Trans R Soc Trop Med Hyg 2015; **109**: 469–476
doi:10.1093/trstmh/trv034 Advance Access publication 14 May 2015

Higher sensitivity of immunohistochemistry for bona fide diagnosis of dog *Leishmania* (*Viannia*) *braziliensis*-driven American tegumentary leishmaniasis: description of an optimized immunohistochemistry method

Isabele Barbieri dos Santos[a,*], Rogerio Tortelly[b], Leonardo Pereira Quintella[c], Maria de Fátima Madeira[d], Luisa Helena Monteiro de Miranda[e], Fabiano Borges Figueiredo[e], Raquel de Vasconcellos Carvalhaes de Oliveira[f] and Tânia Maria Pacheco Schubach[e]

[a]Programa de Epidemiologia e Vigilância em Saúde (PEPIVS), FIOCRUZ BRASILIA, Avenida L3 Norte, s-n, campus Universitario Darcy Ribeiro, Gleba A, CEP: 70910–000–Brasilia–DF; [b]Serviço de Anatomia Patológica Doutor Jefferson Andrade dos Santos - Faculdade de Veterinária - Universidade Federal Fluminense, Niterói, RJ, Brazil; [c]Serviço de Anatomia Patológica, Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil; [d]Serviço de Parasitologia, Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil; [e]Serviço de Dermatozoonoses, Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil; [f]Serviço de Epidemiologia, Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil

*Corresponding author: Tel: +55 21 33294791; Fax: +55 21 33294500; E-mail: belebarbieri@yahoo.com.br; isabele.santos@ioc.fiocruz.br

Received 19 January 2015; revised 1 April 2015; accepted 7 April 2015

Background: The in situ detection of parasite antigens in tissue sections by immunohistochemistry (IHC) is a diagnostic alternative for human American tegumentary leishmaniasis (ATL), but has not been used for the diagnosis of cutaneous lesions in dogs with ATL. This study describes the results of IHC for the detection of amastigote forms and other *Leishmania* sp. antigen-positive cells and compares the results of IHC, histopathology and cytopathology for the diagnosis of canine ATL. In addition, possible cross-reactivity with sporotrichosis is analyzed.

Methods: Forty paraffin-embedded biopsies and 40 smears of cutaneous lesions from dogs with ATL, confirmed by isolation and characterization of *Leishmania* (*Viannia*) *braziliensis*, and 40 paraffin-embedded biopsies of cutaneous lesions from dogs with sporotrichosis, confirmed by isolation of *Sporothrix schenckii* in culture (control group), were studied.

Results: Immunohistochemistry was more sensitive in detecting amastigote forms than cytopathology and histopathology, with a positivity rate of 70% (n=28) versus 37.5% and 22.5% for histopathology and cytopathology, respectively. Cytoplasmic staining of mononuclear and endothelial cells was detected by IHC, which was highly specific since no cytoplasmic staining of these cells or staining of fungal structures was observed in sporotrichosis fragments.

Conclusions: In view of the higher sensitivity of IHC in detecting *Leishmania* sp. antigen and patterns of positivity for *Leishmania* sp. antigen compared to histopathology or cytopathology and the absence of cross-reactions with sporotrichosis, we recommend this technique for the diagnosis of canine tegumentary leishmaniasis.

Keywords: American tegumentary leishmaniasis, Brazil, Dogs, Immunohistochemistry

Introduction

Leishmaniasis is a parasitic infectious disease caused by protozoa of the genus *Leishmania* which affects humans and animals. The causal agent of leishmaniasis is transmitted by pool blood feeding sand flies that are hosting Leishmania metacyclic promastigotes in the anterior part of the mid gut and the foregut. Leishmaniasis is classified into the tegumentary (cutaneous, mucocutaneous and mucosal) and visceral form. Clinical symptoms vary according to the *Leishmania* species involved, which can belong to the subgenus *Leishmania* or *Viannia*.[1,2] Leishmaniasis is an important public health problem in several countries and is one of the six leading epidemic diseases in the world.[3]

American tegumentary leishmaniasis (ATL) caused by *Leishmania (Viannia) braziliensis* is a widespread zoonosis in Brazil. In the state of Rio de Janeiro, American tegumentary

leishmaniasis (ATL) in dogs and humans is caused by *Leishmania (Viannia) braziliensis*. Domestic or peri-domestic transmission predominates in the State of Rio de Janeiro as a result of adaptation of the sand fly population *Lutzomyia intermedia*, to the environment modified by human activities.[4,5] One feature of both human and canine ATL is the presence of either cutaneous lesions or mucosal lesions. In dogs, ATL is characterized by the presence of ulcerated cutaneous lesions on the ears, nose, scrotum, and limbs. Cutaneous lesions are generally single, sometimes multiple, painless ulcers with raised edges and a shallow granular center which can be purulent or not. Crust formation might be observed.[6–9]

The definitive diagnosis of canine ATL is based on, either the demonstration of amastigotes forms in cutaneous smears or biopsies by means of cytological or histological analysis, or the growth of promastigote in axenic culture medium from the sampled lesions.[4,5,9,10] However, these 'classical' parasitological methods used alone or in combination are far from being optimal, a feature reflecting the low *L. (V) braziliensis* load in both human and canine ATL. Moreover, for both human and dog populations there is a need to differentiate between sporotrichosis and ATL. Briefly, in Rio de Janeiro as observed in human disease, dogs with sporotrichosis may present a leishmanin positive intradermal skin test and/or serum immunoglobulins that bind *L. (V) braziliensis* antigens.[11–13] Thus, for optimizing the dog ATL diagnosis, we decided to monitor and validate an alternative diagnosis method designed for human ATL,[14–16] namely in situ detection of *L. (V) braziliensis* antigens.

