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Serological survey and comparison of two polymerase chain reaction (PCR) assays with enzyme-linked immunosorbent assay (ELISA) for the diagnosis of canine visceral leishmaniasis in dogs

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Visceral leishmaniasis (VL) is systemic zoonotic parasitic infection that is a health problem in some tropical and subtropical countries. The purpose of our study is to determine the seroprevalence of canine visceral leishmaniasis (CVL) in owned dogs of the Sarab area and to identify the species of *Leishmania* isolated from these dogs. We also compared the sensitivities and specificities of two polymerase chain reaction (PCR) assays (kDNA and ITS1) used for *Leishmania infantum* identification with culture, microscopic detection and enzyme-linked immunosorbent assay (ELISA) methods as well as validate the PCR techniques for the molecular diagnosis of CVL. Sera from 384 dogs of 30 villages around Sarab, were tested by ELISA and buffy coat blood fractions after sampling tested with PCR by specific primers (kDNA, ITS-18sRNA). Thirty-five dogs were seropositive by ELISA. The seroprevalence rate (SPR) of CVL was 9.1% (CI, 95% 6.6 -12.4). The most important serological result was a high proportion of seropositivity for leishmaniasis. Out of 361 (94%) asymptomatic dogs, 31 (8.6%) were seropositive, and out of 23 (6%) symptomatic dogs, 4 (17.4%) were seropositive. Agreement between the ELISA test and clinical signs was 86.7%. Each assay was performed on 60 blood samples. PCR of kDNA (7/60 positives, 11.8%) was the most sensitive of the assays examined, followed by ELISA (3/60, 5%) and ITS1-PCR (2/60, 3.4 %). All diagnostic assays were highly specific (100 %) and had positive predictive values (PPV) >90% and negative predictive values (NPV) >88% for CVL. As expected, kDNA-PCR proved to be the most sensitive (87.5 %) assay for leishmanial DNA in peripheral blood. This study shows that kDNA-PCR is significantly more sensitive than the other parasitological and serological methods, allowing the identification of infected dogs even before the appearance of serum *L. infantum* antibodies. Because kDNA-PCR is the most reliable, sensitive, and also a rapid diagnostic assay for CVL, it should be employed as the new standard for routine diagnosis.

Key words: *Leishmania infantum*, polymerase chain reaction, kinetoplast DNA, enzyme-linked immunosorbent assay, Visceral leishmaniasis, dogs, prevalence.

INTRODUCTION

Infection with the protozoan parasite *Leishmania* spp. (Kinetoplastida: Trypanosomatidae) can lead to three different forms of disease: cutaneous, mucocutaneous, and visceral leishmaniasis (VL). Visceral leishmaniasis,

also called Kala-azar, is the most severe form of leishmaniasis caused by the *Leishmania donovani* complex species of *Leishmania* (*L. donovani, L. d. infantum, and L. d. chagasi*) (Farajnia et al; 2008). Visceral leishmaniasis is a systemic zoonotic infection recognized as an emerging endemic disease in more than 80 tropical and subtropical countries. Up to half a million new cases of VL occur worldwide every year (Noyes et al., 1998). A prevalence of 1 - 37% has been reported in the Mediterranean and Middle East, and the World Health Organization has named VL as one of the top six infectious diseases of humans (Bettini et al., 1986; Gradoni et al., 1995). Canine visceral leishmaniasis (CVL) is a zoonotic disease in endemic areas, which is more common in the Mediterranean and Middle East (Belazzoug; 1992; Gradoni et al., 1995; Godal et al., 1996; Mohebali et al., 2005; Edrissian, 1990; Nadim et al., 1994; WHO, 1993). The presence of leishmaniasis depends on a variety of ecological and biological factors. Because the various Leishmania species depend as much on specific reservoirs as on specific vector species, a Leishmania focus can only exist if suitable ecological conditions are present for both the host animal species and the vector sand fly species. The topography and the climate are essential for the maintenance of the parasite life cycle. The transmission of the parasite and maintenance of the infectious cycle are only possible if the reservoir and the vector live close enough together. Mediterranean-type *Leishmania infantum* is the agent of VL in Iran. Dogs (*Canis familaris*) act as domesticated hosts, and jackals, foxes and wolves are the major wild reservoir hosts for CVL (Mohebali et al., 2005; Fakhar et al., 2004; Edrissian, 1990; Moshfe et al., 2008; Mazloumi et al., 2007). Sand fly vectors belonging to *Phlebotomus* spp. and *Lutzoumyia* spp. are responsible for transmission of *Leishmania* spp. between humans and animal reservoirs (Abranches et al., 1991; Belazzoug, 1992; Mohebali et al., 2005; Molina et al., 1994). Asymptomatic dogs are the most important source of sand fly vectors for parasite transmission to humans (Mohebali et al., 2005). Thus far, at least four endemic disease foci have been studied and reported in some areas of Iran such as the

