

## UTILITY OF LYMPH NODE ASPIRATION IN THE DIAGNOSIS OF VISCERAL LEISHMANIASIS IN SUDAN

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**Abstract.** We evaluated lymph node aspiration (LNA) as a simple diagnostic procedure for visceral leishmaniasis (VL). Lymph node aspiration was compared with the direct agglutination test (DAT) using a diagnostic titer  $\geq 1:6,400$  in 7,880 suspected VL patients in eastern Sudan. Compared with DAT, LNA had a sensitivity of 65.1% (95% confidence interval = 63.5–66.6%). Parasite density in LNA correlated strongly with DAT titers ( $P < 0.0001$ ), and low parasite density accounted for 78.1% of positive LNA results with DAT titers  $< 1:6,400$  ( $n = 782$ ). Risk factors predictive of a positive LNA result were an age of 1–29 years, male sex, a hemoglobin level  $< 10.0$  g/dL, a DAT titer  $\geq 1:800$ , and a location with a higher prevalence of VL. Lymph node and splenic aspirations were similarly accurate as tests of cure after treatment of 50 VL patients in southern Sudan. Pre-treatment LNA results were negative in 20 cases of severe post kala-azar dermal leishmaniasis.

### INTRODUCTION

Visceral leishmaniasis (VL or kala-azar) is a parasitic disease that clinically resembles a number of other infections. It is usually fatal if undiagnosed and untreated, but if treated usually leads to life-long immunity. This disease is classically diagnosed by demonstrating parasites in smears from spleen or bone marrow, and occasionally in buffy coat, lymph nodes, or liver biopsy specimens. Because of concerns of fatal intra-abdominal hemorrhage, splenic aspiration is sometimes avoided in remote rural settings.<sup>1</sup> Bone marrow aspiration, although safe, is a tedious and sometimes painful procedure and requires costly disposable bone marrow needles.<sup>2,3</sup>

The high prevalence of generalized lymphadenopathy among Sudanese VL patients suggests lymph node aspiration (LNA) might be useful in establishing a parasitologic diagnosis of VL.<sup>4,5</sup> The splenomegaly of VL disappears at the end of treatment in 78% of Sudanese patients.<sup>6</sup> Because 90% of patients will still have palpable lymph nodes,<sup>4</sup> LNA might also be useful in assessing initial parasite clearance. However, parasites are reportedly found in groin lymph nodes in some patients with post-kala-azar dermal leishmaniasis (PKDL),<sup>3</sup> which might give a false impression that the patient had VL.

Among other serodiagnostic tests, the direct agglutination test (DAT) has been extensively used under field conditions to diagnose VL.<sup>7,8</sup> A meta-analysis including 30 studies on DAT showed a sensitivity of 94.8% and a specificity of 85.9% in clinically suspected VL patients.<sup>9</sup>

To establish the usefulness of LNA in the diagnosis of VL, we analyzed clinical and laboratory data collected from suspected cases of VL seen during a 43-month period at the Médecins Sans Frontières (MSF)–Holland VL treatment centers in Gedaref State in eastern Sudan. We report validity estimates of LNA in comparison to DAT. We also examined biologic and environmental factors that could predict the results of LNA.

Additionally, in the MSF–Holland VL treatment center in

Jonglei Province in southern Sudan, we sought to establish whether LNA might provide useful results when used as a test of parasite clearance at the end of treatment for VL. We compared the performance of LNA and splenic aspiration (SA) in VL patients who received their first course of treatment. We also performed LNA in patients with severe PKDL before treatment to assess the presence of parasites in such aspirates.

### MATERIALS AND METHODS

#### LNA versus DAT in patients suspected of having VL.

**Study area.** Visceral leishmaniasis is endemic in the southern part of Gedaref State in eastern Sudan. This is a remote but fertile farming region characterized by woodlands of *Acacia seyal* and *Balanites aegyptica*, soft black soil, and dry savannah climate with a short rainy season (July–October). *Leishmania donovani* is the parasite and *Phlebotomus orientalis* is the vector responsible for VL transmission in this region.<sup>3</sup>

**Médecins Sans Frontières VL treatment centers.** The Um Elkheir and Kassab centers in eastern Sudan were run by MSF to provide treatment for VL for a rural population of approximately 500,000. The medical department of (MSF)–Holland reviewed and approved the study.

