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EVALUATION OF A NEW RECOMBINANT K39 RAPID DIAGNOSTIC TEST FOR SUDANESE VISCERAL LEISHMANIASIS

KOERT RITMEIJER,* YOSEPH MELAKU, MARIUS MUELLER, SAMMY KIPNGETICH, CAROLINE O'KEEFFE, AND ROBERT N. DAVIDSON

Médecins sans Frontières-Holland, Amsterdam, The Netherlands; Department of Infection and Tropical Medicine, Northwick Park Hospital, Harrow, United Kingdom

Abstract. A new rK39 rapid diagnostic dipstick test (DiaMed-IT-Leish®) was compared with aspiration and a direct agglutination test (DAT) for diagnosis of visceral leishmaniasis (VL) in 201 parasitologically confirmed cases, 133 endemic controls, and in 356 clinical suspects in disease-endemic and -epidemic areas in Sudan. The sensitivity of the rK39 test in parasitologically confirmed VL cases was 90%, whereas the specificity in disease-endemic controls was 99%. The sensitivity of the DAT was 98%. In clinically suspected cases, the sensitivity of the rK39 test was 81% and the specificity was 97%. When compared with the diagnostic protocol based on the DAT and aspiration used by Médecins sans Frontières in epidemic situations, the positive predictive value was 98%, and the negative predictive value was 71%. This rK39 rapid diagnostic test is suitable for screening as well as diagnosis of VL. Further diagnostic work-up of dipstick-negative patients with clinically suspected VL is important. The ease and convenience of the dipstick test will allow decentralization and improved access to care in disease-endemic areas in Sudan.

INTRODUCTION

Clinical diagnosis of visceral leishmaniasis (VL, kala-azar) is inaccurate. In our experience in Sudan, approximately 50% of patients meeting the clinical case definition of VL have the disease; the remainder are diagnosed with other illnesses such as malaria, typhoid, tuberculosis.

The ultimate confirmation of visceral leishmaniasis is by demonstration of *Leishmania donovani* amastigotes in stained smears from spleen, lymph glands, or bone marrow aspirates. Splenic aspirates are more sensitive (96%) than aspirates of bone marrow (70%) or lymph nodes (58%).[1] During the last 15 years, Médecins Sans Frontières (MSF)–Holland has treated more than 58,000 cases of VL in Sudan under harsh field conditions. The use of aspirates in these epidemic and highly endemic settings with large numbers of patients is limited because of the time and skills required in preparing and reading the slides. Due to the small but definite risk of internal bleeding, the use of splenic aspirates in this setting is further restricted. Bone marrow and lymph node aspirates are safer, but the material obtained is more dilute than in a splenic aspirate and thus less sensitive diagnostically. Moreover, bone marrow sampling is inconvenient and often painful for the patient. Many patients would be denied life-saving treatment on the basis of false-negative aspirates if bone marrow or lymph node aspirations were the only diagnostic tool. These characteristics make diagnostic aspiration of any tissue unsuitable as a routine screening test for VL for large numbers of patients in a field setting.

Serologic tests have been developed for VL, and the direct agglutination test (DAT) is used in the Sudan as the first-line diagnostic tool.[2] DAT titers ≥1:3,200 have a sensitivity of 94% and a specificity of 72% for active disease.[1] Lower titers are less specific because of non-specific agglutination and high prevalence of antibodies to *Leishmania* because of subclinical and/or past infections with *Leishmania* species in this area. Longitudinal studies in a highly endemic village in eastern Sudan between 1991 and 1996 show a ratio of clinical to subclinical infection between 1.2:1 and 2.6:1, in which *Leishmania* antibodies are present without illness.[3,4] When large numbers of patients are being assessed for kala-azar, e.g., during epidemics, high DAT titers (≥1:6,400) plus a typical clinical picture may be used as a surrogate method for diagnosing kala-azar without aspiration, giving a high predictive value. Conversely, a low DAT titer (≤1:400), effectively rules out kala-azar.[5] Thus, the DAT can be used at a high titer as a diagnostic test, and at a low titer as a screening test. In the situation where the patient has a DAT titer >1:400 but <1:6,400, aspirations are done to confirm a diagnosis of leishmaniasis. The MSF uses this diagnostic protocol, which reduces the number of aspirates by approximately 80%.[6]

