Sensitivity and Specificity of In Situ Hybridization for Diagnosis of Cutaneous Infection by Leishmania infantum in Dogs

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Leishmaniasis is a worldwide disease caused by protozoa of the genus Leishmania which infect wild and domestic mammals, including humans (1). The spectrum of clinical forms of leishmaniasis can vary from focal cutaneous to disseminated visceral disease (1). There are 12 Leishmania species infecting dogs, L. donovani, L. infantum (syn. L. chagasi), L. major, L. arabica, and L. tropica in the Old World and L. infantum, L. colombiensis, L. panamensis, L. mexicana, L. braziliensis, L. pereviana, L. pijanoi, and L. amazonensis in the New World (1–7). Although many species of Leishmania infect dogs, dogs are considered a proven reservoir only for L. infantum (1, 2). The species L. infantum can also infect humans, cats, and wild mammals and is the cause of zoonotic visceral leishmaniasis (VL) (1, 2, 8).

In many countries, zoonotic VL constitutes a significant public health problem, especially due to its prevalence, high mortality rates, mainly in children, and emergency rate in patients infected by the human immunodeficiency virus (1, 9). Transmission in areas of endemcity is usually via bites of infected sand flies, with dogs as the main domestic reservoir of the parasite (1, 9). Therefore, a rapid and accurate diagnosis of the infection of dogs with L. infantum is fundamental for the control of zoonotic VL transmission.

The clinical diagnosis of canine VL is difficult, with many animals being asymptomatic. Therefore, a variety of serological, parasitological, and molecular laboratory methods have been developed to detect infection by L. infantum in dogs (10, 11). Serological assays, PCR, and parasitological culture are the most sensitive methods for the diagnosis of L. infantum infection (12–18). However, these three methods do not allow visualization of the intact amastigotes within the tissue and correlation of the parasites with associated lesions, which is possible by histopathology (HP) and immunohistochemistry (IHC) (19). HP and IHC are frequently used in the current routine for the histological diagnosis of L. infantum in dogs, but these methods have limited accuracy and do not allow species identification (16, 18, 20). The identification of species of Leishmania is currently only possible by parasitological culture followed by multilocus enzyme electrophoresis (MLEE), which is the reference method, and by PCR (1). Hence, alternative histological methods are necessary to improve the accuracy of diagnosing infection of dogs with L. infantum.

A recently established chromogenic in situ hybridization (ISH) technique is a promising method for the diagnosis of canine VL because it permits the highly specific identification of Leishmania in formalin-fixed, paraffin-embedded (FFPE) surgical biopsy specimens of dogs (19). However, the previously published probe for ISH was unable to determine the Leishmania species (19). In addition, the published ISH protocol was based on manual labeling, which presents lower efficiency and productivity than automation (21). Thus, the present study aimed to evaluate the sensitivity and specificity of automated ISH for the diagnosis of canine cutaneous infection caused by L. infantum in routinely processed surgical biopsy samples using the previously published generic

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other fragment was fixed in 10% neutral buffered formalin and processed with antimicrobials (22) and submitted for parasitological culture. The was divided into two samples. One of them was immersed in sterile saline obtained from the intact skin over the scapula after disinfection with 70% alcohol and local anesthesia with 2% lidocaine. Each specimen obtained was divided into two samples. One of them was immersed in sterile saline with antimicrobials and processed for routine paraffin embedding (23). The paraffin blocks were processed for IHC, IH, and HP. The IH, HP, and parasitological culture with identification of the species of trypanosomatids by MLEE were performed at the Evandro Chagas Clinical Research Institute, FIOCRUZ, Brazil. The ISH was performed at the Diagnostic Center for Population and Animal Health, Michigan State University. The biopsy procedure performed on animals was approved by the Ethics Committee on the Use of Animals, FIOCRUZ, Brazil (license L-038/08).

**Study design.** ISH using a previously published generic (ISH-GP) and a newly developed specific (ISH-SP) oligonucleotide probe for the diagnosis of *L. infantum* infection in dogs was evaluated. In order to calculate the number of samples required for this study, the estimated values for the ISH were 70% sensitivity/specificity according to preliminary tests, 17% absolute error in sensitivity/specificity, and 5% alpha. Considering the loss of samples during processing, the calculated number was increased by 4%. As a result, 51 dogs positive for *L. infantum* infection in the parasitological culture and 51 dogs negative for *L. infantum* infection in the parasitological culture were randomly selected from 2,066 surgical skin biopsy specimens of dogs collected between the years 2009 and 2012. The dogs originated from seven cities in Brazil with endemic canine VL: Niterói, Rio de Janeiro; Rio de Janeiro, Rio de Janeiro; Bauru, São Paulo; Brasília, Distrito Federal; Cuiabá, Mato Grosso; Palmas, Tocantins; and Fortaleza, Ceará. Skin samples were selected for this study since they are easy to obtain and have been shown to be a good target for the confirmation of canine VL by parasitological culture (14).

