EVALUATING DIAGNOSTICS

Evaluation of rapid diagnostic tests: visceral leishmaniasis

Marleen Boelaert, Sujit Bhattacharya, François Chappuis, Sayda H. El Safi, Asrat Hailu, Dinesh Mondal, Suman Rijal, Shyam Sundar, Monique Wasunna and Rosanna W. Peeling

Visceral leishmaniasis (VL) is a severe infectious disease caused by a protozoan parasite: *Leishmania donovani* in East Africa and the Indian subcontinent and *Leishmania infantum* in Latin America and the Mediterranean basin. Not all leishmanial infections lead to overt clinical disease, but in those infected persons who do develop the disease, multiplication of the parasite in the reticulo-endothelial system causes prolonged fever, anaemia, hepatosplenomegaly and weight loss. VL is fatal if it is not adequately treated. The drugs currently used to treat VL can have severe side effects and the clinical presentation of VL is not sufficiently specific to guide treatment. Highly accurate (both sensitive and specific), cheap and simple rapid diagnostic tests (RDTs) are therefore crucial for case-management of VL. Early case detection followed by adequate treatment is also central to control of VL because, as yet, no vaccine is available and the long-term impact of vector control is unclear.

Although the need for accurate VL diagnostics is obvious, innovation in this field has been slow. Since the 1980s, the main objective of VL diagnostics development has been to replace the direct demonstration of parasites in tissue smears, a technique that is invasive and requires considerable expertise, by a 'field test' that is more appropriate for use in a VL-endemic context. Several serological tests have been developed, but none are specific for VL disease as such, although they have proved useful in combination with a clinical case definition.

New diagnostic tools are needed for more than just the confirmation of VL disease. No alternatives to parasitological methods are yet available to establish test of cure in treated VL patients. Clinicians do not have the tools to distinguish re-infection from relapse in cases of recurrence, and control programmes do not have validated assays for the surveillance of drug resistance in parasites. Furthermore, in the context of the VL elimination initiative, it would be desirable to have better markers of leishmanial infection at the population level.

Any evaluation of a new diagnostic device should carefully identify its intended purpose. Too often developers and researchers confuse a device for the detection of leishmanial infection with a device for the confirmation of VL disease, and this is particularly the case for nucleic-acid-based assays. PCR is usually highly sensitive for detection of leishmanial infection, but this does not mean PCR will be useful for the confirmation of acute VL disease in patients in endemic areas, as many carriers of the infection in these areas will be PCR-positive without developing VL disease. This article will focus specifically on the evaluation of RDTs for confirmation of VL disease.

I. CURRENT DIAGNOSTIC TESTS FOR VL DISEASE

The World Health Organization (WHO) established the clinical case definition of VL as persistent fever (≥2 weeks) and splenomegaly in a person residing in an VL-endemic area[1]. The combination of both signs is found in the majority of VL cases, though splenomegaly is not always present[2]. Some VL control programmes therefore add other clinical signs or symptoms to this definition, such as wasting, anaemia and lymphadenopathy. Unfortunately, these clinical definitions lack specificity as such signs are common in other diseases that can be prevalent in VL-endemic areas, such as malaria, hyper-reactive malarial splenomegaly, enteric fever, disseminated tuberculosis, brucellosis and haematological malignancies. Given the high cost and toxicity of the current therapeutic options for VL, starting a course of anti-leishmanial treatment solely on the basis of clinical suspicion is not acceptable. Confirmatory diagnostic tests must therefore be used, particularly in first-line health services, where the prior probability of disease is lower than in referral centres. Below, we discuss the existing options for confirmation of diagnosis, with the emphasis on those techniques that are suitable for field use.

1. Parasite-detection methods

The identification of parasite amastigotes in tissue smears or culture has been the recommended method of VL diagnosis for many years but has variable sensitivity, depending on the type of aspirate that is used. The most sensitive technique, splenic aspiration, can only be used under highly controlled conditions (see below), and is not suitable for decentralized use in first-line health services.

1.1. Direct microscopic examination. The amastigote forms of the parasite (called 'LD bodies') can be seen intracellularly in monocytes or macrophages on microscopic examination of Giemsa-stained blood or aspirates from lymph nodes, bone marrow or spleen. Amastigotes are round or oval bodies, 2–4 μm in diameter, with characteristic organelles (nucleus and kinetoplast). The identification of amastigotes requires expertise and training and the accuracy is dependent on the microscopist.

The sensitivity of direct microscopic examination varies, but it is lowest in peripheral blood smears, as parasitaemia in immunocompetent individuals with VL is low. The reported sensitivity of direct microscopic examination of lymph node aspirates ranges from 52% to 58%[2,3], and for bone marrow aspirates from 52% to 85%[2,4,5]. Enlarged lymph nodes are typically observed in VL patients in Sudan, but are rare in patients from other countries. Spleen aspiration has been shown to be the most sensitive aspirate assay (93.1%–98.7%)[2,3,6]. Parasite density in

Table 1 | **Ideal performance and operational characteristics of diagnostics tests for VL**

Purpose of test	Level of use	Sensitivity	Specificity	Time to result	Comments
Case detection	Field and laboratory use	≥95%	≥98%	30 min	
Test of cure	Field and laboratory use	≥95%	≥95%	Does not have to be rapid	
Relapse	Field and laboratory use	≥95%	≥98%	30 min	Should be able to distinguish between past and active infection
Surveillance	Laboratory use	N/A	≥98%	Does not have to be rapid	Used to monitor transmission
Detection of drug resistance	Laboratory use	N/A	N/A	Does not have to be rapid	
Certify elimination	Laboratory use	N/A	≥98%	Does not have to be rapid	

N/A, not applicable; VL, visceral leishmaniasis.

splenic or lymph node aspirate smears can be graded on a logarithmic scale (from 0 to 6+), allowing the response to treatment to be evaluated, and slow responders can be distinguished from non-responders by using sequential smears[7]. A safe procedure for splenic aspiration has been developed in Kenya[8], but it remains an invasive and complex technique[1,5]. After the procedure the patient must be observed in the recumbent position for a minimum of 8 hours in a facility where blood transfusion is available. Splenic aspiration is not possible in non-cooperative children, is difficult in those without a palpable spleen and is contra-indicated in persons with active bleeding, thrombocytopenia, severe anaemia or jaundice, those in a moribund state, non-cooperative individuals and pregnant women. There is a small risk of fatal haemorrhage[7] and several authors have reported iatrogenic morbidity and mortality[9,10]. One death was observed in a series of 671 splenic aspirates in Kenya[8], and 3 in a series of 3,000 in India[11]. Two episodes of fatal bleeding occurred following 9,612 splenic aspirates (0.02%) in a specialised treatment centre in India[12]. In conclusion, splenic aspirate is highly sensitive and specific, but can only be carried out under strictly controlled conditions, and is not suitable for use in first-line health centres.

