

Original Article

Characterization of *in Vitro* Cultivated Amastigote like of *Leishmania major*: A Substitution for *in Vivo* Studies

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Abstract

Background: Promastigotes of *Leishmania* spp. have been readily cultured, but the axenic culture of amastigotes has been successful in *L. donovani*, *L. infantum*, *L. mexicana* and *L. pifanoi*. However, some species such as *L. major*, is much less amenable to axenic cultivation. In present study, we describe an *in vitro* culture system for the generation and propagation of axenic amastigotes form of *L. major*.

Methods: Promastigotes of *L. major* were cultivated in a biphasic NNN medium. The liquid phase was Schneider's medium, pH 3.5, supplemented by 25% FCS (fetal calf serum). The cultures were maintained at 33-34°C for 120 hours.

Results: Fine structure analysis of these *in vitro*-grown amastigotes by electron microscopy, demonstrated that they have a pear-shaped body with abortive short terminal flagellum. The *in vitro*-grown cells are agglutinated by peanut lectin. SDS-PAGE pattern of these axenic amastigotes showed a 66-kDa band, which was not present in promastigotes. The axenic grown amastigotes were able to infect peritoneum macrophages of BALB/c mice. In supernatant of culture, biochemical analysis showed decreased protein and acid phosphate activity.

Conclusion: These amastigotes like cells might serve as a suitable strain for the study of amastigote biochemistry, *in vitro* drug testing, and immunology of *L. major*.

Keywords: *Leishmania major*, *Amastigotes*, *Characterization*, *in vitro*, *Culture*

Introduction

Leishmania major is a causative agent of acute cutaneous leishmaniasis in many parts of the world. It is transmitted to human beings by the bite of female sand flies. The infective stage of the parasites in the form of promastigotes is injected into the skin of patient by insect bite and entered phagolysosomes of macrophages (1). Acidic environment or elevated temperature changes the promastigote to amastigote form

(2). Amastigotes are responsible for all clinical manifestations in the vertebrate host; therefore vaccines and chemotherapeutic target need to be developed against this stage of parasite (3, 4). During the past few years, several laboratories have succeeded in axenic culture of *Leishmania* amastigotes by combining both acidic pH and elevated temperature. Bates et al. 1992 were the first who successfully indeed differentiated *L. mexicana* promastigotes into axenic amastigotes (5).

Characterization of axenic amastigote of the new world *Leishmania* species has demonstrated that they resemble animal derived amastigotes (5-8). Axenic amastigotes of some of *Leishmania* species such as *L. donovani*, *L. mexicana* and *L. pifanoi* have been characterized extensively and found to be identical to *bona fide* so far. Transformation of the promastigotes of *Leishmania infantum* into amastigotes and their characterization has also been reported (9). However, amastigotes of some species such as *L. major* have been much less amenable to axenic cultivation (10).

This paper describes *in vitro* cultivation and characterization of *L. major* amastigotes, which resemble to animal derived amastigotes in their morphology and characteristics, using electron microscopy and biochemical approaches.

Materials & Methods

Cell culture

L. major (MRHO/IR/75/ER) was used in this study. Promastigotes were cultured in NNN medium followed by adding RPMI¹⁶⁴⁰ and Schneider's Drosophila medium supplemented with 10% fetal calf serum (FCS) at 25 °C. The pH of culture medium was changed to 3.5, 4 and 5 by adding sterile 10mM succinic acid. Promastigotes of *L. major* (1×10^6 cell/ml) were added to 18 tubes containing NNN medium and to 18 tubes without NNN medium. The tubes were divided into 12 groups and different media was added into each tube as follows:

In first 3 tubes with NNN and second 3 tubes without NNN, 2 ml of RPMI, pH 5, was added.

In third 3 tubes with NNN and fourth 3 tubes without NNN, 2 ml of RPMI, pH 4, was added.

In fifth 3 tubes with NNN and sixth 3 tubes without NNN, 2 ml of RPMI, pH 4, was added.

In seventh 3 tubes with NNN and eighth 3 tubes without NNN, 2 ml of Schneider's Drosophila medium, pH 5, was added. In ninth 3 tubes with NNN and tenth 3 tubes without NNN, 2 ml of Schneider's Drosophila medium, pH 4, was added.

In eleventh 3 tubes with NNN and twelfth 3

tubes without NNN, 2 ml of Schneider's medium, pH 3.5, was added.

