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Selective elimination of *Leptomonas* from the *in vitro* co-culture with *Leishmania*

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ABSTRACT

Leishmania and *Leptomonas* are protozoan parasites of the family Trypanosomatidae. *Leishmania donovani* causes the fatal visceral leishmaniasis (VL; kala-azar) in mammals and is transmitted by sand fly vector. Certain VL-cured human populations in India and Sudan develop post kala-azar dermal leishmaniasis (PKDL) due to the same parasite. Although *Leptomonas* is parasitic mainly in insects, several recent reports on the clinical isolates of *L. donovani* from VL and PKDL patients in India confirm co-infection of *Leptomonas seymouri*, probably due to immune suppression in those individuals. Detection of *L. seymouri* in the *in vitro* cultures of *L. donovani* from clinical origin is difficult due to many similarities between *L. seymouri* and *L. donovani*. We describe here ways to detect *L. seymouri* and *L. donovani* in co-culture. In addition, based on our observation regarding the growth of *L. seymouri* in different culture conditions, we report here a novel procedure, which can selectively eliminate *L. seymouri* from the *in vitro* co-culture with *L. donovani*. This would be beneficial to researchers who prefer to deal with pure populations of *Leishmania* parasites for various downstream immunological and genetic studies.
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1. Introduction

Protozoan parasites of the genus *Leishmania* belong to the family Trypanosomatidae of the order Kinetoplastida. Many of them cause a spectrum of diseases known as Leishmaniasis found in nearly 88 tropical countries. Among the various clinical manifestations of leishmaniasis, visceral leishmaniasis (VL) due to *L. donovani* or *L. infantum* is fatal if left untreated. More than 90% of VLs are reported from Bangladesh, India, Brazil and Sudan with nearly 60,000 deaths per annum [1]. Medications take a long time to cure, are expensive and generate drug-resistant parasites [2]. Few VL (due to *L. donovani*)-cured populations in India and Sudan develop post kala-azar dermal leishmaniasis (PKDL) [3]. No vaccine is yet available for human leishmaniasis [4], although a few first-generation vaccines are available for canine VL [5]. Intensive investigations are underway worldwide as many laboratories to look for vaccines or new drugs against VL.

Researchers study *L. donovani* isolated clinically from infected humans. However, unusual cases (~17% of cases) of VL and PKDL were often noticed with isolates of *L. donovani* showing *Leptomonas seymouri* (a lower trypanosomatid [6]) as a co-infectant in the Indian subcontinent [7,8]. In regions of antimony drug failure in leishmaniasis in India, *L. seymouri* was observed to contribute to growing incidents of VL/PKDL [8]. Recently, a next-generation SOLiD™ platform identified *Leptomonas*, while sequencing the genome of parasites isolated from clinical cases of VL in India [9]. The occurrence of *Leptomonas* in the VL or PKDL cases is probably due to *L. donovani* inducing a strong immunosuppression in humans in this region. As an opportunistic infection, *Leptomonas* has been identified from the parasite cultures obtained from the bone marrow aspirate of an HIV patient presented with VL [10]. Whether the presence of *L. seymouri* cells in the clinical isolates of *L. donovani* is a recent occurrence or existed in past and was never explored previously is a moot point.

In the life cycle of *Leishmania*, the 'promastigote' form replicates extracellularly in the insect vector gut, whereas the 'amastigote' form multiplies intracellularly in the host cells (e.g., macrophages). With appropriate *in vitro* culture conditions, these two stages can be cultivated in laboratories. Cell doubling time for the promastigote stage of *L. seymouri* in the *in vitro* culture is shorter than the promastigotes of *L. donovani*. Hence, the former outgrows the latter soon in co-culture *in vitro* [7]. *Leptomonas*, except for minor differences, has many of its features including most of the genomic sequences, organization and antigenicity identical to those of *Leishmania* [9,11–13]. In common with *L. donovani*, *L. seymouri* has been known to be susceptible to many of the leishmanicides [9], making it difficult to eliminate *L. seymouri* from the mixed cultures, compounding the challenges for studies towards treatment/eradication of VL. Hence, the utmost importance is to recognize the presence of *L. seymouri* in *L. donovani* cultures originating from clinical samples and eliminate it in order to focus the studies on *L. donovani*. Here, we describe a unique and rapid cell culture approach

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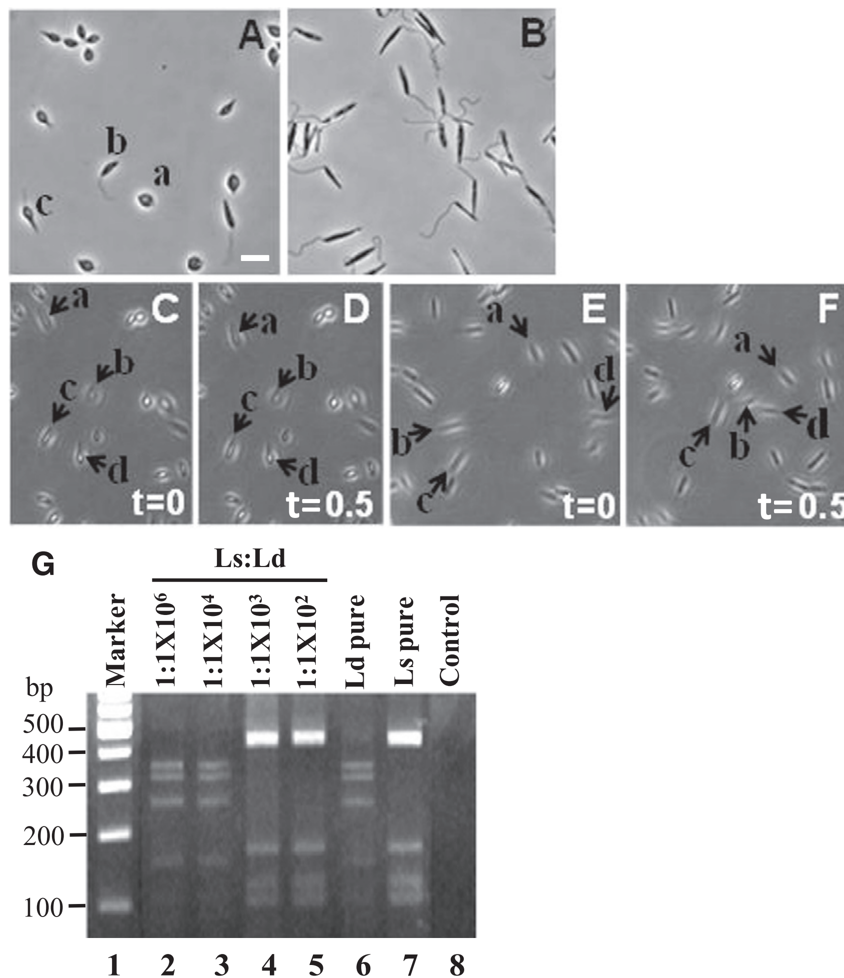