Sporotrichosis is a fungal disease caused by species of the dimorphic fungus from the *Sporothrix schenckii* complex. The disease has a worldwide distribution[17] and has been recently referred as an emerging health problem.[18] In Rio de Janeiro sporotrichosis is considered an endemic disease, with high transmission rate from infected cats to humans and dogs. Between 1998 and 2009, 2200 cases in humans, 3244 cases in cats and 120 cases in dogs were diagnosed at the Evandro Chagas Clinical Research Institute (IPEC), Oswaldo Cruz Foundation (FIOCRUZ).[19]

In Rio de Janeiro, sporotrichosis has become the main differential diagnosis of ATL in dogs due to the similarity of laboratory alterations and location of cutaneous lesions in different stages of infection, as well as overlapping endemic areas of the two diseases. Preliminary studies have shown that, as observed in human disease, dogs with sporotrichosis may present a positive intradermal skin test[11] and positive serology for leishmaniasis.[12,13] These results suggest that the demonstration of the etiological agent in material obtained from cutaneous or mucosal lesions is necessary for the differential diagnosis between ATL and sporotrichosis.[5,8,12,20–22]

This study describes the results of IHC based methods for the detection of amastigote forms and other *Leishmania* sp. antigen-positive cells and compares IHC datasets to the datasets obtained by classical cytological and histological diagnosis methods.

Materials and methods

Collection of biological samples from dogs

Forty paraffin-embedded biopsies of cutaneous lesions and 40 smears of cutaneous lesions from dogs with ATL, confirmed by isolation and characterization of *L.* (*V.*) *braziliensis*[23] (group 1) were studied. Forty paraffin-embedded biopsies of cutaneous lesions from dogs with sporotrichosis, confirmed by isolation of *Sporothrix schenckii* in culture,[24] were included as a control group for cross-reactions. The control group were used for analysis of differential diagnosis between ATL and sporotrichosis.

The paraffin-embedded biopsies from group 1 were subjected to histopathology and IHC and the smears were subjected to cytopathological evaluation. The paraffin-embedded biopsies from control group were subjected to IHC.

The cutaneous lesion biopsies were obtained through punch-biopsies from dogs treated at the clinic of the Laboratory for Clinical Research on Dermatozoonoses in Domestic Animals (Laboratório de Pesquisa Clínica em Dermatozoonoses em Animais Domésticos, LAPCLIN-ZOONOSE), Evandro Chagas Clinical Research Institute, Oswaldo Cruz Foundation (Instituto de Pesquisa Clínica Evandro Chagas [IPEC]–Fundação OswaldoCruz), Rio de Janeiro, Brazil.

All procedures were approved by the Ethics Committee for Animal Use (Comite de Ética em Uso de Animais, CEUA) of Fundação Oswaldo Cruz (Protocol No. P.0238–05).

Characterization of the parasitic isolates

Multi-locus enzyme electrophoresis was used for characterization of the isolates according to Cupolillo et al.[23] The isolates were analyzed by agarose gel electrophoresis, by using four enzymatic systems: nucleosidase (NH1 and NH2, E.C.3.2.2.1), glucose-6-phosphate dehydrogenase (G6PDH, E.C.1.1.1.49), glucose phosphate isomerase (GPI, E.C.5.3.1.9), and 6-phosphogluconate dehydrogenase (6PGDH,E.C.1.1.1.43). *Leishmania* (*V.*) *braziliensis* (MHOM/BR/75/M2903), *L.* (*L.*) *chagasi* (MHOM/BR/74/PP75) and *L.* (*L.*) *amazonensis* (IFLA/BR/67/PH8) were used as reference samples.

Cutaneous smear cytological analysis

Giemsa-stained smears from group 1 were examined with light microscope under oil immersion at 1000X magnification. Each field was examined in order to identify structures morphologically consistent with amastigotes (considering shape, size and location inside macrophage vacuoles or host cell free amastigotes). The result was considered to be positive when at least one intact form was detected.

Cutaneous lesion sample histological analysis

Each paraffin block containing biopsies of cutaneous lesions from group 1 was cut into 5 μm thick sections with a microtome and stained with hematoxylin-eosin (HE).[25] The histopathological examination was performed with basis on the methods previously described by Miranda et al.[26,27] Briefly, the slides were examined under a light microscope, observing the type of inflammatory infiltrate and the presence or absence of granulomas, multinuclear giant cells, ulcers, acanthosis, collagen destruction, hyperpigmentation. The presence of amastigote forms was also verified. The result was defined as positive adopting the same criterion used for cytopathologic examination.

Immunohistochemistry

For IHC, the paraffin-embedded biopsies of cutaneous lesions from dogs of both groups were cut and mounted on silanized

slides. Next, the sections were deparaffinized by three incubation steps in xylene: incubation for 20 min in an oven at 56°C and for 20 and 2 min at room temperature. The sections were rehydrated in a decreasing alcohol series, followed by washing under running tap water and in distilled water.

Endogenous peroxidase activity was blocked by incubation of the sections in a solution of 13.5% hydrogen peroxide and methanol for 40 min, followed by washing under running tap water and in distilled water. Antigen recovery was performed in citrate buffer, pH 6.0, by boiling the sections in a pressure cooker for 3 min and cooling at room temperature. Next, the sections were incubated in normal chicken serum (1:400) for 20 min and then in 1.5% bovine serum albumin in Molico milk powder for 40 min at room temperature to inhibit nonspecific binding. The sections were then incubated with rabbit polyclonal anti-*L. (L.) chagasi* serum as the primary antibody (1:400) in a moist chamber overnight at 4°C. As negative control, sections were incubated with normal rabbit serum from the same rabbit at the same dilution. After incubation, the sections were washed with Tris-buffered saline at room temperature for mechanical removal of the anti-*L.* (*L.*) *chagasi* serum. This step was repeated three times. A universal biotinylated anti-goat, anti-rabbit and anti-mouse secondary antibody system (Dako LSAB+ System Kit, Dako Corporation, Carpinteria, CA, USA) was applied for 25 min at room temperature and the sections were washed again as described in the previous step. Next, the sections were incubated with the streptavidin-biotin-peroxidase complex (Dako LSAB+ System Kit) for 25 min at room temperature and immediately washed as described in the previous step. The reaction was developed with diaminobenzidine (DAB Tablets, Dako catalog code S300), diluting one tablet in 10 mL distilled water and mixing 2 mL of this solution with 15 μL 3% hydrogen peroxide. Development of the reaction was monitored under a light microscope using a 20x objective using a positive control previously selected for its parasite richness. The reaction was interrupted with distilled water when brown amastigote forms appeared in the tissue. The sections were counterstained with Harris hematoxylin, dehydrated in an increasing series of alcohol and xylene, and mounted on slides with coverslips and balsam.

