Application of Specific Monoclonal Antibodies for Characterization of Leishmania spp. Promastigotes Using Indirect Immunofluorescent and Immunoperoxidase Tests

Seyed Mahmoud Sadjjadi[1*], Sadreddin Mohseni Ardehali[1], Reza Adibmanesh[1], Ezzatollah Basiri[2]

[1]Department of Parasitology and Mycology, [2]Bacteriology and Virology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.

ABSTRACT

Background: Different methods have been used for characterization of *Leishmania* promastigotes. Monoclonal antibodies are useful in characterization of *Leishmania spp*. both amastogotes and promastigotes. **Objective:** Comparing the characterization of *Leishmania spp.* promastigotes with immunoperoxidase test (Avidin-Biotin) techniques and an indirect immunofluorescent assay (IFA). **Methods:** Application of specific monoclonal antibodies for characterization of different *Leishmania* species. Immunoperoxidase tests (Avidin-Biotin) and indirect immunofluorescent assay (IFA) were employed for characterization of different *Leishmnia* promastigotes from culture. Five monoclonal antibodies including 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*, XLVI-5B8- B3 (T1) specific for *L. major*, and T7 reactive to both *L. major* and *L. tropica* as well as an anti GP63 mAb were used. **Results:** The best result was obtained with the dilution of 1:50 for both mAb and conjugate. One hundred percent sensitivity and specificity was achieved for characterization of *Leishmania* promastogotes with both methods. **Conclusion:** As immunoperoxidase method needs less equipments compared to IFA technique, it would be a preferred method for characterization of promastigotes.

Keywords: Immunofluorescent, Immunoperoxidase, Leishmania, Monoclonal Antibody, Promastogote

*Corresponding Author: Dr. Seyed Mahmoud Sadjjadi, Department of Parasitology and Mycology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran. Tel/Fax: (+) 98 711 235 9847, (+) 98 711 230 5291, P.O.Box: 71345-1735, e-mail: parasito@sums.ac.ir

INTRODUCTION

Leishmania genus includes many different species which infect a wide variety of mammalian hosts. The precise identification of leishmanial parasites at the species level is very important for determining whether an organism, causing the disease in a given area, is of the same biotype as that found in suspected vector (1). The identification of *Leishmania* species has been traditionally based on clinical manifestations, epidemiological parameters and geographical distribution (2). These methods are no longer valid to be used for precise identification. There are now several well established methods available including isoenzyme, PCR and monoclonal antibodies (3-6). Monoclonal antibodies have been employed extensively for the identification of *Leishmania* species, development of diagnostic tests and in the characterization of defined leishmanial antigens (7-9).

Different methods have been used for characterizing *Leishmania* promastigotes including ELISA, IFA and Radio-Immuno Assay (10-12), but each of them has its limitations.

The appearance of promastigotes following culturing of the biopsy tissue on NNN medium also provides an unequivocal diagnosis of leishmaniasis (13,14).

In present investigation, with the use of monoclonal antibodies, attempts were made to characterize the promastigotes of *Leishmania* with immunoperoxidase test (Avidin-Biotin) techniques and compare the results with those of an indirect immunofluorescent assay (IFA).

MATERIALS AND METHODS

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: *L. 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 Programme 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*, XLVI-5B8- B3 (T1) specific for *L. major*, and T7 reactive to both *L. major* and *L. tropica* was used as a positive control.

An anti GP63 mAb produced in immunology department at Shiraz University of Medical Sciences, was also used (15).

All these mAbs were provided as lyophilized mouse ascites fluids. A 1:1000 dilution of pooled normal mouse serum was used as a negative control.

Preparation of Antigen for IFA. The promastigotes were washed thrice in PBS, pH 7.2. The supernatant was discarded and the pellet was resuspended in 1 ml of PBS. The parasites were counted in a haemocytometer and adjusted to a final concentration of 10^6 per ml of PBS. Then, 10 ml of the parasite suspension containing 10^4 organisms

were added to each well on slides previously washed with detergent, dried and marked. These slides were fixed with acetone for 10 min and stored at -20°C until used.

IFA Test. For IFA test the prepared slides were thawed and blocked with PBS containing 5% heat-inactivated FCS for 15 min subsequently they were washed, and a 1:1000 working dilution of monoclonal antibodies was added to each well. The slides were then incubated in a humid chamber for 30 min at 37°C and washed three times with PBS pH 7.2 and dried. Anti-mouse polyvalent immunoglobulins-fluorescein conjugate (Sigma, Dorset, UK) at a 1:40 dilution was added to each well and incubated at room temperature for 30 min. The slides were washed three times, dried, and mounted with 90% glycerol in PBS pH 8.0. These slides were then examined under the fluorescence microscope using a 40X objective.

