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Identification and characterization of *Leishmania* amastigote and axenic form antigens

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Abstract. This study was aimed to identify and characterize *Leishmania* amastigote, and axenic form antigens. Two *in vitro* techniques were used to change *leishmania* parasite isolates from promastigote form to amastigotes and amastigote like (axenic) forms. The main strategy relied upon *in vitro* infection of murine macrophages cell line J774 with *leishmania* promastigote, at 37°C with 5% CO_2, while the second technique relied upon the culture of promastigote at 37°C with low pH (5.5), and 5-10% CO_2. Proteins were extracted and fractionated utilizing 12% Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE). Antigens were recognized using both immune dot blot and western blot procedures. PCR was performed for recognition of *leishmania* parasites in infected J774 macrophages. *L. major* was quicker in infectivity of macrophages cell line than *L. donovani*. Shared proteins ranging from 26-116 kDa were identified by SDS PAGE in all stages. Immune Dot-blot method showed positive outcomes, while western blot identified an exceptional antigen band of 16 kDa in amastigote, this unique band could be of value in diagnosis and vaccination of leishmaniasis. PCR results confirmed presence of both isolates demonstrating that co-infection is conceivable, and no indications of hereditary recombination at kinetoplast DNA (kDNA) were identified in macrophages simultaneously infected by *L. major* and *L. donovani*.

INTRODUCTION

Leishmaniasis is a noteworthy general medical issue in several regions of the world, where in excess of 12 million people are infected with a sum of 350 million individuals in danger of being infected. Every year, 1.5 million new cases of cutaneous leishmaniasis, 500,000 new cases of visceral leishmaniasis are assessed, and around 20-30 thousands deaths (WHO 2018).

Two phases of the parasite, the intracellular amastigote stage found in the vertebrate host, and the promastigote form predominately found in the insect vector.

The amastigote can be exhibited after inoculation of research facility animals, (for example, hamsters, mice or guinea pigs) with infective phase (Magill *et al.*, 1993) and after that intra-celluler amastigotes were isolated from the animal spleen 6-8 weeks post infection (Dwyer 1976).

Amastigotes segregated from infected tissues represent heterologous populations at any given time, which varies probably with respect to their age and phase of improvement in their cell cycles (Joshi *et al.*, 1993).

For development and creation of *in vitro* amastigote, Maria *et al.* (1998) built up an *in vitro* formwork for identification of

purified amastigote types of *L. amazonensis* from monolayers murine macrophage J774 G8, which is originally derived from macroghage of peritoneal exudates of BALB/c mice. Their consequences of separating amastigote forms from this cell line enable the likelihood of working with huge amounts of the parasites to perform studies with the intracellular phase of the *Leishmania* species including protein synthesis, gene expression, ultra auxiliary changes, components of medication activity and the advancement of techniques for infection treatment and counteractive action.

The exposure of *Leishmania* promastigotes to 37°C induce morphological changes, like those occurring naturally inside mammalian macrophages (Eperon and McMahon-Pratt 1989).

Several endeavors were made to culture amastigote-like types of various *leishmania* species (Castilla *et al*., 1995; Doyle *et al*., 1991; Hodgkinson *et al*., 1996; Pan *et al*., 1993). These specialists have utilized for the most part temperature and in some cases pH to trigger the *in vitro* parasite change.

As of late, *in vitro* axenic culture of *L. donovani* (Strain Dd8) was proven (Gupta *et al*., 1996a), and the membranes of transferred cells were characterized (Gupta *et al*., 1996b). The outcomes detected a solid similarity between the axenic and intracellular amastigotes. The comparative metabolite profile of different parasite forms uncovers that axenic amastigotes appear to represent an intermediate stage between promastigotes and intracellular amastigotes regardless of their solid similarity of intracellular amastigotes in morphology, infectivity, biochemical reaction and even in the appearance of amastigote specific A2 protein (Gupta *et al*., 2001).

Alian *et al*. (2004) depicted *in vitro* culture formwork for the generation of axenic form from the well-characterized 1S-CL2D line of *leishmania donovani*.

Fine structural examination of these *in vitro* developed amastigotes exhibited that they had morphological highlights similar to *L. donovani* tissue-determined amastigotes.

MATERIALS AND METHODS

Two *Leishmania* species were used in this study, *L. donovani*, which was isolated from a visceral leishmaniasis (VL) case from Gadarif state, Eastern Sudan and *L. major*, which was obtained from a cutaneous leishmaniasis (CL) patient from Khartoum state, Central Sudan. Both were characterized by iso-enzyme as MON 82 and MON 74, and by molecular techniques as *L. donovani* and *L. major* individually.

