EVALUATION OF LYMPH NODE AND BONE MARROW CYTOLOGY IN THE DIAGNOSIS OF CANINE LEISHMANIASIS (LEISHMANIA INFANTUM) IN SYMPTOMATIC AND ASYMPTOMATIC DOGS

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Abstract. The sensitivity and specificity of lymph node and bone marrow smear microscopy for the diagnosis of Leishmania infantum–infected dogs was evaluated in 79 dogs with leishmaniasis (Group A), 52 asymptomatically infected dogs (Group B), and 44 healthy noninfected dogs (Group C). Light microscopy examination included 10 to 1,000 oil immersion fields, and the density of Leishmania amastigotes was scored by a 0 to +6 scale. Using polymerase chain reaction as the gold standard, the specificity of lymph node and bone marrow cytology was 100%, whereas sensitivity ranged from 7.8% to 92.6%, being significantly higher in Group A compared with Group B. The amastigote scores were also significantly higher in Group A compared with Group B. These results indicate that lymph node and bone marrow cytology is a highly sensitive and specific method for the diagnosis of canine patent leishmaniasis, whereas its sensitivity is relatively low in asymptomatic infections.

INTRODUCTION

Canine leishmaniasis (CL), caused by Leishmania infantum, is an important zoonotic disease in the Mediterranean countries, where dog is considered the main reservoir. Prompt diagnosis of infected dogs is mandatory, not only for a successful treatment outcome, but also to decrease their infectivity to sandfly vectors through chemotherapy and insect repellent application. Microscopic examination of fine-needle biopsy smears of lymph node (LN) and aspiration smears of bone marrow (BM), along with serology and polymerase chain reaction (PCR), are the most frequently used diagnostic methods for CL in the clinical setting.

Despite its widespread use, only scarce and sometimes conflicting data are available on the accuracy of microscopy for the diagnosis of CL. Although it is generally considered 100% specific, reported sensitivity rates range from less than 30% to 100% for LN fine-needle biopsy smears and from 6.2% to 100% for BM aspiration smears. Furthermore, while it is currently widely accepted that many infected dogs do not present clinical manifestations of CL to the best of our knowledge, there are no published data comparing the sensitivity of microscopy between asymptomatically infected dogs and their symptomatic counterparts.

The aim of this study was to evaluate, in a blind design, the relative sensitivity and specificity of LN and BM cytology for the diagnosis of canine infection by L. infantum, using BM PCR as the gold standard, in both symptomatically and asymptomatically infected dogs.

MATERIALS AND METHODS

Animals. The 175 privately owned dogs used in this study, after their owner’s informed consent was obtained, were allocated into three groups. Group A included 79 dogs with a clinical diagnosis of CL that was subsequently confirmed by positive BM PCR, according to a validated protocol, using primers T2 and B4 to amplify a 250-bp fragment of the rRNA gene of Leishmania spp. Group B comprised 52 clinically healthy but BM PCR positive dogs that were considered as asymptomatically infected by L. infantum, and Group C included 44 clinically healthy noninfected dogs (negative BM PCR). PCR amplification was carried out according to the guidelines described by Kwok and Higuchi and DEPC-treated H2O was used, instead of DNA, as a negative control, to exclude the possibility of contamination. All these dogs had been admitted to our clinic due to their disease manifestations (Group A) or to be examined, on a prophylactic basis, for a possible infection by L. infantum.

Group A included 47 (59.5%) male and 32 (40.5%) female dogs, with an age and body weight ranging from 9 months to 10.5 years (median: 4 years), and from 7.8 to 41 kg (median: 21.3 kg), respectively. Forty-five (57%) of these dogs belonged to 15 different pure breeds, 23 (29.1%) were crossbreeds, and 11 (13.9%) mongrels. The most common CL-compatible clinical signs included peripheral lymphadenomegaly, poor body condition, masticatory muscle atrophy, and exfoliative dermatitis. Of the 79 Group A dogs, 77 (97.5%) were serologically positive using indirect immunofluorescence antibody testing (IFAT; cutoff titer ≥ 1/200).