Immunoperoxidase Test. Promastigotes of *Leishmania* were characterized by Labeled Avidin Biotin (LAB) method. For immunoperoxidase test on promastogotes, the prepared slides were thawed. The slides were fixed in cold acetone for 10 min followed by the application of H_2O_2/Methanol 0.6% and kept for 10 min. Mouse anti-*Leishmania* antibody was used for 120 min and the slides were washed with PBS for 20 min. Biotinylated rabbit anti-mouse immunoglobulins were applied on slides and kept for 60 min followed by two successive washing in PBS for 20 and 60 min. In the next step, peroxidase-conjugated avidin was applied and kept for 60 min followed by another washing in PBS for 20 min. Diaminobenzidin-H_2O_2 reaction (1 ml DAB + 7µl H_2O_2 30%) was completed on slides for 10-15 min and the slides were washed in distilled water for 5-10 min. The slides were then counter stained with

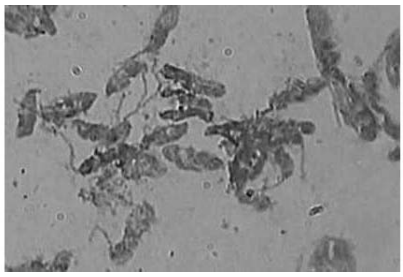

Figure 1. Avidin-Biotin immunoperoxidase staining of Leishmania promastigotes (positive 1250X)

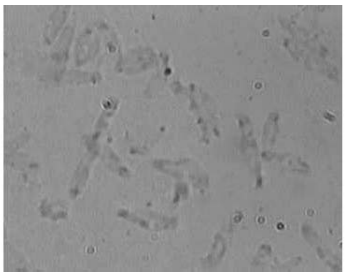

Figure 2. Avidin-Biotin immunoperoxidase staining of Leishmania promastigotes (Negative 1250X)

Haematoxyline-Eosin (H & E) and finally mounted with Canada Balsam. These slides were then examined under the light microscope. The positive reaction defined as a dark brown color in promastigotes. No color was observed in negative controls (Fig. 1-2).

RESULTS

In the present investigation, the results for application of monoclonal antibodies for identification and characterization of *Leishmania* promastigotes recovered from culture are shown.

Different dilutions for anti GP63, mAb with different dilutions of conjugate anti

Table 1. IFA test using different dilutions of anti GP63 and anti-mouse conjugates

	Primary Antibody (titer)						
Conjugate anti-mouse Ab (titer)	Non diluted antibody	25	50	100	200	400	Normal mouse serum
25	+4	+3	+3	+3	+2	+1	-
50	+4	+3	+3	+2	+2	+1	-
100	+3	+3	+3	+2	+1	+1	-
200	+2	+2	+2	+2	+1	+1	-
400	+2	+1	+1	+1	+1	+1	-

L. tropica promastigotes were used as antigens

mouse Ab were used. The best result was obtaind with the application of non–diluted mAb with a 1:50 dilution of conjugate (Table 1).

The tests were also carried out with T7 mAb which reacts with both *L. tropica/L. major* promastigotes.

The results for IFA test using different dilutions of T7 mAb and anti–mouse conjugate on *L. tropica* promastigotes are shown in Table 2. The best result was obtained with the dilution of 1:50 for both mAb and conjugate. The results for IFA test using different dilutions of T7 mAb and anti–mouse conjugate for *L. major* promastigote are shown in Table 3. According to this test, the best result was obtained with 1:50 dilution for both T7 mAb and conjugate. In IFA test more positivity was observed by *L. major* promestigotes. In immunoperoxidase test, a 1:50 dilution for both T7 mAb and avidin-biotin using *L.major* promastigotes as antigen was used.

A total of 19 isolates of promastigotes were used in this study. Table 4 shows the results of immunoperoxidase test on these promastigotes. Out of 19 promastigote isolates studied, 9 reacted with anti-*L. tropica* mAb (A11), 7 with anti-*L. major* (T1) and three with anti-*L. donovani*/*L. infantum* mAb (D2). However, a total of 16 out of 19 specimens reacted with anti *L. tropica*/*L. major* (T7) which was used as the positive control.

