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AN IMMUNOHISTOCHEMIC ASSAY TO LOCALIZE LEPTOSPIRES IN TISSUE SPECIMENS

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SUMMARY

An Immunoperoxidase technique for identification of leptospires in formalin fixed, paraffin embedded kidney sections is presented, using peroxidase-antiperoxidase complex. The anti-leptospiral antibody was raised in rabbit. Possible applications of this technique are discussed.

KEY WORDS: Leptospirosis — Immunodiagnosis — Immunohistochemistry — Kidney Sections.

INTRODUCTION

Leptospirosis, a spirochetal infection of zoonotic origin, is most prevalent in the humid tropics [2]. While in North Hemisphere countries this disease is included as an occupacional risk [3], in Brasil its occurrence is endemic or in epidemic bouts during raining periods [3,5].

The detection of leptospires in tissue specimens through the usual silver impregnation techniques has disadvantages such as low sensitivity and impregnation of structures other than the microorganisms.

The purpose of this study is the development and testing of an immunoperoxidase technique, designed to obtain a highly specific and sensitive identification of **Leptospira interrogans** serovar **icterohaemorrhagiae.**

MATERIAL AND METHODS

Preparation of rabbit antiserum:

The antigen (**Leptospira interrogans,** serovar **icterohaemorrhagiae** strain R 192) was obtained from 7 to 10 day cultures at 28°C in semi-solid Fletcher medium (Difco) enriched with 10% normal rabbit serum. The antigen was previously examined to assure absence of contamination.

The antiserum was prepared by hyperimmunising adult rabbits with sequential doses of 1,2,4 and 4ml of antigen, at weekly intervals. Seven days after the last inoculation, a large blood sample was drawn. The microscopic agglutination test revealed a 1:3200 titer.

In order to avoid false-positive results from avidity of other components of the serum to kidney structures, 1ml of the anti-serum was absorbed with 100mg of guinea-pig kidney powder overnight, at a temperature of 4°C. Afterwards, the serum was centrifuged at 3.000 r.p.m. and supernatant was utilised as the primary antibody. In this experiment the dilutions tested were 1/40, 1/80, 1/160 and 1/240.

Tissue specimens examined:

A preliminary test experiment, inoculating guinea-pigs weighing 180-200 g with a culture of **L. interrogans** serovar **icterohaemorrhagiae** was

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run in order to enhance virulence. Afterwards, a guinea-pig of similar weight was inoculated, by intraperitoneal route, with 2 ml of blood of a previously infected animal.

Five days after this inoculation, when the concentration of leptospires in kidney tissue was significantly enhanced, the guiea-pig was sacrificed and autopsy was immediately performed. Kidney sections 3 mm-thick were routinely fixed in 10% neutral-buffered formalin during 24 hours, followed by dehydration in alcohols, clearing in xylene, embedded paraffin and cut at 4-μ- thick.

Immunoperoxidase test:

The method was modified from the original Sternberger report[3], following some technical suggestions from Ellis et al.[6] in their study on leptospires in porcine kidneys. The rabbit antiserum to leptospire prepared in our laboratories was used to label the spirochetae in tissue. Linking antibody was swine anti-rabbit IgG (Dako), used in excess, and the enzyme-label was peroxidase-antiperoxidase complex (Dako). The technique is presented in detail in Table I.

TABLE I
Immunoperoxidase staining technique to localise leptospires in fixed tissues

1. Remove paraffin from tissue sections (xylene 2 x 10 min, ethanol absolute, 95%, 80% and 50%, 3 min each).
2. Remove formalin pigment (10% NH_4OH in 95% alcohol for 10 min).
3. Destroy tissue peroxidases: (3% H_2O_2 in absolute methanol for 20 min at room temperature).
4. Wash sections in buffer (PBS 0,01M pH: 7,4, 3 x 5 min).
5. Non-specific receptor blocking (cover sections with normal swine serum for 15 min, wipping off the excess).
6. Apply rabbit anti-leptospire antiserum during 30 min in moist chamber, at room temperature.
7. Wash 3 x 5 min with buffer
8. Apply swine anti-rabbit immunoglobulin G* 1/30 dilution for 30 min in moist chamber, at room temperature.
9. Wash 3 x 5 min with buffer.
10. Apply peroxidase-antiperoxidase complex (PAP)* 1/100 dilution for 30 min in moist chamber at room temperature.
11. Wash 3 x 5 min with buffer.
12. Apply made up substrate for 3 minutes under microscope control (100 ml PBS 0,01M, pH: 7,4; 15 mg 3,3 — Diaminobenzidine +; 0,6 ml 6% H_2O_2).
13. Wash thoroughly in running water to stop reaction.
14. Counterstain with Mayer's haematoxylin for 5 min.
15. Blue in running water for 10 min.
16. Dehydrate, clear and mount.

