

ORIGINAL ARTICLE

Importance of *L. Infantum* H2B Recombinant Antigen for Serodiagnosis of Visceral Leishmaniasis

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ABSTRACT

Background: Visceral leishmaniasis (VL) can lead to death in more than 95% of cases if left untreated. Accurate and early diagnosis has an important role in reducing mortality rate of this disease. **Objective:** To express recombinant H2B antigen from an Iranian isolate of *Leishmania Infantum* and evaluate its efficacy in the diagnosis of VL. **Methods:** The recombinant H2B antigen was produced in a prokaryotic system, and its efficacy for VL diagnosis was evaluated by ELISA. The serum samples from 80 VL patients, 100 individuals from endemic and non-endemic regions of VL, and 58 non-VL patients were collected. VL cases were confirmed based on the clinical sign, positive IFAT (>64), real time PCR, and response to treatment. **Results:** The H2B gene sequence of the Iranian *L. infantum* isolate had about 4% diversity in comparison with the H2B gene of the *L. infantum* counterpart. ELISA, using the produced H2B recombinant antigen, showed sensitivity of 71.25% (95% CI: 60.05%-80.82%) and specificity of 69.62% (95% CI: 61.81%-76.68%) regarding VL diagnosis. **Conclusion:** Recombinant H2B antigen expressed in the prokaryotic system had suboptimal performance for the serological diagnosis of VL. It seems that the production and expression of recombinant H2B antigen in a eukaryotic system may enhance the performance of this antigen in the diagnosis of VL in Iran.

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INTRODUCTION

Visceral leishmaniasis (VL), caused by the *Leishmania donovani* complex, is a parasitic infectious disease that, in the absence of treatment, can lead to death in symptomatic patients (1). In Iran, VL has been reported in most provinces, predominantly in the north (Ardebil, East Azerbaijan, and North Khorasan provinces) and in the south (Fars province) (2). In a hospital-based study, a total of 380 VL cases were recorded during a 16-year period in Fars province, southwestern Iran. The majority of the cases (91.5%) were <5 years old (3). A definitive diagnosis of VL is based on the observation of parasites in samples obtained from the liver, spleen, or bone marrow (4). Invasive, painful and not having satisfactory sensitivity, this diagnostic procedure is unsuitable for the diagnosis of VL (5-6). A high level of antibodies, usually generated during the VL infection, can serve as a good marker in the serologic diagnosis of this disease (7). So far, several serological methods, including Direct Agglutination Test (DAT), IFA, and ELISA, have been developed with different antigens for the diagnosis of VL (8-10). However, these tests are not easy to routinely perform, are time-consuming, and require some special equipment. In 1993, Burn *et al.* introduced the K39 antigen for the serological diagnosis of VL. They used an ELISA system with a sensitivity of 92.8% and 100% for Brazilian and Sudanese VL patients, respectively (11). Later, different brands of immunochromatographic test (ICT) were manufactured based on the K39 antigen (6). Contrary to other serological tests such as IFA, ELISA, and immunoblotting, ICTs are simple, quick and reliable assays (12-13). Nevertheless, the sensitivity of the ICTs vary throughout different regions of the world (14). A previous study, conducted in southern Iran, reported a sensitivity of 82% for the InBios ICT regarding the diagnosis of VL (15). Also, the diagnosis of all VL patients using a single recombinant antigen in the serological assay was reported to be unlikely (16-18). The results of several previous studies have shown that histone antigens are highly immunogenic and can serve as a suitable diagnostic antigen for VL diagnosis (19-21). Present in both promastigote and amastigote forms of the parasite, *Leishmania* H2B antigen is a small protein with 12 kDa weight. A previous study reported a sensitivity of 100% and specificity of 92% for recombinant H2B antigen in the diagnosis of VL (21). The current study aimed to express recombinant H2B antigen from an Iranian isolate of *L. infantum* and evaluate its efficacy in terms of VL diagnosis.