Table 2. IFA test using different dilutions of T7 mAb and anti-mouse conjugates

	T7 monoclonal Ab (titer)					
Conjugate anti-mouse Ab (titer)	25	50	100	200	400	Normal mouse serum
25	+3	+3	+2	+2	+1	-
50	+4	+4	+2	+1	+1	-
100	+2	+2	+1	+1	+1	-
200	+1	+1	+1	+1	+1	-
400	+1	+1	+1	+1	+1	-

L. tropica promastigotes were used as antigens.

Table 3. Immunoperoxidase test using different dilutions of T7 mAb and biotinylated anti-mouse immunoglobulins and preoxidase conjugated avidin

Dilutions of avidin and biotin	T7 monoclonal Ab (titer)					
	25	50	100	200	400	Normal mouse serum
25	+3	+3	+3	+2	+2	-
50	+4	+4	+3	+2	+2	-
100	+3	+3	+3	+2	+1	-
200	+2	+2	+2	+1	+1	-
400	+2	+1	+1	+1	+1	-

L. tropica promastigotes were used as antigens.

Table 4. Results of immunoperoxidase test using promastigotes with different monoclonal antibodies

Promastigote stocks	Monoclonal antibodies				
	L. major (T1)	*L. tropica* (A11)	*L. infantum* (D2)	Positive control T7	PBS
stock 1 to 9	-	+	-	+	-
stock 10 to 16	+	-	-	+	-
stock 17 to 19	-	-	+	-	-
total	7	9	3	16	19

DISCUSSION

Characterization of *Leishmania* has been carried out by different methods, each method has advantages and limitations (1). Monoclonal antibodies have been employed extensively for identification of *Leishmania* species, development of diagnostic tests, investigation of molecules associated with parasite virulence and/or pathogenicity, and in the characterization of defined *Leishmania* antigens that should be able to produce immunoprotection against human leishmaniasis following vaccination (8).

The present investigation applied monoclonal antibodies for characterization of *Leishmania spp.* promastigotes recovered from the culture. High sensitivity was observed for characterization of promastogotes with both IFA and immunoperoxidase tests. Sharifi et al. showed that from 90 isolates discriminated by IFA, 82 cases (91.1%) were *L. tropica* and 8 cases (8.9%) were *L. major* (16). In the same study, they applied ELISA test for characterization of 28 species in which 19 cases (67.9%) and 5 cases (17.9%) were *L. tropica* and *L. major*, respectively, while 4 cases (14.2%) reacted equally with anti-*L. tropica* T14 and anti-*L. major* T1. This was the only discordance obtained in the results with the IFA and ELISA so that, they were discriminated as *L. tropica* by IFA and *L. tropica/L. major* by ELISA. No comparison has been

made in the mentioned study between the sensitivity and specificity of IFA and ELISA for characterizing *Leishmania* promastigotes. However, our results showed that using mAb for characterization of *Leishmania* promastgotes with both IFA and immunoperoxidase methods was very sensitive and specific.

Another study on characterization of cutaneous *Leishmania* in Iran with the species specific mAbs using IFA and ELISA tests showed that out of 156 isolates of *Leishmania* from patients with cutaneous leishmaniasis and one isolate from gerbil, a total of 63, 72 and three *Leishmania* isolates preferentially reacted with anti-*Leishmania tropica* mAb (A11), anti- *Leishmania major* mAb (T1) and anti-*Leishmania infantum* mAb (D2), respectively (4). However, no comparison was made between the two methods for characterizing *Leishmania* promastigotes. Hejazi et al. used monoclonal antibodies for characterization of *Leishmania* parasites isolated from patients with cutaneous leishmaniasis in Isfahan (17). They reported *L. major* as the causative agent of the disease in 100 cases and *L. tropica* in 8 patients. In 12 cases, the results were inconclusive as the antigens reacted with either 2 or 3 specific monoclonal antibodies. Anthony et al. used a genus-specific monoclonal antibody with indirect immunofluorescent antibody assay (IFA) for identification of *Leishmania spp.* They 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 yielded promastigotes upon culture in vitro studies using specific antibodies for identification of *Leishmania* in New World showed 100% sensitivity by IFA (14). A high relationship has been reported for the tests IPA, IFA and RTA with no cross-reaction with malarial infection (18). Identification of New World promastigotes of *Leishmania* has been carried out using monoclonal antibodies (7).

Application of mAb should be useful for identifying sand fly species involved in transmitting different species of *Leishmania*, and for rapid assessment of leishmanial infection rates in endemic regions (1).

Our results for identification and characterization of promastigotes showed a high sensitivity and specificity. The results showed no difference between IFA and immunoperoxidase methods for characterizing of *Leishmania* promastigotes. Therefore, both tests could be very helpful when promastigotes are used for characterization of *Leishmania* promastigotes. As immunoperoxidase needs less equipments than IFA technique; this technique would be preferred for characterization of promastigotes and could be employed for characterization of promastigotes in *Leishmania* vectors involved in transmitting different species of *Leishmania* to human. Immunoperoxidase test has also been suggested as a reliable, substitution for IFA test in diagnosis of toxoplasma (19).

This test could also be used for rapid assessment of leishmanial infection rates of sand fly vectors in endemic areas. So, immunoperoxidase test using *Leishmania* promastigotes as antigens could be a very suitable substitute for IFA test especially in less equipped laboratories.

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