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Abbreviations: VL, Visceral leishmaniasis; **CVL,** canine visceral leishmaniasis; **HVL,** human visceral leishmaniasis; **ELISA,** enzyme-linked immunosorbent assay; **EDTA,** ethylenediaminetetraacetic acid; **PCR,** polymerase chain reaction; **kDNA,** kinetoplast DNA; **ITS,** internal transcribed spacer; **MEM-α,** minimum essential medium alpha; **UV,** ultraviolet; **TN,** true negative; **TP,** true positive; **FN,** false negative; **FP,** false positive; **SPR,** seroprevalence rate; **IFAT,** indirect immuno fluorescence antibody test; **PPV,** positive predictive values; **NPV,** negative predictive values.

provinces of Ardebil (Meshkin Shahr, Moghan, Ghermi, Pars Abad and Bilesavar), East Azerbaijan (Kaleybar, Ahar and Azarshahr), Fars (Jahrom, Ghir and Kazeron), Semnan, Bushehr or Qom city and also, the provinces of Kerman and Karaj (Fakhar et al., 2004). Sporadic cases of VL are also reported every year in other parts of Iran (Mohebali et al., 2005). To determine the type of *L. infantum*, *Leishmania* samples were isolated from animal reservoirs and tested by biochemical typing (isozyme) of LON49, in the provinces of Ardebil and East Azerbaijan (Mohebali et al., 2005). In many cases, human visceral leishmaniasis (HVL), caused by the *Leishmania* strains complexes of the above mentioned provinces, is lethal if not promptly diagnosed and treated (Mohebali et al., 2005). Yet in the Mediterranean and Middle East, only a small percentage of infections progress to clinical disease (Fakhar et al., 2004). HVL in this region is primarily a disease of young children; however, its epidemiology is changing. In Southern Europe, 50% of new cases are in adults co-infected with HIV (Edrissian et al., 1993). Thus, CVLs are considered as important animal reservoirs for mentioned human disease (Abranches et al., 1991; Mohebali et al., 2005). Diagnosis of leishmaniasis can be difficult due to the wide spectrum of clinical signs and symptoms that are similar to other diseases caused by different etiological factors (Harris et al., 1998). In fact, microscopic examination and diagnosis is usually based on routine Giemsa-stained tissue smears or *in vitro* parasite cultivation. On one hand, although, microscopic examination is fast, cheap and easy, it lacks the necessary sensitivity when the number of parasites present in the tissue is low. On the other hand, differential diagnosis of Leishmania species from samples of bone marrow, lymph nodes and spleen is very aggressive, and special skills are required (Reale et al., 1999; Singh et al., 1999; Aransay et al., 2000; Da silva et al., 2001; Nasereddin et al., 2006). Enzyme-linked immunosorbent assay (ELISA) is also used widely to detect CVL and HVL; however, the sensitivity and specificity of this technique depends on the antigen used. Many types of antigens, including crude and recombinant proteins (dp72, gp70, rK39, and HSP70) have been tested, but an antigen solution such as antigen retrieval with Tris/EDTA (ethylenediaminetetraacetic acid) pH 9.0 buffer is suitable for most antigens. Sodium citrate pH 6.0 is also widely used, despite cross-reactivity with other diseases (Ashford et al., 1995). Anti-*Leishmania* antibody titres are typically high during the acute disease, and this feature has been exploited for the serodiagnosis of VL using different methods (Farajnia et al., 2004). Unfortunately, there is no single widely accepted standard procedure currently that can be used as a basis for evaluating new molecular diagnostic assays for leishmaniasis; however, polymerase chain reaction (PCR) methods using either genomic or kinetoplast DNA (kDNA) are now frequently cast in this role. Therefore, reliable diagnostic tests for diagnosis in symptomatic and asymptomatic dogs are needed (Nasereddin, et al; 2006). PCR has the highest specificity and sensitivity of
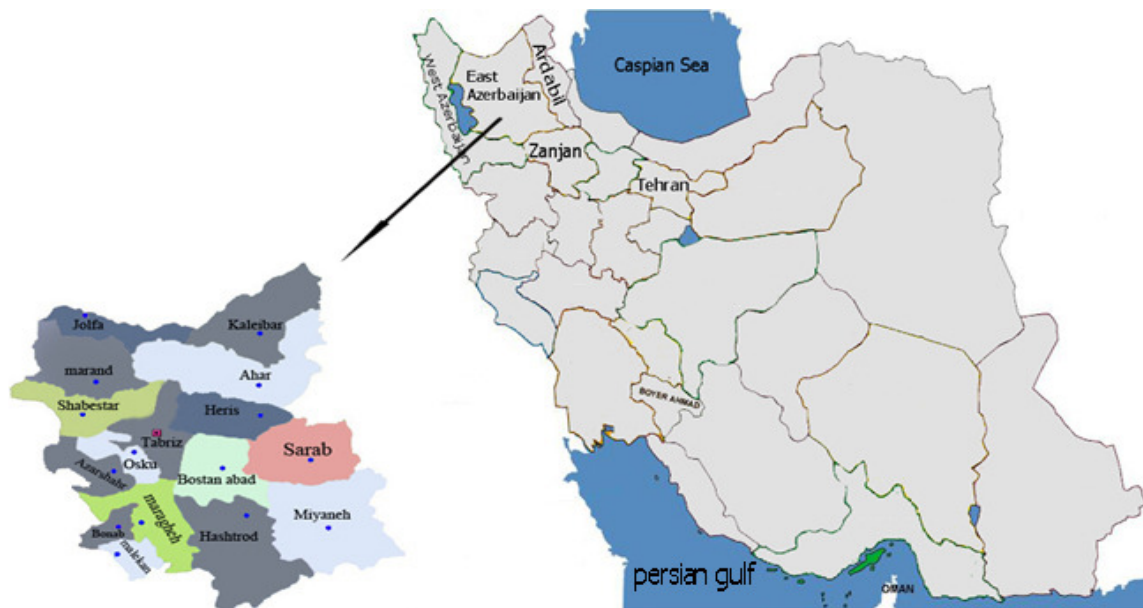
Figure 1. Geographical map of Sarab district in East Azerbaijan province, North West of Iran. The study area is shown by its zooming.

