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Recombinant *Leishmania* (*Leishmania*) *infantum* Ecto-Nucleoside Triphosphate Diphosphohydrolase NTPDase-2 as a new antigen in canine visceral leishmaniasis diagnosis

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ABSTRACT

Canine visceral leishmaniasis is an important public health concern. In the epidemiological context of human visceral leishmaniasis, dogs are considered the main reservoir of *Leishmania* parasites; therefore, dogs must be epidemiologically monitored constantly in endemic areas. Furthermore, dog to human transmission has been correlated with emerging urbanization and increasing rates of leishmaniasis infection worldwide. *Leishmania* (*Leishmania*) *infantum* (*L. chagasi*) is the etiologic agent of visceral leishmaniasis in the New World. In this work, a new *L.* (*L.*) *infantum* (*L. chagasi*) recombinant antigen, named ATP diphosphohydrolase (rLic-NTPDase-2), intended for use in the immunodiagnosis of CVL was produced and validated. The extracellular domain of ATP diphosphohydrolase was cloned and expressed in the pET21b-*Escherichia coli* expression system. Indirect ELISA assays were used to detect the purified rLic-NTPDase-2 antigen using a standard canine sera library. This library contained CVL-positive samples, leishmaniasis-negative samples and samples from *Trypanosoma cruzi*-infected dogs. The results show a high sensitivity of 100% (95% CI = 92.60–100.0%) and a high specificity of 100% (95% CI = 86.77–100.0%), with a high degree of confidence ($k = 1$). These findings demonstrate the potential use of this recombinant protein in immune diagnosis of canine leishmaniasis and open the possibility of its application to other diagnostic approaches, such as immunochromatography fast lateral flow assays and human leishmaniasis diagnosis.

1. Introduction

Visceral leishmaniasis (VL) affects millions of people worldwide. In the New World, VL is a zoonosis caused by *Leishmania* (*Leishmania*) *infantum* (*L. chagasi*) (Mauricio et al., 2000; Dantas-Torres and Brandão-Filho, 2006). Recent publications have reported the expansion of VL to areas previously considered non-endemic, such as North America and Europe (Petersen, 2009; Ready, 2010). The increase in both the number of VL human cases and its prevalence in the New World have been linked to environmental changes. These changes are a result of human actions, such as migration between endemic and non-endemic regions, and adaptation of the vector *Lutzomyia longipalpis* that allow it to persist in domestic locations and domestic reservoirs, such as the dog (Palatnik-de-Sousa et al., 2001; Dantas-Torres and Brandão-Filho, 2006). Dogs are the primary domestic reservoir of *Leishmania* parasites in endemic areas in the New World (Tesh, 1995; Mauricio et al., 2000; Lainson and Rangel, 2005).

Some endemic regions such as Brazil have used euthanasia of infected dogs as a strategy to control canine visceral leishmaniasis (CVL). These practices, however, have not been effective in reducing the incidence of human infection. There are two reasons why these efforts may not be succeeding: the delay between blood collection, sample analysis, and sacrificing the infected dog and the

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persistence of false-negative tests for CVL due to the low sensitivity of the current methods of diagnosis (Dantas-Torres, 2007; Lemos et al., 2008).

Diagnostic techniques based on visualization of the parasite in smears of bone marrow, spleen, liver, and lymph node aspirates are invasive, time-consuming, and inappropriate for epidemiological surveillance (Piarroux et al., 1994; Ikonomopoulos et al., 2003). Currently, the serological tests used for diagnosis include *D*irect *A*gglutination *T*est (DAT), *I*ndirect *I*mmunofluorescence *A*ssays (IFA) and Enzyme-Linked Immunosorbent Assay (ELISA) (Gomes et al., 2008). These conventional methods, however, generate false-positive results in dogs infected with other parasites, and cross-reactivity with antigens from co-infected parasites limits the specificity of these serological tests. In Chagas disease-endemic areas, the cross-reactivity between *Leishmania* and *Trypanosoma cruzi* antigens is a serious concern. Because these organisms are closely related and most testing utilizes partial or total parasite extract as the antigenic source, these diagnostic techniques can lead to inconsistent results (El Amin et al., 1986; Harith et al., 1987; Barbosa-De-Deus et al., 2002). In endemic areas of Brazil, an alternative approach using biochemically purified *Leishmania* ribosomal proteins (LPRs) from promastigotes in ELISA assays provides better results, showing a sensitivity and specificity of 100% and 98.2%, respectively (Coelho et al., 2009). Nonetheless, the manipulation of live parasites to purify these antigens introduces the risk of accidental infection. It also increases the difficulty in obtaining standard lots of purified antigen because of the natural fluctuations in the expression of general proteins, as well as LPR.

