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Research paper

Leishmania infantum nucleoside triphosphate diphosphohydrolase 1 (NTPDase 1) B-domain: Antibody antiproliferative effect on the promastigotes and IgG subclass responses in canine visceral leishmaniasis

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ABSTRACT

A nucleoside triphosphate diphosphohydrolase-1 (NTPDase 1) was identified on the surface, flagellum and kinetoplast from *L. infantum* promastigotes by immunocytochemistry and confocal laser scanning microscopy, using immune sera that recognized specifically the B domain of NTPDase 1 and produced against synthetic peptides (LbB1LJ and LbB2LJ) derived from this domain. The polyclonal antibodies had effective antileishmanial effect, reducing significantly *in vitro* promastigotes growth (21–25%), an antiproliferative effect also demonstrated by immune sera produced against recombinant r-pot B domain, and two other synthetic peptides (potB1LJ and potB2LJ). In addition, using these biomolecules in ELISA technique, IgG1 and IgG2 subclasses reactivities of either healthy dogs or infected by *L. infantum* and classified clinically as asymptomatic, oligosymptomatic and symptomatic were tested. Analysis of distinct IgG1 and IgG2 seropositivities patterns suggested antibody subclasses binding epitopes along B domain for protection against infection, indicating this domain as a new tool for prophylactic and immunotherapeutic investigations.

1. Introduction

Leishmaniasis is a set of diseases caused by *ca* 20 pathogenic protozoa parasites of the genus *Leishmania*, endemic in 98 countries, which are transmitted to mammalian host by the bite of infected female sandflies of the genus *Phlebotomus* or *Lutzomyia,* constituting important worldwide public-health problem. Visceral leishmaniasis, caused mainly by *Leishmania infantum,* is the most severe form of the disease affecting several mammals including human, and dogs contribute for widespread of accessing the parasites to biting insects (Gradoni, 2015; Iborra et al., 2018; Ribeiro et al., 2018). Chemotherapy is the main strategy for leishmaniasis treatment but it increased parasite resistance and has elevated toxicity (De Brito et al., 2018; Iborra et al., 2018; Ribeiro et al., 2018). Genetically modified live vaccines or individual antigens originated from the parasite or insect vector are currently investigated, and some veterinary vaccines with partial protective efficacy are already commercialized (Araújo et al., 2009; Reis et al., 2010; Gradoni, 2015; Iborra et al., 2018; Ribeiro et al., 2018). Peptide vaccines for leishmaniasis are under investigation, and discovery of new specific epitopes could be useful for designing new prophylactic methods with greater effectiveness (De Brito et al., 2018).

Leishmania spp. have two NTPDases (nucleoside triphosphate

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diphosphohydrolases; EC 3.6.1.5) with predicted size of approximately 47 kDa and 74 kDa containing five apyrase conserved regions (ACRs) characteristic of the NTPDase family but with low identity and similarity between them (Peacock et al., 2007). These proteins hydrolyze di- and triphosphate nucleosides under activation of a divalent ion, and both catalytic activity and localization have been studied in different species of pathogenic parasites. Besides involvement in the purines recovery, they were associated to parasite defense mechanisms modulating nucleotides concentration, cellular adhesion and inflammatory immune response, and favoring infectivity and/or virulence (Santos et al., 2009; Tan et al., 2010, 2011; Vasconcellos et al., 2014; Figueiredo et al., 2016; Marconato et al., 2017; Luo et al., 2017; Paes-Vieira et al., 2018).

Antigenic and catalytically active NTPDase 1 proteins from the *L. braziliensis* (Rezende-Soares et al., 2010; Porcino et al., 2012), *L. amazonensis* (Coimbra et al., 2008; Detoni et al., 2013) and *L. infantum* (Maia et al., 2013) were characterized in promastigote forms. In addition, by *in silico* analysis including T- and B-cell epitopes prediction, an antigenic domain of 40 amino acids, named B domain, of high identity among these *Leishmania* species and highly conserved in plant proteins was identified (Faria-Pinto et al., 2008; Maia et al., 2011), and its antigenicity was proved by means of assays using a recombinant polypeptide and synthetic peptides (Rezende-Soares et al., 2010; Maia et al., 2011; Porcino et al., 2012; Detoni et al., 2013; Maia et al., 2013).