diagnostic techniques for leishmaniasis (Ashford, et al; 1995). Nuclear DNA and kDNA are both used as targets for PCR as an example of targets that include minicircle kDNA, small subunit rRNA (Lemarini et al., 2002) spliced leader miniexon (Harris et al., 1998) and internal transcribed spacer (ITS) (Nasereddin et al., 2006). All of these targets have proven to be suitable for diagnosis, although, kDNA-PCR appears to be the most sensitive assay because kDNA minicircles are present as 10,000 copies per cell (Strauss-ayali et al., 2004). The aim of the present study are to determine the seroprevalence of CVL in owned dogs of the Sarab area and to identify the species of *Leishmania* isolated from dogs in this region. We compared the sensitivities and specificities of two PCR assays (kDNA and ITS1) with culture, microscopic detec-tion and ELISA methods for *L. infantum* identification and validated these PCR techniques for the molecular diagnosis of CVL.

MATERIALS AND METHODS

Study area, dog population and sampling

Sarab district is located in East Azerbaijan Province in North-western Iran with moderate mountainous climate. It covers an area of approximately 18/3452 km², including 168 villages, and its population is estimated to be 148,831 of which 43% is settled in urban areas and 57% is settled in rural areas. Most of the inhabitants of Sarab district are involved in agriculture and animal husbandry, live in mud or stone houses, and maintain domestic animals, such as sheep, goats, chickens, and dogs. The city of Sarab is situated at an altitude of 1650 m above the sea level and is the closest city to Sabalan Mountain (Figure 1).

The study method was descriptive cross-section, and the sampling method was multi-stage cluster random sampling. Out of 168 villages in Sarab district, 30 villages (cluster) were selected randomly. Based on previous studies, the level of infection of dogs in endemic areas (Meshkin Shahr) were determined lowest (at least 4%) by the number of symptomatic dogs (Moshfe et al., 2008). We tested 384 dogs with a 95% confidence level and less than 2% error and studied endemic cases, approximately with ten humans and one dog per study area. The following information was obtained for each dog using a questionnaire: Owner name, age, gender, hair colour, size, habitancy location, presence or absence of visceral leishmaniasis symptoms (skin abnormalities including dry exfoliative dermatitis, periorbital alopecia, hair loss, cachexia, lethargy and onychogryphosis, local or general lymphadenomegaly, keratoconjunctivitis, big belly, diarrhoea and splenomegaly) and environmental data with local information as well as other distinctive characteristics of each animal owner. Each dog was assigned a number for identification purposes. From each dog, 10 cc of peripheral blood was taken. Before blood clotting, 3 - 4 smears were quickly prepared, and 5 ml of blood was dispensed into poly-propylene tubes. To prevent lysis of blood samples after 6 -10 h, blood samples were immediately taken to the Sarab Hospital laboratory, and sera were isolated by centrifugation at 2000 rpm for 20 min. Five millilitres of blood was dispensed into tubes containing an anticoagulant substance (0.01 ml of EDTA /ml blood) for isolation. Samples were centrifuged at 1500 g for 10 min to separate the buffy coat layer (~300 µl). The isolated buffy coat fraction was washed with phosphate buffered saline (PBS, pH 7.4) and then frozen at -20°C in micro centrifuge tubes until use (Nasereddin et al., 2006). All seropositive dogs were autopsied, and spleen, liver and bone marrow samples were removed for parasite culture.

