Screening for subclinical *Leishmania* infection in HIV-infected patients living in eastern Spain

Javier Ena, Francisco Pasquau, María del Mar López-Perezagua, Carmen Martinez-Peinado, Francisco Arjona

Department of Internal Medicine, Division of Infectious Diseases; Department of Clinical Microbiology, Hospital Marina Baixa, Villajoyosa, Alicante, Spain

Background: We anticipated that patients with HIV infection living in endemic areas were at greater risk of infection which can reactivate due to immunosuppression; therefore, we analyzed the prevalence of latent *Leishmania infantum* infection in patients infected with HIV.

Methods: A total of 179 patients with HIV infection were screened for the presence of anti-*Leishmania* antibodies using indirect immunofluorescent antibody test (IFAT) (*Leishmania*-spot IF; bioMérieux, Marcy l'Etoile, France). All patients were followed up for at least 1 year. The primary end-point was to confirm the presence of *Leishmania* infection.

Results: Significant titer of antibodies to *Leishmania* was detected in six (3%; 95% confidence interval: 0.5–5.5%) asymptomatic patients. Two of them had visceral leishmaniasis that was confirmed by parasite visualization in clinical samples, the presence of *Leishmania* promastigotes in Novy–MacNeal–Nicolle culture, polymerase chain reaction (PCR)-based methods, and/or urinary antigen test. Among 173 patients with indirect immunofluorescent antibody test below 1 : 40, one HIV-infected patient severely immunosuppressed, confirmed negative by IFAT, was diagnosed of visceral leishmaniasis.

Conclusion: The use of indirect immunofluorescent antibody test for *Leishmania* screening is not justified in asymptomatic patients with HIV infection living in endemic areas due to the small rate of significant antibody titer and the low frequency of clinical disease.

Keywords: *Leishmania*, Serology, Screening, HIV-1 infection

Introduction

Visceral leishmaniasis caused by *Leishmania infantum* has emerged as a significant protozoan infection in patients infected with human immunodeficiency virus living in the Mediterranean basin.[1] It has been estimated that 2–9% of patients with AIDS in that particular area suffer from primary or reactivated visceral leishmaniasis.[2] The clinical presentation of visceral leishmaniasis in patients with HIV infection is not especially different from that found in immunocompetent patients.[3] The age of patients with *Leishmania* and HIV co-infection ranges from 29 to 33 years: 80–85% are male, often with past history of intravenous drug use. Diagnosis often requires an invasive procedure to obtain a sample for tissue culture or direct examination where *Leishmania* promastigotes or amastigotes, respectively, are visualized. At diagnosis, most patients show profound immunosuppression and have suffered from an AIDS indicator disease, but there is a considerable proportion of asymptomatic cases among patients with HIV infection.[4]

There are a number of non-invasive methods that have been developed to assist in the diagnosis of visceral leishmaniasis. The leishmanin skin test is performed by intradermal inoculation of a suspension of promastigotes, but it lacks in many cases of uniformity in type and dose of antigen used.[5] The detection of serum antibodies to *Leishmania* have high diagnostic value in immunocompetent patients,[6] but the performance of detection of serum antibodies to *Leishmania* in patients with HIV infection has been a matter of controversy. Patients with severe immunosupression may not have antibodies detectable by standard techniques.[7,8] Antigen detection methods are useful in patients with a deficient antibody production, such as patients with AIDS. A latex agglutination test has been developed for detecting *Leishmania* antigen in urine samples. However, in the absence of clinical symptoms, the detection of parasite antigens in urine has low specificity.[9] Over the past 10 years, several molecular techniques targeting various parasitic genes have been developed to the diagnosis of visceral

Correspondence to: J. Ena, HIV Unit, Hospital Marina Baixa, Av Alcalde Jaime Botella Mayor, 7, Villajoyosa, 03570 Alicante, Spain. Email: ena_jav@gva.es

© W. S. Maney & Son Ltd 2014
DOI 10.1179/2047773214Y.0000000164

leishmaniasis. The polymerase chain reaction (PCR)-based method seems specially promising in immunosuppressed patients.[10,11] PCR-based methods have proved in some studies to be more sensitive, and specific than the traditional diagnostic methods.[8]

Since patients with HIV infection need to be evaluated for latent co-infection such as cytomegalovirus, hepatitis B virus, hepatitis C virus, syphilis, toxoplasma, and tuberculosis,[12] we have included a routine serology testing for *Leishmania* infection in our endemic area for visceral leishmaniasis.

