Axenic amastigote cultivation and *in vitro* development of *Leishmania orientalis*

Wetpisit Chanmol[1] • Narissara Jariyapan[2] • Pradya Somboon[2] • Michelle D. Bates[3] • Paul A. Bates[3]

✉ Narissara Jariyapan

njariyapan@gmail.com

[1]Graduate PhD's Degree Program in Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand

[2]Center of Insect Vector Study, Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand

[3]Division of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University, UK

Abstract

Leishmania (*Mundinia*) *orientalis* is a recently described new species that causes leishmaniasis in Thailand. To facilitate characterization of this new species an *in vitro* culture system to generate *L. orientalis* axenic amastigotes was developed. *In vitro* culture conditions of the axenic culture-derived amastigotes were optimized by manipulation of temperature and pH. Four criteria were used to evaluate the resulting *L. orientalis* axenic amastigotes, i.e., morphology, zymographic analysis of nucleases, cyclic transformation, and infectivity to the human monocytic cell line (THP-1) cells. Results revealed that the best culture condition for *L. orientalis* axenic amastigotes was Grace's insect medium supplemented with FCS 20%, 2% human urine, 1% BME vitamins, and 25 µg/ml gentamicin sulfate, pH 5.5 at 35 °C. For promastigotes, the condition was M199 medium, 10% FCS supplemented with 2% human urine, 1% BME vitamins, and 25 µg/ml gentamicin sulfate, pH 6.8 at 26 °C. Morphological characterization revealed six main stages of the parasites including amastigotes, procyclic promastigotes, nectomonad promastigotes, leptomonad promastigotes, metacyclic promastigotes, and paramastigotes. Also, changes in morphology during the cycle were accompanied by changes in zymographic profiles of nucleases. The developmental cycle of *L. orientalis in vitro* was complete in 12 days using both culture systems. The infectivity to THP-1 macrophages and intracellular growth of the axenic amastigotes was similar to that of THP-1 derived intracellular amastigotes. These results confirmed the successful axenic cultivation of *L. orientalis* amastigotes. The axenic amastigotes and promastigotes can be used for further study on infection in permissive vectors and animals.

Keywords *Leishmania* · Thailand · Axenic amastigote · Promastigote · Culture · Zymography

Introduction

At least 21 species of *Leishmania* parasites are known to cause leishmaniasis in humans. Infection results in three major types of disease: cutaneous leishmaniasis (CL); mucocutaneous leishmaniasis (MCL); and visceral leishmaniasis (VL), with about 0.9-1.3 million new cases and 20,000-30,000 deaths being reported annually (Akhoundi et al. 2017). *Leishmania* is a dixenous parasite, with its life cycle comprising extracellular promastigote stages in an insect vector and intracellular amastigote stages in macrophages of a vertebrate host (Ghosh et al. 2003). Transmission from the insect vector to a mammalian host is achieved by inoculation of metacyclic promastigotes (Bates 2007). Promastigote stages can be relatively easily axenically cultivated in various types of media, whereas amastigote stages are more difficult to culture or obtain in large numbers free from host cell contaminants (Schuster and Sullivan 2002). However, the ability to culture amastigotes in axenic culture is extremely useful for studies on the biochemistry and molecular biology of the parasites, and for examining other aspects of their biology such as developmental cycle, infection, pathogenicity, and drug resistance.

Several methods and criteria have been used to characterize axenic amastigotes, comparing their properties to *in vivo* and/or *in vitro* promastigotes and/or intracellular amastigotes. For example, light microscopy (LM) and electron microscopy have been used to observe similarity in morphology (Hodgkinson et al. 1996). Changed status of lectin-mediated agglutination and membrane-bound enzymes, proteinase and nuclease activities, determination of total protein content, production of secretory acid phosphatase, and incorporation of thymidine, uridine and proline have all been used in analysis of biochemical properties (Bates 1994; Saar et al. 1998). In immunochemistry analyses, recognition by amastigote-specific monoclonal antibodies (mAbs) and differential expression of stage-specific antigens and cysteine proteinase-specific antigens have been reported. Genes encoding beta-tubulin and p100/11E or stage-specific genes such as Gp46, A2 and β-tubulin, have been used to characterize molecular properties (Rainey et al. 1991; Li et al. 2017). However, crucial criteria that should also be included in testing of axenic amastigote properties are cyclic transformation or expression of developmentally regulated gene(s) and infectivity or antigenic epitopes (Gupta et al. 2001). To date, axenic amastigotes that have been characterized extensively and are found to be essentially indistinguishable from genuine intracellular amastigotes include those of *Leishmania pifanoi*, *Leishmania mexicana*, and *Leishmania donovani* (Rainey et al. 1991; Bates et al. 1992; Debrabant et al. 2004).

Different culture conditions including temperature, pH and concentration of fetal calf serum (FCS) are required to be optimized for each species or strain (Teixeira et al. 2002). For example, *Leishmania braziliensis* and *Leishmania amazonensis* amastigotes required a relatively low pH (5.4) in the medium to transform, but not *Leishmania infantum chagasi*. Some reports describe the serial cultivation of *Leishmania* axenic amastigotes in cell-free medium with a complex composition, including a mixture of nucleotides and vitamins (Pan 1984), or with different protein sources and rabbit blood lysate (al-Bashir et al. 1992).

Leishmania orientalis is a new species that causes leishmaniasis among Thai patients (Jariyapan et al. 2018). The parasite is in the new subgenus *Mundinia*, previously called "*Leishmania siamensis*" (Espinosa et al. 2018). To facilitate characterization of this new species this study was undertaken in which first an *in vitro* culture system to generate *L. orientalis* axenic amastigotes was developed. *In vitro* culture conditions of the axenic culture-derived amastigotes were optimized by manipulation of temperature and pH. The resulting *L. orientalis* axenic amastigotes were evaluated by four criteria: (1) morphology, (2) zymographic analysis of nucleases, (3) cyclic transformation, and (4) infectivity to the human monocytic cell line (THP-1) cells, to confirm successful establishment of axenic amastigotes.

Materials and methods

Parasite strain

L. orientalis parasites (MHOM/TH/2014/LSCM4) were originally isolated from a skin lesion on the face of Thai woman patient (Jariyapan et al. 2018). The isolated parasites were initially grown as promastigotes in Schneider's insect medium (Sigma-Aldrich, St Louis, MO, USA), pH 6.8 supplemented with 20% (v/v) FCS (Life Technologies-Gibco, Grand Island, NY, USA) at 26 °C. Some were then cryopreserved as parasite culture stock at the Department of Parasitology, Faculty of Medicine, Chiang Mai University. As a routine promastigote culture, parasites were grown at 26 °C in M199 medium, pH 6.8 with Hank's balance salt solution (HBSS) (Hyclone, Logan, UT, USA) supplemented with 10% (v/v) FCS, 2% (v/v) healthy human urine, 1% (v/v) Basal Medium Eagle (BME) vitamins (Sigma-Aldrich, St Louis, MO, USA) and 25 μg/ml gentamicin sulfate (Sigma-Aldrich, St Louis, MO, USA). Promastigotes were subpassaged to fresh medium every 4 days to maintain growth and viability of the parasites.