MATERIALS AND METHODS

Parasite. An Iranian strain of *L. infantum* (MCAN/IR/14/M14), obtained from a domestic dog in Meshkin-Shahr area in northwestern Iran in 2015, was used in this study. Promastigotes were cultured in RPMI 1640 medium (Gibco) supplemented with 15% heat-inactivated fetal calf serum (FCS). They were kept at 25 °C until a stationary phase was reached.

Isolation of DNA and Amplification of H2B gene of *L. infantum*. Pellets of 10^8 *L. infantum* promastigotes were centrifuged, suspended in 250 µl of PBS, and re-suspended in a tube containing 250 µl of TNNT buffer (0.5% Tween 20, 0.5% Nonidet P-40, 10 mM NaOH, 10 mM Tris, pH 7.2). Next, they were supplemented with 10 µl of proteinase K (10 µg/ml) and incubated at 56°C overnight. DNA extraction was performed through the use of phenol-chloroform method. The extracted DNA was

dissolved in 50 μ l of TE buffer and stored at -20°C until use. The H2B-596 forward (5'-CTTCATCAAACCTTCCTAT-3') and H2B-596 reverse (5'-AAGAAACAAGAAATCTCTAT-3') primers were designed for the amplification of a 596 bp fragment of the H2B gene by PCR. For PCR amplification, we prepared the final volume of 25 μ l reaction, containing 1X Taq DNA polymerase master mix RED (Amplicon), 10 pmol of each primer, and 10 ng of total genomic DNA. The thermal cycle program was set as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 5 min in Eppendorf thermocycler. PCR products were separated by electrophoresis on 1.5% agarose gel and excised from the agarose gel and purified using a QIAquick® Gel Extraction Kit according to the manufacturer's instructions.

Cloning of the *L. infantum* H2B Gene. Purified PCR product was cloned into pTZ57R/T vector using InsTAclone™ PCR Cloning Kit based on the manufacturer's instructions. Via heat shock procedure, 1 μ l of the ligated product was transformed into *Escherichia coli* DH5 α competent cells, suspended in 500 μ l of Luria Bertani (LB) broth, and incubated for 1 hour at 37°C . Afterwards, it was plated on LB agar and supplemented with ampicillin (100 $\mu\text{g}/\text{mL}$), X-gal (20 mg/mL) and IPTG (100 mM) at 37°C overnight. Multiple white colonies were selected for PCR colony using the H2B-596 forward and reverse primers. Plasmid preparations from an overnight culture of these white colonies were transferred for DNA sequencing using vector universal (M₁₃uni-43 and M₁₃rev-29) primers.

Sequence Analysis and Sub-cloning of the H2B Gene into an Expression Vector. H2B sequence with antigenic property was 336 bp, located within the 596 bp sequence. Sequence analysis was performed using the CLC sequence viewer. A BLAST analysis was done in order to compare the Iranian *L. infantum* H2B sequence with that of available *Leishmania* spp. H2B sequences in the GenBank. The 336 bp sequence of H2B sequence, with antigenic properties, was used for sequence analysis, codon optimization and subsequent subcloning into the PET28b+ expression vector containing a C-terminal hexahistidine tag tail for affinity purification. Subcloning was performed by Biomatik Company (Biomatik, Ontario, Canada).

Expression and Purification of *L. infantum* H2B Recombinant Antigen. The recombinant plasmid was transformed into competent cells of *E. coli* BL21 (DE3). Following transformation, the cells were cultured on LB agar medium containing 50 mg/L kanamycin. A single colony of the recombinant bacteria was inoculated in 5 ml of LB broth supplemented with 100 $\mu\text{g}/\text{ml}$ of kanamycin in a shaking incubator at 30°C overnight. Five ml of the culture was inoculated into 200 ml of LB broth and placed in a shaking incubator at 30°C until an optical density (OD₆₀₀) of 0.6 was reached. Protein expression induction was performed through adding 0.1 mM isopropyl β -d-1-thiogalactopyranoside (IPTG, Thermo Scientific, Waltham, MA) for 3 hours at 30°C in a shaking incubator. The recombinant bacterial cells were then harvested by centrifugation for 10 min at 7500 g and 4°C ; the pellet was suspended in 6 ml of lysis buffer (20 mM phosphate buffer pH 7.4 and 8 M urea), supplemented with a protease inhibitor cocktail (Roche Diagnostics, Deutschland GMBH, Mannheim, Germany) and sonicated (5 \times 30 s) on ice. Later, the lysed cell was centrifuged for 30 min at 15000 g and 4°C . Subsequently, the supernatant was applied onto the equilibrated 2 ml Ni-NTA agarose column. After two steps of washing by different concentrations of imidazole (10 mM and 20 mM) in phosphate buffer, bound proteins were eluted using a high

