JOURNAL OF CLINICAL MICROBIOLOGY, May 2010, p. 1932–1934 Vol. 48, No. 5
0095-1137/10/$12.00 doi:10.1128/JCM.01733-09

Evaluation of Blood Agar Microtiter Plates for Culturing *Leishmania* Parasites To Titrate Parasite Burden in Spleen and Peripheral Blood of Patients with Visceral Leishmaniasis▽

Radheshyam Maurya,[1,3] Sanjana Mehrotra,[1] Vijay Kumar Prajapati,[1] Susanne Nylén,[2] David Sacks,[2] and Shyam Sundar[1]*

Infectious Disease Research Laboratory, Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221 005, India[1]; Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892[2]; and Department of Animal Sciences, University of Hyderabad, Hyderabad, India[3]

Received 3 September 2009/Returned for modification 25 November 2009/Accepted 16 March 2010

Serial dilution of blood and spleen biopsy specimens, plated on Novy-MacNeal-Nicolle (NNN) blood agar using microtiter culture plates, is a sensitive and reproducible method for detection and growth of *Leishmania* parasites. Plates could be easily monitored, and growth could be rapidly detected. Moreover, parasite number may be estimated using this technique.

Demonstration of parasites in tissue biopsy specimens by microscopy is the conventional and most reliable method for diagnosing visceral leishmaniasis (VL), also known as kala-azar. The sensitivity of detection of amastigotes in the splenic aspirate (SA) smear by microscopy is 96 to 98%, and this sensitivity is regarded as the gold standard for diagnosis of VL (6, 8, 11). Sensitivities for bone marrow (BM) and lymph node (LN) aspirate smears are lower, around 70% and 58%, respectively (2, 3, 11). The parasite density score is determined using a logarithmic scale ranging from 0 (no parasites per 1,000 oil immersion fields) to +6 (>100 parasites per field) (4) and is sometimes used as an indication of severity of infection, e.g., in drug evaluation trials.

An improved, affordable diagnostic procedure for VL is desirable, as splenic aspiration is an invasive procedure associated with risk of severe hemorrhage. Bone marrow aspiration, on the other hand, requires anesthesia and is painful for the patient (9, 10). Patients' peripheral blood contains living amastigotes (7) and may be a good specimen for diagnosis of VL, if sufficiently high sensitivity similar to that for invasive tissue biopsy specimens (BM, LN, SA) can be achieved. However, the isolation of parasites from the patients' blood may be difficult because of low parasitemia, and it is not very well documented.

Culturing of *Leishmania* parasites from patient biopsy specimens is usually done using Novy-MacNeal-Nicolle (NNN) blood agar (BA) in tubes. The sensitivity for SA culture in an NNN tube is around 97 to 100%, whereas the sensitivities for bone marrow and lymph node aspirates are estimated at 70 to 80% and 60 to 70%, respectively.

In this study, we have evaluated a serial dilution microtiter culture technique for the isolation and detection of *Leishmania* parasites from SA and white blood cells (WBC) (peripheral blood mononuclear cells [PBMC] and other buffy coat cells) isolated from 2 to 3 ml of peripheral blood. Experimentally, this method has been used to estimate parasite burden in infected animals.

Sixty-eight microscopically confirmed cases of VL with an average splenic score of +2 were included in the study. Patients were from the Kala-azar Medical Research Centre, Muzafarpur, India. The Ethical Committee at the Centre approved the study.

Splenic aspirates were collected in heparinized tubes containing RPMI 1640 supplemented with serum and antibiotics. Peripheral blood was collected in heparinized Vacutainer tubes. Samples were transported to a central laboratory at 15 to 20°C (blood) or 4 to 8°C (splenic aspirates). All samples were processed in less than 24 h from the time of collection. Buffy coat cells (WBC) were isolated from 3 ml of blood by centrifugation at 1,300 × *g* for 15 min; red blood cells in the buffy coat layer were reduced by lysis using hypotonic (0.2%) NaCl solution for 20 s, and, to achieve an isotonic solution, an equal volume of hypertonic (1.6%) NaCl solution was added. Mononuclear cells (PBMC) were isolated from 2 ml of blood by Ficoll gradient centrifugation according to manufacturer's instructions (GE Healthcare).

