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Evaluation of immunohistochemistry for the diagnosis of sporotrichosis in dogs

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ABSTRACT

The aim of this study was to apply immunohistochemistry (IHC) for the diagnosis of canine sporotrichosis and to compare this method with the Grocott's silver stain (GSS) and periodic acid Schiff (PAS) techniques. Eighty-seven dogs with sporotrichosis (group 1) and 35 with American tegumentary leishmaniosis (ATL) (group 2) were studied. The fungus was detected in group 1 by GSS, PAS and IHC. IHC was also applied to group 2 to evaluate the occurrence of cross-reactions. PAS, GSS and IHC detected yeast cells in 19.5%, 43.7% and 65.5% of the group 1 cases, respectively. The detection of intracellular antigens of *Sporothrix schenckii* by IHC increased the sensitivity of the histological diagnosis to 80.5%. No positive reaction was observed in ATL lesions. The results suggest that IHC may be indicated for the diagnosis of sporotrichosis because of its higher diagnostic sensitivity.

Introduction

Sporotrichosis is a subcutaneous mycosis caused by the dimorphic fungus *Sporothrix schenckii*, which infects humans and several animal species (Schell, 1998). Canine sporotrichosis is considered to be rare and presents a low zoonotic potential (Santos et al., 2007) but has reached epidemic proportions in Rio de Janeiro since 1998, affecting humans, dogs and cats (Schubach et al., 2008). The increasing number of cases emphasizes the importance of fast and efficient diagnostic methods for the disease. American tegumentary leishmaniosis (ATL) has become the main differential diagnosis for sporotrichosis in humans and dogs in Rio de Janeiro (Barros et al., 2005; Santos et al., 2007), and, in addition to their clinical similarity, the endemic areas for the diseases overlap and cross-reactions are observed in serological tests.

The reference method for the diagnosis of sporotrichosis is the isolation and identification of the fungus in culture (Kwon-Chung and Bennett, 1992). However, no fungal growth may be observed, generally because of inadequate transport of the material or contamination with saprophytic microorganisms (Moore and Ackerman, 1946; Schwarz, 1982). Additionally, the fungus may present slow growth, requiring maintenance of the culture for a long period (Schwarz, 1982).

Identification of *S. schenckii* in paraffin blocks is frequently the only method available, particularly when fungal infection was not suspected at the time of biopsy (Moskowitz et al., 1986; Werner and Werner, 1994). Histochemical techniques for the detection of fungal structures in histological sections, such as Grocott's silver stain (GSS) and periodic acid Schiff (PAS), are not specific and present low sensitivity for the diagnosis of sporotrichosis in humans and dogs because of the scarcity of *S. schenckii* yeast cells in skin lesions (Marques et al., 1992; Barros et al., 2004, 2005; Schubach et al., 2006; Santos et al., 2007).

IHC has shown improved sensitivity in the diagnosis of human sporotrichosis (Marques et al., 1992; Rodriguez and Sarmiento, 1998), but its application to the diagnosis of the disease in dogs has not been standardized. The aim of the present study was to apply IHC for the diagnosis of canine sporotrichosis and to compare this method with the GSS and PAS techniques.

Materials and methods

Samples

Active skin lesions obtained from dogs seen at the Evandro Chagas Clinical Research Institute, Oswaldo Cruz Foundation, Instituto de Pesquisa Clínica Evandro Chagas (IPEC), Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, between 2001 and 2008 were studied. The study was approved by the Ethics Committee on the Use of Animals, FIOCRUZ (protocol P.00060-00).

The samples were embedded in paraffin blocks and divided into two groups: group 1 consisted of skin lesions from 87 dogs with sporotrichosis confirmed by isolation of *S. schenckii* in culture; group 2 consisted of skin lesions from 35 dogs with

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ATL confirmed by isolation and characterization of *Leishmania (Viannia) braziliensis*. The isolation of the agent in culture is considered the gold standard for the diagnosis of both diseases.

Histochemical techniques

The GSS and PAS techniques were applied to group 1 for the detection of fungal elements. The cases were qualitatively classified as positive or negative. Cases were considered positive when yeast forms consistent with *S. schenckii* were found, and negative when no yeast cell was observed after analyzing a range of 50 microscopic fields of 400× magnification.