In this work, NTPDase 1 was identified and localized in *L. infantum* promastigotes by immunocytochemistry and confocal laser scanning microscopy using the specific antibodies produced against synthetic peptides (LbB1LJ and LbB2LJ), which belong to the B domain from the *Leishmania* NTPDase 1. *In vitro* antiproliferative effects on the promastigotes were tested using these sera and, additionally, by those produced against r-pot B domain recombinant polypeptide, and potB1LJ and potB2LJ synthetic peptides, all them belonging to the B domain from the potato (*Solanum tuberosum*) apyrase. IgG1 and IgG2 subclasses of healthy or naturally infected dogs by *L. infantum* clinically classified in asymptomatic, oligosymptomatic and symptomatic were tested by ELISA technique using these biomolecules as antigen.

2. Materials and methods

2.1. r-pot B domain recombinant polypeptide

The recombinant r-pot B domain (5493 Da) was generated by the fusion of a six-histidine-linked tag at the N-terminal portion of the conserved domain (r78-117) from the potato apyrase (accession number U58597.1) and expressed according earlier described (Maia et al., 2011, 2013). It has 55% identity and 67% similarity with B domain counterpart (r83-122) from the *L. infantum* NTPDase 1 (CAM66723.1), and no significant identity with its *L. infantum* NTPDase 2 counterpart (Peacock et al., 2007; CAM66031.1; 74 kDa; r236–276), as shown in Graphical Abstract (Maia et al., 2011).

2.2. Synthetic peptides

Synthetic peptides were obtained by solid-phase synthesis and purified as earlier described (Korkmaz et al., 2008). The molecular mass and purity of synthesized peptides were confirmed by amino acid analysis and by MALDI-TOF using a Microflex-LT mass spectrometer (Bruker-Daltonics, Billerica, MA, USA). The LbB1LJ (r82-103; RERFKRIEPGLSSFATDQEGAK^{22}; 86% identity and 95% similarity over 22 amino acids) and LbB2LJ (r102-121; AKQSLAGLLRFAEKAVPRSY^{20}; 80% identity and 90% similarity over 20 amino acids) synthetic peptides belong to the N- and C- terminal portions, respectively, from conserved B domain (82–121) from the *L. braziliensis* NTPDase 1 (Peacock et al., 2007; CAM42020.1; 47,689 Da), and have high identity with their *L. infantum* NTPDase 1 counterparts (Rezende Soares et al., 2010; Porcino et al., 2012; Maia et al., 2013; Detoni et al., 2013). The potB1LJ (r77-98; IEYFMATEPGLSSYAEDPKAAA^{22}; 55% identity and 68% similarity over 22 amino acids) and potB2LJ (r97-116; AANSLEPLLDGAEGVVPQEL^{20}; 50% identity and 60% similarity over 20 amino acids) synthetic peptides belong to the N- and C- terminal portions, respectively, from conserved B domain from the potato apyrase, and have high identity with their *L. infantum* NTPDase 1 counterparts (Rezende-Soares et al., 2010; Maia et al., 2011). No significant identity with their *L. infantum* NTPDase 2 counterparts (Peacock et al., 2007; CAM66031.1) was found.

2.3. Polyclonal antiserum

Polyclonal immune serum anti-r-pot B domain (Maia et al., 2011) and those produced against synthetic peptides (Porcino et al., 2012; Detoni et al., 2013; Maia et al., 2013) were obtained from seven-week-old BALB/c mice that were inoculated by a peritoneal route emulsified in Freund's complete and incomplete adjuvant, delivered in 15-day interval. The sera were stored at −20 °C.

2.4. Confocal laser scanning microscopy

Promastigotes from *L. infantum* MHOM/BR/1972/BH46 strain cultured in LIT medium for 7 days were obtained according earlier described (Reis et al., 2006). *Leishmania infantum* promastigotes were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 5 min, blocked using 4% fetal bovine serum (FBS) in PBS for 60 min at room temperature. After washing, parasites were incubated for 12 h at 4 °C with mouse polyclonal antibodies anti-LbB1LJ or anti-LbB2LJ diluted 1:200 in blocking solution, and washed again. Afterward, samples were incubated for 1 h at 37 °C with anti-mouse IgG antibody conjugated to Alexa Fluor 488 (Invitrogen) diluted 1:800 in blocking solution, washed, and incubated 30 min with 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI; Santa Cruz Biotechnology, Dallas, TX, USA) in darkness. To avoid loss of fluorescence an anti-fading agent was used (Fluoro-Gel w/Anti-Fading Agent; Electron Microscopy Sciences; Hatfield, PA, USA). Normal mouse serum or only the secondary antibody was used as control. Signs were analyzed on a Leica TCS-SP2 confocal microscope (Leica Microsystems, Germany), adjusted to 405 nm (diode laser) for DAPI (blue) or 488 nm (argon laser) for Alexa Fluor (green), and Leica Application Suite software (LAS 260). Images were also captured in DIC (Differential Interference).

