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Infection Dynamics of a *Leishmania tropica* Isolate in the BALB/c Mouse Model: Effects of Pre-infection Culture Conditions on Experimental Infectivity

Leishmania tropica İzolatının BALB/c Fare Modelinde Enfeksiyon Dinamikleri: Enfeksiyon Öncesi Kültür Koşullarının Deneysel Enfektivite Üzerine Etkisi

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ABSTRACT

Objective: To evaluate the effects of distinct pre-infection culture conditions [RPMI-1640 and Schneider's insect medium (SIM)] on the experimental infectivity of a clinical *Leishmania tropica* isolate and to determine how culture-associated differences are reflected in infection dynamics in the BALB/c mouse model.

Methods: A clinical *Leishmania* isolate, obtained from a patient with cutaneous leishmaniasis and cryopreserved in liquid nitrogen, was recovered in NNN medium. Species identification was performed using an internal transcribed spacer 1-targeted probe-based real-time polymerase chain reaction followed by melting-curve analysis. Following post-thaw recovery, logarithmic-phase promastigotes were transferred to RPMI-1640 or SIM and maintained for five days without serial subpassaging before experimental infection. BALB/c mice (n=5 per group) were subcutaneously inoculated in the right hind footpad with 1×10^7 promastigotes. Infection dynamics were evaluated over six weeks by monitoring footpad diameter, amastigote burden, and promastigote recovery.

Results: Molecular analysis identified the clinical isolate as *L. tropica* (MHOM/TR/2025/CBU152). Mice infected with SIM-derived promastigotes developed visible lesions from week 2. At the same time, longitudinal footpad measurements differed significantly between the SIM and RPMI groups from week 1 onward (Sidak-adjusted $p<0.0001$ at each time point). By week 6, the mean absolute footpad diameter reached 4.22 mm in the SIM group, compared with 3.52 mm in the RPMI group. Amastigote burden was significantly higher in the SIM group than in the RPMI group [median (interquartile range [IQR]): 6 (5-6) vs. 2 (2-2); p=0.0079]. Promastigote recovery on day 7 was also significantly higher in the SIM group [median (IQR): 1.15 (1.12-1.18) vs. 0.15 (0.14-0.16)$\times10^6$/mL; p=0.0079]. No evidence of infection was observed in the control group.

Conclusion: Pre-infection culture conditions significantly influence the experimental infectivity of an *L. tropica* isolate in BALB/c mice. SIM-derived promastigotes exhibited a more infectious phenotype than RPMI-derived promastigotes, supporting SIM preconditioning as a useful approach for establishing experimental *L. tropica* infection in BALB/c mice.

Keywords: BALB/c mouse model, cutaneous leishmaniasis; *Leishmania tropica*, experimental infectivity, Schneider's insect medium

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ÖZ

Amaç: Bu çalışmada, farklı enfeksiyon öncesi kültür koşullarının [RPMI-1640 ve Schneider's insect medium (SIM)] klinik bir *Leishmania tropica* izolatının deneysel enfektivitesi üzerindeki etkisinin değerlendirilmesi ve kültür koşullarıyla ilişkili farklılıkların BALB/c fare modelindeki enfeksiyon dinamiklerine nasıl yansıdığının belirlenmesi amaçlanmıştır.

Yöntemler: Kutanöz leishmaniasisli bir hastadan elde edilerek sıvı azotta kriyoprezerve edilen klinik *Leishmania* izolatı NNN besiyerinde canlandırılmıştır. Tür tanımlaması, transkripsiyonlar arası bölge 1 bölgesini hedefleyen prob tabanlı gerçek zamanlı polimeraz zincir reaksiyonu ve ardından erime eğrisi analizi kullanılarak gerçekleştirilmiştir. Kriyoprezervasyon sonrası canlandırılan logaritmik fazdaki promastigotlar RPMI-1640 veya SIM besiyerine aktarılmış ve deneysel enfeksiyon öncesinde seri alt pasaj yapılmaksızın beş gün süreyle kültüre edilmiştir. BALB/c farelere (her grupta n=5) sağ arka ayak tabanından subkütan yolla 1×10^7 promastigot inoküle edilmiştir. Enfeksiyon dinamikleri; ayak tabanı çapı, amastigot yükü ve promastigot geri kazanımı değerlendirilerek altı hafta boyunca izlenmiştir.

Bulgular: Moleküler analiz sonucunda klinik izolat *L. tropica* (MHOM/TR/2025/CBU152) olarak tanımlanmıştır. SIM kaynaklı promastigotlarla enfekte edilen farelerde gözle görülür lezyon gelişiminin 2. haftadan itibaren ortaya çıktığı belirlenmiştir. Longitudinal ayak tabanı ölçümlerinin ise SIM ve RPMI grupları arasında 1. haftadan itibaren anlamlı farklılık gösterdiği saptanmıştır (her bir zaman noktası için Sidak-düzeltilmiş p<0,0001). Altıncı haftada ortalama mutlak ayak tabanı çapı SIM grubunda 4,22 mm, RPMI grubunda ise 3,52 mm olarak belirlenmiştir. Amastigot yükünün SIM grubunda RPMI grubuna kıyasla anlamlı düzeyde daha yüksek olduğu saptanmıştır [medyan (çeyrekler arası aralık [ÇAA]): 6 (5-6) ve 2 (2-2); p=0,0079]. Yedinci gündeki promastigot geri kazanımının da SIM grubunda anlamlı düzeyde daha yüksek olduğu belirlenmiştir [medyan (ÇAA): 1,15 (1,12-1,18) ve 0,15 (0,14-0,16) $\times10^6$/mL; p=0,0079]. Kontrol grubunda enfeksiyon bulgusuna rastlanmamıştır.