In the present study, we evaluated the usefulness of a routine serology screening at baseline for detecting latent *Leishmania* infection in patients with HIV infection attending a single centre clinic in Alicante, Spain.

Methods

Setting

Hospital Marina Baixa is a 280-bed institution belonging to the National Health System (Agencia Valenciana de Salud) attending a semi-urban population of 210 000 inhabitants living in Eastern Spain.

Type of study

Cross-sectional study with 1-year follow-up.

Population studied

Asymptomatic patients with HIV infection attending for first time the outpatient clinic from January 2009 to December 2012. Patients' information, including demographic variables, HIV infection stage, conventional laboratory, and immunological data, was recorded. Screening for *Leishmania* infection was carried out in all patients by indirect immunofluorescent antibody test (IFAT) (*Leishmania*-spot IF; bioMérieux, Marcy l'Etoile, France). Patients with past history of cutaneous or visceral leishmaniasis were excluded.

Laboratory tests

For indirect IFAT, we used a commercially available test (Leishmania-spot IF; bioMérieux) in which promatisgotes in stationary phase are dispensed in 15-well immunofluorescence slides. Patient and control sera were assayed in serial two fold dilutions from 1 : 10 to 1 : 640 and incubated with parasites for 30 minutes at 37°C. After three washes in PBS, antibody fixation was revealed with fluorescein isothiocyanate-conjugated sheep anti-human IgG (heavy plus light chains) (ICN, Aurora, OH, USA) diluted at 1 : 150 in 0.01% Evans blue for counterstaining. The slides were then incubated for 30 minutes at 37°C, washed, and examined under with a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). The IFAT results were considered significant when a 1 : 40 dilution of the serum gave fluorescence.

Patients with a significant titer of anti-*Leishmania* antibodies underwent bone marrow aspirate or tissue biopsy for direct visualization of the parasite and culture in Novy–Nicolle–McNeal's medium (NNN), PCR testing, and/or *Leishmania* antigen test in urine sample.

Definitions

Symptomatic visceral leishmaniasis was defined as the presence of fever lasting more than 2 weeks, with splenomegaly and confirmed by demonstration of *Leishmania* amastigotes from bone marrow aspirates or other tissue samples, by isolation of parasite in NNN or by a *Leishmania*-positive antigen test in urine.

Subclinical visceral leishmaniasis infection was defined by demonstration of *Leishmania* amastigotes from bone marrow aspirates or other tissue samples or by isolation of parasite in NNN medium in a patient in whom fever, splenomegaly, and a hemoglobin level <9 g/dl were absent.

Latent visceral leishmaniasis was defined as the presence of significant titers of antibody anti-*Leishmania* in a patient in whom fever, splenomegaly, and a hemoglobin level <9 g/dl were absent.

Non infected individuals were defined as those with no significant titers of antibody anti-*Leishmania* and absence of fever, splenomegaly, and a hemoglobin level <9 g/dl.

Statistical analysis

To estimate a prevalence of latent *Leishmania* infection in our high endemic area with a 95% confidence limit, we hypothesized a frequency of 25–50% according literature data and an error or ±7%. These parameters called for a sample size between 196 and 147 subjects, respectively. The sample size estimation was carried out using Openepi (Open Source Epidemiologic Statistics for Public Health).[13]

Results

One hundred and seventy-nine HIV-infected subjects were included in the study, 143 males and 36 females. The study population included patients with HIV infection acquired by sexual ($n=168$) and non-sexual ($n=11$) transmission routes. A total of 73 (41%) patients had a lymphocyte CD4 count less than 200 cells/μl at the time of the study and 18 (10%) patients had suffered an AIDS indicator disease.

