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Cloning and Expression of *Leishmania major* Superoxide Dismutase B1: A Potential Target Antigen for Serodiagnosis of Leishmaniasis

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ABSTRACT

Background: Leishmaniasis -a neglected public health problem- is a group of diseases affecting an estimated 12 million people worldwide. **Objective:** In the present study, recombinant *Leishmania major* superoxide dismutase B1 (rLmSODB1) has been utilized as a potential antigen for the serodiagnosis of human cutaneous (CL) and visceral leishmaniasis (VL) in the endemic regions of southern part of Iran. Additionally, the sensitivity and specificity of ELISA-based serodiagnosis using rLmSODB1 and the soluble *Leishmania* antigen (SLA) were compared. **Methods:** For the first time, rLmSODB1 has been cloned successfully and used for ELISA-based serodiagnosis. Sera from 30 CL and 24 VL cases were included in this study. Additional studies were also done for the evaluation of cross-reactivity using sera from 41 endemic controls including normal endemic donors (n=20), systemic lupus erythematosus patients (n=5), rheumatoid arthritis patients (n=5), and patients with tuberculosis (n=11). **Results:** Analysis indicated that rLmSODB1 was recognized by 62.5% and 13.3% of sera from patients with VL and CL, showing a sensitivity of 72.7% and 53.6%, respectively. However 95.8% of VL and 30% of CL sera reacted with SLA, revealing sensitivities of 96% and 58.8%, respectively. Additionally, from 41 sera collected either from healthy subjects or patients affected with other diseases, 97.5% were negative with SLA or rLmSODB1 (specificity 97.6%). **Conclusion:** These results show that rLmSODB1 almost does not react with sera from patients with tuberculosis and autoimmune diseases and may be considered as a candidate antigen for the specific immunodiagnosis of visceral leishmaniasis.

Keywords: Visceral leishmaniasis, Cutaneous leishmaniasis, ELISA, Superoxide dismutase

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INTRODUCTION

Leishmania are obligate intracellular parasites that infect mononuclear phagocyte lineage in their vertebrate hosts. These parasites are the causative agents of leishmaniasis, a group of diseases affecting an estimated 12 million people worldwide (1). There are 2 million new cases each year and another 350 million people are at risk of infection (1). Infection with *Leishmania spp*. can lead to a number of clinically different yet sometimes overlapping forms of disease in humans. Clinical manifestations of leishmaniasis range from self-healing cutaneous ulcers to potentially lethal visceral spread. CL represents approximately 90% of all cases of leishmaniasis (1). VL or Kala-azar is the most severe form of leishmaniasis. The causative agent of VL in the Indian subcontinent, Asia and Africa is *Leishmania* (*L) donovani*, while the chief culprit in Mediterranean region, south west and central Asia and South America are *L. infantum* or *L. chagasi* (2).

The diagnosis of VL is difficult due to the similar clinical features with other commonly occurring diseases such as tuberculosis, malaria, and typhoid (3). Parasitological diagnosis for leishmaniasis is the usual and golden standard method and relies on either the microscopical demonstration of Leishman body in specimens or culturing. The drawbacks of these invasive methods are painful and is not practical to use in most rural areas. Furthermore, these methods are not sensitive enough to detect all patients (4). Due to these limitations, several serological techniques have been developed for the diagnosis of VL. Although, crude parasite extracts are very sensitive in serodiagnosis of leishmaniasis, but they yield a significant number of false positive results, especially when sera from patients with some other infections such as *Trypanosoma cruzi are* tested (5). In order to improve the specificity of the serodiagnostic tests, many different candidate antigens have been tested. Among them identification of *L. chagasi* recombinant antigen K39 has been a major progress in the diagnosis of VL (6). Even though the use of *L. chagasi* rK39 protein for serodiagnosis of New World VL was reliable (7,8) unfortunately its use for diagnosis of Old World VL caused by *L. Infantum* or *L. donovani* infections has shown variable results and several studies showed that the sensitivity and specificity of the test were not as high as reported for the diagnosis of New World VL (9,10). Therefore, the need to develop a specific method for diagnose and confirm such diseases is of utmost value.

