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Optimization of the Parasite Rescue and Transformation Assay for *Leishmania tropica*: Analysis of Preconditioning and Back Transformation Dynamics

Leishmania tropica için Parazit Kurtarma ve Dönüştürme Test Protokolünün Optimizasyonu: Ön Koşullandırma ve Geri Dönüşüm Dinamiklerinin Analizi

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ABSTRACT

Objective: This study aimed to compare the effects of three culture media [RPMI-1640, Schneider's insect medium (SIM), and M199] on *Leishmania tropica* promastigote propagation, preconditioning, and back-transformation of intracellular amastigotes, with the aim of establishing a standardized parasite rescue and transformation assay (PRTA) capable of consistently generating infective metacyclic forms.
Methods: Nine experimental groups were designed by combining the three media during the preconditioning and back-transformation phases. Promastigotes were expanded in the test media and then used to infect differentiated THP-1 macrophages to obtain intracellular amastigotes. Back-transformation was monitored morphologically under an inverted phase-contrast microscope, and metabolic activity was quantified using the ATP-based CellTiter-Glo assay.
Results: The highest back-transformation efficiency was observed in test 5 (SIM→SIM) [relative luminescence units (RLU): 49.567±9.099, $p<0.05$], with microscopic analysis revealing abundant viable transformed promastigotes. In contrast, other medium combinations produced either limited or no transformation. Test 9 (M199→M199; RLU: 0.089±0.071) and test 1 (RPMI→RPMI; RLU: 0.118±0.080) showed minimal back-transformation without statistical significance ($p>0.05$).
Conclusion: These findings demonstrate that culture medium composition is a key determinant of *L. tropica* promastigote differentiation. SIM medium supported the most efficient generation of metacyclic promastigotes, likely because it approximates vector-like conditions. The PRTA model described here offers a reproducible *in vitro* system for studying parasite differentiation and provides a practical framework to support infection biology and antileishmanial drug development.
Keywords: Back-transformation, culture medium, *Leishmania tropica*, parasite rescue, preconditioning, Schneider's insect medium

ÖZ

Amaç: Bu çalışmada, *Leishmania tropica* izolatı kullanılarak farklı kültür ortamlarının [RPMI-1640, SIM (Schneider's insect medium], M199) promastigot çoğaltımı, ön koşullandırma ve amastigotlardan promastigotlara dönüşüm süreçleri üzerindeki etkileri karşılaştırmalı olarak değerlendirilerek; enfektif metasiklik formların yüksek verimlilikle elde edilebildiği, biyolojik açıdan güvenilir ve metodolojik bakımdan standardize edilebilir bir parazit kurtarma ve dönüştürme test (PRTA) protokolünün geliştirilmesi amaçlanmıştır.
Yöntemler: *L. tropica* hücre içi amastigotlarının promastigot forma dönüşümünü değerlendirmek amacıyla, RPMI-1640, SIM ve M199 besiyerlerinin ön koşullandırma ve geri dönüşüm aşamalarında farklı kombinasyonlarla kullanıldığı dokuz test grubu

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oluşturulmuştur. NNN besiyerinden elde edilen promastigotlar üç farklı besiyerinde çoğaltılmış, farklılaşmış THP-1 makrofaj hücrelerinde enfeksiyon modeli oluşturulmuştur. Elde edilen hücre içi amastigotlar, PRTA protokolü kapsamında kültüre alınarak dönüşüm süreçleri morfolojik olarak invert mikroskopla izlenmiş; metabolik aktiviteleri ATP temelli CellTiter-Glo testi ile kantitatif olarak değerlendirilmiştir.

Bulgular: En yüksek dönüşüm verimi test 5 (SIM→SIM); grubunda elde edilmiştir [göreli lüminesans birimleri (RLU): 49,567±9,099, p<0,05]. Bu grupta mikroskobik incelemelerde yüksek yoğunlukta ve canlı dönüşen promastigotlar gözlemlenmiştir. Diğer kültür ortamı kombinasyonlarında ise dönüşüm ya düşük düzeyde kalmış ya da hiç gerçekleşmemiştir. Özellikle test 9 (M199→M199; RLU: 0,089±0,071) ve test 1 (RPMI→RPMI; RLU: 0,118±0,080) gruplarında dönüşüm başarısı düşük kalmıştır (p>0,05).

