Detection of *Leishmania infantum* infections in dog reservoirs

by using a multiepitope recombinant protein (PQ10)

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**Abstract**

Visceral leishmaniasis is a neglected disease caused by *Leishmania infantum* and transmitted via female sand flies. Canine visceral leishmaniasis diagnosis should be performed as soon as possible, even on the basis of only a few or even a single clinical sign, to enhance the prediction of disease and to avoid both dog and human transmission and unnecessary euthanasia of apparently positive dogs. In the present work, we examined whether PQ10 recombinant protein could be suitable for immunological detection of *Leishmania infantum* infection. The coding sequence of PQ10 recombinant protein was sub-cloned in pET28 expression vector and was commercially synthesized by GENERAY Biotechnology, China. In the following process, sequencing with proper primers was done and the expression, optimization of expression and protein purification were performed. The efficacy of PQ10 for serodiagnosis was evaluated with 100 serum samples collected from dogs living in the visceral leishmaniasis endemic areas of Iran. Samples (n=20) of the dogs with other infectious disease were also be collected. The synthesized clones verified by the sequencing
with proper primers. In the following process, expression, optimization of expression and protein purification performed and the purified recombinant protein confirmed by western blot. The ELISA was performed with PQ10 recombinant protein. The sensitivity of ELISA that was evaluated with sera from naturally infected dogs was 94%. The specificity value of the ELISA was determined with sera from healthy dogs and from dogs with other infectious diseases was 86%. The positive predictive value (PPV) and negative predictive value (NPV) were determined 87.03% and 93.47% respectively. Our findings indicated to the potential use of this recombinant protein in the diagnosis of canine visceral leishmaniasis.

**Keywords:** visceral leishmaniasis, *Leishmania infantum*, PQ10, recombinant protein, serodiagnosis, dog.

**Introduction**

Visceral leishmaniasis is a neglected disease caused by *Leishmania infantum* and transmitted via female sand flies (Desjeux, 2004). Nicolle and Comte first described canine leishmaniasis in Tunisia (Nicolle and Comte, 1908). Domestic dogs (*Canis familiaris*) are the main reservoir hosts for *L. infantum* and play a critical role in the transmission cycle of visceral leishmaniasis (Travi et al., 2018). Sand fly vectors could take infection from both symptomatic and asymptomatic dogs (Gradoni et al., 1987; Molina et al., 1994). The risk of parasite transmission from dogs to sand flies and then to humans could be increased by close contact between dogs and human populations (Miro et al., 2008). Canine leishmaniasis cause a wide variety of clinical forms ranging from mild popular skin diseases to severe forms characterized by keratoconjunctivitis, cachexia, alopecia, anorexia, weight loss, and death (Costa et al., 2003; Farahmand et al., 2015; ORDEIX et al., 2005). While visceral leishmaniasis is endemic in northwestern and southern areas of the Iran (Edrissian et al., 1998; Edrissian, 1996; Mohebali et al., 2006), but the report of human disease is sporadic and about 100–300 new cases reported annually (Mohebali, 2013).
Different numbers of *Leishmania* antigens such as A2 antigen from amastigote forms of *Leishmania infantum*, Ecto-Nucleoside Triphosphate Diphosphohydrolase NTPDase-2, *Leishmania* amastigote-specific protein and Synthetic Peptides have been investigated for serodiagnostic purposes (Akhoundi et al., 2013; Faria et al., 2011; Francisco et al., 2013; Martins et al., 2013; Moura et al., 2014). Studies indicated that high values of sensitivity and specificity are very essential for these antigens and, however, if the objective is a screening test, high sensitivity is desirable and if a confirmatory test is being developed, high specificity becomes more important in this case (Faria et al., 2015). Faria et al indicated that 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 (Faria et al., 2015). In this study, we developed and evaluated a multiepitope recombinant protein (PQ10) for the detection of *Leishmania infantum* infections in asymptomatic and symptomatic dog reservoirs in endemic area of visceral leishmaniasis in Iran. This recombinant protein was constituted by ten antigenic peptides with epitopes previously identified and showed good results in canine visceral leishmaniasis ELISA with accuracy up to 0.94 (Costa et al., 2011; Faria et al., 2015).

**Materials and methods**

**Dog sera collecting.** Dog sera that were confirmed as positive by the direct agglutination test (DAT) for visceral leishmaniasis prepared as follows: for asymptomatic serum samples (Group A, n=25), bloods were taken from Kordan region of Alborz Province, an endemic area for visceral leishmaniasis. The dog symptomatic serum samples (Group B, n=25) had been collected from different endemic region of Iran during the time in the Parasitology Laboratory, School of Public Health, Tehran University of Medical Sciences. The dog sera that were confirmed as negative for visceral leishmaniasis prepared as follows: 50 serum samples were collected from healthy dogs that were living 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 dogs with other infectious disease (Canine distemper virus, Leptospirosis and Parvavirus infection) that were living in non-\textemdash\textit{Leishmania} endemic areas in Iran (group D). Thirty of other negative samples and twenty of the serum samples from dogs with other infectious disease were used to calculate specificity. All negative samples were confirmed for absence of anti-\textit{Leishmania} antibodies by DAT.

