

Short communication

Nucleoside hydrolase from *Leishmania (L.) donovani* is an antigen diagnostic for visceral leishmaniasis[☆]

Débora M. Santana ^{a,b}, Gulnara P. Borja-Cabrera ^b, Edilma Paraguai de Souza ^b, Nancy R. Sturm ^a, Clarisa B. Palatnik de Sousa ^b, David A. Campbell ^{a,*}

^a Department of Microbiology, Immunology and Molecular Genetics, School of Medicine, University of California, 10833 Le Conte Avenue, Los Angeles, CA 90095-1747, USA

^b Instituto de Microbiologia—Professor Paulo de Góes, CCS, Cidade Universitária, Ilha do Fundão, UFRJ, CP 68040, CEP 21944-590 Rio de Janeiro, RJ, Brazil

Received 13 November 2001; accepted in revised form 10 January 2002

Keywords: Recombinant antigen; Kala-azar; Zoonotic visceral leishmaniasis; Diagnosis

In this study, we have isolated a gene encoding the nucleoside hydrolase (NH) of *Leishmania (L.) donovani* which is a component of the GP36 antigen. We show that recombinant LdNH is useful for the diagnosis of clinical kala-azar in dogs since it reacts preferentially with the IgG1 subtype of immunoglobulin that is enhanced in kala-azar susceptible dogs and in advanced disease.

In the New World kala-azar (visceral leishmaniasis, VL) is a canid zoonosis and one of the most important emerging diseases [1]. In human and canine kala-azar antibodies to *Leishmania* appear in the circulation soon after infection during an early subclinical period [2]. The control of zoonotic VL (ZVL) in northeastern Brazil has centered on the detection of infected dogs by serological assays using immunofluorescence (IF) and elimination of seropositive dogs [3]. Seropositivity detected by IF usually correlates well with parasitological evidence of infection [3–5]. However due to its low sensitivity this method underestimates the true prevalence of canine infection. Enzyme linked immunosorbent assay (ELISA) [3,6,7], dot-ELISA [8], Western blot [9], rapid

immunochromatographic tests [10] and DNA-based techniques [3,11] represent improved and more sensitive methods of detection.

The fucose–mannose ligand (FML) is an antigenic glycoprotein complex from the promastigote form of *L. (L.) donovani* that is used for the diagnosis, prognosis and blood bank control of human kala-azar [12–14] and the diagnosis and prognosis of canine VL [15]. In addition, vaccination with FML conferred protection in mice [16,17], hamsters [18] and dogs [19]. The major immunoprotective component of FML is a 36-kDa glycoprotein fraction designated GP36 [20] that is recognized specifically by sera from human kala-azar patients [21]. Thus, the GP36 fraction of FML is a useful antigen for diagnostic assays [21] and as a potential candidate for a second-generation vaccine [20].

To obtain recombinant protein for further studies on the antigenicity and vaccine potential of GP36, we cloned the gene that encodes the immunogenic component of the GP36 fraction of FML. Briefly, the GP36 fraction was isolated from FML [20] and the sequence of a tryptic peptide, EVGTKPAAFMLQILDFYTK, was obtained. Hybridization screening of a *L. (L.) donovani* genomic library with two degenerate oligonucleotides yielded a double-positive cosmid clone. The cosmid harbored a hybridizing, single-copy gene whose ORF translated into a 314-amino acid protein that contained the entire sequenced peptide fragment (data not shown). The sequence showed 96.2% similarity to the *Leishmania major* NH [22] and other kinetoplastid NHs.

The LdNH was expressed as a maltose-binding protein fusion protein in *Escherichia coli*. The LdNH fusion protein was purified by affinity chromatography,

Abbreviations: FML, fucose–mannose ligand; GP 36, 36-kDa glycoprotein; IF, immunofluorescence; Ld, *Leishmania donovani*; NH, nucleoside hydrolase; rNH, recombinant NH; VL, visceral leishmaniasis; ZVL, zoonotic VL.

[☆] Nucleotide sequence data reported in this paper are available in the EMBL, GenBank[™] and DDJB data bases under the accession number: AY007193.

* Corresponding author. Tel.: +1-310-206-5556; fax: +1-310-206-3865.

E-mail address: dc@ucla.edu (D.A. Campbell).

followed by Factor Xa cleavage. Further purification of the rLdNH by ion exchange chromatography yielded a high purity product that was recognized in Western blot analysis by polyclonal anti-GP36 serum and sera from symptomatic dogs (data not shown). Thus, we conclude that LdNH presents epitopes that are recognized in cases of kala-azar.

