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Short communication

# A microculture technique for isolating live *Leishmania* parasites from peripheral blood of visceral leishmaniasis patients

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**Abstract**

Current procedures for diagnosing *Leishmania* parasites from patients involve invasive and dangerous tissue aspiration. We have developed a non-invasive and highly sensitive microculture method that can isolate parasites from the buffy coat of the patient's peripheral blood. The parasites were cultured in 96-well culture plates. Nineteen parasitologically proven visceral leishmaniasis (VL) patients were included in the study. Using this technique, we were able to isolate parasites from 16 (84%) samples. However, all 19 (100%) samples were positive on culture of splenic aspirates. We conclude that this technique is useful for the isolation and cryoconservation of parasites from patients' blood. This simple method could be tried as a first-instance alternative before other more sensitive procedures such as splenic aspirate; however, negative results should be confirmed by tests with higher sensitivity.


*Keywords:* *Leishmania*; Parasite isolation; Patient peripheral blood; Buffy coat; Non-invasive method

## 1. Introduction

Indian kala-azar or visceral leishmaniasis (VL) caused by *Leishmania donovani* is a potentially fatal disease. It is endemic in the eastern part of India and often turns epidemic. Worldwide, nearly 500,000 new cases of VL occur every year (Desjeux, 1992) with 100,000 cases of VL estimated to occur annually in India; the state of Bihar accounts for majority of these 100,000 cases (Bora, 1999). Although demonstration of parasites in tissue smears is often used for the diagnosis of VL, culture improves the specificity and sensitivity. Usually tissues from either bone marrow or spleen are used; lymphadenopathy is rare in Indian VL. Sensitivity of tissues from bone marrow is often very low, which makes it unsuitable for parasite cultivation (Chulay and Bryceson, 1983). Splenic aspiration is highly sensitive for the purposes of parasite isolation, but it is a risky procedure and may occasionally be associated with fatal haemorrhage (Sundar et al., 1991; Sundar and Rai, 2002). Patients' peripheral blood contains living amastigotes (Lachaud et al., 2000; Osman et al., 1998), which could be a very good source for diagnosis by culture. However, the isolation of parasites from the patients' blood may be difficult because of low parasitemia, and it is not very well documented. In this paper, we describe a microculture technique for the isolation of *Leishmania*

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parasites from the buffy coat of VL patients' peripheral blood. This culture method was thoroughly optimized for the appropriate quantity of buffy coat and culture medium. Parasite strains isolated using this method have also been cultured and cryoconserved for further studies.

## 2. Material and methods

### 2.1. Human blood samples

This study was conducted at the Infectious Diseases Research Laboratory, Banaras Hindu University, Varanasi, India. The Ethical Committee of Institute of Medical Science, Banaras Hindu University, approved the study. Informed written consent was obtained from all patients. Nineteen parasitologically proven patients (by microscopy of splenic smear) were selected for the study.

### 2.2. Splenic smear

Smears were prepared by spreading splenic aspirates on a glass slide. Smears were air-dried, fixed with methanol and stained with a Giemsa solution. The parasite density score for pre- and post-treatment aspirates was graded on the basis of the results of blinded microscopic analysis using a conventional logarithmic scale of 0, which indicated 0 parasites per oil-immersion field (magnification, 1000) to +6, which indicated 1100 amastigotes per field (Chulay and Bryceson, 1983).

### 2.3. Micro-isolation

Five millilitres of peripheral blood were drawn from the patients and centrifuged at $1000 \times g$ for 15 min in order to isolate the buffy coat. The Tobie's blood-agar (or NNN medium) was prepared by dissolving 15 g Bacto-tryptose (BD Difco™, Franklin Lakes, NJ 07417, USA), 15 g Bactoagar (BD Difco™), 4 g NaCl, 5 g $Na_3PO_4 \cdot 12H_2O$ and 0.4 g KCl in 1 L of distilled water. The pH was adjusted to 7.6. The medium was autoclaved at 120 °C for 15 min prior to use. The RPMI 1640 medium (Lonza Ltd., Switzerland) was supplemented with 20% heat-inactivated fetal calf serum (FCS) (Lonza Ltd.), 100 units/mL penicillin (Sigma–Aldrich, Inc.), 100 μg/mL streptomycin (Sigma–Aldrich, Inc.), 1% L-glutamine (Lonza Ltd.), 0.5% gentamycin (Invitrogen Corporation) and 5% filtered human urine (donor: Dr. M. Hide).

