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Comparative assessment of skin reactivity to thimerosal- or phenol-preserved Imunoleish© antigen in dogs with suspected American Tegumentary Leishmaniasis in an endemic area of the state of Rio de Janeiro, Brazil

Estudo comparativo da intradermorreação com antígeno Imunoleish® conservado em timerosal ou em fenol em cães com suspeita de Leishmaniose Tegumentar Americana em área endêmica do Estado do Rio de Janeiro, Brasil

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ABSTRACT

The leishmanin skin test (LST), which is an in vivo test that assesses the cellular immune responses to *Leishmania*-derived antigens, is an important tool in the laboratory diagnosis of American tegumentary leishmaniasis (ATL). This study aimed to compare the results obtained in LST employing the Imunoleish® antigen preserved with thimerosal (AgT) or phenol (AgP) and serological techniques to detect a possible infection caused by *Leishmania (Viannia) braziliensis* in dogs. The study included 172 dogs from an area endemic for ATL in the municipality of Paracambi, state of Rio de Janeiro, Brazil. The results obtained with Imunoleish® antigen preserved with thimerosal (AgT) or phenol (AgP) and serological tests were compared. Each dog received, intradermally, 0.1 mL of each antigen on the inner side of the right (AgT) and left (AgP) thighs. Five (2.7%) dogs presented ATL lesions. Of these, two were reactive to both formulations and three were reactive only to AgT. Among the 172 dogs, 68 (39.5%) were reactive only to AgT, 16 (9.3%) only to AgP, and 11 (6.4%) to both formulations. Twenty-one (12.2%) sera samples were reactive by immunofluorescent antibody test (IFAT) and 21 enzyme-linked immunosorbent assay (ELISA). However, in only two dogs out of the five which *Leishmania* was isolated from, serological tests were positive. The LST and serological tests could be a useful tool in the diagnosis of *L. (V.) braziliensis* infection in dogs. Standardization of the techniques and reagents used could allow comparative studies on sensitivity, specificity, and positive and negative predictive values in dogs from different regions.

Keywords: American Tegumentary Leishmaniasis, Leishmanin skin test, Diagnosis, Dogs, Host

RESUMO

A intradermorreação à leishmanina é um teste in vivo de avaliação da reação imune celular contra *Leishmania* e constitui uma importante ferramenta no diagnóstico da Leishmaniose Tegumentar Americana (LTA). Este estudo teve o objetivo de comparar os resultados obtidos através da intradermorreação à leishmanina utilizando Imunoleish® preservado em timerosal ou fenol e técnicas sorológicas para detectar a possibilidade de infecção por *Leishmania (Viannia) braziliensis* em cães. O estudo incluiu 172 cães de área endêmica de LTA no município de Paracambi, Estado do Rio de Janeiro, Brasil. Foram comparados os resultados obtidos com o antígeno Imunoleish® conservado em timerosal (AgT) ou em fenol (AgF) e os resultados dos testes sorológicos. Em cada cão foi injetado, por via intradérmica, 0,1 mL de cada reativo, na face interna da coxa direita (AgT) e na face interna da coxa esquerda (AgF). Cinco (2,7%) cães apresentaram lesões cutâneas de LTA. Destes, 2 reagiram a ambas as formulações e 3 somente ao AgT. Dentre os 172 cães, 68 (39,5%) reagiram apenas ao AgT, 16 (9,3%) ao AgF e 11 (6,4%) a ambos. Vinte e uma amostras de soro (12,2%) foram reativas ao teste de imunofluorescência (IFAT) e 21 ao ELISA. No entanto, em apenas dois cães dos cinco nos quais *Leishmania* foi isolada, os testes sorológicos foram positivos. A intradermorreação com leishmanina e técnicas sorológicas podem ser ferramentas úteis para o diagnóstico da infecção por *L. (V.) braziliensis* em cães. A padronização das técnicas e dos reativos utilizados poderia permitir a realização de estudos comparativos sobre sensibilidade, especificidade e valor preditivo positivo e negativo em diferentes regiões.