A total of 0.5 ml FCS was added to each tube. The whole tubes were divided into 3 groups, each of 12 tubes, and incubated at 31 °C, 33 °C and 35 °C for 120 h. The tubes were checked regularly for any changes in cultivated parasites.

Electron microscopy

For structural analysis, the grown parasites were checked by transmission-electron microscopy where a pellet of cultured cells was fixed in 3% cacodylate buffered glutaraldehyde, followed by fixation in 1% osmium tetroxide and dehydrated in graded series of ethanol. The cells were embedded in agar 100 resin semithin sections (1µm thick) and stained with toluidine blue. Ultra-thin sections of the samples (50 nm thick) were made and stained with uranyl acetate and lead citrate. The sections were examined in a LEO 906 transmission electron microscope.

Lectin assay

A cell suspension containing 10^6 parasites/ml was provided by 3 times washing in phosphate buffered saline (PBS). An equal volume (50 µL) of cell suspensions and peanut lectin (PNA), diluted in PBS (1/160), was mixed in a well of micro titer plate and left at 34 °C for one hour. The cells were stained with Giemsa and the agglutinated cells were studied by light microscopy, using 400X magnification. Same assay was performed with promastigotes at room temperature.

Infecting of peritoneal macrophages

The peritoneal macrophages obtained from BALB/c mice were suspended in RPMI¹⁶⁴⁰ growth medium supplemented with 10% (v/v) FCS and incubated at 37 °C for 24 h. The culture supernatant was removed after 7 days and the adherent cells were infected with grown amastigotes at a ratio of 2/1 (amastigotes/macrophages). The infected cells were incubated at 33 °C for 48 h and used for further studies.

Acid Phosphatase Assay

Acid phosphatase assay was performed as described by Doyle and Dwyer (11). Briefly, supernatant of 80×10^6 amastigote-like and pro-

mastigote culture were used for the assay. P-nitrophenyl phosphate was used as substrate. Enzyme activities were reported as the number of nonmoles of substrate hydrolyzed to P-nitrophenol per minute at 30 °C. Protein concentration was determined by the Bradford method (12).

SDS-PAGE

A total of 4×10^7 *L. major* amastigotes like and 4×10^7 promastigotes were washed three times, and 1 ml of PBS was added. Three times freeze and thaw were made on cells followed by sonication for 20 min, using 3-5 pulse/sec. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on vertical slab gel (Mini protein, Bio Rad) with 10% separating gel and 3.5% stacking gel (13).

Results

The methods devised in this work for *in vitro* transformation of *L. major* promastigotes to amastigotes mimic *in situ* process. *L. major* promastigotes exposed to three different pH [3.5, 4 and 5] and temperatures (31, 33 and 35 °C) in RPMI, Schneider's and NNN media. Differentiation was completed within 5 days. Differentiation to amastigote shaped cells fully completed in biphasic NNN medium with Schneider's as liquid with pH 3.5 at 33 °C (Fig. 1). Transformation of promastigotes to amastigotes in liquid medium could not be completed at pH above 4 or at temperatures 31 °C and 35 °C. changing the pH and the temperature rather than pH 3.5 at 33 °C failed to induce promastigotes to fully differentiated amastigotes (Fig 2, 3).

Amastigotes were induced to differentiate back to promastigotes by shifting them to grow at pH 7 at 25 °C. Under this condition, parasites diffe-

rentiate back to promastigotes within 48 h. The results indicated that under the conditions outlined above *L. major* promastigotes were successfully differentiated into amastigote forms. Differentiated parasites were observed by light microscopy and appeared to be immobile and closely look like animal-derived amastigotes (Fig. 1).

Electron micrograph of amastigote form of *L. major* isolated from culture media showed a pear shaped cell body with abortive short terminal flagellum with exocytose vesicles, flagella pocket, micro bodies and rare mitochondrion (Fig.4).

The axenic grown amastigotes and promastigotes were differentially express surface components as indicated by lectin-mediated agglutination assays. Axenic amastigotes were selectively agglutinated by PNA at concentration of 6 µg/ml while no agglutination was observed with promastigotes (Fig. 5 and 6).