THP-1 macrophage differentiation

The human monocytic cell line (gift from Dr. Sirida Yangshim) was maintained in RPMI-1640 medium supplemented with 10% (v/v) FCS at pH 7.4 and 37 °C in a 5% CO_2 incubator. To maintain differentiation ability, the cells were subpassaged every 3 days to prevent cell density from exceeding 1×10^6 cells/ml. For cell differentiation, phorbol 12-myristate 13-acetate (PMA) was added into the THP-1 cell culture (2.5×10^5 cells/ml) on day 3 at a final concentration of 10 ng/ml (Jain et al. 2012). Following this, 200 μl of PMA-treated cells was dispensed to each well of 8-well Lab-Tek culture chamber slides. The cells were incubated at 37 °C, 5% CO_2 for 48 h to allow complete differentiation of the cells. After the 48 h incubation period, the cells were washed with pre-warmed culture medium and then used for infection assays.

Generation of THP-1-derived intracellular amastigotes

To generate THP-1-derived intracellular amastigotes, promastigotes in the stationary growth phase were used to infect THP-1 macrophages at a ratio of 10:1 parasites/cell. Cells were then incubated in tissue culture flasks at 35 °C, 5% CO_2. After incubation for 8 h, the non-internalized promastigotes were removed by washing three times with pre-warmed RPMI-1640 medium and incubated in the same medium supplemented with 10% FCS for an additional 72 h. The cells were then washed with serum-free RPMI-1640 medium and removed from the culture plate by using a cell scraper. The cells were then washed and suspended in the serum-free RPMI-1640 medium, and passaged 10 times through a sterile 26-gauge needle. The homogenized suspension was centrifuged for 3 min at 300×*g*. The resulting supernatant was recovered and centrifuged at 1,000×*g* for 5 min to collect the amastigotes. The pellet of amastigotes (~ 5×10^6 cells) was resuspended in the RPMI-1640 medium supplemented with 10% FCS and 25 μg/ml of gentamicin sulfate for subsequent experiments.

Ficoll density gradient centrifugation for enrichment of *L. orientalis* nectomonad promastigotes, leptomonad promastigotes, and metacyclic promastigotes

Ficoll density gradient centrifugation for enrichment of *L. orientalis* nectomonad promastigotes, leptomonad promastigotes, and metacyclic promastigotes was adapted from methods described by Späth and Beverley (2001) and Yao et al. (2008) as shown in Fig. 1. Stages of *L. orientalis* parasites were assigned according to the classification described by Rogers et al. (2002). To prepare a 10%-20%-40% discontinuous Ficoll density gradient, a 40% stock solution of Ficoll Type 400 (Sigma, UK) was prepared in phosphate buffered saline (PBS), stored at 4 °C and used within a month. Twenty percent of Ficoll in Schneider's insect medium without serum and 10% Ficoll in M199 medium without serum were prepared on the day that they were used by diluting from the stock solution. The working solutions were then filtered through a 0.22 μm cellulose acetate filter, separately. The 10-20-40% discontinuous Ficoll density gradient was prepared in a 15 ml conical tube by carefully loading 2 ml of 40% Ficoll-PBS at the bottom, then 2 ml of 20% Ficoll- Schneider's insect medium in the middle, and 2 ml of 10% Ficoll-M199 medium on the top. The Ficoll density gradient was used immediately to maximize the homogenicity of nectomonad promastigotes, leptomonad promastigotes, or metacyclic promastigotes. Samples of each promastigote form were collected as follows. Initially, THP-1-derived intracellular amastigotes were cultured in M199 medium, 10% FCS supplemented with 2% human urine, 1% BME vitamins, and 25 μg/ml gentamicin sulfate, pH 6.8 at 26 °C. A culture with a high proportion of nectomonad promastigotes was collected on day 3 (passage [P] 0). To produce higher numbers of leptomonad promastigotes and metacyclic promastigotes the promastigote culture (P0) at day 3 was subpassaged into fresh medium and incubated for further 5 days to collect culture with a high proportion of leptomonad promastigotes (P1) and 7 days for a high proportion of metacyclic promastigotes (P1). Each sample of culture parasites was pelleted at 2,000×*g* for 10 min at room temperature. Each pellet sample was resuspended in 2 ml Schneider's insect medium to adjust parasite density to 2×10^8 cells/ml and gently applied to the top

of the Ficoll density gradient. The gradient was then centrifuged for 15 min at 400×*g* at room temperature. Nectomonad promastigote-enriched and leptomonad promastigote-enriched populations were recovered between 10 and 20% discontinuous gradients, and a metacyclic promastigote-enriched population was found between 0 and 10% discontinuous gradients of the Ficoll interfaces. Each enriched population was carefully collected, transferred into a 1.5 ml sterile microcentrifuge tube, and centrifuged at 2,000×*g* for 10 min at room temperature. The supernatant was discarded and the pellet was stored at -20 °C until used. The percentage of the promastigote-enriched population of each form was quantified by staining with 5% Giemsa's solution and counted under a light microscope.

Optimization of amastigote culture conditions and growth kinetics of *L. orientalis* axenic amastigotes

The culture conditions for generating axenic amastigotes were optimized by varying parameters including temperature and pH. In these experiments, Grace's insect medium was used to investigate the optimum temperature for transformation of promastigotes to amastigotes. Promastigotes (2×10^6 cells/ml) at the stationary phase were used as initial cells. They were subpassaged into 5 ml of the tested media at pH 5.5 and incubated at 32, 34 or 36 °C for 96 h. All media used in this study were supplemented with FCS 20% (v/v), 2% (v/v) human urine, 1% (v/v) BME vitamins, and 25 µg/ml gentamicin sulfate. After 96 h incubation, all cultures were examined for amastigote-like forms under an inverted microscope (Olympus, Tokyo, Japan). After obtaining the temperature that facilitated promastigote transformation to amastigote forms, experiments to optimize pH were performed. Acidity of the culture media was adjusted from acidic to neutral pH levels, at pH 5.5, 6.0, 6.5 and 7.0. All experimental cultures were incubated at the optimum temperature for 5 days. Small aliquots of each culture were collected daily and parasite counts were performed using a hemocytometer (Precicolor HBG, Germany) for the percentage of parasite forms (% parasite forms) and parasite density. Morphological criteria used to identify a typical amastigote form in the culture included an ovoid body form with no flagellum protruding from the flagellar pocket (Rogers et al. 2002). A trypan blue dye exclusion test (Fluka, Buchs, Switzerland) was used to evaluate cell viability.