Sonuç: Enfeksiyon öncesi kültür koşulları, bir *L. tropica* izolatının BALB/c farelerindeki deneysel enfektivitesini anlamlı düzeyde etkilemektedir. SIM kaynaklı promastigotlar, RPMI kaynaklı promastigotlara kıyasla daha enfektif bir fenotip sergilemiş ve bu bulgular, SIM'de enfeksiyon öncesi kültürlemenin BALB/c farelerinde deneysel *L. tropica* enfeksiyonunun oluşturulmasında yararlı bir yaklaşım olduğunu desteklemiştir.

Anahtar Kelimeler: BALB/c fare modeli, kutanöz leishmaniasis, *Leishmania tropica*, deneysel enfektivite, Schneider's insect medium

INTRODUCTION

Cutaneous leishmaniasis (CL) is a major vector-borne disease caused by protozoan parasites of the genus *Leishmania* and transmitted by infected female sand flies (*Phlebotomus* spp. in the Old World) (1). Among the Old World species, *Leishmania tropica* is the principal cause of anthroponotic CL across the Middle East, North Africa, Central Asia, and the Mediterranean basin. Its persistent and often disfiguring skin lesions contribute substantially to chronic morbidity, while current treatments remain limited by toxicity, variable efficacy, cost, and emerging drug resistance (2).

The infectivity of *Leishmania* is determined largely by metacyclogenesis, the differentiation of non-infective procyclic promastigotes into infective metacyclic forms within the sand fly vector. This process is accompanied by morphological, biochemical, and molecular changes that enhance parasite survival and establishment in the mammalian host (3). Under *in vitro* conditions, promastigote differentiation is influenced by multiple culture-associated factors, including medium composition, pH, nutrient availability, serum supplementation, parasite density, growth phase, and passage history (4-6). Consequently, the biological state of culture-derived promastigotes represents an important experimental variable in animal infection studies.

This concept is particularly relevant for *L. tropica,* for which reproducible infection in conventional murine models has historically been more difficult to achieve than in the classical *L. major*-BALB/c model. Rather than producing rapidly progressive lesions, experimental *L. tropica* infection typically follows a chronic, slowly developing, and predominantly non-ulcerative course. Disease outcome is strongly influenced by parasite strain, inoculum dose, developmental stage, route of inoculation, and host-related factors (7,8). Consistent with this variability, a previous study using a human-derived *L. tropica* isolate from Türkiye reported footpad lesions in only four of ten BALB/c mice following subcutaneous inoculation of 2×10^7 culture-derived promastigotes, with lesions appearing approximately 45 days after infection (9).

Pre-infection culture conditions may contribute to this variability by altering the developmental composition and infectivity-related phenotype of promastigote populations. RPMI-1640 is widely used for routine cultivation of *Leishmania* promastigotes, whereas Schneider's insect medium (SIM) provides a physicochemically distinct environment, originally developed for insect cell culture. Experimental studies have shown that manipulation of culture medium and extracellular pH can enhance metacyclic differentiation and modify infectivity-related phenotypes in several *Leishmania* species, although these effects remain species- and condition-dependent (4-6). More recently, optimized acidic SIM was shown to increase metacyclic promastigote production and enhance complement resistance and macrophage infectivity in *Leishmania (Mundinia) orientalis* (4). However, whether similar culture-dependent effects influence the experimental infectivity of clinical *L. tropica* isolates has not been established.

This study aimed to compare the experimental infectivity of a clinical *L. tropica* isolate after pre-infection cultivation in RPMI-1640 versus SIM, and to determine whether these culture-associated differences are reflected in infection dynamics in BALB/c mice, as assessed by longitudinal footpad changes, tissue amastigote burden, and promastigote recovery.

METHODS

Ethical Approval

This study was approved by the Local Ethics Committee for Animal Experiments, Faculty of Medicine, Manisa Celal Bayar University (date: 29.07.2025, decision no: 77.637.435/330). Written informed consent was obtained from the patient at the time of the original clinical sample collection. The archived parasite isolate used in the present study was de-identified, and no identifiable patient information was accessed.

Preparation of Culture Media

(I) NNN Medium: To prepare NNN medium, 5 g of agar, 2 g of peptone, and 1 g of NaCl were dissolved in 200 mL of distilled water, sterilized by autoclaving at 121 °C for 20 minutes, and

subsequently cooled to room temperature. The solidified base was enriched with 1% gentamicin, 1% penicillin/streptomycin, and 30 mL of defibrinated rabbit blood, then dispensed into sterile slanted tubes and stored at 4 °C for at least 48 hours to allow proper conditioning.

(II) RPMI-1640 Medium: RPMI-1640 was supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, and 1% gentamicin. The pH was monitored using a calibrated pH meter and adjusted to 7.2 by the gradual addition of 1 M HCl or 1 M NaOH, as required. The medium was stored at 4 °C and brought to room temperature before use.

