JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1994, p. 746–749
0095-1137/94/$04.00+0

Vol. 32, No. 3

Comparison of PCR with Direct Examination of Bone Marrow Aspiration, Myeloculture, and Serology for Diagnosis of Visceral Leishmaniasis in Immunocompromised Patients

RENAUD PIARROUX,[1]* FRANÇOISE GAMBARELLI,[1] HENRI DUMON,[1] MICHEL FONTES,[2] SYLVIANE DUNAN,[1] CHARLES MARY,[1] BELLA TOGA,[1] AND MICHEL QUILICI[1]

Laboratoire de Parasitologie-Mycologie[1] and Institut National de la Santé et de la Recherche Médicale Unité 242,[2] Faculté de Médecine de la Timone, 13385 Marseille Cedex 5, France

Received 8 July 1993/Returned for modification 9 September 1993/Accepted 9 December 1993

A PCR assay amplifying a repeated sequence from the *Leishmania infantum* genome was compared with direct examination of bone marrow aspirate, myeloculture, and serology for the diagnosis of visceral leishmaniasis in immunocompromised patients. Of 73 patients living in an area endemic for leishmaniasis and where visceral leishmaniasis was suspected by physicians, only 10 had an indisputable diagnosis of visceral leishmaniasis. None of the diagnostic tests performed in the study achieved 100% sensitivity for diagnosing visceral leishmaniasis. PCR exhibited superior sensitivity (82%) in comparison with bone marrow aspirate examination (55%) and myeloculture (55%). Our PCR assay also showed good specificity (97%), negative predictive value (97%), and positive predictive value (82%) even when all unconfirmed PCR results were scored as false positives. Serology exhibited good sensitivity (80%) and excellent specificity (100%), negative predictive value (98%), and positive predictive value (100%) in diagnosing new cases of visceral leishmaniasis but failed to diagnose relapses. We also observed consistent negative serological results using several different immunological detection methods for 2 of the 10 patients with confirmed cases of visceral leishmaniasis. This lack of serological reactivity persisted throughout the course of their infections. These results demonstrate the importance of using PCR as an aid in the diagnosis of visceral leishmaniasis in immunocompromised patients.

Protozoa of the genus *Leishmania* are a group of morphologically similar parasites which cause a number of disease manifestations in humans, e.g., cutaneous, mucocutaneous, and visceral leishmaniasis. The major features of visceral leishmaniasis (kala azar) are intermittent fever, enlargement of the spleen, and pancytopenia. The disease leads to death if it is not treated. The agents responsible for kala azar are *L. infantum*, *L. chagasi*, and *L. donovani*. Visceral leishmaniasis has been reported to be an opportunistic infection in immunosuppressed patients (8) and in immunocompromised subjects such as patients with human immunodeficiency virus infection (1, 2, 10, 11, 14, 17) living in areas in which visceral leishmaniasis is endemic. Unfortunately, diagnosis of visceral leishmaniasis is difficult in such patients; serologic diagnosis is not reliable for immunosuppressed patients, direct diagnosis is not sensitive, and culture of *Leishmania* cells is insensitive and time-consuming. Two different approaches have been used to improve the diagnosis of visceral leishmaniasis. Mary et al. (9) proposed a means of diagnosis based on the Western blot (WB; immunoblot) technique, which is more sensitive than other serological techniques (9). Another approach is the use of DNA-specific probes or amplification of a specific sequence of *Leishmania* DNA by the PCR technique. Although different authors have described PCR assays that could be useful for the diagnosis of visceral leishmaniasis (4, 12, 13, 15, 16), no study has been performed to compare the reliability of PCR with that of serology, culture, or direct examination of bone marrow aspirate (BMA). We report here the results of a prospective study on the diagnosis of leishmaniasis in 73 immunocompromised patients by using PCR amplification of a repetitive target sequence specific for all causative agents of visceral leishmaniasis (*L. infantum*, *L. chagasi*, and *L. donovani*), serology, and myeloculture.

* Corresponding author. Mailing address: Laboratoire de Parasitologie-Mycologie, Faculté de Médecine, 27 Boulevard Jean Moulin, 13385 Marseille Cedex 5, France. Phone: (33) 91 78 14 54. Fax: (33) 91 79 60 63.

