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Original Article

Designing and Evaluation of a Recombinant Multiepitope Protein by Using ELISA for Diagnosis of *Leishmania infantum* Infected in Dogs

Parisa Yaghoubi [1], Mojgan Bandehpour [2,3], Mehdi Mohebali [4,5], Behnaz Akhoundi [4], *Bahram Kazemi [1,2,3]

1. Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
2. Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
3. Department of Biotechnology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
4. Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
5. Center for Research of Endemic Parasites of Iran, Tehran University of Medical Sciences, Tehran, Iran

Abstract
Background: Visceral leishmaniasis (VL) is the most severe form of leishmaniasis. Correct identification of infected patients and reservoirs is vital to control the spread of VL. One important step in the control of Zoonotic Visceral leishmaniasis (ZVL) is the identification of infected dogs, which are the main domestic reservoir hosts of *Leishmania infantum*. We aimed to prepare and evaluate a new recombinant antigen using Bioinformatics tools for diagnosis of ZVL in domestic dogs.

Methods: The present study was carried out in Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran during 2015- 2018. Three *L. infantum* (JPCM5 strain) proteins were analyzed as follows: Nucleotide sequences of the surface proteins, putative amastin-like surface protein (P1), surface antigen protein 2 precursor (P2) and surface antigen-like protein (P3). The epitopes were predicted by several different bioinformatics servers using different methods. The predicted epitopes were selected with the highest immunogenic potential (P1P2P3) linked to each other with linkers (Gly, Se) and synthesized. Then the expression and protein purification were performed. In total, 114 serum samples were collected at 7 months. Positive and negative sera were confirmed using direct agglutination test (DAT). These recombinant antigens from *L. infantum* were used by indirect ELISA.

Results: Considering the cut-off point of 0.23, the test showed a sensitivity of 98% (95%CI=89.50%-99.90%) and a specificity of 95.31% (95%CI=87.10%-98.72%). Kappa analysis indicated very good agreement (kappa=0.831) between ELISA and DAT (*P<0.05*).

Conclusion: ELISA using the recombinant protein P1P2P3 has great potential for the diagnosis of canine visceral leishmaniasis (CVL).

Introduction

*L*eishmaniasis is a complex of infectious diseases caused by protozoan parasites of the genus *Leishmania* and transmitted by Phlebotominae vectors. Visceral leishmaniasis (VL) is one of the most important clinical forms of leishmaniasis in humans as well as canines with high levels of morbidity and mortality. VL is usually caused by *L. donovani* and *L. infantum* (1). In Iran and the Middle East, VL is caused by *L. infantum*. Ardabil, East Azerbaijan, Fars, Bushehr, Kerman, Qom and North Khorasan provinces of Iran are endemic areas of VL (2,3).

Early and accurate diagnosis of VL is important to reduce mortality because the disease is fatal if left untreated (4). One of the important steps in control of canine visceral leishmaniasis (CVL) is the identification of infected dogs, which are the main reservoir of *L. infantum* (5).

The clinical symptoms of CVL vary from asymptomatic forms to restricted and deadly infection. Moreover, the incubation period lasts from several months to several years, depending on the virulence of the parasite and host genetic characteristics (6, 7). Thus, development of precise diagnostic methods for canine infection is essential for VL surveillance programs, as well as to understand immunological responses in resistant or susceptible animals (8, 9).

Improving serological tests for the diagnosis of VL is important because they are quick, easy, non-invasive, allow the screening of multiple samples and are capable of early detection, before the formation of lesions in VL (10). False-positive results especially in crude antigens are often seen in the sera of humans and dogs infected with *L. infantum*. Furthermore, the different strains of parasites and the protocols used for the preparation of crude antigens cause variations that can affect the sensitivity of this method (11).

Trying to obtain high sensitivities and specificities in testing, an alternative method is the use of multiepitope recombinant proteins. The use of synthetic peptides has low costs, high specificity and increases the features of safety tests relative to crude antigens. Bioinformatics and consequently identification of B-cell epitopes could play a major role in the development of synthetic vaccines, diagnostic tests and therapeutic products.

The present study reported for the first time the use of three surface antigens of *L. infantum* (P1,P2,P3).