(III) SIM: SIM was was prepared according to the manufacturer's instructions and supplemented with 20% FCS, 1% L-glutamine, and 1% penicillin/streptomycin. The pH was monitored using a calibrated pH meter and adjusted to 5.5 by gradual addition of 1 M HCl or 1 M NaOH, as required. The medium was subsequently sterilized through a 0.22-µm membrane filter, stored at 4 °C, and equilibrated to room temperature before inoculation.

In vitro Maintenance of the Clinical Leishmania Isolate

The clinical *Leishmania* isolate, originally obtained from a cutaneous lesion on the right cheek of a patient with CL in Türkiye and cryopreserved in the Parasite Bank of the Faculty of Medicine at Manisa Celal Bayar University, was retrieved from liquid nitrogen and rapidly thawed in a 37 °C water bath. Following thawing, the promastigotes were immediately transferred onto freshly prepared NNN medium and cultured at 26 °C to allow initial recovery and growth. Promastigote growth was assessed by routine light microscopy, and motile promastigotes in the logarithmic growth phase were confirmed through evaluations performed every 2-3 days (10). No serial passaging was performed in NNN medium. Logarithmic-phase promastigotes obtained from this single post-thaw recovery culture were used directly in the subsequent experimental preconditioning procedure.

Molecular Identification of the Clinical Leishmania Isolate

Molecular species identification of the clinical *Leishmania* isolate was performed using internal transcribed spacer 1 (ITS1) -targeted probe-based real-time polymerase chain reaction (PCR) followed by melting-curve analysis. Briefly, genomic DNA was prepared using the Template Preparation Kit (Roche, Germany) according to the manufacturer's instructions. Molecular identification was performed using a probe-based real-time PCR assay targeting the ITS1 region located between the genes encoding SSU rRNA and 5.8S rRNA, using specific primers and probes as previously described (11). Melting-curve analysis was performed using a Rotor-Gene real-time PCR system. Four reference strains, *L. tropica* (MHOM/AZ/1974/SAF-K27), *L. major* (MHOM/SU/1973/5ASKH), *L. infantum* (MHOM/TN/1980/IPT1), and *L. donovani* (MHOM/IN/1980/DD8), were included as positive controls for species identification.

In vitro Preconditioning of Promastigotes for Experimental Infection

To evaluate whether pre-infection culture conditions influence subsequent experimental infectivity, logarithmic-phase promastigotes of the clinical *Leishmania* isolate, which was initially recovered in NNN medium, were transferred to either RPMI-1640 supplemented with 10% FCS or SIM supplemented with 20% FCS. Cultures were incubated at 26 °C for five days, during which parasite proliferation, motility, and morphological integrity were evaluated daily by light microscopy (12). During this five-day preconditioning period, no further subpassaging was performed in either SIM or RPMI-1640. Following the five-day culture period, stationary-phase promastigotes from the SIM and RPMI-1640 cultures were harvested separately and washed twice with sterile phosphate-buffered saline (PBS) by centrifugation at 1,000× g for 10 min. After each centrifugation step, the supernatant was carefully removed. Following the final wash, the parasite pellets were resuspended in sterile physiological saline. Promastigotes were counted using a hemocytometer, and the parasite suspensions were adjusted to a final concentration of 1×10^8 promastigotes/mL. The same harvesting, washing, counting, and resuspension procedure was applied to both experimental groups to standardize inoculum preparation and minimize carryover of residual culture medium and serum components.

Experimental Infection of BALB/c Mice with the Clinical Leishmania Isolate

Male BALB/c mice (6-8 weeks old; n=15) were randomly allocated to three experimental groups (SIM, RPMI, and control; n=5 per group) using a simple lottery method based on coded lots assigned to individually identified animals. The SIM and RPMI groups received promastigotes of the clinical *Leishmania* isolate that had been previously cultured in SIM (pH 5.5; 20% FCS) and RPMI-1640 (pH 7.2; 10% FCS), respectively. These media represented two complete pre-infection culture conditions. The individual effects of pH, serum concentration, and basal medium composition were not evaluated independently. Before inoculation, promastigotes from both cultures were washed twice with sterile PBS and resuspended in sterile physiological saline. Each mouse was inoculated subcutaneously in the plantar surface of the right hind footpad with 100 µL of the standardized suspension (1×10^8 promastigotes/mL), corresponding to 1×10^7 promastigotes per mouse. Control mice received 100 µL of sterile physiological saline. Animals were monitored for six weeks post-infection under standardized housing and welfare conditions (13).

Evaluation of Experimental Infection Parameters

Footpad Diameter Measurement

The absolute diameter of the inoculated right hind footpad was measured weekly with a digital caliper and recorded in millimeters (mm) to monitor changes over time. Measurements represent the absolute footpad diameter and were not calculated by subtracting the contralateral footpad measurement (14). Each mouse was treated as an independent biological replicate, and group means and standard deviations (SD) were calculated directly from the individual animal-level measurements (n=5 per group) at each time point. No technical replicate measurements were treated as independent observations. To minimize measurement bias, weekly footpad diameter measurements were performed using a digital caliper by an observer blinded to the experimental group allocation.

