Identification and Characterization of *Leishmania spp.* in Impression Smears of Patients with Cutaneous Leishmaniasis

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ABSTRACT

Background: Monoclonal antibodies have been employed extensively for the identification of *Leishmania* species, development of diagnostic tests and in the characterization of defined leishmanial antigens. **Objectives:** Identification and characterization of *Leishmania spp*. directly from cutaneous lesions of infected individuals. **Methods:** An immunoperoxidase test (Avidin-Biotin technique) using monoclonal antibodies was used for this purpose. One hundred and fifty individuals referring to Dermatology Clinic or Parasitology and Mycology Department of Shiraz University of Medical Sciences were chosen of whom a total of 28 individuals whose smears showed a large number of amastigotes after staining with Giemsa were included in this study. Five monoclonal antibodies designated: D2 (against *L. donovani*), A11 and T10 (against *L. tropica*), T1 (against *L. major*) and T7 (against *L. tropica* and *L. major*) were used. Amastigotes were identified by Labeled Avidin Biotin (LAB) method. **Results:** LAB method for identification of amastigotes in impression smears of patient lesions showed that 20 out of 28 cases (71%) were positive. Among these 12 (60%) and 7(35%) were identified as *L. tropica* and *L. major* respectively. **Conclusion:** The results showed that immunoperoxidase is suitable for *in situ* identification and characterization of *Leishmania spp*. at the species level.

Keywords: Immunoperoxidase, Leishmania, Monoclonal antibodies

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INTRODUCTION

Diverse clinical manifestations of leishmaniasis have been reported from different parts of Iran. The cutaneous forms vary from a simple nodule to non-healing disfiguring lupoid forms (1, 2). Complete visceralization or involvement of lymph nodes without any other clinical signs or symptoms is also present (3, 4). Detection of *Leishmania* amastigotes in host tissue has relied upon staining of impression smears (e.g. with Giemsa's stain) or on culture and biopsied tissue. Where the tissue impression smear or lesion aspirate contains reasonable numbers of parasites, Giemsa staining has provided the best and simplest diagnostic method. The appearance of promastigotes after culture of biopsy tissue on NNN medium also provides an unequivocal diagnosis of leishmaniasis. However, many patients with cutaneous leishmaniasis are slow to present to the clinic, by that time the lesions may be several months old and harbor few parasites (5, 6).
A rapid, sensitive and specific technique which could allow unequivocal identification of parasites is needed, especially when they are present in small numbers in the lesions.
Characterization of *Leishmania* appears to be necessary in many cases to treat the patients or not. As treatment of cutaneous leishmaniasis is usually a lengthy, painful and expensive procedure. Monoclonal antibodies have been employed extensively for the identification of *Leishmania* species, development of diagnostic tests and in the characterization of defined leishmanial antigens (7).
In the present investigation, with the use of monoclonal antibodies, attempts were made to characterize the organisms from patients with cutaneous leishmaniasis from various parts of Fars province, Iran. For this purpose an indirect sandwich procedure using monoclonal antibodies and peroxidase labeled antibodies on amastgote from impression smears of patients were performed.

MATERIALS AND METHODS

Source of organisms and preparation of antigen for immunoperoxidase test. Patients clinically suspected to cutaneous leishmaniasis were referred by dermatologists to the relevant laboratories in Shiraz for diagnosis. Slit-skin technique was used for obtaining the sample from patients, which was spread on a pre-cleaned slide. Three slides were prepared from each patient and in each slide two smears were provided. One slide was used for Giemsa staining, and two for immunoperoxidase test. Negative controls were also performed in each set of experiment using normal mouse sera.

Reference strains. The following reference strains were obtained from Dr David Evans, WHO International *Leishmania* Reference Center, at the London School of Hygiene and Tropical Medicine: *Leishmania major* (MHOM/SU/73/5ASKH), *Leishmania tropica* (MHOM/SU/74/K27), *Leishmania infantum* (MHOM/SU/TN/80/IPT1), and *Leishmania donovani* (MHOM/IN/80/DD8). These organisms were maintained in the laboratory by cryopreservation.