Group B consisted of 27 (51.9%) males and 25 (48.1%) females, with an age and body weight ranging from 7 months to 7 years (median: 2 years), and from 11 to 35 kg (median: 19.5 kg), respectively. Of the 52 Group B dogs, 48 (92.3%) belonged to 7 different pure breeds, whereas 2 (3.8%) were crossbreeds, and 2 (3.8%) mongrels. Most Group B dogs (48 of 52; 92.3%) were IFAT negative.

Group C included 24 (54.5%) male and 20 (45.5%) female dogs that belonged to 5 different pure breeds (43 of 44; 97.7%) or were crossbreeds (1 of 44; 2.3%). Their age ranged from 8 months to 9 years (median: 1.6 years) and their body weight from 10 to 28 kg (median: 18 kg). All 24 Group C dogs were IFAT negative.

Biopsy. One popliteal or prescapular LN was sampled from all dogs with a nonaspiration fine-needle biopsy technique...
using a 21-gauge needle attached to a 10-mL syringe; smears prepared with the squash method were Giemsa-stained. Bone marrow was obtained after iliac crest puncture with a Rosenthal needle and a 10-mL syringe containing 0.2 mL sterile 3% EDTA/isotonic saline solution. One smear was immediately prepared and stained, as described for LN, with the exception of the doubled staining time. Microscopy. Light microscopy examination of LN and BM smears was carried out blindly (i.e., without knowledge of the animals’ Group), in manolayer areas of adequate cellularity and staining quality. Leishmania amastigotes were recognized as round to oval organisms, with a diameter of 2–5 μm, an eccentric nucleus, a kinetoplast with more intense basophilic staining compared with the nucleus, and a visible cellular membrane. Microscopic examination included a total of 10 to 1,000 oil immersion fields (OIFs, ×1,000), depending on the detection and density of amastigotes; the latter was scored, according to a scale proposed by Chulay and Bryce-son, as 0 (no amastigotes per 1,000 OIFs), +1 (1–9 amastigotes per 1,000 OIFs), +2 (1–9 amastigotes per 100 OIFs), +3 (10–99 amastigotes per 100 OIFs), +4 (10–99 amastigotes per 10 OIFs), +5 (100–999 amastigotes per 10 OIFs), and +6 (more than 1,000 amastigotes per 10 OIFs). Statistical analysis. Relative sensitivities and specificities were calculated for the microscopic examination of up to 100 OIFs and up to 1,000 OIFs, of LN and BM smears, as well as for their combination (when both LN and BM smears were interpretable). Sensitivity (%) was separately calculated for Groups A and B, as the number of positive smears/total number of smears examined × 100, whereas, specificity (%) was calculated in the Group C, as the number of negative smears/total number of smears examined × 100. Sensitivity was compared between Groups A and B by the χ² test and within each of these two groups separately, between LN smears, BM smears, and their combination by McNemar’s χ² test for symmetry. The same test was also applied to compare, the sensitivity of the examination of up to 100 OIFs versus the examination of up to 1,000 OIFs, within Groups A and B separately. LN and BM amastigote scores were compared between Groups A and B using the Mann-Whitney U test, whereas marginal homogeneity test was used for the comparison of LN and BM amastigote scores, in the same animal group. Finally, the observation or not of Leishmania amastigotes in LN and BM cytology was associated with the breed (purebreds, crossbreds or mongrels), the age, the sex of the dogs (Groups A and B), and the presence of peripheral lymphadenomegaly (Group A dogs, only) in multiple logistic regression models. Analyses were carried out in SAS, ver. 8.1, and evaluated for significance at the 5% level.

RESULTS
Not all the 175 LN and 175 BM smears had an adequate number of suitable microscopic fields to complete the examination. In particular, 29 of 175 (16.6%) and 47 of 175 (26.9%) LN smears had less than 100 and 1,000 interpretable OIFs, respectively, whereas, the relevant figures for BM smears were 11 of 175 (6.3%) and 17 of 175 (9.7%). The main reason for the relatively high percentage of useless LN smears was the small and hardly palpable LN’s of some dogs, especially from Groups B and C (clinically healthy dogs), rendering fine-needle biopsy a difficult task.

