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OPERATIONAL VALIDATION OF THE DIRECT AGGLUTINATION TEST FOR DIAGNOSIS OF VISCERAL LEISHMANIASIS

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Abstract. The validity of the direct agglutination test (DAT) for visceral leishmaniasis (VL) was studied with a standardized field kit on 148 clinically suspected persons and 176 healthy controls recruited between 1993 and 1994 from an endemic area in Gedaref State, Sudan. A sensitivity of 95.9% and a specificity of 99.4% were found at a 1:8,000 cut-off titer when parasitologically confirmed cases were compared with healthy controls. While corroborating previously reported sensitivity and specificity estimates of this serodiagnostic test, this study examined the bias generated by commonly used test validation procedures. The fundamental methodologic problem in VL test validation is the absence of a reliable gold standard. Moreover, any operational guideline on DAT use has to consider the critical dependency of the predictive values of the test on VL prevalence rates. The DAT diagnostic cut-off titer depends upon many external factors, among which the prevalence of disease in the area and the case mix seem the most important.

Visceral leishmaniasis (VL) is a potentially fatal disease that affects an estimated 500,000 persons per year.[1] Ninety percent of the cases occur in Bangladesh, India, Nepal, and Sudan. Both the disease incidence and its severity are linked to poverty: malnutrition was found to be associated with a 8.7 times higher risk for VL,[2] and high case fatality rates have been reported from war-torn southern Sudan.[3] Since the disease occurs mainly in areas where health services are poorly developed, research has been focused on the development of a simple, cheap, and reliable diagnostic test for VL.

Allain and Kagan first described a direct agglutination test (DAT) for VL.[4] El Harith and others adapted the method[5,6] according to the card-agglutination test for trypanosomiasis antigen procedures,[7] and recommended the DAT for clinical work in hospitals and health centers,[5] for seroepidemiologic studies,[5,8] and for mass VL population screening.[6,8] Several investigators reported high accuracy of the test under laboratory conditions for VL due to *Leishmania donovani*,[9–11] as well as *L. infantum*.[12] Among the serologic assays recently developed, the DAT seemed most appropriate for use in the field.[11,13] The occurrence of disastrous VL epidemics in southern Sudan in the late 1980s created a pressing demand for appropriate diagnostic tests.[14] The DAT method was rapidly taken into the field and proved valuable to field workers.[13,15–17] Before officially endorsing the use of the DAT in VL surveillance and control programs, the UNDP/World Bank/World Health Organization Special Program for Research and Training in Tropical Disease decided to evaluate the operational validity and reproducibility of this test for VL through a multicenter study in Sudan, Kenya, and Nepal. This paper reports on part of this multicenter evaluation: the operational validity of the DAT as documented in Sudan. It also highlights the particular problems encountered when this theoretically effective diagnostic test was validated at the community level in this country.

MATERIALS AND METHODS

Direct agglutination test antigen. The DAT antigen was prepared at the Prince Leopold Institute of Tropical Medicine in Antwerp (ITMA) using a modification of the method described by El Harith and others.[5,6] Log phase promastigotes of *L.* (*L*) *donovani* 1-S at a concentration of $1–2 \times 10^7$ parasites/ml in glucose lactalbumin serum hemoglobin[18] were harvested by centrifugation at $1,590 \times g$ at 2°C for 20 min and washed twice in phosphate-glucose-saccharose buffer solution (PGS), pH 8.0, before cell surface digestion with 0.4% trypsin for 60 min at 37.0°C with gentle orbital stirring (90 rpm). A 1% promastigote suspension in cold PGS was fixed with 4% formaldehyde at 0°C for 20 hr with stirring. Fixative was removed through repeated washing in phosphate-buffered saline citrate (PBSC), and promastigotes were stained with 0.025% Coomassie brilliant blue in PBSC for 20 hr at 4°C with stirring. A final 5×10^7 parasites/ml suspension was made in formaldehyde-citrate saline. The ready-to-use antigen batches were stored at 4°C until use. Antigen was sent to the University of Khartoum Central Laboratory (UKCL) in 5-ml vials in a kit containing reagents and supplies for 660 tests. The kit was designed to suit field and laboratory conditions. After approval by the local review board, the study protocol was reviewed and approved by the Secretariat Committee on Research Involving Human Subjects of the World Health Organization.