First, 10 μL defibrinated rabbit blood was put in 30 wells of a 96-well culture plate (TPP®, Trasadingen, Switzerland) (Fig. 1); 100 μL of warmed NNN medium (40 °C) was added to these wells. Then 50 μL of complete medium was added to each well. With one 96-well culture plate, 10 wells were prepared for each blood sample and 3 samples were treated in the same plate. All wells without culture were filled with sterile water to avoid evaporation during incubation. Finally, 50 μL of buffy coat was inoculated in 10 wells and the 96-well culture plate was sealed with parafilm and kept in an incubator at 27 °C. In parallel, several wells were inoculated with a tiny amount of splenic aspirate material in order to compare both methods. Cultures were examined on alternate days. After detection of positive culture, promastigotes were transferred in complete medium. Cultures were examined every day under the microscope.

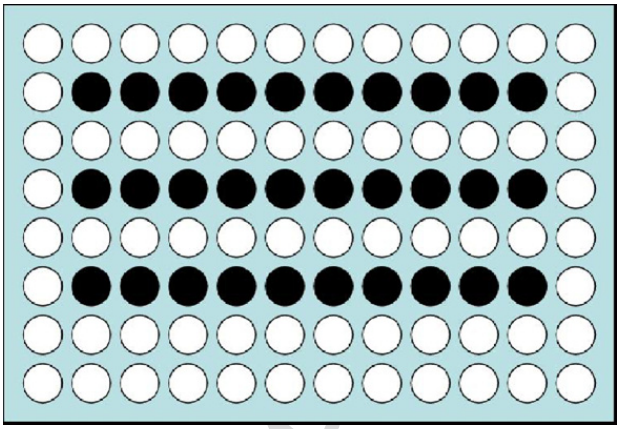

Fig. 1. The 96-well plate disposition. In the 30 black wells, 10 μL defibrinated rabbit blood was completed with 100 μL warmed NNN medium. Then 50 μL complete RPMI was added to each well. The other wells (white) were filled with 250 μL sterile water.

### 2.4. Cryo-conservation

After observation of living parasites, the culture was maintained for 1 or 2 weeks more (until the well was full of parasites) and then inoculated in a culture tube (Tube Nunclon, tissue culture, round polystyrene, radiation sterilised, screw cap, 100 mm × 14 mm, Nunc™) containing 500 μL of NNN medium (with 50 μL of defibrinated rabbit blood) and 250 μL of complete RPMI. The culture was slowly diluted by adding 250 μL of medium every 2–3 days. After 2–3 weeks, the parasites were cryopreserved in nitrogen after addition of 33 μL of DMSO in 1 mL of culture or cultured in mass.

## 3. Results and discussion

Promastigotes were detected in 16 (84%) buffy coat samples, whereas all 19 (100%) samples were posi-

tive on splenic aspirate culture. The most important difference observed in buffy coat and splenic aspirate culture was that the time necessary to observe live parasites was longer in the buffy coat preparation than in splenic aspirate culture. In general, positive cultures were detected within 7–20 days using the buffy coat, whereas only 5–10 days were required for splenic aspirates. This observation might stem from relatively low parasite density in peripheral blood. We also tried to culture parasites using peripheral blood mononuclear cells (PBMCs), but we could not detect parasites in any samples (data not shown). This last method concentrates parasites in a lower volume but requires more experiments and time. This lower efficiency has already been pointed out by several authors (Riera et al., 2004).

