MAJOR ARTICLE

Clinical Use of Polymerase Chain Reaction Performed on Peripheral Blood and Bone Marrow Samples for the Diagnosis and Monitoring of Visceral Leishmaniasis in HIV-Infected and HIV-Uninfected Patients: A Single-Center, 8-Year Experience in Italy and Review of the Literature

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Background. To overcome some of the limitations of conventional microbiologic techniques, polymerase chain reaction (PCR)–based assays are proposed as useful tools for the diagnosis of visceral leishmaniasis.

Patients and methods. A comparative study using conventional microbiologic techniques (i.e., serologic testing, microscopic examination, and culture) and a *Leishmania* species–specific PCR assay, using peripheral blood and bone marrow aspirate samples as templates, was conducted during an 8-year period. The study cohort consisted of 594 Italian immunocompetent (adult and pediatric) and immunocompromised (adult) patients experiencing febrile syndromes associated with hematologic alterations and/or hepatosplenomegaly. Identification of the infecting protozoa at the species level was directly obtained by PCR of peripheral blood samples, followed by restriction fragment–length polymorphism analysis of the amplified products, and the results were compared with those of isoenzyme typing of *Leishmania* species strains from patients, which were isolated in vitro.

Results. Sixty-eight patients (11.4%) had a confirmed diagnosis of visceral leishmaniasis. Eleven cases were observed in human immunodeficiency virus (HIV)–uninfected adults, 20 cases were observed in HIV-infected adults, and the remaining 37 cases were diagnosed in HIV-uninfected children. In the diagnosis of primary visceral leishmaniasis, the sensitivities of the *Leishmania* species–specific PCR were 95.7% for bone marrow aspirate samples and 98.5% for peripheral blood samples versus sensitivities of 76.2%, 85.5%, and 90.2% for bone marrow aspirate isolation, serologic testing, and microscopic examination of bone marrow biopsy specimens, respectively. None of 229 healthy blood donors or 25 patients with imported malaria who were used as negative control subjects had PCR results positive for *Leishmania* species in peripheral blood samples (i.e., specificity of *Leishmania* species–specific PCR, 100%). PCR and restriction fragment–length polymorphism analysis for *Leishmania* species identification revealed 100% concordance with isoenzyme typing in the 19 patients for whom the latter data were available.

Conclusions. PCR assay is a highly sensitive and specific tool for the diagnosis of visceral leishmaniasis in both immunocompetent and immunocompromised patients and can be reliably used for rapid parasite identification at the species level.

Visceral leishmaniasis (VL) is a systemic, vector-borne parasitic disease caused by obligate intracellular protozoa of the genus *Leishmania* and transmitted by the bite of sandflies. The disease is endemic in 62 countries worldwide [1], with a total of 200 million people at risk, and it is fatal if not diagnosed and left untreated.

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Table 1. Demographic characteristics of and diagnostic findings in 68 consecutive patients with visceral leishmaniasis.

Variable	Group A (n = 11)	Group B (n = 20)	Group C (n = 37)	Total (n = 68)
Sex				
Male	10	16	15	41
Female	1	4	22	27
Age, median value (range)	49 years (32–72 years)	38.5 years (27–47 years)	15 months (4–156 months)	
Lymphocyte $CD4^+$ cell count, median cells/μL (range)	NP	81 (4–190)	NP	NP
Positive *Leishmania* serologic test result	5/7 (71.4)	11/18 (61.1)	37/37 (100)	53/62 (85.5)
Positive findings of microscopic examination of BMA sample	9/10 (90)	14/15 (93.3)	32/36 (88.8)	55/61 (90.2)
Positive BMA culture	3/3 (100)	6/9 (62.5)	23/30 (76.6)	32/42 (76.2)
Positive PB culture	NP	3/4 (75)	NP	3/4 (75)
Positive result of PCR of PB sample	11/11 (100)	20/20 (100)	33/34 (97.1)	64/65 (98.5)
Positive result of PCR of BMA sample	5/5 (100)	13/14 (92.8)	27/28 (96.4)	45/47 (95.7)

NOTE. Data are no. (%) of patients, unless otherwise indicated. Group A includes HIV-uninfected adults, group B includes HIV-infected adults, and group C includes HIV-uninfected children. BMA, bone marrow aspirate; NP, not performed; PB, peripheral blood.