SDS-PAGE zymography of nucleases

SDS-PAGE zymography described by Joshi et al. (2012) with some modifications was used to analyze nuclease activity against polyadenylic acid (poly (A)) of *L. orientalis* THP-1-derived intracellular amastigotes, promastigote forms, and axenic amastigotes. Cell lysate (~ 5×10^6 cells) of each sample was lysed in a lysis buffer containing 1% SDS, 25 mM Tris/192 mM glycine pH 8.5, 50 µg/ml leupeptin, mixed with a non-reducing buffer for SDS-PAGE and boiled for 5 min. The prepared sample was loaded in each well and separated on 12.5% (w/v) SDS polyacrylamide gels containing poly (A) as a substrate under non-reducing conditions at the final concentration of 0.3 mg/ml. Electrophoresis was carried out under constant voltage (120 V) at 25 °C for 2 h 30 min. After the separation, gels were washed at room temperature with

renaturation buffer (100 mM HEPES, 0.1% (v/v) Triton X-100, pH 8.5) for four times and then incubated in the same buffer for 45 min at 37 °C. Subsequently, gels were rinsed and fixed with 7.5% (v/v) acetic acid aqueous solution before staining with Toluidine Blue O and de-staining with deionized water. Activity of nucleases against poly (A) was visible as clear bands within a blue staining gel.

Developmental cycle of *L. orientalis in vitro*

To investigate the developmental cycle of *L. orientalis in vitro*, initially, axenic amastigotes (1×10^6 cells/ml) were cultured in M199 medium supplemented with 10% (v/v) FCS, 2% (v/v) human urine, 1% (v/v) BME vitamins, and 25 µg/ml gentamicin sulfate, pH 6.8 at 26 °C. When the parasite population was largely composed of metacyclic promastigotes, the parasites (1×10^6 cells/ml) were then transferred to the optimal culture medium for axenic amastigotes. During cultivation, aliquots to prepare cell lysates of each culture were harvested daily by centrifugation of parasites at 2,000×*g* for 10 min, and washed three times with PBS. The collected samples were used for SDS-PAGE zymography for nucleases as described here. Also, cell density was recorded daily throughout the period of cultivation. Experiments were performed in triplicate.

Light microscopy

The morphological characteristics of the parasites including cell body length and width, flagellum length, and nuclear-kinetoplast position were measured and analyzed using an Olympus CX31 light microscope (Olympus America Inc., USA) at ×1,000 magnification. Five microliters of culture collected from the axenic culture were smeared on microscopic slides. The slides were air-dried, fixed in methanol and stained with 5% (v/v) Giemsa's stain solution. Morphological forms based on previous descriptions by Rogers et al. (2002) were used to classify the parasites into categories. A minimum of 200 parasites were examined and classified at each time point.

SEM and TEM

For SEM, parasites were collected from the exponential phase of the culture. Parasites were allowed to settle on poly-L-lysine coated glass coverslips and fixed with 2.5% glutaraldehyde in 0.1 cacodylate buffer (pH 7.2) for 1 h at 4 °C. After washing with the same buffer, the cells were dehydrated in a graded series of ethanol (50%, 70%, 90%, 95% for 10 min each and then twice with 100% ethanol for 30 min each), followed by critical point drying in liquid CO_2 and coated with gold particles. The gold-coated preparations were examined under a scanning electron microscope, JEOL JSM-5910 (JEOL, Tokyo, Japan), at 25-30 kV.

For TEM, the harvested culture parasites were pelleted and washed twice with PBS pH 7.2. The samples were fixed in 2.5% glutaraldehyde and 5 mM $CaCl_2$ in 0.1 M cacodylate buffer pH 7.2 for 1 h or overnight at 4 °C and then

washed in the same buffer and post fixed in 1% osmium tetraoxide, 0.8% potassium ferrocyanide and 5 mM $CaCl_2$ in 0.1 M cacodylate buffer pH 7.2. The samples were then washed with 0.1 M cacodylate buffer pH 7.2 and subsequently dehydrated in a graded acetone series (50%, 70%, 90%, 95% for 10 min each and then twice with 100% acetone for 30 min each). Finally, the samples were embedded in a mixture of Araldite-Epon. Ultra-thin sections were cut using Leica Ultramicrotome UCT (Leica, Austria). Sections were stained with lead citrate and 1% uranyl acetate. The stained sections were examined under a transmission electron microscope, JEOL JEM-2200 FS (JEOL, Tokyo, Japan), at 80 kV.

Comparison of infectivity in THP-1 cells between THP-1-derived intracellular amastigotes and axenic amastigotes

THP-1 macrophages were grown in eight-chamber Lab-Tek tissue culture slides and infected at a 5:1 parasite to macrophage ratio with either the THP-1-derived intracellular amastigotes or the axenic amastigotes for 8 h at 35 °C in 5% CO_2. Subsequently, these cultures were washed three times with pre-warmed RPMI-1640 medium to remove non-internalized parasites. The chamber slides containing the infected macrophages were incubated at 35 °C, 5% CO_2. Then slides were incubated for an additional 16, 40 or 64 h, fixed, stained with 5% (v/v) Giemsa's stain solution and processed for light microscopy (Debrabant et al. 2004). Experiments were performed in triplicate. A minimum of 200 macrophages was counted from each chamber. Results of these experiments were expressed as the percentage of infected macrophages, the average number of amastigotes per macrophage, and the infection index. The infection index was determined by multiplying the percentage of infected macrophages by the average number of amastigotes per macrophage (Paladi et al. 2012).

***In vitro* intracellular amastigote growth**

Evaluation of amastigote replication was performed at 8, 24, 48, and 72 h post infection by light microscopic determination of average infection index of 200 Giemsa-stained macrophages. Experiments were performed in triplicate. To allow comparison, the infectivity at 8 h post-infection was used as an internal baseline control (T0). Amastigote multiplication ratio was calculated using the following formula:

$$\text{Amastigote multiplication ratio} = \frac{\text{no. of amastigotes at Tx}}{\text{no. of amastigote at T0}}$$

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6.0 software. Statistical differences between intracellular and axenic amastigotes were determined using two-way ANOVA with Bonferroni's *post hoc* multiple comparisons for infection index and intracellular multiplication ratio. Tests were considered statistically significant if $P < 0.05$.

Results

Zymographic analysis of nucleases of THP-1-derived intracellular amastigotes and promastigote forms of *L. orientalis*

To search for markers to differentiate between amastigotes and promastigote forms, SDS-PAGE zymography of nucleases was used to analyze nuclease profiles of pure *L. orientalis* intracellular amastigotes harvested from infected THP-1 macrophages (100% purity), procyclic promastigotes collected on day 2 (~ 89% purity), nectomonad promastigote-enriched population (~ 85% purity), leptomonad promastigote-enriched population (~ 88% purity), and metacyclic promastigote-enriched population (~ 84% purity) (Table 1). The nuclease profile of the THP-1-derived intracellular amastigotes was detected on the gel with molecular masses of 27, 29, and 32 kDa while the promastigote forms processed nuclease bands with molecular masses of 27, 29, 30, and 32 kDa with different intensities except for the metacyclic promastigote-enriched population in which no 29 kDa band was observed (Fig. 2). Therefore, the differences of the zymographic profiles of nucleases between the intracellular amastigotes and promastigote forms were used as markers to identify *in vitro* axenic amastigotes in further experiments.

