The biological diagnosis of leishmaniasis in HIV-infected patients

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This review emphasises the particular difficulties encountered in confirming a suspected case of cutaneous or visceral leishmaniasis when that case is co-infected with HIV. HIV infection appears to have a more profound impact on the development of visceral leishmaniasis than on the evolution of the purely cutaneous disease. The various techniques available for immunological, parasitological and molecular diagnosis are presented and evaluated. The value of serodiagnosis for the detection of antileishmanial antibodies is in part dependent on the antigens used. Western blots may have a use not only in diagnosis but also in predicting the cases of HIV infection that are at most risk of developing symptomatic leishmaniasis. The presence of leishmanial parasites may still only be demonstrated incontrovertibly by the microscopical examination of smears or the culture of blood or biopsy samples. The use of cultures not only permits diagnosis but also detailed study of the parasites. The potential use of PCR in diagnosis is explored and related to other possible tests. A recommended, standardized procedure for the diagnosis of leishmaniasis in HIV-infected patients is presented.

In HIV-infected cases, diagnosis of leishmaniasis may be delayed because the clinical manifestations typical of the disease in the immunocompetent do not develop. The co-infected cases are also far more likely to respond poorly to antileishmanial treatment. Biological diagnosis and follow-up are therefore particularly important for the clinicians who have to manage such cases. Serodiagnosis may be a useful first step despite the immunodeficiencies in some HIV-positive patients. The demonstration of leishmanial parasites, by microscopy or culture, confirms a suspected diagnosis of leishmaniasis and may be a useful way of detecting the treatment failures. Culture also permits detailed study of the causative Leishmania species (in terms of identification, epidemiology, phylogeny, susceptibility to drugs, molecular biology and biochemistry) and the cryopreservation of isolates for future comparisons and experiments. Molecular diagnosis has recently been applied to leishmaniasis and may offer some significant advantages compared with the older techniques.

In the immunocompetent, visceral leishmaniasis (VL) is almost always marked by haematological and immunological abnormalities: normochromic anaemia with hyperplastic bone-marrow and rapid turn-over of erythrocytes, leucopenia, thrombocytopenia,
a marked dysproteinaemia, with a reversed albumin:globulin ratio, and a raised titre of polyclonal IgG (Mardsen and Jones, 1985). Unfortunately, these modifications are not specific to leishmaniasis and may have numerous aetiologies. In HIV-positive patients, they may even be caused by the drugs given to control the virus or other opportunistic infections.

The possible methods of diagnosing leishmaniasis and the particular problems of diagnosing the disease in those who are HIV-positive are reviewed below.

IMMUNOLOGICAL DIAGNOSIS

Cellular Immunity

The use of leishmanin skin (Montenegro) tests to investigate cellular immunity to leishmanial parasites has proven useful in the detection of cutaneous and mucocutaneous leishmaniasis, whether for screening populations or for individual diagnosis. Although not commercially available, the leishmanin antigen may be obtained from the Istituto Superiore di Sanità in Rome, Wellcome in London or the Institut Pasteur in Tehran. This antigen was standardized by Weigle et al. (1991). All three sources supply the antigen as a suspension of $5 \times 10^6$ promastigotes in 1 ml 0.5% phenol–saline. For each test, 0.1 ml of this suspension is inoculated intradermally and the development, 48 h later, of a papule that is at least 5 mm in diameter is taken as a positive result. Healthy individuals who give a positive result may have asymptomatic infections and indicate active foci of transmission in endemic areas (Marty et al., 1994).

Immunocompetent individuals give negative results in skin tests when they are in the acute phase of VL or diffused cutaneous leishmaniasis but become skin-test positive several months after healing (Marty et al., 1994). The lack of a commercial source has limited the use of leishmanin for diagnosis and very little is known about leishmanin reactivity in cases of Leishmania/HIV co-infection.

Serodiagnosis

Serological tests may be particularly useful in the diagnosis of visceral or mucocutaneous leishmaniasis. They have been found to have a high predictive value in the diagnosis of VL in immunocompetent individuals (Kar, 1995). Of the serodiagnostic tests available, IFAT are the most frequently used. The antigens used in the IFAT are either axenic cultures of promastigotes — as used in the Leishmania-spot IF (bioMérieux, Marcy l’Etoile, France) and other commercial kits — or freshly prepared smears of organs containing amastigotes. Although the use of amastigotes may give slightly higher sensitivity, it seems more important to use, as the antigen, a recent isolate from the focus under investigation (unpubl. obs.). The threshold titre for sero-positivity is generally about 1/80. An IFAT should only be considered positive if the whole parasites fluoresce but sera giving fluorescence limited to the parasites’ nuclei — generally the result of cross-reactivity but occasionally indicative of an asymptomatic leishmanial infection — need to be checked further, by western blotting (see below).

Direct agglutination tests (DAT) are easy to perform and need no sophisticated equipment. The specificity and sensitivity of DAT may be enhanced by pre-treating the antigens with β-mercaptoethanol and incorporating urea in the reaction mixture (El Harith et al., 1996). El Harith et al. (1996) set their threshold titres for DAT positivity at 1/1600–1/3200. At least one commercial test for the diagnosis of leishmaniasis — the Leish-KIT® (Meredith et al., 1995), which comes with lyophilized antigens — is based on the DAT.