To determine the usefulness of the cloned antigen in a diagnostic assay, the ability of LdNH and FML to be recognized by sera from canine kala-azar cases was compared by a microtiter plate ELISA assay. Titrations of the antigens (2–0.03125 µg per well) against pools of normal, asymptomatic and symptomatic kala-azar dog sera were performed. All FML and LdNH antigen concentrations were able to distinguish the three groups of sera (data not shown). Fig. 1 shows the response of pools of sera against 2 µg of each antigen. While FML antigen reacted strongly with sera from all infected animals (either symptomatic or asymptomatic), rLdNH was recognized mainly by sera from kala-azar symptomatic dogs. A minor response against rLdNH was developed by sera from asymptomatic dogs while normal healthy animals remain below the cut-off for FML. The mean average of symptomatic sera developed

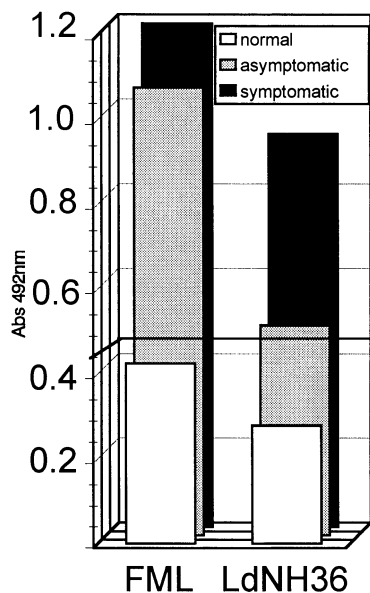


Fig. 1. ELISA reactivity with sera from dogs with parasitologically-confirmed VL from an endemic area of Brazil. Sera are indicated for symptomatic dogs, asymptomatic dogs, and normal healthy dogs from a non-endemic area. The y-axis values represents the mean average of absorbency values (ABS 492 nm) of triplicates of pooled sera ($n = 25$) at a 1:100 dilution. The LdNH antigen and the FML antigen of *L. (L.) donovani* were used at a concentration of 2 µg per well. Protein A peroxidase conjugate (1:32000) was used for detection of total IgG antibodies. The horizontal line represents the cut-off of the FML-ELISA assay for canine kala-azar [15]. According to the standardization of the assay, sera of normal healthy dogs develop absorbency values below 0.433. Sera with absorbencies above this value are considered reactive.

against 2 µg of rLdNH (Abs 492 nm = 0.927) was lower than that developed by FML antigen at the same concentration (Abs 492 nm = 1.189). A possible explanation for this result is that each antigen may react with different antibody fractions.

To analyze the immunoglobulin response, the individual total IgG, IgG1 and IgG2 reactivities of each symptomatic dog sera sample against the LdNH antigen were determined in the ELISA assay. The absorbency values corresponding to IgG1 and IgG2 antibodies of symptomatic dogs are represented in Fig. 2. The levels of IgG1 antibodies were higher than IgG2 in the majority of the samples (18/25) (mean average of absorbencies for IgG = 0.951 ± 0.458 and for IgG2 = 0.611 ± 0.368) indicating that LdNH is an antigenic marker mainly recognized by IgG1, the immunoglobulin subtype related to the advancement of disease [23]. Fig. 3 shows that the IgG1 absorbency values increase with the number of dog symptoms. The correlation coefficient analysis (Pearson bivariate) gave a significant result ($P = 0.035$), while no correlation was detected between levels of IgG2 and total IgG, whether the LdNH was used at 0.5 or 1 µg per well. Also, the IgG1 rate was correlated with IgG2 ($P = 0.001$) but not with total IgG ($P = 0.926$). In contrast, IgG2 was correlated to total IgG ($P = 0.003$).

In the present study we have isolated the gene for LdNH, which we demonstrate is an antigenic component of the GP36 fraction of FML [20,21]. Sera from symptomatic dogs show a predominant IgG1 antibody response against NH in the majority of animals. This result and the association of an IgG1 response with symptomatic disease [23,24] supports further development of FML-derived vaccine and diagnostic reagents. In addition to its potential for diagnosis and vaccination, the absence of NH activity in mammals [25] makes it a potential target for selective drug design.

