (not shown), hence indicating that no loss of assay sensitivity occurs when the multiplexed ELISA is used.

To begin the clinical validation of the optimized multiplexed assay, we used urine samples from VL patients from Kenya. In addition, several control samples from non-VL patients who had other infectious diseases (cutaneous leishmaniasis,  $n = 6$ ; Chagas disease,  $n = 6$ ; schistosomiasis,  $n = 6$ ; and tuberculosis,  $n = 12$ ) were also tested. Unfortunately, at the time of the conclusion of this validation, we did not have access to a large panel of urine samples from VL patients from India. Once we can circumvent the logistical difficulties that exist for the shipment of biological samples from India to other countries, we will include them in this validation. The results of the experiments performed with urine samples from VL patients from Kenya are illustrated in Fig. 7 and show that the multiplexed assay greatly increased the sensitivity of the diagnostic test to 82.2%. Also important was the demonstration that the multiplexed assay had a specificity of 100%, as no positive result was observed with urine from healthy control subjects or from patients having several other infectious diseases. However, a future and more stringent evaluation of the test specificity will have to include urine samples from patients with other tropical infectious diseases, e.g., malaria and African trypanosomiasis.

**DISCUSSION**

Our study has discovered and validated two *L. donovani* proteins as diagnostic biomarkers of VL. These proteins, which were found in the patients’ urine, are the maoc



**FIG 7** Initial clinical validation of a multiplexed assay for the diagnosis of Old World VL. ELISA plate wells were coated with a pool of affinity-purified antibodies specific for *Li-isd1*, *Li-txn1*, *Li-ntf2*, *Ld-mao1*, and *Ld-ppi1*, followed by blocking and overnight incubation with urine samples from VL patients ( $n = 45$ ) or control urine samples from local healthy control subjects ( $n = 24$ ) from Kenya. Urine samples from non-VL patients were from Brazil and included samples from patients with cutaneous leishmaniasis (CL;  $n = 6$ ), Chagas disease (CD;  $n = 6$ ), schistosomiasis (Sch;  $n = 6$ ), and tuberculosis (TB;  $n = 12$ ). The plates were washed and the wells were incubated with a second pool containing biotinylated antibodies specific for all five leishmanial antigens. The wells were then incubated with streptavidin-labeled peroxidase, the substrate  $H_2O_2$ , and the chromophore TMB. The OD was then read at 450 nm. The red dashed line represents the cutoff value (0.289), calculated as described in the legend to Fig. 1. The results presented here are representative of those from at least three experiments performed at different times with the same urine samples.

family dehydratase-like protein (*Ld-mao1*; GenBank accession number [XP\\_003858460](#)) and peptidyl-prolyl *cis-trans* isomerase/rotamase (*Ld-ppi1*; GenBank accession number [XP\\_003858557](#)). Two other potential biomarkers were also discovered (ATP-dependent Clp protease subunit, heat shock protein 78 [*Ld-clp1*; GenBank accession number [XP\\_003857963](#)] and malate dehydrogenase [*Ld-mad1*; GenBank accession number [XP\\_003864180](#)]). Unfortunately, we have not yet validated the diagnostic utility of the last two biomarkers due to difficulties in producing the highly purified recombinant proteins needed for the initial production of specific antibodies for assay development and validation. However, we believe that the diagnostic potential of malate dehydrogenase (*Ld-mad1*) as an additional marker would be worth investigating because of the solid data that we obtained during its discovery. The mass spectroscopy (MS) analysis of the VL patients' urine revealed the presence of at least four different peptides with high XCorr values spanning the full length of the amino acid sequence of *Ld-mad1* with a molecular weight (MW) of 33 kDa. Moreover, these peptides were found in 3/6 and 6/9 urine samples from VL patients from Kenya and India, respectively, demonstrating that this molecule is abundantly present in the urine of these patients. We are currently producing monoclonal antibodies (MAbs) to *Ld-mad1* in the hope that we can circumvent the nonspecific reactivity observed with the rabbit and chicken antibodies generated against this molecule (not shown). Selection of MAbs that will be highly specific for *Ld-mad1* should be relatively easy regardless of the poor purity of the recombinant protein used to immunize the mice for MAb production. On the other hand, ATP-dependent Clp protease (*Ld-clp1*) presents tangible complications as a biomarker candidate. Although MS revealed that the three discovered peptide sequences that match *Ld-clp1* also had high XCorr values, the protein has a very high MW (90.7 kDa), no pure recombinant protein could be prepared, and no peptides matching *Ld-clp1* were found in any of the urine samples from VL patients from Kenya. Therefore, we have at this point deprioritized this protein for further validation. Finally, this study also confirmed in the urine of the patients from Kenya and India the presence of *Li-isd1*, *Li-txn1*, and *Li-ntf2*, the three biomarkers that we previously discovered in the urine of

New World VL patients (28, 29). The MS findings for these three biomarkers were very strong, and they were abundantly present in the urine of the VL patients from Kenya and India (Table 1).