Although the DAT was specifically developed for epidemic field situations, it is still difficult to use in remote conditions. Performance of this test requires a proper laboratory with skilled technicians to perform correct titer setting, cross-checking with controls, meticulous implementation, and overnight incubation. In the field where a laboratory cannot be established, samples have to be transported to a central laboratory, typically resulting in a delay in starting treatment of 1–2 weeks. Improvements have been made by the introduction of freeze-dried antigen, which replaces the unstable aqueous antigen.[7] However, there is an urgent need for a simpler, faster, non-invasive, but reliable test to screen for and diagnose kala-azar at the bedside.

Serologic tests have been developed using the cloned antigen rK39 instead of whole *Leishmania* parasites. The rK39 antigen, which consists of 39 amino acid repeats of a kinesin-like gene found in *L. chagasi*, is used in an enzyme-linked immunosorbent assay (ELISA) format and has shown satisfactory results in India, Brazil, and the Mediterranean.[8–13] Several recombinant K39 antigen-based dipstick formats have been developed for rapid field diagnostics by different companies (InBios Inc., Seattle, WA; Arista Biologicals Inc., Allentown, PA; and Amrad ICT, Richmond, Victoria, Australia). However, the latter two companies no longer produce these tests. These dipstick formats showed high sensitivity and specificity in India, Nepal, Bangladesh, and Brazil.[14–20] In Sudan, previous versions of rK39 antigen-based tests showed less satisfactory results. An rK39-based ELISA had a sensitivity of 93% in Sudanese VL patients.[21] However, different

* Address correspondence to Koert Ritmeijer, Médecins sans Frontiéres-Holland, P.O. Box 10014, 1001 EA, Amsterdam, The Netherlands. E-mail: koert.ritmeijer@amsterdam.msf.org

dipstick formats showed either good specificity but poor sensitivity,[22] or good sensitivity but poor specificity,[23] making these rapid tests unreliable for diagnosis of kala-azar under field conditions. The performance of different K39-based tests in different regions is summarized in Table 1. We evaluated a commercially produced, rK39 antigen-based, rapid diagnostic test in highly endemic and epidemic regions in Sudan.

MATERIALS AND METHODS

Study sites. The evaluation was carried out in three MSF-Holland kala-azar treatment centers in eastern and southern Sudan (Um el Kher in Gedaref State, Lankien in Bieh State, and Bimbim in Latjor State) between November 2003 and April 2004. These centers are in highly endemic areas, where regular epidemic outbreaks occur.[24] During the six months of the study these three centers treated 3,342 new cases of kala-azar.

Patients. Patients fulfilling the clinical case definition for suspected kala-azar (fever for >2 weeks with malaria excluded, wasting, and either splenomegaly or lymphadenopathy) were further evaluated. In this study, we only considered patients with no history of previous treatment of kala-azar. All patients were tested by the DAT. A DAT titer ≥1:6,400 in patients satisfying the clinical case definition was regarded as diagnostic of VL. Those with borderline DAT titers (1:800–1:3,200) underwent further parasitologic examination by aspiration of spleen or lymph nodes. In addition, extra measures were taken for patients who appeared severely ill to make a diagnosis as quickly as possible: they underwent aspiration on the same day as the DAT, and if the initial aspirate was negative, a repeat aspiration was performed the following day with or without a repeat DAT after one week. Kala-azar was diagnosed after a positive aspirate and/or a positive DAT (≥1:6,400). Patients were treated according to our standard protocol.[6,25] Patients with a negative DAT titer (≤1:400) suspected of having VL were evaluated for alternative diagnoses.