Materials and Methods

The present study was carried out in Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran during 2015- 2018. Surface proteins are major targets of diagnosis research due to their utility as cellular markers and their extracellular accessibility for diagnosis intervention. The surface proteins comprising epitopes are potentially able to more interaction with B-cells than intracellular proteins, in addition to induce antibodies against them. For this reason, we chose three surface antigens. Three *L. infantum* [*JPCM5* strain] proteins were analyzed as follows: Nucleotide sequences of the surface proteins, putative amastin-like surface protein (P1) (GI: 134072905), surface antigen protein 2 precursor (P2) (GI: 339897242) and surface antigen-like protein (P3) (GI: 146076154), were obtained from the National Centre for Biotechnology Information (NCBI) Nucleotide Database (www.ncbi.nlm.nih.gov). The *L. infantum* JPCM5 strain was used in this study. The *L. infantum* JPC(MCAN/ES/98/LLM-724) strain was isolated in the WHO Collaborating Centre for Leishmaniasis, ISCIII, Madrid, Spain from the spleen of a naturally-infected dog resident in the area in 1998 (12).

Multiepitope protein prediction

To predict B-cell epitopes in the P1, P2, and P3 protein, the IEDB, BepiPred, ABCpred, BcePred, and CBTOPE web servers were used. Physicochemical properties were analyzed by using the ExPASy's ProtParam server (http://web.expasy.org/protparam/). As well as signal peptide were analyzed. Evaluation of the secondary structure was analyzed by SOPMA server. The predicted epitopes were analyzed and were selected as the highest immunogenic potential. For the P1, P2, and P3 proteins, 19, 46, and 48 amino acids respectively were selected and used for in silico concatenation. Afterwards, they were fused to each other with proper linkers. The recombinant protein P1P2P3 contains 148 amino acids. The tertiary structure of final construct was modeled by I-TASSER server. The predicted model was validated with ProSA, VERIFY3D and Ramachandran plot. The modeling quality values showed the quality of the modeled as satisfactory. The sequence was synthesized and cloned into the expression vector pET-28a by SBS Genetech Co. (China) to produce recombinant expression plasmid P1P2P3.

Expression and purification of the recombinant polypeptide

The recombinant plasmid was transformed into *E. coli* BL21 (DE3). The single colonies of *E. coli* BL21 (DE3) containing the recombinant plasmid were cultured overnight in Luria-Bertani broth with 30 µg/ml kanamycin in a shaker incubator (200 rpm) at 37 °C. Then it was subcultured in LB medium and incubated with a shaker at 37 °C. One mM of Isopropyl-β-D-thiogalactopyranoside (IPTG) was added when the OD600 was around 0.6-0.8 and cultures were grown for a further 2, 4 and 6 h at 37 °C with agitation (200 rpm). Bacterial cells were centrifuged at 8,000× g for 5 min. The induced cells were lysed in lysis buffer and sonication. The induced samples were analyzed by SDS-PAGE with 15% resolving gel, followed by Coomassie Brilliant Blue G-250 staining. Then the expression of recombinant protein was confirmed by Western blotting. The recombinant protein purification was carried out by Ni-NTA column as specified by the manufacturer's instructions (Novagen, USA). The purified recombinant protein was confirmed by Western blot. Protein concentration measured using the Bradford method.

Ethical approval

This study was approved by the Ethical Committee of Shahid Beheshti University of Medical Sciences (Ethical code no IR.SBMU.SM.REC.1394.66) in accordance with the Helsinki Declaration.

Samples

For ELISA assays 100 serum samples of two different clinical groups from the School of Public Health, Tehran University of Medical Sciences, Tehran, Iran were prepared. Fifty sera from *L. infantum* naturally infected dogs and 50 sera of healthy dogs (control group) were used. Additionally, in order to evaluate cross-reactivity on ELISA assays, we also used 14 samples of dogs sera infected by other infections including cutaneous leishmaniasis (n=3), toxocariasis (n=3), babesiosis (n=2), toxoplasmosis (n=2), brucellosis (n=1), and pneumonia (n=3) collected from the Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran. All collected samples were tested by using DAT (Gold standard) in the leishmaniasis Laboratory of the School of Public Health, Tehran University of Medical Sciences, Tehran, Iran for Anti-*Leishmania* antibodies. In total, 114 serum samples were collected during 7 months. The infected and uninfected dogs were confirmed by DAT (13, 14).

ELISA

In order to get the best results from ELISA assay, all ELISA procedures were optimized with regard to antigen concentrations, dilu-

tions of sera and conjugated antibody by checkerboard titration. The wells of 96-well ELISA flat bottom microplates (Greiner) were coated with 1 µg/well of the purified protein diluted in 100 µl PBS buffer and incubated overnight at 4 °C. All the samples were analyzed by ELISA (15).