Assessment of Amastigote Burden in Touch Preparations

At week six, animals were euthanized and infected tissue samples were collected. Touch preparations were obtained by gently pressing tissue sections onto clean glass slides, followed by air-drying and staining with 10% Giemsa (pH 7.2) for 30 min. Amastigote burden was assessed using a semi-quantitative scoring system. Smears were examined under oil immersion (1000× magnification), and parasite burden was classified according to the following criteria: 6+ (>100 parasites per field), 5+ (10-100 parasites per field), 4+ (1-10 parasites per field), 3+ (1-10 parasites per 10 fields), 2+ (1-10 parasites per 100 fields), 1+ (1-10 parasites per 1000 fields), and 0 (no parasites observed in 1000 fields) (15). To minimize observer bias, microscopic evaluation and semi-quantitative amastigote scoring were performed by an observer blinded to the experimental group allocation.

Assessment of Promastigote Recovery in NNN Medium

Additional tissue fragments were aseptically minced and inoculated into NNN medium. Cultures were incubated at 26±1 °C, and promastigote recovery was quantitatively assessed on day 7 post-culture. Approximately 0.5 mL of the liquid phase was withdrawn from each NNN culture. To enhance detection sensitivity, the collected samples were centrifuged, and the resulting pellet was resuspended in 0.2 mL RPMI to concentrate the parasites. Promastigotes were counted using a Thoma hemocytometer after fixation with 10% formaldehyde. Viability was assessed by trypan blue exclusion, and only viable promastigotes were included in the quantitative analysis (16).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 9 (GraphPad Software, USA). Weekly footpad measurements were analysed using two-way repeated-measures analysis of variance (ANOVA), with experimental group as the between-subject factor and time as the within-subject repeated-measures factor, followed by Sidak's multiple-comparisons test for comparisons between SIM and RPMI at individual time points. Amastigote burden scores were treated as ordinal data and were presented as median [interquartile range (IQR)]. Promastigote recovery data are also presented as median (IQR). For amastigote burden and promastigote recovery, inferential comparisons were restricted to the two experimentally infected groups (SIM and RPMI) and performed using two-tailed Mann-Whitney U tests. The saline-inoculated group served as a negative control for these outcomes, and the outcomes were summarized descriptively. All statistical tests were two-sided, and $p<0.05$ was considered statistically significant.

RESULTS

Molecular Identification of the Clinical Isolate

ITS1-targeted probe-based real-time PCR followed by melting-curve analysis identified the clinical isolate MHOM/TR/2025/CBU152 as *Leishmania tropica*, based on its molecular profile in comparison with the reference strains.

Lesion Development in BALB/c Mice

Longitudinal analysis of weekly footpad measurements, using a two-way repeated-measures ANOVA, demonstrated significant effects of experimental group, time, and the group-by-time interaction (all $p<0.0001$), indicating distinct lesion progression trajectories between the SIM and RPMI groups over the six-week observation period. Post-hoc multiple comparisons showed no significant difference between the groups at baseline (week 0); however, a significant difference was detected at week 1 and persisted through weeks 2-6 (adjusted $p<0.0001$ for each comparison). Despite this early quantitative difference, visible footpad swelling and lesion development were first observed in the SIM group at approximately week 2, whereas in the RPMI group these findings appeared later and remained less pronounced throughout follow-up. By week 6, the mean ± SD absolute footpad diameter was 4.22±0.01 mm in the SIM group and 3.52±0.01 mm in the RPMI group. No visible inflammatory response or lesion formation was observed in the saline control group. Under the conditions tested, SIM-derived promastigotes were associated with greater longitudinal footpad enlargement, higher amastigote burden, and greater parasite recovery than RPMI-derived promastigotes (Figures 1A-C and 2; Table 1).

Amastigote Burden in Touch Preparations

At week six post-infection, necropsy was performed and infected footpad tissues were collected for microscopic examination. Touch preparations were made on clean glass slides, air-dried, and stained with 10% Giemsa solution (pH 7.2) for 30 min, following standard protocols. Slides were examined under oil immersion at 1000× magnification using a bright-field light microscope. Amastigote burden was significantly higher in the SIM group than in the RPMI group [median (IQR): 6 (5-6) vs. 2 (2-2); Mann-Whitney U=0, exact two-tailed p=0.0079]. No amastigotes were detected in tissue samples from control mice [0 (0-0)] (Figure 1D-F; Table 1).

Promastigote Recovery in NNN Cultures

Following necropsy at week 6 post-infection, fragments of infected plantar tissue were aseptically inoculated into NNN medium and monitored for promastigote proliferation. Promastigote recovery on day 7 was significantly greater in cultures established from the SIM group than in those established from the RPMI group [median (IQR): 1.15 (1.12-1.18)×10^6/mL vs. 0.15 (0.14-0.16)×10^6/mL; Mann-Whitney U=0, exact two-tailed p=0.0079]. No promastigotes were recovered from the saline-inoculated control group (Figure 1G-I; Table 1).