For the collection of samples, one 3-mm punch biopsy specimen was obtained from the intact skin over the scapula after disinfection with 70% alcohol and local anesthesia with 2% lidocaine. Each specimen obtained was divided into two samples. One of them was immersed in sterile saline with antimicrobials (22) and submitted for parasitological culture. The other fragment was fixed in 10% neutral buffered formalin and processed for routine paraffin embedding (23). The paraffin blocks were processed for ISH, IH, and HP. The IH, HP, and parasitological culture with identification of the species of trypanosomatids by MLEE were performed at the Evandro Chagas Clinical Research Institute, FIOCRUZ, Brazil. The ISH was performed at the Diagnostic Center for Population and Animal Health, Michigan State University. The biopsy procedure performed on animals was approved by the Ethics Committee on the Use of Animals, FIOCRUZ, Brazil (license L-038/08).

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**Parasitological culture and characterization by MLEE.** Skin samples collected in saline were seeded in the biphasic medium culture NNN (Novy, MacNeal and Nicolle)-Schneider’s insect medium (Sigma–Aldrich Co., St. Louis, MO) containing 10% fetal bovine serum and were incubated at 26 to 28°C. The *Leishmania* promastigotes isolated were identified by MLEE using five enzymatic systems (24).

**Immunohistochemistry and histopathology.** For immunohistochemistry, serial sections of 5 μm were obtained on silane-treated slides and processed according to a previously described protocol (20), with some modifications. The antigen expression was performed by incubation of the sections in a sodium citrate buffer (pH = 6.0) at 100°C for 20 min in steam. Then, the sections were incubated with rabbit anti-*Leishmania* polyclonal serum at a dilution of 1:500. Histological sections with numer-
controls. Infection by *L. infantum* in these controls had been confirmed by parasitological culture and MLEE. For the reagent negative controls, sections were treated only with RibonHybe hybridization buffer. The total duration of slide processing for ISH-GP was 8:17 h and for ISH-SP was 8:25 h using the Discovery XT.

**Probe validation.** Before testing the ISH on the selected canine skin samples, validation of each probe was performed to confirm the specificity of the generic probe to *Leishmania* and the specificity of the specific probe to *L. infantum* by excluding cross-hybridization with selected pathogenic fungi and protozoa. For this purpose, FFPE tissue samples infected with various protozoa and fungi and FFPE pellets of some of these microorganisms obtained by centrifugation of cultured pathogens were tested. Validation samples of protozoa consisted of *L. infantum* (skin of dog and pellet of promastigote forms), *L. braziliensis* (skin of dog and hamster and pellet of promastigote forms), *L. amazonensis* (skin of mouse and pellet of promastigote forms), *Trypanosoma caninum* (pellet of epimastigote, spheromastigote, and trypomastigote forms), *Trypanosoma cruzi* (heart of mouse and pellet of epimastigote and trypomastigote forms), *Neospora caninum* (lung of rat and pellet of tachyzoites), *Toxoplasma gondii* (lung and heart of dog, brain of mouse, and intestine and lymph nodes of wallaby), *Sarcocystis neurona* (brain of horse), and *Rangelia vitalii* (heart and kidney of dog). The methods used for the diagnosis of protozoa were isolation in parasitological culture and MLEE, except for *T. gondii* and *N. caninum*, which were identified by PCR and IHC, and *R. vitalii*, which was detected by HP in tissues of a dog with the characteristic clinical and pathological alterations caused by this protozoon (27). Validation samples of fungi consisted of Blastomyces dermatitidis (lung of dog), Cryptococcus neoformans (nasal mucosa of horse), Sporothrix (skin of dog and cat), and Histoplasma capsulatum (spleen and kidney of dog and pellet of mycelial form). The diagnosis of all these fungi was based on mycological culture and Grocott’s methenamine silver stain. In all pellets and tissue samples used for validation, microorganisms were easily visible by light microscopy.

**Statistical analysis.** Data obtained were stored in the EpiData software and then analyzed using the Statistical Package for Social Sciences software (version 16.0) for Windows. The sensitivity and the respective 95% confidence interval (95% CI), the specificity, and the accuracy of ISH-GP, ISH-SP, IHC, and HP were compared to the reference standard (parasitological culture). The comparisons between the sensitivity and specificity of ISH-GP, ISH-SP, IHC, and HP were descriptive.

### RESULTS

The ISH using both the generic (ISH-GP) and specific (ISH-SP) probes clearly detected amastigote forms of *Leishmania* with a dark blue signal that was slightly stronger for the ISH-GP (Fig. 1A). The ISH-SP instead showed less background and a better signal-to-noise ratio (Fig. 1B). There was no cross-hybridization of either probe with any of the other microorganisms tested. However, there was cross-reaction of the polyclonal antitoxoplasma antibody (Sigma-Aldrich) used for ISH-GP with cysts and tachyzoites of *Toxoplasma gondii*. This problem was solved by replacing this antibody with the same monoclonal antitoxoidinogenin antibody (Invitrogen) used for ISH-SP. All species of *Leishmania* tested were detected by ISH-SP (Fig. 1A and D). The ISH-SP detected only *L. infantum* (Fig. 1B, E, and F).

