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Original Article

Preparation and Development of An Immunochromatographic Test for early Detection of Canine Visceral Leishmaniasis: A Preliminary Study

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Abstract

Background: Canine visceral leishmaniasis (CVL), caused by *Leishmania infantum* (*L. infantum*), is a serious parasitic disease. Domestic dogs in endemic regions act as primary reservoirs for the parasite. Early diagnosis, control, and regular screening of dogs are essential for effective disease management. This study aims to develop a practical, low-cost immunochromatographic test (ICT) for detecting specific anti-*Leishmania* antibodies in domestic dogs.

Methods: Overall, 93 canine serum samples were collected from endemic and non-endemic areas of CVL in Iran. Rabbit anti-canine antibodies were conjugated with gold nanoparticles, and strips were coated with *Leishmania* antigens. A drop of serum was added to each strip, and a positive result was indicated by two red lines. The validity of ICT for the detection of CVL in the field was determined with comparing to direct agglutination test (DAT) as gold serological test on 40 sera with anti-*Leishmania* antibodies at titers ≥1:320 considered as positive control as well as 53 sera with no anti-*Leishmania* antibodies including 10 collected from healthy dogs and 43 from other infectious diseases considered as negative control sera.

Results: A sensitivity of 82.5% (CI 95%.78.1-86.9) and specificity of 90.5% (CI 95%. 87.7-92.5) were found at a 1:320 cut off titer when DAT confirmed cases were compared with negative control. The agreement (0. 871) was found between ICT and DAT using kappa analysis.

Conclusion: A relatively good agreement was found between ICT and DAT. Further researches on test validation with larger populations in endemic and non-endemic areas of CVL, is recommended.

Introduction

Leishmaniasis is a heterogenous group of diseases and an ongoing public health crisis caused by intracellular protozoan parasites of the genus *Leishmania* (1). Leishmaniasis has different clinical manifestations, including cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL) (2). VL also known as kala-azar, is a life-threatening parasitic disease caused by various *Leishmania* species and transmitted by the bit of infected female Phlebotomine sandflies (3). VL is endemic in approximately 98 countries, affecting about 12 million people annually (4). Clinical manifestations can range from asymptomatic to fatal, with over 350 million individuals at risk globally (3). Key risk factors for leishmaniasis include poverty, malnutrition, climate change, and environmental factors that promote the proliferation of sand-fly vectors (5). CVL is a complex of infectious; sever zoonotic disease of veterinary and public health importance caused predominantly by *L. infantum* (6). In the Mediterranean region, including Iran, domestic dogs (Canis familiaris) serve as primary reservoir hosts for zoonotic VL (7). Dogs frequently show no clinical signs; however, when symptomatic, they may range from anemia and weight loss to hepatosplenomegaly, renal alterations, and onychogryphosis. Without appropriate treatment, these conditions can be fatal. Moreover, even after receiving therapy, infected dogs may continue to serve as a source of infection for vectors, thereby sustaining the transmission of the disease in urban environments and posing a significant public health concern (6). Dogs can be transmitted the parasite to humans through the bite of infected sandflies. Children living in close proximity to infected dogs are particularly vulnerable in endemic areas (8). Despite advances in diagnosis and treatment, efforts to control VL remain challenging due to insufficient resources for vector control, diagnosis, and treatment in endemic regions (9, 10). Most VL cases occur in regions with poor socio-economic conditions, including Brazil, India, Ethiopia, and Sudan, which together account for more than 90% of global cases (11, 12).

DAT is considered the gold standard for diagnosing CVL (13-15). However, the implementation of DAT and other diagnostic methods is hindered by the lack of rapid, cost-effective, and accurate testing tools (3, 16, 17). Recent advancements in immunological techniques, such as ICTs, offer a promising approach for early detection and rapid screening of CVL in endemic areas (18, 19). These tests have the potential to enhance surveillance and control measures, ultimately reducing the human burden of VL (18, 20).