2.5. In vitro antileishmanial activity

Effects of the polyclonal anti-r-pot B domain, anti-LbB1LJ, anti-LbB2LJ, anti-potB1LJ or anti-potB2LJ antibodies were tested on the L. infantum promastigotes by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method based on tetrazolium salt reduction by mitochondrial dehydrogenases, as earlier described (Porcino et al., 2012). Briefly, promastigotes (3×10^6 cells/mL) from a logarithmic phase growth cultured in LIT medium supplemented with 10% FBS at 24 °C were incubated with mouse polyclonal immune serum at final dilution of 1:200 for 24 h, 48 h or 72 h. Medium alone, and serum sample (diluted 1:200) from healthy rabbit or pooled serum samples obtained from BALB/c mice pre-inoculated only with complete and incomplete Freund adjuvants (mouse control serum; diluted 1:200) were also tested in this assay. All serum samples were previously incubated at 56 °C for 30 min for the complete inactivation of the complement system. Absorbance was measured at 570 nm (Multiskan MS microplate reader, LabSystems Oy, Helsink, Finland). Amphotericin B (Cristália, São Paulo, Brazil) was used as a control. Three independent experiments in triplicate were made. Results were analyzed statistically by the GraFit v.5 (Erithacus Software Ltd., Horley, UK) with the level of significance set at $P < 0.05$, and represented as mean (optical density; OD) ± standard deviation (SD).

2.6. Selection of serum samples from dogs

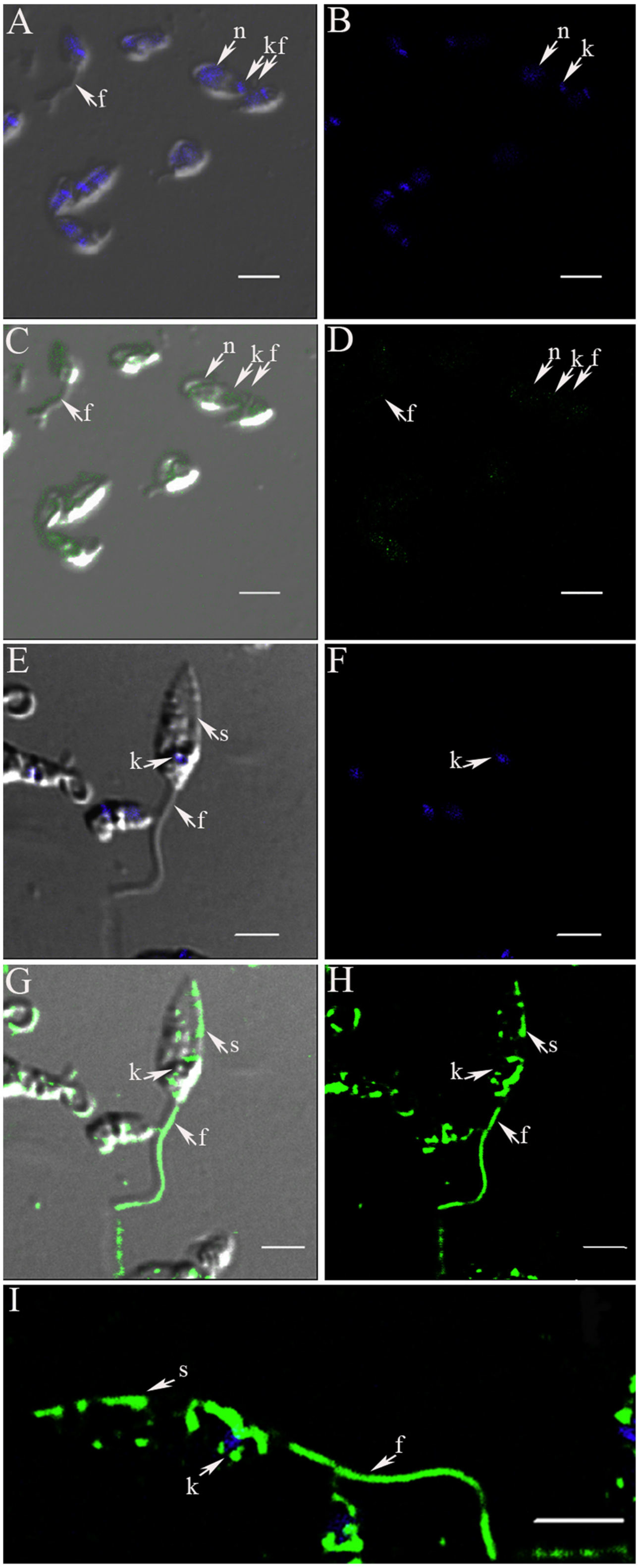
Sera from adult dogs not infected (n = 17) or with visceral leishmaniasis (n = 38), of both genders aging from 2- to 6-years-old, were selected from serum collections (Reis et al., 2006), whose diagnosis was obtained by IFAT using *L. chagasi* (MHOM/BR/1972/BH46) and *Leishmania amazonensis* (MHOM/BR/1960/BH6) promastigotes antigens, ELISA technique using promastigotes extract or r-K39 as coating antigen, and/or parasitological examination (Reis et al., 2006). These dogs were clinically classified according to the presence/absence of clinical signs into three distinct categories, including asymptomatic (AD; n = 11), with no suggestive signs of the disease; oligosymptomatic (OD; n = 12), with maximum three clinical signs, including opaque bristles and/or localized alopecia, and/or moderate loss of weight; symptomatic (SD; n = 15), with characteristics clinical signs of visceral leishmaniasis, such as opaque bristles, severe loss of weight, onychogryphosis, cutaneous lesions, apathy and keratoconjunctivitis (Reis et al., 2006; Neto et al., 2010). The healthy dogs from the endemic area (HEA; n = 17) were classified by clinical exams, and confirmed by negative IFAT and negative parasitological exams (Reis et al., 2006). Healthy adult dogs (C; n = 10; both genders aging from 2- to 8-years-old) from Juiz de Fora/MG, a non-endemic area for *Leishmania* spp. in Brazil, were examined using the same parameters. The study protocols complied with the regulations of the Brazilian National Council of Research in Animals and were approved by the Ethical Committee for Animal Research of the Federal University of Juiz de Fora, Juiz de Fora, MG, Brazil (process no.141/2006).