The results of LN and BM cytology in terms of finding or not Leishmania amastigotes are shown on Table 1. Using BM PCR as the gold standard, the specificity of both LN and BM microscopy was 100%, as no amastigotes were found in Group C. On the other hand, sensitivity ranged from 7.8% (examination of up to 100 BM OIFs in Group B) to 94.7% (examination of up to 1,000 LN and 1,000 BM OIFs in Group A). No association was found between the signalment (breed, age, sex) of the dogs, or the presence or absence of peripheral lymphadenomegaly, and the positive outcome of LN and BM cytology. Sensitivity was significantly higher (P < 0.001) in Group A compared with Group B, for all the possible comparisons (examination of up to 100 or 1,000 OIFs of LN smears, BM smears, and their combination) (Table 1). No difference in terms of sensitivity was found between LN and BM smears, irrespective of the number of OIFs examined (up to 100 or 1,000), for both Groups A and B. The combined examination of LN and BM smears, was more sensitive than the examination of LN (P = 0.031) or BM (P < 0.001) smears alone, only in Group A dogs, when up to 100 OIFs were examined (Table 1). Finally, the examination of up to 1,000 OIFs was more sensitive than the examination of up to 100 OIFs in Group A dogs only, for both LN (P = 0.031) and BM (P = 0.002) smears, but not for their combination.

The amastigote scores in LN smears ranged from 0 to 6 (median: 3) and from 0 to 3 (median: 0) in Groups A and B, respectively, and were significantly higher (P < 0.001) in the former (Figure 1). Similarly, amastigote scores in BM smears (Figure 2) were higher (P < 0.001) in Group A (range 0–5; median 2) compared with Group B (range 0–2; median 0). Amastigote scores were higher in LN smears compared with their BM counterparts in Group A (P = 0.011), but not in Group B (P = 0.634).

DISCUSSION
Specificity of LN fine-needle biopsy smears and BM aspiration smears for the diagnosis of L. infantum infection is heavily dependent on the examiner’s skills, as natural pigments, such as hemosiderin, and stain precipitates may occasionally mimic Leishmania amastigotes. This may be especially true when amastigotes are scarce, and could account for the less than absolute sensitivity (94.8%) of bone marrow and spleen aspiration smear cytology found in a recent study of human visceral leishmaniasis, using latent class analysis of the data. Strict adherence to the morphologic criteria established for the identification of Leishmania amastigotes resulted in 100% specificity in this study, a figure comparable with those already reported for cytology in general, as well as for LN and BM cytology, in particular. In contrast, false-positive results may sometimes occur with both PCR and serology. Therefore, finding amastigotes in tissue smears, when feasible, should be considered a very accurate diagnostic method for the canine L. infantum infection, similarly to the situation in human visceral leishmaniasis.

Sensitivity, apart from the examiner’s efficiency, depends on the quality of the smear and the number of microscopic fields screened. In this study, LN smears of poor cellularity and/or inadequate numbers of interpretable OIFs, were frequently obtained from dogs without lymphadenomegaly, ir-
Table 1
Frequency of Leishmania amastigote microscopic observation (× 1,000) in lymph node and bone marrow smears, in dogs with clinical leishmaniasis (Group A), in asymptotically infected dogs (Group B), and noninfected dogs (Group C)

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
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<tbody>
<tr>
<td></td>
<td>(N = 79)</td>
<td>(N = 52)</td>
<td>(N = 44)</td>
</tr>
<tr>
<td>Lymph node fine-needle biopsy smears</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 oil immersion fields</td>
<td>57/68 (83.8%)</td>
<td>5/40 (12.5%)</td>
<td>0/38 (0%)</td>
</tr>
<tr>
<td>1,000 oil immersion fields</td>
<td>63/68 (92.6%)</td>
<td>8/31 (25.8%)</td>
<td>0/29 (0%)</td>
</tr>
<tr>
<td>Bone marrow aspiration smears</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 oil immersion fields</td>
<td>50/69 (72.5%)</td>
<td>4/51 (7.8%)</td>
<td>0/44 (0%)</td>
</tr>
<tr>
<td>1,000 oil immersion fields</td>
<td>60/68 (88.2%)</td>
<td>7/48 (14.6%)</td>
<td>0/42 (0%)</td>
</tr>
<tr>
<td>Lymph node fine-needle biopsy and bone marrow aspiration smears in combination</td>
<td></td>
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</tr>
<tr>
<td>100 oil immersion fields</td>
<td>54/58 (93.1%)</td>
<td>7/39 (17.9%)</td>
<td>0/38 (0%)</td>
</tr>
<tr>
<td>1,000 oil immersion fields</td>
<td>54/57 (94.7%)</td>
<td>8/29 (27.6%)</td>
<td>0/29 (0%)</td>
</tr>
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a,b,c: different superscripts in the same row denote significant difference between Groups A and B (χ² test, P < 0.05).