Axenic amastigotes and promastigotes which were harvested 5 days after culturing, were subjected to SDS-PAGE under reducing conditions, The promastigotes SDS-PAGE banding pattern were 29, 36, 45, 60 kDa bands while amastigotes revealed a clearly visible 66 kDa band (Fig. 7). These results indicate the differences between axenic amastigotes and promastigotes in the view of biochemical analysis. The enzyme activity of promastigote was higher than that of amastigotes (Table 1).

Macrophage infectivity

The fourth criterion employed for evaluating of the axenically grown amastigotes was the ability of cells to infect peritoneal macrophages of BALB/c. Results showed that a total of 70% of macrophages were infected with amastigotes 48 h after exposure.

Table 1: The enzyme activity (acid phosphatase) of amastigotes and promastigotes

Sample	Protein concentration mg/L	Ratio of acid phosphatase to protein
Supernatant of <i>L.major</i> amastigotes	36	0.2 ± 0.04
Supernatant of <i>L.major</i> promastigotes	22	0.4 ± 0.04

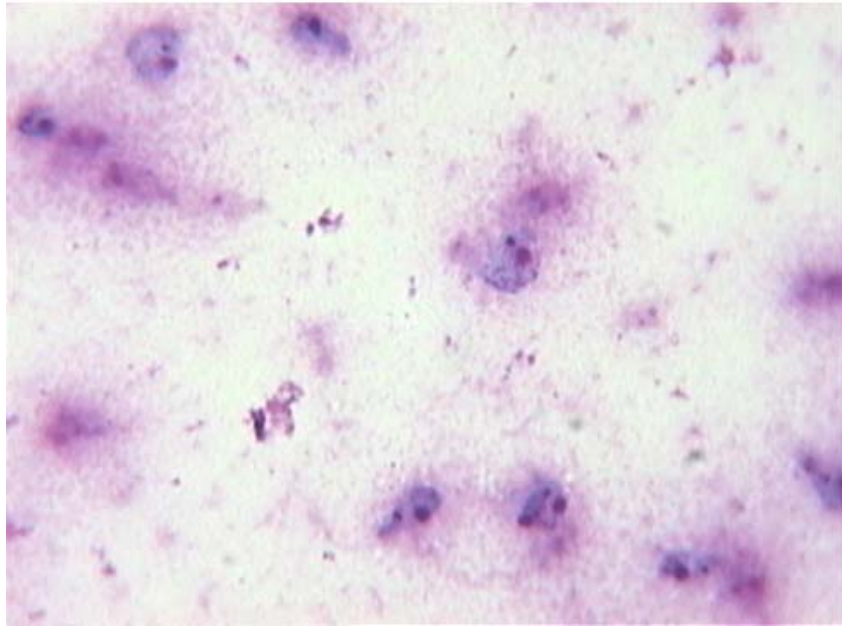


Fig 1: Morphological features of axenic *Leishmania major* amastigote (MRHO/IR/75/ER) after 120 hours in biphasic NNN medium with Schneider's pH 3.5 at 33 °C-34 °C

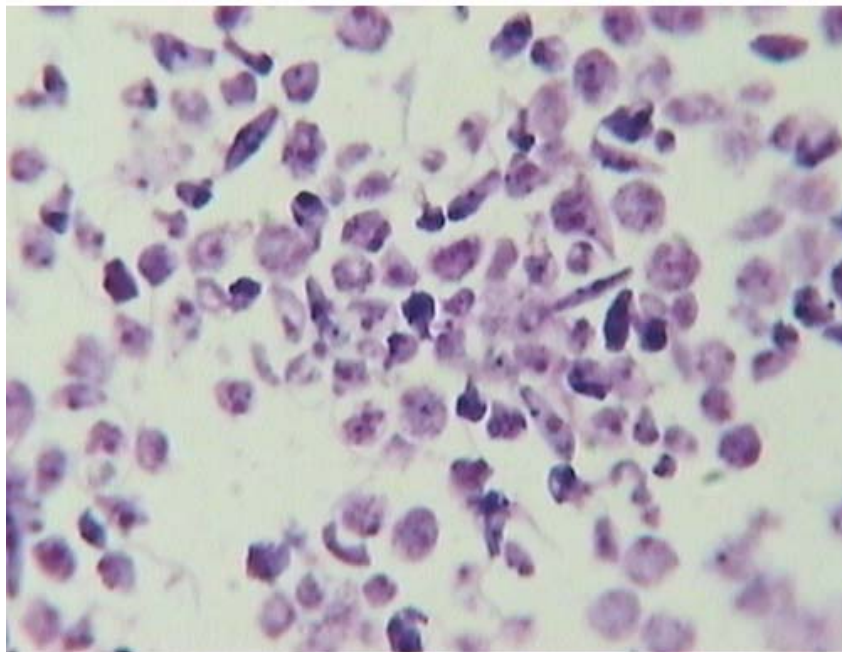


Fig 2: Axenic *Leishmania major* (MRHO/IR/75/ER) amastigote morphological features after 120 hours in with Schneider's liquid medium pH 4 at 33 °C-34 °C.

