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# Immunoperoxidase method of identification of *Leishmania* in routinely prepared histological sections

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**Summary.** An indirect immunoperoxidase technique was applied for identification of *Leishmania* in routinely prepared histological sections.

Paraffin embedded, formalin-fixed slide preparations of skin (1 case), bone marrow (2 cases) and lymph node (1 case) were examined. The tissues were obtained from one patient with cutaneous leishmaniasis and from three patients with visceral leishmaniasis. Specific rabbit antisera against *L. donovani, L. tropica* and *L. mexicana* were used as primary reagents. Positive controls were performed simultaneously and included *L. tropica* cultures in blood-agar (NNN media) – free promastigotes and amastigotes within macrophages.

Strongly positive brown staining was localized specifically in Leishman-Donovan (LD) bodies only. This method increases the probability of microscopic diagnosis of leishmaniasis and helps to prevent confusion of *Leishmania* with other infective agents in histological sections.

**Key words:** Immunoperoxidase – Leishmania

## Introduction

Identification of *Leishmania* in histological sections by traditional stain is sometimes difficult by light microscopy (Abdalla 1980; Latif et al. 1979; Matzner et al. 1979; Singer et al. 1975) and immunological identification by immunofluorescence demands the use of costly equipment (Taylor 1978; Wick et al. 1978).

We have tried to apply a relatively simple, less expensive and highly specific technique of indirect immunoperoxidase staining (Taylor 1978) for visualization and immunologic identification of *Leishmania* in routinely prepared histological sections, in cases of cutaneous and visceral leishmaniasis.

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## Materials and methods

The indirect immunoperoxidase method was used in the present study. Rabbit antibodies to *L. donovani, L. tropica,* and *L. mexicana* were used in the primary reaction.

*Preparation of rabbit antibodies to leishmania.* Rabbits were inoculated six times at five day intervals by either *L. donovani, L. tropica,* or *L. mexicana* using living promastigotes. Several days after the last inoculation the blood samples were obtained and antibody titer against Leishmania Excreted Factor (LEF) (El-On et al. 1979) was measured by means of immunodiffusion. Antisera were obtained from rabbits showing highest antibody titers against *L. donovani, L. tropica,* and *L. mexicana.*

*Light microscopic immunoperoxidase method.* A single skin biopsy specimen, taken during the acute stage of skin leishmaniasis in 1 patient, was fixed in 10% formalin, embedded in paraffin blocks, and stored at room temperature. Two bone marrow biopsy specimens and one lymph node biopsy specimen from three patients with visceral leishmaniasis were fixed, prepared and stored as described above.

Sections, 6 μ in thickness, were treated with xylene to remove paraffin. The endogenous peroxidase was blocked by methanol-hydrogen peroxide solution, applied for 30 min. Non-specific background staining was reduced by incubation with 0.05 ml of diluted 1:10 swine normal serum for ten minutes.

The sections were then incubated with 1:10 solution (in phosphate-buffered saline – PBS) of rabbit antibody to *L. donovani, L. tropica,* and *L. mexicana* on consequent preparations, washed thoroughly in PBS and reacted for thirty minutes with anti-rabbit immunoglobulin IgG peroxidase conjugated, produced in swine (dilution 1:40 in PBS).

The sections were washed in PBS and were reacted in 3,3′-diaminobenzidine (DAB)-hydrogen peroxide solution for five minutes to visualize the peroxidase reaction. The DAB-hydrogen peroxide solution was prepared just before use by dissolving 0.25 mg of 3,3′-diaminobenzidine tetrachloride II (Sigma) in 50 cc of PBS and adding 0.25 ml of 30% solution of hydrogen peroxide.

*Controls.* As a control, 1:20 solution of normal rabbit serum was used instead of primary antibodies. Glass slides with neutral formalin fixed preparations of *L. donovani* promastigotes cultured in NNN media as well as *L. tropica* amastigotes within mice macrophages were used as a positive control instead of tissues, presumably containing *Leishmania* antigen.

## Results

Light microscopic examination easily revealed characteristic dark brown peroxidase staining which was limited to LD bodies only in the macrophages of the skin (one case; Fig. 1), bone marrow (2 cases; Fig. 2) and lymph node (1 case; Fig. 3).

There was no background staining. No staining was observed in the negative control series prepared simultaneously to each experiment using normal rabbit serum. The positive control series of infected mice macrophages and free promastigotes dispersed in NNN media were strongly positive with *L. donovani, L. tropica,* and *L. mexicana* rabbit antisera respectively (Fig. 4). The same preparations were negative with normal rabbit serum, used instead of primary antibodies. It was difficult to determine which strain of *Leishmania* reacted most intensely with either *L. donovani, L. tropica* or *L. mexicana* antisera.

