Tehran University of Medical Sciences Publication
http://tums.ac.ir

Iran J Parasitol

Open access Journal at
http:// ijpa.tums.ac.ir

Iranian Society of Parasitology
http://isp.tums.ac.ir

Original Article

Development of a Multi-Epitope Recombinant Protein for the Diagnosis of Human Visceral Leishmaniasis

Farnoosh JAMEIE [1], *Abdolhossein DALIMI [1], Majid PIRESTANI [1], Mehdi MOHEBALI [2]

1. *Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran*
2. *Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*

Received 21 Feb 2019
Accepted 10 May 2019

Keywords:
Visceral leishmaniasis;
Leishmania infantum;
PQ10;
Protein;
Serodiagnosis;
Human;
Iran

***Correspondence Email:**
dalimi_a@modares.ac.ir

Abstract

Background: Iran is one of the endemic areas of Mediterranean Visceral Leishmaniasis, a disease caused by *Leishmania infantum*. In this work, we examined whether Proteína quimérica 10 (PQ10) recombinant protein is suitable for immunological diagnosis of human visceral leishmaniasis.

Methods: The study was carried out in Tarbiat Modares University during 2016-2018. The coding sequence of PQ10 recombinant protein was sub-cloned in pET28 expression vector and was commercially synthesized by GENERAY Biotechnology, China. Sequencing with proper primers was done, the expression, optimization of expression and protein purification were performed, and the purified recombinant protein was confirmed by western blot. The efficacy of PQ10 for serodiagnosis was evaluated with 50 positive and 50 negative serum samples, which confirmed by the direct agglutination test and collected from individuals living in the visceral leishmaniasis endemic areas of Iran. ELISA was performed with the PQ10 recombinant protein.

Results: The 95% CI sensitivity of ELISA that was evaluated with sera from naturally infected individuals was 84%. The 95% CI specificity value of the ELISA determined with sera from healthy individuals (50 serum samples) and from individuals with other infectious diseases was 82%. The 95% CI positive predictive value (PPV) and negative predictive value (NPV) were exterminated 82.35% and 83.67%, respectively.

Conclusion: We have used a recombinant synthetic protein to improve serodiagnosis of human visceral leishmaniasis. PQ10 could be useful for diagnosis of asymptomatic cases, as well as in the early phase of infections.

Copyright © 2021 Jameie et al. Published by Tehran University of Medical Sciences.
This work is licensed under a Creative Commons Attribution-NonCommercial 4.0 International license
(https://creativecommons.org/licenses/by-nc/4.0/). Non-commercial uses of the work are permitted, provided the original work is properly cited.

Introduction

Leishmaniasis is a majorly neglected tropical disease that its victims are among the poorest people that has been acknowledged by the WHO (1). While visceral leishmaniasis is endemic in northwestern and southern areas of the Iran, but the report of disease is sporadic and about 100–300 new cases reported annually (2).

The successful control of the disease requires efficient and reliable methods of diagnosis and for this purpose enormous efforts have been directed toward developing diagnostic tests (3). But current serodiagnostic techniques are not satisfactory because they do not discriminate between disease and asymptomatic infection (4). For example, immunological assays such as ELISA and indirect immunofluorescence assay (IFA) offer moderate sensitivity and specificity or as for direct agglutination test (DAT), cost, multiple steps, incubation, and antigenic variations are the limiting factors (5), thus leading to the misdiagnosis of infected animals cases in endemic areas (6).

In recent studies, different numbers of *Leishmania* antigens have been investigated for serodiagnostic purposes (7–11). The direct agglutination test and rk39 dipstick have made a progress in detecting visceral leishmaniasis. Some other kinesin-related protein, including K26, K28, and KE16 provided promisingly diagnostic accuracy in the diagnosis of visceral leishmaniasis (12), but despite these promising results, tests with other multiepitope proteins, especially antigens that could be used to detect visceral leishmaniasis in an early phase, are still needed in human with different clinical status to follow up of human infection. High sensitivities and specificities in these tests can be achieved by using multiepitope proteins, which have been shown to be a valuable tool in canine visceral leishmaniasis diagnosis (13).