Sonuç: Kültür ortamı bileşiminin *L. tropica*'nın promastigot dönüşümünde belirleyici bir faktör olduğu gösterilmiştir. SIM besiyeri, vektör benzeri koşulları taklit etme kapasitesi sayesinde metasiklik promastigot üretiminde en yüksek verimliliği sağlamıştır. Bu çalışma, enfeksiyon yeteneği yüksek promastigotların elde edilmesini mümkün kılan güvenilir bir *in vitro* model sunarak enfeksiyon biyolojisi ve antileishmanial ilaç araştırmaları için güçlü bir deneysel altyapı sağlamaktadır.

Anahtar Kelimeler: Geri dönüşüm, kültür ortamı, *Leishmania tropica*, parazit kurtarma , ön koşullandırma, Schneider's insect medium

INTRODUCTION

Leishmaniasis, a vector-borne disease caused by obligate intracellular protozoa of the genus *Leishmania,* remains a major public health concern in tropical and subtropical regions. According to the World Health Organization, nearly one million new cases are reported annually, and the disease continues to impose a substantial global burden due to high morbidity, permanent sequelae, and significant socioeconomic impact (1). Cutaneous leishmaniasis (CL) is the most common clinical form; if left untreated, it may lead to ulcerative skin lesions and disfiguring scars. *Leishmania tropica*, a causative agent of CL in the Mediterranean basin, the Middle East, and South Asia, represents a notable regional threat (2). Disease control is further complicated by limited therapeutic options, increasing drug resistance, and the growing number of unresponsive field isolates (3).

The life cycle of *Leishmania* involves morphological transitions between flagellated promastigotes in the sand fly vector and non-flagellated amastigotes within mammalian phagocytic cells. These stage conversions are regulated by environmental cues such as temperature, pH, oxygen availability, and nutrient content (4). *In vitro* modeling of these developmental transitions not only provides critical insights into parasite biology but also permits the isolation of biologically competent, infective forms. Nevertheless, propagation of promastigotes in culture is strongly influenced by the composition of the growth medium and surrounding environmental parameters, and *in vitro*-derived forms do not invariably exhibit infectivity *in vivo* (5). The induction of the metacyclic promastigote stage—the developmental form that initiates infection in mammalian hosts—necessitates culture conditions that accurately recapitulate the physiological transition from vector to host (6). Preconditioning, defined as the gradual exposure of parasites to host-like conditions prior to infection, has therefore been recognized as a critical step in enhancing infectivity (7).

To overcome inconsistencies in promastigote recovery and differentiation, the parasite rescue and transformation assay (PRTA) has been established as a robust analytical platform for quantitative assessment of parasite viability, infectivity, and the efficiency of metacyclic differentiation. Nonetheless, the assay's analytical sensitivity and inter-assay reproducibility remain critically dependent on the physicochemical properties of the culture medium and on the precision and efficacy of both preconditioning and back-transformation protocols (8). Previous studies have highlighted substantial interspecies and inter-isolate variation in culture adaptation, with delays in transformation and reduced differentiation rates frequently reported (6,9,10). These limitations raise concerns regarding the biological relevance of *in vitro*-derived promastigotes and underscore the need for standardized protocols that yield infective and functionally valid forms.

In this study, we investigated the effects of three culture media (RPMI, SIM, and M199) on the propagation, preconditioning, and back-transformation of *L. tropica* promastigotes from intracellular amastigotes. The aim was to develop a reproducible and standardized PRTA protocol capable of reliably generating metacyclic promastigotes, thereby providing a robust platform for infection biology research and antileishmanial drug development.

METHODS

Ethical Approval

This study was conducted with the approval of the Health Sciences Ethics Committee of the Manisa Celal Bayar University Faculty of Medicine (date: 07.05.2025, decision no: 20.478.486/3098). Patient consent was not required, as the study was performed using *L. tropica* strains and THP-1 cells obtained from a parasite bank, without direct use of human clinical specimens.