The ultimate goal of this study was to find leishmanial biomarkers present in the urine of patients with VL caused by *L. donovani* that could increase the sensitivity of a multiplexed assay that we initially developed for the diagnosis of VL that occurs in Brazil, which is caused by *L. infantum* (28). Although this multiplexed assay is highly sensitive and specific for the diagnosis of VL caused by *L. infantum*, it has a poor sensitivity for the diagnosis of VL caused by *L. donovani*. That occurred despite the fact that the three biomarkers are highly conserved between *L. infantum* and *L. donovani*. However, as discussed earlier, these results could be explained on the grounds of the known different serological, pathological, and clinical manifestations that exist between the VLs that are caused by these two different species of parasites (31, 32, 34–39).

Expression and purification of the recombinant proteins *Ld-mao1* and *Ld-ppi1* were achieved with no major difficulties, and these proteins were validated as genuine parasite molecules by Western blot analysis. This approach clearly demonstrated that these molecules are abundantly produced by *L. donovani* and *L. infantum* cultured *in vitro* because the rabbit-specific antisera used in the Western blot analysis recognized a single band of the expected MW of either *Ld-mao1* or *Ld-ppi1* in promastigote lysates from both *L. donovani* and *L. infantum*. The fact that both markers were detected in both *L. donovani* and *L. infantum* is not surprising, as the genes coding for *Ld-mao1* and *Ld-ppi1* in *L. donovani* are 100% identical to the corresponding genes in *L. infantum*. Also expected was the fact that the recombinant molecules migrate slightly slower than the native molecules because of their slightly higher MW than the native molecules due to the presence of the 6× His tag.

The initial validation of the ELISAs assembled to detect *Ld-mao1* and *Ld-ppi1* confirmed that the combination of chicken IgY and rabbit IgG antibodies is a reliable permutation for the development of a reproducible and sensitive urine-based capture ELISA. The biochemical sensitivity of the ELISAs assembled with these antibodies is approximately of 15 to 45 pg/ml for both antigens. Importantly, urine has little or no interference with the assay sensitivity. These assay formats yielded a clinical sensitivity of 44.4% for the detection of *Ld-mao1*. In contrast, the clinical sensitivity for the detection of *Ld-ppi1* was 28.8%. However, several urine samples were uniquely positive, with only one of the two assays yielding a combined specificity of 53%. This result coincides with our previous observations that indicated that a highly sensitive test requires the simultaneous detection of several VL biomarkers, either separately or in a multiplex-formatted test (28).

Indeed, the multiplexed assay assembled to detect the markers originally defined using urine samples from VL patients from Brazil plus the new markers of VL caused by *L. donovani* described in this work showed an increase in the overall sensitivity of the assay to nearly clinically acceptable levels of 82.2%. This multiplexed assay showed excellent specificity when tested with urine samples not only from healthy control subjects but also from non-VL patients who had other infectious diseases (cutaneous leishmaniasis, Chagas disease, schistosomiasis, and tuberculosis).

We recognize that, thus far, the sensitivity and specificity of the assembled multiplexed assay are based on a limited number of urine samples from both patients and controls. Notwithstanding this fact, these results are very encouraging. In fact, we are confident that the addition and/or replacement of one or more of the current markers of the multiplexed assay with newer markers will result in the development of a highly sensitive and specific test for the diagnosis of VL from both the New and Old Worlds. As we mentioned before, an example of a strong candidate for such a marker is *Ld-mad1*, which we report in this work (Table 1). We will soon evaluate its potential to increase the sensitivity of the developed multiplexed assay.

However, the ideal way to increase the sensitivity and specificity of the test would be to assemble the assay with MAbs specific to the discovered markers. Indeed, we have finished the production and purification of MAbs specific to *Li-isd1*, *Li-txn1*, *Li-ntf2*,

*Ld-mao1*, and *Ld-ppi1*. Moreover, MAbs specific to *Ld-mad1* will soon be available. Together, these MAbs will help with the development of a highly sensitive and specific multiplexed test for the diagnosis of VL caused by either *L. infantum* or *L. donovani*.

Finally, because antigen detection tests are, by definition, dependent on the release of specific molecules by actively multiplying microorganisms, they are of particular interest, in that they are efficacious as markers of active VL, post-kala-azar dermal leishmaniasis, VL-HIV coinfection, etc. Moreover, the developed multiplexed capture ELISA is a very promising tool for following the efficacy of VL treatment, as antigen abundance decreases concomitantly with the elimination of the parasites, and we have preliminary evidence to support this proposed utility (30).

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