The result was defined as positive when at least one brown structure morphologically consistent with an amastigote form was observed. Cytoplasmic staining of mononuclear, endothelial and neural cells (dermal nerves) was also investigated and was classified as cellular, vascular and neural staining pattern, respectively.

The IHC technique was performed based on the study described by Quintella et al.[28] The use of anti-*L. (L.) chagasi* serum has been previously standardized for the diagnosis of ATL in humans, with a high-performance detection of amastigote forms of *Leishmania (v.) braziliensis*.

Statistical analysis

The Fisher exact test (nonparametric) was used to determine whether the proportions in the comparison between the tests commonly used (histopathology and cytopathology) and IHC were fully random or if there was a relationship between the positive/negative results of the two tests. The hypothesis of independence (random occurrence) is expected to be rejected at the 5% level of significance since histopathology and cytopathology are tests already consolidated in the investigation of leishmaniasis. In addition, it was determined which diagnostic test showed the highest sensitivity for leishmaniasis. The McNemar test was used to determine differences in the proportion of positive results, adopting a level of significance of 5%. This test should provide the same results as the Fisher exact test. Simple Kappa statistics were used to evaluate agreement between test results.

Results

Clinical presentation of dogs with ATL

All the dogs presented round cutaneous ulcers with elevated borders and a granular bottom, sometimes covered with crusts. The number of lesions ranged from 1 to 5 (median=2). Of the 40 dogs 16 (40%) had one lesion, nine (23%) had two lesions, ten (25%) had three lesions and five (13%,) had four to five lesions. The lesions were observed on the ears in 53% (21) of the cases, on the nose in 53% (21), on the forelegs in 15% (6), on the face in 13% (5), on the hind limbs in 8% (3) and on the scrotum in 5% (2). Twenty-seven (68%) dogs had lesions in a single location, ten (25%) in two locations and three (8%) had lesions in multiple locations. The size of the cutaneous lesions ranged from 1.0 to 2.5 cm in diameter.

Cutaneous smear cytological analysis

Amastigote forms (inside macrophages and free-living) were detected in nine (23%) out of 40 smears (Figure 1).

Cutaneous lesion sample histological analysis

Epidermal changes observed in the 40 cutaneous lesion biopsies of dogs with ATL included ulcers in 75% (30), acanthosis in 50% (20), and melanin hyperpigmentation in 15% (6). In the dermis, the presence of a diffuse granulomatous infiltrate of epithelioid cells (poorly formed granuloma) was observed in 35% (14) of

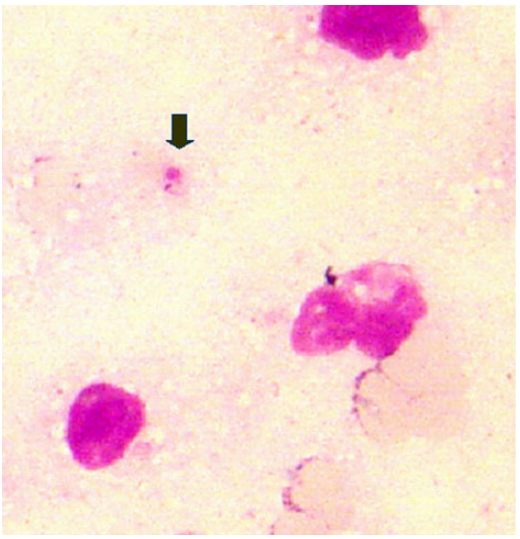

Figure 1. Cutaneous lesions, American tegumentary leishmaniasis, dog. Free amastigote form was visualized in smear. Giemsa. Bar 10 μm. This figure is available in black and white in print and in color at Transactions online.

the cases, a diffuse inflammatory infiltrate of epithelioid cells arranged in granulomas in 5% (2), a diffuse mononuclear inflammatory infiltrate of epithelioid cells and plasma cells in 45% (18), a diffuse mononuclear inflammatory infiltrate of plasma cells in 15% (6), granulomas surrounded by plasma cells in 5% (2), multinuclear giant cells in 5% (2), and pyocytes in 15% (6). The inflammatory infiltrated caused collagen destruction in 33% (13) of the cases and melanin hyperpigmentation in the papillary dermis was seen in 15% (6).

Amastigote forms (inside macrophage vacuoles and free-living) were detected in 38% (15/40) of cases (Figure 2). In six of the 31 cases in which amastigotes were not detected by cytopathology, these forms were visualized by histopathology.

Diseased skin biopsy sections processed by immunohistochemistry allows extending the tool box for optimized dog ATL diagnosis

Streptavidin-biotin-peroxidase IHC detected the presence of amastigote forms in 28 samples (70%, 28/40) (Figure 3A and B).

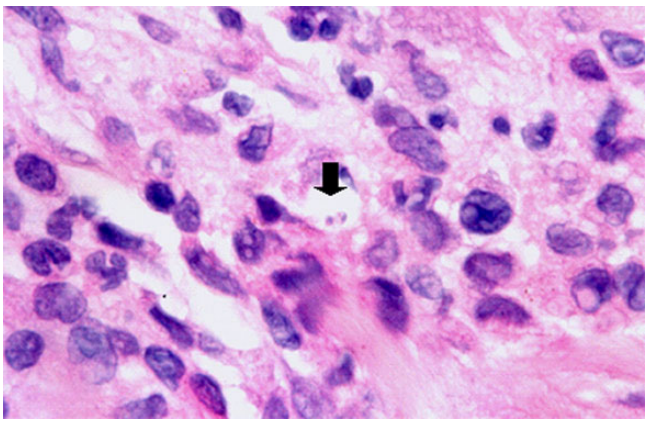

Figure 2. Cutaneous lesions, American tegumentary leishmaniasis, dog. The amastigote forms within macrophage vacuoles. Hematoxylin-eosin. Bar 10 μm. This figure is available in black and white in print and in color at Transactions online.