Media and Solutions

To support the optimal *in vitro* growth of *L. tropica* promastigotes and THP-1 macrophages, all culture media and buffer solutions were prepared under aseptic conditions and stored in accordance with international laboratory standards.

NNN Medium: NNN medium was prepared by dissolving 5g of agar, 2g of peptone, and 1g of NaCl in 200mL of distilled water, followed by autoclaving at 121 °C for 20 minutes. After cooling to room temperature, the medium was supplemented with 1% gentamicin, 1% penicillin/streptomycin, and 30 mL of defibrinated rabbit blood. Aliquots were dispensed into sterile slant tubes and stored at 4 °C for a minimum of 48 hours to allow stabilization before use.

RPMI-1640 Medium: RPMI-1640 was supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, and 1% gentamicin. The pH was adjusted to 7.2. The medium was stored at 4 °C and equilibrated to room temperature before use in cell cultures and promastigote cultures.

Schneider's insect medium (SIM): SIM was prepared according to the manufacturer's protocol and supplemented with 20% FCS, 1% L-glutamine, and 1% penicillin/streptomycin. The pH was adjusted to 5.5 to mimic insect midgut conditions. The medium was sterilized using a 0.22 µm filter, stored at 4 °C, and equilibrated to room temperature before use.

M199 Medium: M199 was enriched with 10% FCS, 1% penicillin/streptomycin, and 1% gentamicin to support the proliferation of host cells and promastigotes. The pH was adjusted to 7.4. Following sterile preparation, the medium was stored at 4 °C and equilibrated to room temperature prior to application.

Sterile 0.05% Sodium Dodecyl Sulfate (SDS): For controlled lysis in PRTAs, a 0.05% SDS solution was prepared by dissolving 0.05 g of SDS in 100 mL of sterile distilled water. The solution was mixed until fully dissolved, sterilized through a 0.22 µm membrane filter, and stored at 4 °C until use.

Sterile 1xPBS: 1xPBS was prepared by dissolving 8.0 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4 \cdot 2H_2O$, and 0.24 g KH_2PO_4 in approximately 800 mL of distilled water. The pH was adjusted to 7.2-7.4, and the final volume was brought to 1 L. The solution was sterilized by filtration through a 0.22 µm membrane filter and stored at 4 °C until use.

In vitro culture of L. tropica

L. tropica (MHOM/TR/2022/CBU121), isolated from the lesion on the right cheek of a CL patient in Türkiye and subsequently maintained in the Parasite Bank of the Faculty of Medicine, Manisa Celal Bayar University, was rapidly thawed in a 37 °C water bath after removal from liquid nitrogen. The thawed suspension was inoculated into NNN medium for initial expansion and incubated at 26 °C. Parasite proliferation was monitored regularly under a light microscope, and viable promastigotes in the logarithmic growth phase were obtained through periodic observations every 2-3 days (11).

THP-1 Macrophage Cell Culture

The THP-1 monocytic cell line was removed from liquid nitrogen and rapidly thawed in a 37 °C water bath. Cells were centrifuged at 1500 rpm for 10 minutes to obtain a pellet, which was then resuspended in 4 mL of RPMI-1640 medium. A 1 mL aliquot of the suspension was transferred into culture flasks containing 9 mL of fresh RPMI-1640. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Cell morphology and viability were monitored regularly under an inverted microscope, with viability assessed using trypan blue exclusion. Cell counts were performed before experimental use, using a Thoma chamber, after a 1:3 dilution. Cultures were passaged regularly, and the medium was replaced every other day. The medium was supplemented with 100 ng/mL phorbol-12-myristate-13-acetate for differentiation into macrophage-like cells and incubated for 24 hours (12).