BA was prepared by mixing 3 parts defibrinated rabbit blood with 7 parts warmed (43°C) sterile NNN medium (5 g Bacto beef, 2 g neopeptone, 2 g Bacto agar, and 0.5 g NaCl per 100 ml water, pH 7.4). Fifty microliters of blood agar was added to each well of a 96-well plate, which was tilted at a 45° angle until the blood agar coagulated. This generates an open window that allows examination of parasite growth using an inverted microscope. M199 medium (100 μl), supplemented with serum, antibiotics, and trace amounts of hemin, adenine, biotin, and triethanolamine, as previously described (1), was added to each well except for those in the first vertical row of the plate, which were inoculated with 150 μl of splenic aspirate, WBC, or PBMC. A serial 3-fold dilution from the 1st to the 12th well was made. The culture plate was sealed and incubated at 27°C.

* Corresponding author. Mailing address: Infectious Disease Research Laboratory, Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221 005, India. Phone: 91-542-2367795. Fax: 91-542-2367568. E-mail: drshyamsundar@hotmail.com.

▽ Published ahead of print on 24 March 2010.

TABLE 1. NNN tube and blood agar microtiter culture technique using splenic aspirates, WBC/buffy coat cells, and PBMC

Parameter	Result for:			
	NNN tube culture of splenic aspirates	Blood agar microplate culture of:		
		Splenic aspirates	Buffy coat cells/WBC	PBMC
Time for detection of parasite growth (days)	5–10	3–7	5–15	3–7
No. of samples	68	68	68	47
No. of *Leishmania*-positive samples	67	68	58	43
Sensitivity (%)	98.5	100	85.3	91.5
Average end titer in BA plate	NA[d]	8.9	6.3	4.7
Dilution factor (1:3)		$3^{8.86}$	$3^{6.27}$	$3^{4.7}$
No. of parasites/ml specimen		~196,830[a]	~243[b]	~121[c]

[a] SA (around 100 μl) was collected in 900 μl of complete RPMI (cRPMI) medium. The 10-fold-diluted SA (150 μl) was plated in the 1st well of a 96-well microtiter plate, followed by serial 1:3 dilutions out to the 12th well.
[b] WBC/buffy coat cells were separated from 3 ml of peripheral blood, resuspended in 150 μl of cRPMI medium, and plated in serial dilution (1:3) out to the 12th well.
[c] PBMC were separated from 2 ml of peripheral blood, resuspended in 150 μl of cRPMI medium, and plated in serial dilution (1:3) out to the 12th well.
[d] NA, not applicable.

Cultures were examined for growth on every second day using an inverted microscope.

Culturing parasites from leishmaniasis patients is sometimes needed to confirm the diagnosis, particularly when microscopic evaluation is uncertain and when species determination is desired. Sensitive and rapid outgrowth of parasites in cultures is thus helpful for diagnostic purposes. To determine if plates were better than tubes for growing out *Leishmania* and suitable for routine diagnosis of kala-azar, the sensitivity, parasite load, and time to detection were evaluated.

This serial dilution method had a sensitivity of 100% for detection of parasites in splenic aspirates. The sensitivity for detection of parasites in WBC was 85%, which is close to what Hide et al. found (84%), using a similar technique (5). In contrast to results by Hide et al., who were unable to culture parasites from PBMC, we were able to grow out parasites from 91% of PBMC samples (Table 1). The culture success in WBC and PBMC cultures was better than or equivalent to results for BM and LN aspirates. The time to detection in titrated cultures was shorter than that using the larger NNN culture tubes, for which samples are not diluted.

Titration of parasites allows a rough estimation of the parasite load in peripheral blood, which may give an indication of the severity of infection. There was, however, no correlation between splenic score and the parasite load as determined by titration ($R^2 = 0.00005$; Pearson test). The average numbers, determined by serial dilution of parasites in the different blood or tissue preparations, are shown in Table 1. The numbers are, however, underestimated, as no end titration (i.e., growth detected in the last well of the titration) (Table 2) was determined in 43% of SA cultures and in 16% of buffy coat/WBC cultures. The levels of parasites found in blood were low compared to those in the spleen. However, it is clear that in most cases it is feasible to diagnose VL by culturing either PBMC or buffy coat cells/WBC isolated from a small volume of blood. Whole blood cannot be used (not shown). In addition to concentrating the sample in the enriched leukocyte preparations, the removal of serum/plasma is probably required for transformation and growth, as promastigotes are complement sensitive.