Trypanosomatids, including different species of *Leishmania*, have superoxide dismutases (SODs) to protect themselves against oxidative stress (11). SODs from parasitic protozoa have only iron cofactors and structurally differed from SOD of the vertebrates. nBLAST and pBLAST analysis of the *Leishmania* SODs against the non-redundant (NR) sequence database revealed that no homology exists between these molecules and human proteins. Moreover, previous studies have discovered that some SODs from parasitic protozoa are highly immunogenic for human and dog (12-14). As a result, these data will raise the possibility of using SOD in the diagnosis of human leishmaniasis. Previous studies have shown that SODs excreted by *Trypanosoma cruzi* are highly immunogenic and antibodies to SODe were detectable in sera to a dilution of 1:2500 by Western blot (12). More recently in a study by Marin et al., an excreted SODe by the promastigote form of *L. infantum* was also used for the diagnosis of *L. infantum* in naturally infected dogs (14).

The goal of this study was to determine the usefulness of the rLmSODB1 in diagnosing human CL and VL. The results of the present study show that serodiagnosis based on

rLmSODB1 is highly specific and may be of great benefit in the diagnosis of VL cases. This study represents preliminary information for developing more sensitive and specific serodiagnostic tests, for the diagnosis of leishmaniasis.

MATERIALS AND METHODS

Source of Sera. Sera from 30 CL and 24 VL patients were used in this study. VL patients were selected according to their clinical manifestation and a positive rK39 based on a serological test. Sampling from CL patients was done in active phase of the disease while no chemotherapy was started for their cure. Moreover, existence of leishman bodies were confirmed microscopically in the lesion smears prepared from CL patients. Forty one endemic control sera collected from individuals without any history of leishmaniasis and were also included in the present study. The source of the control sera were normal endemic donors (n=20), systemic lupus erythematosus (SLE) patients (n=5), rheumatoid arthritis (RA) patients (n=5), and patients with tuberculosis (TB) (n=11). In all cases the sera were obtained from blood collected by vein puncture according to the regulation of ethical committee of Shiraz University of Medical Sciences, Iran, and kept at -70°C until use.

Parasite Cultivation. The *L. major* strain MHRO/IR/75/ER (IR75) was used in this study. Promastigotes were cultured at 26 °C in RPMI 1640 medium with glutamine (Biosera, UK) supplemented with 10% heat-inactivated FCS (Biosera, UK).

DNA Amplification. Genomic DNA was isolated as described previously (15) with some modification. Briefly, logarithmic phase promastigotes were disrupted in lysis buffer (50 mM NaCl, 50 mM EDTA, 1% SDS, 50 mM Tris–HCl, pH 8.0), and then incubated with proteinase K (100 mg/ml, Sigma-Aldrich, USA) at 37 °C for 4h. DNA was purified further by phenol-chloroform extraction and ethanol precipitation. The coding region of LmSODB1 was amplified by PCR using *Pfu* DNA polymerase (Fermentas, Lithuania) with forward primer, 5'-GTGCCATATGCCGTTCGCTGTTCA-3' which contained an *Nde*I (Fermentas, Lithuania) site and an initiation codon and a reverse primer 5'- AACCCTCGAGCTGGCTAGAGGCGAAA -3' which contained an *Xho*I (Fermentas, Lithuania) site without a stop codon. DNA amplification was performed in a final volume of 30 µl containing 75 ng of genomic DNA, 5 pM of each primer, 375 µM of dNTPs, 2.5 mM $MgCl_2$, and 1.5 unit of *Pfu* DNA polymerase in a 1X amplification buffer. The PCR mixture was submitted to a denaturation step (4 min at 95°C), followed by 35 cycles of amplification (60s of denaturation at 95°C, 60s of annealing at 62°C, and 60s of extension at 72°C) and further extension (10 min at 72°C) steps.

Expression and Purification of Recombinant SODB1. Amplified DNA fragments were purified using PCR clean up Nucleospin Kit (Macherey-Nagel, Germany) and cloned directly in to pGEM-T easy T vector TA cloning (Invitrogen, USA). The insert was removed by *Nde*I and *Xho*I digestion and ligated into the *Nde*I-*Xho*I insertion site of expression vector pET22-b (Novagen, USA) to create the plasmid clone pET-LmSODB1. Two recombinant clones were sequenced using M13 forward and reveres primers. Sequencing with these vector based primers showed that the His-tag protein was in frame. The construct was transformed into *E.coli* BL21 (DE3) and grown at 37˚C in LB medium containing 100 µg/ml Ampicilin. Hexa-His-tag fusion protein was expressed by induction of log-phase culture (OD 280=0.6) with 0.2 mM Isopropyl-b-D-

