JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1997, p. 2551–2555 Vol. 35, No. 10
0095-1137/97/$04.00+0
Copyright © 1997, American Society for Microbiology

Rapid Identification of Causative Species in Patients with Old World Leishmaniasis

P. MINODIER,[1]* R. PIARROUX,[1,2] F. GAMBARELLI,[1,3] C. JOBLET,[3] AND H. DUMON[1,3]

Laboratoire de Parasitologie-Mycologie, CHU Timone, 13385 Marseille Cedex 05,[1] Laboratoire de Parasitologie-Mycologie, CHU Jean Minjoz, 25000 Besançon,[2] and Unité INSERM 399, Faculté de Médecine, 13005 Marseille,[3] France

Received 31 March 1997/Returned for modification 13 May 1997/Accepted 8 July 1997

Conventional methods for the identification of species of *Leishmania* parasite causing infections have limitations. By using a DNA-based alternative, the present study tries to develop a new tool for this purpose. Thirty-three patients living in Marseilles (in the south of France) were suffering from visceral or cutaneous leishmaniasis. DNA of the parasite in clinical samples (bone marrow, peripheral blood, or skin) from these patients were amplified by PCR and were directly sequenced. The sequences observed were compared to these of 30 strains of the genus causing Old World leishmaniasis collected in Europe, Africa, or Asia. In the analysis of the sequences of the strains, two different sequence patterns for *Leishmania infantum*, one sequence for *Leishmania donovani*, one sequence for *Leishmania major*, two sequences for *Leishmania tropica*, and one sequence for *Leishmania aethiopica* were obtained. Four sequences were observed among the strains from the patients: one was similar to the sequence for the *L. major* strains, two were identical to the sequences for the *L. infantum* strains, and the last sequence was not observed within the strains but had a high degree of homology with the sequences of the *L. infantum* and *L. donovani* strains. The *L. infantum* strains from all immunocompetent patients had the same sequence. The *L. infantum* strains from immunodeficient patients suffering from visceral leishmaniasis had three different sequences. This fact might signify that some variants of *L. infantum* acquire pathogenicity exclusively in immunocompromised patients. To dispense with the sequencing step, a restriction assay with *Hae*III was used. Some restriction patterns might support genetic exchanges in members of the genus *Leishmania*.

The protozoan parasites of the genus *Leishmania* are the causative agents of various diseases, ranging from isolated cutaneous lesions to fatal systemic diseases (14).

In the south of France, and especially in the suburban foci, both visceral and cutaneous forms of leishmaniasis are observed in humans. The endemic species *Leishmania infantum* is the causative agent of the visceral form; both *L. infantum* strains and the imported *Leishmania major* and *Leishmania tropica* strains may be isolated from the lesions of the skin. Many people traveling between Marseilles and North Africa may indeed be infected on one or the other side of the Mediterranean Sea.

The cutaneous lesions, induced by the dermatotropic *L. infantum* strains, may enter the viscera. This process is, however, not found with *L. tropica* or *L. major*. The identification of the causative species is therefore essential for choosing the correct therapy.

We have recently isolated a repetitive sequence from *Leishmania* nuclear DNA and have developed a PCR-based diagnostic test specific for visceral and Old World cutaneous leishmaniases (15, 16). Also, the PCR products of some representative strains causing Old World leishmaniasis have been sequenced after molecular cloning and dendrograms have been constructed (17).

In the present work, we have developed a rapid test for the detection of specific *Leishmania* species. It is based on a comparison of the sequences of the PCR products obtained from the parasites from the patients and the sequences of a genomic library. We have used direct sequencing of the PCR products, without DNA extraction. An restriction analysis assay is also proposed.

MATERIALS AND METHODS

Patients and samples. Samples were obtained from 33 patients suffering from visceral (31 samples) or cutaneous (2 samples) leishmaniasis during the period from March 1993 to July 1996. Eighteen bone marrow samples, 13 blood samples, and 2 samples from skin lesions were obtained after the patients provided consent. The bone marrow and blood samples were recollected in tubes containing EDTA. The samples of the skin lesions were placed in 0.9% sodium chloride. The diagnosis of the disease was confirmed by direct microscopic examination or by culture in RPMI medium supplemented with 10% fetal bovine serum, as described previously (16).