Amastigotes were detected by IHC in 19 cases that were negative by cytopathology and in 13 that were negative by histopathology.

Cytoplasmic staining of macrophage-like cells (Figure 4A and B) was seen in four cases and amastigote forms were not detected in two of them. Cytoplasmic staining of endothelial cells was observed in two cases (Figure 5A and B), with no detection of amastigote forms in one. There was no cytoplasmic staining of nerve fibers.

In the control group, no staining of yeast-like structures was observed in endothelial cells, macrophage-like cells or nerve fibers (Figure 6A and B).

Analysis of the association between diagnostic methods

Analysis of the association between cytopathology and IHC by the Fisher exact test (p=0.01) and McNemar test (p=0.00) revealed some degree of association between the two methods in the diagnosis of leishmaniasis, as expected. IHC was more sensitive than cytopathology, with 100% of the cases with a positive cytopathologic diagnosis also being positive by IHC, whereas 61% (19/31) of the cases with a negative cytopathologic result had a positive IHC diagnosis (Figure 7).

The Fisher exact test (p=0.01) and McNemar test (p=0.00) also showed some degree of association between histopathology and IHC in the diagnosis of leishmaniasis. IHC was more sensitive than histopathology, with 100% of the cases with a positive histopathologic diagnosis being also positive by IHC, whereas 52% (13/25) of the cases with a negative histopathologic result had a positive IHC diagnosis (Figure 7).

The kappa coefficient of agreement was 0.441 between histopathology and IHC, but only 0.221 between cytopathology and IHC, corresponding to fair and poor agreement, respectively.[29]

Discussion

Tegumentary leishmaniasis is endemic in several Brazilian states and therefore represents an important public health problem for the country. Within this context, the domestic dog (*Canis familiaris*) is an emerging concern since its true importance for the transmission cycle of leishmaniasis has not been established.

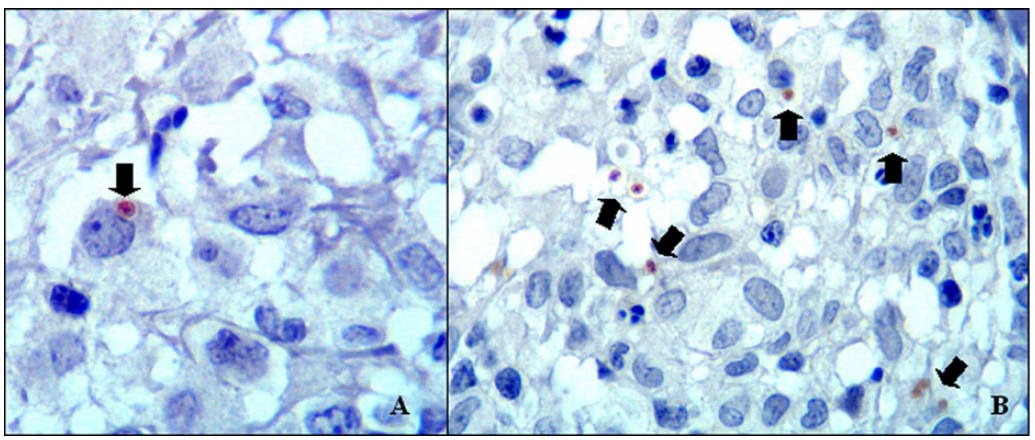

Figure 3. A and B: Cutaneous lesions, American tegumentary leishmaniasis, dog. Amastigote forms. Immunohistochemistry (streptavidin-biotin-peroxidase). Bar 10 μm. This figure is available in black and white in print and in color at Transactions online.

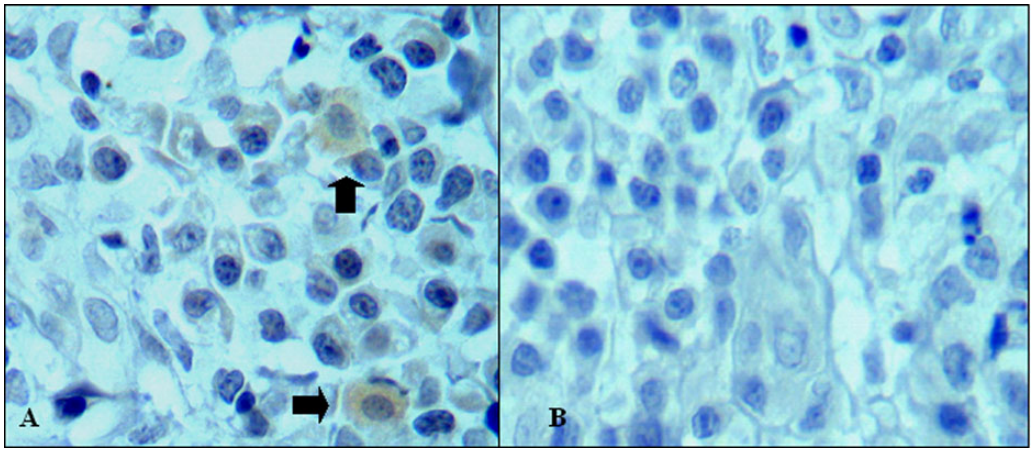

Figure 4. Cutaneous lesions, American tegumentary leishmaniasis, dog. (A) Cytoplasm positiveness for mononuclear cells of monocytic lineage (macrophages), Immunohistochemistry (streptavidin-biotin-peroxidase) incubated in 1:400 rabbit polyclonal anti-*Leishmania Leishmania chagasi* serum. (B) Section was incubated in normal rabbit serum at the same dilution as negative reaction controls for this case. Bar 10 μm. This figure is available in black and white in print and in color at Transactions online.