Parasite culture

Parasite isolates were grown in RPMI 1640 containing 25mM Hepes pH 7.4, 10% heat-inactivated fetal calf serum (FCS), streptomycin and penicillin at 5U/ml (complete media). Cultures were grown in sterile 25 ml tissue culture flask and incubated at 26°C.

Axenic form culture

Stationary stage promastigote was harvested at 1200 rpm/10 min; sub-cultured in 25 ml tissue culture flask containing 10 ml of complete media as mentioned above, at pH 5.5, the pH was balanced with concentrated HCl and incubated in an environment containing 10% CO_2 at 37°C.

J774 macrophage culture

Murine macrophages cell line J774 were cultured in 25 ml tissue Culture flasks containing RPMI 1640 enhanced with 20% FCS and incubated in a climate of 5% CO_2 at 37°C, the medium was changed each 3-4 days.

***In vitro* macrophage infection**

Growing J774 cells were infected by stationary-stage promastigotes at ratio 1:10, infected cultures were incubated in 5% CO_2 atmosphere at 37°C. Nonadherent parasites were washed away with RPMI (3 times), following three days of infection.

***In vitro* macrophages co-infection**

Growing J774 cells were infected by stationary-stage promastigotes of two *leishmania* parasite species *L. major* and

L. donovani, 2ml (1x10^7/ml) were taken from every parasite culture, well mixed, and added to the growing macrophages (as co-infection step), cultures were incubated in 5% CO_2 atmosphere at 37°C. Nonadherent parasites were washed away with RPMI, and infected macrophages were harvested for protein fractionation, and genomic DNA extraction.

Detection of macrophage infection
From infected culture, the smear was prepared at several interims: 24h, 48h, 72h, 96h and 6, 8 days post-infection. The smears were fixed with 70% methanol and stained with 10% Geimsa stain, for 10 minutes. The prepared film was photographed, for infection rate observing.

Harvest of parasites and infected macrophages
This was done by centrifugation of cultures at 1200 rpm/10 min at 4°C. the supernatant was disposed of, and the pellet was suspended in sterile phosphate buffer saline (PBS).

Lysis of infected macrophages
This was done by freeze-thawing for three times in liquid nitrogen, water bath at 37°C, and vortexing, cell debris was removed. The supernatant was collected, tested for antigen reactivity, and fractionated on 12% Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE).

Western blot analysis
Protein tests were set up, and isolated on 12% SDS/PAGE, transferred to nitrocellulose paper, and analysed by immunoblotting for *leishmania* antigens utilizing human serum with high antibody titer in Direct Agglutination Test (DAT) as depicted by Bahador *et al.* (2015).

Detection of co-infection by kDNA PCR
Extraction of genomic DNA was done by phenol-chloroform technique as depicted by Van Eys *et al.* (1992). PCR was carried out using genus-specific primers for mini-circle kinetoplastd DNA (kDNA) using the AJS3 5'ggggTTggTgTAAAATAgggC-3', and DBY 5'CCAGTTTCCCgCCCCggAg-3' primers as described by Barker *et al.* (1992). We used this technique for its specificity, and we didn't have transfected parasite in our lab for both species at the time of experiment.

Ethical approval
Not Applicable

RESULTS

In this study *in vitro* infection of J774 macrophages cell line by *leishmania* parasite, showed that the duration of attachment, invasion, and the colonization of the cell varied between *L. major* (the length time went from 3-5 days), and *L. donovani* (span time ran from 7-9 days) (Fig. 1, 2, 3).

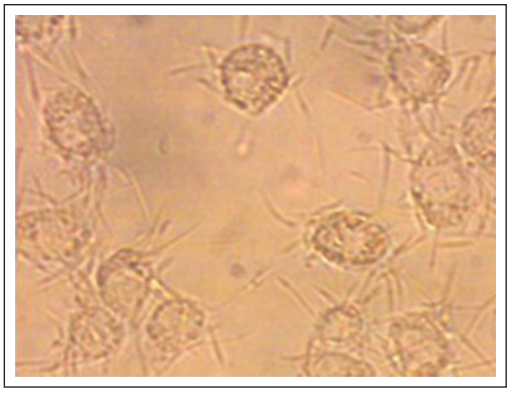

Figure 1. Immediate attachment of *L. major* promastigotes to macrophages J774 cell line after incubation at 37°C after immediate infection (x400).