Tests based on indirect haemagglutination lack sensitivity and specificity. In contrast, some of the ELISA that have been developed for the diagnosis or detection of leishmanial infection are both sensitive and specific. Two commercial ELISA are available: one produced by Bordier Affinity Products (Crissier, Switzerland) and another, the ELISA-Melotest-Leishmania®, produced by Immunotech (Marseille, France). Among
the many antigens used in ELISA for the diagnosis of leishmaniasis are total soluble antigen (Hommel et al., 1978), dp72 and gp 70-2 (Jaffe and Zalis, 1988), gp 63 (Okong’o-Odera et al., 1993b), a 70-kDa antigen (Okong’o-Odera et al., 1993a), and rK39, a recombinant, kinesin-related protein (Burns et al., 1993) marketed by LMD Laboratories (Carlsbad, CA). This list of possible antigens, which is by no means exhaustive, illustrates the lack of standardization. Both the antigen used and the HIV status of the subject affect the performance of the assay. Among HIV-positives, immunodiagnostic tests for leishmanial infection have sensitivities varying between 5% and 82% (Montalbán et al., 1989; Altés et al., 1991; Medrano et al., 1992, 1998; Gradoni et al., 1993) and may often be complicated by cross-reactions with other opportunistic diseases.

The choice of which test to employ has to be tailored to the setting and to be based not only on the test’s performance but also on its ease of use, direct costs and the costs of any necessary equipment that is not already available.

**A COMPARATIVE EVALUATION**

In an attempt to make a valid comparison between the various serological tests that could be used to diagnose VL in HIV-positive patients, six such tests have been used, in parallel, to investigate sera from 45 Spanish cases of *L. infantum*/HIV co-infection (C. Cañavate, unpubl. obs.). The sera were collected, between January 1995 and June 1998, from patients whose leishmaniasis had been parasitologically confirmed. Their HIV infection was detected and staged and their AIDS status was assessed using the standard criteria of the Centers for Disease Control and Prevention in Atlanta (CDC). The 73 control sera used came from HIV-infected patients who had been found free of *Leishmania* infection at autopsy, healthy individuals living in an endemic area, immunocompetent but parasitologically confirmed cases of VL, or patients with disorders that might, by cross-reacting, give false-positive results. The six tests assessed were:

1. **IFAT**, following a standard method (Bray, 1985). The antigen was prepared from promastigotes of the international reference strain of *L. infantum* zymodeme MON-1 (MHOM/FR/78/LEM-75). Antibody binding was revealed with a conjugate of fluorescein iso-thiocyanate and sheep anti-human-IgG (heavy plus light chains). The threshold titre giving the highest sensitivity and specificity in separating the immunocompetent VL patients from the healthy subjects from VL-endemic areas, 1/80, was set as the general cut-off for seropositivity.

2. **BAP-ELISA**. All the sera were tested using the eight-well ELISA strips, sensitised with *L. infantum* soluble antigens, that are manufactured by Bordier Affinity Products (BAP). The test was carried out according to the manufacturer’s recommendations. Each serum sample was tested in duplicate (at a dilution of 1/100) and positive and negative controls were included in each plate. Bound antibody was detected using goat anti-human-IgG–biotin and streptavidin–horseradish-peroxidase conjugates. The optimum dilutions for the test sera and conjugates were determined by checkerboard titration. The optical density (OD) used as the cut-off for seropositivity, in this and all the other ELISA, was the mean OD for the sera from healthy subjects from VL-endemic areas plus four s.d.

3. **SLA-ELISA**. The sera were investigated using an experimental ELISA in which a promastigote lysate serves as the antigen source. Microtitre plates were coated with soluble *L. infantum* antigen (1 μg/well), prepared as described by Scott et al. (1987). This ‘SLA-ELISA’ then continued like the BAP-ELISA.
(4) rK39-ELISA. Other ELISA were used to determine the titres of antibodies reacting with the rK39 antigen (Burns et al., 1993). This antigen, a recombinant protein, containing a 39-amino-acid repeat, that is based on *L. chagasi* kinesin, was kindly provided by Dr S. G. Reed. Micro-assay plates were sensitised with 50 ng rK39/well. Sera were diluted 1/100 and bound antibody was detected with protein-A-conjugated horseradish peroxidase.

(5) rK39-based dipsticks (supplied by the World Health Organization), which use a protein-A–colloidal-gold conjugate to give a visible result. Each dipstick is a membrane of nitrocellulose pre-coated with the recombinant *Leishmania* antigen in the test-line region and an anti-protein-A antibody in the control-line region. Two red lines, in the antigen and control-line regions, indicate a positive result, whereas one red (control) line indicates a negative result and must always appear.

(6) ACON® *Leishmania* one-step test. Like the World Health Organization's dipsticks (the two test procedures are identical), this rapid chromatographic immuno-assay, manufactured by Acon Laboratories (Bethlehem, PA), uses a combination of a protein-A–colloidal-gold conjugate and rK39 antigen, on a dipstick, to detect antibody to *Leishmania*.