\textbf{Design of synthetic gene constructs.} The PQ10 multiepitope synthetic gene was designed according to Faria et al (Faria et al., 2015). First, 10 coding sequences of antigenic peptides (Faria et al., 2011) were joined, resulting in PQ10. A flexible linker (Gly-Ser-Gly-Ser-Gly) coding sequence was used as a spacer between epitope sequences (Robinson and Sauer, 1998). NdeI and NotI restriction sites were added to the 5’ and 3’ ends, respectively, of 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 synthetic gene. The sequence was codon-optimized for \textit{Escherichia coli} expression. Three dimensional structure of PQ10 protein was predicted by I-TASSER server (https://zhanglab.ccmb.med.umich.edu/ITASSER/). The PQ10 gene was commercially synthesized by GENERAY Biotechnology, China. The synthesized gene was cloned into the NdeI and NotI restriction sites of a pET28 expression vector, resulting in pET-PQ10. Sequence analysis of the cloned fragments were done for confirmation of the correct fusion and the orientation of the insert.

\textbf{Expression, production, purification and confirmation of recombinant protein.} Recombinant plasmid was transformed to \textit{E.coli} \textit{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. Then, expression of PQ10 recombinant protein was induced by adding 1 mM IPTG (isopropyl-Beta-D-thiogalactopyranoside) to medium on a rotary shaker (180 rpm) at 37°C. Induction time was 5 hours, which the expression levels were assessed at one-hour 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-hour 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 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-dog IgG (whole molecule)-HRP (from rabbit) (Riz Pad tan Parse, Iran) diluted 1:3000 in PBS with 0.05% Tween20 (PBS-T) and DAB staining.

**Checkerboard titration.** The best concentrations of the antigen, sera and conjugates were optimized for the immunoassay by checkerboard titration. The Maxisorp microplate (Nunc, Denmark) was coated with antigen at concentrations of 0.06, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 64µg/ml. These plates were incubated with pools of sera from dog infected with *L. infantum* and uninfected animals at different dilutions (1/50, 1/100, 1/200, 1/400, 1/800, 1/1600, 1/3200). Binding of the antibodies was detected using anti-dog IgG-HRP (from Rabbit) (Sigma, reference A6792) in different dilutions (1/5000, 1/10000, 1/20000).

**Immunoassays with dog sera.** ELISA procedures were done to evaluate the antigenicity of multiepitope PQ10 protein. Maxisorp microplate (Nunc, Denmark) was coated overnight with 2 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$_2$HPO$_4$; 1.37 mM KH$_2$PO$_4$; 146 mM NaCl; 2.64 mM KCl, pH 7.4, containing 0.05% Tween 20), 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-dog IgG-HRP (from Rabbit) (Sigma, reference A6792), diluted 1:10000 in PBS, at 37 °C for 1 h. After washing five times, reactions were developed with Tetra Methyl Benzidine (TMB) (Biolegend, reference 421101) and the plates were incubated for 20 min in the dark room. Reactions were stopped with 2 M H$_2$SO$_4$, 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 was as follows: first, mass cultivation of promastigotes of *L. infantum* Lon49 (Iranian strain) were performed in RPMI1640 medium (supplemented with 10 % fetal bovine serum) and then the promastigotes were centrifuged at 4000 g for 10 min at 4°C. After washing (x5) with Locke’s solution, the parasites were trypsinized and then fixed with 2 % formaldehyde. Finally, the fixed parasites stained with Coomassie Brilliant blue and resuspended in citrate saline containing 0.4% formaldehyde and stored until use (el Harith et al., 1989; WHO, 1996).

For performing the test, initially, serums screened by 1: 80 dilution. The samples with titers 1: 80 were diluted further to give end-point titers of 1: 20480. Antigen control well (antigen only) and negative and positive control sera (the positive serum was prepared from dogs with *L. infantum* infection from the endemic areas confirmed by microscopy, culture and DAT titers of 1: 20480) were used in all procedures. The highest dilution which agglutination was still visible, compared with negative control wells which had clear blue dots, considered as the cut off titer (WHO, 1996).
**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 dogs. 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% (Tjitra et al., 1999). Statistics were performed using SPSS version 25 and differences 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 (Faria et al., 2015) for PQ10 protein expression in *Escherichia coli* was commercially synthesized successfully (Figure 1) and confirmed by sequencing with PET-28 vector specific primers.