IHC using rabbit anti-S. schenckii polyclonal serum

The polyclonal serum was obtained according to protocol by Lopes-Alves et al. (1994). Paraffin blocks were cut and mounted on silanated slides. After deparaffinization and rehydration, endogenous peroxidase activity was blocked with 30% hydrogen peroxide in 40 mL/100 mL (v/v) methanol solution. Nonspecific reactions were inhibited with normal swine serum (Novocastra) in 1.5% bovine serum albumin (BSA) (1:20 dilution), followed by incubation in a solution of 0.1 g/mL milk powder in 3.0% BSA.

Sections were then incubated in a moist chamber overnight at 4 °C with anti-*S. schenckii* serum in 1.5% BSA (1:4000 dilution); next they were washed with Tris-buffered saline and incubated with universal biotinylated secondary antibody and the streptavidin–biotin–peroxidase complex (DakoCytomation). The reaction was developed using diaminobenzidine (DakoCytomation) as chromogen. The histological sections were counterstained with Mayer's hematoxylin and dehydrated.

The case used as positive control was confirmed by isolation of *S. schenckii* in culture and was known to contain yeast cells on histopathological analysis. The same case was also used as negative control by incubating with normal rabbit serum (DakoCytomation) and BSA to control for nonspecific reactions and for endogenous biotin and peroxidase activity, respectively. The analysis was conducted as described for the GSS and PAS techniques. The pathologist was not blinded to the diagnosis of the cases at the time of the analysis.

Data analysis

Data were stored and analyzed with SPSS for Windows, version 16.0. For the analysis, the cases were classified as positive or negative based on the presence of yeast forms consistent with *S. schenckii*. All cases included were previously confirmed as sporotrichosis by isolation of *S. schenckii* in culture. The sensitivity of the methods (GSS, PAS and IHC) was calculated by comparing them to the culture results, which were considered the gold standard. The results were compared using the McNemar test. $P < 0.05$ was considered significant.

Results

The sensitivity of GSS staining (43.7%) was higher than that of PAS staining (19.5%) ($P < 0.001$) for lesions of the 87 group 1 dogs (sporotrichosis). GSS was positive in all PAS-positive cases. Yeast cells were detected by IHC in 65.5% of the lesions (Fig. 1). Of these,

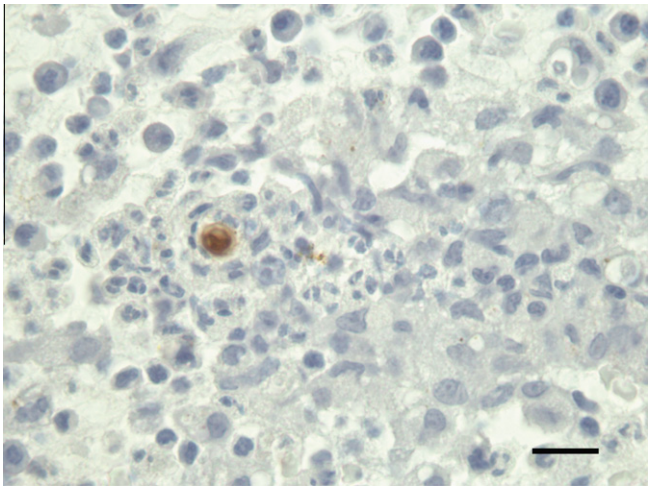

Fig. 1. Skin of a dog with sporotrichosis. *S. schenckii* yeast cells inside a suppurative granuloma. Immunohistochemistry (anti-*S. schenckii* anti-serum). Bar = 10 μm.

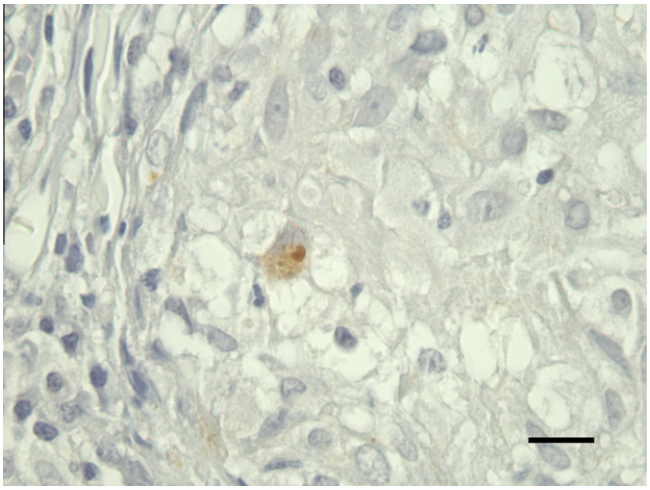

Fig. 2. Skin of a dog with sporotrichosis. Intracellular antigens of *S. schenckii* inside granulomas. Immunohistochemistry (anti-*S. schenckii* anti-serum). Bar = 10 μm.

