

Characterization of Novel *Leishmania infantum* Recombinant Proteins Encoded by Genes from Five Families with Distinct Capacities for Serodiagnosis of Canine and Human Visceral Leishmaniasis

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Abstract. To expand the available panel of recombinant proteins that can be useful for identifying *Leishmania*-infected dogs and for diagnosing human visceral leishmaniasis (VL), we selected recombinant antigens from *L. infantum*, cDNA, and genomic libraries by using pools of serum samples from infected dogs and humans. The selected DNA fragments encoded homologs of a cytoplasmic heat-shock protein 70, a kinesin, a polyubiquitin, and two novel hypothetical proteins. Histidine-tagged recombinant proteins were produced after subcloning these DNA fragments and evaluated by using an enzyme-linked immunosorbent assays with panels of canine and human serum samples. The enzyme-linked immunosorbent assays with different recombinant proteins had different sensitivities (67.4–93.0% and 36.4–97.2%) and specificities (76.1–100% and 90.4–97.3%) when tested with serum samples from *Leishmania*-infected dogs and human patients with VL. Overall, no single recombinant antigen was sufficient to serodiagnosis all canine or human VL cases.

INTRODUCTION

Visceral leishmaniasis (VL) is a neglected disease with an annual worldwide incidence of 500,000 human cases and which, in epidemiologic terms, can be classified into anthroponotic and zoonotic types.¹ The predominant causal agent of the anthroponotic type of VL is *Leishmania donovani*, and the major species that cause zoonotic VL are *L. chagasi* in the Western Hemisphere and *L. infantum* in the other areas of the world.^{2,3} These two species that cause zoonotic VL are believed to be indistinguishable from each other.^{4,5} A subclinical form of the infection develops in most persons exposed to *L. infantum* and *L. donovani*,^{6–9} and the proportion of dogs that remain asymptomatic after being exposed to the parasite is not known.

The gold standard method for the diagnosis of human VL is the search for *Leishmania* amastigotes in smears of needle aspirates from splenic or bone marrow tissues. However, the sample collection procedure used in the method is invasive and may pose risks to patients, and the method sensitivity varies considerably.^{10–12} Detection of canine infection and/or disease essentially can be carried out with the methods mentioned above.^{13–16} However, because most persons with the disease produces antibodies against *Leishmania*, diagnoses of clinically suspected human cases are often confirmed or the infection in dogs is indicated by serologic immunoassays.^{17–23} These assays are carried out mainly with antigens from cultured *Leishmania* promastigote forms, which may also react with antibodies associated with other infectious diseases such as Chagas' disease and malaria, and thus produce false-positive results.²⁴

In the past two decades, there has been a considerable effort to produce defined antigens, especially recombinant antigens, to be used in the serodiagnosis of human and canine VL.²⁵ Many recombinant antigens have been selected and tested for

the serodiagnosis of VL (including rGP63, rHSP70, rHSP90, rK39, HASBP1, PSA, Lepp12, pape22, LiPs, and histones).²⁵ Among these antigens, a fragment of a kinesin protein, known as K39,²⁶ has enabled development of assays that have shown good performance in the serodiagnosis of human VL in most disease-endemic areas.²⁷ However, it is unlikely that one recombinant antigen is recognized by antibodies from all infected or sick persons.

The repertoire of the antibody specificities against *L. infantum* in dogs or humans may vary with distinct conditions.^{28–31} Moreover, even in persons with apparently the same clinical status, the fine specificity of the individual immune response is unlikely to be identical. For instance, in a panel of nine serum samples from patients with LV, no single antigen in an *L. infantum* extract was clearly recognized by antibodies from all serum samples when tested by using Western blotting.³² In accordance with this finding, none of the commercially available *L. infantum* recombinant antigen-based immunoassays has a sensitivity of 100% in different disease-endemic regions.^{33–36} For these reasons, it is important to expand the existing *L. infantum* recombinant antigen panel³⁷ and evaluate new recombinant *Leishmania* proteins to exploit the full potential of recombinant antigens in the serodiagnosis of VL.

With the purpose of enlarging the current panel of recombinant antigens that may be useful for the serodiagnosis, Teixeira and others screened *L. infantum* cDNA and genomic libraries with pools of serum from infected dogs or human patients with VL.³² In the present study, four previously obtained³² and one new recombinant antigen, encoded by five genes/families, were characterized and their reactivity was tested against serum from different canine and human populations.

MATERIALS AND METHODS

Parasites and recombinant antigens. *Leishmania infantum* promastigotes and amastigotes were generated from the MHOM/BR2000/Merivaldo2 strain and maintained as described.³⁸ Total parasite lysate was obtained by sonication of log-phase parasites and its protein content was quantified by using the Bradford method before use in enzyme-linked

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immunosorbent assays (ELISAs). The lambda bacteriophage clones described in this study were isolated by two consecutive screenings of cDNA or genomic *L. infantum* libraries with a pool of serum from four dogs and a pool of serum from three human patients with confirmed infection by *L. infantum* as reported.³² The four dogs were clinically healthy mongrel animals from a visceral leishmaniasis-endemic area (Jequié, Bahia, Brazil), which in addition to antibodies against *L. infantum*, had delayed skin hypersensitivity reactions to Montenegro's antigen. The three humans lived in Terezina, Piauí, Brazil, and had antibodies that recognized antigens in an *L. infantum* lysate but did not recognize previously obtained recombinant antigens.³² Thirty-two antibody-reactive isolated recombinant clones were studied, 30 from the first screening with canine serum and 2 from the subsequent screening with human serum.

Sequencing and characterization of selected inserts. From the selected lambda bacteriophage clones, corresponding plasmid vectors (pBK-CMV) were excised according to the manufacturer's instructions (Stratagene, La Jolla, CA). Partial (at the 5' and 3' ends: *Lci1A* and *Lci5A*, respectively) or full nucleotide sequences (*Lci2B*, *Lci3A*, and *Lci4A*) of plasmid inserts from each clone were determined and compared by using the Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) nucleotide or deduced amino acid sequences with those deposited in *Leishmania* and *Trypanosoma* genomic databases (GeneDB, <http://www.genedb.org> and Genbank, <http://www.ncbi.nlm.nih.gov/>).