The number of OIFs examined before the outcome of cytology is considered negative is rarely specified in the various studies of CL. In the current study, an upper limit of 1,000 OIFs was set out, corresponding to approximately 60 and 45 minutes of microscopy for LN and BM smears, respectively. In statistical terms, a higher diagnostic sensitivity by examining up to 1,000 OIFs versus the 10-fold less time consuming 100 OIFs, was documented only in dogs with clinical signs of CL (Group A), but was negated when the combination of LN and BM smears was considered. However, sensitivity was always numerically higher, though not significantly, when up to 1,000 OIFs were examined, for both animal groups and types of smears. Therefore, for the individual infected dog the continuation of microscopy until up to 1,000, or even more, OIFs have been examined may be justified to prove the infection, when the result is negative at 100 OIFs.

It is generally accepted that BM and spleen are the body tissues with the highest density of Leishmania amastigotes and, therefore, BM cytology is considered superior to that of LN in both CL and human visceral leishmaniasis. In the current study, however, no significant difference was found between LN and BM smears in terms of sensitivity, and the amastigote count was higher in the former, in Group A. This discrepancy may be attributed to the effect of hemodilution, which is inevitable during BM aspiration. Anyhow, in the clinical setting, LN fine-needle biopsy smears should be obtained first and the more invasive BM aspiration reserved for those cases where a definitive diagnosis is still elusive.

The clinical status of the dogs appeared as the most important parameter affecting the sensitivity of cytology and the population density of Leishmania amastigotes. In dogs with overt CL the sensitivity of microscopy may be as high as 94.7% if 1,000 OIFs of both LN and BM smears are screened, due to the high number of amastigotes present, whereas the relevant figure for Group B dogs is less than 30%. This finding is in agreement with the observation of a lower sensitivity of microscopy in asymptotically infected dogs and easily explained by their relatively low parasitic load, similar to the human cases of asymptomatic visceral leishmaniasis. These dogs, which may constitute a large percentage of the canine population in endemic areas, represent a nonhomogeneous group in respect to their immunologic response to L. infantum and the perspective of their infection: a subset of them will eventually go on to develop CL (susceptible dogs), whereas the resistant ones will remain asymptomatic with the potential of self-cure. At the macrophage level, susceptible dogs show impaired phagocytosis and intracellular killing of amastigotes. Therefore, it is logical to assume that Group

Figure 1. Leishmania amastigote scores in lymph node fine-needle biopsy smears from dogs with clinical leishmaniasis (Group A) and from asymptotically infected dogs (Group B).

Figure 2. Leishmania amastigote scores in bone marrow aspiration smears from dogs with clinical leishmaniasis (Group A) and from asymptotically infected dogs (Group B).
B dogs with numerous amastigotes in LN and BM smears probably represent, similar to Group A dogs, susceptible individuals that permit massive multiplication of Leishmania and will probably develop CL after a certain period of time.

In Group A dogs, no association was found between when the presence of peripheral lymphadenomegaly and the outcome of LN and BM microscopy, which is in contrast with the situation in acute canine monocytic ehrlichiosis, where Ehrlichia canis morulae are more easily observed in smears taken from enlarged LNs. Therefore, LN cytology is a worthwhile examination in CL, even in the absence of peripheral lymphadenomegaly.

Lymph node and BM smear microscopy is a highly sensitive and specific method for the diagnosis of CL. However, its sensitivity for the diagnosis of asymptomatic canine infections by Leishmania is lower than 30% even when up to 1,000 OIFs of both LN and BM smears are examined.

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