Parasitology International 64 (2015) 1–5

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

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

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ARTICLE INFO

Article history:
Received 16 July 2014
Received in revised form 30 December 2014
Accepted 2 January 2015
Available online 9 January 2015

Keywords:
Leishmania
Leptomonas
elimination
Promastigote
Amastigote
Selective medium

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.

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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http://dx.doi.org/10.1016/j.parint.2015.01.003
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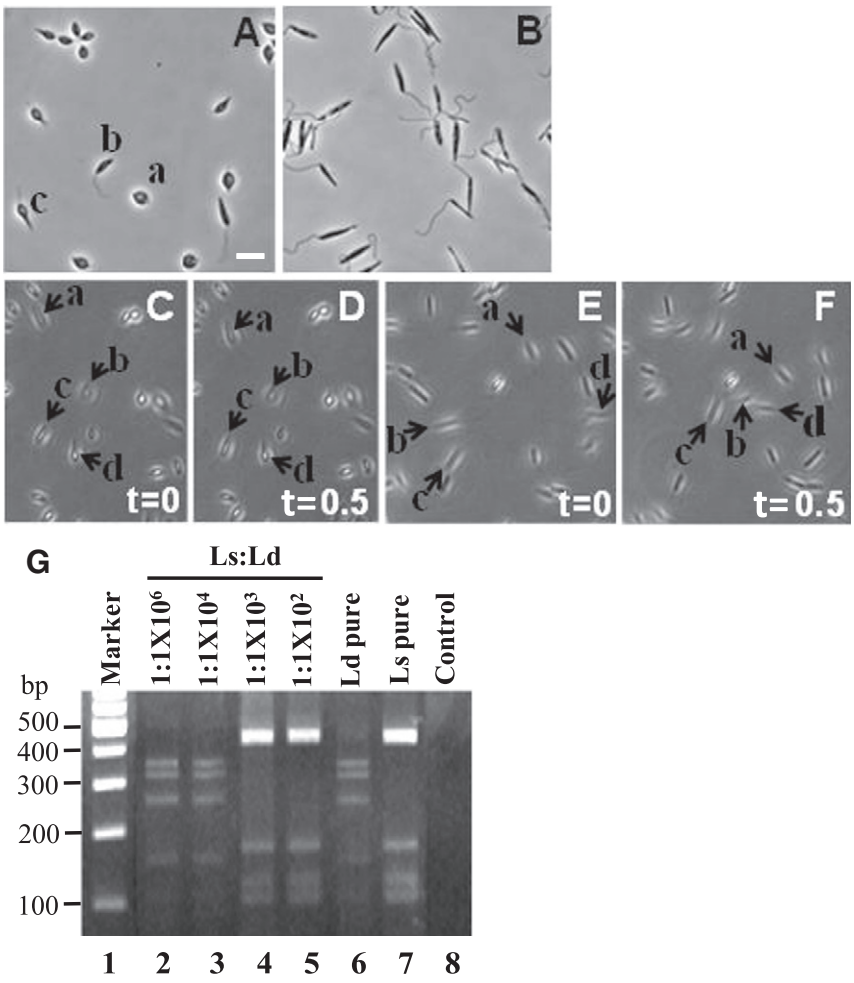

Fig. 1. Comparison of the morphology of *L. donovani* and *L. seymouri* in an *in vitro* culture. (A and B) Phase-contrast image comparison of the morphology of the parasites in the promastigote medium. (A) The variably shaped cell morphology of *L. donovani* (at least 3 different shapes are shown by 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.

