A SENSITIVE NEW MICROCULTURE METHOD FOR DIAGNOSIS OF CUTANEOUS LEISHMANIASIS

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Abstract. A sensitive microcapillary culture method (MCM) was developed for rapid diagnosis of cutaneous leishmaniasis (CL). The MCM is superior to the traditional culture method (TCM) as determined by the smaller inoculum size, the higher sensitivity for detection of promastigotes, and the more rapid time for emergence of promastigotes. With lesion amastigote loads from grade III to 0, the positive rates and the periods for promastigote emergence were 3−4-fold higher and faster with the MCM than with the TCM, e.g., 83−97% positive in 4−7 days versus 20–40% positive in 15−30 days ($P = 0.0001$). The higher PCO$_2$ and lower PO$_2$ and pH presumably encourage a rapid amastigote-to-promastigote differentiation and/or the survival or growth of the latter. This MCM has the advantage of simplicity, and may be suitable for diagnostic use and for parasite retrieval in many other endemic sites where parasites are known to be difficult to grow.

INTRODUCTION

Cutaneous leishmaniasis (CL) is a major problem in many tropical and subtropical countries. The causative agent of CL in southeastern Turkey is mainly *Leishmania tropica*. The clinical diagnosis of CL depends on finding parasites by microscopy in smears or tissue biopsy specimens and cultivation whose sensitivities are 33−60% and 50%, respectively. Although many different methods have been developed for serodiagnosis of CL, their sensitivities vary significantly. The sensitivity of an enzyme-linked immunosorbent assay was reported to be as low as 20%. Recently, a polymerase chain reaction (PCR) technique opened the possibility of directly detecting parasites with a high specificity and sensitivity. The sensitivity of this method is 86−95% in patients with acute lesions, but it may decrease to 45.5% in chronic cases.

A timely and definitive diagnosis of CL is important for initiating appropriate clinical management and treatment of this disease. Microscopy may be the simplest method of achieving this, but the high rate of undiagnosed cases associated with this method shows its deficiency. The only rapid method for the diagnosis of CL is the PCR; however, it is not yet available outside the research setting and remains expensive for field deployment.

Diagnosis of CL by cultivation in liquid media has several advantages. The major one is the possibility of examining the entire sample collected in a closed system for the emergence of a few motile promastigotes. For decades, blood-based biphasic media of various formulations have been used for this purpose with variable degrees of success. The culture often becomes positive when inoculated with lesion aspirates from patients with a large number of amastigotes. Also, a prolonged incubation of 15−30 days is often required for a positive result. Efficient culture techniques for diagnosis of leishmaniasis require an in vitro environment for rapid conversion of a small number of amastigotes into a population of motile promastigotes visible by microscopy. We report here the development of a microcapillary culture method (MCM) that is simpler to use and more sensitive than the conventional Novy, McNeal, Nicolle (NNN) tube cultures for diagnosis of CL in the Adana area of Turkey.

MATERIALS AND METHODS

Patients. Samples were obtained from the lesions of 139 patients with suspected CL at the Cukurova University Tropical Diseases Research Center in Adana. The study was reviewed and approved by the University of Cukurova Faculty of Medicine Local Ethics Council. All patients agreed to provide samples after filling out and signing standard informed consent forms. Patients were divided into two groups according to the clinical types of lesions: acute lesions (duration of lesion ≤ 12 months) and chronic lesions (duration of lesion > 12 months). The results obtained by microscopy and cultures were correlated with the clinical types of the lesions.

Sampling and diagnostic procedures. We compared two methods, the traditional culture method (TCM) and the MCM, using 16 × 110 mm tissue culture tubes and 1 × 75 mm microhematocrit capillary tubes, respectively. Lesions were cleansed with 70% ethanol before sample aspiration. A 26-gauge needle and syringe containing 0.1−0.2 mL of sterile saline was then inserted intradermally into the outer border of the lesion. The syringe was rotated and the tissue fluids gently aspirated into the needle as they were withdrawn. The aspirated materials were divided equally and inoculated into tubes for incubation under the following conditions. Culture tubes contained either 1) 3.5 mL of NNN medium (blood agar base; Difco, Detroit, MI) with 15% defibrinated rabbit blood; 2) 2.5−3.0 mL of RPMI 1640 medium with 15% HEPES (Sigma) or without HEPES (for measurement of PCO$_2$, PO$_2$, and pH in culture media); 2 mM NaHCO$_3$ supplemented with 15% fetal bovine serum (FBS) (Sigma); and gentamicin (80 μg/mL); or 3) Schneider’s *Drosophila* medium (SDM) (Sigma) supplemented with 15% FBS (heat-inactivated at 56°C for 30 minutes) and gentamicin (80 μg/mL). Microhematocrit capillary tubes were loaded with 25−50 μL of the same medium (mixed at a 1:1 ratio with lesion aspirates), i.e., RPMI 1640, SDM, or heat-liquefied NNN agar, using a 1.0-mL syringe. The ends of the capillary tubes were sealed with melted candle wax.