The PQ10 multiepitope protein was successfully expressed by *E.coli* BL21 DE3 strain under the conditions described above in materials and methods. Three dimensional structure of PQ10 protein that was predicted by I-TASSER server are shown in Figure 2.

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. Figure 3 shows the expression of PQ10 induced by IPTG 1mM in different times. There was a difference in quantitative expression of protein among 1- to 5 hours induced cells. The maximized protein expression was after 4 hours after cells induced. PQ10 recombinant protein was found mainly in the insoluble fraction of the cell lysate. Figure 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 150µg/ml by the Bradford assay. Western blotting was utilized to examine affinity of purified PQ10 protein against dog
sera. Western blots of purified PQ10 demonstrated that recombinant protein reacted with dog's sera (Figure 4).

**Checkerboard titration**

**Immunodassays with dog sera.** 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 2µg / ml concentration, with the dog sera diluted at 1/50 and the conjugate diluted at 1/10000 (Figure 5,6).

**Sensitivity and specificity determination of PQ10-Indirect ELISA.** According to the Table 1, the results showed that 6% out of 50 confirmed subjects, which were infected by *Leishmania infantum*, didn't react with PQ10 antigen and 14% out of 50 healthy subjects reacted with PQ10. The PQ10 showed reactivity with 1 sample from healthy sera and cross reaction was observed with 6 of samples collected from other infectious diseases. The results of antibody detection by DAT and PQ10-Indirect ELISA testing are compared in Table 1. The PQ10-Indirect ELISA test was sensitive (94%) and specific (86%) for the diagnosis of canine visceral leishmaniasis, with a PPV and a NPV of 87.03 and 93.47%, respectively. Additionally, PQ10 multiepitope protein was able to detect 92% of asymptomatic and 96% of symptomatic infected dog, which was confirmed with DAT.

From 20 serum samples that were collected from dogs with other infectious disease, six samples from dogs with Leptospirosis, Parvavirus infection and Distemper infection were false positive. The kappa index was calculated for PQ10 antigen to find the level of agreement with DAT. This index was calculated 0.800 that showed excellent agreement with DAT. The Youden's index is a single statistic that captures the performance of a dichotomous diagnostic test. The Youden J index for PQ10-ELISA test was calculated 0.8. The McNemar test result was calculated 0.344 that indicated with 95% confidence interval, there is no
significant difference between the results of DAT and PQ10-ELISA in the diagnosis of dogs that with infected with *Leishmania infantum*.

**Discussion**

Canine visceral leishmaniasis diagnosis should be performed as soon as possible, even on the basis of only a few or even a single clinical sign, to enhance the prediction of disease and to avoid both dog and human transmission and unnecessary euthanasia of apparently positive dogs (false positive dogs) (Gharbi et al., 2015). Because of the high proportion of asymptomatic dogs and the absence of obvious clinical signs, the diagnosis of infected dogs depends on laboratory diagnostic tests support (Ribeiro et al., 2018). The most of the antileishmanial drugs are toxic, thus an ideal diagnostic test should be able to differentiate between acute disease and asymptomatic infection (Srivastava et al., 2011). Unfortunately, at now, a method with satisfactory VL diagnosis efficiency is not yet available. For diagnosis and consequently disease control, the use of accurate methods that are cheap and easy to use in the field, is crucial (Mary et al., 1992).

Direct methods of diagnosis such as microscopy examination, culture, or the inoculation of hamsters with biopsy materials have limitations to detect parasites in dogs, thus anti-*Leishmania* antibodies are usually employed as a marker of infection (Dye et al., 1993; Quinnell et al., 1997).

The immunodiagnosis assays have become important alternatives for the above tests (Elmahallawy et al., 2014; Srivastava et al., 2011). Serological methods are powerful tools for diagnosis of visceral leishmaniasis (Faria et al., 2015) and in order to make a diagnosis of the visceral leishmaniasis, several serological techniques based on immunologic response such as direct agglutination test (DAT), indirect immunofluorescence test (IFAT) and ELISA have been broadly created (Seyyedtabaei et al., 2017).
The direct agglutination test (DAT) has long incubation times and requires some level of expertise. Immunofluorescent antibody test (IFAT) is a diagnostic method that commonly is used for canine visceral leishmaniasis detection, but this technique has main limitations such as cross-reactivity with other diseases and low sensitivity in identifying asymptomatic dogs (Mancianti et al., 1995). Enzyme-linked immunosorbent assay (ELISA) has been used as an appropriate diagnostic method for almost all infectious diseases, including leishmaniasis (Sundar and Rai, 2002) and this technique requires a highly specific antigen in order to capture a specific antibody (Sakkas et al., 2016). ELISA in contrast to IFAT is readily conformable to large-scale seroepidemiological researches and has demonstrated that can be at least as sensitive and specific as IFAT (Evans et al., 1990; Paranhos-Silva et al., 1996). On the other hand, it is reported that ELISA also has cross-reactivity with other canine infectious disease (Mancianti et al., 1996; Roffi et al., 1980).