Direct agglutination test. Blood samples were collected in triplicate on filter paper. All DAT titers in this paper are expressed as plain blood titers from a serial dilution (1:500–1:512,000). We explicitly draw the attention of the reader to the fact that dilutions and titers in this paper are plain blood dilutions. They correspond to two-thirds of the actual serum dilution if one takes into account a mean hematocrit value of 30% (1:500 plain blood = 1:750 serum). In contrast to El Harith and others,[5] the expression of the dilution in this paper takes account of the volume of antigen added in the well of the microtiter plate. Accordingly, 1:500 plain blood dilution in this paper corresponds to a 1:375 serum titer expressed by El Harith and others.[5,8] The 1:3,200 serum titer recommended as a cut-off value by El Harith and others (serum dilution before addition of antigen)[5] corresponds in this paper to a titer of 1:4,000. A conversion table is provided in Table 1.

All subjects were screened at the field site by the DAT at

TABLE 1

Correspondence between blood and serum titers of the direct agglutination test (DAT) with and without consideration of added antigen volume

DAT titers mentioned in this study, on volume of diluted blood plus antigen	DAT titers mentioned by El Harith and others[6] on diluted blood volume only	DAT titers mentioned by El Harith and others[5] on diluted serum only	DAT titers on volume of diluted serum plus antigen
1:200	1:100	1:150	1:300
1:500	1:250	1:375	1:750
1:1,000	1:500	1:750	1:1,500
1:2,000	1:1,000	1:1,500	1:3,000
1:4,000	1:2,000	1:3,000	1:6,000
1:8,000	1:4,000	1:6,000	1:12,000
1:16,000	1:8,000	1:12,000	1:24,000
1:32,000	1:16,000	1:24,000	1:48,000
1:64,000	1:32,000	1:48,000	1:96,000
1:128,000	1:64,000	1:96,000	1:192,000
1:256,000	1:128,000	1:192,000	1:384,000
1:512,000	1:256,000	1:384,000	1:768,000

a dilution of 1:500. The test was carried out in microtiter plates (V-shaped wells) with the necessary positive and negative controls. In the UKCL laboratory, field samples were retested at a dilution of 1:500 and full serial titration was performed whenever positive (1:500–1:512,000 plain blood). Serology was repeated at the ITMA at a dilution of 1:500, followed by serial titration when positive.

The test was read visually against a white background. The end point titer was taken as the last well where agglutination was seen, i.e., the well with the dilution immediately before the well with a clear, sharp-edged, blue spot identical to the one observed in the negative control well.

Clinical examination. Between April 1993 and December 1994, 164 clinically suspected persons and 183 healthy persons were recruited from Ban-Dighaiw village in endemic Gedaref State in Sudan, an area free from trypanosomiasis. The aims of the study were discussed with community leaders. The objectives and procedures of the study were explained to individuals and their informed consent was obtained before participation in the study. All persons were examined clinically and blood samples for thick films and for DAT serology were obtained. Thick films were stained with Giemsa and examined microscopically for malaria.

A clinically suspected individual was a person with fever for a minimum of two weeks and splenomegaly at the clinical examination in whom malaria could be excluded (blood film negative). A healthy person was defined as a person who did not present any of the above symptoms or signs at the time of recruitment (t_0). Six drops (30μl/drop) of blood were collected from each person by fingerprick onto three filter papers. One filter paper was for DAT screening in the field, one for serologic analysis at the UKCL, and the third filter paper was sent to the ITMA for serologic analysis.

Bone marrow and lymph node (when conspicuous) was aspirated for parasitologic examination from every clinically suspected individual and from healthy persons with a positive DAT field screening result (DAT titer ≥ 1:500). The aspirates were smeared onto microscopic slides, dried, and fixed with methanol. Smears were read in the field without prior knowledge of the DAT result and checked at the UKCL. Quality control analysis of the parasitologic readings was done by the Laboratory for Protozoology at the ITMA.

Parasitologically positive cases and clinically suspected individuals with a positive DAT reading in the field (≥ 1:500) were immediately treated with sodium stibogluconate (10 mg of pentavalent antimony/kg for 30 days (Pentostam®; Wellcome, Manchester, United Kingdom). Of the 164 clinically suspected individuals examined, 15 were excluded because of signs of post kala-azar dermal leishmaniasis and one because of a missing result. Of the 183 healthy persons examined, two were excluded because of a history of VL and three because of missing data. Thus, 148 clinically suspected individuals and 178 healthy persons were enrolled in the study. Clinically suspected individuals and healthy persons were revisited at six and 12 months after the time of enrollment (t_0). This follow-up had the specific purpose of excluding persons with VL at t_0 from the control series.

