Iranian J Parasitol: Vol.1, No.1, 2006, pp. 15-25

Application of Direct Agglutination Test (DAT) for the Diagnosis and Seroepidemiological Studies of Visceral Leishmaniasis in Iran

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(Received 4 July 2006; accepted 18 Sep 2006)

Abstract

Visceral leishmaniasis (VL) is one of the most important parasitic diseases which is endemic in different parts of Iran. Serological studies were conducted by direct agglutination test (DAT) on 12144 human serum samples, collected from four geographical zones of Iran. Sero prevalence, geographical distribution, clinical signs and symptoms for human visceral leishmaniasis based on DAT for the period of 2002 through 2005 were determined. From 516 kala-azar cases detected: 50.6% were from Meshkin-shahr and Moghan districts in Ardabil Province, northwest of Iran and 49.4% were detected from other areas of Iran. In physical examination of seropositive cases, which were detected by DAT with anti-leishmanial antibodies at titers of 1: 3200 to 1: 102400, almost 50% of suspected individuals showed the classical kala-azar signs and symptoms. Predominant signs and symptoms in 233 hospitalized patients with anti-*Leishmania* antibodies at 1:3200 and higher, were fever (88.0%) and splenomegaly (84.5%). Statistically significant difference was found between males (58%) and females (42%) ($P< 0.01$). Moreover, 93.6% of the VL patients were $\leq$ 5 yr of age, and 6.4% were older than 5 yr that this difference was statistically significant ($P< 0.01$). From 1383 serum samples collected from domestic dogs in the villages that are known as endemic foci of human leishmaniasis, 152 (11.0%) were positive by DAT ($\geq$ 1:320). Parasitological and serological examinations that were performed in 30 wild canines showed that 10% of these animals were infected by *L. infantum. L. infantum* Lon49 is the principal agent of the disease in human as well as animal reservoir hosts in different parts of Iran. For the first time in Iran, *L. tropica* isolated from both skin lesions in the face and bone marrow aspiration in a HIV^+ man who co-infected with VL as well as in an infected dog from Ardabil Province.

Keywords: *Visceral leishmaniasis, Seroepidemiology, Diagnosis, Direct agglutination test, Iran*

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Introduction

Zoonotic Visceral leishmaniasis (ZVL) is a potentially fatal protozoan infection usually caused by *Leishmania infantum/ L. chagasi*. It is broadly distributed in the old and new worlds (1, 2). In Iran, the disease is maintained in nature in a complex epidemiological cycle that may include both domestic and wild components reservoirs including domestic dogs, foxes, jackals and wolves (3-6). The epidemiological studies have showed that the Mediterranean type of kala-azar is occurred in different parts of Iran that is caused by *L. infantum* (3). Our studies in the last decade showed that *L. infantum* Lon 49 was the principal agent of the disease in human and animal reservoirs in Iran (7- 9). Based on the recent studies, natural leptomonad infections have been observed in *Phlebotomus kandelakii* and *P. perfiliewi* in north west (10, 11) as well as in *P. major* and *P. keshishiani* in south and central parts of Iran (12, 13).

Direct agglutination test (DAT), based on the method of Allain and Kagan (14) was developed and described as a simple and economical technique in sero-diagnosis and sero-epidemiological studies of visceral leishmaniasis (VL) by Harith *et al*. in 1986 (15). It has been applied, evaluated, occasionally modified, and generally considered as a practical and reliable serological technique for the diagnosis and epidemiological surveys of VL in human as well as animal reservoirs with high validity (16- 18).

In Iran, DAT has been applied in sero-epidemiological studies of VL in human and animal reservoirs in Fars and Ardabil Provinces, where kala-azar is endemic (8). DAT has been also compared to ELISA using intact and soluble antigens of *Leishmania* promastigote and indirect fluorescent antibody test (IFAT) in the diagnosis of VL with satisfactory results (19, 20).

The objective of this study was to report the selected sero-epidemiological data of visceral leishmaniasis from Iran for the four year period of 2002 through 2005, including characteristics of VL cases and the results of serological screening DAT.

Materials and Methods

Study area

The investigation was conducted from 2002 to 2005 in northwest, northeast, south and central parts of Iran where HVL is endemic (8). In these four distinct geographical zones in Iran, samples were collected.