A multiepitope recombinant protein (PQ10= Proteína Quimérica10 or Chimeric Protein10), a protein that consisted of ten antigenic peptides, showed good results in diagnosis of canine visceral leishmaniasis by ELISA, with accuracy of up to 0.94 (13,14).

The present study was carried out to develop and evaluate PQ10 in detecting human visceral leishmaniasis in the early phase.

Materials and Methods

Human sera collecting

The present study was carried out in the Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran during 2016-2018. Human sera that were confirmed as positive by the direct agglutination test (DAT) for visceral leishmaniasis were prepared as follows: for asymptomatic serum samples (Group A, n=25), blood was taken from apparently healthy blood donors (that were found positive for visceral leishmaniasis) from six blood service centers in Ardabil Province, where visceral leishmaniasis cases had been recorded.

The human symptomatic serum samples (Group B, n=25) had been collected during the time in the Parasitology Laboratory, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. Human sera confirmed as negative for visceral leishmaniasis prepared as follows: 50 serum samples were collected from healthy individuals who were residents in visceral leishmaniasis endemic areas in Iran (group C). These negative sera were used to define a cut off value. Moreover, 20 serum samples were collected from patients with other infectious diseases (Toxoplasmosis (4 sample), bartonellosis (2 sample), echinococcosis (1 sample), syphilis (1 sample), strongyloidiasis (1 sample), malaria infection (2 sample), cytomegalovirus infection (2 sample), fasciolosis (1 sample), trichostrongylosis (1 sample), tuberculosis (1 sample), measles (2 sample), hymenolepiasis (1 sample) and toxo-

cariasis (1 sample)) who were residing in non-*Leishmania* endemic areas in Iran (group D). Thirty of the negative samples and twenty of the serum samples from patients with other infectious disease were used to calculate specificity. All negative samples were confirmed for absence of anti-*Leishmania* antibodies by DAT.

Design of synthetic gene constructs

The PQ10 multiepitope synthetic gene was designed according to Faria et al (13). First, 10 coding sequences of antigenic peptides (8) were joined, resulting in PQ10. A flexible linker (Gly-Ser-Gly-Ser-Gly) coding sequence was used as a spacer between epitope sequences (15). NdeI and NotI restriction sites were added to the 5ʹ and 3ʹ ends, respectively, of the synthetic gene to aid in cloning. For affinity purification of recombinant protein, a 6xHIS tag coding sequence was added upstream of the stop codon of the synthetic gene. The sequence was codon-optimized for *Escherichia coli* expression. The *PQ10* gene was commercially synthesized by GENERAY Biotechnology, China. A synthetic gene was cloned into the NdeI and NotI restriction sites of a pET28 expression vector, resulting in pET-PQ10. Sequence analyses of the cloned fragments were done for confirmation of the correct fusion and the orientation of the insert.

Expression, production, purification and confirmation of recombinant protein

Recombinant plasmid was transformed to *E.coli BL21 DE3* expression host and protein expression was carried out by inoculating 500 ml of Luria Bertani medium containing 0.05 mg/ml kanamycin with an overnight bacterial culture. All cultures were incubated on a rotary shaker at 180 rpm at 37 °C. Cultures were grown until an optical density of 0.6 at 600 nm was attained. Then, the expression of PQ10 recombinant protein was induced by adding 1 mM of IPTG (isopropyl-Beta-D-thiogalactopyranoside) to the medium on a rotary shaker (180 rpm) at 37 °C. Induction time was 5 h, the expression levels were assessed at one-h intervals of induction. Induced samples (soluble and insoluble protein) were screened and analyzed by SDS-PAGE (mod.VSTS-3000; AKHTARIAN) with 12.5% resolving gel, followed by Coomassie Brilliant Blue G-250 staining. Cultures (200 ml of 4-h induced) were pelleted and purification of PQ10 protein was performed under denaturing conditions, according to the manufacturer's instructions (The *QIA* expressionist TM, QIAGEN). The eluted proteins were analyzed by SDS-PAGE and were quantified using Bradford method. Western blot analysis was performed on purified protein samples. Proteins were electrotransferred to nitrocellulose membranes (Sigma-Aldrich®) in a semi-dry transfer cell at 15 volts/overnight (mod WB-1100, AKHTARIAN). Detection of antigens was performed by an indirect antibody immunoassay using anti-human IgG (whole molecule)-HRP (from rabbit) (Riz Pad tan Parse) diluted 1:7000 in PBS with 0.05% Tween20 and DAB staining.