In the present study, we demonstrated that the blood of *Leishmania*-infected persons can be used to diagnose visceral leishmaniasis and to isolate parasites. The microculture technique developed is non-invasive and has a significant advantage over the invasive and dangerous splenic aspiration. Furthermore, our purpose in using culture plates instead of culture tubes was to reduce the quantity of culture medium and buffy coat. Parasites grow better in 96-well plates because of the lower volume of the two media and the proportion of air to medium culture appears favourable for *Leishmania* growth. Indeed, a large amount of air in the culture environment is involved in a natural basification of the RPMI, which is not favourable for parasite development. We used sterile human urine along with fetal calf serum, which have both been shown to be good growth enhancers for *Leishmania* (Howard et al., 1991; Singh et al., 2000). Other media such as normal saline and Schneider insect medium have been tested on several samples and have given positive cultures (data not shown).

Moreover, this method makes it possible to obtain live parasite strains, which can then be cultured for further analysis or cryoconserved. In addition, this technique would be also tested on isolate *Leishmania* parasites from the blood of serologically positive, apparently healthy endemic subjects in whom tissue aspiration could not be done for ethical reasons. However, the microculture technique should be considered as complementary to other diagnostic protocols, and negative results should be confirmed by tests with higher sensitivity.

Development of highly sensitive and specific diagnostic tools such as the direct agglutination test (DAT), the rK39 strip test and polymerase chain reaction (PCR) has made it highly likely that the gold standard parasitology that involves invasive and dangerous tissue aspiration will be replaced as the basis for the decision to treat VL patients. Hence, the method described herein is highly relevant to meeting the need for parasites for research purposes. Furthermore, antibody detection tests such as DiaMed-IT LEISH do not reveal the parasite presence but only that the person has been exposed to parasite. However, this method has limited utility for diagnostic purpose since faster tests are available. Consequently, this method appears to be a very harmless alternative to isolate parasite and could be tried in a first instance before other, more sensitive (but more invasive) procedures such as splenic aspirate.

## Conflict of interest

None.

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## References

Bora, D., 1999. Epidemiology of visceral leishmaniasis in India. Natl. Med. J. India 12, 62–68.

Chulay, J.D., Bryceson, A.D., 1983. Quantitation of amastigotes of *Leishmania donovani* in smears of splenic aspirates from patients with visceral leishmaniasis. Am. J. Trop. Med. Hyg. 32, 475–479.

Desjeux, P., 1992. Human leishmaniases: epidemiology and public health aspects. World Health Stat. Q. 45, 267–275.

Howard, M.K., Pharoah, M.M., Ashall, F., Miles, M.A., 1991. Human urine stimulates growth of *Leishmania* in vitro. Trans. R. Soc. Trop. Med. Hyg. 85, 477–479.

Lachaud, L., Dereure, J., Chabbert, E., Reynes, J., Mauboussin, J.M., Oziol, E., Dedet, J.P., Bastien, P., 2000. Optimized PCR using patient blood samples for diagnosis and follow-up of visceral Leishmaniasis, with special reference to AIDS patients. J. Clin. Microbiol. 38, 236–240.

Osman, O.F., Oskam, L., Zijlstra, E.E., el-Hassan, A.M., el-Naeim, D.A., Kager, P.A., 1998. Use of the polymerase chain reaction to assess the success of visceral leishmaniasis treatment. Trans. R. Soc. Trop. Med. Hyg. 92, 397–400.

Riera, C., Fisa, R., Udina, M., Gallego, M., Portus, M., 2004. Detection of *Leishmania infantum* cryptic infection in asymptomatic blood donors living in an endemic area (Eivissa, Balearic Islands, Spain)

by different diagnostic methods. Trans. R. Soc. Trop. Med. Hyg. 98, 102–110.

Singh, S., Mohapatra, D.P., Sivakumar, R., 2000. Successful replacement of fetal calf serum with human urine for in vitro culture of *Leishmania donovani*. J. Commun. Dis. 32, 289–294.

Sundar, S., Kumar, K., Singh, V.P., Mahopatra, T.M., 1991. Diagnostic lag period in kala-azar: test for early diagnosis needed. J. Assoc. Physicians India 39, 651.

Sundar, S., Rai, M., 2002. Laboratory diagnosis of visceral leishmaniasis. Clin. Diagn. Lab. Immunol. 9, 951–958.