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Canine leishmaniasis in Crete, Greece: epidemiology, diagnosis and control of the disease

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Abstract. Canine leishmaniasis is a zoonotic parasitic disease, caused by an endocellular protozoa called *Leishmania infantum*, transmitted by hematophagous vectors, known as phlebotomine sand flies, which transmit the disease through their bite. Leishmaniasis is an endemic disease of public interest, because it affects not only wild and domestic animals but also humans. Therefore, a major attention should be given to the prevention of this disease, which evolves endemic in Crete. From 2014 to 2016, 43 cases with different clinical signs, diagnosed in a Cretan veterinary clinic, were studied. All patients were diagnosed with rapid tests, which detect anti-*Leishmania infantum* antibodies using the method of immunochromatography. The tests have 98% sensitivity and 100% specificity. In positive cases, the therapeutic protocol was a combination of miltefosine (milteforan) 1 ml/10 kg, once per day, for 28 days, with allopurinol (zylapour) 15 mg/kg, twice a day, as lifelong therapy. The dose of allopurinol was adjusted after 6 months of treatment depending on the health status of each patient. A clinical cure was observed in the vast majority of patients but not a parasitological one, as the latest is extremely rare to be achieved.

Keywords: Canine leishmaniasis; Diagnosis; Treatment; Prevention.

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Leishmanioza canină în Creta, Grecia: epidemiologie, diagnostic şi controlul bolii

Rezumat. Leishmanioza canină este o boală parazitară zoonotică, produsă de protozoarul intracelular, *Leishmania infantum*, transmisă de vectori hematofagi, cunoscuţi sub numele de flebotomi, care transmit patogenul prin înţepătură. Leishmanioza este o boală endemică, de interes public, deoarece afectează nu numai animalele sălbatice şi domestice, ci şi oamenii. Prin urmare, o atenţie deosebită ar trebui acordată prevenirii acestei boli, care evoluează endemic în Creta. Între anii 2014-2016, au fost examinate 43 de cazuri de leishmanioză canină cu diferite semne clinice, toate cazurile au fost diagnosticate într-o clinică veterinară cretană. Toţi câinii au fost diagnosticaţi cu ajutorul testelor rapide, care au detectat anticorpi anti-*Leishmania infantum* utilizând metoda imunocromatografiei. Testele au o sensibilitate de 98% şi o specificitate de 100%. Cazurile pozitive au fost tratate cu o combinaţie de miltefosină (milteforan), 1 ml/10 kg, o dată pe zi, timp de 28 de zile, cu alopurinol (zylapour) 15 mg/kg, de două ori pe zi, pe tot parcursul vieţii. Doza de alopurinol a fost ajustată după 6 luni de tratament, în funcţie de starea de sănătate a fiecărui pacient. Vindecarea clinică a fost observată la marea majoritate a pacienţilor, dar nu şi cea parazitologică, deziderat greu, chiar imposibil de atins.

Cuvinte cheie: Leishmanioza canină; Diagnostic; Tratament; Prevenţie.

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Introduction

Canine leishmaniasis is a vector-borne, zoonotic, endemic disease caused by parasites of the genus *Leishmania* (phylum *Sarcomastigophora,* class *Kinetoplastida,* family *Trypanostomatidae)* and is transmitted by the bite of an infected female phlebotomine sandfly (Diptera, Psychodidae) to dogs and humans (Dantas-Torres et al., 2012). *Leishmania infantum* has a mandatory endocellular parasitic life and the presence of both mammalian hosts (human, dog etc.) and vectors (phlebotomies) is necessary for the conduction of the biological cycle. It is presented under two distinct morphological forms, the amastigote (in phagocytes of the host) and the promastigote form (in the gastrointestinal tract of phlebotomies) (Solano-Gallego et al., 2009). In the Mediterranean basin, *Leishmania infantum* is the main cause of canine leishmaniasis (CL) and visceral leishmaniasis (VL) in humans. Asymptomatic animals are of high importance, because they constitute a source for phlebotomine infection (Alvar et al., 1994). In Crete, VL was firstly recorded in 1907, with a high incidence in human (Adler et al., 1938) and the canine population (Chaniotis and Tselentis, 1994). Twenty-five years ago, leishmaniasis was not considered a health problem in Crete, although, before 1940, the visceral and cutaneous forms were widespread. The use of DDT against malaria vectors drastically reduced the phlebotomine populations (Hertig, 1949; Hadjinicolaou, 1958). However, the annual increase of seropositive dogs (Antoniou et al., 2009) was followed by an annual increase in the number of human VL and CL cases. The disease tends to move northwards due to: technology, socio-economic reasons, demographic and climate changes, the creation of new habitats, import of infected hosts, traveling (Solano-Gallego et al., 2009; Cortes et al., 2012). Other factors include the distribution of vectors of the genus *Phlebotomus* and *Lytzomya*, the transmission of the protozoa from generation to generation, it's survival capacity, the vector feeding mode of about 100 bites/hour, and its period of activity which is conditioned by temperature and humidity. No predisposition for sex or age is mentioned. A sensitizing factor is poor health and hygiene conditions. However, a genetic predisposition for Boxer, Cocker Spaniel, Rottweiller and German Shepherd breeds was

