Utility of the Microculture Method in Non-Invasive Samples Obtained from an Experimental Murine Model with Asymptomatic Leishmaniasis

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Abstract. The sensitivity of diagnostic methods for visceral leishmaniasis (VL) decreases because of the low number of parasites and antibody amounts in asymptomatic healthy donors who are not suitable for invasive sample acquisition procedures. Therefore, new studies are urgently needed to improve the sensitivity and specificity of the diagnostic approaches in non-invasive samples. In this study, the sensitivity of the microculture method (MCM) was compared with polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), and immunofluorescent antibody test (IFAT) methods in an experimental murine model with asymptomatic leishmaniasis. Results showed that the percent of positive samples in ELISA, IFAT, and peripheral blood (PB) -PCR tests were 17.64%, 8.82%, and 5.88%, respectively, whereas 100% positive results were obtained with MCM and MCM-PCR methods. Thus, this study, for the first time, showed that MCM is more sensitive, specific, and economic than other methods, and the sensitivity of PCR that was performed to samples obtained from MCM was higher than sensitivity of the PCR method sampled by PB.

INTRODUCTION

Leishmaniasis is a zoonotic disease transmitted to humans by *Leishmania*-infected sandflies that are previously infected by reservoir animal hosts such as dogs, mice, foxes, and cats.¹ However, humans can also be reservoirs for some *Leishmania* species, and the disease can be asymptotically transmitted.²

In endemic regions, animal leishmaniasis is very important in terms of veterinary and human health, because the ratio of asymptomatic dogs is more than 85%.³ Generally, the methods used for diagnosis of symptomatic leishmaniasis are same as the diagnostic methods for determining asymptomatic leishmaniasis. These methods include microscopy, culture, serology, and molecular methods.

The most important advantage of using culture methods in diagnosis of leishmaniasis is their capacity for showing active parasite in samples obtained from patients. However, the culture often becomes positive when inoculated with samples from patients with a large number of amastigotes. For these reasons, the sensitivity of these methods for asymptomatic donors is low (0.4–4.4%), despite the high (61–75%) sensitivity of symptomatic patients.^{4–7}

There are several serological methods for the diagnosis of VL such as enzyme-linked immunosorbent assay (ELISA), immunofluorescent antibody test (IFAT), and direct agglutination test (DAT).^{8–10} The specificity and sensitivity of these methods are considerably high, especially for symptomatic leishmaniasis, and they are considered important for epidemiologic studies. The most important advantage of these methods is that they are non-invasive. However, these currently used methods may give false-positive results. In addition, sensitivity of these methods decreases because of low antibody levels in bloods of asymptomatic patients.^{6,11,12}

One of the most sensitive and specific methods in the diagnosis of VL is polymerase chain reaction (PCR). For diagnosing patients with clinical symptoms, PCR has 90% and greater sensitivity,¹³ whereas microscopic methods and blood culture

have 70.5% and 74.5% sensitivity, respectively.¹⁴ It is also known that positivity of the PCR method in asymptomatic donor samples is very low (6.8%).¹⁵

Microscopic methods, which are considered to be the simplest and most specific methods for diagnosis, can be applied to samples obtained from blood, bone marrow, spleen, and liver aspirates. However, it is known that invasive methods are not convenient to apply to asymptomatic donors, and the sensitivities of microscopic methods are very low for non-invasive samples. In one study, it was shown that sensitivity of microscopic methods was at 1.3% in asymptomatic donors.¹⁶ Hence, to date, the sensitivity of diagnostic methods of leishmaniasis depends on the number of parasites in the obtained sample, and sensitivity is high in symptomatic patients, despite low sensitivity in asymptomatic donors. Therefore, it is very difficult to determine asymptomatic infections because of the lack of a gold standard for that purpose.¹⁷ Previously, we developed the microculture method (MCM) for the diagnosis of VL with high sensitivity independent of the number of parasites or type of used culture medium.¹⁸ The MCM method has also achieved the same sensitivity in other endemic areas for leishmaniasis. Although MCM is used in the diagnosis of symptomatic leishmaniasis, there is no information regarding its application and sensitivity in asymptomatic VL cases.¹⁸ For this reason, the aim of this study is to create a mouse model with asymptomatic leishmaniasis, compare the sensitivity of MCM methods with other methods, and identify the more sensitive and specific non-invasive method for diagnosis of asymptomatic leishmaniasis.