Checkerboard titration

The best concentrations of the antigen, sera and conjugates were optimized for the immunoassay by checkerboard titration. The microplate was coated with antigen at concentrations of 0.06, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 64 μg/ml. These plates were incubated with pools of sera from humans infected and uninfected with *L.infantum* in different dilutions (1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200). Binding of the antibodies was detected using anti-human IgG-HRP (from goat) (Sigma, reference A8667) in different dilutions (1:15000, 1:30000, 1:45000).

Immunoassays with human sera

ELISA procedures were done to evaluate the antigenicity of multiepitope PQ10 protein. Maxisorp microplate (Nunc, Denmark) was coated overnight with 16 μg/ml PQ10 protein diluted in 0.1 M carbonate buffer (pH 9.6) at 4 °C. After three washes with PBS-0.05% Tween-20 (PBST) (PBS: 10.14 mM Na_2HPO4;

1.37 mMKH$_2$PO4; 146 mM NaCl; 2.64 mM KCl, pH 7.4, containing 0.05%Tween20), wells were blocked with 200 µl/well of 1% Bovine Serum Albumin (BSA) in PBS at 37 ˚C for 2 h. Serum samples, diluted 1:100 in PBS-0.05% Tween-20 containing 0.5% BSA, were added and incubated at 37 ˚C for 1 h. After three washes, microplate was incubated with anti-human IgG-HRP (from goat) (Sigma, reference A8667), diluted 1:30000 in PBS at 37 ºC for 1 h. After washing five times, reactions were developed with Tetra Methyl Benzidine (TMB) (Biolegend, reference 421101) and the microplates were incubated for 20 min in the dark room. Reactions were stopped with 2M H_2SO4, and microplate was read at 450 nm in a DYNEX (MRX II) ELISA reader.

Direct Agglutination Test (DAT) assay

The principal procedures for preparing the DAT antigen were performed according to the previous researches (16,17). To perform the test, sera were screened by 1: 800 dilution. The samples with titers 1: 800 were diluted further to give end-point titers of 1: 102400. Antigen control well (antigen only), in addition to negative and positive control sera (the positive serum was prepared from VL patients with *L. infantum* infection from the endemic areas confirmed by microscopy, culture and DAT titers of 1: 102400) were used in all procedures. The highest dilution which agglutination was still visible, compared with negative control wells which had clear blue dots was considered as the cut off titer(17).

Ethics Statement

During the sample collection, informed consents had been obtained from all participants and all clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. The project was approved by the Ethical Committee of Tarbiat Modares University with code No. IR.TMU. REC.1394.241, based on the Specific National Ethical Guidelines for Biomedical Research issued by the Research and Technology Deputy of Ministry of Health and Medicinal Education of Iran.

Statistical analysis

The cut-off value of PQ10 protein was calculated as the mean values + 3SD of OD value of the negative sera that were obtained from healthy individuals. Consequently, OD values higher than the mean value + 3 SD+10% were considered as positive. The sensitivity, specificity, PPV and NPV were calculated as follows: Sensitivity = TP / (TP + FN) × 100%, specificity = TN / (TN +FP) × 100%, PPV = TP / (TP + FP) × 100%, NPV = TN /(TN + FN) × 100% (18). The kappa index (which is used to find the level of agreement of PQ10 antigen with DAT), the Youden's index (which is a single statistic that captures the performance of a dichotomous diagnostic test) and the McNemar test were calculated. Statistics were performed using SPSS(Chicago, IL, USA) version 16 and the differences between the results of PQ10-ELISA and DAT tests were considered statistically significant when $P < 0.05$.