Several different *Leishmania* antigens have been identified and characterized that has been used for the development of new enzymatic immunoassays (Faria et al., 2015). For example to detect subclinical and clinical infections, crude promastigote antigens are highly sensitive but have lower specificity than other techniques that use other antigens (Gomez-Ochoa et al., 2003; Rodríguez-Cortés et al., 2010). Because of easily adsorbed of recombinant proteins on several scaffold surfaces and yield reproducible results, they are considered as good candidate antigens for field diagnosis (Lauricella et al., 2016; Pattabhi et al., 2010; Santarém et al., 2010; Venturin et al., 2015). Among the recombinant proteins, rK39 was the most successful antigen, which has good efficacy to detect active canine visceral leishmaniasis and showing high sensitivity (90 to 100%) in studies from the Mediterranean basin and South America, but this protein has less sensitivity and specificity for detecting asymptomatic canine visceral leishmaniasis in contrast to other serological methods (Badaro et al., 1996; Gomez-Ochoa et al., 2003; Mettler
et al., 2005; Ozensoy et al., 1998; Rhalem et al., 1999; Santarém et al., 2010; Scalone et al., 2002). rKLO8 and rK26 are other antigens that could 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 with their combination which exhibited higher sensitivity (85%) and specificity (93%)(Abad et al., 2017).

To improve the detection of asymptomatic infected dogs, chimeric multi-epitope proteins are being designed, and here, we have also produced a multiepitope recombinant antigen, that according to previous researches(Faria et al., 2015) the main advantages of using it are low cost, easy automation in ELISA tests and the ability to detect circulating antibodies in the early phase of infection(Faria et al., 2015).

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 dogs. It provided values of 94% and 86% from sensitivity and specificity, respectively. A sensitive and specific antigen for the detection of asymptomatic infections would be highly desirable, because the important limitation in VL control is the inability to identify asymptomatic infections. The observation of false positives related to the cross-reaction samples (six samples from dogs with Leptospirosis, Distemper infection and Parvavirus infection) raises the suspicion of cross reaction with other infectious diseases. The PQ10 multiepitope protein was able to detect 92% of asymptomatic infected dog that is its advantage in performing control programs. The epidemiological, clinical, and laboratory aspects of canine visceral leishmaniasis are very variable, thus complete diagnosis and then treatment and control of the disease, especially due to the lack of more effective drugs and vaccines is difficult(Ribeiro et al., 2018). Here, we have used a recombinant synthetic protein to improve dog visceral leishmaniasis serodiagnosis. Our results showed that PQ10 could be useful for diagnosis of asymptomatic,
as well as, early phase of infections. It is necessary to perform more studies in more different target population of dogs in endemic area of disease to find accurate efficiency of using this recombinant protein.

Ethics

Informed consents were 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 Research Advisory Committee of Tarbiat Modares University 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.

Author Contributions

Study concept and design: Abdolhossein Dalimi, Mehdi Mohebali, Majid Pirestani and Farnoosh Jameie; perform, Analysis, interpretation of data, writing the manuscript and Statistical analysis: Farnoosh Jameie.

Conflict of Interest

The authors declare that they have no conflict of interests.

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Table 1. Diagnostic performance of anti-PQ10 indirect ELISA assay and comparison

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<th>PQ10 indirect ELISA</th>
<th>Positive ELISA result</th>
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<tr>
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<tr>
<td>Direct Agglutination Test</td>
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<td>DAT Positive sera</td>
<td>47</td>
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<td>3</td>
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<tr>
<td>DAT Negative sera</td>
<td>7</td>
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between ELISA using recombinant protein PQ10 (cut-off = 0.701) and DAT result.

PQ10 indirect ELISA had † (94%) sensitivity and ‡ (86%) specificity for *Leishmania infantum*
Figure 1. Schematic picture of pET-28-PQ10 structure.
Figure 2. Three dimensional structure of PQ10 protein.
Figure 3.
Figure 4. Nitrocellulose membrane after western blotting with anti-dog IgG. M: Molecular marker in kDa.

Figure 5. Checkerboard titration of different dilution of positive serum and different concentration of PQ10 antigen.
Figure 6. Checkerboard titration of different dilution of negative serum and different concentration of PQ10 antigen.

**Research Highlights**

- PQ10 is a suitable antigen for the detection of Visceral Leishmaniasis.

- PQ10 could be useful for diagnosis of asymptomatic, as well as, early phase of infections.

- PQ10 multiepitope protein was able to detect 92% of asymptomatic infected dogs that is its advantage in performing control programs.