2.7. Antibody analyses by enzyme-linked immunosorbent assays (ELISA)

Aliquot (10 µg/well) of either r-pot B domain, LbB1LJ, LbB2LJ, potB1LJ or potB2LJ in 0.1 M $NaHCO_3$, pH 9.6, was absorbed overnight onto flat-bottomed microtiter plates (Costar 3590; Corning Inc., Corning, NY, USA). After blocking step (0.15 M phosphate buffer solution, pH 7.2 plus 0.3% Tween-20 and 2% casein), sera diluted 1:50 (for synthetic peptides) or 1:100 (for r-pot B domain) were tested in duplicate, for 4 h at room temperature. Antibodies bound to the antigen-plate were detected using peroxidase conjugated anti-dog IgG1 or anti-dog IgG2 (Bethyl Laboratories Inc.; Montgomery, TX, USA) and o-phenylenediamine dihydrochloride (OPD; Sigma Chemical Co.; St. Louis, MO, USA)/H_2O_2 as substrate. The subsequent color reaction was read at 492 nm on a microplate reader (Molecular Devices Corp.; Menlo Park, CA, USA). The considered values of A492 nm were the means of 4 determinations with a variation of no more 15% between them. GraphPad Prism Software (version 4) was used for statistical analysis. The median and the 95% confidence interval were calculated, and the data were analyzed using the Mann-Whitney test to compare 2 groups, or Kruskal-Wallis test to compare > 3 groups. P values < 0.05 were considered significant. Antibody reactivity of dogs (O.D.) and individual antibody reactivity expressed in ELISA unit (U) are shown in Supplementary Fig. 1 (SF1).

3. Results

3.1. Antibodies against B domain recognize L. infantum promastigote NTPDase 1



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Immune serum produced against LbB2LJ (A–D) or LbB1LJ (E–I) synthetic peptide identified the NTPDase 1 expression in *L. infantum* promastigotes (Fig. 1). This protein was evidenced by the fluorescent green points on the plasmatic membrane (s), flagelum (f), and kinetoplast (c) of the parasite, as revealed by the secondary anti-mouse IgG-Alexa Fluor 488 (C, D, G, H, I), which are also shown by differential interference contrast (DIC; C, G). Nucleus (n) and kinetoplast (k) of promastigotes are shown by DAPI staining (A, B, E, F, I), a marker of nucleus and mitochondrial DNA (blue), also displayed by DIC (Fig. 1; A, E). In addition, the NTPDase 1 identification is clarified by merge (I) of the image of DAPI staining (F) and respective image of Alexa Fluor 488 (Fig. 1; H). No signal was detected when only secondary antibodies or control mouse serum were used (data not shown).