In the initial tests, 55 sera from HIV-positive patients (45 from VL cases and 10 from non-VL cases) were investigated. The numbers of sera found truly positive and negative and falsely positive and negative are shown, for each test, in Table 1. In terms of the combined number of false-negative and false-positive results, the SLA-ELISA appeared to have the greatest specificity. No false-positive results were obtained with the IFAT, rK39 dipstick or ACON *Leishmania* tests, but both dipstick assays gave high numbers of false-negative results. The sensitivity, specificity, positive and negative predictive values and efficiency of each test are given in Table 2.

Four of the control sera, from HIV-infected patients without VL, gave false-positive results in one or two of the tests: two (one from a patient with disseminated tuberculosis and the other from a patient with no apparent concomitant opportunistic infection) were positive in the BAP-ELISA, one (from a patient suffering hepatic cirrhosis, which is known to produce polyclonal hypergammaglobulinaemia) was positive in both the BAP- and SLA-ELISA, and one (from another patient with disseminated tuberculosis) was positive in both the BAP- and rK39-ELISA.

To explore the problem of cross-reactivity further, 53 sera from patients with tuberculosis, leprosy, African or American trypanosomiasis, malaria or an auto-immune disorder were screened in each of the six tests (Table 3). Just two of these sera, both from patients with American trypanosomiasis (Chagas disease), were found positive in the IFAT, and then

<table>
<thead>
<tr>
<th>Test</th>
<th>Truly positive</th>
<th>Falsely positive</th>
<th>Truly negative</th>
<th>Falsely negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFAT</td>
<td>30</td>
<td>0</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>BAP-ELISA</td>
<td>34</td>
<td>4</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>SLA-ELISA</td>
<td>40</td>
<td>1</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>rK39-ELISA</td>
<td>28</td>
<td>1</td>
<td>9</td>
<td>17</td>
</tr>
<tr>
<td>rK39 dipstick</td>
<td>9</td>
<td>0</td>
<td>10</td>
<td>36</td>
</tr>
<tr>
<td>ACON <em>Leishmania</em></td>
<td>10</td>
<td>0</td>
<td>10</td>
<td>35</td>
</tr>
</tbody>
</table>

**TABLE 1.** The test results obtained with six serological methods for the diagnosis of visceral leishmaniasis in HIV-infected patients from southern Europe.
TABLE 2. The performance of six serological tests in the diagnosis of visceral leishmaniasis among the HIV-infected

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive</th>
<th>Negative</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFAT</td>
<td>67</td>
<td>100</td>
<td>100</td>
<td>40</td>
<td>73</td>
</tr>
<tr>
<td>BAP-ELISA</td>
<td>76</td>
<td>60</td>
<td>89</td>
<td>35</td>
<td>73</td>
</tr>
<tr>
<td>SLA-ELISA</td>
<td>89</td>
<td>90</td>
<td>98</td>
<td>64</td>
<td>89</td>
</tr>
<tr>
<td>rK39-ELISA</td>
<td>62</td>
<td>90</td>
<td>97</td>
<td>35</td>
<td>67</td>
</tr>
<tr>
<td>rK39 dipstick</td>
<td>20</td>
<td>100</td>
<td>100</td>
<td>22</td>
<td>34</td>
</tr>
<tr>
<td>ACON Leishmania</td>
<td>22</td>
<td>100</td>
<td>100</td>
<td>22</td>
<td>36</td>
</tr>
</tbody>
</table>

TABLE 3. The cross-reactivity of six serodiagnostic tests for visceral leishmaniasis, with sera from patients with other diseases

<table>
<thead>
<tr>
<th>Disease</th>
<th>No. of sera tested</th>
<th>IFAT</th>
<th>BAP-ELISA</th>
<th>SLA-ELISA</th>
<th>rK39-ELISA</th>
<th>rK39 dipstick</th>
<th>ACON Leishmania</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tuberculosis</td>
<td>5</td>
<td>0 (0)</td>
<td>1 (20)</td>
<td>1 (20)</td>
<td>1 (20)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Leprosy</td>
<td>10</td>
<td>0 (0)</td>
<td>9 (90)</td>
<td>6 (60)</td>
<td>2 (20)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>African trypanosomiasis</td>
<td>8</td>
<td>0 (0)</td>
<td>3 (37)</td>
<td>3 (37)</td>
<td>1 (12)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Chagas disease</td>
<td>10</td>
<td>2 (20)</td>
<td>6 (60)</td>
<td>6 (60)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Malaria</td>
<td>10</td>
<td>0 (0)</td>
<td>3 (30)</td>
<td>4 (40)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Auto-immune disease</td>
<td>10</td>
<td>0 (0)</td>
<td>4 (40)</td>
<td>1 (10)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Any</td>
<td>53</td>
<td>2 (4)</td>
<td>26 (49)</td>
<td>21 (40)</td>
<td>4 (8)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

only at the titre used as the threshold for seropositivity (1/80). Neither of the two dipstick assays (rK39 and ACON Leishmania) gave any positive results with the heterologous sera but the two ELISA based on crude antigens each gave more than 20 false-positives. The rK39-ELISA appeared to be the best of the ELISA, giving just four false-positives. All three ELISA gave a false-positive result for a serum sample from a tuberculosis patient.

