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Original Article

Preparation of a K39sub Recombinant Antigen for the Detection of *Leishmania infantum* Antibodies in Human: a Comparative Study with an Immunochromatographic Test and Direct Agglutination

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Abstract

Background: The Mediterranean type of kala-azar is occurred in different parts of Iran and caused by *Leishmania infantum*. A rapid and valid test for early detection of visceral leishmaniasis in human would be highly desirable because it could decrease mortality rate of the disease. In this study, we aimed to compare the results of K39sub antigen with an commercial immunochromatographic dipstick rk39 test (Cypress Diagnostic Company, Belgium) for early detection of *L. infantum* infection in human.

Methods: K39sub recombinant antigen of *L. infantum* LON49 was expressed in prokaryotic system and evaluated for the diagnosis of human visceral leishmaniasis. This study evaluated the performance of recombinant K39sub antigen by ELISA and an commercial immunochromatographic dipstick rk39 test for the detection of *L. infantum* antibodies in 43 clinically infected patients with direct agglutination test (DAT) at a 1: 3200 cut off titer and higher. Controls included 69 healthy volunteers and 28 patients with other diseases including malaria (n=5), tuberculosis (n= 3), toxoplasmosis (n= 4), cystic hydatidosis (n= 5) and cutaneous leishmaniasis (n= 11).

Results: The sensitivity of the K39sub antigen and an immunochromatographic dipstick rk39 test was 90.7%, and 97.7%, respectively, while the specificity was 95.6% and 97.9%, correspondingly. A good concordance was found between k39sub antigen and commercial dipstick rk39 strips (k= 96.4%).

Conclusion: The accuracy of the K39sub antigen in the detection of *L. infantum* antibodies in human infection is confirmed.

Keywords: *K39sub Antigen, Serodiagnosis, Commercial dipstick rk39, Direct agglutination test, Human visceral leishmaniasis*

Introduction

Protozoan parasites of the genus *Leishmania* are widely distributed and transmitted by the bite of sandflies (1). In the vertebrate host, the infecting promastigotes differentiate into and replicate as amastigotes within macrophages (1). Depending on the species of *Leishmania*, infection can lead to a variety of manifestations including fever, splenomegaly, hepatomegaly, weight loss, anemia (1, 2). Visceral leishmaniasis (VL), also known as kala-azar, is commonly caused by *L. infantum* (Mediterranean), *L. donovani* (India and Africa), *L. archibaldii* (Africa) and *L. chagasi* (South America) (2). Although human is the sole reservoir host for some *Leishmania* species, in most cases animals play a major role in the maintenance of infections (2). VL is an endemic disease in some areas of northwest and south of Iran while in other parts of the country

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the disease occurs sporadically (3). The causative agent of VL is *L. infantum* and dog is the main animal reservoir in Iran (3, 4). Microscopic examination, mostly based on bone marrow aspirate, and specific serological tests such as indirect fluorescent antibody test (IFAT) and direct agglutination test (DAT) have been used on a large scale for diagnosis and seroepidemiological studies of VL (4, 5). Although demonstration of the parasite is conclusive for the diagnosis of VL, but it is an invasive tool, is not high sensitive, and its performance is not practical in most VL endemic areas (3-5). DAT and IFAT are routinely used for the detection of *Leishmania* specific antibodies. DAT in spite of high sensitivity and specificity is time consuming (5, 6). IFAT lacks sensitivity or specificity, requires technological expertise and specialized laboratory equipment, and can be labor-intensive, and is difficult to standardize and to interpret (7, 8). Immunoenzymatic assays such as the enzyme-linked immunosorbent assay (ELISA) are easier to standardize and more practical as routine laboratory tools (2). The performance of ELISA tests is greatly affected by the quality of the antigens used; however, test specificity limitations are the main drawback when crude antigen preparations are used (2, 9). Recombinant technology, together with the characterization of specific immunodominant antigens at the genetic level, allowed the development of a second generation of diagnostic immunoassays, and the recent validation of a recombinant k39-ELISA as a diagnostic marker for canine visceral leishmaniasis represents a good example (9). The k39 antigen is a repetitive of 39 amino acid residues in visceral *Leishmania* isolates examined so far (10, 11) and is mainly expressed in the amastigote stage and elicits a strong immunoresponse in both asymptomatic and clinically infected dogs (12).