DISCUSSION

CL is primarily shaped by cell-mediated immune mechanisms, and its clinical spectrum varies markedly across *Leishmania* species—including *L. major, L. tropica, L. mexicana,* and *L. amazonensis*—as well as by host genetic background and qualitative differences in immune responses (17). This diversity, ranging from self-resolving lesions to chronic non-healing disease, underscores the importance of reliable animal models for dissecting CL immunopathology and evaluating candidate interventions (18). BALB/c and C57BL/6 mice remain central to this work, as their distinct immunological profiles—Th2-driven susceptibility in BALB/c and Th1-mediated resistance in C57BL/6—provide

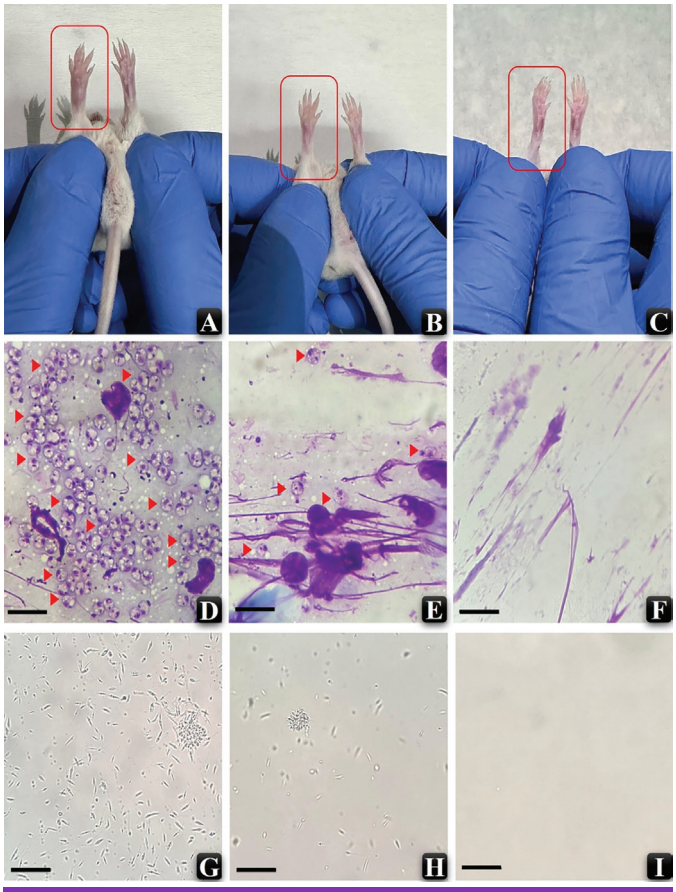

Figure 1. Macroscopic and light microscopic findings from footpad lesions of BALB/c mice six weeks after experimental *Leishmania tropica* infection

(A-C) Absolute footpad diameter: promastigotes derived from SIM cultures induced greater footpad swelling with well-defined edema and erythema **(A)**, whereas RPMI-derived parasites produced only mild to moderate enlargement **(B)**. Saline-injected controls exhibited no visible pathology **(C)**. **(D-F)** Amastigote burden: Giemsa-stained impression smears revealed a dense intracellular amastigote burden in the SIM group **(D)**, a lower burden in the RPMI group **(E)**, and no detectable parasites in the control group **(F)**. Arrowheads denote intracellular amastigotes. **(G-I)** Promastigote recovery: representative microscopic fields from NNN cultures established from footpad tissues of the SIM **(G)**, RPMI **(H)**, and control **(I)** groups on day 7. Promastigotes were observed in cultures from both infected groups, whereas none were detected in control cultures. Scale bar, D-F: 10 µm; G-I: 10 µm

SIM: Schneider's insect medium

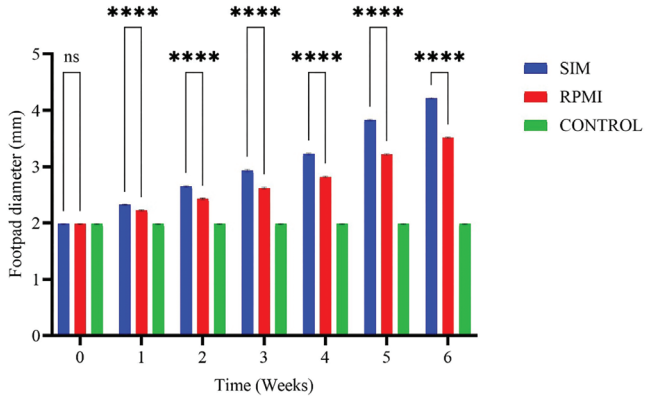

Figure 2. Longitudinal changes in absolute footpad diameter in BALB/c mice infected with *Leishmania tropica* promastigotes cultured under different *in vitro* conditions. Footpad diameter was measured weekly using a digital caliper in mice inoculated with SIM- or RPMI-derived promastigotes and in uninfected controls. Data are presented as mean ± SD (n=5 per group). Statistical analysis was performed using two-way repeated-measures ANOVA followed by Sidak's multiple-comparisons test for prespecified SIM-RPMI comparisons at each time point. No significant differences were detected at week 0, whereas significant differences were observed from week 1 through week 6

*****: Adjusted p<0.0001, ns: Not significant, SIM: Schneider's insect medium, SD: Standard deviation, ANOVA: Analysis of variance*

valuable contrasts for interrogating cellular processes including Th1/Th2 polarization, macrophage activation, cytokine dynamics, and parasite dissemination (19). Within this broader framework, *L. tropica*, the principal agent of anthroponotic CL in the Old World, presents particular experimental challenges because it typically produces only small, non-ulcerative nodules and modest delayed-type hypersensitivity responses in mice, limiting its capacity to generate progressive disease in standard murine models (20). This has historically hampered detailed pathogenicity studies. In the present study, we addressed this limitation by testing whether pre-infection culture conditions could enhance the infectivity of a clinical *L. tropica* isolate in highly susceptible BALB/c mice.