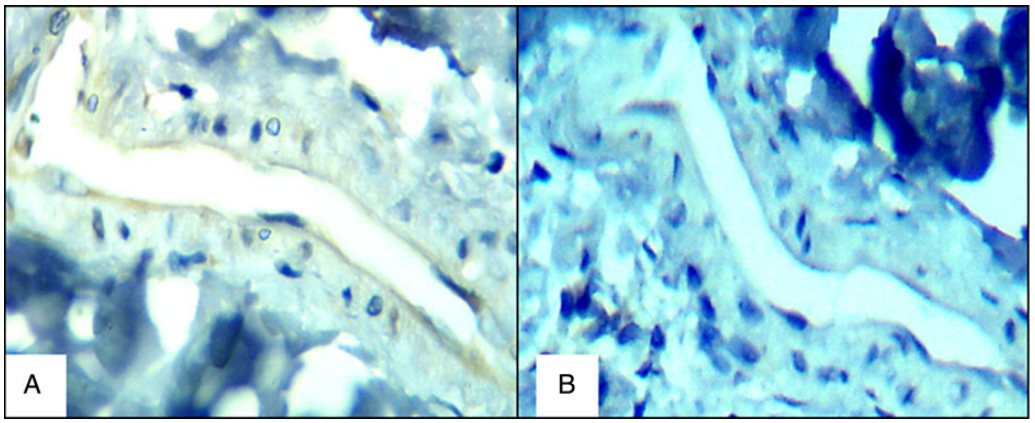

Figure 5. Cutaneous lesions, LTA, dog. (A) Endothelial cell cytoplasm labeling, Immunohistochemistry(streptavidin-biotin-peroxidase) incubated in 1:400 rabbit polyclonal anti-*Leishmania Leishmania chagasi* serum. (B) Section was incubated in normal rabbit serum at the same dilution as negative reaction controls for this case. Bar 10 μm. This figure is available in black and white in print and in color at Transactions online.

However, in the State of Rio de Janeiro, euthanasia of seroreactive dogs is performed without confirmation of infection by parasitological tests such as histopathology, culture or cytopathology of skin lesions.[10,30,31]

In the present study, histopathologic examination of active ATL lesions demonstrated the predominance of a chronic inflammatory process characterized by the presence of plasma cells and epithelioid cells, as well as acanthosis and ulcers. These findings agree with those reported by other authors.[8,27,33] Pirmez et al. observed granulomas in cutaneous lesions of dogs with ATL 3 to 24 months after experimental infection, indicating that granulomas occur during the early stages of canine disease, in contrast to what is observed in humans.[34]

In the present study, well-formed granulomas were seen in a small number of cases. Miranda et al.[27] also described a predominance of poorly formed granulomas. It was not possible to associate their formation with the time since lesion onset since most dogs were from peri-urban or rural areas and their owners did not know when the lesions had occurred. Different findings were described by Madeira et al.[10] with nonspecific inflammation in all of the 25 dogs in the study. Dos Santos et al.[12] also found the predominance of a chronic inflammatory infiltrate with granuloma formation, but whether the granulomas were well-formed or poorly-formed was not mentioned. It should be considered that there are a few studies describing histological changes in skin lesions related to infection with *Leishmania (V.) braziliensis* and most of them do not contain the evaluation of the type of the granulomas or even the criteria applied for the analysis.

In humans, Quintella et al.[28] proposed a predictive rule based on histopathological changes to differentiate skin lesions of ATL and sporotrichosis in humans, even when the aetiological agent is not found. The presence of aggregates of macrophages, tuberculoid granulomas and extracellular matrix degeneration were useful for the differential diagnosis between ATL and sporotrichosis.

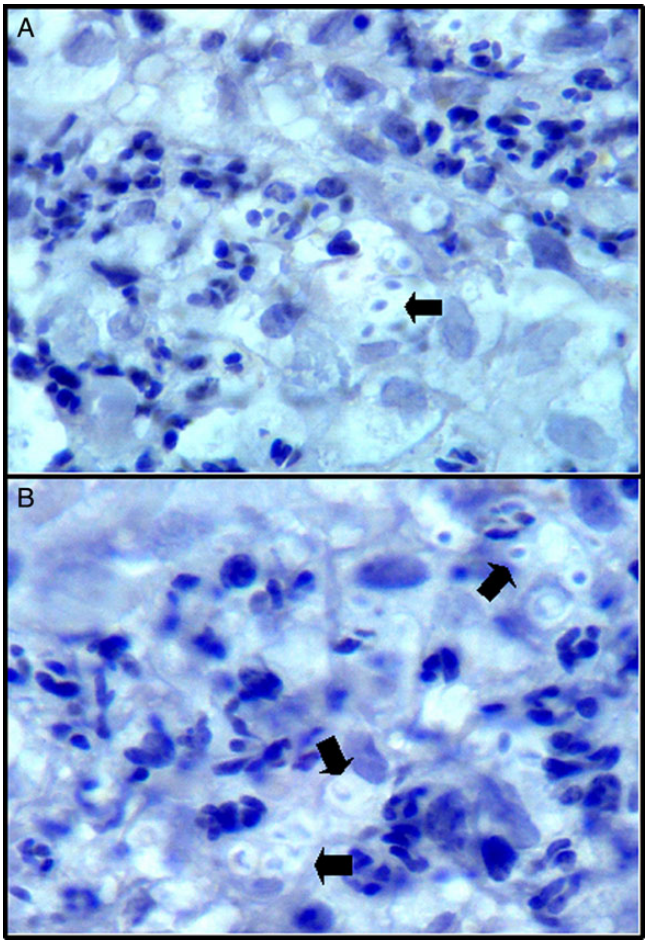

Figure 6. A and B: Cutaneous lesions, sporotrichosis, dog. No marking of yeast-form structures, endothelial cells, macrophages or neural threads. Immunohistochemistry (streptavidin-biotin-peroxidase) incubated in 1:400 rabbit polyclonal anti-*Leishmania Leishmania chagasi* serum. Bar 10 μm. This figure is available in black and white in print and in color at Transactions online.