The purified rLdNH obtained from *E. coli* maintained its antigenicity, as evidenced by its reaction with sera from symptomatic dogs. Further, an ELISA assay using low concentrations of antigen showed that while FML reacts with sera of both symptomatic and asymptomatic dogs, the LdNH recognizes primarily cases of clinical symptomatic confirmed disease. This result suggests that the LdNH exposes epitopes reacting specifically with the antibody fraction synthesized during the intensification of the disease. This antibody fraction was characterized as the IgG1 subtype and, as found in other studies (see below), the level of anti-LdNH IgG1 antibodies in infected animals is correlated with the increase in the number of symptoms due to advancement of the disease.

The control of VL in Northeastern Brazil in the past 30 years has centered on the detection of infected dogs by serological methods and the elimination of seropositive dogs. The optimization of the control campaign

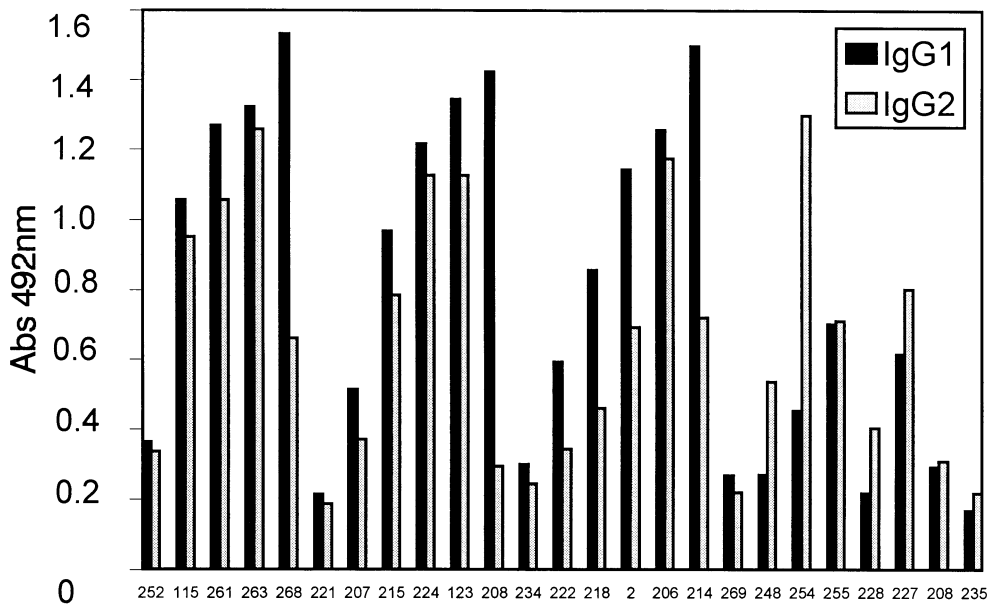


Fig. 2. Anti-*LdNH* IgG1 and IgG2 antibodies in sera of naturally infected symptomatic kala-azar dogs. *LdNH* 0.5 μ g were reacted with 25 sera (1:100 dilution; animal numbers are indicated in the *x*-axis) in an ELISA assay. The IgG1 and IgG2 peroxidase conjugates were previously titrated and used at a final dilution of 1:2000. The *y*-axis values represents the mean average of absorbency values (ABS 492 nm) of triplicates of each sera sample.

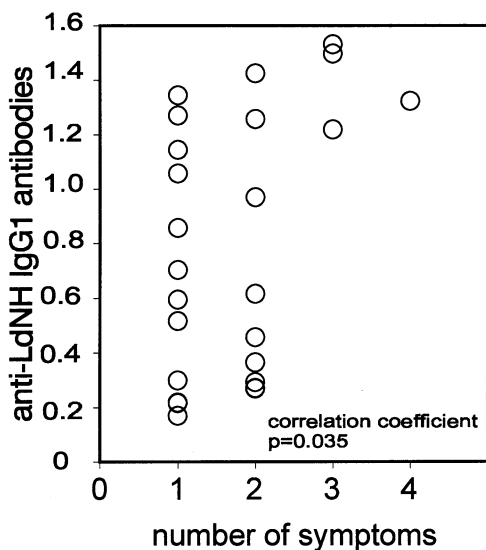


Fig. 3. Correlation analysis of the anti-*LdNH* IgG1 antibody titers and the number of symptoms in naturally infected symptomatic kala-azar dogs. The *y*-axis values represents the mean average of absorbency values (ABS 492 nm) of triplicates of each sera sample. The *x*-axis values represent the number of kala-azar symptoms. The coefficient analysis was determined on a Pearson bivariate, two-tailed test of significance.

relies on the improvement of the sensitivity of the diagnostic tool used [6–8,15,26]. Some seropositive dogs are infected and asymptomatic or oligosymptomatic [2,4]; some infected dogs spontaneously recover [27]. Furthermore, evidence correlating dog symptoms and infectiousness to sandflies is controversial.