concentration of imidazole (250 mM) in phosphate buffer. Bradford assay was utilized to determine the concentration of the eluted protein.

SDS-PAGE and Western Blotting. The presence of the purified recombinant H2B protein was tested by SDS-PAGE and confirmed via Western blotting. Briefly, the protein was separated on 15% polyacrylamide gels under reducing conditions; subsequently, it was stained by Coomassie Brilliant Blue (G 250, Merck) and transferred onto a 0.45- μ m nitrocellulose membrane (Bio-Rad), blocked with 5% non-fat dry milk in Tris-buffered saline (TBS; 0.1 M Tris-HCl, pH 7.5, 2.5 M NaCl); incubation was then performed with anti-his-tag alkaline phosphatase conjugate (Bio-Rad, Cat No. 1396A). The membrane was washed with TBS containing 0.05% Tween-20 and incubated with substrate solution and developer ((0.1 M Tris, pH 9.5, 0.1 M NaCl, and 5 mM MgCl₂), NBT and BCIP). The reaction was stopped with H₂O.

Human Serum Samples. A total of 238 serum samples obtained from 80 VL patients, 50 endemics, 50 non-endemic healthy controls, and 58 non-VL patients were utilized in this study. VL patients comprised 80 children (53% male and 47% female) with an age range of 6 months to 16 years; all subjects had a history of 14-day fever, 90% with hepatosplenomegaly and 95% with anemia. The patients were from endemic regions and admitted to Namazee Hospital, Shiraz University of Medical Sciences, Shiraz, Iran. The subjects had a positive IFAT titer (>64), all cured and afebrile following treatment with antimonial or amphotericin B therapy. Moreover, decreased spleen size was observed at the end of the treatment, and the VL cases were further confirmed by real time PCR. To evaluate the probability of cross-reactivity with other diseases, we also included sera from the patients previously diagnosed with other diseases, including cutaneous leishmaniasis (n=11), malaria (n=11), toxoplasmosis (n=10), fascioliasis (n=10), hydatidosis (n=10), toxocariasis (n=1), scleroderma (n=1), hymenolepiasis (n=1), systemic lupus erythematosus (n=1), myocardial infarction (n=1), and purpura (n=1). Further included were the sera obtained from 100 endemic (from Sarmashhad area, the known focus of VL in southern Iran) and non-endemic healthy individuals (healthy subjects attending different laboratories in Shiraz, capital of Fars province for routine checkup). These sera were all negative by IFA (titer<64). This study received ethical approval from the Ethics Committee of Shiraz University of Medical Sciences (SUMS), Shiraz, Iran (ethical reference: IR.SUMS.REC.1396.S494).

ELISA Using *L. infantum* rH2B Antigen. 96-well flat-bottom microplates (Maxi binding, SPL Life Sciences, Eumhyeon, South Korea) were coated with 0.5 μ g/ml of rH2B in 0.1 M carbonate/bicarbonate buffer (pH 9.6), overnight at 4°C. Each well was blocked with 400 μ l of 5% skimmed milk in phosphate-buffered saline for 2 hours at room temperature. Diluted serum (100 μ l of 1:150 dilution in PBS containing skimmed milk) was added to each well and incubated for 1 hour at ambient temperature. The wells were then washed three times with 350 μ l of PBS-Tween (0.01 M, pH 7.4). Afterwards, 100 μ l of horseradish peroxidase-conjugated goat anti-human IgG (Jackson ImmunoResearch), at 1:40,000 dilution in PBS-Tween (0.01 M, pH 7.4), was applied to the wells and incubated for 1 hour at ambient temperature. After five times washing as before, 100 μ l/well of substrate solution (TMB; Thermo Scientific) was added. The reaction was stopped with 100 μ l/well of 1 N H₂SO₄. The absorbance was measured at 450 nm and 630 nm with a microplate reader (Epoch, BioTek Instrument Inc, USA).