All inoculated tubes were incubated at 27°C under standard atmospheric conditions and examined every 2−3 days under an inverted microscope (CK 2; Olympus, Melville, NY) with a 10× ocular lens and a 20× objective. Samples of overlay from the blood agar medium were placed in sterile capillary tubes for microscopic examination. All cultures were incu-
bated and examined for 15–30 days before being considered negative. Patients were positively diagnosed for CL when actively motile promastigotes were seen in culture.

**Detection of amastigotes.** Smears were prepared by the skin scraping method. A small amount of lesion material was obtained for spreading on a glass slide. Smears were air-dried, fixed with methanol, and stained with a Giemsa solution. Amastigotes were counted for grading their density according to the method of Chulay and Bryceceon.23 Patients were divided into groups I, II, or III according to increasing parasite density (I = grades 4–6; II = grades 1–3; III = grade 0).

**Determination of P CO2, P O2, and pH.** The P CO2, P O2, and pH of the cultures were determined using the AVL OMNI modular system apparatus (AVL Medical Instruments AG, Schaffhausen, Switzerland). This analyzer measures gases in blood and other solutions. Samples (0.5 mL) were taken from traditional cultures with 1.0-mL plastic syringes. The MCM capillary tubes were used directly for analysis. Caution was exercised to avoid the formation of any air bubbles during sample preparation. All procedures for gas phase analysis were carried out according to the Operator’s Manual OMNITM version 1–9 Modular System (CH 3534 Rev 11.0, September 2000).

**Data processing and statistical analysis.** Quantitative variables were compared using the Student’s t-test and Wilcoxon test for comparison of proportions in two related samples (distribution using the Statistical Packages of Social Sciences version 9.0 for Windows; SPSS, Inc., Chicago, IL). P values < 0.05 were considered statistically significant.

**RESULTS**

The relative sensitivities of the two culture methods (TCM and MCM using different media) used for the diagnosis of CL are shown in Table 1. The MCM was more sensitive than the TCM, as determined by evaluating specific parameters, especially the number of amastigotes in the samples and time of detection of promastigotes in cultures. The average time period of incubation needed to detect promastigotes was much shorter with the MCM than the TCM, i.e., 2–7 days versus 2–30 days. The emergence of promastigotes in the TCM showed a correlation with the number of amastigotes seen on thin smears of the samples. Parasites were detected at a much higher rate with the TCM when the parasites observed in smears were abundant in number. The sensitivity of the TCM to detect promastigotes in culture decreased significantly from group I (smear positive with a large number of amastigotes) to group III (smear negative with undetectable amastigotes). The TCM has a sensitivity comparable with the MCM only in group I patients and only with two of the media, i.e., SDM and NNN agar. In contrast, the MCM was sensitive for all groups and independent of amastigote density in the lesions.

Detection of promastigotes also depends on the culture media in both methods, especially in the TCM (Table 1). The ratio of detection of promastigotes in the TCM with SDM, RPMI 1640, and NNN agar varied according to the density of parasites in smears between 55.7% and 93.3%, 19.1% and 60%, and 42.9% and 96.6%, respectively. The sensitivity of detecting of promastigotes was higher in the MCM than in the TCM with all types of media and varied between 83.3% and 100% (P = 0.0001, by Wilcoxon test). The transformation of amastigotes to promastigotes was generally more rapid in the MCM with most types of media, but this was true under both culture conditions when especially SDM was used. Promastigotes were physically more active in SDM than in the other media. This medium diagnosed CL in patients with microscopically negative smears.

Detection of promastigotes in culture showed a correlation with lesion types in addition to the methods of culture, number of amastigotes in samples (group II and III), and media used. Detection of promastigotes decreased significantly from 54.1% to 40.0% in the TCM when acute lesions were compared with chronic lesions, while it remained very high in the MCM, i.e., 100% and 93.3% for acute and chronic lesions, respectively. Consequently, the MCM is far more sensitive than the TCM in detecting promastigotes in different media in cases with acute and chronic lesions in Groups II and III (P = 0.0001, by Wilcoxon test).