Table 1
Sensitivity of the different techniques, alone or in combination, for the diagnosis of canine sporotrichosis.

Technique	Sensitivity ($n = 87$)
PAS	19.5% (17)
GSS	43.7% (38)
IHC (yeast)	65.5% (57)
IHQ (Ag)	27.6% (24)
IHC (yeast + Ag)	73.6% (64)
IHC (yeast) + GSS	74.7% (65)
IHC (yeast + Ag) + GSS	80.5% (70)

PAS, periodic acid Schiff; GSS, Grocott's silver stain; IHC, immunohistochemistry; Ag, antigens.

Table 2
Comparison of the sensitivity of the different techniques, alone or in combination, for the diagnosis of canine sporotrichosis.

Technique(s)	McNemar test (P^*)
GSS × PAS	<0.001
GSS × IHC (yeast)	0.002
PAS × IHC (yeast)	<0.001
GSS × IHC (yeast + Ag)	<0.001
PAS × IHC (yeast + Ag)	<0.001
IHC (yeast) × IHC(yeast + Ag)	0.016
GSS × IHC (yeast) + GSS	<0.001
IHC (yeast) × IHC (yeast) + GSS	0.008
GSS × IHC (yeast + Ag) + GSS	<0.001
IHC (yeast) × IHC (yeast + Ag) + GSS	<0.001

PAS, periodic acid Schiff; GSS, Grocott's silver stain; IHC, immunohistochemistry; Ag, antigens; *, $P < 0.05$ was considered statistically significant.

47.4% were negative by GSS. Eight of the 30 IHC-negative cases were positive by GSS. The sensitivity of IHC was higher than that of GSS ($P = 0.002$) and PAS ($P < 0.001$).

Intracellular antigens were detected by IHC in 27.6% of the cases (Fig. 2). Among the cases in which yeast cells were not detected by GSS or IHC ($n = 22$), five presented intracellular antigens by IHC. Considering the detection of yeast cells and intracellular antigens, the combined application of IHC and GSS increased the sensitivity (80.5%) when compared to either technique alone.

The sensitivities of the single and combined methods are shown in Table 1 and the sensitivities of the different techniques are compared in Table 2. No positive reaction was observed for the

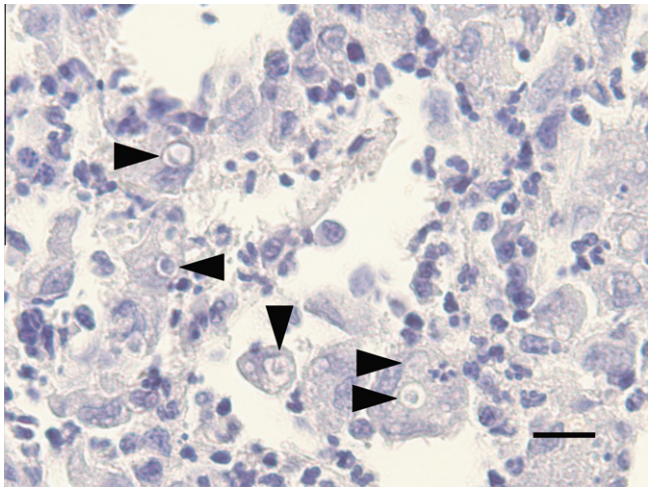

Fig. 3. Skin of a dog with sporotrichosis. Intracellular *S. schenckii* yeast cells (arrowheads) without positive reaction. Immunohistochemistry negative control (normal rabbit serum). Bar = 10 μm.

negative controls (Fig. 3). Cross-reactions with *Leishmania* spp. amastigote forms or their antigens were not observed in lesions of the 35 group 2 dogs (ATL).

