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Evaluation of canine anti-Leishmania IgG subclasses and their relation with skin signs in naturally infected dogs in the northwest of Iran

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Abstract: Canine leishmaniasis (CanL) is a severe, intracellular parasitic zoonosis and is an endemic complaint in Iran. Dogs are considered the reservoir host for *Leishmania infantum* in the Old World. The aims of this study were to evaluate the relation between anti-*Leishmania* antibody titers (IgG) and skin symptoms of CanL in naturally affected dogs and to assess the correlation of IgG_1 and IgG_2 subclasses of titers with these symptoms. Anti-*Leishmania* antibody titers were evaluated in 100 dogs' blood samples with the direct agglutination test (DAT) and enzyme-linked immunosorbent assay (ELISA) methods. Based on DAT results, 45 samples were positive. Twenty-five of the positive dogs had cutaneous signs of leishmaniasis and 20 of them were asymptomatic (DAT of 1:320 and above). In the dogs, high levels of total IgG were found in symptomatic dogs. In ELISA, we found an apparent correlation between IgG_1 and symptomatic dogs and between IgG_2 and asymptomatic dogs. It seems that measurements of IgG subclasses can be used as gold-standard markers for evaluation of susceptibility or resistance to this lethal chronic infectious disease in dogs.

Key words: Canine leishmaniasis, immunoglobulin G, direct agglutination test, ELISA

1. Introduction

Canine leishmaniasis (CanL) is a severe, intracellular parasitic zoonotic disease that occurs worldwide. Leishmaniasis is an old disease and it still happens in Europe, Asia, Africa, and the Americas (1,2). Unfortunately, *Leishmania* is an endemic complaint in Iran, the Middle East, and Mediterranean areas (1,3). In the Old World, dogs (*Canis lupus familiaris*) are considered the reservoir host for *Leishmania infantum* (1). Dogs are major reservoir hosts for zoonotic visceral leishmaniasis in the world and in Iran (1,3). Northwestern and southern parts of Iran are the most endemic areas of this fatal disease (3,4). The incubation period may last for months to years, during which the parasite can distribute itself in different body organs (5,6). The widely accepted view of CanL highlights the similarity of canine visceral leishmaniasis with human visceral leishmaniasis.

Symptomatic and asymptomatic dogs are reported in CanL, but cutaneous lesions are present in approximately 90% of the infected dogs (7). Skin lesions are main manifestations of *Leishmania infantum* infection in dogs, especially in the Mediterranean region (7). The clinical signs of CanL are variable and dogs can show a variety of clinical symptoms, ranging from asymptomatic to polysymptomatic (5,7). While most symptomatic dogs show dermal symptoms, some may present other signs in combination with or without skin lesions (5,7). In asymptomatic dogs, in which these signs are neglected, serological confirmation of infestation is essential (7).

In addition to weight loss, polyuria, polydipsia, muscle wasting, depression, vomiting, diarrhea, cough, epistaxis, splenomegaly, and lymphadenopathy, cutaneous lesions such as hyperkeratosis, scaling, thickening, mucocutaneous ulcers, and intradermal nodules on the muzzle, pinnae, ears, and footpads are also seen in the infected dogs (7). Furthermore, leishmaniasis can be established by identifying the causative organisms through cytology, histopathology, detection of antibodies against *Leishmania* in serum (such as indirect fluorescence antibody test, direct agglutination test [DAT], and enzyme-linked immunosorbent assay [ELISA]), and polymerase chain reaction (PCR) assay (5).

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It seems that high antibody levels are generally related with symptomatic illness or constant symptomatic infection in carrier dogs and low antibody levels are found in exposed dogs that have not shown clinical disease (1).

The aims of this study were to evaluate the relation between cutaneous symptoms of CanL and anti-*Leishmania* antibody titers and to assess the correlation of IgG_1 and IgG_2 subclasses of titers with these symptoms.

2. Materials and methods

2.1. Sample collection

In this study, 100 dogs that lived in different parts of the endemic area of northwestern Iran (Meshkin Shahr) were studied. All dogs lived at near distances to each other. Negative controls were chosen from 10 laboratory dogs that were kept at Tabriz Azad University (nonendemic region). In addition to clinical manifestation of leishmaniasis, the results of DAT and ELISA testing were negative in the control group.

From all dogs, 5 mL of whole blood samples was collected from available veins (especially the cephalic vein).

