INTRODUCTION

Visceral Leishmaniasis (VL or Kala-azar) is a systemic parasitic disease transmitted by phlebotomine sand flies. The causative parasite in east Africa is Leishmania donovani. Visceral leishmaniasis is endemic in some regions in the southern and northern lowlands of Ethiopia. Since 1997, Médecins Sans Frontières (MSF) has established two VL treatment centers in the northwestern leishmaniasis-endemic region of Ethiopia, where 500–2,000 VL patients are treated annually. Because access to treatment is limited, incidence is probably much higher. The prevalence of human immunodeficiency virus (HIV) in the counseling and testing centers in the area is 11–13% (MSF data) and 29% among VL patients.¹

Clinical diagnosis of VL is inaccurate. The experience of MSF in eastern Africa is extensive, with more than 80,000 VL cases treated since 1989. In our centers, only approximately 50% of patients fulfilling the clinical case definition for VL (history of fever for > 2 weeks with splenomegaly and/or lymphadenopathy) have confirmed VL; the rest have diseases including malaria, typhoid, or disseminated tuberculosis. Accurate diagnosis is essential. Visceral leishmaniasis is generally fatal if untreated, and sodium stibogluconate, the most commonly used drug for treatment of patients with VL, has serious toxicity. Early diagnosis of VL requires an accurate test that is simple, rapid, does not require a laboratory or refrigeration, non-invasive, and suitable for use in remote settings.

Parasitologic diagnosis by direct microscopy of spleen aspirates has a reported sensitivity of 96%,² but spleen aspirates are impractical and potentially unsafe in remote settings with large numbers of suspected cases. Lymph node aspiration is safe but among 8,000 patients suspected of having VL, we found a sensitivity of only 65%.³ We have had disappointing experience with bone marrow aspirates (MSF, unpublished).

Serologic tests have been developed for diagnosis of VL. The direct agglutination test (DAT) is standard in most MSF VL treatment centers. Although specifically developed for field conditions, the DAT requires a sufficiently equipped laboratory with skilled technicians, meticulous implementation, and overnight incubation. Samples need to be transported to a central laboratory if a peripheral one cannot be established, resulting in treatment delay of 1–2 weeks.

DAT titers ≥ 1:3,200 have a high sensitivity and can be used to confirm a diagnosis of VL. Conversely, a low DAT titer (≤ 1:400) effectively rules out VL.⁴ If the patient has a DAT titer of > 1:400 but < 1:3,200, an aspiration is performed to confirm a diagnosis of VL. The MSF uses this diagnostic protocol, which reduces the number of aspirates by approximately 80%.⁵

Lower and even negative titers for antibodies to Leishmania spp. have been observed in the Mediterranean region in persons with advanced HIV infection and VL.⁶,⁷ In Ethiopia, the overall reported sensitivity of the DAT (56% of VL patients were HIV infected) was 97.7%. Among parasitologically confirmed VL cases, a false-negative DAT result was obtained in only 2 (3.9%) of the 51 cases that were HIV co-infected and in none of the 40 HIV-negative VL cases.⁸ In a clinical study in Ethiopia, no difference was found in mean DAT titers between HIV-positive and HIV-negative VL patients.¹ Thus, the DAT appears to be reasonably sensitive in the diagnosis of VL in patients co-infected with HIV in Ethiopia.

Dipstick serologic tests have been developed by using the cloned antigen of the 39-amino acid repeats of a kinesin-like gene (rK39) found in Leishmania chagasi. The DiaMed-IT-Leish® (DiaMed AG, Cressier-sur-Morat, Switzerland) was evaluated in VL patients in Sudan and showed a sensitivity of 90%, a specificity of 99%, positive predictive value of 98%, and a negative predictive value of 71%.⁹ In Uganda, it showed sensitivity of 97% and a specificity of 99%.¹⁰ In India, it showed a sensitivity of 99–100% and a specificity of 95–100%.¹¹

Field Evaluation of rK39 Test and Direct Agglutination Test for Diagnosis of Visceral Leishmaniasis in a Population with High Prevalence of Human Immunodeficiency Virus in Ethiopia