Optimization of conditions for *L. orientalis* axenic amastigote cultivation

To generate axenic amastigotes of *L. orientalis*, optimizations of temperature and pH for cultivation were performed. Initially, to optimize temperature that allows promastigotes to transform to amastigotes, Grace's insect medium supplemented with FCS 20% (v/v), 2% (v/v) human urine, 1% (v/v) BME vitamins, and 25 μg/ml gentamicin sulfate and adjusted pH to 5.5 was used and temperature was varied at 32, 34 and 36 °C. Results showed that after incubation for 96 h at 32 °C, all parasites were in promastigote forms (still in possession of an external flagellum). No amastigote-like form was found. At 34 °C some amastigote-like forms, characterized by an ovoid body form with no flagellum protruding from the flagellar pocket, were observed using LM (Fig. 3a). Ultrastructural morphology of the axenic amastigotes was revealed by TEM (Fig. 3b). General features of *L. orientalis* amastigotes included an ovoid shape with a body width of 2-4 μm, a short non-emergent flagellum, and the absence of a paraflagellar rod. At 36 °C most parasites died.

The acidity of culture media was also varied by adjusting the pH of the culture medium from acidic to neutral levels at pH 5.5, 6.0, 6.5 and 7.0. Promastigote transformation into amastigotes occurred within 24 h in all tested pH at 34 °C. However, it was only in the media with pH 5.5 and 6.0 that all promastigotes had completely transformed into amastigotes on day 5 of cultivation with final cell density 22.72×10^6 and 21.76×10^6 cells/ml, respectively. At 34 °C, pH 5.5 on day 5, cell viability evaluated by trypan blue dye began to fall (lower than 95%), unless cultures were supplemented with fresh medium. When the cultured amastigotes from these conditions were subcultured in fresh culture medium, pH 5.5 and incubated at 34 °C, a low growth rate was obtained (a mean doubling time = 153.9 ± 19.57 h) with

approximately 98% of viable cells. However, when the duplicated cultured amastigotes were incubated at 35 °C, the growth rate of the parasites increased with a mean doubling time of 22.76 ± 0.07 h. Therefore, the axenic amastigotes were subpassaged every 4 days in fresh Grace's insect medium, supplemented with FCS 20% (v/v), 2% (v/v) human urine, 1% (v/v) BME vitamins, and 25 µg/ml of gentamicin sulfate, pH 5.5 and incubated at 35 °C. Under these culture conditions, it was possible to maintain amastigote cultures continuously for a period of at least 3 months, involving more than 22 subpassages.

Zymographic analysis of nucleases of *L. orientalis* axenic amastigotes

The zymographic profiles of nucleases of the axenic amastigotes (P0, P1, P5, P10, and P20) (Fig. 4) were similar to that of the intracellular amastigotes derived from infected THP-1 macrophages but different from *in vitro* promastigotes (as shown in Fig. 2).

Cyclic transformation and development of *L. orientalis* in axenic culture

To investigate cyclic transformation and generate a complete life cycle of *L. orientalis in vitro*, two culture systems for cultivation of amastigotes and promastigotes were combined. Axenic amastigotes (1×10^6/ml) were used as initial cells and cultured in M199 medium supplemented with 10% (v/v) FCS at pH 6.8, and incubated at 26 °C. Under these conditions the amastigotes transformed to promastigote forms within 24 h. During days 1-7 of cultivation, various promastigote forms were observed and identified. These included procyclic promastigotes, nectomonad promastigotes, leptomonad promastigotes, metacyclic promastigotes and paramastigotes. Morphological categories and the fine structure of six developmental forms of *L. orientalis* in the axenic culture are shown in Table 2 and Fig. 5. Morphometric data including body length, width, flagellum length and nucleus-kinetoplast position of the parasites at each developmental stage are shown in Table 3.

The relative percentages of amastigotes and several developmental forms of promastigotes were determined from Giemsa-stained smears (Fig. 6a). Procyclic promastigotes were observed at the highest proportion on day 1 after subculturing (~ 78%). The procyclic population was higher than other forms on days 1-4 (~ 70%) and decreased after day 5 until its proportion was near zero on day 7. Nectomonad promastigotes were observed from days 2-7 with a proportion of ~ 20% on day 2 and the highest population on day 3 (~ 25%) and then the proportion had decreased to ~ 10% on day 7. Leptomonad promastigotes were found on days 3-7 but dominated on days 5-7 with the proportion of ~ 40-50%. Metacyclic promastigotes were observed from day 5 and increased continuously until day 7. Paramastigotes were observed from day 6 but the peak of population was found on day 7 (~ 27%). When the promastigotes were left in the same culture for 10 days the paramastigote population started to decrease to ~ 10% and ~ 3% on day 8 and day 10, respectively. At the stationary phase on day 7, the highest population of metacyclic promastigote forms (~ 20%) was

obtained (Fig. 6a). Then, these parasites (1×10^6/ml) were subcultured into Grace's insect medium supplemented with FCS 20% (v/v), 2% (v/v) human urine, 1% (v/v) BME vitamins, and 25 μg/ml gentamicin sulfate, pH 5.5 and incubated at 35°C (the optimal medium and condition for axenic amastigotes). The metacyclic promastigotes resorbed their flagella and adopted a rounded morphology within 24 h. On day 8, ~ 15 % of the rounded aflagellate population and ~ 85% of an intermediate form population (round body cells with a flagellum shorter than body length or a short stump) were observed. The amastigote-like forms subsequently propagated in the axenic culture. Amastigote-like forms accounted for 100% of the parasite population were observed on day 12 onwards (Fig. 6a). Parasite density in the cultures from days 1-12 is shown in Fig. 6b.

Nuclease zymographic profiles were used to assist in defining life cycle forms of *L. orientalis*. Therefore, changes in the zymographic profiles of the parasites harvested daily during their developmental cycle in the *in vitro* cultures were analyzed. The axenic amastigotes (day 0) possessed three prominent bands of the apparent molecular masses 27, 29 and 32 kDa. Upon transformation to promastigotes, the intensity of the bands was decreased from days 1-6 and on day 7, the 29 kDa band was undetectable. In addition, from days 4-7 an additional band with a molecular mass of 30 kDa was observed when the promastigote population was predominant. After transferring the promastigotes to the Grace's insect medium under the optimum condition for amastigote culture, the 30 kDa band completely disappeared on day 8. Also, a marked increase in intensity of the 27, 29 and 32 kDa bands was noted, thus the nuclease profile of the amastigotes was restored from days 8-12 (Fig. 7).

Comparison of infectivity in THP-1 cells and intracellular growth between axenic amastigotes and THP-1-derived intracellular amastigotes

Infectivity of the axenic amastigotes and the THP-1-derived intracellular amastigotes in THP-1 cells was determined and compared (Fig. 8a). The infection index of axenic amastigotes was relatively higher than that of the intracellular amastigotes during the first 48 h of infection. However, at the 72 h after infection, the infection indices of both axenic amastigotes and intracellular amastigotes were not statistically different and the average number of parasites per cell was similar (approximately four parasites/cell). An increase in the amastigote multiplication ratio was observed at all time points in both axenic and THP-1 derived amastigotes but no statistical difference in the ratios was found (Fig. 8b).