The number of cases with a positive histopathologic diagnosis was larger than that of cases with a positive cytopathologic result. No data are available in the literature for dogs with ATL. On the other hand, the use of these methods for the diagnosis of human ATL is a matter of discussion, with some authors favoring cytopathology and others histopathology in terms of the sensitivity in detecting *Leishmania* sp. amastigotes.[15,17,36]

In the present study, IHC was more sensitive than histopathology or cytopathology since, in some cases, the immunohistochemical staining obtained made the use of morphological criteria unnecessary to identify the parasite. Thus, in cases in which the parasite could not be identified by HE or Giemsa staining because it appeared incomplete in tissue sections or smears, immunohistochemical staining of the parasite permitted the identification of its shape, location and size. Immunohistochemistry is particularly important when few amastigote forms are present in the tissue because of the excellent contrast between the dark brown color of the positive reaction and the light blue background.

On the other hand, IHC is an expensive diagnostic technique and is more time consuming than Giemsa or HE staining. Immunohistochemistry (streptavidin-biotin-peroxidase) requires preparation of the hyperimmune polyclonal serum and the reaction takes 2 days, whereas Giemsa and HE staining requires 40 and 5 min, respectively, and no serum is needed. Another advantage of IHC is the easy handling of the material. The tissue sections should be fixed in 10% neutral formalin or other fixative and no other special treatment such as refrigeration (necessary for isolation in culture) is required, facilitating field work and excluding the need for immediate dispatch to the laboratory. Furthermore, retrospective surveys can be performed using paraffin-embedded material, as done in this study.

The production of anti-*Leishmania* sp. antibodies is fundamental for the application of immunological diagnostic techniques. In the present study, rabbits were used for the production of anti-*L.* (*L.*) *chagasi* antibodies. These animals are widely used and present a good yield of antibodies.[37] Quintella et al.[28] reported the effectiveness of an immunoperoxidase technique using an anti-*Leishmania (L.) chagasi* hyperimmune serum from rabbits in the diagnosis of culture-confirmed cases of American tegumentary leishmaniasis. Alves et al.[16]reported the effectiveness of an immunohistochemical protocol for the detection of Leishmania in human skin tissues samples with ATL in which canine hyperimmune serum from a dog naturally infected with *Leishmania* (*L.*) *infantum* was employed as a primary antibody. Some investigators have already described the use of hyperimmune serum from seropositive dogs as primary anti-*Leishmania* sp. antibody for the immunohistochemical diagnosis in tissues samples of dogs naturally infected with *Leishmania chagasi*.[3–40]

The present study also verified immunohistochemical staining patterns in mononuclear cells and vessels, as reported for humans with ATL.[15] These patterns were considered suggestive of infection with *Leishmania* sp., since they were not detected in the control group. However, it should be stated that the use of serum from rabbits as primary antibody may lead to unspecific staining as previously reported by Quintella et al.[28] The use of control groups and the replacement of hyperimmune serum by normal rabbit serum as performed in this study are mandatory to evaluate the specificity of these findings. Nevertheless, the disagreement between different studies emphasizes the need for further information concerning the diagnostic value of these staining patterns when amastigote forms are not observed. The use of hyperimmune serum directed to other pathogens and of healthy tissue as negative controls could be an alternative.

In Rio de Janeiro, euthanasia of *Leishmania*-seroreactive dogs is performed without isolation and identification of the parasite species. This fact is a matter of concern since it may lead to the unnecessary culling of dogs with sporotrichosis that are seroreactive for *Leishmania* due to the existence of cross-reactivity.[11–13] Therefore, the differential diagnosis of the two diseases should be performed by demonstrating the respective etiological agent in material obtained from ulcerated skin lesions.[15]

Cross-reactivity between ATL and sporotrichosis in IHC (streptavidin-biotin-peroxidase) using polyclonal serum has been reported for humans, i.e., the chromogen also stained yeast-like structures and cellular and vascular patterns were seen in active skin lesions of humans with sporotrichosis.[15] However, in the present study, no staining of yeast-like structures or specific IHC patterns were observed in biopsies of active skin lesions of dogs with sporotrichosis. This fact makes IHC (streptavidin-biotin-peroxidase) a reliable diagnostic technique for canine ATL since it

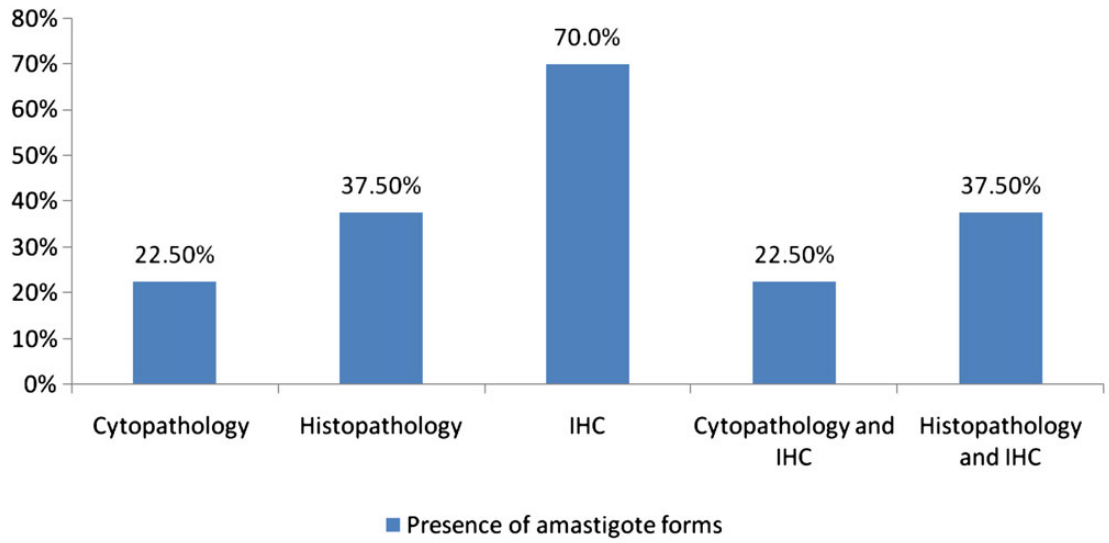

Figure 7. Analysis of the association between diagnostic methods–cytopathology, histopathology and immunohistochemistry (IHC). This figure is available in black and white in print and in color at Transactions online.

permits demonstration of the etiological agent in the absence of cross-reactions with sporotrichosis, the main differential diagnosis.