noticed, whereas the Ibizian Hound breed is refractory. These theories have demonstrated that there is a S111c1 gene (Solute carrier family 11 member c1) that is associated with susceptibility to CanL (Solano-Gallego et al., 2009). Transplacental and venereal infection has rarely been implicated (Boggiato et al., 2011; Teichmann et al., 2011). Infected canine blood products increase the risk of transmission (Dey and Singh, 2006). Depending on the clinical signs and the results of the hematological and biochemistry profile, dogs are included in one of the four stages, from stage I, meaning mild infection, to stage IV, meaning severe infection. Host resistance is due to intense cellular response in which Th1 or a mix of Th2/Th1 cells predominate, whereas the clinical manifestation of the disease is correlated with Th2 cell predominance. The therapeutic protocol depends on the severity of clinical signs, serology results and laboratory results (Solano-Gallego et al., 2011). As prevention methods, pyriproxyfen, deltamethrin, permethrin, imidacloprid, alone or in combination, are found in different products (collars, spot-on or sprays) and are used for the control of the vector. Furthermore, two vaccines are available on the market, each one with a different protocol of application.

Material and methods

The study was conducted on the island of Crete (35°19'40 N, 25°8'36 E), Greece's largest island and the 5th largest island in the Mediterranean area. The weather is characterized by mild but rainy winters and hot and dry summers. There are significant differences between the coastal and the mountain area, as well as between the west and east of Crete, when it comes to weather conditions (Panagos et al., 2014). Between 2014 and 2016, 43 dogs, pure or mixed breed, with different ages, have been studied. Animals originated from various parts of Crete and a case from Sweden, but born in Crete, respectively. Except for an animal that came from a shelter, the rest of them had owners. The dogs included in this research were patients at Ktiniatriko Kentro Vet 365 in Iraklion, Crete, during the aforementioned period. There were collected 43 blood samples from these dogs. The blood was stored in EDTA, heparin, and Eppendorf tubes for serum. Serum was sent to Micoanalysi Iatriki Athinon, a laboratory in Athens in order to determinate the antibody titer using indirect immunofluorescence (IFAT). The samples are serially diluted and tested to establish the maximum reaction titer. The cut-off titer being 1:100 and the endpoint titer being 1:1600. All samples were stored in the freezer at -18°C and, during transportation, they were stored in ice using a polystyrene package. For each patient enrolled in the study, the methods used to establish the diagnosis were: clinical examination, hematological profile, biochemical profile, speed Leish K rapid test, speed Leish K/Ehrli Double Speed Test and indirect immunofluorescence. The results were calculated using the cross-multiply method. By analogy in a 100 of animals, number x (where x stands for parameters) was determined, where x is corresponding to a percentage (x%).

Clinical examination

It consisted in the evaluation of the general clinical condition of the animals, facies, attitude, temperament, skin and fur, the appearing of mucosal membranes, capillary refill time (TRC), exploratory lymph nodes (submandibular and taken about the condition of the animal in popliteal), body temperature and the presence or absence of appetite. Anamnesis was the last 2 to 3 weeks (animal behavior, condition, presence of appetite or not, mode of urination / defecation and, in the case of hunting animals, their resistance during hunting).

Hematological profile

For 34 patients, the hematological profile was performed using the SCIL VET ABC classic (Pethealth) analyzer. The hematological values and their normal interval are presented in table 1.

Rapid immunochromatographic test

The test is based on the identification of anti-*Leishmania infantum* antibodies for kinesin, the responsible protein for the cellular metabolism of *Leishmania*. These antibodies are synthesized in all the evolutionary stages within the definitive host. Blood samples were tested for

the presence of circulating antibodies using *Speed Leish K* or *SpeedDuo Leish K/Ehrli* rapid test according to the manufacturer's instructions. Briefly, the reagents were brought to room temperature and after opening the package and placing the test on a flat surface, a single drop of blood was transferred to the circular well of the test until it was completely absorbed into the pad. Then, 5 drops of reagent were added to the circular well. After 20 minutes, the result was read from the test zone band, compared to the control zone band and recorded as negative or positive.