Discussion

In this study, the axenic cultivation of *L. orientalis* amastigotes was successfully established for the first time, which has also not been previously reported for any other member of the new subgenus *Mundinia*. This *in vitro* culture system was devised to mimic some of the environmental conditions that intracellular amastigotes would encounter within the phagolysosomal system of macrophages of vertebrate hosts including temperature and acidic pH. Since *L. orientalis*

causes CL (Jariyapan et al. 2018), the optimum temperature obtained in this study for growing axenic amastigotes was relevant to its clinical manifestation. *L. orientalis* amastigote-like forms were observed after incubation for 96 h at 34 °C but all promastigotes completed their transformation to amastigotes at 35 °C. Not only the temperature but also the acidity of the culture medium is an essential factor for transformation and retention of the amastigote morphology. *L. orientalis* was able to retain the morphology as amastigotes at pH 5.5. These finding were similar to that demonstrated in other species of *Leishmania* (Debrabant et al. 2004). This adaptation may partly account for the ability of amastigotes to survive and multiply within the acidic environment of the phagolysosomes *in vivo*. Several physiological activities of amastigotes such as respiration, catabolism of energy substrates and incorporation of precursors into macromolecules are carried out optimally at pH 4.5-5.5, whereas these activities are optimal at or near neutral pH for promastigotes (Moradin and Descoteaux 2012). Therefore, the optimum condition for generation of *L. orientalis* axenic amastigotes in this study was using Grace's insect medium, supplemented with FCS 20% (v/v), 2% (v/v) human urine, 1% (v/v) BME vitamins, and 25 µg/ml of gentamicin sulfate, pH 5.5, incubated at 35 °C. As revealed by LM and TEM *L. orientalis* axenic amastigotes processed general morphological features (ovoid in shape, 2-4 µm on the body width, a short non-emergent flagellum, and no paraflagellar rod) similar to that reported for other *Leishmania* species (Gupta et al. 2001; Sunter and Gull 2017).

Leishmania species that cause the same or different form of disease may require specific culture conditions, for example, *L. mexicana*, a causative agent of CL, amastigotes can be cultivated axenically under conditions of 32 °C, pH 5.5 (Bates 1994). In *Leishmania* species that cause VL, for example, *L donovani* and *Leishmania tropica*, these species required incubation at 37 °C, pH 5.5 with CO_2 to transform into axenic amastigote-like form (Debrabant et al. 2004). In the case of *L. amazonensis*, a species associated with cutaneous and diffuse cutaneous leishmaniasis, extracellular amastigote-like form can be maintained in axenic culture at 32 °C, pH 4.6. It grows in continuous culture at a lower temperature and pH than any other species characterized to date (Hodgkinson et al. 1996).

Analysis of the nuclease profile of *L. orientalis* axenic amastigotes at subpassages P0, P1, P5, P10, and P20 by SDS-PAGE zymography revealed that for survival and development the axenic amastigotes processed only three nuclease bands at molecular masses of 27, 29, and 32 kDa with high intensity in all subpassages as found in the THP-1-derived intracellular amastigotes. Procyclic promastigotes, nectomonad promastigotes, and leptomonad promastigotes expressed the nuclease activity at molecular masses of 27, 29, 30, and 32 kDa apart from metacyclic promastigotes in which the 29 kDa band was undetectable. These results indicate that the 30 kDa nuclease was specific in all promastigote forms and might be important for their development. In the case of the 29 kDa nuclease, it might be unnecessary for metacyclic promastigotes in their development and/or other functions, for example, infection. Identification and characterization of these enzymes in their type, kinetics and other properties should be performed to investigate their roles in each parasite forms and compare to other *Leishmania* species.

One of the important criteria used to characterize axenic amastigotes is ability of the axenic amastigotes to

differentiate back to the promastigote forms on transfer to promastigote growth conditions and *vice versa*. This study demonstrated that *L. orientalis* axenic amastigotes could transform to promastigote forms within 24 h and multiply in the M199 medium, pH 6.8 at 26 °C and the promastigotes that had converted back from amastigotes could also transform to amastigote form in the Grace's insect medium, pH 5.5 at 35 °C and stay in the cycle *in vitro* for at least 20 passages.

The *in vitro* developmental cycle of *L. orientalis* was completed within 12 days. The sequence of morphological forms of promastigotes in the cycle of *L. orientalis* resembled the forms in the natural life cycle of *L. mexicana* in a sand fly vector (*Lutzomyia longipalpis*) (Rogers et al. 2002). The complete developmental cycle was initiated with amastigotes which subsequently differentiated into procyclic forms on day 1. The procyclic population was higher on day 1-4 than other forms present, i.e., amastigotes, nectomonad promastigotes and leptomonad promastigotes, and also dividing procyclic promastigotes were observed indicating that the procyclic promastigotes multiplied in the culture. This form is responsible for the initial establishment of infection in sand flies. Then, some of them developed to be nectomonad promastigotes from day 2-7 with the highest population on day 3 and then the proportion had dropped to ~ 10% on day 7. This is correlated with a biological property of nectomonad promastigotes in sand flies that this form is not a proliferative stage as reported by Rogers et al. (2002).

On day 3, the nectomonad promastigotes began to transform into leptomonad form. The leptomonad promastigotes dominated on day 5-7 and its proportion peaked on day 6 while the proportion of the procyclic promastigotes and the nectomonad promastigotes decreased to be less than 10% and 20%, respectively. Leptomonad promastigotes were also found in both dividing and rosette forms. The results indicated that the leptomonad form was responsible for parasite propagation. Finally, the leptomonad promastigotes differentiated into metacyclic form from day 5. The highest proportion of the metacyclic promastigotes was found on day 7 at a proportion of ~ 20%. However, on day 7 the proportion of the leptomonad promastigotes was ~ 43%. It is consistent with the study by Rogers et al. (2002) that leptomonad promastigotes are found ~ 30-40% after metacyclic promastigotes begin to dominate in sand flies. Leptomonad promastigotes have an important role in generation of the promastigote secretory gel (PSG). The PSG blocks the anterior parts of the sand fly midgut coincident with the accumulation of metacyclic promastigotes.

In addition, in this study, paramastigotes were observed from day 6 but the peak of the paramastigote population was found on day 7 at the proportion of ~ 27%. Then, the proportion fell to 3% when they were left until day 10 (data not shown). Paramastigotes could be aberrant cells, where the kinetoplast is adjacent to the nucleus (Sunter and Gull 2017). The role of paramastigotes in the sand fly vector remains unknown. In this study, no haptomonad promastigotes were found *in vitro*. Haptomonad form has been reported only in insect vectors (Rogers et al. 2002; Bates 2018). In sand flies, haptomonad promastigotes are anchored to the chitin surface of the anterior midgut by their flagella. These promastigote forms, together with the PSG, create the blocked fly that is essential for transmission (Bates 2018).