Fig. 1. Identification of the NTPDase 1 of *L. infantum* promastigotes by immunocytochemistry using anti-LbB2LJ (A–D) and anti-LbB1LJ (E–I) antibodies. Promastigotes previously fixed and permeabilized were incubated with immune serum (dilution 1:200), secondary anti-IgG antibodies conjugated to Alexa Fluor 488 and DAPI. Analysis was performed by confocal scanning microscopy under diode laser (405 nm) for DAPI (blue; A, B, E, F, I) and argon laser (488 nm) for Alexa Fluor 488 (green; C, D, G, H, I). The general appearance of promastigotes is evidenced by DIC (A, C, E, G). Fluorescent green points (C, D, G, H, I; arrows) are observed on the surface (s), flagelum (f), and kinetoplast (k) of the parasite, better visualized in enlarged image (I), merge of DAPI staining (F) and respective image of Alexa Fluor 488 (H). Nucleus (n); A–D, 400 ×. E–H and I, zoom in random area. Scale bar: 5 μm (A–D) and 3 μm (E–H). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.2. Antibodies against B domain have in vitro antileishmanial activity

Antiproliferative effects of the immune serum produced against r-pot B domain recombinant polypeptide, or those produced against synthetic peptides (diluted 1:200) were assayed. In general, it was not possible to observe statistically significant differences at 24 h or 48 h. At 72 h, polyclonal antibodies anti-r-pot B domain (A; 28%; $P = 0.0417$), anti-potB1LJ (B; 37%; $P = 0.0215$), anti-potB2LJ (C; 29%; $P = 0.0204$), anti-LbB1LJ (D; 21%; $P = 0.0365$) and anti-LbB2LJ (E; 25%; $P = 0.0031$) were effective antileishmanials, reducing significantly parasite growth when compared with the mouse control serum (Fig. 2; C3; 100%). The growth of promastigotes in the culture medium, and either in the presence of normal rabbit serum or mouse control serum was similar between them (Fig. 2).

3.3. IgG1 and IgG2 subclasses binding-epitopes within B domain

IgG1 seropositivity for r-pot B domain recombinant polypeptide was found in healthy dogs from the endemic area (HEA; 47%), which was similarly maintained in asymptomatic (AD; 45%) and oligosymptomatic (OD; 42%) dogs groups and, although not statistically significant, reduced in symptomatic (SD; 27%), group of sick dogs with visceral leishmaniasis (Fig. 3A; SF1). The highest IgG2 seropositivity was observed in AD (91%) and OD (67%), which was also reduced in SD (53%), all of them higher than that found in HEA dogs group (Fig. 3B; SF1; 35%). These results generated the first indication that antigenic B domain from the NTPDase 1 could be involved in immune response modulation during canine visceral leishmaniasis (CVL).

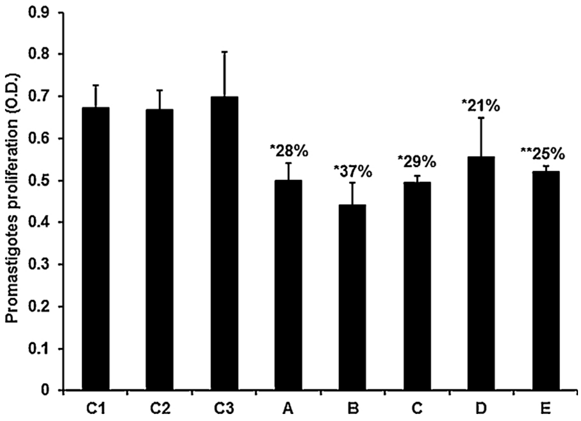

Fig. 2. *In vitro* antileishmanial activity of polyclonal antibodies produced against r-pot B domain or synthetic peptides. The promastigotes from a logarithmic phase growth were incubated for 72 h at 24 °C with polyclonal anti-r-pot B domain (A), anti-potB1LJ (B), anti-potB2LJ (C), anti-LbB1LJ (D) or anti-LbB2LJ (E) antibodies diluted 1:200. Medium alone (C1), serum sample from healthy rabbit (C2; diluted 1:200) and mouse control serum (C3; diluted 1:200) were used as controls. The antileishmanial activity was determined by the colorimetric MTT method. Three independent experiments in triplicate were made, and results are represented as mean (O.D.) ± SD. Statistical analyses were performed using GraFit v.5 (Erithacus Software Ltd., Horley, UK) to compare mouse control serum (C3; 100%) and immune serum, and percentage of inhibition is also shown. $P < 0.05$* or 0.01**.