The aim of the present study was to produce a recombinant subunit of k39 (k39sub) antigen from Iranian strain of *L. infantum* (*L. infantum* LON 49) and it was evaluated for the detection of human *L. infantum* infection for the first time in Iran. An ideal test would therefore employ a combination of relevant epitopes in a single recombinant antigen, more specific than crude antigen preparation and more sensitive than single epitope-based ELISA. In the last decade, several *Leishmania* antigens have been genetically and antigenically characterized. Some of them have been shown to be expressed in the amastigote stage, thus representing a pool of potential markers during vertebrate infection. Recombinant K39 antigen (rK39) is a 39-amino-acid-repetitive immunodominant B-cell epitope of the 230-kDa kinesin-related protein of *L. chagasi* (10, 11). The rK39 dipsticks have been demonstrated suitable for detection of human VL (10, 13-16) and of both clinical and asymptomatic canine VL (9, 17). K39 and K26 are two related hydrophilic antigens of *L. chagasi* that differ for the presence of 11 copies of a 14-amino-acid repeat in the open reading frame of K26 (18).

In this study, we aimed to compare the results of K39sub antigen test with an commercial immunochromatographic dipstick rk39 test (Cypress Diagnostic Company, Belgium) in the detection of *L. infantum* antibodies in 43 clinically infected patients with direct agglutination test (DAT) positive at a 1: 3200 cut off titer and higher and 101 healthy controls.

Materials and Methods

DNA isolation, PCR, cloning, expression and purification of recombinant protein

DNA was extracted from promastigotes of the Iranian reference strain of *L. infantum* LON49 that was isolated from an infected dog in central of Iran (4). Primers were synthesized for the amplification of the 3'-terminal gene fragment encoding a single 39-amino-acid unit of k39 (k39sub here after) (GenBank accession number L07879; fw 3146 to 3169, rv 3248 to 3265). Oligonucleotides contained restriction sites for *Bam* HI (sense) and *Hind* III (antisense) to facilitate cloning and stop codon. Amplification of the target gene was carried out in a 50-µl reaction volume containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 2 mM MgCl2, 200 µM deoxynucleoside triphosphates, 10 pmol of each primer, 200 ng of the template,

and 1 U of *PFU* DNA polymerase (Gibco). PCR was performed in 14 cycles of 95 °C for 30 s, 66.4-0.5 for 30 s, and 72 °C for 20 s and 20 cycles of 95 °C for 30 s, 59.4 for 30 s, and 72 °C for 20 s. Amplified products of the expected length were subcloned into pBluescript II SK (+) (Stratagene), miniprepared, digested with appropriate restriction enzymes, and cloned into pMAL-p2 expression vector (New England Biolabs, Inc) in frame with maltose-binding protein (MBP) fusion. To express recombinant protein, early log-phase cultures of positive clones were induced for 5 h with 0.5 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) under agitation. Bacterial cells were recovered by centrifugation and lysed by conventional physicochemical methods. Recombinant fusion proteins were recovered in the soluble fraction and purified by affinity chromatography on amylase columns according to the supplier's method (New England Biolabs). The expected molecular mass was 47 kDa for MBP-K39sub. To avoid false-positive reactions to the carrier moiety, recombinant MBP was expressed and purified under the same conditions and used in the ELISA procedure as a negative antigen. The purity and yield of antigen was estimated by SDS-PAGE and Bradford methods (19).

Patients

For evaluating the recombinant K39sub antigen compared to dipstick rk39 test, serum samples were obtained from 43 consecutive patients with clinical manifestations and DAT positive at a 1: 3200 cut off titer and higher from 2004 to 2006 and stored at -70 °C. For each patient, a medical history was obtained and a complete physical examination was performed. In addition, control serums included 69 healthy volunteers and 28 patients with other diseases: malaria including (n= 5), tuberculosis (n= 3), toxoplasmosis (n= 4), cystic hydatidosis (n= 5) and cutaneous leishmaniasis (n= 11) were similarly stored. All diagnoses were confirmed by DAT positive (1: 3200 and higher) accompanied specific clinical manifestations in-cluding fever, splenomegaly, hepatomegaly and anemia. Some patients underwent further parasitologic examination by aspiration of bone marrow microscopy. The negative controls were defined as healthy volunteers and patients with other diseases who had no history of previous treatment of kala-azar, and a negative DAT test result.

