

LEISHMANIASIS CONTROL STRATEGIES

A CRITICAL EVALUATION OF
IDRC-SUPPORTED RESEARCH

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Leishmaniasis control strategies

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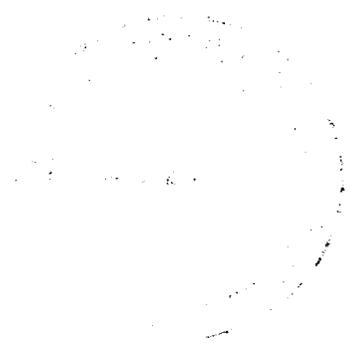
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Immunoenzymatic Method Dot-ELISA in the Detection of Potential Reservoir Hosts of Leishmaniasis in Peruvian Rural Endemic Regions

By

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Introduction

To succeed in the control of leishmaniasis, the study and analysis of the vector, parasite and reservoir host must be done. Since all the New World Leishmaniasis are zoonosis, to better understand and assess the control of this disease, serological studies of domestic and wild potential reservoir hosts, in endemic regions are essential.

Diagnostic methods have been applied in leishmaniasis research but some methods which are appropriate for reference laboratories tend not to be appropriate for use in rural areas, where the disease is endemic. While serodiagnostic methods similar to those used in human leishmaniasis can be applied to the study of wild and domestic potential reservoirs, difficulties of conducting serodiagnosis in the field impairs such studies. There is a need to find an adequate and reliable method to work with.

Work has not been done at the serological level in reservoir hosts in Peruvian endemic regions. To know the existing condition in potential reservoir hosts, and applying an improved low costimmunoenzymatic method, Dot-ELISA, we carried out a survey among dogs living in endemic regions with this method.

Since 1913 when Pedroso documented the first infected dog in Peru many other experimental studies, at the parasitological level, have been undertaken in endemic regions. Opossums, rats, foxes, edentates, armadillos, horses and dogs have been studied to isolate of parasites. Attempts to detect infected animals, as evidence of active peridomestic transmission, have also been conducted. Despite the complexity of the risk of infection and the evidence of the parasite, the serological study of reservoir hosts in endemic regions can complement parasitological studies, giving an initial indication of the situation in situ.

Serodiagnosis

Serology is widely used in the diagnosis and research of human leishmaniasis, and to detect antibodies, in vitro, against the parasite. There are several available methods, varying in complexity and cost: Immunofluorescence Antibodies Test, IFAT; Complement Fixation Test, CFT; Immunoenzymatic Assays, ELISA; Direct Agglutination Test, DAT. The specificity and sensitivity of the methods depends on several factors such as antibody

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concentration in sera-severity of the disease, nutritional status of the host and the utilization of specific, partially purified or characterized leishmanial antigens.

It is known that improved serological methods would help in studying the risk factors and reservoir epidemiology. Applying some of these methods to perform a survey detecting antibodies against leishmania parasites in dog's sera would be useful in the search of potential reservoir hosts in places where these animals may play an important role in the dissemination of the disease.

There are simple and sophisticated methods. However there are difficulties to be solved first before the survey starts: the stability of reagents, the high cost of the laboratory equipment and the lack of well trained workers. At our laboratory, we had improved a low-cost, reliable, versatile immunoenzymatic method to detect antibodies against leishmania to be used in the field.

Diagnosis at Field Level

Working in serodiagnosis in the rural areas, one faces unpredictable technical and physical difficulties. Therefore, to achieve ones goal, one should be able to solve all of them at once.

In many Peruvian rural endemic areas, facilities to undertake such work are difficult: very bad roads, no power supply or distilled water. Variable climatic conditions are an additional stress with very hot, high humidity in the jungle and cold and dry conditions in the Andean region. Working with delicate reagents, antigens, conjugates and immunologicals under these difficult conditions, requires extreme care.

Faced with these obstacles, and trying, in some way, to understand the risk of infection and the dissemination of leishmaniasis, we improved an immunoenzymatic method, originally reported by Pappas in 1984 to detect antibodies against *Leishmania* parasites in sera.