Statistical Analysis. The optical density of *L. infantum* rH2B-ELISA results was employed to construct a receiver-operator characteristic (ROC) curve in GraphPad Prism and calculate the sensitivity and specificity for different cut off points. The

concordance rate between *L. infantum* rH2B-ELISA and IFA was determined through calculating Kappa (k) and interpreted, according to reported literature (22-23), that is, negligible, weak, moderate, good and excellent if the k value is 0.00–0.20, 0.21–0.40, 0.41–0.60, 0.61–0.80 and 0.81–1.00, respectively. All the calculations were estimated with a 95% confidence interval.

RESULTS

Amplification and Sequencing of *L. infantum* H2B Gene.

PCR amplification of the coding region for *L. infantum* H2B gene using H2B-596 forward and reverse primers resulted in a 596 bp size product (Figure 1A). Sequencing results revealed that the 336 bp of *L. infantum* H2B sequence had 96.36% identity with 336 bp of H2B available sequence for *L. infantum* in NCBI database reported by Soto *et al.* (19). When this sequence was translated to protein, the Iranian *L. infantum* H2B sequence revealed 5 amino acid differences (4.5%) in comparison with *L. infantum* (LEM75, zymodem1) H2B sequences of Soto *et al.* (Figure 1B).

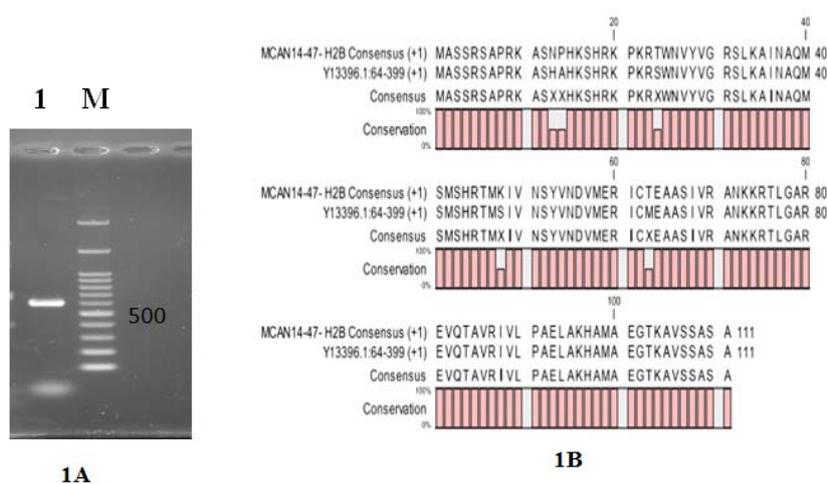


Figure 1. 1A: lane 1: the amplified H2B gene (596 bp) from promastigotes of an Iranian isolate of *L. infantum*. M: 1 kb molecular weight marker. **1B:** Amino acid alignment of the corresponding fragment of the translated reference Y13396 (19) with the sequences from the H2B sequence from Iranian isolate of *L. infantum*.

Expression and Purification of rH2B Antigen.

H2B sequence of 336bp size was synthesized and subcloned into the pET28b+ expression vector. The cloned gene, encoding a protein of 111 amino acid residues with approximately 13 kDa weight protein, was purified using Ni-NTA resin. The presence of protein and its purity were confirmed by SDS-PAGE analysis and subsequent Coomassie blue staining (Figure 2A). Final confirmation was made by Western blotting using an anti-his-tag antibody (Figure 2B).

Diagnostic Performance of the Li-rH2B in ELISA.