In the sand fly vector, *Leishmania* parasites undergo a series of differentiation steps—including the procyclic, nectomonad, leptomonad, haptomonad, and metacyclic stages—culminating in the production of metacyclic promastigotes, the infective form transmitted to mammals (21). Following inoculation, these metacyclics rapidly enter macrophages and transform into intracellular amastigotes, establishing infection (22). Metacyclogenesis is therefore a key determinant of virulence, as metacyclic promastigotes possess characteristic adaptations including elongated flagella, a dense LPG coat, and upregulated gp63, all of which enhance complement resistance and early survival within host phagocytes (23). The extent to which metacyclics are represented in an inoculum directly influences infection outcomes. While metacyclogenesis occurs physiologically in the sand fly gut, it can also be induced *in vitro* by environmental cues, particularly nutrient limitation and pH reduction—stressors that mimic those encountered during blood meal digestion in the vector (24). Under axenic conditions, promastigotes similarly undergo partial metacyclogenesis as cultures reach the stationary phase. However, the efficiency of differentiation varies widely among species and strains, with some producing only limited numbers of metacyclics and others exhibiting strong stress-induced differentiation responses (12).

To investigate whether pre-infection culture conditions could alter subsequent experimental infectivity, promastigotes of the clinical *L. tropica* isolate were maintained in SIM (pH 5.5, 20% FCS) or

Table 1. Quantitative assessment of footpad measurements, amastigote burden, and promastigote recovery in BALB/c mice infected with *Leishmania tropica* promastigotes cultured under different *in vitro* conditions (SIM and RPMI)

Group	Footpad diameter[a] mm, (mean ± SD)	Amastigote burden[b] median (IQR)	Promastigote recovery[c] x10^6/mL median (IQR)	n[d]
SIM	4.22±0.01	6 (5-6)	1.15 (1.12-1.18)	5
RPMI	3.52±0.01	2 (2-2)	0.15 (0.14-0.16)	5
Control	1.98±0.01	0 (0-0)	0 (0-0)	5

[a]: Absolute footpad diameter was measured at the inoculated right hind footpad using a digital caliper. Each mouse represented one independent biological replicate, and values are presented as mean ± SD calculated from the five individual animal-level measurements in each group (n=5)
[b]: Amastigote burden was assessed in Giemsa-stained touch preparations using the following ordinal semi-quantitative scoring system: 6+ (>100 parasites/field), 5+ (10-100 parasites/field), 4+ (1-10 parasites/field), 3+ (1-10 parasites/10 fields), 2+ (1-10 parasites/100 fields),1+ (1-10 parasites/1000 fields), and 0 (no parasites detected in 1000 fields). Because amastigote burden represents ordinal data, values are presented as median (IQR). The prespecified comparison between the SIM and RPMI groups was performed using a two-tailed Mann-Whitney U test.
[c]: Promastigote densities (×10^6/mL) were quantified on day 7 following incubation in NNN medium and presented as median (IQR). The prespecified comparison between the SIM and RPMI groups was performed using a two-tailed Mann-Whitney U test.
n[d]: Number of animals included in each experimental group
mm: Millimeters, IQR: Interquartile range, SD: Standard deviation, SIM: Schneider's insect medium

RPMI-1640 (pH 7.2, 10% FCS) before inoculation into BALB/c mice. These conditions were selected because previous studies in other *Leishmania* species have shown that environmental factors, including acidic pH and nutrient composition, can influence promastigote differentiation and infectivity. Under the conditions tested, the *in vivo* results demonstrated that SIM-derived promastigotes exhibited greater experimental infectivity in BALB/c mice than RPMI-derived promastigotes. SIM-cultured promastigotes induced earlier lesion development (detectable by week 2) and greater absolute footpad diameter by week 6 (4.22±0.01 mm vs. 3.52±0.01 mm; Sidak-adjusted $p<0.0001$), and a significantly higher amastigote burden in the SIM group than in the RPMI group [median (IQR): 6 (5-6) vs. 2 (2-2); $p=0.0079$]. Promastigote recovery on day 7 was also significantly greater in cultures established from the SIM group than in those established from the RPMI group [median (IQR): 1.15 (1.12-1.18)×10^6/mL vs. 0.15 (0.14-0.16)×10^6/mL; $p=0.0079$]. Collectively, in the SIM group, the earlier lesion onset, greater lesion development, higher amastigote burden, and enhanced parasite recovery provide consistent evidence that SIM-derived promastigotes have greater experimental infectivity than RPMI-derived promastigotes.

These findings are consistent with previous studies demonstrating that acidic culture conditions can promote metacyclic differentiation and increase infectivity in several *Leishmania* species. Bates and Tetley (25) showed that *L. mexicana* cultured in SIM at pH 5.5 developed a predominantly metacyclic-like population characterized by increased macrophage infectivity and complement resistance (25). Similarly, Zakai et al. (26) reported increased proportions of metacyclic promastigotes and enhanced infectivity under acidic culture conditions in several *Leishmania* species. These observations provide a biologically plausible framework for interpreting the greater experimental infectivity observed in our SIM-derived *L. tropica* promastigotes. In the present study, metacyclic promastigotes were not directly quantified; therefore, the increased infectivity cannot be specifically attributed to enhanced metacyclogenesis. Instead, our findings demonstrate that SIM preconditioning is associated with a more infectious phenotype in this clinical *L. tropica* isolate under the experimental conditions tested.