Preconditioning of Promastigotes

To enhance the infectivity of *L. tropica* promastigotes, a preconditioning protocol was implemented. This process involved controlled exposure to environmental stressors that mimic the transition from the sandfly vector to the mammalian host. Promastigotes in the logarithmic phase, initially cultured in NNN medium, were transferred to RPMI-1640 (10% FCS), SIM (20% FCS), and M199 (10% FCS) and incubated at 26 °C for five days. Throughout the incubation period, cultures were monitored daily by light microscopy to assess cell morphology and viability. This approach aimed to obtain biologically active and infective promastigotes prior to host-cell infection. The impact of each medium on the preconditioning process was comparatively evaluated (7).

Infection of THP-1 Macrophages with L. tropica

Differentiated THP-1 macrophages were harvested using a cell scraper upon reaching confluency in the culture flasks, and then centrifuged at 1500 rpm for 10 minutes. The resulting cell suspension was counted and adjusted to a concentration of 5×10^5 cells/mL, and then seeded into fresh RPMI-1640-containing flasks. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 24 hours. Subsequently, infection was carried out using *L. tropica* promastigotes at a 1:10 (cell:parasite) ratio (13). The promastigotes had reached the logarithmic growth phase in RPMI-1640, SIM, or M199. After infection, non-internalized promastigotes were removed by washing with sterile 1xPBS, and fresh RPMI-1640 medium was added. Infected cultures were then incubated for 48 hours at 37 °C under 5% CO_2.

Back-transformation of Amastigotes to Promastigotes

The back-transformation capacity of *L. tropica* amastigotes developed within macrophages was systematically evaluated under defined *in vitro* conditions. This process serves as a functional indicator of the parasite's adaptability to extracellular environments and enables a comparative analysis of how culture media modulate promastigote re-differentiation efficiency.

Controlled Lysis and Rescue of L. tropica Amastigotes

Infected macrophage cultures in 96-well plates were washed three times with sterile 1× PBS and then 20 µL of 0.05% SDS was added to each well to induce controlled host-cell lysis. Plates were gently agitated for 30 seconds to ensure efficient lysis while preserving the viability of released amastigotes. Subsequently, 180 µL of complete medium (RPMI-1640, SIM, or M199) was added to each well. Plates were incubated at 26 °C for 96 hours to evaluate the transformation of intracellular amastigotes into promastigotes.

Quantitative ATP Assay for Back-transformed L. tropica Promastigotes

Following 96 hours of incubation at 26 °C, viable amastigotes successfully differentiated into promastigote forms. Subsequently, 100 µL of CellTiter-Glo® reagent was added to each well, and the plates were incubated at room temperature for 10 minutes. Luminescence signals were recorded using a luminometer and expressed as relative luminescence units (RLU). This ATP-based assay provides a quantitative measure of promastigote metabolic activity, offering insight into their energy production capacity and proliferative potential under specific culture conditions.

Morphological Evaluation of Back-transformed L. tropica Promastigotes

For morphological evaluation of the transformation into promastigote forms, cultured amastigotes were examined at the end of the incubation period using an inverted phase-contrast microscope. Cultures were divided into nine test groups (tests 1-9) based on different medium combinations used during the preconditioning and recovery phases (Table 1). After 96 hours of incubation, promastigotes in each group were examined microscopically, and the images were organized accordingly. Observations focused on assessing promastigote morphological integrity, cell density, and motility.

Table 1. Distribution of experimental groups (test 1-9) according to the culture media used in the preconditioning and back transformation phases for the morphological evaluation of *Leishmania tropica* promastigote transformation

Test	Preconditioning	Back transformation
Test 1	RPMI	RPMI
Test 2	RPMI	SIM
Test 3	RPMI	M199
Test 4	SIM	RPMI
Test 5	SIM	SIM
Test 6	SIM	M199
Test 7	M199	RPMI
Test 8	M199	SIM
Test 9	M199	M199

Statistical Analysis

Statistical analyses were performed to evaluate differences in transformation efficiency among nine test groups, each defined by distinct combinations of preconditioning and back-transformation media (RPMI-1640, SIM, and M199). RLU values obtained from the CellTiter-Glo® assay were expressed as mean ± standard deviation. One-way analysis of variance followed by Tukey's post-hoc test was used to identify statistically significant differences between groups, with significance set at $p<0.05$. All analyses were conducted using GraphPad Prism version 9. Additionally, morphological evaluations were performed using inverted phase-contrast microscopy to assess promastigote integrity, density, and motility after a 96-hour incubation.