A parasitologically confirmed case of kala-azar was defined as a patient with positive parasitologic results on either a spleen or lymph node aspiration smear. A control was defined as a patient admitted to the MSF health centers for other reasons than kala-azar, and who had no clinical signs and symptoms of VL, no history of previous treatment of kala-azar, and a negative DAT test result. The studies were performed after review and approval from the regional health authorities. Informed consent was obtained from the study subjects.

RK39 rapid dipstick test. The rK39 rapid diagnostic test kit (DiaMed-IT Leish®; DiaMed AG, Cressier sur Morat, Switzerland) is composed of a nitrocellulose membrane coated with a line of recombinant antigen K39 across the strip. Antibodies to *Leishmania* present in a blood sample react with K39 antigen, and their presence is shown by mouse anti-human antibody conjugated to an indicator. A finger prick sample (20 μL) of blood is added to a well and mixed with a drop of buffer. A DiaMed-IT Leish test strip is placed vertically in the well, and the diluted blood rises up the nitrocellulose strip. After the blood is completely wicked up, the strip is transferred to the next well, which contains a few drops of wash buffer, and allowed to clear. The entire process takes approximately 20 minutes and results are read visually. A control line at the top of the strip verifies that the test strip is functional. If this is the only line that appears, then the test result is considered negative; two lines are positive for VL.

Parasitologic diagnosis. Parasitologic confirmation of VL was established by microscopic demonstration of *Leishmania* amastigotes in Giemsa-stained smears from either spleen or lymph node aspirates. A parasite density score was determined using a scale ranging from 0 (no parasites per 1,000 oil-immersion fields) to +6 (>100 parasites per field).[26]

Direct agglutination test. The DAT was performed as previously described using freeze-dried antigen (*L. donovani*

TABLE 1
Overview of rK39 antigen-based test results for visceral leishmaniasis (VL)*

Location/Name	Year	Country	Format	Company	Total sample	VL cases	Sensitivity (%)	Specificity (%)
South Asia								
Sundar S	1998	India	Dipstick	Arista	344	127	100	98
Bern C	2000	Nepal	Dipstick	Inbios	127	14	100	100
Qu JQ	2000	China	Dipstick	Inbios	13	13	100	
Singh S	2002	India	Dipstick	Inbios	308	228	100	100
Sarker CB	2003	Bangladesh	Dipstick	Amrad	180	60	97	98
Chappuis F	2003	Nepal	Dipstick	Inbios	184	139	97	71
Boelaert M	2004	Nepal	Dipstick	Inbios	310		87	93
Latin America								
Delgado O	2001	Venezuela	Dipstick	Inbios	117	41	88	100
Braz RF	2002	Brazil	ELISA	Inbios	208	120	93	99
Carvalho SG	2003	Brazil	Dipstick	Inbios	188	128	90	100
Mediterranean								
Ozensoy S	1998	Turkey	ELISA		71	24	96	94
Maalej IA	2003	Tunisia	ELISA	Inbios	146	38	100	97
Sudan								
Zijlstra EE	1998	Sudan	ELISA	Inbios	15	15	93	
Zijlstra EE	2001	Sudan	Dipstick	Arista	116	55	67	97
Veeken H	2003	Sudan	Dipstick	Amrad	77	50	92	59
MSF unpublished	1999	Sudan	Dipstick	Inbios	91	60	67	87
Present study	2004	Sudan	Dipstick	DiaMed	338	201	90	99

* ELISA = enzyme-linked immunosorbent assay.

promastigotes) and control sera with known titers from the Royal Tropical Institute (Amsterdam, The Netherlands).[7] Samples with known DAT titers were included as standards. The titer of the sample is expressed as the highest dilution at which agglutination is still visible.