VL is caused by the *Leishmania donovani* complex, which includes the following 3 species with partly overlapping geographical distribution: *L. donovani* (found in India and Africa), *Leishmania infantum* (found in the Mediterranean basin, the Middle East, and Asia), and *Leishmania chagasi* (found in South America), which is now considered to be identical to *L. infantum* [2].

VL is characterized by prolonged fever, weight loss, progressive anemia or pancytopenia, and hypergammaglobulinemia. The diagnosis of VL is traditionally based on serologic testing results and the direct demonstration of *Leishmania* parasites by microscopic examination and in vitro isolation [3]. Accepted limits of *Leishmania* serologic testing are reduced sensitivity in patients with severe underlying immunosuppression, the absence of discriminating power between active or past infections, and, consequently, lack of value in monitoring the parasitological response to specific therapy. On the other hand, microscopic examination requires invasive procedures, such as spleen and bone marrow aspiration (BMA). Splenic aspirate cytologic testing has an excellent sensitivity (95%) but is associated with the risk of severe complications when performed by inexperienced health care workers. The sensitivity of microscopic examination of BMA samples varies among different studies from 60% to 85% [3]. Finally, in vitro parasite isolation still remains the reference method to unequivocally identify the parasite at the species level, but it is expensive, cumbersome, and generally unhelpful in clinical practice because of the long duration of time (days to weeks) required to obtain a definitive result.

In the past decade, PCR has been increasingly used to detect *Leishmania* DNA in clinical samples, such as BMA and peripheral blood (PB) samples, for the diagnosis of VL and for parasite species characterization. Several target sequences, such as ribosomal RNA (rRNA) genes, the mini-exon derived RNA gene, the kinetoplast minicircles, and repetitive nuclear DNA sequences, have been used in published PCR assays [4–11]. In the present study, we performed a comparative analysis of microscopic, serologic, and PCR-based diagnostic methods for detection of *Leishmania* species in a consecutive series of 68 adult and pediatric cases of VL that were observed in both immunocompetent and immunocompromised Italian patients.

PATIENTS AND METHODS

An 8-year prospective study was performed in Milan (Lombardy region, northern Italy) and Palermo (Sicily region, southern Italy) from January 1997 through January 2005. Five hundred ninety-four consecutive patients living in the Mediterranean area in Italy who presented with clinical signs and symptoms compatible with VL were included in the study. All VL cases were confirmed by serologic testing, direct examination, and/or culture (at least 1 positive criteria was required for confirmation)

The majority of patients enrolled at the Department of Infectious and Tropical Diseases at the L. Sacco University Hospital of Milan were HIV-positive adults (447 patients; 83.5%), and the individuals who were observed at the Institute of Infectious Pathology and Virology at the G. di Cristina Hospital of Palermo were exclusively immunocompetent children (55 children). In addition, 254 control subjects were studied to assess the specificity of the PCR assay. These included 229 healthy blood donors who were recruited from the Immune-Hematology Department of L. Sacco Hospital and 25 patients with a confirmed diagnosis of imported malaria.

Molecular studies. Details of specimen collection, processing, DNA extraction, and qualitative and semiquantitative PCR amplification of a 359–base pair fragment of the small rRNA gene of *Leishmania* species are described elsewhere [4,

Table 2. Reported sensitivities and specificities of different PCR protocols in the diagnosis of visceral leishmaniasis.