Blood sampling

The finger prick blood samples were collected by trained health workers (Behwars) from suspected kala-azar patients in the laboratory and tested using DAT by the trained technicians. The sero-positive patients were referred to pediatricians or general physicians in district hospitals or health centers for physical examination and treatment of the seropositive cases who had clinical manifestations. Active case finding and reservoirs studies were carried out in areas that the cases of visceral leishmaniasis were considerable.

Canine blood samples (2.5 ml) were taken from 1383 domestic dogs from four geographical zones of Iran including northwest, northeast, south and intermediate regions where HVL is endemic. Additionally, samples were taken from 30 wild canines in these areas: 10 jackals, 10 foxes, and 10 wolves. Blood samples were taken by venapuncture, poured into 10 ml polypropylene tubes, and processed 4-10 h after collection. The blood was centrifuged at 800 g for 5-10 min, and sera were separated and stored at -20 °C. All the serum samples were tested by DAT.

DAT antigen and serological tests

DAT *Leishmania* antigen is made in the Protozoology Unit of the School of Public Health and Institute of Public Health research, Tehran University of Medical Sciences (17). The principal phases of the procedure for making DAT antigen were mass production of the Iranian strain of *L. infantum* Lon 49 that was isolated from an infected domestic dog in central part (Shahrekord) of Iran, cultured in RPMI 1640 plus 10% fetal bovine serum, tripsinization of the parasites, staining with Coommassie brilliant blue and fixing with formaldehyde 2% (15, 16). Several batches of the locally made DAT antigen were sent to WHO reference laboratories in the Royal Tropical Institute and the Dept. of Medical Microbiology in Amsterdam, through the TDR, for quality control in 1993. These samples were reported as similar and comparable to the standard DAT *Leishmania* antigen available in the above laboratories.

All the collected serum samples were tested by DAT according to the methods described earlier (15, 16). Initially, for screening purposes, two dilutions of 1: 800 and 1: 3200 were made and tested. If both of these two dilutions were positive, the samples with titers 1: 800 were diluted further to give end-point titers of 1: 102400. Negative control wells (antigen only) and known negative and positive controls were tested in each plate daily. The cut off titer was defined as the highest dilution at which agglutination was still visible as blue dot, compared with negative control wells, which had clear blue dots. The positive standard control serum was prepared from VL patients and dogs with *L. infantum* infection from the endemic areas confirmed by microscopy, culture and DAT titers of 1: 102400. The cut off was determined in previous study by experimental infection. Sensitivity and specificity of the DAT were estimated with exact binomial 95% confidence limits. To study the optimal DAT cut off level, a receiver-operator characteristics curve (ROC) was constructed (22). Moreover, specific *Leishmania* antibodies at a titer of 1: 3200 and upper were considered as positive in previous studies for human and a titer of 1: 320 and higher for domestic and wild canines too (17- 20). Therefore, we considered anti-*Leishmania* antibodies titers at $\geq$ 1:3200 and $\geq$ 1: 320 as human and canine *Leishmania* infection in this investigation, respectively.

Parasitological study

The seropositive cases (1$\geq$ 320 and above) were clinically examined for signs of *Leishmania* infection. If they had clinical signs and symptoms of VL, they were considered as kala-azar patients and treated with Glucantime^{R} and if the clinically suspected in some of them bone marrow aspiration was carried out. The prepared smears were fixed with methanol, stained with Giemsa and examined microscopically for the presence of amastigotes. In necessary occasion, culture was carried out into biphasic culture media (prepared from nutrient agar containing 10% whole rabbit blood overlaid with liver infusion tryptose broth (LIT) containing 100-200 UI/ ml penicillin G and 1 ug/ ml streptomycin). The inoculated cultures were incubated at 21 °C for up to six weeks and examined weekly for the presence of promastigotes. Meanwhile, for mass production of promastigotes, Schneider Insect (HIMEDIA) and RPMI1640 (GIBCO) media were used.

Molecular characterization

Some *Leishmania* promastigotes which had been isolated from VL patients and dogs following mass production in RPMI1640 media, were analyzed by RAPD- PCR techniques and compared the results with standard species of *Leishmania infantum,* (MCAN/IR/96/LON49), *L. tropica* (MHOM/IR/01/Yazd) and *L. major* (MRHO/IR/75/ER) in the School of Public Health, Te-

hran University of Medical Sciences and izoenzyme analysis at the London School of Hygiene and Tropical Medicine, UK and Shiraz University of Medical Sciences. (9, 23- 26). Dr KP Chang from Chicago University, USA, confirmed some of the *Leishmania* identification with PCR-RFLP using nagt gene primers.