When the parasites on day 7 were subcultured into the medium for amastigote culture, Grace's insect medium,

pH 5.5 at 35 °C, the metacyclic promastigotes transformed to the amastigote-like form within 24 h and propagated until they accounted for 100% of the parasite population on day 12. This result was similar to the previous study in *L. mexicana* (Bates 1994).

Zymographic profiles of nucleases were used as biochemical markers to assist in defining the developmental cycle forms of *L. orientalis*. In this study, the change in morphological form of parasites was correlated with changes in the profiles of the enzymes. As discussed above, the nuclease with the apparent molecular mass of 30 kDa was found specifically in the promastigote forms of *L. orientalis*. It correlates with the results of the nucleases expressed by parasites harvested on day 4-7 in which only promastigote forms were observed. More characterization of the stage specific nuclease is required as the enzyme might be involved in different metabolisms between promastigotes and amastigotes.

To investigate another biological property with regard to infectivity, the ability to infect THP-1 macrophages between the axenic amastigotes and the THP-1-derived intracellular amastigotes was compared. Results revealed that both axenic amastigotes and intracellular amastigotes had the similar infection indices, average number of parasites per cell and amastigote multiplication ratio at 72 h post infection indicating that the *L. orientalis* axenic amastigotes had significant infectivity and intracellular growth *in vitro* in human macrophages.

In summary, the axenic cultivation of *L. orientalis* amastigotes was successfully established. The developmental cycle of *L. orientalis in vitro* was complete in 12 days using two culture systems: (1) Grace's insect medium supplemented with FCS 20%, 2% human urine, 1% BME vitamins, and 25 μg/ml gentamicin sulfate, pH 5.5 at 35 °C for amastigotes and (2) M199 medium, 10% fetal calf serum supplemented with 2% human urine, 1% BME vitamins, and 25 μg/ml gentamicin sulfate, pH 6.8 at 26 °C for promastigotes. All analyzed properties of the *L. orientalis* axenic amastigotes including morphology, biochemical properties, cyclic transformation, and infectivity to THP-1 cells were similar to the THP-1-derived intracellular amastigotes.

Leishmania parasites in the new subgenus *Mundinia* include *Leishmania enriettii, L. martiniquensis, Leishmania macropodum* (previously called '*Leishmania* sp. AM-2004'), and *L. orientalis* (previously called '*L. siamensis*') (Barratt et al. 2017; Jariyapan et al. 2018; Espinosa et al. 2018). Only *L. martiniquensis* and *L. orientalis* have been reported to infect humans (Jariyapan et al. 2018; Pothirat et al. 2014; Chiewchanvit et al. 2015). So far, few studies regarding *L. orientalis* have been conducted (Siripattanapipong et al. 2018). These authors have detected '*L. siamensis*' DNA in one female sand fly, *Sergentomyia iyengari*, however, no development of infection has been observed and transmission through the sand fly bite has not been determined. Although the proven vectors of *Leishmania* parasites are all sand flies of various species (Bates, 2007), *L. enriettii* can develop late stage infections in the biting midge *Culicoides sonorensis* and grows aggressively, producing large, ulcerated, tumor-like lesions, in guinea pigs (Seblova et al. 2015). Successful infection of *C. sonorensis* with *L. enriettii* after feeding on the ears and nose of these guinea pigs highlights that vector(s) other than sand flies should be considered on parasites belonging to the members of the subgenus *Mundinia*. Further

investigations of *L. orientalis*'s development in permissive vectors, such as *L. longipalpis* and *C. sonorensis*, would provide a clue for speculation on vector(s) of the parasites in nature. The availability of large quantities of uniform populations (axenic amastigotes) of *L. orientalis* would be beneficial for studies on infection and transmission mechanisms of this parasite species.

In conclusion, to our knowledge, this study is the first successful generation and continuous propagation of axenic amastigotes of *L. orientalis*. Also, its complete developmental sequence in the axenic culture was described. Both *in vitro* culture systems would provide a useful tool for the generation of large amounts of pure and viable parasite populations of each stages or forms for further studies on cell and molecular biology, biochemistry, and others, especially, mechanisms involved in infection, survival and development in permissive vector(s) and host(s). These results would be useful and invaluable in increasing the understanding of *L. orientalis* biology and for developing strategies to control and eliminate *Leishmania* parasites.

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Compliance with ethical standards

Conflict of interests

The authors declare that they have no conflict of interests.

Author contributions

NJ and PAB conceived and designed study. WC and MDB performed research. NJ, PS, and WC analyzed data. NJ, WC and PAB wrote the paper. All authors read and approved the final version of the manuscript.

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Table 1 Percent purity of parasite forms of *L. orientalis* expressed as mean ± standard deviation

Form	Days of collection				
	0[a]	2[b]	3[b]	5[c]	7[c]
Intracellular amastigote	100	10.33 ± 1.53	0.33 ± 0.58	0	0
Procyclic promastigote	0	89.67 ± 1.53	7.33 ± 0.58	0	0
Nectomonad promastigote[d]	0	0	85.67 ± 1.53	3.67 ± 1.53	9.00 ± 2.00
Leptomonad promastigote[d]	0	0	6.67 ± 0.58	88.00 ± 1.73	6.33 ± 1.15
Metacyclic promastigote[d]	0	0	0	8.33 ± 2.52	84.67 ± 3.06

[a]Pure intracellular amastigotes harvested from infected THP-1 macrophages

[b]Day of collection from passage 0

[c]Day of collection from passage 1

[d]Parasitic form enriched by discontinuous Ficoll gradient centrifugation

Table 2 Morphological categories of *L. orientalis*

Morphological category[a]	Criteria
Amastigote	Oval body form, no flagellum protruding from flagellar pocket
Procyclic promastigote	Body length 8-11.5 um flagellum < body length
Nectomonad promastigote	Body length ≥ 12.5 μm, body width and flagellum length varied
Leptomonad promastigote	Body length 8.0-11.5 μm flagellum ≥ body length
Metacyclic promastigote	Body length < 12.5 μm, body width ≤ 1.5 μm, flagellum > body length
Paramastigote	Kinetoplast adjacent to nucleus, external flagellum present

[a]Based on descriptions by (Rogers et al. 2002)

Table 3 Morphological features of *L. orientalis* including cell body length and width, flagellum length, and nuclear-kinetoplast position measured and expressed as mean ± standard deviation

Morphological category	Morphological feature				
	Body length (μm)	Body width (μm)	Flagellum length (μm)	Anterior-kinetoplast (μm)	Anterior-nucleus (μm)
Amastigote	4.90 ± 0.45	2.34 ± 0.33	-	-	-
Procyclic promastigote	9.33 ± 1.45	2.23 ± 0.35	7.55 ± 2.19	2.28 ± 0.66	4.24 ± 0.67
Nectomonad promastigote	15.10 ± 2.07	2.15 ± 0.30	19.65 ± 3.95	2.60 ± 0.70	5.41 ± 0.79
Leptomonad promastigote	10.68 ± 1.00	2.35 ± 0.32	15.20 ± 3.42	2.24 ± 0.32	4.39 ± 0.54
Metacyclic promastigote	10.04 ± 1.59	1.11 ± 0.22	16.93 ± 2.54	2.15 ± 0.52	4.22 ± 0.76
Paramastigote	8.43 ± 1.58	2.89 ± 0.40	14.63 ± 3.13	2.97 ± 0.73	3.43 ± 0.70