ELISA procedure

For ELISA, microtiter plates were coated overnight with 20µg/ml of MBP-k39sub and MBP in 100µl of 0.1M bicarbonate buffer, pH 9.0. Following overnight incubation at 4 °C and subsequent washes with PBS- 0.05%Tween 20 (phosphate-buffered saline- 0.05%Tween 20) excess protein binding sites were blocked at room temperature for 3 h with 250 µl /well of PBS containing 1% bovine serum albumin. After three washes, 100 µl of 1: 200 patient sera in serum diluent (PBS containing 0.1% bovine serum albumin and 0.05%Tween 20) was added to duplicate wells. After three washes, 100µl of anti-human conjugated with alkaline phosphatase (Sigma Co.) with dilution 1/1000 was added to each well and incubated for 3 min. after three washes, 100µl p-nitro phenyl phosphate (Sigma Co.) in diethyl amine buffer was added to each well and incubated for 20 min, the reaction was stopped with 100µl of 3 N NaOH and the absorbance at 405 nm was measured. The ELISA reader (Anthos 2020) was set to subtract the reading of MBP as the negative antigen from that of MBP-k39sub. An OD reading of ≥0.45 unit was taken as positive on the basis of values from negative samples. For each serum sample, ELISA net absorbance was obtained by subtracting the absorbance against negative antigen from the absorbance against K39sub antigen and expressed as the percentage of reactivity of the positive reference serum. Concordance between chimeric ELISA and DAT was evaluated using R software and Kappa (k) test.

Dipstick

The dipstick test (Cypress Diagnostic Company, Belgium) was carried out according to the manufacturer's instructions. The dipsticks were briefly placed into 50 µl of serum. After 1-4 min a red control line and, if positive, a second line appeared on the test field. The test was based on a

combination of protein-A colloidal gold conjugate and rk39 Leishmania antigen to detect anti-*Leishmania* antibody in serum or plasma.

Direct agglutination test

The *L. infantum* antigen for this study was prepared in the Protozoology Unit of the School of Public Health Tehran University of Medical Sciences, Iran. The principal phases of the procedure for making DAT antigen were mass production of promastigotes of *L. infantum* LON49 in RPMI1640 plus 10% fetal bovine serum, trypsinization of the parasites, staining with Coomassie brilliant blue and fixing with formaldehyde 2% (4, 6, 20). The serum samples were tested by DAT according to the methods described earlier (6). Specific *Leishmania* antibodies at a 1: 3200 and above were considered as positive (4- 6, 20).

Parasitologic diagnosis

Bone marrow aspirates were obtained from 15 patients with DAT positive for HVL (consent was refused in the remaining cases). The procedure was done in Pediatric Center of Tehran University of Medical Sciences. Parasitologic confirmation of VL was established by microscopic demonstration of *Leishmania* amastigotes in Giemsa-stained smears from bone marrow aspirates. Parasitologic examination (n= 15) was only performed against DAT positive patients for approving DAT results.

Data analysis

Sensitivity and specificity for each diagnostic test and concordance between both tests were calculated as earlier stated (21, 22).

Results

Patients had symptoms including fever (87.3%), splenomegaly (74.7%), hepatomegaly (63.5%), weight loss (25%) and anemia (75%).

The mean age of patients was 16 yr (range 3 mo -50 yr); and 57% were male.

MBP and MBP-k39sub were highly expressed, found in the soluble fraction, and successfully purified by affinity chromatography (Fig. 1).

The frequency distribution of DAT titers in patients and their comparison results with k39sub-ELISA and dipstick rk39 are summarized in Table 1 and 2.

Three and 1 out of 69 healthy controls and 1 and 1 out of 28 patients with other diseases were positive using the k39sub-ELISA and dipstick rk39 respectively. Four and 1 out of 43 positive controls were negative by using the k39sub-ELISA and dipstick rk39, respectively.

Positive controls had signs including fever, splenomegaly, hepatomegaly, weight loss and anemia. Using a cut off value of 1: 3200 for DAT and that of 0.45 for k39sub-ELISA the concordance between DAT with k39sub-ELISA and dipstick rk39 tests was 93.8 and 97.9, respectively ($P< 0.05$). The most concordance rate between DAT and two tests was occurred in 1: 3200 DAT cut off value. The validity of DAT regarding the complete series of clinically infected cases and concordance between DAT and two tests were analyzed separately in Tables 3 and 4. A good concordance (k=96.4%) was found between k39sub antigen and dipstick rk39 strips.

A total of 12 parasitologically confirmed VL cases were evaluated with the k39sub-ELISA and dipstick rk39 (Table 5).