Twenty-six patients were adults, and seven patients were children under age 15 years. The patients' ages ranged from 11 months to 86 years (mean, 33 years; standard deviation, 15 years). The sex ratio was 3.7. Twenty patients were coinfected with human immunodeficiency virus (HIV) (mean age, 37 years; male/female ratio, 9). Among the 13 HIV-negative patients (mean age, 27 years; sex ratio, 1.6), 11 were immunocompetent and 2 were immunocompromised secondary to renal transplantation.

Strains. Thirty strains that cause Old World leishmaniasis and that had been collected in Europe, Africa, or Asia were analyzed by PCR and their DNAs were sequenced. Twenty-one strains were previously characterized as *L. infantum*, one was characterized as *Leishmania donovani*, two were characterized as *L. major*, five were characterized as *L. tropica*, and one was characterized as *Leishmania aethiopica*. Table 1 lists the most important features of the strains.

PCR amplification. The samples (100 μl) were washed twice in 0.3% NaCl and were then lysed by heating at 95°C for 20 min with 400 μl of a mixture containing 1% polyoxyethylenesorbitan monolaurate (Tween 20; Sigma Chemical Co., St. Louis, Mo.), 1% Nonidet P-40 (BDH Laboratory Supplies, Poole, England), and 10% Chelex 100 resin (Bio-Rad Laboratories, Hercules, Calif.) in sterile water (DNA-free) as described previously (17).

Primers T2 (5′-CGGCTTCGCACCATGCGGTG-3′) and B4 (5′-ACATCC CTGCCCACATACGC-3′) were used for the amplification and were used as described previously (16).

Each PCR mixture contained 5 μl of the sample, 1× DNA polymerase buffer (Boehringer, Indianapolis, Ind.), 100 μM (each) dATP, dCTP, dGTP, and dTTP, 0.2 μM (each) primer, and 1 U of *Taq* polymerase (Eurobio Taq; Boehringer), and the mixture was overlaid with 50 μl of mineral oil. After a 5-min incubation at 96°C, 40 cycles of amplification (with 1 cycle consisting of 30 s at 96°C, 30 s at

* Corresponding author.

Downloaded from http://jcm.asm.org/ on October 25, 2020 by guest

TABLE 1. Main Features of 30 *Leishmania* strains collected in the old world

Species[a]	International code	Zymodeme	Source
L. infantum (1)	MCAN/FR/73/LPM 56	MON 1	Provence (France)
L. infantum (2)	MHOM/FR/88/LPM 74	MON 186	Provence (France)
L. infantum (3)	MHOM/FR/90/LPM 81	MON 33	Provence (France)
L. infantum (4)	MHOM/FR/91/LPM 84	MON 1	Provence (France)
L. infantum (5)	MHOM/FR/91/LPM 85	MON 1	Corsica (France)
L. infantum (6)	MHOM/FR/91/LPM 86	MON 1	Provence (France)
L. infantum (7)	MHOM/FR/91/LPM 87	MON 1	Provence (France)
L. infantum (8)	MHOM/FR/91/LPM 88	MON 1	Greece
L. infantum (9)	MHOM/FR/92/LPM 92	MON 1	Portugal
L. infantum (10)	MHOM/FR/93/LPM 95	MON 1	Provence (France)
L. infantum (11)	MHOM/FR/93/LPM 104	MON 1	Provence (France)
L. infantum (12)	MHOM/FR/93/LPM 106	MON 1	Provence (France)
L. infantum (13)	MHOM/FR/95/LPM 136	MON 33	Provence (France)
L. infantum (14)	MHOM/FR/95/LPM 137	MON 1	Provence (France)
L. infantum (15)	MHOM/FR/95/LPM 143	MON 186	Provence (France)
L. infantum (16)	MHOM/FR/95/LPM 144	MON 1	Provence (France)
L. infantum (17)	MHOM/FR/95/LPM 148	MON 1	Provence (France)
L. infantum (18)	MHOM/ES/81/BCN 1	MON 29	Spain
L. infantum (19)	MHOM/FR/80/LEM189	MON 11	Languedoc (France)
L. infantum (20)	MHOM/SD/62/3 S	MON 81	Sudan
L. infantum (21)	MHOM/IT/85/ISS 176	MON 24	Italy
L. donovani (1)	MHOM/ET/67/HV 3	MON 18	Ethiopia
L. major (1)	MHOM/YD/76/LEM 62	MON 26	Yemen
L. major (2)	MHOM/MA/81/LEM 265	MON 25	Morocco
L. tropica (1)	MRAT/IQ/73/Adhanis 1	MON 5	Iraq
L. tropica (2)	MHOM/TN/80/LEM 163	MON 8	Tunisia
L. tropica (3)	MHOM/SU/75/K 27	MON 60	Former USSR
L. tropica (4)	MHOM/GR/80/GR-L 35	MON 56	Greece
L. tropica (5)	MHOM/MA/87/FAT 1	MON 112	Morocco
L. aethiopica (1)	MHOM/ET/67/L 86	MON 106	Ethiopia

[a] Numbers in parentheses refer to the strains shown in Fig. 1.

