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An Evaluation of the Performance of Direct Agglutination Test on Filter Paper Blood Sample for the Diagnosis of Visceral Leishmaniasis

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Abstract. The attack phase of the visceral leishmaniasis (VL) elimination program in Bangladesh aims to decrease the burden of VL incidence from close to 20 cases to less than one case per 10,000 at sub-district level. The consolidation phase will aim to confirm no increase in VL in endemic areas through active surveillance. During this phase, a reliable diagnostic tool for mass screening is required. Here, we report the diagnostic sensitivity and specificity of a filter paper-based agglutination test (FP-DAT) for diagnosis of VL in patients admitted to an upazila health complex in Mymensingh, a VL-endemic region of Bangladesh. The sensitivity of both the conventional direct agglutination test (DAT) and FP-DAT were 100% and 96%, respectively. The specificity of both assays was 100%. However, when the performances of the two assays were compared using McNamar's test, neither the sensitivity nor the specificity of the FP-DAT differed significantly from conventional DAT.

INTRODUCTION

Under the initiative of the World Health Organization (WHO), the Governments of Bangladesh, India, and Nepal committed to eliminate visceral leishmaniasis (VL) or Kala-azar (KA) by the year 2015.[1] This elimination program was conceived in four phases (preparatory, attack, consolidation, and maintenance) for South-East Asia. In Bangladesh, the attack phase is coming to an end and the consolidation phase will begin very soon.[2] Bangladesh has already made remarkable achievement by decreasing the number of active cases from 9,379 in 2006 to 1,902 in 2012 (Director General Health Services, Bangladesh, 2013). To maintain and accelerate this promising trend, early diagnosis of VL cases by active case detection and periodic mass screening will be crucial. Because this disease occurs primarily in the most resource-limited regions of endemic countries, there remains a high risk of underreporting.[3] Rigorous active case detection during mass screenings will require a thoroughly evaluated diagnostic tool.

Among all the diagnostic tools for VL, the direct agglutination test (DAT) has been extensively validated in most VL-endemic areas for high sensitivity and specificity (94.8% and 97.1%, respectively).[4] However, the DAT requires centralized laboratory support. In resource-limited contexts, blood sample transportation from the field to the laboratory presents a critical challenge for diagnosis and screening. Blood sample transportation requires trained personnel, must occur in a specific timeframe, and remains sensitive to environmental conditions like extreme heat. These problems can be addressed by drying the blood sample on filter paper before transporting. It has been shown that this simple solution can dramatically improve the logistics of laboratory testing for genetic, hormonal, immunological, and biochemical analyses in epidemiological surveys without compromising the accuracy or precision of results.[5–9] For this reason, we evaluated the performance of the DAT performed on blood samples dried on filter paper before transportation and compared it to that of the conventional DAT using liquid blood samples.

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MATERIALS AND METHODS

Study site and study period. The study was conducted in the Upazila Health Complex of Muktagacha a sub-district of Mymensingh in Bangladesh between May and December of 2012.

Study participants. Patients diagnosed with VL in the Muktagacha Health Complex during the study period were invited to participate in the study. Age and sex matched healthy individuals from the same community were invited to participate as controls. Written informed consent was obtained before case and control subject enrollment. Inclusion and exclusion criteria were derived from the National Guideline for VL diagnosis. These criteria are as follows:

1. Inclusion criteria for a VL case: Patients with fever more than 2 weeks, splenomegaly, rK-39 test positive, no past history of VL or post kala-azar dermal leishmaniasis (PKDL), and who inhabited the endemic area were considered cases of VL, which were confirmed when response to the anti-leishmanial drug was observed.
2. Exclusion criteria for a VL case: Patients lacking one of the previously mentioned inclusion criteria were excluded. (e.g., fever < 2 weeks, no splenomegaly, rK-39 test negative and/or who were inhabitants of non-endemic areas).
3. Inclusion criteria for a healthy control: Participants who were not suffering from fever, having no splenomegaly, rK-39 test negative, and inhabitants of endemic zones were included as healthy controls.
4. Exclusion criteria for a healthy control: Participants were excluded as healthy controls for presenting with any one of the previously mentioned inclusion criteria (e.g., suffering from fever, having splenomegaly, rK-39 test positive and/or who were inhabitants of non-endemic areas).