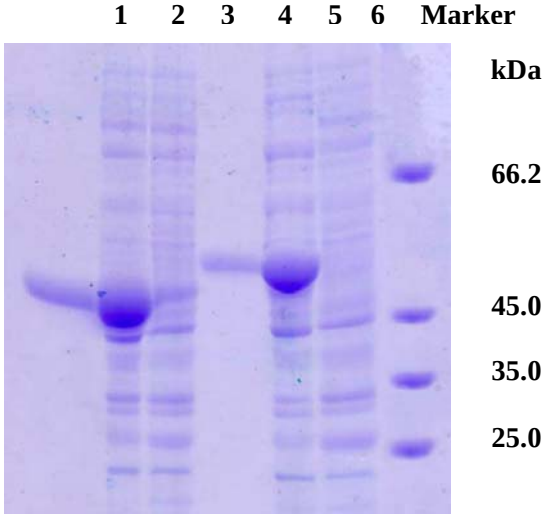

Fig. 1: SDS-PAGE result showing expression and purification of recombinant antigens in *E. coli*. M, molecular-weight standard; lanes 1, 2, 4, 5, total noninduced and induced bacterial lysates expressing MBP-K39sub, and MBP, respectively; lanes 3 and 6, MBP-K39sub, and affinity-purified MBP, respectively.

Table 1: Comparison of k39sub-ELISA and DAT in diagnosis of human *L. infantum* infection

DAT	k39 sub-ELISA					
	Positive		Negative		Total	
	No.	%	No.	%	No.	%
Negative	3	7.0	90	92.7	93	66.4
1:1600	1	2.3	3	3.1	4	2.8
1:3200	7	16.1	2	1.2	9	6.4
1:6400	6	14.0	1	1.0	7	5.0
1:12800	5	11.5	1	1.0	6	4.3
1:25600	7	16.1	0	0	7	5.0
1:51200	3	7.0	0	0	3	2.1
1:102400	6	14.0	0	0	6	4.3
1:204800	5	11.5	0	0	5	3.6
Total	43	100	97	100	140	100

Table 2: Comparison of dipstick rk39 and DAT in the diagnosis of human *L. infantum* infection

DAT	Dipstick rk39					
	Positive		Negative		Total	
	No.	%	No.	%	No.	%
Negative	1	2.3	92	95.8	93	66.4
1:1600	1	2.3	3	3.2	4	2.8
1:3200	8	18.4	1	1.0	9	6.4
1:6400	7	16.1	0	0	7	5.0
1:12800	6	13.8	0	0	6	4.3
1:25600	7	16.1	0	0	7	5.0
1:51200	3	6.9	0	0	3	2.1
1:102400	6	13.8	0	0	6	4.3
1:204800	5	11.5	0	0	5	3.6
Total	44	100	96	100	140	100

Table 3: Sensitivity, specificity and concordance values of k39sub-ELISA compared with DAT according to the different cut off titers

DAT cut off	Sensitivity(range)	Specificity(range)	Concordance(range)
1:1600	85.1(78.5-91.7)	96.9(93.7-100)	91.9(86.8-97.0)
1:3200	90.7(85.3-96.1)	95.6(91.8-99.4)	93.8(89.3-98.3)
1:6400	94.1(89.8-98.4)	87.2(81.0-93.4)	89.3(83.6-95.0)
1:12800	96.3(92.8-99.8)	81.2(74.0-88.4)	84.8(78.2-91.4)
1:25600	100	76.9(69.1-84.7)	81.2(74.0-88.4)
1:51200	100	71.4(63.3-79.8)	75.0(67.0-83.0)
1:102400	100	69.3(60.4-78.2)	72.3(66.0-80.6)
1:204800	100	65.4(56.6-74.2)	67.0(59.2-74.8)

Values are binomial 95% confidence limits

Table 4: Sensitivity, specificity and concordance values of dipstick rk39 compared with DAT according to the different cut off titers

DAT cut off	Sensitivity(range)	Specificity(range)	Concordance(range)
1:1600	91.5(89.1-93.9)	98.9(97.2-100)	96.4(93.3-99.5)
1:3200	97.7(95.2-100)	97.9(95.5-100)	97.9(95.5-100)
1:6400	100	90.6(85.8-95.4)	92.9(86.0-99.8)
1:12800	100	85.0(79.1-90.9)	87.9(82.5-93.4)
1:25600	100	80.7(74.2-87.2)	90.7(85.9-95.5)
1:51200	100	76.2(69.1-83.3)	78.6(71.7-85.5)
1:102400	100	74.4(67.2-81.6)	76.4(69.4-83.4)
1:204800	100	71.1(63.6-78.6)	72.1(64.7-79.5)