Statistical analysis. Data entry and statistical analysis was done using Epi-Info (Centers for Disease Control and Prevention, Atlanta, GA). Proportions were compared using the chi-square test with Yates' correction, or Fisher's exact test, where appropriate. Sensitivity, specificity, and positive predictive values were estimated with exact binomial 95% confidence intervals (CIs).

RESULTS

Performance of rK39 dipstick in confirmed VL cases and endemic controls. A total of 201 parasitologically confirmed VL cases (152 in Um-el-Kher and 49 in Lankien; 157 by lymph node aspirate and 44 by splenic aspirate) were evaluated with the dipstick; 180 of 201 were positive. Thus, the sensitivity of the dipstick in these parasitologically confirmed cases was 89.6% (95% CI = 84.5–93.4%). No significant difference observed in sensitivity between the Um-el-Kher and Lankien sites (91.4% versus 83.7%; $P = 0.20$).

Among the 201 parasitologically confirmed cases, 197 had a positive DAT test result (titer ≥ 1:6,400). Thus, the sensitivity of the DAT at high titer was 98.0% (95% CI = 95.0–99.5%), which was significantly higher than the rK39 dipstick ($P = 0.001$). Using a titer > 1:400, the sensitivity of the DAT was 99.0% (199 of 201).

A total of 133 endemic controls were recruited. Only one control had a positive rK39 dipstick test result, but this control also had a negative lymph node aspirate and a negative DAT result. Thus, the specificity was 99.2% (95% CI = 95.9–100%) (Table 2).

All cases and controls at the Um-el-Kher site were systematically checked for malaria by blood smear. Of the confirmed cases, 21.7% had malaria parasitemias. Of the endemic controls, 29.0% had malaria parasitemias ($P = 0.34$). Malaria prevalence was identical in persons with true-positive and false-positive rK39 dipstick results ($P = 1.00$).

Performance of rK39 dipstick in suspected VL cases. A total of 356 consecutive, unselected clinically suspected kala-azar cases in Lankien and Latjor sites were prospectively evaluated. Fifteen suspected cases with borderline positive DAT results (titers = 1:800–1:3,200) were excluded from the analysis because their diagnosis remained uncertain; no aspiration could be done under prevailing field conditions. Among the remaining 341 individuals the rK39 dipstick was compared with diagnosis by high-titer DAT, positive aspirate, or both. The dipstick had a sensitivity of 80.7% (95% CI = 75.2–85.6%), a specificity of 97.3% (95% CI = 92.4–99.4%), a positive predictive value of 98.4% (95% CI = 95.4–99.7%), and a negative predictive value of 71.4% (95% CI = 63.6–78.4%) (Table 3).

TABLE 2

Results of the rK39 dipstick test in 201 confirmed visceral leishmaniasis cases and 133 endemic controls

Dipstick	Cases	Controls
Positive	180	1
Negative	21	132
Total	201	133

DISCUSSION

When evaluating a new test, it is difficult to be sure whether disagreements arise from inaccuracy in the new test or in existing tests. The high sensitivity (90%) of the DiaMed-IT Leish rK39 rapid diagnostic test in parasitologically confirmed cases means that it is a good screening test for kala-azar in the Sudan, although is significantly less sensitive than the DAT (sensitivity = 98–100% in this study). Sensitivity is not influenced by presence of malaria. The lower sensitivity of the dipstick test when compared with the standard MSF diagnostic protocol (based on the DAT and aspiration) than if compared with parasitologically confirmed VL cases may be attributable to the specificity of the DAT test. Several studies in Sudanese kala-azar have shown specificity estimates of the DAT between 72% and 99%, which may result in significant overdiagnosis among clinically suspected patients.[5,22,27–29]

The rK39 dipstick has high specificity (99% in endemic controls and 97% in clinically suspected patients), making overdiagnosis of kala-azar unlikely, despite the high background prevalence of antibodies against *L. donovani* in this region.[3,4] The lower specificity found in aspirate-negative clinical suspects may be attributable to the low sensitivity of lymph node aspirates,[1] which results in possible false-negative aspirate results.