Gold standard. National Guideline for VL diagnosis along with effective treatment response is considered as the gold standard. According to the guideline, any one will be considered as a VL patient if he/she is a habitant of a VL-endemic area, having fever > 2 weeks, and splenomegaly along with a rK-39 strip test positive. A patient is considered as finally cured if fever has subsided and spleen size returned to the normal size.

Sample collection and storage. Venous Blood samples were collected once from cases before treatment and once from

controls to perform conventional DAT and filter paper-based agglutination test (FP-DAT). The 3 mL of venous blood from the antecubital vein was collected using all aseptic precautions. For the filter paper samples, three drops of blood sample from the syringe were spotted on Whatman filter paper (GE Healthcare, Little Chalfont, United Kingdom) where each drop contained ~50 μL. The filter paper blood sample was then air dried and stored at room temperature (20 to 25°C). The remaining whole blood sample was centrifuged at a rate of 3,500 rpm for 5 minutes; between 1 and 1.5 mL of serum was separated and then stored at –20°C.

Reagents. All reagents for the DAT were prepared by Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium, in the Applied Technology and Production Unit and kindly donated for this study. Freeze-dried suspensions of purified, trypsin-treated, fixed and stained promastigotes of *Leishmania donovani* strain 1-S were used to prepare DAT antigen. Freeze dried serum in phosphate buffered saline (PBS) was used as a positive control. A freeze-dried solution of bovine albumin was used as a negative control and for the preparation of both DAT buffer and DAT diluents PBS supplemented with protein (bovine serum albumin) was used. DAT antigens along with positive and negative controls were stored at –20°C. The DAT buffer and DAT diluents were stored between 2 and 8°C.

Reconstitution of reagents. First, 2.5 mL of DAT buffer was added to a vial of freeze-dried DAT antigen and shaken gently for a few seconds to obtain a homogeneous suspension. Next, 0.6 mL DAT buffer was added to positive and negative control vials. Finally, 0.24 mL, 2-Metacarpo-Ethanol (ME) was added to the vial of DAT diluents. Blood protein eluates were prepared from the filter papers in V-shaped microplates.

Preparation of filter paper eluates. From each blood spot, a 5-mm disc was punched out and placed in a well of a microplate. 125 μL of DAT buffer was pipetted into each well. The plate was covered to avoid evaporation and incubated overnight at 4°C. Assuming a mean hematocrit volume of 50% of the total, a 5-mm disc of blood-impregnated filter paper contains ~5-μL blood and 2.5 μL serum. The eluate in 125 μL of DAT buffer thus corresponds to an approximate serum dilution of 1:50.

Dilution of blood eluates and serum. From the filter paper blood eluates, serial 2-fold dilutions starting at 1:100 and ending at 1:51,200 were made in fresh DAT buffer. Serum samples were thawed and homogenized through gentle mixing. Serial 2-fold dilutions of 1:100 up to 1:51,200 were made in a fresh DAT buffer in V-shaped microplates.

Test procedure. The filter paper DAT and the liquid sample DATs were performed simultaneously. To every fifth plate, a positive and a negative control serum was added. For each test sample, an "antigen control," containing 50 μL of diluent per well, was included; 50 μL of the antigen was pipetted into the wells containing the test sample dilutions and to the control wells. The microplates were covered and incubated overnight at ambient temperature. The results were read after 18 hours. The cut-off value of antibody was considered as 1:3,200.

Calculation of sample size and statistical analysis. Assuming expected sensitivity of FP-DAT was not < 90% along with precision ± 10% and confidence interval 95%; our calculated sample size was a minimum 35 pairs. However, we took 50 pairs upon the availability of cases and controls within the suggested period of study. All data were computed and analyzed using the SPSS software (version 16.01, SPSS, Inc., Chicago, IL) and McNemar's test was performed to estimate the difference between the two protocols. The sensitivity, specificity, predictive values (positive and negative), and diagnostic accuracy were mathematically calculated as follows: sensitivity = (true positive/true positive + false negative) × 100; Specificity = (true negative/true negative + false positive) × 100; Positive predictive value (PPV) = (true positive/total positive) × 100; Negative predictive value (NPV) = (true negative/total negative) × 100; Diagnostic accuracy = (true positive + true negative/grand total) × 100. True positive and negative were defined by the previously mentioned clinical parameters.