Study (year)	Study cohort	No. of patients with VL	Type of PCR (bp): *Leishmania* species	Sensitivity of PCR, %		Specificity, %
				Whole blood specimen	BMA specimen	
Adhya et al. [15] (1995)	39 Indian patients	31	MedRNA (180 bp): *Leishmania donovani*	96.8	NR	100
Nuzum et al. [16] (1995)	63 Indian, Kenyan, and Brazilian patients	63	kDNA (nr): *L. donovani*	90[a]	NR	100
Mathis et al. [17] (1995)	51 Swiss patients (43 with HIV infection)	11	ssU-rRNA (603 bp): *Leishmania* species	72.7	100	100
Piarroux et al. [19] (1996)	252 French Id patients with HIV infection	50	Repetitive nuclear sequence (140 bp): *Leishmania infantum*	NR	84	99
Costa et al. [20] (1996)	90 French Id patients with HIV infection	13	ssu-rRNA (nr): *L. donovani*	98	98	100
Andresen et al. [21] (1997)	40 Sudanese patients	40	kDNA (800 bp): *L. donovani*	92.5[b]	100	100
Osman et al. [22] (1997)	492 Sudanese patients	47	ssU-rRNA (nr): *Leishmania* species	70[c]	100	100
Katakura et al. [11] (1998)	13 Chinese patients (including 12 children)	13	n-PCR mini-exon gene (450 bp): *L. donovani*	NR[d]	83.3	100
Singh et al. [23] (1999)	100 Indian patients	17	kDNA (204 bp): *L. donovani*	82.3	NR	100
Lachaud et al. [7] (2000)	237 French patients	36[e]	ssU-rRNA (603 bp): *L. infantum*	97	100	100
Campino et al. [24] (2000)	24 Portuguese patients (18 with HIV infection)	20	ssU-rRNA (600 bp): *L. infantum*	75[f]	NR	NR
Hu et al. [25] (2000)	22 Chinese patients	22	kDNA (297bp): *L. donovani*	68.8	91	100
Salotra et al. [26] (2001)	51 Indian patients	51	kDNA (792 bp): *L. donovani*	96	100	96
Pizzuto et al. [9] (2001)	76 Italian adult HIV-infected patients	10	ssU-rRNA (359 bp): *L. infantum*	100	100	100
Fisa et al. [27] (2002)	45 Spanish HIV-infected patients (2 were uninfected)	33	n-PCR (100 bp): *L. infantum*	100	100	100
Cruz et al. [28] (2002)	89 Spanish Id, HIV-infected adults	44	ssU-rRNA n-PCR (358 bp): *L. infantum*	95.4	100	100
Cascio et al. [14] (2002)	14 Italian Ic children	10	ssU-rRNA (359 bp): *L. infantum*	100	100	100
Martin-Sanchez et al. [29] (2002)	56 Spanish patients (13 with HIV infection)	24	kDNA- Elisa (nr): *L. infantum*	100	67	100
Disch et al. [30] (2003)	68 Brazilian Ic adults	53	kDNA (120 bp): *Leishmania chagasi*	91	NR	100
Deniau et al. [31] (2003)	69 French HIV-infected patients	14	nDNA (250 bp): *Leishmania* species	86	93	100
Bossolasco et al. [32] (2003)	25 Italian Id, HIV-infected adults	10	ssU-rRNA real-time: *L. infantum*	100	NR	100
Fissore et al. [33] (2004)	21 French Ic adults and children and 12 French Id patients (10 with HIV infection, 1 with BmT, and 1 with Ly)	33	kDNA (139 bp): *L. infantum*	NR[g]	NR	95
Cortes et al. [34] (2004)	22 Portuguese patients	8	kDNA (447 bp): *L. infantum*	NR	100	100
Gatti et al. [35] (2004)	22 Italian Id patients (12 with HIV infection, 4 with Sot, and 6 with mal) and 20 Italian Ic adults	41	ssU-rRNA (215 bp): *Leishmania* species	93.3 for Ic patients and 100 for Id patients	NR	100

(continued)

Table 2. ***(Continued.)***

Study (year)	Study cohort	No. of patients with VL	Type of PCR (bp): *Leishmania* species	Sensitivity of PCR, %		Specificity, %
				Whole blood specimen	BMA specimen	
Pal et al. [36] (2004)	20 Indian patients	14	kDNA (790 bp): *Leishmania* species	100	83	100
Da Silva et al. [37] (2004)	85 Brazilian patients	85	kDNA (120 bp): *L. chagasi*	84.7	100	100
De Doncker et al. [38] (2005)	99 Nepalese Ic adults	56	ssU-rRNA	73.2 by conventional PCR and 83.9 by ELISA PCR	NR	87.2
Maurya et al. [39] (2005)	251 Indian patients	101	kDNA (600 bp): *L. donovani*	99	NR	100
Cruz et al. [40] (2006)	25 Spanish children	25	ssU-rRNA n-PCR (358 bp): *L. infantum*	79	100	100

NOTE. BMA, bone marrow aspirate; BmT, bone marrow transplant; bp, base pair; Ic, immunocompetent; Id, immunodepressed; kDNA, kinetoplast DNA; Ly, lymphoma; mal, malignancy; medRNA, multicopy mini-exon RNA; n-PCR, nested PCR; NR, not reported; Ra, rheumatoid arthritis; Sot, solid organ transplant; ssU-rRNA, small subunit ribosomal RNA.