Rachel ter Horst, Tewodros Tefera, Gessesse Assefa, Abdurazik Z. Ebrahim, Robert N. Davidson, and Koert Ritmeijer* Médecins Sans Frontières, Humera, Ethiopia; Kassay Abersa Hospital, Humera, Ethiopia; Ethiopian Health and Nutrition Research Institute, Addis Ababa, Ethiopia; Department of Infection and Tropical Medicine, Northwick Park Hospital, Harrow, United Kingdom; Médecins Sans Frontières, Amsterdam, The Netherlands

Abstract. Accuracy of an rK39 rapid diagnostic test (DiaMed-IT-Leish®) for visceral leishmaniasis (VL) was compared with splenic aspiration and the direct agglutination test (DAT) in a population with a high prevalence of infection with human immunodeficiency virus (HIV) in Ethiopia. There were 699 patients clinically suspected of having VL (153 parasitologically confirmed, 482 DAT confirmed, and 130 DAT negative), and 97 DAT-negative controls. A total of 84% were tested for HIV and 34% were HIV positive. Sensitivity of the rK39 test in parasitologically confirmed VL patients was 84% (77% in HIV positive and 87% in HIV negative; $P = 0.25$). Sensitivity of the DAT was higher (94%; $P = 0.01$), 89% in HIV-positive patients and 95% in HIV-negative patients; $P = 0.27$). Specificity of the rK39 test was 99% in DAT-negative controls and 92% in DAT-negative patients clinically suspected of having VL. A diagnostic algorithm combining DAT and the rK39 test had a sensitivity of 98% in HIV-positive VL patients and 99% in HIV-negative VL patients. Despite the lower sensitivity in a population with a high prevalence of HIV, the DiaMed-IT-Leish® rK39 test enables decentralization of diagnosis. Patients clinically suspected of having VL who show negative results on the rK39 antigen test should undergo follow-up DAT testing, especially if they are HIV positive.
A meta-analysis of the diagnostic performance of the DAT and rK39 test, which did not include the above mentioned newer DiaMed-IT-Leish test results, showed sensitivities of 94.8% for the DAT and 93.9% for rK39 in HIV-negative parasitologically confirmed VL cases.\textsuperscript{12} Regional differences were noted; the tests are more sensitive in south Asia than in Sudan, which highlights the importance of validating new diagnostic tests in leishmaniasis-endemic areas. An Ethiopian study\textsuperscript{13} showed a sensitivity of 95.3% for the DAT and 71.7% for rK39 (Kalazar Detect Rapid Test\textsuperscript{6}; InBios International Inc., Seattle, WA) (active case detection for most cases, no HIV testing performed). Data on 38 passively recruited patients in Ethiopia clinically suspected of having VL in another study\textsuperscript{14} showed a sensitivity of 94.0% and a specificity of 93.6% for the DAT and a sensitivity of 75.4% and a specificity of 70.0% for rK39 (Kalazar Detect Rapid Test\textsuperscript{6}; InBios).

Few studies have assessed the role of HIV infection on the performance of the rK39 test, and high globulin titers found in patients infected with HIV might reduce specificity and lower titers of antibodies to *Leishmania* spp. might reduce the sensitivity of the test. Because of the increasing geographic overlap of HIV and VL in east Africa, it is important to determine the accuracy of the rK39 test in HIV-positive VL patients. Thus, we compared the performance of the rK39 rapid test (DiaMed-IT-Leish\textsuperscript{6}) for the diagnosis of VL in the northwest region of Ethiopia with the DAT and splenic aspirate in HIV-positive and HIV-negative VL patients.

**METHODS**

**Study site.** We conducted the study in the MSF VL treatment center at the Kahsay Abera Hospital in Humera in northwestern Ethiopia from June 2006 through January 2007.

**Patients and routine diagnostic algorithm.** Patients without previous VL treatment who fulfilled the clinical VL case definition were eligible to join the study. All consenting patients were tested with the DAT, the rK39 test, and, where appropriate, splenic aspiration. Aspirations were performed by an MSF physician. Contraindications to splenic aspiration were severe anemia (hemoglobin level < 6 g/dL), active bleeding, jaundice, advanced pregnancy, impalpable spleen, or inability to lie still during and after aspiration.