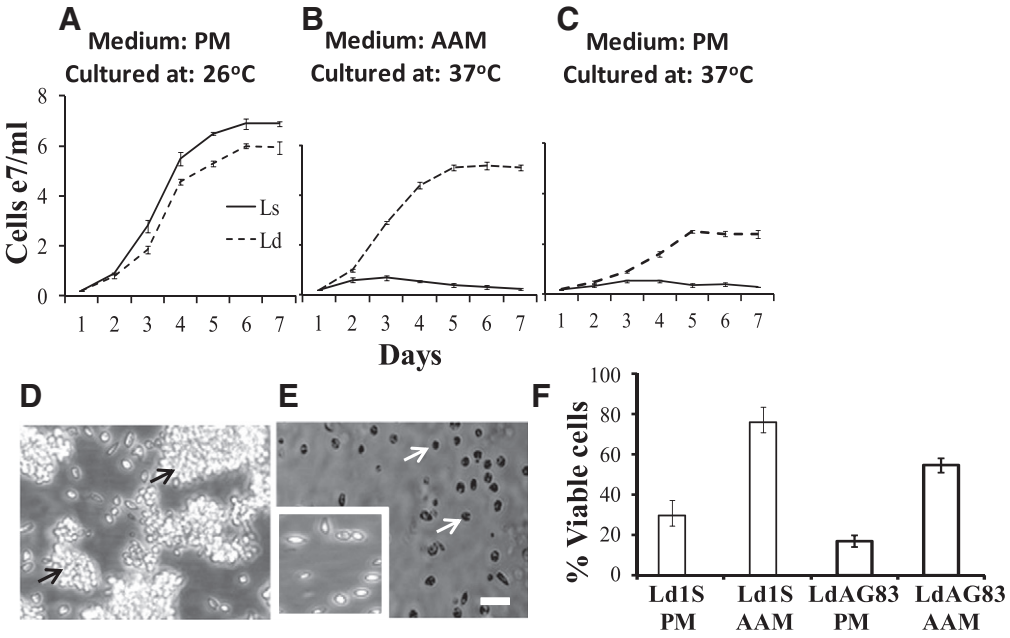

Fig. 2. Growth comparison of *L. donovani* and *L. seymouri* in culture *in vitro*. (A–C) *In vitro* growth comparison of *L. donovani* (dashed line) and *L. seymouri* (solid line). Growth of the parasites at 26 °C in the promastigote medium (A), at 37 °C in the axenic amastigote medium (B) and at 37 °C in the promastigote medium (C) are shown. (D and E) Comparison of the morphology of parasites in the axenic amastigote medium on day 7 in culture. (D) Trypan blue-negative live cells of *L donovani*. The black arrows indicate aggregated live cell clumps of axenic amastigotes. (E) Trypan blue-positive dead cells of *L. seymouri* on day 7 in culture. The inset shows *L. seymouri* cells after differentiation into amastigote-like rounded cells on day 3 in culture. The white arrows indicate dead cells. Scale bar: 20 μm. (F) Percent viable cells of *L. donovani* 1S (Ld1S) and *L. donovani* AG83 (LdAG83) grown either in the promastigote medium or in the axenic amastigote medium at 37 °C and counted after staining with trypan blue on day 5. The data represent the means ± S.D. of three independent experiments. PM, promastigote medium; AAM, axenic amastigote medium.

Differential DNA fragments obtained suggested the target as either *Leptomonas* or *Leishmania*.

3. Results and discussion

3.1. Detection of Leptomonas at the promastigote stage by comparing its morphology and mobility with L. donovani promastigotes in culture

Since we observed *Leptomonas* contamination in the *Leishmania in vitro* cultures, we used the promastigote and axenic amastigote media of *Leishmania* to culture *Leptomonas* to compare their morphology and physiology with *Leishmania*. We examined *L. seymouri* and *L. donovani* cells microscopically to see the difference in morphology. All promastigote cells of *L. seymouri* were ellipsoidal and uniformly elongated at both log stage and stationary stage (Fig. 1B). In contrast, *L. donovani* promastigotes were heterogeneous in shape ranging from shorter ellipsoidal (Fig. 1Aa), to longer ellipsoidal (Fig. 1Ab) and tadpole-like (Fig. 1Ac). *L. seymouri* cells displayed 3.4 times faster mobility compared to *L. donovani* cells in culture (average measured from at least 30 cells in each case from the still images obtained from videos [data not shown]), when observed under a microscope (Fig. 1C–F).