DISCUSSION. IFAT has been shown to be a highly sensitive and specific method for diagnosing VL in immunocompetent patients. In the Spanish study, it appeared to have a relatively low sensitivity (74%) but 100% specificity when used on HIV-infected patients. Similarly low sensitivities have been seen before, when sera from co-infected patients have been tested in other IFAT based on freshly prepared antigen (Gradoni et al., 1993; Gari-Toussaint et al., 1994). Although both the commercially available ELISA and the experimental ELISA based on soluble *L. infantum* antigens had higher sensitivities than the IFAT, they also had lower specificities. Overall, the experimental ELISA had better diagnostic indexes than the BAP-ELISA, perhaps because the latter assay was not based on fresh antigen. The manufacturers of the BAP-ELISA do not state the antigen concentration used for coating the strips. The rK39-ELISA had a lower sensitivity (62%) than the ELISA based on crude antigen preparations, perhaps because of genetically restricted responses to a single antigen. Houghton et al. (1998), however, reported a higher sensitivity (82%) when they used the rK39-ELISA to investigate 56 co-infected patients.

Both immunochromatographic dipstick assays appeared insensitive, though very specific. At least in southern Europe, they
are clearly not reliable tools for the diagnosis of VL in HIV-infected patients. Among immunocompetent subjects in India, such rK39-based dipsticks, specifically designed for rapid diagnosis under field conditions, have proved to be both highly sensitive and highly specific in the serodiagnosis of VL (Sundar et al., 1998) and post-kala-azar dermal leishmaniasis (Salotra et al., 2001). They have performed much less well in southern Europe (Jelinek et al., 1999) and Sudan (Zijlstra et al., 2001).

Serological cross-reactivity between *Leishmania* and other infectious agents, such as *Trypanosoma* and mycobacteria, has been widely reported (Hommel et al., 1978; Pappas et al., 1983; Badaró et al., 1986). When using a commercial IFAT for the serodiagnosis of VL, López-Vélez et al. (1998) found that 10 (20%) of 51 patients with active tuberculosis (25 of whom were HIV-positive) gave a false-positive result. In another study of IFAT, Gallardo et al. (1996) found that 5% of HIV-infected patients with tuberculosis were falsely positive for VL.

If a test is to be used to screen patients, before a positive result is confirmed using another, more accurate method, a few false-positive results are less of a problem than the false-negative results. The negative predictive values of the six tests compared in Spain are generally low but indicate that IFAT and the ELISA would be better screening methods than the dipsticks (Table 2).

**WESTERN BLOTS**

Western blots can help to confirm a positive result from another serodiagnostic test, although their production may be too complicated for some clinical laboratories were VL is endemic. Valid comparison between the results of studies on the use of western blotting to confirm leishmanial infection is made difficult because of variation in the methodology, polyacrylamide gels and antigen preparations used. It is clear, however, that western blotting can be a highly sensitive technique. Bands representing antigens of particular sizes may confirm a diagnosis, the ‘best’ bands varying with the methodology and parasite. Sera reacting with leishmanial antigens of 230, 75, 66, 50, 42, 18, 14 and 12 kDa (Hoerauf et al., 1992), 65–66, 42, 14 and 12–13 kDa (Bogdan et al., 1990), 23 and 31 kDa (Rolland et al., 1994) or 14, 16, 21, 23 and 31 kDa (Marty et al., 1995) may only come from cases of leishmaniasis. In southern Europe, antibodies reacting with *L. infantum* antigens of 14 and/or 16–18 kDa are not only present in acute VL but, in asymptomatic individuals, may also represent specific evidence of delayed-type antileishmanial hypersensitivity; Marty et al. (1994) observed 82% concordance between a positive result in a leishmanin skin test and the presence of antibodies reacting with the bands at 14 and/or 16–18 kDa. Bands at 14 and 16–18 kDa (Mary et al., 1992) and particularly bands at 18, 21, 23 and 31 kDa (Marty et al., 1995) are good markers of the symptomatic VL caused by *L. infantum*, the species most frequently associated with HIV infection. The way in which bands at these positions appear, disappear or fade may be useful indicators of response to antileishmanial (and antiretroviral) treatment.

The specificities and sensitivities of western blotting in the diagnosis of VL among HIV-positive patients were recently investigated in southern France (P. Marty, unpubl. obs.). The sera used came from 100 cases of VL (69 HIV-negative and 31 HIV-positive), 72 asymptomatic HIV-positive patients without VL, and 23 patients with mycobacteria/HIV co-infection. In the western blots, bands at 18, 21, 23 and 31 kDa (the ‘full profile’) were seen with all (100%) of the VL patients who were HIV-negative, and 23 (74%) of the cases of *Leishmania*/HIV co-infection. None of the other sera tested gave the full profile (specificity = 100%). Among the co-infected cases, western blotting and IFAT each gave sensitivities of 74% but the results of the two tests were not entirely concordant; assuming a case was positive for leishmaniasis if his or her serum was found ‘full-profile’-positive by western blotting and/or positive by IFAT increased the overall sensitivity to 84%.
In a longitudinal study in Nice, France, Kubar et al. (1998) collected multiple serum samples from 236 HIV-infected patients over periods ranging from 1.5 to 7 years. At the beginning of the study, 12 of the patients had patent leishmaniasis and 20 of the other 224 probably had asymptomatic leishmanial infections, as they had antibodies that reacted with the 14- and/or 18-kDa antigens of *L. infantum* in western blots (two of these 20 patients developed symptomatic leishmaniasis during their follow-up). The western-blot results for the 14 patients who developed clinical leishmaniasis during their follow-up are summarized in Table 4. Seven patients of these 14 were asymptomatic, though positive for antibodies to the 14- and 18-kDa antigens, at the beginning of the study and had ‘full-profile’ sera when they developed symptomatic VL; their VL was assumed to result from the re-activation of a latent infections (RE-VL). Another five patients were asymptomatic and negative for antibodies to the 14- and 18-kDa antigens at the beginning of the study yet had ‘full-profile’ sera when they developed symptomatic VL; their VL was considered to result from primary *Leishmania* infections (PI-VL). One patient had ‘full-profile’ serum and confirmed leishmaniasis at the beginning of the study. The final patient was consistently seronegative over 7 years of follow-up.