The DAT was performed on a blood spot from a fingerprick. A DAT titer ≥ 1:3,200 in patients suspected of having VL was regarded as diagnostic, and a titer ≤ 1:400 as exclusion of VL. In patients with titers ≤ 1:400, other diagnoses were sought or the DAT was repeated in a week. Those patients with DAT titers between these values were considered indeterminate; these patients underwent spleen aspiration. Patients who appeared severely ill were additionally treated so that a diagnosis was made as quickly as possible. These patients underwent an aspiration on the same day as the DAT. If the initial aspirate result was negative, a repeat aspiration was performed the next day with or without a repeat DAT in those patients with negative results and clinically suspected of having VL. An experienced MSF laboratory technician performed all DAT examinations, aspirate slide readings, and rK39 tests during the study period (blinded for the patient’s previous results). Each dipstick was read by a different laboratory technician blinded to the results of the first technician to assess inter-reader variability.

A parasitologically confirmed VL case was defined as a patient with a positive splenic aspiration smear. A negative control was defined as patients seen in Humera Hospital for reasons other than VL, with no signs and symptoms of VL, a negative DAT result, and no history of previous treatment for VL.

**rK39 rapid diagnostic test.** The diagnostic test kit (rK39 rapid test) was obtained from DiaMed AG, Switzerland. A fingerprick sample of blood was used and the result is available in 20 minutes. The procedures have been described elsewhere.\textsuperscript{9}

**Direct agglutination test.** The DAT was performed by using whole dry blood spots on Whatman 3 filter paper (Whatman, Maidstone, United Kingdom) and freeze-dried antigen containing *L. donovani* promastigotes (supplied by the Royal Tropical Institute, Amsterdam, The Netherlands). The quality of the DAT antigen was controlled by comparing it with a set of serum samples with known titers supplied by the Royal Tropical Institute. The DAT titer of the sample is the highest dilution at which agglutination is still visible.

**Parasitologic diagnosis.** Spleen aspirates were performed by the MSF physicians in charge of the patient. Giemsa-stained smears from spleen aspirates were examined by our laboratory technicians using oil-immersion microscopy. Parasite density was graded on a scale from 0 to 6+.\textsuperscript{15}

**HIV counseling and testing.** We offered HIV counseling and testing with informed consent to all adult VL patients. The HIV testing was performed by nurse counselors using two rapid tests (HIV-Determine; Abbott Diagnostics, Abbott Park, IL and UniGold; Trinity Biotech, Bray, Ireland) run in parallel. Results were confirmed with repeat testing in the laboratory using the same tests on a second sample by a trained technician. The HIV status was not known for all study participants. Those patients in whom VL was excluded were not routinely offered HIV testing. In addition, a few VL patients refused testing and some died before testing could be offered. The HIV-positive patients were referred to the free medical HIV/AIDS care center for ongoing care.

**Tuberculosis and malaria co-infection.** We tested all patients suspected of having VL for malaria by blood film or rapid diagnostic test (Paracheck\textsuperscript{7}; Orchid Biomedical Systems, Goa, India and/or Parascreen\textsuperscript{8}; Zephyr Biomedical Systems, Goa, India). We recorded a history of tuberculosis at enrollment and assessed for tuberculosis if clinically indicated during hospitalization.

**Statistical analysis.** We entered data into an Excel\textsuperscript{8} (Microsoft, Redmond, WA) spreadsheet. We performed statistical analysis using SPSS version 11 software (SPSS Inc., Chicago, IL) by chi-square test with Yates’ correction or Fisher’s exact test as appropriate.

**Quality control.** External quality control of splenic aspirates was performed sequentially by three independent laboratories. The International Central Laboratories in Addis Ababa reviewed 26% of the positive slides and 32% of negative slides, and the Ethiopian Health and Nutrition Research Institute in Addis Ababa and our senior MSF laboratory technician reviewed a random sample of these results. External quality control of DAT was performed on dried blood spot filter papers of 20% of negative and intermediate DAT results.

**Ethical considerations.** All medical care was provided free of charge. We conducted the study in collaboration with the Regional Bureau of Health in Tigray, the Ethiopian Health and Nutrition Research Institute, and the director of Kahsay...
Abera Hospital in Humera. The only modification of routine care involved a fingerprick for the rK39 test. All eligible patients provided written informed consent. Visceral leishmaniasis nurses explained the study to patients in the local language. Ethical approval was obtained from the Ethiopian Science and Technology Committee, the Tigray Regional Bureau of Health, the Humera Hospital director, and the MSF Ethics Review Board.

