Effect of Human Urine on Cell Cycle and Infectivity of *Leishmania* Species Promastigotes *In Vitro*

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Abstract. *In vitro* cultivation of *Leishmania* parasites plays an important role in diagnosis and treatment of leishmaniasis and in vaccine and drug development studies. Conversely, long-term cultivation of *Leishmania* parasites usually results in decreased infectivity potential. Some studies reported a stimulatory effect of human urine in *Leishmania* promastigotes. However, there is no information about the effects of urine within culture on the infectivity of *Leishmania* parasites. Analysis of the effect of urine have showed that proliferation indexes were significantly increased in culture medium supplemented with human urine (*L. tropica* = 38.17 ± 5.12 , *L. donovani* = 34.74 ± 5.6 , *L. major* = 34.22 ± 4.66 , and *L. infantum* 35.88 ± 6.40) than in controls. Infection indexes were 13 ± 1.7 for *L. tropica*, 55 ± 2.2 for *L. infantum*, 41 ± 3.14 for *L. donovani*, and 49 ± 3.26 for *L. major*. Our results showed that human urine increased the infectivity and proliferation of *Leishmania* parasites.

INTRODUCTION

Leishmaniasis is a parasitic disease that affects large populations in many areas of the world.¹ The disease is caused by protozoan parasites belonging to the genus *Leishmania*, which are transmitted by *Phlebotomus* spp. sand flies.¹ The genus *Leishmania* contains a large number of pathogenic species and subspecies, which are responsible for cutaneous, mucocutaneous, and visceral forms of leishmaniasis.^{1,2}

In vitro cultivation of *Leishmania* parasites plays an important role in vaccine and drug development studies and diagnosis and treatment of leishmaniasis.³ These parasites, which have a procyclic form in *Phlebotomus*, change into a metacyclic form by metacyclogenesis and acquire infectivity potential. Conversely, promastigotes maintained in *in vitro* culture progressively lose their infectivity potential during extended culture (from the third to the tenth passages). Therefore, most parasites in the culture become procyclic promastigotes. However, metacyclic promastigotes can only be present in the stationary phase and the ratio of metacyclic promastigotes significantly decreases.⁴ This situation causes problems in the *in vitro* and *in vivo* infectivity studies with long-term cultivated *Leishmania* promastigotes.⁴ Therefore, investigations have been performed to enhance the infectivity and virulence of *Leishmania* parasites *in vitro* culture.^{4–6} In one study, it was shown that infectivity and virulence changed dependent on the parasite species and the medium that was used.⁵ In another study, infectivity of *Leishmania brasiliensis* promastigotes was found to be increased when they were exposed to heat shock.⁶

Several studies have shown the stimulatory effect of human urine on *Leishmania* promastigotes.^{7–11} When supplemented with human urine, Schneider's *Drosophila* culture media was found to increase the proliferation of different *Leishmania* parasite species. In the same study, it was found that culturing amastigotes that were isolated from *Leishmania*-infected hamsters in a culture environment containing urine increased promastigote differentiation and proliferation compared with controls.⁷ Another study questioned whether urine from a healthy human donor, a patient with nephritis, and several

types of animals could be an alternative to fetal calf serum (FCS) for *in vitro* *L. donovani* culture. Results have indicated that urine was not a valid alternative to FCS, but can be used to increase the proliferation in *L. donovani* cultures.⁹ In one study, it was determined that xanthine can stimulate parasite growth in cultures containing urine.¹¹ However, use of human urine as a supplementary material for *Leishmania* and *Trypanosoma* culture have been reported in many studies.^{12–15}

There is no information on the effects of urine on the infectivity of *Leishmania* parasites. Therefore, the aim of this study was to investigate the effects of culture medium containing human urine on the infectivity of different species of *Leishmania* parasites (*L. tropica*, *L. infantum*, *L. donovani*, and *L. major*).