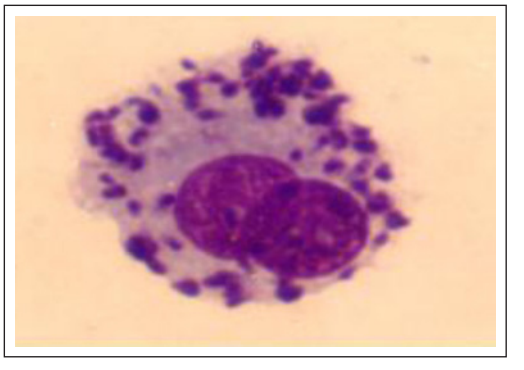

Figure 2. Macrophages J774 cell line infected with *L. donovani* promastigotes (8 days post infection Geimsa stain x1000).

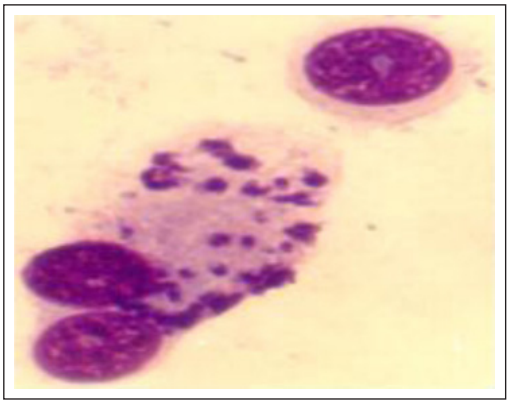

Figure 3. Macrophages infected with *L. major* promastigotes (5 days post infection Geimsa stain x1000).

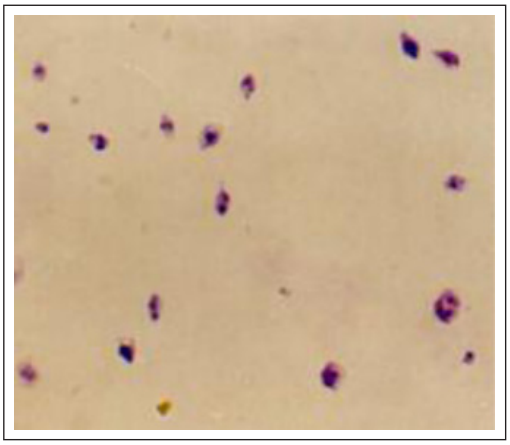

Figure 4. Axenic form *L. donovani* after incubation for 96 hours in RPMI 1640, 10% FBS, pH 5.5, 10% CO_2 and 37°C, showing Kinetplast and Nucleolus (Geimsa stain x1000), the promastigotes became round-ovale shape, lost their flagellum, and non-motile.

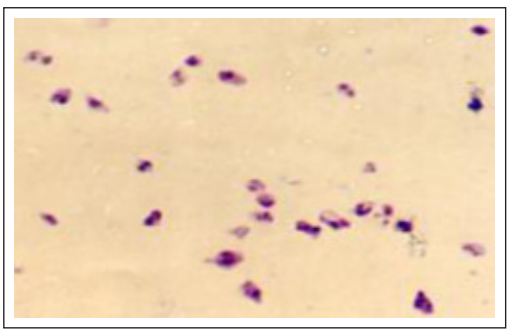

Figure 5. Axenic forms of *L. major* after incubation for 96 hours in RPMI 1640, 10% FBS, pH 5.5, 10% CO_2 & 37°C, Showing Kinetplast and Nucleolus (Geimsa stain x1000), the promastigotes became round-ovale shape, lost their flagellum, and non-motile.

Change of promastigote to axenic form was accomplished 48-96 hours when the parasite was cultured in RPMI 1640, 10% FCS, pH 5.5, 10% CO_2 and 37°C. Parasite changed to round form, was nonmotile, lost its flagellum with the noticeable nucleolus and kinetoplast (Fig. 4, 5).

For screening of amastigote antigens, immune-dot blot test was performed, sera of visceral leishmaniasis (VL) patients were responsive with lysate of macrophage infected with *L. major* and *L. donovani* (Fig. 6). Immunoblotting of proteins concentrates of promastigote, amastigote, and axenic structures with VL positive sera distinguished antigens ranging between 16-110 kDa. Regular antigens shared among amastigote and axenic structures were (75, 66, 45 and 29 kDa), and among amastigote and promastigote were (97, 75, 66, 45 and 29 kDa) (Fig. 7, 8).