RESULTS

We enrolled 699 patients fulfilling the VL clinical case definition without previous VL treatment. Of these, 491 (70%) had confirmed VL: 153 had a positive splenic aspirate, 482 had a DAT titer ≥ 1:3200, and 144 had both of these results. A total of 130 patients suspected of having VL had VL excluded by a DAT titer ≤ 1:400. The mean ± SD age of the total sample population was 25.4 ± 10.1 years. The male-to-female ratio was 15:1 (reflecting the large male migrant worker population), the mean ± SD body mass index was 16.4 ± 2.5 kg/m², and the mean ± SD spleen size was 9.1 ± 5.5 cm.

Among parasitologically confirmed VL cases, 128 (83.7%) of 153 underwent HIV testing and 44 (34.4%) of these 128 patients were HIV positive. Among serologically confirmed VL cases, 224 (46.5%) of 482 underwent HIV testing, and 60 (26.8%) of 224 were HIV positive. Co-infection with tuberculosis was confirmed in 21 VL patients (4.4%), 8 (38%) of whom were co-infected with HIV. Tuberculosis was clinically suspected in an additional 28 patients (5.8%), 7 (25%) of whom were co-infected with HIV. Malaria co-infection was found in 26 VL patients (5.4%), 1 (3.8%) of whom was co-infected with HIV.

Sensitivity of rK39 test and DAT in parasitologically confirmed VL patients. The rK39 test result was positive in 129 of 153 parasitologically confirmed VL patients (sensitivity = 84.3%), with no significant difference between those patients who were HIV positive (34 of 44, 77.3%) and those patients who were HIV negative (73 of 84, 86.9%) (P = 0.25) (Table 1).

The DAT showed a diagnosis of VL (titer ≥ 1:3200) in 144 of 153 parasitologically confirmed VL cases (sensitivity = 94.1%), with no significant difference between those patients who were HIV positive (39 of 44, 88.6%) and those patients who were HIV negative (80 of 84, 95.2%) (P = 0.27). Overall sensitivity of the rK39 test was significantly lower than that of the DAT (odds ratio = 0.34, 95% confidence interval = 0.14–0.79, P = 0.01).

Mean ± SD DAT titer and distribution in parasitologically confirmed HIV/VL co-infected patients (titer 8.8 ± 2.1) and HIV-negative VL patients (9.3 ± 1.9) was not significantly different (P = 0.2) (Table 2 and Figure 1). However, parasite density was significantly higher in HIV/VL co-infected patients than in HIV-negative patients (mean ± SD grade = 3.7 ± 1.7 versus 2.5 ± 1.3; P = 0.0001) (Table 2 and Figure 2). This 1.2 grade difference is equivalent to a nearly 16-fold increased parasite density in aspirates from HIV co-infected patients.

The diagnosis of VL by either a positive rK39 test result or a positive DAT result yielded a high sensitivity in all parasite-positive patients (98.7%), whether HIV negative (sensitivity = 98.8%) or HIV positive (sensitivity = 97.7%) (Table 1).

Specificity of the rK39 test in DAT-negative patients and in DAT-negative controls. The rK39 test result was negative in 119 of 130 patients clinically suspected of having VL who had DAT titers ≤ 1:400, giving a specificity of 91.5% for the DAT. Among 97 DAT-negative controls, 96 were rK39 negative (specificity = 99.0%) (Table 3). Specificity of the rK39 test in patients with intermediate DAT titers and negative splenic aspires was 83% (25 of 30).

Comparison of the rK39 test to the combination of the DAT and splenic aspiration. In Ethiopia, a diagnosis of VL is made if DAT titers are ≥ 1:3200 or a splenic aspirate is positive for leishmanial parasites. Visceral leishmaniasis is excluded either by DAT titers ≤ 1:400 or DAT titers < 1:3200 in combination with a negative aspirate result. Using this definition of VL, we determined that the rK39 test had a sensitivity of 64.6% (42 of 65) and a specificity of 66.7% (4 of 6) in patients co-infected with HIV. In HIV-negative patients, the sensitivity was 81.0% (136 of 168) and the specificity was 81.8% (9 of 11). The positive predictive value of the rK39 test was 95.5% in patients co-infected with HIV and 98.6% in HIV-negative patients (Table 4).