**Study population.** All patients clinically suspected of having VL who came to the MSF treatment centers at Um Elkheir and Kassab from February 1998 to August 2001 were included in the study. We included individuals who had not been previously treated for VL and who had a fever for  $> 2$  weeks with either splenomegaly or lymphadenopathy. We provided diagnosis and treatment of malaria while investigating for possible VL. Patients who were previously treated for VL were excluded because the DAT does not differentiate between past or current VL. Similarly, patients with PKDL were excluded.

**Direct agglutination test.** Aqueous *Leishmania* antigen was obtained from the Prince Leopold Institute of Tropical Medicine (Antwerp, Belgium) and the Royal Tropical Institute (Amsterdam, The Netherlands). Each batch of antigen was standardized with freeze-dried positive controls of known titers. Whole blood samples were collected by finger prick on

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Whatman (Brentford, United Kingdom) 3 filter paper and left to air-dry. Briefly, 5-mm disks (5  $\mu$ L of blood) were punched from the filter paper and placed in microtiter plates containing 96 V-shaped wells. Samples were eluted and serially diluted to titers  $\geq$  1:102,400. After overnight incubation at ambient temperature, plates were read for agglutination. The titer recorded was the highest dilution at which agglutination was visible.

**Lymph node aspiration.** The LNA was performed by our laboratory technicians; the entire procedure was performed in < 1 minute without local anesthesia. Aspirations produced a similar degree of discomfort as that from taking a blood sample. The patient lay on a local bed (*angareb*) in a screened area of the laboratory. The overlying skin was cleaned and the largest palpable inguinal lymph node was held firmly between thumb and index fingers while a 21-gauge 1.25-inch hypodermic needle was inserted. Lymph was encouraged to flow up the needle by twirling it and gently milking the lymph node. The needle was then withdrawn with the hub being sealed by the index finger. The aspirate was expelled onto a glass slide, smeared, air-dried, fixed with 100% methanol, and stained with Giemsa.

The density of *L. donovani* parasites was determined according to the method of Chulay and Bryceson.<sup>1</sup> The grades used were: Grade 6: > 100 amastigotes/high-power field (HPF); Grade 5: 10–100 amastigotes/HPF; Grade 4: 1–10 amastigotes/HPF; Grade 3: 1–10 amastigotes/10 HPFs; Grade 2: 1–10 amastigotes/100 HPFs; Grade 1: 1–10 (amastigotes/1,000 HPFs; Negative: no amastigotes seen in 1,000 HPFs.

**Diagnostic protocol.** Figure 1 shows the diagnostic protocol we used. All cases suspected of having primary VL cases underwent the DAT. A DAT titer  $\geq$  1:6,400 in patients satisfying the VL clinical case definition was regarded as diagnostic.<sup>10</sup> Those with borderline DAT titers (1:800–1:3,200)

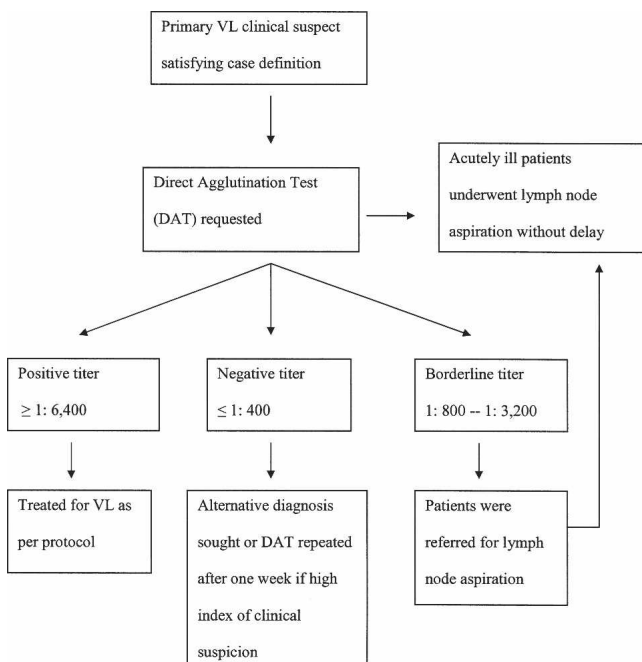


FIGURE 1. Diagnostic flow chart for cases suspected of having visceral leishmaniasis (VL) seen at the Médecins Sans Frontières–Holland treatment centers in eastern Sudan.

underwent further testing by LNA. Patients suspected of having VL who appeared acutely unwell were further tested to obtain a diagnosis as quickly as possible: they underwent LNA on the same day as the DAT, with repeat LNA and DAT after 1 day and 1 week, respectively. Patients suspected of having VL with a negative DAT titer ( $\leq$  1:400) were evaluated to obtain alternative diagnoses. On average, it took two working days for the DAT results to be obtained. Diagnostic procedures were always explained and verbal consent was obtained from the patients or their guardians. Laboratories in each treatment center followed standardized operating procedures and were subject to routine periodic quality control assessments by MSF.