Our standard diagnostic work-up has been based on microscopy and the DAT, both of which require laboratory services with well-trained laboratory technicians. In situations where field laboratories cannot be established (for reasons of security or capacity), transportation of filter papers containing blood samples for DAT testing from peripheral units to centralized laboratories often results in an unacceptable treatment delay. Simple, rapid, and cheap serologic dipstick tests are therefore urgently required. The DiaMed-IT Leish can be used with whole capillary blood, does not require a cold chain, and can be stored for least one year under the high field temperatures in the Sudan (25–40°C). Community health workers in peripheral health units can easily perform the test. A positive dipstick test result in a clinically suspected case of kala-azar can be considered confirmation of the disease with a high level of certainty, and the patient can be treated immediately. Especially in southern Sudan, where access to kala-azar care is very poor, kala-azar patients often come for treatment after a prolonged symptomatic period in a critical

TABLE 3

Results of the rK39 dipstick test compared with Médecins Sans Frontières diagnostic protocol (DAT ≥ 1:6,400 or aspirate positive) in 341 clinical suspects*

Dipstick	DAT positive (≥ 1:6,400) and/or positive aspirate	DAT negative (≤ 1:400) or DAT borderline with negative aspirate	Total
Positive	184	3	187
Negative	44	110	154
Total	228	113	341

* DAT = direct agglutination test.

condition.[30] Decentralized diagnosis will allow active case-finding, reduce delays, and improve chances of survival. Because the sensitivity of the rK39 dipstick is 90%, 10% of kala-azar patients (dipstick false-negative results) may die without treatment if the disease is not otherwise confirmed. Therefore, a backup protocol (using the DAT and/or aspiration) remains essential for clinically suspect VL cases with a negative rK39 dipstick test results. Therefore, rapid parasite antigen tests or *Leishmania* DNA/RNA detection tests might be an important improvement in serodiagnosis. This is particularly important in view of increasing human immunodeficiency virus/VL co-infection rates in eastern Africa, which are expected to reduce the validity of serologic tests.[11]

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Authors' addresses: Koert Ritmeijer, Médecins sans Frontières-Holland, P.O. Box 10014, 1001 EA, Amsterdam, The Netherlands, Telephone: 31-20-5208700, Fax: 31-20-6205170, E-mail: koert.ritmeijer@amsterdam.msf.org. Yoseph Melaku and Sammy Kipngetich, Médecins sans Frontières-Holland, Fourth Floor, Chaka Place off Argwings Khodhek Road, Hurlingham, P.O. Box 40643, Nairobi, Kenya. Marius Mueller and Caroline O'Keeffe, Médecins sans Frontières-Holland, Steet 33, Amarat, P.O. Box 3319, Khartoum, Sudan. Robert N. Davidson, Infection and Tropical Medicine, Northwick Park Hospital, Harrow HA1 3UJ, United Kingdom.