Data analysis

Chi-squared was used to compare sero-prevalence values relative to gender, age, geographical zone, and tribe. Analyses were with Epi-Info software, with a *P* value of < 0.05 or < 0.01 as statistically significant.

Results

Sero-epidemiological survey

The sero-prevalence rate (SPR) in titers 1: 3200 and above was 6.2% (Table 1, Fig. 1). About half of them were showed at least one clinical manifestations including fever, anemia, splenomegaly and hepatomegaly who referred for complete physical examination and appropriate treatment. Of the 6558 serum samples prepared randomly from children under 12 yr old among residents in the villages of 6 provinces of Iran, 168 cases (2.6%) showed anti-*Leishmania* antibodies in titers of ≥ 1: 3200 by DAT (Table 2). Twenty five (14.9%) patients had signs and symptoms of kala-azar .All of them were seropositive with DAT analysis (titers ≥ 1: 3200).

As shown in Table 3; 23(1.7%) of children belonging to nomads had *Leishmania* infection while 45 (0.9%) from settled populations were infected. Statistically significant difference between these two groups of children, were seen ($P= 0.006$).

Four distinct geographical zones of Iran could be defined by seropositivity including: northwestern (4.4%), northeastern (4.6%), central (1.9%) and southwestern (1.5%) (Table 4). A significant difference was observed between the northwest and northeast with and the other two locations ($P< 0.01$).

Anti-*Leishmania* specific antibodies, using the cut-off value of 1: 3200 and above were detected in male and female individuals. The seroprevalence values between male and female were 58% and 42%, respectively. Statistically significant gender differences in human *Leishmania* infection were observed ($P< 0.01$).

Referring to human age groups, the highest seroprevalence (93.6%) was found in children younger than 5 yr old and the lowest values (6.4%) in individuals higher than 5 yr old (Fig. 2). Strong statistical significance was observed between these two age groups ($P< 0.01$).

Fifty-three of the 223 of sero-positive individuals showed at least one clinical sign including fiver, anaemia, splenomegaly, anorexia and so on (Table 5). Predominant signs and symptoms in 233 hospitalized patients with anti-*Leishmania* anti-bodies at 1: 3200 and higher, were fever (88.0%), splenomegaly (84.5%), and anemia (82.8%).

Altogether, 156 of examined dogs (11.0%) were positive by DAT (1: 320 and above). The highest prevalence of canine *Leishmania* infection (46.4%) related to Shirvan & Bojnoord districts, northeast of Iran (Table 6).

From 10 jackals (*Canis aureus*), 10 foxes (*Vulpes vulpes*) and 10 wolves (*Canis lupus*) was parasitologically and serologically positive one of each animal *L. infantum* was only principal agent that isolated from these animals. Visceral leishmaniasis caused by *L. infantum* in a wolf from northwestern Iran was reported for the first time.

Parasitological study

During this study, 25 of the seropositive domestic dogs (1: 320 and higher) were dissected after obtaining owner consent, and all of them were parasitologically positive.

Characterization

Ten out of 11 isolates from infected dogs were identified as *L. infantum* by RAPD-

PCR analysis techniques. The only isolate of a *Leishmania* sp. isolated from both skin lesions in the face and bone marrow aspiration in a HIV^+ man who co-infected with VL was determined as *L. tropica* by RAPD-PCR and confirmed by RFLP analysis of PCR amplified from the sample using specific restriction enzyme discrimination. This was the first report for isolation of *L. tropica* from viscera of dogs in Iran.

Table 1: Serological reactivity (≥1:3200) in 5586 serum samples tested for human visceral leishmaniasis by direct agglutination test (DAT) in patients referred to remote laboratories from studied areas (2002-2005)

Province	No. of tested	Positive*	
		n	%
East Azerbaijan*	1814	144	7.9
Ardabil*	3638	173	4.7
Khorassan**	15	5	33.3
Bushehr **	28	8	28.6
Tehran and other areas**	91	18	19.8
Total	5586	348	6.2

*Suspected patients in endemic areas
**Clinically suspected hospitalized patients

Table 2: Sero-prevalence of human visceral *Leishmania* infection by direct agglutination test (DAT) with specific antibodies against *Leishmania infantum* in titers 1:3200 to 1:102400 in endemic areas of Iran from 2002 to 2005