MATERIALS AND METHODS

Patients and samples. Clinical specimens were obtained from 73 patients admitted to hospitals in the Marseille, France, area from January 1992 to May 1993. All patients were

TABLE 1. Test results for patients with no previous history of leishmaniasis

Patient no.	Direct BMA examination	Culture	Serology	PCR	Final diagnosis
1	+	+	+	+	Visceral leishmaniasis
2[a]	+	+	+	+	Visceral leishmaniasis
3	+	+	+	+	Visceral leishmaniasis
4	−	−	+	+	Visceral leishmaniasis
5	− and then +	−	−	+	Visceral leishmaniasis (confirmed by meticulous reexamination of BMA samples)
6	−	−	−	+	Unknown (considered false-positive result of PCR)
7	−	−	−	+	Tuberculosis (?) (considered false-positive result of PCR)
8–64	−	−	−	−	No visceral leishmaniasis

[a] This patient experienced a relapse during the time of the study (see Table 2).

TABLE 2. Test results for patients with a history of treated leishmaniasis

Patient no.	Direct BMA examination	Culture	Serology	PCR	Final diagnosis
65	+	+	−	+	Relapse
66	+	+	Not done[a]	+	Relapse
67	−	−	+	+	Relapse (confirmed by testing another BMA sample)
2	−	−	+	+	Relapse (confirmed by testing another BMA sample)
68	+	+	+	−	Relapse
69	−	−	+	−	Relapse (*Leishmania* cells were visualized in a ganglionic biopsy specimen)
70–73	−	−	+	−	No evidence of relapse

[a] The patient died before receiving treatment; thus, no serology could be performed.

immunocompromised (70 had human immunodeficiency virus infection with fewer than 0.2×10^9 $CD4^+$ T cells per ml, 2 were renal transplantation recipients, and 1 had pulmonary neoplasty). The mean age of the subjects was 35 ± 7 years (range, 16 to 75 years). Patients were divided into two groups according to their histories, as follows: Group I (n = 64) included patients without a previous history of leishmaniasis and group II (n = 10) included those with a previous history of treated visceral leishmaniasis. Clinical features presented by patients in both groups were fever (n = 63), multiple adenopathies (n = 20), splenomegaly (n = 15), and hepatomegaly (n = 13). Serum abnormalities included hemoglobinemia of less than 120 g/l (n = 60), neutrophil count of less than 1.5×10^9/ml (n = 40), and platelet count of less than 1×10^{11}/ml (n = 37). BMAs were collected with a syringe containing heparin to avoid coagulation, and BMAs were transported to the laboratory at room temperature.

Immunological detection methods. The presence of *L. infantum*-specific antibodies was determined by four different tests: indirect immunofluorescent-antibody test (IFA), counterelectrophoresis (CEP), enzyme-linked immunosorbent assay (ELISA), and WB. IFA and CEP were performed by using *L. infantum* promastigotes (MCAN/FR/73/LPM 56) as antigens (9). The IFA result was regarded as positive when a 1:100 dilution of the serum gave fluorescence. CEP was performed as described by Gentilini et al. (5) by using promastigotes lysed by ultrasonic treatment; CEP was considered positive when at least one arc of precipitation formed. The preparation of *L. infantum* antigens from a lysate of promastigotes (MCAN/82/GR/MON 497) and the methods used to perform ELISA and WB tests have been described previously (6, 9). ELISA was regarded as positive when the optical density at 620 nm was more than 0.50, and WB was considered positive when sera from patients recognized either the 14- or the 16-kDa antigen.

Direct examination of BMAs. The slides were stained with Giemsa and examined with a ×10 eyepiece and a ×100 oil objective. Each sample was examined at least twice for 45 min each time by two senior parasitologists (representing more than 1,000 microscopic fields examined) before confirming or determining a negative result.

Culture of *Leishmania* cells. Each bone marrow sample (0.5 ml) was mixed with 3 ml of culture medium (10% fetal bovine serum [Sigma, St. Louis, Mo.] in RPMI 1640 medium [Sigma]) that was heated for 30 min at 56°C. The mixture was then incubated for 7 days at 24°C and was examined by microscopy. When no *Leishmania* cells were found, 1 ml of the culture sample was subcultured onto the same medium for another 7 days at 24°C before confirming a negative result.

Preparation of *Leishmania* DNA for PCR amplification. A 100-μl aliquot of BMA was added to 300 μl of specimen preparation buffer consisting of 20% Chelex (Bio-Rad, Paris, France), 1% Nonidet P-40 (Sigma), and 1% Tween 20 (Sigma) in distilled water. This mixture was then heated for 20 min at 96°C and was centrifuged at 13,000 × g for 10 min at room temperature. The subsequent supernatant was collected and was used immediately for PCR amplification or was stored at −20°C until use.