MATERIALS AND METHODS

Parasite culture. *Leishmania tropica*, *L. infantum*, *L. donovani*, and *L. major* promastigotes were cultured at 27°C in culture flasks containing RPMI 1640 medium supplemented with 10% FCS (Sigma, St. Louis, MO), L-glutamine (Sigma), and gentamicin (80 µg/mL). Cultures were passaged after four days of incubation. The growth of promastigotes was monitored every day by using an inverted microscope (CK40; Olympus, Tokyo, Japan). Samples of promastigote culture (2×10^6 parasites/mL) were transferred to culture flasks containing 7 mL of RPMI 1640 medium plus 10% FCS. The parasites were counted by using a hemocytometer with a 20× objective under standard light microscopy. Before parasites were counted, they were mixed by using a pipette for homogeneous separation. Parasites were killed with 2% formalin (v/v) in phosphate-buffered saline (PBS) before counting.¹⁶

Urine sample collection and cultivation. Urine samples were collected from 20 healthy adolescent volunteers who had similar living conditions. Urine samples were taken in the morning before any food or drink was consumed. All urine samples were sterilized by filtration (0.22-µm disposable sterile filters; Millipore, Billerica, MA) and stored in sterile falcon tubes at 4°C. An aliquot of 1×10^6 parasites/mL were inoculated into 2 mL of medium (RPMI 1640 medium plus 10% fetal bovine serum). After incubation for 24 hours, media of experiment group cultures were supplemented with different concentrations of urine. Phosphate-buffered saline was used as a control. Parasites were counted every 24 hours.

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In vitro Leishmania infection of macrophages. Stationary phase promastigotes of *L. tropica*, *L. infantum*, *L. donovani*, and *L. major* promastigotes were washed twice by centrifugation ($1,500 \times g$ for 5 minutes) in PBS, pH 7.2, and their concentration was adjusted to 10^7 parasites/mL. Infection of macrophages was based on a ratio of 10 parasites per macrophage, and this procedure was used in triplicate for each strain. Therefore, the infection index of promastigotes in medium supplemented with 25% urine was based on the mean of these triplicate values. After 24 hours of macrophage-*Leishmania* interaction in culture, coverslips containing promastigotes were washed in PBS, fixed in absolute methyl alcohol (5 minutes), and stained with Giemsa for determining the infection index.¹⁷

Determination of infection index. The number of infected macrophages and amastigotes per macrophage was determined for 100 cells on a coverslip for each strain examined. The infection index was expressed as the percentage of infected macrophages multiplied by the average number of amastigotes per macrophage.¹⁸ In addition, the final infection index of promastigotes was represented by the mean of infection index values found in the different *Leishmania* species examined.

Effect of urine on the cell cycle. Parasites were incubated in medium supplemented with 25% urine for 96 hours at 27°C. A solution of 25% PBS was used as a control. Cells were fixed in 100% methanol (pre-chilled at -20°C) and kept at -20°C until analysis. After washing the cells in PBS, the resultant pellet was resuspended in 500 mL of Nuclear Isolation Media 4'-6-diamidino-2-phenylindole (NIM)-DAPI staining solution (NIM-DAPI; NPE Systems, Pembroke Pines, FL) and incubated in the dark for 20 minutes at 27°C. NIM-DAPI-stained samples were processed in a Cell Laboratory Quanta

flow analyzer fitted with a mercury arc lamp, 365/546 nm dual exciter, a 565DCXR splitter (long pass), and a 450AF55 nm emission filter. A minimum of 10,000 nuclear signals were collected in list mode files.¹⁹ G0/G1, S, and G2/M values were determined by flow cytometry analysis. These values were then used to calculate proliferation index as described: proliferation index = $(S + G2/M) \times 100 / (G0/G1 + S + G2/M)$.²⁰

Data processing and statistical analysis. All experiments were repeated at least three times. The results were expressed as the mean $\pm$ SD. A parametric test (paired Student's *t* test, analysis of variance, F test, and Tukey's post-hoc test) was used to evaluate the significance of the results. All data were analyzed by using SPSS version 16.0 for Windows (SPSS, Inc., Chicago, IL) and *P* values < 0.05 were considered statistically significant.