Data analysis. The titers obtained at the ITMA were used for the validity analysis. The DAT titers for 49 VL cases and 176 controls were compared. A VL case was defined as a clinically suspected individual with positive parasitologic results on bone marrow or lymph node aspirates at the UKCL. A control was defined as a person healthy at t_0 who showed no clinical evidence of VL at a six-month follow-up visit. Sensitivity and specificity of the DAT were estimated with exact binomial 95% confidence limits. To study the optimal DAT cut-off level, a receiver-operator characteristics curve (ROC) was constructed.[19] Predictive values were calculated for a range of VL prevalence rates using Bayes' theorem.[19]

RESULTS

Amastigotes were found in 49 (33.1%) of 148 samples from clinically suspected cases. Three of these 49 were parasitologically and serologically negative at t_0, but were parasitologically positive six months later. Of the 178 healthy persons at t_0, 144 had 12 months of follow-up without clinical evidence of VL. Two were excluded from the analysis because they had developed clinical symptoms corresponding to the clinical case definition for VL at six months. Thirty-two healthy persons were lost during follow-up. Notwithstanding the fact that some of them might have subsequently developed VL, we decided to include them in the control series for two reasons: first, distribution of DAT titers at t_0 between the 32 persons without complete follow-up and the 144 with complete follow-up were comparable ($P = 0.47$, by Mann-Whitney test), and second, results on DAT validity including the 32 healthy persons without complete follow-up were similar to an analysis that excluded them.

Figure 1 shows the distribution of DAT titers in 49 parasitologically confirmed cases, 99 parasitologically negative clinically suspected persons, and 176 controls. The median DAT titer for the VL cases was a 1:64,000 plain blood dilution. For parasitologically negative clinically suspected persons and for controls, the median titer was ≤ 1:500. A DAT cut-off titer at a dilution of 1:500 had a sensitivity of 100% (49 of 49 cases detected). Table 2 shows the DAT sensitivity and specificity in a range of cut-off levels for 49 VL cases compared with 176 controls. The 1:8,000 cut-off titer in this study, which corresponded best to the most recent recommendation of a 1:6,400 titer for a DAT with serum by El Harith and others,[6] had a sensitivity of 95.9% (47

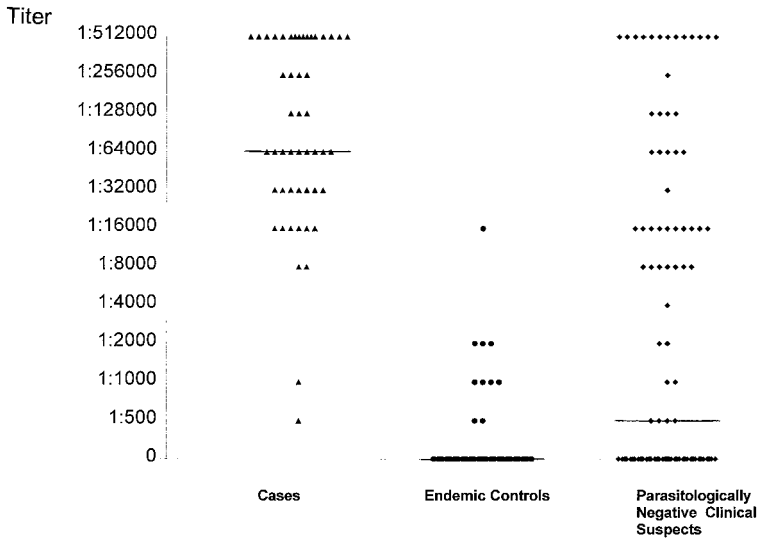

FIGURE 1. Distribution of direct agglutination test titers in cases of visceral leishmaniasis (▲), healthy endemic controls (●), and parasitologically negative persons (♦). The medians are represented by horizontal bars.

of 49). The ROC analysis (Figure 2) showed that in our study, the cut-off titer that optimizes both sensitivity and specificity is from 1:4,000 to 1:8,000, with a sensitivity of 95.9% (47 of 49 cases) and a specificity of 99.4% (175 of 176 controls).

The validity of DAT regarding the complete series of clinically suspected persons was analyzed separately in the course of the discussion of the present results.