56°C, and 20 s at 72°C) were performed in an automatic thermocycler (Biometra; Eurogentec, Tampa, Fla.). Then, 8 μl of the reaction mixture was analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide.

Sequence analysis. PCR products were purified on MicroSpin S-300 HR columns (Pharmacia Biotech) by using a 2-min centrifugation at 735 × *g*. For sequence determinations, the AmpliCycle sequencing kit (Perkin-Elmer) was used, following the recommendations of the supplier. An amount of 250 to 500 ng of DNA of the PCR products was added to 4 μl of 10× Cycling Mix containing AmpliTaq DNA polymerase CS, 3 pmol of each fluorescein-labelled primer (T2-FLU [5′-CTTCGCACCATGCGGTGGGG-3′] and B4-FLU [5′-TCCCTGCCCACATACGCCCA-3′]), and 30 μl of sterile water (DNA-free). A total of 6 μl of this mixture was added to 2 μl of each Termination Mix (G Termination Mix, A Termination Mix, T Termination Mix, and C Termination Mix). After 1 min of preheating to 95°C (reducing the amount of nonspecific primer binding sites being extended by the *Taq* polymerase), 25 amplification cycles (with 1 cycle consisting of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C) and an extra elongation step (5 min at 72°C) were performed. Then, 4 μl of Stop solution was added. The templates were stored at −20°C or were used immediately.

Sequence analysis was performed in an automated sequencer (ALF; Pharmacia) by using denaturation by heating (5 min at 95°C), and electrophoresis was performed on an acrylamide-bisacrylamide gel (ReadyMix Gel; ALF grade; Pharmacia Biotech).

Restriction enzyme analysis. For the restriction enzyme analysis, *Hae*III (Eurogentec, Seraing, Belgium) was chosen. This enzyme can identify the sequence 5′-GG$^{\downarrow}$CC-3′. Its incubation temperature is 37°C. Following the recommendations of the supplier, 17 μl of the PCR products was added to 2 μl of the enzyme buffer and 1 μl (10 U) of the enzyme. This mixture was incubated for 3 h at 37°C.

RESULTS

Genomic amplification and sequences. All samples gave an amplified fragment of about 250 bp. Four different sequences were obtained among the isolates from the patients: strains from 29 patients (88%) had the same sequence, called type A; strains from 2 others patients (6%) had similar sequences, called type B; a strain from 1 patient (3%) had the sequence called type C; and the strain from 1 patient (3%) had another sequence, called type D. By sequencing of the *Leishmania* strains, two sequence patterns were obtained for the *L. infantum* species, one pattern was obtained for the *L. donovani* species, one pattern was obtained for the *L. major* species, two patterns were obtained for the *L. tropica* species, and one pattern was obtained for the *L. aethiopica* species. All these sequences were quite different. All the sequences are presented in Fig. 1.

The sequences of strains of types A and C from the patients were similar to the two sequences obtained for the *L. infantum* strains. Zymodeme analysis performed with strains from certain patients, confirmed the species identifications. The sequences of strains of type B had a high degree of homology with sequences of *L. infantum* and *L. donovani* strains, but the results of zymodeme analysis are not yet available. The sequence of the type D strain was similar to the sequences of *L. major* strains.

Restriction enzyme analysis with *Hae*III. Different patterns of electrophoresis were observed for the *Leishmania* species and the strains from the patients. A single 250-bp band was obtained for the first group of *L. infantum* strains (type A sequence), and three bands of 250, 180, and 70 bp were obtained for the second group of *L. infantum* strains (type C sequence). Two bands of 180 and 70 bp for the *L. donovani* strains, four bands of 215, 155, 95, and 35 bp for the *L. major* strains, and two bands of 215 and 35 bp for *L. tropica* and *L. aethiopica* strains were obtained. For the type B sequences of strains from the patients (species unknown), the same patterns of electrophoresis as for the type C sequence were obtained. Fig. 2 shows the different patterns.