Subcloning of selected inserts for production of recombinant antigens. To produce N-terminally histidine (His)-tagged recombinant proteins (NH6), whole or partial selected inserts were isolated from original pBK-CMV-derived plasmids and subcloned into prokaryotic expression vectors of the pRSET series (Invitrogen, Carlsbad, CA). The pRSET vector introduces at the N-terminus of recombinant proteins an approximately 30-amino acid peptide that includes a six histidine tag (His-tag). Inserts corresponding to only a fragment of the original insert in pBK-CMV were named with an additional letter and/or number (e.g., *Lci3A-R3*, *Lci5A-I*). Subcloning strategies used for each insert were defined by the available restriction enzyme sites and are summarized in Table 1. The only exception was *Lci5A*, which had to be amplified by using a polymerase chain reaction and primers flanked by *Kpn I/EcoR I* (forward primer 5'-CGAGGTACCGGCGCAGCGTGAGGAGCAGGC-3'; reverse primer 5'-CGAGAATTCCACCGGTGGCTCCTCCTGCTG-3'; restriction sites are underlined), before cloning into the pGEM-T Easy plasmid (Promega, Madison, WI) and sequencing and subcloning into the pRSET expression vector.

Production and purification of recombinant proteins. To produce His-tag recombinant proteins, pRSET-derived plasmids were used to transform BL21(DE3)pLysS *Escherichia coli* (Invitrogen). Transformed bacteria were grown in Luria-Bertani medium and induced for protein expression with isopropyl β -D-thiogalactoside. Induced cells were centrifuged, resuspended in phosphate-buffered saline (PBS), and lysed by sonication. Protein purification was performed as described by using Ni-NTA agarose (QIAGEN, Hilden, Germany).³⁹ Protein products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a denaturing 15% polyacrylamide gel and staining of proteins with R-250 Coomassie blue. For estimation of recombinant protein concentrations, densities of bands in Coomassie blue-stained gels were compared with those of known concentrations of bovine serum albumin.

Serum samples. Canine serum samples were obtained from two groups of dogs. The first group contained 46 dogs naturally infected with *Leishmania*, which was detected by splenic aspirate and culture in the disease-endemic area of Jequié (Bahia, Brazil). These dogs were polysymptomatic (21 dogs), oligosymptomatic (21 dogs), or asymptomatic (4 dogs) on the basis of defined clinical criteria.⁴⁰ The second group contained 31 dogs from *Leishmania*-free areas in Recife, Pernambuco, Brazil, being 7, 4 and 20 dogs with demodicosis, babesiosis, and ehrlichiosis, respectively. All diseases were parasitologically confirmed. Because the 31 animals from the *Leishmania*-free areas were not subjected to parasitologic examination by means of splenic aspiration, the possibility that they may have had a subclinical *Leishmania* infection, although unlikely, cannot be ruled out. In addition, serum from 15 healthy mongrel dogs from another non-endemic area (Salvador, Brazil) and which had negative results in splenic tissue cultures for *Leishmania* amastigotes, were used. The dogs were handled in accordance with the Oswaldo Cruz Foundation guidelines for experimentation on animals.

Human serum samples were obtained from three groups. The first group contained 39 clinically and parasitologically diagnosed VL patients (36 from Feira de Santana, Brazil and three from Teresina, Brazil, whose serum samples were kindly provided by Dr. Aldina Barral, Fundação Oswaldo Cruz, Salvador, Brazil). The second group contained 26 parasitologically confirmed patients with cutaneous leishmaniasis (CL). The third group contained 40 serologically confirmed chronic Chagas' disease patients. Serum samples were also obtained from 50 healthy persons of various ages from non-endemic areas in Brazil. For ethical reasons, patients with Chagas' disease, patients with CL, and healthy controls were

TABLE 1
Characterization of antigens used in immunoassays*

Group of clones/inserts	Protein encoded by homolog genes of inserts	Gene DB accession nos. of homolog genes	Subcloned DNA fragment of selected inserts	Fusion polypeptides used in ELISA
<i>Lci1</i>	Heat shock protein HSP70	LinJ28_V3.2950; LinJ28_V3.2960; LinJ28_V3.3000; LinJ28_V3.3060	~3,000 bp, <i>Bam</i> H I/ <i>Xho</i> I	rLci1A-NH6
<i>Lci2</i>	N-kinesin	LinJ14_V3.1180	1,285 bp, <i>Bam</i> H I/ <i>Kpn</i> I	rLci2B-NH6
<i>Lci3</i>	Hypothetical protein with repetitive motifs	Sequence similar to: LinJ34_V3.0700; LinJ34_V3.0710	~1,500 bp, <i>Sac</i> I/ <i>Kpn</i> I	rLci3A-R3-NH6
<i>Lci4</i>	Poly-ubiquitin	LinJ36_V3.3690	2,259 bp, <i>Bam</i> H I/ <i>Kpn</i> I	rLci4A-NH6
<i>Lci5</i>	Hypothetical protein with repetitive motifs	LinJ33_V3.3230. (contains other coding DNA sequences out of frame)	915 bp, <i>Kpn</i> I/ <i>EcoR</i> I	rLci5A-I-NH6

* ELISA = enzyme-linked immunosorbent assay; bp = basepairs.

not subjected to bone marrow or splenic aspirations for investigation of *Leishmania* infection. The study was approved by the appropriate Ethics Committee, and informed consent was obtained from all adults or legal guardians of children before blood was collected.

ELISA. Protein samples were diluted in coating buffer (15 mM Na₂HCO₃, 28 mM NaHCO₃, pH 9.6), placed into wells of 96-well microtiter plates (1 µg of parasite lysate or 0.5 µg of each recombinant antigen per well), incubated overnight at 4°C, and blocked with 0.15 M PBS, pH 7.2, containing 0.05% Tween 20 and 10% dry non-fat milk. Wells were incubated with the selected serum samples at a dilution of 1:200 (canine serum) or 1:600 (human serum). These dilutions had been shown to constitute the best compromise in terms of producing fewer false-negative and false-positive results. Wells were then washed with 0.15 M PBS, pH 7.2, containing 0.05% Tween 20. Peroxidase-conjugated goat anti-dog IgG (diluted 1:1,200) or anti-human IgG (diluted 1:15,000) (Sigma-Aldrich, St. Louis, MO) depending on the serum sample tested was added and plates were incubated for one hour at room temperature. Enzymatic activity was detected by using 0.01% hydrogen peroxide and 0.01% *o*-phenylenediamine (Sigma-Aldrich) in 0.1 M phosphate-citrate buffer, pH 5.0 and read in a spectrophotometer with a 490 nm filter. The cutoff values for the ELISAs were defined as means of results obtained with serum samples from healthy donors plus 3 SD. Intensities of reactivities obtained in the ELISAs were arbitrarily classified as weak (OD values ≤ 0.299), moderate (OD = 0.300–0.899), or strong (OD values > 0.899). Means of the OD values and individual values for each serum sample are shown in the figures.

RESULTS

Selection and identification of *L. infantum* antigens. After their excision from plasmids, sequencing, and identification, inserts from 29 clones isolated during the first screening with canine serum (one clone was lost during the insert isolation process) were shown to correspond to segments of four distinct genes or gene families of *L. infantum*. These gene families were named *Lci1* (24 clones), *Lci2* (2 clones), *Lci3* (2 clones), and *Lci4* (1 clone). Two additional clones isolated in a second screening with a pool of three serum samples from VL patients, which in preliminary tests had not reacted with recombinant polypeptides selected in first screening,³² yielded identical inserts that corresponded to a fifth gene, which was named *Lci5*. All five genes were selected for further studies of the potential of the corresponding recombinant proteins for diagnosis of canine and human VL.