We found a low prevalence of latent *Leishmania* infection in the study population. Only six (3%) patients out from 179 showed significant IFAT anti-*Leishmania* titers. Among six patients with significant IFAT titers, two had *Leishmania* infection confirmed by visualization of parasite in bone marrow aspirates, the presence of promastigotes in NNN culture medium, and positive results for PCR. In the remaining four patients, a clinical infection was ruled out and they persisted asymptomatic for at least 1 year of follow-up (Table 1). Patients with symptomatic

visceral leishmaniasis were cured with five doses of liposomal amphotericin B followed by once-a-month liposomal amphotericin B as secondary prophylaxis to prevent relapses.

On the other hand, among 173 subjects with IFAT serology titers below 1 : 40, we found one patient who developed signs and symptoms of visceral leishmaniasis one month after his first evaluation (Table 1, case 7). This patient was a 40-year-old male with high risk sexual behavior that recently tested positive for HIV infection. At diagnosis, his laboratory test results showed hemoglobin 14.4 g/dl, platelet count 266 000/mm^3, white cell count 4350/mm^3, a HIV viral load 68 900 copies/ml, and CD4 cell count 28 cells/mm^3. At that time, *Leishmania* IFAT serology was below 1 : 40. He started treatment with tenofovir disoproxil fumarate, emtricitabine, darunavir, and ritonavir as well as prophylaxis with timethoprim-sulphametoxazole. One month after starting with antiretroviral treatment, the patient suffered from fever up to 38.5°C, rash and myalgia. The patient was treated with prednisone for a possible adverse drug reaction. However, the patient continued with fever and 8 weeks later the physical examination showed enlargement of the liver and the spleen. His blood test showed hemoglobin 13.4 g/dl, platelet count 78 000/mm^3, and white cell count 2000/mm^3. A repeated *Leishmania* IFAT titer was below 1 : 40. A bone marrow aspiration was carried out as a work-up for fever of unknown etiology. *Leishmania* infection was confirmed in the bone marrow aspirate by the visualization of *Leishmania* amastigotes, the presence of *Leishmania* promastigotes in NNN medium culture, and by PCR of the bone marrow aspirate (PCR-RFLP targeting the internal transcribed spacer-1 region) that was positive for *Leishmania infantum*. The patient was treated with five doses of liposomal amphotericin B (total accumulated dose of 1.725 g) and then he received secondary prophylaxis with liposomal amphotericin B. The patient remained asymptomatic after a total follow-up of 26 months.

Discussion

We carried out a baseline screening for *Leishmania* infection by IFAT in patients with HIV infection attending our clinic. We found six patients (3% prevalence; 95% confidence interval: 0.5–5.5%) with significant antibody titer against *Leishmania*. Among the six asymptomatic patients, two had subclinical infection confirmed by parasite visualization of bone marrow aspirate or skin biopsy, positive culture in NNN medium, positive PCR-based method, or positive urine antigen testing. The remaining four patients had latent leishmaniasis with no signs of clinical infection during the follow-up.

Several methods have been developed to detect anti-*Leishmania* antibodies. Among them, there are enzyme-linked immunosorbent assay (ELISA) to antigen K39 or antigens extracted from promastigotes, indirect IFAT based on cultures of *L. infantum*, immunoblotting (Blot) to detect antibodies to the 14-kD antigen, or direct agglutination test (DAT) based on whole promastigotes of *L. donovani* or *L. infantum.* A recent comparison on the performance of serologic methods in patients with HIV-infection indicated superiority of Blot over IFAT and ELISA; and superiority of DAT over IFAT or ELISA, being Blot and DAT equivalent techniques.[8] A meta-analysis showed that IFAT in HIV-infected patients had an overall sensitivity of 51% and a specificity of 93% when it was compared with parasite direct visualization in tissue samples or bone marrow aspirate or culture of blood or bone marrow aspirate. In our study, we used IFAT because it was the method ready available in our laboratory. The detection of *Leishmania* antigen in urine has proven an appropriate technique for the diagnosis of subjects suspected of having visceral leishmaniasis and for monitoring the efficacy of treatment.[14–16] However, for asymptomatic HIV-infected patients, *Leishmania* antigen is not detected in urine probably due to the low level of circulating parasites.[17]

