

Thick Smear Is a Good Substitute for the Thin Smear in Parasitological Confirmation of Canine Visceral Leishmaniasis

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Abstract. Although direct examination methods are important for diagnosing leishmaniasis, such methods are often neglected because of their low sensitivity relative to other techniques. Our study aimed to evaluate the performance of bone marrow (BM) thick smears and cyto centrifugation tests as alternatives to direct examination for diagnosing canine visceral leishmaniasis (CVL). Ninety-two dogs exhibiting leishmaniasis seroreactivity were evaluated. The animals were euthanized; and healthy skin, spleen, popliteal lymph node, and BM puncture samples were cultured. BM cultures were used as the reference standard. Of the 92 dogs studied, 85.9% exhibited positive cultures, and *Leishmania infantum* (synonym *Leishmania chagasi*) was confirmed in all positive culture cases. The sensitivity rates for cyto centrifugation as well as thin and thick smears were 47.1%, 52.8%, and 77%, respectively. However, no association between the dogs' clinical status and culture or direct examination results was found. To our knowledge, this was the first study to use thick smears and cyto centrifugation for diagnosing CVL. Our results indicate that BM thick smears have a good sensitivity and their use reduces the time required to read slides. Therefore, thick smears can provide a rapid and safe alternative to parasitological confirmation of seroreactive dogs.

INTRODUCTION

The domestic dog is main reservoir of *Leishmania infantum* (synonym *L. chagasi*) and is an important link in the transmission cycle in urban environments.¹ In Brazil, visceral leishmaniasis (VL) is spreading; therefore, efforts have been made to improve the diagnostic accuracy in dogs.^{2,3} Currently, the dual path platform (DPP) rapid immunochromatographic assay and enzyme-linked immunosorbent assay (ELISA; Canine VL [CVL]) are used for diagnostic screening and confirmation respectively in these animals; positive animals are then euthanized in an attempt to stop the transmission cycle.^{1,4} This strategy is applied in areas with confirmed cases of human or CVL where parasitological examinations are not conducted because of the extensive amount of work that is required. However, to identify new areas of transmission, parasitological confirmation and identification of the etiological agent are necessary. Direct examinations can be used in this context because they are easy to perform and inexpensive, representing a useful alternative, especially in areas with limited resources. However, the time required to read the slides and low sensitivity remain major disadvantages.⁵ Therefore, techniques that concentrate clinical samples and reduce the reading area would provide alternatives to counteract these drawbacks. Techniques, such as cyto centrifugation, which are commonly used with biological fluids for the diagnosis of various diseases, have already been established.^{6–8} According to analyzed clinical samples, hemolyzing solutions can also be added to cell suspensions to provide better disposal of material to be examined after staining.^{6,9,10} Despite these advantages, cyto centrifugation remains largely unexplored as a method of

parasitological diagnosis for diseases such as malaria, filariasis, and leishmaniasis.¹⁰

The thick smear test is another method of concentrating clinical materials, especially peripheral blood samples. This technique was first described in 1903 and uses a concentrated sample on a small area of the slide (approximately 1 cm²) that is subsequently hemolyzed and stained. Although its dedicated use is the diagnosis of malaria and filariasis, this test could potentially be used to diagnose blood parasites.^{11,12}

Different clinical specimens can also be used for the parasitological diagnosis of CVL; however, selection of the best sample remains challenging and there is no consensus in this literature. This lack of consensus is related to the clinical diversity of CVL and the animal's disease stage.^{13–18} For example, bone marrow (BM) is a widely used specimen in many different diagnostic techniques and provides acceptable sensitivity values for both symptomatic and asymptomatic dogs.^{14,17} Given the importance of direct examination, this study aimed to assess different concentration techniques, such as thick smear and cyto centrifugation, using BM for parasitological confirmation of CVL.

METHODS AND MATERIALS

Collection of biological samples. Ninety-two dogs exhibiting leishmaniasis seroreactivity and originating from a survey carried out in the municipalities of Barra Mansa and Rio de Janeiro, Brazil were included in this study. The animals were diagnosed using a DPP assay and an ELISA and euthanized in the Laboratory of Clinical Research on Dermatозoonosis in Domestic Animals (LAPCLIN DERMZOO; Instituto Nacional de Infectologia, Fundação Oswaldo Cruz) according to the recommendations of the Brazilian Program for VL Surveillance and Control.¹ Initially, the dogs were sedated with ketamine (10 mg/kg) and acepromazine (0.2 mg/kg) for clinical examination. Animals without characteristic signs of

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TABLE 1
Parasitological cultures performed with different clinical specimens and the clinical status of seropositive dogs