Characterization of selected *L. infantum* antigens. Inserts of the *Lci1* group encode members of the 70-kD heat shock protein (HSP70) family. The prototype clone, *Lci1A*, which was selected for further studies, included the full length open-reading frame encoding one of the members of the 70-kD cytosolic heat shock protein (HSP70) and additional 5' untranslated region (UTR) and 3'UTR DNA segments (Table 1 and Figure 1A), which are known to be encoded by multiple genes within the *L. infantum* genome.⁴¹

The two *Lci2* inserts, *Lci2A* and *Lci2B*, encode segments of a *L. infantum* kinesin that is homologous to the C-terminus of the *LinJ14_V3.1180* gene product (Table 1). Comparison between the *LinJ14_V3.1180*-encoded protein, which is 3,279

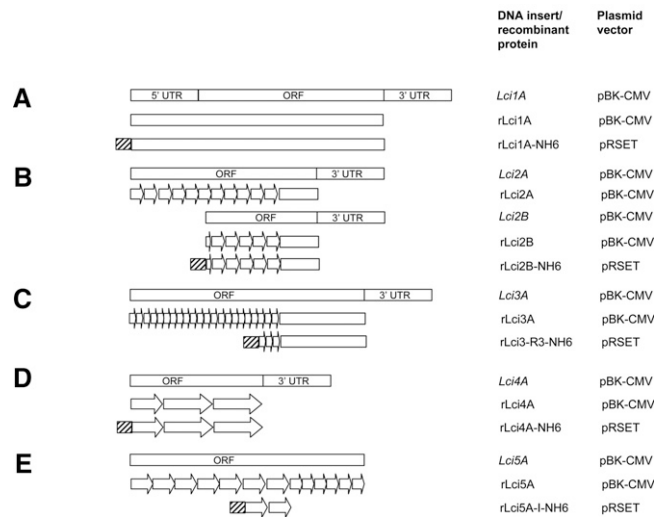


FIGURE 1. Schematic representation of various cDNAs and corresponding deduced recombinant proteins evaluated in this study. Maps were derived from sequences produced after direct sequencing of the *Lci2A* and *Lci2B*, *Lci3A* and *Lci4A* cDNAs and/or from the coding genomic sequences of the *Lci1A* and *Lci5A*, available at the GeneDB (www.genedb.org). **A**, *Lci1A* cDNA, including the 5' untranslated region (UTR) segment, which is included in the recombinant protein r*Lci1A*-NH6. **B**, *Lci2A* cDNA, highlighting the 11 39-amino acid repetitive segments and the segment common to *Lci2B* cDNA and which is present in recombinant protein r*Lci2B*-NH6. **C**, *Lci3A* cDNA highlighting the 21 repetitive 14-amino acid motif segments and recombinant protein r*Lci3A*-R3-NH6, which contain the last three repetitive segments. **D**, *Lci4A* cDNA sequence emphasizing the last two repeats and part of the third repeat and recombinant protein r*Lci4A*-NH6. **E**, *Lci5A*-coding sequence and the recombinant protein r*Lci5A*-I-NH6. **Arrows** and **open boxes**, which have not been drawn to represent protein segments in scale, represent repetitive amino acid motifs and non-repetitive polypeptide segments. Hatched represent His-tag provided by the vector. ORF = open reading frame.

amino acids, and the *Lci2A*-encoded polypeptide (524 amino acids; *Lci2B* completely overlapped the 3' end of *Lci2A*), showed near complete homology at the C-terminus (Figure 2), except for four amino acid differences (2,782 S → A, 2,983 E → D, 2,984 L → V, and 2,991 A → T). The *Lci2*-encoded polypeptides possess 39-amino acid multiple tandem repetitive



FIGURE 2. Schematic representation of the full-length sequences or sequence fragments from selected kinesins from the *Leishmania donovani* complex. Sequences from LdK39 (kinesin of *L. donovani*¹⁸), the protein encoded by LinJ14_V3.1180 (kinesin of *L. infantum*, GeneDB), rLcKin and rk39 (*L. chagasi* kinesin fragments described by Evans and others⁷), and the r*Lci2A* and r*Lci2B* kinesin fragments reported here are represented. Open boxes represent overlapping regions, and closed boxes indicate sequences in protein encoded by LinJ14_V3.1180 that are absent in LdK39. The numbers indicate the position of the amino acids at the start and end of the polypeptides.

various his-tagged polypeptides by using ELISAs with serum from dogs naturally infected with *L. infantum* ($n = 46$), healthy controls ($n = 15$), and dogs with other infectious diseases ($n = 31$). Serum samples were assayed against total *Leishmania* antigens. As expected, mean reactivities of serum samples from *L. infantum*-infected dogs and healthy controls were strong and weak, respectively. The reactivity of serum samples from dogs with other infectious diseases was similar to that of the healthy control group, although 5 of 31 dogs in this group showed reactions above the cutoff value (Figure 5).

Mean reactivities of serum samples from dogs infected with *L. infantum* were evaluated with recombinant antigens and showed strong reactivity with rLci1A-NH6 ($n = 43$: 27 strong, 9 moderate, and 7 weak), rLci2B-NH6 ($n = 44$: 29 strong, 10 moderate, and 5 weak), and rLci4A-NH6 ($n = 37$: 33 strong and 4 moderate) antigens and moderate reactivity with rLci3A-R3-NH6 ($n = 37$: 8 strong, 14 moderate, and 13 poor) and rLci5A-I-NH6 ($n = 46$: 12 strong, 14 moderate, and 23 poor) antigens (Figure 5). No significant differences between reactivities of serum samples from four asymptomatic *Leishmania*-infected dogs and 42 symptomatic dogs were observed. Mean reactivity of serum samples from healthy con-

trol dogs was mostly weak for the different antigens (rLci1A-NH6, rLci2B-NH6, rLci3A-R3-NH6, and rLci5A-I-NH6), with the exception of rLci4A-NH6, which showed a result classified as moderate ($n = 15$: 8 moderate and 7 poor). Mean binding activities were weak for all recombinant antigens with serum samples from dogs with other infectious diseases. Sensitivities and specificities of *Leishmania* lysate antigens and rLci1A-NH6, rLci2B-NH6, rLci3A-R3-NH6, rLci4A-NH6, and rLci5A-I-NH6 (used to detect natural infections in dogs) are shown in Table 2. Interestingly, recombinant antigens that showed the best performance with dog serum samples were rLci3A-R3-NH6 and rLci4A-NH6, which showed sensitivities and specificities $> 90\%$ (Table 2).