This study aims to evaluate the diagnostic performance of ICT compare to the standard DAT method for detecting CVL, using serum samples collected from dogs under controlled laboratory condition.

Materials and Methods

Ethical considerations

This study was approved by the Ethical Committee of Tehran University (Ethical code: IR.UT.VETMED.REC.1402.042). Dog sera were collected with the coordination of the Iran Veterinary Organization after obtaining informed consent from the owners.

Parasite and culture

The *L. infantum* strain (MCAN/IR/14/M14, GenBank Accession number KT201383) was isolated from a VL-infected dog in Meshkin-Shahr. Promastigote culture was conducted according to previously described methods (21).

DAT

In this study, we used DAT as the gold serological test to detect anti-*L. infantum* antibodies in dogs from CVL (13). Altogether, 40 sera with anti-*Leishmania* antibodies at titers ≥1:320 considered as positive control as well as 53 sera with no anti-*Leishmania* antibodies including 10 collected from healthy dogs and 43 from other infectious diseases considered as negative control sera. DAT was performed using a titer cutoff of ≥1:320 and samples

with titers at or above this threshold were classified as positive (22, 23).

SDS-PAGE of L. infantum exosome

SDS-PAGE was conducted to analyze the protein profile of *L. infantum* promastigotes lysates (24). Protein concentration was determined using the Bradford assay. Electrophoresis was performed according to the Laemmli method on 12% tris-glycine SDS-PAGE gels (25). For each lane, 20 µL of sample containing 1 mg/mL of *Leishmania* lysate proteins was mixed with 20 µL of sample buffer. The mixture was loaded onto the gel, and electrophoresis was carried out at 80 V for 90 minutes. Following electrophoresis, the protein bands were stained with Coomassie Brilliant Blue R-250 and de-stained using a solution of 10% acetic acid, 50% methanol, and 40% distilled water (20).

Gelatin Zymography

Gelatin Zymography was performed to evaluate the metalloprotease activity of Gp63 in *Leishmania* lysates (24). Proteins were separated on polyacrylamide gels containing 0.1% gelatin. After electrophoresis, gels were incubated in 2% Triton X-100 for 30 minutes to remove SDS, followed by incubation in 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM CaCl2 at 37 °C for 18 hours to activate proteolytic enzymes. Gels were then stained with Coomassie Brilliant Blue R-250 and destained with 10% acetic acid, 50% methanol, and 40% distilled water for at least 2 hours to reveal clear bands indicative of gelatinase activity (26, 27).

ICT (Antibody detection)

Immunochromatographic assays were developed for the detection of anti-*Leishmania* antibodies in dog serum samples. To produce anti-dog antibodies, two rabbits were immunized with purified dog immunoglobulins (28). The resulting rabbit anti-dog antibodies were conjugated with gold nanoparticles using a standard colloidal gold conjugation protocol (29-31).

Following conjugation, the gold-labeled anti-dog antibodies were dispensed into microvials, which served as sample wells for the immunochromatography test (29). The immunochromatographic strips, pre-coated with *Leishmania* promastigotes antigens, were placed into the microvials containing the dog serum. Upon application, the reaction mixture migrated along the strip by capillary action. A positive result was indicated by the appearance of two distinct red lines: one at the test line corresponding to the presence of anti-*Leishmania* antibodies (indicating the presence of anti-*Leishmania* antibodies) and one at the control line, ensuring the validity of the test procedure (18, 32).

Statistical Analysis

The agreement between DAT and the ICT was evaluated using SPSS version 23. Sensitivity and specificity were calculated as TP / (TP + FN) × 100% and TN / (TN + FP) × 100%, respectively. Kappa coefficient was used to assess repeatability, with 1.0 indicating perfect agreement. A p-value ≤ 0.05 was considered significant. An appropriate concordance (87.1%) was observed between DAT and ICT methods (Table 1).