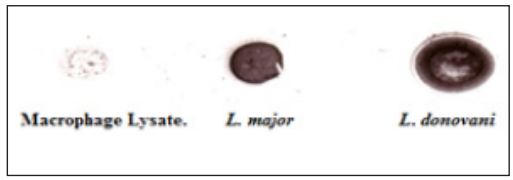

Figure 6. Immune-dot blot test showing reactive antigens of lysate of J774 macrophages infected with *leishmania* parasites, with VL patient serum.

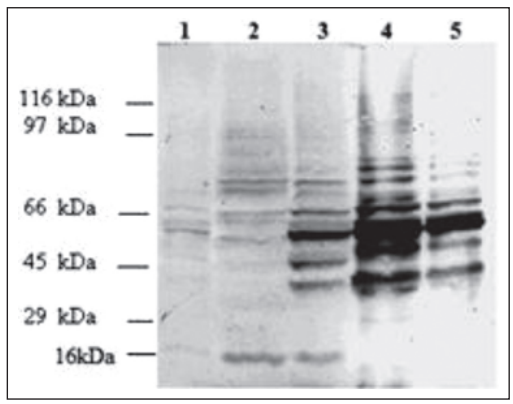

Figure 7. Western blot analysis of reactive *leishmania* promastigotes and amastigote antigens, with VL pateint serum. (1, 3: *L. donovani* amastigotes, 2: *L. major* amastigotes, 4: *L. donovani* promastigotes, 5: *L. major* promastigotes). The positions of molecular weight markers were indicated.

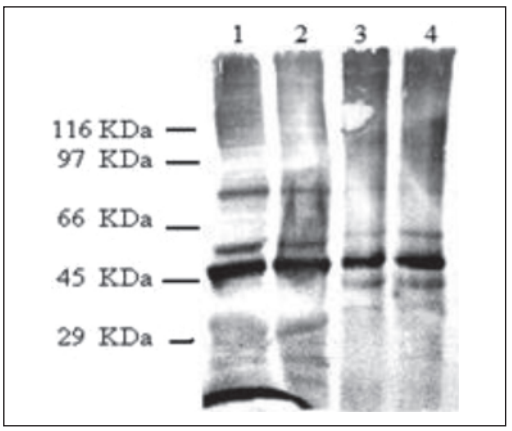

Figure 8. Western blot analysis of reactive *leishmania* promastigotes and axenic form antigens, with VL pateint serum. (1: *L. major* promastigote, 2: *L. donovani* promastigote, 3: *L. major* axenic form, 4: *L. donovani* axenic form). The positions of molecular weight markers were indicated.

Concurrent infection of murine J774 cell line by *L. major* and *L. donovani* promastigote had no impact on the productivity of connection and intrusion of macrophages. Anyway, macrophages all the while co infected by the two *leishmania* species, hinted at vacuolation and demise 6 days post infection figure. kDNA PCR: in light of the measure of the DNA intensified band, utilizing *leishmania* family particular preliminary (AJS3/DBY) *L. donovani* complex was related to an amplicon of 800bp, and *L. major* with an amplicon of 700bp. Two enhanced DNA bands of 700bp and 800bp were recognized in macrophages at the same time infected with *L. major* and *L. donovani* (Fig. 9).

DISCUSSION

The vast majority of the learning about leishmania antigens is identified with the promastigote stage that lives in the insect vector and can be cultured in vitro at 26°. Little information is accessible about the intracellular stage (amastigotes), or, in other words, the infection in the mammalian host.

In the current examination, fruitful *in vitro* culture formwork for the age and nonstop spread of substantial amounts of amastigotes and axenic form was accomplished for both *L. major* and *L. donovani*.