RESULTS

The back-transformation capacity of *L. tropica* amastigotes was assessed across nine experimental groups that combined different culture media during the preconditioning and back-transformation phases (Figure 1). Quantitative analysis demonstrated the highest efficiency in test 5 (SIM→SIM), which yielded a significantly elevated RLU value (49.567±9.099; $p<0.05$) compared with all other groups. Microscopic examination confirmed these findings, revealing dense cultures of motile promastigotes with characteristic morphology (Figure 2E).

When RPMI was used for preconditioning, notable transformation was observed only when SIM served as the recovery medium (test 2), with moderate RLU levels and corresponding microscopic evidence of viable promastigotes (Figure 2B). In contrast, test 1 (RPMI→RPMI) and test 3 (RPMI→M199) failed to support transformation, with negligible RLU values and no detectable promastigotes under microscopy (Figures 2A, 2C) (Table 2).

SIM-based preconditioning combined with RPMI recovery (test 4) resulted in partial back-transformation, as evidenced by moderate RLU values and visible promastigotes (Figure 2D). However, test 6 (SIM→M199) yielded markedly reduced transformation (RLU: 0.196±0.219), consistent with the presence of only a few motile promastigotes (Figure 2F; Table 2).

Overall transformation success was lowest in groups preconditioned with M199. Test 9 (M199→M199) yielded only minimal RLU values (0.089±0.071) and displayed no microscopically appreciable promastigote proliferation (Figure 2I). Similarly, test 7 (M199→RPMI) remained at very low levels (Figure 2G), while test 8 (M199→SIM) displayed slightly improved transformation (RLU: 1.018±0.547) with low-to-moderate promastigote density (Figure 2H; Table 2).

Taken together, quantitative and microscopic data indicate that SIM, when applied during the preconditioning and back-transformation phases, most efficiently supports the back-transformation of *L. tropica*. By contrast, RPMI and M199 alone were insufficient, with M199 showing the poorest performance across test groups.

DISCUSSION

Leishmania spp. are digenetic parasites that alternate between the insect vector and the mammalian host, undergoing distinct morphological transitions during their life cycle. Modeling these developmental stages under laboratory conditions contributes substantially to understanding infection biology and to advancing therapeutic strategies. However, the biological relevance of such models is largely determined by the influence of the culture medium on parasite differentiation and viability (14,15). In clinical diagnosis, microscopic examination and *in vitro* culture are among the most frequently employed direct approaches. However, their diagnostic sensitivity may exhibit substantial variability, largely influenced by sample quality, the experience of the operator, and the degree of optimization of the culture systems used (16). Although culture isolation is theoretically considered the diagnostic gold standard, its practical yield is influenced by multiple biological and technical factors. These include the composition of the culture medium, the virulence characteristics of the isolate, and the initial parasite burden in

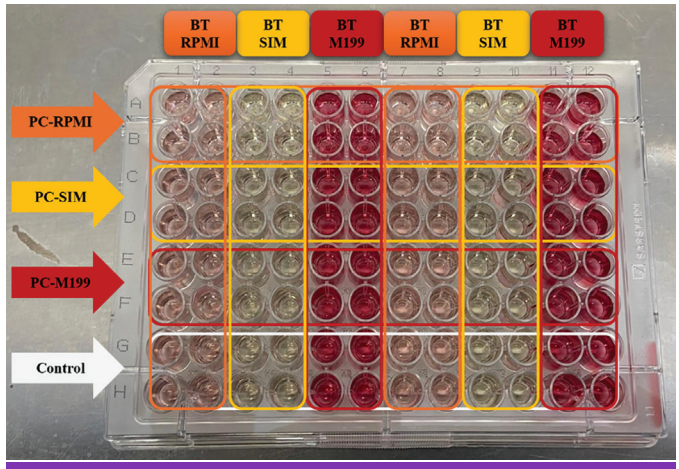

Figure 1. Layout of a 96-well microtiter plate used to assess the back-transformation of *Leishmania tropica* amastigotes into promastigote forms. [The plate was organized into nine experimental groups representing distinct combinations of culture media applied during the preconditioning (PC) and back-transformation (BT) phases. PC conditions (left axis) included RPMI (orange), SIM (yellow), and M199 (red), while BT conditions (top axis) consisted of RPMI (orange), SIM (yellow), and M199 (red). The white row served as the negative control]. Each condition was tested in triplicate technical replicates