Enzyme-linked immunosorbent assay

ELISA indirect kits (ID Screen® Leishmania Indirect, Code: LEIS, Paris, France) were used for detection of canine antibodies against *L. infantum*. First, the *L. infantum* diagnostic kits were retrieved from refrigerator and antigen-coated micro plates prepared in raised laboratory temperature (5 ± 21°C). A Biolinx Dynatech ELISA

Table 1. Result of ELISA Indirect kits.

Position	Result
Negative	Percent sample (S/P%)≤40%
Doubtful	50% > Percent sample (S/P%)>40%
Positive	Percent sample (S/P%)≥50%

reader (Dynatech Laboratories, Roseville, Canada) was then used to read the plates at 450 nm. The final optical density of each sample was calculated according to the manufacturer's instructions using the following formula:

OD positive control / OD negative control > 3

Percent sample (S / P %) = OD sample / OD positive control × 100

Percent sample (S / P %) ≥ 50% was positive (Table 1).

Parasite isolation

Buffy coat fractions from 384 samples were subjected to PCR with specific primers (kDNA, ITS-18sRNA), and after it, seropositive dogs were autopsied to determine parasite species. Spleen biopsies and bone marrow aspirates were cultured in specific media for the Novy-MacNeal-Nicolle (NNN) medium supplemented with 10% heat-inactivated foetal calf serum (Sigma-Aldrich, Dorset, UK), RPMI 1640 or Schneider's Drosophila Medium (Gibco Invitrogen, USA). Cultures were studied after 48 h to assess the presence and growth of promastigotes. This practice was repeated once a week for five weeks if results were negative. If growth was not observed after this period, cultures were presumed to be negative. But, when NNN cultures were positive, those transferred to minimum essential medium alpha (MEM-α) fluid medium for a large-scale culture once again. These cultures were transferred to the Drug Applied Research Centre at Tabriz University of Medical Sciences for PCR testing (kDNA, ITS-18sRNA). The primers used to amplify kDNA were 5′-TCGCAGAACGCCCCTACC-3′ (forward); and 3′-AGGGGTTGGTGTAAAATAGG (reverse) and the primers used to amplify ITS-18sRNA were 5′-CTGGATCATTTTCCGATG-3′ (forward) and 3′-ACACTCAGGTCTGTAAAC (reverse). DNA from *L. infantum* was used as a positive control and sample containing all PCR materials without DNA used as a negative control. ITS-18sRNA PCR products were separated on 1.5% agarose-acrylamide gels in 1X tris-acetate-EDTA (TAE) buffer at 12 V/cm, and kDNA PCR products were separated 17 V/cm (GeneR*uler™* DNA Ladders*, © Fermentas*). All gels were stained with ethidium bromide (10 mg/ml in ddH$_2$O), visualised, and images were captured using a ultraviolet (UV) transilluminator imaging system (UV- BIO-RAD™ und Sequi-Gen™ sind eingetragene Warenzeichen von BIO-RAD Laboratories). A suitable marker was used to determine the molecular weights of the PCR products. Parasite species were identified by comparing the electrophoresis pattern (fingerprint) with reference strains of *L. infantum*, (MCAN/IR/96/LON49), *L. tropica,* (MHOM/IR102/Mash4) and *L. major* (MRHO/IR/75/ER) (Mohebali et al., 2002, 2004, 2005). Seropositive samples conformed to *L. infantum* using primers specific, and PCR products were analysed by electrophoresis.

DNA isolation and PCR amplification

Genomic DNA was isolated as described previously (Godal et al., 1996). 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 overnight with proteinase K (100 mg/ml, Sigma-Aldrich) at 37°C. DNA was purified further by phenol-chloroform extraction and ethanol precipitation. An Eppendorf DNA thermal cycler and Taq DNA polymerase (Roche, Mannheim, Germany) were used to amplify the desired gene. The reaction mixture included 10 pmol of each primer, 200 mM dNTPs and 1.5 mM MgCl$_2$. PCR conditions were as follows: Initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min (Farajnia et al., 2004).

Statistical analysis

The sensitivity and specificity of tests were determined as follows: A: Sensitivity=TP/ (TP+FN) ×100; B: Specificity=TN/ (TN+FP) ×100, C: Positive predictive value=TP/ (TP+FP) ×100, D: Negative predictive value=TN/ (TN+FN) ×100. TN represents true negative, TP is true positive, FN is false negative and FP is false positive. The degree of agreement between ELISA, ITS and kDNA determined by calculating Kappa (k), were significant (P < 0.001). Statistical significance of sensitivity and specificity between tests were evalua-ted using the chi-square (χ2) and Fisher's exact test. Analyses of seroprevalence values relative to gender, age and clinical signs were conducted using the Statistical Package for the Social Sciences (SPSS) software version 13.5 (SPSS Inc., Chicago, Ill), with a probability (p) value of <0.05 considered as statistically significant.