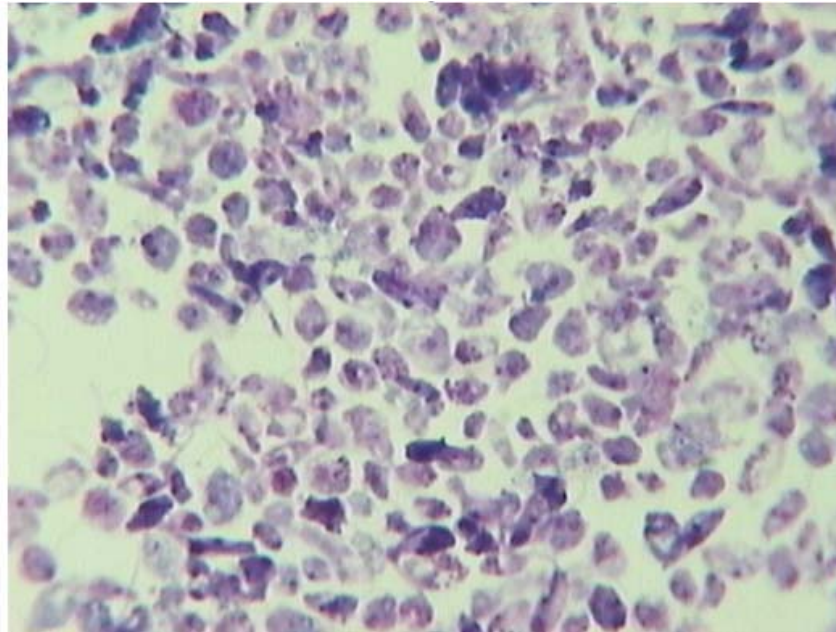


Fig 3: Axenic *Leishmania major* (MRHO/IR/75/ER) amastigote morphological features after 120 in with Schineider's liquid medium pH 3.5 at 33 °C-34 °C

