Detection of *Leishmania infantum* by cytocentrifugation in peripheral blood from *Leishmania* positive PCR dogs

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SUMMARY

The veterinary guidelines for blood transfusion suggest *Leishmania infantum* screening by PCR techniques among potential blood donors for evaluating the infection risk. The aim of the present study was to investigate whether circulating amastigotes in peripheral blood may be detected using a cytocentrifugation technique in dogs with *Leishmania* infection confirmed by blood nested-PCR. For that, 20 untreated positive blood n-PCR dogs with serum anti-*Leishmania* antibodies (evidenced by IFAT) were included in this study, clinically examined and some conventional haematological and biochemical parameters were also determined leading to classify dogs into symptomatic (at least 3 anomalies, n = 6), oligosymptomatic (1 to 3 anomalies, n = 9) and asymptomatic (no detected anomaly, n = 5) groups. Prevalence of amastigotes determined by cytocentrifugation, mainly located into neutrophils, was 50% and 1 to 3 parasites by cytospot were evidenced in 3 symptomatic, 4 oligosymptomatic and in 3 asymptomatic dogs. In conclusion, blood n-PCR positive dogs reasonably harbour amastigotes. Furthermore transfusion medicine has a pivotal part in veterinary emergency and critical care medicine. In these cases an appropriate screening of blood donors should be performed.

Keywords: Dog, *Leishmania infantum*, cytocentrifugation, nested PCR, amastigotes, blood transfusion, risk.

Introduction

Blood transfusion is a common therapeutic option in veterinary practice and the request for blood products is continuously increasing. Donors should be accurately screened for pathogens responsible for blood-borne infections [23]. The risk of *Leishmania* transmission in dogs via blood transfusion is mainly based on indirect evidences [3]. Even if *Leishmania* PCR positivity on blood specimens is not a rare finding [14] no thorough investigations have been done to see if asymptomatic dogs who are positive for *Leishmania* via nested PCR (n-PCR) have circulating amastigotes. The presence of intact protozoa is considered as an unusual feature reported in dogs living in hyperendemic areas as in Central-Southern Italy [7, 8, 17].

Canine leishmaniasis due to *Leishmania infantum* could be included among such infections considering also that hamsters inoculated with whole blood or monocytes from infected dogs develop visceral disease [3]. The veterinary guidelines for blood transfusion suggest a dog screening by PCR techniques to be included in an integrated approach to evaluate *L. infantum* infection among potential blood donors, considering that *L. infantum* infection is spread in endemic and non-endemic areas [13, 21]. This is relevant, because asymptomatic dogs are usually not monitored for leishmaniasis although they may harbour amastigotes. Furthermore transfusion medicine has a pivotal part in veterinary emergency and critical care medicine. In these cases an appropriate screening of blood donors should be performed.

The aim of the present paper was to investigate whether intact amastigotes may be detectable in peripheral blood of dogs living in canine leishmaniasis endemic foci from Tuscany (Italy) using a cytocentrifugation technique. Cytocentrifugation is a simple, quick and inexpensive tool that might be easier to use to detect the infection status and visualize intact organisms.

RÉSUMÉ

Emploi de la cytocentrifugation pour la détection de *Leishmania infantum* dans le sang périphérique de chiens leishmaniens positifs par PCR

Les directives vétérinaires concernant la transfusion sanguine chez le chien préconisent des techniques de PCR pour diagnostiquer la présence de *Leishmania infantum* parmi les donneurs potentiels. Le but de cette étude est de vérifier la présence d’amastigotes circulants dans le sang périphérique par une technique de cytocentrifugation chez des chiens positifs par nested-PCR. Pour cela, 20 chiens non traités positifs par n-PCR et possédant des anticorps sériques anti-*Leishmania* (mis en évidence par immunofluorescence indirecte) ont été inclus dans cette étude et un examen clinique et plusieurs paramètres hématologiques et biochimiques conventionnels ont été déterminés en parallèle pour classer les chiens en 3 groupes, symptomatique (présentant au moins 3 anomalies, n = 6), oligosymptomatique (de 1 à 3 anomalies, n = 9) et asymptomatique (aucune anomalie détectable, n = 5). La prévalence des amastigotes, le plus souvent localisés dans les neutrophiles, a été de 50 % et 1 à 3 parasites par cytospot ont été mis en évidence chez 3 chiens symptomatiques, chez 4 oligosymptomatiques et chez 3 chiens asymptomatiques. En conclusion, la présence des amastigotes de Leishmania dans le sang périphérique doit être suspectée chez les chiens positifs par n-PCR et puisque des chiens asymptomatiques peuvent héberger des amastigotes circulants, il est recommandé de tester et d’exclure tout chien positif par n-PCR d’un programme de don du sang.