Fig. 1. Comparison of the morphology of *L. donovani* and *L. seymouri* in an *in vitro* culture. (A and B) Phase-contrast image comparison of the morphology of the parasites in the promastigote medium. (A) The variably shaped cell morphology of *L. donovani* (at least 3 different shapes are shown by lowercase alphabets) in promastigote medium. (B) The near uniform shaped *L. seymouri* cells. (C–F) The mobility of promastigote cells were imaged from the *in vitro* culture; $t = 0$: time zero; $t = 0.5$: 0.5 s after. (C and D) Selected still images of *L. donovani* from video (not included): (E and F) Selected still images of *L. seymouri* from video (not included). Each representative cell (marked in lowercase alphabets) is displayed to follow mobility in 0.5 s. (G) Agarose gel showing the detection of *L. seymouri* via PCR-RFLP analysis. RFLP was carried out from the combined cells of known number of *L. seymouri* (Ls) and *L. donovani* (Ld) as the ratios mentioned in lanes 2–5. Ld pure, only *L. donovani* cells; Ls pure, only *L. seymouri* cells; Control, no DNA. Scale bar: 10 μm. Data are representative of three independent experiments.

that makes use of differential culture conditions to selectively eliminate *L. seymouri* from the *in vitro* spiked mixed cultures with *L. donovani*. Elimination of *L. seymouri* from the *in vitro* co-culture with *L. donovani* as reported here would be useful in laboratories that prefer to deal with pure populations of *Leishmania* cells for various follow up research activities.

2. Materials and methods

Leishmania donovani 1S (a cloned line from strain 1S, WHO designation: MHOM/SD/62/1S), *L. donovani* DD8 (ATCC #50212), *L. donovani* AG83 [14], *L. donovani* $HP^{+/-}$ (Kavita et al., unpublished) and *L. seymouri* (ATCC #30220) were used in all experiments. Among the *Leishmania* species, strain 1S was used for most experiments unless otherwise mentioned.

Promastigote forms of all the *L. donovani* strains were grown *in vitro* in T25 cm^2 culture flasks (Corning) at 26 °C in medium 199 (Sigma) [15] (pH 6.8) with 8 μM 6-Biotin, 25 mM Hepes (*N*-[2-hydroxyethyl]piperazine-*N*0-[2-ethanesulfonic acid; Sigma], 0.1 mM adenine (Sigma; in 25 mM Hepes), 8 μM hemin (4 mM stock made in 50% triethanolamine), 100 U/ml each of penicillin G and streptomycin (Gibco) and 10% (v/v) heat-inactivated fetal bovine serum (Gibco). Axenic amastigotes were grown in T25 cm^2 culture flasks at 37 °C with 5% CO_2 in RPMI-based medium [16] (pH 5.6) containing 15 mM KCl, 114.6 mM KH_2PO_4, 10.38 mM $K_2HPO_4 \cdot 3H_2O$, 0.5 mM $MgSO_4 \cdot 7H_2O$ and 24 mM $NaHCO_3$, 1× liquid RPMI-1640 vitamin mix (Sigma); 1× liquid RPMI-1640 amino acid mix (Sigma), 4 mM L-glutamine (Gibco), 25 mM adenosine (Sigma), 23 μM folic acid (23 mM stock made in 1 N KOH Sigma), 100 U/ml each of penicillin G and streptomycin (Gibco), 1× liquid phenol-red (Gibco), 22 mM D-glucose (Sigma), 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES, Sigma) and 20% heat-inactivated fetal bovine serum (Gibco). Growth of both promastigotes and amastigotes were measured as described previously [17].

Cells were examined under a microscope (Nikon (DIAPHOT-200), Tokyo, Japan) and images were processed using Adobe Photoshop 7.0.1 (Adobe Systems Inc., Mountain View, CA). Cell viability was determined by staining with 0.4% trypan blue (Sigma Aldrich) and counted by haemocytometer (Sigma Aldrich).

Genomic DNA isolation from the parasites, PCR, restriction digestion and other routine molecular biological procedures were carried out as described previously [18]. *Leptomonas* or *Leishmania* strains were differentially diagnosed by restriction fragment length polymorphism (RFLP) analysis of Hsp70 as described by others [7,19,20]. Briefly, a 1,420-bp fragment of Hsp70 gene was amplified, digested with *Hae*III restriction enzyme (New England Biolabs) and resolved on 3% agarose gel.

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