According to Gomes et al. (2008), the specificity of serological tests has recently improved with the use of purified recombinant antigens. Scalone et al. (2002) used recombinant K39 in ELISA that reached a sensitivity and specificity of 97.1% and 98.8%, respectively. The K39 antigen, however, did not demonstrate promising results in other regions of the world including Africa (Boelaert et al., 2008). The use of recombinant antigens in CVL is an open field of research because there is heterogeneity in the successful use of antigens around the world, and new antigens are still needed (Gomes et al., 2008).

The *E*cto-*N*ucleoside *T*riphosphate *D*iphosphohydrolases (E-NTPDases) are promising new antigens because they are expressed in the infectious forms of trypanosomatides (Fietto et al., 2004; Maioli et al., 2004; Marques-da-Silva et al., 2008; Santos et al., 2009; De Souza et al., 2010). E-NTPDases, also called ecto-apyrase or ATPDases, are enzymes that act in the conversion of extracellular triphosphate and diphosphate nucleotides to monophosphate nucleotides, which have numerous physiological functions in vertebrate hosts, including the regulation of immune responses (Zimmermann, 2000). *Leishmania* species that have sequenced genomes (Smith et al., 2007) show two genes encoding E-NTPDases: guanosine diphosphatase, referred to as NTPDase-1 due to its similarity with the previously described NTPDase-1 from *T. cruzi* (Fietto et al., 2004; Santos et al., 2009) and ATP diphosphohydrolase or nucleoside diphosphatase (referred to in this work as NTPDase-2).

In this work, the *L.* (*L.*) *infantum* (*L. chagasi*) NTPDase-2 soluble ecto-domain was cloned and expressed in an *Escherichia coli* system, producing a recombinant protein of 43.9 kDa. This recombinant protein (rLic-NTPDase-2) was then used as an antigen in ELISA assays, demonstrating its potential for application to CVL diagnosis.

2. Materials and methods

2.1. Serum samples

CVL IFA-positive sera samples isolated from 48 naturally infected dogs found in a CVL-endemic area (Caratinga, MG, Brazil) were used. The positive samples were grouped as follows: 16 asymptomatic, 16 oligosymptomatic and 16 polysymptomatic, classified according to Mancianti et al. (1988). Sera from dogs experimentally infected with *T. cruzi* from a non-endemic area (30 samples) were assayed in cross-reactivity tests. All samples were obtained from the sera library of the Federal University of Ouro Preto, Ouro Preto, MG, Brazil and were kindly provided by Dr. George Luiz Lins Machado Coelho. *T. cruzi*-positive sera were collected from experimentally infected dogs that had positive parasitological and serological assays. Negative sera were obtained from non-infected animals that had negative results in both parasitological and serological assays.