The B domain has 40 amino acids, and it was of interest to verify IgG1 and IgG2 subclasses reactivities with its N-terminal portion represented here by the potB1LJ, and with its C-terminal portion represented by the LbB2LJ and potB2LJ homologous peptides. High IgG1 seropositivity for potB1LJ was found in HEA (71%), similar to those observed in AD (73%) and OD (75%), and reduces in SD (47%) dogs group (Fig. 3C; SF1). IgG2 subclass was highly reactive in AD (91%) and OD (92%), in opposite to that observed in HEA (35%), and reduces significantly in SD dogs group (Fig. 3D; SF1; 27%), corroborating the results obtained with r-pot B domain.

IgG1 or IgG2 seropositivity for potB2LJ and LbB2LJ homologous peptides was similar, an indication that both peptides maintained their shared epitopes similarly exposed in the Elisa technique. High IgG1 seropositivity for either potB2LJ (E) or LbB2LJ (G) was found in HEA (94–100%) dogs group, maintained in AD (82%) and OD (92–100%), whereas significantly reduces (20–27%) in SD dogs group (Fig. 3; SF1), now indicating that IgG1 subclass also binds epitopes within C-terminal portion from the B domain. High IgG2 seropositivity for both potB2LJ (F) and LbB2LJ (H) was detected in AD (91%) and OD (100%), and reduced in SD dogs group (27–40%), similar to that results observed for potB1LJ (D). However, elevated IgG2 seropositivity (88–94%) for potB2LJ (F) and LbB2LJ (H) in HEA was also found, a distinct result to that observed for N-terminal portion from the B domain (Fig. 3; SF1; D)

4. Discussion

Antigenicity and catalytic activity of the *L. infantum* NTPDase 1, isolated in its native form of promastigotes preparation, were previously reported (Maia et al., 2013). In this work, specific NTPDase 1 was located in *L. infantum* promastigotes by immune sera anti-LbB1LJ and anti-LbB2LJ, which are of high affinity and specificity for recognizing of *Leishmania* NTPDase 1 as demonstrated by Western blots of homogenized parasites, immunoprecipitation assays and inhibition of phosphohydrolytic activity (Porcino et al., 2012; Detoni et al., 2013; Maia et al., 2013). Recently, NTPDase 1 was also shown in intracellular amastigotes of *L. infantum*-infected macrophage using these same antibodies (Maia et al., 2019). No reactivity was observed on the surface or subcellular organelles of macrophages, suggesting that these immune sera do not recognize mammals NTPDases reinforcing their specificity for parasite protein (Maia et al., 2019). In addition, by ultrastructural immunocytochemical microscopy and using these same immune sera, NTPDase 1 of *L. braziliensis* (Porcino et al., 2012) or *L. amazonensis* (Detoni et al., 2013) promastigotes was identified at the surface of plasma membrane, mitochondria and kinetoplast, and at flagellar pocket and flagellum, corroborating this wide distribution of the NTPDase 1 of *L. infantum* promastigotes shown here.

The polyclonal antibodies produced against B domain from the *L. infantum* NTPDase 1 are antileishmanials, significantly reducing the *in vitro* promastigote growth. Effects of antibodies produced against parasites NTPDases are frequently used for investigate structure and function of these proteins. Previously, we show that polyclonal anti-r-pot B domain, anti-LbB1LJ and anti-LbB2LJ antibodies were capable of significantly inhibiting (44–99%) phosphohydrolytic activity from the *L. infantum* promastigotes preparation (Maia et al., 2013), and similar results were obtained for *L. braziliensis* NTPDase 1 (Maia et al., 2011; Porcino et al., 2012). *In vitro* reductions of both adhesion and infection of macrophages by *L. infantum* promastigotes were demonstrated using a polyclonal immune serum that recognizes both NTPDase 1 and NTPDase 2 (Vasconcellos et al., 2014). A polyclonal immune serum against NTPDase 1 of *Trypanosoma cruzi* trypomastigotes significantly inhibited its infectivity (Santos et al., 2009). Monoclonal antibodies produced against *T. gondii* NTPase II inhibit enzyme activity and