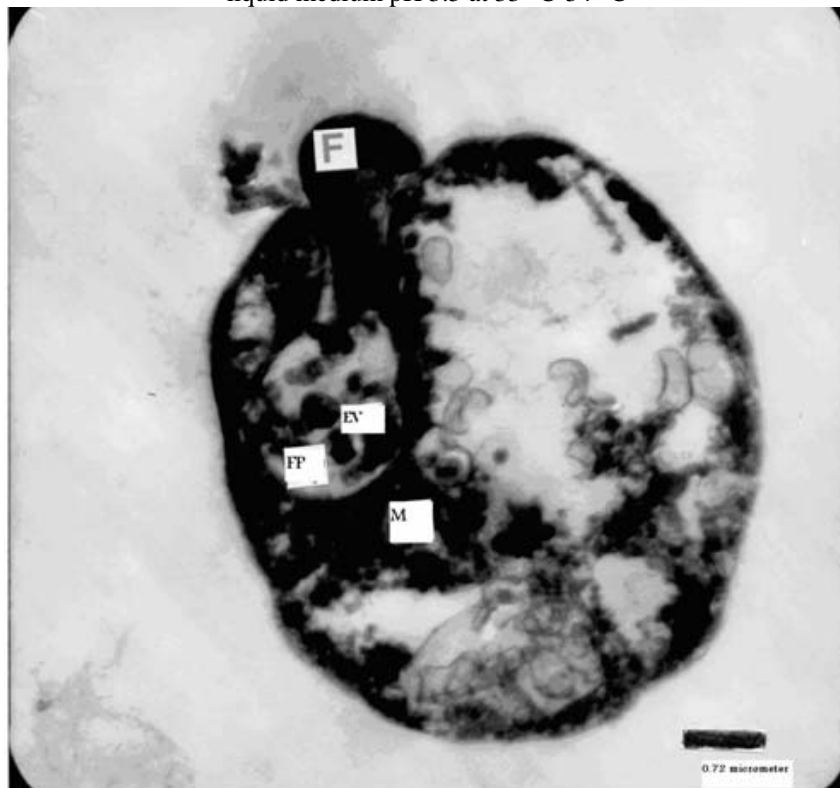


Fig 4: Electron micrograph of amastigote form of *Leishmania major* isolated from culture media showing pear shape cell body with abortive short terminal flagellum (f) which contains exocytotic vesicles (ev), flagellar pocket (fp), microbodies (b) and rare mitochondrion (m). (Uranium acetate, lead citrate, original magnification $\times 16700$ Scale bar = 0.72 μ m)

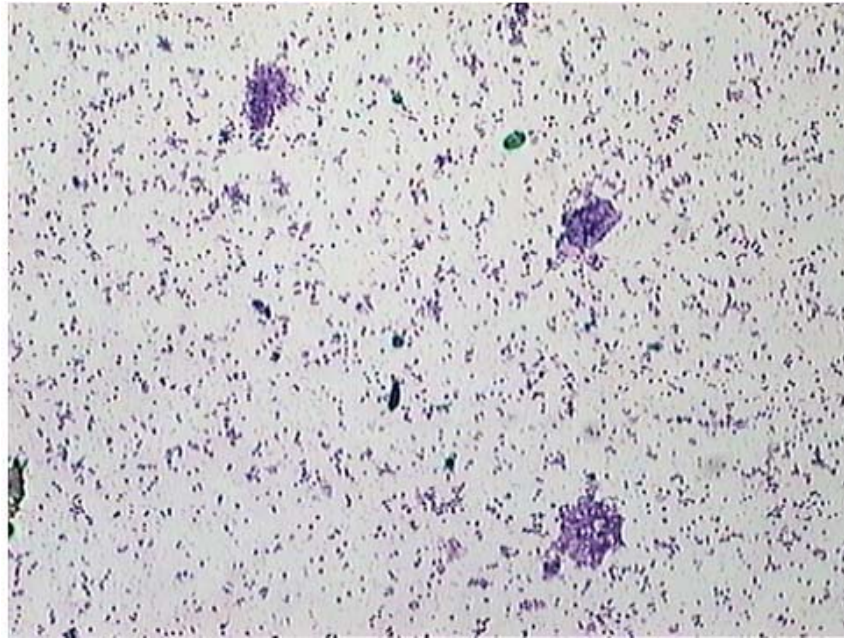


Fig. 5: Axenic amastigotes of *Leishmania major* (MRHO/IR/75/ER), agglutination with PNA lectin.



Fig 6: Axenic promastigotes of *Leishmania major* (MRHO/IR/75/ER) no agglutination with PNA lectin.

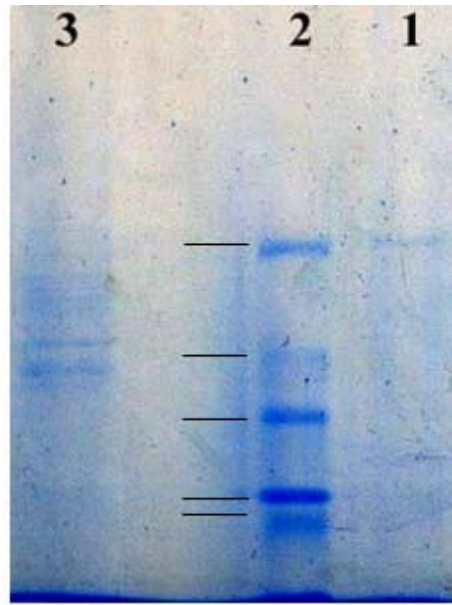


Fig. 7: SDS PAGE analysis of various forms of *Leishmania major*

Lane 1: Axenic amastigotes of *L. major*; lane 2: Low molecular weight marker (6.5, 14.5, 20, 24, 29, 36, 45 and 66 kDa); Lane 3: promastigotes of *L. major*.