2.2. rLic-NTPDase-2 cloning

Genomic DNA from the *L.* (*L.*) *infantum* (*L. chagasi*) M2682 strain was purified using the phenol method and ethanol precipitation (Sambrook and Russell, 2001). The full-length Lic-NTPDase-2 coding region (1278 bp) was amplified by PCR and cloned into the cloning vector pGEM-T Easy (Promega). The following primers were designed based on the predicted E-NTPDase sequence from *L.* (*L.*) *infantum* JPCM5 ATP diphosphohydrolase (gi 146081774): forward primer 5′ cta gct agc atg cgt ccg tac tcc tcg 3′ and reverse primer 5′ gga att ccg ttc cat ctt gag cag gga 3′. The bold bases indicate endonuclease restriction sites for *NheI* and *EcoRI*, respectively. The PCR reaction (20 pmol of each primer, 0.2 mM dNTP mix, 90 nmol of genomic DNA) was performed in GoTaq Green Master Mix and GoTaq (Promega) according to the manufacturer's instructions. PCR steps: one cycle at 94 °C 5 min, 34 cycles of 94 °C for 60 s, 50 °C for 60 s, and 72 °C for 90 s; and a final amplification step at 74 °C for 5 min. After gel electrophoresis separation, the PCR product was purified using the PureLink™ Gel Extraction Kit (Invitrogen) and cloned into a pGEM-T Easy vector. The recombinant plasmid was transformed into *E. coli* strain DH5α (Sambrook and Russell, 2001), and transformed clones were confirmed by PCR and sequencing. To express only the putative extracellular soluble domain of NTPDase-2, we amplified the region codifying the soluble domain (from L41 to E425) using the primers: forward primer 5′ agtagctagcatgctgctctcccca 3′ and reverse primer 5′ agctcgagttccatcttgagcaggaa 3′. The 5′ regions flanking the ecto-domain of both primers have restriction sites for *Nhe*I and *Xho*I endonucleases (in bold). The PCR reaction used the same conditions described for full-length amplification. The expected ampliconSambrook and Russell (2001) containing 1155 bp was separated by agarose gel electrophoresis (Sambrook and Russell, 2001) and purified using a PureLinkTM Quick Gel Extraction Kit (Invitrogen). The purified amplicon and pET21b vector were digested with the aforementioned endonucleases. The amplicon was then cloned into pET21b using T4 DNA ligase (Promega). Chemically competent cells of *E. coli* DH5α were transformed with the recombinant plasmid (pET21b + rLicNTPDase-2) using the chemical-heat shock method (Sambrook and Russell, 2001). The transformant clones were identified by colony PCR, endonuclease digestion, and sequencing. All general, non-specific molecular biology techniques were performed following Sambrook and Russell (2001). The nucleotide and amino acid sequences of NTPdase-2 from the *L.* (*L.*) *infantum* (*L. chagasi*) strain M2682 were deposited in database (GenBank ID: JX075891).

2.3. Expression and purification of rLic-NTPDase-2

The pET21b-rLic-NTPDase-2 construction was used to transform *E. coli* BL21(DE3). Transformed cells were grown in 5 mL of Luria Bertani medium containing 50 μg/mL of ampicillin for 16 h at 37 °C under 180 rpm. The culture was then transferred to 500 mL of SOC medium, and the cells were incubated until

log phase ($O.D._{600\,nm}$ = 0.6). Protein expression was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 h at 37 °C under 180 rpm. Thereafter, the culture was centrifuged at 4000 × *g* for 10 min at 4 °C and cell pellets were lysed in 3 mL of lysis buffer (50 mM Tris, pH 8.00, 100 mM NaCl) supplemented with protease inhibitors (1 mg/mL aprotinin, 1 g/mL pepstatin, 10 mg/mL leupeptin, 1 mg/mL PMSF, and 1 mg/mL lysozyme) using Misonix (Ultrasonic Liquid Processor) for 6 cycles for 10 s. The supernatant was removed by centrifugation at 12,000 × *g* for 30 min at 4 °C. The pellet was washed with 50 mM Tris supplemented with 500 mM NaCl, 10 mM 2-mercaptoethanol, and 2 M urea. The supernatant was removed by centrifugation as described above. The inclusion bodies were dissolved in 10 mL of extraction buffer (50 mM Tris supplemented with 500 mM NaCl, 10 mM 2-Mercaptoethanol, and 8 M urea). The rLic-NTPDase-2 purification was performed using a batch method or by Fast Protein Liquid Chromatography – FPLC (Akta-Purifier UPC 100 from GE). For the batch purification assay, 10 mL of soluble inclusion bodies was mixed with 500 μL Ni-NTA agarose gel matrix (HIS-Select™ Nickel Affinity Gel – SIGMA) and incubated for 1 h at 4 °C under gentle agitation. The resin was packed in a manual column and washed with 10 column volumes of 50 mM Tris, pH 8.0, and 500 mM NaCl. Bound protein was eluted with 10 column volumes of elution buffer (50 mM Tris, pH 8.0, supplemented with 300 mM NaCl and 250 mM Imidazol). FPLC purification was performed using the same protocol described in batch purification with the exception of utilization of 1-mL HisTrap FF crude nickel ion affinity column and 8 M urea in all used buffers. The purified protein was dialyzed in PBS and analyzed by 10% SDS-PAGE (Sambrook and Russell, 2001) and capillary electrophoresis (Agilent 2100 Bioanalyzer) in accordance with the manufacturer's instructions.