Figure captions

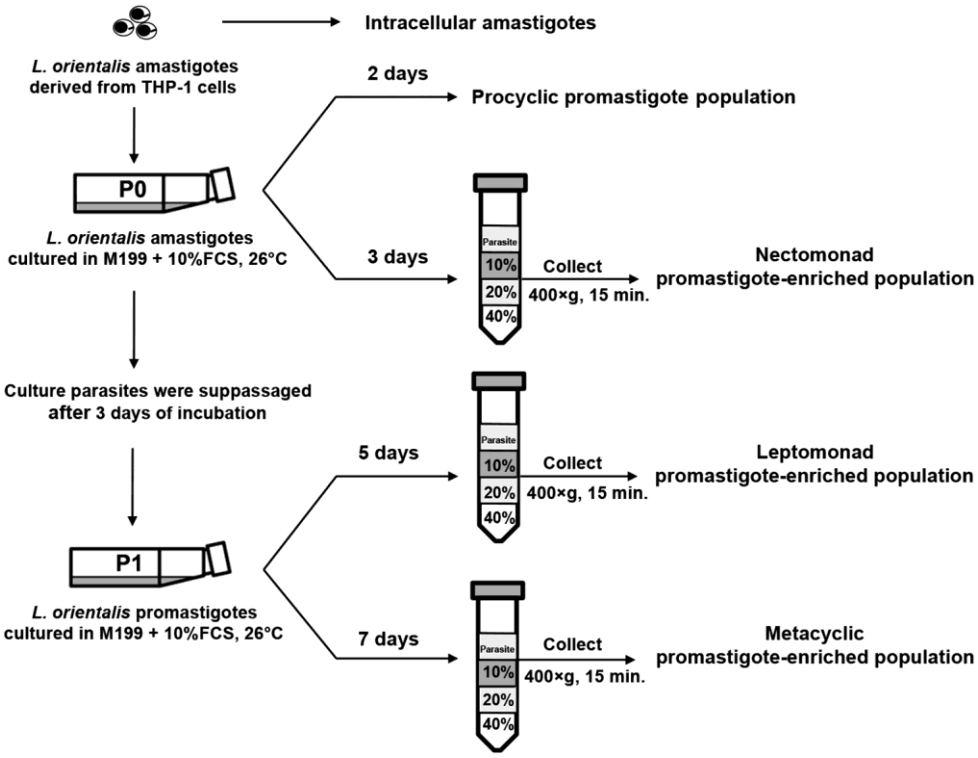


Fig. 1 Schematic illustration for enrichment of *L. orientalis* nectomonad promastigotes, leptomonad promastigotes, and metacylic promastigotes using discontinuous Ficoll gradient centrifugation and collection of samples for zymographic analysis of nucleases

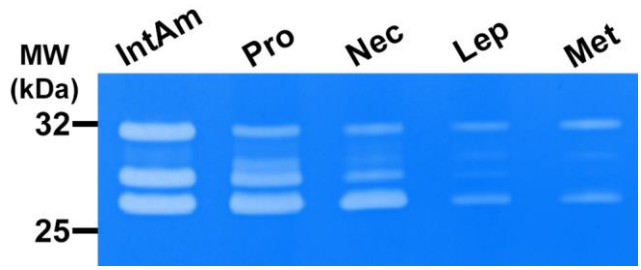


Fig. 2 Zymographic profiles of nucleases of THP-1 derived intracellular amastigotes (IntAm), procyclic promastigote-enriched population (Pro), nectomonad promastigote-enriched population (Nec), leptomonad promastigote-enriched population (Lep), and metacyclic promastigote-enriched population (Met)

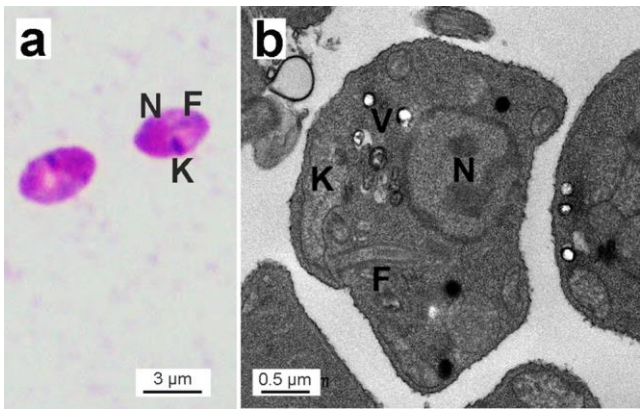


Fig. 3 *L. orientalis* axenic amastigotes showing kinetoplast (K), nucleus (N), flagellum (F), and vacuole (V). **a** LM. **b** TEM

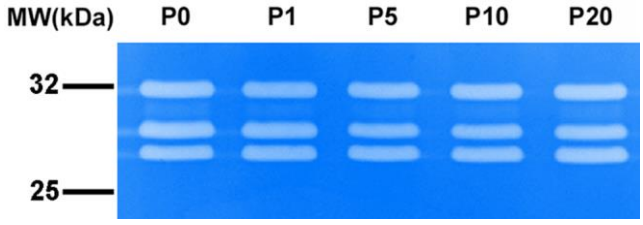


Fig. 4 Zymographic profiles of nucleases of axenic amastigotes from different parasite subpassages (P0, P1, P5, P10, and P20)

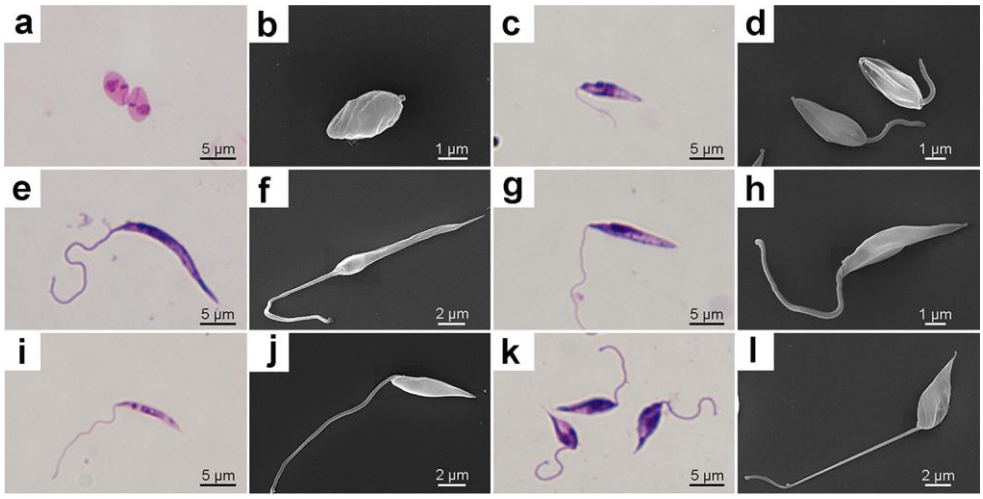


Fig. 5 Six developmental forms of *L. orientalis* in the axenic culture including amastigotes (**a**, **b**); procyclic promastigotes (**c**, **d**); nectomonad promastigotes (**e**, **f**); leptomonad promastigotes (**g**, **h**); metacyclic promastigotes (**i**, **j**); and paramastigotes (**k**, **l**). LM micrographs = **a**, **c**, **e**, **g**, **i**, and **k**. SEM micrographs = **b**, **d**, **f**, **h**, **j**, and **l**