Downloaded from http://jcm.asm.org/ on October 25, 2020 by guest

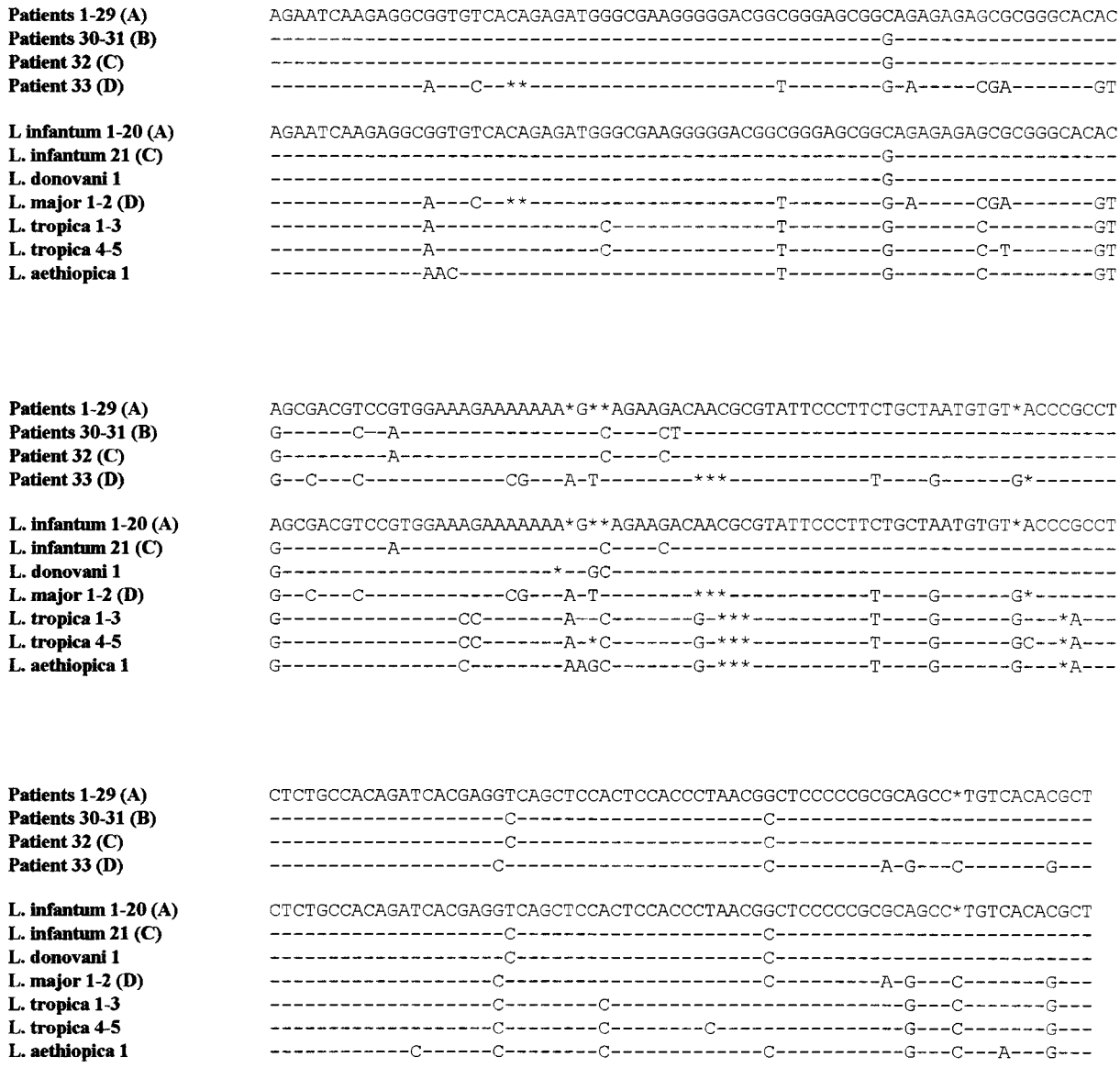

FIG. 1. Alignment of a repetitive genomic sequence of *Leishmania* strains from 33 patients (sequence types A, B, C, and D) and 30 representative strains causing Old World leishmaniasis.