Results

SDS-PAGE Analysis

SDS-PAGE analysis of *Leishmania* promastigotes lysate revealed multiple protein bands with molecular weights ranging from 10 kDa to over 245 kDa (Fig. 1).

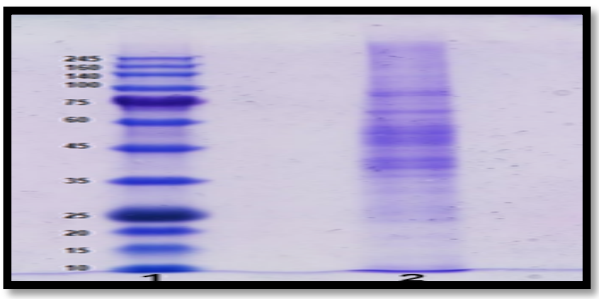

Fig. 1: SDS-PAGE profile: **Lane 1:** Molecular weight marker, and **Lane 2:** *Leishmania* promastigotes lysate.

Zymography Analysis

Zymographic analysis of *Leishmania* promastigotes lysate revealed a prominent metalloproteinase band with an approximate molecular weight of 47 kDa (Fig. 2). It should be noted that, to identify the molecular weight and function of key parasitic proteins, SDS-PAGE and Zymography were initially performed.

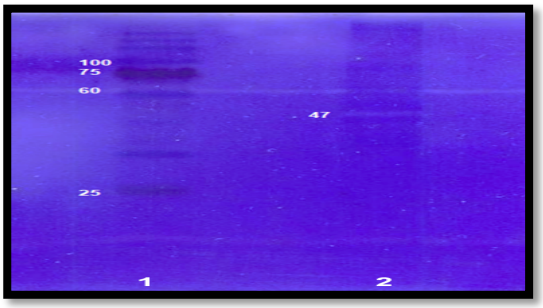

Fig. 2: Zymographic profile of metalloproteinase: **Lane 1:** Molecular weight markers, and **Lane 2:** *Leishmania* promastigotes lysate

Comparison between DAT and ICT

The comparative results between the immunochromatographic rapid test and the gold standard DAT are presented in Table 1 and Fig. 3. Among the 40 DAT positive samples, 33 were also positive by ICT and 7 were negative. Among 53 DAT negative samples, 48 were negative and 5 were false positive by ICT. A sensitivity of 82.5% (CI 95%.78.1-86.9) and specificity of 90.5% (CI 95%. 87.7-92.5) were found at a 1:320 cut off titer when DAT confirmed cases were compared with negative control. The agreement (0. 871) was found between ICT and DAT using kappa analysis.

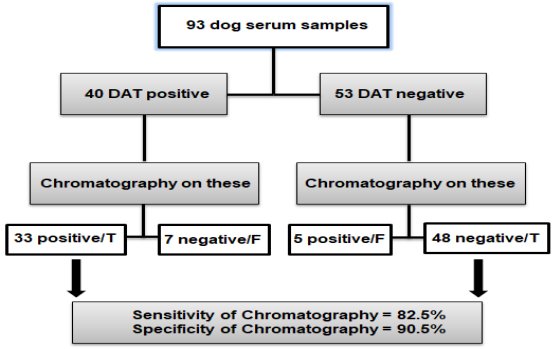

Fig. 3: Flowchart summarizing the evaluating of DAT and ICT tests on 93 dog serum samples. T: True/ F: False

Table 1: Distribution of anti-*Leishmania* antibodies using DAT (cut-off= 1:320) in comparison ICT results

Comparison of DAT and ICT on 40 positive dog sera			
DAT positive titers	**DAT Positive samples**	**ICT positive samples**	**ICT negative samples**
1:320	2	2	3
1:640	5	5	2
1:1280	13	8	2
1:2560	20	18	0
Total	40	33	7