Proposed algorithm for rK39 test use for patients suspected of having VL in Ethiopia. We assessed the performance of the rK39 test in a diagnostic algorithm (Figure 3). If 200 patients clinically suspected of having VL come to a clinic, approximately 100 will have VL (assumed sensitivity of 50% of the clinical case definition). If immediately tested by using the rK39 test, 84 will be correctly diagnosed (sensitivity = 84.3%) and 8 will be incorrectly diagnosed (specificity = 91.5%). The remaining 108 patients will be further investigated, of whom 16 will have a positive DAT result. Fifteen of these patients will be correctly diagnosed by a positive DAT result (sensitivity = 95%) and one will be incorrectly diagnosed (specificity = 95%). Two patients with indeterminate DAT titers will undergo splenic aspiration, with 1 of them being positive. A negative DAT result

### Table 1

<table>
<thead>
<tr>
<th>Test</th>
<th>Overall, no positive/no tested, sensitivity (95% CI)</th>
<th>HIV negative, no positive/no tested, sensitivity (95% CI)</th>
<th>HIV positive, no positive/no tested, sensitivity (95% CI)</th>
<th>Odds ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>rK39 DiaMed-IT-Leish&lt;sup&gt;®&lt;/sup&gt; test</td>
<td>129/153, 84.3% (77.6–89.7%)</td>
<td>73/84, 86.9% (77.8–93.3%)</td>
<td>34/44, 77.3% (62.2–88.5%)</td>
<td>0.51 (0.18–1.46)</td>
<td>0.253</td>
</tr>
<tr>
<td>DAT (titer ≥ 1:3200)</td>
<td>144/153, 94.1% (89.1–97.3%)</td>
<td>80/84, 95.2% (88.3–98.7%)</td>
<td>39/44, 88.6% (75.4–96.2%)</td>
<td>0.39 (0.08–1.80)</td>
<td>0.273</td>
</tr>
<tr>
<td>rK39 test plus DAT</td>
<td>151/153, 98.7% (95.4–99.8%)</td>
<td>83/84, 98.8% (93.5–100%)</td>
<td>43/44, 97.7% (88.0–99.9%)</td>
<td>0.52</td>
<td>1.00</td>
</tr>
</tbody>
</table>

* CI = confidence interval; HIV = human immunodeficiency virus; DAT = direct agglutination test.

### Table 2

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Sample SD</th>
<th>HIV positive</th>
<th>HIV negative</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± DAT titer</td>
<td>8.8 ± 2.1</td>
<td>9.3 ± 1.9</td>
<td>0.216</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD parasite grade</td>
<td>3.7 ± 1.7</td>
<td>2.5 ± 1.3</td>
<td>0.0001</td>
<td></td>
</tr>
</tbody>
</table>
in 98 patients will correctly eliminate VL in 97 patients and falsely eliminate VL in 1 patient (sensitivity = 99%).

**Quality control.** There was 100% agreement among two laboratory technicians who read the rK39 test dipsticks independently and blinded for results. There was 97.5% agreement between splenic aspirate results of the Humera Laboratory and the aforementioned quality control laboratories. For DAT results, agreement was 96.3%.

**DISCUSSION**

We have demonstrated that HIV-positive VL patients in Ethiopia DAT titers similar to those of HIV-negative VL patients, a result that was previously reported, which indicates preservation of antibody responses to *Leishmania* spp. promastigotes. Reassuringly, 88.6% of HIV co-infected patients can be diagnosed non-invasively by the DAT. The rK39 test diagnosed only 77.3% of HIV co-infected patients. This test has good specificity in controls (99.0%) and in DAT-negative patients suspected of having VL (91.5%). Our rK39 test specificity estimation is possibly slightly overestimating true specificity because there might have been DAT false-negative controls because of imperfect sensitivity of the DAT. The predictive value of a positive rK39 test result was high (> 95%) in clinically suspected cases irrespective of HIV status.