Although a correlation between increasing symptoms and infectiousness has recently been reported [28], both asymptomatic and symptomatic naturally-infected dogs proved to be infective to sandflies [4]. Cabral et al. [29] and Pinelli et al. [30] described stronger antibody synthesis only in symptomatic dogs, both naturally and experimentally infected. Solano-Gallego et al. [24] found higher titers in symptomatic than in asymptomatic dogs. As observed for humans [31], the differential increase in several IgG immunoglobulin subclasses might be marker of overt kala-azar. Dogs infected with *L. infantum* produced both IgG1 and IgG2 antibodies with IgG1 being associated with disease (symptomatic dogs, non- or low-responsive to chemotherapy) and IgG2 being associated to asymptomatic infections [23]. Solano-Gallego et al. [24] analyzed the *L. infantum*-specific IgG, IgG1 and IgG2 antibody response in healthy and ill dogs of an endemic area of Spain. Although a great variation of antibody levels was recorded, the authors observed that in a group of naturally infected asymptomatic dogs, only IgG1 remained stable for a 5 years period. Furthermore, IgG1 levels were especially decreased in the group of symptomatic dogs that were responsive to chemotherapy treatment and remain stable in the unresponsive group suggesting a correlation between IgG1 increase, symptoms and disease.

Since infectiousness to sandflies may be related to dog symptoms [28] and the development of clinical disease is related to the increase of IgG1 subtype of anti-*L. donovani* antibodies [23,24], the identification of an *L.*

donovani antigen that could discriminate anti-*Leishmania* IgG1 specific antibodies in sera of dogs from endemic areas contributes to the improvement of tools used in epidemiological control of kala-azar. Serological testing of dogs in endemic areas using the FML–ELISA assay and the *LdNH* ELISA will help in elucidating the real incidence of canine disease.

Future studies on the *LdNH* potential to discriminate dog infectiousness might also be helpful, improving decisions for control programs. The molecular cloning of *LdNH* has provided insights into the immune response to ZVL and constitutes a promising advance for the development of a battery of quality molecular diagnostic tests to be used in the control of this emerging disease.

Acknowledgements

C.B. Palatnik de Sousa. received financial support from: National Foundation of Health (Brazilian Ministry of Health), PCDEN, PNUD-FNS, FINEP, CNPQ, CAPES, MCT/PRONEX (Brazilian Ministry of Science and Technology), RHAEC-CNPQ, 'José Bonifácio' University Foundation of the Federal University of Rio de Janeiro, FAPERJ, and CEPG-UFRJ. D.M. Santana received fellowships from CAPES, RSG-UNDP/World Bank/WHO—Special Program for Research and Training in Tropical Diseases (TDR ID no. 981147), and 'José Bonifácio' University Foundation of the Federal University of Rio de Janeiro. D.A. Campbell received support from Margaret H. Moffat. We thank Buddy Ullman for the cosmid library; Sabrina Dyall, Larry Feldman, Otávio Nóbrega, Kumaran Ramamurthy, Guy Roberts, Arun Venkatesan, Mike Yu and Gusti Zeiner for suggestions; and Dr L.Y. Hashimoto Okada from Clínica Veterinária de Cães e Gatos, Araçatuba, São Paulo, Brazil for the donation of dog sample sera.