Discussion

VL, or kala-azar, is a systemic disease caused by *Leishmania* parasites (33). The incidence of the disease varies across regions and is influenced by climatic conditions, diagnostic capabilities and environmental factors (34). Diagnostic methods include parasitological, serological, molecular, and biopsy-based approaches (35). Although parasitological techniques are considered the gold standard, they are invasive, require skilled personnel, and are often impractical in resource limited endemic settings (36, 37). Thus, alternative methods have gained increasing attention. This study aimed to develop a field-friendly ICT for screening CVL in endemic areas. Among 93 dog sera tested, 40 were confirmed positive by DAT. The ICT identified 33 true positives, 7 false negatives, and 5 false positives, resulting in a sensitivity of 82.5%, specificity of 90.5% (32-34). Molecular methods such as PCR offer high sensitivity for the detection and species-specific identification of *Leishmania* DNA (18). However, due to its cost and technical requirements, PCR is less suitable for large-scale screening, although it remains valuable for confirming active infections and monitoring treatment responses. Serological methods like DAT, ELISA, and ICT remain to be the most practical tools for field applications in endemic regions (36, 38). In this study, DAT and ICT were employed to detect anti-*Leishmania* antibodies in canine sera. ICT proved advantageous in terms of speed, cost-effectiveness and ease of use, making it suitable for field screening in resource limited settings. Additionally, SDS-PAGE and Zymography were used to analyze *Leishmania* lysates, identifying GP63 metalloprotease (~47 kDa) as a key protein (27, 39). Sequence alignment of GP63 from *L. major* and *L. infantum* revealed conserved regions indicating potential cross-reactivity and broad diagnostic applicability (25, 27). These findings support the use of GP63 as both a diagnostic biomarker and candidate antigen for vaccine development. The immunochromatographic assay demonstrated consistent results across multiple trials, confirming its reproducibility.

VL remains a neglected tropical disease with significant public health implication in endemic regions, including Iran (3). Early and accurate diagnosis is crucial for detecting asymptomatic carriers, co-infections, and differentiating between healthy and infected individuals (40).

This study demonstrated 82.5% sensitivity and 90.5% specificity, with 87% agreement with DAT ($P< 0.05$), confirming ICT's diagnostic potential. Previous research using rk39 and rk26 antigens reported high diagnostic performance in both humans and dogs (19). Sensitivities of 98.9% in dogs and 95.2% in humans further support the value of these antigens in serodiagnosis (19). Dogs serve as primary reservoir hosts for *L. infantum*, playing a pivotal role in sustaining the parasite transmission cycle (41). Screening dogs is essential to control human and canine VL, especially in both endemic and non-endemic regions particularly where asymptomatic cases are present (13, 17). Although parasitological methods offer near perfect specificity, they are often impractical for routine use (42). Given the need for practical and cost-effective diagnostic tools, ICT represents a promising strategy for screening of canine population (37). Similar studies have reported sensitivity of 95.8% and specificity of 98.7% using antigen-based ICTs (18, 40). Both antibody and antigen detection methods are valuable for screening/identifying past and current infections, and their operational simplicity for epidemiological surveillance makes them ideal (43-45). Despite the promising results obtaining in designing and evaluating the ICT strip for the diagnosis of CVL several limitations should be taken when interpreting the findings. First, the sample size was relatively small and the results may not be fully generalizable to larger populations. Also, only the DAT test was used as a reference test for comparison. Additionally, ICT was evaluated only under controlled laboratory conditions, and its stability and perfor-

mance in various environmental settings (such as high temperature, humidity, or prolonged storage) were not assessed.

Conclusion

This study showed that the potential of ICTs, affordable, and practical tools for field screening of CVL, especially in endemic areas. A relative good concordance was found between ICT and DAT. Further research on test validation with larger populations in endemic and non-endemic areas of CVL is recommended. Additionally, the identification of GP63 as a key antigen supports its role in future diagnostic development**.** While ICT may not fully replace molecular methods like PCR, its ease of use and cost-effectiveness make it suitable for mass surveillance in resource-limited settings**.**

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Conflict of interests

The authors declare that they have no competing interests.

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