Province	District	No. of tested	Positive		clinical signs	
			n	%	n	%
Ardabil	Germi	1155	32	2.8	4	12.5
	Meshkin-Shahr	885	32	3.6	5	15.6
	Ardabil, Parsabad Khalkhal	470	24	5.1	4	16.7
Chaharmahal	kohrang	554	13	2.3	5	38.5
Fars	Mamasani	321	6	1.9	3	50.0
Khorasan	Bognourd,Shirvan	1088	5	0.46	3	60.0
Lorestan	Poshtkooh	457	6	1.3	1	16.7
Kohkoloyeh	Yassuj	1628	24	1.5	-	-
Total		6558	168	2.6	25	14.9

Table 3: Sero-prevalence of human *Leishmania* infection in nomadic tribal regions and settled areas (2002-2005)

Nomad/Settled	No. of tested	No. of DAT Positive	Prevalence (%) (≥1: 3200)
Nomadic	1345	23	1. 7
Settled	5213	45	0.9
Total	6558	68	1.0

Table 4: Sero-prevalence of human *Leishmania* infection by geographical zones of Iran (2002-2005)

Zone	No. of tested	No. of DAT positive	Prevalence (%) (≥1:3200)
Northwest	2510	111	4. 4
Northeast	1088	50	4.6
Central	1011	19	1.9
Southwest	1949	30	1.5
Total	6558	210	3.2

Table 5: Frequency of signs & symptoms in 233 hospitalized patients in Ardabil and Bushehr Province

Signs/Symptoms	n	%
Fever	205	88.0
Anemia	193	82.8
Lymphodenopathy	19	8.1
Splenomegaly	197	84.5
Hepatomegaly	85	36.5
Anorexia	125	53.6
Vomiting	53	22.7
Diarrhea	58	24.9
Cachexi	21	9.0
Malnutrition	105	45.0
Cough	88	37.8
Bleeding	14	6.0
Oedema	12	5.1
Ascite	6	2.6

Table 6: Sero-prevalence of canine visceral *Leishmania* infection by DAT (≥1:320) in studied areas (2002-2005)

Province	District	No. of tested	Positive (≥1:320)	Prevalence (%)	*Leishmania* sp*
Ardabil	Germi	22	3	13.6	*L. infantum*
	Ardabil, Pars-abad & Meshkin-hahr	268	27	10.1	*L. infantum & L. tropica* (first report)
East Azerbaijan	Ahar	80	29	36.2	*L.infantum*
Lorestan	Eastern Miankooh	12	1	8.3	No isolated
Khouzestan	Ahwaz	210	10	4.8	No isolated
Chaharmahal	Kohrang	301	33	11.0	No isolated
Fars	Noorabad-Mamasani	31	3	9.7	*L.infantum*
Fars	Kazerun	431	33	7.7	No isolated
Khorassan	Shirvan & Bojnoord	28	13	46.4	*L.infantum*
Total		1383	152	11 .0	*L.infantum & L. tropica*