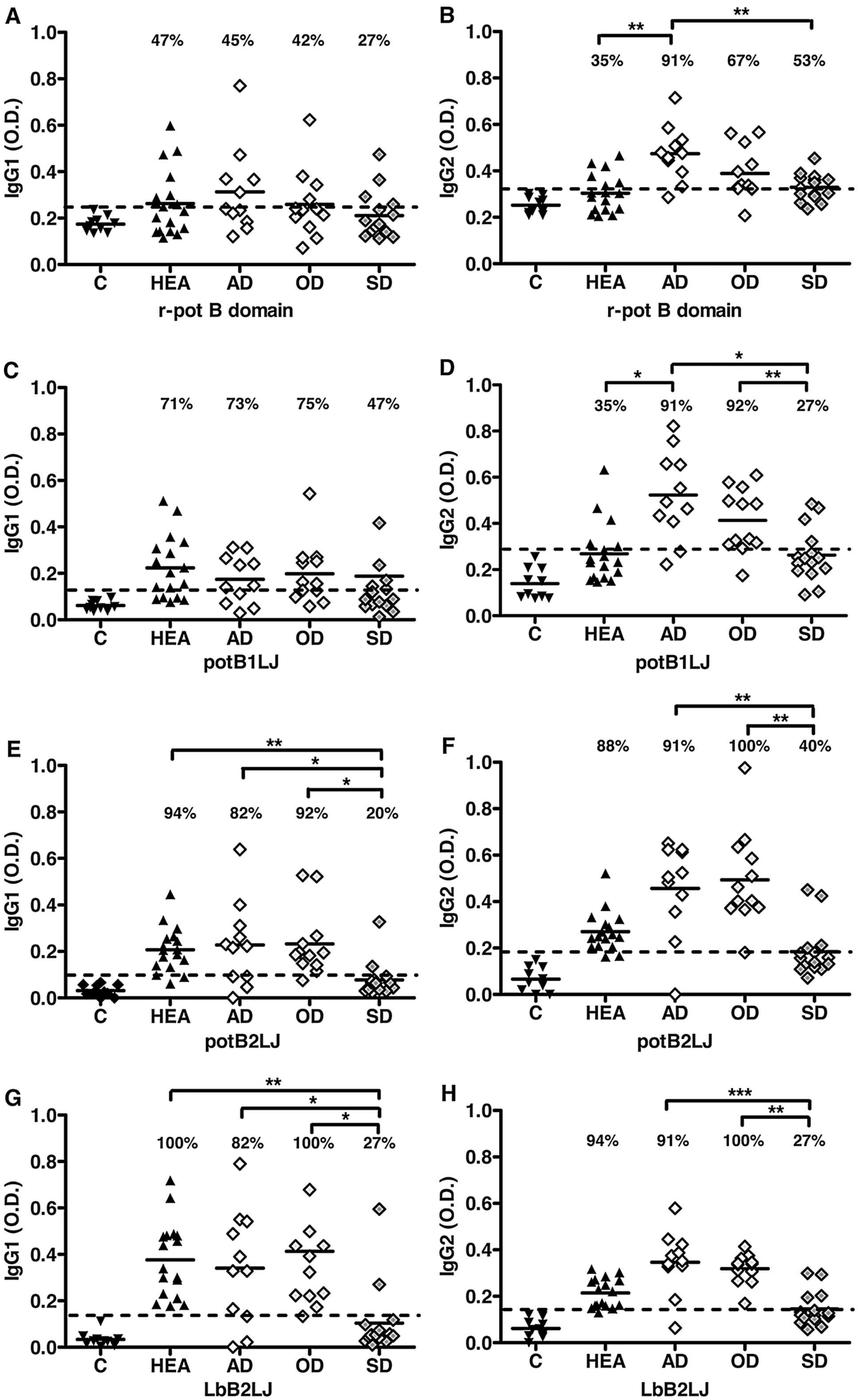

Fig. 3. IgG1 and IgG2 subclasses reactivity with B domain from the *L. infantum* NTPDase 1. IgG1 (A, C, E, G) and IgG2 (B, D, F, H) subclasses levels were quantified in sera diluted 1:50 (for synthetic peptides) or 1:100 (for r-pot B domain) of healthy dogs (HEA; n = 18) and naturally *L. infantum* infected dogs (n = 38) classified clinically as asymptomatic (AD; n = 11), oligosymptomatic (OD; n = 12) and symptomatic (SD; n = 15), all of them domiciled in same endemic area, using recombinant r-pot B domain (A, B), and potB1LJ (C, D), potB2LJ (E, F) and LbB2LJ (G, H) synthetic peptides as coating antigen in ELISA technique. The results are expressed in optical density. The horizontal line represents the mean of the optical density of the control sera (C) plus two times the standard deviation, being that value greater than this cut-off point was considered seropositive. Statistical analysis of the groups was determined using the Mann-Whitney test and Kruskal-Wallis test. The P value is < 0.05*, 0.01** or 0.001***.