In the past decade, PCR-based methods to detect *Leishmania* DNA in peripheral blood or bone marrow aspirates have increased the accuracy of the diagnosis of this protozoan infection. In the published PCR assays, several target sequences such as ribosomal RNA genes, the mini-exon-derived RNA gene, the kineoplast minicircles, and repetitive nuclear sequences have been used.[18–25] A recently published meta-analysis showed that PCR-based methods have an overall sensitivity of 92% in peripheral blood samples, and 93% in bone marrow aspirates, with a specificity of 96%.[8] The sensitivity of PCR-based methods remains high in HIV-infected patients either with symptomatic or cryptic *Leishmania* infection regardless the degree of immunosuppression.[11,26] PCR-based methods have proven to be useful to monitor the parasitemia after specific treatment.[27] The major limitation of PCR-based methods for disease survey is availability of the procedure in low-resource facilities. In our study, we used PCR (PCR restriction-fragment-length polymorphism analysis targeting the internal transcribed spacer-1 region of the ribosomal genes) only to confirm the presence of visceral leishmaniasis. The procedure was carried out in bone marrow aspirate samples that were sent to a reference laboratory.

Survey studies carried out in our country show that the prevalence of significant titer to *Leishmania* using ELISA ranged in healthy individuals or blood donors from 0 to 5%.[28–30] When the survey was carried out in subjects at risk, such as intravenous drug users

Table 1 Characteristics of HIV-infected patients with *Leishmania* infection

Case	Age	Sex	HIV 2 transmission category	Hb (g/dl)	Platelet (count/mm^3)	White cell (count/mm^3)	CD4 (cells/mm^3)	HIV- RNA (copies/ml)	IFAT title	Type of infection	Diagnosis	Follow-up
1	34	Male	MSM	9.0	280 000	5600	180	1 640 000	1 : 80	Subclinical visceral leishmaniasis	Bone marrow aspirate: no amastigotes Skin biopsy: presence of Leishmania amastigotes Urine antigen detection: (KAtex, Kalon Biological, Guildford, UK) positive agglutination Bone marrow Leishmania PCR-RFLP (ITS-1 region) positive for *Leishmania infantum*	Treatment with liposomal Amphotericin B Secondary prophylaxis with once a month prophylaxis with amphotericin B for 48 mo. No relapse Follow-up: 48 mo
2	49	Male	MSM	10.7	266 000	4630	65	750 000	1 : 640	Subclinical visceral leishmaniasis	Bone marrow aspirate: presence of *Leishmania* amastigotes Presence of *Leishmania* promastigotes in NNN culture	Treatment with liposomal Amphotericin B Secondary prophylaxis with once a month prophylaxis with amphotericin B for 48 mo. No relapse Follow-up: 48 mo
3	52	Male	IVDU	13.0	109 000	2400	213	159 000	1 : 160	Latent visceral leishmaniasis	Bone marrow aspirate: absence of *Leishmania* amastigotes. Absence of *Leishmania* promastigotes in NNN culture Urine antigen detection: (KAtex, Kalon Biological) negative agglutination	No symptoms of *Leishmania* infection Follow-up: 36 mo
4	47	Male	Heterosexual	13.7	231 000	4880	360	Below detection limit	1 : 80	Latent visceral leishmaniasis	Bone marrow aspirate: no amastigotes	No symptoms of *Leishmania* infection Follow-up 48 mo
5	54	Male	Heterosexual	9.1	30 000	3850	231	Below detection limit	1:40	Latent visceral leishmaniasis	Bone marrow aspirate: absence of *Leishmania* amastigotes Absence of *Leishmania* promastigotes in NNN culture	No symptoms of *Leishmania* infection Follow-up: 36 mo

Table 1 Continued

Case	Age	Sex	HIV 2 transmission category	Hb (g/dl)	Platelet (count/mm^3)	White cell (count/mm^3)	CD4 (cells/mm^3)	HIV- RNA (copies/ml)	IFAT title	Type of infection	Diagnosis	Follow-up
6	47	Female	IVDU	8.1	69 000	3500	217	Below detection limit	1:40	Latent visceral leishmaniasis	Urine antigen detection: (KAtex, Kalon Biological) negative agglutination	No symptoms of *Leishmania* infection Follow-up 24 mo
7	40	Male	Heterosexual	14.4	266 000	4350	28	68 900	<1:40	Symptomatic visceral leishmaniasis	Bone marrow aspirate: presence of *Leishmania* amastigotes Presence of *Leishmania* promastigotes in NNN culture Bone marrow *Leishmania* PCR-RFLP (ITS-1 region) positive for *Leishmania infantum*	Treatment with liposomal Amphotericin B Secondary prophylaxis with once a month prophylaxis with amphotericin B for 48 mo. No relapse Follow-up: 18 mo