**Post-treatment LNA versus SA in VL patients.** Primary VL patients initially diagnosed by DAT or aspirate underwent simultaneous lymph node and spleen aspiration to assess parasite clearance at the end of their treatment for VL at the MSF center in Lankien, Jonglei province in southern Sudan. Post-treatment aspirates were performed on day 28 of treatment with sodium stibogluconate (20 mg/kg).

To perform splenic aspiration, a 21-gauge hypodermic needle attached to a 5-mL syringe was inserted in the midline of the anterior surface of the spleen. The needle is inserted quickly to its full depth (1.25 inches) into the spleen while a vacuum is being created and maintained by pulling the plunger back. The needle is removed rapidly from the spleen. The (scanty) aspirate is expressed onto a slide, smeared, air-dried, fixed with 100% methanol, and stained with Giemsa. Slides were independently assessed and parasites on LNAs and SAs were quantified as per the method of Chulay and Bryceson.<sup>1</sup>

**Pre-treatment LNA in PKDL patients.** Post kala-azar dermal leishmaniasis was diagnosed on clinical grounds by a recent history of VL and a classic rash. Twenty patients with severe PKDL who came to the MSF VL treatment center in Lankien, Jonglei Province in southern Sudan agreed to undergo LNA before starting treatment.

**Data processing and analysis.** Laboratory data for patients suspected of having VL were routinely recorded in ledgers. Data included date, treatment center, name, age, sex, hemoglobin concentration, DAT titer, and parasite grade on LNA. These data were entered into Microsoft (Redmond, WA) Excel®. Range and consistency checks were performed and a coding scheme was subsequently developed. Missing values were recoded, the database was cleaned and saved as a tab-delimited text file, and transferred into STATA version 7.0 (STAT Corp., College Station, TX) for analysis. We also collected data from laboratory records in southern Sudan that contained information on post-treatment simultaneous SAs and LNAs, as well as results of pre-treatment LNAs in PKDL patients.

The chi-square test was used to assess the association between categorical variables. A logistic regression model was constructed to predict which suspected VL cases were more likely to have positive LNA results. We constructed a hierarchical order for variables to allow for proper control of distant and proximate potential confounders.<sup>11</sup> Age, sex, season, treatment center, hemoglobin level, and DAT titer were serially fitted into the regression model. The Mantel-Haenzel odds ratios with 95% confidence intervals (CIs) were used to express the strength of associations between biologic and environmental factors and results of LNA.

## RESULTS

**LNA versus DAT in patients suspected of having VL.**

Overall, 22,540 patients clinically suspected of having VL underwent the DAT during the study period. A total of 7,880 subjects underwent simultaneous DAT and LNA. If either DAT or LNA was repeated, only the first result was analyzed.

A total of 9,328 subjects underwent LNA. Of this group, 1448 subjects underwent LNA without DAT because of an interruption of DAT supplies to the field sites. Poor-quality lymph node samples were obtained in 8 (< 0.1%) of 9,328 LNAs and these were excluded from further analysis. Using a cut-off DAT titer  $\geq$  1:6,400 (Table 1), validity estimates of LNA among 7,880 patients undergoing simultaneous DAT and LNA testing yielded a sensitivity of 65.1% (95% CI = 63.5–66.6%).

Table 2 shows a strong correlation between parasite load on LNAs and the corresponding DAT titers ( $P < 0.0001$ , by chi-square test for trend). To provide larger groups, we simplified parasite scores to low density (grades 1 and 2), medium density (grades 3 and 4), and high density (grades 5 and 6). A total of 782 patients had positive LNAs but their corresponding DAT titers were < 1:6,400. Overall, this group accounted for 25.3% (782 of 3,092) of all positive LNA results. Further breakdown of positive LNA results with DAT titers < 1:6,400 showed that a low parasite density accounted for 78.1% (611 of 782) of the results, medium density accounted for 21.0% (164 of 782), and high density accounted for 0.9% (7 of 782).