Mots clés : Chien, *Leishmania infantum*, cytocentrifugation, nested PCR, amastigotes, transfusion sanguine, risque.
Material and Methods

A preliminary survey was carried out on peripheral blood samples from 37 immunofluorescence antibody test (IFAT) positive untreated dogs of different breed, gender and age living in inland areas of Pisa and Leghorn provinces, where canine leishmaniasis is reported with an average prevalence of 15% [15]. Dogs were checked for anti-Leishmania antibodies by IFAT as previously described [12]. All subjects scored positive for amastigotes in lymph-node biopsy, by microscopic examination of Giemsa stained smears. The animals were also examined by n-PCR as a suggestive marker of Leishmania DNA presence in peripheral blood but not of intact and viable amastigotes [10]. Total genomic DNA was extracted from 350 µL of peripheral blood using the Easy-DNA kit (Invitrogen, San Diego, CA) according to the manufacturer’s instructions. After extraction, DNA was stored at -20°C until used. In the first step, 50 pmol of the kinetoplastid-specific primers: R221 (GGTTCTTTTCCTGATTACG) and R332 (GGCGGTAT-AAGGCGGATAG) were added to genomic DNA (10 µL) and thereafter the primary PCR products (3 µL) were added as a template for the second amplification step, with 3 pmol of the following Leishmania-specific primers: R223 (TCCCA-TGCACACCTCGGT) and R333 (AAAGCGGCGCGGC- TGCTG) [22]. Contamination by amplicons was avoided using separate rooms and materials as well as decontamination procedures (UV exposure and bleaching of materials and surfaces). Cross-contamination was monitored using negative controls (no DNA) for sample extraction and PCR solutions. Amplification reactions were analysed by 1.5% agarose gel electrophoresis, and visualized under UV light. A total of 22 samples were scored as positive when a n-PCR product of 358 base pair (bp) was detected, but among this group, one dog died and another one ran away during the trial, so subsequent analysis could be performed on the 20 remaining animals.

All n-PCR positive dogs were submitted to clinical examination for the presence of signs attributable to Leishmania infection (i.e. lymphadenopathy, dermatitis, skin ulcers, alopecia, ocular lesion, onychogryphosis and weight loss) and to evaluation of non specific laboratory analyses such as red blood cells, total white blood cells, plates counts, total proteins and serum protein electrophoresis. A three-level classification combining clinical and laboratory findings was defined as follows: symptomatic, at least 3 clinical and/or laboratory abnormalities (n = 6); oligosymptomatic, 1 to 3 abnormalities (n = 9); asymptomatic, with no discernible alteration (n = 5).

Peripheral blood samples from the 20 positive n-PCR dogs were collected in sterile EDTA-microtubes to perform microscopic examination of smears and to detect amastigotes by cytocentrifugation. Blood smears were stained with Giemsa and 100 fields for each slide were examined at a 1200 magnification by the same observer. The cytocentrifugation with Cytospin® chambers, with glass microscope slide and Cytofunnel® were assembled as recommended by the manufacturer. Cytospin® sample chambers were then placed into the Cytospin® head, and spun at 200 g for 12 minutes. The samples were air-dried and Giemsa stained. Each slide was observed at great magnification (x 1200).

Results

The results of the investigations carried out on the peripheral blood samples from the 20 n-PCR positive dogs are reported in Table I. No protozoa were observed using classical microscopic procedures in all blood smears. Nevertheless, 10 samples (50%) gave positive results by Cytospin® for circulating amastigotes in buffy coat. Amastigotes were recovered from 3/5 asymptomatic, 4/9 oligosymptomatic and 3/6 symptomatic dogs. Scanty (1 to 3) amastigotes per cytospot were recorded and parasites were free (in 5 dogs) and/or intraneutrophilic (in 9 dogs). All positive animals by cytocentrifugation also exhibited IFAT titres ranging from 1/160 to 1/640.