*determined by RAPD-PCR &RFLP-PCR

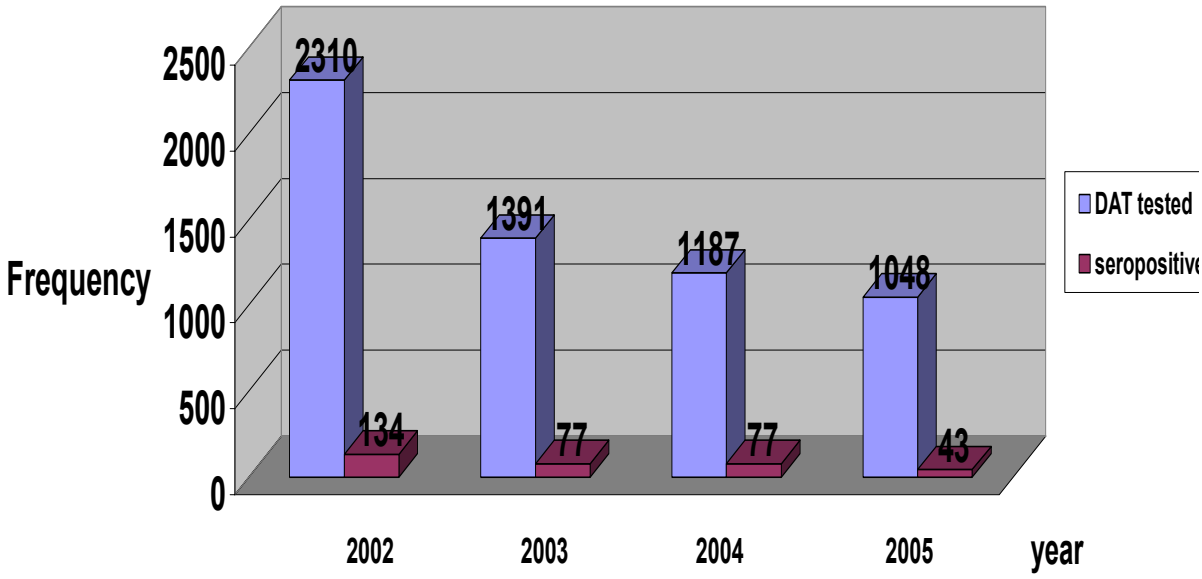

Fig. 1: Frequency of anti-*Leshmania* antibodies (≥1:3200) using DAT in suspected individuals referrd to kala-azar laboratories in the endemic regions of northwest of Iran during of 2002-2005

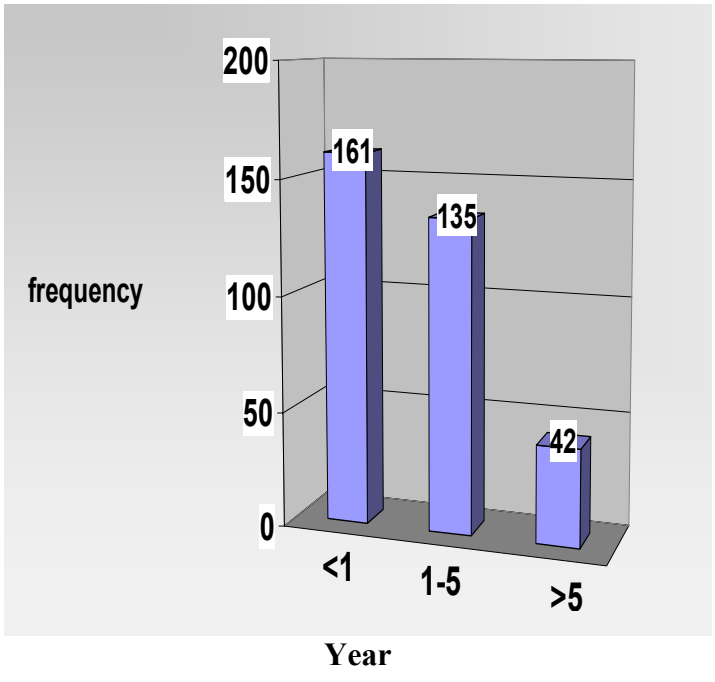

Fig. 2: Distribution of human visceral *Leishmania infantum* infection by age group in patients referred to remote laboratories of endemic areas from northwest of Iran (2002-2005)

Discussion

Among the specific serological tests, DAT was found to be more specific (72-100%), sensitive (92-100%), and practical particularly in endemic areas of the world (17, 18). The results of the DAT for detection of *L. infantum* infection in humans and dogs were excellent. Therefore, we used of the DAT for the determination of seroprevalence of human and canine *Leishmania* infection.

As the delay in the diagnosis and treatment of kala-azar causes high mortality in the patients thus, serological surveillance using DAT, and treatment of seropositive cases (*Leishmania* specific antibodies at 1: 3200 and above) who have clinical signs and symptoms are carrying out with cooperation of Provincial Health Services in the endemic foci in northwest, northeast and south parts of Iran since 1997 (21).

In this study, serological surveys using DAT analysis showed that 2.6% (active case detection) and 6.2% (passive cease detection) of the population in the study areas had anti-*Leishmania* antibodies in titers of 1: 3200, respectively, 93.6% of them were children under 5 yr old. A considerable number of seropositive cases were found among children with no previous history of kala- azar. The peak number of cases was in children 1-2 yr old and the seropositive rate decreased with increasing of age of the children. Prior studies in the Islamic Republic of Iran have shown a seropositive rate of about 50% in the age group 1-2 yr and 96% of seropositive cases in children up to 8 yr old (8, 27). No anti-*Leishmania* antibodies were detected with titers 1: 3200 in adults.