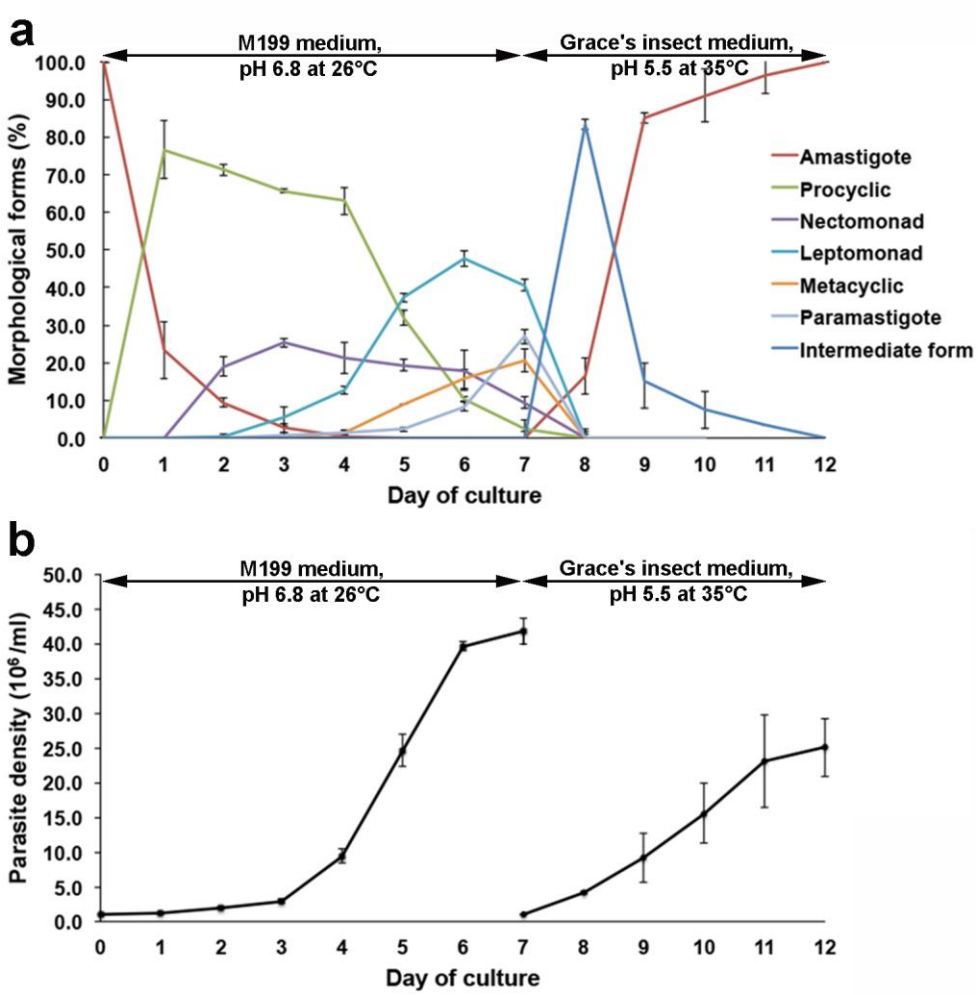


Fig. 6 Sequential development of *L. orientalis* parasites in the culture. **a** Morphological forms present on day 1-12 **b** Parasite density in the cultures from day 1-12

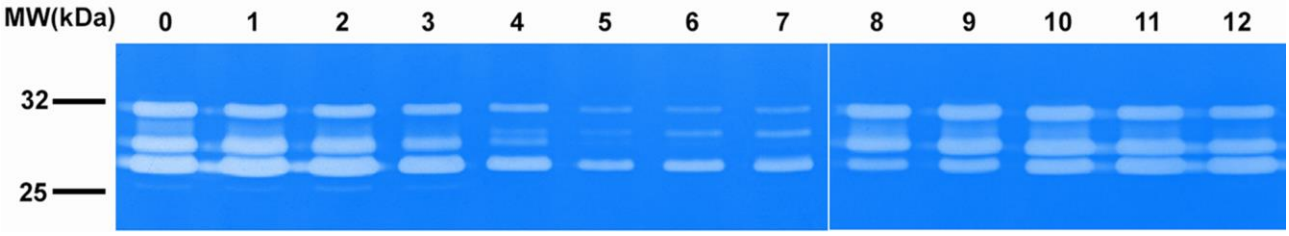
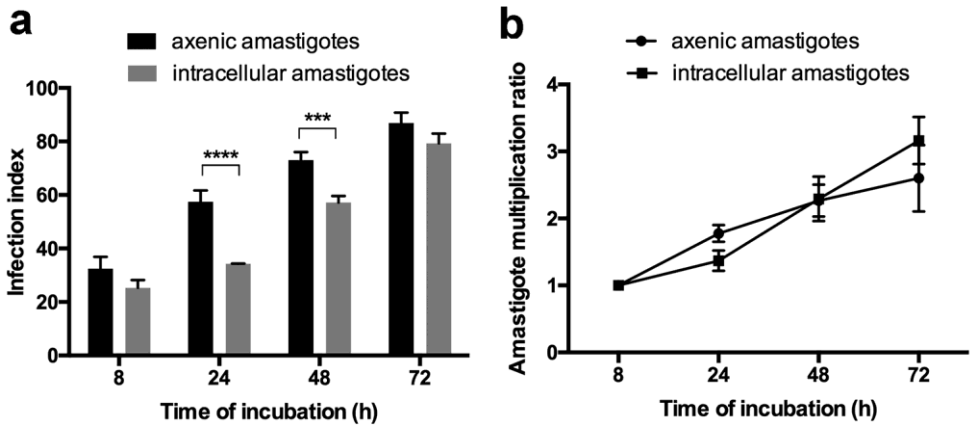


Fig. 7 Nuclease zymographic profiles of parasite lysates from cells harvested throughout the development in axenic culture (day 0-12). Noted that on day 0-7 the parasites were in the M199 medium and on day 8-12 the parasites were in the Grace's insect medium



560

561 **Fig. 8 a** Infection index of *L. orientalis* axenic and THP-1-derived intracellular amastigotes to THP-1 cells at different

562 time points. Results are expressed as mean ± standard deviation and based on three independent replicates. ***P < 0.001;

563 ****P < 0.0001. **b** Amastigote multiplication ratio of *L. orientalis* axenic and intracellular amastigotes in THP-1 cells.

564 Results are expressed as mean ± standard deviation and based on three independent replicates