**DISCUSSION.** For studies on VL, in Europe at least, western blotting clearly has some advantages. Its sensitivity and specificity make it a good tool for diagnosis. Moreover, it may help predict the risk of an immunocompromised subject (who may have a latent leishmanial infection) developing symptomatic VL. The observations made by Kubar et al. (1998) indicate that, in longitudinal investigations, western blots may help distinguish episodes of VL caused by primary infections from those caused by the recrudescence of older infections. Such uses clearly have epidemiological significance. Although the titres of antileishmanial antibodies observed in IFAT and ELISA may gradually fall with time, peaking again whenever there is a relapse, these changes are less clear-cut than those seen in western blots. Each serological test has its limitations but the effects of HIV infection weaken the usefulness of certain tests more than others. It is evident that a large percentage of HIV-infected individuals with VL cannot develop an adequate humoral immune response to the parasite. The often low titres of antileishmanial antibodies observed in co-infected patients can be expected, since HIV infection is associated with pronounced disorders of the immune system, including polyclonal B-cell activation and impairment of antigen presentation.

**PARASITOLOGICAL DIAGNOSIS**

Whatever the immunological or clinical status of a patient infected with *Leishmania*, the characteristic amastigotes may often be detected, microscopically or by culture, in

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Duration of follow-up (years)</th>
<th>Results of western blotting</th>
<th>Categorized as:</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>1.5–7</td>
<td>14- to 18-kDa profile in the first serum and ‘full profile’ at VL diagnosis</td>
<td>RE-VL</td>
</tr>
<tr>
<td>5</td>
<td>1–5</td>
<td>Negative in the first serum and ‘full profile’ at VL diagnosis</td>
<td>PI-VL</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>‘Full-profile’ first serum and concurrent VL</td>
<td>Initial VL</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>Always negative</td>
<td>False-negative</td>
</tr>
</tbody>
</table>

RE-VL, The episode of visceral leishmaniasis diagnosed during follow-up was a re-activation of a latent infection; PI-VL, the episode of visceral leishmaniasis diagnosed during follow-up was the result of a primary infection.
samples of bone marrow, lymph node, liver or spleen obtained by needle aspiration or biopsy. They may also be seen under the microscope in samples of blood, especially if smears of leucocyte concentrates are prepared. The production and examination of histological sections, usually stained with haematoxylin–eosin, may give useful information on the host-tissue reactions.

**Bone Marrow**

In southern Europe, bone-marrow aspirates (BMA) are routinely collected from suspected cases of VL and examined as Giemsa-stained smears. Amastigotes can be detected in 48%–100% of such samples from HIV-positives, the level of success largely being dependent on parasite burden, which varies considerably in co-infected patients (Montalbán et al., 1990). Bone-marrow aspiration is an invasive and painful technique that is rarely repeated on the same individual. Use of BMA in post-treatment follow-up is made difficult by the often low parasite burdens and the poor co-operation of the patients.

**Blood**

The demonstration of amastigotes in blood may be particularly easy in HIV-infected patients, who often carry many more amastigotes in their peripheral circulation than their HIV-negative counterparts (Fillola et al., 1992; Martínez et al., 1993). As most of the amastigotes lie within phagocytic leucocytes, the probability of detecting leishmanial infection in blood samples may be greatly increased by making leucocyte concentrates, using methods that are easy, fast and inexpensive. The older leucoconcentration method (Izri et al., 1993; Box 1) does not require a cytocentrifuge but the newer leuco cytoconcentration (LCC) method (Izri et al., 1996; Box 2 and Figure 1) permits the parasite burden in the blood (and any post-treatment changes in it) to be quantified. The sensitivity of examining LCC smears for amastigotes appears at least as high as the examination of BMA smears (unpubl. obs.) and patients are far happier having blood samples collected than BMA.

Molina et al. (1992) described the use of laboratory-bred sandflies of the vector species to collect blood samples. By maintaining the blood-fed flies for several days and then dissecting them, leishmanial infection in the bloodmeal source can often be revealed. This xenodiagnostic technique appears sensitive, and isolation of the parasites, by subculture of samples from the sandfly gut, is possible. It is a fairly complex technique, however, and the production of infected sandflies clearly carries the risk of human infection. The use of a sealed insectary with double doors and the careful killing of all the insects used are recommended by the CDC.

**Other Biopsies**

The efficiency of examining other biopsies for amastigotes, under the microscope, varies widely. It depends on the species of *Leishmania* involved and the host’s responses to the infection (and therefore on HIV status). In HIV-positives co-infected with *Leishmania*, the leishmanial parasites are often widespread
and often in sites, such as the digestive tract, healthy skin, and broncho-alveolar fluid, that would be very unusual in the immunocompetent. It is sometimes the symptoms caused by infection in these odd localities that lead to the leishmaniasis and the HIV infection first being detected.

