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THE VALUE OF A NEW MICROCULTURE METHOD FOR DIAGNOSIS OF VISCERAL LEISHMANIASIS BY USING BONE MARROW AND PERIPHERAL BLOOD

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Abstract. We have demonstrated that the microculture method (MCM) enables the diagnosis of visceral leishmaniasis (VL) with samples from both the bone marrow (BM) and peripheral blood (PB). The MCM is superior to the traditional culture method (TCM) as determined by its higher sensitivity in the detection of promastigotes and the more rapid time for emergence of promastigotes. The sensitivity of MCM (100% in BMs and 77.8–100% in PB) was considerably higher than that of the TCM (37.5–100% in BMs and 0–100% in PB) according to decreasing parasite density ($P < 0.05$). The concentration of parasites in buffy coats has increased the sensitivity of both methods, especially that of the MCM. Detection of promastigotes by MCM requires lower amounts of culture media (25–50 μL) and shorter incubation periods (2–7 days) than TCM (2.5–3.5 mL and 15–35 days, respectively). MCM was found to be valuable with the advantages of simplicity and sensitivity, in addition to being cost-effective in the routine diagnosis for VL in Adana Turkey.

INTRODUCTION

Visceral leishmaniasis (VL) or *kala-azar*, is caused by the intracellular protozoan *Leishmania infantum* in countries of the Mediterranean basin and the Middle East.[1–4] An estimated 200 million people are at risk of contracting the disease worldwide, with approximately 500,000 new cases annually.[5,6] During the past 15 years, the number of people with leishmaniasis is believed to have increased mainly due to the extension of *Leishmania*-HIV coinfections, especially in southern Europe and Africa.[7–9] Left untreated, VL has a mortality approaching 100%. Therefore, rapid and accurate methods for diagnosis is mandatory.[10,11] Although many methods have been developed for the diagnosis of VL, the sensitivities reported have been variable and insufficient.[12–16]

The most sensitive method for the diagnosis of VL is the polymerase chain reaction (PCR). Currently, PCR is regarded as a promising tool, with the potential advantage of employing blood specimens rather than the conventional invasive procedures such as splenic aspirate, bone marrow, and liver biopsy.[17–20] The sensitivity of PCR using peripheral blood (75%) was considerably higher than the sensitivity of microscopy (26.3%) and blood culture (42.3%).[21] However, PCR is not yet available outside the research setting and still remains expensive for field deployment. Moreover, sometimes PCR gives negative results for blood samples from culture-positive asymptomatic blood donors.[22] PCR was able to detect 10 parasites/mL.[17]

In a previous study, we described a new sensitive microcapillary culture method (MCM) for the rapid diagnosis of cutaneous leishmaniasis.[23] In the current study, we compared the sensitivities of the traditional culture methods (TCM) and MCM to reveal the value of MCM for diagnosing VL using peripheral blood and bone marrow in patients from the region of Adana.

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MATERIALS AND METHODS

Patients. Twenty-five children on admission at the Infectious Diseases Unit of the Department of Pediatrics, Faculty of Medicine, Cukurova University, were enrolled in the study. All patients were referred for diagnostic analysis because of an abnormally enlarged liver and/or spleen at presentation with or without fever. We examined the peripheral blood samples (PBs) and bone marrow samples (BMs) of all patients with the following methods. Approval for this study was obtained from the Ethics Committee of the Faculty of Medicine, Cukurova University.

Sampling and diagnostic procedures. *Peripheral blood.* Five milliliters of PBs were taken by sterile venipuncture into sterile EDTA-coated 10-mL polypropylene tubes and mixed.

Bone marrow. Two to 3 mL of BMs were aspirated from iliac crest of all patients. Aspirated BMs were immediately transferred into EDTA-coated, 10-mL polypropylene tubes and mixed. Substances known to inhibit growth of promastigotes in BMs were removed by washing with a medium of RPMI-1640 by centrifugation as described below.

Concentration of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) were isolated by the Ficoll-Paque (PAA Laboratories GmbH, Linz, Austria) in PBs. Preparations of PBMC were immediately used for cultures as described below.