The sensitivity of the DiaMed-IT Leish® rK39 test was lower than that reported in previous studies. Some of this difference is related to the lower sensitivity in HIV co-infected patients. However, these patients did not have lower titers of antibodies to *Leishmania* spp., as indicated by DAT titer. Antibodies detected by the rK39 test, although present, may have had lower affinities, and thus less able to react in a rapid reaction (dipstick format) than in overnight incubation (as in the DAT test). The sensitivity we found in HIV-negative VL patients (87%) is similar to the sensitivity of 90% we reported in a study using the same dipstick in patients from southern Sudan who had a low prevalence of HIV. When comparing studies, it is important to note which commercially available version of the rK39 test is being used. For example, we found poor performance in Sudan using a different prototype of the rK39 test. There are also important geographic differences that can affect this test. Chappuis and others, Diro and others, and Boelaert and others reported disappointing results in Africa with the Kalazar Detect Rapid Test® (InBios). However, this test showed good performance in India.

In contrast to the observations made for patients co-infected with HIV and VL in Europe, the DAT in Ethiopia remains reasonably sensitive in the diagnosis of VL in patients co-infected with HIV. The DAT titer distributions were similar in parasitologically confirmed VL cases who were HIV positive and those who were HIV negative. The high sensitivity of the DAT we observed in patients co-infected with HIV and VL (89%) confirms previous findings in patients in Ethiopia co-infected with HIV and VL. The nearly 16-fold higher parasite density found in splenic aspirates of patients co-infected with HIV implies that diagnosis by microscopy is sensitive in such patients. Thus, microscopy should remain the gold standard until non-invasive *Leishmania* antigen- or DNA-based tests are suitable for field use. We encountered no complications with this procedure.

An important finding is that, despite suboptimal sensitivities of the rK39 test, the combination of both serologic tests in HIV-positive, parasitologically confirmed VL cases showed a high sensitivity (97.7%, 95% confidence interval = 88.0–99.9%). This finding demonstrates that apparently a significant part of the sensitivity of each separate test does not overlap. Therefore, use of the DAT after a negative rK39 result is obtained for a clinically suspected VL patient co-infected with HIV patient will improve VL detection, and the number of splenic aspirates can be greatly reduced. The ease of use of the rK39 dipstick test and its low cost enable early diagnosis of VL in decentralized settings and thereby improve access to early life-saving treatment.
Performance of rK39 test compared with the MSF diagnostic algorithm of DAT and aspirate (positive = DAT titer ≥ 1:3,200 and/or aspirate positive; negative = DAT titer < 1:3,200 and aspirate negative)*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HIV negative</th>
<th>HIV positive</th>
<th>Odds ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>179</td>
<td>71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity, % (95% CI)</td>
<td>81.0 (74.2–86.8)</td>
<td>64.6 (51.8–76.1)</td>
<td>0.43 (0.22–0.85)</td>
<td>0.014</td>
</tr>
<tr>
<td>Specificity, % (95% CI)</td>
<td>81.8 (48.2–97.7)</td>
<td>66.7 (22.3–95.7)</td>
<td>0.44 (0.03–6.96)</td>
<td>0.58</td>
</tr>
<tr>
<td>PPV, % (95% CI)</td>
<td>98.6 (94.9–99.8)</td>
<td>95.5 (84.5–99.4)</td>
<td>0.31 (0.03–3.18)</td>
<td>0.25</td>
</tr>
<tr>
<td>NPV, % (95% CI)</td>
<td>22.0 (10.6–37.6)</td>
<td>14.8 (4.2–33.7)</td>
<td>0.62 (0.14–2.59)</td>
<td>0.68</td>
</tr>
</tbody>
</table>

*MSF = Médecins Sans Frontières; DAT = direct agglutination test; HIV = human immunodeficiency virus; CI = confidence interval; PPV = positive predictive value; NPV = negative predictive value.

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Authors’ addresses: Rachel ter Horst and Koert Ritmeijer, Médecins Sans Frontières Holland, PO Box 10014, 1001EA, Amsterdam, The Netherlands, E-mail: Koert.ritmeijer@amsterdam.msf.org. Tewodros Tefera, Kahsay Abera Hospital Humera, Tigray Regional Bureau
of Health, PO Box 7, Mekele, Ethiopia. Gessesse Assefa, Ethiopian Health and Nutrition Research Institute, PO Box 9, Humera, Ethiopia. Robert N. Davidson, Department of Infection and Tropical Medicine, Lister Unit, Northwick Park Hospital, Harrow, Middlesex, HA1 3UJ, United Kingdom.

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