REFERENCES

1. Zijlstra EE, Siddig Ali M, El-Hassan AM, El-Toum IA, Satti M, Ghalib HW, Kager PA, 1992. Kala-azar: a comparative study of parasitological methods and the direct agglutination test in diagnosis. *Trans R Soc Trop Med Hyg 86:* 505–507.
2. Harith AE, Kolk AH, Kager PA, Leeuwenburg J, Muigai R, Kiugu S, Laarman JJ, 1986. A simple and economical direct agglutination test for the serodiagnosis and sero-epidemiological studies of visceral leishmaniasis. *Trans R Soc Trop Med Hyg 80:* 583–586.
3. Zijlstra EE, El-Hassan AM, Ismael A, Ghalib HW, 1994. Endemic kala-azar in eastern Sudan: a longitudinal study on the incidence of clinical and subclinical infection and post kala-azar dermal leishmaniasis. *Am J Trop Med Hyg 51:* 826–836.
4. Khalil EAG, Zijlstra EE, Kager PA, El Hassan AM, 2002. Epidemiology and clinical manifestations of *Leishmania donovani* infection in two villages in an endemic area in eastern Sudan. *Trop Med Int Health 7:* 35–44.
5. Boelaert M, El Safi S, Jacquet D, De Muynk A, van der Stuyft P, Le Ray D, 1999. Operational validation of the DAT for diagnosis of visceral leishmaniasis. *Am J Trop Med Hyg 60:* 129–134.
6. Medecins sans Frontieres, 2004. *MSF Manual for the Diagnosis and Treatment of Visceral Leishmaniasis (Kala-Azar) under Field Conditions*. Amsterdam: MSF-Holland.
7. Meredith SE, Kroon NC, Sondorp E, Seaman J, Goris MG, Ingen van CW, Oosting H, Schoone GJ, Terpstra WJ, Oskam L, 1995. Leish–KIT, a stable direct agglutination test based on freeze dried antigen for serodiagnosis of visceral leishmaniasis. *J Clin Microbiol 33:* 1742–1745.
8. Singh S, Gilman-Sachs A, Chang KP, Reed SG, 1995. Diagnostic and prognostic value of K39 recombinant antigen in Indian leishmaniasis. *J Parasitol 81:* 1000–1003.
9. Badaro R, Benson D, Eulalio MC, Freire M, Cunha S, Netto EM, Pedral-Sampaio D, Madureira C, Burns JM, Houghton RL, David JR, Reed SG, 1996. rK39: a cloned antigen of *Leishmania chagasi* that predicts active visceral leishmaniasis. *J Infect Dis 173:* 758–761.
10. Ozensoy S, Ozbel Y, Turgay N, Alkan MZ, Gul K, Gilman-Sachs A, Chang KP, Reed SG, Ozcel MA, 1998. Serodiagnosis and epidemiology of visceral leishmaniasis in Turkey. *Am J Trop Med Hyg 59:* 363–369.
11. Medrano FJ, Canavate C, Leal M, Rey C, Lissen E, Alvar J, 1998. The role of serology in the diagnosis and prognosis of visceral leishmaniasis in patients co-infected with human immunodeficiency virus type 1. *Am J Trop Med Hyg 59:* 155–162.
12. Houghton RL, Petrescu M, Benson DR, Skeiky YA, Scalone A, Badaro R, Reed SG, Gradoni L, 1998. A cloned antigen (recombinant K39) of *Leishmania chagasi* diagnostic for visceral leishmaniasis in human immunodeficiency virus type 1 patients and a prognostic indicator for monitoring patients undergoing drug therapy. *J Infect Dis 177:* 1339–1344.
13. Maalej IA, Chenik M, Louzir H, Ben Salah A, Bahloul C, Amri F, Dellagi K, 2003. Comparative evaluation of ELISAs based on ten recombinant or purified *Leishmania* antigens for the serodiagnosis of Mediterranean visceral leishmaniasis. *Am J Trop Med Hyg 68:* 312–320.
14. Sundar S, Reed SG, Singh VP, Kumar PC, Murray HW, 1998. Rapid accurate field diagnosis of Indian visceral leishmaniasis. *Lancet 351:* 563–565.
15. Chappuis F, Rijal S, Singh R, Acharya P, Karki BMS, Das ML, Bovier PA, Desjeux P, Le Ray D, Koirala S, Loutan L, 2003. Prospective evaluation and comparison of the direct agglutination test and an rK39-antigen-based dipstick test for the diagnosis of suspected kala-azar in Nepal. *Trop Med Int Health 8:* 277–285.
16. Singh S, Kumari V, Singh N, 2002. Predicting kala-azar disease manifestations in asymptomatic patients with latent *Leishmania donovani* infection by detection of antibody against recombinant K39 antigen. *Clin Diagn Lab Immunol 9:* 568–572.
17. Bern C, Jha SN, Joshi AB, Thakur GD, Bista MB, 2000. Use of the recombinant K39 dipstick test and the direct agglutination test in a setting endemic for visceral leishmaniasis in Nepal. *Am J Trop Med Hyg 63:* 153–157.
18. Boelaert M, Rijal S, Regmi S, Singh R, Karki B, Jacquet D, Chappuis F, Campino L, Desjeux P, Le Ray D, Koirala S, van der Stuyft P, 2004. A comparative study of the effectiveness of diagnostic tests for visceral leishmaniasis. *Am J Trop Med Hyg 70:* 72–77.
19. Sarker CB, Momen A, Jamal MF, Siddiqui NI, Siddiqui FM, Chowdhury KS, Rahman S, Talukder SI, 2003. Immunochromatographic (rK39) strip test in the diagnosis of visceral leishmaniasis in Bangladesh. *Mymensingh Med J 12:* 93–97.
20. Carvalho SF, Lemos EM, Corey R, Dietze R, 2003. Performance of recombinant K39 antigen in the diagnosis of Brazilian visceral leishmaniasis. *Am J Trop Med Hyg 68:* 321–324.
21. Zijlstra EE, Daifalla NS, Kager PA, Khalil EA, El Hassan AM, Reed SG, Ghalib HW, 1998. rK39 enzyme-linked immunosorbent assay for diagnosis of *Leishmania donovani* infection. *Clin Diagn Lab Immunol 5:* 717–720.
22. Zijlstra EE, Nur Y, Desjeux P, Khalil EA, El-Hassan AM, Groen J, 2001. Diagnosing visceral leishmaniasis with the recombinant K39 strip test: experience from the Sudan. *Trop Med Int Health 6:* 108–113.
23. Veeken H, Ritmeijer K, Seaman J, Davidson RN, 2003. Comparison of an rK39 dipstick rapid test with direct agglutination test and splenic aspiration for the diagnosis of kala-azar in Sudan. *Trop Med Int Health 8:* 164–167.
24. Ritmeijer K, Davidson RN, 2003. Médecins Sans Frontières interventions against kala-azar in the Sudan, 1989–2003. *Trans R Soc Trop Med Hyg 97:* 609–613.
25. World Health Organization, 1996. *Manual on Visceral Leishma-*