2.2. Diagnostic techniques

In all dogs, a physical examination was performed for detection of cutaneous signs of leishmaniasis. Cutaneous lesions like hyperkeratosis and exfoliated dermatitis; mucocutaneous ulcers, nails, and nail-bed involvement; alopecia; ocular signs; and any abnormalities on the muzzle, pinnae, ears, and footpads were checked in the dogs. In addition to cutaneous manifestations, systemic signs of the disease such as anorexia, weight loss or emaciation, muscle atrophy, depression, epistaxis, splenomegaly, and lymphadenopathy were also checked in all dogs.

Anti-*Leishmania* antibody titers of all samples were evaluated by DAT and ELISA methods.

2.2.1 Direct agglutination test

The *L. infantum* antigens for this study were prepared in the Protozoology Unit of the Public Health Faculty of Tehran Medical University. Samples were tested by DAT according to the methods described by El Harith et al. (8). Sera with antibody titers of 1:320 and above were considered positive (9).

2.2.2. Enzyme-linked immunosorbent assay

ELISA was performed as described by Riera et al. (10). The IgG kits of this study were manufactured by Bethyl Co. (Bethyl Laboratories, Montgomery, TX, USA, Catalog No: E40-121). Briefly, a crude *L. infantum* antigen was prepared from sonicated *L. infantum* LON_ 49 as described by Gradoni and Gramiccia (11). Flat-bottomed 96-well plates were coated at 4 °C with a 20 µg/mL concentration of *L. infantum* antigens in 0.1 mL of coating buffer (0.1 M carbonate bicarbonate, pH 9.6). Sera were diluted with phosphate-buffered saline and Tween 1% milk (PBS-TM) at a 1:400 concentration. One hundred microliters of dog diluted sera per well was added and incubated for 2 h at 37 °C. After the plates were washed 3 times with PBS-TM and 1 time with PBS, 100 U/L of goat antidog IgG-horseradish peroxidase (HRP) conjugate was added. The conjugate working dilution was 1:10,000 in PBS-TM for antidog IgG peroxidase. This conjugate was incubated for 1 h at 37 °C and the plates were washed 3 times. The substrate solution tetramethyl benzidine (Sigma, USA) was added to each well. After a 15-min incubation period, reactions were stopped by addition with 50 U/L of stopping solution (2 M H_2SO_4). Absorbance values were read at 450 nm in an automatic micro-ELISA reader with point-to-point method. The reaction was quantified in ELISA units (U) by reference sera from dogs with clinical signs parasitologically confirmed as positive controls and by sera from healthy dogs as negative controls. These positive sera used calibrators and were arbitrarily set at 94 U. The cut-off was established at 18 U (mean ± 6 standard deviations of 10 dogs from Tabriz, a nonendemic region).

2.3. Statistical analysis

Mann–Whitney–Wilcoxon testing (MWW or independent t-test) was performed by using SPSS 13.

3. Results

Twenty-five of the examined dogs showed cutaneous lesions of leishmaniasis. All dogs were checked for exfoliated dermatitis, mucocutaneous lesions, abnormality of nails and nail-beds, alopecia, and ocular signs of the disease. The results of cutaneous signs of leishmaniasis are listed in the Table. Anorexia, emaciation, muscle atrophy, depression, and epistaxis were detected in 15, 17, 15, 12, and 2 of the dogs, respectively.

Based on DAT results, 45 samples were seropositive. From among the seropositive dogs, 25 dogs showed cutaneous lesions of leishmaniasis and 20 of them were asymptomatic (DAT of 1:320 and above). In clinical examinations, 25 dogs showed cutaneous lesions and all of

Table. The results of cutaneous signs of leishmaniasis in examined dogs.

Cutaneous signs	Number of dogs
Skin involvement	25
Exfoliate dermatitis	15
Mucocutaneous ulcers	10
Nail abnormalities	5
Alopecia	18
Ocular signs	5

were seropositive based on DAT results.

In ELISA, high levels of total serum IgG were found in dogs with cutaneous lesions ($P < 0.05$). Furthermore, higher titers belonged to polysymptomatic dogs. These dogs also showed general signs of leishmaniasis such as lymphadenopathies, enlarged spleen, epistaxis, onychogryphosis, ocular lesions, and cachexia.

Significant correlation was seen between skin lesions and IgG subclass levels in symptomatic and asymptomatic dogs ($P < 0.05$). IgG_1 levels showed significant differences between symptomatic and asymptomatic dogs ($P < 0.05$) and IgG_1 titers were higher in symptomatic dogs. IgG_2 levels were significantly higher in asymptomatic dogs ($P < 0.05$).