tachyzoite replication in infected host cells (Tan et al., 2010), and a recombinant form of this protein (Tan et al., 2011) as well as RNA vaccine-encoding NTPase II gene (Luo et al., 2017) induce significant protection against toxoplasmosis in murine models. Taken all together, our results suggest that antibodies could has a direct effect on the B domain from the NTPDase 1, also identified in amastigote forms (Maia et al., 2019), possibly involved in stabilization of the catalytic site of this protein, disrupting its enzyme activity and causing the death of the parasite.

In addition, we determine IgG1 and IgG2 subclasses reactivities for B domain from the antigenic NTPDase 1. We used the same serum samples of healthy or *L. infantum* infected dogs from the sera collection previously tested with promastigotes preparation, whose results showed a positive correlation between IgG1 subclass and asymptomatic dogs, low parasite density, and reduced morbidity suggesting association of this antibody subclass with immunoprotector mechanisms in CVL, while IgG2 subclass was better associated with symptomatic dogs, higher parasite density and disease morbidity (Reis et al., 2006; Neto et al., 2010). As here shown, high antibody affinity for synthetic peptides representing N- and C-terminal portions from the B domain was observed, even greater than that demonstrated for r-pot B domain recombinant polypeptide, allowing a more accurate analysis on this antigenic domain. High IgG1 seropositivity for potB1LJ, potB2LJ or LbB2LJ was found in HEA, also maintained in AD and OD dogs groups, in the which signs of leishmaniasis yet are not pathognomonic, suggesting involvement of IgG1 reactivity for B domain in protection against CVL. The highest IgG2 seropositivity for potB1LJ was observed in AD and OD, and lower in HEA dogs group suggesting its association to the disease progression. In addition, elevated IgG2 seropositivity for potB2LJ and LbB2LJ was also found in HEA, besides elevated in AD and OD dogs groups. Therefore, using a single domain which makes assays more accurate, an additional protective aspect on this IgG2 antibody subclass could be suggested, not observed when the same serum samples were tested with promastigotes preparation (Reis et al., 2006; Neto et al., 2010). These results enable us to speculate that high IgG1 and IgG2 reactivities with B domain from the NTPDase 1 contribute against *Leishmania* infection and also during disease progression, possibly inhibiting parasite proliferation.

It is relevant point out that both IgG1 and IgG2 subclasses of dogs could be associated to immune protective mechanisms against *L. infantum* infection, and high levels of them are demonstrated for several vaccine candidates (Araújo et al., 2009; Reis et al., 2010). In addition, antibodies with ability for neutralizing parasite virulence factors blocking replication inside macrophages were suggested as potential immunotherapeutic approach against visceral leishmaniasis (Rodrigues et al., 2016). Peptides have been used in vaccine composition with promising results (De Brito et al., 2018), and low identity between amino acid sequences of B-domains from the parasite NTPDases 1 and mammalian NTPDases and absence of cross-immunoreactivity between anti-B domain antibodies and mice tissues by confocal fluorescence microscopy were previously reported (Faria-Pinto et al., 2008; Maia et al., 2011; Mendes et al., 2011). Therefore, immune sera and biomolecules shown here, or derivatives thereof, could be tested in prophylactic or immunotherapeutic experimental protocols of the CVL for evaluation of their capacity to induce immune cellular response, promote production of blocking antibody and reduce the parasite load and lesions.

5. Conclusion

Antibodies produced against B domain from the *L. infantum* NTPDase 1 recognize this protein on the surface of promastigotes, besides kinetoplast and flagellum. These antibodies have *in vitro* antiproliferative effects on the promastigotes, possibly associated to inhibition of its phosphohydrolytic activity. In addition, IgG1 and IgG2 subclasses binding-epitopes within B domain seems are involved in protector mechanisms against CVL, indicating that B domain from the NTPDase 1 is a new tool for prophylactic and immunotherapeutic investigations.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2019.06.004.

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