Furthermore, we emphasize herein the potential of the IHC method as a robust diagnostic tool on further epidemiological studies in endemic areas. Little is known so far concerning the epidemiological impact of both asymptomatic and symptomatic dogs on the frequency of *L. intermedia* adult females carrying *L. (V.) braziliensis*. However, it has already been described in experimental models the involvment of *L. intermedia* saliva exposure on the development of immune response to *Leishmania (V.) braziliensis*.[41] Whether the previous exposure to sand fly saliva impacts on the immune response to ATL in dogs is still to be discussed. In this context, the IHC method may represent a rapid and reliable tool to analyse how the microenviroment in the skin lesions of canine ATL may correlate with the parasitic burden and, consequently, on their pontential as a source of transmission to the vector.

Conclusions

The higher sensitivity of IHC in detecting *Leishmania* sp. antigen compared to histopathology or cytopathology and the absence of cross-reactions with sporotrichosis suggest the use of this technique for the diagnosis of canine tegumentary leishmaniasis.

Authors' contributions: IBS, RT and TMPS conceived the study; IBS, RT, MFM, RVCO, FBF and TMPS designed the study protocol; IBS and FBF carried out the clinical assessment and samples collection; IBS, RT, LHMM, LPQ and MFM carried out the histopathology, immunohistochemistry and parasitological culture; IBS and RVCO performed statistical analysis and interpretation of these data; IBS and LHMM drafted the manuscript; TMPS, LPQ and MFM critically revised the manuscript for intellectual content. All authors read and approved the final manuscript. TMPS is guarantor of the paper.

Acknowledgements: The authors thank Armando de Oliveira Schubach, Thais Okamoto, Marina de Carvalho Furtado, Isabella Dib Ferreira Gremião, Marco Francisco, Antônio Correia, Antônio Carlos, Rodrigo de Almeida Paes, Leandra Faria dos Santos, Tatiana Cristina Vieira de Carvalho, Cristiane Leal, and Cibele Baptista for technical support.

Funding: This study was partially supported by Programa de Apoio a Pesquisa Estratégica em Saúde (PAPES 3-400255/2006-5). Tânia Schubach was the recipient of a fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-482395/2007-9).

Competing interests: None declared.

Ethical approval: All procedures were approved by the Ethics Committee for Animal Use (Comite de Ética em Uso de Animais, CEUA) of Fundação Oswaldo Cruz (Protocol No. P.0238-05).

References

1 Molyneux DH, Killick-Kendrick R. In: Peters W, Killick-Kendrick R (editors). The Leishmaniases in Biology and Medicine. Vol 1. London: Academic Press; 1987, 121–76.

2 Lainson R, Shaw J. Evolution, classification and geographical distribution. In: Peters W, Killick-Kendrick., editors. The leishmaniases in Biology and Medicine. Vol. 1. London: Academic Press; 1987, 1–120.

3 WHO. Health topics: Leishmaniasis. Geneva: World Health Organization; 2005. http://www.who.int/leishmaniasis/en/ [accessed 10 October 2014].

4 Marzochi MAC, Marzochi KBF. Tegumentary and visceral leishmaniases in Brazil. Emerging anthropozoonosis and possibilities for their control. Cad Saúde Pública 1994;10:359–75.

5 Oliveira-Neto MP, Pirmez C, Rangel E et al. An outbreak of American cutaneous leishmaniasis (*Leishmania braziliensis braziliensis*) in a periurban area of Rio de Janeiro city, Brazil: clinical and epidemiological studies. Mem Inst Oswaldo Cruz 1988;83:427–35.

6 Padilla AM, Marco JD, Diosque P et al. Canine infection and the possible role of dogs in the transmission of American tegumentary leishmaniosis in Salta, Argentina. Vet Parasitol 2002;110:1–10.

7 Madeira MF, Uchôa CM, Leal CA et al. *Leishmania (Viannia) braziliensis* in naturally infected dogs. Rev Soc Bras Med Trop 2003;36:551–5.

8 Pirmez C, Coutinho SG, Marzochi MC et al. Canine American cutaneous leishmaniasis: a clinical and immunological study in dogs naturally infected with *Leishmania braziliensis braziliensis* in an endemic area of Rio de Janeiro, Brazil. Am J Trop Med Hyg 1988;38:52–8.

9 Castro EA, Thomaz-Soccol V, Augur C, Luz E. *Leishmania (Viannia) braziliensis*: epidemiology of canine cutaneous leishmaniasis in the State of Paraná (Brazil). Exp Parasitol 2007;117:13–21.

10 De Fátima Madeira M, de O Schubach A, Schubach TM et al. Post mortem parasitological evaluation of dogs seroreactive for Leishmania from Rio de Janeiro, Brazil. Vet Parasitol 2006;138:366–70.

11 Barros M.B.L, Schubach A, Francesconi-do-Valle AC et al. Positive Montenegro skin test among patients with sporotrichosis in Rio de Janeiro. Acta Trop. (2005) 93:41–47.

12 Dos Santos IB, Schubach TM, Leme LR et al. Sporotrichosis: the main differential diagnosis with tegumentary leishmaniosis in dogs from Rio de Janeiro, Brazil. Vet Parasitol 2007;143:1–6.

13 Ribeiro FC, de O Schubach A, Mouta-Confort E et al. Use of ELISA employing *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) chagasi* antigens for the detection of IgG and IgG1 and IgG2 subclasses in the diagnosis of American tegumentary leishmaniasis in dogs. Vet Parasitol 2007;148:200–6.

14 Salinas G, Valderrama L, Palma G et al. Detection of amastigotes in cutaneous and mucocutaneous leishmaniasis using the immunoperoxidase method, using polyclonal antibody: sensibility and specificity compared with conventional methods of diagnosis. Mem Inst Oswaldo Cruz 1989; 84:53–60.