Recognition of recombinant proteins by human serum samples. We evaluated human serum samples with different antigens to investigate the potential of these antigens for diagnosis of VL. Mean reactivities of serum samples from patients with VL and from healthy controls to total *Leishmania* antigens were strong and weak, respectively, as expected (Figure 6). Mean reactivities of serum samples from patients with CL and Chagas' disease were classified as moderate. The mean reactivities of serum samples from patients with VL were

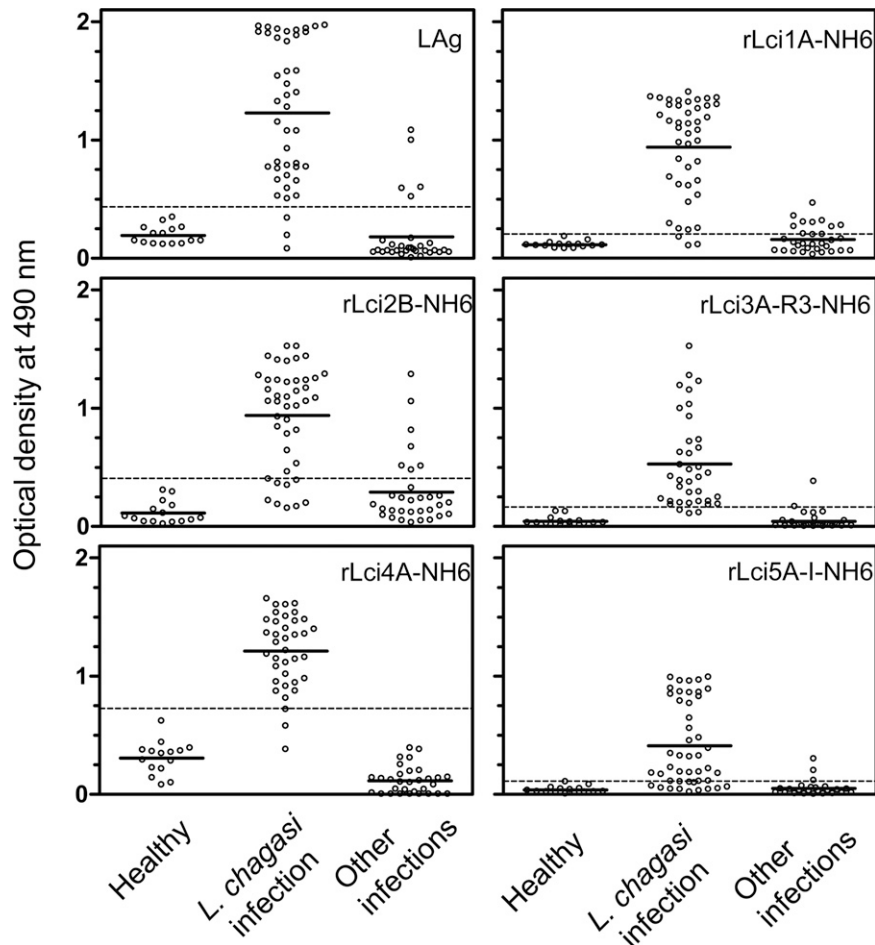


FIGURE 5. Reactivity of a panel of canine serum with *Leishmania* recombinant antigens assessed by an enzyme-linked immunosorbent assay. Serum samples from 46 dogs with visceral leishmaniasis (*L. chagasi*-infected), 31 dogs with other infections (4 with babesiosis, 20 with ehrlichiosis, and 7 with demodicosis) and 15 healthy control animals were assayed with the various recombinant antigens produced in *Escherichia coli* (rLci1A-NH6, rLci2B-NH6, rLci3A-R3-NH6, rLci4A-NH6, and rLci5A-I-NH6) or with total *L. chagasi* lysate as antigen (LAg). Each symbol corresponds to the result obtained with an individual serum. Solid horizontal lines indicate mean optical densities. Dashed horizontal lines indicate cutoff values, which were calculated as described in the Materials and Methods.

TABLE 2
Serodiagnostic performance of recombinant antigens with canine serum samples*

Fusion polypeptides used in ELISA	Serum from naturally <i>Leishmania</i> -infected dogs			Serum from healthy controls or dogs with other infectious diseases		
	No. samples tested	No. positive samples	Sensitivity (%)	No. samples tested	No. positive samples	Specificity (%)
LAg	43	41	95.3	44	4	89.1
rLci1A-NH6	43	40	93.0	46	11	76.1
rLci2B-NH6	43	36	83.7	46	7	84.8
rLci3A-R3-NH6	37	34	91.9	46	2	95.7
rLci4A-NH6	37	34	91.9	46	0	100
rLci5A-I-NH6	46	31	67.4	39	2	94.9

*ELISA = enzyme-linked immunosorbent assay.

moderate with rLci1A-NH6 (n = 36: 9 strong, 18 moderate, and 9 poor), rLci2B-NH6 (n = 36: 17 strong, 17 moderate, and 2 poor), and rLci3A-R3-NH6 (n = 21: 7 strong, 3 moderate, and 11) and strong with rLci4A-NH6 (n = 22: 15 strong, moderate 5, and 2 poor) and rLci5A-I-NH6 (n = 22: 16 strong, 3 moderate, and 3). In contrast, mean binding of antibodies from serum samples of healthy controls, patients with CL, and patients with Chagas' disease was weak for recombinant antigens tested, with the exception of rLci4A-NH6 and rLci5A-I-NH6, which showed results defined as moderate with serum samples from patients with Chagas' disease (Figure 6). Sensitivities and specificities of *Leishmania* lysate antigens, rLci1A-NH6, rLci2B-NH6, rLci3A-R3-NH6, rLci4A-NH6,

and rLci5A-I-NH6 in detecting antibodies in serum samples (diluted 1:600) from patients with VL are shown in Table 3. Recombinant antigen rLci2B-NH6 had the best overall performance (sensitivity and specificity > 90%), and all recombinant antigens tested had a high specificity > 90%.