3.2. Sensitivity of PCR-RFLP for identification of L. seymouri in co-culture with L. donovani

A PCR-RFLP-based procedure targeting heat shock protein (Hsp70) gene to discriminate *Leishmania* species in the New and Old World [19,20] has recently been followed by many researchers to further discriminate between *Leishmania* and *Leptomonas* species [7–9], although few other fluorescence 'melt curve' qPCR methods to discriminate between these parasites have been suggested [21]. However, the detection level of the parasite in the *in vitro* co-culture with *Leishmania* is not known. Here, we determined the lowest level of detection of *L. seymouri* in *L. donovani* cultures by following the Hsp70 targeting PCR-RFLP analysis using DNA extracted from *Leishmania* promastigotes spiked with varying numbers of *L. seymouri* cells, viz., ratio *L. seymouri:L. donovani*; $1:1 \times 10^6$, $1:1 \times 10^4$, $1:1 \times 10^3$, $1:1 \times 10^2$. At $1:1 \times 10^3$ ratio, only *Leptomonas* was detected by RFLP (Fig. 1G, lane 4), whereas at $1:1 \times 10^4$ ratio or less cells of *L. seymouri* per million of *L. donovani*, only *Leishmania* was detected (Fig. 1G, lanes 2 and 3). The reason for the dominant selective amplification of *Leptomonas* DNA even at $1:1 \times 10^3$ ratio is not clear but could be due to selective annealing of the primers to the target DNA of *Leptomonas* over *Leishmania* or due to the presence of the PCR target in *Leptomonas* as high copy number in the genome compared to *Leishmania*. However, it is clear from the study that even though the immediate promastigote culture isolates from clinical samples resemble *Leishmania* under the microscope, as little as 1 *Leptomonas* in 1000 *Leishmania* cells can be detected by this method.

3.3. Growth comparison of promastigotes and axenic amastigotes of L. donovani and L. seymouri in culture

We monitored the growth of the parasites individually in different culture media. When grown in *Leishmania's* promastigote medium at 26 °C, *L. seymouri* showed slightly faster growth rate compared to *L. donovani* (Fig. 2A) as also reported by others [7]. Since the replication of *L. seymouri* in the mammalian tissue has not been well studied, we tested its growth as axenic amastigotes in the amastigote medium at 37 °C. *L. seymouri* showed growth arrest (Fig. 2B) with rounded, unstained amastigote-like cells on day 3 (Fig. 2E, inset) and dead, trypan blue-stained cells on day 7 (Fig. 2E). Axenic amastigotes of *L. donovani* 1S displayed its normal growth (Fig. 2B) [17] with all living (negative to trypan blue staining) and aggregated cells on day 7 (Fig. 2D). The 7-day old culture of *L. seymouri* cells from axenic amastigote medium did not replicate/recover into promastigotes when transferred to promastigote medium (data not shown), confirming that all cells were dead. Such *in vitro* growth arrest of *L. seymouri* axenic amastigotes resembled the growth of *Leptomonas costoris*, a parasite of water striders, which when infected *ex vivo* in hamster macrophages at 35 °C,