Results

Constructing molecular sequences, recombinant protein expression and confirmation of protein production

A sequence that was codon-optimized (13) for PQ10 protein expression in *Escherichia coli* was commercially synthesized successfully (Fig. 1) and confirmed by sequencing with PET-28 vector specific primers.

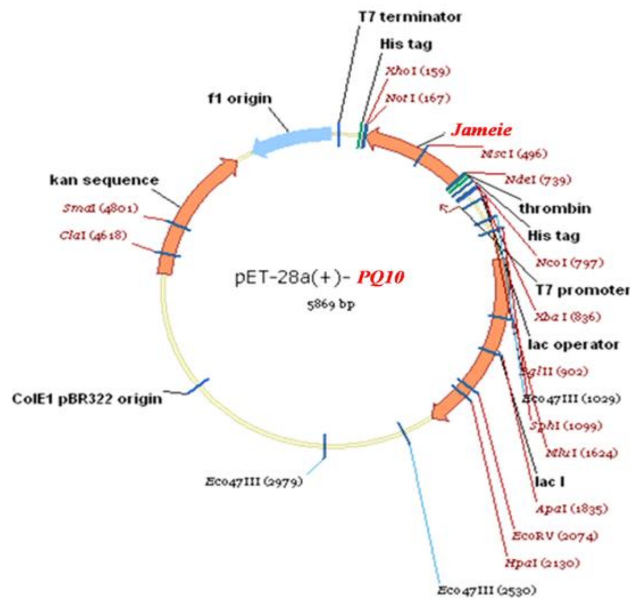

Fig. 1: Schematic picture of pET-28-PQ10 structure (Original)

The PQ10 multiepitope protein was successfully expressed by E.coli BL21 DE3 strain under the conditions described in methods. Three-dimensional structure of PQ10 protein designed by I-TASSER server are shown in Fig. 2.

Fig. 2: Three-dimensional structure of PQ10 protein

SDS-PAGE analysis of *E. coli* BL21 (DE3), which was transformed with pET28- PQ10 and induced with IPTG, showed the expected 21.4 kDa band of PQ10 protein. Fig. 3 shows the expression of PQ10 induced by IPTG 1 mM in different times. There was a difference in quantitative expression of protein among 1- to 5 h induced cells. The maximized protein expression was after 4 h after cells induced. PQ10 recombinant protein was found mainly in the insoluble fraction of the cell lysate. Fig. 3 shows PQ10 protein in cell lysate and its

purified form at the expected sizes (21.4 kDa). Concentration of the purified PQ10 protein was calculated as 150 µg/ml by the Bradford assay.