[a] Sensitivity of PCR of whole blood specimens was 80% for Brazilian patients, 90% for Indian patients, and 100% for Kenyan patients.
[b] Sensitivity of PCR of lymph node samples was 100%.
[c] Sensitivity of PCR of lymph node samples was 86.8%.
[d] Sensitivity of PCR of buffy coat was 53.8%.
[e] Seven Ic adults, 5 Ic children, and Id adults (15 with HIV infection, 3 with Sot, and 1 with Ra).
[f] Overall sensitivity was 95.5%.
[g] Sensitivity of PCR of serum was 97%.

9]. Molecular identification of Old World *Leishmania* species was performed by restriction fragment–length polymorphism analysis of a PCR-amplified specific nuclear repetitive genomic sequence, exactly as described by Minodier et al. [12].

In vitro isolation. *Leishmania* cultures were performed by seeding BMA specimens in blood agar media (Evans modified, Tobie's medium, and/or Sloppy Evans medium). Positive culture results were further characterized by the electrophoretic analysis of 15 enzymatic loci, using the techniques and zymodeme nomenclature of the World Health Organization Collaborating Centre of Montpellier [13].

Serologic testing. The serologic diagnosis was performed by indirect immunofluorescence antibody (the threshold titer for positivity was $1 \geqslant 40$), using an in-house manufactured antigen preparation from the World Health Organization reference strain of *L. infantum* or a commercial kit (*Leishmania* spot IF; BioMèrieux). In particular, serologic testing of samples from all children was performed with the in-house preparation at the Laboratorio di Parassitologia at the Istituto Superiore di Sanità (Rome, Italy). All of the other samples were analyzed with the commercial kit.

Statistical analysis was performed using the Mann-Whitney *U* test. All *P* values $\leqslant .05$ were considered to be statistically significant. This study followed the appropriate Italian ethical requirements and was approved by the local ethical committees. The parents gave informed consent before the enrollment of their children in the study.

RESULTS

During the study, a total of 1678 samples (1483 PB samples and 195 BMA samples) obtained from 594 patients were processed for *Leishmania* DNA PCR at our laboratory, either for the primary diagnosis or the follow-up of VL.

Overall, 68 patients (11.4%) received a diagnosis of VL, including 11 HIV-uninfected adults (group A), 20 HIV-infected adults (group B), and 37 immunocompetent HIV-uninfected children (group C) (table 1). Preliminary results of examination of 10 HIV-positive patients and 10 immunocompetent children in this series have been previously reported [9, 14]. Among the 447 HIV-infected patients, 89 had atypical mycobacteriosis, 67 had disseminated and/or pulmonary tuberculosis, 49 had non-Hodgkin lymphoma, 49 had bacterial sepsis, 31 had pneumocystosis, 27 had Kaposi sarcoma, 23 had disseminated cytomegalovirus disease, 76 had other infectious or neoplastic diseases, and 36 had an unidentified cause of fever. Among the HIV-uninfected adult patients, 23 had non-Hodgkin lymphoma, 12 had Hodgkin lymphoma, 8 had autoimmune diseases, 4 had acute leukemia, and 11 had an unidentified cause of fever. Among the remaining 18 pediatric patients, 13 had acute leukemia, 2 had infectious mononucleosis, and 3 had non-Hodgkin lymphoma.

A total of 536 PB samples and 74 BMA samples from patients with confirmed VL were available for investigation by PCR during the study period. Specimens were distributed as follows: 69 PB specimens (range, 2–16 specimens per patient) and 6 BMA specimens (range, 1–2 specimens per patient) for group A, 306 PB specimens (range, 1–101 specimens per patient) and 24 BMA specimens (range, 1–4 specimens per patient) for group B, and 159 PB specimens (range, 2–15 specimens per patient) and 44 BMA specimens (range, 1–3 specimens per patient) for group C. Overall, the sensitivity of PCR in the primary diagnosis of VL was 95.7% (for BMA specimens) and

Table 3. Summary of clinical studies of parasitological monitoring of visceral leishmaniasis treatment using PCR.