Fluid from tissue aspirates can be inoculated in Novy–MacNeal–Nicolle medium for culture, which increases the sensitivity, but parasite culture is costly and time-consuming, and requires expertise and expensive equipment. Its use is therefore restricted to referral hospitals or research centres.

1.2. Molecular diagnosis. Molecular approaches to diagnosis have recently been reviewed by Reithinger and Dujardin[13]. These techniques remain complex and expensive, and in most VL-endemic countries they are therefore restricted to a few teaching hospitals and research centres.

2. Antigen-detection methods

Recently, Sarkari *et al.* described a urinary leishmanial antigen, a low-molecular-weight, heat-stable carbohydrate that was detected in the urine of VL patients[14]. An agglutination test to detect this antigen has been evaluated in laboratory trials, using urine collected from well-defined cases and controls from endemic and non-endemic regions. This test showed 100% specificity and sensitivity between 64% and 100%[15]. However, the sensitivity of this test was disappointingly low in clinically suspect patients in a VL-endemic area in Nepal[16]. Further work is ongoing, as this technique holds promise as a test of cure, for which none of the current serological tests is appropriate.

3. Serological methods

Several antibody-detection tests have been developed for field diagnosis of VL, but, as mentioned before, none is sufficiently specific for acute VL disease to be used as a stand-alone test. In a VL-endemic region, asymptomatically infected persons can also be positive in these antibody-detection tests, but they do not require treatment. This used to be the reason why many control programmes restricted treatment to parasitologically confirmed patients. However, since the late 1990s, ample evidence has been generated that a combination of the WHO clinical case definition for VL and a positive antibody test is an adequate and safe basis for the decision to treat[17]. Nonetheless, the limitations of these antibody-detection tests in clinical practice should be acknowledged. Assessment of cure is necessary at the immediate end of treatment (which usually lasts 21–28 days) and also at 3 and 6 months post-treatment, at a time when antibody levels have not yet waned. This also limits the usefulness of the current antibody-detection tests in persons with a previous history of VL who present with recurrence of fever and splenomegaly, as these tests cannot discriminate between a case of VL relapse and other pathologies.

Conventional methods such as gel-diffusion immunoelectrophoresis, a complement-fixation test, indirect haemagglutination test and counter-current immunoelectrophoresis have limited diagnostic accuracy and/or feasibility for field use[18–20]. Indirect fluorescence antibody (IFA) tests showed acceptable estimates for sensitivity (87–100%) and specificity (77–100%)[21,22] but the need for a fluorescence microscope restricts their use to reference laboratories. So far, only two antibody-detection tests have been extensively evaluated for field use: the direct agglutination test (DAT) and the rK39 immunochromatographic test (ICT).

3.1 DAT. In 1985, El Harith *et al.* developed a DAT for VL with high sensitivity and specificity[23], and these values have been confirmed by other laboratories[21,24–27]. The test is semi-quantitative and uses microtitre plates with V-shaped wells in which increasing dilutions of serum or blood eluted from filter paper are mixed with stained killed *L. donovani* promastigotes. As the ongoing VL epidemic in Sudan[28] created a pressing demand, the DAT was rapidly taken to the field. Contradictory reports on its performance were soon published[29,30]. A multi-centre study reported low reproducibility owing to problems reading the results and the heat- and shock sensitivity of the liquid antigen[31]. A freeze-dried version of the test was developed to circumvent the latter problem, and this version showed similar diagnostic performance to the liquid version[32–34].

Since 1986, the DAT has been extensively validated in most VL-endemic areas. Thirty studies were included in a recent meta-analysis, showing sensitivity and specificity estimates of 94.8% (95% confidence intervals (CI), 92.7–96.4) and 97.1% (95% CI, 93.9–98.7), respectively[17]. The performance of the DAT was not dependent on the region nor on the *Leishmania* species. DAT antigen production was initiated in some endemic countries but the production could not always be

sustained, and quality control remained an issue. The cost of the antigen is in the range of €1–2 per test. Although highly sensitive and specific, the DAT requires substantial manipulation, and can only be read after a minimum of 8 hours incubation.

3.2. rK39 ICT. A test based on a 39-amino-acid-repeat recombinant leishmanial antigen from *Leishmania chagasi* (rK39) has been introduced into an enzyme-linked immunosorbent assay (ELISA)[35,36] and, later, an immunochromatographic strip test[37]. The latter is easy to use in the field and results are available after 15 minutes. The initial study showed 100% sensitivity and 98% specificity[37], but this particular format (Arista Biologicals, Allentown, PA, USA) is no longer commercially available. An evaluation in Sudan of an ICT from the same producer showed only 67% sensitivity[38]. An ICT produced by a different company (INBIOS, Seattle, WA, USA) proved to be a good diagnostic guide in suspected VL cases in India[39] and in Bangladesh, Sarker *et al*. found excellent sensitivity and specificity with this ICT[40]. In Nepal, an early prototype showed a specificity of only 71% in controls with clinical signs of VL[41]; however, better specificity was obtained with later generations of the InBios ICT and with an ICT produced by DiaMed AG, Switzerland[22,42].

II. THE NEED FOR EVALUATION OF VL RDTs

An expert meeting on VL diagnostics convened by TDR in Nairobi, Kenya in January 2006 identified multiple challenges in the development of VL diagnostics.