RESULTS

Effect of human urine in culture medium on growth of promastigotes. The effect of human urine of different concentrations on different species of *Leishmania* promastigotes in culture is shown in Figure 1. Although there was a statistically significant difference in *L. donovani* ($P = 0.043$, $n = 20$) and *L. major* ($P = 0.047$; $n = 20$) promastigotes compared with controls in culture containing 5% urine, this difference started at a urine concentration of 10% for *L. infantum* ($P = 0.038$; $n = 20$), and *L. tropica* ($P = 0.023$; $n = 20$) promastigotes, respectively (by paired Student's *t* test). It was determined that compared with controls, the highest increase in parasite concentration was observed in the 25% urine group for *L. tropica* promastigotes. Conversely, the 30% urine

FIGURE 1. Growth of different *Leishmania* promastigotes in increasing concentrations of urine in RPMI 1640 medium supplemented with 10% fetal calf serum. **A**, *L. donovani*; **B**, *L. infantum*; **C**, *L. major*; **D**, *L. tropica*. Addition of human urine (5%, 10%, 15%, 20%, 25%, and 30%) to the medium significantly enhances the growth rate. Bars correspond to different incubation periods (24, 48, 72, and 96 hours). Experiments were repeated three times. Error bars show the mean $\pm$ SD.

TABLE 1
Effect of urine on the cell cycle of *Leishmania* spp. promastigotes*

Species	G0/G1		S		G2/M		Proliferation index	
	Control	Urine	Control	Urine	Control	Urine	Control	Urine
<i>L. tropica</i>	87.97 ± 2	61.83 ± 4	9.77 ± 2	25.35 ± 5†	2.26 ± 0.5	12.82 ± 1	12.03 ± 3.20	38.17 ± 5.12†
<i>L. infantum</i>	76.12 ± 2	64.12 ± 2	17.8 ± 1	29.33 ± 3†	6.70 ± 2	6.55 ± 2	24.05 ± 4.35	35.88 ± 6.40†
<i>L. donovani</i>	79.74 ± 3	65.26 ± 5	15.71 ± 3	27.39 ± 5†	4.55 ± 1	7.35 ± 2	20.26 ± 4.1	34.74 ± 5.6†
<i>L. major</i>	83.33 ± 4	65.78 ± 4	11.42 ± 3	26.84 ± 4†	5.25 ± 2	7.38 ± 2	16.67 ± 3.76	34.22 ± 4.66†

*Values are mean ± SD cell cycle parameters and proliferation indexes of *L. tropica*, *L. infantum*, *L. donovani*, and *L. major* parasites in 25% urine-supplemented medium. There was a significant increase in the percentage of parasites for S phases compared with control groups ($P < 0.05$; $n = 20$) after incubation for 96 hours. Proliferation indexes for all *Leishmania* species were significantly different compared with those of controls.

† $P < 0.05$ ($n = 20$) versus corresponding control value.

concentration resulted in a decrease in parasite volume when compared with values for the 25% urine group.

Proliferative effect of urine on *Leishmania* promastigotes.

Cell cycle parameters and proliferation index of *L. tropica*, *L. infantum*, *L. donovani*, and *L. major* parasites in medium supplemented with 25% urine are shown in Table 1. After 96 hours of incubation, there was a significant increase in the percentage of the parasites in S phases in cultures containing 25% urine in contrast to the control groups ($P < 0.05$; $n = 20$). These findings of a high percentage of cells in the S phase and at a high proliferation index indicates a stimulatory effect of 25% urine on cell proliferation.

In addition, results showed that for all *Leishmania* species, proliferation indexes in culture medium containing urine were significantly different than those for controls ($P < 0.05$; $n = 20$). However, no significant differences in the proliferation indexes were observed among different *Leishmania* species that were cultured in the presence of urine ($P = 0.134$, by Tukey's post-hoc test, $n = 20$).

Infection index. Results showed that the infection index for *L. tropica* ($P = 0.037$; $n = 20$), *L. infantum* ($P = 0.021$; $n = 20$), *L. donovani* ($P = 0.009$; $n = 20$), and *L. major* ($P = 0.001$; $n = 20$) promastigotes exposed to urine was greater than those for the controls (by paired Student's *t* test; Table 2). In addition, the infection indexes for *L. infantum* ($P = 0.049$; $n = 20$), *L. donovani* ($P = 0.038$; $n = 20$), and *L. major* ($P = 0.041$; $n = 20$) promastigotes were different from those for *L. tropica* promastigotes (by paired Student's *t* test). The presence of urine also caused significant difference in infection indexes of *L. infantum* and *L. donovani* parasites that cause visceral leishmaniasis. Likewise, similar results were observed between infection indexes of *L. tropica* and *L. major* promastigotes that cause cutaneous leishmaniasis.