In this study *L. major* was more quicker in attachment, invasion and infectivity to macrophage *in vitro* than *L. donovani*, this was most likely because of the high motility,

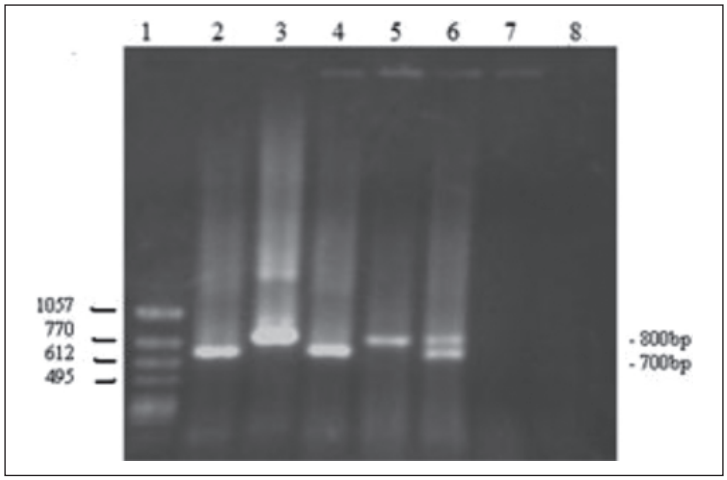

Figure 9. PCR amplification of *leishmania* kDNA. DNA of *leishmania* extracted from culture and J774 macrophages infected with *leishmania* parasites. EB stained agarose gel electrophoreses of PCR amplified kDNA. (Lane 1: DNA marker, Lane 2: *L. major* promastigote, Lane 3: *L. donovani* promastigote, Lane 4: *L. major* amastigote, Lane 5: *L. donovani* amastigote, Lane 6: co-infection, Lane 7: macrophage, Lane 8: negative control).

and augmentation of *L. major* versus *L. donovani*, despite the fact that *L. major* real utilize indistinguishable receptor ligands from *L. donovani*, on the grounds that the association between *Leishmania* parasite and the macrophage cell line *in vitro* is, for the most part, rely on direct acknowledgment of parasite surface molecules Lipophosphoglycan (LPG), Glycoprotein63 (GP63), and the cell receptors.

The age of axenic form is of significance; as a result of it is comparability to tissue determined amastigotes and for the troubles in acquiring an expansive measure of feasible amastigotes free of host proteins. Encourage amastigotes detached from infected tissues to represent heterogeneous populations at some random time amid infection, which contrasts probably as to their age and phase of improvement in their cell cycle (Joshi *et al*., 1993).

In our examination noteworthy comparability between the axenic and intracellular amastigote was seen as detailed already by (Gupta *et al*., 1996a; Gupta *et al*., 1996b). Proficient parasite change to axenic form was accomplished at pH 5.5, 10% CO_2 and 37°C, when changes in pH, CO_2 or temperature brought about wasteful change with recognizable promastigote form (Alian *et al*., 2004). Immune-dot blot test was performed, amastigote isolated from infected macrophages of *L. donovani* and *L. major*, gave a positive outcome against VL patient sera Figure [5], this implies there was shared vital antigens between the two *Leishmania* species (Handman**,** and Hocking 1982) in spite of the fact that there was no role of humoral immunity to *L. major* (El-Hassan and Zijlstra 2001).

Immunoblotting of proteins concentrates of promastigote, amastigote, and axenic structures with VL sera recognized antigens ranging between 16-110 kDa, the majority of the responsive antigens were likewise identified in coomassie blue stained gels. Regular antigens shared among amastigote and axenic structures, and among amastigote and promastigote. The outcome was in agreement with results mentioned by Gupta *et al.* (2001). An extra band of around 16 kDa was detected in amastigote form Figure [6], which was for the most part, emitted or shedded antigens from infected macrophages. The criticalness of this antigens isn't yet known, but it could be of value for diagnosis and vaccination of leishmaniasis (Coelho *et al*., 2012; Rachel *et al*., 2014).

The lysates staged from co infection of macrophages demonstrated comparable protein similar to single infected cells by either *L. major* or *L. donovani*, when electrophoretically isolated on SDS PAGE. These discoveries propose that simultaneous infection in spite of the fact that could be deadly to macrophages it didn't create new antigens. The discoveries were reinforced by the consequences of western blots, when VL sera perceived comparative antigens in J774 cell line co infected or single infected by *L. major* or *L. donovani*.

No indications of hereditary recombination were distinguished in macrophages co infected by *L. major* and *L. donovani*. Two intensified DNA bands of 800 pb and 700 pb, were detected in macrophages co-infected by *L. donovani* and *L. major* individually, when broke down by kDNA PCR (Fig. 9), this show there was no transcendence in macrophages co infected by both *L. donovani* and *L. major*, and furthermore demonstrate that both *L. donovani* and *L. major* utilize a similar receptor ligands in *in vitro* macrophages infection.

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DISCLOSURE

The authors declare that they have no competing interests.

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