Discussion

Canine leishmaniasis is an emergent disease that is clearly expanding in several regions in the world and the domestic dog is an important reservoir in urban areas. Dogs affected by the disease present an extremely variable incubation period and a large spectrum of clinical characteristics. This fact complicates identification of infected animals, particularly asymptomatic ones that constitute nearly 40% of those harbouring the parasite [6].

In this report all the animals were scored positive by IFAT, but direct relationship between presence of amastigotes in blood and serological titre could not be ascertained: indeed, cytospin results were not positively correlated with IFAT titres because among dogs presenting the same antibody titre, some were positive and others negative for amastigotes after cytocentrifugation. Since any dog included in the survey was IFAT-negative, it is impossible to state if IFAT positivity could predict the presence of intact protozoa [14]. The presence of intact amastigotes by parasitological techniques suggests the viability of the parasites, in agreement with the infecting potential of peripheral blood as demonstrated by DE FREITAS et al. [3]. No correlation between the presence of amastigotes using cytocentrifugation and the clinical/laboratory status was obtained, considering that symptomatic, oligosymptomatic and asymptomatic animals may score positive.

The detection of Leishmania amastigotes in the peripheral blood of infected dogs represents a quite rare finding when a direct examination of smears is performed [5, 7, 8]. The parasite can spread and colonize a number of organs and tissues, due to dissemination by the lymphatic way or, less frequently, the blood route [5]. REALE et al. [17] detected amastigotes in peripheral blood from 36 to a total of 52 dogs, with the results of microscopic examination only partially confirmed by
DETECTION OF AMASTIGOTES BY CYTOCENTRIFUGATION IN BLOOD SAMPLES FROM N-PCR POSITIVE DOGS

A positive culture (14 positive blood cultures out of 52). This massive presence of circulating parasites could be explained, pointing out that all the animals included in the study were kept in an area characterized by high seroprevalences [1]. The parasites were free and/or intraneutrophilic, as reported in the single cases described in literature [7, 20]. DE GOPEGUI and ESPADA [4] reported the presence of protozoa into monocytes from an infected dog, indicating that different white blood cells can harbour *Leishmania* amastigotes. The ability of neutrophils to induce a macrophage microbicide activity was demonstrated in human infection with *Leishmania major* [18] indicating the involvement of these cells in intracellular killing of parasites.

The cytocentrifugation technique was firstly applied to human visceral leishmaniasis in HIV-positive patients [9] and in immunocompetent children [2]. To the best of our knowledge the present report represents the first application of cytocentrifugation techniques to canine leishmaniasis. Molecular tools indicate the presence of *Leishmania* DNA but are not able to indicate the viability of the parasites since undamaged amastigotes were detected in 50% of PCR positive dogs. On the basis of this observation, a true parasitaemia in blood n-PCR positive dogs could be hypothesized. The cytocentrifugation test yielded a sensitivity of 50%, in agreement with the results of CHEMLI et al. [2] who recovered amastigotes from 56% of hospitalized pediatric immunocompetent patients. The amastigotes were obtained from a small amount of buffy coat (0.5 mL), and probably a greater volume would have enhanced the sensitivity of the test. It is therefore noteworthy that, during a transfusion, recipient dogs can receive a considerable amount of blood, increasing in this way the infection risk. Microscopic methods have a low sensitivity when used on peripheral blood, while PCR has demonstrated that *Leishmania* presence in a dog from endemic areas is not a rare finding [11]. Furthermore TABAR et al. [21] detected *L. infantum* by real time PCR in specimens from a canine blood bank.

Blood n-PCR positive dogs reasonably harbour *Leishmania* organisms in peripheral blood. Considered that 3 out of 5 asymptomatic n-PCR positive dogs exhibited amastigotes in blood, it is strongly recommended to carry out an integrated approach comprehensive for *Leishmania* PCR survey prior to the inclusion of a subject in a canine blood bank, and the exclusion of all blood positive n-PCR dogs as blood donors is strongly advisable. Although amastigotes were only found via cytocentrifugation in 50% of the subjects, the amount of blood examined in this test is small and the risk of transmission with transfusion of larger volumes is possible. Although cytocentrifugation is a simple test, following the conditions described in the present work it is not sensitive enough to be used in order to screen blood donors for inclusion in a canine blood bank.