Age, sex, hemoglobin level, season, location of treatment center, and DAT titer were strongly associated with a positive outcome of LNA ( $P < 0.0001$ , by chi-square test; data available upon request). Table 3 shows the results of multivariate analysis using a hierarchical model for these variables. Male sex and young age were linked to increased odds of a positive LNA result. The odds of obtaining a positive LNA result correlated with severity of anemia. We found a strong positive correlation between DAT titer and the likelihood of obtaining a positive LNA result. The LNA results were more likely to be positive in Um Elkheir than in Kassab. Less lymph node aspirates were positive in April–June compared with our baseline period of July–September.

**Post-treatment LNA versus SA in VL patients.** Fifty primary VL patients in southern Sudan underwent simultaneous LNA and SA to assess parasite clearance at the end of their treatment for VL. Overall, both LNA and SA identified 43 patients as negative and 6 patients as positive for *Leishmania* parasites. However, in one case, the SA result was negative but a corresponding LNA result was positive with grade 3 parasite density.

TABLE 1

Performance of lymph node aspiration and direct agglutination test in patients with suspected VL at MSF–Holland VL treatment centers in eastern Sudan, February 1998–August 2001\*

		Direct agglutination test		Total
		Titers $\geq$ 1:6,400	Titers < 1:6,400	
Lymph node aspiration	Positive	2,310	782	3,092
	Negative	1,241	3,547	4,788
	Total	3,551	4,329	7,880

\* VL = visceral leishmaniasis; MSF = Médecins Sans Frontières.

TABLE 2

Grades of parasite density in lymph node aspirates and the corresponding titers of the direct agglutination test among patients suspected of having primary VL seen at Médecins Sans Frontières–Holland treatment centers in eastern Sudan from February 1998 through August 2001\*

Grade of lymph node aspirate	Direct agglutination test			Total
	Titers $\leq$ 1:400	Titers 1:800–1:3,200	Titers $\geq$ 1:6,400	
Negative	2,126	1,421	1,241	4,788
Grade 1	66	249	372	687
Grade 2	54	242	793	1,089
Grade 3	27	98	621	746
Grade 4	8	31	334	373
Grade 5	3	4	155	162
Grade 6	0	0	35	35
Total	2,284	2,045	3,551	7,880

\* VL = visceral leishmaniasis.  $P < 0.0001$  by chi-square test for trend.

**Pre-treatment LNA in PKDL patients.** No parasites were found in lymph node aspirates obtained from 20 patients with severe PKDL in southern Sudan.

## DISCUSSION

Lymph node aspiration has rarely been studied as a method for diagnosing VL. In a series of 30 confirmed Sudanese VL cases,<sup>2</sup> *Leishmania* parasites were found in 30 of 30 LNAs. In two cases, parasites were found in LNAs and bone marrow aspirates (BMAs) but not in SAs, and in one case they were found in LNAs and SAs but not in BMAs. In another series of confirmed Kenyan VL patients,<sup>12</sup> parasites were found in 20 of 31 LNAs, 18 of 19 liver biopsy samples, and 39 of 40 SAs. When compared with SAs in the Sudan, two small studies found LNAs to have low sensitivities of 56% ( $n = 19$ ) and 54% ( $n = 87$ ), respectively.<sup>13,14</sup>

In our experience, LNAs produced good quality material in > 99.9% of the subjects. We used the DAT as the reference test in this study, although it provides only indirect evidence of the parasite. The slight risk of causing bleeding by unnecessary SAs made it difficult for us to use SAs as the gold standard against which to evaluate LNAs. Furthermore, patients who underwent both LNAs and SAs to establish a diagnosis of VL were inevitably those in whom the LNA had been done first and had failed to demonstrate parasites. Diagnostic SAs were rarely done in our centers and only when all other results were inconclusive (SAs were performed in 353 of 22,540 VL suspects seen over a period of 43 months in eastern Sudan).

When LNA was compared with the DAT, LNA had a sensitivity of 65%. It is important to note that we gathered these data in an operational setting where the prevalence of VL was approximately 45% among our clinical suspects. Our diagnostic algorithm contained an inherent bias that led to underestimating the sensitivity of LNA: 22,540 subjects underwent DAT whereas only 9,328 subjects underwent LNA and only 7,880 underwent both procedures simultaneously. Patients with strongly positive DAT titers would have been treated for VL without undergoing further LNA, and we found that these are the patients in whom the LNA result was most likely to be positive (adjusted odds ratio = 28.3).