About 58% of seropositive individuals were male. Statistically significant difference (P<0.01) was observed between males and females. Previous cross-sectional IFA and DAT serological surveys of VL in endemic foci of Iran showed that females were exposed and become infected at least as much as males. However, sub clinical forms of the disease may be more common in females than males. In some rural areas, the rate of active kala-azar cases in males may be higher than females (27).

Out of 223 diagnosed cases of kala-azar found in this study, fever, anemia and splenomegaly were the predominant clinical features. These signs and symptoms are the same as those found in other clinical studies (5, 27). Examination of the history of kala-azar patients showed that kala-azar cases were much more frequent in nomads, and most seropositive cases were found among nomadic children because of large dog populations existed with them. The four distinct established zones of mean seroprevalence (northwestern 4.4%), (northeastern 4.6%), (centeral 1.9%) and (south 1.5%) show the geographical regions with various weathers.

High levels of human *Leishmania* infection in the cold northwestern and northeastern parts of Iran were shown to be the most important focus of visceral leishmaniasis in the country. Dogs from this zone seem to have the most important role with the disease because of large dog populations (7 dogs/ 100 humans in Meshkinshahr area was found previously) and their heavy infections that sometimes reached to 20% in some of the villages (29-33). The dog populations and *Leishmania* spp. infection rate of dogs in the hot south zone was low (5).

The animal reservoir hosts were found in this investigation to be the Canidae family (dog, jackal, fox, and wolf). Dogs and wild canines are the domestic reservoir for *L. infantum* in both the old and new worlds. Determination of the prevalence of canine *Leishmania* infection is necessary to define control measures for zoonotic visceral leishmaniosis (28). In this study,

we isolated *L. infantum* from 10 domestic dogs, one Jackal, one fox and one wolf. Previous studies in the same areas have found that parasites infecting humans and dogs are the same zymodeme of the parasite (8, 28). Therefore, the Canidae family especially domestic dogs are the most important source of *L. infantum* infection for human. One of the *Leishmania* sp. that was isolated from a domestic dog in northwestern Iran near the Republic Azerbaijan was identified as *L. tropica*. This species of *Leishmania* is reported in Iran for the first time. This *Leishmania* species as a cause of visceral leishmaniasis in humans and dog was described recently (6, 29). Results of the present study demonstrate that wild canines including jackal, fox and wolf were infected by *L. infantum* and it seems that these carnivores have the possible role of secondary reservoirs in endemic areas particularly in villages located in mountainous regions where the transmission cycle takes place. In previous studies, *Leishmania* spp. were isolated from the jackal and fox (3, 4) but species of the isolated parasites had not been determined. In this survey, we have identified all the *Leishmania* spp. that were isolated from wild canines and this is the first record of *L. infantum* infection in a wolf out of the10 which were shot and examined around Ahar city where HVL and CVL are highly endemic (6).

In conclusion, As Mediterranean type of visceral leishmaniasis has been proved by various studies. DAT is helpful for rapid case finding; establish surveillance and seroepidemiological studies of visceral leishmaniasis in various parts of Iran.

To control VL in Iran, we suggest the following measures: eliminating stray dogs; identifying suspect leashed dogs by periodic DAT serological tests and exterminating those found infected; and identifying human cases using practical serological tests such as DAT and treating infected individuals in order to decrease the mortality rate. Nevertheless, further investigations are needed to discover the main vector of the disease in this area and applying vector control measures.

Acknowledgements

Some parts of this study received financial support from the Institute of Public Health Research, Tehran University of Medical Sciences (Projects No: 241/74/3, 241/82/54/60, 241/74/81/3/5).

We thank Dr KP Chang for the performance of PCR-RFLP by the primers, designed from nagt gene at the Chicago University, USA, as well as the staff of the District Health Centers in Ardabil, Meshkin-Shahr, Parsabad, Tabriz, Kalibar and Ahar from the northwest, Dashti, Dashtestan, Yassuj and Marvdasht from the south, Bojnoord an Shirvan from the northeast, Lorestan and Shahrekord from the central parts of Iran.

We wish to thank Dr MH Motazedian, Dr H Ourmazdi, Dr B Sarkari, M Mahami, N Pedram, Dr Changizi, Dr Y Hamzavi, MT Satvat, A Chegeni-sharafi, and our colleagues in local leishma-niasis laboratories areas in the VL endemic studied areas in Iran for this sincere collaboration.

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