MATERIALS AND METHODS

Parasite culture. *L. donovani* (HOM/IN/83/AG83) promastigotes were cultured at 27°C in culture flasks as described previously.¹⁹ The strain was provided by Kwang-Poo Chang (Chicago Medical School/Rosalind Franklin University of Medicine and Science [RFUMS]). The growth of promastigotes was monitored every day using an inverted microscope (Olympus CK 40). The parasites were counted using a hemocytometer with a 20× objective under standard light microscopy.

Ethics statement. Thirty-four 5- to 6-week-old inbred BALB/c mice were used for this study. The animal protocol

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adhered to the University of Connecticut Health Center for Laboratory Animal Care, Standard Operating Procedure for Tail Cut Bleeding. The ethical clearance and approval of the animal protocol for conducting experiments on animals was granted from Yeditepe University in Turkey by the Yeditepe University Experimental Animal Ethics Committee (YUDHEK).

Infection of animals. The animals were subcutaneously injected with 1×10^7 stationary *L. donovani* promastigotes in the abdominal area; 10 uninfected mice were used as negative controls. The peripheral blood (PB) samples were obtained on the days 2, 15, and 30 after infection by cutting the tail about 2 cm from the tip to permit a free flow of blood. Obtained blood samples were used for the following described experiments.

Giemsa staining. Buffy coat smears were prepared on clean glass slides and air-dried.²⁰ They were fixed in methanol for 3–5 min. After fixation, the slides were washed with tap water. The slides were stained in Giemsa dye for 25–30 minutes. Washed and dried slides were observed under light microscopy at $\times 1,000$ magnification using immersion oil.

MCM. PB (100- μ L aliquots) samples were transferred into sterile citrate-coated eppendorf tubes and mixed. The samples were washed two to three times with RPMI 1640 medium (Gibco, Gaithersburg, MD) (in the ratio of 1:3) and centrifuged for 10 minutes at $1,800 \times g$. The last centrifugation was performed for 30 minutes at $3,220 \times g$. The supernatant was removed, and 40–50 μ L upper phase were filled directly into hematocrit capillary tubes. The capillary tubes were centrifuged for 10 minutes at $900 \times g$ in a hematocrit centrifuge (Heraeus Pico 17). The buffy coat, which includes PB mononuclear cells, was collected and mixed with 100 μ L Schneider's *Drosophila* medium (SDM; Sigma Chemical Co., St. Louis, MO) supplemented with 15% fetal bovine serum (FBS; Gibco BRL, New York, NY) and gentamisin (Sigma Chemical Co., St. Louis, MO) (80 μ g/mL). The mixture was pipetted into the microcapillary tubes. The ends of the capillary tubes were sealed with melted candle wax. After the tubes were incubated at 27°C under atmospheric conditions, they were examined under the inverted microscope (Olympus CKX 41) at 200 magnification over 1–7 days.¹⁸ MCM can detect even 1 active parasite per 100 μ L microscopically.

IFAT. The presence of anti-*Leishmania* immunoglobulin M (IgM) antibodies was measured by IFAT using the *L. donovani* as the antigen and a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgM (Millipore Corporation, Bedford, MA) as the conjugate. Titers of 1/32 or over are considered to be significant, and 1/128 titers are taken as diagnostic.^{21,22} All wells on the antigen slides included 3×10^5 parasites, which were washed for several times in 0.9 NaCl. The mice sera (1/32, 1/64, and 1/128 dilutions) were coated and incubated at 37°C for 45 minutes followed by 15 minutes washing in phosphate-buffered saline (PBS; pH = 7.4). The slides were overlaid by diluted anti-mouse IgM for 45 minutes and washed in PBS again. The slides were then examined under a fluorescent microscope at $\times 200$ magnification.