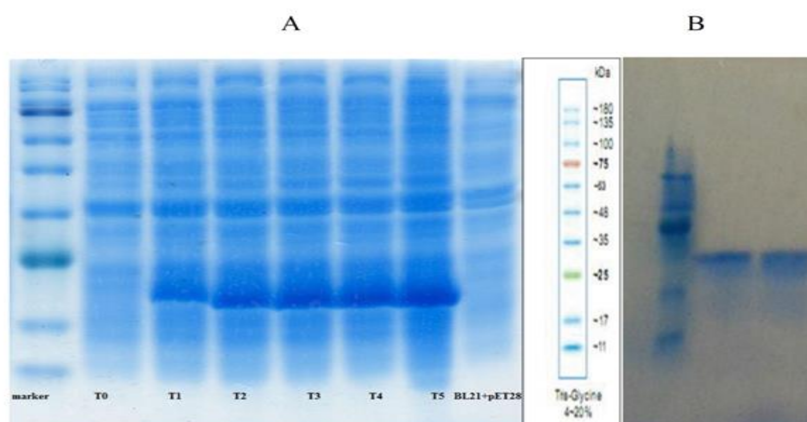

Fig. 3: Cell lysate fractions from IPTG-induced bacterial cultures in different times (A), T0: Non-induced BL21-pET28-PQ10; T1: 1-h induced BL21-pET28-PQ10; T2: 2-h induced BL21-pET28-PQ10; T3: 3-h induced BL21-pET28-PQ10; T4: 4-h induced BL21-pET28-PQ10; T5: 5-h induced BL21-pET28-PQ10 and BL21-pET28 as negative control and purified PQ10 protein (B), showing bands in the expected size (21.4 kDa) for the PQ10 recombinant protein. 12% SDS-PAGE gel stained with Coomassie blue-stained. M: molecular weight marker in kDa

Western blotting was used to examine affinity of purified PQ10 protein against the patient's sera. Western blots of purified PQ10 demonstrated that recombinant protein reacted with the patient's sera (Fig. 4).

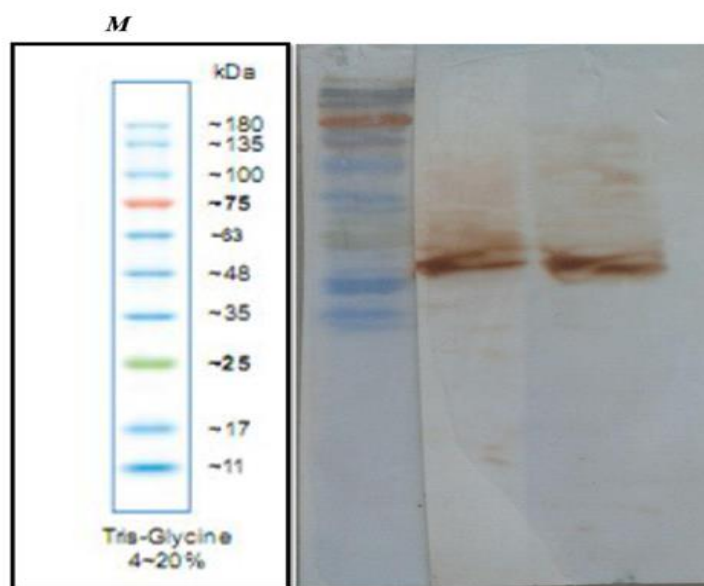

Fig. 4: Nitrocellulose membrane after western blotting with anti-human IgG. M: Molecular marker in kDa

Immunoassays with human sera

Checkerboard titration

The standardization assay performed to determine the best concentration of the PQ10, as well as the best dilutions of the primary and secondary antibodies. It was observed that the best results were obtained by coating the wells with the PQ10 recombinant protein at 16 µg / ml concentration, with the human sera diluted at 1:100 and the conjugate diluted at 1:30000.

Sensitivity and specificity determination of PQ10-Indirect ELISA

According to Table 1, 16% (8 samples) out of 50 confirmed subjects, who were infected by *L. infantum*, did not react with PQ10 antigen and 18% (9 samples) out of 50 healthy subjects reacted with PQ10. The PQ10 showed reactivity with 2 samples from healthy sera and cross reaction was observed in 7 of samples collected from other infectious diseases. The results of antibody detection by DAT and PQ10-Indirect ELISA testing are compared in Table 1.

Table1: The diagnostic performance of anti-PQ10 indirect ELISA assay and comparison between ELISA using recombinant protein PQ10 (cut-off=0.555) and DAT result
PQ10 indirect ELISA had (84%)* sensitivity and (82%) ** specificity for *Leishmania infantum*

Direct Agglutination Test		***PQ10 indirect ELISA***					
		Positive ELISA result		Negative ELISA result		Total	
		Number	Percentage	Number	Percentage	Number	Percentage
DAT sera	Positive	42	**84***	8	16	50	100
DAT sera	Negative	9	18	41	**82***	50	100