The P CO2 (Figure 1A), P O2 (Figure 1B), and pH (Figure 1C) differed in the MCM and the TCM when different media were used for inoculation with aspirated materials from group I patients (with smear-positive grades 4–6). In all cases, these parameters were significantly different in capillary tubes than in the large tubes (P = 0.001, by Student’s t-test). The P CO2 was approximately 45% higher and the P O2 was approximately 35% lower in the MCM compared with the TCM at the end of cultivation. The pH decreased by an average of 0.1 units.

**DISCUSSION**

We have demonstrated in the present study that the MCM is more sensitive than the TCM in the diagnosis of CL. The

| Table 1 | Comparison the sensitivity of the culture methods (TCM and MCM by using different medium) in the diagnosis of the patients with suspected cutaneous leishmaniasis* |
|-------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Media                                     | Group I (TCM)  | Group MCM | Group II (TCM) | Group MCM | Group III (TCM) | Group MCM |
| SDM + 15% FBS                             | 28/30 (93.3%)  | 30/30 (100%) | 34/67 (50.7%)  | 67/67 (100%) | 15/42 (35.7%)   | 41/42 (97.6%) |
| RPMI 1640 + 15% FBS                       | 18/30 (60%)    | 30/30 (100%) | 15/67 (22.3%)  | 67/67 (100%) | 10/42 (19.1%)   | 38/42 (90.5%) |
| NNN agar + 15% DRB                        | 29/30 (96.6%)  | 30/30 (100%) | 36/67 (53.7%)  | 56/67 (83.3%) | 18/42 (42.9%)   | 35/42 (83.3%) |

*TCM = traditional culture method; MCM = microcapillary culture method; SDM = Schneider’s Drosophila medium; FBS = fetal bovine serum; DRB = defibrinated rabbit blood. Group I = smears are positive (grade 4+; 5+, 6+); Group II = smears are positive (grade 1+, 2+, 3+); Group III = smears are negative (grade 0).
sensitivity of the TCM varies with different reports, depending on the lesions types, parasite strains, and localities, even within the same region.\textsuperscript{2,14} Positive diagnosis of CL by the TCM also varies greatly with the number of amastigotes in aspirate materials and sometimes requires an incubation period of up to 20–30 days.\textsuperscript{15,21} In comparison, the MCM is much less susceptible to these parameters and provides a more rapid reading. Navin and others used culture tubes containing 1.0-mL agar slants and 0.5 mL of liquid overlay (SDM with 20% FBS).\textsuperscript{16} They successfully diagnosed CL in patients with smear-negative lesions. However, the sensitivity in their study (27%) was significantly lower than that obtained in this study with the MCM (93.6%). The sensitivity for the TCM in this study was 35.7%. The finding that the SDM was superior to the other media used is consistent with previous work using macroculture methods.\textsuperscript{24–27}

The high sensitivity of the MCM may be explained by the use of capillary tubes, which concentrate the sample material and provide microaerophilic conditions favorable for transformation of the amastigotes to promastigotes. This is not achieved under the conditions used for the TCM. The Pco2 was significantly higher ($P = 0.001$), concomitant with a significant reduction in the Po2 and pH ($P = 0.01$) in the capillary tube than in the TCM culture tubes. These results are consistent with results obtained by others.\textsuperscript{28–32} High CO2 levels may act as the trigger for amastigote-to-promastigote transformation and favor the survival of promastigotes.\textsuperscript{33,34} It is known that CO2 and low concentrations of O2 have regulatory effects on glucose metabolism in promastigotes.\textsuperscript{35} Capillary tubes have not been used previously for diagnosis, although they were used for cloning promastigotes from continuous culture, obtaining samples from lesions, and assaying the chemotactic responses of \textit{Leishmania} promastigotes.\textsuperscript{16,36,37}

In summary, a new microculture method was found to have the advantages of simplicity and sensitivity, in addition to being cost-effective in the routine diagnosis of CL in Adana, Turkey. This method awaits further evaluation for its usefulness in the diagnosis of CL in other endemic sites.

Received August 1, 2003. Accepted for publication November 13, 2003.

Acknowledgments: We thank K.-P. Chang (University of Health Sciences/Chicago Medical School, Chicago, IL) for reviewing the manuscript.

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