Detection of antibodies to *Leishmania* antigens by ELISA. The humoral immune response was evaluated by measuring polyclonal antibodies (pAb) level against mouse by indirect ELISA. Serum samples were collected from tail vein of 34 healthy male BALB/c mice (6–8 weeks of age) and mixed with (ethylenedinitrilo)tetraacetic acid (EDTA). Briefly,

96-well plates were coated with whole *Leishmania* antigen (1 μ g/well) and incubated overnight at 4°C. After blocking with 4% gelatin (Sigma, St. Louis, MO) and incubation at 37°C, plates were washed three times. Serum samples with 1:100 dilution were added and incubated at 37°C for 2 hours followed by three washes and the addition of 100 μ L pAb conjugated with alkaline phosphatase (dilution 1:1,500). They were incubated again for 1 hour at 37°C, and then, the substrates were added; absorbance was read on an ELISA reader (Bio-Rad Benchmark Plus, Hercules, CA). The positivity ELISA cut-off (0.350) was determined as the mean plus three SDs of the values in 10 uninfected mice.²³

DNA extraction. For PCR analysis, the same DNA extraction method was applied to obtain samples from PB and MCM samples. DNA extraction was performed using a phenol-chloroform-isoamylalcohol method; 300 μ L lysis buffer (100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.5% sodium dodecyl sulfate [SDS]) and 1 μ L proteinase K (100 mg/mL; Invitrogen, Carlsbad, CA) were added into the 100- μ L samples, and they were incubated at 56°C overnight. Then, two sample volumes of phenol-chloroform-isoamylalcohol were added, and the mixture was gently vortexed for 30 seconds. The mixture was centrifuged at $13,400 \times g$ at 4°C for 10 minutes, and the supernatant was collected. Two sample volumes of chloroform were added to one volume of supernatant, and they were mixed by pipetting up and down and kept at 4°C for 30 minutes. The mixture was centrifuged at $13,400 \times g$ at 4°C for 15 minutes. After centrifugation, supernatant was transferred to a new collection tube and treated with 1 mL ice-cold ethanol. The mixture was gently mixed (5–10 times) and kept at –20°C overnight. The sample was centrifuged at $15,800 \times g$ for 10 minutes, and the pellet was washed with cold 70% ethanol and then dissolved in 20 μ L TE buffer 10/0.1 (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).²⁴

Samples for diagnosis by PCR. PCR has been performed for the amplification of *Leishmania*-specific gene from mini-exon sequences by using DNA extracted from previously infected BALB/c PB and MCM samples. PCR was set up in a final volume of 25 μ L with the 2 $\times$ Master Mix (100 pM; Fermentas) for each primer (Fme 5' TAT TGG TAT GCG AAA CTT CCG 3' and Rme 5' ACA GAA ACT GAT ACT TAT ATA CGC3') used for the detection and identification of *Leishmania* parasites. This primer pair amplifies a 378- to 424-bp DNA fragment. The reaction mixture included 1 μ L template DNA. Forty-five cycles were performed in a thermocycler (Techne). Each cycle consisted of 94°C initial denaturation for 5 minutes, 94°C denaturation (1 minute), 54°C annealing (1 minute), 72°C elongation (1.5 minutes), and 72°C post elongation (7 minutes). In all assays, positive controls containing *L. donovani* DNA and a negative control without DNA were included; 10 μ L reaction mixture were visualized by 2% agarose gel electrophoresis.²⁴ Our previously experiments showed that the analytical sensitivity of PCR method was 100 parasites in evaluated samples (unpublished results).

