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Cell-block Immunohistochemistry of Bone Marrow Aspirates: a Novel Tool to Improve the Diagnosis of *Leishmania* Infection in Dogs

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Summary

Parasitological methods are the most specific procedures used for the diagnosis of *Leishmania* spp. infection, but their limited sensitivity poses a disadvantage and prompts the need for alternatives. The choice of site for sample collection influences diagnostic sensitivity. The combination of an accurate diagnostic method and a technique that allows large-scale field studies is highly desirable to enhance the investigation of *Leishmania* spp. infection in dogs, especially in endemic regions. The bone marrow is a good target for the detection of *Leishmania* spp. in dogs. In this context, bone marrow aspiration is rapid and less invasive compared with biopsy procedures, and also enables cell block processing, paraffin wax embedding and the sectioning of samples for further histological and immunohistochemical analyses. The aim of this study was to describe for the first time parasitological methods (immunohistochemistry [IHC] and histopathology) using the cell block technique with bone marrow aspirates for the diagnosis of *Leishmania* spp. infection in dogs. Bone marrow aspiration was performed in 45 dogs from an area endemic for visceral leishmaniosis for parasitological culture and the cell block technique (histopathology and IHC). Fourteen (31.1%) dogs tested positive for *Leishmania* spp. by IHC, six (13.3%) by parasitological culture and four (8.9%) by histopathology. Cell block IHC was a useful tool for the diagnosis of canine visceral leishmaniosis. Further studies should be conducted to validate this method for routine epidemiological screening.

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Zoonotic visceral leishmaniosis (ZVL) caused by *Leishmania infantum* (syn. *L. chagasi*) affects man, dogs, cats and wild mammals. This disease is a serious public health problem in many regions of the world owing to its wide geographical distribution, large number of cases and severity of clinical forms (WHO, 2010). In Brazil, ZVL is transmitted by the phlebotomine sand flies *Lutzomyia longipalpis* and *Lutzomyia cruzi*. Domestic dogs (*Canis familiaris*) are the reservoir host of the parasite. Together with the vector, dogs contribute to the maintenance cycle of the disease (Brasil, 2006).

Serological screening of dogs for diagnosis followed by humane destruction of positive cases is the main control measure for ZVL in Brazil (Brasil, 2006). However, this measure is very controversial because of disagreement regarding the sensitivity and specificity of the diagnostic techniques used (Brasil, 2006; Figueiredo *et al*., 2010; Romero and Boelaert, 2010). Although parasitological methods such as parasitological culture, cytopathology,

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histopathology, in-situ hybridization and immunohistochemistry (IHC) are the most specific procedures used in the diagnosis of *Leishmania* spp. infection in dogs, they have limited sensitivity (Maia and Campino, 2008; Menezes *et al.*, 2013). Parasitological culture followed by multilocus enzyme electrophoresis (MLEE) is the reference standard for the identification of species of *Leishmania* (Madeira *et al.*, 2009; WHO, 2010), but the complexity and susceptibility to microbiological contamination of this method, especially in samples collected in the field, prevent it from being used routinely by public health services (Brasil, 2006; Maia and Campino, 2008; WHO, 2010). Therefore, more sensitive parasitological methods for the diagnosis of *Leishmania* infection in dogs need to be developed, not only as tools for the confirmation of positive cases, but also as reference standards for the development of canine serology panels that would permit better validation of serological test kits. IHC is a parasitological method that has proven to be sensitive and specific (Menezes *et al.*, 2013) and, for this reason, it can enhance the accuracy of diagnosis.

The cell block technique is used widely in human medicine for the diagnosis of various diseases using cells isolated from body fluids or aspirates (Nathan *et al.*, 2000; Nga *et al.*, 2005; Dharan, 2010). This technique has also been used in veterinary medicine in the diagnosis of neoplastic diseases (Zanoni et al., 2012; Fernandes *et al.*, 2015). The cells are concentrated by centrifugation or filtration and the resulting cell pellet is processed as a tissue sample, allowing examination by histopathology and IHC with excellent preservation of cell morphology (Nathan *et al.*, 2000). Despite its wide applicability using different samples, the cell block technique has not yet been tested for the diagnosis of *Leishmania* spp. infection in dogs.

In dogs with ZVL, the bone marrow is one of the sites in which the parasite is more likely to be found, especially in asymptomatic animals (Almeida *et al.*, 2011; Paparcone *et al.*, 2013). In this context, bone marrow aspirate samples could represent a valuable alternative for the application of the cell block technique in a rapid and less invasive manner compared with biopsy procedures (Almeida *et al.*, 2011; Paparcone *et al.*, 2013). The combination of an accurate diagnostic method, such as IHC, and a technique that allows large-scale field studies is highly desirable to enhance the investigation of *Leishmania* spp. infection in dogs, especially in endemic regions.

Forty-five dogs were selected randomly from a survey conducted in a ZVL endemic area of Brazil. Bone marrow aspirate samples were collected for parasitological culture and for the cell block technique for histopathology and IHC. These samples were selected for this study because they are a good biological target for the detection of *L. infantum* in dogs by parasitological methods (Moreira *et al.*, 2007; Toplu and Aydogan, 2011; Paparcone *et al.*, 2013).

For sample collection, the animals were sedated by intramuscular administration of ketamine hydrochloride (10 mg/kg) and acepromazine (0.2 mg/kg). After shaving, asepsis and local anaesthesia with 2% lidocaine, bone marrow was aspirated from the sternal manubrium using a 20 ml syringe and a 40 × 12 mm needle. The material collected was divided into two parts: 1 ml was stored in a sterile tube containing EDTA for culture and possible parasite isolation, and 1.5–2.0 ml was transferred to a sterile tube containing EDTA for histopathology and IHC to detect amastigote forms of *Leishmania* spp. The samples were stored in a refrigerator and sent to the laboratory to be processed within 6 h after collection.

For parasite isolation, 0.6 ml of bone marrow was seeded into three tubes containing culture medium. The cultures were incubated at 26–28°C and examined weekly for 30 days (fresh samples) for the demonstration of promastigote forms, which were identified to the species level by MLEE (Cupolillo *et al.*, 1994).

For histopathology and IHC, the tubes containing the bone marrow aspirates were centrifuged at 1,350 g for 10 min and the supernatant was discarded. Next, cell block fixative solution (850 ml absolute alcohol, 100 ml 37% formaldehyde and 50 ml glacial acetic acid) was added to the sediment containing the leucocyte layer and red blood cells until a volume of 5 ml was reached. The mixture was left to stand for 24 h for complete fixation and formation of the cell block. The cell block was then cut and processed for embedding in paraffin wax. Sections (5 μm) were mounted on silane-treated slides. For histopathology, these sections were stained with haematoxylin and eosin (HE). For IHC, these sections were processed according to a previously described protocol (Menezes *et al.*, 2013).

All animal procedures in this study were approved by the Ethics Committee on Animal Use of FIOCRUZ (permit number L-038/08).

Among the 45 dogs from which bone marrow aspirates were evaluated, 14 (31.1%) tested positive for *Leishmania* spp. by IHC, six (13.3%) by parasitological culture and four (8.9%) by histopathology. Figs. 1A and B show how leucocytes are concentrated in histological sections of bone marrow processed by the cell block technique and the dark-brown staining of *Leishmania* spp. amastigotes by IHC, respectively.

All cases that were negative by IHC were also negative by parasitological culture and histopathology.

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