SIM: Schneider's insect medium

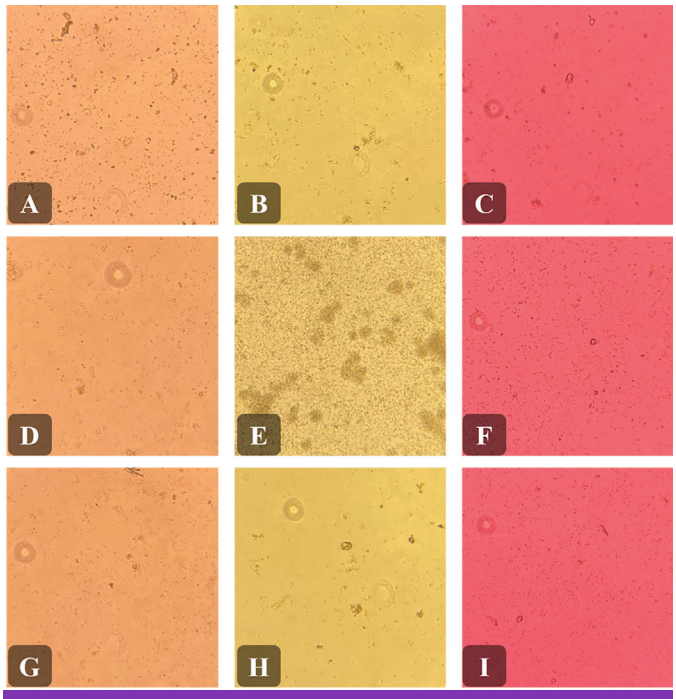

Figure 2. Representative inverted phase-contrast micrographs showing *Leishmania tropica* growth following back-transformation of intracellular amastigotes into promastigotes under different culture conditions. [Test groups were defined by the culture media used during the preconditioning and back-transformation phases: (A) test 1: RPMI→RPMI, (B) test 2: RPMI→SIM, (C) test 3: RPMI→M199, (D) test 4: SIM→RPMI, (E) test 5: SIM→SIM, (F) test 6: SIM→M199, (G) test 7: M199→RPMI, (H) test 8: M199→SIM, (I) test 9: M199→M199]

SIM: Schneider's insect medium

Table 2. Mean relative luminescence unit (RLU ± SD) values reflecting the back-transformation efficiency of *Leishmania tropica* amastigotes into promastigotes under different preconditioning and back-transformation medium combinations. [Test groups are defined as follows: test 1 (RPMI→RPMI), test 2 (RPMI→SIM), test 3 (RPMI→M199), test 4 (SIM→RPMI), test 5 (SIM→SIM), test 6 (SIM→M199), test 7 (M199→RPMI), test 8 (M199→SIM), and test 9 (M199→M199)]

Test	RLU (mean ± SD)
Test 1	0.118±0.080
Test 2	2.671±2.636
Test 3	0.010±0.008
Test 4	2.404±1.015
Test 5	49.567±9.099
Test 6	0.196±0.219
Test 7	0.107±0.051
Test 8	1.018±0.547
Test 9	0.089±0.071

SD: Standard deviation, RLU: Relative luminescence units

the sample (17). Therefore, culture systems should be optimized not only for diagnostic purposes but also to enhance their physiological relevance and standardization for translational applications, including vaccine development, drug susceptibility testing, and experimental infection modeling (18). In this context, our study compared culture conditions that influence the production of infective metacyclic promastigotes of *L. tropica* using RPMI-1640, SIM, and M199 media. The findings suggest that culture medium composition is a critical determinant, affecting not only the parasite's capacity for *in vitro* proliferation but also the differentiation processes governing the transition from promastigote to infective phenotypes.