2.4. ELISA assays

Micro plates with 96 wells (Nunc MaxiSorp™) were coated with the rLic-NTPDase-2 (0.5 μg/well) in coating buffer (0.1 M Na_2CO_3/$NaHCO_3$, pH 9.6) for 18 h at 4 °C. The plates were washed four times with PBS–0.05% Tween 20 and blocked with PBS–3% BSA for 1 h at room temperature. The dog serum (diluted 1:40 in PBS–1% BSA) was added and incubated for 1 h at room temperature. After washing with PBS–0.05% Tween 20, a 1:5000 dilution of horseradish peroxidase-conjugated anti-dog IgG antibody (Sigma Aldrich) was added and incubated for 1 h at room temperature. The plates were washed four times with PBS–0.05% Tween 20 and developed with 100 μL/well of developing solution (H_2O_2 30 (v/v) 0.05%, ortho-phenylenediamine 6 mg in citrate-phosphate buffer Na_2HPO_4 0.2 M; acid citric 0.1 M, pH 5.0). Development was performed for 15 min in the dark and stopped by the addition of 32 μL/well of 2.5 M H_2SO_4. The absorbance was measured at 492 nm in an automated BioTek Plate Reader (Synergy HT). Each sample was assayed in duplicate.

2.5. ELISA reproducibility test

Thirty percent of the CVL ELISA-positive samples, previously detected by ELISA using rLic-NTPDase-2, and 50% of the negative samples were randomly selected to evaluate the reproducibility of the ELISA results. The assays were performed in triplicate using the same procedure described above for the ELISA assay.

2.6. Statistical analysis

Sensitivity and specificity were calculated using GraphPad Prism (version 5 for Windows). The following formulas were used: Positive Predictive Value = TP/(TP + FP) × 100, Negative Predictive Value = TN/(TN + FN) × 100, and Accuracy = TP + FP/(TP + FP + TN + FN) × 100. TN, TP, FN, and FP represent true negative, true positive, false negative, and false positive, respectively. The degree of agreement between ELISA assays using rLic-NTPDase-2 and the results from the standardized sera library (previously assayed using the Biomanguinhos test) was determined by the Kappa (*k*) values with 95% confidence intervals (Faria et al., 2011). The cut-off was established using the ROC curve. Statistical significance was determined by analysis of variance (ANOVA) and the Tukey test. *P*-values <0.001 were considered statistically significant.

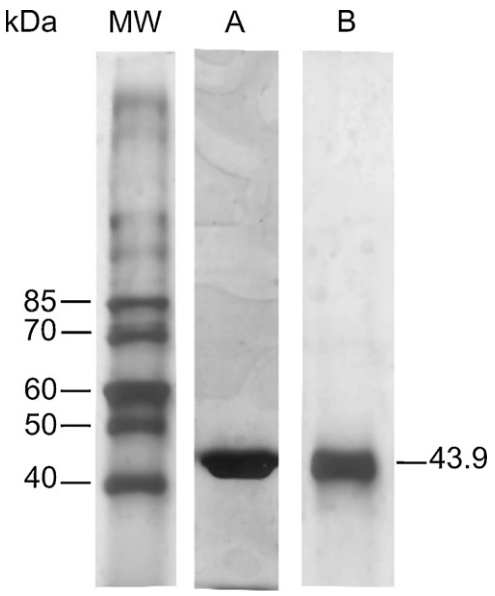
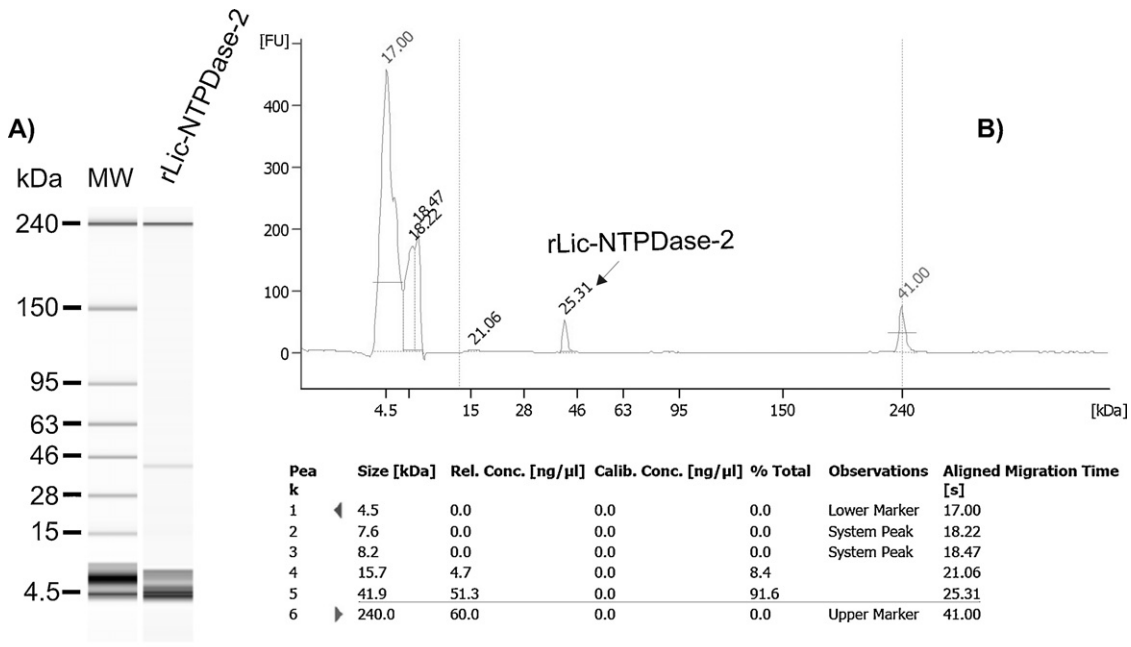