Study (year)	Study cohort	Type of PCR (bp): *Leishmania* species	Treatment	Proportion (%) of patients with negative results	
				1 Month after treatment	2 Months after treatment
Nuzum et al. [16] (1995)	13 Indian patients	kDNA (nr)	Sodium stibogluconate	6/7 (85.7)	4/4 (100)
Osman et al. [22] (1998)	49 Sudanese patients	ssU-rRNA (560 bp): *Leishmania* species	Sodium stibogluconate	10/49 (20.4)[a]	NR
Lachaud et al. [7] (2000)	23 French patients (19 patients with AIDS, 3 with Sot, and 1 with Ra)	ssU-rRNA (603 bp): *Leishmania infantum*	NR	78/134 (58.2)	NR
Pizzuto et al. [9] (2001)	10 Italian patients with AIDS	ssU-rRNA (359 bp): *L. infantum*	LaB	9/10 (90)	9/10 (90)
Cascio et al. [14] (2002)	10 Italian children	ssU-rRNA (359 bp): *L. infantum*	LaB and meglumine antimoniate	10/10 (100)	9/10 (90)
Cruz et al. [28] (2002)	38 Spanish patients with AIDS	ssU-rRNA (358 bp): *L. infantum*	Amphotericin B lipid complex and pentavalent antimonials	8/38 (21), by PCR of PB specimens, and 3/38 (7.9), by PCR of BMA specimens	NR
Fisa et al. [27] (2002)	10 Spanish patients (8 with AIDS and 2 immunocompetent patients)	n-PCR (100 bp): *L. infantum*	NR	3/10 (30)	0/10 (0)[b]
Bossolasco et al. [32] (2003)	10 Italian patients with AIDS	Real-time PCR ssU-rRNA: *L. infantum*	LaB	1/7 (14.3)	1/2 (50)
Disch et al. [41] (2004)	17 Brazilian patients	KDNA (120 bp): *Leishmania chagasi*	Meglumine antimoniate	16/17 (94.1)	15/16 (93.7)
Pal et al. [35] (2004)	5 Italian patients	ssU-rRNA (215 bp): *L. infantum*	NR	0/5 (0)	5/5 (100)
Maurya et al. [39] (2005)	60 Indian patients	kDNA (600 bp): *Leishmania donovani*	NR	51/60 (85)[c]	58/60 (96.7)[b]
Cruz et al. [40] (2006)	20 Spanish children	ssU-rRNA (358 bp): *L. infantum*	LaB	19/20 (95), by PCR of PB specimens, and 11/19 (57.9), by PCR of BMA specimens	NR

NOTE. BMA, bone marrow aspirate; bp, base pair; kDNA, kinetoplast DNA; LaB, liposomal amphotericin B; n-PCR, nested PCR; NR, not reported; PB, peripheral blood; Ra, rheumatoid arthritis; Sot, solid organ transplant; ssU-rRNA, small subunit ribosomal RNA.

[a] n-PCR performed on lymph node.
[b] At 3 months after treatment.
[c] At first day after treatment.