Concentration of infected cells in PBs and BMs. PBs and BMs were mixed with the medium RPMI-1640 (in the ratio of 1:3) and washed 2–3 times by centrifugation for 10 minutes at 1500 × *g*. The supernatant was removed and the sediment resuspended with RPMI-1640 and separated into two parts:

1. One hundred to 200 μL of resuspended material was added directly to culture tubes containing different media.
2. Two to 3 mL of resuspended material was centrifuged for 30 minutes at 2000 × *g* and the supernatant removed, after which the remaining pellet (upper layer) was divided into two parts: i) 1–2 mL cell suspensions (with 2–3% hematocrit) were prepared from the pellet and 100–200 μL inoculated into culture tubes containing different media, ii) 80–90 μL of the pellets were filled directly into hematocrit

capillary tubes and centrifuged for 5 minutes in a hematocrit centrifuge system. Buffy coat samples (BCs) in the capillary tubes were then separated and mixed with 100–200 μL of suitable media (i.e., RPMI-1640 or SDM, M199 and heat-liquified NNN) and used for cultivation.

Cultivation procedure. Samples prepared from the buffy coat or peripheral blood and BM aspirates as described above were inoculated into tubes under the following conditions:[23]

1. TCM: Culture tubes, each containing i) ~3.5 mL of NNN medium (Difco blood agar base) with 15% defibrinated rabbit blood (DRB), ii) 2.5–3.0 mL of Roswell Park Memorial Institute-1640 (RPMI-1640) with L-glutamine (Sigma) [buffered with 25 mM HEPES (Sigma), 2 mM $NaHCO_2$ supplemented with 15% fetal bovine serum (FBS) (Sigma) and gentamicin 80 μg/mL], iii) Schneider's *Drosophila* medium (SDM) (Sigma), and iv) Medium 199 (M199) supplemented with 15% FBS (Sigma) (heat-inactivated at 56°C for 30 minutes) and gentamicin at 80 μg/mL.
2. MCM: Microhematocrit capillary tubes, each filled with 25–50 μL of the same medium (mixed with PBMCs, BCs, whole blood concentrated by centrifugation, and BMs), that is, RPMI-1640, SDM, M199, and heat-liquefied NNN using 1.0 mL syringes. The ends of the capillary tubes were sealed by a heat-melted candle.

All inoculated tubes were incubated at 27°C under standard atmospheric conditions and examined every 1–3 days under an inverted microscope (Olympus CK 2) with a 10× ocular lens and a 20× objective. Samples of overlay from the blood-agar media were taken with sterile capillary tubes for microscopic examinations. All cultures were kept and examined for 15–35 days before being considered negative. Patients were positively diagnosed for VL when actively motile promastigotes were seen in culture.

Detection of amastigotes. Smears were prepared from the BMs, whole blood, PBMCs, and BCs. The smears were air-dried, fixed with methanol, and stained with the Giemsa dye. Amastigotes were counted for grading their density according to Chulay and Bryceson.[24] Patients were divided into Groups I to III according to decreasing parasite density of Grades 2–3, Grade 1, and Grade 0, respectively, in smears of BMs.

Data processing and statistics. Quantitative variables were compared using the Student's *t* test and Wilcoxon test for comparison of proportions in two related samples (using the Statistical Packages for the Social Sciences, ver. 12.0 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 BMs and PBs for the diagnosis of VL are shown in Tables 1 and 2. The MCM was more sensitive than the TCM, as determined by the number of amastigotes in the samples and time taken to demonstrate presence of promastigotes in cultures. The average time period of incubation needed to detect promastigotes was much shorter in the MCM than in the TCM (i.e., 2–7 days versus 4–35 day). There was a correlation between demonstration of promastigotes in the TCM and the number of amotigotes seen on thin smears from BMs. Parasites was detected at a much higher rate with the TCM when the parasites were observed in smears. In both groups (PBs and BMs), the sensitivity of TCM to detect promastigotes decreased significantly from Group I (smear-positive with the most number of amastigotes) to Group III (smear-negative with undetectable amastigotes). The TCM had a sensitivity comparable with the MCM only in Group I using BMs and PBS and only with SDM + 15% FBS. In contrast, the MCM was sensitive for all groups (I–III) independent of amastigote density in the samples and also independent of type of culture medium. However, the sensitivity of TCM using the PBs was lower than that of TCM using BMs (Tables 1 and 2). Differences also existed between the sensitivities of TCM in biphasic and monophasic media. The sensitivity in the biphasic medium was higher than that of the monophasic medium especially for Groups I–II in the TCM ($P < 0.05$, by Wilcoxon test).