DISCUSSION

In this study, lambda bacteriophage clones encoding five gene or gene family members (*Lci1*, *Lci2*, *Lci3*, *Lci4*, and *Lci5*) and selected from an *L. infantum* cDNA or genomic library were characterized and evaluated for their reactivity against a panel of canine or human serum samples. Four of these

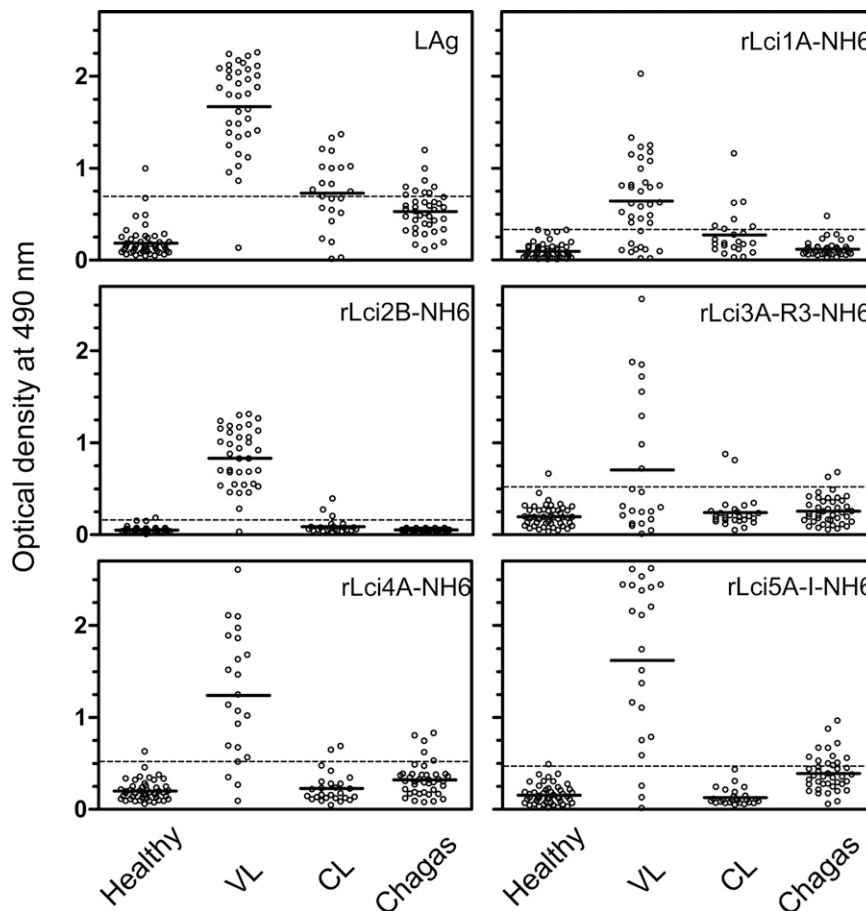


FIGURE 6. Reactivity of a panel of human sera with *Leishmania* recombinant antigens assessed by an enzyme-linked immunosorbent assay. Serum samples 36 patients with visceral leishmaniasis (VL), 26 patients with cutaneous leishmaniasis (CL), 40 patients with Chagas' disease (Chagas) and 50 healthy controls were assayed with the same set of antigens evaluated in Figure 5. Each symbol corresponds to the result obtained with an individual serum. Solid horizontal lines indicate mean optical densities. Dashed horizontal lines indicate cutoff values, which were calculated as described in the Materials and Methods.

TABLE 3
Serodiagnostic performance of recombinant antigens with human serum samples*

Fusion polypeptides used in ELISA	Serum from patients with VL			Serum from healthy controls or patients with CL or Chagas' disease		
	No. samples tested	No. positive samples	Sensitivity (%)	No. samples tested	No. positive samples	Specificity (%)
LAg	36	35	97.2	113	21	81.4
rLci1A-NH6	36	26	72.2	113	7	93.8
rLci2B-NH6	36	35	97.2	113	3	97.3
rLci3A-R3-NH6	22	8	36.4	115	4	96.5
rLci4A-NH6	22	18	81.8	115	7	93.9
rLci5A-I-NH6	22	19	86.4	115	11	90.4

* ELISA = enzyme-linked immunosorbent assay; VL = visceral leishmaniasis; CL = cutaneous leishmaniasis.

clones (*Lci2*, *Lci3*, *Lci4*, and *Lci5*) encode proteins that have tandem amino acid repeats, an observation reported by other investigators using a different approach.^{37,42} The only exception we identified was *Lci1*, which encodes a protein homolog to members of the cytoplasmic HSP70 family⁴³ and has also been identified in *L. donovani* and *L. infantum* using similar approaches.⁴⁴⁻⁴⁷ Cytoplasmic HSP70s are highly conserved proteins in eukaryotes,⁴³ and canine and human HSP70s have each approximately 70% identity with *L. infantum* HSP70.^{47,48} Recombinant protein rLci1A-NH6 reacted strongly with antibodies from most dogs naturally infected with *Leishmania*.

Although this protein also cross-reacted with antibodies from a certain proportion of dog with other infectious diseases, the OD values observed were low. In contrast, it reacted only moderately with antibodies from most patients with VL, which resulted in an ELISA with much less sensitivity than the ELISA for *L. infantum*-infected dog serum samples. Low cross-reactivity was observed with some serum samples from patients with CL and patients with Chagas' disease.

The *Lci2* inserts encode C-terminal fragments of the ≈358-kD protein that belongs to the kinesin superfamily of motor proteins,⁴⁹ of which the complete coding sequence has been recently determined from a *L. donovani* genomic cosmid library clone.⁵⁰ The N-terminal segments of this protein correspond to the recombinant proteins K39 and Lckin, which were isolated from a *L. infantum* genomic library.²⁶ These N terminally derived kinesin fragments have been used to develop immunodiagnostic assays that showed good performance (high sensitivity and specificity).^{9,27,51,52} The performance of the C-terminal region of the kinesin (*Lci2*) as ELISA antigen in the serodiagnosis of human VL, as shown in the present study, also showed good performance. Serum samples from 3 of 26 patients with CL showed a positive result in the *Lci2*-based ELISA. However, this result is consistent with results obtained with rK39, which reacted with 1 of 13 serum samples from patients with CL.³⁴ The *Lci2*-based ELISA performance with canine serum samples was inferior (in sensitivity and specificity) compared with that observed with human serum samples and also with other recombinant antigens evaluated in this study.

The *Lci3* inserts are encoded by a gene within a dicistronic operon that is conserved in *L. major*, *L. infantum*, and *L. braziliensis*; the second cistron is also homologous to that of *Lci3*. On the basis of the non-repetitive C-terminus, several related proteins were found and showed various degrees of homology with various trypanosomatid species. A possible ortholog from *Trypanosoma cruzi* (TCR3) is also composed of 14-amino acid repeats and a non-repetitive C-terminus homologous to the one found in *Lci3*.⁵³ Ortholog TCR3 is located in the paraflagellar region, between the body of the parasite and the flagellum.⁵⁴

Likewise, the *Lci3* ortholog in *T. brucei* has been classified as a flagellar attachment zone protein (*Tb927.4.3740*). The ELISA with rLci3A-R3-NH6 antigen displayed high sensitivity and specificity for dogs naturally infected with *Leishmania*. However, among all ELISAs evaluated, it had the poorest capacity for the diagnosis of patients with VL.