With the 95% CI, the PQ10-Indirect ELISA test was sensitive (84%) and specific (82%) for the diagnosis of visceral leishmaniasis, with a PPV and an NPV of 82.35 and 83.67%, respectively. Additionally, PQ10 multiepitope protein was able to detect 92% of asymptomatic and 76% of symptomatic infected human, which was confirmed with DAT. From 20 serum samples that were collected from patients with other infectious disease, seven samples from patients with Toxoplasmosis, Syphilis, Malaria infection, Cytomegalovirus infection, Tuberculosis, Measles and Hymenolepiasis were false positive. The kappa index was calculated 0.660 that showed good agreement with DAT. The Youden J index for PQ10-ELISA test was calculated 0.66. The McNemar test result was calculated 1 that indicated with 95% confidence interval, there is no significant difference between the results of DAT and PQ10-ELISA in the diagnosis of people that with infected with *L. infantum*.

Discussion

Visceral leishmaniasis has common clinical features with other commonly occurring diseases of human such as malaria, typhoid, and tuberculosis, thus its diagnosis is complex (5). As because the most of the antileishmanial drugs are toxic, ideally, a diagnostic test should make the distinction between acute disease and asymptomatic infection (19).

The current methods that are used for diagnosis of visceral leishmaniasis are splenic aspiration, in vitro culture of bone marrow and direct observation, but unfortunately these

methods are time consuming, invasive, life threatening, and are unable to detect the infected asymptomatic persons who may serve as a reservoir of VL (20). Diagnostic methods based on molecular tools such as PCR and real-time PCR have some disadvantages like being too cumbersome to perform and high cost (21).

The immunodiagnosis assays have become important alternatives for the above tests (19,22) and several different *Leishmania* antigens have been identified and characterized that has been used for the development of new enzymatic immunoassays (13). The number of false-positives will be increased when only one antigen is used in screening and confirmation diagnostic tests of VL (23). But, we have produced a multiepitope recombinant antigen, which is low cost in preparation, easy automation in ELISA tests and have high ability to detect circulating antibodies in the early phase of infection, according to previous researches (13).

ELISA has been used as an appropriate diagnostic method for almost all infectious diseases, including leishmaniasis (5) and this technique requires a highly specific antigen in order to capture a specific antibody; therefore, several antigenic molecules, such as surface antigens, ribosomal or nuclear proteins, histones and kinesin-related proteins were used for this purpose (24). The sensitivity and specificity of crude soluble antigen (CSA), that is one of the most commonly used antigens, ranges from 80 to 100% and 84 to 95%, respectively, but cross-reactivity among patients with tuberculosis, trypanosomiasis and toxoplasmosis has also been reported (5,22).

rK39 among the recombinant proteins, was the most successful antigen, which has good efficacy to detect active canine visceral leishmaniasis. The rK39 showed high sensitivity (90% ±100%) in detecting the disease from the Mediterranean basin and South America, but it has less sensitivity and specificity for detecting asymptomatic canine visceral leishmaniasis (25–31). rKLO8 and rK26 are other antigens that it is shown to be able to enhance the diagnostic accuracy of canine visceral leishmaniasis. The individual use of these two antigens indicated lower sensitivity and specificity (rKLO8, 68% and 92%, respectively; rK26, 77% and 91%, respectively) compared to their combination which exhibited higher sensitivity (85%) and specificity (93%) (32).

In some studies, the chimeric proteins has been used for diagnosis of *L. infantum,* with 79% sensitivity and 96% specificity in infected (n =59) and uninfected dogs (n= 15) respectively. In another study on 232 animals infected with *Leishmania* that had been confirmed by parasitological examination or RIFI method, the sensitivity and specificity of their chimeric antigen in diagnosis of visceral leishmaniasis were 96% and 99%, respectively (33,34).

In our study, the cut off value was calculated by adding three standard deviation values to the mean absorbance of 50 negative serum samples from healthy individuals. It provided values of 84% and 82% from sensitivity and specificity, respectively. The observation of false positives related to the cross-reaction samples (seven samples from patients with toxoplasmosis, syphilis, malaria infection, cytomegalovirus infection, tuberculosis, measles and hymenolepiasis) raises the suspicion of cross-reaction with other infectious diseases. PQ10 multiepitope protein was able to detect 92% of asymptomatic infected human that is its advantage in performing control programs.