Values are binomial 95% confidence limits

Table 5: Results of k39sub-ELISA, dipstick rk39 and DAT compared to 15 bone marrow aspirated patients

Parasitology	k39sub-ELISA		dipstick rk39		DAT	
	Positive	Negative	Positive	Negative	Positive	Negative
	No.	No.	No.	No.	No.	No.
Positive	11	1	12	0	12	0
Negative	3	0	3	0	3	0

Discussion

The development of serodiagnostic tests for VL seems to be a show case of the multiple methodological pitfalls in test validation (22, 23). The most important question is the imperfect standard for diagnosing VL and the absence of such a reference test (gold standard) for infection with the parasite that causes VL. The definite diagnosis of VL depends upon the demonstration of leishmanial amastigotes in bone marrow or biopsy material (spleen, lymph nodes, liver). These procedures are invasive. Moreover, they have poor sensitivity (24). Parasitological confirmation might be the best standard for diagnosing VL, but not for diagnosing infection with *L. donovani* complex in a community at risk (24).
According to previous studies (4, 6, 20, 25), the performance of the DAT for detection of *L. infantum* infection in humans and dogs was practical and with high sensitivity and specificity. Therefore, we used of DAT as the valid test and compared the results of two tests with DAT.
The serodiagnosis potential of k39 for VL, has been shown previously with subjects from Brazil, Sudan, China, Pakistan and Iran (10, 15-17). Findings indicate not only the conservation of the K39 epitope among visceralizing species of *Leishmania* in different geographical regions, but also its high antigenicity (15). A recent development has been the identification of the K39 *Leishmania* antigen, a member of the kinesin family of proteins. Detection of IgG antibodies to this antigen has been found to be extremely sensitive and specific in the diagnosis of VL. The cloning of the K39 antigen has resulted in the synthesis of rK39 antigen, which was then placed on cellulose strips to be used in the field for the diagnosis of VL (15). The rK39 antigen is used in an enzyme-linked immunosorbent assay

(ELISA) format and has shown satisfactory results in India, Brazil, and the Mediterranean (11). In India, the rK39 antigen, impregnated on nitrocellulose paper, was used as a dipstick in rapid field diagnosis of VL with good sensitivity and specificity (24). The "rK39 Dipstick" test and DAT were evaluated in bone marrow parasitologically-positive cases of VL and control patients from Nepal (26). With the dipstick test, both sensitivity and specificity were 100%. With DAT, the sensitivity was 100% while the specificity was 93% (24). In another comparative evaluation performed in Nepal, DAT showed slightly better results than those with rK39 in the confirmatory diagnosis of VL cases (26). The dipstick rK39 test and DAT were also used in diagnosis of VL in parasitologically-positive and apparently cured patients in Sudan. Both tests showed limited specificity and sensitivity, and the dipstick test remained positive to a lesser extent after treatment. Nevertheless, the dipstick test was reported as ideal for use in the field (16, 27). The specificity of the dipstick rK39 test in non visceral leishmaniasis infections was also 100% in Iran (16). Also in the study was shown DAT seropositivity rate in clinically suspected VL patients and children from VL endemic areas was higher than that with rK39 (16). In Brazil the sensitivity of the rK39 antigen strip test and the crude antigen ELISA was 90% and 89%, respectively, while the specificities were 100% and 98%, respectively (28). A new rK39 rapid diagnostic dipstick test (DiaMed-IT-Leish-) was compared with aspiration and DAT for diagnosis of VL in Sudan (29). The sensitivity of the rK39 test in parasitologically confirmed VL cases was 90%, whereas the specificity in disease-endemic controls was 99%.

There are several hypotheses that could explain this regional variation of the results of the rK39 antigen. First, there may be differences in the test accuracy between subspecies of the *L. donovani* complex. Similarly, within these subspecies, there may be regional differences as a result of variations in the rK39 antigen. Another possible explanation involves genetic differences in individual patients or in racial subgroups.

Our results demonstrated that k39sub antigen and rk39 carry highly reactive and diagnostically relevant epitopes. The sensitivity of k39sub antigen was lower than that of dipstick rk39 and one parasitologically positive patient serum gave negative result in k39sub antigen but positive result in rk39 antigen. These were resulted from not only a higher density of immunodominant epitope but also from a complete subset of overlapping epitopes which might be lost if a single repetition was to be used. The k39sub ELISA and dipstick rk39 showed the good agreement with the DAT results. K39sub obtained from Iranian isolate is valid for serodiagnosis of HVL caused by this strain in comparison with K39sub obtained from other isolates.

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