References

- [1] Tesh R. Control of zoonotic visceral leishmaniasis. Is it time to change strategies. *Am J Trop Med Hyg* 1995;52:287–92.
- [2] Oliveira GGS, Santoro F, Sadigursky M. The subclinical form of experimental visceral leishmaniasis. *Mem Inst Oswaldo Cruz MEMIOC* 1993;88:243–8.
- [3] Evans TG, Vasconcelos IAB, Lima JW, Teixeira JM, Mc Aullife IT, Lopes UG, Pearson RD, Vasconcelos AW. Canine visceral leishmaniasis in northeast Brazil: assessment of serodiagnostic methods. *Am J Trop Med Hyg* 1990;42:118–23.
- [4] Alvar J, Molina R, San Andrés M, Tesouro M, Nieto J, Vitutia M, González F, San Andrés MD, Boggio J, Rodríguez F, Sainz A, Escacena C. Canine leishmaniasis: clinical, parasitological and entomological follow-up after chemotherapy. *Ann Trop Med Parasitol* 1994;88:371–8.
- [5] Marzochi MCA, Coutinho SG, de Souza WJS, Toledo LM, Grimaldi G, Jr, Momen H, Pacheco RDS, Sabroza PC, de Souza MA, Rangel FB, Jr, Tramontano NC. Canine visceral leishmaniasis in Rio de Janeiro, Brazil. Clinical, epidemiological, therapeutical and epidemiological findings (1977–1983). *Mem Inst Oswaldo Cruz* 1985;80:349–57.
- [6] Ashford DA, David JR, Freire M, David R, Sherlock I, Eulálio MC, Pedral Sampaio D, Badaro R. Studies on control of visceral leishmaniasis: impact of dog control on canine and human visceral leishmaniasis in Jacobina, Bahia, Brazil. *Am J Trop Med Hyg* 1998;59:53–7.
- [7] Badaró R, Benson D, Eulálio MC, Freire M, Cunha S, Netto EM, Pedral Sampaio C, Madureira C, Burns JM, Houghton RL, David J, Reed SG. k39: A cloned antigen of *Leishmania (L.) chagasi* that predicts active visceral leishmaniasis. *J Infect Dis* 1996;173:758–61.
- [8] Dietze R, Falqueto A, Valli LCP, Rodrigues TP, Boulos M, Corey R. Diagnosis of canine visceral leishmaniasis with a dot-enzyme-linked immunosorbent assay. *Am J Trop Med Hyg* 1995;53:40–2.
- [9] Rachamim N, Jaffe CL, Abranches P, Silva-Pereira MC, Schnur LF, Jacobson RL. Serodiagnosis of canine visceral leishmaniasis in Portugal: comparison of three methods. *Ann Trop Med Parasitol* 1991;85:503–8.
- [10] Zijlstra EE, Nur Y, Desyeux P, Khalil EA, El-Hassan AM, Groen J. Diagnosing visceral leishmaniasis with the recombinant K39 st test: experience from Sudan. *Trop Med Int Health* 2001;6:108–13.
- [11] Ashford DA, Bozza M, Freire M, Miranda JC, Sherlock I, Eulálio C, Lopes UG, Fernandes O, Degraive W, Barker RH, Jr, Badaró R, David JR. Comparison of the polymerase chain reaction and serology for the detection of canine visceral leishmaniasis. *Am J Trop Med Hyg* 1995;53:251–5.
- [12] Palatnik de Sousa CB, Gomes EM, Paraguai de Souza E, Palatnik M, Luz KG, Borojevic R. The fucose mannose ligand of *Leishmania donovani* in diagnosis and prognosis of visceral leishmaniasis (kala-azar). *Trans R Soc Trop Med Hyg* 1995;89:390–3.
- [13] Luz KG, Gomes EM, da Silva VO, Machado FCS, Araújo MAF, Fonseca HEM, Freire T, d'Almeida JB, Palatnik M, Palatnik de Sousa CB. Prevalence of anti-*Leishmania donovani* antibody among Brazilian blood donors and polytransfused hemodialyzed patients. *Am J Trop Med Hyg* 1997;57:168–71.
- [14] Otero ACS, da Silva VO, Luz KG, Palatnik M, Pirmez C, Fernandes O, Palatnik de Sousa CB. Occurrence of *Leishmania donovani* DNA in seroreactive Brazilian blood donors. *Am J Trop Med Hyg* 2000;62:128–31.
- [15] Borja-Cabrera GP, da Silva VO, da Costa RT, Barbosa Reis A, Mayrink W, Genaro O, Palatnik de Sousa CB. The fucose–mannose ligand-ELISA in the diagnosis and prognosis of canine visceral leishmaniasis in Brazil. *Am J Trop Med Hyg* 1999;61:296–301.
- [16] Palatnik de Sousa CB, Paraguai de Souza E, Gomes EM, Borojevic R. Experimental murine *Leishmania donovani* infection: immunoprotection by the fucose–mannose ligand (FML). *Braz J Med Biol Res* 1994;27:547–51.
- [17] Santos WR, Paraguai de Souza E, Palatnik M, Palatnik de Sousa CB. Vaccination of Swiss Albino mice against experimental visceral leishmaniasis with the FML antigen of *Leishmania donovani*. *Vaccine* 1999;17:2554–61.
- [18] Palatnik de Sousa CB, Moreno MMB, Paraguai-de-Souza E, Borojevic R. The FML vaccine (fucose–mannose ligand) protects hamsters from experimental kala-azar. *Braz J Assoc Adv Sci Ciência Cultura* 1994;46:290–6.
- [19] da Silva VO, Borja-Cabrera GP, Correia Pontes NN, Paraguai de Souza E, Luz KG, Palatnik M, Palatnik de Sousa CB. A phase III trial of efficacy of the FML-vaccine against canine kala-azar in an endemic area of Brazil (São Gonçalo do Amarante, RN). *Vaccine* 2001;19:1068–81.