The IHC clearly detected amastigote forms of *Leishmania* with a dark brown signal and a good signal-to-noise ratio (Fig. 1C).

The sensitivity and accuracy results of ISH-SP, ISH-GP, IHC, and HP for detecting *L. infantum* are listed in Table 1. The specificity of ISH-SP, ISH-GP, IHC, and HP was 100%.

Of the 51 skin samples positive for *L. infantum* by parasitological culture, 36 were detected by ISH-SP. Fifteen skin samples positive by parasitological culture were not detected by ISH-SP.

### DISCUSSION

The ISH-SP and ISH-GP were both sensitive and specific methods for the diagnosis of *Leishmania* in dogs, showing satisfactory accuracy compared to the reference standard. The values of sensitivity of both ISH methods were very close to those of IHC, with ISH-SP being the most sensitive, followed by ISH-GP, IHC, and HP. The somewhat higher sensitivity of ISH-SP in spite of the slightly lower signal intensity compared to that of ISH-GP may be due to the better signal-to-noise ratio of ISH-SP. The use of ISH and IHC increased the number of correctly diagnosed positive cases in comparison to the results for HP similarly to what has been reported by other authors when using IHC (16, 18, 28, 29).

The results of the current study confirm ISH as an accurate method for the diagnosis of *L. infantum* infection in dogs. Nonetheless, it is less sensitive for the diagnosis of *L. infantum* infection in dogs than parasitological culture, PCR on frozen skin samples, and some serological assays (12, 14–18). ISH failed to detect between 25.5% and 29.4% of the parasitological-culture-positive cases in the current study. In the previous study using the same generic ISH probe (19), ISH was negative in 3 dogs out of 6 positive for *L. infantum* by PCR. In a systematic review (17), the majority of studies on serological assays for the diagnosis of *L. infantum* infection in dogs found sensitivities higher than 75%. Nonetheless, parasitological culture, PCR, and serological assays present disadvantages that prevent their use alone for the routine diagnosis of *L. infantum* infection in dogs. The parasitological culture is time consuming, taking from 5 to 30 days (on average, 15 days) to be completed, and there are only a small number of reference centers worldwide currently using MLEE (1, 22). In addition, this method is susceptible to microbiologic contamination, which in many cases prevents its use in samples collected in the field, where proper storage and sterile conditions may be difficult to attain (1, 10). Furthermore, parasitological culture may be difficult to perform due to poor adaptation of some isolates to the medium (1, 10). The drawbacks of PCR are lack of standardization of the different protocols used among laboratories, possibility of contamination, and the fact that it does not necessarily indicate infection with live *Leishmania* (1, 13, 18, 30). Serologic assays may yield false-positive results due to cross-reactivity with sera of dogs infected with *L. braziliensis*, *T. cruzi*, *T. caninum*, and *Ehrlichia canis* (15, 31–33). Also, they do not necessarily indicate current infection (10) and do not differentiate positive results produced by natural infection from those induced by vaccines (17).

The main advantage of ISH over parasitological culture, PCR, serological assays, IHC, and HP for the diagnosis of *Leishmania* infection in dogs is that it simultaneously allows visualization of the intact amastigotes within the tissue and species identification of them as *Leishmania infantum*, as demonstrated in the present study by ISH-SP. This observation of amastigotes within the tissue, which is not possible by parasitological culture, PCR, and serological assays, offers the possibility to correlate parasites with the associated lesions and, also, to semiquantify the parasite load (16, 19, 34). Similarly to ISH, IHC and HP also link amastigotes of *Leishmania* to lesions (16); however, they are not able to discriminate *L. infantum* from other *Leishmania* species. An important
The advantage of ISH compared to IHC and HP is therefore the higher specificity of ISH. Extensive testing of the specificity of ISH has shown no cross-reaction of the *Leishmania* probes with other histomorphologically similar organisms (19), which was confirmed in the current study. Although IHC in the present study was specific for the diagnosis of *Leishmania*, cross-reactivity with histomorphologically similar fungi, such as *Histoplasma capsulatum*, has been demonstrated (20). In addition, parasitic organisms such as *Histoplasma* and *Trypanosoma*, are difficult to differentiate from *Leishmania* by HP; thus, wrong or inconclusive etiologic diagnoses may occur using this method (19, 35). Furthermore, commercially available anti-*Leishmania* antibodies for IHC that work well on FFPE tissues are currently lacking (19, 35). The possibility of automation is another advantage of ISH (21), which was
ISH-GP and ISH-SP should be implemented in the laboratory as a high-sensitivity and specificity, improving the histological diagnosis, possibly warranting the design of additional probes.

**REFERENCES**