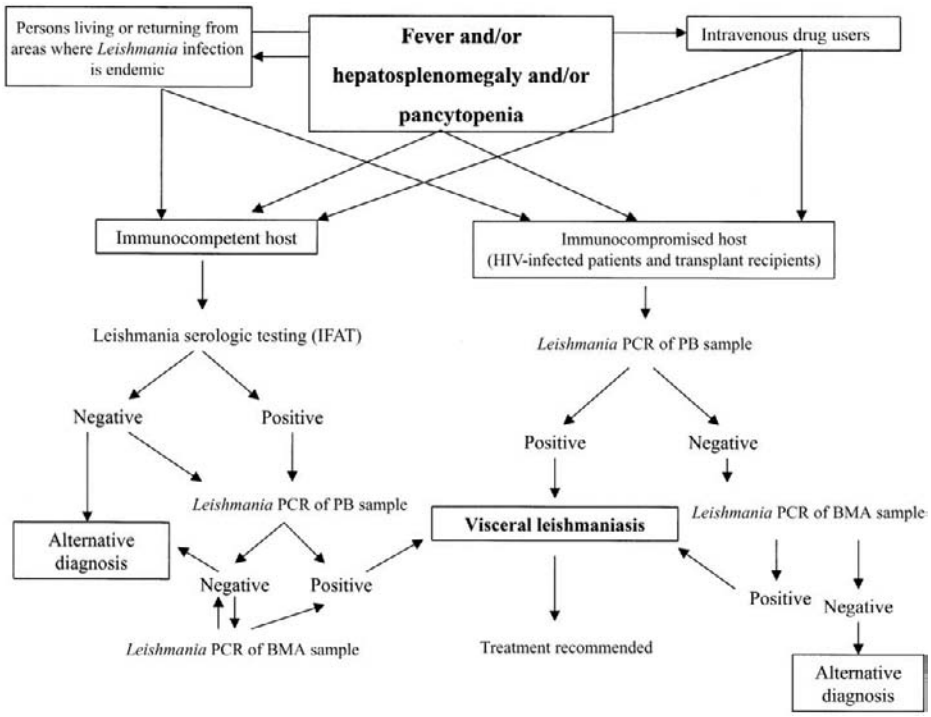

Figure 1. Proposed clinical algorithm for the use of PCR in the primary diagnosis of visceral leishmaniasis. *Leishmania* serologic testing in immunocompromised patients is generally of little value, especially if performed with commercial kits, and, therefore, should be used as initial screening only in immunocompetent patients. *Leishmania* PCR, when available, has been shown to be extremely sensitive and specific, and it should be used to confirm the primary diagnosis of VL. Bone marrow aspirate (BMA) samples should be obtained only when PCR of a peripheral blood (PB) sample is negative for VL, to definitively exclude VL or to make an alternative diagnosis.

98.5% (for PB specimens). In all of the cases, the diagnosis of VL was obtained from a single initial sample.

On the other hand, the sensitivities of in vitro isolation, *Leishmania* serologic testing, and microscopic examination of BMA samples were 76.2%, 85.5%, and 90.2%, respectively (table 1). In all of the study groups, PCR of PB samples exhibited the highest sensitivity, ranging from 97.1% (in group C) to 100% (in groups A and B). *Leishmania* DNA was not detected by PCR in any sample from a negative control subject (i.e., 229 blood donors and 25 patients with imported malaria); therefore, the specificity of the assay was assumed to be 100%.

At diagnosis of primary VL, the median *Leishmania* DNA burden was higher in BMA samples than in PB samples ($P < .001$). In more detail, HIV-uninfected adults exhibited a 2 $\log_{10}$ higher parasite load in BMA samples, compared with that in PB samples ($P = .01$), and HIV-infected adults had comparable levels of *Leishmania* DNA in the 2 anatomical districts. In HIV-negative children, the median *Leishmania* DNA levels were 1 $\log_{10}$ higher in BMA samples ($P = .001$). Finally , significantly higher levels of parasitaemia were observed in adults than in children ($P < .009$).

Fifty-four patients were prospectively monitored using semiquantitative PCR of PB samples during periods ranging from 1 to 84 months (median duration, 6 months). In particular, median periods of molecular monitoring were 3 months for patients in group A, 13 months for patients in group B, and 6 months for patients in group C.

Clinical and parasitologic relapses were detected in 13 of 54 individuals, including 11 adults with AIDS (55%) and 2 HIV-uninfected children (5%; $P < .001$). In particular, 4 subjects with HIV infection experienced multiple relapses (range, 2–6 relapses). No recrudescences of VL were observed in HIV-uninfected adult patients (group A).

Immunocompetent children and adults cleared *Leishmania* DNA from PB within a few days from the start of treatment (median duration, 9 and 12 days, respectively). By contrast, the duration of time to *Leishmania* PCR negativity following the first cycle of specific therapy was consistently longer in individuals coinfected with HIV (median duration, 17.5 days). In particular, 4 (23.5 %) of 17 HIV-infected patients had undetectable *Leishmania* DNA in PB within 8 days from the start of treatment; 5 of these patients cleared *Leishmania* DNA within 5 weeks, 4 patients cleared *Leishmania* DNA within 8 weeks, and the remaining 3 patients cleared *Leishmania* DNA within 21 weeks.