DISCUSSION

Leishmaniasis is a public health concern in many countries of the world. Diagnosis of the disease requires the identification of the strain, especially in the cutaneous forms of the disease, whose clinical characteristics do not allow clinicians to establish a prognosis. Several species may indeed be causative agents, and some of them (e.g., *L. infantum*) may enter the viscera. The morphologies of the various *Leishmania* species on direct microscopic examination are very homogeneous, and this method cannot be used to differentiate the various species. Isoenzyme analysis is the standard method for the identification and classification of *Leishmania* strains (19, 20) and has been used for the characterization of the species causing both Old and New World leishmaniases (2, 8–11, 18, 21). This tool requires culturing of the parasites, however. It is time-consuming (excluding the possibility of rapid typing), the results vary with the electrophoresis conditions, and it may be performed by only a few laboratories in the world. The zymodeme characterization is often obtained several months after strain isolation, which is not suitable for clinical practice.

In order to type the species, some investigators have been interested in the DNA of *Leishmania* species. Some of them have used nuclear or kinetoplast DNA probes (4, 6, 23), but the probes are very taxon specific. The use of several radioactive probes is often necessary for species identification. PCR tests, based either on the kinetoplast DNA (12, 13, 21, 22) or on rRNA genes or their spacers (3, 24), were also developed. The kinetoplast DNA, located in minicircles, is naturally repeated in the genome, but it has a high degree of polymorphism; to increase the sensitivity of the test, primers specific for each species must be synthesized. The RNA genes (and their spacers) are repeated about 200 times in the cell, and because of their low level of polymorphism, they should be an interesting PCR target. Analysis of the PCR products, however, can-

Downloaded from http://jcm.asm.org/ on October 25, 2020 by guest

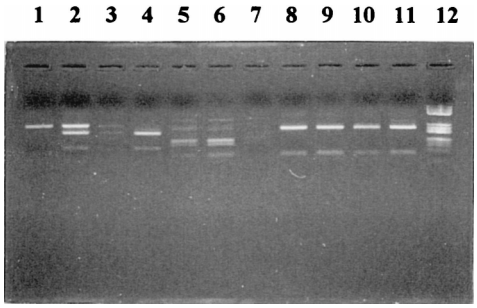

FIG. 2. Restriction enzyme analysis of a repetitive genomic sequence of *Leishmania* in different strains and patients with *Hae*III. Lane 1, *L. infantum* type A (strain MCAN/FR/73/LPM 56); lane 2, *L. infantum* type C (strain MHOM/IT/85/ISS 176); lane 3, *L. infantum* type C (from patient 32); lane 4, *L. donovani* (strain MHOM/ET/67/HV 3); lane 5, *L. major* (strain MHOM/YD/76/LEM 62); lane 6, *L. major* (strain MHOM/MA/81/LEM 265); lane 7, *L. major* (from patient 33); lane 8, *L. tropica* type A (strain MRAT/IQ/73/Adhanis 1); lane 9, *L. tropica* type A (strain MHOM/TN/80/LEM 163); lane 10, *L. tropica* type B (strain MHOM/GR/80/GR-L 35); lane 11, *L. aethiopica* (strain MHOM/ET/67/L 86); lane 12, DNA size marker.

not differentiate between the strains, and the sequences of the PCR products of the 18S rRNA genes are just able to be used to compare members of the genus *Leishmania* to members of other related genera (25). Recently, some investigators have used DNA analysis (analysis of mini-exon-derived gene spacers [5] or repetitive sequences [15]) for the diagnosis of the disease.

We analyzed a repetitive sequence to study the phylogenetic relationships among species causing Old World leishmaniasis (17). This molecular biology-based approach fit very well with the clinical and epidemiological classification; visceral and cutaneous strains were clearly separate. Three groups were distinguished among the cutaneous *Leishmania* species, in agreement with epidemiological characters. This approach to classifying *Leishmania*, however, required cloning, which is difficult and expensive.

In order to use the observed differences for identification purposes, we have focused on the direct sequencing of the PCR products. In this way, the cloning step is not necessary, which saves time and money. Culturing of the parasites may also be avoided. Indeed, culturing may not be always carried out because of low numbers of parasites in the templates, the presence of smears in the initial samples, and poor temperature and culture conditions, but it is essential, however, for isoenzyme analysis. When culture fails to increase the numbers of parasites, isoenzyme analysis may not be performed and the species will remain unknown. Moreover, culture may select for the variants if several strains coexist in the same host or if certain strains are not commonly able to grow in culture. On the other hand, isoenzyme analysis takes a very long time to perform (because of the culture conditions), even when our methodology allows the species to be known 48 h after the template is obtained, even if blood is sampled.