The clinical evaluation of new tests is fraught with difficulties. The lack of a gold standard has made diagnostic accuracy studies for VL extremely complex[43]. A gold standard in VL diagnosis exists — VL culture from splenic aspirate. However, obtaining splenic aspirates is invasive, and culture techniques are often not available in VL-endemic areas. The clinical presentation of the leishmaniasis syndromes varies considerably in different regions, and the current RDTs behave differently in the Indian subcontinent compared with East Africa[17]. It is therefore essential to evaluate any RDT in the region in which it will be used and greater uniformity in such diagnostic evaluations is important. The fact that substandard and/or counterfeit products have been circulating in endemic regions only adds to the need for rigorous evaluation and quality assurance. Last but not least, the variable performance of VL diagnostics in VL–HIV co-infected patients poses new challenges to test evaluation[44].

In addition to confirmatory tests for diagnosis, a marker indicating the prognosis in treated patients, a test of cure after therapy, a marker of asymptomatic infection and assays that allow easier surveillance of parasite drug resistance are also needed. The ideal performance and operational characteristics for the different VL diagnostic tests that are required are summarized in TABLE 1.

The purpose of the test being evaluated should guide the design of the trial as the operational and performance characteristics of a test can vary depending on the purpose of the test. It is of utmost importance in the evaluation of diagnostic devices for leishmaniasis to distinguish the detection of infection from the diagnosis of VL disease.

III. GENERAL ISSUES IN STUDY DESIGN

Past evaluations of RDTs have concentrated too often only on sensitivity and specificity. A proper evaluation of an RDT should address its performance (sensitivity, specificity and reproducibility) as well as its operational characteristics (user-friendliness and stability) and cost (see *Evaluation of diagnostic tests for infectious diseases: general principles* in this supplement). Also, it should be acknowledged that the development of a diagnostic test involves several phases, from early proof-of-principle and laboratory-based studies on archived samples to, eventually, clinical evaluation on prospectively recruited patients. Before a VL test can be recommended for clinical use, its clinical benefits should have been demonstrated in a prospective study that evaluated the test on a representative sample of the target population. For VL RDTs, these are the patients on whom the RDT will be used in the future; that is, persons with signs and symptoms that make them clinically suspect for VL. Zhou *et al*. distinguish three phases in the evaluation of diagnostics; this is useful as the study design will depend on the phase of evaluation[45] (BOX 1) .

Below, we discuss the essential elements in the design of a protocol to determine the diagnostic accuracy of RDTs for VL.

1. Rationale for the study

In the introduction to the evaluation protocol it should clearly state the rationale for the evaluation and the objectives of the study, describing what is already known about the issue, and how the new diagnostic test might contribute. The specific indication for the new diagnostic test should be described. Is this a test to be used in sick patients to confirm their diagnosis, or is it a marker of infection to be used for epidemiological work at the population level? Is the test a marker of acute disease? Can it be used as a prognostic marker or test of cure? In which phase of development is the test?

2. Study site

The local VL epidemiology (causative species, endemicity and most affected age groups), the climatic conditions and the workplace conditions at the study site should be described. Will the study be carried out in a research laboratory (proof-of-principle and case-control designs) or in the clinical setting (in a first-line health centre or in a

Box 1 | **Phases in the evaluation of a diagnostic test for VL**

Phase 1

The aim of Phase 1, early exploratory studies, is to provide proof-of-principle that the marker is strongly associated with visceral leishmaniasis (VL). This requires a relatively small number (10–100) of samples, usually archived specimens.

Phase 2

In Phase 2 studies, the candidate test is evaluated in a case-control design, on several series of subjects (or archived samples of subjects), who are enrolled on the basis of their status: VL or control. The sample size should be a minimum of 100 subjects in each series. The control subjects should be of different kinds: healthy non-endemic, healthy endemic and patients with potentially cross-reacting diseases.

Healthy non-endemic controls are healthy persons living in a region where no leishmanial transmission occurs and who have not been exposed to it by travel. Healthy endemic controls are persons living in an endemic region without signs or symptoms of leishmanial disease. Controls with potentially cross-reacting diseases are patients with a confirmed diagnosis of a disease that might induce a false-positive reaction to the serological test. These include malaria, African trypanomosomiasis, Chagas disease and tuberculosis.

Phase 3

Phase 3 studies are large-scale prospective studies validating the test in the target population, requiring a sufficiently large (usually minimum 300) and representative sample of consecutively enrolled or randomly selected patients. In this case, all persons who are clinically suspect for VL (febrile for more than 2 weeks and presenting with splenomegaly) should be recruited consecutively from the clinical setting where the test will be used in the future.

Table 2 | **Recommended reference test for the evaluation of an RDT for detection of active VL disease**

Reference standard	Specimen(s)	Problems
Direct smears and culture of tissue aspirate, including splenic aspirate	Splenic aspirate, or lymph node or bone marrow aspirates	Splenic aspirates can only be carried out under controlled conditions (risk 0.1%)
		Will yield only minor misclassification bias, which should be adjusted for
If splenic aspirates cannot be obtained, use latent class analysis, based on one or more of the following: other parasitology; validated serology (rK39, or DAT); response to treatment (if other markers available); specific clinical signs (pancytopenia, darkened skin)	Lymph node or bone marrow buffy coat; serum or capillary blood	Latent class analysis requires good prior knowledge of the markers included in the model or the inclusion of a sufficient number of markers for identifiability; response to narrow-spectrum drug and no drug resistance/ requires standardization of assessment
If splenic aspirates cannot be obtained, use a composite reference standard based on one or more of the following: other parasitology; validated serology (rK39 or DAT); response to treatment (if other markers available)	Lymph node or bone marrow buffy coat; serum or capillary blood	A composite reference standard requires good prior knowledge of the markers included and adjustment for the amount of misclassification bias; response to narrow-spectrum drug and no drug resistance/ requires standardization of assessment

DAT, direct agglutination test; VL, visceral leishmaniasis.

specialized VL treatment centre)? Describe the type of infrastructure, and the type of staff conducting the test.