* Dako Corp., U.S.A.
\+ Sigma Co., U.S.A.

The semi-quantitative microscopic analysis of staining intensity was reported as follows: |—|: absent; + light; ++ moderate; +++ intense.

As controls, one section from the same infected guinea-pig kidney was submitted to the whole procedure, except for the ommission of the primary antibody incubation; another negative control included the whole reaction in a section of a normal guinea-pig kidney section.

RESULTS

Since diamiobenzidine was used as chromogen substrate, leptospires were stained in granular brown colour, in a clear contrast to the blue haematoxylin counterstaining of the tissue structures (Fig. 1). Leptospires were localized, preferentially, lining renal tubules or

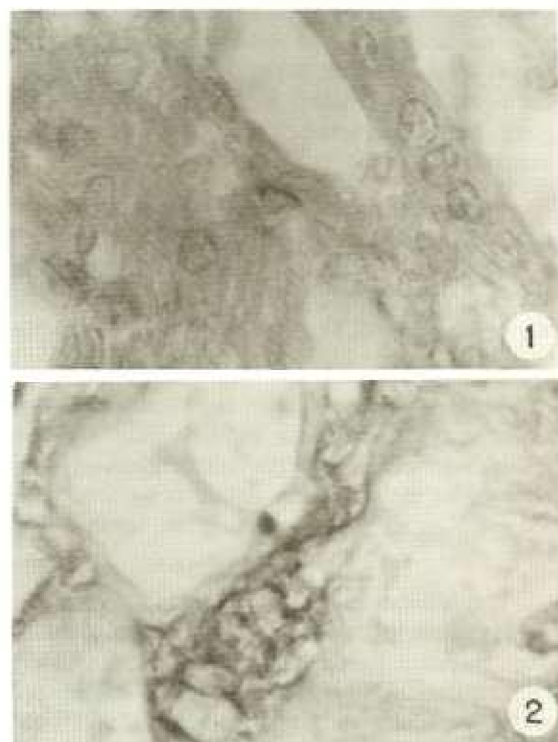

Fig. 1 — Immunoperoxidase stained kidney section. Leptospires are seen in edematous interstitium adjacent to renal tubules (PAP counterstained with haematoxylin, x 400).
Fig. 2 — Detail from another kidney section. Leptospires form a tightly tangled mass around renal tubules (PAP counterstained with haematoxylin, x 1.000).

free in their lumina, forming groups around small vessels and, sometimes, dispersed through the edematous interstitium.

Comparative results of the different dilutions of the primary antibody are presented at Table II.

TABLE II

Dilution	Intensity of positive specific staining	Intensity of back ground staining	Contrast achieved
1/40	+++	++	+
1/80	+++	+	++
1/160	++/+++	—	++/+++
1/240	++	—	++
Negative controls:			
primary antibody ommitted	—	—	—
Normal kidney section	—	—	—

(—: negative; +: light; ++: moderate; +++: intense)

DISCUSSION

This immunoperoxidase staining technique has been shown to be an accurate procedure applicable to formalin-fixed and paraffin embedded tissue specimens. The primary anti-leptospiral antibody was raised following conventional schemes of rabbit hyperimmunisation and its specificity could be demonstrated by the fully negative results obtained with negative controls.

Comparison of the several dilutions shows that, although the most concentrated antibody solutions (1/40 and 1/80) were associated with strong staining of leptospires, background was also high and, so, the contrast was not intense.

On the other hand, the most diluted solution (1/240) presented no background but leptospiral staining was rather weak. The best result was achieved with 1/160 dilution, since leptospiral staining was still intense and background was minimal, yielding an intense contrast.

The aplication of this technique to localize leptospires and its antigen fractions in formalin fixed paraffin embedded tissues will be, in the near future, an important tool for the diagnosis of leptospirosis in human biopsies and autopsies submitted routinely to histopathology. Its introduction in official laboratories will enable public health authorities to identify the real prevalence and lethality of this disease, endemic in Brasil and in several other countries. It will also contribute in clearing out some doubts that persist on pathogenetic pathways, especially regarding the real role of a toxin or an immunopathological reaction as the main mechanism in leptospiral lesions in the kidneys [1,2,4,7,9] and also in the recently discovered cardiac manifestations (T. De Brito, unpublished data).

RESUMO

Método Imunohistoquímico para localização de leptospiras em amostras teciduais.

Os autores apresentam uma técnica de imunoperoxidase para a identificação de leptospiras em cortes de rim fixados em formol e emblocados em parafina. A variante metodológica inclui a utilização do complexo peroxidase-antiperoxidase, conferindo alta sensibilidade à reação. São, ainda, discutidas algumas possíveis aplicações do método ao estudo do acometimento renal na leptospirose.

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