TABLE 2. Last well titration in which parasites were detected

Technique	Total no. of patients	No. of patients for which last well of titration (in blood agar microplate culture) was:				
		0[a]	1–3	4–6	7–9	10–12
SA culture	68		1	12	26	29
Buffy coat culture	68	10	1	23	23	11
PBMC culture	47	4	8	23	12	0

[a] "0" represents no growth.

The number of parasites was lower in PBMC than in buffy coat cells/WBC, as determined by serial dilution; however, the sensitivity was slightly better and, more importantly, the time to detection was faster using purified PBMC. Similar observations were made when mononuclear cells (MNC) from SA were cultured (S. Sundar, unpublished observation). Due to limitations in sample volumes, a proper comparison using larger samples of splenic MNC has not been made.

From our results it is evident that dilution of the samples may benefit detection of parasites. *Leishmania* did not always grow in the least-diluted wells and were often first detected in the 4th or 5th well (dilution factors of 243 and 729, respectively). The serial dilution may improve parasite growth because the high concentration of blood cells dying as a result of culture conditions optimized for promastigote growth produces an environment toxic to the parasites.

Replacing the traditional agar tubes with plates may be advantageous in several respects. The method consumes less medium, and microtiter plates can be monitored for growth while in culture, so time to detection is shortened. Moreover, it can give a crude estimate of infection burden, which may be more accurate than the traditional 1 to 6 scoring system.

We thank the hospital staff at the KMRC, Muzaffarpur, for their assistance in the collection of patient samples.

This work was supported by the Extramural Research Program of the NIAID, NIH, USA grant number 1P50AI074321. S.M. and V.K.P. were funded by CSIR and ICMR, New Delhi, India, respectively.

REFERENCES

1. **Belkaid, Y., S. Mendez, R. Lira, N. Kadambi, G. Milon, and D. Sacks.** 2000. A natural model of Leishmania major infection reveals a prolonged "silent" phase of parasite amplification in the skin before the onset of lesion formation and immunity. J. Immunol. **165:**969–977.

2. **Boelaert, M., K. Aoun, J. Liinev, E. Goetghebeur, and P. Van der Stuyft.** 1999. The potential of latent class analysis in diagnostic test validation for canine Leishmania infantum infection. Epidemiol. Infect. **123:**499–506.
3. **Chowdhury, M. S., A. el Harith, A. al Massum, E. al Karim, and A. al Rahman.** 1993. Prevalence of agglutinating anti-Leishmania antibodies in two multi-thousand Bengali communities. Parasitol. Res. **79:**444–450.
4. **Chulay, J. D., and A. D. Bryceson.** 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.
5. **Hide, M., R. Singh, B. Kumar, A. L. Bañuls, and S. Sundar.** 2007. A microculture technique for isolating live Leishmania parasites from peripheral blood of visceral leishmaniasis patients. Acta Trop. **102:**197–200.
6. **Kager, P. A., P. H. Rees, F. M. Manguyu, K. M. Bhatt, and S. M. Bhatt.** 1983. Splenic aspiration; experience in Kenya. Trop. Geogr. Med. **35:**125–131.
7. **Lachaud, L., J. Dereure, E. Chabbert, J. Reynes, J. M. Mauboussin, E. Oziol, J. P. Dedet, and P. Bastien.** 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.
8. **Marsden, P. D., and T. C. Jones.** 1985. Clinical manifestation, diagnosis and treatment of leishmaniasis, p. 183–198. *In* K. P. Chang and R. S. Bray (ed.), Leishmaniasis. Elsevier Science Publishers, Amsterdam, Netherlands.
9. **Sundar, S., and M. Rai.** 2002. Laboratory diagnosis of visceral leishmaniasis. Clin. Diagn. Lab. Immunol. **9:**951–958.
10. **Sundar, S., K. Kumar, V. P. Singh, and T. M. Mahopatra.** 1991. Diagnostic lag period in kala-azar: test for early diagnosis needed. J. Assoc. Physicians India **39:**651.
11. **Zijlstra, E. E., M. S. Ali, A. M. el-Hassan, I. A. el-Toum, M. Satti, H. W. Ghalib, and P. A. Kager.** 1992. Kala-azar: a comparative study of parasitological methods and the direct agglutination test in diagnosis. Trans. R Soc. Trop. Med. Hyg. **86:**505–507.