With regard to LNA specificity, microscopic demonstration of parasites is 100% specific. Interestingly, we observed that

TABLE 3

Multivariate analysis of predictors of a positive LNA in patients suspected of having primary VL seen at Médecins Sans Frontières–Holland treatment centers in eastern Sudan from February 1998 through August 2001\*

Characteristic	Total	Positive LNAs	Adjusted odds ratio†	95% confidence interval	P
Age group (years)					
< 1	74	29	1.88	0.99–3.57	NS
1–4	1,240	517	2.38	1.76–3.21	< 0.0001
5–14	3,341	1,592	1.97	1.51–2.58	< 0.0001
15–29	2,780	1,087	1.42	1.08–1.86	< 0.02
30–44	1,196	327	1.16	0.86–1.56	NS
≥ 45	614	114	1.00		
Sex					
Male	5,998	2,436	1.28	1.14–1.44	< 0.0001
Female	3,316	1,253	1.00		
Hemoglobin level (g/dL)					
< 6.0	1,038	433	2.68	2.18–3.31	< 0.0001
6.0–7.9	2,312	1,275	2.50	2.13–2.93	< 0.0001
8.0–9.9	2,737	1,175	1.73	1.49–2.01	< 0.0001
≥ 10.0	3,060	758	1.00		
Season					
October–December	3,007	1,210	1.05	0.89–1.25	NS
January–March	3,142	1,301	0.89	0.75–1.06	NS
April–June	1,885	668	0.71	0.59–0.86	< 0.0002
July–September	1,286	512	1.00		
Treatment center					
Um Elkheir	4,670	2,112	2.62	2.32–2.97	< 0.0001
Kassab	4,650	1,579	1.00		
DAT titer					
≥ 1:6,400	3,551	2,310	28.32	23.3–34.33	< 0.0001
1:800–1:3,200	2,045	624	6.12	5.02–7.46	< 0.0001
≤ 1:400	2,284	158	1.00		

\* LNA = lymph node aspiration; VL = visceral leishmaniasis; NS = not significant ( $P > 0.05$ ); DAT = direct agglutination titer.

† Adjusted for all other factors listed.

25% (782 of 3,092) of our parasitologically confirmed VL cases were missed by the DAT when a diagnostic cut-off titer  $\geq 1:6,400$  was used. Most of these subjects (78.1%) had low parasite densities in their LNAs, which suggested that a cut-off titer  $\geq 1:6,400$  for the DAT is less sensitive in identifying VL cases in the early or mild stages of disease, assuming this shows a correlation with low parasite load.

We found a strong correlation between parasite density in LNAs and the corresponding DAT titers in our 7,880 patients. This is in contrast to a previous study in 49 patients,<sup>14</sup> in which no correlation was found between DAT titers and parasite load in splenic aspirates. The correlation between DAT titers and parasite load in LNAs shown in this study suggests that these diagnostic methods are complementary. One estimate is that the sole use of parasitologic methods could avert only 53% of deaths attributable to VL but that combining the DAT and LNA would prevent 85% of mortality attributable to VL.<sup>15</sup>

We do not think that co-infection with human immunodeficiency virus (HIV) had much influence on our results. The United Nations AIDS program estimated an HIV prevalence rate of 1.9% (range = 0.7–5.2%) for Sudanese adults 15–49 years of age by the end of 2001,<sup>16</sup> but accurate estimates for other age groups were lacking. We also find it reassuring that co-infection with HIV in east African patients does not adversely affect the performance of the DAT, unlike the situation in southern Europe.<sup>17,18</sup>

Our multivariate analysis indicated that VL is more likely in those with a compatible illness if they are < 30 years of age, male, and anemic. The difference observed between our two treatment centers in eastern Sudan in terms of the likelihood

of obtaining a positive LNA result is probably due to a higher level of VL endemicity in Um Elkheir.

We found only one important discrepancy in 50 patients undergoing simultaneous LNA and SA at the end of VL treatment. This probably represents a false-negative SA result, rather than a false-positive LNA result, because of the significant number of parasites detected. Although there have been reports of parasites in lymph nodes in patients with PKDL, we did not find parasites in 20 such LNAs.

In conclusion, we found LNA to be a useful diagnostic tool for VL in the Sudan. Parasitologic confirmation with LNA in patients suspected of having VL with borderline DAT titers ( $> 1:400$  but  $< 1:6,400$ ) is imperative. Performances of LNAs and SAs were similar in assessing parasite clearance after adequate treatment for VL. We also found no evidence of parasites in LNAs obtained from patients with severe PKDL.

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