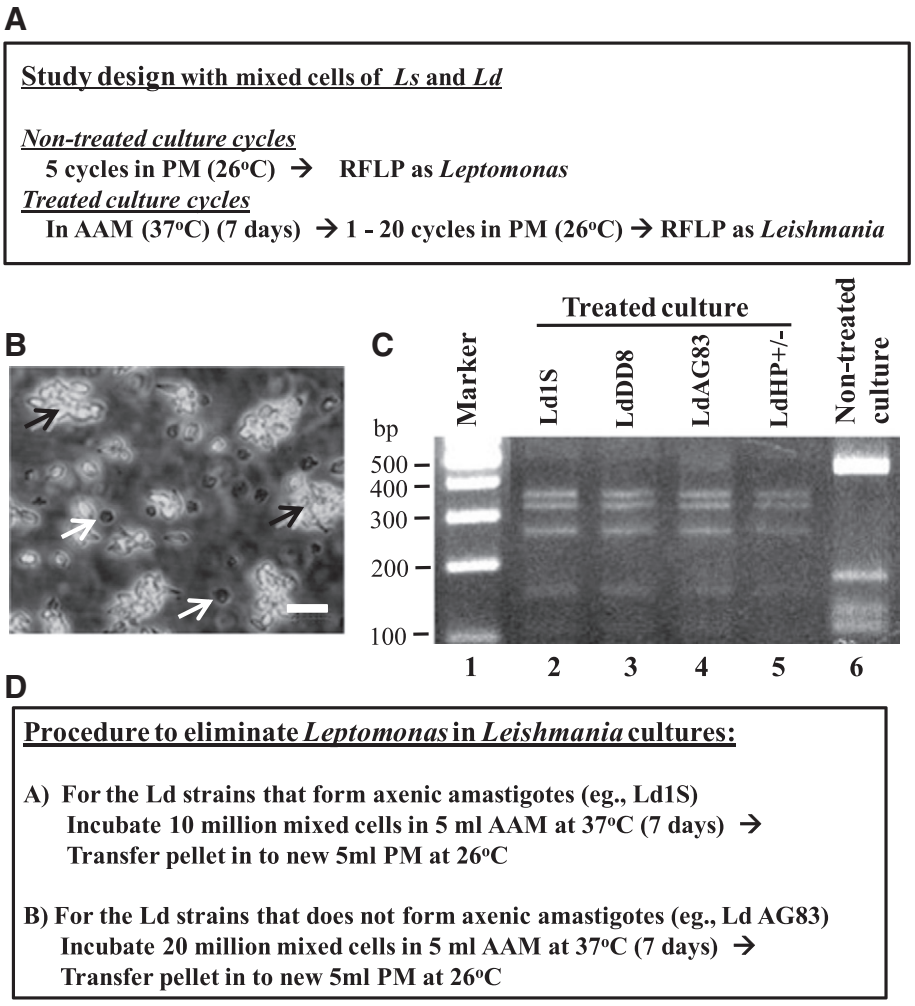

Fig. 3. Study design and procedure to eliminate *Leptomonas* in *Leishmania* cultures. (A) Study design to eliminate *L. seymouri* in the experimental culture. (B) Co-culture of *L. donovani* and *L. seymouri* showing the live and dead cells of *L. donovani* and *L. seymouri*, respectively, on day 7 in culture in the axenic amastigote medium at 37 °C. The black and white arrows indicate live and dead cells, respectively. Scale bar: 20 μm. (C) Agarose gel confirming elimination of *L. seymouri* from *L. donovani* culture *in vitro*. The non-treated culture subsequently overtaken by the growth of *L. seymouri* (after 5th culture cycle) is shown in lane 6. The treated culture of *L. donovani* after elimination of *L. seymouri* after 20 culture cycles that displays *Leishmania* pattern in RFLP analysis is shown in lanes 2–5. Data are representative of at least ten independent experiments. (D) Description of procedure for *Leptomonas* elimination in the mixed co-culture with *Leishmania*. PM, promastigote medium; AAM, axenic amastigote medium; Ls, *L. seymouri*; Ld, *L. donovani*.

transformed into amastigotes but did not survive [22]. In order to confirm whether the growth arrest of *L. seymouri* is due to pH and/or temperature stresses, we also tested its growth in the promastigote medium at 37 °C instead of 26 °C. Even in this medium (with pH 6.8), the cells of *L. seymouri* displayed complete growth arrest (Fig. 2C), confirming that elevated culture temperature could be the sole lethal factor for its growth. However, the growth of *L. donovani* in this medium, although not arrested, showed a reduced growth rate when compared to its growth in the axenic amastigote medium at 37 °C. Moreover, on day 5 in culture, the viable *L. donovani* 1S cells that excluded trypan blue were only 31% when grown at 37 °C in the promastigote medium, whereas it showed 78% viability when grown as axenic amastigotes (Fig. 2F). A similar trend was observed with *L. donovani* AG83 (a strain that does not propagate as axenic amastigotes *in vitro*), but with reduced survivability at this temperature compared to the strain 1S, viz., 17% and 55% in promastigote and axenic amastigote media, respectively (Fig. 2F). These results confirm that the cell death of *L. donovani* at 37 °C was minimal in the axenic amastigote medium compared to its growth in the promastigote medium. The results also indicate that *L. seymouri* can grow optimally only at 26 °C. Whether the death of *Leptomonas* in culture at 37 °C is due to necrosis or apoptosis needs further investigation. These results indicate that *L. seymouri* parasites in the *Leishmania* cultures can be selectively and efficiently killed, when the culture is grown for 7 days in *Leishmania*'s axenic amastigote medium at 37 °C.

3.4. Elimination of *L. seymouri* from the mixed in vitro culture with *L. donovani*

In order to obtain pure *L. donovani* cultures devoid of *L. seymouri* contamination originating from clinical samples, we devised a simple *in vitro* culture procedure as in Fig. 3A (treated culture cycles), based on our observation that *Leptomonas* does not grow in *Leishmania*'s axenic amastigote culture conditions. The mixed cell cultures of *L. seymouri* and *L. donovani* at either $1{:}1 \times 10^6$ or $1 \times 10^6{:}1$ cell ratios were grown initially in the axenic amastigote medium and the culture continued at 37 °C for 7 days in order to completely kill *L. seymouri* cells (as in Fig. 3B). On day 7, cells from the axenic amastigote culture were transferred to promastigote medium and then subcultured every 3 days in the promastigote medium for 20 cycles. The cells either at the 1st or after 20 continuous culture cycles in the promastigote medium did not show fast moving *Leptomonas* under the microscope and the RFLP from such cultures confirmed the presence of only *Leishmania* parasites (Fig. 3C, lane 2). The mixed population that was not cultured under *L. donovani* amastigote culture conditions but cultured continuously in

