Agar barrier method for removal of yeast and other fungal contamination from *Leishmania* promastigotes culture

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The present study explains a novel method of *Leishmania* promastigotes culture decontamination. The method is based on motility of *Leishmania* promastigotes across agar barrier which facilitates decontamination from yeast and other non motile contamination. This is inexpensive, easy, rapid and reliable physical method and is able to save valuable isolates in culture.

Keywords: Contamination, decontamination, *Leishmania* promastigotes, agar, physical method

Indian visceral leishmaniasis is a neglected but lethal infectious disease if left untreated. Since there is no vaccine, costly anti-leishmanial drugs with long treatment regimen are the only preventive measures. Further, emerging cases of resistance to existing drugs and co-infection of *Leishmania* with HIV warrants regular research to overcome this disease. Gold standard visceral leishmaniasis diagnosis is still relying on direct demonstration of parasites under microscopy. Continuous availability of live parasite is required for drug sensitivity test and other research activities. But ordinary culture labs dare not to grow the parasites possibly, due to chances of yeast and other fungal contamination. Despite rigorous aseptic precautions, it is almost impossible to avoid contamination¹ especially when working in field conditions and collecting samples from vectors and hosts. Removal of contamination, if occur, is of prime importance to get pure culture for further utilities. The regular use of existing antimicrobial agents like amphoterecin-B, ketoconazole, nystatin (mycostatin), griseofulvin, and 5-fluorocytosine in culture media is not in practice, due to their lethal effect on the promastigotes.^{2,3} Besides, it exerts selective pressure on sensitive promastigotes that may lead induction for development of resistance.¹ Well tolerated 5-fluorocytosine *Leishmania* promastigotes are reported to induce mutation.⁴ Further, the decontamination process using anti fungal agent is torturing, cumbersome and requires continuous subpassaging.

Agar plating method⁵ allows cloned promastigotes which may differ from original properties of archived sample. In connection to the above facts, an inexpensive, easy to perform, rapid and reliable, physical method has been developed to remove yeast and other fungal contamination, which is able to save valuable isolates in culture.

In this study, sterile *Leishmania* promastigotes culture was isolated from kala-azar patient and maintained in complete biphasic medium. Yeast and other fungal culture were maintained separately in the same medium. A pure culture of *L. donovani* promastigotes (10^7 /ml) and yeast (*Sachromycess spp*) with other fungal cells usually grow in culture as contamination (10^7 /ml) were mixed in Locke's solution in the ratio of 2:1, 1:1, and 1:2 (v/v). The mixed culture of *Leishmania* and fungus was maintained in Biological Oxygen Demand (BOD) incubator and subpassaged.

One and half milliliter of above culture ratios were poured into the bottom of different sterile screw capped 10 ml glass culture tubes. Any contact of culture bearing pipette with the parts of tube other than bottom was essentially avoided. These culture-containing vials were kept at 4°C to facilitate solidification of molten agar and to minimize heat shock effect on *Leishmania* parasite during introduction of molten agar interface. The development of interface 0.5, 0.8, and 1% agar (Himedia RM-666) was prepared with distilled water and autoclaved. The temperature of molten agar was maintained at ~48°C for further use. The molten agar was overlaid slowly on the cooled culture by using pasteur pipette up to 1.5 cm thickness (Fig. 1). Formation of air

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Figure 1 Figure showing the technique used for removal of contamination from *L. donovani* promastigotes culture. (A) 1.5 ml of contaminated culture. (B) 1.5 cm agar interface. (C) Overlay of 1.5 ml Locke's solution.

bubbles in the molten agar was avoided. The vials were left undisturbed for few minutes at room temperature for complete solidification. One and half milliliter of Locke's solution was added on solidified agar interface in each vial and incubated at $24 \pm 1^\circ\text{C}$ for different time intervals (6, 12, and 18 hours).

It was observed microscopically that 1.5 cm thick 1% agar interface allowed only promastigotes from the all selected concentration of promastigotes and fungal contamination within 6-hour incubation (Table 1). The isolated parasite from upper phase of the test system was subpassaged 10 times on every 3–4 days in the fresh medium to see the contamination

and found complete pure culture of promastigotes without any doubt. Additionally, a small amount of pure culture of the promastigotes obtained by this technique was inoculated on blood agar [10% defibrinated rabbit blood (v/v) in commercial blood agar base; Hi-media laboratories, Mumbai, India] by sterile L-spreader to confirm the removal of yeast and other fungal contaminants. The plate was incubated at $24 \pm 1^\circ\text{C}$ for 15 days with regular microscopic examination.⁵ Some distinct colonies (Pale-white, dome shaped and mucoid) were observed. A colony was picked and diluted in balanced salt solution for microscopic examination.

In the present study, agar was selected as interface, as it provides nutrition as well as pores to pass motile organism. The development of agar layer interface using agar gradient allowed upside travel of motile promastigotes, while the yeast cells and other fungal contamination were restricted below the interface in a specified time. A few other physical as well as biological methods for the removal of yeast and other fungal contamination, had been explored such as rapid ion-exchange chromatography,⁶ culture in sterilized U-tubes,¹ agar plating method.⁵ These methods were, however, more cumbersome and time-consuming as well as less reproducible. The method described in the present study is novel and quite convenient for removal of fungal contamination from *Leishmania* culture at a single attempt in limited time to save important isolates. This agar barrier for the removal of yeast and fungal contamination from *Leishmania* culture may also be applicable for other motile microorganism for removal of contamination.

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Table 1 Data showing feasibility of isolation of pure promastigotes culture (+) from yeast and other fungal contaminated *L. donovani* culture passing through 1.5 cm agar (1%) barrier within 6 hours of incubation

Promastigotes: yeast and other fungal cells	2:1	1:1	1:2
Experiment I			
Replicate	+/-	+/-	+/-
Replicate	+/-	+/-	+/-
Replicate	+/-	+/-	+/-
Replicate	+/-	+/-	+/-
Replicate	+/-	+/-	+/-
Experiment II			
Replicate	+/-	+/-	+/-
Replicate	+/-	+/-	+/-
Replicate	+/-	+/-	+/-
Replicate	+/-	+/-	+/-
Replicate	+/-	+/-	+/-
Experiment III			
Replicate	+/-	+/-	+/-
Replicate	+/-	+/-	+/-
Replicate	+/-	+/-	+/-
Replicate	+/-	+/-	+/-
Replicate	+/-	+/-	+/-
	15/0 (100%)	14/1 (93.3%)	14/1 (93.3%)

Note: Different concentration (2:1, 1:1, 1:2) of promastigotes culture versus yeast and other fungal contamination were used as control during experimentation.

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