Note: IFAT: indirect immunofluorescent antibody test; MSM: men having sex with men; NNN culture: Novy–Nicolle–McNeal's medium. PCR restriction-fragment-length polymorphism analysis targeting the internal transcribed spacer-1 (ITS1) region.

or patients with HIV infection, the prevalence of significant antibody titer was found in up 43–64%.[24,25] When the screening method was a PCR-based technique in patients with HIV infection, the prevalence of *Leishmania* infection ranged from 13.5 to 30.4%.[31,32]

According to the literature, we anticipated a 25–50% rate of significant IFAT anti-*Leishmania* titer in the studied population with HIV infection. In our case, the low percentage rate (3%) of positive anti-*Leishmania* titer translated into a Number Needed to Test of 85 to confirm one patient with subclinical *Leishmania* infection. Moreover, a patient repeatedly testing negative with IFAT was found to have symptomatic visceral leishmaniasis shortly after the diagnosis of HIV infection. These results underline the useless of IFAT for screening of subclinical leishmaniasis in patients with HIV infection. Currently, the use of PCR-based methods in peripheral blood samples has been proposed as the first diagnostic test to be used in symptomatic patients with fever and/or hepastosplenomegaly and/or pancytopenia who are intravenous drug users or have immunosuppression caused by HIV or are transplant recipients.[11] However, the utility of PCR in asymptomatic HIV-co-infected patients may be of limited use because the detection of *Leishmania* is not always correlated with clinical disease.[33,34]

In the same period, a total of six cases of symptomatic visceral leishmaniasis were confirmed by our laboratory (incidence: 0.95 cases per 100 000 inhabitants), three of the cases were found in immusuppressed patients with no documented HIV infection (one patient with ulcerative colitis treated with infliximab, one patient with giant cell arteritis treated with prednisone, and one patient with brain metastases treated with dexamethasone), and the remaining three cases occurred in patients with HIV infection and symptomatic visceral leishmaniasis.

In summary, the low performance of IFAT for *Leishmania* does not justify its routine use as screening method in patients with HIV infection living in endemic areas.

Disclaimer Statements

Contributors Conception and design (JE, FP), acquisition of data (MMLP, CMP, FA), analysis and interpretation of data (JE, FP, MMLP, CMP, FA), drafting the article (JE), revising it critically for important intellectual content (JE, FP, MMLP, CMP, FA), and final approval of the version to be published (JE, FP, MMLP, CMP, FA).

Funding The study was funded in part by AMBEMI.

Conflicts of interest None.

Ethics approval The study was approved by the Institutional Review Board of our center (Comisión de Investigación y Biblioteca). The Institutional Review Board waived the requirement for the investigator to obtain a signed consent.

Acknowledgments

Division de Parasitologıa del Centro Nacional de Microbiologıa del Instituto de Salud Carlos III (Madrid, Spain) for performing the PCR-based methods.

References

1 Pasquau F, Ena J, Sanchez R, Cuadrado JM, Amador C, Flores J, *et al.*; Leishmania HIV Mediterreanean Co-operative Group. Leishmaniasis as an opportunistic infection in HIV-infected patients: determinants of relapse and mortality in a collaborative study of 228 episodes in a Mediterranean region. Eur J Clin Microbiol Infect Dis. 2005;24:411–8.

2 Alvar J, Cañavate C, Gutiérrez-Solar B, Jiménez M, Laguna F, López-Vélez R, *et al. Leishmania* and human immunodeficiency virus coinfection: the first 10 years. Clin Microbiol Rev. 1997;10:298–319.

3 Pintado V, Martín-Rabadán P, Rivera ML, Moreno S, Bouza E. Visceral leishmaniasis in human immunodeficiency virus (HIV)-infected and non-HIV-infected patients. A comparative study. Medicine (Baltimore). 2001;80:54–73.