the promastigote medium for 5 culture generations (non-treated cycles; Fig. 3A) was enriched in *Leptomonas* as confirmed by their uniform cell morphology, their rapid motility as well as by RFLP analysis (Fig. 3C, lane 6). The *Leishmania* cells shown in the data here were of *L. donovani* 1S. Since not all the strains of *L. donovani* could be cultured as axenic amastigotes, we also included three other strains of *L. donovani* viz.: *L donovani* DD8, which exhibits moderate growth, and *L. donovani* AG83 and *L. donovani* $HP^{+/-}$, which show poor growth in the axenic amastigote culture condition. We similarly successfully confirmed the elimination of *Leptomonas* from the manually mixed cultures with these strains of *L. donovani* (Fig. 3C, lanes 3–5). The incubation of mixed parasites in the promastigote medium at 37 °C, although it selectively killed only *L. seymouri*, led to poor growth of *L. donovani* 1S (Fig. 2C) when compared to its growth in the amastigote medium at 37 °C (Fig. 2B). Hence, we chose axenic amastigote medium and its growth conditions to promote optimal growth of *L. donovani* and at the same time eliminate *L. seymouri*.

The procedure developed by us to eliminate *Leptomonas* from cultures of these strains of *Leishmania* is briefly summarized in Fig. 3D. Alternatively, through the previously described procedures, *Leptomonas* can be eliminated from the mixed culture by either directly plating the cells over noble-agar plates containing the same growth ingredients or culturing by limiting dilutions in multiwall plates [17]. However, the growth of the derived clonal populations and confirmation of elimination of *L. seymouri* contamination from *Leishmania* cultures can take as long as 3 weeks. There are many occasions when researchers might not want clonally selected populations of *Leishmania* through clonal selection and might like to study the entire cell population of a clinical isolate of *L. donovani*, since it is known that amastigotes isolated from infected tissues represent a heterogeneous population [23]. Another previously described procedure for elimination of *Leptomonas* spp., which is via infecting the mixed parasite population in animals in order to isolate pure *L. donovani* amastigotes from their organs after a few weeks, may not be ideal, since a recent report revealed the isolation of *Leptomonas* from the spleen of mice infected previously with *Leptomonas* spp. [7]. Additionally, since no drug is available that can selectively kill only *L. seymouri*, the growth conditions at specific temperature that selectively allow only the growth of *L. donovani* and kill *L. seymouri* can be a recommended procedure to eliminate the latter from the mixed culture originating from clinical samples. Moreover, since *L. donovani* axenic amastigotes were also known to retain infectivity in animals [16,24], the promastigotes recovered via axenic amastigote culture can also be used for infectivity purposes.

4. Conclusion

In Trypanosomatidae, *Leptomonas* sp. is known to infect mainly insects, whereas *Leishmania* is known to infect both insects and mammals including humans with distinct clinical manifestations [11,13]. However, several recent reports revealed the presence of *L. seymouri* along with *L. donovani* in clinical isolates from VL and PKDL patients in the Indian subcontinent. The contribution of *L. seymouri* to either VL or PKDL along with *L. donovani* has still not been ascertained [8,9]. Hence, until the involvement of *L. seymouri* in VL or PKDL is proven, it is pertinent to eliminate *L. seymouri* from *Leishmania* cultures in order to focus studies on *L. donovani*. Therefore, we suggest here an easy-to-follow *in vitro* procedure to selectively eliminate *L. seymouri* in the *L. donovani* cultures based on our observation that *L. seymouri* does not grow *in vitro* at 37 °C. This procedure is simple and takes only a week to obtain pure cultures of *L. donovani*. In this process, we have also investigated ways to discriminate the parasite *L. seymouri* in the mixed cultures with *L. donovani*.

Acknowledgements

This work was partly supported by DBT, India grant #BT/PR441/MED/15/71/2011. We acknowledge Niti Puri, Jawaharlal Nehru University and Vibha Taneja, Department of Research, Sir Gangaram Hospital, New Delhi, India for critical reading of the manuscript.

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