Palavras-chave: Leishmaniose Tegumentar Americana, Teste intradérmico, Diagnóstico, Cães, Hospedeiro

Introduction

Brazil is currently one of the largest producers andTegumentary Leishmaniasis (TL) is an infectious disease caused by protozoa of genus *Leishmania* and transmitted by sandfly vectors. According to the World Health Organization (WHO), TL is among the main and most

worrying neglected diseases in the world, with an estimated occurrence of 700,000 to 1 million new cases annually. TL affects mainly the poorer populations and is associated with precarious housing, lack of financial resources (WHO, 2020). Control actions can only be implemented through the use of accurate diagnostic methods.

The leishmanin skin test (LST) is an in vivo test that assesses the cellular immune responses to *Leishmania*-derived antigens, is an important tool in the clinical diagnosis of human TL. The LST started being used in the 1920s (Montenegro, 1926). Because this is a low-cost test that can be easily performed, it was used in the diagnosis of American tegumentary leishmaniasis (ATL) for many years. Although the LST is still included in the Epidemiologic Surveillance Manual of the Brazilian Ministry of Health (2017) as an alternative for the laboratory diagnosis of ATL, production of reagents for human testing was discontinued in 2012, and the last batch produced was distributed in 2015.

The test consists in intradermally injecting 0.1 mL of *Leishmania* antigen (leishmanin) and observing the area after 48 h. The test result is considered positive if induration with diameter >5 mm is observed (Barbosa-Santos et al., 1998; Brasil. Ministério da Saúde, 2017; Marzochi & Barbosa-Santos, 1988). Test reactivity appears within the first weeks of active disease and may continue present for many years after lesion healing. The LST can also be used as an indicator of unapparent infection by *Leishmania* and has been widely used in epidemiological surveys (Brasil. Ministério da Saúde, 2017).

Originally, leishmanin for human use was prepared with a suspension of promastigote forms of a dermotropic *Leishmania* isolate preserved in phenol diluted in saline solution. From the observation of positive skin reactivity to phenol and absence of positive skin reactivity to thimerosal in individuals tested simultaneously with both solutions, thimerosal started being used more often in the preparation of leishmanin (Imperato et al., 1974). However, it was later verified that thimerosal, applied in intradermal tests in humans, is capable of inducing cutaneous delayed-type hypersensitivity (Fagundes et al., 2007; Marzochi et al., 1998). Consequently, the different leishmanin formulations used in humans vary in specificity and sensitivity, thus hindering comparison between different studies. The antigens used are crude solutions, at different concentrations, produced through the sonication of promastigotes of a single species or a pool of different *Leishmania* species preserved with thimerosal or phenol (Fagundes et al., 2007).

In the present study, Imunoleish®, antigen preserved with thimerosal (AgT) or phenol (AgP), and serological tests, were used to detect a possible infection by *Leishmania (Viannia) braziliensis* in dogs from the municipality of Paracambi, state of Rio de Janeiro, Brazil.

Material and methods

Selection of domiciles and dogs

A canine survey was conducted in the homes of patients diagnosed with ATL recorded in the municipality of Paracambi, state of Rio de Janeiro, between 1990 and 2002. Paracambi is a municipality located in the Brazilian state of Rio de Janeiro at the coordinates of 22º 35' 22" South latitude and 43º 40' 43" West latitude. It is located in the western portion of the State of Rio de Janeiro and the Northwest limit of the Metropolitan Region of the capital.

The majority of dogs were not a defined breed and puppies aged <6 months and untamed or difficult-to-contain animals were excluded from the investigation. The participating cases were named index domiciles. For each index domicile, the domicile located immediately to its right was selected, and in case of absence, the one located immediately to its left, or the nearest one, was selected. The domiciles of patients that had moved after the disease were also included in the survey, considering the addresses on the notification forms and their respective adjacent domiciles, as

long as they were inhabited. The domicile owners were informed about the study procedures and, after consent, their dogs were registered in the study.