TABLE 1

Comparison the sensitivity of the culture methods (TCM versus MCM by various media) in the diagnosis of the patients with suspected VL using BMs

	Ratio of culture-positive cases to total number of cases in each group (%) and time (days) to become positive					
	Group I		Group II		Group III	
Media	TCM	MCM	TCM	MCM	TCM	MCM
SDM + 15% FBS	8/8 (100%) 4–6*	8/8 (100%) 1–2	5/9 (55.6%) 7–20	9/9 (100%) 2–4	3/8 (37.5%) 15–24	8/8 (100%) 3–5
RPMI-1640 + 15% FBS	6/8 (75%) 7–12	8/8 (100%) 1–2	4/9 (44.4%) 7–25	5/5 (100%) 2–5	3/8 (37.5%) 15–30	8/8 (100%) 3–5
Medium 199 + 15% FBS	5/8 (62.3%) 7–25	8/8 (100%) 2–4	2/9 (22.2%) 7–35	9/9 (100%) 4–6	2/8 (25%) 15–35	8/8 (100%) 3–7
NNN-agar + SDM + 15% DRB	8/8 (100%) 3–7	–	7/9 (77.7%) 7–15	–	5/8 (62.5%) 15–25	–
NNN-agar + RPMI-1640 + 15% DRB	7/8 (87.5%) 4–10	–	6/9 (66.7%) 7–20	–	4/8 (50.0%) 15–25	–
NNN-agar + 199 + 15% DRB	6/8 (75%) 7–25	–	4/9 (44.4%) 10–30	–	4/8 (50.0%) 15–35	–

TCM, traditional culture method; MCM, microcapillary culture method; BMs, bone marrow samples; SDM, Schneider's Drosophila medium; FBS, fetal bovine serum; DRB, defibrinated rabbit blood. Group I, smears are positive (Grade 2+, 3+); Group II, smears are positive (Grade 1+); Group III, smears are negative (Grade 0).
Patients were divided into Groups I, II, or III according to decreasing parasite density in BMs smears.
* Days for culture to become positive.

TABLE 2
Comparison of the sensitivity of culture methods (TCM versus MCM in various culture media) in the diagnosis of patients with suspected VL using PBs before and after concentration procedure in capillary tubes

Medium	Ratio of the culture-positive cases to total number of cases in each group (%) and time (days) to become positive											
	Group I				Group II				Group III			
	Before concentration		After concentration		Before concentration		After concentration		Before concentration		After concentration	
	TCM	MCM	TCM	MCM	TCM	MCM	TCM	MCM	TCM	MCM	TCM	MCM
NNN-agar + 15% DRB	8/8 (100%) 7–15*	–	8/8 (100%) 4–7	–	2/9 (22.2%) 15–30	–	4/9 (44.4%) 4–10	–	0/9 (0%) 30	–	4/9 (44.4%) 10–20	–
SDM + 15% FBS	8/8 (100%) 4–7	8/8 (100%) 2–3	8/8 (100%) 4–6	8/8 (100%) 2–3	0/9 (0%) 30	7/9 (77.8%) 3–5	4/9 (44.4%) 15–30	9/9 (100%) 2–4	0/9 (0%) 30	6/9 (66.7%) 4–7	5/9 (55.6%) 30	9/9 (100%) 2–4

TCM, traditional culture method; MCM, microcapillary culture method; PBs, peripheral blood samples; SDM, Schneider's Drosophila medium; FBS, fetal bovine serum; DRB, defibrinated rabbit blood. Group I, smears are positive (Grade 2+, 3+); Group II, smears are positive (Grade 1+); Group III, smears are negative (Grade 0).
Patients were divided into Groups I, II, or III according to decreasing parasite density in bone marrow sample smears.
* Days for culture to become positive.

The effects of the concentration of parasites on the sensitivities of TCM and MCM in PBs are showed in Figure 1. As shown, the concentration of parasites using different methods increased the sensitivities of both TCM and MCM. However, the capillary tubes (i.e., buffy coat) method of concentration increased the sensitivities of TCM and in particular that of the MCM than the other methods ($P < 0.05$, by Student's t test). Table 2 compares the results of sensitivities of TCM and MCM before and after concentration of parasites in PBs using the capillary method. The rate of detection of promastigotes decreased from Group I to III before concentration in both methods. The decrease was more significant in TCM ($P < 0.05$, by Wilcoxon test). However, after concentration, the sensitivities of both methods, especially that of MCM, increased in all groups (i.e., there were any differences in the rate of detection of parasites in groups).