DISCUSSION

The development of serodiagnostic tests for VL seems to be a showcase of the multiple methodologic pitfalls in test validation.[19–21] The results obtained in Sudan allowed us to address straightforwardly at least some of those problems. The most important question is the imperfect standard for diagnosing VL and the absence of such a reference test (gold standard) for infection with the parasite that causes VL.[15]

The definite diagnosis of VL depends upon the demonstration of leishmanial amastigotes in bone marrow or other biopsy material (spleen, lymph nodes, liver). These procedures are invasive, and thus ethically acceptable only in case of clinical suspicion of the disease. Moreover, they have poor sensitivity.[22] Zijlstra and others found a sensitivity of 93.1%, 67.8%, and 56.3% for splenic, bone marrow, and lymph node aspiration, respectively, in a study of 91 hospital patients.[23] In an extensive community survey in Bangladesh, only 29 (23%) of 125 clinically suspected persons were parasitologically proven, and all responded favorably to treatment.[24]

TABLE 2

Sensitivity and specificity values of the direct agglutination test according to the cut-off titer*

Titer	Sensitivity (no. of cases = 49)	Specificity (no. of controls = 176)
1:500	100% (92.7–100)	94.3% (89.8–97.2)
1:1,000	98.0% (89.1–99.9)	95.5% (91.24–98.0)
1:2,000	95.9% (86.0–99.5)	97.7% (94.3–99.4)
1:4,000	95.9% (86.0–99.5)	99.4% (96.9–100)
1:8,000	95.9% (86.0–99.5)	99.4% (96.9–100)
1:16,000	91.8% (80.4–97.7)	99.4% (96.9–100)
1:32,000	79.6% (65.7–89.7)	100% (97.3–100)
1:64,000	65.3% (50.4–78.3)	100% (97.3–100)
1:128,000	46.9% (32.5–61.7)	100% (97.3–100)
1:256,000	40.8% (27.0–55.8)	100% (97.3–100)
1:512,000	32.7% (19.9–47.5)	100% (97.3–100)

* Values in parentheses are exact binomial 95% confidence limits.

Although response to treatment is probably the best methodologic option in establishing a gold standard for VL, few related studies have been reported. The DAT was validated by Abdel-Hameed and others in a series of 49 patients who were either parasitologically positive (40 of 49) or responded well to treatment (9 of 49), and they reported a sensitivity of 100% for this test.[25] Specificity was not assessed. These investigators stated '' . . . The protocol followed for parasitological diagnosis is probably the most that could be done with the facilities available in Sudan.'' The DAT has been validated mainly by laboratory-based testing on banked sera from parasitologically proven VL patients of different geographic origins, from controls from nonendemic areas, and from patients with potentially cross-reacting diseases such as malaria, tuberculosis, leprosy, and trypanosomiasis. The latter was the only disease showing significant cross-reactivity in the DAT.[5,11] Parasitologic confirmation might be the second-best standard for diagnosing VL, but not for diagnosing infection with *L. donovani* in a community at risk. Neither parasitology nor response to treatment are acceptable as a gold standard for *L. donovani* infection in humans, given the substantial proportion of asymptomatic infections in the community[26–28] and, to a lesser degree, the persistence of the serologic response in treated VL-patients.[29]

In this study, we evaluated DAT validity for VL, and not for infection with the parasites that cause VL, in a series of VL patients and in persons without VL symptoms living in the same area at risk, i.e., the case-mix representative of an endemic area. Persons with prior VL were excluded from our control series. The gold standard was a combination of