Sample collection

The dogs were physically contained, examined for skin lesions suggestive of ATL, and submitted to the LST. Animals with skin lesions suggestive of ATL were sedated with an intramuscular injection of ketamine (10 mg/ kg) associated with acepromazine (0,2 mg/kg). After that, they were subjected to local anesthesia with 2% lidocaine for subsequent 4 mm punch skin biopsy of the lesion border. The fragments were preserved in sterile saline solution containing 50μg 5'fluorocytosine, 1000 UI penicillin, and 200μg streptomycin per mL, and then stored at 4 ºC for 24 h. Subsequently, the fragments were transferred aseptically to tubes containing biphasic culture medium (NNN medium plus Schneider's medium supplemented with 10% fetal bovine serum) and stored at 26-28 ºC (Miranda et al., 2019). The fresh culture was observed at weekly intervals for thirty days. The *Leishmania* isolates were characterized by isoenzyme electrophoresis (Miranda et al., 2019).

Skin test

Two batches of Imunoleish®, provided by Bio-Manguinhos-FIOCRUZ, were prepared with the *L. (V.) braziliensis* antigen (MHO/BR/86/DCB-02) containing 200 μg of protein per 0.1 mL of saline solution. One of them was preserved with thimerosal (1:10000) (AgT) whereas the other was conserved with 0.4% phenol (AgP). One tenth mL of each antigen was injected intradermally into the inner surface of the right (AgT) and left (AgP) thighs of each dog. Skin reactivity was measured after 48 h according to the Sokal (1975) method. The test result was considered positive if a local induration with diameter >5 mm was observed (Barbosa-Santos et al., 1998; Brasil. Ministério da Saúde, 2017; Marzochi & Barbosa-Santos, 1988).

Serological diagnosis

After alcohol (70%) local asepsis, 5 mL of peripheral blood was collected for serological tests. The immunofluorescent antibody test (IFAT) was performed using *L. major* antigen and anti-dog IgG conjugate (Biomanguinhos/FIOCRUZ, Rio de Janeiro, Brazil). Titers ≥1:40 were considered positive. The enzyme-linked immunosorbent assay (ELISA) was performed using *L. major*-like antigen. Reactivity was defined as readings higher than the average of the absorbances of the normal sera plus two standard sera (Voller et al., 1976).

Statistical analysis

The means of skin reactivity to AgT- and AgP-preserved Imunoleish® antigen were compared using the Student's t-test. A significance level of 5% ($p \leq 0.05$) was adopted for all statistical analyses.

Ethical statement

The means of skin reactivity to AgT- and AgP-presThis study was approved by the Committee on Ethics in the Use of Animals (CEUA/ FIOCRUZ) under protocol no. P0158-03.

Results

A total of 203 dogs, 115 males and 88 females an age range of seven months to ten years, were found in 74 (85%) of the 87 surveyed domiciles. Thirty-one (15.3%) dogs were excluded from the investigation: 18 (8.9%) for being puppies aged <6 months and 13 (6.4%) for being untamed or difficult-to-contain animals, or for having died before the survey was conducted. The remaining 172 (84.7%) dogs were included in the study.

The 172 dogs included in the survey were tested with both conserved antigen (AgT and AgP) and 95 were reactive: 68 (39.5%) only to AgT, 16 (9.3%) only to AgP and 11 (6.4%) to both formulations (AgT and AgP). Ten of the 172 dogs

assessed presented ulcerative lesions suggestive of ATL, located on the genitalia, snout and ears. *Leishmania* was isolated in culture in five (2.9%) of the dogs, but in only one was identified the species, *L. (V.) braziliensis*, most comum *Leishmania* species in Rio de Janeiro (Miranda et al., 2019). Of these, two animals were reactive to both formulations (AgT and AgP) and three were reactive only to AgT.