Discussion

Among the histological techniques studied, GSS showed higher sensitivity than PAS for the diagnosis of canine sporotrichosis. Similar results have been reported by Marques et al. (1992) and Moskowitz et al. (1986) for humans. However, artifacts are frequent in silver stains and might be mistaken for fungal structures, particularly when the latter are scarce in the tissue (Moskowitz et al., 1986).

In the present study, IHC showed higher sensitivity than GSS and PAS in the detection of yeast cells in canine sporotrichosis lesions, a finding already described for human sporotrichosis by Marques et al. (1992). Moskowitz et al. (1986) did not observe differences in sensitivity between silver staining and IHC. Even when scarce, *S. schenckii* yeast cells are easily detectable by IHC because they acquire a brown color that contrasts on the blue background of the tissue (Moskowitz et al., 1986; Marques et al., 1992).

The combination of GSS and IHC significantly increased sensitivity when compared to IHC alone. Marques et al. (1992) also found improved sensitivity in the diagnosis of human sporotrichosis when these two techniques were combined. However, despite the improved sensitivity, it should be stated that the GSS stain is not a specific method and is not indicated for differentiate yeast cells of *S. schenckii* from other fungal species. Regarding the IHC method, the results may vary according to the source of the antibody used, since this is not a commercially available antibody.

The IHC technique used in this study permitted the staining of *S. schenckii* yeast cells and intracellular antigens. The labeling of intracellular antigens by IHC has been described for leishmaniosis lesions and is referred to as 'cellular pattern' (Schubach et al., 2001). The detection of a sporotrichosis 'cellular pattern' in lesions with a suspicion of sporotrichosis might be useful in cases in which intact yeasts are not observed (Marques et al., 1992). However, the diagnostic value of this finding requires further evaluation.

The sporotrichosis 'cellular pattern' frequently observed in the present study increased the sensitivity of IHC to 73.6%. Thus, considering the detection of yeast cells and of the 'cellular pattern', the combination of IHC and GSS increased the sensitivity of histological diagnosis to 80.5%. This improved sensitivity was statistically significant when compared to the results obtained for the methods alone or in combination. Marques et al. (1992) also obtained improved sensitivity of detection of the 'cellular pattern' and *S. schenckii* yeast cells by IHC and emphasized the possible contribution of these findings to the study of the pathogenesis of sporotrichosis. Rodriguez and Sarmiento (1998) observed a sporotrichosis 'cellular pattern' in human sporotrichosis lesions and related this finding to fungal degradation by phagocytosis.

The relevance of the negative controls should be emphasized. The 'cellular pattern' was considered to be specific in this study due to its absence in the negative controls. Additionally, anti-*S. schenckii* serum did not react with *Leishmania* spp. in canine ATL lesions. Previous studies in humans have reported cross-reactions between *S. schenckii* and *Leishmania* spp. when anti-*Leishmania* serum was used for IHC (Schubach et al., 2001; Quintella et al., 2009). In dogs, the differential diagnosis between sporotrichosis and ATL is usually difficult, because the diseases share clinical and epidemiological features and present cross-reactions in serological tests (Santos et al., 2007). Based on this evidence, the application of a method which accurately differentiates the diseases is necessary.

In the present study, the IHC method, using the anti-*S. schenckii* serum, showed specificity when applied on canine ATL lesions used as negative controls, since the amastigotes and antigens of Leishmania were not visualized. Therefore, the technique described might be a useful alternative or complementary technique for the differential diagnosis between these diseases.

Since yeast cells are rare in most cases, the investigation of serial sections obtained from the same fragment may increase the chance of detection of the agent. In the present study, two sections per case were analyzed by each technique, providing a higher sensitivity when compared to other studies (Moraes and Miranda, 1964; Barros et al., 2005; Schubach et al. 2006; Santos et al., 2007). Other authors reported a larger number of positive results when multiple sections were analyzed (Moraes and Miranda, 1964; Itoh et al., 1986). According to Marques et al. (1992), the deeper the skin fragments, the higher the possibility of detecting the agent. However, skin biopsy fragments are usually small and may not be sufficient to obtain serial sections.

Conclusions

GSS staining should be used if histopathological aspects consistent with sporotrichosis are observed. IHC is a complementary method that can be applied, if available, to improve the sensitivity of the histological diagnosis.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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