15 Schubach AO, Cuzzi-Maya T, Oliveira AV et al. Leishmanial antigens in the diagnosis of active lesions and ancient scars of American tegumentary leishmaniasis patients. Mem Inst Oswaldo Cruz 2001;96:987–96.

16 Alves CF, Alves CF, Figueiredo MM et al. American tegumentary leishmaniasis: effectiveness of an immunohistochemical protocol for the detection of Leishmania in skin. PLoS One 2013;8:633–43.

17 Schell W. Agents of chromoblastomycosis and sporotrichosis. In: Ajello L, Hay RJ. Topley & Wilson's Microbiology and Microbial Infections. London: Arnold; 1998, 315–36.

18 López-Romero E, Reyes-Montes M,, del R, Pérez-Torres A et al. *Sporothrix schenckii* complex and sporotrichosis, an emerging health problem. Future Microbiol 2011;6:85–102. doi:10.2217/fmb.10.157.

19 Barros MB, Schubach TP, Coll JO et al. Sporotrichosis: development and challenges of an epidemic. Rev Panam Salud Publica 2010;27:455–60.

20 Heller HM, Swartz MN. Nodular lymphangitis: clinical features, differential diagnosis and management. Curr Clin Top Infect Dis 1994;14:142–58.

21 Livni N, Abramowitz A, Londner M et al. Immunoperoxidase method of identification of *Leishmania* in routinely prepared histological sections. Virchows Arch A Pathol Anat Histopathol 1983;401:147–51.

22 Miranda LH, Quintella LP, Menezes RC et al. Evaluation of immunohistochemistry for the diagnosis of sporotrichosis in dogs. Vet J 2011;190:408–11.

23 Cupolillo E, Grimaldi G Jr, Momen H. A general classification of New World Leishmania using numerical zymotaxonomy. Am J Trop Med Hyg 1994;50:296–311.

24 Werner A, Werner B. Sporotrichosis in man and animal. Int Dermatol 1994;33:692–700.

25 Montenegro MR, Franco M. Patologia processos gerais. Rio de Janeiro, São Paulo, Riberão Preto e Belo Horizonte: Atheneu; 2004.

26 de Miranda LH, Quintella LP, dos Santos IB et al. Histopathology of canine sporotrichosis: a morphological study of 86 cases from Rio de Janeiro (2001–2007). Mycopathologia 2009;168:79–87.

27 Miranda LH, Quintella LP, Santos IB et al. Comparative histopathological study of sporotrichosis and American tegumentary leishmaniasis in dogs from Rio de Janeiro. J Comp Pathol 2010;143:1–7.

28 Quintella LP, Cuzzi T, Madeira MF et al. Immunoperoxidase technique using an anti-*Leishmania (L.) chagasi* hyperimmune serum in the diagnosis of culture-confirmed American tegumentary leishmaniasis. Rev Inst Med Trop S Paulo 2009;51:83–6.

29 Landis JR., Koch GG. The measurement of observer agreement for categorical data. Biometrics 1977;33:159–74.

30 Lopes UG, Momen H, Grimaldi G Jr et al. Schizodeme and zymodeme characterization of *Leishmania* in the investigation of foci of visceral and cutaneous leishmaniasis. J Parasitol 1984;70:89–98.

31 Marzochi MC, Coutinho SG, Souza WJ et al. Canine visceral leishmaniasis in Rio de Janeiro, Brazil. Clinical, parasitological, therapeutical and epidemiological findings (1977–1983). Mem Inst Oswaldo Cruz 1985;80:349–57.

32 Pirmez C., Marzochi M.C., Coutinho S.G., Experimental canine mucocutaneous leishmaniasis (*Leishmania braziliensis braziliensis*), Mem. Inst. Oswaldo Cruz (1988a) 83:145–151.

33 Lima WS, Costa CA, Michalick MSM et al. Histopatologia e terapêutica da leishmaniose tegumentar canina, naturalmente adquirida. Arq Bras Med Vet Zoo 1986;38:34–41.

34 Magalhães AV, Morais MAP, Raick AN et al. Histopatologia da leishmaniose tegumentar por *Leishmania braziliensis braziliensis*. 1. Padrões histopatológicos e estudo evolutivo das lesões. Rev Inst Med Trop S Paulo 1986;28:253–62.

35 Quintella LP, Passos SR, de Miranda LH et al. Proposal of a histopathological predictive rule for the differential diagnosis between American tegumentary leishmaniasis and sporotrichosis skin lesions. Br J Dermatol, 2012;167:837–46.

36 Faber WR, Oskam L, van Gool T et al. Value of diagnostic techniques for cutaneous leishmaniasis. J Am Acad Dermatol 2003;49:70–74.

37 Froes AM, dos Santos CV, Penha-Filho ML et al. Sub-clinical infection as an effective protocol for obtaining anti-*Leishmania chagasi* amastigote antibodies of different animal species. Vet Immunol Immunopathol 2004;99:135–41.

38 Tafuri WL, Santos RL, Arantes RM et al. An alternative immunohistochemical method for detecting Leishmania amastigotes in paraffin-embedded canine tissues. J Immunol Methods 2004;292:17–23.

39 Xavier SC, Andrade HM, Hadad S et al. Comparation of paraffin-embedded skin biopsies from different anatomical regions as sampling methods for detecting Leishmania infection in dogs using histological, immunohistochemical and PCR methods. BMC Vet Res 2006;2:1746–56.

40 Pinto AJ, Figueiredo MM, Silva FL et al. Histopathological and parasitological study of the gastrointestinal tract of dogs naturally infected with Leishmania infantum. Acta Vet Scand 2011;53:67.

41 Weinkopff T, de Oliveira CI, de Carvalho AM et al. Repeated exposure to Lutzomyia intermedia sand fly saliva induces local expression of interferon-inducible genes both at the site of injection in mice and in human blood. PLoS Negl Trop Dis 2014;2618:2627.