Fig. 1. Purified rLic-NTPDase-2. rLic-NTPDase-2 was expressed using the pET21b *E. coli* BL21-codon plus system and purified by Ni-agarose affinity chromatography. Purified protein was analyzed by SDS-PAGE 10% stained with silver. MW (molecular weight marker) shown in kDa. (Lane A) 10 μg of rLic-NTPDase-2 purified by batch adsorption. (Lane B) 10 μg of rLic-NTPDase-2 purified by FPLC.

3. Results

3.1. Cloning, expression and purification of recombinant rLic-NTPDase-2 antigen

In the first step of the cloning strategy, the full-length coding region of *L.* (*L.*) *infantum* (*L. chagasi*) was amplified by PCR and cloned into the amplification vector pGEM. Sequencing of the full coding region (gi JX075891) showed 99% similarity to the GenBank ATP diphosphohydrolase (gi 146081774). Simple translation analyses showed that this divergence led to a change in only one amino acid at the 419 position: a phenylalanine in the JPCM5 strain where in the M2682 strain, there is a serine. The pGEM-full-rLicNTPDase-2 was used as a template to amplify the region encoding the predicted soluble ecto-domain of Lic-NTPDase-2 that was cloned into the expression vector pET21b. All amplification and cloning results were successful (data not shown). As shown in Fig. 1, the recombinant protein with a predicted molecular weight of 43.9 kDa, was successfully expressed and purified. We used two different strategies to purify the recombinant protein: manual purification by batch adsorption (Fig. 1A) and automated purification by FPLC (Fig. 1B). For each of these methods, SDS-PAGE analysis revealed the presence of only one protein band. The high purity of rLic-NTPDase-2 was confirmed by highly sensitive capillary electrophoresis coupled with fluorescence detection of proteins (Fig. 2). The purified antigen accounted for 91.6% of the total protein in



Peak	Size [kDa]	Rel. Conc. [ng/µl]	Calib. Conc. [ng/µl]	% Total	Observations	Aligned Migration Time [s]
1	4.5	0.0	0.0	0.0	Lower Marker	17.00
2	7.6	0.0	0.0	0.0	System Peak	18.22
3	8.2	0.0	0.0	0.0	System Peak	18.47
4	15.7	4.7	0.0	8.4		21.06
5	41.9	51.3	0.0	91.6		25.31
6	240.0	60.0	0.0	0.0	Upper Marker	41.00

Fig. 2. Analysis of rLic-NTPDase-2 purity by capillary electrophoresis and fluorescence stain. The purity of rLic-NTPDase-2 was analyzed using 51.3 ng/µL of FPLC-purified rLic-NTPDase-2 and the LabChip® Agilent Protein 230 Kit (Agilent 2100 Bioanalyzer). Panel A shows the electrophoresis result after SDS-PAGE gel visualization. Only one protein band is observed in the rLic-NTPDase-2 lane. Panel B shows quantification of these results and reveals that rLic-NTPDase-2 protein band represents 91.6% total protein in sample.

the sample (Fig. 2). This purified antigen was then tested in CVL immunodiagnosis using the ELISA technique as described below.