Clinical status*	Culture results % (no. of positive cases/no. of examined samples)/(CI: 95%)				
	Skin (N = 42)	Spleen (N = 91)	Bone marrow (N = 73)	Lymph node (N = 34)	Total† (N = 92)
Asymptomatic, N = 27	61.5% (8/13) (90.1–56.0)	73.1% (19/26) (90.1–56.0)	68.2% (15/22) (87.6–48.7)	70% (7/10) (98.4–41.6)	85.2% (22/27) (78.3–92.0)
Oligosymptomatic, N = 49	69.6% (15/23) (60.0–79.2)	79.6% (38/49) (73.8–85.3)	74.4% (28/39) (67.4–81.4)	78.9% (14/19) (69.5–88.3)	87.8% (42/49) (83.1–92.7)
Symptomatic, N = 12	100% (6/6) (100–100)	83.3% (10/12) (100–62.2)	87.5% (7/8) (100–64.6)	100% (5/5) (100–100)	100% (12/12) (100–100)

CI = confidence interval.

*Missing = 4.

†Total: positivity in any of the evaluated sites/number of tested animals.

CVL were classified as asymptomatic, animals exhibiting one to three CVL signs were oligosymptomatic, and animals exhibiting more than three signs were considered symptomatic according to Mancianti and others.¹⁹ After euthanizing the animals with an overdose of 5% sodium thiopental, intact skin, spleen and lymph node fragments, and BM puncture samples were collected for parasitological tests. All procedures performed during this study were approved by the Oswaldo Cruz Foundation's Ethics Committee for Animal Use under protocol number LW-54/13.

Cultures and etiologic characterization. The intact skin, spleen and lymph nodes fragments, and BM puncture samples were cultured in the biphasic Novy–Neal–Nicolle medium and Schneider's *Drosophila* medium containing 10% fetal calf serum. After samples were inoculated, the media were incubated at 26–28°C and examined weekly for a maximum of 30 days according to protocols described by Madeira and others.²⁰ BM culture was used as the reference standard in the analysis.

Positive cultures were grown to obtain parasite mass and then characterized by enzyme electrophoresis as outlined by Cupolillo and others.²¹ At least one isolate from each animal was characterized using five enzyme systems: 6-phosphogluconate dehydrogenase (EC.1.1.1.43), phosphoglucose isomerase (EC.5.3.1.9), nucleoside hydrolase (EC.3.2.2.1), glucose-6-phosphate dehydrogenase (EC.1.1.1.49), and phosphoglucomutase (EC.1.4.1.9). Reference strains of *Leishmania* (*Viannia*) *braziliensis* (MHOM/BR/75/M2903), *Leishmania* (*Leishmania*) *chagasi* (synonym *L. infantum*) (MHOM/BR/74/PP75) and *Leishmania* (*Leishmania*) *amazonensis* (IFLA/BR/67/PH8) were used in all electrophoretic runs.

Direct examination: thin and thick smears and cyto-centrifugation. Approximately 1 mL of BM was collected via puncture from the sternal bone of each dog and added to ethylenediamine tetraacetic acid. Thin smears were prepared with approximately 1 µL of BM evenly distributed on a microscope slide. To prepare a thick smear, 5 µL of BM were distributed over a 1 cm² area of the slide with the aid of a printed card containing the defined area. Once completely dry, the thick smear slide was subjected to both dehemoglobinization and Giemsa staining. For cyto-centrifugation, 5 µL of BM was suspended in 195 µL of ammonium chloride (NH₄Cl, 0.87%) and kept at room temperature for 5 minutes for hemolysis. Subsequently, cyto-centrifugation (2,500 rpm/5 minutes) was performed to deposit the cells on a 6 mm² area. The slides containing the thin smears and cyto-centrifuged material were dried and subsequently fixed with methanol for 5 minutes then Giemsa stained.

The preparations were analyzed on a light microscope using an immersion objective (×1,000); all microscopic fields were considered for the thick smear and cyto-centrifugation samples. For thin smear slides at least 2,000 fields were observed for thin smear slides.

Data analysis. Data were analyzed using the Statistical Package for Social Sciences software (SPSS), version 16.0 (SPSS Inc., Chicago, IL). χ^2 and Fisher's exact tests were performed to estimate the associations between clinical signs and parasitological test positivity. The positivity percent rates and respective confidence intervals (95% CI) were obtained for different clinical specimen cultures. The sensitivity and simple agreement were calculated for the direct examinations considering the BM culture as the standard reference.