3. Study population
The choice of the study population will depend on the phase of development of the test (see above). If the study is carried out using archived samples, provide as much detail as possible on the origins of the samples, as well as the methods that were used to reach the diagnosis. Describe how these samples were stored and for how long. If the study requires prospective recruitment of patients in a clinical setting, carefully describe the inclusion and exclusion criteria. Standardized clinical case definitions should be used for enrolment, preferably the WHO case definition (see above). The minimum age of the participants should be included. Concomitant illness might confound the study results and some should be considered as exclusion criteria. The design of the evaluation should consider recent treatment of cases; although recent treatment will have little impact on the results of serological RDTs, as antibody-based tests usually remain positive for several months after treatment, it might affect the evaluation of antigen-detection tests.

4. Co-morbidities
The performance of VL diagnostic tests is highly influenced by HIV co-infection[3]. HIV co-infected patients typically have lower antibody and higher parasitaemia levels. Future studies of VL diagnostics should specify the HIV status of the study population and, if possible, assess the HIV status of the study subjects to allow for a separate estimate of test performance in HIV-positive and -negative patients. Due consideration should be given to all of the ethical aspects of HIV testing.

5. Recruitment process
Persons who give informed consent should undergo an interview and a physical examination according to clinical best-practice guidelines, as well as the work-up for case ascertainment if they are clinically suspect for VL. Information should be collected about sex, age, duration of illness, previous history of VL and onset of symptoms, as suggested in the sample clinic data collection form in APPENDIX 1.

6. Tests under evaluation
Record all details of the RDTs that will be evaluated, including: manufacturer (company name, site of manufacture), batch number, date of manufacture, packaging type and inclusion of desiccant, lancets or capillary tubes. Note whether the product is under evaluation for regulatory purposes or is already commercially available.

7. Reference standard
Several published VL diagnostic accuracy studies suffer from reference test bias. Researchers comparing a new test to a reference standard with high specificity but low sensitivity, such as bone marrow or lymph smears, will underestimate the true specificity of the new test. This kind of sub-optimal reference standard misses many true VL cases that test positive with the new test[1]. Moreover, lymph-node-positive VL patients probably comprise only a sub-set of all VL patients in a given region, and this might again bias the sensitivity estimates. All of the tissue aspirate assays have another inherent problem: they cannot be applied indiscriminately to healthy controls, which complicates the ascertainment of control status in Phase II studies.

The demonstration of parasite amastigotes in smears or culture from splenic aspirates should be used as the reference standard in VL diagnostic accuracy studies, if the procedure can be carried out safely. Given flawless technical execution, it will be both specific (~100%) and sensitive (>95%). Some centres use the sequence of lymph node and/or bone marrow aspirates, followed by splenic aspiration if the other aspirates are negative. This has the advantage of limiting the number of splenic aspirations while maintaining high sensitivity and specificity.

In cases where splenic aspiration cannot be used, researchers can opt to use either a composite reference standard (CRS) or latent class analysis (LCA)[46]. Both involve the use of several diagnostic tests as comparators for the test under evaluation, the former being an empirical definition of disease status and the latter a mathematical approach based on the probability of disease given the observed test pattern. Notwithstanding their inadequate specificity for acute disease, serological tests for VL can be included in the panels of tests used in CRS or LCA, but cannot be considered as a reference standard for stand-alone use. In the past, response to specific VL treatment was used to confirm that a diagnosis was correct, as antimonials have a very narrow spectrum. With other drugs, for example, amphotericin B, this criterion becomes less specific. TABLE 2 gives an overview of the acceptable reference standards in the evaluation of VL diagnostic tests.

8. Organization of testing
Consideration should be given in the protocol to who will perform the tests and, in the case of an RDT, whether the results will be

Box 2 | **General guidelines for the use of test kits**

- Note the test number and expiry date: a test kit should not be used beyond the expiry date
- Ensure correct storage conditions: if a desiccant is included in the package, do not use the kit if the desiccant has changed colour
- If test kits are stored in the refrigerator, they should be brought to room temperature (about 30 minutes) before use. The use of cold test kits can lead to false-negative results
- Damaged kits should be discarded
- Use test kits immediately after opening
- Reagents from one kit should not be used with those of another kit
- Tests should be performed exactly as described in the product insert

read by one or multiple readers. For prospective evaluations in populations for whom the test is intended, it is important that the tests be performed by clinic staff or outreach workers who will provide the diagnostic testing in that population in the future. The protocol should describe the qualifications the staff require and the training they need.

IV. CONDUCTING THE EVALUATION

1. Obtaining informed consent

See the discussion of informed consent in the generic guidelines *Evaluation of diagnostic tests for infectious diseases: general principles* in this supplement and the sample informed consent forms in APPENDICES 2 AND 3.

2. Specimen sampling and preparation

Venous or capillary blood or serum can be used for most RDTs. The manufacturer's instructions should be carefully respected. However, if there is evidence which allows deviation from the manufacturer's instructions, such deviations can be followed. For example, the package insert from InBios specifies the use of serum for VL detection using the rK39 ICT, however, there is now sufficient evidence that for active VL, whole blood obtained through a finger prick produces similar results. This is extremely important from a programmatic point of view, as the necessity to centrifuge the blood to obtain serum is likely to pose great problems in field conditions. Most RDTs specify that the results should be read within 15–20 minutes after the application of the specimen. This might not always be possible in a busy clinic. It might be useful to include in the evaluation protocol a reading after 1 hour to determine whether the test results remain the same. This would certainly increase the usefulness of the RDT.

3. Transport and storage of specimens for RDTs

A major effect of specimen sampling has been observed for the latex urine antigen-detection test: the test performed very poorly on stored urine. The manufacturer's storage instructions should therefore be followed carefully, tests should be kept out of direct sunlight and the cold-chain requirements should be respected. Keep records of the date of manufacture, expiry date, duration of storage on site, temperature and humidity of storage, the state and type of packaging, and the time to complete use from opening.

4. Use of test kits

The general guidelines for the use of test kits outlined in BOX 2 should be adopted and implemented. All tests should be performed according to the manufacturer's instructions. Any deviation from the recommended procedure should be recorded.