thiogalactoside (IPTG) for 4 h at 37 ˚C, with vigorous shaking (200 rpm). Bacteria was harvested (10,000 X g, for 10 min), resuspended in lysis buffer (50 mM NaH_2PO4, 300 mM NaCl, 10 mM imidazole, pH 8), incubated for 30 min on ice and disrupted by sonication (five 45s pulses interrupted with cooling on ice). Inclusion bodies were isolated by centrifugation at 12,000 X g for 15 min at 4 ˚C, washed with PBS containing 1% Triton X-100 and solubilized in a buffer containing urea (50 mM Tris–HCl, 300 mM NaCl, 8 M Urea, pH 8). Recombinant protein containing the histidine tag was affinity purified using IDA-chelating sepharose (Amersham Pharmacia Biotech AB, Sweden) according to the manufacturer's protocol with some modifications. Briefly, to charge the resin 25 ml IDA-chelating sepharose were mixed with 75 ml of 100 mM NiSO4. The mixture was shaken for 4 h and washed with Milli Q water. Solubilized inclusion bodies were passed through a Fast Performance Liquid Chromatography column containing 25 ml charged IDA-chelating sepharose resin (flow rate; 1,3 ml/min), washed with solubilization buffer containing 10 and 20 mM imidazole and the bound material was eluted with the solubilization buffer containing 300 mM imidazole. Purification of recombinant protein was assessed by SDS-PAGE analysis. Purified recombinant protein was gradually dialyzed against guanidine chloride-containing PBS buffer. The concentration of guanidine chloride reached 0.5 mM at the end of the experiment.

Preparation of Soluble Leishmania Antigen. SLA was prepared from the stationary-phase promastigotes of *L. major* (IR75) harvested after five passages and washed 3 times in ice-cold PBS (pH 7.2) and eventually suspended in PBS buffer at a concentration of 1x 10^7/ml. The suspension was sonicated 5 times, 30 sec each time, followed by a 30 sec interval on ice and the lysate was centrifuged at 12,000 × g for 15 min at 4°C. The supernatant was collected and stored at −70°C. The protein concentration was estimated by the Bradford method.

Enzyme–Linked Immunosorbent Assay (ELISA). The ELISA analysis was performed according to a standard method. Briefly, a titration curve was established and the optimal concentration was found to be 400 ng/well for rSODB1 and 200 ng/well for SLA. ELISA plates (Nunc, Denmark) were coated overnight at 4°C. The wells were blocked with 3% bovine serum albumin for 16 h at 4°C, washed 3 times with PBS containing 0.05 % tween 20 (PBS-T), and incubated for 1.5 h at 37°C with 50 µl of patients sera at a 1/50 dilution. Wells were washed 4 times with the same buffer and incubated with 50 µl of mouse anti-human total antibody conjugated with HRP for 1 h. This step was followed by 5 rinses with PBS-T and the reaction was developed using 100 µl of TMB as substrate. The reaction was stopped with 50 µl of 3N H2SO4 and the OD of each well was measured at 450 nm in an ELISA reader. A serological test for *Leishmania* antigens was considered positive when the OD of unknown sample was higher than cut off value, which represents the mean plus three standard deviations of the ODs for healthy subjects. All samples were analyzed in triplicate.

Statistical Analysis. The performances of the tests were calculated as follows: sensitivity= [true positive/true positive + false negative] × 100 and specificity= [true negative/true negative+ false positive] × 100. In addition; positive predictive values (PPV) and negative predictive values (NPV) were determined according to the following formulas: PPV= [true positive/true positive + false positive] × 100 and NPV= [True negative/true negative+ false negative] × 100.