RESULTS

The promastigote form of *Leishmania* was isolated in at least one type of culture sample (e.g., intact skin, spleen, lymph node, or BM) from 85.9% (N = 79) of the dogs studied. Seventy-three isolates were subjected to etiologic identification, and all exhibited an electrophoretic pattern compatible with *L. infantum*. Regarding the clinical status of the dogs, 13% (N = 12) were considered symptomatic, 29.3% (N = 27) asymptomatic, and 53.3% (N = 49) oligosymptomatic. The clinical data records of four animals could not be recovered. Of the 13 dogs with negative cultures, seven were classified as oligosymptomatic, five as asymptomatic, and one as indeterminate. The results of the parasitological cultures and the clinical status of the seropositive dogs are presented in Table 1.

Regarding direct examinations of BM, 48 dogs exhibited positive results via thick smear, 34 via cyto-centrifugation, and 31 via thin smear. The specificity of direct examination

TABLE 2

Sensitivity of direct parasitological examinations (thin smear, thick smear, and cyto-centrifugation) compared with the reference standard (bone marrow culture)

Direct examination	Sensitivity % (no. of positive/no. of samples)/(CI: 95%)	% Simple agreement
Cyto-centrifugation	47.1% (25/53) (40.24–53.9)	56.1
Thick smear	77% (41/53)* (65.6–88.3)	75.3
Thin smear	52.8% (28/53) (39.3–66.2)	57.5

CI = confidence interval.

Sensitivity: number of positive direct examinations/number of positives in the bone marrow culture × 100; % simple agreement: (number of positives + number of negatives in both tests/total × 100).

*Sensitivity of thick smear was significantly higher than that of cyto-centrifugation and thin smear (P < 0.05).

was 100%. The sensitivity and simple agreement results of direct examinations are shown in Table 2. No association was found between the animals' clinical status and positivity in either cultures or direct examinations ($P > 0.05$).

The visual and microscopic appearances of the preparations are shown in Figure 1. On the thick smear and cytocentrifugation slides, the cells became more concentrated, as expected, but exhibited good dispersion. The hemolysis step was satisfactory in both cases and did not prevent the staining and identification of amastigotes.

DISCUSSION

In this article, we evaluated the performance of techniques used for clinical sample concentration for the parasitological examination of CVL while using cultures as a reference standard. Although serological tools are the most widely used for CVL diagnosis, parasitological data are equally important, particularly when identifying new areas of *L. infantum* circulation. Parasitological confirmation is complex and there is no consensus about the choice of clinical samples and lab-

oratory techniques in the literature. In this study, although cultures were used to evaluate different clinical specimens, *L. infantum* was isolated in 85.9% of the cases and there was no observed association between this result and the animals' clinical status.

Direct examination is an important approach for the diagnosis of leishmaniasis; however, such methods are frequently neglected because of low sensitivity relative to other techniques. In this study, the sensitivity of the thick smear was higher than the sensitivity obtained with cytocentrifugation or thin smears (at a 5% significance level). We chose to use the BM culture as the reference standard (instead of all the clinical specimens) since the distribution of the parasite in different animal's organs is not homogeneous, and the direct examinations were prepared with BM puncture, thus this comparison is the most suitable.

Some aspects that influence the sensitivity of direct examination include the number of slides and fields examined.^{22,23} For example, Regina-Silva and others²⁴ reported a 71% sensitivity rate after examining slides prepared from four different samples (skin, spleen, lymph node, and BM) collected from dogs with suspected infection. In this study, we obtained a 77%