As the interpretation of RDT results is subjective, it is recommended that at least two individuals read the test results independently. The results of RDTs performed in the clinic can also be evaluated against RDTs performed by trained laboratory technicians to assess the feasibility of using these tests in field settings, executed by auxiliary staff. In this type of agreement study, blinding is necessary to ensure the independence of test results in the evaluation. Laboratory staff should be blinded to the RDT results at the clinic and vice versa. To avoid any potential bias in the interpretation of the results, laboratory technicians and readers of RDTs should be blinded to the clinical status of the patient, his or her reference standard results and the results of other RDTs.

5. Training and choice of technicians, test preparation and interpretation

Training and experience of technicians can affect the test performance because reading of an RDT result is not always unequivocal. Sometimes the bands are faint, but these do indicate a positive test and it is a common mistake to read these as negative or doubtful. Similarly, if a dent is produced on the strips owing to manufacturing or handling error, a coloured line can appear but this is generally located in the wrong place on the strip or is very thin. In such circumstances, it is prudent to repeat the test. A company-prepared buffer is supplied with the strips, and it is extremely important to use that buffer only. If for some reasons the buffer runs out, it is best to ask for replacement buffer.

6. Laboratory facilities and testing sites

The reference laboratory that will conduct the evaluation should establish clear Standard Operating Procedures (SOPs) for both the reference standard and the RDT being evaluated.

7. Biosafety issues

The general biosafety guidelines for clinic and laboratory staff outlined in BOX 3 should be adopted and implemented

V. QUALITY ASSURANCE

Teams that engage in the evaluation of RDTs for VL should subscribe to existing processes for laboratory quality assurance.

VI. RECORDING OF RESULTS AND ARCHIVING OF SPECIMENS

The results of the two readings of the RDT under evaluation should be recorded in separate notebooks to ensure independent interpretation of the results. Both the results of the RDT and the results of the reference standard should then be entered into a spreadsheet, together with the information on the sex and age of the subject and a

Box 3 | **General biosafety guidelines**

- Treat all specimens as potentially infectious
- Wear protective gloves and laboratory gown while handling specimens
- Do not eat, drink or smoke in the laboratory
- Do not wear open-toe footwear in the laboratory
- Dispose of sharp objects such as lancets and needles in appropriate sharps containers
- Clean up spills using appropriate disinfectants (e.g. 1% bleach)
- Decontaminate all waste materials with an appropriate disinfectant
- Dispose of all waste, including test kits, in a biohazard container and autoclave, if available

Box 4 | **Minimum standards for field evaluation of VL RDTs**

In addition to the general criteria for diagnostics trials outlined in *Evaluation of diagnostic tests for infectious diseases: general principles* in this supplement, the points listed below should be considered in the design and conduct of all evaluations of comparative field trials of VL RDTs and should be documented. This information should also be recorded in published trials.

Checklist for study design and analysis of results*

Rationale for the evaluation and for what indication, for example:
- ❑ Need rapid test to assess who to put on VL therapy in a clinical setting or for screening a population
- ❑ Need to evaluate performance and ease of use in a field setting

Record details of RDT used:
- ❑ Manufacturer (company name, site of manufacture)
- ❑ Batch number
- ❑ Packaging type (sealed individually, multiple strips in same canister, etc)
- ❑ Inclusion of desiccant with strips
- ❑ Inclusion of lancets/capillary tubes needed to perform test (or otherwise note the items used)
- ❑ Is product under evaluation for regulatory purposes or is it commercially available?

Describe the trial site:
- ❑ Climatic conditions (mean local temperature and humidity)
- ❑ Workplace conditions (type of facility, lighting used for reading RDTs)
- ❑ Local VL epidemiology: endemic/epidemic, causative species
- ❑ Type of facility that will conduct the study: a dedicated VL treatment centre or a general out-patient department?

Describe the study population:
- ❑ Inclusion criteria (symptoms and signs if any, age)
- ❑ Exclusion criteria
- ❑ Demographics (age, sex)
- ❑ Duration of illness (months)
- ❑ Recent anti-VL treatment
- ❑ Describe the controls: healthy endemic, healthy non-endemic, clinical suspects in whom VL disease was ruled out, or patients with confirmed cross-reacting diseases

Describe the recruitment process:
- ❑ Who will be responsible for recruitment?
- ❑ Will recruitment be 'prospective' and 'consecutive'?
- ❑ Specify process: passive or active case detection?
- ❑ Describe informed consent process — remember to obtain consent for specimen storage for future test evaluations

Record organization and training of test readers/technicians:
- ❑ One or multiple readers
- ❑ Same technician/ reader per test type, or alternating
- ❑ Blinding to reference standard, to results of other RDT readers, and to clinical presentation
- ❑ Identity of technicians/readers for later analysis (can be coded)
- ❑ Training/experience in use of test (including date of training and validation of quality of training) and comparison with intended end-users of the RDT

Describe reference standard:
- ❑ Reagents used
- ❑ Staining method
- ❑ Time from preparation to staining
- ❑ Parasite grading according to WHO criteria
- ❑ Proficiency and training of technicians (subscription to external quality assurance programmes)
- ❑ Mechanisms for blinding to RDT results
- ❑ Consider collecting blood dried on filter paper or in EDTA to allow for additional testing as necessary

Describe methods for data analysis:
- ❑ The case definitions for a true VL case and for a control should be clearly stated
- ❑ Sensitivity and specificity estimates should be presented with a 95% confidence interval
- ❑ Positive and negative predictive values with a 95% confidence interval in case of prospective studies

Checklist for conduct of diagnostic evaluation

Describe storage/transport conditions of RDT since receipt at evaluation site:
- ❑ Date of manufacture
- ❑ Date of expiry
- ❑ Duration of storage on site
- ❑ State and type of packaging, and whether canisters of test strips or reagent bottles have been opened before the start of the study (tests in damaged packaging should not be used)
- ❑ General temperature and humidity conditions at storage (monitoring of temperature and humidity if possible). Tests should be stored away from direct sunlight
- ❑ Time to complete use from opening of canister or bottled reagents (when dipsticks with this type of packaging are used)