Biochemical profile

Based on the results of the rapid test, the biochemical profile was performed in a total of 34 patients after the owner's agreement. The tracking parameters are shown in table 2.

Table 1. The haematological parameters and their normal values

Hematological parameters	Normal values
White Blood Cells (WBC)	6,0 -12,0 * 10^3/μL
Red Blood Cells (RBC)	5,5-8,5 * 10^6/μL
Hemoglobin (HGB)	14-20 g/dl
Hematocrit (HCT)	40-57 %
Mean Corpuscular Volume (MCV)	60-77 fl
Mean Corpuscular Hemoglobin(MCH)	17-23 pg
Mean Corpuscular Hemoglobin Concentration (MCHC)	31-36 g/dl
Platelets (PLT)	200-460 x10^3/μL

Table 2. The biochemical parameters and their normal values

Biochemical parameters	Normal values
Blood Urea Nitrogen (Bun)	7-27 mg/dl
Creatinine (Crea)	0,5-1,8 mg/dl
Aspartate Aminotransferase (AST)	23-66 UI
Alanine Aminotransferase (ALT)	10-94 UI
Alkaline Phosphatase (ALP)	23-212 U/L

Indirect immunofluorescence

Blood sample was collected from 8 patients in Eppendorf tubes of 2 ml for the determination of the antibody titer. For the transportation, the samples were stored in ice inside a polystyrene package and sent in a specialized laboratory in Athens (Microanalysi medical Athens s.a.).

Treatment

Out of the total of 43 patients, 36 received allopurinol and miltefosine while the rest of them were euthanized due to the severity of clinical signs. The commercial product Milteforan (miltefosine) was administered per os, for 28 days, once per day, at a dose of 2 mg/kg and Zylapour (allopurinol) was given per os, twice a day, for the first 6 months, at a dose of 15 mg/kg. In addition to basic medication, other medicines have been used to combat clinical signs such as vomiting, low hematocrit, edema, and skin modifications (Lasix 20 or 40 mg, hemovet, calopet, urinovet, cerenia, dexadreson, Medrol, vetoskin etc.). Two patients received only allopurinol twice a day at the same dose as the rest of the patients treated with the combination of allopurinol and miltefosine.

Results

Out of the 43 patients, 25 were males and 18 females. The study shows that the the disease is most frequent diagnosed in dogs between 2 and 4 years of age. Using the cross-multiply method, the most common clinical manifestation was apathy and fatigue (65.11%) followed by weight loss (58.14%), lymphadenopathy (23.25%), lameness (18.60%), hematuria (6.98%), fever (4.65%) and rhinorrhea (2.32%), respectively. Among skin manifestations, the most common lesion was the presence of scales (53.49%), followed by crusts and erythema (51.16%),

onychogryphosis (20.93%), periocular alopecia and/or alopecia (9.30%), hyperkeratosis and nummular alopecia (6.98%), and at cutaneous and paw edema (4.65%). In the apparent mucous membranes, oral ulcers were observed in a percentage of 4.65%. Out of 42 patients, 40 of them were positive (95.23%) by means of anti-*Leishmania infantum* antibodies rapid test, 1 was positive for both leishmaniasis and ehrlichiosis (2.38%) and 1 was equivocal (2.38%). Of the total number of positive dogs, 2 patients were vaccinated against canine leishmaniosis, 1 year and a half and 2 years, respectively, prior to the day that they had a positive result at the rapid test. For a total of 34 patients, hematological profile was performed. Among hematological modifications, 85.29% had a decrease of the hematocrit, 76.47% showed a decreased hemoglobin, 47.05% presented thrombocytopenia, 44.11% erythrocytopenia and 38.23% leukopenia, respectively. In the case of biochemical changes, 17.64% of the patients had increased creatinine and 11.76% increased aspartate aminotransferase, respectively. Some patients had undergone indirect immunofluorescence, to assess their condition. We obtained antibody titers from 1/100 to 1/1600 and observed that most of the patients (62.5%) had a titer of 1/1600. After the end of the treatment, 33 out of the 36 patients had an admirable clinical development, with a remarkable improvement of the clinical signs and hematological and biochemical values. In one of the 2 patients who were treated only with allopurinol, after 3 months, the clinical signs regressed. Following the re-evaluation and the re-titration of the antibodies for a single patient after one month, in which the confirmation line from the rapid test was of weak intensity and the antibody titer was 1/100 (equivocal result), we observed a negative test result and a 1/90 antibody titer (negative result). Upon the re-examination of the patient, the animal was treated with allopurinol, which was stopped 10 days before the re-examination.