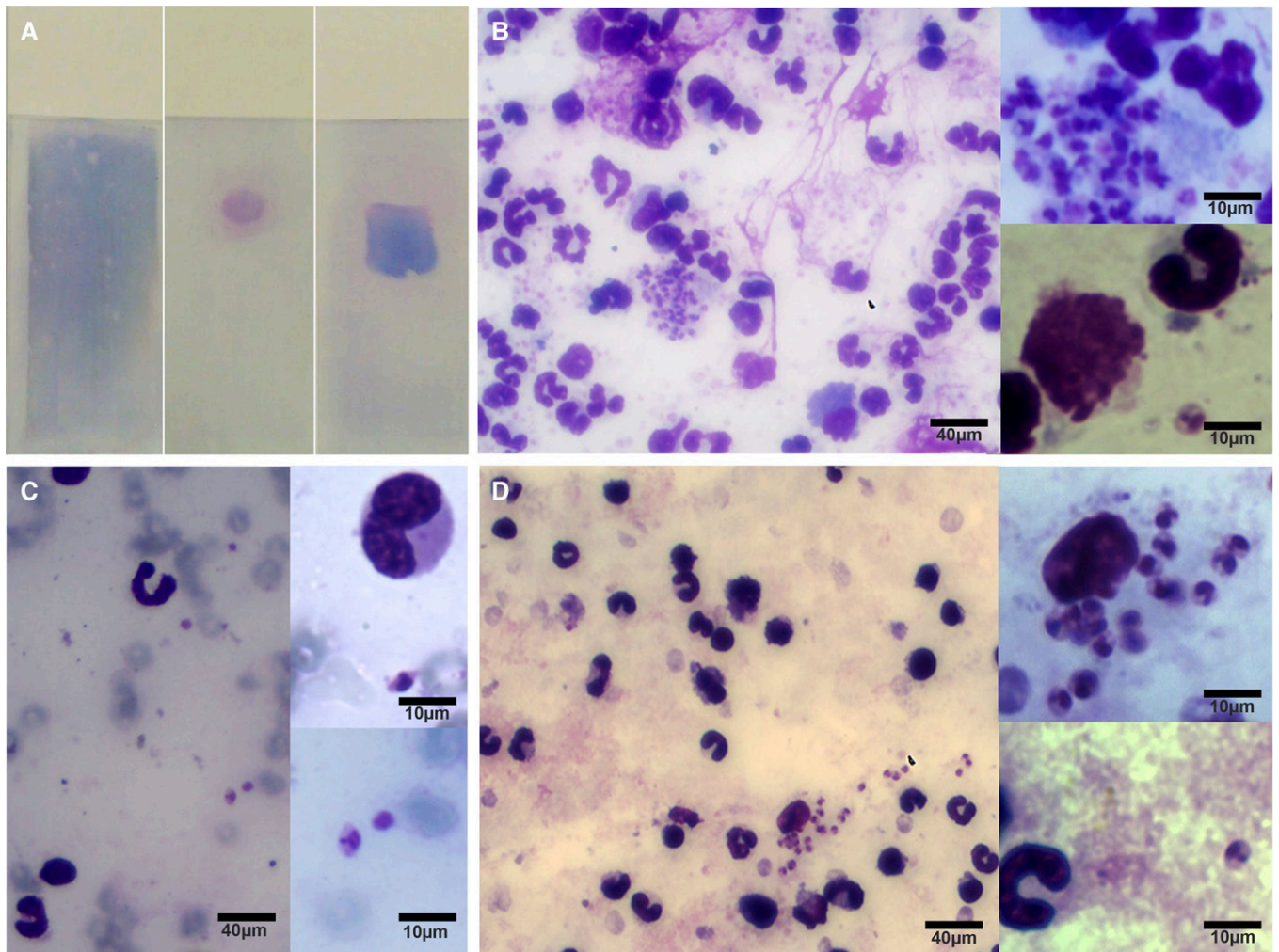


FIGURE 1. (A) Visual aspects of bone marrow preparations for direct examination: thin smear, cytocentrifugation, and thick smear (left to right). Microscope images of the (B) cytocentrifugation, (C) thin smear, and (D) thick smear preparations. Amastigotes are observed in all preparations.

sensitivity rate for BM thick smears and it is worth noting that only a 1 cm² area was examined on each slide. Furthermore, thick smears were prepared as easily and rapidly as thin smears, no fixation step was required, and the dehemoglobinization and coloration steps were performed simultaneously.

In contrast, in addition to exhibiting low sensitivity, cyto-centrifugation requires a preprocessing step and a special centrifuge, which increases the time required for slide preparation and test costs, thus making it more expensive than a thin smear.²⁵ The low sensitivity observed with cyto-centrifugation (relative to other direct examinations) may be explained by the nature of processing (hemolysis and subsequent centrifugation), which might destroy the amastigotes. In addition, we observed that the cells exhibited somewhat contracted cell morphology in this preparation. The thick smear and cyto-centrifugation techniques allowed a 5-fold increase in clinical material concentration on a small area of the slide, thus, not only increasing the likelihood of finding the parasite, but also reducing the time required for the examination and thereby reducing the professional workload required. In general, we observed a one-third decrease in the time required to read the thick smear and cyto-centrifugation tests relative to the thin smear test. These observations demonstrated that thick smear is a promising technique as it combines speed, ease of use, low cost, and good sensitivity in a single examination. Therefore, it is worth mentioning that the thick smear can also be adapted for use with other biological samples, such as lymph node punctures, or used for the diagnosis of human VL.

Serological surveys are useful when measuring diseases, such as VL, that require rapid intervention.¹ However, in certain situations, the parasitological confirmation and characterization of *Leishmania* species requires the same speed. Therefore, the use of concentration techniques for direct examination, as described herein, may be useful. In addition, as the etiologic agent must be identified, the thick smear and cyto-centrifugation preparations can also be used in molecular assays.^{7,26}

To our knowledge, this is the first study to use thick smears and cyto-centrifugation for CVL diagnosis. In addition, our results show that such tests, particularly the thick smear, can be a rapid and safe alternative for the parasitological confirmation of seroreactive dogs.

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