Describe the evaluation procedure:
- ❑ Time of strip or device package opening to time of use
- ❑ Blood collection (venous or finger prick using lancet or capillary)
- ❑ Specimen processing (whole blood or serum, blood transfer to test device or strip, device provided by manufacturer or pipette, etc)
- ❑ Time from blood collection to placing sample on RDT; and how whole blood or serum is stored in this interval
- ❑ Time taken to obtain reading (as per manufacturer's instructions, or, if delayed, how long and reason for delay)
- ❑ Record each line on the test strips separately, starting with the control line. A record of intensity is not necessary as the test is not quantitative

Record significant difficulties encountered with testing:
- ❑ Significant or recurrent problems encountered in kit preparation or specimen collection (including opening of packaging, etc)
- ❑ Any deviations from manufacturer's instructions
- ❑ Record what is done with indeterminate results, how many tests had to be repeated
- ❑ Conduct a formal independent qualitative appraisal of 'ease of use' of product by each technician

*Issues concerning ethics and patient consent are detailed in *Evaluation of diagnostic tests for selected infectious diseases: general principles* in this supplement. See APPENDICES 1–3 for sample data-collection and informed consent forms.

limited set of variables (including treatment status and duration of symptoms). Double entry of data is recommended to minimize errors. The collected information as well as the frozen serum samples should be kept until the study has ended and the results have been published.

VIII. ANALYSIS OF RESULTS

The sensitivity, specificity and 95% CIs should be calculated for each RDT compared to the results obtained by the reference standard. In Phase III studies with prospective recruitment of patients, positive and negative predictive values of the new test should be given, but not in case-control studies as the frequency of disease in such studies is artificially determined, and does not reflect the real prevalence or allow a meaningful interpretation of predictive values.

BOX 4 contains a checklist with all of the points that should be considered in the design and conduct of evaluations of RDTs for VL.

Marleen Boelaert is at the Institute of Tropical Medicine, Nationalestraat 155, 2000 Antwerp, Belgium.

Sujit Bhattacharya is at the Indian Council of Medical Research, Ansari Nagar, New Delhi 110029, India.

François Chappuis is at the Hôpital Universitaire de Genève, 24 rue Micheli-du-Crest, CH-1211, Geneva 14, Switzerland.

Sayda H. El Safi is at the Faculty of Medical Laboratory Sciences, PO Box 102, Khartoum University, Khartoum, Sudan

Asrat Hailu is at the Faculty of Medicine, Addis Ababa University, Black Lion Hospital, PO Box 9086, Addis Ababa, Ethiopia.

Dinesh Mondal is at LSD, ICDDRB, Mohakhali, Dhaka 1212, Bangladesh.

Suman Rijal is at the B.P. Koirala Institute of Health Sciences, Dharan, Nepal.

Shyam Sundar is at the Institute of Medical Sciences, Banaras Hindu University, Varanasi 221 005, India.

Monique Wasunna is at the Centre for Clinical Research (CCR), Kenya Medical Research Institute (KEMRI), PO Box 20778, 00202 Nairobi, Kenya

Rosanna W. Peeling is at the UNICEF/UNDP/World Bank/ WHO Special Programme for Research and Training in Tropical Diseases (TDR), World Health Organization, 20 Avenue Appia, CH-1211 Geneva 27, Switzerland.

For further details contact: Marleen Boelaert (e-mail: mboelaert@itg.be) or Rosanna W. Peeling (e-mail peelingr@who.int)

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APPENDIX 1 | SAMPLE CLINIC DATA COLLECTION FORM FOR THE EVALUATION OF VL RDTs

Study number: _____

Name of clinic: _____ **Date of clinic visit:** _____ (day/month/year)

Date of birth (day/month/year): _____ **Sex:** Male ❑ Female ❑

Clinical signs and symptoms:
Duration of fever: ___ weeks
Weight: ___ (kg)
Height: ___ (cm)

Tick all that are applicable below:

	Yes	No		Yes	No
Past history of VL	❑	❑	Oedema	❑	❑
History of bleeding	❑	❑	Ascites	❑	❑
Skin pallor	❑	❑	Hepatomegaly	❑	❑
Jaundice	❑	❑	Splenomegaly	❑	❑
Bleeding signs	❑	❑	Lymph nodes	❑	❑

Laboratory investigations:
Haemoglobin: _____ (g dl^{-1})
White blood cells: _____
Platelets: _____
Thick smear for malaria: Positive ❑ Negative ❑

Bone marrow aspiration:
Positive for LD bodies; grading (1–6): ❑ _____
Negative for LD bodies ❑
Not done ❑

Spleen aspiration:
Positive for LD bodies; grading (1–6): ❑ _____
Negative for LD bodies ❑
Not done ❑

Final diagnosis at discharge from hospital:
Confirmed kala-azar ❑
Probable kala-azar ❑
Other : _____
If other, what was the evidence: _____

Outcome at discharge from hospital:
Clinical improvement ❑
Defaulted ❑
No or poor response ❑
Death ❑

APPENDIX 2 | SAMPLE INFORMED CONSENT FORM

(Should be translated into the local language for field trials)
(A separate patient information sheet containing this information should also be provided)

Principal Investigator: _____
Organization: _____
Sponsor: _____
Study title: _____

INTRODUCTION

We are doing research on visceral leishmaniasis (VL), which is very common in this part of the country. The diagnosis of VL is not easy and we would like to do some research to improve the tests. We suspect that you/your child might suffer from this disease. I am going to give you information and invite you/your child to be part of this research. You do not have to decide today whether or not you/your child will participate in the study. Before you decide, you can talk to anyone you feel comfortable with about the research. There might be some words that you do not understand. Please ask me to stop as we go through the information and I will take time to explain. If you have questions later, you can ask the study doctor, the staff or me.

A | PURPOSE OF THE STUDY

VL is an infection with the *Leishmania* parasite that is transmitted by sandflies. The disease can result in death if it is not treated. VL patients should be diagnosed correctly otherwise they will not get the right treatment. Today, the disease is diagnosed by searching for the parasite in a drop of fluid taken from the lymph glands, bone marrow or spleen. New tests using blood or urine have been developed but we do not know how well they work. We invite you/your child to provide some blood and urine for us so that we can evaluate these new tests.

Another purpose of our research is to check these new tests in patients who are also infected with HIV. This part of the study will only be done in adult patients (>15 years old), so it does/ does not apply to you/your child. We will ask these persons specifically, with a separate consent form, if they agree to be tested for HIV before we test them.