**SKIN**

Samples of skin are probably best obtained, after local anaesthesia with 1% xylocaine, with a 5-mm punch that takes all layers of the tissue. If the biopsy is to be used for culture, the patient’s skin should first be cleaned with 70% ethanol (not iodized antisepsics, which can kill the parasites). When there is an ulcerated lesion, the sample should be collected from the raised edge of the lesion. Histological examination and the isolation of parasites by culture, with a minimum risk of bacterial contamination, are generally possible. Scraping or aspirates...
are used when punch biopsy is impossible. For an aspirate, a little sterile saline is injected into the dermis and then drawn back.

LIVER AND SPLEEN
Whether or not the patient is HIV-positive, liver and spleen biopsies can be particularly dangerous because of thrombopenia, and should only attempted as a last resort, when all other methods of diagnosis have failed.

LYMPH NODES
In HIV-positive patients presenting with a lymphadenopathy, the microscopical examination of lymph-node aspirates or biopsies offers a sensitivity similar to that of BMA smears (Alvar et al., 1997).

UNUSUAL TISSUES
It would clearly not be rational to take samples of other tissues unless there are signs or symptoms of unknown origin, indicating the involvement of the tissues in the observed morbidity. Biopsies from other tissues may offer the first hint that an HIV-infected patient has leishmaniasis or, if amastigotes are found, that the patient is HIV-positive. It should be noted that zymodemes of *L. infantum* that are only dermatropic in the immunocompetent may be viscerotropic in the HIV-positive, and vice versa (Pratlong et al., 1995).

Isolation of Parasites
Wherever and whenever possible, attempts should be made to culture the parasites causing each case of VL, so that they can be investigated in more detail and perhaps cryopreserved. As a diagnostic method, culture is generally more sensitive that the microscopical examination of smears of the same tissues or fluids. Cultures can be made directly, using human samples, or indirectly, using samples from laboratory hosts that have been inoculated with samples from the cases of VL. The parasites are almost always cultured as promastigotes, at about 27°C.

CULTURE
Although liquid media such as RPMI or Schneider’s *Drosophila* medium, both supplemented with heat-inactivated foetal calf serum (FCS), are good media for the maintenance of parasites in the laboratory, they are generally found to be inferior to very rich, diphasic blood-agar media when parasites are first being isolated (López-Vélez et al., 1995; Dereure et al., 1998). In France, NNN (Novy, McNeal, Nicolle) medium, made of agar containing 10% defibrinated rabbit blood, with an overlay of Schneider’s medium supplemented with antibiotics and FCS is currently the first choice (Box 3). The use of a mix of FCS and Schneider’s medium (with antibiotics) as an overlay dramatically enhances the probability of a successful isolation, especially for samples collected from HIV-positives and/or after antileishmanial treatment. Even strains known for their difficult growth, such as *L. braziliensis*, have been successfully isolated in this medium. Once a culture has been set up, duplicate subcultures every 7 days seem to offer the best chance of isolating the parasites. For direct culture, samples of bone marrow, whole blood or buffy coats are usually inoculated. Punch biopsies of healthy skin or skin lesions may also often be successful but some biopsies, such as those of the gut, usually have too much bacterial or fungal contamination for direct culture.

Whole blood or buffy coats are easy to obtain from HIV-patients and give good results in VL co-infection; cultures set up with 1 ml citrated blood seem to be slower to develop than those set up with (a typically smaller sample) of BMA but are more likely to be found positive (unpubl. obs.).

SUBCULTURE AFTER ANIMAL INOCULATION
Samples from suspected cases, even those with too much bacterial or fungal contamination
For direct culture, may be used to inoculate susceptible laboratory hosts — usually golden hamsters (*Mesocricetus auratus*). Ideally, for VL, 0.5 ml citrated blood or 0.2 ml BMA should be injected into the hamster intraperitoneally. When cutaneous or mucocutaneous leishmaniasis is suspected, a skin biopsy is ground in saline supplemented with antibiotics and a sample of the suspension (0.1 ml) is injected into the foot or nose of a hamster. Ideally, the mode of inoculation should reflect the type of parasite involved (i.e. whether it is likely to be dermatotropic or viscerotropic, given the symptoms of the case and the geographical region), but this may not be obvious, especially if the suspected case is immunocompromised. For HIV-positive cases, intraperitoneal and intra-dermal inoculations in different hamsters are advisable. The parasites, which multiply in the hamster, are usually recovered 6 months (range = 3–12 months) post-inoculation. Use of hamsters and their maintenance for several months makes indirect culture much more expensive than direct. It does, however, allow small number of parasites to multiply into large numbers and allow the in-vivo behaviour of the parasites to be determined before they have been modified by in-vitro culture.