Statistical analysis. The results of the different diagnostic methods were submitted to statistical analysis using the SPSS software (version 19.0) for Windows. To evaluate the concordance index between two serological diagnostic methods, κ -values were ranked as low ($0.2 < \kappa < 0.4$), moderate ($0.4 < \kappa < 0.6$), good ($0.6 < \kappa < 0.8$), or excellent ($\kappa > 0.8$). The results were from the different diagnostic methods using the Pearson

TABLE 1
Measure of agreement among the results of IFAT and ELISA
Leishmania tests calculated for 34 mice

Test and result	Number of mice with ELISA result 15 days after infection		Number of mice with ELISA result 30 days after infection	
	Negative	Positive	Negative	Positive
IFAT negative	29	2	28	3
IFAT positive	0	3	0	3

For IFAT vs. ELISA, the concordance of the results was good for 15 days after infection ($\kappa = 0.719$; $P < 0.001$) and good for 30 days after infection ($\kappa = 0.622$; $P < 0.001$).

χ^2 test. The Fisher test was applied when the expected values were below five, and the results were considered significant when showing at P value < 0.05 .

RESULTS

Clinical follow-up of infected animals. After infection with long-term culture of stationary phase *L. donovani* promastigotes, mice remained with subclinical infection. No clinical signs of VL, such as loss of weight, cachexia, alopecia, onychogryphosis, or any other disease signs, were observed during the experimental period in mice.²⁵

Detection of intracellular amastigotes by Giemsa method. Amastigotes of *L. donovani* could not be detected in the buffy coat microscopically at any observation time point during the 30 days of the experiments.

Detection of anti-*Leishmania* antibodies in mouse sera. For the detection of antileishmanial antibodies, serum samples were obtained from mice at days 2, 15, and 30 of infection. ELISA and IFAT were used for serological diagnosis. Anti-*Leishmania* antibody was determined in five samples (14.7%) at day 15 of infection and six samples (17.64%) at day 30 of infection when the samples were investigated with ELISA. Presence of the antileishmanial antibody was detected in 3 of 34 serum samples when tested with IFA (1/128) at days 15 and 30 of infection. The rate of concordance between the results of IFAT and the results of ELISA was good after 15 ($\kappa = 0.719$; $P < 0.001$) and 30 days of infection ($\kappa = 0.622$; $P < 0.001$) (Table 1). MCM and MCM-PCR showed a significant difference from serological methods ($P < 0.001$) (Table 2).

Detection of extracellular promastigotes by MCM. The presence of parasites in infected BALB/c mice was also investigated by MCM on days 2, 15, and 30 of the experiment. MCM is based on the fact that intracellular amastigotes can turn into extracellular promastigotes in a microaerophilic environment, and these motile promastigotes can be detected under the inverted microscope. In this study, we determined

TABLE 2
Comparison of different diagnostic methods with MCM and MCM-PCR 30 days after infection

Diagnostic method	MCM and MCM-PCR result		P value
	Number of positive (%)	Number of negative (%)	
	34 (100)	0 (0)	
PB-PCR	2 (5.88)	32 (94.12)	< 0.001
ELISA	6 (17.65)	28 (82.35)	< 0.001
IFAT	3 (8.82)	31 (91.18)	< 0.001

MCM = microculture method; MCM-PCR = PCR obtained from microculture; PB-PCR = PCR obtained from peripheral blood.

motile promastigotes in 33 of 34 mice (97.05%) at day 2 of infection and in all of the mice at days 15 and 30 of infection by microcultures prepared from mice showing subclinical symptoms (Figure 1).

Detection of intracellular amastigotes by PCR. Of 34 samples analyzed by PCR, specific DNA amplifications of parasite were detected in one sample (2.94%) at day 2 of infection and in two samples (5.88%) at days 15 and 30 of infection. Samples that were prepared from the same PB and cultured in capillary tubes at 27°C for 3 days were analyzed by PCR. According to the data, specific DNA amplifications of parasite were determined in 32 of 34 mice (94.11%) at day 2 of infection and in all mice (100%) at days 15 and 30 of infection. There was significant difference between PCR samples that were prepared from PB and MCM according to statistical analysis (Table 2).