PCR. We used two oligonucleotide primers derived from a repetitive sequence of the *L. infantum* genome specific for the three agents of visceral leishmaniasis (*L. infantum*, *L. chagasi*, and *L. donovani*) (11): 5′-ACG AGG TCA GCT CCA CTC C-3′ and 5′-CTG CAA CGC CTG TGT CTA CG-3′. The reaction mixture consisted of 1× DNA polymerase buffer (Boehringer, Meylan, France), 0.2 μM (each) dATP, dCTP, dGTP, and dTTP, and 2 μM (each) primer in a final volume of 50 μl. Each reaction was overlaid with 50 μl of mineral oil. *Taq* polymerase (1.5 U; Boehringer) was added after a step of "hot start," which consisted of 5 min of incubation at 96°C and cooling to 55°C (3). After 35 cycles of amplification (30 s at 94°C, 30 s at 59°C, and 30 s at 72°C) in a thermocycler (Biometra; Eurogentec, Liège, Belgium), 20-μl samples of the reaction mixture were visualized by 2% agarose gel electrophoresis.

Diagnostic criteria for determining visceral leishmaniasis. The diagnosis of visceral leishmaniasis was confirmed when *Leishmania* cells could be visualized on direct examination or after culturing. We also considered those patients with no previous history of leishmaniasis whose sera tested positive by the four serological methods as true-positive cases even when direct examination of BMAs and myeloculture yielded negative results.

RESULTS

Of the 64 patients in group I (with no previous history of leishmaniasis), 5 had undisputed leishmaniasis. No *Leishmania* cells were found in BMAs from one of these patients, but all of

TABLE 3. Results of PCR diagnosis in both groups of patients

Patient group	PCR positive (no. of patients true positive/no. false positive)	PCR negative (no. of patients false negative/no. true negative)	No. of positive patients/total no. of patients (%)			
			Sensitivity	Specificity	Positive predictive value	Negative predictive value
Group I	5/2	0/57	5/5 (100)	57/59 (97)	5/7 (71)	57/57 (100)
Group II	4/0	2/4	4/6 (67)	4/4 (100)	4/4 (100)	4/6 (67)
Both groups	9/2	2/61	9/11 (82)	61/63 (97)	9/11 (82)	61/63 (97)

TABLE 4. Results of direct examination of BMAs in both groups of patients

Patient group	Positive examination (no. of patients true positive/no. false positive	Negative examination (no. of patients false negative/no. true negative)	No. of positive patients/total no. of patients (%)			
			Sensitivity	Specificity	Positive predictive value	Negative predictive value
Group I	3/0	2/59	3/5 (60)	59/59 (100)	3/3 (100)	59/61 (97)
Group II	3/0	3/4	3/6 (50)	4/4 (100)	3/3 (100)	4/7 (57)
Both groups	6/0	5/63	6/11 (55)	63/63 (100)	6/6 (100)	63/68 (93)

the immunological detection methods were positive. Results of direct BMA examination, myeloculture, serology, and PCR are given in Table 1.

Of the 10 patients in group II (people with a previous history of treated leishmaniasis), 6 had indisputable relapses. Results of direct BMA examination, myeloculture, serology, and PCR are given in Table 2. Our PCR-based diagnoses exhibited superior sensitivity and negative predictive value (Table 3) than of those diagnoses determined by direct examination of BMAs (Table 4) or myeloculture (Table 5). By our definition, direct examination and culture cannot yield false-negative results; hence, the two methods had specificities and positive predictive values of 100%. Immunological analysis was of interest for patients with no previous history of leishmaniasis (Table 6) but not for patients with relapses, because serological results for patients with a previous history of leishmaniasis typically remained positive. In patients with a previous history of leishmaniasis, only a high antibody titer signaled a relapse; the antibody titer exceeded 1/800 by IFA and 1 by ELISA for four of the six patients experiencing relapses, whereas it was less than 1/200 by IFA and less than 1 by ELISA for patients without relapses. In addition, sera from 2 of the 10 patients with confirmed cases of visceral leishmaniasis maintained consistent negative results by all four immunological detection methods.