In *Leishmania* spp., the process of metacyclogenesis is largely regulated by the specific environmental conditions of the thoracic midgut of the vector. In permissive vectors such as *Lutzomyia longipalpis*, the acidic pH environment of the thoracic midgut has been suggested to facilitate the development of infective metacyclic forms (19). In our study, a notable increase was observed in the proportion of promastigotes exhibiting morphological traits consistent with metacyclogenesis when cultured in SIM medium at pH 5.5, despite the relatively low overall parasite density. This observation suggests a possible association between acidic conditions and the *in vitro* differentiation process of *L. tropica*. These findings are consistent with the report by Siripattanapipong et al. (15), who demonstrated a high rate of metacyclic differentiation of *L. martiniquensis* in SIM medium. Both studies indicate that SIM medium, through its ability to mimic environmental cues that trigger the transition to the metacyclic phenotype, appears to exert a pronounced inductive effect on differentiation. Furthermore, this observation is consistent with reports describing natural mechanisms of metacyclogenesis in *L. mexicana*, *L. braziliensis*, *L. donovani*, and *L. major*, where spontaneous acidification of the medium during the stationary phase has been shown to promote differentiation (6). In our study, the markedly elevated RLU observed in test 5 (SIM→SIM) (49.567±9.099; $p<0.05$) suggests that SIM medium exerts a stimulatory effect on metacyclogenesis. This finding is consistent with the notion that low pH and the compositional factors of SIM may collectively generate favorable signals that promote differentiation. Specific components within SIM may further enhance this effect by more effectively mimicking the environmental cues that drive the transition to the metacyclic phenotype. By contrast, culture media commonly used, such as RPMI-1640 (pH 7.2) and M199 (pH 7.4), exhibited markedly lower back-transformation efficiencies. For instance, test 1 (RPMI→RPMI), test 3 (RPMI→M199), test 6 (SIM→M199), test 7 (M199→RPMI), and test 9 (M199→M199) all yielded very low RLU values, suggesting that these media do not adequately reproduce the environmental stress conditions required for successful differentiation of field isolates. Although such media are frequently employed, their use may contribute to reduced infectivity potential during laboratory adaptation, particularly given the heightened sensitivity of field isolates to environmental stress.

The declining response rates to first-line therapies across all clinical forms of leishmaniasis represent a growing public health concern that threatens the effectiveness of current standard treatment regimens. This trend underscores the critical need for systematic monitoring of drug resistance and the assessment of resistance rates, particularly in endemic regions (20). This

underscores the need to standardize and strengthen *in vitro* drug susceptibility assays based on field isolates. As molecular markers of resistance are not yet fully defined or clinically validated, assays employing viable *Leishmania* stages remain the primary tool for the epidemiological surveillance of drug resistance. However, the reliable assessment of drug susceptibility in patient-derived field isolates continues to face major methodological limitations due to the lack of standardized, reproducible, and physiologically relevant test systems. These shortcomings hinder the ability to predict treatment outcomes and pose significant challenges for the early detection of therapeutic failure. Consequently, there remains an urgent need for more robust and standardized *in vitro* systems that can provide biologically meaningful and reliable evaluations of clinical *Leishmania* isolates (7). In this study, the PRTA was employed as an *in vitro* system to evaluate the controlled back-transformation of amastigotes isolated from infected host cells into promastigotes. The assay represents a platform with potential utility in both diagnostic and translational research contexts. The differentiated THP-1 macrophage model used herein demonstrates a high degree of biological relevance. Specifically, motile promastigotes were observed microscopically within 24 hours after transferring infected host cells from 37 °C to 26 °C, and by 96 hours, a high density of promastigotes was confirmed, both freely proliferating in extracellular culture and still residing intracellularly (21). These findings suggest that the PRTA protocol can model the transition of *Leishmania* to the infective stage under laboratory conditions in a biologically relevant manner and may serve as a suitable platform for drug susceptibility testing.