In the five dogs with *Leishmania* isolates, the means of skin reactivity to AgT were positive for >12 mm induration. In the remaining reactive dogs without ATL lesions, no statistically significant difference was observed between the means of skin reactivity to AgT (9.29 mm) and AgP (9.27 mm). In the dogs without ATL lesions reactive to both AgT and AgP, statistically significant difference was also not observed between the means of skin reactivity, 9.9 mm and 9.2 mm induration, respectively.

Twenty-one of sera samples (12.2%) were reactive by IFAT and ELISA techniques, with six being positive in both tests simultaneously. In the five dogs with *Leishmania* isolates two were positive IFAT and ELISA tests and three were negative by serological tests.

Discussion

Amastigote forms of *Leishmania* have been described in tissues of dogs and other animal species in endemic areas for *L. (V.) braziliensis* (Ferreira et al., 2015; Sasani et al., 2016; Marquez et al., 2017; Lago et al., 2019). The same leishmanin used for diagnosis in humans was used, without success, in the diagnosis of ATL in dogs from endemic areas of the city of Rio de Janeiro (Pirmez et al., 1988), suggesting the need for a more suitable leishmanin for use in this species (Marzochi & Barbosa-Santos, 1988). In this study, skin tests were performed using Imunoleish®, a reagent developed and used for the diagnosis of ATL in dogs (Marzochi & Barbosa-Santos, 1988).

In this study, Imunoleish® was preserved in thimerosal or phenol to compare the effects of these preservatives on test results. Among the animals, 39.5% were reactive only to AgT and 9.3% were reactive only to AgP. In these dogs, there was no statistically significant difference between the means of skin reactivity to both AgT and AgP. Other studies conducted with dogs in southeastern Brazil using thimerosal-preserved antigens have reported high positivity rates, ranging from 10 to 66.7% (Marzochi & Barbosa-Santos, 1988; Paranhos-Silva et al., 2001; Fagundes et al., 2007). A study in an area endemic for visceral leishmaniasis in the state of Bahia, Brazil, found positive skin reactivity in 19.6% of the 56 dogs tested with a 1:10000 thimerosal solution and suggested that this preservative could lead to a larger number of false positive results in this species (Paranhos-Silva et al., 2001).

Another study conducted with humans using leishmanin preserved with thimerosal demonstrated that reading of the standard skin test did not discriminate a result positive for leishmanin from reactivity to thimerosal, indicating the possibility of false positive test results when thimerosal was used as preservative (Fagundes et al., 2007).

Other authors have reported positive reactions to *Leishmania*-derived antigens preserved with phenol in dogs and humans (Weigle et al., 1991; Solano-Gallego et al., 2001). Some authors found positive reactivity to 19% phenol solution in 37 dogs with positive skin reactivity to leishmanin. Those authors highlighted that phenol is used to inactivate *Leishmania* promastigotes in the preparation of different leishmanin formulations and that it could promote delayed-type hypersensitivity response (Pineda et al., 2001).

The large number of positive serological tests in asymptomatic animals suggests that prevalence of infection in the canine species can be underestimated. In this study, only 2.9% of the dogs presented ulcerative lesions with *Leishmania* isolated in culture but 12.2% from sera samples were reactive by IFAT and ELISA techniques. However, in only two dogs out of the five which *Leishmania* was isolated from, IFAT

and ELISA tests were positive and in another three animals serological tests were negative. The use of serological tests of dogs is limited due to the possibility of cross-reactivity. Studies described *Trypanosoma caninum* from seroreactive dogs in *Leishmania* suspected cases (Barros et al., 2012; Madeira et al., 2009; Pinto et al., 2010).

Similarly to what occurs in humans, skin reactivity using leishmanin could be a useful tool in the diagnosis of infection by *L. (V.) braziliensis* in dogs. However, the resulted standardization of the technique and reagents used could allow comparative studies on sensitivity, specificity, and positive and negative predictive values in different regions. In addition, serological tests can also represent an important tool for the diagnosis of ATL in dogs, together with the intradermal test, but it is important to rule out the possibility of cross-reaction with other agents..

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Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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