Results of *Leishmania* PCR of PB samples reverted to positive in 11 HIV-infected patients, 7 of whom thereafter had persistently positive results during the follow-up period (median du-

ration, 21 months; range, 3–72 months). Six of these 7 patients experienced at least 1 relapse of VL during the follow-up period.

PCR and restriction fragment-length polymorphism analysis for *Leishmania* species identification was performed for all patients with the following results: 10 patients had *L. infantum* infection and 1 patient had *L. donovani* infection in group A, 16 patients had *L. infantum* infection and 4 patients had *L. donovani* infection in group B, and 37 patients had *L. infantum* infection in group C. One hundred percent perfect concordance was observed with the isoenzyme typing method in the 19 patients for whom the method was available (1 patient had *L. infantum* infection and 1 patient had *L. donovani* infection in group A, 2 patients had *L. donovani* infection in group B, and 15 patients had *L. infantum* infection in group C).

DISCUSSION

The application of PCR to detect parasite DNA for the diagnosis of cutaneous leishmaniasis and VL in tissues has been reported since the early 1990s [5, 10]. The use of PCR of PB samples to diagnose VL was first reported in 1995 by 4 investigators [15–18] who employed this technique in patients with confirmed VL in India [15, 16], Brazil, Kenya [16, 18], and Switzerland (the patients in Switzerland had imported disease) [17]. Since then, at least 29 studies have been published with the description of different protocols that use, as the target of PCR amplification, either the *Leishmania* kinetoplast DNA minicircle, which is present at thousands of copies per parasite [16, 21, 23, 25–27, 29, 30, 31, 33, 34, 36, 37, 39, 41] , or the small subunit rRNA gene, which is repeated >100 times in the *Leishmania* genome [6, 9, 14, 17, 20, 22, 24, 28, 32, 35, 38, 40] (table 2).

In our study, we chose the small subunit rRNA gene of the parasite as the target sequence. The primers were those originally described by van Eys et al. [4], which have been shown to specifically amplify all known human pathogenic *Leishmania* species.

When compared with serologic testing, in vitro culture, and direct bone marrow examination, the PCR performed on whole blood samples appeared, in our study, to be more sensitive, with an overall sensitivity of 98.5%, considering all categories of patients studied. Moreover, the PCR of PB samples performed slightly better than PCR of BMA samples with regard to sensitivity (98.5% vs. 95.7%). Our results are in good agreement with those reported by several investigators who studied different groups of French, Spanish, and Indian patients [7, 26, 28, 33, 39]. However, in some other studies, the sensitivity of PCR of PB samples was unsatisfactory, ranging from as low as 54% (in a small study performed with Chinese children [11]) to 70%–85% [22–25, 37, 38, 40]. These discrepancies have been ascribed either to differences in the volume of blood tested in the PCR [22], to differences in the timing of sample collection [6, 15, 22], or to differences pertaining to the species of *Leishmania* parasites, some of which may indeed circulate less frequently or at lower levels in the infected host [22]. In addition, in a study by Singh et al. [23], it was speculated that some patients with negative results of PCR of PB samples might have undergone a previous private treatment before admission to the hospital. Finally, in at least 2 studies, the use of blood samples spotted on filter paper might have negatively influenced the results [26, 39].

It is also worth noting that the sensitivity of *Leishmania* PCR of PB samples was superior to all the conventional diagnostic techniques in HIV-infected and HIV-uninfected adults but was inferior to serologic testing in HIV-negative children (sensitivity of PCR of PB samples vs. sensitivity of serologic testing, 97.1% vs. 100%). A high sensitivity of *Leishmania* serologic testing was also recently reported by Cruz et al. [40] in a study involving 25 Spanish children. However, in the same study, the authors reported a lower sensitivity of *Leishmania* PCR of PB samples, compared with our present and past experience [14].

The specificity of our PCR assay was also excellent (100%), because no false-positive result was obtained either in the negative control tubes or from negative control patients. Overall specificities of 100% were reported in 23 of 28 studies [7, 9, 11, 14–17, 20–23, 25, 27–30, 32, 34–37, 39, 40], with the lowest values being those reported in the study by De Doncker et al. [38]. Of note, De Doncker et al. [38] found a 100% specificity using Belgian blood donors as negative controls, compared with 87.2% specificity when individuals living in an area where VL was endemic were used as negative controls.