Discussion

Both canine and human leishmaniasis are endemic diseases in Greece that seem to be spreading fast. In the last 25 years, Crete appears to be a re-emerging area of canine leishmaniasis. Concerning the rest of the endemic countries, two major epidemiological concepts emerge. The first concept says that the infection is widespread in endemic countries but not all infected dogs develop the disease. The second concept says that infection spreads more quickly in the presence of favorable conditions, such as an increasing number of sandflies or infected animals. In non-endemic countries, studies have shown that the incidence of canine leishmaniasis is increasing especially in the northern and eastern European countries. There are countries such as Republic of Macedonia, Albania and Bosnia-Herzegovina, where there is still insufficient information on the prevalence status. In Greece, the reservoir is primarily represented by rodents followed by the fox and the wolf. Diagnosis of the disease using a quantitative serological technique is always recommended because it provides more information on the level of antibodies (Solano-Gallego et al., 2009; Solano-Gallego et al., 2011). According to a study by Solano-Gallego in 2014 *Speed Leish K* showed a high specificity (1,000), but the sensitivity was small (0.636), although another study showed that rapid qualitative tests have low sensitivity in infected subclinical dogs (Mettler et al., 2005). One of the major disadvantages of serological tests is the possibility of cross-reaction with other pathogens or other clinical entities (Schallig et al., 2004; Santarém et al., 2010). In the same study by Solano-Gallego in 2014, a total of 14 samples from IFAT-tested animals with a positive result for other pathogens were selected to determine the specificity and the only serological tests with a 100% specificity *were Leiscan®, ID Screen®* and *Speed Leish K®*. Cross-reaction with *L. infantum* is more common in dogs infected with other species of leishmania or other protozoa, such as *Trypanosoma cruzi*, but they are predominant in America (Ferreira et al., 2007; Porrozzi et al., 2007).

Our results are in agreement with those described by previous studies. The characteristic systemic clinical signs of the disease are: apathy, weight loss, 'old dog' appearance and hyperthermia. Lymphadenopathy and splenomegaly are also

observed. Uveitis, keratitis or conjunctivitis can occur at the eye level. The most frequent renal symptom observed is glomerulonephritis. From the hematological and biochemical point of view, anemia, leukopoiesis followed by leucopenia, thrombocytopenia, hyperproteinemia, hypergammaglobulinemia and hypoalbuminemia occur (Guaguere and Prelaud, 2008). Clinical dermatological signs are polymorphic and not pruritic. Diffuse alopecia, exfoliative dermatitis, onycogryphosis, nasal depigmentation associated with ulcers and crusts, paws and muzzle with bleeding tendency and ulcerative lesions at the pressure points (elbow, hip, jar) are most common. Atypical clinical signs include nodular and pustular forms. In mucosa may appear ulcers. Epistaxis, rhinitis, sialorrhea, pain during mastication, conjunctival secretions and hemorrhagic diarrhea may also occur.

In the present study, leukopenia, low red blood cell counts, low hemoglobin, low hematocrit and thrombocytopenia occurred within the hematological results. The combination of allopurinol and miltefosine was used for the treatment. The treatment was initiated on the day of confirmation of the disease, with the exception of 5 cases where miltefosine was not available on the commercial market at the time of diagnosis, and the treatment was initiated after the drug was available. Around 92% of the patients (allopurinol in combination with miltefosine) had a very good clinical evolution, with improvement in clinical, hematological and biochemical signs. The rest of the patients (8%) showed signs of relapse shortly (approximately 6 months after the end of the therapy). All patients, even if clinical recovery was evident, continued with allopurinol. No adverse reactions were observed in any patient. In addition to specific therapy, symptomatic therapy was also performed, depending on the clinical signs present and the needs of each patient.

Conclusions

The results of this study highlight that leishmaniasis represents a severe, possible life-threatening conditions, for dogs in Crete. Moreover, taking in consideration the zoonotic character of the disease and the fact that the canine leishmaniasis is not just sporadic diagnosed in Crete, is of particular importance from the public health perspective. In this frame, more epidemiological studies evaluating prevalence of the disease in dog populations and investigation on other possible source for human contamination are needed.

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