DISCUSSION

We sought to develop a PCR-based diagnostic test for visceral leishmaniasis because none of the usual biological exams were capable of diagnosing all cases of leishmaniasis in immunocompromised hosts. We noted that the presence of specific antibodies against *L. infantum* were undetectable in two patients. Several investigators have already reported the absence of a serological response in immunocompromised hosts with visceral leishmaniasis (2, 7, 10), but they did not use WB analysis, which is a more sensitive technique than other serological techniques (9). For such patients, serological analysis may lead to false-negative results. Use of immunological detection methods are also difficult for the diagnosis of relapse in patients with a previous history of visceral leishmaniasis. Indeed, these patients often preserve a positive serology that would interfere with the unambiguous detection of a relapse. Previously, the diagnosis of visceral leishmaniasis in immunocompromised hosts required direct examination of BMAs or cultivation of *Leishmania* cells from a BMA. As seen in the present study, even meticulous examination of BMAs can yield false-negative results because of the very low number of *Leishmania* cells in bone marrow or because of hemodiluted samples. Culture of *Leishmania* cells from BMAs may improve the direct diagnosis of visceral leishmaniasis in such patients (2). Unfortunately, this time-consuming method can also yield false-negative results. In particular, the culture results are often negative when samples are hemodiluted or when patients are treated with pentamidine or amphotericin B for pneumocystosis or mycosis. Thus, in previous studies, establishing the diagnosis required the use of invasive techniques such as liver biopsy or splenectomy (10). In our study, PCR confirmed the diagnosis of visceral leishmaniasis in 3 of the 10 patients. In one patient (patient 5), the diagnosis was not established because all of the tests except PCR-based diagnosis were negative, but meticulous reexamination of BMAs then confirmed it to be a true-positive result. In two other patients (patients 67 and 68), a diagnosis of relapse was suspected because antibody levels were high and results of PCR were positive, whereas results of direct examination and culture were negative. In these two patients, diagnosis was confirmed by testing another BMA. The significance of the positive PCR results associated with negative serology, myeloculture, and BMA examination was impossible to determine in two patients (patients 6 and 7) because the patients refused to undergo a second bone marrow sampling. A false-positive result because of contamination is unlikely, but it cannot be excluded.

Because our objective was to develop a fast and simple method for use in routine diagnosis, we used a sample preparation procedure that was simpler than the classical phenol-chloroform extraction method. Samples for PCR can be prepared in only 1 h, and risks of sample cross-contamination are diminished. This method of specimen preparation also reduces the cost of PCR assays because all of the reagents involved are inexpensive. Even with a simplified sampling procedure, PCR-based diagnosis was more sensitive than direct examination of BMAs and myeloculture. We have previously demonstrated that our PCR assay is able to detect a single *Leishmania* cell without a hybridization step, because the primers we used recognize a repetitive sequence in the *L. infantum* genome (12).

TABLE 5. Results of BMA culture diagnosis in both groups of patients

Patient group	Positive culture (no. of patients true positive/no. false positive)	Negative culture (no. of patients false negative/no. true negative)	No. of positive patients/total no. of patients (%)			
			Sensitivity	Specificity	Positive predictive value	Negative predictive value
Group I	3/0	2/59	3/5 (60)	59/59 (100)	3/3 (100)	59/61 (97)
Group II	3/0	3/4	3/6 (50)	4/4 (100)	3/3 (100)	4/7 (57)
Both groups	6/0	5/63	6/11 (55)	63/63 (100)	6/6 (100)	63/68 (93)

TABLE 6. Results of serological diagnosis in both groups of patients

Patient group	Positive serology (no. of patients true positive/no. false positive)	Negative serology (no. of patients false negative/no. true negative)	No. of positive patients/total no. of patients (%)			
			Sensitivity	Specificity	Positive predictive value	Negative predictive value
Group I	4/0	1/59	4/5 (80)	59/59 (100)	4/4 (100)	59/60 (98)
Group II	4/4	1/0	4/5 (80)	0/4 (0)	4/8 (50)	0/1 (0)
Both groups	8/4	2/59	8/10 (80)	59/63 (94)	8/12 (67)	59/61 (97)

The sensitivity, specificity, and ease of PCR-based diagnosis make PCR a good candidate for the initial screening of BMAs sent to the laboratory when immunocompromised patients have a fever. When serological results are negative or when patients have a previous history of visceral leishmaniasis, a positive result must be confirmed by reexamination of a BMAs or by the acquisition of new samples before beginning specific treatment. Myeloculture is not performed when PCR, serology, and direct examination of BMAs are negative because it is a difficult technique that does not improve the sensitivity of PCR-based diagnosis. In contrast, myeloculture is indicated when PCR, serology, or direct examination of BMAs is positive because it is currently the only way to obtain precise identification of strains and to test the susceptibilities of *Leishmania* organisms to antimonial drugs.

Finally, easier and rapid detection of *Leishmania* organisms by PCR can lead to investigations of the role of *L. infantum* in infections of immunocompromised patients living in areas where the organism is endemic, such as southern Europe, and can facilitate improved patient care.