RESULTS

Cloning and Expression of L.major SODB1. The *lmsodb1* gene was amplified by PCR and cloned into a pGEM-T easy T vector and sequenced. Then, the insert was subcloned into the pET-22b for high-level expression in *E.coli*. The *lmsodb1* contains an open reading frame of 588 nucleotides, encoding a 195-amino-acid protein with a molecular weight of 21287 Da. Comparison of the Alignment of LmSODB1 with the sequence of different SODs of some Trypanosomatids revealed that LmSODB1 has a high homology with them (Figure 1). IPTG Induction of bacteria transformed with recombinant vector resulted in a high yield recombinant protein expression. Different concentrations of IPTG was examined to identify the highest yield (Figure 2.A.). After Purification, imidazole was removed by gradually dialyzing the recombinant protein against guanidine chloride-containing PBS buffer, and at the end the purified rLmSODB1 was solubilized in 0.5 mM guanidine chloride (Figure 2.B. lane 1).


```
LiSODB2         ....................................N.......G.......... 60
LdSODB2         ....................................N.......G.......... 60
LmSODB2         ....................................T.......A.......... 60
LiSODB1         ....................................N.......G.......... 60
LdSODB1         ....................................N.......G.......... 60
LmSODB1         MPFAVQPLPYPHDALASKGMSKEQVTFHHEKHHKGYAVKLTAAAESNSALASKSLVDIIK 60

LiSODB2         ............F..D........R..SK.H.EI....V......AS..K...D..... 120
LdSODB2         ............F..D........R..SK.H.EI....V......AS..K...D..... 120
LmSODB2         ............Y..D........R..SK.H.EI....I......SN..K...D..... 120
LiSODB1         ............F..-........R..GE.S.PL....V......AS..K...D..... 119
LdSODB1         ............F..D........R..GE.S.PL....V......AS..K...D..... 120
LmSODB1         SEKGPAFNCAAQIYNHDFFWRCLSPCGGGEPSGNLASAIIDSFGSFSNFKEEFTAAANGH 120

LiSODB2         ................L...........N.....T..I.........R.......N 180
LdSODB2         ................L...........N.....T..I.........R.......N 180
LmSODB2         ................F...........D.....A..V.........R.......S 180
LiSODB1         ................L...........N.....T..I.........R.......N 179
LdSODB1         ................L...........N.....T..I.........R.......N 180
LmSODB1         FGSGWAWLVKDKSSGKLKVFQTHDAGCPLTEPDLVPILTCDVWEHAYYIDYKNDRASYVS 180

LiSODB2         ......N.SH.NRCYRAAGGSHYVN.D. 208
LdSODB2         ......N.SH.NRCYRAAGGSHYVN.D. 208
LmSODB2         ......N.SH.NHCYRAAGGSHYVN.D. 208
LiSODB1         ......D.DF.S-------------.Q. 194
LdSODB1         ......D.DF.S-------------.Q. 195
LmSODB1         AFWNMVDWNFAS-------------.QL 195
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Figure 1. Alignment of the amino acid of sequence *Leishmania major* SOD1 with those of *L. infantum* and *L.donovani.* This comparison shows a high homology between different SODs of *Leishmania* parasites. This alignment was done using the Clustal W program.

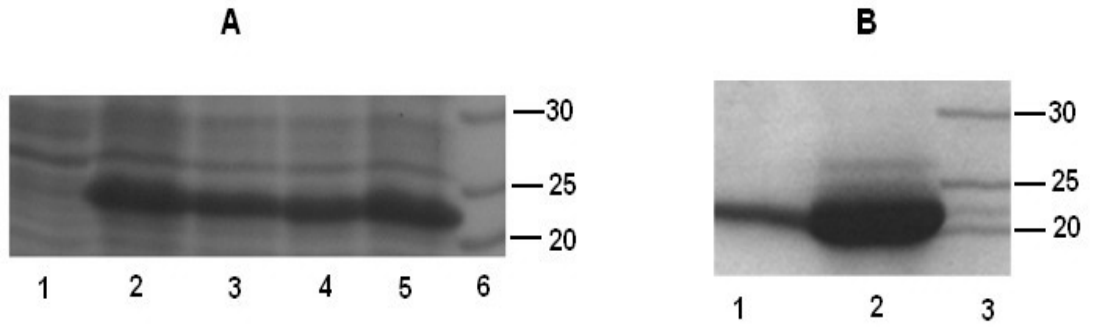

Figure 2. Expression of rSODB1 in bacteria. A: Expression of rSODB1 was induced with various concentrations of IPTG in BL21 (DE3) bacteria containing the expression construct, pET-*sodb1*. Total bacterial protein was analyzed by SDS-PAGE followed by Coomassie Blue staining. Lane 1: total bacterial lysate before induction; lanes 2–5: total bacterial lysate induced with 1mM, 0.7mM, 0.5mM and 0.2mM of IPTG, respectively; Lane 6: molecular weight markers. B: purity of rSODB1 after purification and dialysis. Lane 1: purified rSODB1 after dialysis, lane 2: purified rSODB1before dialysis, and lane 3 is the molecular weight markers.