In research conducted with recombinant proteins usually a concentration less than 5 μg/ml was used in ELISA, but in this study, the 16μg/ml concentration of PQ10 recombinant protein was shown to be the best.

Conclusion

We have used a recombinant synthetic protein to improve human visceral leishmaniasis serodiagnosis. PQ10 could be used as a good antigen for diagnosing asymptomatic cases, as well as in the early phase of the infections.

Acknowledgements

The present work is part of PhD. thesis, supported financially by Faculty of Medical Sciences Tarbiat Modares University (Grant No. 52.112071).

Conflict of interest

The authors declare that there is no conflict of interest.

References

1. WHO. Control of the leishmaniases. World Health Organization technical report series. Geneva: World Health Organization; 2010.
2. Mohebali M. Visceral leishmaniasis in Iran: Review of the Epidemiological and Clinical Features. Iran J Parasitol. 2013; 8(3):348–58.
3. Motazedian M, Fakhar M, Motazedian MH, et al. A urine-based polymerase chain reaction method for the diagnosis of visceral leishmaniasis in immunocompetent patients. Diagn Microbiol Infect Dis. 2008; 60(2):151-4.
4. Sarkari B, Chance M, Hommel M. A capture ELISA for the diagnosis of visceral leishmaniasis using a monoclonal antibody against a leishmanial. Iran Biomed J. 2005; 9(3):117–22.
5. Sundar S, Rai M. Laboratory diagnosis of visceral leishmaniasis. Clin Diagn Lab Immunol.2002;9(5):951–8.
6. Fraga DB, da Silva ED, Pacheco LV, et al. A multicentric evaluation of the recombinant *Leishmania infantum* antigen-based immunochromatographic assay for the serodiagnosis of canine visceral leishmaniasis. Parasit Vectors 2014;7:136.
7. Martins VT, Chávez-Fumagalli MA, Costa LE, et al. Antigenicity and protective efficacy of a *Leishmania* amastigote-specific protein, member of the super-oxygenase family, against visceral leishmaniasis. PLoS Negl Trop Dis. 2013; 7(3):e2148.
8. Faria R, Giusta S, Jr GG, et al. High-throughput analysis of synthetic peptides for the immunodiagnosis of canine visceral leishmaniasis. PLoS Negl Trop Dis. 2011; 5(9): e1310.
9. Moura A, Rosa A, Tenório F, et al. Acta Tropica Evaluation of three recombinant *Leishmania infantum* antigens in human and canine visceral leishmaniasis diagnosis. Acta Trop. 2014; 137:25–30.
10. Akhoundi B, Mohebali M, Shojaee S, et al. Rapid detection of human and canine visceral leishmaniasis : Assessment of a latex agglutination test based on the A2 antigen from amastigote forms of *Leishmania infantum*. Exp Parasitol .2013; 133(3):307–13.
11. de Souza FR, Santos YLD, Vasconcellos RS, et al. Recombinant *Leishmania (Leishmania) infantum* Ecto-Nucleoside Triphosphate Diphosphohydrolase NTPDase-2 as a new antigen in canine visceral leishmaniasis diagnosis. Acta Trop. 2013; 125(1):60–6.
12. Shahriari Rad B, Rezaei Z, Mohebali M. Immunodiagnosis of visceral leishmaniasis: current status and challenges: A review article. Iran J Parasitol. 2018; 13(3):331–41.
13. Faria R, Veloso LDC, Coura-vital W, et al. Novel recombinant multiepitope proteins for the diagnosis of asymptomatic *Leishmania infantum*- infected dogs. PLoS Negl Trop Dis. 2015; 9(1):e3429.
14. Costa MM, Andrade HM, Bartholomeu DC, et al. Analysis of *Leishmania chagasi* by 2-D difference gel eletrophoresis (2-D DIGE) and immunoproteomic: identification of novel candidate antigens for diagnostic tests and vaccine. J Proteome Res. 2011; 10(5):2172–84.
15. Robinson CR, Sauer RT. Optimizing the stability of single-chain proteins by linker length and composition mutagenesis. Proc Natl Acad Sci U S A. 1998; 95(11):5929–34.
16. el Harith A, Slappendel RJ, Reiter I, et al. Application of a direct agglutination test for detection of specific anti-*Leishmania* antibodies in the canine reservoir. J Clin Microbiol. 1989; 27(10):2252–7.
17. WHO, Manual on visceral leishmaniasis control. First edition. Desjeux DP, editor: Geneva: World Health Organization; 1996; 79, 84 p.
18. Tjitra E, Suprianto S, Dyer M, et al. Field evaluation of the ICT malaria Pf/Pv immunochromatographic test for detection of *Plasmodium falciparum* and *Plasmodium vivax* in patients with a presumptive clinical diagnosis of malaria in Eastern Indonesia. J Clin Microbiol. 1999; 37(8):2412–7.