We have used the methodology under clinical conditions. For instance, the type D sequence (from a strain from patient 33) was amplified from the skin of a patient infected with HIV living in Marseilles and in Algeria and suffering from an extensive cutaneous lesion. Culture always failed to increase the numbers of parasites. This tool was the only way to characterize the strain as an *L. major* strain, which the patient contracted in North Africa. The species identification was confirmed by the fact that the patient never had a visceral infection.

On the other hand, this new approach contributes to a better understanding of the epidemiology of the disease. The strains detected in our Mediterranean suburban focus were quite monomorphic by a zymodeme approach (*L. infantum*, zymodeme MON 1). We have amplified three different sequences in the samples from patients suffering from visceral leishmaniasis. Two of these patterns (sequence types B and C) were exclusively obtained for strains from immunocompromised patients. Strains from all immunocompetent patients were similar and had the same sequence type (type A). These data may indicate that some variants of *Leishmania* are pathogenic exclusively in immunocompromised subjects.

Restriction analysis is useful for reducing the cost of the methodology or when molecular biology tools are not available. It avoids the sequencing step, which is very practical under field conditions. By using *Hae*III, the three different species in the Mediterranean Basin, i.e., *L. infantum*, *L. major*, and *L. tropica*, can be distinguished. It was surprising, however, to obtain three bands of 250, 180, and 70 bp in the *Hae*III restriction enzyme analysis of the sequences of types B and C. This could have been the consequence of a partial restriction, but the electrophoresis patterns did not change when a longer incubation was used. An initial miscellany of sequences, either in the parasite population or within the repetitive sequences, could explain the uncut band. Such a possibility would support the existence of genetic exchanges in the species of genus *Leishmania*, as suggested by other investigators (1, 7).

In summary, PCR amplification and sequencing may contribute to the detection of the species causing leishmaniasis. Some variants may be pathogenic only in immunosuppressed patients. Restriction analysis suggests that the DNA sequences may be mixed in the same strain, as seen from the existence of genetic crossovers.

REFERENCES

1. **Bastien, P., C. Blaineau, M. Taminh, J. A. Rioux, G. Roizès, and M. Pagès.** 1990. Interclonal variations in molecular karyotype in *Leishmania infantum* imply a 'mosaic' strain structure. Mol. Biochem. Parasitol. **40:**53–62.
2. **Grimaldi, G., Jr., H. Momen, R. D. Naiff, D. McMahon-Pratt, and T. V. Barrett.** 1991. Characterization and classification of leishmanial parasites from humans, wild mammals and sand flies in the Amazon region of Brazil. Am. J. Trop. Med. Hyg. **44:**645–661.
3. **Guevara, P., G. Alonso, J. F. Da Silveira, M. De Mello, J. V. Scorza, N. Anez, and J. L. Ramirez.** 1992. Identification of New World *Leishmania* using ribosomal gene spacer probes. Mol. Biochem. Parasitol. **56:**15–26.
4. **Guizani, I., G. J. J. M. Van Eys, R. Ben Ismail, and K. Dellagi.** 1994. Use of recombinant DNA probes for species identification of Old World *Leishmania* isolates. Am. J. Trop. Med. Hyg. **50:**632–640.
5. **Hassan, M. D. Q., A. Ghosh, S. S. Ghosh, M. Gupta, D Basu, K. K. Mallik, and S. Adhya.** 1993. Enzymatic amplification of mini-exon-derived gene spacers of *Leishmania donovani*: primers and probes for DNA diagnosis. Parasitology **107:**509–517.
6. **Howard, M. K., W. Ogunkolade, A. D. M. Bryceson, R. N. Davidson, A. H. Moody, and M. A. Miles.** 1992. A DNA probe for human visceral Leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. **86:**35–36.
7. **Lanotte, G., and J. A. Rioux.** 1990. Fusion cellulaire chez les *Leishmania* (Kinetoplastida, Trypanosomatidae). C. R. Acad. Sci. **310:**285–288.
8. **Leblancq, S. M., L. F. Schnur and W. Peters.** 1986. *Leishmania* in the Old World. 1. The geographical and hostal distribution of *L. major* zymodemes. Trans. R. Soc. Trop. Med. Hyg. **80:**99–112.
9. **Leblancq, S. M., and W. Peters.** 1986. *Leishmania* in the Old World. 2. Heterogeneity among *L. tropica* zymodemes. Trans. R. Soc. Trop. Med. Hyg. **80:**113–119.
10. **Leblancq, S. M., A. Belehu, and W. Peters.** 1986. *Leishmania* in the Old World. 3. The distribution of *L. aethiopica* zymodemes. Trans. R. Soc. Trop. Med. Hyg. **80:**360–366.
11. **Leblancq, S. M., and W. Peters.** 1986. *Leishmania* in the Old World. 4. The distribution of *L. donovani sensu lato* zymodemes. Trans. R. Soc. Trop. Med. Hyg. **80:**367–377.
12. **Lopez, M., R. Inga, M. Cangalaya, J. Echevarria, A. Llanos-Cuentas, C. Orrego, and J. Arevalo.** 1993. Diagnosis of *Leishmania* using the polymerase chain reaction: a simplified procedure for field work. Am. J. Trop. Med. Hyg. **49:**348–356.
13. **Nuzum, E., F. White III, C. Thakur, R. Dietze, J. Wages, M. Grogl, and J. Berman.** 1995. Diagnosis of symptomatic visceral leishmaniasis by use of the