It is generally accepted that, in patients with VL, the parasite load in PB samples is lower than that in BMA samples [7]. In addition, it is commonly believed that the parasite burden in immunocompetent patients is lower than that in patients with VL and concomitant HIV infection [32, 38]. However, these assumptions are thus far only speculative and, to our knowledge, have not been formally demonstrated. The hypothesis that higher levels of parasitaemia occur in HIV-infected patients was indirectly inferred by the observation that, in these patients, *Leishmania* parasites could be readily isolated from PB samples [41]. In our study, using semiquantitative PCR, we demonstrated that, if we considered all patients, irrespective of age and immune status, the median parasite DNA load detected in BMA samples was 10 times higher than that detected in PB samples. This is also confirmed by the analysis of HIV-uninfected children and adults. However, HIV-infected patients did not have statistically significant differences in parasite burden measured in simultaneously obtained PB and BMA samples. In addition, the parasite load detected in PB samples was significantly higher in HIV-infected patients than in HIV-uninfected children but was not significantly higher in HIV-infected patients than it was in HIV-uninfected adults.

With regard to the usefulness of PCR in the monitoring of patients undergoing specific treatment, to date, 13 studies have been published [7, 9, 14, 16, 22, 27, 28, 30, 32, 35, 39–41], of which only 7 have specifically addressed the issue [7, 9, 14, 28, 30, 39, 41] (table 3). Immunocompetent patients rapidly clear *Leishmania* DNA from peripheral blood [14, 16, 30, 39], confirming a good concordance between clinical cure and PCR results. However, few possible exceptions to the rule have been reported [30, 39].

On the other hand, patients with VL and concomitant HIV infection have a completely different course of *Leishmania* DNAemia while receiving specific therapy. Indeed, the majority of HIV-infected patients do not clear *Leishmania* DNA from PB [9, 27,28, 32] or show a rapid reappearance of *Leishmania* DNA when specific therapy is interrupted. Both situations are generally—albeit, not invariably—associated with clinical relapse [28, 42]. It remains to be determined if it will be possible to define a cutoff value for *Leishmania* DNAemia that will allow researchers to predict the future development of clinical relapses of the disease in HIV-infected patients.

Isoenzyme analysis is currently considered to be the gold standard for the differentiation of *Leishmania* parasites at the species level, but this technique is laborious and time-consuming, is available only in a few laboratories, and, above all, requires prior isolation of the parasite. In our study, using a PCR and restriction fragment–length polymorphism protocol that was originally described by Minodier et al. [12], we readily identified, at the species level, all 68 cases of VL. Interestingly, we identified 5 cases of VL in which *L. donovani* was implicated. The first case was observed in a immunocompetent Italian patient who had lived and traveled in India for 7 years, and the remaining 4 cases occurred in HIV-infected intravenous drug users. In 2 of the latter cases, no evidence of travel history to areas where *L. donovani* is endemic was found, which lends support to the hypothesis of the introduction of the parasite in parenteral drug users through the artificial epidemic cycle [43]. Finally, for the 19 cases for which standard isoenzyme characterization of *Leishmania* species was available, we found perfect concordance between the 2 techniques that we used. Thus, molecular characterization of *Leishmania* species may overcome the need for parasite isolation. However, it should be noted that the PCR and restriction fragment–length polymorphism protocol we used in our study is unable to discriminate, at the species level, *Leishmania* parasites of the New World belonging to *Leishmania braziliensis* (e.g., *Leishmania braziliensis* and *Leishmania peruviana)* and the *Leishmania guyanensis* complex (e.g., *Leishmania guyanensis* and *Leishmania panamensis)*.

In summary, on the basis of the results of our study and of previous reports, we propose the algorithm reported in figure 1 for the primary diagnosis of VL in patients presenting with fever, hepatosplenomegaly, and pancytopenia. Because the diagnostic yield of PB appears to be at least equivalent to that of BMA when *Leishmania* PCR is used in both immunocompromised and immunocompetent patients, few justifications remain to perform first-line BMA in clinical practice when the diagnosis of VL is suspected.

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