DISCUSSION

This study has shown that human urine in parasite culture affected the infectivity and proliferation of all four types of

Leishmania parasites that were investigated *in vitro*. To the best of our knowledge, no similar methods or data have been reported.

The native growth conditions for amastigotes that are found in mammalian hosts are different than those for promastigotes in vectors.²¹ Infected macrophages of mammalian hosts include factors that provide optimal growth conditions for amastigote replication.²² Conversely, in sand flies, growth conditions originate from enzymatic digestion of protein macromolecules in blood obtained from the mammalian host into smaller peptides and amino acids and can play an important role in proliferation and transmission of parasites from amastigote to promastigote forms. It has been demonstrated that transmission of procyclic parasites into infective metacyclic promastigotes changed, depending on the type of blood obtained from the host.²¹ This finding is caused by variations in protein contents of different vertebrate blood types.²³ Blood of all hosts have specific effects in differentiation of each *Leishmania* species. Reservoir host blood plays a key role in transmission of parasites into the infective stage.²¹ Therefore, specific molecules are required for differentiation of procyclic promastigotes into metacyclic forms in *Phlebotomus* spp. sand flies and in *in vitro* culture medium.

In our study, differences in infectivity between control and urine-containing cultures may induce metacyclic parasite growth by molecules in urine. Differentiation of *Leishmania* parasites varies from species to species.²⁴ Formation of metacyclic promastigotes varies for each species because of environmental conditions. Similarly, in our study, the effect of urine-containing medium on infectivity was different for each *Leishmania* species. The infection indexes were 13 ± 1.7 for *L. tropica*, 55 ± 2.2 for *L. infantum*, 41 ± 3.14 for *L. donovani*, and 49 ± 3.26 for *L. major*. In addition, differences have been detected in infectivity indexes between types of *Leishmania* parasites and clinical forms. These differences may be caused by different biological properties of different parasite species. Conversely, decreases in infectivity indexes may be related to long-term cultivation of parasites after isolation.

TABLE 2
Influence of human urine on infection of macrophages by *Leishmania* spp. promastigotes*

Species	% Infected cells		No. parasites/cell		Infection index	
	Control	Urine	Control	Urine	Control	Urine
<i>L. tropica</i>	15 ± 0.5	26 ± 0.1	0.43 ± 0.02	0.49 ± 0.02	6 ± 0.1	13 ± 1.7
<i>L. infantum</i>	26.05 ± 1.1	39.25 ± 2.1	0.78 ± 0.06	1.4 ± 0.15	20 ± 1.3	55 ± 2.2
<i>L. donovani</i>	25.16 ± 1.8	37.44 ± 1.24	0.65 ± 0.05	1.1 ± 0.1	16 ± 1.62	41 ± 3.14
<i>L. major</i>	21.14 ± 1.68	41.18 ± 2.87	0.56 ± 0.02	1.2 ± 0.18	12 ± 1.28	49 ± 3.26

*Values are mean ± SD for infected macrophages of controls and the 25% concentration of human urine-exposed *Leishmania* promastigotes *in vitro*. Exposed parasites and the cultures were examined after 24 hours. Infection index of *L. tropica* ($P = 0.037$; $n = 20$), *L. infantum* ($P = 0.021$; $n = 20$), *L. donovani* ($P = 0.009$; $n = 20$), and *L. major* ($P = 0.001$; $n = 20$) was greater than that of controls. Statistical analysis (by paired Student's *t* test) indicates a significant difference ($P < 0.001$) compared with controls.

Different media (biphasic, semi-solid, or liquid) are being used for *in vitro* culture of *Leishmania* promastigotes. These media must contain defibrinated rabbit blood and fetal bovine serum or fetal calf serum as an essential factor for survival and replication of the parasites.^{25,26} However, in long-term *in vitro* cultivation of parasites with these media, infectivity was found to decrease with time, although proliferation of parasites was supported.^{27,28} Parasites isolated from animal models have been found to have increased infectivity *in vitro*. However, infectivity of these parasites starts to decrease after two passages.²⁷ Infectivity of parasites decreased in hamsters, even though parasites proliferated in Novy-McNeal-Nicolle agar culture that contained rabbit blood.²⁹