Serodiagnosis of Leishmaniasis Using rSODB1. In order to verify the serodiagnostic value of *L. major* rSODB1 antigen, the sensitivity and specificity of rLm-SODB1-based ELISA were analyzed and judged against the sensitivity and specificity of SLA. To achieve this aim, 30 individual sera from active patients with CL and 24 individual sera from active VL patients were analyzed by ELISA using the purified rSODB1 and SLA extracted from metacyclic *L. major* as antigens. The specificity of the rSODB- or SLA-based ELISA was evaluated using sera from 41 controls without any history of leishmaniasis.

According to Table 1, sera of 32 out of 54 leishmaniasis patients (VL, n=24 and CL, n=30) were positive in SLA-based ELISA (59.2%), whereas 19 out of 54 patients' sera (35.2%) were reactive with rSODB1. When cross-reactivity was tested, none of the sera from patients with SLE (n=5) or RA (n=5) recognized either SLA or rSODB1 and only one of the 11 tested TB sera was positive in both ELISA tests. Surprisingly, none of the 20 individual controls living in the endemic area showed reactivity with SLA or rSODB1. Table 2 shows the sensitivity and specificity of the two ELISA-based tests for diagnosis of leishmaniasis. The sensitivity of SLA-based ELISA for all infected cases (VL plus CL) was 71% which was higher than the sensitivity of rSODB1-based ELISA (60.7%). The specificities of the two tests were equal (97.6%). In addition, SLA-based ELISA showed more sensitivity for VL sera than rSODB1-based ELISA (96% vs. 72.7%). The optical density distribution of rSODB-based ELISA and the SLA-based ELISA in VL and CL patients and different control groups are shown in Figure 3.

Table 1. Reactivity of sera from VL and CL patients, healthy controls, and patients with tuberculosis with either rLmSODB1 or SLA

		SLA		SODB1	
	N	Pos N (%)	Neg N (%)	Pos N (%)	Neg N (%)
CL + VL	54	32 (59.2%)	22 (40.8%)	19 (35.2%)	35 (64.8%)
VL	24	23 (95.8%)	1 (4.2%)	15 (62.5%)	9 (37.5%)
CL	30	9 (30%)	21 (70%)	4 (13.3%)	26 (86.7%)
Healthy Control	20	0 (0%)	20 (100%)	0 (0%)	20 (100%)
SLE	5	0 (0%)	5 (100%)	0 (0%)	5 (100%)
RA	5	0 (0%)	5 (100%)	0 (0%)	5 (100%)
TB	11	1 (9.1%)	10 (90.9%)	1 (9.1%)	10 (90.9%)
Total Control	41	1(2.5%)	40 (97.5%)	1(2.5%)	40 (97.5%)

Table 2. Sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) of the ELISA assay for diagnosis of VL and CL

	Sensitivity %			Specificity %	PPV %			NPV %		
	CL+VL	VL	CL	All	CL+VL	VL	CL	CL+VL	VL	CL
SLA	71	96	58.8	97.6	98.2	96	96.8	65	97.6	66.1
SODB1	60.7	72.7	53.6	97.6	98.2	96	96.8	53.9	82	61.2

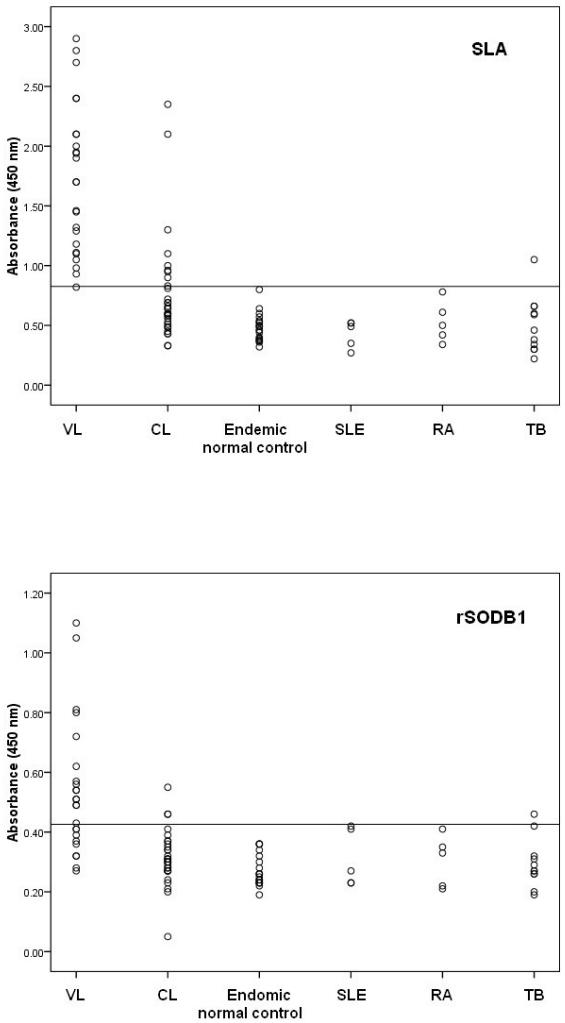

Figure 3. The optical density distribution of SLA and rSODB1based ELISA in visceral leishmaniasis (n = 24) and cutaneous leishmaniasis (n = 30) patients and different control groups including endemic normal control (n = 20) subjects, SLE (n = 5), RA (n = 5) and TB (n = 11) patients. The cut-off value was defined as the mean optical density plus three standard deviations for the values obtained from sera of healthy control subjects.

DISCUSSION

The completion of the *Leishmania major* genome sequence (16) in combination with proteomics studies (17) have identified all potential proteins that may be targets for drug and vaccine development or might be useful in diagnosis of the disease. In recent years major advances have been made in the development of a number of diagnostic methods

such as ELISA and direct agglutination test (18-21). Despite the large number of serological techniques that are used, there is still no golden standard diagnostic test. This is in part due to the fact that none of the available tests have proved to be 100% sensitive and specific. Furthermore, the increase of *Leishmania*/HIV co-infection cases complicates the use of the serological tests as a result of low or lack of antibody responses in these patients (1). In addition, use of whole parasite extracts in these serologic tests is limited because of low assay specificity mainly due to the