Discussion

Amastigotes are the form of *Leishmania* parasites in tissues. Vaccines and chemotherapeutic targets need to be against this stage of the parasite life cycle. Amastigotes have been obtained either from short term *in vitro* cultivation in murine peritoneal macrophage or other macrophage cell lines (5, 14). The traditional approach, implemented to produce amastigote like form in culture includes progressive temperature and pH modification. Such methodology requires using individual conditions for each species (5, 7-10, 14, 15). It is very difficult to produce high number of homogeneous, aflagellated population of parasites in a short period of time (10). Our results showed that optimal temperature range for propagation *L. major* amastigotes was 33 °C to 34 °C with pH equal to 3.5. This condition is different with other *Leishmania* species. The first amastigotes culture was developed for *L. pifanoi* by adaptation of parasite to 33°C or 35 °C temperature (16) and latterly for *L. panamensis* at 32 °C, *L. braziliensis* at 28 °C (17), *L.*

mexicana at 32 °C–33 °C (5), *L. mexicana* at 33 °C– 35 °C (3, 18), *L. donovani* at 37 °C (19) and *L. infantum* at 36.5 °C (10).

In our study optimal temperature rang for cultivation of *L. major* was 33 °C to 34 °C. However, other studies indicated comparable temperatures of 33 °C–35 °C for species causing cutaneous leishmaniasis (3, 18, 20-22). The effect of pH on *Leishmania* parasites in relation to growth of amastigote-like forms has been reported before (5). Our attempts to axenically culture of the *L. major* amastigotes led to progressive appearance of promastigotes and intermediate forms in cultures. Our results demonstrated that retention of the morphology of *L. major* amastigotes required a pH below 4.5. However, pH 3.5 was selected as the optimum condition in our experiments using RPMI and Schneider's Drosophila with NNN biphasic media. Schneider's and RPMI, as liquid medium, permitted the adaptation and sub-cultivation of promastigotes to amastigotes form with different pH and temperature. A round shape *Leishmania* parasite without flagellum was grown in

culture media with biphasic NNN medium and Schneider's as liquid medium as demonstrated by light microscopy examination. The change of promastigotes to amastigotes did not take place completely in cultures with RPMI or only Schneider's liquid medium without NNN. These findings again emphasize that optimal condition for propagation of axenic amastigotes vary and have to be determined for each *Leishmania* species isolates (16).

The grown *L. major* amastigotes were morphologically characterized and were similar to intracellular amastigotes. The parasites were round to ovoid aflagellate with lack of pocket flagellar rod. The short terminal flagellum in axenic amastigotes was clearly confirmed by transmission electron microscopic examination. We have also shown that amastigotes and promastigotes are differentially express surface components. This was shown by lectin binding capacities of amastigotes and decreasing acid phosphatase activity. In general, agglutination profiles of promastigotes were in agreement with previous findings (23-25). Promastigotes did not react with PNA. In contrast, axenic amastigotes were selectively agglutinated with low concentration of PNA (6 µg/ml). These results indicate the different sugar binding capacities of amastigotes and promastigotes and imply the expressing of different membrane glycoconjugate in two developmental stages of *Leishmania* parasites. Promastigotes from of a variety of species of *Leishmania* release a soluble acid phosphatase activity during *in vitro* cultivation (26). These results indicate decrease of releasing acid phosphatase activity in amastigote form in addition protein amount in supernatant of amastigote like cell decrease. Compared with promastigotes, like their intracellular counterparts, axenic amastigotes display decreased total protein content, secretory acid phosphatase. (10). SDS-PAGE analysis of grown amastigotes indicated different banding patterns in amastigote like cells and promastigotes of *L. major*. The prominent band in axenic amastigote was a 66 kDa band, which was not present in promastigotes.

However, it has been reported that protein content decreases in amastigotes (10).

Immunofluorescence studies suggested that the 66-kDa polypeptide is associated with intracytoplasmic vesicles (27). The ability of *L. major* amastigotes to infect peritoneal macrophages was also examined in this study. Axenic amastigotes were shown to infect peritoneal derived BALB/c mice macrophages. Several known virulent factors have been described on *Leishmania* macrophage interactions (28). The macrophage infectivity rate in our study was higher than reported earlier on promastigotes of *L. major* (29) as well as amastigote like form of *L. braziliensis* (7). In the present study, infection rate of macrophages was 70% after 48 h.

In conclusion, results of this preliminary research indicates that the method developed in our laboratory for *in vitro* differentiation of *L. major* promastigotes to amastigotes is efficient and yields organisms closely resembling to animal derived amastigotes. It could also serve as a suitable method for *in vitro* studies on *L. major* amastigote biochemistry, drug testing and immunology.

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