**Table I:** Signalment, classification and cytocentrifugation results (presence of amastigotes) in 20 canine leishmaniasis seropositive and nested-PCR (n-PCR) positive dogs.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Sex</th>
<th>Age (year)</th>
<th>Classification</th>
<th>IFAT titres</th>
<th>Score1 / Count / Localisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bloodhound</td>
<td>M</td>
<td>2</td>
<td>S</td>
<td>1/2560</td>
<td>Negative</td>
</tr>
<tr>
<td>English Setter</td>
<td>M</td>
<td>4</td>
<td>S</td>
<td>1/320</td>
<td>Negative</td>
</tr>
<tr>
<td>English Setter</td>
<td>F</td>
<td>2</td>
<td>S</td>
<td>1/320</td>
<td>Negative</td>
</tr>
<tr>
<td>Bloodhound</td>
<td>F</td>
<td>1</td>
<td>S</td>
<td>1/640</td>
<td>Positive / F and IN</td>
</tr>
<tr>
<td>Crossbreed</td>
<td>M</td>
<td>3</td>
<td>S</td>
<td>1/640</td>
<td>Positive / IN</td>
</tr>
<tr>
<td>Crossbreed</td>
<td>F</td>
<td>3</td>
<td>S</td>
<td>1/640</td>
<td>Positive / IN</td>
</tr>
<tr>
<td>Crossbreed</td>
<td>M</td>
<td>6</td>
<td>OS</td>
<td>1/80</td>
<td>Negative</td>
</tr>
<tr>
<td>Crossbreed</td>
<td>M</td>
<td>8</td>
<td>OS</td>
<td>1/160</td>
<td>Negative</td>
</tr>
<tr>
<td>Crossbreed</td>
<td>M</td>
<td>5</td>
<td>OS</td>
<td>1/160</td>
<td>Negative</td>
</tr>
<tr>
<td>Springer Spaniel</td>
<td>F</td>
<td>2.5</td>
<td>OS</td>
<td>1/640</td>
<td>Negative</td>
</tr>
<tr>
<td>W.P.G.</td>
<td>M</td>
<td>7</td>
<td>OS</td>
<td>1/160</td>
<td>Negative</td>
</tr>
<tr>
<td>Crossbreed</td>
<td>F</td>
<td>4</td>
<td>OS</td>
<td>1/320</td>
<td>Positive / F and IN</td>
</tr>
<tr>
<td>Crossbreed</td>
<td>M</td>
<td>4</td>
<td>OS</td>
<td>1/160</td>
<td>Positive / F and IN</td>
</tr>
<tr>
<td>W.P.G.</td>
<td>M</td>
<td>5</td>
<td>OS</td>
<td>1/320</td>
<td>Positive / F and IN</td>
</tr>
<tr>
<td>Crossbreed</td>
<td>M</td>
<td>5</td>
<td>OS</td>
<td>1/160</td>
<td>Positive / F and IN</td>
</tr>
<tr>
<td>Pointer</td>
<td>F</td>
<td>13</td>
<td>AS</td>
<td>1/80</td>
<td>Negative</td>
</tr>
<tr>
<td>Crossbreed</td>
<td>F</td>
<td>5</td>
<td>AS</td>
<td>1/320</td>
<td>Negative</td>
</tr>
<tr>
<td>Rottweiler</td>
<td>M</td>
<td>3</td>
<td>AS</td>
<td>1/640</td>
<td>Positive / IN</td>
</tr>
<tr>
<td>Crossbreed</td>
<td>F</td>
<td>4</td>
<td>AS</td>
<td>1/320</td>
<td>Positive / F and IN</td>
</tr>
<tr>
<td>Crossbreed</td>
<td>M</td>
<td>5</td>
<td>AS</td>
<td>1/320</td>
<td>Positive / F and IN</td>
</tr>
</tbody>
</table>

W.P.G.: Wirehaired Pointing Griffon; M: male; F: Female; Score1: presence of intact amastigotes; S: symptomatic (at least 3 clinical and/or laboratory anomalies); OS: oligosymptomatic (1 to 3 clinical and/or laboratory anomalies); AS: asymptomatic (no clinical and/or laboratory anomalies); IFAT: immunofluorescence antibody test; F: free amastigotes; IN: amastigotes located in neutrophils.

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To the best of our knowledge, the application of this technique in veterinary transfusion medicine has not been investigated yet. It is noteworthy to underline that the use of leukodepletion filters to remove amastigotes from blood as reported by RIERA et al. [19] in human blood donors appeared to be an effective method to reduce the risk of transfusion-transmitted leishmaniasis. Further studies are required to determine the effectiveness and reliability of such promising tools in canine transfusion medicine.

References