The antigen: A culture of *Leishmania braziliensis braziliensis* (MHOM/BR/5/M2903) growing in bifasic medium was prepared according to Romero et al (1987). The parasites were washed three times in sterile phosphate buffer saline (pH 7.4) and brought to a concentration of 5×10^8 /ml. The parasites were dotted onto nitrocellulose filter paper disks of 5 mm in diameter and put into microtiter plates of 96 flat bottom wells. The microtiter plates, containing the disks with the fixed parasites, were carefully kept, sealed in plastic bag under vacuum with silica gel, and stored at -20°C until use. The antigen, kept in this condition, under normal laboratory conditions, was recognized by its corresponding antibody without losing reactivity for up to one year.

Sera and conjugate: The sera collected from the individuals in the field were mixed with sodium azide 0.01 % and then stored in the dark until use. When possible the samples were kept at -20°C with a small difference in the final chromogenic reaction. For each assay, positive and negative control sera were used. The conjugate used in this assay was anti-human IgG alkaline phosphatase purchased from SIGMA. The conjugate was dispensed in small, "ready to use" plastic vials.

Dot-ELISA Procedure: The disks dotted with the antigen, were treated with HCl 0.1 N for 30 minutes and washed twice with phosphate buffered saline (PBS pH 7.2). The disks were blocked with PBS-Tween-20 0.05 % containing 8% non-fat milk and incubated for 45 minutes at environment temperature (ET). Disks were washed three times with PBS for 10 minutes. Serum samples at several dilutions were prepared using PBS-Tween-20 containing 2% non-fat milk and incubated for 45 minutes at ET. The microtiter plate containing disks were washed with PBS, and the conjugated anti-human Ig G at 1/800 dilution was added.

The microplate was incubated for 45 minutes at ET, washed three times and incubated for one minute only with NaCl 1.16 M. The plates had a final wash three times with PBS-Tween-20. The substrate was prepared using 4.8 mgs of nitro blue Tetrazolium and 2.4 mgs of bromo-chloro-indolil phosphate, dissolved in 100 µl of N-N dimethyl formamide, and brought to 10 ml of alkaline phosphate buffer (pH 9.5).

By washing the microplates with distilled water the enzymatic reaction was stopped. The positive samples showed a dark blue spot on the white paper, and the negative samples showed no visible colour.

Results and Discussion

Ninety sera samples from dogs were collected by the field team lead by Dr. Alejandro Llanos from Huayllacullan Valley in the midst of the country where leishmaniasis is endemic. Twenty-five percent of the collected sera were positive by the Dot-ELISA. Our positive working dilution, after filtration with the positive and negative control, was 1/100. This dilution was used as a limit to distinguish the positive from the negative sera. However, this dilution could be replaced by the next immediate dilution (1/200) to sharply distinguish the samples with high antibodies concentration and diminish the chance of cross reaction. At this dilution the variation between the positive sera was not significant because 23.3% of the samples with this dilution were also positive.

We should mention that the feces of dogs in this study were not examined for parasites or other infectious agents. Because antibodies against worms and other parasites can interfere, giving cross reaction with leishmania (Luis Guevara unpublished results) this may affect the real status of infection.

To avoid the increase of false positives, we are in process of searching for specific antigens to diminish or eliminate the risk of having high percentage of positive samples. Preliminary results obtained in collaboration with Dr. Angel Hernandez in Caracas show the strong possibility of having a glycoprotein with molecular weight around 50-52 Kd to use in the diagnosis and recognition of an infected individual.

Since the risk of human infection cases by leishmania parasites could increase, as long seropositive cases of infected dogs in endemic regions exists, the control of the reservoir hosts should be pursued. To reduce the risk of transmission of leishmaniasis by wild animals, the majority of the potential reservoir hosts should be tested. In the Amazonian region this should include primates, opossums, armadillos, rats, etc.

Having a versatile and low cost (each sample costs \$0.20 USD) immunoenzymatic method, the study of prevention and control of leishmaniasis looks promising. This serodiagnostic tool for use in the field work is ready at our laboratory in a kit design and should be available for the peripheral field worker soon. It must be emphasized that these diagnostic testing kits should be taken where the disease is, and it can be applied to other diseases.

This very preliminary and first report in Peruvian endemic regions shows a relatively high percentage of positive dogs living in endemic areas. These results demonstrate the importance of developing diagnostic tests for the study, prevention and control of the leishmaniasis in Peruvian endemic regions.

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