- [20] Paraguai de Souza E, Bernardo RR, Palatnik M, Palatnik de Sousa CB. Vaccination of Balb/c mice against experimental visceral leishmaniasis with the GP36 glycoprotein antigen of *Leishmania donovani*. *Vaccine* 2001;19(23–24):3104–15.
- [21] Palatnik de Sousa CB, Gomes EM, Paraguai de Souza E, Santos WR, Macedo SR, Medeiros LV, Luz KG. The FML (fucose mannose ligand) of *Leishmania donovani*. A new tool in diagnosis, prognosis, transfusional control and vaccination against human kala-azar. *Rev Soc Bras Med Trop* 1996;29:153–63.
- [22] Shi W, Schramm VL, Almo SC. Nucleoside hydrolase from *Leishmania major*. Cloning, expression, catalytic properties, transition state inhibitors, and the 2.5-Å crystal structure. *J Biol Chem* 1999;274:21114–20.
- [23] Deplazes P, Smith NC, Arnold P, Lutz H, Eckert J. Specific IgG1 and IgG2 antibody responses of dogs to *Leishmania infantum* and other parasites. *Parasite Immunol* 1995;17:451–8.
- [24] Solano-Gallego L, Riera C, Roura X, Iniesta L, Gallego M, Valladares JE, Fisa R, Castillejo AS, Alberola J, Ferrer L, Arboix M, Portus M. *Leishmania infantum*-specific IgG, IgG1 and IgG2 antibody responses in healthy and ill dogs from endemic areas. Evolution in the course of infection and after treatment. *Vet Parasitol* 2001;96:265–76.
- [25] Parkin DW, Horenstein BA, Abdulah DR, Estupiñán, Schramm VL. Nucleoside hydrolase from *Crithidia fasciculata*. *J Biol Chem* 1991;266:20658–65.
- [26] Palatnik-de-Sousa CB, Santos WR, França-Silva JC, Costa RT, Barbosa Reis A, Palatnik M, Mayrink W, Genaro O. Impact of canine control on the epidemiology of canine and human visceral leishmaniasis in Brazil. *Am J Trop Med Hyg* 2001;65:510–7.
- [27] Genaro O, Costa CA, Breyner ET, Reis AB, Silva AR, Tropa AR, Tafuri WL, Dias M, Mayrink W. The course of experimental visceral leishmaniasis in dogs. *Mem Inst Oswaldo Cruz* 1992;87:105.
- [28] Travi BL, Tabares CJ, Cadena H, Ferro C, Osorio Y. Canine visceral leishmaniasis in Colombia: relationship between clinical and parasitologic status and infectivity for sand flies. *Am J Trop Med Hyg* 2001;64:119–24.
- [29] Cabral M, O'Gradi JE, Alexander J. Demonstration of *Leishmania* specific cell mediated and humoral immunity in asymptomatic dogs. *Parasite Immunol* 1992;14:531–9.
- [30] Pinelli E, Killick-Kendrick R, Wagennar E, Barnadina W, del Real G, Ruitenber J. Cellular and humoral immune responses in dogs experimentally and naturally infected with *Leishmania (L.) infantum*. *Infect Immunol* 1994;62:229–35.
- [31] Elasad AMS, Younis SA, Siddig M, Garyson J, Petersen E, Ghalib HW. The significance of blood levels of IgM, IgA, IgG and IgG subclasses in Sudanese visceral leishmaniasis patients. *Clin Exp Immunol* 1994;95:294–9.