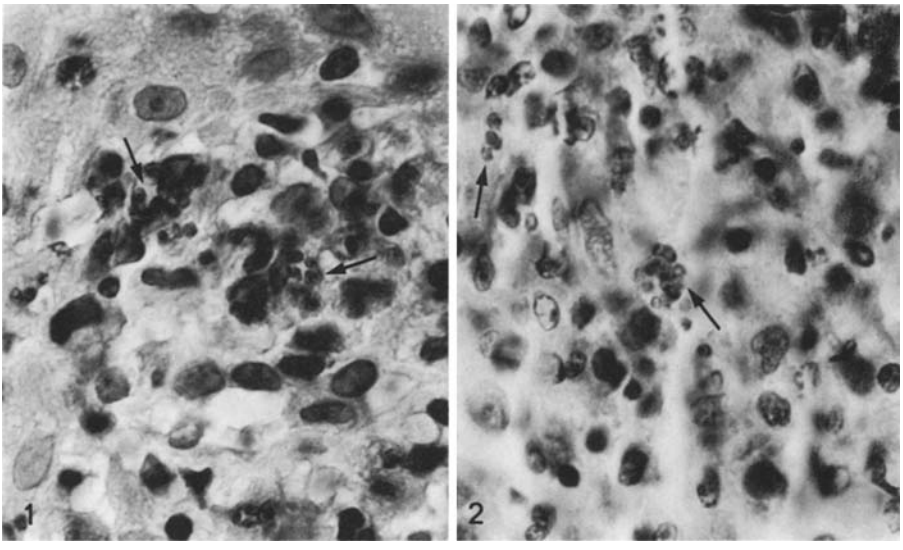


**Fig. 1.** Cutaneous leishmaniasis. LD bodies within macrophages of the dermis *(arrows)*. (Immunoperoxidase staining. Counterstain – haematoxylin × 1,050)

**Fig. 2.** Visceral leishmaniasis. LD bodies within histiocytes of the bone marrow *(arrows)*. (Immunoperoxidase staining. Counterstain – haematoxylin × 1,050)

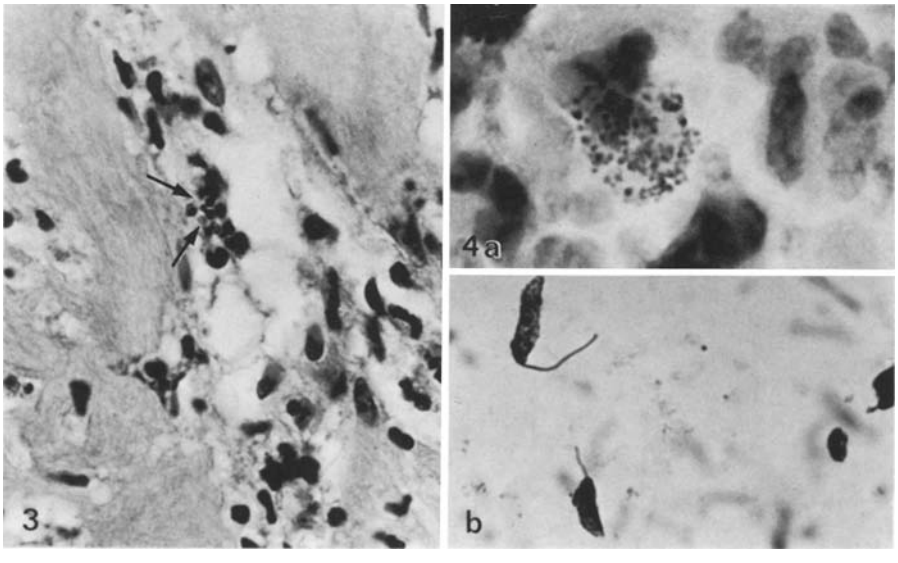


**Fig. 3.** Visceral leishmaniasis. LD bodies within macrophages of the lymph node *(arrows)*. (Immunoperoxidase staining. Counterstain – haematoxylin × 800)

**Fig. 4A–B.** Positive Control **A** *L. tropica* amastigotes within mice macrophages *(arrows)*. **B** *L. donovani* promastigotes in NNN media. (Immunoperoxidase staining. Counterstain – haematoxylin × 1,050)

## Discussion

It is well known to pathologists that histological diagnosis of leishmaniasis in tissues may be complicated by difficulties of visualization of parasites by accepted methods (Abdalla 1980; Latif et al. 1979; Matzner et al. 1979; Singer et al. 1975). There are reports of failures in histological diagnosis (Abdalla 1980; Matzner et al. 1979), due to those difficulties, which have led to clinical misdiagnosis of leishmaniasis and grave consequences to patients (Veress et al. 1974). This may be especially important in the cases of visceral leishmaniasis, which not infrequently mimicks other diseases (Daneshbod 1978; Matzner et al. 1979; Naik et al. 1979).

Immunofluorescence methods for visualization of antigens is costly due to the need for a specialized microscope. Pitfalls of this method include poor visualization of histological structures and the need for special conditions for storage of specimens (Taylor 1978; Wick et al. 1978).

The immunoperoxidase method presented is simple, does not need special equipment, is highly sensitive and specific to *Leishmania* species. Characteristic brown peroxidase staining contrasts perfectly with blueish counterstain by haematoxylin, making visualization in the light microscope a quick and decisive process. Another advantage may be in avoiding confusion with other infective agents, which have no common antigenic determinants with *Leishmania* species. This may be especially important in the differentiation from *Toxoplasma* and *Histoplasma capsulatum* which may be misdiagnosed histologically as *Leishmania* (Chatak et al. 1970; Daneshbod 1978).

Since Leishmaniata are antigenically closely related, there is cross immunity (Behforouz et al. 1976, Bray and Lainson 1965, Latif et al. 1979) which does not permit immunological differentiation between various *Leishmania* species in tissues. However, because of the advantages noted above we recommend the method described here for routine use in immunohistological diagnosis of leishmaniasis. It may be especially important in the countries where leishmaniasis is a major problem.

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