The optical density (OD) was measured at 450 nm using ELISA reader. Positive and negative serum samples of Anti-*Leishmania infantum* IgG antibodies were tested by using Indirect ELISA method. The results of ELISA method were analyzed by statistical analysis.

Statistical analysis

All the statistical analyses were processed with GraphPad Prism release 7.0, and SPSS (Chicago, IL, USA) software version 16.0. A cut off point for optimal sensitivity and specificity was determined using ROC analysis. The cut-off was obtained according to the point, which provides the maximum of sensitivity and specificity (16). Test was calculated for sensitivity (SE), specificity (SP), positive predictive value (PPV), negative predictive value (NPV), area under curve (AUC), accuracy, validity, and Kappa. Kappa values were determined with a confidence interval of 95% to determine the degree of agreement between the ELISA result in comparison to DAT. Kappa values classified according to the Fleiss scale: 0.00–0.20, poor; 0.21–0.40, fair; 0.41–0.60, moderate; 0. 61–0.80, good; 0.81–0.99, very good and 1.00, perfect. A *P*-value of less than 0.05 was considered to indicate statistical significance.

Results

Based on bioinformatics analysis, B-cell linear and conformational epitopes of P1, P2, and P3 antigens were performed. Evaluations of the Secondary & Tertiary Structure were analyzed by several servers (Fig. 1). The recombinant plasmid was confirmed by PCR and restriction enzyme digestion (Fig. 2). In SDS-PAGE of recombinant protein a band of approximately 22 kDa was detected, the recombinant protein and purified recombinant protein were confirmed by Western blot (Fig. 3). All sera were tested for the presence of anti-leishmanias IgG antibodies by polyantigenic protein (Table 1,2). The results of ELISA were analyzed by statistical analysis.
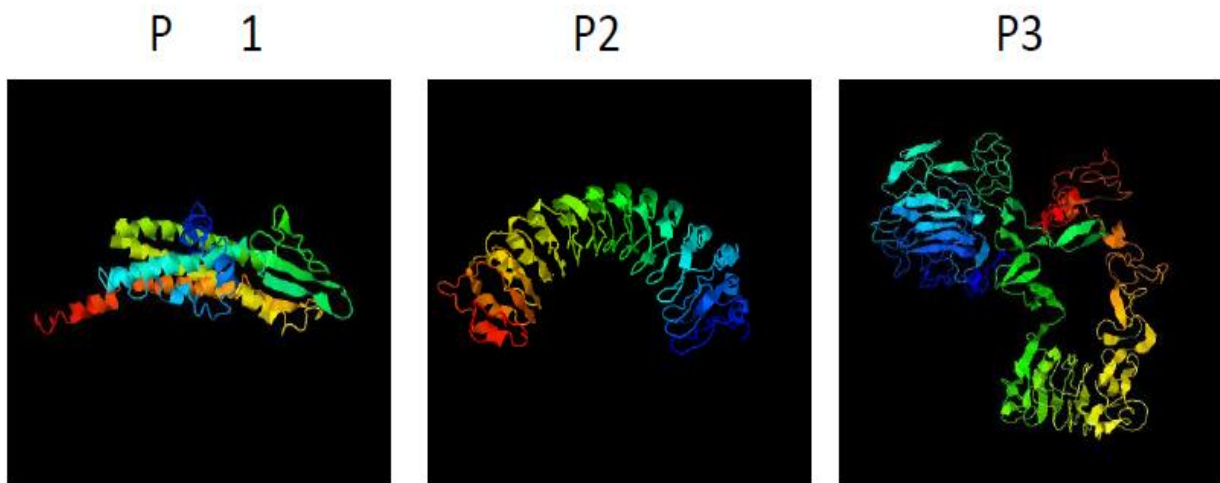