DISCUSSION

Diagnosis of leishmaniasis 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 few motile promastigotes. Diagnosis of VL by the TCM has been described previously.[12,13] However, the sensitivity of TCM is considerably low (42.3%) and therefore is rarely needed in routine clinical practice.[12,21] Although laboratory examination of splenic aspirates offers the highest sensitivity, it is associated with the risk of life-threatening hemorrhage in cases with profound thrombocytopenia.[25–27] Our results demonstrated that the new culture method, the MCM, is more sensitive than the TCM in the diagnosis of VL. The sensitivity of the TCM varies greatly with the number of amastigotes in aspirated materials that sometimes requires very long incubation periods (between 20 and 30 days).[20,28–30] Also, in blood samples from asymptomatic subjects (blood donors) who have low parasite load, a long incubation period (1 to 6 months) may be necessary to detect parasites.[22,20] By comparison, MCM is much less susceptible to these variable parameters and provides a more rapid and accurate reading.

FIGURE 1. The effects of the concentration of parasites in peripheral blood samples (by using different methods of concentration) on the sensitivity of TCM and MCM ($P < 0.05$ by Student's t test). TCM, traditional culture method; MCM, microcapillary culture method.

In this study, we have also compared the effects of monophasic and biphasic media on the growth of parasites. We revealed that TCM in which the biphasic medium (NNN medium + overlays of SDM, RPMI-1640, or M199) was used had a higher sensitive than that with the monophasic medium (SDM, RPMI-1640, or M199). Similar results have been reported by Barrouin–Melo and others who used the biphasic medium (SDM with 20% FBS) with spleen aspirates.[31] However, in this study, the sensitivity of the biphasic medium with spleen aspirates was significantly lower (73.4%) than that obtained in our study using the MCM with BMs (100%).

In both methods (TCM and MCM), we found that the SDM and RPMI-1640 were superior to other media. This superiority of the SDM over the other media used is consistent with previous works using the macroculture methods.[23,32–35] The M199 containing FBS has been reported by Sundar and others to be more effective in supporting culture growth and amplification of parasite numbers.[36]

Another objective of this research was to replace BMs with PBs for demonstrating promastigotes in culture because of the ease and convenience of this type of sampling considering its noninvasive nature. The MCM was shown to be much more sensitive than the TCM for diagnosing VL using PBs. Detection of promastigotes by macrocultures requires higher amounts of medium (5–25 mL) and longer incubation periods (1–6 months) than the MCM (25–50 μL and 2–7 days, respectively).[20,37] However, its sensitivity is still much lower (26.3%)[21] than that of the MCM (77.8–100%).

Bearing in mind that the parasite load in circulating blood is lower than in BM, we have tried to develop the most sensitive method possible. Most of the amastigotes live within phagocytic leukocytes, the probability of detecting leishmanial infection in blood samples may be greatly increased by making leukocyte concentrates. We therefore compared the effects of different methods of concentration on the sensitivity TCM and MCM (Figure 1). In both methods (MCM and TCM), the sensitivity was higher when capillary tubes were used for concentration of parasites than when centrifugation and Ficoll-Paque were used. However, after concentration of BCs by capillary tubes, the sensitivity of MCM was significantly higher than that of TCM. Similarly, Riera and others in their analysis of PBMCs and BCs from asymptomatic subjects in TCM cultures found that BCs were superior to PBMCs for the detection of parasites.[20] The value of parasite concentration in BCs had been described previously for the isolation of protozoan parasites or DNA from PB.[17,20,38–42] We used the capillary method as described in a previous research by the authors.[38,39] The current study showed that this method was also more efficient than the other methods for concentrating the amastigotes before cultivation (Figure 1). The leuko-concentration and leuko-cytoconcentration methods have been used by Izri and others.[43,44] Despite the ease and quick yield of this method, we prefer using the BCs because our experience has shown that parasites generally do not exist in the leuko-concentration materials. Moreover, these methods are time consuming and require more processes.

The mechanism of high sensitivity of the MCM was explained in our previous research.[23] The concentration of sample material by the microcapillary method provides a microaerophilic condition during the period of cultivation, which also facilitates the transformation of amastigotes to promastigotes. Moreover, the high parasite load induces a high concentration of autocrine growth–regulating factors in the medium.[45]

In conclusion, the microculture method was found to be valuable with the advantages of simplicity and sensitivity, in addition to being cost-effective for the routine diagnosis of VL in Adana, Turkey. The method also seems to have a significant potential for replacing the invasive methods and for mass screening in endemic areas with its usage in peripheral blood.

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