RESULTS

Serological results

In this study, samples were taken from 384 dogs from 30 villages around Sarab and tested with ELISA. Thirty-five dogs were seropositive (Figure 2). The seroprevalence rate (SPR) of CVL was 9.1% (CI, 95% 6.6 - 12.4). The most important serological result was a high proportion of seropositivity for leishmaniasis. Out of 361 (94%) asymptomatic dogs, 31 (8.6%) were seropositive, and out of 23 (6%) symptomatic dogs, 4 (17.4%) were seropositive. 306 (79.7%) dogs were male and 78 (20.3%) were female; of these, 28 (9.2%) male dogs and 7 (9%) female dogs were seropositive. Significant statistical differences between sex and seroprevalence were not observed (P = 0.962). Of the studied dogs, the largest age group was 2 - 4 years, with 170 (44.3%) dogs. However, only 18 dogs (10.5%) from mentioned age group were seropositive. Agreement between the ELISA test and clinical signs was 86.7%. A significant difference in the presence of antibodies against *Leishmania* was seen between symptomatic and asymptomatic dogs (P = 0.015). The highest rate of infection was the village of Jalde Bakhan, with 9 infected dogs (33.3% of dogs in the village of Jalde Bakhan). The lowest rate of infection was Asb Froshan, with only 1 infected dog (1.4%). Because some dog owners were disinclined to perform test or sampling, we tested 28 animals from between 35 volunteered sero-positive dogs for parasitological examination. For each dog, 20 impression smear slides from spleen, liver and
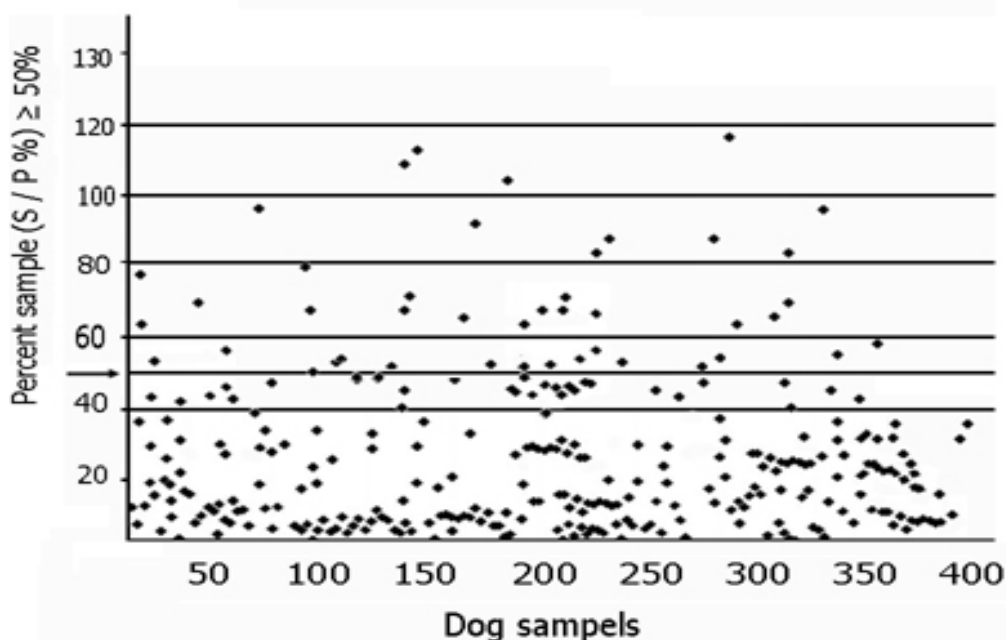
Figure 2. ELISA for antileishmanial antibodies against *L. infantum* antigen in sera of 384 dogs from 30 villages in the Sarab district. Arrow indicates the cut-off value.
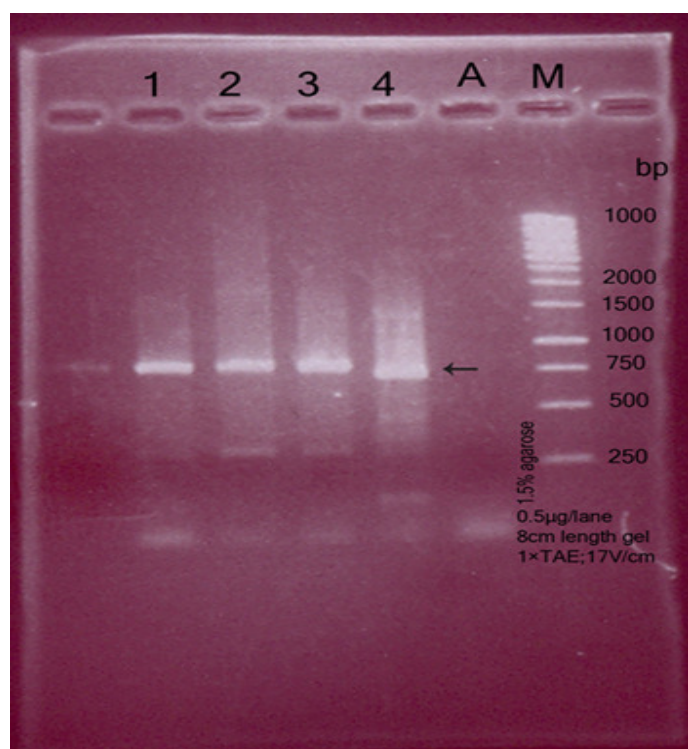