4 Pineda JA, Hernández-Quero J, Gallardo MA, López-Ruz MA, Martinez-Pérez MA, Macías J, *et al.* Frequency of subclinical visceral leishmaniasis in HIV-infected patients in Spain. Eur J Clin Microbiol Infect Dis. 1996;15:263–4

5 WHO. Manual of visceral leishmaniasis control. Geneva: World Health Organization Division of Control of Tropical Diseases; 1996. p. 1–84. Available from: http://www.who.int/leishmaniasis/surveillance/training/en/WHO_LEISH_96.40.pdf

6 Kar K. Serodiagnosis of leishmaniasis. Crit Rev Microbiol. 1995;21:123–52.

7 Medrano FJ, Cañavate C, Leal M, Rey C, Lissen E, Alvar J. The role of serology in the diagnosis and prognosis of visceral leishmaniasis in patients coinfected with human immunodeficiency virus type-1. Am J Trop Med Hyg. 1998;59:155–62.

8 Cota GF, de Sousa MR, Demarqui FN, Rabello A. The diagnostic accuracy of serologic and molecular methods for detecting visceral leishmaniasis in HIV infected patients: meta-analysis. PLoS Negl Trop Dis. 2012;6(5):e1665.

9 Riera C, Fisa R, Udina M, Gállego M, Portus M. Detection of *Leishmania infantum* cryptic infection in asymptomatic blood donors living in an endemic area (Eivissa, Balearic Islands, Spain) by different diagnostic methods. Trans R Soc Trop Med Hyg. 2004;98:102–10.

10 de Ruiter CM, van der Veer C, Leeflang MM, Deborggraeve S, Lucas C, Adams ER. Molecular tools for diagnosis of visceral leishmaniasis: systematic review and meta-analysis of diagnostic test accuracy. J Clin Microbiol. 2014;52:3147–55.

11 Antinori S, Calattini S, Longhi E, Bestetti G, Piolini R, Magni C, *et al.* 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. Clin Infect Dis. 2007;44:1602–10.

12 Panel de expertos de GeSIDA; Plan Nacional sobre el Sida. GeSIDA/National AIDS Plan: consensus document on antiretroviral therapy in adults infected by the human immunodeficiency virus (Updated January 2014). Enferm Infecc Microbiol Clin. 2014;32:446.e1–42. Spanish.

13 Dean AG, Sullivan KM, Soe MM. OpenEpi: open source epidemiologic statistics for public health. Available from: http://www.OpenEpi.com [accessed 4 September 2014].

14 Attar ZJ, Chance ML, el-Safi S, Carney J, Azazy A, El-Hadi M, *et al.* Latex agglutination test for the detection of urinary antigens in visceral leishmaniasis. Acta Trop. 200;78:11–6.

15 de Colmenares M, Portus M, Riera C, Gallego M, Aisa MJ, Torras S, *et al.* Short report: detection of 72-75-kD and 123-kD fractions of *Leishmania* antigen in urine of patients with visceral leishmaniasis. Am J Trop Med Hyg. 1995;52:427–8.

16 Riera C, Fisa R, Lopez P, Ribera E, Carrió J, Falcó V, *et al.* Evaluation of a latex agglutination test (KAtex) for detection of *Leishmania* antigen in urine of patients with HIV–*Leishmania* coinfection: value in diagnosis and post-treatment follow-up. Eur J Clin Microbiol Infect Dis. 2004;23:899–904.

17 García-García JA, Martín-Sánchez J, Gállego M, Rivero-Román A, Camacho A, Riera C, *et al.* Use of noninvasive markers to detect *Leishmania* infection in asymptomatic human immunodeficiency virus-infected patients. J Clin Microbiol. 2006;44:4455–8.

18 van Eys GJ, Schoone GJ, Kroon NC, Ebeling SB. Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of *Leishmania* parasites. Mol Biochem Parasitol. 1992;51:133–42.

19 Rodgers MR, Popper SJ, Wirth DF. Amplification of kinetoplast DNA as a tool in the detection and diagnosis of *Leishmania*. Exp Parasitol. 1990;71:267–75.