Fig. 1a: Tertiary structure B-cell constructs proteins P1, P2, and P3 predicted by I-TASSER server
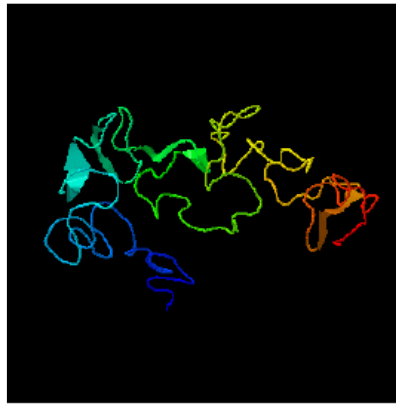
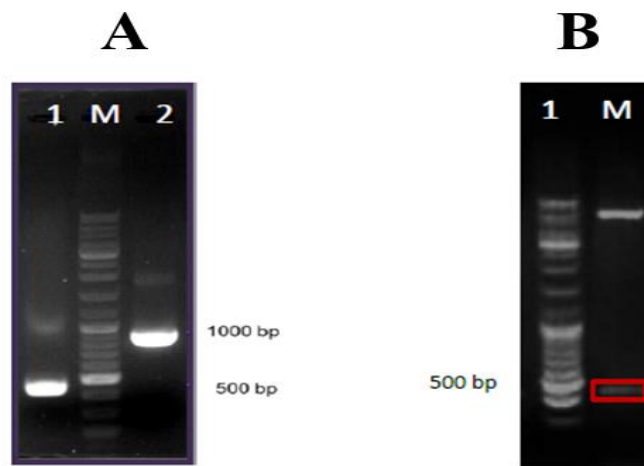**Fig. 1b:** Tertiary structure B-cell construct Recombinant protein P1 P2 P3 predicted by I-TASSER server

Fig. 2: A: Confirmation of the recombinant plasmid by the PCR. Lane 1, PCR product without fragment plasmid; Lane M, DNA1kb marker; Lane 2, PCR product; **B**: Confirmation of the recombinant plasmid by restriction enzyme; Lane 1: PET28a / P1P2P3 plasmid digested with Sac1 and HindIII enzymes; Lane M, DNA1kb marker
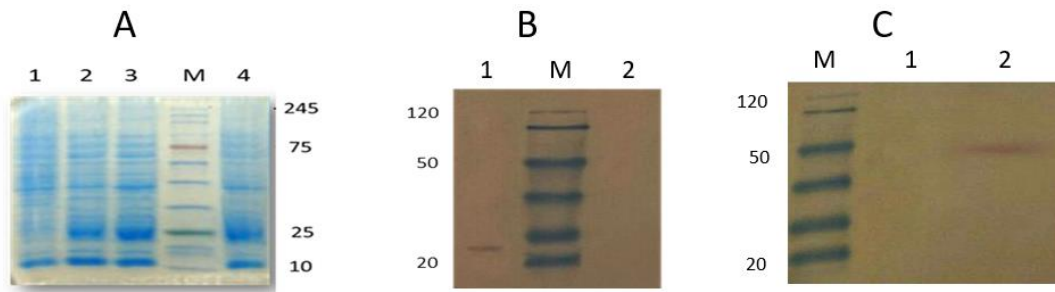

Fig. 3: A: SDS-PAGE of recombinant protein P1P2P3 : lane 1, BL21 (negative control); lane 2, induced cells for 2 h; lane3, induced cells for 4 h; Lane M, protein size marker (SMOBIO); lane 4, induced cells for 6 h by 1 mM IPTG was separated in 15% SDS-PAGE. **B**: Western blot analysis of recombinant protein P1P2P3 with anti His-tag antibody. Lane 1 recombinant protein; Lane M, protein size marker (Fermentas); Lane 2, BL21 (negative control). **C**: Western blot analysis of recombinant purified protein P1P2P3 with anti His-tag antibody. Lane M, protein size marker (Fermentas); Lane 1, BL21 (negative control); Lane 2, recombinant purified protein

Table 1: Results of positive serum for anti-*Leishmania* IgG antibody using recombinant P1P2P3 antigen

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.20	0.49	0.26	0.45	0.57	0.69	0.61	0.91	0.81	0.70	0.54	0.69
B	0.24	0.82	0.75	0.62	0.51	0.55	0.81	0.70	0.47	0.95	0.41	0.79
C	0.25	0.81	0.79	0.42	0.72	0.77	0.43	0.72	0.82	0.42	0.54	0.61
D	0.52	0.61	0.46	0.98	0.52	0.41	0.64	0.75	0.55	0.98	0.59	0.99
E	0.51	0.39									0.03	0.04

A1-E2: positive serums
E11, E12: blank

Table 2: Results of negative serum for anti-*leishmania* IgG antibody using recombinant P1P2P3 antigen

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.01	0.17	0.14	0.03	0.05	0.14	0.15	0.02	0.19	0.20	0.16	0.18
B	0.07	0.02	0.17	0.07	0.20	0.17	0.23	0.18	0.01	0.21	0.19	0.17
C	0.12	0.17	0.05	0.01	0.12	0.01	0.02	0.17	0.19	0.14	0.15	0.19
D	0.19	0.09	0.24	0.09	0.04	0.06	0.18	0.14	0.24	0.15	0.20	0.12
E	0.24	0.05	0.12	0.15	0.02	0.04	0.18	0.05	0.14	0.20	0.19	0.02
F	0.07	0.09	0.17	0.14							0.02	0.05