Ethical considerations. The Ethical Review Committee of the respected institutions approved the protocol for this study. Written informed consent was obtained from each study participant and from parents or a legal guardian for all child participants.

Table 1
Results regarding conventional DAT

	Conventional DAT
Sensitivity	100%
Specificity	100%
Positive predictive value	100%
Negative predictive value	100%
Accuracy	100%

DAT = direct agglutination test; FP-DAT = filter paper-based agglutination test.

RESULTS

Participants' particulars. A total 50 cases were enrolled in the study and among them 60% were male and the rest were female with a mean age of 28.88 ± 23.38 years and spleen size of 4.78 ± 2.31 cm. All participants were from the endemic areas and had a history of fever for more than 2 weeks and also a rK-39 strip test positive (Table 1). Again, 50 controls were enrolled among them 60% were male and the rest were female with a mean age of 29.11 ± 22.33 years. All participants had no history of fever, absence of splenomegaly, and rK-39 negative.

Performance of FP-DAT and conventional DAT for diagnosis of VL. The performance of FP-DAT and conventional DAT are shown in Tables 1 and 2. Among 50 cases, FP-DAT showed a positive result of 48 samples, whereas conventional DAT showed a positive result to all samples. The McNemar's test showed no significant difference between these two procedures (P value = 0.1573), which means that the performance of the two procedures were the same (Table 3). Again, the calculated specificity, positive predictive value, negative predictive value, and accuracy of FP-DAT were

Table 2
Results regarding FP-DAT*

	FP-DAT
Sensitivity	96%
Specificity	100%
Positive predictive value	100%
Negative predictive value	96.15%
Accuracy	98%

*FP-DAT = filter paper-based agglutination test.

TABLE 3

Comparison between DAT on filter paper and venous blood samples for the detection of *Leishmania* antibodies in confirmed human samples*

	Filter paper blood samples		
Venous blood samples	Positive (n)	Negative (n)	Total (n)
Positive (n)	48	02	50
Negative (n)	00	00	00
Total (n)	48	02	50

McNemar test: x^2: 2; df: 1; *P* value: 0.1573

*df = degrees-of-freedom.

96%, 100%, 100%, 96.15%, and 98%, respectively, although the all-estimated values of conventional DAT were 100%. We calculated the different validity parameters of the two diagnostic tests taking the cut-off value of Ab titer one-fold below (1:1,600) the cut-off value (1:3,200) but no difference was revealed.

DISCUSSION

The most important finding of our work was that the widely used conventional DAT and the DAT performed using blood spots dried in the field on filter paper do not differ significantly in their ability to diagnose VL. Visceral leishmaniasis mainly occurs among the poorest people in rural-endemic areas, where health care resources are limited.[10] Therefore, developing and validating a sensitive and specific diagnostic test that is also practical in these settings is an important step toward successful long-term surveillance of VL in these regions. The conventional DAT has been used as a well-regarded sero-diagnostic test for VL for the last two decades,[11] but there exist inherent limitations in field applications that require handling and transport of liquid blood. In this study, the performance of DAT on filter paper blood samples was evaluated and compared with the conventional DAT. In this study, the FP-DAT showed a specificity of 100% and a sensitivity of 96%, which is consistent with other studies using the conventional DAT method.[12] Therefore, when considering the different study results, we may suggest that the quality of the opportunity is to introduce this practical change in methodology for mass surveillance of VL. It is important to note that some studies have suggested that rK-39 strip testing is the best field-level diagnostic tool based on results and cost effectiveness.[13] However, rK-39 testing shows disparate results depending on manufacturer and region. The DAT has not been shown to suffer from these limitations and inconsistencies.[14] In this regard, the DAT is preferable to rK-39 strip testing. We further show that the DAT can be better adapted to the field using the filter paper sample collection with no or minimal effect on results.[9,13–14] However, a further limitation of our study is the sample size. Although the results are encouraging, a large-scale study would provide more reliable data about the comparison of the conventional approach to DAT and our modified FP-DAT.

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