19. Srivastava P, Dayama A, Mehrotra S, et al. Diagnosis of visceral leishmaniasis. Trans R Soc Trop Med Hyg. 2011; 105(1):1–6.
20. Mary C, Lamouroux D, Dunan S, et al. 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. 1992; 47(6):764–71.
21. Deborggraeve S, Laurent T, Espinosa D, et al. A simplified and standardized polymerase chain reaction format for the diagnosis of leishmaniasis. J Infect Dis. 2008; 198(10):1565–72.
22. Elmahallawy EK, Martinez AS, Rodriguez-Granger J, et al. Diagnosis of leishmaniasis. J Infect Dev Ctries. 2014; 8(8):961–72.
23. Costa CHN, Vieira JBF. Changes in the control program of visceral leishmaniasis in Brazil. Rev Soc Bras Med Trop. 2001; 34(2):223–8.
24. Sakkas H, Gartzonika C, Levidiotou S. Laboratory diagnosis of human visceral leishmaniasis. J Vector Borne Dis. 2016; 53(1):8-16.
25. Mettler M, Grimm F, Capelli G, et al. 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. 2005; 43(11):5515–9.
26. Gomez-Ochoa P, Castillo JA, Lucientes J, et al. Modified direct agglutination test for simplified serologic diagnosis of leishmaniasis. Clin Diagn Lab Immunol. 2003; 10(5):967–8.
27. Rhalem A, Sahibi H, Guessous-Idrissi N, et al. Immune response against *Leishmania* antigens in dogs naturally and experimentally infected with *Leishmania infantum*. Vet Parasitol. 1999; 81(3):173–84.
28. Scalone A, De Luna R, Oliva G, et al. 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. 2002; 104(4):275–85.
29. Badaro R, Benson D, Eulalio MC, et al. rK39: a cloned antigen of *Leishmania chagasi* that predicts active visceral leishmaniasis. J Infect Dis. 1996; 173(3):758–61.
30. Ozensoy S, Ozbel Y, Turgay N, et al. Serodiagnosis and epidemiology of visceral leishmaniasis in Turkey. Am J Trop Med Hyg. 1998; 59(3):363–9.
31. Santarém N, Silvestre R, Cardoso L, et al. Application of an improved enzyme-linked immunosorbent assay method for serological diagnosis of canine leishmaniasis. J Clin Microbiol. 2010; 48(5):1866–74.
32. Abad LPM, Almeida CS, Mattos AMM, et al. Diagnostic accuracy of rKLO8 versus rK26 ELISAs for screening of canine visceral leishmaniasis. Acta Trop. 2017; 166:133–8.
33. Soto M, Requena JM, Quijada L, et al. Multicomponent chimeric antigen for serodiagnosis of canine visceral leishmaniasis. J Clin Microbiol. 1998; 36(1):58–63.
34. Boarino A, Scalone A, Gradoni L, et al. Development of recombinant chimeric antigen expressing immunodominant B epitopes of *Leishmania infantum* for serodiagnosis of visceral leishmaniasis. Clin Diagn Lab Immunol. 2005; 12(5):647–53.