The ubiquitin gene product is one of the most conserved proteins among eukaryotes,⁵⁵ and the *L. infantum* sequence shares 97% identity with canine and human ubiquitin.⁵⁶ If one considers only one repeat, *L. infantum* polyubiquitin has only two and four amino acid differences (at positions 14, 52, 70, and 77) relative to canine and human ubiquitin, respectively. Nevertheless, it has been shown that patients with chronic Chagas' disease produce antibodies against *T. cruzi* ubiquitin,⁵⁷ the monomer of which has only three amino acid differences relative to human ubiquitin. The ELISA developed with the ubiquitin-derived rLci4A-NH6 antigen showed the best performance in serodiagnosis of VL in dogs. However, it also showed moderate cross-reactivity with serum samples from a few patients with CL or Chagas' disease or healthy control persons.

The hypothetical protein encoded by the *Lci5* gene has not been described in the literature, apart from the automatic annotation performed with sequenced trypanosomatid genomes. This conserved protein showed ≈30% identity in the N-terminal half with *L. major* and *T. brucei* orthologs (whose sequences are known). The rLci5A-I-NH6 protein showed strong reactivity in an ELISA with serum samples from patients with VL, although it also showed cross-reactivity with 10 of 40 serum samples from patients with Chagas' disease and with 1 of 49 serum samples from healthy controls. In contrast, its ELISA performance was much lower with canine serum samples (lower sensitivity and weaker reactivities).

Sensitivities and specificities of rLci1A-NH6, rLci2B-NH6, rLci3A-NH6, rLci4A-NH6, and rLci5A-I-NH6 ranged from 36.4% to 97.2% and 90.4% to 97.3% in detecting human VL and from 67.4% to 93.0% and 76.1% to 100% in detecting canine infection or disease, respectively. The recombinant antigen that showed the best performance for serodiagnosis of human disease by ELISA was rLci2B-NH6 (sensitivity = 97.2% and specificity = 97.3%). The recombinant antigens that showed the best performance for serodiagnosis of canine infection or disease were rLci3A-NH6 and the rLci4A-NH6 (sensitivities = 91.9% and specificities = 95.7% and 100.0%, respectively). Specificities and sensitivities of the ELISAs with these recombinant antigens were comparable with those of other ELISAs with recombinant antigens previously evaluated for serodiagnosis of canine and human VL.^{52,58-61}

Three of the recombinant antigens in the present study were derived from fragments of genes that encode relatively large

native *Leishmania* proteins (*Lci2*, *Lci3*, and *Lci5*). To the best of our knowledge, these large proteins have not been identified as antigens by Western blotting performed with parasite lysates and patient serum samples. This finding is in contrast to the fact that rK39, rLci2, rLci3, and rLci5 are clearly recognized by antibodies in patient serum samples and could be explained by denaturation of native proteins, possible cleavage during preparation of parasite antigens, or relatively low efficiency of transfer of high molecular mass proteins from polyacrylamide gels to nitrocellulose membranes.

As detailed above, the recombinant proteins evaluated in this work displayed different capacities to react with antibodies from dogs naturally infected with *L. infantum* and from humans with VL. The present work and data reported by other investigators⁶² suggest that repertoires of humoral immune responses in dogs and humans infected with *L. infantum* are different, and that this difference should be taken into account when developing serodiagnostic methods. Because no antigen, even if it has a high sensitivity and specificity in ELISAs for detection of antibodies against *L. infantum*, was able to detect all dogs or humans that acquired the infection and produced specific antibodies, immunodiagnostic assays should be developed on a platform appropriate to show reactivity to several recombinant antigens simultaneously (<http://www.chembio.com/newtechnologies.html>). A single platform could be potentially useful with human and canine serum samples. Alternatively, species-specific platforms could be developed that differ for those antigens that induce stronger and more specific humoral immune responses in different host species.

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REFERENCES

- Desjeux P, 2004. Leishmaniasis: current situation and new perspectives. *Comp Immunol Microbiol Infect Dis* 27: 305–318.
- Ministry of Health, Brazil, 2003. *Manual de Vigilância e Controle da Leishmaniose Visceral*. Brasília, Brazil: Ministry of Health.
- Desjeux P, 2001. The increase in risk factors for leishmaniasis worldwide. *Trans R Soc Trop Med Hyg* 95: 239–243.
- Mauricio IL, Howard MK, Stothard JR, Miles MA, 1999. Genomic diversity in the *Leishmania donovani* complex. *Parasitology* 119: 237–246.
- Lukes J, Mauricio IL, Schonian G, Dujardin JC, Soteriadou K, Dedet JP, Kuhls K, Tintaya KW, Jirku M, Chocholova E, Haralambous C, Pratloug F, Obornik M, Horak A, Ayala FJ, Miles MA, 2007. Evolutionary and geographical history of the *Leishmania donovani* complex with a revision of current taxonomy. *Proc Natl Acad Sci USA* 104: 9375–9380.
- Badaro R, Jones TC, Lorenc R, Cerf BJ, Sampaio D, Carvalho EM, Rocha H, Teixeira R, Johnson WD Jr, 1986. A prospective study of visceral leishmaniasis in an endemic area of Brazil. *J Infect Dis* 154: 639–649.
- Evans TG, Teixeira MJ, McAuliffe IT, Vasconcelos I, Vasconcelos AW, Sousa Ade A, Lima JW, Pearson RD, 1992. Epidemiology of visceral leishmaniasis in northeast Brazil. *J Infect Dis* 166: 1124–1132.
- Moral L, Rubio EM, Moya M, 2002. A leishmanin skin test survey in the human population of l'Alacanti region (Spain): implications for the epidemiology of *Leishmania infantum* infection in southern Europe. *Trans R Soc Trop Med Hyg* 96: 129–132.
- Sundar S, Maurya R, Singh RK, Bharti K, Chakravarty J, Parekh A, Rai M, Kumar K, Murray HW, 2006. Rapid, noninvasive diagnosis of visceral leishmaniasis in India: comparison of two immunochromatographic strip tests for detection of anti-K39 antibody. *J Clin Microbiol* 44: 251–253.
- da Silva MR, Stewart JM, Costa CH, 2005. Sensitivity of bone marrow aspirates in the diagnosis of visceral leishmaniasis. *Am J Trop Med Hyg* 72: 811–814.
- Thakur CP, 1997. A comparison of intercostal and abdominal routes of splenic aspiration and bone marrow aspiration in the diagnosis of visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 91: 668–670.
- Zijlstra EE, Ali MS, el-Hassan AM, el-Toum IA, Satti M, Ghalib HW, Kager PA, 1992. Kala-azar: a comparative study of parasitological methods and the direct agglutination test in diagnosis. *Trans R Soc Trop Med Hyg* 86: 505–507.
- Ciaramella P, Oliva G, Luna RD, Gradoni L, Ambrosio R, Cortese L, Scalone A, Persechino A, 1997. A retrospective clinical study of canine leishmaniasis in 150 dogs naturally infected by *Leishmania infantum*. *Vet Rec* 141: 539–543.
- Dos-Santos WL, Jesus EE, Paranhos-Silva M, Pereira AM, Santos JC, Baleeiro CO, Nascimento EG, Moreira ED, Oliveira GG, Pontes-de-Carvalho LC, 2008. Associations among immunological, parasitological and clinical parameters in canine visceral leishmaniasis: emaciation, spleen parasitism, specific antibodies and leishmanin skin test reaction. *Vet Immunol Immunopathol* 123: 251–259.
- Koutinas AF, Polizopoulou ZS, Saridomichelakis MN, Argyriadis D, Fytianou A, Plevraki KG, 1999. Clinical considerations on canine visceral leishmaniasis in Greece: a retrospective study of 158 cases (1989–1996). *J Am Anim Hosp Assoc* 35: 376–383.
- Saridomichelakis MN, Mylonakis ME, Leontides LS, Koutinas AF, Billinis C, Kontos VI, 2005. Evaluation of lymph node and bone marrow cytology in the diagnosis of canine leishmaniasis (*Leishmania infantum*) in symptomatic and asymptomatic dogs. *Am J Trop Med Hyg* 73: 82–86.
- Pedras MJ, de Gouveia Viana L, de Oliveira EJ, Rabello A, 2008. Comparative evaluation of direct agglutination test, rK39 and soluble antigen ELISA and IFAT for the diagnosis of visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 102: 172–178.
- Scalone A, De Luna R, Oliva G, Baldi L, Satta G, Vesco G, Mignone W, Turilli C, Mondesire RR, Simpson D, Donoghue AR, Frank GR, Gradoni L, 2002. Evaluation of the *Leishmania* recombinant K39 antigen as a diagnostic marker for canine leishmaniasis and validation of a standardized enzyme-linked immunosorbent assay. *Vet Parasitol* 104: 275–285.
- Shaw JJ, Voller A, 1964. The detection of circulating antibody to kala-azar by means of immunofluorescent techniques. *Trans R Soc Trop Med Hyg* 58: 349–352.
- Bartlett A, Bidwell DE, 1976. Enzyme immunoassays for parasitic diseases. *Trans R Soc Trop Med Hyg* 70: 98–106.
- Harith AE, Kolk AH, Kager PA, Leeuwenburg J, Muigai R, Kiugu S, Laarman JJ, 1986. A simple and economical direct