PRTA is an *in vitro* technique used to evaluate the reversion of parasites isolated from infected host cells to proliferative forms. The reliability of PRTA depends on the careful execution of several key procedural steps. One critical step is the effective removal of non-internalized *Leishmania* promastigotes from the culture after infection. Infected THP-1 macrophage cultures were washed multiple times with PBS, ensuring that only intracellular parasites remained in the assay system. This washing step minimizes the risk of false-positive results and enables the generation of more reliable data in subsequent analyses (8-21). The second critical step is the lysis stage, which aims to release intracellular amastigotes. This is achieved by subjecting infected THP-1 cells to a brief lysis with a buffer containing 0.05% SDS, allowing controlled disruption of host cell membranes. Such controlled lysis facilitates the release of intact amastigotes and is intended to enhance the overall sensitivity of the assay (8,22). In this study, the transformation of viable amastigotes into promastigotes progressed gradually following the transfer of infected macrophage cultures from 37 °C to 26 °C. Motile promastigotes of *L. tropica* were microscopically detected as early as 24 hours after incubation at 26 °C, both within the cytoplasm of host macrophages and in the extracellular culture medium. Over time, parasites entered an active proliferation phase; by 96 hours, large numbers of extracellular promastigotes were observed, either freely motile in suspension or clustered in colony-like aggregates. Notably, a subset of promastigotes remained within macrophages even at 96 hours, as confirmed by microscopic examination. These findings demonstrate that the back-transformation from intracellular amastigotes to promastigotes can be reliably monitored under controlled culture conditions and further support the biological relevance of the PRTA system. Both critical procedural steps can be adapted to automated platforms, allowing the assay to retain reproducibility and high efficiency through standardization. In addition, re-washing infected cells after treatment with drugs or test compounds eliminates residual free promastigotes from the culture medium. Collectively, these strategies enhance the reliability of PRTA and facilitate its integration into large-scale screening applications (8). Such cell-based models not only provide a more accurate representation of host–parasite interactions but are also well suited for translational research due to their compatibility with high-throughput screening systems. Considering the homogeneity, standardizability, and ethical sustainability of the cell source, differentiated THP-1 macrophages represent a valuable model system for intracellular drug screening targeting the amastigote form of *Leishmania*. Nevertheless, this study has certain methodological limitations. First, only three culture media were evaluated, and a broader parametric analysis encompassing different medium components (e.g., serum types, glucose concentrations, or ionic compositions) was not addressed. Second, the assessment relied solely on RLU measurements without complementary phenotypic or molecular characterization of metacyclic differentiation. Future studies should integrate multi-level approaches, including LPG analysis, infectivity validation through *in vitro* macrophage infections, and gene expression profiling, to provide a more comprehensive understanding

CONCLUSION

This study compared the effects of different culture media (RPMI-1640, SIM, and M199) on promastigote production, preconditioning, and back-transformation of *L. tropica* amastigotes. The data indicate that culture medium composition is a critical determinant, particularly in the generation of metacyclic promastigotes. Among the tested media, SIM emerged as the most effective medium for producing infective promastigotes under experimental conditions, with significantly higher efficiency than the other media ($p<0.05$). Supporting the PRTA protocol with SIM-based conditions enhanced the physiological relevance of experimental infection models and improved the reliability of *in vitro* drug susceptibility analyses. Moreover, the ability of this model to dynamically track the transformation of intracellular amastigotes highlights its potential as a useful tool in translational research. Overall, the findings underscore the need for standardized and biologically relevant test systems for the evaluation of clinical field isolates. This work provides novel insights that may contribute to the refinement of experimental approaches in Leishmania biology and the optimization of therapeutic strategies.

*Ethics

Ethics Committee Approval: This study was conducted with the approval of the Health Sciences Ethics Committee of the Manisa Celal Bayar University Faculty of Medicine (date: 07.05.2025, decision no: 20.478.486/3098).

Informed Consent: Patient consent was not required, as the study was performed using *Leishmania tropica* strains and THP-1 cells obtained from a parasite bank, without direct use of human clinical specimens.

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Footnotes

**Authorship Contributions*

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

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