Culture and animal inoculation are often complementary (Table 5). Whatever the species or variant of *Leishmania* involved,

<table>
<thead>
<tr>
<th>Hamster inoculation</th>
<th>Direct culture</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>109</td>
</tr>
<tr>
<td>Negative</td>
<td>Positive</td>
<td>38</td>
</tr>
<tr>
<td>Positive</td>
<td>Negative</td>
<td>40</td>
</tr>
<tr>
<td>Negative</td>
<td>Negative</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 5. Comparative performances of the inoculation of hamsters and the direct culture of samples in NNN medium, observed when 187 samples collected, at first diagnosis of visceral leishmaniasis and during follow-up, from 35 patients with *Leishmania infantum/HIV* co-infection were tested.
some parasites do not grow directly on NNN and can only be obtained by animal inoculation. Conversely, a few viscerotropic isolates that fail to visceralize in hamsters or other susceptible animals (such as BALB/c mice for example) can be isolated by direct culture.

**Definitions**

Although the above discussion of parasitological diagnosis mentions few particular *Leishmania* species by name, this was intentional. In those co-infected with HIV, the designation of a particular species or zymodeme as ‘dermatotropic’ or ‘viscerotropic’ can be misleading, and the relationship between the host and the parasite must often be reconsidered. In particular, the way in which parasites behave after antileishmanial treatment (i.e. during a relapse) appears to depend more on the host’s immunological status than on the species or variant of *Leishmania* present. Although, in HIV-infected individuals, just two species — *L. donovani* and particularly *L. infantum* — account for most cases of generalized leishmaniasis, other species have been implicated as the causes of the visceral or localized disease (Pratlong *et al*., 1995).

**Molecular Diagnosis**

Molecular diagnosis consists of the detection, by hybridization or, more often than not, by amplification, of *Leishmania* nucleic acids.

**Hybridization**

Leishmanial DNA may be detected *in situ* by hybridization with radio-isotope- or enzyme-labelled probes. The performance of this technique, which is suitable for detecting parasites in smears of human samples and sandflies and in histological preparations of human tissue, may be enhanced by genomic amplification (i.e. in-situ PCR) and/or the use of a chemiluminescent detection system (Wilson *et al*., 1992).

**Genomic Amplification by PCR**

The potential usefulness of PCR-based genomic amplification in the diagnosis and follow-up of leishmaniasis in immunocompetent individuals was demonstrated by Weiss (1995). The use of such amplification in the diagnosis of *Leishmania* infection in the HIV-positive has, however, only recently been explored.

**Detection of Circulating Antigens**

The routine diagnosis of leishmaniasis has not been based on the serological detection of *Leishmania*-specific antigens, although such antigens have been found in blood and urine samples. Mary *et al.* (1993), for example, identified a 51-kDa leishmanial protein in 30% of blood samples from VL cases. Two polypeptide fractions, of 72–75 kDa, were detected in 14 out of 15 urine samples from confirmed cases of VL (De Colmenares *et al*., 1995). These preliminary results merit further investigation, indicating that antigen-detection assays may be useful in diagnosis and that decreasing concentrations of leishmanial antigens may be good markers of the efficacy of chemotherapy.
as previously described (Piarroux et al., 1994). Briefly, a 100-μl test sample was mixed with 300 μl preparation buffer consisting of 20% Chelex, 1% Nonidet P-40 (Sigma) and 1% Tween 20 (Sigma) in distilled water. This mixture was then heated for 20 min at 96°C before being centrifuged at 13,000 × g for 10 min at room temperature. The supernatant solution was then either used immediately for PCR amplification or stored at −20°C.

The two primers used for the genomic amplification recognized a 250-bp sequence of nuclear DNA present in all Old-World Leishmania species and L. chagasi (Piarroux et al., 1995). DNA from L. braziliensis, which should not be amplified by the primers, was used as a negative control in each set of amplifications (Minodier et al., 1997).

RESULTS. Some of the samples investigated came from 69 HIV-infected patients who had no previous history of VL. Fourteen of the patients were, however, suffering from their first episode of VL when the samples were collected but the other 55 were found negative in all tests used for the diagnosis of VL, including the PCR (Table 6). The combined results of direct examination and culture were used as the ‘gold standard’. That is, only the 14 cases who were found BMA- and/or blood-positive for leishmanial infection by direct examination and/or culture were considered true positives. PCR gave three false-negative results (two for blood and one for BMA) but no false-positive. The positive predictive value and specificity of the PCR were both therefore 100%. The three false-negative results probably resulted from the presence of inhibitory factors in the samples. The PCR’s sensitivity was better for BMA (93%) than for blood (86%) but neither of these sensitivities was as high as the corresponding values for culture (100% for BMA and 93% for blood).

Further samples of BMA and blood were collected from 77 cases of Leishmania/HIV co-infection when they were suspected to be suffering from VL relapses. The combined results of direct examination and culture, which indicated 15 true positives, were again used as the ‘gold standard’ (Table 7). Again, the PCR gave no false-positive results for either blood or BMA and therefore had a positive predictive value and specificity of 100%. However, the sensitivity of the PCR in the tests of the ‘relapse’ samples was higher than that of direct examination or culture, whether the samples tested were of blood or BMA. All of the samples found false-negative (by PCR, direct examination or culture) had been collected relatively late in the relapse and 5 days after the first treatment of the relapse with an antileishmanial drug. Ideally, blood samples and BMA for the diagnosis of VL should be collected before any antileishmanial treatment, and blood samples for the confirmation of a VL relapse should be collected while the patient is febrile.