3.2. ELISA assay analysis using rLic-NTPDase-2

To evaluate the applicability of recombinant rLic-NTPDase-2 in CVL diagnosis, the purified protein was used as the antigen to coat ELISA plates and perform the assays. The first step in this approach was to determine the optimal amount of recombinant antigen to sensitize the ELISA plates. We tested 0.25, 0.5 and 1 µg, using 3 sera samples from known positives and 3 sera samples from the negative group of samples in the sera library (CVL-negative and CVL-positive, respectively) at the same dilution factor (1:40). We observed that 0.5 µg provided better differentiation between the groups of samples (data not shown). Using 0.5 µg of rLic-NTPDase-2, we tested the reactivity of the standardized sera library containing 48 CVL-positive sera (16 asymptomatic, 16 oligosymptomatic, and 16 polysymptomatic), 26 CVL-negative sera and 30 sera positive for infection by *T. cruzi*. The rLic-NTPDase-2 ELISA results showed that the 48 true positive sera reacted 100% above the cut-off value (Figs. 3 and 4, and Table 1). In this first assay, sera samples were tested in duplicate using recombinant protein purified manually by batch adsorption. Next, a blind validation assay (reproducibility test) was performed using a set of 30% of the CVL-positive samples and 50% of the CVL-negative/Chagas-positive samples. The assay was performed in triplicate using the FPLC-purified recombinant protein. These results are shown in Fig. 3, panel B, and Table 1, and they confirm the significant differences between the groups of samples observed in the full library assay (Fig. 3, panel A, and Table 1). Statistical analyses showed a high degree of confidence ($k = 1$) between the predicted data (standard sera library) and the rLic-ELISA data and also revealed high levels of sensitivity and specificity when using this new recombinant antigen (Table 1). To analyze the existence of any correlation between CVL disease progression and the results from the ELISA assay using rLic-NTPDase-2, we separated the 48 positive dogs according to their clinical characteristics as asymptomatic, oligosymptomatic

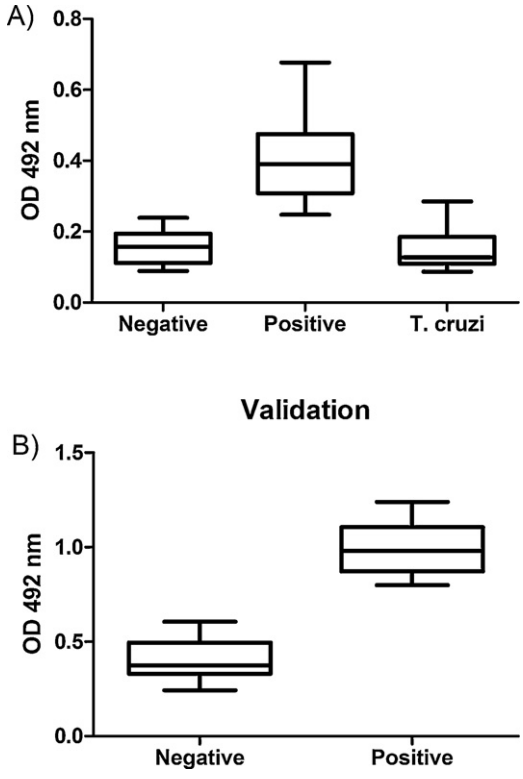


Fig. 3. Box plot analysis of ELISA assay using rLic-NTPDase-2 as antigen. (Panel A) ELISA assay using batch-purified recombinant protein and full sera library containing CVL-negative samples from healthy dogs ("negative" ($n = 26$)), CVL-positive samples from endemic area ("positive" ($n = 48$)), and CVL-negative *T. cruzi*-infected samples from dogs from non-endemic area ("*T. cruzi*" ($n = 48$)). (Panel B) Reproducibility assay (validation) performed using recombinant protein purified by FPLC along with 30% of positive samples and 50% of negative samples chosen blindly and assayed in triplicate. All sera samples were diluted 1:40 and assayed using 0.5 µg of purified rLic-NTPDase-2.