Monoclonal antibodies (mAb). The following antibodies were kindly provided by Special Program for Research and Training in Tropical Disease (TDR), WHO: LXXVIII-2E5- A8 (D2) specific for *L. Donovani*/*L. infantum*, IS2-2B4 (A11) specific for *L. tropica*, XCIV-H2- AB (T10) specific for *L. tropica*, and XLVI-5B8- B3 (T1) specific for *L. major*, and T7 reactive to both *L. major* and *L. tropica* was used as a positive control. All these mAbs were provided in the form of lyophilized mouse ascites fluids. A 1:50 dilution of mAbs as well as Biotinylated Rabbit anti-mouse immunoglobulins was used in the present investigation. A 1:1000 dilution of pooled normal mouse serum was used as a negative control.

Immunoperoxidase test. *Leishmania* parasites (amastigotes) were characterized by Labeled Avidin Biotin (LAB) method. For test on amastigotes the prepared slides were taken out of the freezer, thawed and the following procedure used: The slides were fixed in cold aceton for ten minutes followed by application of H_2O_2/Methanol 0.6% for ten minutes. Mouse anti-*Leishmania* antibody was used for 120 minutes and then PBS washed the slides for 20 minutes. Biotinylated rabbit anti-mouse immunoglobulins were used on slides for 60 minutes followed by two successive washing in PBS for 20 and 60 minutes. In the next step peroxidase -conjugated avidin were applied for 60 minutes followed by another washing in PBS for 20 minutes. Diaminobenzidin- H_2O_2 reaction (1 ml DAB+ 7 µl H_2O_2 %30) was done on slides for 10-15 minutes followed by washing in distilled water for 5-10 minutes. The slides then were counter stained with Haematoxyline-Eosin (H & E) and in the last step were mounted with Canada Balsam. These slides were then examined under the light microscope. The positive reaction defined as a dark brown color in amastigotes. However, no color was observed in negative controls.

RESULTS

One hundred fifty patients were included in this study of whom a total of

28 individuals whose direct smears using Giemsa stain showed a large number of amastigotes were selected for the immunoperoxidase test. The age range of the patients varied from infants to adults and the number of

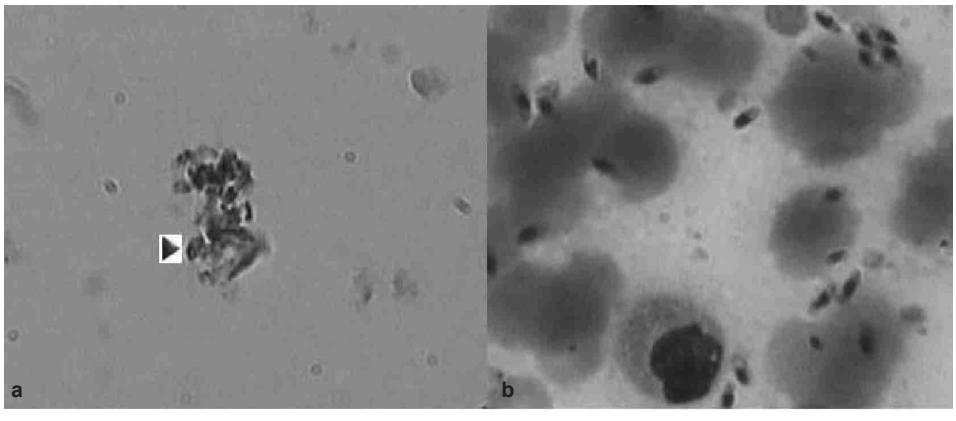

Figure 1. Leishmania amastigotes in impression smear: a) Avidin-Biotin immunoperoxidase staining (1250X). b) Giemsa staining (1250X).

lesions was ranging from 1 to 3. Lesions were on the face, hands and legs. The lesions on the face were mainly on the cheeks, nose, chin and eyelids. *In situ* characterization of cutaneous *Leishmania* in slit sections of 28 patients showed that a total of 20 out of 28 reacted with different mAbs (Figure 1a). Giemsa staining preparation of *in situ* smears presented in Figure1b. However 8 specimens showed no rection with any mAbs used.

TABLE 1. Results of immunoperoxidase test on the isolated amastigotes using different monoclonal antibodies.