Discussion

PCR-based techniques appear reliable for the diagnosis of VL, offering sensitivities at least equal and often superior to those of the other techniques available. In the diagnosis of VL in HIV-positives, they should be preferred to the serodiagnostic methods that have their performance reduced as the result of the HIV-induced immunosuppression.

For the PCR-based diagnosis of VL, no consensus exists on the best target sequence (Weiss, 1995). Although the repeated sequences of the minicircle (Ashford et al., 1995) or maxicircle kinetoplast DNA (Lee et al., 1995) are often selected, they may show considerable inter-isolate variation. The repeated nuclear DNA sequences tend to show less intra-specific variation and are therefore probably more useful for diagnosis (Piarroux et al., 1994). Many other targets, such as the Leishmania-specific sequences coding for gp 63 (Button and McMaster, 1988) and a 51-kDa antigen (Berrahal et al., 1996), have been tested. Primers based on the small-subunit ribosomal RNA gene, which can be used to identify the species of
### TABLE 6. The performances of direct microscopical examination, culture and PCR in the diagnosis of first episodes of visceral leishmaniasis in patients co-infected with HIV

<table>
<thead>
<tr>
<th>Sample</th>
<th>Method</th>
<th>No. of patients</th>
<th>Performance index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>True positive</td>
<td>Truly negative</td>
<td>Falsely positive</td>
</tr>
<tr>
<td>Blood</td>
<td>Direct examination</td>
<td>11</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Culture</td>
<td>13</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>12</td>
<td>55</td>
</tr>
<tr>
<td>Bone-marrow aspirate</td>
<td>Direct examination</td>
<td>12</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Culture</td>
<td>14</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>13</td>
<td>55</td>
</tr>
</tbody>
</table>

### TABLE 7. The performances of direct microscopical examination, culture and PCR in the diagnosis of visceral leishmaniasis relapses in patients co-infected with HIV

<table>
<thead>
<tr>
<th>Sample</th>
<th>Method</th>
<th>No. of patients</th>
<th>Performance index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>True positive</td>
<td>Truly negative</td>
<td>Falsely positive</td>
</tr>
<tr>
<td>Blood</td>
<td>Direct examination</td>
<td>10</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Culture</td>
<td>12</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>14</td>
<td>62</td>
</tr>
<tr>
<td>Bone-marrow aspirate</td>
<td>Direct examination</td>
<td>12</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Culture</td>
<td>12</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>14</td>
<td>62</td>
</tr>
</tbody>
</table>
Leishmania that is present, have also been proposed (Looker et al., 1988; Van Eys et al., 1992; Costa et al., 1996). The choice of target sequence depends in part on the aims of the study (e.g. individual diagnosis or epidemiological investigation) and sometimes whether good sensitivity or good specificity is the more important. Whatever the primers used, it is important that the numbers of false-positives and particularly the numbers of false-negatives are kept low and that a standardized protocol is carefully followed. The use of enzymatic decontamination and an internal control are also essential. It is possible to optimise a PCR-based diagnostic method by a combination of ELISA and microtitre-plate hybridization (Costa et al., 1996). A ‘real-time’ PCR has recently been developed to evaluate the parasite burden in particular organs (Bretagne et al., 2001) and may perhaps form the basis of a fast and reliable diagnostic test.

As blood samples appear as useful as BMA for PCR (Nuzum et al., 1995; Costa et al., 1996; Piarroux et al., 1996), there seems little justification in collecting BMA for genomic amplification, especially from AIDS patients who require numerous, painful and invasive investigations for their multiple pathologies. Although PCR-based diagnosis may be relatively expensive, its cost has to be weighed against its good sensitivity and rapidity and the use of blood samples (that can be more simply and less painfully collected) rather than bone-marrow or other aspirates.

CONCLUSIONS

The biological diagnosis of leishmaniasis is possible in patients co-infected with HIV and often no more difficult than in HIV-negative individuals. The unusually wide dissemination of large numbers of parasites seen in many HIV-positive cases may, in fact, increase the probability that the parasites will be found on smears or by culture. In VL-endemic areas, clinicians faced with an ill HIV-positive patient should always consider leishmaniasis, even if the typical symptoms of VL in the immunocompetent are absent, and request the relevant laboratory tests. Unlike serodiagnosis, PCR-based techniques may give equally reliable results whether the patient is co-infected with HIV or not. Although IFAT and the rK39-ELISA appeared less sensitive when used on co-infected cases than ELISA based on crude leishmanial antigens, they were highly specific and could still be useful tools for diagnosing VL in HIV-positive patients. A positive result in a serodiagnostic or PCR-based test should ideally be confirmed by the demonstration of the parasites themselves, by direct examination, culture and/or animal inoculation. In any given setting, the choice of which serological technique to employ, if any, depends in part on the laboratory equipment and money available. If diagnosis is to be serological, a combination of two complementary techniques, such as IFAT and western blotting, is recommended.

Those who must decide which procedure to use for the parasitological diagnosis of leishmaniasis must consider the performance, ease of use and cost–benefit ratio of each technique and the possibility of culture. Figure 2 indicates a reasonable protocol, for samples from HIV-positives or HIV-negatives.

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FIG. 2. A recommended procedure for the parasitological diagnosis of leishmaniasis.
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blotting as a valuable tool to differentiate human visceral leishmaniasis from lymphoproliferative disorders and other clinically similar diseases. *Research in Immunology, 143*, 375–383.