REFERENCES

1. **Alvar, J., B. Gutierrez-Solar, R. Molina, R. Lopez-Velez, A. Garcia-Camacho, P. Martinez, F. Laguna, E. Cercenado, and A. Galmes.** 1992. Prevalence of *Leishmania* infection among AIDS patients. Lancet **339:**1427.
2. **Berenguer, J., S. Moreno, E. Cerdenaro, J. C. L. Bernaldo de Quiros, A. Garcia de la Fuente, and E. Bouza.** 1989. Visceral leishmaniasis in patients infected with human immunodeficiency virus (HIV). Ann. Intern. Med. **111:**129–132.
3. **Chou, Q., M. Russell, D. E. Birch, J. Raymond, and W. Bloch.** 1992. Prevention of pre-PCR mispriming and primer dimerization improves low-copy-number amplifications. Nucleic Acids Res. **20:**1717–1723.
4. **de Bruijn, M. H. L., and D. C. Barker.** 1992. Diagnosis of New World leishmaniasis: specific detection of the *Leishmania braziliensis* complex by amplification of kinetoplast DNA. Acta Trop. **52:**44–58.
5. **Gentilini, M., J. M. Pinon, and G. Niel.** 1972. Immunoélectrodiffusion sur membrane d'acétate de cellulose: applications en parasitologie. (A propos de 600 essais). Bull. Soc. Pathol. Exot. **65:**60–66.
6. **Hommel, M., W. Peters, J. Ranque, M. Quilici, and G. Lanotte.** 1978. The micro-ELISA technique in the serodiagnosis of visceral leishmaniasis. Ann. Trop. Med. Parasitol. **72:**213–218.
7. **Lortholary, O., D. Christiaens, D. Mechali, and P. Babinet.** 1991. Leishmaniose viscérale et immunodépression. Ann. Med. Intern. **142:**215–217.
8. **Ma, D. D. F., A. J. Concannon, and J. Hayes.** 1979. Fatal leishmaniasis in renal-transplant recipient. Lancet **ii:**311–312.
9. **Mary, C., D. Lamouroux, S. Dunan, and M. Quilici.** 1992. Western blot analysis of antibodies to *L. infantum* antigens: potential of the 14-kD and 16-kD antigens for diagnosis and epidemiologic purposes. Am. J. Trop. Med. Hyg. **47:**764–771.
10. **Montalban, C., J. L. Calleja, A. Erice, F. Laguna, B. Clotet, D. Podzamczer, J. Cobo, J. Mallolas, M. Yebra, A. Gallego, and the Cooperative Group for the Study of Leishmaniasis in AIDS.** 1990. Visceral leishmaniasis in patients infected with human immunodeficiency virus. J. Infect. **21:**261–270.
11. **Peters, B. S., D. Fish, R. Golden, D. A. Evans, A. D. M. Bryceson, and A. Pinching.** 1990. Visceral leishmaniasis in HIV infection and AIDS: clinical features and response to therapy. Q. J. Med. **283:**1101–1111.
12. **Piarroux, R., R. Azaiez, A. M. Lossi, P. Reynier, F. Muscatelli, F. Gambarelli, M. Fontes, H. Dumon, and M. Quilici.** 1993. Isolation and characterization of a repetitive sequence from *Leishmania infantum*: development of a visceral leishmaniasis polymerase chain reaction. Am. J. Trop. Med. Hyg. **49:**364–369.
13. **Rodgers, M. R., S. J. Popper, and D. F. Wirth.** 1990. Amplification of kinetoplast DNA as a tool in the detection and diagnosis of Leishmania. Exp. Parasitol. **71:**267–275.
14. **Senaldi, G., G. Cadeo, G. Carnevale, G. di Perri, and G. Carosi.** 1986. Visceral leishmaniasis as an opportunistic infection. Lancet **i:**1094.
15. **Uliana, S. R., M. H. Affonso, E. P. Camargo, and L. M. Floeter-Winter.** 1991. Leishmania: genus identification based on a specific sequence of the 18S ribosomal RNA sequence. Exp. Parasitol. **72:**157–163.
16. **van Eys, G. J. J. M., G. J. Schoone, N. C. M. Kroon, and S. B. Ebeling.** 1992. Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of Leishmania parasites. Mol. Biochem. Parasitol. **51:**133–142.
17. **Vigevani, G. M., M. Galli, G. Rizzardini, S. Antinori, M. Almaviva, and M. Coen.** 1989. Visceral leishmaniasis in HIV infection: a report of three cases. AIDS **3:**674–675.