Figure 3. Amplification of parasite DNA of PCR products (KDNA), DNA (1 ng) extracted from cultures of parasite isolates was used for PCR. Lane M: Marker, 100 bp ladder; Lane A: negative control; Lane 4 *L. infantum*, (Standard strain *L. infantum*, (MCAN/IR/96/LON49) School of Public Health, Tehran University of Medical Sciences), and Lanes 1, 2, 3: DNA (1 ng) *L. infantum* isolated from parasite cultures (750 bp).

bone marrow tissues were prepared, Giemsa-stained and examined for Leishman-Donovan bodies. These bodies were observed in the autopsy slides as well as impression smear slides of liver and spleen tissues from 21 (75%) dogs. Of the impression smear slides, 100% of spleen and 85.7% of liver samples were positive (Figures 3 and 4).

Evaluation of PCR and ELISA for CVL detection

Buffy coat samples from 60 dogs were used to compare the sensitivity and specificity of the PCR and ELISA based assays. Seven (11.8%) dogs were positive by kDNA-PCR, three (5%) were positive by ELISA, and two (3.4%) were positive by ITS-PCR. Because no single test can be designated as the gold standard for CVL diagnosis, assessment of test performance was based on the assumption that a dog was positive when at least one assay was positive for *Leishmania* and considered as a confirmed negative if all three assays were negative (Nasereddin et al., 2006; Marfurt et al., 2003). These confirmed values were used as the "gold standard" against which each individual diagnostic assay was measured. All diagnostic assays were highly specific (100%) and had positive predictive values (PPV) >90 % and negative predictive values (NPV) >88% for CVL (Table 2). As expected, kDNA-PCR proved to be the most sensitive (87.5%) assay for Leishmania DNA in peripheral blood. Using kDNA-PCR, 7 (10.1%) and 52 (86.6%) samples were identified as true positives and negatives, respectively.
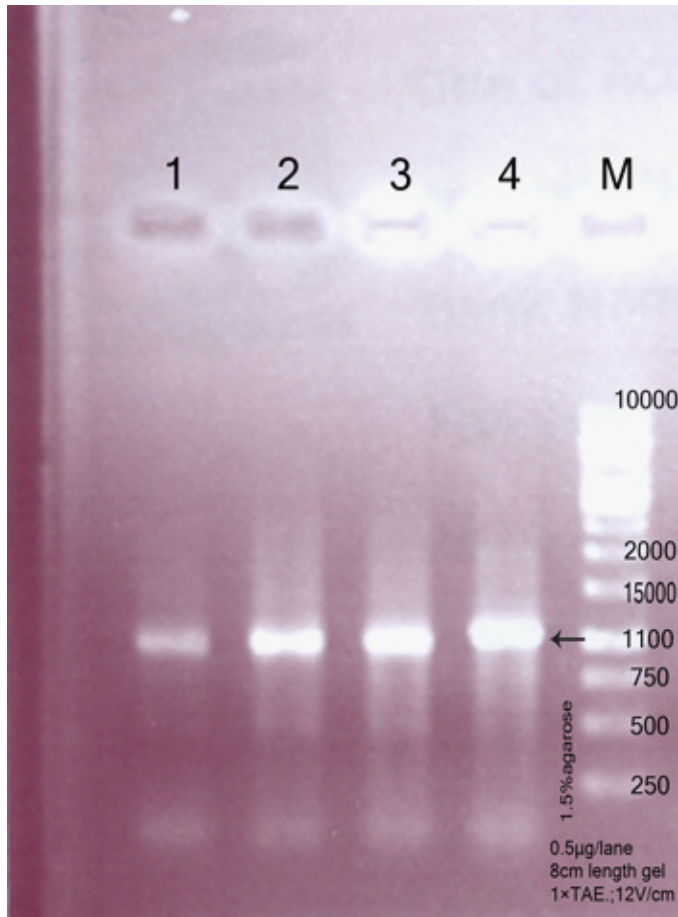
Figure 4. Amplification of parasite DNA of PCR products (ITS-18sRNA), DNA (1 ng) extracted from cultures of parasite isolates was used for PCR. Lane M: Marker, 100 bp ladder. Lane 4 *L. infantum*, (Standard strain *L. infantum,* (MCAN/IR/96/LON49) School of Public Health, Tehran University of Medical Sciences), and Lanes 1, 2, 3: DNA (1 ng) *L. infantum* isolated from parasite cultures (1100 bp).