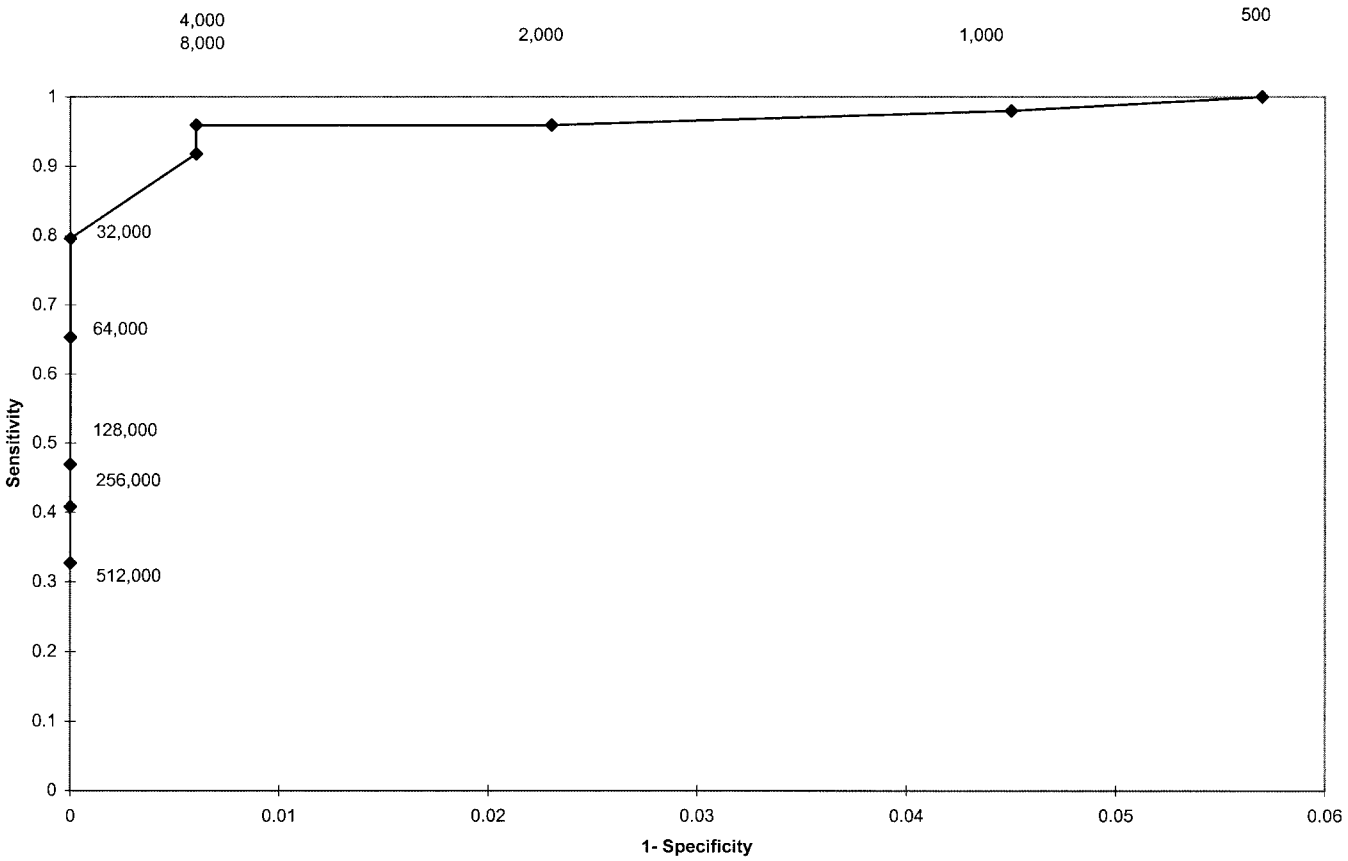

FIGURE 2. Sensitivity and specificity of 11 cut-off titers for the direct agglutination test determined at the Prince Leopold Institute of Tropical Medicine in Antwerp: comparison of cases with endemic controls.

parasitologic examinations of bone marrow and lymph node aspirates because splenic aspirates could not be implemented safely under the field conditions of the study.

According to our data, the performance of the DAT for the detection of VL was excellent. However, the sensitivity of 95.9% and the specificity of 99.4% we recorded at a dilution of 1:8,000 give an overly optimistic picture of the actual situation because the validation method we used includes two sources of uncertainty. First, the sensitivity of the DAT for VL could not be correctly assessed because the series of parasitologically negative clinical suspects (n = 99), assuming a false-negative rate of at least 20%,[23] probably contained at least 12 cases of VL. Second, subjects with cross-reacting heterologous diseases may be more frequent in a series of persons with clinical symptoms than in a series of healthy ones.[19] Thus, the specificity for VL estimated in healthy controls is likely to be an overestimate. This can be easily demonstrated if we assume that parasitology is the actual gold standard. If we compare the DAT results of the parasitologically positive and negative groups in the series of clinically suspected persons, the sensitivity of the DAT for a parasitologically positive smear is 95.4%, but the specificity is only 57.6%. Zijlstra and others found a sensitivity of 94% and a specificity of 72% in a similar way,[15] and Masum and others found a sensitivity of 100% and a specificity of 25% in a small series of clinically suspected persons (n = 53).[17] Such a dependence of the serologic specificity on the case-mix implies that in any clinical setting in which the DAT is applied to clinical suspects, the specificity of DAT for VL will definitely be lower than in our study. How much lower is difficult to assess, given the actual uncertainty about the gold standard.