niasis Control. Geneva: World Health Organization. WHO/Leish/96.40.

26. Chulay JD, Bryceson AD, 1983. Quantitation of amastigotes of *Leishmania donovani* in smears of splenic aspirates from patients with visceral leishmaniasis. *Am J Trop Med Hyg 32:* 475–479.
27. Zijlstra EE, Ali MS, el-Hassan AM, el-Toum IA, Satti M, Ghalib HW, Kager PA, 1991. Direct agglutination test for diagnosis and sero-epidemiological survey of kala-azar in the Sudan. *Trans R Soc Trop Med Hyg 85:* 474–476.
28. Boelaert M, el Safi S, Goetghebeur E, Gomes-Pereira S, Le Ray D, Van der Stuyft P, 1999. Latent class analysis permits unbiased estimates of the validity of DAT for the diagnosis of visceral leishmaniasis. *Trop Med Int Health 4:* 395–401.
29. Zijlstra EE, el-Hassan AM, 2001. Leishmaniasis in Sudan. Visceral leishmaniasis. *Trans R Soc Trop Med Hyg 95(Suppl 1):* S27–S58.
30. Collin S, Davidson RN, Ritmeijer K, Keus K, Melaku Y, Kipngetich S, Davies C, 2004. Conflict and kala-azar: determinants of adverse outcomes of kala-azar among patients in southern Sudan. *Clin Infect Dis 38:* 612–619.