One sample, identified as positive by ELISA, was missed by kDNA-PCR. The PPV was 99% and the NPV was 98% for kDNA-PCR. The level of agreement between kDNA-PCR and the confirmed results (the kappa coefficient [K] ± standard error [SE] = 0.075 ± 0.924) was excellent, where 0 is chance agreement and 1 is perfect agreement (Nasereddin et al., 2006). ITS1-PCR gave the poorest result of any of the assays used. Only 2/8 (20%) true positives were correctly diagnosed by this test, with 6 (80%) false negatives. The sensitivity was very low (25%), and the PPV and NPV were 91 and 89%, respectively. Agreement between ITS1-PCR and the confirmed golden standard was slight (K ± SE = 0.366 ± 0.190). No DNA product was observed in any of the negative control PCRs. ELISA serology was somewhat better than ITS1-PCR, giving 3 (5%) true positives, 52 (86.6%) true negatives and 10 (16.6%) false negatives that were identified as positive by at least one PCR assay. The

sensitivity of ELISA was low (37.5%). The PPV for the ELISA assay was 94% and the NPV was 91%. Agreement between the confirmed results and ELISA was moderate (K ± SE = 0.183 ± 0.51).

DISCUSSION

The overall seroprevalence rate of CVL was 9.1% in the Sarab district (East Azerbaijan province). Similar results were found in Portugal (Molina et al., 1994), Italy (Gradoni et al., 1995), Morocco (Lemarini et al., 2002), Greece (Ikonomopoulos, et al., 2003; Sideris et al., 1996), and Iran (Edrissian et al., 1993; Mohebali et al., 2005; Fakhar et al., 2004; Mazloumi et al., 2007; Bokai et al., 1998; Soleimanzadeh et al., 1993; Edrissian et al., 1996; Hamidi et al.,1982). Because the annual prevalence of CVL in endemic regions frequently fluctuates and can vary among adjacent villages, additional surveys are needed to establish the trend of CVL in the region studied. Nevertheless, our results imply that the prevalence of CVL may be an important risk factor for human disease in this region (Gavgani et al., 2002). The most important serological result was a high proportion of seropositivity for leishmaniasis (8.6%) among asymptomatic dogs. These data are extremely important because owned asymptomatic dogs can play a significant role in the epidemiology of this zoonotic disease. Furthermore, the domestic dog population could be helpful sentinels to follow the progress of the disease in endemic areas. Epidemiological studies on the reservoir hosts of VL have been discouraged by the lack of sensitive and practical methods to detect infections in the various species. Negative parasitological results do not rule out *Leishmania* infection in dogs; therefore, a combination of clinical, parasitological, serological, and therapeutic tests must often be done to confirm *Leishmania* infection in this reservoir host (Harith et al., 1989). Several diagnostic tests, such as the indirect immunofluorescence antibody test (IFAT), ELISA, microscopic examination of smears, and cultural isolation are routinely used for diagnosis. The gold standard for VL diagnosis is the detection of parasite in specimens of infected organs. However, these samples must be obtained by invasive procedures, such as bone marrow, lymph node or spleen aspirations (Mohebali et al., 2004). *In vitro* cultivation has a good sensitivity but is carried out only in specialised centres; if the results are negative or the number of parasites is insufficient, then the results can be obtained only after several weeks. Specific serology is unreliable for immunocompromised patients, and false negative results cannot be excluded. PCR has been shown to be as good as or better than these diagnostic methods, with the advantage that it provides a more rapid result. A number of PCR assays for the diagnosis of VL due to *L. infantum* have been developed over the past few years (Kazemi et al., 2008). Blood has been used as source of parasite for

Table 2. Comparison of ITS1 and kDNA-PCR with ELISA results for the diagnosis of canine VL.

Method	Result	Confirmed result			NPV (%)	PPV (%)	SP (%)	SN (%)	Kappa*
		Negative	Positive	Total					
I TS1	Negative	52 (86.6)	6 (10)	58 (96.6)	89	91	100	25	0.366 ± 0.190
	Positive	0	2 (3.4)	2 (3.4)					
	Total	52 (86.6)	8 (13.4)	60 (100)					
Elisa	Negative	52 (86.6)	5 (8.4)	57 (95)	91	94	100	37.5	0.183 ± 0.51
	Positive	0	3 (5)	3 (5)					
	Total	52 (86.6)	8 (13.4)	60 (100)					
kDNA	Negative	52 (86.6)	1 (1.6)	53 (89.9)	98	99	100	87.5	0.075 ± 0.924
	Positive	7 (10.1)	7 (11.8)	0					
	Total	52 (86.6)	8 (13.4)	60 (100)					

* All kappa calculations were significant (P> 0.001).