B | STUDY PROCEDURES

If you agree to participate, a medical doctor will examine you/your child and ask you some questions. Because we suspect that you/your child have VL he/she will request some tests to confirm this. These tests are the routine procedure for every patient suspected of having VL in this country. First, a small amount of fluid will be taken from a lymph node using a needle. This procedure is simple and does not need any anaesthesia. If we cannot find *Leishmania* in that sample, we will take a very small amount of tissue from the bone of your hip, under local anaesthesia. If we cannot find parasites in it, we will take a tiny bit of tissue from your spleen. These tests will be done whether you decide to participate in the study or not. (NB. The specific procedures for the diagnosis of VL can differ from country to country. For example in Kenya, patients with VL do not present with lymphadenopathy so there would be no indication for lymph node aspirate. Depending on the regulations of the country the procedure can be done as indicated above or amended to suit national requirements.)

If you agree to participate, we will take an extra blood sample from your/your child's arm using a syringe and a needle. We will take about a tablespoon of blood. All these procedures will be done by qualified personnel in our team. If the diagnosis of VL is confirmed, you will receive all the drugs and medical care needed.

Your/your child's sample will only be used to do tests on VL. Any left-over sample will be stored at –20°C for 10 years. This might be used for future research to improve diagnostic tests for VL

C | VOLUNTARY PARTICIPATION

Your/your child's participation in this research is entirely voluntary. It is your choice whether you/your child will participate or not. Even after you agree to participate, you can change your mind later and stop your/your child's participation, without any prejudice to your medical care and treatment.

D | DISCOMFORTS AND RISKS

The only additional procedure for this study is to take a blood sample from your/your child's arm. The staff are familiar with this procedure and any risks are rarely anticipated. Possible discomfort you/your child will feel is pain from the needle stick.

However, some of the routine tests that are necessary for the diagnostic work-up of VL carry some risk we would like you to be aware of. If it is necessary to do a bone marrow puncture, we will perform it under local anaesthesia, so you/your child should feel no pain, but only slight discomfort. If splenic aspiration is necessary, you should know this can sometimes lead to internal bleeding, and, in the worst case, rupture of the spleen. The probability that life-threatening bleeding occurs as a complication has been estimated to be 1 in 1,000 patients undergoing the procedure. There are tests which help us determine if the procedure will be too risky, for example, we will determine you/your child's haemoglobin level and exclude any bleeding tendencies or problems of blood clotting.

E | BENEFITS

You/your child will not obtain any direct benefits for participation in this study. If we have good results at the end of the study, our patients in the community will benefit, as they will no longer need to undergo the bone marrow and spleen aspirates.

F | COMPENSATION

It will not cost you/your child anything to participate in this study. You/your child will not be paid for participating.

G | CONFIDENTIALITY STATEMENT

The information that we collect from this research project will be kept confidential. Your/your child's personal information collected during the research will not be identified by name but by a number. Only the researchers will know what your/your child's number is and they will lock that information up with a lock and key. It will not be shared with or given to anyone.

Your/your child's medical data will only be recorded by the medical personnel and will be stored in a confidential, centralized electronic database. You have the right at any time to have access to these data and to have them adapted if applicable. Your/your child's name will not be used to identify samples, only your study number. Your/your child's name or any confidential information will not be used in written reports. We will not be sharing the identity of those participating in the research with anyone.

H | QUESTIONS AND FREEDOM TO WITHDRAW FROM THE STUDY

You/your child do not have to take part in this study if you do not want to. If you decide that you/your child will not participate, this will not change the medical care you will receive. You/your child can stop participating in the research at any time. If you have any questions you can ask them now or later. (Please give the contact name, address and telephone number of the contact person for each site)

I | RESULTS PUBLICATION

The knowledge that we get from doing this research will be shared with the staff of the clinic and with you before it is made widely available. We will communicate the results of the study in a poster we will put on display at the clinic. Confidential information will not be shared. The results of the study will be written in a report to be submitted to _____ _____, the organization that is funding this study. We will publish the results in a medical journal so that other interested people can learn from our research.

J | PARTICIPANT STATEMENT

I have been invited to participate in the above mentioned research on VL and I understand that it will involve blood and/or urine being taken. I have been informed that the risks are minimal. I am aware that there will be no benefit to me personally and that I will not be compensated. I have been provided with the name of a researcher who can be easily contacted using the number and address I was given. I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate in this research and understand that I have the right to withdraw from the research at any time without in any way affecting my medical care.

Date: _____

Name of participant: _____
Signature (or thumb print or cross if illiterate) of participant: _____

Date: _____
Name of witness (if illiterate participant):_____

Signature of witness: _____

K | INVESTIGATOR'S STATEMENT

I, the undersigned, have defined and explained to the volunteer in a language he/she understands, the procedures of this study, its aims and the risks and benefits associated with his/her participation. I have informed the volunteer that confidentiality will be preserved, that he/she is free to withdraw from the trial without affecting the care he/she will receive at the clinic. Following my definitions and explanations, the volunteer agrees to participate in this study.

Date:_____
Name of investigator who gave the information about the study: _____
Signature: _____

APPENDIX 3 | SAMPLE HIV INFORMATION AND CONSENT FORM

Principal Investigator: _____
Organization: _____
Sponsor: _____
Proposal title and version: _____

PART I: INFORMATION SHEET

[Informed Consent Form for _____]
Name the group of individuals for whom this consent is written. Because research for a single project is often carried out with a number of different groups of individuals — for example healthcare workers, patients, and parents of patients — it is important that you identify which group this particular consent is for.
(e.g. This Informed Consent Form is for men and women who attend clinic Z, and who we are inviting to participate in research X.)

YOU MUST READ THIS BEFORE THE TEST IS DONE. IF YOU HAVE ANY PROBLEMS UNDERSTANDING THIS, ASK THE NURSE OR DOCTOR TO EXPLAIN TO YOU

INTRODUCTION

This document contains the information that you have a right to know, before agreeing to be tested for HIV antibodies. The HIV antibody test (sometimes called an 'AIDS test') is a test that will tell you whether or not you have been infected with HIV, 'the AIDS virus'. Below, we set out your rights with respect to this test, information about HIV and AIDS, and the AIDS test.