cross-reactivity with other diseases such as Chagas' disease and tuberculosis that may be present along with VL in tropical and subtropical regions (3,22,23). Thus, developing a new laboratory diagnosis test plays a vital role in the confirmation of clinical diagnosis of leishmaniasis. In this respect, sequence comparisons of the *fesodb1* genes in the different species of *Leishmania* revealed more than 86% identity (Figure 1), suggesting a high degree of conservation of *fesodb* genes in *Leishmania*. Therefore, using one of these molecules might be useful in serodiagnosis of leishmaniasis. To resolve whether SODB1 could be utilized for this purpose, we investigated the possible usefulness of rLmSODB1 as the antigen in a diagnostic ELISA and verified the sensitivity and specificity of this test in comparison with SLA-based ELISA.

In the present project, 35.2 % of all cases with leishmaniasis were positive with rSODB1 while 59.2% were positive with SLA (Table 2; 60.7 % vs. 71% sensitivity, respectively). In addition, the results of the rSODB1-based ELISA and SLA-based ELISA for leishmaniasis showed that both tests had similar specificities (97.6 %).

The potential cross-reactivity of SLA with sera from patients with other infectious diseases (specially Chagas' disease) is the major obstacle in serodiagnosis of VL using this crude antigen (5,22,24). However, the results of the present study show that in the non-endemic regions for trypanosomiasis, SLA could be used as a valuable antigen in serodiagnosis of VL. In fact, SLA-base ELISA had a higher sensitivity than rSODB1-based ELISA in VL patients (96% Vs 72.7%, respectively), while they were similar in their specificities. Considering the presence of different *Leishmania*-derived antigens in SLA preparation and the possibility of reaction between different anti-*Leishmania* antibodies and SLA, higher sensitivity of SLA-based ELISA compared to rSODB1 is conceivable in the diagnosis of VL.

However, the significance of SOD for determination of leishmaniasis has been shown in previous studies (13,14). In this respect, native excreted SOD from *Leishmania infantum* was used for diagnosis of VL infected dogs by western blot, showing that seroprevalence values of SODe based western blot were significantly higher than other serological methods (Indirect fluorescent assay and ELISA) using whole parasite antigen extract (14). In addition, this test showed enough specificity to discriminate the *L. infantum* infected dogs from those infected with *T. cruzi*. Therefore, authors came to this conclusion that SODe can be used in the diagnosis of canine leishmaniasis. In another study native excreted SODe from *T. cruzi* used for screening the Chagas' disease in Mexico (12) revealed that the seroprevalence values for SODe by ELISA and western blot were considerably higher in the endemic areas for Chagas' disease than those tests using whole *Leishmania* cells. However, in the present study rSODB1 from *Leishmania major* did not show the proper sensitivity for the diagnosis of leishmaniasis in humans. This controversy could be explained by the nature of antigens used in these studies. As a matter of fact, due to the lack of native conformation in recombinant proteins produced in prokaryotes, immunodominant epitopes may be lost. Using native excreted SOD

ensures that raised antibodies can react with their own epitopes. On the other hand, the low sensitivity in VL diagnosis test might be due to the probable differences between the dominant epitopes of rLmSODB1 and SODB1 of *L. infantum* (i.e., heterologous parasite). Hence, the serodiagnosis tests based on the rSODB1 of *L.infantum* may have better performance in diagnosis. In this respect, a comparative study showed that use of recombinant proteins from *L.infantum* rather than *L.major* yields better ELISA result (19). In addition, the results showed that rSODB1 cannot be of much use in identifying CL patients, as merely 13.3% (4 of 30) of the sera from patients with CL were positive when tested with rSODB1-based ELISA. In fact, due to localized characteristics of CL, humoral immune responses are not strong enough to screen CL patients.