CVL diagnosis in recent studies. In a previous study in Bahia, Brazil, Ashford et al. (1995) used serology to detect *Leishmania* infection in dogs and suggest that PCR might serve as a better gold standard to define *Leishmania* infection than culture or hamster inoculation (Ashford et al., 1995). Pilatti et al. (2009) compared four PCR assays for the detection of *Leishmania* DNA in conjunctival swab samples and showed that kDNA based methods had significantly higher sensitivity. Additionally, Chargui et al. (2009) conducted a study in Kairouan (central Tunisia) and showed that PCR was more sensitive than IFAT and *in vitro* culture. While the choice of method will depend on the kind of information available for diagnosis, several studies have suggested that PCR has the best potential to a direct successful diagnosis or sensitive and species-specific diagnosis (Mohebali, et al; 2002, Reithinger et al., 2002). The second objective of this study is to compare two diagnostic PCR assays with ELISA using peripheral blood to detect CVL. Blood sampling is less invasive, easily repeatable, and more readily accepted by dog owners than bone marrow, spleen, or lymph node aspiration. Because *Leishmania* spp. are intracellular parasites of monocytes and macrophages, use of the buffy coat fraction rather than whole blood should increase assay sensitivity and reduce potential inhibitors. PCR has slowly become the preferred method for diagnosing leishmaniasis because conventional parasitological methods are not sufficiently sensitive (Nasereddin et al., 2006; Strauss-ayali et al., 2004). However, the sensitivity of this assay varies considerably depending on the source of the DNA used for PCR. Accordingly, we found ITS1-PCR to be significantly less sensitive in diagnosing CVL when the DNA used for amplification was purified from either Buffy coat or whole blood of seropositive dogs, as opposed to conjunctiva, spleen, and lymph node biopsies. For this reason, kDNA-PCR should be especially useful in regions in which only one species, *L. infantum*, causes leishmaniasis in dogs. The latter PCR enabled the identification of infected dogs even before the appea-

rance of serum *L. infantum* antibodies (Nasereddin et al., 2006). In many studies, PCR has been found to be more sensitive than microscopy, and it even proved to be more sensitive compared to serology (100% vs. 63%) (Ashford et al., 1995; Osman et al., 1997). Studies have shown that kDNA-PCR is significantly more sensitive than conventional parasitological methods. In agreement with previous reports, the present study found kDNA-PCR testing of peripheral blood to be particularly encouraging, allowing for routine diagnosis of CVL (Ikonomopoulos et al., 2003). We found kDNA-PCR to be highly sensitive, identifying cases missed by ITS1-PCR and/or ELISA. The excellent sensitivity of kDNA-PCR is due to the high copy number of the target kDNA (10,000 copies/cell); while, nuclear DNA targets such as ITS1 are present in only a few hundred copies per parasite cell. In addition to copy number, other parameters can affect PCR sensitivity, such as secondary structure and amplicon size (Nasereddin et al., 2006; Leishmaniasis in Meshkin-Shahr District, 2008; Mazloumi-Gavgani, 2007). Unfolded highly sensitivity of kDNA-PCR of our study is in agreement with previous studies that showed PCR could detect parasite DNA in the tissues and/or blood of seronegative naturally infected or experimentally infected dogs (Gallego et al., 2001). Experimental CVL shows that a 2- to 4-month delay occurs between parasite infection and ELISA seroconversion (Nasereddin et al., 2006; Quinnell et al., 2001). This delay also occurs in natural infections, during which dogs that are parasite-positive by culture remain seronegative for long prepatent periods (Quinnell et al., 2001). Therefore, in the present study, the 5 (8.4%) PCR positive/ELISA negative dogs were infected and, due to the absence of a humoral immune response against the parasite, can be considered as asymptomatic carriers. Our finding that 1 (3.3%) ELISA-positive dog was PCR-negative suggests that both assays may be required to accurately assess CVL prevalence in endemic areas. One possible explanation for the negative PCR reactions in seropositive dogs is that the amount of parasites in peripheral blood is low or

even temporarily absent, and that the blood parasite load fluctuates dramatically over time, especially in asymptomatic or oligosymptomatic dogs (Nasereddin et al., 2006). ITS1-PCR using buffy coat DNA isolated from experimentally infected oligosymptomatic dogs showed significant fluctuations in the number of PCR-positive dogs over a 30 day period following seroconversion (Strauss-ayali et al., 2004). Nasereddin et al. (2006) showed that kDNA-PCR is significantly more sensitive than conventional parasitological methods. Quinnell et al. (2001) showed that the number of positive parasite cultures from bone marrow peaked in naturally infected dogs at two months post-seroconversion and then decreased. Thus, our results are similar to those reported by other researchers (Reale et al., 1999; Ikonomopoulos et al., 2003). kDNA is the most sensitive and reliable diagnostic assay for CL and should therefore be employed as the new standard for routine diagnosis when species identification is not required (Bensoussan et al., 2006).

Conclusions

This study shows that CVL is a problem in Sarab district and that infected dogs may play a role in the incidence of human disease. Finally, kDNA using peripheral blood can be used as sensitive, specific, and rapid screening test for CVL.

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