Downloaded from http://jcm.asm.org/ on October 25, 2020 by guest

polymerase chain reaction on patient blood. J. Infect. Dis. **171:**751–754.
14. **Organisation Mondiale de la Santé.** 1990. Lutte contre les leishmanioses: rapport d'un Comité OMS d'experts. Série de Rapports Techniques 793. Organisation Mondiale de la Santé, Geneva, Switzerland.
15. **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 DNA sequence from *Leishmania infantum*: development of a visceral leishmaniasis polymerase chain reaction. Am. J. Trop. Med. Hyg. **49:**364–369.
16. **Piarroux, R., M. Fontes, R. Perasso, F. Gambarelli, C. Joblet, H. Dumon, and M. Quilici.** 1995. Phylogenetic relationships between Old World *Leishmania* strains revealed by analysis of a repetitive DNA sequence. Mol. Biochem. Parasitol. **73:**249–252.
17. **Piarroux, R., F. Gambarelli, H. Dumon, M. Fontes, S. Dunan, C. Mary, B. Toga, and M. Quilici.** 1994. Comparison of PCR with direct examination of bone marrow aspiration, myeloculture, and serology for diagnosis of visceral leishmaniasis in immunocompromised patients. J. Clin. Microbiol. **32:**746–749.
18. **Pratlong, F., J. P. Dedet, P. Marty, M. Portùs, M. Deniau, J. Dereure, P. Abranches, J. Reynes, A. Martini, M. Lefebvre, and J. A. Rioux.** 1995. *Leishmania*-human immunodeficiency virus coinfection in the Mediterranean Basin: isoenzymatic characterization of 100 isolates of the *Leishmania infantum* complex. J. Infect. Dis. **172:**323–326.
19. **Rioux, J. A., G. Lanotte, R. Maazoun, and N. Pasteur.** 1985. L'électrophorèse des enzymes dans le genre *Leishmania* Ross, 1903. Parassitologia **27:** 141–156.
20. **Rioux, J. A., G. Lanotte, E. Serres, F. Pratlong, P. Bastien, and J. Perieres.** 1990. Taxonomy of *Leishmania*. Use of isoenzymes. Suggestions for a new classification. Ann. Parasitol. Hum. Comp. **65:**111–125.
21. **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.
22. **Smyth, A. J., A. Ghosh, M. Q. Hassan, D. Basu, M. H. L. De Bruijn, S. Adhya, K. K. Mallik, and D. C. Barker.** 1992. Rapid and sensitive detection of *Leishmania* kinetoplast DNA from spleen and blood samples of kala-azar patients. Parasitology **105:**183–192.
23. **Spithill, T. W., and R. J. Grumont.** 1984. Identification of species, strains and clones of *Leishmania* by characterization of kinetoplast DNA minicircles. Mol. Biochem. Parasitol. **12:**217–236.
24. **Uliana, S. R. B., M. T. H. Affonso, E. P. Camargo, and L. M. Floeter-Winter.** 1991. *Leishmania*: genus identification based on a specific sequence of 18S ribosomal RNA sequence. Exp. Parasitol. **72:**157–163.
25. **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.

Downloaded from http://jcm.asm.org/ on October 25, 2020 by guest