The diagnostic performance of Li-rH2B antigen was assessed in ELISA with sera belonging to 80 VL patients, 50 endemic healthy controls, 50 non-endemic healthy controls, and 58 non-VL patients (Table 1).

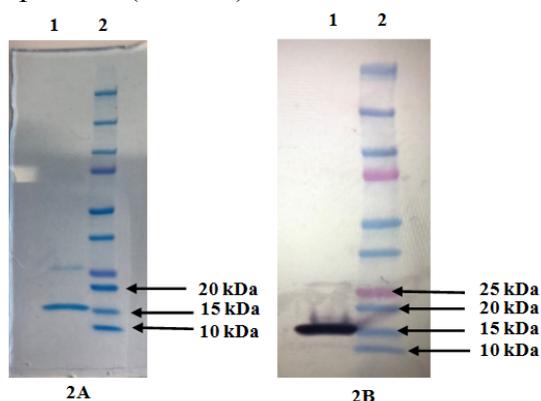


Figure 2. SDS-PAGE and Western blotting showing the rH2B protein after the purification on the Ni-NTA. Panel 2A: SDS-PAGE and Coomassie blue-stained gel; lane 1: eluted rH2B protein; lane 2: 10-245 kDa protein marker. 2B: Western blotting; lane 1: rH2B eluted fraction, lane 2: 10-245 kDa protein marker.

With IFAT as a reference test and O.D. 0.765 cut-off corresponding with the highest Youden index ($J = \text{sensitivity} + \text{specificity} - 1$), the *Li-rH2B* antigen showed a sensitivity of 71.25% (95% CI: 60.05% - 80.82%) and a specificity of 69.62% (95% CI: 61.81% - 76.68%), yielding an AUC of 0.739 (Figure 3).

Table 1. ELISA results of the different groups of patients and healthy subjects, using Li-rH2B antigen.

Sera Samples	Positive		Negative		Total
	No.	Percent	No.	Percent	
VL patients	56	70	24	30	80
Endemic healthy controls	16	32	34	68	50
Non-endemic healthy controls	13	26	37	74	50
Non-VL patients	19	32.76	39	67.24	58

Of the endemic, non-endemic and non-VL patients, 16, 13 and 19 individuals, respectively, had a positive ELISA results. Of the 19 non-VL patients, with a positive reaction in ELISA, seven were cutaneous leishmaniasis, six were malaria, four were toxoplasmosis, and two were hydatidosis patients. The agreement between Li-rH2B ELISA and IFA was weak ($k=0.37$).

DISCUSSION

Visceral leishmaniasis can lead to death in more than 95% of cases if left untreated. Therefore, accurate and prompt diagnosis of the disease has a special role in the treatment, control, prevention and reduction of corresponding morbidity and mortality (13). The main diagnostic method for VL is serological methods including DAT, IFAT, ELISA, and immunoblotting which have some limitations (4,24-25).

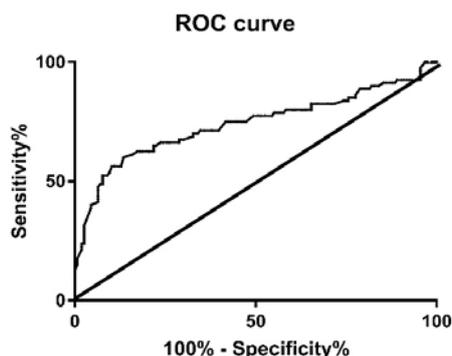


Figure 3. Receiver operator characteristic curve constructed from ELISA results.