- agglutination test for serodiagnosis and sero-epidemiological studies of visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 80: 583–36.
22. el Harith A, Slappendel RJ, Reiter I, van Knapen F, de Korte P, Huigen E, Kolk AH, 1989. Application of a direct agglutination test for detection of specific anti-*Leishmania* antibodies in the canine reservoir. *J Clin Microbiol* 27: 2252–2257.
 23. Sundar S, Singh RK, Maurya R, Kumar B, Chhabra A, Singh V, Rai M, 2006. Serological diagnosis of Indian visceral leishmaniasis: direct agglutination test versus rK39 strip test. *Trans R Soc Trop Med Hyg* 100: 533–537.
 24. Kar K, 1995. Serodiagnosis of leishmaniasis. *Crit Rev Microbiol* 21: 123–152.
 25. Kubar J, Fragaki K, 2005. Recombinant DNA-derived *Leishmania* proteins: from the laboratory to the field. *Lancet Infect Dis* 5: 107–114.
 26. Burns JM Jr, Shreffler WG, Benson DR, Ghalib HW, Badaro R, Reed SG, 1993. Molecular characterization of a kinesin-related antigen of *Leishmania chagasi* that detects specific antibody in African and American visceral leishmaniasis. *Proc Natl Acad Sci USA* 90: 775–779.
 27. Chappuis F, Rijal S, Soto A, Menten J, Boelaert M, 2006. A meta-analysis of the diagnostic performance of the direct agglutination test and rK39 dipstick for visceral leishmaniasis. *BMJ* 333: 723.
 28. Evans TG, Krug EC, Wilson ME, Vasconcelos AW, de Alencar JE, Pearson RD, 1989. Evaluation of antibody responses in American visceral leishmaniasis by ELISA and immunoblot. *Mem Inst Oswaldo Cruz* 84: 157–166.
 29. Mary C, Lamouroux D, Dunan S, Quilici M, 1992. Western blot analysis of antibodies to *Leishmania infantum* antigens: potential of the 14-kD and 16-kD antigens for diagnosis and epidemiologic purposes. *Am J Trop Med Hyg* 47: 764–771.
 30. Aisa MJ, Castillejo S, Gallego M, Fisa R, Riera MC, de Colmenares M, Torras S, Roura X, Sentis J, Portus M, 1998. Diagnostic potential of Western blot analysis of sera from dogs with leishmaniasis in endemic areas and significance of the pattern. *Am J Trop Med Hyg* 58: 154–159.
 31. le Fichoux Y, Quaranta JF, Aufeuve JP, Lelievre A, Marty P, Suffia I, Rousseau D, Kubar J, 1999. Occurrence of *Leishmania infantum* parasitemia in asymptomatic blood donors living in an area of endemicity in southern France. *J Clin Microbiol* 37: 1953–1957.
 32. Teixeira MC, Oliveira GG, Silvany MA, Alcantara-Neves NM, Soares MB, Ribeiro-Dos-Santos R, Jeronimo SM, Costa CH, Dos-Santos WL, Eichinger D, Pontes-de-Carvalho L, 2007. A strategy for identifying serodiagnostically relevant antigens of *Leishmania* or other pathogens in genetic libraries. *Biologicals* 35: 51–54.
 33. Carvalho SF, Lemos EM, Corey R, Dietze R, 2003. Performance of recombinant K39 antigen in the diagnosis of Brazilian visceral leishmaniasis. *Am J Trop Med Hyg* 68: 321–324.
 34. Braz RF, Nascimento ET, Martins DR, Wilson ME, Pearson RD, Reed SG, Jeronimo SM, 2002. The sensitivity and specificity of *Leishmania chagasi* recombinant K39 antigen in the diagnosis of American visceral leishmaniasis and in differentiating active from subclinical infection. *Am J Trop Med Hyg* 67: 344–348.
 35. Lemos EM, Laurenti MD, Moreira MA, Reis AB, Giunchetti RC, Raychaudhuri S, Dietze R, 2008. Canine visceral leishmaniasis: performance of a rapid diagnostic test (Kalazar Detect) in dogs with and without signs of the disease. *Acta Trop* 107: 205–207.
 36. Reithinger R, Quinnell RJ, Alexander B, Davies CR, 2002. Rapid detection of *Leishmania infantum* infection in dogs: comparative study using an immunochromatographic dipstick test, enzyme-linked immunosorbent assay, and PCR. *J Clin Microbiol* 40: 2352–2356.
 37. Goto Y, Coler RN, Guderian J, Mohamath R, Reed SG, 2006. Cloning, characterization, and serodiagnostic evaluation of *Leishmania infantum* tandem repeat proteins. *Infect Immun* 74: 3939–3945.
 38. Baleeiro CO, Paranhos-Silva M, Dos Santos JC, Oliveira GG, Nascimento EG, de Carvalho LP, Dos-Santos WL, 2006. Montenegro's skin reactions and antibodies against different *Leishmania* species in dogs from a visceral leishmaniasis endemic area. *Vet Parasitol* 139: 21–28.
 39. de Melo Neto OP, Standart N, Martins de Sa C, 1995. Autoregulation of poly(A)-binding protein synthesis *in vitro*. *Nucleic Acids Res* 23: 2198–2205.
 40. Reis AB, Teixeira-Carvalho A, Vale AM, Marques MJ, Giunchetti RC, Mayrink W, Guerra LL, Andrade RA, Correa-Oliveira R, Martins-Filho OA, 2006. Isotype patterns of immunoglobulins: hallmarks for clinical status and tissue parasite density in Brazilian dogs naturally infected by *Leishmania (Leishmania) chagasi*. *Vet Immunol Immunopathol* 112: 102–116.
 41. Folgueira C, Requena JM, 2007. A postgenomic view of the heat shock proteins in kinetoplastids. *FEMS Microbiol Rev* 31: 359–377.
 42. Goto Y, Coler RN, Reed SG, 2007. Bioinformatic identification of tandem repeat antigens of the *Leishmania donovani* complex. *Infect Immun* 75: 846–851.
 43. Germot A, Philippe H, 1999. Critical analysis of eukaryotic phylogeny: a case study based on the HSP70 family. *J Eukaryot Microbiol* 46: 116–124.
 44. MacFarlane J, Blaxter ML, Bishop RP, Miles MA, Kelly JM, 1990. Identification and characterisation of a *Leishmania donovani* antigen belonging to the 70-kDa heat-shock protein family. *Eur J Biochem* 190: 377–384.
 45. de Andrade CR, Kirchhoff LV, Donelson JE, Otsu K, 1992. Recombinant *Leishmania* Hsp90 and Hsp70 are recognized by sera from visceral leishmaniasis patients but not Chagas' disease patients. *J Clin Microbiol* 30: 330–335.
 46. Arora SK, Melby PC, Sehgal S, 1995. Lack of serological specificity of recombinant heat shock protein of *Leishmania donovani*. *Immunol Cell Biol* 73: 446–451.
 47. Quijada L, Requena JM, Soto M, Alonso C, 1996. During canine viscerocutaneous leishmaniasis the anti-Hsp70 antibodies are specifically elicited by the parasite protein. *Parasitology* 112: 277–284.
 48. Kano R, Abe K, Hasegawa A, 2004. cDNA of canine heat shock protein 70 (HSP70). *Vet Res Commun* 28: 395–405.
 49. Miki H, Okada Y, Hirokawa N, 2005. Analysis of the kinesin superfamily: insights into structure and function. *Trends Cell Biol* 15: 467–476.
 50. Gerald NJ, Coppens I, Dwyer DM, 2007. Molecular dissection and expression of the LdK39 kinesin in the human pathogen, *Leishmania donovani*. *Mol Microbiol* 63: 962–979.
 51. Sivakumar R, Sharma P, Chang KP, Singh S, 2006. Cloning, expression, and purification of a novel recombinant antigen from *Leishmania donovani*. *Protein Expr Purif* 46: 156–165.
 52. Takagi H, Islam MZ, Itoh M, Islam AU, Saifuddin Ekram AR, Hussain SM, Hashiguchi Y, Kimura E, 2007. Short report: production of recombinant kinesin-related protein of *Leishmania donovani* and its application in the serodiagnosis of visceral leishmaniasis. *Am J Trop Med Hyg* 76: 902–905.
 53. Hoft DF, Kim KS, Otsu K, Moser DR, Yost WJ, Blumin JH, Donelson JE, Kirchhoff LV, 1989. *Trypanosoma cruzi* expresses diverse repetitive protein antigens. *Infect Immun* 57: 1959–1967.
 54. Hoft DF, Donelson JE, Kirchhoff LV, 1995. Repetitive protein antigens of *Trypanosoma cruzi* have diverse intracellular locations. *J Parasitol* 81: 549–554.
 55. Nei M, Rogozin IB, Piontkivska H, 2000. Purifying selection and birth-and-death evolution in the ubiquitin gene family. *Proc Natl Acad Sci USA* 97: 10866–10871.
 56. Wiborg O, Pedersen MS, Wind A, Berglund LE, Marcker KA, Vuust J, 1985. The human ubiquitin multigene family: some genes contain multiple directly repeated ubiquitin coding sequences. *EMBO J* 4: 755–759.
 57. Telles S, Abate T, Slezzynger TC, Henriquez DA, 1999. *Trypanosoma cruzi* and human ubiquitin are immunologically distinct proteins despite only three amino acid difference in their primary sequence. *FEMS Immunol Med Microbiol* 24: 123–130.
 58. Badaro R, Benson D, Eulalio MC, Freire M, Cunha S, Netto EM, Pedral-Sampaio D, Madureira C, Burns JM, Houghton RL, David JR, Reed SG, 1996. rK39: a cloned antigen of *Leishmania chagasi* that predicts active visceral leishmaniasis. *J Infect Dis* 173: 758–761.