WHAT ARE MY RIGHTS?

Your rights are:

1. Not to be tested for the AIDS virus without your free and informed consent.
2. To be given all the material information on the harm, risks and benefits of taking, or not taking, the AIDS test.
3. To refuse to take the test.
4. To receive pre-test counselling upon request. This is private and confidential, and will give you more information about the test and its implications before you consent to the test. Should you in any way be unfamiliar with the issues involved, you are strongly advised to seek pre-test counselling.
5. To have your test result treated confidentially. The result will be made available to your doctor only with your prior consent.
6. To post-test counselling.

WHAT IS HIV?

HIV is the virus that causes AIDS and is sometimes called 'the AIDS virus'. While infected with HIV, and before a person develops AIDS, he or she will feel well and healthy. During this time, the person will be able to infect other people with the virus.

WHAT IS AIDS?

AIDS is a number of illnesses that develop as a result of being infected with HIV. The AIDS virus attacks the immune system, makes the body weak, and leaves it unable to fight various germs and illnesses. More than half the people infected with the AIDS virus will develop the illness within ten years of infection. When you are sick with AIDS, you can usually no longer work. AIDS is a serious disease that eventually leads to death.

WHAT IS THE HIV TEST?

The HIV test checks if you have been infected by the AIDS virus. It does not tell you if you have the disease. The test cannot tell you the date when you were infected, or by whom you were infected. A sample of blood will be drawn from you. It will be sent to a pathologist's laboratory, where it will be tested.

HOW DO I BECOME INFECTED WITH THE VIRUS THAT CAUSES AIDS?

Almost all cases of infection results from sexual intercourse. The AIDS virus is transmitted in this way from one person to another through semen and vaginal fluids. The AIDS virus can also be passed on to babies through the mother's blood or through breastfeeding. Although rare, the AIDS virus can be transmitted by contact with infected blood; for example, through blood transfusions and sharing needles during drug use. Most cases of infection are transmitted either from women to men, or from men to women. Men and women of all ages, races and religious beliefs can be infected with the AIDS virus. Homosexual transmission also occurs. It is important for you to know that the disease is NOT spread by kissing, shaking or holding hands or by sharing meals.

IS THERE A CURE FOR THE AIDS VIRUS?

There is no known cure for HIV and AIDS. Modern medical science, as well as traditional healers, have searched for cures for the AIDS virus. So far, these efforts have been unsuccessful. However, should you be positive, by adopting a healthy lifestyle and having your HIV managed properly by healthcare workers, you can greatly enhance your quality of life before AIDS sets in. It is therefore of the utmost importance that you keep yourself both mentally and physically healthy in spite of being HIV positive. It is also possible that a cure may be found over this time. In the unlikely event you are found to be positive for the AIDS virus, we will refer you to the clinic that looks after people with this virus. There, you will receive counselling and treatment if and when required.

IS THE TEST ALWAYS CORRECT? CAN THERE BE MISTAKES?

The tests used are very accurate, and are performed by registered pathology laboratories. If your test result shows that you are infected with the AIDS virus, you can have this confirmed by having further tests done called a western blot.

WHAT DOES IT MEAN IF THE TEST IS NEGATIVE?

If your test result is negative, it means that at this time, you do not have the virus in your blood. There is a period of approximately six weeks after infection when an HIV test will not detect the AIDS virus. This happens because the test for antibodies cannot detect them at such a short interval after infection. This time is called the 'window period' If you are in the window period, you can arrange to be tested again in three or more month's time.

If your test result is negative now, this does not mean that you will not be infected in the future. If you engage in unprotected sex you may be infected at some time in the future. You should think very seriously about the ways in which you can ensure that you are not infected in the future, in particular, you should consider using safer sexual practices, such as a condom.

WHAT DOES IT MEAN IF THE TEST IS POSITIVE?

If your test is positive, this means that you have been infected with the AIDS virus. It does not mean that you have the illness AIDS. After the test is done you will be counselled again whatever the result might be.

WHAT IS THE HARM AND RISK OF THE AIDS TEST?

Many people do not understand the facts about infection with the AIDS virus. This has led to people infected with the AIDS virus being stigmatized and isolated by their families and communities. Psychological difficulties may also arise.

WHAT ARE THE BENEFITS OF THE AIDS TEST?

If the test is negative, this can reassure you and help you to make sure you do not become infected with the AIDS virus. A positive test result can offer an opportunity to get early treatment, to change life plans and to prevent infection of your sexual partners. You also have a chance to get referred to an HIV treatment centre for management of your HIV/AIDS.

RIGHT TO REFUSE

You do not have to take part in this testing. Counselling is available to you before you make the decision to participate in this testing. If you refuse HIV testing, it will not change the medical care you will receive at this hospital/clinic. Please feel free to ask us any questions about the test, about HIV infection or AIDS, or in general about your health.

PART 2. CERTIFICATE OF CONSENT

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I understand that HIV testing is necessary for taking part in the research and I consent voluntarily to be tested for HIV. I understand that I will receive pre- and post-test counselling, and also understand that I have the right to withdraw consent for HIV testing after the counselling without in any way affecting my further medical care.

Print name of participant: _____ Signature of participant: _____
Date: _____ (Day/month/year)

If *illiterate, a literate witness must sign (if possible, this person should be selected by the participant and should have no connection to the research team).*
I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of witness: _____ Signature of witness: _____
Date: _____ (Day/month/year)

I have accurately read, or have witnessed the accurate reading, of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of researcher: _____ Signature of researcher: _____
Date: _____ (Day/month/year)

A copy of this Informed Consent Form has been provided to participant _____
(initialled by the researcher/assistant)

DECLARATION: HIV TEST CONSENT

I, _____ agree to an HIV test being performed on my blood. I have been adequately counselled on this test by _____ (name of informant) and I understand and accept the 'Information document for HIV testing' handed to me in connection with this trial.
I freely consent to the above procedure.

Signed:_____ Date:_____
Subject

Signed: _____ Date:_____
Witness

Signed: _____ Date:_____
Informant