20 Hassan MQ, Ghosh A, Ghosh SS, Gupta M, Basu D, Mallik KK, *et al.* Enzymatic amplification of mini-exon-derived RNA gene spacers of *Leishmania donovani*: primers and probes for DNA diagnosis. Parasitology. 1993;107(Pt 5):509–17.

21 Lachaud L, Dereure J, Chabbert E, Reynes J, Mauboussin JM, Oziol E, *et al.* Optimized PCR using patient blood samples for diagnosis and follow-up of visceral Leishmaniasis, with special reference to AIDS patients. J Clin Microbiol. 2000;38:236–40.

22 Harris E, Kropp G, Belli A, Rodriguez B, Agabian N. Single-step multiplex PCR assay for characterization of New World *Leishmania* complexes. J Clin Microbiol. 1998;36:1989–95.

23 Pizzuto M, Piazza M, Senese D, Scalamogna C, Calattini S, Corsico L, *et al.* Role of PCR in diagnosis and prognosis of visceral leishmaniasis in patients coinfected with human immunodeficiency virus type 1. J Clin Microbiol. 2001;39:357–61.

24 Smyth AJ, Ghosh A, Hassan MQ, Basu D, de Bruijn MH, Adhya S, *et al.* Rapid and sensitive detection of *Leishmania* kinetoplast DNA from spleen and blood samples of kala-azar patients. Parasitology. 1992;105(Pt 2):183–92.

25 Katakura K, Kawazu SI, Sanjyoba C, Naya T, Matsumoto Y, Ito M, *et al. Leishmania* mini-exon genes for molecular epidemiology of leishmaniasis in China and Ecuador. Tokai J Exp Clin Med. 1998;23:393–9.

26 Colomba C, Saporito L, Vitale F, Reale S, Vitale G, Casuccio A, *et al.* Cryptic *Leishmania infantum* infection in Italian HIV infected patients. BMC Infect Dis. 2009;9:199.

27 Cascio A, Calattini S, Colomba C, Scalamogna C, Galazzi M, Pizzuto M, *et al.* Polymerase chain reaction in the diagnosis and prognosis of Mediterranean visceral leishmaniasis in immunocompetent children. Pediatrics. 2002;109:E27.

28 Gutiérrez J, Maroto MC, Piédrola G, Higuera A. [Prevalence of anti-Leishmania antibodies in parenteral drug addicts. Yield value of 2 study techniques]. Med Clin (Barc). 1993;100:168–70. Spanish.

29 Garrote JI, Gutiérrez MP, Izquierdo RL, Dueñas MA, Zarzosa P, Cañavate C, *et al.* Seroepidemiologic study of *Leishmania infantum* infection in Castilla-Leon, Spain. Am J Trop Med Hyg. 2004;71:403–6.

30 Riera C, Fisa R, López-Chejade P, Serra T, Girona E, Jiménez M, *et al.* Asymptomatic infection by Leishmania infantum in blood donors from the Balearic Islands (Spain). Transfusion. 2008;48:1383–9.

31 Martín-Sánchez J, Pineda JA, Morillas-Márquez F, García-García JA, Acedo C, Macías J. Detection of *Leishmania infantum* kinetoplast DNA in peripheral blood from asymptomatic individuals at risk for parenterally transmitted infections: relationship between polymerase chain reaction results and other *Leishmania* infection markers. Am J Trop Med Hyg. 2004;70:545–8.

32 García-García JA, Martín-Sánchez J, Gállego M, Rivero-Román A, Camacho A, Riera C, *et al.* Use of noninvasive markers to detect *Leishmania* infection in asymptomatic human immunodeficiency virus-infected patients. J Clin Microbiol. 2006;44:4455–8.

33 Riera C, Fisa R, Ribera E, Carrió J, Falcó V, Gállego M, *et al.* Value of culture and nested polymerase chain reaction of blood in the prediction of relapses in patients co-infected with *Leishmania* and human immunodeficiency virus. Am J Trop Med Hyg. 2005;73:1012–5.

34 Silva LA, Romero HD, Fagundes A, Nehme N, Fernandes O, Rodrigues V, *et al.* Use of the polymerase chain reaction for the diagnosis of asymptomatic *Leishmania* infection in a visceral leishmaniasis-endemic area. Rev Inst Med Trop Sao Paulo. 2013;55:101–4.