The mechanisms underlying the greater infectivity of SIM-derived promastigotes cannot be determined from the present experimental design. SIM and RPMI differed in pH, serum concentration, and basal nutrient composition, all of which may influence parasite physiology and infectivity. Nutrient availability and metabolic stress have been implicated in promastigote differentiation in *Leishmania* spp. (27) while previous studies also support an important role for acidic culture conditions in modulating parasite differentiation and infectivity (25). However, because these variables were not independently controlled in the present study, the greater infectivity observed after SIM preconditioning cannot be attributed specifically to acidic pH or to any single medium component. Rather, our findings indicate that the overall SIM culture environment was associated with a more infective phenotype in this clinical *L. tropica* isolate.

Study Limitations

The present study has several limitations. Metacyclic promastigotes were not directly identified or quantified; therefore, the greater infectivity observed after SIM preconditioning cannot be attributed specifically to enhanced metacyclogenesis, and this requires confirmation using stage-specific approaches. In addition, SIM and RPMI-1640 differed in pH, FCS concentration, and basal medium composition, preventing determination of the individual contributions of culture-associated factors. The exploratory design included a relatively small sample size (n=5 animals per group), lacked a formal a priori power calculation, and evaluated only a single clinical *L. tropica* isolate; therefore, the findings should be considered isolate-specific and not generalized to other isolates without further validation. Future studies using larger cohorts, additional clinical isolates, controlled culture conditions, and complementary stage-specific, histopathological, and immunological analyses are warranted to confirm and extend these findings.

CONCLUSION

Our findings demonstrate that pre-infection culture conditions significantly influence the experimental infectivity of a clinical *L. tropica* isolate in BALB/c mice. SIM-derived promastigotes exhibited a more infective phenotype than RPMI-derived promastigotes, as evidenced by earlier lesion development, greater tissue amastigote burden, and enhanced parasite recovery. Although the underlying mechanisms remain to be elucidated, these findings support SIM preconditioning as a useful approach for establishing robust experimental *L. tropica* infection and provide a basis for further studies investigating culture-associated promastigote differentiation and infectivity.

**Ethics*

Ethics Committee Approval: This study was approved by the Local Ethics Committee for Animal Experiments, Faculty of Medicine, Manisa Celal Bayar University (date: 29.07.2025, decision no: 77.637.435/330).

Informed Consent: Written informed consent was obtained from the patient at the time of the original clinical sample collection. The archived parasite isolate used in the present study was de-identified, and no identifiable patient information was accessed.

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Footnotes

**Authorship Contributions*

Surgical and Medical Practices: A.Ö., A.Y., Concept: A.Y, T.A., İ.Ç.Ş., Design: A.Y., T.A., Data Collection or Processing: A.Ö., A.Y., İ.Ç.Ş., Analysis or Interpretation: A.Ö., A.Y., T.A., Literature Search: A.Y., T.A., İ.Ç.Ş., Writing: A.Y., T.A.

Conflict of Interest:

One of the authors of this article (A.E.) is a member of the Editorial Board of this journal. He was completely blinded to the peer review process of the article.