4. Discussion

The differentiation and proliferation of B lymphocytes are directly regulated by T cells and lymphokines; hence, the measurement of IgG subclasses concentrations perhaps indirectly determines the shifts of immune response in symptomatic and asymptomatic patients (e.g., TH1 or TH2). It seems that high levels of IgG production were seen in the acute phase of leishmaniasis and pathological reaction incidences (9,12). It was proven that, like human visceral leishmaniasis, canine visceral leishmaniasis is associated with high levels of polyclonal specific IgG (12,13). Different studies on canine visceral leishmaniasis suggested an obvious correlation between clinical status and levels of IgG subclasses (13–16).

Specific anti-*Leishmania* immunoglobulins (e.g., IgG subclasses) were first described in dogs with the clinical disease and in treated dogs by Deplazes et al. in 1995 (14). This researcher suggested that specific anti-*Leishmania* IgG_1 and IgG_2 titers are more reliable indicators of the disease status than total IgG. Furthermore, some authors reported the association between types of IgG subclasses with the presence of clinical or subclinical leishmaniasis in dogs (13,16,17). With attention to this hypothesis, clinical leishmaniasis is associated with high levels of IgG_1 and, reversely, IgG_2 is related to subclinical infections.

Based on our results, there is a correlation between incidences of clinical signs of CanL and IgG_1 levels (Figure). Our results showed that the levels of the IgG_1/IgG_2 ratio in symptomatic dogs were significantly higher than in asymptomatic ones ($P < 0.05$). Some studies reported the correlation between levels of IgG subclasses and CanL clinical manifestation, and our results were compatible with these studies (13,14,16). However, based on reports by Bourdoiseau et al. (18) and Nieto et al. (15), there is no correlation between the incidence of clinical leishmaniasis and the type of IgG subclasses, and both IgG subclasses are high in experimental leishmaniasis.

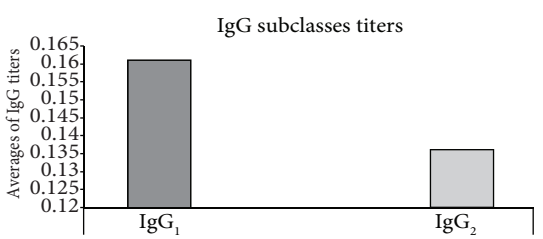

Figure. Comparison of IgG subclass titers in affected dogs.

This matter may differ between natural and experimental leishmaniasis because in field conditions an infected dog faces 2 major groups of factors that affect immune responses: first, host-dependent factors, and second, stimulation of the immune system and proper secretion of different cytokines at the same time with environmental indicators as an important factor. Factors such as stress, malnutrition, parasitic infestations, and concurrent infections can lead to different immune responses. For example, in humans, the prevalence of visceral leishmaniasis among AIDS patients is significantly higher (100 to 2320 times higher) than in immunocompetent individuals (19). This complexity may explain the intensive paradox of host immune responses and the correlation with clinical manifestation.

Some researchers believed that Th1 and Th2 are more of a paradox than a paradigm in leishmaniasis (20,21). The research of de Oliveira Mendes et al. showed higher IgG_1 absorbencies in infected dogs, while the IgG_2 subclass was predominant in preimmune sera or in vaccinated animals (22). They suggested that a high IgG_1/IgG_2 ratio (>1) would be characteristic of the progression of the patient to production of clinical disease (Th2 responses), while a low ratio (<1) would characterize protected animals (Th1 responses in vaccinated dogs). Our results were similar to those of the study by de Oliveira Mendes et al. (22) and verified that the levels of the IgG_1/IgG_2 ratio were significantly higher in symptomatic dogs ($P < 0.05$).

Regardless of all of these conflicting results, it seems that IgG subclasses are a reliable marker in the analysis of patients' immune response and clinical status, and maybe they can be used for prognosis of chemotherapy follow-up, especially in human patients. In spite of some research that showed contrasting results and failed to offer any correlation of IgG subclasses with the production of apparent clinical disease (15,18), the predominance of levels of specific IgG subclasses should be carefully studied along with Th1- or Th2-like activity associations. A mixed Th1-Th2 response or other unknown immunological mechanism is probably involved. We strongly suggest a complete study on the correlation of immune responses and CanL clinical manifestations with different cytokine productions and the associations of these cytokines with IgG subclasses. Lack of canine commercial cytokine kits is a major problem in such studies.

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