DISCUSSION

In this study herein, sensitivity of the methods used for diagnosis of leishmaniasis was compared with the recently developed MCM method in non-invasive samples obtained from mice with asymptomatic leishmaniasis. Obtained results showed that MCM was the more sensitive method compared with other methods for the diagnosis of asymptomatic VL. There are different studies in various endemic regions of the world for detection of the most sensitive method for asymptomatic VL. However, none of these methods has provided a comparison between MCM and the other methods, despite the fact that MCM has been used in different endemic regions for diagnosis of symptomatic VL.

In our study, the buffy coat of PB was used, and no parasite was determined microscopically in any of the Giemsa-stained blood samples of 34 asymptomatic mice. In the Bihar Region of India, where VL is endemic, researchers detected *Leishmania* amastigotes in 6 of 450 asymptomatic healthy people when smears prepared from PB of individuals were examined.¹⁶ In another study, amastigote existence in different tissues and organs of dogs was investigated by hematoxylin and eosin staining, and the presence of amastigotes was detected at the ratio of 9/23 (39.13%), 8/23 (34.78%), and 3/23 (13.04%) in popliteal lymph node, spleen, and bone marrow and liver, respectively.²⁶ In another study, it was shown that the sensitivity of microscopic methods was 60–85% in samples obtained from bone marrow and more than 95% in spleen samples in diagnosis of symptomatic VL.²⁷ Our previous studies have indicated that the sensitivity of microscopic methods is high in samples obtained by invasive methods independent of the number of parasites. Therefore, the sensitivity of microscopic methods highly depends on the number of parasites in the samples, despite their simplicity and specificity. However, asymptomatic donors are not suitable for invasive procedures, and microscopic methods are not appropriate for routine diagnosis of asymptomatic VL.

Determination of specific *Leishmania* antibodies in serum samples is an important diagnostic method, because it is not invasive. Therefore, serological tests such as IFAT,²⁸ DAT,²⁹ ELISA,³⁰ dot-ELISA,³¹ and Western blots³² are generally used. However, in these studies, it was also reported that sensitivity of the serological tests was very low in asymptomatic humans and animals, despite the high sensitivity and specificity in symptomatic patients.

FIGURE 1. Percentage of positive samples by polymerase chain reaction (PCR) and microculture. Numbers above the bars indicate the percentage of positive samples. MCM = microculture method; MCM-PCR = PCR obtained from microculture; PB-PCR = PCR obtained from peripheral blood.

IFAT, because of its high sensitivity and specificity, is used as a gold standard in the diagnosis of canine leishmaniasis in several countries.³³ However, in one study, IFAT was shown to have 90% sensitivity in symptomatic dogs, despite the fact that sensitivity of asymptomatic dogs was 29.4%.⁹ In another study, presence of anti-*Leishmania* antibody was detected in only 79 of 831 dogs (9.5%) with IFAT.¹⁰ Accordingly, in our study, presence of the anti-*Leishmania* antibody was detected in 3 of 34 serum samples of mice that showed asymptomatic indications when tested with IFAT at days 15 and 30 of infection. Therefore, it was revealed that IFAT was insufficient to confirm the diagnosis of asymptomatic leishmaniasis.

In several studies, it has been determined that ELISA was the more sensitive method than IFAT in the diagnosis of asymptomatic leishmaniasis.²⁸ Specific antibodies were detected with an ELISA method in 143 of 837 (17.1%) dogs in a study of asymptomatic dogs.²⁸ However, in our study, the anti-*Leishmania* antibody was determined in five samples (14.7%) at day 15 of infection and six samples (17.64%) at day 30 of infection when 34 serum samples were investigated with ELISA.