A1-E2: negative serums
E3-F4: negative serums (other infections)
F11, F12: blank

Serum samples were tested by ELISA, considering the cut-off point of 0.23, the test showed a sensitivity of 98% (95%CI=89.50%-99.90%) and a specificity of 95.31% (95%CI=87.10%-98.72%) The Indirect ELISA test with the 95% CI, showed PPV(94.2), NPV(98.4), Accuracy(96.5), and Validity(96.7). The results of comparison of ELISA tests with recombinant antigens against DAT test as a standard method can be seen in the (Table 3).Statistical analysis of the data showed the very good agreement (kappa=0.831) between ELISA and DAT. Considering this cut-off value, 49 samples of 50 cases were diagnosed correctly as positive and one was negative. Moreover, 61 samples of 64 cases of control and other infection groups were diagnosed as negative and three were positive by ELISA.

Table 3: Comparision of results between direct agglutination test (DAT) (1: 80 threshold) and ELISA of recombinant antigen on 114 dog serum samples in the diagnosis of *Leishmania infantum* infection in dogs

	ELISA		
DAT	Positive N(%)	Negative N(%)	Total N(%)
Positive($\geq$ 1:320)	49 (94.2)	1(1.6)	50 (43.9)
Negative(<1:80)	3 (5.8)	61 (98.4)	64 (56.1)
Total	52 (100)	62 (100)	114 (100)

Discussion

The early and correct diagnosis of leishmaniasis is essential for disease control and treatment. Important step in the control of VL is the identification of infected dogs, which are the main domestic reservoir of *L. infantum* (17, 18). Serological methods are strong tools in CVL diagnosis that often used for canine mass screening (19, 20).

When crude antigens are used, Increases the number of false-positive results and cross-reactivity with other canine pathogens (21, 22). The use of native antigens for diagnostic tests is expensive, time-consuming, and has many restrictions. To control these limitations recombinant antigen by using bioinformatics tools can standardize diagnostic methods. An alternative method to resolve these limitations is peptide synthesis by using bioinformatics tools. In recent years synthetic peptides used as antigens have shown high sensitivity and specificity as well as decrease in cross-reactivity (23-25).

Sensitivities and specificities found using synthetic peptides or recombinant proteins in ELISA were, respectively, 88% and 95% (20), 75% and 90% (26), 72.7% and 87.3% (27), and 100% and 98% (28).

In this study for the first time, a predicted recombinant protein from *L.infantem* B-cell epitopes of three surface antigens (P1, P2, and P3) was successfully expressed and analyzed for CVL diagnos. We chose surface proteins because they contain epitopes that are potentially able to more interaction with B-cells than intracellular proteins and to induce antibodies against them. Appropriate levels of sensitivity and specificity are especially desirable to avoid false-negative and false-positive reactions. This problem can lead to disease transmission and unnecessary euthanasia of healthy dogs (29).

Several online software analysis methods such as IEDB, BepiPred, ABCpred, BcePred and CBTOPE were used to predict B-cell epitopes. Moreover the tertiary structure of the recombinant protein was predicted by I-TASSER server. Peptides that included regions rich in B-cell epitopes and had higher antigenicity were considered. Finally, to achieve the best immunization in construct, selected epitopes were fused to each other with appropriate linkers (Gly, Ser). The sequence was synthesized, and then cloned into the expression vector pET-28a to produce recombinant expression plasmid P1P2P3. Multiepitope recombinant plasmid was verified by PCR and restriction enzyme digestion. Afterward expression and purification of protein performed and the purified recombinant protein confirmed by Western blot. Potential of recombinant protein as diagnostic antigens for CVL was assessed by ELISA.

Statistical analysis of the data showed very good agreement (kappa=0.831) between ELISA and DAT. This finding showed different and higher density of immunodominant epitopes of recombinant protein P1P2P3 causing more sensitivity and better assay reproducibility.

Conclusion

We have produced and validated new recombinant antigens from *L.infantem* B-cell epitopes for CVL diagnosis. Our work suggested that synthetic peptide-based ELISA strategy may be useful for the development of a sensitive and highly specific serodiagnosis for CVL.

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Research Center, Department of Medical Parasitology and Mycology of Shahid Beheshti University of Medical Sciences, and leishmaniasis laboratory of the School of Public Health, Tehran University of Medical Sciences.

Conflict of interest

The authors declare that there is no conflict of interest.

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