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REFERENCES

1. Blaizot R, Pasquier G, Kone AK, Duvignaud A, Demar M. Cutaneous leishmaniasis in sub-Saharan Africa: a systematic review of *Leishmania* species, vectors and reservoirs. Parasit Vectors. 2024; 17: 318.
2. Knight CA, Harris DR, Alshammari SO, Gugssa A, Young T, Lee CM. Leishmaniasis: recent epidemiological studies in the Middle East. Front Microbiol. 2023; 13: 1052478.
3. Mendes BP, da Silva IA, DaMata JP, Castro-Gomes T, Vieira LQ, Ribeiro-Dias F, et al. Metacyclogenesis of *Leishmania (Viannia) guyanensis*: a comprehensive study of the main transformation features in axenic culture and purification of metacyclic promastigotes by negative selection with *Bauhinia purpurea* lectin. Parasitology. 2019; 146: 716-27.
4. Chanmol W, Jariyapan N, Preativatanyou K, Mano C, Tippawangkosol P, Somboon P, et al. Stimulation of metacyclogenesis in *Leishmania (Mundinia) orientalis* for mass production of metacyclic promastigotes. Front Cell Infect Microbiol. 2022; 12: 992741.
5. Siripattanapipong S, Boontanom P, Leelayoova S, Mungthin M, Tan-Ariya P. *In vitro* growth characteristics and morphological differentiation of *Leishmania martiniquensis* promastigotes in different culture media. Acta Trop. 2019; 197: 105039.
6. Hendricks L, Wright N. Diagnosis of cutaneous leishmaniasis by *in vitro* cultivation of saline aspirates in Schneider's Drosophila medium. Am J Trop Med Hyg. 1979; 28: 962-4.
7. Rostamian M, Niknam HM. *Leishmania tropica*: what we know from its experimental models. Adv Parasitol. 2019; 104: 1-38.
8. Lira R, Méndez S, Carrera L, Jaffe C, Neva F, Sacks D. *Leishmania tropica*: the identification and purification of metacyclic promastigotes and use in establishing mouse and hamster models of cutaneous and visceral disease. Exp Parasitol. 1998; 89: 331-42.
9. Girginkardeşler N, Balcioğlu IC, Yereli K, Ozbilgin A, Ozbel Y. Cutaneous leishmaniasis infection in Balb/c mice using a *Leishmania tropica* strain isolated from Turkey. J Parasitol. 2001; 87: 1177-8.
10. Yıldırım A, Özbilgin A, Yereli K. *Trypanosoma cruzi*, *Leishmania tropica* ve *Toxoplasma gondii* Parazitlerinin J774, Vero ve HeLa hücre hatlarında *ex vivo* kültivasyon potansiyellerinin değerlendirilmesi [Evaluation of *ex vivo* cultivation potentials of *Trypanosoma cruzi*, *Leishmania tropica* ve *Toxoplasma gondii* parasites in J774, Vero and HeLa Cell Lines]. Mikrobiyol Bul. 2023; 57: 71-82.
11. Toz SO, Nasereddin A, Ozbel Y, Ertabaklar H, Culha G, Sevil N, et al. Leishmaniasis in Turkey: molecular characterization of *Leishmania* from human and canine clinical samples. Trop Med Int Health. 2009; 14: 1401-6.
12. da Luz RI, Vermeersch M, Dujardin JC, Cos P, Maes L. In vitro sensitivity testing of *Leishmania* clinical field isolates: preconditioning of promastigotes enhances infectivity for macrophage host cells. Antimicrob Agents Chemother. 2009; 53: 5197-203.
13. Yardley V, Croft SL. Animal models of cutaneous leishmaniasis. Handbook of Animal Models of Infection. 1999;775-81.
14. Attia H, Bali A, Sghaier RM, Martinez PAL, Mkannez G, Atri C, et al. Comparison between plethysmometer and caliper methods to monitor lesion-size induced by *Leishmania major* infection in BALB/c mouse experimental model. J Antivir Antiretrovir. 2015; 3: 179-85.
15. Ramírez JR, Agudelo S, Muskus C, Alzate JF, Berberich C, Barker D, et al. Diagnosis of cutaneous leishmaniasis in Colombia: the sampling site within lesions influences the sensitivity of parasitologic diagnosis. J Clin Microbiol. 2000; 38: 3768-73.
16. Ladopoulos T, Ntais P, Tsirigotakis N, Dokianakis E, Antoniou M. The proliferation potential of promastigotes of the main *Leishmania* species of the old world in NNN culture medium prepared using blood of four different mammals. Exp Parasitol. 2015; 157: 124-7.
17. Nateghi-Rostami M, Lipoldová M, Sohrabi Y. Improving reproducibility and translational potential of mouse models: lessons from studying leishmaniasis. Front Immunol. 2025; 16: 1559907.
18. Muñoz-Durango N, Gómez A, García-Valencia N, Roldán M, Ochoa M, Bautista-Erazo DE, et al. A mouse model of ulcerative cutaneous leishmaniasis by *Leishmania (Viannia) panamensis* to investigate infection, pathogenesis, immunity, and therapeutics. Front Microbiol. 2022; 13: 907631.
19. Restrepo CM, Llanes A, Herrera L, Ellis E, Lleonart R, Fernández PL. Gene expression patterns associated with *Leishmania panamensis* infection in macrophages from BALB/c and C57BL/6 mice. PLoS Negl Trop Dis. 2021; 15: e0009225.
20. Mears ER, Modabber F, Don R, Johnson GE. A review: the current *in vivo* models for the discovery and utility of new anti-leishmanial drugs targeting cutaneous leishmaniasis. PLoS Negl Trop Dis. 2015; 9: e0003889.
21. Becvar T, Vojtkova B, Siriyasatien P, Votypka J, Modry D, Jahn P, et al. Experimental transmission of *Leishmania (Mundinia)* parasites by biting midges (Diptera: Ceratopogonidae). PLoS Pathog. 2021; 17: e1009654.
22. Liévin-Le Moal V, Loiseau PM. *Leishmania* hijacking of the macrophage intracellular compartments. FEBS J. 2016; 283: 598-607.
23. da Silva IA Jr, Morato CI, Quixabeira VB, Pereira LI, Dorta ML, de Oliveira MA, et al. *In vitro* metacyclogenesis of *Leishmania (Viannia)* braziliensis and *Leishmania (Leishmania) amazonensis* clinical field isolates, as evaluated by morphology, complement resistance, and infectivity to human macrophages. Biomed Res Int. 2015; 2015: 393049.
24. Serafim TD, Figueiredo AB, Costa PA, Marques-da-Silva EA, Gonçalves R, de Moura SA, et al. *Leishmania* metacyclogenesis is promoted in the absence of purines. PLoS Negl Trop Dis. 2012; 6:e1833.
25. Bates PA, Tetley L. *Leishmania mexicana*: induction of metacyclogenesis by cultivation of promastigotes at acidic pH. Exp Parasitol. 1993; 76: 412-23.
26. Zakai HA, Chance ML, Bates PA. *In vitro* stimulation of metacyclogenesis in *Leishmania braziliensis*, *L. donovani, L. major* and *L. mexicana*. Parasitology. 1998; 116: 305-9.
27. Abdi Ghavidel A, Aghamiri S, Jajarmi V, Bandehpour M, Kazemi B. The influence of different culture media on the growth and recombinant protein production of iranian lizard *Leishmania* promastigote. Iran J Parasitol. 2022; 17: 543-53.