Table 1
Sensitivity, specificity and predicted value of the ELISA assay performed with rLic-NTPDase-2 antigen of *L.* (*L.*) *infantum chagasi*.

Test	Sensitivity (%)	Specificity (%)	PP[a] (%)	PN[b] (%)	Kappa
Full serum library[c]	100	100	100	100	1
Validation[d]	100	100	100	100	1

[a] PP (Predicted Positive).
[b] PN (Predicted Negative).
[c] Full sera library test was performed with all sera samples (48 CVL-positive, 26 CVL-negative, and 48 Chagasic-positive and CVL-negative) in duplicate, using recombinant protein purified by batch adsorption.
[d] Validation test was performed with 30% of the CVL-positive samples and 50% of the CVL-negative samples blindly chosen and assayed in triplicate using recombinant protein purified by FPLC.

or polysymptomatic. The rLic-NTPDase-2 ELISA mean absorbance values were 0.391, 0.375 and 0.415 for asymptomatic, oligosymptomatic and polysymptomatic samples, respectively. The results were statistically analyzed but did not show significant differences that could relate the detection of symptoms in animals to the rLic-NTPDase-2 ELISA results.

The specificity of the rLic-NTPDase-2 ELISA assay was determined using the results obtained for control sera from healthy dogs (true negatives) and sera from dogs with Chagas disease. The results showed 99% specificity; only one chagasic serum was detected as positive (Fig. 3). Table 1 shows the sensitivity, specificity, positive predictive value and negative predictive value of these tests. The results demonstrate that rLic-NTPDase-2 shows very low cross-reactivity with *T. cruzi*-infected samples.

4. Discussion

The NTPDase-2 gene from *L.* (*L.*) *infantum* (*L. chagasi*) was cloned, and its expression in a bacterial system led to the production of a 43.9-kDa recombinant protein. In using a recombinant antigen produced in a bacterial system, it is very important to avoid non-specific cross-reactivity. Such cross-reactivity may result from the presence of host proteins in the expression system (in this case *E. coli*); therefore, the use of recombinant protein with high purity is ideal and achieved in this work (Figs. 1 and 2). rLic-NTPDase-2 was used as the antigen in ELISA assays to assess its potential use in the serodiagnosis of CVL.

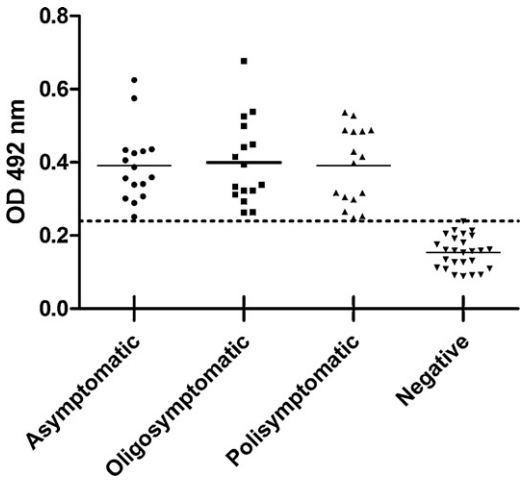


Fig. 4. Analysis of correlation of ELISA assay using rLic-NTPDase-2 and CVL progression. The ELISA results were separated by CVL disease stage into asymptomatic (without symptoms), oligosymptomatic (lower levels of symptoms) and polysymptomatic (severe symptoms). Sera samples were diluted 1:40 and assayed in duplicate using 0.5 μg of batch-purified rLic-NTPDase-2. The dashed line represents the cut-off value calculated using a ROC curve. Non-significant differences were observed between disease stage groups.