Another serological test used in endemic areas of the disease is rapid diagnostic tests based on several recombinant antigens of the VL causing agents. Such tests, unlike other serological tests, are fast and simple, and require no special equipment. One of these common rapid diagnostic tests is based on the recombinant K39 antigen (11). Depending on the geographical distribution of VL, previous studies have shown significant differences in the sensitivity and specificity of rK39-based, DAT and rK26 diagnostic, for the diagnosis of VL (8-10,14). The reported sensitivities for immunochromatographic assay, using rK39 antigen are between 67.6% to 100% and the specificities are between 59% to 100% (14). In 2006, a study conducted in the south of Iran, where the present study has been conducted, revealed the sensitivity and specificity of the InBios brand of VL rapid diagnostic test to be 82.4% and 100%, respectively (15). Thus, other tests such as IFAT and real-time PCR which are both pricey and time-consuming have to be done to diagnose all patients. Results of a few studies have shown that *Leishmania* histone antigens can stimulate the immune system of the VL patients and might be a suitable candidate for the diagnosis of VL (17,26). In the present study, the H2B antigen of Iranian isolate of *L. infantum* was expressed in bacteria and showed a sensitivity and specificity of 71.25% and 69.62%, respectively in the diagnosis of VL. In 1999, Soto *et al.* produced the rH2B antigen of *L. infantum* in *E. coli* with a reported sensitivity and specificity of 63% and 100%, respectively for the diagnosis of canine VL (19). Maalej *et al.*, produced *L. infantum* rH2B antigen in *E. coli* with a reported sensitivity and specificity of 100% and 92%, for the diagnosis of human VL (21). Lakhali *et al.*, utilized the crude histone of *L. infantum* for serological diagnosis of VL with a reported sensitivity and specificity of 97.6% and 100% (20). Recombinant protein can be produced in prokaryotic or eukaryotic organisms with their advantages and drawbacks (27). A major drawback of the heterologous recombinant expression

system is codon bias and lack of post-translational modifications process (28). It should be noted that previous studies have shown that *Leishmania* histone proteins undergo post-translational modifications such as acetylation, methylation, and phosphorylation (29-30). These post-translation modifications occur on N-terminal part of the *Leishmania* histone protein and interestingly, Soto *et al.*, by producing small peptide fragments of *Leishmania* histone protein, demonstrated that the N-terminal domain of the *Leishmania* histone protein has more antigenic properties than other parts of the protein (31-32) which can influence both sensitivity and specificity of this antigen in serological diagnosis of VL. Therefore, it seems that the extraction of *Leishmania* crude histone proteins or the expression of each histone, separately in a eukaryotic system, will considerably improve their diagnostic efficacy. Therefore, it seems that the lack of post-translational modification of this antigen in the prokaryotic expression system has affected both the sensitivity and specificity of the rH2B-ELISA. In one hand, it reduces the antigenic activity and, as a result, reduces the sensitivity of the rH2B-ELISA in the diagnosis of all cases of VL, and on the other hand, it provide an antigenic structure that reacts with a wide range of general antibodies, present in the sera of some healthy individuals or non-VL patients. However, it should be noted that a significant percentage of people living in the endemic area of VL of the present study have had antibodies against *Leishmania* antigens (33). Seventeen individuals suffering from cutaneous leishmaniasis, malaria, and toxoplasmosis in the present study showed a positive result in ELISA. Cross reactivity between the toxoplasmosis, cutaneous leishmaniasis and malaria infection and *L. infantum* antigens has previously been documented (34). However, the probability of past infection, asymptomatic infection and pre-clinical VL status of these patients should not be ignored. Meanwhile, it should not be forgotten that the other causative agent of VL in Iran is *L. tropica*, which mainly cause CL in Iran (35-36). A higher specificity (92%) for rH2B antigen has been reported in Maalej *et al.* study (21). One of the differences between our study and their study is the difference between the populations of non-VL patients. In our study, most of the non-VL patients were with parasitic diseases while in their study only one parasitic disease (i.e., hydatidosis) was used to calculate the specificity, and the rest of the non-VL patients suffered from diseases other than parasitic disease. In conclusion, findings of the current study revealed that the recombinant H2B antigen expressed in the prokaryotic system has a substantial low sensitivity and specificity resulted in a suboptimal performance for the serological diagnosis of VL in Iran. It seems that the production and expression of recombinant H2B antigens in a eukaryotic system may increase the performance of this antigen in the diagnosis of VL in Iran. Moreover, co-expression of the H2B along with other antigens, particularly rK39 and rK26, may improve the diagnostic efficacy of these antigens for the diagnosis of VL.

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