Furthermore, diagnostic accuracy is critically dependent on the choice of the DAT cut-off titer for positivity. El Harith and others initially proposed a reciprocal titer of a 1:3,200 serum dilution as the best trade-off between sensitivity and specificity.[5] This value was subsequently used in community surveys in Sudan,[15,27] Kenya,[26,30] and Bangladesh,[24] as well as in clinical work in Sudan,[14,25] Ethiopia, [29,31] and Bangladesh.[17] In Somalia, Shiddo and others used a cut-off titer of 1:800 for population screening because it optimized sensitivity and specificity.[32] Nevertheless, the choice of a cut-off value cannot be based only on sensitivity and specificity because the predictive values of the test also depend on the epidemiologic context, as demonstrated in Table 3, in which positive (PPV) and negative (NPV) predictive values of a 1:8,000 cut-off titer were calculated according to prevalence rates ranging from 0.1% to 3%.

When the prevalence rate is approximately 0.1%, the PPV will be 14%. If the prevalence rate is somewhat higher (0.5%), 44 of 100 DAT-positive persons with clinical symptoms will actually have VL. The NPV is excellent throughout the whole range of prevalence rates.

The cost of a false-positive result and a false-negative classification should be considered for the individual as well as for the community in the decision on a cut-off titer.[19] A

TABLE 3

Positive predictive (PPV) and negative (NPV) predictive values for a 1:8,000 direct agglutination test cut-off titer (sensitivity = 95.9%, specificity = 99.4%), according to prevalence rate

Prevalence rate	PPV	NPV
0.1%	14%	99.9%
0.5%	44%	99.9%
1%	62%	99.9%
2%	76%	99.9%
3%	83%	99.9%

false-positive diagnosis of VL has the implication of subjecting someone to an expensive although relatively safe treatment regimen. A false-negative diagnosis is also harmful in that sense that it will cause a delay of treatment, assuming patients will come back to the health service if they do not feel better. In view of these considerations, the decision on a DAT cut-off titer has to be made in the context of the country and of the health services concerned. We strongly warn against the use of a single, universal, DAT cut-off titer for all purposes.

Based on the available evidence, we can formulate some preliminary recommendations about the use of the DAT provided the reproducibility of the test is confirmed. The data presented here were based on DAT results obtained at the ITMA laboratory, which functioned under highly controlled and favorable conditions: the DAT antigen was not transported under risky conditions, and cold storage was without interruption. The DAT has so far proved to be a highly sensitive and specific test at the ITMA. However, although the cases and controls were selected from an endemic area, the spectrum of these cases and controls is not representative of a clinic situation in which a test for VL will be applied. If the performance of the DAT can be reliably reproduced in the health service, we can recommend its use to screen clinically suspected cases for VL. In clinically suspected persons with a negative DAT result, the disease can be ruled out with a high degree of certainty and there will be no need for parasitologic analysis or scheduled follow-up. However, if a clinically suspected person has a positive DAT result, we cannot, with the presently available evidence, recommend treatment based only on the DAT result in an endemic situation. In an epidemic situation, treatment based only on the DAT result might be justified. The weakness of the specificity of the DAT in clinically suspected persons is partially due to inherent weakness of the validation methodology, given the imperfect gold standard. Several methodologic improvements seem possible: better clinical case definition, validation of the DAT by a carefully designed study with response to treatment as a gold standard, and more advanced mathematical methods should be explored.[33] The detection of infection with *L. donovani,* including the diagnosis of asymptomatic carriers and subclinical cases of VL, should be addressed in a straightforward manner, comparing the DAT with other serologic assays in carefully designed prospective studies.[34]

In conclusion, we can state that notwithstanding the highlighted methodologic difficulties, the DAT can be regarded as having a fairly high validity for detection of VL in persons with clinical symptoms. Further research on test validation is needed, with careful attention given to the choice of the gold standard. Whenever the DAT is included in a decision tree, the cut-off titer for diagnosis should be carefully weighted against its predictive values according to the prevailing prevalence rates. The cut-off titer for decision making also depends on the case-mix, and might vary depending on whether the test is used for active case detection in the community, or for passive case detection at a first-line or intermediate level health facility. Last but not least, the reliability of the DAT in terms of reproducibility still needs to be formally evaluated.[35]

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