	Monoclonal antibodies				
	L. major (T1)	**L. tropica (A11)**	**L. infantum (D2)**	**Positive control T7**	**PBS**
Patients no.					
Case 1 to 12	-	+	-	+	-
Case 13 to 20	+	-	-	+	-
Case 21 to 28	-	-	-	-	-
Total	12	8	0	20	28

Of 20 reacted smears with mAbs, 12 reacted with anti-*L. tropica* mAb (A11 and T10) and 8 with anti-*L. major* (T1). 20 out of 20 specimens reacted with anti *L. tropica*/ *L. major* (T7) which was used as positive control. No smear showed positive reaction with anti *L. donovani*/*infantum* mAb (D2) (Table1). Geographical distribution of the isolates studied in Shiraz showed that from 12 patients with *L. tropica* 9 patients were residents of Shiraz and

the other 3 were from rural areas of Shiraz. From eight patients with *L. major* only one person was resident of Shiraz; however one case was resident of rural areas of Shiraz.

DISCUSSION

In the present investigation application of monoclonal antibodies for identification and characterization of *Leishmania* in impression smears of patients are shown. A total of 71% positivity was observed in impression smear. The application of mAbs with appropriately selected specificities allows the unequivocal immunological detection of *Leishmania* in the lesions, and also provides the means for the *in situ* differentiation of the species or subspecies of the protozoan in biopsies. (5). Apparently most researches of *in situ* characterization of *Leishmania* has been done on tissue biopsies as paraffin embedded or frozen sections which have yielded 90-95% positivity (6, 7, 8, 9, 10, 11). No work has been done on *in situ* identification of cutaneous *Leishmania* in impression smears so far.
Using a genus-specific monoclonal antibody with indirect immunofluorescent antibody assay (IFA), Anthony et al. identified 9 of 9 biopsies and in 11 of 12 needle aspirates taken from human lesions. In contrast, only 5 of the biopsies and 4 of the aspirates of yielded promastigotes upon culture in vitro (6).
It seems that when a large number of amastigotes present in the impression smears it is easier to identify and characterize the *Leishmania* species. Low positivity for identification of *Leishmania* amastigotes in impression smears may be due to the nature of impression smears that could be unsuitable for identification of *Leishmania*. It does not also constitute a monolayer cell on the slide which itself may be necessary for contacting mAbs to the cells. Decrease of positivity in our impression smears amastigotes as compared to paraffin embedded or frozen sections could be due to non-specific reactions in some mAbs when ABC-peroxidase was used as noted by Ismail *et al*. (11). Also there may be some strains that do not react with the available mAbs. In other words if we use more specific mAbs on impression smears more amastigotes could be identified.
This test could be a suitable substitution to IFA tests for identification and characterization of *Leishmania*. It has been suggested that immunoperoxidase test could be a reliable test for substitution to IFA test for diagnosis of toxoplasma (12). A rate of 71% positivity in impression smears in our results could be comparable with results of Ismail *et al*. (11). These investigations showed 88% positivity when tissue biopsy was used for characterization of *Leishmania*. In some observations 20% positivity were shown when

haematoxyline eosine with standard pathological method was used for identification of *Leishmania* in tissue biopsies of the patients. Using IFA test on frozen tissues 88.46% and in paraffin embedded tissues 89.28% positivity was shown (13).

The most recent studies about characterization of cutaneous *Leishmania* in Iran with the species specific mAbs using IFA and ELISA tests showed that from 156 isolates of *Leishmania* from patients with cutaneous Leishmaniasis and one isolate from gerbil, a total of 63, 72, and 3 *Leishmania* promastigote isolates preferentially reacted with anti- *Leishmania tropica* mAb (A11), anti- *Leishmania major* mAb (T1) and anti- *Leishmania infantum* mAb (D2), respectively (14).

So, immunoperoxidase test could be a very suitable substitute to IFA test especially in less equipped laboratories. Although immunoperoxidase test could result in a high positivity with impression smears, but it seems that tissue biopsies will yield a higher positivity in identification and characterization of *Leishmania*. Comparing to the H & E in tissue biopsies which has yielded a 20-30% positivity, identification and characterization of *Leishmania* in impression smears is less expensive and less invasive procedure which with its higher positivity the patients could benefit its application.

The majority of cases in Shiraz have showed *L. tropica* although *L. major* has also been detected in our study which is similar to the work of Ardehali *et al*. (14).

Treatment of cutaneous leishmaniasis is usually lengthy, painful and expensive procedure. On the other hand *L. major* which is a causative agent for cutaneous *Leishmaniasis* could result a cross immunity to *L. tropica*. Some lesions may also be in some sites of patient's body which the physician suggests not to be treated. Therefore, *in situ* characterization of *Leishmania* which could detect the causal organisms in cutaneous leishmaniasis may help this task.

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