Additionally, the specificity of SLA-based ELISA which is developed in the present study was slightly higher than previous studies non-endemic controls (18,25). This observation could be explained by a variation in the nature of the control subjects used for calculating the cut-off value. In most of previous studies subjects from non-endemic areas were used as control groups, while in the current study all of the subjects (including healthy controls and patients with SLE, RA and TB) were selected from individuals living in the endemic area. Indeed, valuable serological tests should be able to discriminate patients and normal individuals in the endemic area, where some healthy subjects have antibodies without any clinical symptom or history of leishmaniasis. In this line, Alborzi et al. have shown that nearly 25 percent of healthy individuals in endemic area of Fars province (south of Iran) have kDNA PCR-ELISA positive results in combination with IFAT seroreactivity (26). Moreover, the results of current study are in agreement with a pervious study using endemic controls (19).

It has previously been shown that the use of recombinant proteins in serologic tests for diagnosis of leishmaniasis in human cases would be hindered by the existence of cross-reactivity against desired recombinant antigens in sera from patients with autoimmune diseases (27,28). Of interest, our preliminary evaluations have revealed that none of the sera from individuals with either SLE (n=5) or RA (n=5) reacted with rSODB1. However, considering the low number of autoimmune sera in the present study, further research is needed to corroborate this result.

In order to identify the best antigen among various *Leishmania* antigens, few comparative analyses have been made among serodiagnosis tests (25). Among several recombinant or purified antigens introduced for serodiagnosis of VL cases, rK39 has the best performance. rK39 is a cloned antigen of 39-amino acid repeat that is part of a kinesin-related protein in *Leishmania chagasi* (6). An rK39-based ELISA showed excellent sensitivity (90–99%) and specificity (89–98%) in many VL-endemic countries (29,30). In a comparative evolution study, a panel of ten different recombinant or purified *Leishmania* antigens was analyzed(19). Among them, only rK39 introduced significant improvement over the classic SLA. Indeed, rK39 gave the best results in terms of specificity (97%) and positive predictive value (92%). In comparison, rLmSODB1-based ELISA showed excellent specificity and moderate sensitivity for serodiagnosis of VL cases in the endemic area of south Iran.

In conclusion, the results of this study showed that rLmSODB1 is highly immunogenic and antibody produced against this antigen is specifically raised in leishmaniasis patients. However, the diagnostic sensitivity of VL using rSODB1 derived from *L.major* was fairly low, therefore it might be more useful to use rSODB1 from *L. infantum* in order to increase the sensitivity of VL diagnosis. In addition, combination of different

recombinant antigens in a multiple-epitope format could further increase the sensitivity of a single –well ELISA for the serodiagnosis of VL. Our results also indicate that classical SLA antigen represents a very good performance, which may be of interest for developing a serodiagnostic panel, especially in regions that are not endemic for trypanosomiasis.

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