59. Soto M, Requena JM, Quijada L, Angel SO, Gomez LC, Guzman F, Patarroyo ME, Alonso C, 1995. During active viscerocutaneous leishmaniasis the anti-P2 humoral response is specifically triggered by the parasite P proteins. *Clin Exp Immunol* 100: 246–252.
60. Maalej IA, Chenik M, Louzir H, Ben Salah A, Bahloul C, Amri F, Dellagi K, 2003. Comparative evaluation of ELISAs based on ten recombinant or purified *Leishmania* antigens for the serodiagnosis of Mediterranean visceral leishmaniasis. *Am J Trop Med Hyg* 68: 312–320.
61. Mettler M, Grimm F, Capelli G, Camp H, Deplazes P, 2005. Evaluation of enzyme-linked immunosorbent assays, an immunofluorescent-antibody test, and two rapid tests (immunochromatographic-dipstick and gel tests) for serological diagnosis of symptomatic and asymptomatic *Leishmania* infections in dogs. *J Clin Microbiol* 43: 5515–5519.
62. Goto Y, Howard RF, Bhatia A, Trigo J, Nakatani M, Netto EM, Reed SG, 2009. Distinct antigen recognition pattern during zoonotic visceral leishmaniasis in humans and dogs. *Vet Parasitol* 160: 215–220.