In several studies, researchers have tried to increase the infectivity of *Leishmania* parasites that were long-term cultured *in vitro* and had low infectivity.^{4-6,16,17,30-33} Promastigotes of *L. braziliensis* were exposed to heat shock by changing the temperature of their culture from 26°C to 34°C.⁶ Completely defined serum-free medium was used for conservation of infectivity *in vitro*.¹⁶ Attempts were made to increase infectivity of parasites by co-culture with J774 macrophages.⁴

All of these studies showed that culture medium should contain factors that enable development of non-infective procyclic promastigotes into infective metacyclics. NMR-based metabolomics analysis of urine samples that were taken from healthy persons showed that urine was composed of 3-hydroxybutyrate, acetate, succinate, alanine, citrate, creatine, creatinine, dimethylamine, formate, glycine, hippurate, histidine, indoxyl sulfate, lactate, N-acetyl groups from glycoproteins, phenylalanine, taurine, and trimethylamine N-oxide.³⁴ Our study showed that proliferation and infectivity of parasites were increased in urine-containing medium. Another study reported detection of intracellular metabolites of *L. donovani* by ¹H NMR spectroscopy, and that *Leishmania* parasites contained hydroxybutyrate, acetate, lactate, alanine, and succinate, which are also present in urine.³⁵ It is likely that these metabolites are also present in human urine and are important for successful metabolism of parasites.

In another study, components synthesized from glycine via mitochondrial glycine cleavage complex were shown to play important roles in metabolism and virulence of *L. major* parasites.³⁶ In a study of *Trypanosoma cruzi* and *T. rangeli*, an increase in the number of infective tripomastigotes was detected in urine-containing culture.³⁷ In our study, infectivity of *Leishmania* promastigotes increased in cultures containing urine compared with control cultures that did not contain urine. Results were similar to the effects of urine in cultures of *Trypanosoma* spp. Our results also demonstrated that changes in proliferation and infectivity of parasites may be affected by components found in urine.

Leishmania parasites have digenetic life cycles. They first differentiate and increase in size as procyclic promastigotes within the gut of *Phlebotomus* spp. sand flies. They then exit the cell cycle and differentiate as metacyclic promastigotes (infective form).³⁸ Growth curves of procyclic and metacyclic forms of promastigotes can change in *in vitro* culture conditions, and some differences also occur during life cycles of parasites. Therefore, differences in infectivity may be attributed to differences in the cell cycle.³⁸ Analysis of the effect of urine on the cell cycle phases (G0/G1, S, and G2/M) has showed that proliferation indexes were increased in culture media supple-

mented with human urine (*L. tropica* 38.17 ± 5.12; *L. donovani* 34.74 ± 5.6; *L. major* 34.22 ± 4.66; and *L. infantum* 35.88 ± 6.40) compared with controls.

Leishmania promastigotes initially grow in length and have a constant amount of DNA (G1). DNA synthesis then starts and DNA is duplicated while the cell length remains constant (S phase). Finally, the cell is reduced in length and retains the duplicated DNA content (G2 and mitosis) before cytokinesis returns the cell to start a new cell cycle.³⁹ However, *Leishmania* promastigote length shows a wide distribution (6–12 μm) and no significant difference between culture densities. Similarly, total DNA content has a two-fold range and no significant difference between culture densities during the cell cycle.³⁹ On the basis of flow cytometry data, urine-containing culture media create differences in analysis of the cell cycle. Although a decrease in G0/G1 phases was observed compared with controls, there was an increase in G2/M, S phases and the proliferative index. Results have shown that, similar to serum that contains numerous unknown growth factors, urine also contains some undefined factors that increase parasite proliferation and facilitate the transition from G0/G1 to S phases more quickly.

Another observation in our study was that urine concentrations greater than 5% can inhibit proliferation of *Leishmania* parasites.^{7,10,37} Our results typically have shown that promastigotes could efficiently proliferate and grow in culture media containing relatively high urine concentrations. In our study, distinct from the other studies, the reason for increased development of parasites in culture containing 25% urine may be dependent on use of different parasite species and types of culture medium.

In conclusion, we report that human urine in parasite cultures differentially affected the infectivity and proliferation of *Leishmania* species promastigotes. Further identification of components found in urine that play a role in infectivity of parasites may be important for understanding transmission mechanisms of parasites into infective stages.

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