Antibodies against rLic-NTPDase 2 were detected in 100% of the samples isolated from dogs infected with CVL (Table 1). This value is higher than or quite similar to those reported for other recombinant antigens such as recombinant K39, which have been shown to have a sensitivity of 97% in a study using blood isolated from dogs in Italy (Scalone et al., 2002). The multicomponent protein chimera *L.* (*L.*) *infantum* PQ was previously shown to have a sensitivity of 93% in sera of dogs from Spain (Soto et al., 1998). Cysteine proteinase antigen rLdccys1 presented 98% sensitivity in sera samples from CVL-infected dogs found in the Brazilian endemic regions of Teresina, Piauí (Pinheiro et al., 2009). For protein chimera (epitopes K9, K26 and K39), the sensitivity was 96% (Boarino et al., 2005). This is comparable to results using the extract of *Leishmania* ribosomal proteins (LPRs), which showed a sensitivity of 100% in ELISA assays (Coelho et al., 2009). Furthermore, the level of sensitivity with rLic-NTPDase-2 was significantly higher than those reported with the antigen *L.* (*L.*) *donovani* A2, which showed a sensitivity of 87% (Carvalho et al., 2002). In general, the use of recombinant proteins in diagnostic tests has many advantages over the use of non-recombinant proteins. The use of recombinant proteins yields higher specificity, which is associated with the high purity and homogeneity of its preparations; there is no risk of infection during sample manipulation because the pathogenic agent is not used in the production of the recombinant antigen, and there is less cross-reactivity with other disease antigens that do not have a high degree of similarity with the antigen at the molecular level. Despite the existence of other recombinant antigens in the literature, the discovery of additional recombinant antigens, such as rLic-NTPDase-2, is necessary. New recombinant antigens can be used to differentiate vaccinated dogs from infected dogs when another recombinant antigen is already used for vaccination programs in endemic areas, and the new antigen can be used in combination with other antigens to improve existing diagnostic tests. Furthermore, these additional recombinant proteins might be used in regions of the world where the antigen currently in use is producing inadequate results due to molecular divergences between the antigen in prevalent strains (Boelaert et al., 2008).

The use of rLic-NTPDase-2 as the antigen in ELISA assays was not able to distinguish between CVL progression states, as shown in Fig. 3 (panel B). Therefore, it seems that rLic-NTPDase-2 may not be useful as a marker of CVL prognosis. This result can be explained by the fact that this protein may be expressed at similar levels in all infected dogs; its expression may be independent of disease stage. Alternatively, the result may be explained by the capacity of this antigen to induce the production of significant, elevated levels of specific antibodies in all infected dogs. Given the role of this protein in *Leishmania* infection and virulence (Marques-da-Silva et al., 2008; De Souza et al., 2010), and the high absorbances observed in ELISA assays, both of these explanations are possible.

The percentage of cross-reactivity with sera isolated from dogs with Chagas disease, an infection found in endemic areas of CVL,

was comparable to those observed for other recombinant proteins analyzed for use in immunodiagnosis (Soto et al., 1998; Carvalho et al., 2002; Scalone et al., 2002; Rosati et al., 2003; Boarino et al., 2005; Pinheiro et al., 2009). This high specificity may be due to the absence of the NTPDas-2 isoform in the *T. cruzi* genome. Until now, only one E-NTPDase, referred to as NTPDase-1, was described in *T. cruzi*, and two isoforms are predicted in *Leishmania* parasites: NTPDase-1 and NTPDase-2 (Fietto et al., 2004; Santos et al., 2009). The amino acid identity between the *T. cruzi* NTPDase-1 (GenBank entry GI: 45685733) and the isoform NTPDase-2 from *L.* (*L.*) *infantum* is very low (28%, data not shown). This discrepancy could explain the specificity of this antigen and the low cross-reactivity in chagasic samples. More importantly, this advantage lends further support to the use of rLic-NTPDase-2 as a diagnostic antigen.

As a whole, our data indicate that the recombinant protein rLic-NTPDase-2 may be used in serodiagnostic tests for CVL, allowing the detection of both subclinical and clinical forms of the disease. In this context, its use in the diagnosis of CVL could help in identifying the asymptomatic dogs in endemic areas. As these canines serve as a major reservoir for the parasite, this would be critical for the epidemiological control of this disease.

5. Conclusions

These results show the great potential of this recombinant protein for use in the immune diagnosis of CVL. Furthermore, the results open new doors for use in other diagnostic approaches such as immunochromatography (IC) fast lateral flow assays that can be used to control CVL. Because IC assays are faster (results in approximately 10 min) and easy-to-perform tests they can be applied as a point-of-care diagnostic method in endemic areas in order to select CVL-positive dogs and isolate the domestic reservoirs contributing to human and canine leishmaniasis-control programs. Additional potential applications are the use of this antigen in human visceral leishmaniasis diagnosis, epidemiological inquiries or the development of new recombinant vaccines. All of these uses would contribute to the control of this serious but neglected disease.

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