Prevalence of asymptomatic infection has been changed between 2.4% and 17% in studies based on serology.^{11,34–36} An explanation may be that there are low antibody levels of these samples and low circulation of parasites within the body compared with symptomatic patients.³⁵

Additionally, in dogs, asymptomatic leishmaniasis can be accurately diagnosed by *in vitro* isolation of parasites and then its culture and by using PCR to determine DNAs from biopsy samples obtained from bone marrow or lymph nodes.^{9,37} In biopsy samples taken from lymph nodes of asymptomatic dogs by invasive methods, sensitivity of PCR was measured as high as 95.65%.²⁶ However, its sensitivity in asymptomatic dogs was very low when non-invasive methods were used.³⁸

In our study, according to the results of PCR analysis of PBs taken from tail veins of 34 mice that showed subclinical indications at days 2, 15, and 30 of infection, specific DNA amplifications of parasite were detected in one sample (2.94%) at day 2 of infection and two samples (5.88%) at

days 15 and 30 after infection. The most likely reason for this finding is the probability of low circulation levels of parasites in the blood of asymptomatic *Leishmania* carriers, and the sensitivity of PCR is not enough to detect the parasites.

It was known that the sensitivity of PCR was expected to be higher for *Leishmania*–human immunodeficiency virus (HIV) asymptomatic coinfections. However, in a Spanish study, parasite existence was detected in only 28 of 92 HIV-infected patients (30.4%) that showed subclinical symptoms according to PCR analysis of PBs.^{39,40} However, invasive methods generally cannot be used for asymptomatic VL patients.⁴¹ In addition, as mentioned above, sensitivity of non-invasive methods that are used in diagnosis of VL is typically very low.

Developing new non-invasive methods with high specificity and sensitivity is, therefore, very important for detecting parasites in asymptomatic healthy patients; numbers have been seriously and continuously increasing. For this purpose, we investigated the sensitivity of MCM in our asymptomatic mouse model compared with other methods, and we obtained significant results on the subject of detecting asymptomatic *Leishmania* infections. Motile promastigotes were detected in 33 of 34 mice (97.05%) at day 2 of infection and all of the mice at days 15 and 30 of infection. The most important reason for using MCM methods in this study was that MCM showed high sensitivity independent of the number of parasites and type of culture medium; also, it was a very simple and economic method. Additionally, sensitivity of this method was shown to be independent of the number of parasites in other studies that were performed in different endemic regions of world. In contrast, the sensitivity of the traditional culture method (TCM) varies greatly with the number of amastigotes in samples, and it sometimes require very long incubation periods.⁶ Also, it is known that long incubation periods (1–6 months) may be necessary for the detection of parasites in donor blood samples from asymptomatic patients with low parasite load.^{6,42} In comparison, MCM is much less susceptible to these variable parameters and provides a more rapid and accurate reading. The high

sensitivity of the MCM can 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 result is not achieved under the conditions used for the TCM.^{18,43}

Also in this study, PCR analysis of PB samples was compared with PCR analysis of MCM samples, and it was determined that PCR samples of asymptomatic mice, prepared from MCM, had 100% positivity, whereas positivity of PCR samples, prepared from PB, was 5.88% (χ^2 test, $P < 0.001$); 100% positivity of PCR analysis from MCM samples may be very important for identification studies in terms of asymptomatic infections. In our previous study, we showed that the PCR method could detect at least 100 parasites in evaluated samples. This finding is the main reason of low sensitivity of PCR for mice models. Conversely, microscopic determination of only one parasite in blood samples is sufficient to reveal the positivity of MCM. Hence, the sensitivity of MCM is very high. Obtained blood samples were incubated at 27°C for 7 days. The most important reason for higher sensitivity of MCM than PB is the enhancement of parasite numbers within microculture conditions during this period.

In conclusion, the present study, for the first time, showed that, in non-invasive samples of asymptomatic infections, parasites can be determined by MCM with high specificity and sensitivity. This study also revealed a new potential application of MCM, which has been used in diagnosis of clinically suspected cutaneous leishmaniasis (CL) and VL cases. Obtained results also revealed that, in endemic and non-endemic regions, MCM can also provide the detection of the presence of asymptomatic leishmaniasis in humans and animals that are reservoirs of leishmaniasis.

Received January 16, 2012. Accepted for publication April 22, 2012.

Acknowledgments: The authors gratefully acknowledge the Scientific and Technological Research Council of Turkey (TUBITAK) for financial support (Project Number 108S170).

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